The Role of Adenosine in Vascular Steal in Peripheral Vascular Disease

by

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TO MY WIFE AND CHILDREN

Abstract

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Peripheral vascular disease (PVD) is a disease of older men. In a Scottish survey, men predominated at younger age groups, but once over 70 years old the prevalence of PVD was 6% in both men and women. There is no effective drug treatment, though oxpentifylline has been widely touted. Vasodilators were originally tried, but have been shown to divert blood away from ischaemic muscle to less ischaemic areas, giving rise to the vascular steal hypothesis. Ischaemic areas of skeletal muscle are supplied by critically stenosed blood vessels. Blood vessels distal to the stenosis are already maximally vasodilated. During exercise the healthier blood vessels elsewhere are able to dilate further, but if there is a more proximal stenosis, the blood flow in the limb will already be limited. The net effect is that blood is diverted from the most ischaemic areas to the healthier areas and exercise is limited by ischaemic pain. Treatment with a vasodilator will have a similar effect to exercise, preferentially dilating healthier vascular beds at the expense of the ischaemic areas, therefore paradoxically reducing exercise tolerance. A more logical approach would be to selectively reduce exercise hyperaemia and hence reduce vascular steal during exercise, hopefully improving exercise tolerance.

Adenosine is a metabolic intermediate, which is believed to link metabolic requirements of muscle to local blood flow. Via its A_{2A} receptor it is a vasodilator in skeletal muscle arteries and increased tissue levels have been found during exercise, particularly ischaemic exercise. One adenosine A_{2A} receptor antagonist has been shown to reduce active (exercise-induced) hyperaemia in animals. Theophylline is a non-selective adenosine antagonist licensed for treatment of asthma. I have shown that oral theophylline antagonises adenosine-induced vasodilatation in the human forearm at therapeutic levels. In volunteers I demonstrated paradoxical enhancement of active hyperaemia, but no change in reactive (post-ischaemic) hyperaemia. I also noted that at these doses theophylline increased baseline forearm blood flow and in subsequent studies I found evidence that theophylline might be inhibiting phosphodiesterases (PDEs). Oxpentifylline has proportionately greater potency as a PDE inhibitor and less potency as an adenosine antagonist than theophylline. I found no effect of oxpentifylline on baseline or hyperaemic flows in volunteers, perhaps because oxpentifylline has a different spectrum of PDE inhibition. In the final study I observed the effect of low-dose theophylline, compared to placebo and standard-dose theophylline in patients with peripheral vascular disease. The available literature suggested that low-dose theophylline should still inhibit adenosine in the forearm, but have little activity as a PDE inhibitor. I found as predicted that low-dose reduced active hyperaemia whereas standard-dose theophylline enhanced exercise hyperaemia, however neither of these results achieved significance probably due to the variability of the data. Adenosine A_{2A} receptor antagonists hold out some promise in the treatment of PVD. The liabilities of this approach are discussed.

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I performed all forearm blood flow measurements whilst at Zeneca. Technical staff helped me to apply the equipment and took blood and stored it and helped prepare infusions. Amanda Wilcox (Zeneca Pharmaceuticals) measured basal and stimulated TNF production by mononuclear cells ex vivo. Statistical staff headed by Stuart Ellis advised on the processing of the results. Chris West (Department of Public Health, University of Liverpool) assisted me in my first faltering steps in statistical computer programming with SAS™. All members of the Clinical Pharmacology Unit at Zeneca, in particular Dr Andrew Williams, contributed to the design of experiments and the interpretation of results, though I was responsible for execution of the protocols.

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Abbreviations

AI	acceleration index
ANOVA	analysis of variance
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AUC	area under the curve
BOMED	brand name for impedance cardiograph
BP	blood pressure
cAMP	cyclic adenosine monophosphate
cCMP	cyclic cytosine monophosphate
cGMP	cyclic guanosine monophosphate
CI	cardiac index
CO	cardiac output
cTMP	cyclic thymidine monophosphate
CV	coefficient of variation
ECG	electrocardiograph
EDI	end-diastolic index
EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
EDV	end-diastolic volume
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
ER	ejection ratio
FBF	forearm blood flow
HR	heart rate
HRP	heart rate period
IC	index of contractility
IMP	inosine monophosphate
LPS	lipopolysaccharide
MAP	mean arterial pressure
MUGA	multi-gated radionuclide scan
MVC	maximum voluntary contraction
NIRS	near infra-red absorbance spectroscopy
pCO_2	partial pressure of carbon dioxide
PDE	phosphodiesterase
PEP	pre-ejection period
PF	peak (aortic blood) flow
PFBF	mean positive forearm blood flow
PFI	peak flow index
pO_2	partial pressure of oxygen
SD	standard deviation
SE	standard error (of the mean)
SI	stroke index
SNP	sodium nitroprusside

Abbreviations

Std.	standard
STR	systolic time ratio
SV	stroke volume
TEB	transthoracic electrical bioimpedance
TFI	thoracic fluid index
t_{max}	time to maximal plasma concentration after dosing
TNF	α-tumour necrosis factor
Xe	Xenon

Chapter 1

Introduction

1.1 Peripheral vascular disease

Peripheral vascular disease (PVD) is caused by atherosclerosis in large and medium-sized arteries supplying skeletal muscle characteristically sparing the arteries in the upper limbs. The atheromatous stenoses restrict flow during periods of high flow, such as during exercise, for example whilst walking. Patients present with ischaemic muscular pain during exercise which passes promptly once the patient rests. This is known as intermittent claudication and is most commonly felt in the calves, but sometimes in the thighs or the buttocks depending on the positioning of critical stenoses and availability of collateral blood supply.

Analysis of Framingham data collected over 26 years of surveillance of 5,209 subjects demonstrated a marked male preponderance and rising incidence of intermittent claudication with age, plateauing around 75 years of age. In the age range 65-74 years the biennial incidences for PVD in men and women were 1.4% and 0.8% respectively[1]. Intermittent claudication was identified in a Scottish study surveying 1592 subjects using a questionnaire and again showed age and gender differences, but by 70-74 years average the prevalence for both men and women was in excess of 6%[2]. Analysis of the 14 year follow-up data from the Framingham study had disclosed evidence of increased mortality from coronary artery disease in patients with PVD, but no deaths directly attributable to infarction of the legs[3]. This has been supported by more recent studies. Of 565 people examined by Doppler ultrasound, average age 66 years, 67 (12%) were found to have PVD. Over10 years 62% of men and 33% of women with PVD died giving a 3-

fold relative mortality compared to an age, sex and risk factor adjusted control group without PVD. There was a 6-fold excess mortality rate from coronary disease even when patients with symptomatic coronary disease at the outset were excluded[4].

Treatment with vasodilators would seem logical. Unfortunately, whilst vasodilators increased blood flow in unaffected legs, they more often reduced blood flow in ischaemic legs[5]. In another study radiolabelled tracer clearance was reduced in diseased legs by nylidrin a ß-agonist causing skeletal muscle vasodilatation[6]. It was suggested that vasodilatation in vascular beds supplied by healthier arteries reduced perfusion pressure in the diseased arterial segments, which were unable to dilate due to atheroma. Effectively vasodilators diverted blood from ischaemic tissues to tissues supplied by healthier arteries. Vasodilators can precipitate vascular steal.

1.2 Active hyperaemia

Exercise was first noted to cause an increase in blood flow in the exercising muscle during and after exercise as far back as 1877[7]. In the same report it was noted that exercise-induced hyperaemia still occurred in denervated muscle. This increase in blood flow caused by exercise has become known as active hyperaemia. Active hyperaemia is clearly beneficial: the increased blood flow helping to meet the increased metabolic demands of exercising muscle and preventing undue accumulation of heat and the products of increased muscle metabolism. In muscle supplied by arteries narrowed by atheroma, active hyperaemia will act in the same way as vasodilators, creating a vascular steal at the expense of already ischaemic tissue at a time when metabolic demands are increased.

1.2.1 Metabolite hypothesis

The idea that the flow responses were linked to the metabolic activity of the muscle by release of vasodilating substances generated during contraction originated soon after active hyperaemia was first observed[8]. The idea is attractive because it closes the feedback loop for homeostatic, autoregulation of skeletal muscle blood flow according to its metabolic needs. Despite considerable effort there is still no clear understanding of the factors linking exercise with active hyperaemia. Maybe it is because there are a number of contributory factors and it seems likely that the importance of their contribution may vary by species, the type and duration of exercise, the type of muscle, and during the time-course of an individual response.

This hypothesis is supported by many crossed-perfusion experiments where the venous effluent from one muscle is perfused through another muscle bed in the same animal[9,10] or even another animal[11]. Exercise hyperaemia in the donor muscle is associated with similar hyperaemia in the recipient muscle bed. In the first study of this type the vasodilator substance was found to be stable in anticoagulated blood for upto 30 minutes[9], however this finding has not been replicated and it seems likely that this was in some way artefactual. A more recent study using venous effluent from heart and kidney concluded that the substance concerned is evanescent, all activity being lost within 2 to 10 minutes[12].

Some doubt has arisen concerning the metabolite hypothesis, because the concentration of a diffusible substance would be expected to fall if blood flow were increased by a vasodilator, reducing the hyperaemic response to exercise. This was not the case in the forearm in which blood flow was increased between 2 and 3 fold by continuous infusion of adrenaline, acetylcholine or histamine. The flows were increased uniformly throughout periods of active and

reactive hyperaemia, but if *excess* flow was considered the saline control response was superimposable on the response in the presence of vasodilator[13,14]. Conversely, when active and reactive hyperaemic responses were measured using a water-filled plethysmograph under pressure, the time profiles during active and reactive hyperaemia was uniformly depressed. Though some diminution of initial flow would be expected, it would also be expected that duration of the response would be greater as the metabolite would not be flushed away as quickly[14]. It could be argued that external compression will just reduce skin flows with no effect on muscle flows, however external compression reduced basal forearm blood flow even after abolition of skin flow by adrenaline[15]. A more plausible explanation is that the metabolite is produced in tissues during ischaemia or exercise and has to diffuse through the vascular smooth muscle and endothelium, where it acts, *before* it reaches the bloodstream. The rate of blood flow would be expected to have little effect if diffusion from the site of action of the metabolite and the blood stream is slow so that the concentration gradient across the vessel wall is steep. The concentration in the vessel wall will be relatively independent of the blood concentration and hence blood flow.

1.2.2 Neurogenic hypothesis

It has been suggested that nerves may have an important role in active hyperaemia, though obviously nerves cannot play a role in vasodilatation noted in cross-perfusion experiments. In feline gracilis cocaine has been shown to abolish active hyperaemia before it fully inhibited the tetanic response due to nerve stimulation. Vasodilatation to acetylcholine remained unimpaired, showing that the vascular smooth muscle was still responsive[16]. In the same paper the effects of pretreatment of feline gracilis with botulinum toxin (a specific, irreversible antagonist of acetylcholine at the skeletal muscle neuromuscular junction) were described. Responses to nerve

stimulation were abolished, but tetany in response to direct, electrical stimulation of the muscle was still possible, yet the hyperaemic response could not be elicited until the botulinum toxin wore off. During this vasodilatation in response to acetylcholine was increased if anything. Could acetylcholine be the neurotransmitter involved? As already outlined, infusion of acetylcholine during active hyperaemia in the forearm showed an additive response, suggesting that the action of acetylcholine and that underlying active hyperaemia are unlikely to be acting via the same receptors[13]. In feline hindlimb atropine infusion did not influence active hyperaemia[16]. Nicotine can induce axon reflexes in post-ganglionic sympathetic fibres and higher concentrations can abolish the reflex. Lower doses infused into cat gastrocnemius caused vasoconstriction in some individuals and vasodilatation in others. Sympathetic ganglion ablation 8 days prior prevented nicotine-induced vasoconstriction and ganglion blocking drugs greatly reduced the nicotine-induced vasodilatation. Large doses of nicotine abolished vasoconstriction and vasodilatation by subsequent, lower doses of nicotine, but had no effect on acetylcholine-induced vasodilatation. Large doses also abolished active hyperaemia, suggesting the participation of a local axon reflex[17].

Detailed analysis of the evidence for the neurogenic hypothesis is presented by Honig[18]. Two weeks after denervation, when degeneration of the axons of extrinsic nerves is complete, nerve fibres can still be seen originating from cell bodies situated in walls of small arteries and arterioles, which stain for acetylcholinesterase[19]. Crossed-perfusion experiments in canine hindlimbs demonstrated rapid onset vasodilatation in the donor leg following contractions and slow-onset relaxation in the recipient limb, however pretreatment of the donor limb with lignocaine or quinidine at concentrations known to block nerve conduction abolished rapid-onset active hyperaemia, leaving only the gradual onset type of hyperaemia seen in recipient limbs[18].

Histamine levels have been shown to increase in venous effluent from canine gracilis during tetany[20], however infusion of histamine was additive with active hyperaemia in the forearm[13] and mepyramine (an antihistamine) had no effect on active hyperaemia[16]. Similar findings have been obtained for adrenaline, prior infusion was only additive in the forearm[14] and phentolamine had no effect on active hyperaemia[16].

Could initiation of voluntary movement in the central nervous system contribute to active hyperaemia via the sympathetic nervous system? There was no hyperaemia when suxamethonium was used to block the neuromuscular junction in one forearm of volunteers provided willed movement was completely blocked, though there was still reflex vasodilatation in response to the stress of mental arithmetic[21]. In feline hindlimb complete isolation of the muscle and division of all the tibial nerve and blood vessels did not change the hyperaemic response following stimulation of the distal end of the tibial nerve, indicating that an axon reflex is involved[16]. Animals in which the lumbar sympathetic chain had been obliterated 2 weeks earlier (to allow Wallerian degeneration) or the lower sensory nerve ganglia removed, showed no change in their active hyperaemic response indicating that neither sympathetic nor sensory nerves are necessary[16]. Initial forearm blood flow following brief forearm muscle contractions were no different in healthy volunteers and in volunteers patients with prior cervical sympathectomy[22]

1.2.3 The myogenic response

The myogenic response relates changes in smooth muscle tone to changes in flow and indirectly to pressure. Vascular smooth muscle tone falls if perfusion pressure is reduced or if flow falls[23]. This provides a means for regulating flow through the vessel, since reductions in perfusion

pressure will reduce flow, and smooth muscle relaxation will reduce the vascular resistance tending to maintain flow. Flow through a vessel is determined both by its radius and by the perfusion pressure, the difference between intravascular pressure and tissue pressure.

Brief tetanic contractions cause transient increases in intramuscular pressure, reducing perfusion pressure, but causes if anything an increase in venous oxygen saturation in the human forearm contrary to expectations, yet there is an active hyperaemic response[24]. In the canine hindlimb perfused at constant, high flow 1 second of tetany did cause a period of hypoxia, however the profile of the fall in end-capillary oxygen saturation was too prolonged compared to the time-profile of active hyperaemia. Could the myogenic response to the sudden decrease in perfusion pressure contribute to exercise hyperaemia? Simulation of the sudden increase in intramuscular pressure was achieved by encasing the canine calf muscles in a cuff, which rapidly and briefly inflated. The cuff caused similar changes in intramuscular pressure to those achieved during brief, tetanic contraction and intriguingly there was a hyperaemic response similar in profile and about half the magnitude of that obtained following brief tetany[25]. The authors advanced the idea that even minor contractions may trigger myogenic responses because resistance vessels closely applied to contracting muscle fibres will experience shear forces capable of twisting-off or nipping them causing transient occlusion and hence a myogenic response. Angiographic studies in dogs have shown reproducible kinking of larger arteries and veins passings between muscle groups, lending support to this hypothesis[26]. However in the human forearm, though contractions as brief as 2 seconds elicited measurable hyperaemia, there was no response to pressurisation of the forearm above and below the strain gauge for upto 5 seconds at pressures of upto 200mmHg[27]. The myogenic response is a short-lived phenomenon, hence it is unlikely to account for anything but the initial peak of hyperaemia following relaxation. It is difficult to

understand how botulinum toxin could block the myogenic response.

1.2.4 Diffusible substances

The idea that nerves are involved in active hyperaemia is not incompatible with the idea that a diffusible substance is also involved, however it broadens the scope beyond just products of metabolism. Adrenaline, histamine and acetylcholine have already been discussed.

Tissue concentrations of a vasodilator contributing to active hyperaemia must change during and after exercise and the change in concentration achieved must be capable of causing a comparable degree of vasodilatation in skeletal muscle to that attained during hyperaemia. If this substance is the main contributor to active hyperaemia then the time profile for the concentration of the substance should correlate well with the shape of the recovery profile of active hyperaemia.

1.2.4.1 Oxygen

It was obvious to consider hypoxia as a cause of exercise hyperaemia as oxygen levels would be expected to fall during exercise as it is consumed by exercising muscle and it would provide a direct link between metabolic need and the means to meet it. The time course of the partial pressure of oxygen in venous blood from the exercising forearm is one problem. Though the pO_2 falls precipitously during exercise it rises rapidly during the initial hyperaemic phase on cessation of exercise, *exceeding* the level prior to contraction. On these grounds oxygen cannot be the sole, determinant of flow[24]. Manipulating the resting venous pO_2 by changing the inspired pO_2 does not support the contention that it is of importance in itself as breathing pure oxygen did not reduce active hyperaemia in the forearm[22], equally decreasing resting venous pO_2 to levels seen during exercise by breathing a reduced oxygen gas mixture did not change resting forearm blood

flow[15]. Breathing a gas mixture containing only 8% oxygen did cause a slight increase in basal forearm blood flow, but this was only additive with active hyperaemia[14].

1.2.4.2 Hypercapnia, lactic acidosis and acidosis

The partial pressure of CO_2 (pCO_2) in the venous effluent of the exercising forearm increases, as would be expected. Magnetic resonance spectroscopy has confirmed intracellular acidosis during muscle contraction in healthy volunteers, as would be expected[28]. Dilution of muscle venous outflow with that from skin can be prevented by iontophoresis of adrenaline into the forearm skin, abolishing skin flow, but with no discernible effect on basal muscle flow. Under these circumstances the increase in CO_2 *production* (and of oxygen consumption) during exercise correlated well with the peak forearm blood flow following exercise[15]. When the venous pCO_2 of the resting forearm is increased by intra-arterial infusion of normal saline with a high pCO_2 it causes vasodilatation, but it also causes an acidosis. The vasodilatation persisted even when sodium bicarbonate was infused, preventing acidosis[29]. Infusion of sodium bicarbonate and the associated alkalosis has been shown to have no effect on resting forearm blood flow[30]. Increases in pCO_2 can cause vasodilatation in the human forearm independent of pH change. The fact that hypercapnic alkalosis and hypercapnic acidosis exert the same influence on forearm blood flow suggests that acidosis is unlikely to contribute to active hyperaemia. Using the relationship between pCO_2 and forearm blood flow just determined, it is evident that the 5mmHg fall in venous pCO_2 measured during forearm exercise is much too small to account for the maximal flow response following exercise[15], though tissue levels are likely to be greater due to dilution by blood from non-exercising tissues. CO_2 could act by causing intracellular acidification and sodium bicarbonate will correct only the extracellular pH, however the alkaline organic amine, tris-hydroxymethyl aminomethane, does penetrate the cell, but even it has little effect on forearm

active hyperaemia[15]. Whilst acidosis is unlikely to have any role in active hyperaemia, hypercapnia does seem likely to play a part, though the magnitude of its contribution is uncertain.

Forearm exercise, even for as little as 5 seconds has been shown to cause an increase in lactate levels in deep venous blood[31]. In patients with an hereditary deficiency of glycogen phosphorylase the venous lactate level and the venous pH are unchanged during exercise. Their active hyperaemic response is similar to that of normal volunteers, indicating that lactate and pH do not play a role in active hyperaemia[32].

1.2.4.3 Phosphate

During vigorous forearm exercise, deep venous blood phosphate rose by 20%, whilst there was little change in the arterial blood phosphate concentration, indicating that increased amounts of phosphate are released by muscle during exercise. Intra-arterial infusion of phosphate, sufficient to increase deep venous phosphate 400% had no effect on resting forearm blood flow[33]. On the basis of these results it seems unlikely that phosphate plays a role in active hyperaemia.

1.2.4.4 Potassium

The concentration of potassium increases during treadmill exercise from resting levels of 3.8mM upto 5mM in healthy volunteers. The same investigators measured a 0.7mM increase in the deep venous concentration of ionised potassium compared to the arterial level during forearm exercise[34]. Other investigators found a 0.35mM increase during fairly mild forearm exercise and a 1.3mM increase if following restricted flow forearm exercise[35]. In the canine hindlimb, following 1 second of tetany the interstitial potassium concentration calculated from venous effluent changes in potassium to increase by 1.3mM or more and the time profile was similar to

that of the associated active hyperaemic response[36].

Isotonic saline solutions containing varying concentrations of potassium chloride produced a concentration-dependent vasodilatation. Potassium was infused at rates of 0.4mMol/min caused cramping pains and doubled forearm blood flow. This rate was calculated to increase the intravascular concentration of potassium to 25mM[37]. Interstitial potassium concentration of 10-15mM have been measured during skeletal muscle contraction[38,39]. This in turn activates the ouabain-sensitive Na^+/K^+ ATPase[40], causing a reduction in the intracellular sodium level in arterial smooth muscle[41]. Ouabain has been shown to inhibit potassium-induced vasodilatation in the canine hindlimb[42] Smooth muscle relaxation could then be triggered either by extrusion of calcium through the Na^+/Ca^{2+} antiport[43] or by decreased calcium entry through the voltage-dependent calcium channel due hyperpolarisation[44]. Responses to the parasympathetic stimulation (the Valsalva manoeuvre) and to infusion of: noradrenaline, acetylcholine and histamine were unchanged by coinfusion of 0.2mM/min potassium[37]. Changes in potassium alone are insufficient to account for active hyperaemia, even in combination with acetylcholine, adrenaline or histamine.

1.2.4.5 Osmolality

Osmolality in deep venous blood has been measured to increase by upto 27mOsm/kg during sustained forearm exercise and this is likely to be an underestimate since it is unlikely that tissue fluid is in equilibrium with venous blood and even deep venous blood during exercise contains some blood drained from skin. Osmolality increased rapidly to reach a plateau within 3 minutes of rhythmic exercise. Intra-arterial infusion of hypertonic solutions of dextrose or xylose increased venous osmolality by upto 15mOsm/kg and were associated with a tripling of forearm

blood flow. There was a correlation between the maximum post-exercise forearm blood flows and the rise in venous osmolality associated with them. There was a similar relationship following infusion of hypertonic solutions. Comparing the two suggested that osmolality may make a significant contribution to the peak post-exercise forearm blood flow, though the authors declined to give a figure[45].

Studies on the constant-flow, blood-perfused dog gracilis muscle have shown synergy between the vasodilator actions of hypoxia, hyperkalaemia and hyperosmolality[46]. In this way it is conceivable that these factors, individually too weak, could account for a major part of the hyperaemic response to exercise.

1.2.4.6 Prostanoids

Indomethacin caused a small reduction in active hyperaemia in the forearm, but no prostaglandin-E like material (by bioassay) could be measured even in the absence of indomethacin in venous blood during exercise[47]. The same group published a subsequent study, using the same dose of indomethacin, in which there was no change in active hyperaemia in the leg during exercise[48]. Others studied the effect on indomethacin on active hyperaemia in the forearm, finding a 16% reduction. This group did find increased PgE_2 production in the exercising forearm that was abolished by indomethacin[49]. The contribution of prostanoids to active hyperaemia is likely to be slight.

1.2.4.7 Bradykinin

It has been suggested that bradykinin might participate in active hyperaemia on the basis that acidosis can activate the kallikrein-kinin system in blood[50] (and acidosis is a feature of exercise)

and bradykinin is known to cause skeletal muscle vasodilatation in man[51]. No increase in venous levels of bradykinin during forearm exercise in volunteers could be detected[52] and in isolated, perfused, canine gracilis muscle carboxypeptidase B, which degrades and inactivates bradykinin, prevented vasodilatation by infused bradykinin, but had no effect on active hyperaemia[53]. Significant levels of the enzyme were detected in lymph draining from the muscle, indicating tissue penetration by the enzyme. On this evidence there is no reason to propose a role for bradykinin in active hyperaemia.

1.2.4.8 Adenosine triphosphate (ATP)

Increased levels of ATP have been measured in venous blood from the ischaemic, exercising forearm[54]. The amounts released are comparable with the amounts needed to be infused to produce a three-fold increase in forearm blood flow[55]. A further study showed elevation in the venous concentration of ATP according to severity of exercise in 5 healthy, young volunteers[56]. The concentrations produced were comparable with those which produced considerable vasodilatation when infused into the brachial artery, however these levels were only reached 5 minutes after exercise. Levels 1 minute after exercise were lower, which would suggest that if ATP does contribute to active hyperaemia, it does so only to the later stages, or it is sequestered. Another study found no change in venous ATP concentration during 3 minutes of vigorous forearm exercise[57], with no change in the ß-thromboglobulin level to indicate confounding ATP release due to platelet activation. This study employed EDTA to stabilise the ATP in blood before assaying it, but previous studies have demonstrated release of ATP from blood cells by EDTA[54,58]. Continuous infusion of ATP, at levels sufficient to increase blood flow 2 to 3 fold, was only additive with active hyperaemia in the forearm. There was no difference in the profile of excess forearm blood flow for post-exercise hyperaemia whether carried out during infusion

of ATP or normal saline infusion[13]. This suggests that there is no interaction between ATP and the factors involved in active hyperaemia. Finally, ATP levels measured by ^{31}P-magnetic resonance spectroscopy were unchanged in muscles of volunteers during ischaemic exercise[28], though this could have obscured a significant change in the interstitial concentration.

1.2.4.9 Adenosine

Adenosine induced vasodilatation was first described in 1929[59]. A role in coupling muscle blood flow to demand in the heart was suggested in 1963[60] and in skeletal muscle the following year[61], but despite much careful experimentation, its importance remains controversial.

1.2.4.9.1 Formation of adenosine

Within the cell adenosine is formed mostly by progressive dephosphorylation of adenine nucleotides (ATP, ADP, AMP)[62]. Finally intracellular 5'-nucleotidase forms adenosine from AMP. A symmetric, facilitated diffusion carrier equilibrates the concentration of adenosine across the cell membrane. Under resting conditions the carrier facilitates net uptake of adenosine into the cell. Adenosine taken up is rapidly phosphorylated to form AMP and some is bound to S-adenosylhomocysteine hydrolase. ATP, ADP and creatine phosphate inhibit 5'-nucleotidase[63], so when the cell is energy replete the rate of formation of adenosine will be low, but it has been observed that a 10% reduction in energy levels in the cell will serve to activate the enzyme and enrich supply of its substrate AMP, doubling the rate of formation of adenosine[63]. The reverse reaction, phosphorylation of adenosine to form AMP is catalysed by adenosine kinase. Adenosine concentrations above 2.5μM inactivate this enzyme[64], so it is possible to envisage a situation in which depletion of the cell's energy reserves activate 5'-nucleotidase increase the adenosine concentration and if this is high enough, the phosphorylation of adenosine is inhibited

providing positive feedback amplification. The level of adenosine is a sensitive index of depleted intracellular energy stores. This has been verified in the isolated, perfused guinea pig heart. The level of adenosine measured in the transudate was found to correlate inversely with the phosphocreatine/phosphate ratio (a measure of cellular energy stores). This applied even when the hearts were stimulated with noradrenaline to reduce the phosphocreatine/phosphate ratio. Interestingly the tissue content of ATP was unchanged despite the fall in the ratio[65].

Also shown in figure 1.1 is the alternative, intracellular pathway for formation of adenosine. Demethylation of S-adenosylmethionine forms S-adenosylhomocysteine which is enzymatically cleaved to form adenosine and L-homocysteine. Although upto 90% of the canine myocyte's contained in S-adenosylhomocysteine complexed with this enzyme, the half-life for dissociation of adenosine is ~2 hours[66], however uptake into the intracellular pool of S-adenosylhomocyteine in guinea pig heart following addition of radiolabelled methionine reached equilibrium in less than a minute indicating rapid forward flux through the pathway[67]. The importance of this pathway in regulation of adenosine formation in response to metabolic needs is uncertain.

Adenosine can be formed extracellularly by the action of ecto-5'-nucleotidase on AMP. Ecto-ATPase and ecto-ADPase have been shown to be attached to the luminal surface of the vascular endothelium[68], so it is feasible for adenosine to be created extracellularly from a variety of adenine nucleotides. Ecto-5'-nucleotidase is inhibited by ATP and especially ADP, so that the production of adenosine is inhibited or at least delayed by release of high-energy adenine nucleotides. Vascular endothelial cells from large, conduit arteries have been shown to release adenine nucleotides in response to a variety of stimuli including hypoxia[69,70] and other cells have been shown to release them when depolarized[71]. Guinea pig coronary vascular endothelial cells

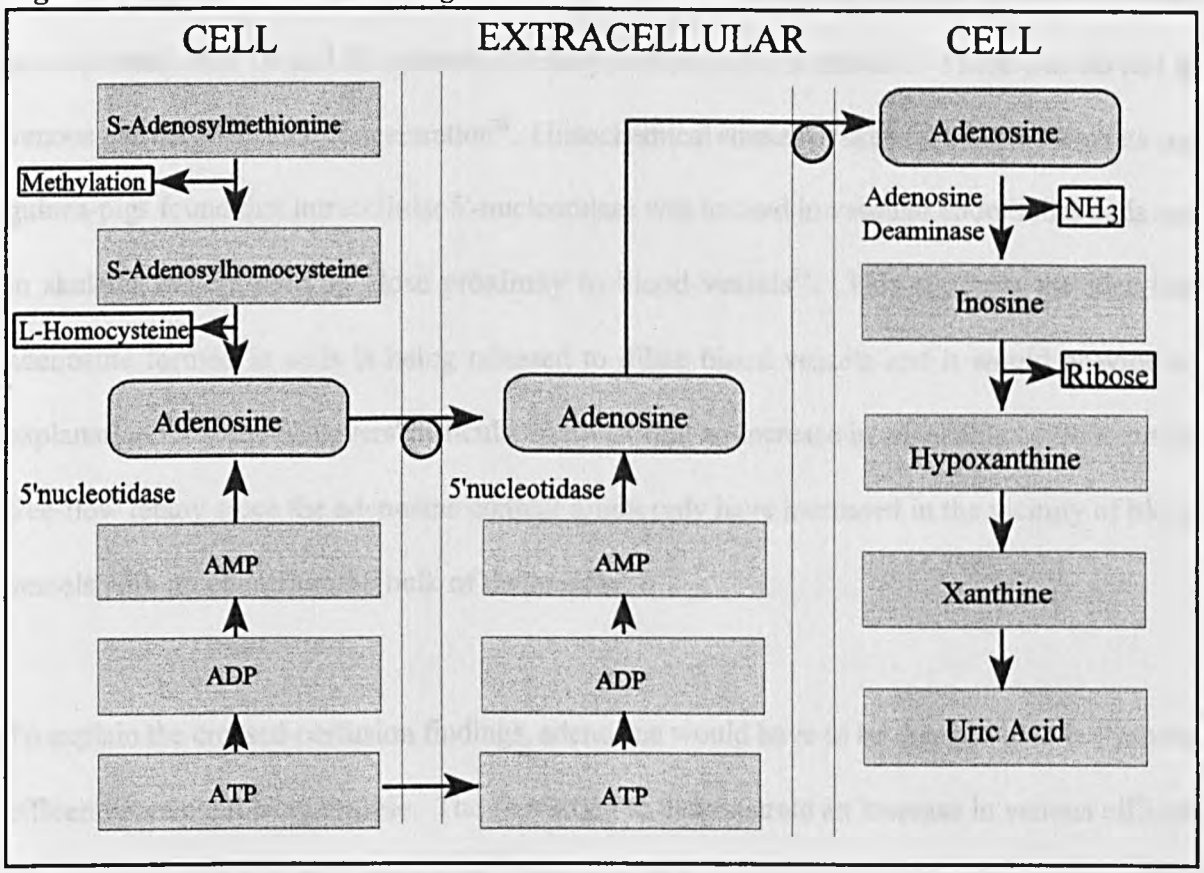
from smaller arteries have not been shown to release adenine nucleotides, although the intracellular concentration of the nucleotides is 3 times greater than guinea pig myocardium. In addition these endothelial cells contain 40 and 50 times higher concentrations of adenosine and inosine and 5'-nucleotidase activity is 7 times as great and adenosine deaminase 7 times less than that measured in whole myocardium[69], implying that their capacity to produce adenosine is much greater than the myocyte. Whilst small-vessel endothelial cells did not release adenine nucleotides, increasing the pCO_2 to 67mmHg increased the rate of adenosine release 14 fold[72]. There were lesser responses to hypoxia, α- or ß-adrenergic agonists, histamine or thrombin.

Inhibition of the nucleoside membrane transport mechanism reduced production of adenosine whereas inhibition of ecto-5'-nucleotidase had no effect on production of adenosine by the perfused rat heart during hypoxia or ischaemia. In this model at least, extracellular production of adenosine cannot be important in coupling metabolic demand to blood flow[73].

1.2.4.9.2 Degradation of adenosine

Infused adenosine is avidly taken up by both the microvascular endothelium and by erythrocytes. The major fate of infused, radiolabelled, adenosine is incorporation into adenine nucleotides in the vascular endothelium, though detectable amounts are degraded by adenosine deaminase present within endothelial cells[74]. Inosine is further degraded to hypoxanthine by deribosylation. Hypoxanthine is hydroxylated twice by xanthine oxidase to form the excretion product uric acid. Inhibition of cellular uptake of adenosine with dipyridamole prolonged the half-life of adenosine added to human blood in vitro and reduced formation of inosine, hypoxanthine, xanthine and uric acid indicating that degradation of adenosine is an important mechanism for disposal of adenosine within blood and the process is initiated at least within cells[75].

Figure 1.1 Formation and degradation of adenosine



1.2.4.9.3 Role of adenosine in active hyperaemia

1.2.4.9.3.1 Tissue content and venous concentration of adenosine

The adenosine and inosine content of feline soleus, but not gracilis muscle increased slightly during tetany with free flow of blood to the muscles[76]. Interestingly feline gracilis had greater adenosine deaminase activity and less 5'-nucleotidase activity than feline soleus[76], suggesting that the metabolic balance in gracilis did not favour utilisation of adenosine for regulation of flow anyway, perhaps because fast-twitch muscle fibres predominate in gracilis in contrast to soleus. Similarly tetanic contraction caused no increase in the adenosine content of canine calf muscles when assayed under free flow conditions, but when the muscles were made hypoxic by restricting flow during tetany the adenosine content increased in proportion to the degree of blood flow

restriction[77]. In canine hindlimb with blood flow restricted to resting flow, the adenosine content was elevated after 10 and 25 minutes of tetany, but not after 5 minutes. There was no rise in venous plasma adenosine concentration[78]. Histochemical studies of skeletal muscle from rats and guinea-pigs found that intracellular 5'-nucleotidase was located in vascular endothelial cells and in skeletal muscle cells in close proximity to blood vessels[79]. This supports the idea that adenosine formed in cells is being released to dilate blood vessels and it would provide an explanation for some observers' difficulty in measuring an increase in adenosine content during free-flow tetany since the adenosine content might only have increased in the vicinity of blood vessels with no change in the bulk of the muscle.

To explain the crossed-perfusion findings, adenosine would have to be detectable in the venous effluent from exercising muscle. The first study to demonstrate an increase in venous effluent adenosine studied the effect of 5 minutes of ischaemic contraction (ie tetany with no blood flow) in canine hindlimb. Adenosine increased 5 fold, but hypoxanthine and inosine increased 22 and 270 fold, whereas more adenosine relative to the metabolites was found in muscle itself, indicating considerable metabolism of adenosine between tissue production and collection for analysis[80]. It was estimated that the adenosine concentration in the interstitial fluid would be 3-fold in excess of that needed to produce maximal dilatation when infused. This experimental model can be criticized as the situation is far from physiological. One study I mentioned earlier failed to detect an increase in the concentration of adenosine in venous effluent during exercise, however other studies have detected increased venous adenosine concentrations in canine gracilis perfused at 180% of resting flow[81] and increased adenosine release, increased tissue and increased venous adenosine concentrations during free-flow exercise[82-85]. Infusion of a 5'-nucleotidase inhibitor (α,β,methylene adenosine 5'-diphosphate) did not prevent the rise in

adenosine caused by exercise[85], implying that adenosine is formed within the cell rather than from adenine nucleotides released by cells. Vasodilatation to greater levels than achieved during active hyperaemia by infusing isoprenaline caused no rise in venous adenosine concentration confirming that vasodilatation alone did not cause the increase in adenosine observed during active hyperaemia[85]. Another group studying the canine hindlimb under constant flow (basal or possibly sub-basal flow) found a fall in the proportion of arterially-infused, radiolabelled adenosine in the venous effluent to 7% of pre-exercise levels coincident with the development of active hyperaemia. Repeating the arterial infusion of radiolabelled adenosine, again at doses too low to cause vasodilatation, but this time coinfusing with sufficient acetylcholine to induce greater vasodilatation than during active hyperaemia caused attenuation of venous hypoxia and some diminution of dilution of radiolabelled adenosine to 39% of the pre-exercise proportion[86]. This work strongly suggests, as flow is held constant, that there is increased adenosine release into blood during exercise and this can only partly be accounted for by vasodilatation itself.

It is worthy of note that dogs differ from humans in that canine erythrocytes lack the adenosine membrane carrier so active in human erythrocytes[87]. In whole, canine blood the adenosine concentration falls by 20% each minute[88], whereas in whole, human blood the adenosine concentration halves every 10 *seconds*[75]. Whilst this makes measurement of adenosine release easier in dogs, it is conceivable that the role of adenosine might also differ.

1.2.4.9.3.2 Effects of adenosine receptor downregulation

An alternative way of determining the importance of adenosine is to flood the system with exogenous adenosine, such that the effect of any endogenously produced will be minimal. Adenosine was infused under free-flow conditions into the canine hindlimb using a

servocontrolled pump keeping the adenosine infusion rate a constant proportion of blood flow. The concentration of adenosine was estimated to be approximately 1000 times that normally measured in blood. Profound vasodilatation occurred, but tachyphylaxis developed over 1 to 3 hours such that the flow returned to resting levels despite the infusion. Active and reactive hyperaemic responses during the adenosine infusion were superimosable on those obtained prior, arguing that endogenously released adenosine could have little role in either[89]. This work assumes that the concentration of adenosine in blood is very little different from that at its site of action and it also assumes that because adenosine induces vasodilatation when infused that it is penetrating to the site of action of endogenously released adenosine and acting in the same way. Neither of these assumptions is safe. Endogenous adenosine could be being released into the perivascular space by muscle cells and it could act on vascular smooth muscle cells in very close proximity before diffusing to the vascular endothelium. Here it could contribute further to vasorelaxation by causing release of nitric oxide, but since the adenosine receptor is on the luminal surface of the endothelial cell, endogenous adenosine would have to traverse the endothelium before it could act[68]. The vascular endothelium has avid uptake mechanisms for adenosine[74,90] and so only a small proportion would be expected to escape to the blood stream.

Vasodilatation due to infused adenosine has been shown to be partially dependent on nitric oxide[91], presumably released by the vascular endothelium, whereas exercise hyperaemia in the feline hindlimb had no dependence on nitric oxide[92]. If only a small proportion of infused adenosine penetrates the endothelium to reach vascular smooth muscle as seems likely[74] and endothelium-dependent vasodilatation by adenosine undergoes tachyphylaxis, but contributes little to vasodilatation caused by endogenously produced adenosine, then these observations would still be consistent with adenosine having a major role in both active and reactive hyperaemia.

1.2.4.9.3 Effects of increased adenosine degradation

Another approach to determining the role of adenosine is to attenuate the effect of endogenous adenosine by use of adenosine deaminase. Arterial infusion of adenosine deaminase, at rates which largely inhibited vasorelaxation by high doses of exogenous adenosine, reduced active hyperaemia by 38% in canine hindlimb perfused at constant, resting flow[93]. However adenosine deaminase had no effect on the initial hyperaemic response. This might represent an underestimate of the role of adenosine as adenosine deaminase might not traverse the vascular endothelium sufficiently well. Adenosine deaminase was superfused over hamster cremaster muscle which was electrically stimulated directly to induce contractions. Exercise increased arteriolar diameters increased to only 89% of their pretreatment levels when adenosine deaminase was added to the superfusate[94]. Since laminar flow is proportional to the fourth power of the diameter, this equates to a 37% reduction in flow. Apart from doubts about tissue penetration use of adenosine deaminase suffers from one other drawback: it degrades adenosine to inosine which at 100μM concentrations acts as a vasodilator[95] and can potentiate adenosine-induced vasodilatation[96].

1.2.4.9.4 Adenosine receptors

Studying the effects of adenosine antagonists circumvents the problems posed by use of adenosine deaminase or adenosine itself. It was realised as early as 1970 that adenosine had actions beyond those accounted for by its place in cell metabolism and that methylxanthines (such as theophylline and caffeine) inhibited these activities[97]. Adenosine receptors were shown to be located on the cell surface as adenosine covalently linked to large stachyose molecules, preventing entry to the cell, still induced vasodilatation[98], subsequent work showing enhancement of vasodilatation by low levels of adenosine by membrane transport inhibitors provided further

support for this idea[99]. These were initially divided into two subtypes (now A_1 and A_2) according to whether adenosine caused stimulation or inhibition of adenylyl cyclase[100,101] and according to the order of potency of a battery of synthetic agonists[99,102]. The A_2 receptor was soon found to have high and low affinity binding subtypes designated A_{2a} and A_{2b} respectively[103] and more recently an A_3 receptor subtype has been discovered which inhibits adenylyl cyclase, like the A_1 receptor, but with low affinity for methylxanthines[104]. Subsequently these receptors have been cloned and their structure determined: all have been shown to be capable of binding G-proteins[105].

Using relative agonist potencies aortic vasodilatation has been attributed to the A_2 adenosine receptor. In guinea-pig coronaries adenosine caused endothelium-dependent vasodilatation attributed to the A_2 adenosine receptor as it was associated with activation of adenylyl cyclase[74]. Human aortic endothelial cells have been shown to possess an A_2 adenosine receptor. Adenosine-induced relaxation of vascular smooth muscle has been attributed to the A_2 adenosine receptor in isolated small arteries from animals[99,106] and man[107]. However these studies were comparing the relative vasorelaxation potency of adenosine agonists when added to the vessels with an intact endothelium, so it is possible that relaxation occurred due to activation of the endothelial A_2 adenosine receptor rather than a smooth muscle receptor. Endothelial removal has been shown to reduce adenosine-induced relaxation in rabbit and canine arterial ring segments[108,109]. Prior infusion of L-N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase in the vascular endothelium, reduced adenosine-induced vasodilatation in the human forearm by nearly 40%[91]. In cultured arterial smooth muscle cells in which the formation of cAMP has been stimulated by forskolin, nanomolar concentrations of adenosine *reduced* cAMP levels, a finding more consistent with activation of an A_1 adenosine receptor[110].

Adenosine could cause vasodilatation by inhibition of noradrenaline release from adrenergic nerve terminals innervating the blood vessel wall. This might have particular relevance in skeletal muscle, which has a particularly high neurogenic, adrenergic tone[111]. During exercise vasoconstriction in response to skeletal muscle adrenergic nerve activity is reduced[112]. Low concentrations of adenosine have been shown to inhibit vasocontrictor responses to adrenergic nerve stimulation, but had no effect on noradrenaline-induced vasoconstriction. In the same study adenosine was shown to inhibit exocytic release of noradrenaline from adrenergic nerve terminals[113].

In the forearm, infused adenosine did ameliorate vasoconstriction due to cold induced sympathetic stimulation more than a control infusion of sodium nitroprusside, however as noradrenaline release was similar, this effect was attributed to postjunctional inhibition of the sympathetic system[114]. The receptor inhibiting vasoconstriction in response to sympathetic nerve stimulation in rabbit portal vein has been classified as an A_1 adenosine receptor[115].

1.2.4.8.3.5 Effects of adenosine receptor antagonists

Theophylline is a methylxanthine, structurally related to adenosine and caffeine. Like caffeine it is found in cocoa, chocolate, tea and coffee. It is a competitive antagonist of adenosine at both A_1 and A_2 receptors[116] with approximately equal potency[117] and is used in the treatment of asthma as a bronchodilator although initially bronchodilation was felt to relate to phosphodiesterase inhibition. Whilst it is a phosphodiesterase inhibitor, its Ki for all phosphodiesterases so far measured is 2 to 10 fold in excess of plasma levels of free drug safely obtainable and which are associated with measurable bronchodilatation[118-120].

Theophylline can be given orally, but in many experiments is infused as the EDTA chelate, aminophylline. Infusion of aminophylline inhibits adenosine-induced vasodilatation in the human forearm, although the tissue level of theophylline was inferred to be lower than that commonly achieved orally, since the infusion rate was titrated to be lower than that which would cause vasodilatation, a property associated with higher oral doses[121-123].

Many experiments have been carried out in animal models to determine the effect of theophylline on active hyperaemia. Theophylline infused at a rate calculated to achieve a concentration of 1mM reduced resistance in the canine hindlimb perfused at resting flow requiring coinfusion of noradrenaline to re-establish resting resistance. Vasodilatation in response to incremental doses of adenosine was inhibited and exercise hyperaemia was significantly reduced, even when vasopressin was substituted for noradrenaline[124]. This finding could not be repeated in the same experimental model again perfused at resting flow. This time the effect of vasodilatation by theophylline were adjusted for by studying the effect of vasodilatation on subsequent vasodilator stimuli and correcting for it.

Vasoconstrictors such as noradrenaline were found to disturb the relationship between resting flows and subsequent vasodilatation, and this was felt to account for the discrepancy[125]. Topical administration of 100μM theophylline had no effect on exercise-induced vasodilatation seen by videomicroscopy in rat cremaster arteries, though it did right-shift the adenosine dose-response relationship approximately 4-fold[126]. In the free-flow canine calf muscle, aminophylline infusion caused a 20-fold rightward shift of the adenosine dose-response relationship and reduced the effect of a single bolus dose given during exercise by 90%, but it had no effect on exercise hyperaemia[82]. In this protocol aminophylline caused initial vasodilatation, though tachyphylaxis

rapidly developed. Flow was allowed to 'decay' back to preinfusion resting flow before experiments commenced.

8-phenyltheophylline is approximately 100 times more potent in vitro than theophylline as an adenosine antagonist[116,127], perhaps 3 fold more potent at the human platelet adenosine A_2 receptor and 700 times more potent as an adenosine A_1 receptor antagonist[117], but arguably less potent than theophylline as a phosphodiesterase inhibitor[127]. 8-phenyltheophylline caused a 40% reduction in the feline hindlimb under conditions of free flow[128]. Another group studied flow in the hindlimbs of conscious dogs using implanted flow probes to measure hyperaemia in response to treadmill exercise. This avoided the possibly confounding effects of general anaesthesia. At doses which blocked adenosine-induced vasodilatation, neither 8-phenyltheophylline nor aminophylline had any effect on active hyperaemia[129].

ZM 241385 is a potent and specific A_{2a} adenosine receptor antagonist[130,131], which reduced active hyperaemic responses in the free-flow cat hindlimb by 27%, similar to the effects of theophylline and 8-phenyltheophylline reported in the same paper[132]. This does not address the issue of whether general anaesthesia is contributing in some way to these observations, but it does at least classify the receptor responsible. The contribution of adenosine to exercise hyperaemia in physiological circumstances is still open therefore, however theophylline significantly increased the maximal power of wrist flexion in volunteers by 19% and also significantly increased the threshold force of contraction above which intracellular acidosis developed due to anaerobic metabolism (measured by ^{31}P-nuclear magnetic resonance spectroscopy)[133]. The mechanisms contributing to exercise hyperaemia in patients with PVD, in areas supplied by stenosed vessels and in those areas relatively free of stenoses have not yet been investigated.

1.3 Reactive (ischaemic) hyperaemia

1.3.1 The contribution of the myogenic response

Vasodilatation following interruption or restriction of blood flow is known as reactive hyperaemia. Bayliss described vasodilatation in the canine hindlimb following interruption of aortic flow in his landmark paper in 1902. He referred to this and other responses as 'reactions'. Bayliss suggested that this particular reaction was due to the myogenic response he described earlier in the same paper, precipitated by the sudden fall in perfusion pressure. By 1925 the term reactive hyperaemia had been coined and it was noted that reactive hyperaemia was unchanged in patients following sympathectomy and the peak, post-occlusion flow increased with increasing duration of ischaemia. Reactive hyperaemia could be thought of as a kind of metabolic 'debt repayment', with the level of metabolite(s) keeping the score. It is hard to explain this purely in terms of the myogenic response. It was hypothesized on the basis of these results that reactive hyperaemia was at least partly due to accumulation of vasodilator metabolites[1]. Reactive hyperaemic flows in the calves of volunteers were reduced when the pressure in the water-filled plethysmograph was raised to 50mmHg and the duration of reactive hyperaemia was reduced[14]. This can be taken as evidence against the metabolite hypothesis unless you allow that the metabolite's diffusion from tissue to blood is rate limiting, hence the tissue concentration will be relatively independent of blood flow.

Further support for the myogenic response contributing to reactive hyperaemia came from forearm experiments in which the forearm was encased in a heated, water-filled plethysmograph in which the pressure could be lowered and normalized suddenly. Negative pressures of upto 100mmHg caused a period of reduced flow lasting for upto a minute. It was felt that this was due to the myogenic response triggered by the sudden increase in transmural pressure[134]. Also noted

was a progressive increase in forearm volume during the period of reduced pressure, perhaps due to tissue fluid accumulation, but also indicative of venous distension. Its possible that some of the reduced flow measured was because of decreased venous compliance due to venous filling. Another study tried to distinguish the myogenic from metabolic components of reactive hyperaemia by comparing hyperaemic responses following arrest of circulation for 5 minutes by venous and by arterial occlusion in volunteers. Venous occlusion caused only slight hyperaemia and so it was considered that the myogenic response was the most important contributor to reactive hyperaemia[135]. Another group were unable to detect hyperaemia even after 20 minutes of venous occlusion[136]. Using absorbtion of 4 wavelengths of near infrared laser light it is possible to monitor non-invasively the oxidation levels of haemoproteins such as haemoglobin, myoglobin and cytochrome a/a3, the final step in the oxidative phosphorylation cascade in the mitochondrion. A tourniquet was used to produce arterial occlusion in one forearm in each of 10 volunteers. There was a progressive decline in the levels of oxyhaemoglobin, oxymyoglobin and cytochrome a/a3 as would be expected, such that cytochrome a/a3 became fully reduced by 6.5 minutes. Venous outflow obstruction for 5 minutes caused an increase in the deoxyhaemoglobin level, but had no effect on the oxidation status of myoglobin or cytochrome a/a3[137], hence venous occlusion would not be expected to trigger a metabolic response to tissue level hypoxia.

1.3.2 The role of diffusible substances

Crossed perfusion experiments in dog hindlimbs showed vasodilatation in the 'bioassay' dog's hindlimb during reactive hyperaemia in the first dog which was proportional to the level of hyperaemia in the first dog and proportional to the duration of arterial occlusion, whereas aortic blood had no effect[11]. Further experiments on canine hindlimb venous effluent during reactive

hyperaemia showed that, whilst hindlimb ischaemia caused forelimb vasodilatation, it caused renal vasocontriction[10]. The pH of hindlimb venous effluent causing forelimb vasodilatation was unchanged, eliminating acidosis as a cause. Interestingly, sudden increases in blood flow in the donor limb caused increases in resistance in the recipient limb, suggesting the presence of a diffusible factor associated with myogenic responses. Adenosine, ATP and AMP caused vasodilatation in hindlimbs and forelimbs, but only AMP and adenosine caused renal vasoconstriction. ATP caused renal vasodilatation when infused directly into one kidney, but when the other kidney was perfused with venous blood from the first kidney, now there was vasoconstriction.

1.3.2.1 Histamine

Raised levels of histamine were measured in venous plasma following 10 to 30 minutes of arterial occlusion[138], and histamine is known to cause dose-dependent vasodilatation in the human forearm[139]. Histamine receptor antagonists infused into the brachial artery at doses inhibiting histamine-induced vasodilatation, had no effect on reactive hyperaemia following 3 minutes of occlusion. Following 10 to 25 minutes of occlusion, there was little change in the initial flow, but a 50% fall in total excess flow (ie the area under the curve) during the subsequent period of hyperaemia[140]. Taken together this evidence suggests that histamine might have a role in prolonged reactive hyperaemia, but not in initial flows following release of occlusion and not if the duration of occlusion.

1.3.2.2 Neurogenic hypothesis

As previously described, large doses of nicotine serve to abolish local axon reflexes conveyed by post-ganglionic sympathetic fibres and in the canine hindlimb abolished or dramatically

diminished reactive hyperaemia following brief periods of arterial occlusion. To confound matters the nicotine also largely abolished the increase in venous pCO_2 and falls in venous pH and pO_2 usually seen[141].

1.3.2.3 Adrenaline

Adrenaline infusion caused vasodilatation in the calves of volunteers, but even allowing for this the time-course for recovery from 2 minutes of arterial occlusion is odd. The initial, peak flow response is if anything reduced by adrenaline compared to control experiments in the same subjects, but then the reactive hyperaemic response is enhanced by adrenaline, diverging for the first minute and narrowing a little by 2 minutes. This contrasts with adrenaline's effect on active hyperaemia illustrated in the same paper, showing slight enhancement throughout, but preserving the same basic profile[14]. Again the mechanism for the initial, peak flow response following a period of arterial occlusion and the subsequent period of recovery appear to have different underlying mechanisms. There is selective enhancement of the maintenance phase of reactive hyperaemia. If adrenaline already played a significant role in this, then a reduced rather than a greater degree of enhancement compared to its effect on basal flow would be expected.

1.3.2.4 Serotonin

Serotonin is present in platelets and can be released when they are activated. Increased levels were noted in venous blood during reactive hyperaemia in canine hindlimb and correlated with the degree of hyperaemia. Intra-arterial infusion of a large amount of serotonin had no effect on blood flow[50]. The role of serotonin in reactive hyperaemia is therefore uncertain.

1.3.2.5 Lactate, acidosis, hypercapnia, hypoxia, osmolality and potassium

Deficiency of glycogen phosphorylase (McArdle's Syndrome) prevents synthesis of lactate and lowered pH during metabolic stress of skeletal muscle. As with active hyperaemia, the reactive hyperaemic profile of a patient with this syndrome was similar to that of normal volunteers[32], suggesting that neither lactate nor pH play a major role in reactive hyperaemia. The venous pCO_2 time profile following 1, 3 and 5 minutes of arterial occlusion correlated well with forearm blood flow in volunteers. Hyperventilation reduced reactive hyperaemia by approximately 40%. Infusion of sodium bicarbonate achieved comparable levels of alkalosis in both arterial and venous blood following arterial occlusion, but only hyperventilation caused a measurable reduction in reactive hyperaemia, suggesting that the pCO_2 is the crucial factor rather than pH[30]. Near infra-red absorbtion spectroscopy confirmed marked tissue-level hypoxia during arterial occlusion in the human forearm[137], but breathing 8% oxygen had no apparent effect on the profile of reactive hyperaemia in the calves of volunteers[14]. On the basis of this evidence it is hard to believe that hypoxia is important in the development of reactive hyperaemia. Unlike active hyperaemia, 5 minutes of arterial occlusion caused no change in venous, plasma osmolality in the fixed-flow canine gracilis muscle[142]. There was only a minor rise in venous potassium concentration (0.3mM).

1.3.2.6 Prostanoids

Indomethacin reduced peak, post-occlusive flow by 23% and total (excess) hyperaemia by 40% after 5 minutes of forearm occlusion in volunteers. The venous concentration of a substance behaving like prostaglandins of the 'E' series was increased in forearm rendered ischaemic and this rise was abolished by indomethacin[47]. On the strength of this evidence prostanoids play an important role in reactive hyperaemia following 5 minutes of occlusion. The same group

repeated this work this time using ibuprofen to inhibit synthesis of prostanoids. Ibuprofen caused significant reductions in peak, post-occlusive flow and total reactive hyperaemia after 3 and 5 minutes of occlusion, but had little effect on either after 1 minute of occlusion and smaller, insignificant reductions were measured after 10 and 20 minutes of occlusion[136].

1.3.2.7 The role of the ATP-sensitive potassium channel

In the coronary circulation in animal models the reactive hyperaemia is reduced by sulphonylureas, which inhibits the ATP-sensitive potassium channel (K_{ATP}). This channel is known to be present in some vascular smooth muscle cells and mediates vasodilatation. Prior treatment of volunteers with the sulphonylurea tolbutamide had no effect on resting forearm blood flow or peak flow, but did significantly reduce total hyperaemia by 25%. Tolbutamide had no effect on the adenosine dose-response relationship in the forearm and neither did it effect submaximal vasodilatation by acetylcholine[143].

1.3.2.8 The role of endothelium-derived relaxing factor (EDRF)

Peak flow and total hyperaemia after 5 minutes of forearm occlusion were both reduced by inhibition of vascular endothelial nitric oxide synthase by coinfusion of N^G-monomethyl-L-arginine, indicating a role for nitric oxide (believed to be EDRF) throughout reactive hyperaemia[144]. The same group also showed that vasodilatation in the forearm by infused adenosine was reduced by 30% over a range of doses by coinfusion of N^G-monomethyl-L-arginine[91]. Others have also demonstrated 20% and 30% reductions in total hyperaemia following 3 and 10 minutes of arterial occlusion in the forearm, but no change in the peak flow immediately following release of arterial occlusion, indicating a role for EDRF in the maintenance, but not the initiation of reactive hyperaemia in man[145].

1.3.2.9 The role of ATP and adenosine

The crossed perfusion experiments in the dog, described earlier revealed an agent, which, like adenosine, caused vasodilatation in skeletal muscle and the coronary circulation, but constricted the renal vasculature. It is conceivable that ATP might achieve this profile by virtue of its conversion to adenosine. Increased levels of inosine, inosine monophosphate and hypoxanthine were found in rabbit muscle during ischaemia; adenosine was undetectable. The levels of ATP, ADP and AMP fell[61]. The plasma concentration of adenosine in deep venous blood after 5 minutes of occlusion of forearm blood flow was unchanged compared to resting levels as were concentrations of inosine, hypoxanthine, xanthine and uric acid[146]. Venous levels of adenosine, hypoxanthine and uric acid were insignificantly elevated following 10 minutes of occlusion of forearm blood flow, but these authors made the point that since blood flow in the first minute of reactive hyperaemia has increased several-fold, then this implies an equivalent increase in formation of adenosine and its metabolites[136]. Finding that the concentration of adenosine in venous blood remains constant with varying degrees of reactive hyperaemia argues that adenosine is either regulating flow or a common factor regulates both in the same way. Prior infusion of theophylline significantly reduced total hyperaemia following 5 minutes of occlusion of forearm blood flow by 35%, at a dose (6mg/Kg) having no effect on resting flow. There were smaller and insignificant falls in total hyperaemia following 1 and 10 minutes of occlusion[136]. There is some evidence for a role for adenosine in reactive hyperaemia, though it is inconclusive.

1.4 Summary and aims

PVD is common, particularly with advancing age. Treatment with vasodilators was unsuccessful, possibly because of exacerbation of vascular steal from ischaemic skeletal muscle during exercise. A treatment which had no effect on basal blood flow in skeletal muscle, but which selectively inhibited active hyperaemia, whilst having little effect on reactive hyperaemia would be ideal. Table 1.1 summarizes the factors considered to be possible contributors to active or reactive hyperaemia in skeletal muscle so far studied. Adenosine is the only factor which could fit the profile. Adenosine seems to be well placed to link tissue blood flow to metabolic needs in skeletal muscle, whether produced by the myocyte or by the vascular endothelium. The development of new A_{2a} receptor adenosine receptor antagonists offer hope of effective drug treatment of PVD with immediate benefit. Unfortunately, no selective antagonists are yet approved for use in man.

The studies in this thesis were conceived to establish whether theophylline, a non-selective adenosine antagonist used for treatment of asthmatics since 1935, acts as an adenosine antagonist when given orally at doses achieving levels within the therapeutic range and given this what effect adenosine antagonism by theophylline has upon active and reactive hyperaemia in healthy volunteers and in patients with PVD.

Table 1.1 Factors contributing to active and reactive hyperaemia in skeletal muscle

Factor	Active Hyperaemia	Species	Reactive Hyperaemia	Species
Myogenic Response	+	Dog	+	Man
Local Axon Reflex	-	Man	ND	
Hypoxia	-	Man	-	Man
Hypercapnia	+	Man	+	Man
Acidosis	-	Man	-	Man
Increased Lactate	-	Man	-	Man
Increased Phosphate	-	Man	ND	
Hyperosmolality	+	Man	-	Man
Adrenaline	-	Man	-?	Man
Acetylcholine	-	Man	ND	
Histamine	-	Man	++	Man
Hyperkalaemia	+	Man	-	Man
Bradykinin	-	Dog	ND	
Prostanoids	+	Man	+	Man
EDRF/Nitric Oxide	-	Man	+	Man
ATP-sens. K^+Channels	ND		+	Man
Serotonin	ND		?	Dog
ATP	?	Man	ND	
Adenosine	+?	Dog/Cat	+?	Man

Key: ND Not Done

? Conflicting evidence

+ Evidence suggests the factor does contribute

- Evidence that the factor does not contribute

Chapter 2

Methods

2.1 Measurement of forearm muscle blood flow

Methods for measurement of forearm muscle blood flow can be classified into invasive or noninvasive, local or global, absolute or relative. Invasive methods are those which involve administration of any substance to the subject (e.g. radio-isotope or dye) or breaking the skin. The terms absolute and relative refer to the units of flow that are produced by the technique. Some techniques produce a value which is applicable only to that individual at that time. They are useful for measuring trends during experiments performed during a single application of the measuring equipment. Techniques which yield absolute values produce results usually measured in mls per 100mls of forearm tissue per minute. This can be thought of as the percentage increase in forearm volume per minute. It is possible to compare these results between days and also between subjects.

2.1.1 Invasive methods

2.1.1.1 Brachial artery blood flow

Several techniques are aimed at measuring brachial artery blood flow, despite the fact that this is only an approximation of forearm muscle blood flow even if hand blood flow is excluded, as blood flow to skin, bone, tendon, nerve and fat is also included. Dissection studies indicate that muscle constitutes 59-64% of the human forearm by volume[147,148]. Two arteries or more were found at the level of the antecubital fossa in 94 of 481 cadaveric arms (~20%)[149], so care must be taken when studying brachial artery flow to ensure that this is not the case, otherwise only one branch will be studied.

Dye dilution involves bolus injection of a known amount of a dye (e.g. Evans Blue) into the brachial artery. By collecting the venous effluent from the muscle and measuring the concentration of the dye it is possible to estimate blood flow to the muscle. One drawback of this technique is that flow measurement takes several minutes and cannot be repeated too often due to recirculation of dye. It is assumed that the dye is not vasoactive, not degraded during passage through the muscle and that it is evenly mixed during passage down the artery and through the muscle. This latter assumption is not safe, because at constant dye infusion rates different concentrations of dye have been measured from different forearm veins. It is possible to achieve more even mixing in the brachial artery before it branches by infusing the dye at high rates through a narrow cannula to create turbulence in the artery. Unfortunately this causes haemolysis[150] with release of powerful vasodilators such as ATP. Radio-iodinated albumin has been used instead of dye[151], this offers the advantage that an instantaneous readout of the dilution curve is available so that many artefacts can be detected early. It suffers from all the other difficulties associated with dye dilution and exposes the subject (in particular the thyroid) to radioactivity.

Thermal dilution is analogous to dye dilution, in this boluses of cool saline are infused into the brachial artery and the exponential temperature profiles in the venous effluent are analyzed to give an estimate of volume and hence blood flow[152]. This shares all of the disadvantages associated with dye dilution, except that the effect of recirculation is likely to be negligible even after frequent measurements at high blood flow. It is assumed that changes in blood temperature will have little effect on muscle blood flow. It is true that increasing core temperature by wrapping subjects in blankets and placing their feet in hot water has no effect on forearm muscle blood flow[153]. Even light exercise can increase intramuscular temperature by 2°C[154], so it is

conceivable that results might be confounded during exercise.

2.1.1.2 Local muscle blood flow

The techniques used for direct measurement of muscle blood flow involve the introduction of a freely diffusible, index substance, not consumed or produced by muscle. The rate of dispersion of the substance gives a measure of flow in mls/100g tissue/min. Since the specific gravity of muscle is very nearly one[155] these blood flow rates are virtually equivalent to those measured in mls/100mls tissue/min - the usual units for plethysmographically determined blood flows. The difficulty with measuring local muscle blood flow is that the between-site variability is high, i.e. the coefficient of variation is 30% or more at best by whatever technique[156]. There is a fundamental difference between measurements made locally in this way and global blood flow measurements. Local blood flow only measures tissue perfusion, whereas global blood flow measurements include blood flowing through arteriovenous shunts and therefore not participating in tissue perfusion.

Using a gamma-emitting radioisotope makes the process easier, because this can be injected into the muscle at the beginning of the procedure and clearance followed by measuring gamma radiation over the site. Initially Sodium[24] was used, but when compared with Xenon[133](Xe), a more lipophilic isotope, it was clear that radiosodium was systematically underestimating blood flows over 2mls/100g/min, probably because sodium has to traverse pores in the capillary basement membrane to enter the vascular compartment, and equilibrium is not achieved at higher flows as this process, rather than blood flow, becomes rate limiting. Values for resting muscle blood flow and for maximal blood flow following ischaemic work using Xe clearance were broadly comparable[155]. Xenon has the additional advantage that it is a gas which can be 'excreted'

via the lungs, however this means that both subject and operative receive a dose of radiation with each procedure.

Dispersion of heat by muscle blood flow can be measured by using a fine probe, 0.9mm in diameter, which has a heating coil at its tip and a thermocouple a 1cm short of the tip. The temperature gradient from tip to thermocouple is proportional to local blood flow. As blood and muscle have similar conductivity, the blood content of the muscle will not be a source of error. Use of two thermojunctions allows compensation for tissue temperature and pre-existing temperature gradient, eliminating error due to changes in tissue temperature[157] for example brought about by exercise[154]. This method gives continuous measurement of local muscle blood flow, suitable for measuring rapidly changing blood flows, such as during reactive hyperaemia. The disadvantages are that results have to be expressed relative to basal blood flow and the problems associated with insertion of a re-usable probe into muscle and retention during exercise. Apparently there is no discomfort during exercise, despite the probe lying within the muscle during contraction. However there must be some possibility of haemorrhage or infection and perhaps even cross-infection.

Another clearance technique has recently been published. It uses ethanol which is dialysed into the muscle continuously via a probe 0.85mm in diameter. An isotonic salt solution containing 5mM ethanol is circulated through the probe at flow rates which are adjusted according to the likely muscle blood flow rates. In volunteers the outflow:inflow ethanol concentration had a strong negative correlation with Xe^{133} measurements made at the same site at rest and during and after exercise[156]. Ethanol at these concentrations does not perturb muscle metabolism and is neither consumed nor produced by muscle. It offers no particular advantage over the calorimetric

method (unless of course the dialysis probes can be made to be disposable), but it shares all the disadvantages.

2.1.2 Noninvasive methods

2.1.2.1 Brachial artery Döppler ultrasound

As this technique is based on measurements made on the brachial artery, it suffers from all of the drawbacks mentioned earlier in connection with invasive studies on the artery: errors will be introduced by the presence of superficial collaterals passing distally at the level of the antecubital fossa and in 20% of forearm it will be technically infeasible due to more proximal bifurcation of the brachial artery.

With the advent of bidirectional pulsed flow Döppler ultrasound probes it is possible to measure absolute blood flow velocity in the brachial artery, because the angle between the direction of the transducer and the main axis of flow can be determined. The between day coefficient of variation for blood velocity determined in this way is 10-20%[158]. Brachial artery diameter can be estimated by using a range-gated time system to measure the distance between the two points at which flow velocities fall, indicating the edges of the vessel. This measurement is precise to within 10%[158]. Knowing these two measurements and the forearm volume (by water displacement) it is possible to estimate forearm blood flow. There was a good correlation between values for resting forearm blood flow measured simultaneously in the same forearm by strain-gauge, venous-occlusion plethysmography and this Döppler technique[158]. Döppler has two distinct advantages: it doesn't require venous occlusion and it provides a continuous measurement of blood flow. Continuous measurement is important when trying to measure transient phenomena such as the initial, instantaneous peak blood flow following a period of occlusion or

following exercise. It has to be assumed however that brachial artery diameter does not change during such measurements. Considering that the diameter is squared during calculation of the cross-sectional area (needed to convert velocity to flow), there is potential for confounding. This assumption is probably safe when blood flow and hence arterial diameter are relatively stable as increases in flow can induce flow-dependent vasodilatation. The between-day variability of resting blood flow measurements can be estimated from the coefficients of variation already mentioned to be 30-40% (as the diameter term is squared during the calculation its coefficient of variation is doubled before adding the two). Despite technical improvements the Döppler technique is probably too variable to make between-day, within subject comparisons in cross-over type studies. Other problems with this technique include the expense, the difficulties of performing it whilst infusing drugs into the same brachial artery and its operator dependence.

2.1.2.2 Near infra-red spectroscopy (NIRS)

Near infrared light penetrates human tissue better than the visible spectrum, up to 6cm in skeletal muscle. The chromophores responsible for its absorption (and re-emission) are haemoproteins. By carefully selecting the wavelengths used and by using an infrared laser to produce very high light intensities it is possible to measure absorption by both oxidised and reduced haemoglobin in human tissue *in vivo*. Since the concentration of total haemoglobin (oxidized plus reduced haemoglobin) can be measured from a blood sample and the average path length of the light is known (by previous experiments on other subjects and assumed to be constant), then it is possible to measure blood flow in the forearm by using the Fick principle. In this case oxyhaemoglobin is used as a tracer. A step change in the concentration of oxyhaemoglobin is induced by asking the subject to take one or two breaths of pure oxygen whilst oxygen saturation is measured using a pulse oximeter. Blood flow can be calculated from the traces displaying oxygen saturation and

the difference in concentrations of oxy- and deoxy-haemoglobin. This assumes that the small increase in tissue oxygenation employed has no effect on blood flow, that the rate of oxygen consumption by the tissue concerned does not change during the measurement and that myoglobin oxygen saturation remains constant (since 10% of the oxy- and deoxy-haemoglobin absorbances are contributed by oxy- and deoxy-myoglobin). Whilst these assumptions are fair during resting blood flow conditions, they would obviously not apply during or following ischaemia or exercise[159].

2.1.2.3 Plethysmography

Plethysmography is the measurement of the rate of flow into an organ by measuring the rate of increase (Greek *plethysmos* - increase) of the volume of the organ when outflow is prevented, usually by clamping the vein. This was applied originally to organs in which the vein(s) could be readily identified. In 1905 it was suggested that this technique could be applied, noninvasively in a limb by use of a ligature sufficiently tight to prevent venous outflow whilst having negligible effect on arterial inflow[160].

2.1.2.3.1 The water-filled plethysmograph

The principle outlined above was used to measure combined forearm and hand blood flow in 1909. The whole of the arm below the elbow was sealed into a rigid box containing warm water and volume displacement measured when a blood pressure cuff on the arm proximal to the apparatus was inflated[161]. The changes in volume had two phases, an initial, linear increase followed by a plateau. The gradient of the initial phase could be prolonged, but not changed, by elevating the apparatus above the level of the heart to allow emptying of the veins between collecting cuff inflations. A similar effect could be achieved by increasing the collecting cuff

pressure. At lower pressures changes in the collecting cuff pressure caused commensurate changes in the level of the plateau, indicating that at these pressures venous pressure was able to rise sufficiently to allow leakage under the collecting cuff. At higher pressures (approaching diastolic blood pressure) the plateau level was largely independent of collecting cuff pressure. This probably indicates that distension of the veins to these pressures is capable of inhibiting arterial inflow. The initial phase, therefore, provides a reliable estimate of blood flow over a wide range of collecting cuff pressures. Subsequent studies have demonstrated that there is no reduction in brachial artery pressure when an arm cuff is inflated to pressures less than diastolic pressure[162]. Increasing venous pressure by 20mmHg has been shown to have no effect on resting forearm blood flow measured by the water-filled plethysmograph[163] and there was no effect on arterial flow rate until venous pressure exceeded 60mmHg. I^{131} labelled albumin confirmed no leakage from the forearm until venous pressure reached collecting cuff pressure[164].

As already stated, the forearm is about 60% muscle by volume. However dissection of the hand showed that it was only 15% muscle and 30% skin by volume[165]. It was originally considered that there was no net flow of venous blood between distal and proximal parts whilst the collecting cuff was inflated. However when hand blood flow (with a high proportion of skin) was measured separately from forearm blood flow by inflation of blood pressure cuff below the forearm plethysmograph to 200mmHg, it became apparent that this was not the case. Often forearm blood flows measured fell when the distal cuff was inflated and in addition much of the fluctuation in resting blood flow was also eliminated. Hand blood flow responses differed to body warming and adrenaline (or momentary stresses - e.g. noise) from those of the forearm. Hand blood flow increased dramatically during body warming, whereas forearm blood flow increased only if the wrist cuff was not inflated. Infusion of adrenaline (or stress) caused

vasoconstriction in the hand, but vasodilatation in the forearm[154]. It has now been shown that inflating wrist cuffs to supravenous pressures, although theoretically sufficient to prevent venous outflow from the hand increasing blood flows measured in the forearm, is not as effective as inflating them to suprasystolic pressures[166].

Inflation of the wrist-cuff causes an apparent increase in forearm blood flow due to blood being displaced from beneath the cuff. Studying the time-course of many resting forearm blood flow readings following inflation of the wrist-cuff has revealed that there is a small, but significant perturbation of blood flow which lasts about 1 minute[167]. Hence a minute should be allowed after wrist-cuff inflation before readings are taken.

The proportion of skin and muscle varies along the length of the forearm. By positioning the plethysmograph and distal cuff accordingly it should be possible to minimise the possibility of confounding by conflicting skin blood flow responses. A further dissection study was carried out, this time quantifying the proportion of skin and muscle in three segments of the forearm. The middle segment (10-15cm distal to the olecranon process) had the best characteristics: 63% muscle and 20% skin and fat by volume, the segment 5cm proximal was similar, but the segment 5cm distal was only 54% muscle. This was born out by results as a 15cm long water-filled plethysmograph covering all 3 segments measured smaller amounts of stress-induced vasodilatation than a 5cm long plethysmograph located over the middle segment[147].

2.1.2.3.2 The air-filled plethysmograph

The water-filled plethysmograph is cumbersome and subject to leaks. It is difficult to apply and it is difficult to achieve thermoneutrality since water has a high specific heat capacity and there

is a natural temperature gradient along the limb. If water is used which closely corresponds to the skin temperature in air, then there is an increased loss of heat from the skin, reducing the core temperature. If a higher temperature is selected (as it usually is), then there is inevitably an increase in skin temperature and a concomitant increase in skin blood flow. Depending on the depth of the water in the apparatus and to some extent on how the volume displacement is measured there is a small pressure exerted on the forearm. Use of air instead of water addresses many of these criticisms, however care must be taken that during measurement of volume changes there is no increase in pressure within the apparatus as both air and forearm tissue are compressible. Direct comparison of the signals produced by air and water plethysmographs simultaneously measuring blood flow in opposite forearm revealed no significant difference[168].

Results from methods such as air-filled or water-filled plethysmography, which measure changes in volume directly are difficult to interpret if there are leaks or if there is any movement between the plethysmograph and the forearm. Some compression of the limb is inevitable if a good seal is to be obtained between the limb and the plethysmograph. This makes them difficult to operate, requiring constant expert supervision. For this reason several techniques have been developed which still utilize the principle of plethysmography, but which measure volume change indirectly with the aim of simplifying the apparatus, simplifying its use and improving the reproducibility of the system.

2.1.2.3.3 Impedance plethysmography

This technique measures volume change in the forearm by measuring the electrical impedance of the tissue to a high frequency current travelling between 4 circumferential electrodes on the forearm. The impedance increases as the forearm swells. Although there was some agreement

between the methods when measuring resting blood flow, impedance plethysmography showed no increase in blood flow following exercise[169]. This may be because exercise causes an increase in blood osmolality reducing the impedance of blood.

2.1.2.3.4 Capacitance plethysmography

This method measures the changes in capacitance of two concentric cuffs of copper wire screen applied around the forearm forming two plates of a capacitor. As the forearm swells during venous occlusion, the length of the two plates increases and hence their capacitance increases. Calibration is carried out using a bladder inserted under the inner cuff in which water can be instilled at a known rate. This method has been compared with strain-gauge plethysmography (q.v.). Several estimates were made of resting blood flow by one method, followed by several further estimates performed on the same arm using the alternative method in random sequence. The two methods produced very similar results[170], however as the authors pointed out, strain-gauges were simpler and quicker to use.

2.1.3 Choice of technique

Plethysmographic techniques in general have a great advantage that they are noninvasive and they measure a global flow, so are not subject to sampling error. More specifically, volume displacement techniques and strain-gauge plethysmography are inexpensive to set-up and use, give absolute values of forearm blood flow and are well-validated against each other and other techniques. We chose strain-gauge plethysmography as it is quicker and easier to use than volume-displacement methods and is in most widespread use. This method is now examined in detail.

2.2 Venous-occlusion, strain-gauge plethysmography

This technique uses venous-occlusion to occlude venous outflow from the forearm, but uses a strain-gauge encircling the forearm to measure the rate of increase in the circumference of the forearm during occlusion rather than measuring volume directly. In addition to the assumptions inherent in plethysmography (already discussed), it also assumes:

i. that changes in circumference of the forearm are proportional to changes in volume.

ii. that length and resistance of the gauge are proportional for the range of lengths encountered during measurements

iii. that forearm tissue is elastic for the range of tensions within the strain-gauge encircling it (i.e. that the reduction in the circumference caused by the gauge is proportional to the tension in the gauge)

iv. as the distal cuff is usually applied at the wrist and the gauge is positioned fairly proximally on the arm, it is assumed that appreciable amounts of blood draining from distal parts of the forearm does not flow proximally during occlusion, artefactually increasing forearm blood flow readings

v. and blood flow to skin, fat, bone and tendon is not appreciable.

I shall deal with each of these assumptions in turn.

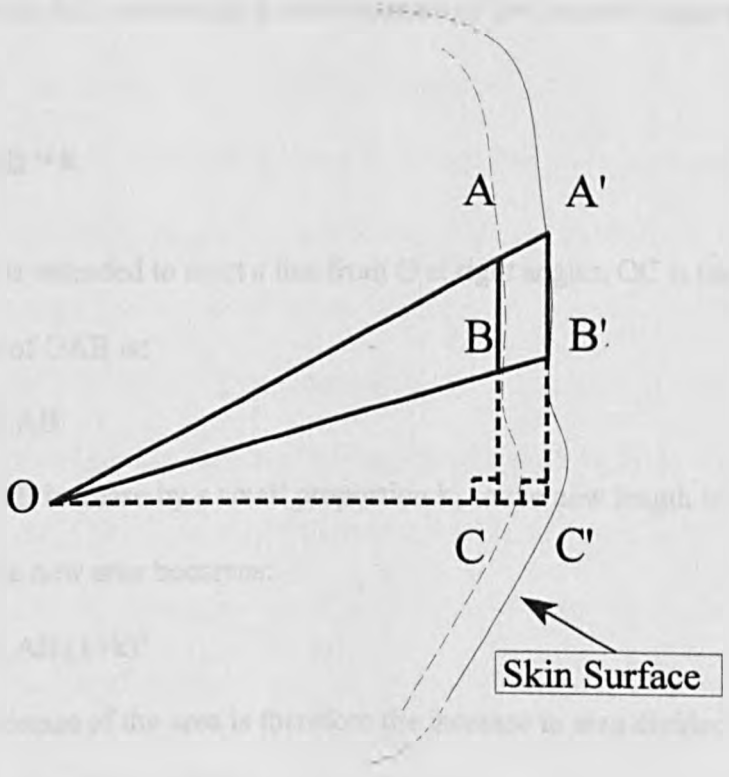
2.2.1 Validation of assumptions

2.2.1.1 Relationship between circumference and volume

This proof is abstracted directly from Whitney's original description of the use of Mercury-in-rubber strain gauges[171]. It is assumed that for small changes in volume (several percent) there is no change in the length of the forearm. Treating the short segment of forearm enclosed by the two strands of the strain-gauge (approximately 1cm) as a cylinder with an irregular rather than

a circular section, as the volume is the product of the length (fixed) and cross-sectional area, volume changes are proportional to changes in cross-sectional area. The problem becomes one of relating small changes in the circumference of an irregular, perhaps even indented, ellipsoid shape to changes in cross-sectional area.

Figure 2.1 Estimation of the area of an irregular ellipsoid



The area of an irregular transverse section can be calculated by breaking it into many triangular elements centred on one point inside the section, as illustrated figure 2.1 above. It is further assumed that the shape of the section remains the same when the volume of the forearm increases. This is likely to be fair providing the gauge is not sited too close to either the elbow (as there will be no expansion at the proximal, subcutaneous surface of the ulna) or the wrist (as both radius and ulna lie close to the surface for much of the circumference). The triangular

element is designated OAB, where O is the point within the section and A and B are two points close to each other on the outer margin of the section. The triangle expands to become OA'B'. As the section expands uniformly the increase in the distance between any two points in the section increases proportionally. That is:

$$\frac{AA'}{OA} = \frac{BB'}{OB} = k$$

Similarly the side AB, comprising a short segment of the circumference will increase by a small proportion, k:

1. $$\frac{A'B'-AB}{AB} = k$$

If the side AB is extended to meet a line from O at right angles, OC is the height of the triangle, hence the area of OAB is:

2. $1/2.OC.AB$

both OC and AB increase by a small proportion k, so the new length is a factor of 1+k greater then the old, the new area becomes:

3. $1/2.OC.AB.(1+k)^2$

the factor of increase of the area is therefore the increase in area divided by the original area or substituting from equations 2 and 3 and gathering terms:

4. $$\frac{1/2.OC.AB((1+k)^2-1)}{1/2.OC.AB} = 2k+k^2 \approx 2k$$ As k is small, k^2 can be neglected.

For small proportional increases in circumference, the proportional increase in area is approximated by twice the proportional increase in circumference. Summing over all elements, let an increase δG in the initial circumference G accompany an increase δH in the initial area H, then:

$$\frac{\delta H}{H} = 2.\frac{\delta G}{G}$$ when δH is small.

Let $\delta V/V$ be the proportionate increase in the volume of the segment, then since there is no change in the length of the forearm, only the cross-sectional area, then $\delta H/H = \delta V/V$, hence

5. $$\frac{\delta V}{V} = 2.\frac{\delta G}{G}$$

i.e. the proportional increase in forearm volume is equal to twice the proportional change in circumference for small proportional increases in volume.

2.2.1.2 Properties, design and use of strain gauges

2.2.1.2.1 Relationship between changes in length and changes in resistance

Cursory consideration would suggest that the resistance of an ideal strain-gauge would increase proportionately with increases in its length. Whitney found this to be the case with his mercury-in-rubber gauges[171]. They are constructed as a long, fine bore rubber tube filled with a thread of mercury in contact with metal plugs occluding either end of the tube. The tube is used as a loop, so that both limbs of the loop traverse the whole circumference of the forearm. Using a loop increases the resistance of the gauge and doubles the change in resistance to be measured for a given change in circumference.

The relationship between the resistance (R) of a conductor, its length (L) and its cross-sectional is:

6. $$R = \frac{\rho.L}{A}$$ where ρ is the resistivity of the conductor.

Provided the temperature of the gauge does not change, the volume (V) of mercury (or indium-gallium alloy used subsequently), should be constant, as liquids are incompressible. Hence

$V = A.L$

substituting in equation 6:

$$R = \frac{\rho . L^2}{V}$$

If the proportional increase in length is given by k and the initial length and resistance are R_0 and L_0 then the new length is $L_0.(1+k)$ and hence the new resistance(R_1) is:

$$R_1 = \frac{p.L0^2}{V}.(1+k)^2 = R_0 + R_0.(2k+k^2)$$

The proportional increase in resistance is:

$\frac{R_1 - R_0}{R_0} = 2k + k^2 \approx 2k$ for small increases in length, i.e.:

$$7. \quad \frac{\delta R}{R} \approx \frac{2\delta L}{L}$$

The proportional increase in resistance has a linear relationship with the proportional increase in length assuming small changes in length.

2.2.1.2.2 The relationship between changes in resistance and changes in forearm volume

The most important relationship is that between changes in strain-gauge resistance and changes in volume. Assuming that the length of the strain-gauge is equal to the circumference of the arm when applied, then the proportional change in length, k, referred to above is equivalent to k referred to in equation 4 earlier, i.e.:

$$8. \quad \frac{\delta R}{R} = 2k + k^2 = \frac{\delta V}{V}$$

The fact that gauge resistance increases according to the square of the increase in length actually makes it ideal for its purpose. Consideration of the relationship between resistance and volume has shown that there is no need for the approximation used by Whitney, nor even to assume that

changes in circumference are small, however it is assumed that increases in the length of the gauge are equal to changes in forearm circumference. This further assumes that the expanding forearm is incompressible!

2.2.1.2.3 The effect of strain-gauge tension on forearm circumference

As the forearm swells during venous occlusion, the gauge is stretched. As the gauge behaves elastically, the tension within it increases in proportion to the increase in length. Using fine-bore elastic tubing filled with a liquid metal ensures that the elastic properties of the gauge are solely those of the silastic. Typically tension increases by 2-3g per 1% increase in the length of the tubing. Although this figure is small, it is never-the-less sufficient to cause some compression of the forearm. If allowance is not made for this then the gauge will systematically underestimate changes in forearm circumference and hence forearm blood flow.

Assuming that the forearm to be a cylinder of elastic material encasing an incompressible core it is possible to derive the following equation:

$$\frac{\delta G}{\delta L} = 1 + \frac{Ke}{GM}$$

where δG is a small increase in circumference of the limb causing a small increase, δL in the length of the gauge. K depends on the proportion of bone at gauge level and is about 3, G is the circumference of the limb at the level the gauge is applied (about 24cm), M is the modulus of elasticity of forearm tissue (estimated to be about 800g/cm^2) and e is the modulus of elasticity of the gauge (typically 200g/cm). Substituting these figures would suggest that the gauge will underestimate the true change in circumference by 3% and hence the forearm blood flow by double that (applying the formula derived earlier). By calibrating the gauge on the arm it is

possible to allow for this effect.

2.2.1.2.3.1 Theory of Mechanical Calibration

Calibration on the arm is achieved by bringing the ends of the gauge together by a small, measurable amount whilst the gauge is in position. This is done by connecting both ends of the loop to a curved, perspex mount, held against the skin of the forearm. The mount incorporates a screw such that turning the screw shortens the mount by the pitch of the screw, which is known. The gauge can be stretched in situ by turning the screw a fixed number of turns. The length of the gauge is increased by a measured amount and the tension in the gauge increases commensurately. The increase in tension causes further compression of the forearm tissue and so the increase in length of the gauge is very slightly less than the change in length calculated from the movement of the screw. This engenders a smaller change in resistance. This change in resistance is used to calibrate the gauge against the change in length used to bring it about. This means of calibration therefore corrects for compression of tissue.

Using this type of gauge it is possible to check the assumption that forearm tissue behaves elastically by measuring the resistance changes in the gauge for a wide variety of length changes imposed on the gauge by use of the screw mechanism. When the change in length was plotted against the resistance change the relationship was linear over all of the values likely to be encountered in forearm blood flow measurements[171]. As the relationship tested by this experiment also depends on the elastic behaviour of the gauge, this was also tested off the arm and found to be elastic over the range likely to be encountered during measurements[171].

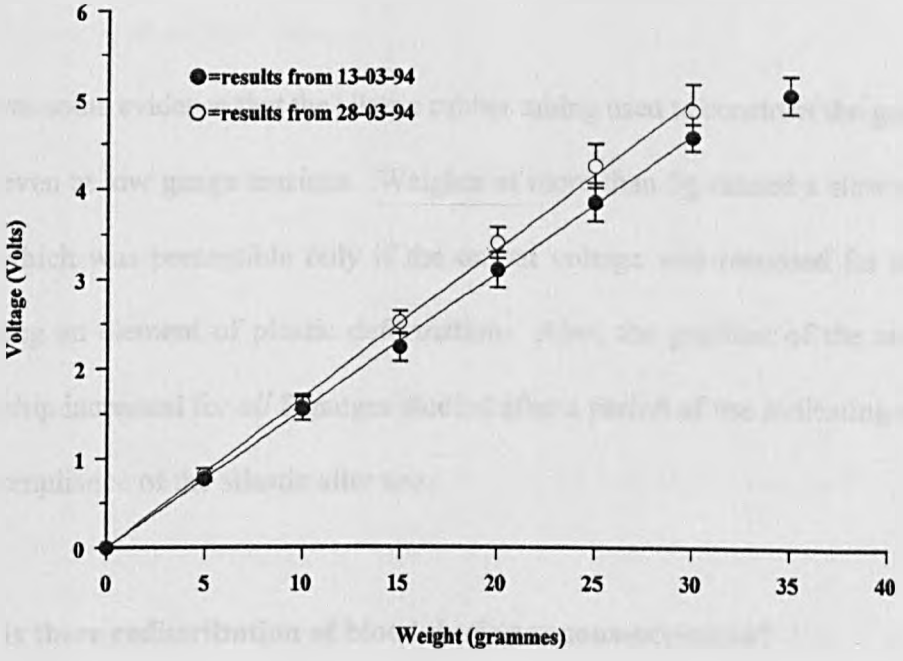
2.2.1.2.3.2 Gauge tension

Whilst the behaviour of the gauge rubber and of the forearm tissue is elastic over a wide range of gauge tension, it is obviously desirable to minimize compression of the forearm tissue by the gauge. The gauge tension used by different groups (when specified) usually lies somewhere between 10 and 30g[171,172]. Others have pragmatically applied the gauges at the lightest tension which will keep the gauge securely in position. This is certainly one constraint, the other is that the tension should be sufficient to ensure that the gauge is in contact with the forearm for the whole of the gauge length. A certain amount of tissue compression is therefore necessary.

It is possible to apply the gauge with a known tension by first measuring the resistance of the gauge when the desired tension is simulated off the limb by hanging that weight on the gauge. Once the gauge is applied, the tension in the gauge is adjusted by changing its length (for example using the screw) until the same resistance and hence the same tension is attained.

I have studied the properties of the gauges I used during these studies (Medasonics™ SP24). I suspended weights between 5 and 35g in 5g increments from the ends of 8 unused gauges and converted changes in the resistance of the gauges to voltages recorded on a chart recorder using the Medasonics™ SPG16 plethysmograph. The results are expressed therefore in volts (V) rather than ohms.

Figure 2.2 Weight-resistance relationship for 8 Medasonics™ strain-gauges before and after use



From figure 2.2 it can be seen that the 8 gauges had a linear response up to 25g when studied before being used and again after a period of use. I therefore chose to apply gauges using a tension of 15g since this allowed the greatest margin for error. As the greatest deflections recorded before venous filling reduces the rate of arterial inflow are of the order of 2% and the 1% calibration signal causes a 95mV deflection, it is possible to estimate the increases is tension which will be experienced by gauges during measurements. The average gradient of the linear segment of the gauge resistance (voltage equivalent) - tension relationship after use was 170mV/g, hence 190mV (equivalent to a 2% increase in resistance) represents an increase in tension of just over 2g. Thus, if the initial tension is set at approximately 15g, this extra tension is unlikely to cause gauge tension to exceed 25g.The only other consideration is whether higher gauge tensions could inhibit venous filling and cause low readings and on the other hand whether there is sufficient gauge tension to keep the gauge adherent for the full circumference

of the arm. With one exception it has been possible to keep the gauge adherent with 15g of tension.

There was some evidence that the silastic rubber tubing used to construct the gauges is not truly elastic even at low gauge tensions. Weights of more than 5g caused a slow extension of the gauge which was perceptible only if the output voltage was recorded for several minutes, suggesting an element of plastic deformation. Also, the gradient of the resistance-tension relationship increased for *all* 8 gauges studied after a period of use indicating a slight increase in the compliance of the silastic after use.

2.2.1.3 Is there redistribution of blood during venous-occlusion?

Whitney noted a gradation in blood flows measured by strain-gauges mounted 3cm apart in the middle third of the forearm. Higher blood flows were measured more proximally[171], though the difference was not found to be statistically significantly different in these experiments. This difference in resting blood flow could be attributable to proximal redistribution of blood during venous occlusion (as noted between hand and forearm[154]), however further experiments make this unlikely. If blood flows proximally during venous occlusion then it may be possible to minimize confounding by skin blood flow by moving the occluding cuff to a point just distal to the gauge. Unfortunately this has not found to be the case as a gauge applied just proximal to the wrist (occlusion) cuff measured the same blood flows whether the proximal, collecting cuff was applied above the elbow or just proximal to the gauge itself[173]. These findings imply that providing hand blood flow is occluded during measurements, the gauge will measure blood flow to the tissue underlying it and not distal to it. To minimize the inflation artefact the proximal cuff should be placed as high up the arm as possible. The distal occlusion cuff can be positioned

anywhere between the wrist and the gauge, but is conventionally applied at the wrist.

2.2.1.4 Blood flow to other tissues

To measure the greatest proportion of skeletal muscle blood flow the strain-gauge should be positioned at the point where the highest proportion of muscle lies. This is born out by experiment, because proximal flow responses differ from distal forearm flow responses. As mentioned earlier proximal flow increases in response to adrenaline, whereas distal flow was virtually unchanged[174] In contrast body warming or direct heating of the forearm in a water jacket caused the greater increase in distal rather than proximal forearm blood flow[173]. This is very much in accord with the comparison of hand and forearm blood flows described earlier[154], indicating that distal forearm blood flow is confounded by skin blood flow despite the use of a wrist cuff.

Skin flow has been estimated by comparison of forearm blood flow before and after the skin has been blanched by iontophoresis of adrenaline adjusting for the relative volumes of skin and muscle in the underlying forearm. Skin blood flow varied in the range 0 to 70mls per 100mls of skin per minute increasing with the temperature of the water in the water-filled plethysmograph (30.5-34.5°C)[148]. At higher temperatures skin blood flow constituted over half of the total forearm blood flow. As adrenaline causes vasodilatation in skeletal muscle, diffusion of adrenaline into the underlying muscle would if anything have reduced the apparent skin blood flow. The mean resting value of skin blood flow was 10mls per 100mls per minute, hence skin blood flow is likely to be a major confounder and source of variability, especially at higher ambient temperatures.

Blood flow in anterior abdominal wall fat was measured at 1.7mls per 100g per minute by Xe^{133} clearance at rest, increasing to 4.5mls per 100g per minute during stimulation by glucagon[175]. No one has ever tried to measure blood flow to tendon, but it is generally assumed to be low. This was thought to be the case for bone, as bone blood flows of 0.5 to 1ml per 100mls per minute were recorded using a water-filled plethysmograph applied around the distal humerus and elbow during arterial occlusion distal to the point at which the nutrient arteries enter the humerus[176]. This probably represents an underestimate as some venous drainage may occur proximal to the occlusion through the periosteum or via the epiphyseal venous plexus[177]. Blood flow in bone has been shown to increase during exercise[177], since bone cannot expand in response to changes in blood flow, the soft tissues surrounding it are likely to do so. By this mechanism it is possible that bone blood flow contributes to the hyperaemic response following exercise.

The simplest way to minimize the contribution of other tissues to forearm blood flow is to mount the gauge at a level on the forearm at which muscle contributes the greatest proportion of the cross-section and conversely, these other tissues contribute least. Dissection studies have shown that the greatest proportion of underlying muscle is in the region between 10-15cm below the olecranon process[147]. This is usually taken to mean the level of maximum circumference since area of skin and bone varies little in cross-sections of the mid-forearm whereas muscle does[147]. The proportions of muscle, residual tissue (skin and fat) and bone measured by computerized tomography are 68%, 23% and 8% respectively at this level[178].

2.2.2 Validation against other techniques

Forearm blood flow measurements by strain-gauge plethysmography have been compared directly with values obtained using the water-filled plethysmograph. Because operation of the

water-filled plethysmograph might interfere with strain-gauges applied to the same arm[171], the strain-gauges have to be applied to contralateral arm to the water-filled plethysmograph. To make the situations entirely comparable a heated water jacket was applied to both arms. Although the results correlated well, the strain-gauges read consistently 9% lower than the water-filled plethysmograph[173]. Interestingly, a modified design of strain-gauge measures readings 9% greater than the conventional design[179]. In this, both strands of the strain-gauge are threaded through a series of 2cm wide flat rests, making a kind of bracelet through which the strain-gauge can pass freely. Assuming the rests occupy over 2/3rds of the circumference of the forearm, comparison of the surface areas of strands and of the bracelet suggests that this will reduce compression of forearm tissue by a factor of 4 or more during each individual measurement and during a prolonged series of measurements during which the gauge tends to indent the forearm.

Near infrared spectroscopy has been compared directly with strain-gauge plethysmography for measurement of basal forearm blood flow. There was a close, linear correlation between measurements by the two methods, though NIRS measurements were marginally greater than those obtained plethysmographically[159]. It was suggested that NIRS may have measured bone blood flow in addition, since bone is penetrated by infrared wavelengths. Alternatively, since the strain-gauge was calibrated electronically it may be that the strain-gauges were underestimating flow by 4-5% by not allowing for tissue compression.

The air-filled plethysmograph and strain-gauge plethysmograph have been compared, this time on opposite calves, measuring resting blood flow and hyperaemic flows following arterial occlusion. There was an extremely good correlation between the two techniques ($r=0.96$). The gradient of the line indicated a that the air-filled plethysmograph measured just 3% greater values

of calf blood flow than the strain-gauge, which was not significantly different from parity[180].

Strain-gauge plethysmography has been compared with blood flow measured using an electromagnetic flow probe installed around the brachial artery during cardiac catheterization. An angiogram performed in each subject confirmed that the flow probe lay proximal to the brachial artery bifurcation and that only a few minor skin collaterals passed distal to the flow probe. There was a very good correlation between forearm blood flow values obtained by the two methods both at rest and immediately following rhythmic exercise, however the plethysmograph read 21% higher. The authors advanced the explanation that blood flowing to skin or bone was bypassing the flow probe, however I think it is more likely due to the fact that flow probe was reflecting the behaviour of the whole forearm, rather than just the tissue underlying the single-strand strain-gauge. As outlined earlier the proximal forearm has a greater proportion of muscle than the whole forearm[147], hence greater blood flows would be expected following exercise when measured by the strain-gauge. This is born out their data showing that whilst the ratio of strain-gauge to flow probe measurements is approximately 1 at low (resting) values of forearm blood flow, it increases progressively as forearm blood flow increases due to exercise.

2.2.3 Refinements

2.2.3.1 Fixed length gauges

It is not necessary for the strain-gauge to span the whole circumference of the forearm, it may just cover a part of it, providing the ends of the strain-gauge do not move in relation to the arm during measurements and providing the segment of the circumference measured reflects the changes in the whole circumference. Under these circumstances if the whole circumference

increases by 1%, then the segment measured by the gauge will also increase by 1%. To minimize the possibility of this kind of sampling error it is advised that such gauges exceed 75% of the circumference of the forearm at the level at which they are applied. This is the design of the Medasonics™ strain-gauge used in the studies described in this thesis. The gauge is attached to one surface of a flexible strap. The free end of the gauge is attached to one end of the strap. This end is applied to the forearm first, then the gauge and the overlying strap are wrapped around the arm. The strap continues after the end of the gauge is reached and this is wrapped around until it encounters the outer surface of the other end of the strap. The two opposing surfaces adhere using Velcro™.

2.2.3.2 Electronic calibration

Resistance in the gauge is measured using a Wheatstone bridge. This comprises 4 resistances each forming sides of a square. Three are fixed and known and the fourth is the resistance to be measured. An increase of 1% in the unknown resistance can be simulated by increasing one of the three known resistances by 1%. This can be achieved by substituting an alternative resistor using a simple switch[181,182]. Resistance in converted by the apparatus to a voltage so using this technique it is possible to determine the voltage change equivalent to a 1% change in resistance. Since blood flow is measured as a percentage change in volume per minute blood flow can be calculated by consideration of the change in voltage with time following venous occlusion. From the gradient of this line the voltage change in one minute is estimated. This divided by the voltage change caused by a 1% change in gauge resistance is the blood flow.

This assumes that a 1% increase in the resistance of the gauge is equivalent to a 0.5% increase in forearm volume. This is not quite true as forearm tissue is compressible. In fact if the gauge

were calibrated by stretching it whilst on the forearm (as previously), compression of the forearm tissue would cause the gauge to extend by approximately 6% less would be expected. The calibration pulse would be commensurately lower. Forearm blood flow measured without this correction for tissue compression will be underestimated therefore by about 6%. Providing comparisons are made between measurements carried out using this type of calibration this systematic error will cancel out.

The originators of the electronic calibration system compared mechanical calibration off the limb with electronic calibration and found good agreement between the two methods in 4 gauges each tested both ways once. They also found good agreement even when the gauges were was looped around a forearm, although no analysis of the results was presented[181]. This suggests that forearm tissue is not significantly compressed at the tensions used, in contrast to Whitney's findings.

The instructions for the Medasonics™ SPG16 recommends calibrating the gauges off the arm, but does not specify whether they should be under tension or not. I compared 12 electronic calibration values measured using the computer(q.v.) when a gauge was tensioned at 15g with values obtained when the gauge was under no tension. There was a small, but significant increase in the calibration value from 433.2 (coefficient of variation (CV) 0.2%) with no tension to 434.9 (CV 0.4%) at 15g tension ($P<0.01$ by unpaired Student's t test). I also compared the chart recorder deflections obtained off the arm using the 1% calibration button of the Medasonics™ SPG16 plethysmograph with measurements obtained by stretching the Medasonics™ SG24 gauges. Apparatus was developed so that the length of the gauges could be measured whilst they were under 15g tension and their length increased by 1 and 2mm. Gauges were flattened out on a perspex mount and one end attached via a screw to the mount. The gauge

was tensioned by attaching a hanging a 15g weight over a pulley at the other end of the mount from the Velcro™ at the free end of the gauge. This end of the gauge was then cross-clamped so that both ends were now fixed and the tension of the gauge was fixed at 15g. The length of the gauge was then maesured using a perspex ruler incorporated in the gauge, before it was stretched by a further 1 and 2mm using the screw. Knowing the length of the gauge and the chart recorder deflection caused by stretching it by known amounts, the deflection corresponding to a 0.5% increase in length (and hence a 1% increase in resistance) was calculated. This was compared with the average of 3 chart recorder deflections measured using electronic calibration when the gauge was on the forearm at 15g tension. 87 measurements were made using 7 gauges and 12 volunteers on up to 4 occasions each. The two results were very similar (90.5mV for manual calibration versus 94mV for electronic calibration) although the overall coefficient of variation for electronic calibration was considerably smaller (0.9% versus 7.6%). The difference was highly significant however, P=0.0001 (Student's t test). I wondered whether the increased temperature of gauges on the arm was contributing to this difference (as the resistivity of a material increases with increasing temperature). This time I compared electronic calibration values measured using the computer off the arm at 15g tension with the average of 3 measurements made the same way on the arm at 15g tension. 37 paired observations obtained from 5 patients using 3 different gauges were considered. The mean calibration values were identical at 434.2 units read from the A/D card within the computer (equivalent to 94.4mV on the chart recorder), however the coefficients of variation were 0.3% for off arm calibration and 1.7% for the mean of 3 calibration values measured on the arm. Taken together this data suggests that electronic calibration is best conducted off the arm. Precision is improved markedly with no change in the accuracy. It improves the accuracy to tension the gauge at the same tension to be used during measurements and this causes a minor reduction in precision. Whilst electronic

calibration on the arm is broadly comparable with mechanical calibration off the arm, electronic calibration yields values which are on average 4% higher. This may be attributable to the resistance of the wires leading to and from the strain gauge. Mechanical calibration will yield a voltage reading proportional to a 0.5% increase in the length of the strain-gauge, equivalent to a 1% increase in resistance of the gauge alone, whereas electronic calibration will yields a voltage change proportional to a 1% increase in resistance of the strain-gauge and its leads. By incorporating an extra pair of wires in the lead it is possible to eliminate this error by effectively extending the corners of the Wheatstone bridge to the ends of the strain-gauge[182].

2.2.4 Interpretation of forearm blood flow traces

Conventionally forearm blood flow traces are analyzed by eye by drawing a straight line through the tips or the bases of the superimposed pulse waves. Some traces are distorted by movement artefact or by a variety of cuff artefacts. Such traces were interpreted according to the method laid down by earlier authors[152,183]. Once the initial arm-cuff inflation artefact (lasting 0.5 to 1 second) is over, the slope between the end of the inflation artefact and 4 seconds after cuff inflation most accurately reflects the true flow[184,185]. Beyond this time the gradient of the trace commonly falls possibly due to venous filling with reduced compliance[184,185].

This is born out by observation of a series of traces taken when the forearm blood flow is relatively constant (e.g. repeated measures of basal flow or during arterial infusion when equilibrium has been reached). Under these circumstances, traces distorted by artefact can be compared with adjacent traces and the segment of the distorted trace discerned which is truly representative. Excluding movement artefacts, this was commonly the first segment of the trace.

At high forearm blood flows the effect of venous filling can be especially marked, so that the trace no longer shows just upward convexity, but actually reaches a plateau. However, traces immediately following exercise (q.v.) show a marked degree of convex distortion, even at low flows, hence venous filling cannot be the explanation for this. This effect is also evident in the work of other authors whenever traces are shown[22,27,172,186], though no one has commented on it. A minority of traces are obviously biphasic, an initial high gradient giving way abruptly to a lower gradient. Mostly however the two phases merge by gradual transition. This makes interpretation very difficult. Observing a series of traces following a period of exercise it is evident that the first phase is initially short and the gradient high and that the second phase is longer and shallower. As time passes the first phase becomes longer and shallower and the second phase commences later and becomes steeper until eventually the first phase occupies the whole of the 8 second trace. The first phase is likely to be the one more truly representative of the forearm blood flow since it follows the expected time profile, starting high and falling progressively and also it conforms to experience with other types of artefact. This has been verified by comparing forearm blood flows estimated from the initial segment of a trace with values obtained using an electromagnetic flowprobe placed around the brachial artery[172].

The first few traces following a period of ischaemia (q.v.) show a similar, more overt, biphasic character to post-exercise traces. If the initial gradient is very high, the first phase can be less than 2 seconds duration and the rest of the trace can consist of a level plateau. This is likely to be due to the high flows encountered following ischaemia causing venous filling, since it is closely associated with the magnitude of the flow and since during occlusion the arm invariably swells, perhaps due to flow through bone circumventing the venous occlusion cuff. Hence the veins are already distended immediately following a period of arterial occlusion. During the first

few measurements the baseline (between venous occlusions) falls progressively, simultaneously the first phase elongates and the second, plateau phase is progressively eliminated.

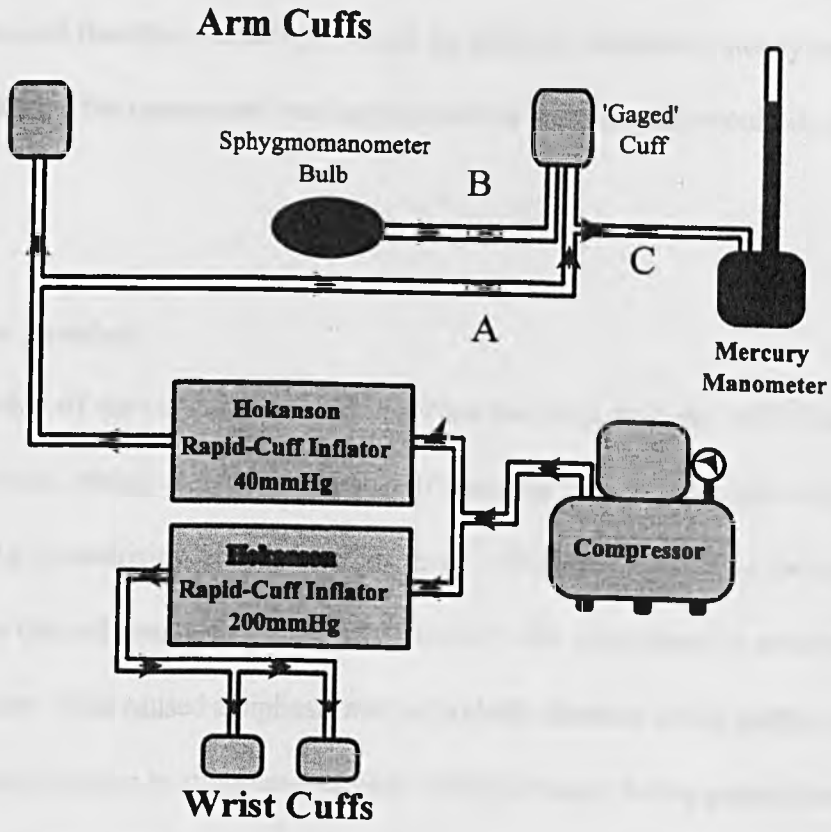
2.2.5 Development and application

2.2.5.1 Ischaemia

Ischaemia was induced in one forearm by occluding arterial inflow for periods of 1, 3 and 5 minutes for the purposes of investigating ischaemic hyperaemia. The maximum duration was chosen to be 5 minutes because it is has been shown that greater durations of occlusion only extend the recovery period rather than increasing the initial flow following release of occlusion[136]. Arterial occlusion was achieved by manually inflating the arm-cuff otherwise used for venous-occlusion during measurement of forearm blood flow to 200mmHg or to 30mmHg plus the systolic blood pressure, whichever is the greater. This abolished the arterial pulsation normally detected by the strain-gauge, however the arm did swell during the occlusion. Increasing the pressure in the arm-cuff had no effect on this swelling however. It was presumed that this swelling was due to continued blood flow through the medullary cavity of the humerus.

To inflate the arm-cuff it was necessary to isolate the arm-cuff from the automatic cuff-inflation circuit using a simple, mechanical valve. The arm-cuff was connected to the sphygmomanometer in the usual way. Mechanical valves were placed on the tubing leading to the mercury manometer and the sphygmomanometer bulb, as illustrated in the figure 2.3 below, so that these could be closed to minimize leaks from the cuff when not in use and to increase the rate of pressurisation during forearm blood flow measurements.

Figure 2.3 System for rapid arm cuff inflation and for creating forearm ischaemia



2.2.5.2 Exercise

It was considered that compression of a sphygmomanometer bulb was the simplest, measurable, forearm exercise we could devise. The release valve was removed from the circuit and the one-way ball valve at the blind end of the bulb, which allows air into the circuit during cuff-inflation was glued shut with cyanoacrylic cement.

In order to reduce the possible variables in the procedure, it was decided to use a the same pressure for all volunteers, rather then to use a proportion of maximal voluntary capacity (MVC). Providing we chose a maximum duration of exercise which all volunteers could manage, the use of a proportion of MVC would offer no advantage, since comparisons were made within rather than between volunteers. Eight male members of staff at Zeneca were tested for the duration for

which they could maintain a pressure of 100mmHg. The least time was 3 minutes and 40 seconds. It was decided therefore volunteers would be asked to maintain a steady pressure of 100mmHg by monitoring the manometer reading themselves and that they would do this for 1, 2 and 3 minutes.

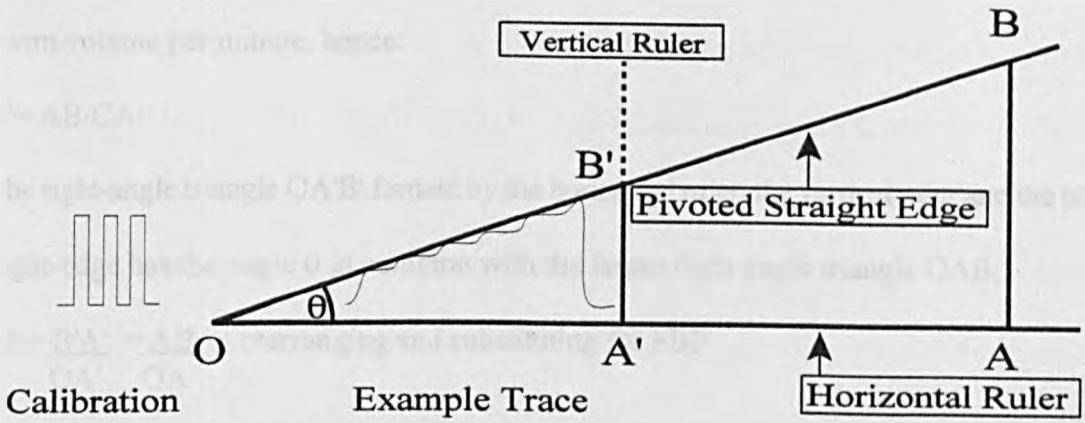
2.2.5.3 Compressor Artefact

Periodic self-activation of the compressor used to inflate the wrist and arm cuffs (Jun Air™) caused an artefact which, though it lasted less than half a second, caused some loss of data. The compressor works by pressurising a reservoir, but there is feedback control to switch off the compressor once the desired pressure is achieved. Similarly the compressor is activated again if pressure falls too low. This caused a biphasic artefact in both channels of the plethysmograph. Using a mains 'cleaning' device to suppress transient voltage changes being transmitted via the mains had no effect. The problem was abolished by installing a 100pF capacitor across the switching circuit to suppress the high frequency signal and by moving the compressor to an adjacent room, connecting the high pressure hose to the cuff inflators (Hokanson™ E20 rapid cuff inflators) via a small hole in the wall. This had the added advantages of improving temperature control and reducing general noise levels as the compressor is both hot and noisy.

2.2.5.4 Data Processing

Forearm blood flow is normally measured from chart recorder traces. The result is calculated from the gradient of the best-fit straight line (judged by eye) through a trace scaled according to the calibration pulse. This process can be streamlined by making a clear plastic frame through which the chart paper can be passed held in fixed orientation to the frame either by guides or by guidelines in the plastic. The principle of its construction has already been described[152]. A

Figure 2.4 Manual Calibration



perspex straight-edge pivoted at the 0cm mark on the upper margin of a ruler held parallel to the direction of movement of the chart paper is used to measure flow. It is rotated and the trace moved through the frame until a line from the pivot of the ruler is judged to be the best-fit line through the trace. A value of forearm blood flow can then be read from a perpendicular ruler which has its origin at the edge of the horizontal ruler. As the displacement of the of vertical ruler along the horizontal is determined by the height of the 1% calibration pulse and the distance the chart paper travels per minute:

$$\text{Displacement} = \frac{\text{Speed (units of length/min)}}{\text{1\% calibration pulse (same units of length)}}$$

The principle of operation of the frame is based on simple trigonometry as illustrated in figure 2.4 above. OA is the distance travelled by the chart paper in one minute so AB is proportional to the increase in forearm volume in one minute. CAL is the height of the 1% calibration pulse.

Forearm blood flow (FBF) is measured in units of mls/100mls/min or percentage change in forearm volume per minute, hence:

FBF= AB/CAL

As the right-angle triangle OA'B' formed by the horizontal ruler, the vertical ruler and the pivoted straight-edge has the angle θ in common with the larger right-angle triangle OAB,

$\tan\theta = \frac{B'A'}{OA'} = \frac{AB}{OA}$ rearranging and substituting for FBF

$FBF = \frac{OA.B'A'}{OA'.CAL}$

If OA', the displacement of the vertical ruler along the horizontal ruler, is set to OA'/CAL expressed in units of length then FBF = B'A' (i.e. the measurement of the vertical ruler at its point of intersection with the straight-edge), measured in the same units of length.

There are two difficulties with this method. The major one is that it is time consuming. Since the first study generated nearly 10,000 data points and each measurement took around 30 seconds, it can be estimated that it would take over 80 hours to process the data. In fact it took 2 people working part-time 4 weeks. As the best-fitting straight-line through the traces is estimated by eye it introduces the possibility of observer bias. Data capture and processing by computer would address both these points.

To save time, our first attempt to capture traces by computer used commercially available software (Codas™) which was embedded in command files written by Research Engineering Laboratories (REL) at Zeneca Pharmaceuticals. Capturing the data was relatively straight forward, unfortunately processing the data was time consuming as it was necessary to position a cursor at the beginning and end of the linear segments of each trace and to exit from the

software and re-enter each time the cursor was positioned. This meant that the time required to process each trace was again about 30 seconds. Comparison of 60 estimates of basal forearm blood flow obtained by this method with those obtained using the frame on chart-recorder traces of the same data demonstrated a good correlation between the two methods ($r = 0.996$). Unfortunately the regression slope was 0.79, suggesting that the computer was systematically underestimating forearm blood flow. Rather than attempt to debug the commercial software, given that little time was saved using this method, it was decided to abandon it.

Software was developed using a mouse and a bitpad to measure the gradients of lines drawn through traces by eye and to read in the height of 1% calibration pulses so that the gradients could be converted to FBF values by the computer connected to the bitpad and stored for analysis. The chart paper was drawn across the surface of the bitpad through a specially constructed channel which held the paper parallel to the lower margin of the bitpad. This method was very simple and much less time consuming than the first method, but it was as prone to observer bias as the fully manual method. It was decided to devote time and energy to the development of a definitive system rather than a stop-gap system. The data from the first study were processed manually using a perspex frame pending development of software capable of recording and processing data.

The first version of the software used a high-performance input-output electronic board (PCI-818, Advantech®) which, when inserted into the computer, converted the continuously varying (or analogue) voltage signals from the two channels of the plethysmograph to 20 points per second per channel of digital data suitable for computer analysis.

Inflation and deflation of the arm cuffs by the rapid cuff inflator was triggered remotely during the first two studies using two digital electronic timers triggering each other in succession. The duration which each was activated for could be set using switches on the front panel of the timing module. One timer controlled the duration for which the arm cuffs were inflated (8 seconds) and the other the duration for which the cuffs were deflated (7 seconds). There was an another, preset timer which halted the process after 3 minutes, so that only 12 cycles could occur. Also located on the front panel was a reset button which could prematurely terminate a sequence. Pulses from the timing mechanism were stepped-up to the voltage required to electrically activate the rapid-cuff inflator for the arm cuffs using a solenoid. During the second study these pulses were also simultaneously led to a digital-input port for the computer to so that the software could sense the times at which the arm cuffs were inflated.

The software was designed so that the computer would not collect data until the calibration sequence had been completed. Since the Medasonics™ SPG16 plethysmograph allowed the 0.1 and 1% calibration pulses to be triggered remotely, it was simple for the computer to do this. It triggered a train of 3 pulses and took an average of the voltages recorded from both channels of the plethysmograph before and during each pulse. The calibration value was taken as the mean of the three changes in voltage. Triggering the calibration pulse for 1 second produced results which were too variable, however using 2 second time intervals produced fairly consistent on-arm, within-volunteer calibration values.

The software displayed the data in two windows on the computer screen and used least squares analysis to fit the best line to the segment of the trace between 0.5 and four seconds after arm cuff inflation, an algorithm already used by other groups[184,185]. The inflation artefact generally takes

up the first half second and the first few seconds of the trace following this are usually most indicative of forearm blood flow as argued earlier. This algorithm was validated by comparing the values of forearm blood flow estimated using the computer with readings obtained manually from traces recorded simultaneously using a chart recorder. The table below shows the gradients of the best-fit straight line through the origin relating the manual (x) and the automated methods (y), it also contains the correlation coefficients.

Table 2.1 Correlation between manual and computer-derived FBFs

ALGORITHM	N	Gradient	r
BASELINE			
0.5-8s fit	99	0.99	0.87
0.5-4s	99	1.03	0.9
0.5-3s	98	1.06	0.87
0.5-2s	98	1.11	0.81
Unified Algorithm	99	1.10	0.99
POST-ISCHAEMIA			
0.5-8s	100	0.72	0.96
0.5-8s, flows <40mls/100mls/min	84	0.95	0.98
0.5-4s	101	1.0	0.997
0.5-3s	101	1.04	0.997
0.5-2s	101	1.06	0.994
Unified Algorithm	101	1.04	0.997
POST-EXERCISE			
0.5-8s	101	0.38	0.24
0.5-8s, flows<15mls/100mls/min	45	0.82	0.93
0.5-4s	101	0.6	0.63
0.5-4s, flows <15 mls/100mls/min	45	1.03	0.99
0.5-3s	101	0.73	0.82
0.5-3s, flow <40mls/100mls/min	84	0.99	0.96
0.5-2s	101	0.93	0.94
0.5-2s, flows <40mls/100mls/min	84	1.12	0.98
Unified Algorithm	101	0.93	0.98
ADENOSINE INFUSION			
0.5-4s	135	0.92	0.94
0.5-4s, flow ≤17mls/100mls/min	100	0.97	0.97
0.5-2s	135	1.06	0.98
Unified Algorithm	134	1.01	0.99
SODIUM NITROPRUSSIDE INFUSION			
0.5-4s	30	0.87	0.89
0.5-4s, flow ≤27mls/100mls/min	27	0.92	0.96
0.5-2s	30	1.00	0.98
Unified Algorithm	30	0.98	0.99

Figure 2.5 Bland-Altman plot of reactive hyperaemia:fitted from 0.5 to 4secs

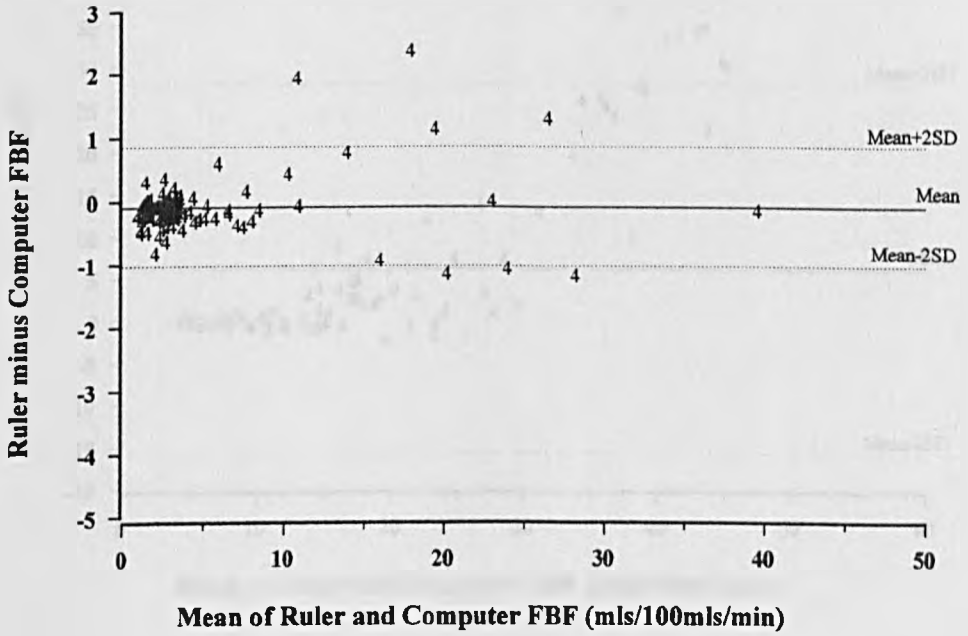


Figure 2.6 Bland-Altman plot of reactive hyperaemia:fitted from 0.5 to 2secs

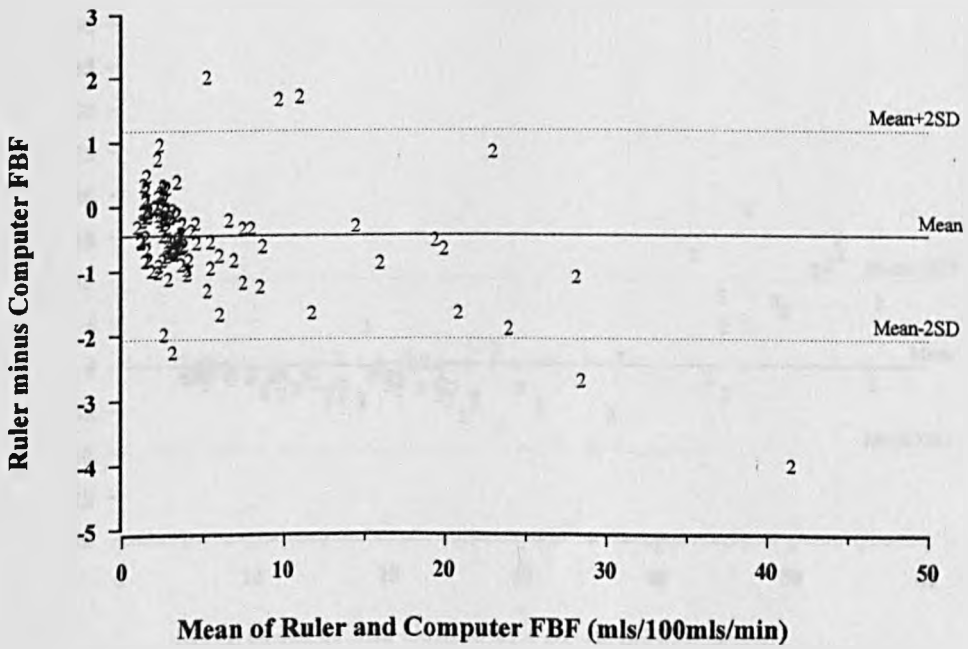


Figure 2.7 Bland-Altman plot of active hyperaemia: fitted from 0.5 to 4secs

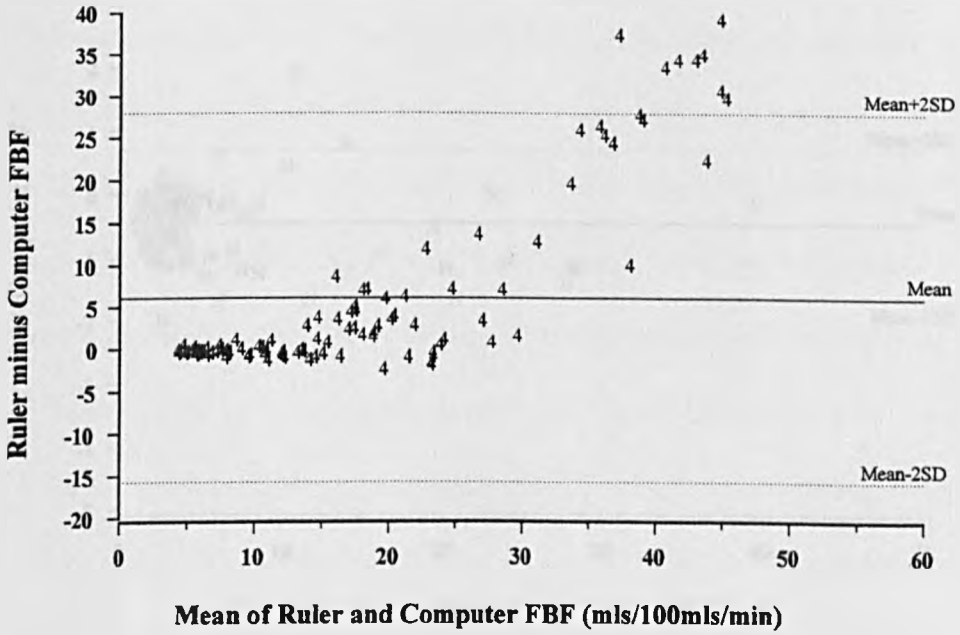


Figure 2.8 Bland-Altman plot of active hyperaemia: fitted from 0.5 to 2 secs

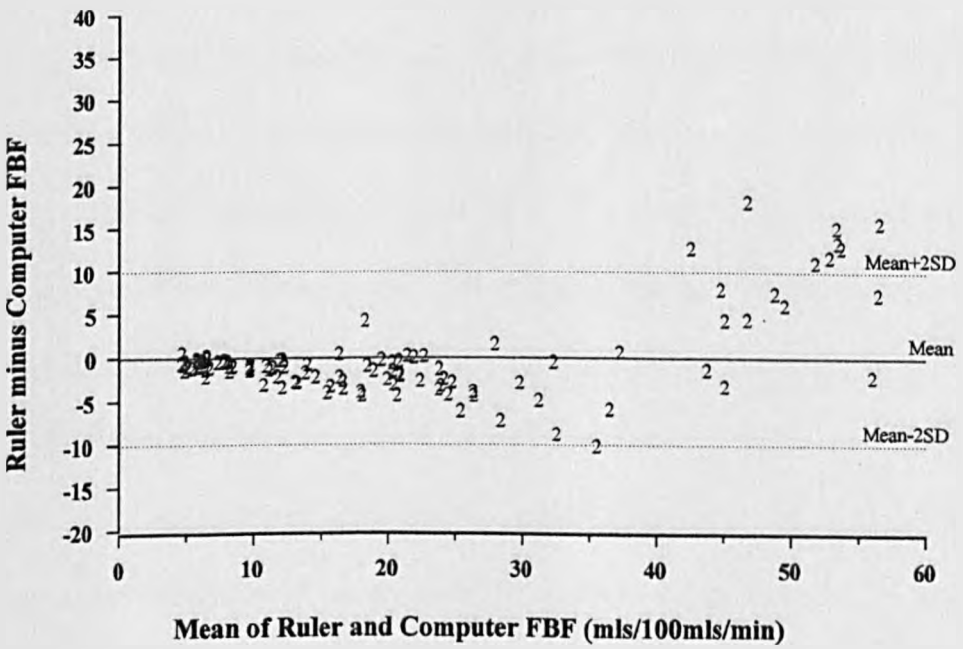


Figure 2.9 Bland-Altman of reactive hyperaemia: new algorithm

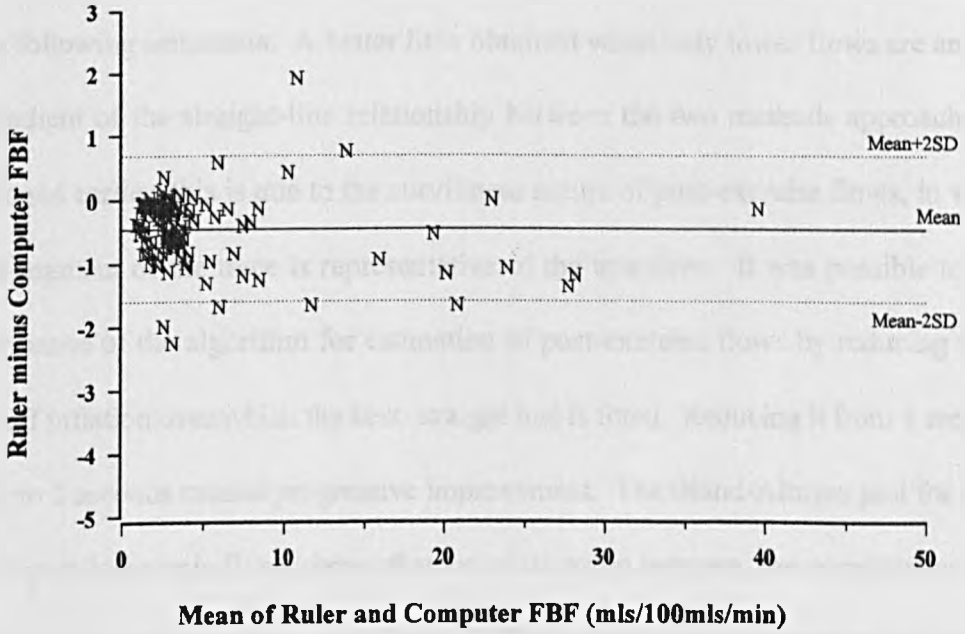
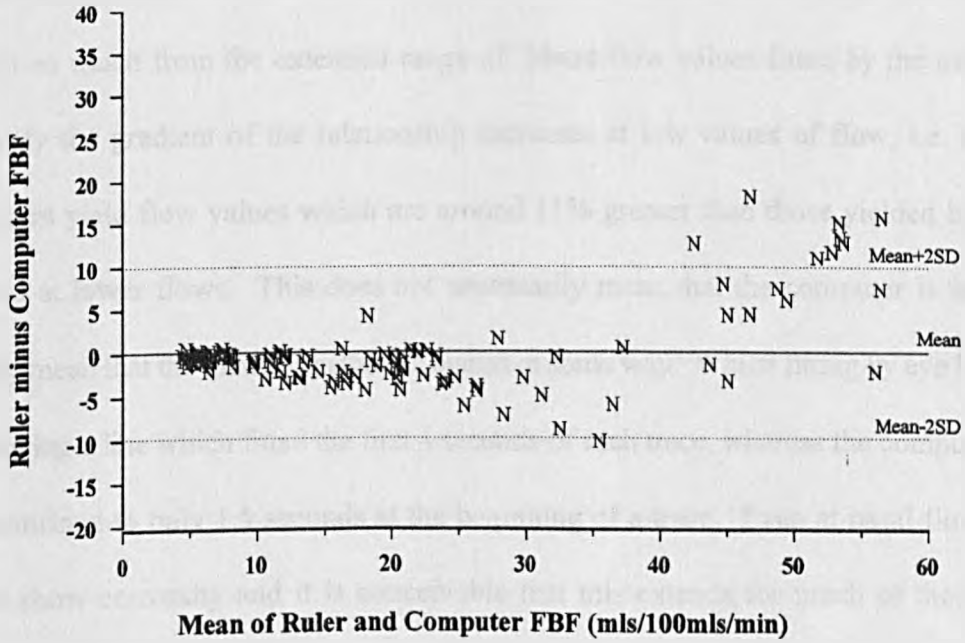


Figure 2.10 Bland-Altman plot of active hyperaemia: new algorithm



As can be seen from the Bland-Altman plots[187] (figures 2.5 -2.10), the computer progressively underestimates forearm blood flow at higher levels of flow following exercise and to a lesser extent following ischaemia. A better fit is obtained when only lower flows are analysed, when the gradient of the straight-line relationship between the two methods approaches unity. As mentioned earlier, this is due to the curvilinear nature of post-exercise flows, in which a short, initial segment of the trace is representative of the true flow. It was possible to improve the performance of the algorithm for estimation of post-exercise flows by reducing the time after arm-cuff inflation over which the best, straight line is fitted. Reducing it from 4 seconds to 3 and finally to 2 seconds caused progressive improvement. The Bland-Altman plot for post-exercise and for post-ischaemic flows shows that the relationship between the computer and the manual method holds good to much greater flows. Unfortunately, as the time over which the line is fitted to each trace is reduced, the effect of the individual cardiac impulses has a proportionately greater effect on the gradient of the line fitted, i.e. the variability of the forearm blood flow values begins to increase dramatically as the width of the line fit falls towards the duration of the cardiac cycle. Hence the correlation coefficient falls for post-ischaemic flows and for basal flows which do not benefit so much from the extended range of blood flow values fitted by the new algorithm. Similarly the gradient of the relationship increases at low values of flow, i.e. the computer algorithm yield flow values which are around 11% greater than those yielded by the manual method at lower flows. This does not necessarily mean that the computer is wrong, it may equally mean that the manual method is biased in some way. Whilst fitting by eye I concentrated on drawing a line which fitted the first 4 seconds of each trace, whereas the computer algorithm was confined to only 1.5 seconds at the beginning of a trace. Even at basal flow rates many traces show convexity and it is conceivable that this extends for much of their length. As reasoned earlier, the initial flow is likely to be most representative of the true flow. It is possible

therefore that the manual method does systematically underestimate forearm blood flow. To retain comparability with other work in the field it was decided to adopt using the 1.5 second fit for post-exercise hyperaemia in the exercised forearm only and to use the 3.5 second fit for all other needs.

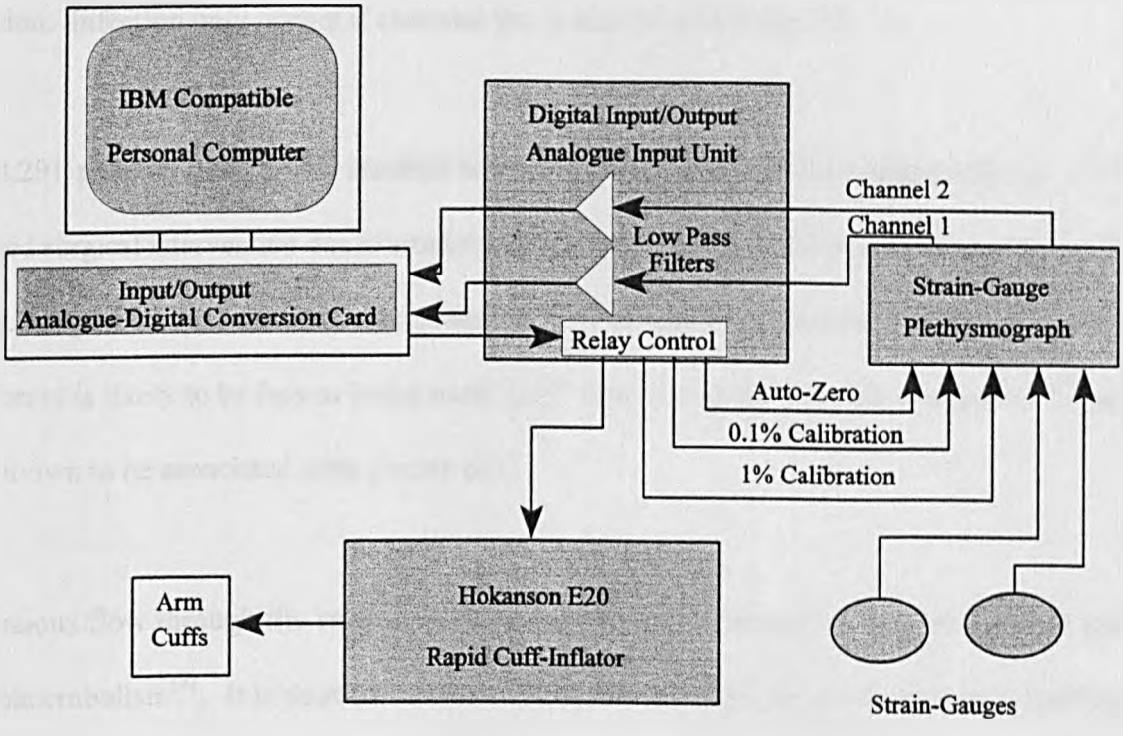
A further unified algorithm has been developed. This was designed to be able to sense changes in gradient. A line is fitted to the first 1.5 seconds (as above), but then the effect of increasing this time by half-second increments to a maximum of 3.5 seconds is studied. If adding a further segment causes the gradient to change by more than 5% then the last increment is discarded and the line is only fitted as far as the previous half-second. As can be seen from the table and the Bland-Altman plots, this algorithm performed similarly to just fitting to 1.5 seconds of trace. This is because 5% is too sensitive and the algorithm rejected further increases in time after the basic 1.5 second fit in many cases at low flows due to detecting a temporary increase in gradient due to the cardiac impulse. On the other hand, the algorithm performed well at higher flows, when the effect of the cardiac impulse is minimal. It also detected movement artefacts and in many cases allowed accurate, automated measurement when traces might otherwise be rejected for analysis. Further development of this algorithm is planned.

The initial version of the software incorporated a preprogrammed sequence of prompts. Before each series of 12 arm-cuff inflations this would display the title of the next block of data required by the protocol. Between periods when data was being captured it was possible to select alternative titles, so that parts of the protocol could be repeated if necessary. Once data capture was complete the software incorporate an editor which at one level allowed blocks of data in the series to be either accepted or rejected and at another level allowed individual pairs of traces from

the control and experimental channels to be accepted or rejected. The block editor also allowed for the duration of fit to be altered for each arm for individual blocks or for all block following a certain point. This allowed fitting between 0.5 and 2 seconds following arm-cuff inflation for the active arm following exercise. Once the data was edited it could then be summarised in a form suitable for processing by SAS™ (Statistical Analysis Software). The software compiled a file containing all of the forearm blood flow values calculated for the control and experimental forearms annotated with the study number, the volunteer and session number and a number indicating the position of the data within the protocol.

In later studies the computer was used to control arm-cuff inflation and so separate software was produced which allowed the series of block titles comprising the protocol to be set The properties of each block were set separately so that the number of arm-cuff inflations could be varied, also the duration of arm-cuff inflation and deflation. Further improvements were instituted to allow for automatic re-zeroing before the first arm-cuff inflation and since this takes 2 seconds, the possibility of a variable time delay was also incorporated. This particular feature was useful following exercise where it was usually necessary to re-zero the equipment because of slight changes in forearm position. Sometimes the inflation-artefact took longer than 0.5 seconds, causing flows to be overestimated. To allow for this the initial period discarded due to artefact was also made variable, both in the protocol editor, but also in the block editor. Care was taken to ensure that all sessions for each volunteer in a study used the same start and finish times relative to arm-cuff inflation for line-fitting. The final configuration of the system is illustrated in the schematic diagram below.

Figure 2.11 Computer integrated arm cuff and forearm blood flow data capture system



2.3 Arterial Needling

2.3.1 Safety

There is no published data on the incidence of complications of brachial artery puncture using ultrafine-bore (27G) needles. The collated experience of over 2000 such procedures from 4 UK centres suggests that the complication rate is very low indeed, much lower than would be expected using larger bore (e.g. 21G) needles or cannulae. There has never been an incidence in which the blood supply to the forearm has been threatened or which has required intervention. If difficulties are encountered they relate only to transient stimulation of the median nerve by the needle tip or possibly the development of a minor haematoma (DJ Webb, personal communication).

The complications encountered with arterial cannulae include: trauma to the artery (haematoma - compartment syndrome, thrombosis, arterial spasm) and to the median nerve, air embolism and infection. Infection only occurs if cannulae are in situ for over 4 days[188].

Of 34,291 patients undergoing brachial artery cannulation for cardiac catheterization, 1.5% required surgical intervention due to arterial trauma. Only 0.12% of those patients having purely diagnostic procedures had such complications[189]. The incidence of complications in healthy volunteers is likely to be less as larger cannulae[190] and greater duration of cannulation[191] have been shown to be associated with greater risk.

Continuous flow through the cannula reduces the risk of thrombosis around the cannula and possible embolism[191]. It is possible to cause retrograde, cerebral air embolism when flushing cannulae[192], however flow rates in excess of 12mls per second are required[192]. Such flows are not attainable through 27G needles.

Median nerve injury with long term sensory loss has occurred following brachial artery sampling for blood gas determination[193]. Subsequent surgical exploration suggested that this was related to the development of a haematoma. Providing sensible precautions are taken, arterial needling using ultrafine-bore needles is a safe procedure.

2.3.2 Procedure

We used 27G unmounted (with no luer-lock hub), 5cm long, steel needles which were made to order (Coopers Needleworks, Birmingham). The needle was flame-sterilized (Labogaz™ burner) and sealed into the tip of an epidural cannula (Portex™) from which the blind end and side exit

ports has been removed by cutting the cannula at the first centimetre graduation using sterile scissors. The blunt end of the needle was then inserted into the epidural cannula and fed back 1 centimetre, indicated by a further graduation mark on the tubing. An airtight seal was formed between the needle and the tubing by dropping molten dental wax onto the joint and passing the needle through the flame again to melt the wax. The needle is only retained securely if molten wax is drawn into the gap between the tubing and needle by capillary action, otherwise the needle can be forced back further into the cannula when it is being inserted into the artery. Occasionally excess wax blocks the proximal end of the needle.

Finally the needle was flushed with sterile saline by connecting the cannula hub to a 50ml syringe with a luer-lock screw fitting, being careful to extrude any air from the syringe prior to making the connection. Once the needle was mounted at the tip of the cannula it was kept sterile by putting it in the plastic casing from a standard, mounted needle. This can be taped down close to the infusion pump for convenience.

The syringe pump was set running prior to cannulation of the brachial artery to minimize delay once the artery was cannulated. We used a Critikon Minuteman 90, which is servo-adjusted to the pressure required to achieve the desired running rate. Because of this feature it will run at the high pressures required for arterial infusion via ultrafine-bore needles without tripping the alarm indicating an occluded line. We ran the infusion pump at 1ml/min as this is the convention and it is sufficient to clear the needle and cannula (dead space 0.4mls) of blood within 30 seconds. If blood remains in the needle for greater than 30seconds there is a great danger that the blood within it will clot. Not only is it virtually impossible to clear such a needle it is undesirable to do so because of the danger of embolism.

Cannulation was performed with the subject supine with their arm resting comfortably over a pillow to maximally extend the elbow joint. The brachial artery was brought to further prominence by supinating the forearm. The course of the brachial artery in the antecubital fossa was carefully delineated and the skin marked to indicate positions of maximal pulsation. 1% lignocaine was infiltrated subcutaneously (but no deeper) and left to act for 5 minutes. Having ascertained that the skin was numb, the needle was inserted just proximal to the distal mark and advanced smoothly and not too slowly or tentatively at a 45° angle to the skin surface towards the more proximal skin mark. There was usually a perceptible increase in resistance as the needle began to enter the artery, followed by a sudden decrease when the lumen was entered. The hub of the cannula was disconnected prior to cannulation so that pulsatile, arterial blood was free to enter the cannula to confirm success. Once a flashback was obtained the needle was advanced at the same angle a further 2 or 3mm so that it could not fall out of the artery. If reflux of blood into the cannula ceased during this then the needle was pulled back slightly to restore flow and advanced at a shallower angle. Once the needle was in position the cannula was reconnected to the syringe pump to flush it. The cannula was taped to the forearm or the wrist cuff in such a way that the angle of entry of the needle to the skin was not changed when the forearm was returned to the position required for plethysmography, with the elbow partly flexed. Sometimes it was necessary to conduct experiments with the elbow largely extended.

The position of the needle was checked whenever the arm was moved and before and after infusions of drug by disconnecting the Luer-lock momentarily. If blood did not reflux promptly into the cannula the needle was repositioned or replaced. On three occasions the needle the needle did fall out when I had failed to advance the needle sufficiently in the arterial lumen due to inexperience. Each time the volunteer complained of an increasing ache and there was

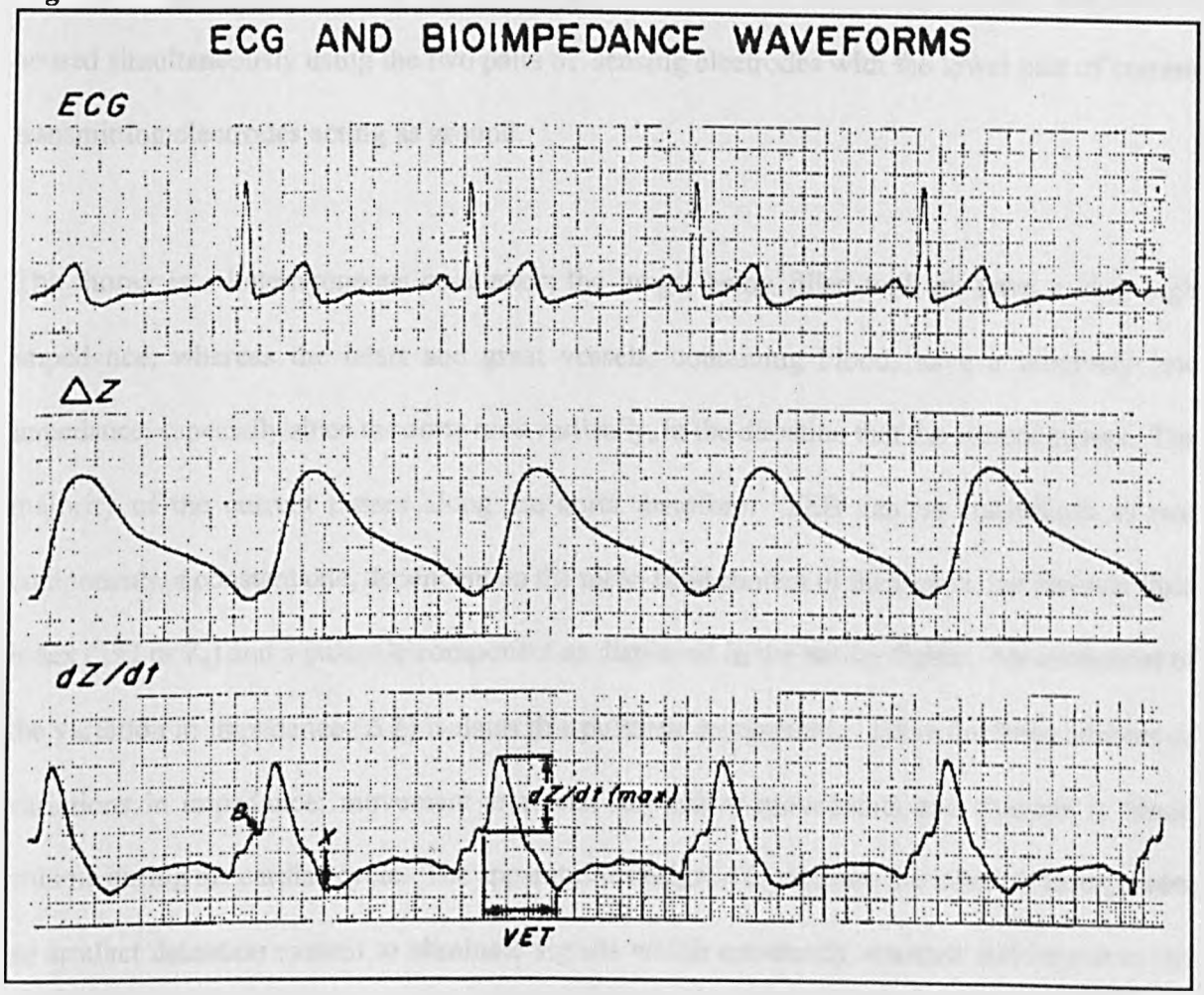
swelling at the infusion site. As tissue pressure is less than arterial pressure, the syringe pump did not alarm. The pain rapidly resolved on discontinuation of the infusion and the swelling resolved within 24hours.

Following removal of the arterial needle, firm pressure was maintained on the site of arterial puncture either manually or by using an elastic, pressure bandage. With this procedure no subjects developed a haematoma following arterial cannulation. Interestingly, none of the three subjects whose arterial needles fell out during the study had any evidence of a haematoma.

2.4 Transthoracic electrical bioimpedance (TEB)

Otherwise called impedance cardiography, this technique is used to measure stroke volume (SV) and cardiac output (CO) noninvasively. A number of other indices of cardiac function can also be derived. It works by measuring changes in the impedance (i.e. the resistance) of the thorax to a small, constant, high-frequency oscillating current. The impedance changes during the cardiac and respiratory cycles with the volume of blood in the chest. A typical waveform can be seen below. We used the BoMed Noninvasive Continuous Cardiac Output Monitor (NCCOM3-R7, BoMed Medical Manufacturing, Irvine, California). This uses the Sramek-Bernstein method of approximating cardiac output[194].

Figure 2.12



From Bernstein[194]

B=opening of aortic valve

X=aortic valve closure

VET=ventricular ejection time

2.4.1 Theory

The impedance of the thorax is measured between two pairs of sensing electrodes, one pair situated symmetrically at the root of the neck and the other at the level of the xiphisternum one on each mid-axillary line. Current passes between two further pairs of electrodes, one pair, connected together, situated 5cm cranial to the sensing electrodes on the neck and another pair,

connected together, 5cm caudal to the sensing electrodes at the base of the thorax. The ECG is sensed simultaneously using the two pairs of sensing electrodes with the lower pair of current transmitting electrodes acting as ground.

The thorax is a heterogeneous conductor; the lungs, being filled with air have a very high impedance, whereas the heart and great vessels, containing blood, have a relatively low impedance, especially since the aorta runs vertically, in the direction that the current passes. The majority of the current passes along the aorta therefore. TEB can be considered as two components, a constant one, dependent on the mean fluid content of the thorax, the thoracic fluid index (TFI or Z_0) and a pulsatile component as displayed in the earlier figure. Measurement of the variation in impedance (ΔZ) isolates this pulsatile component. There are three sources of variations in impedance: movement artefacts, respiratory movements and changes in blood volume during the cardiac cycle. The apparatus is designed to measure the latter. It incorporates an artefact detection system to eliminate signals which are clearly aberrant and improves the signal-to-noise ratio by electronically differentiating the signal and filtering out the low frequency changes, caused for example by respiration. It is possible to enhance further the signal by summing the signals obtained during 16 cardiac cycles together using the ECG signal to gate them. Changes in TEB during the cardiac cycle are brought about in two ways, the predominant being the effects of a sudden increase of pressure in the aorta following aortic valve opening causing a sudden increase in aortic volume and consequently a fall in TEB. The minor component (perhaps 5%[195]) is thought to be due to a decrease in the resistivity of blood during laminar flow along the axis of the flow caused by alignment of the biconcave erythrocytes. This has been shown *in vitro*: during laminar flow the current is able to pass freely in the plasma along the axis of flow, but when there is low or turbulent flow, less erythrocytes, which have a higher

impedance than plasma, are aligned and the current must pursue a longer course[195].

The Sramek-Bernstein method is based on the following assumptions[194]:

1. That the thorax can be regarded as a truncated cone whose base circumference is equal to that of the thorax at the level of the xiphisternum, length L and volume V_c[196].
2. That the volume of this truncated cone approximates 1/3 that of a cylinder of the same length and basal circumference[196].
3. The circumference (C) of the thorax at the level of the xiphisternum is about three times the length of the thorax (L)[196].
4. The thoracic length (L) is approximately 17% of the subject's height (H)[196].
5. The resistivity of the thorax (ρ_c) is twice the resistivity of blood (ρ) in normal volunteers (i.e. $\rho_c \approx 2\rho$)[197].
6. The mean aortic systolic blood flow rate is approximately half of the maximum flow rate, since the time profile of systolic aortic blood flow is roughly triangular[194].

Given these assumptions $V_c = L/3.\pi.(C/2\pi)^2$

substituting C=3L $V_c = 3L^3/4\pi = L^3/4.2$

substituting L=H.0.17 $V_c = (0.17H)^3/4.2$

This is constant for each volunteer and is defined as the volume of electrically participating tissue (V_{EPT}).

7. That the pulsatile component (varying in time with the cardiac cycle) is contributed solely by expansion of the aorta[198].
8. The aorta is an elastic tube of fixed length running the full, vertical length of the thorax[198].
9. The beginning and end of the systolic ejection pattern correspond to the left ventricular ejection time (T_{LVE}), a fact confirmed phonocardiographically[198].

Using these assumptions it is possible to derive a formula for stroke volume:

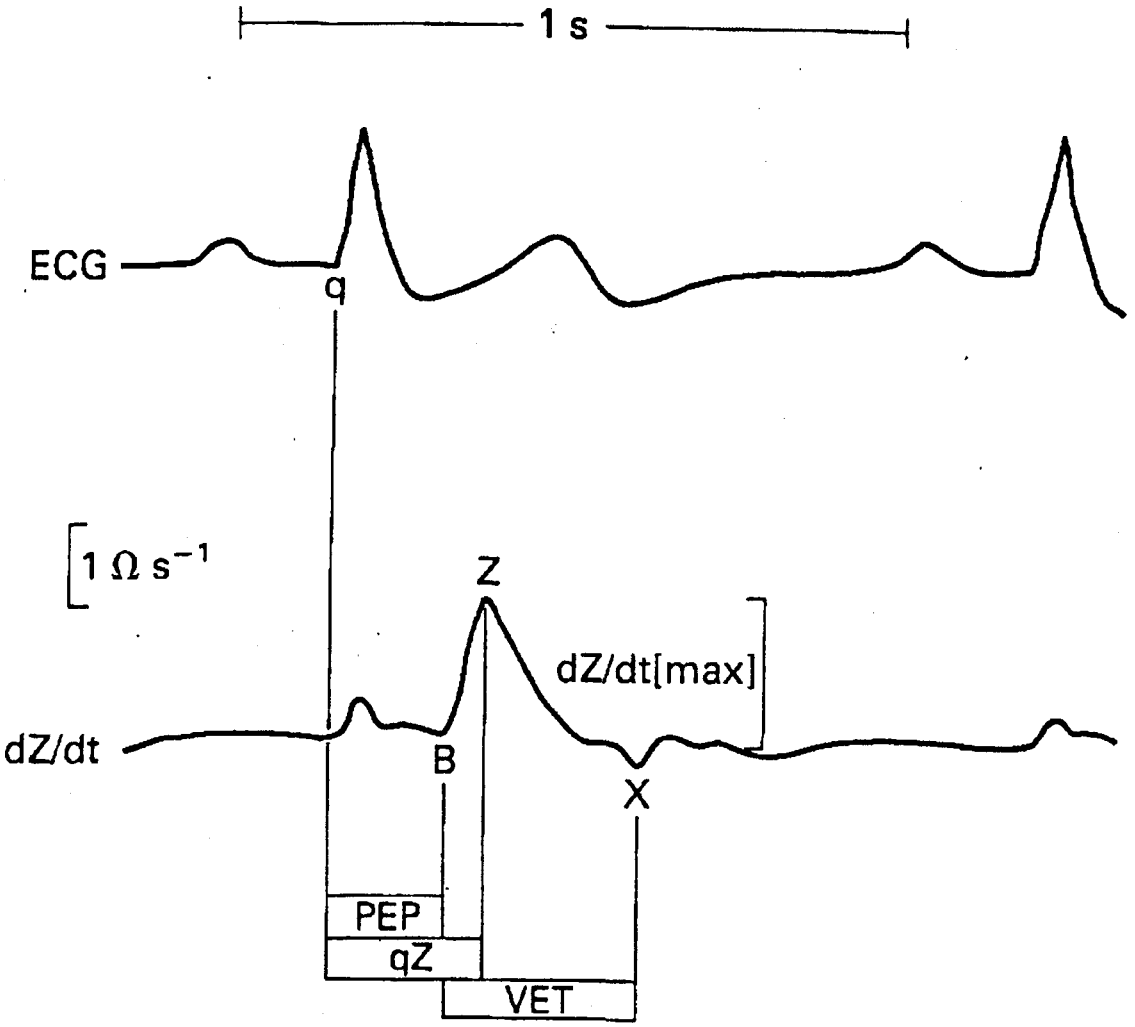
$$SV = V_{EPT}.T_{LVE}.[(dZ/dt)_{max}/Z_0] = [(0.17H)^3/4.2].T_{LVE}.[(dZ/dt)_{max}/Z_0]$$

This formula tends to underestimate SV in obese individuals [194] as the formula only takes account of height and makes no allowance for subtle differences in central haemodynamics with deviation in weight from the norm. A factor δ was introduced based on the proportional deviation in weight from ideal weight for height for gender[198] to correct for differences in SV observed at both extremes of weight[199]. The formula now becomes:

$$\mathbf{SV = [\delta.(0.17H)^3/4.2].T_{LVE}.[(dZ/dt)_{max}/Z_0]}$$

Since the apparatus also senses the ECG it can measure heart rate and hence CO can be derived, the product of SV and heart rate. Although the apparatus is capable of sensing the ECG through the existing leads, two further leads are supplied so that the position of the ECG leads can be varied to optimize the ECG signal without compromising the impedance signal.

Figure 2.13 Thoracic bioimpedance waveform in relation to the cardiac cycle



The ejection fraction is derived from systolic time intervals measured from the raw and differentiated TEB signal. Figure 2.12 shows typical traces of ΔZ and dZ/dt in relation to the ECG. The landmarks for estimation of the pre-ejection period (PEP) (the period between initiation of ventricular contraction and opening of the aortic valve) and the T_{LVE} are shown. The systolic time ratio (STR) is the ratio of PEP to T_{LVE} . T_{LVE} is proportional to SV and decreasing EF causes a decrease in SV, hence T_{LVE} will tend to follow EF. It can be seen from inspection of figure 2.13 that PEP and T_{LVE} together comprise the duration of electromechanical systole, which depends purely on heart rate. So if EF is low then T_{LVE} will also tend to be low,

and conversely PEP is likely to be high. STR, the ratio of the two is likely to be a sensitive (inverse) indicator of EF. EF measured by multi-gated radionuclide (MUGA) scanning has been correlated with STR measured by TEB and the equation for the regression line used for calculation of EF by the NCCOM3-R7[200]:

$EF = 0.84 - (0.64.STR)$ $r=-0.85; p<0.0005.$

End diastolic volume (EDV) is defined as SV/EF.

The maximum rate of change of TEB (dZ/dt_{max}) is proportional to the maximum rate of change of volume in the aorta in other words the peak flow attained (PF). Peak flow is related to myocardial contractility, but will decrease during hypovolaemia. If peak flow is normalized for the thoracic fluid index (TFI), the resultant ratio has dimensions of sec^{-1}, and it is relatively independent of the exact positioning of the sensing electrodes since TFI and PF are similarly dependent on electrode position. This new quantity is referred to as the index of contractility (IC). Despite normalizing for TFI it is still volume dependent.

The peak acceleration of the blood as it emerges from the left ventricle 20msec following the opening of the aortic valve is regarded as the best indicator of myocardial contractility[201] as it is a direct measure of the cardiac impulse following the period of isovolumic, ventricular contraction. This can be derived from the second differential of TEB and although another of its advantages is that it is relatively independent of hydration, it is normalized for TFI for good measure. This is referred to as the acceleration index (ACI). Finally the ejection ratio (ER) is the proportion of time during the cardiac cycle during which the heart is in systole. The duration of each cardiac cycle (the heart rate period - HRP) is 60/heart rate, hence:

$$ER = (T_{LVE} /HRP).100\%.$$

As parameters related to flow increase with body surface area (BSA), the normal ranges for flow-related indices can be reduced by normalising them for BSA, which is simply derived from height and weight. The parameters concerned, CO, SV, EDV and PF, are then referred to as the corresponding index: CI, SI, EDI and PFI.

2.4.1 Validation against accepted techniques

There are two groups of closely related parameters derived by the NNCOM3-R7, those dependent on flow (derived from dZ/dt) and those derived from STIs. EDI is derived from both and HR quite separately from the ECG. TFI (mean thoracic impedance or Z_0) is also unrelated. It is an indicator of the subject's state of hydration and of electrode position (i.e. if it does not fall in the expected range it is worth reviewing the position of the electrodes). Ideally it should stay constant during a study. Comparison studies have concentrated on CI and ER as both of these parameters can be measured by other methods.

The within-day reproducibility of CO in healthy volunteers measured by TEB is 5% with between-day coefficient of variations (CV) of 9% for CO and 11% for SV[202]. The within-day CV of TEB CO in patients undergoing cardiac catheterization is much the same at between 2.7 and 4.9%[203,204].

Correlation of CO measured by TEB with that measured by thermodilution was close in patients on intensive care with r values between 0.83 and 0.95[205-207]. These studies were faulted because in some multiple measurements on single patients were included, in others patients were excluded without explanation. Good correlation was also found in patients undergoing cardiac catheterization[204]. However poorer correlation with thermodilution was found during

pregnancy[208] or in ventilated patients[209].

The accuracy of TEB CO has been assessed by comparison with thermodilution, presuming of course that thermodilution is itself accurate. In some studies TEB overestimated CO[202,205], it underestimated it in one[210] and it agreed with COs measured by thermodilution in others[203,204,206]. It has also been shown to correlate and agree well with results obtained by the Fick and dye dilution methods[204,211].

EF is estimated from STR using a regression equation derived from a correlation based on EFs measured by MUGA scans of 26 selected patients[200]. A subsequent study of 35 patients with cardiac disease found poor agreement and no significant correlation between EFs measured by TEB and by MUGA scanning, even if patients were selected according to the criteria of the original study. No consistent relationship between TEB STR and MUGA scan EF was found, suggesting that for most purposes STR and EF varied independently[212].

Whilst TEB produces a reproducible and fairly accurate measure of CO and related parameters such as SV, SI and CI, the basis for estimation of EF (and hence EDV and EDI) by TEB has been called into question. The exact significance of the other parameters derived by TEB has yet to be determined.

2.4.2 Normal values of TEB indices for healthy, supine, adult men

Table 2.2

Parameter	Normal Range (Mean)	Derivation
CI	3.5-4.7 (4.0) l/min/m^2	dZ/dt & HR
SI	48-90 (69) ml/m^2	dZ/dt
HR	49-78 (61) min^{-1}	ECG
EDI	45-100 ml/min	dZ/dt & STI
PFI	328-495 (415) ml/sec/m^2	dZ/dt
EF	50-72 (64) %	STI
TFI	19-28 (23) Ω	dZ/dt
IC	0.045-0.075 (0.06) sec^{-1}	dZ/dt
ER	28-40 (32.5) %	STI
STR	20-45 (30) %	STI
ACI	1.0-1.6 (1.3) sec^{-2}	dZ/dt

Notes: These values were taken from the operating manual of the NCCOM3-R7 TEB apparatus. Thirty healthy men mean age 37 years (range 23-60), height 1.77m (1.7-1.88) and weight 80kg (57-95) were studied (Sramek,B.B. & DeBow, K.). For each subject a mean of two 16 beat averages was taken for all parameters whilst they were relaxed and supine. Parameters are either derived from dZ/dt or from STIs or from both.

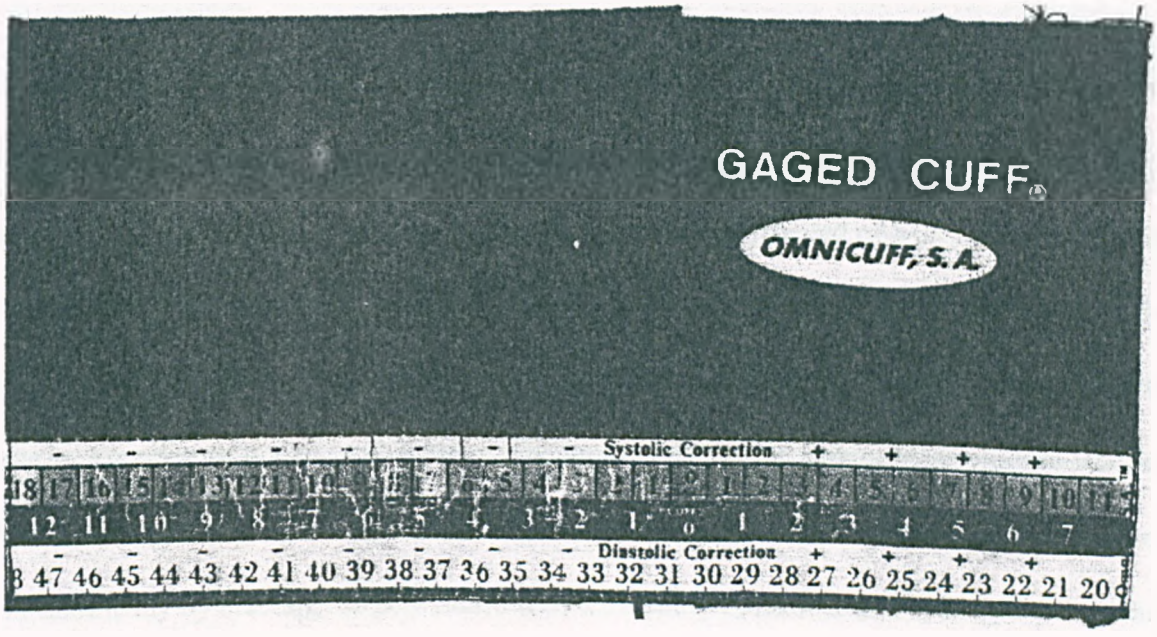
2.5 Measurement of pulse rate and blood pressure

Blood pressure was measured throughout utilising a standard mercury sphygmomanometer using Korotkoff sound V to determine the diastolic pressure. Measurements taken during a study were recorded using the system of valves described earlier to isolate the arm-cuff from the automatic cuff-inflation circuit. In exercise-ischaemia studies measurements were performed on the experimental arm rather than the control arm, whereas in arterial infusion studies the control arm was used to avoid disturbing the arterial needle.

I used the recently developed 'Gaged' sphygmomanometer arm cuff[213], which has a tag appended to the bottom edge of the cuff at the tip of the end first applied to the arm. This protrudes and enables the circumference to be read off a centimetre scale along the lower edge of the outer surface of the cuff. Two other scales (nomograms) are printed alongside the circumference scale. Adjustment factors are read off these which correct both the systolic and diastolic blood pressures measured for deviations of the arm circumference from the ideal for the standard size cuff. These nomograms were developed by comparing blood pressures measured using 3 different lengths of cuffs on each of 1240 obese patients on several clinic visits[214]. A reduced-scale image of the cuff showing the scales can be seen below, figure 2.14.

Pulse rate was measured by palpation of the radial pulse except during studies at Zeneca when it was read from the ECG monitor (Micromon 7141™, Kontron Instruments, Walford, UK) which displays a 5 second moving average calculated from the R-R intervals.

Figure 2.14 Reduced scale image of the Gaged Cuff™



2.6 Measurement of cyclic adenosine monophosphate (cAMP) concentration

This was conducted either by JS Pathology Services (London) under contract or, in the case of the study on patients with peripheral vascular disease, by myself at Liverpool University. The same competitive enzyme-immunoassay was used throughout - Biotrak™ (Amersham International PLC, UK). Information supplied by Amersham indicates that this assay is specific for cAMP, since other cyclic nucleotides (cGMP,cCMP & cTMP), ADP, ATP and theophylline have less than 1% cross-reactivity for both the acetylation and non-acetylation assays. The

sensitivity of the acetylation assay is claimed to be 2fmol/well and for the non-acetylation assay 12fmol/well. Unfortunately the assay is not very precise with maximum, within and between run CVs of 12.4% and 11% for the non-acetylation assay and 6.5% and 12.1% for the acetylation assay.

All standards and unknowns were assayed in duplicate and the results averaged. cAMP added to the reaction mixture either in the standard or unknown competes for limited numbers of binding sites on rabbit anti-cAMP antibodies with peroxidase-labelled cAMP, hence the amount of peroxidase labelled-cAMP bound to the antibody will be inversely proportional to the concentration of added, unlabelled cAMP. The rabbit antibodies are bound within the assay wells by a coating of donkey anti-rabbit antibodies so that excess, unbound peroxidase-labelled cAMP can be rinsed off. The remaining, bound cAMP-labelled peroxidase is measured colorimetrically by reacting the peroxidase with tetramethylbenzidine and hydrogen peroxidase at room temperature for 60 minutes[215]. The reaction is stopped by addition of 1M sulphuric acid. The amount of bound peroxidase is estimated from the absorbance at 450nm using a series of cAMP standards.

The concentration of cAMP in urine is much greater on average than that of plasma (approximately 4mM versus 20nM), so samples of urine were diluted 1:1000 prior to assay. The basic assay allows measurement of cAMP in the range 12.5-3200fmol/well, but due to the lower initial concentrations of cAMP found in plasma, it is recommended that samples and standards are acetylated before competitive binding with the cAMP antibody using a mixture of acetic anhydride and triethylamine. This intensifies the absorbance at 450nm and hence improves the sensitivity of the assay, allowing measurements in the range 2-128fmol/well.

2.6.1 Sample handling

2.6.1.1 Plasma

As cAMP is rapidly degraded in blood, venous blood was collected in cooled, EDTA tubes and kept on ice before centrifugation in a cooled centrifuge at the earliest possible time. The plasma was then decanted and frozen at -20°C or less. To reduce variability due to assay conditions the samples were held in the freezer until completion of the study and submitted as a batch.

2.6.1.2 Urine

On completion of the collection, a 10ml sample was immediately taken and frozen at -20°C for batch assay. The total volume of the sample was recorded so that the total amount of cAMP excreted during the collection period could subsequently be calculated.

2.7 Measurement of plasma theophylline concentration

Related assays were used by the laboratories at Zeneca and Liverpool University to measure theophylline levels. Zeneca used the IMx® assay (Abbott laboratories, USA), which is a competitive protein binding fluorescence polarization immunoassay. The sample is mixed with a solution containing theophylline bound to a fluorophore and with a monoclonal antibody specific for theophylline. The proportion of the fluorophore bound to the antibody depends on the amount of theophylline present in the sample. Monochromatic, polarized light of the appropriate wavelength is used to excite the fluorophore, which fluoresces at a lower wavelength. If the fluorophore is freely mobile (e.g. bound to theophylline from the sample), then the motion of the molecule between excitation and fluorescence will reduce the degree of polarization of the light, however if the fluorophore is relatively immobile due to being complexed with a large protein (the antibody), polarization will be better preserved. By measuring the degree of

polarization of fluorescence and use of appropriate concentration standards and reagent controls it is possible to measure the concentration of theophylline. The assay used at Liverpool University employs the same principle, but uses a polyclonal antibody instead of a monoclonal one (TDX®, Sigma Chemical Co.). Consequently this assay is cheaper, but it is as specific and similarly precise and accurate (comparing information supplied by Abbott with information on file at Liverpool University). Cross-reactivity for both assays is less than 3% with theophylline metabolites and less than 1% with caffeine. The within-run coefficients of variation are less than 3% in both cases and the recovery of theophylline was 97% for the Abbott assay compared with 95% for the Sigma assay.

2.8 *Ex-vivo* measurement of type IV phosphodiesterase inhibition

We used the inhibition of production of α-tumour necrosis factor (TNF) by monocytes in response to lipopolysaccharide (LPS) *ex-vivo* as an indicator of the level of phosphodiesterase (PDE) inhibition achieved by theophylline or oxpentifylline.

Nonspecific PDE inhibitors, including oxpentifylline and theophylline, have been shown to cause concentration-dependent inhibition of TNF production in response to LPS in human monocytes in vitro[216]. Isoenzyme specific inhibitors have shown that the type IV PDE isoenzyme is responsible for this[217]. The intracellular cAMP level increased during PDE inhibition, whereas that of cGMP was barely changed suggesting that cAMP rather than cGMP is the second messenger involved[216].

Production of TNF by monocytes in response to antigen challenge has been developed as a method for measuring the latent activation of monocytes *ex-vivo* for example in patients with

rheumatoid arthritis or to demonstrate impaired monocyte responses in some patients with cancer[218-221]. It has been shown that whole blood can be used rather than isolated monocytes and providing account is taken of the number of monocytes in culture the results using whole blood correlate well with those obtained using isolated monocytes[219]. The within-patient, between-assay coefficient of variation was 5-20% using whole blood, an improvement upon the isolated monocyte technique[219]. Monocytes remain viable for up to 3 days in blood diluted up to five-fold by culture medium and responses remain reproducible[220]. Use of whole blood does not seem to impair the assay in any way, but it is clearly simpler, arguably more physiological and requires much smaller sample volumes. Given the properties of the system, already outlined, this assay can readily be adapted for measurement of PDE inhibition *ex-vivo* in healthy volunteers.

2.8.1 Protocol

Five millilitres of blood were taken from each volunteer using a cooled Vacutainer™ tube and kept cool in an ice-filled container during transit for immediate analysis. The blood was split between a number of wells for subsequent analysis for TNF by enzyme-linked immunosorbent assay (ELISA):

1. Blood alone- to control for immune activation.
2. Blood diluted by vehicle for LPS and dexamethasone-to control for any nonspecific activation of lymphocytes by diluent.
3. 5 wells contained blood to which LPS had been added to reach a final concentration of 0.1μg/ml, which should cause measurable lymphocyte-activation.
4. 5 wells contained blood and LPS - final concentration 10μg/ml, this should cause a higher level of activation of lymphocytes.
5. Blood, LPS 10μg/ml and dexamethasone 1μg/ml. This checks that the TNF response to LPS

can be inhibited by dexamethasone.

After 6 hours incubation at 37°C all wells were assayed for TNF. Providing the control results (1 and 2 above) had undetectable levels of TNF, the response was inhibited by dexamethasone (5)[222] and there was a graded response to LPS, the results were deemed fit for statistical analysis.

Immune activation by, for example, incipient viral illness would clearly confound the assay, so any volunteers who had high circulating levels of TNF were excluded from statistical analysis. Since circulating TNF measured by ELISA is normally below the threshold for detection, this means anyone with detectable levels of TNF. The mean TNF concentration from the 5 wells containing 10μg/ml of LPS was taken as an inverse measure of PDE inhibition

Chapter 3

Reproducibility of reactive and active hyperaemic responses

3.1 Introduction

This first study was conducted at Zeneca pharmaceuticals. When I began working at the University of Liverpool I repeated the study with a slightly modified protocol, testing the method of computerized forearm plethysmography in the new setting, training the clinical research assistant (Mrs Y Tsao) in its use and testing the software and analogue/digital converter card in a different computer.

3.2 Aims

The main aim of this study was to develop a reproducible method for measuring active and reactive hyperaemic responses in the human forearm. I wanted to study the within-volunteer variability of active and reactive hyperaemic responses so that I could systematically analyse the various factors contributing to within and between-session variability when designing further studies. I also wanted to develop appropriate summary measures and suitable methods of analysis to enable me to estimate the number of volunteers required for establishing treatment effects in subsequent studies.

Subsidiary aims included determining the period for which volunteers had to rest supine to allow forearm blood flow (FBF) to settle to basal level, to determine the reproducibility of basal FBFs and to determine whether single-limb exercise or single-limb arterial occlusion disturbed central haemodynamics sufficiently to perturb subsequent FBF measurements, perhaps necessitating

analysing changes in forearm compliance (FBF/mean arterial pressure) rather than forearm blood flow.

3.3 Design

A previous study has shown that there is little further increase in the peak FBF attained during reactive hyperaemia by increasing the period of arterial occlusion beyond 5 minutes[136]. We decided therefore to measure forearm blood flow for 3 minutes following 1, 3 and 5 minutes of occlusion. The time profiles indicated that flow would return to basal levels within 3 minutes, even after 5 minutes of occlusion[136]. It takes longer to return to basal flow levels following exercise, so it was decided to measure active hyperaemic responses after the three periods of reactive hyperaemia to reduce the likelihood of an interaction. As our aim was to develop reproducible measures of reactive and active hyperaemic responses rather than to investigate possible interactions between responses we did not randomize the order of active and reactive hyperaemia, nor did we randomize the order of the durations of exercise or ischaemia. In this way we avoided the need for subgroup analysis and reduced potential sources of variation.

To determine the maximum duration of exercise acceptable, 8 male colleagues from the same department were asked to maintain a handgrip pressure of 100mmHg for as long as they could manage using their nondominant hand. Whilst the maximum duration was in excess of 7 minutes, the minimum was 3 minutes and 40 seconds. I decided therefore that volunteers would be required to exercise for 1, 2 and 3 minutes. Other studies have used dynamic exercise, but I chose isotonic, isometric exercise since this reduced the number of variables that needed to be monitored during exercise, eg the frequency of contractions and the rate of contraction and relaxation. I decided not to try to adjust the level of exercise as a fraction of maximum voluntary

contraction (MVC) as in many exercise studies as this measure will reduce intervolunteer variability, but our main outcome measure is within-volunteer variability, which should not be influenced by the level of exercise providing individual volunteers attain the same level throughout a study.

Impedance cardiographic measurements were taken at 1 minute intervals during forearm blood flow measurements using the 'slow' mode (as described in Chapter 2) averaging the value over 16 beats during which acceptable data was determined by the impedance cardiograph to be collected. All of these values were averaged together for each minute. There were usually three or four 16-beat, impedance cardiograph readings each minute, assigned to that minute according to their start times. These were averaged to obtain the final value for that time point for that volunteer. Additional measurements were recorded during the second reproducibility study at Liverpool *during* periods of exercise or ischaemia.

Blood pressure was recorded twice in the nondominant forearm just before the basal forearm blood flow was recorded prior to the exercise and ischaemia challenges and again after each series of challenges as shown in Table 3.1. It was not feasible to measure blood pressure during blood flow measurements as both arm-cuffs were used for blood flow measurements. Forearm blood flow in the control (inactive) forearm was deemed to be the best way of determining whether ischaemia or exercise had any effect on FBF due to changes in mean artery pressure. It also enabled flow to be analysed as the ratio of the active to the inactive FBF.

The nondominant forearm was chosen to be subjected to interventions (ischaemia or exercise), whilst the dominant forearm was retained as the control. This was because it had been planned

to conduct subsequent arterial infusion studies using the nondominant forearm where possible for safety reasons. I also hoped to minimise any effect that overall fitness or recent exercise might have on hyperaemic responses[223].

To measure within and between-day variability in the principal study end-points I conducted the study protocol twice each on two days a week apart. Everything possible was done to ensure that the two days were as similar as possible. As the timing and nature of meals influences skeletal muscle blood flow[224], I asked volunteers to eat the same breakfast at the same time (2 hours before they were due to be studied) and they were provided with the same lunch, just after their first study on both study days. Baseline FBF shows circadian variation[186,225,226], so I conducted the two FBF measurement sessions at the same times on both study days for each volunteer. Many variables vary slightly according to the day of the week they are measured on, so a gap of one week was adopted. Logistically a one week gap is easier also and it is well in excess of 4.5 elimination half-lives of both microcrystalline theophylline (Nuelin™)[227], which I planned to use subsequently, and slow-release oxpentifylline (Trental™)[228], which I came to use.

The beginning of the first FBF measurement session of the day and the second was separated by 4 hours. As each study takes about 90 minutes this allowed me to assess the reproducibility of a study design in which volunteers were assessed before and after dosing with theophylline or oxpentifylline as the time elapsed from oral dosing to attainment of maximal plasma concentration of these drugs is 2 hours[227] and 3 hours respectively[228]. A 2 hour gap between finishing the first session and starting the second also allowed me to study 2 volunteers each day.

Forearm blood flow results from the first reproducibility study conducted at Zeneca were

unreliable due to strain-gauge failure and possibly also instability of the SPG16 plethysmograph. The strain-gauges, though initially acceptable caused gradual upward drift in the output voltage and a gradual increase in apparent gain of the amplifier causing increased flows. Data with obvious artefacts of this nature were discarded, but some with minor degrees of artefacts were probably retained. This study was repeated in Liverpool with a slightly amended protocol (Table 3.1). This time there was no need for repeating the initial baseline FBF flow measurements and the duration of the protocol was further reduced by decreasing the rest period after baseline measurements and after the measurements following 5 minutes of arterial occlusion. The protocol started once the forearm plethysmography and transthoracic electrical bioimpedance equipment had been applied and tested. This takes over 30 minutes during which the volunteers are resting supine and inactive, so it was felt that no additional time for resting should be allowed.

At the end of each session the volunteers were asked to squeeze a blood pressure bulb as hard as possible on 3 separate occasions with each hand to estimate their maximum voluntary contraction (MVC). The maximum pressure generated during each squeeze was measured in Barr on a manometer connected to the bulb which had a non-return needle so that the maximum pressure generated could be read even when the pressure fell back. It was not possible to measure this using the standard mercury manometer of a sphygmomanometer as all volunteers would be expected to achieve a MVC close to 1 Barr (760mmHg).

Table 3.1 Timing of measurements and interventions

Elapsed time (minutes)	TEB	FBF	Supine pulse & BP	Duration of ischaemia (minutes)	Duration of exercise (minutes)
0-2			+		
2-5	+	+			
7-8	+			1	
8-11	+	+			
13-16	+			3	
16-19	+	+			
21-26	+			5	
26-29	+	+			
29-31			+		
36-38			+		
38-41	+	+			
43-44	+				1
44-47	+	+			
49-51	+				2
51-54	+	+			
56-59	+				3
59-62	+	+			
62-64	+		+		

3.4 Volunteer characteristics

Ten healthy, male, Caucasian recruited from the volunteer panel were studied at Zeneca, aged 24 to 50 years (mean 38 years), height 1.66 to 1.89m (mean 1.77m) and weighing 67 to 81kg (mean 75kg). For the study carried out in Liverpool 12 healthy, male, non-smokers were recruited from the Department of Pharmacology. These comprised 11 Caucasian and one oriental, aged 22 to 42 years (mean 29 years), height 1.56 to 1.86m (mean 1.75m) and weighing 62 to100kg (mean 81kg). In the first study at Zeneca smokers were included, but when the study was repeated in Liverpool, smokers were excluded as smokers have been shown to have reduced levels of reactive hyperaemia[229]. Only males were studied as basal blood flow changes during the menstrual cycle[230]. Analysis of cross-sectional computerised tomographic scans have demonstrated that women have a higher proportion of fat and less muscle in their forearms than men, viz: 58.5% muscle and 29% fat in women versus 72% muscle and 15% fat in men[231].

3.5 Volunteer restrictions

As caffeine is structurally similar to theophylline and is also known to be an adenosine antagonist[232], volunteers were asked to avoid caffeine-containing food and beverages and even decaffeinated products for 40 hours before a study-day, which is comfortably in excess of 4 ½ times the maximum elimination half-life measured[233]. Volunteers were asked to stop smoking and avoid alcohol for 48 hours prior to a study-day. Volunteers requiring regular medication were excluded from the study, although paracetamol was allowed if required. The use of non-steroidal anti-inflammatory drugs, including aspirin was prohibited as they have been shown to reduce both active and reactive hyperaemia[47,49,136,234]. Volunteers were required to avoid strenuous exercise for a day before each study-day in accordance with Zeneca's standard protocol.

3.6 Results

3.6.1 Room temperature

The mean, initial room temperature was 24.3°C (range 21-27°C) and the mean, final temperature was 24.9°C (range 23-27°C). The mean difference between final and initial room temperature was only 0.6°C (range 0-3.5°C). This small increase in temperature is unlikely to have any significant effect on FBF or impedance cardiographic indices, though there was evidence of a trend in some impedance cardiographic indices over the course of a session (qv).

3.6.2 Transthoracic electrical bioimpedance (TEB)

Figures 3.1, 3.2 and 3.3 show the time course of the stroke index (SI), cardiac index (CI) and heart rate(HR) over the course of a session. Table 3.2 details the meaning of the time points. The values shown in the figures are the mean values for all 12 volunteers for each time point and session meaned over the four sessions (2 on each of 2 days) and the error bars represent within-volunteer standard deviation.

Figure 3.1 Time-profile of the stroke index during the protocol

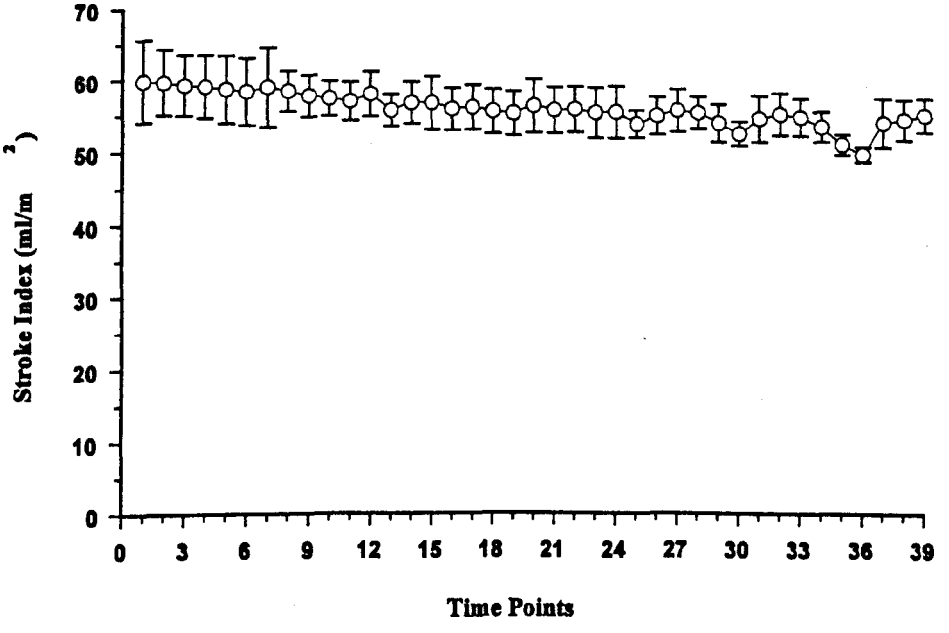


Figure 3.2 Time-profile of the cardiac index during the protocol

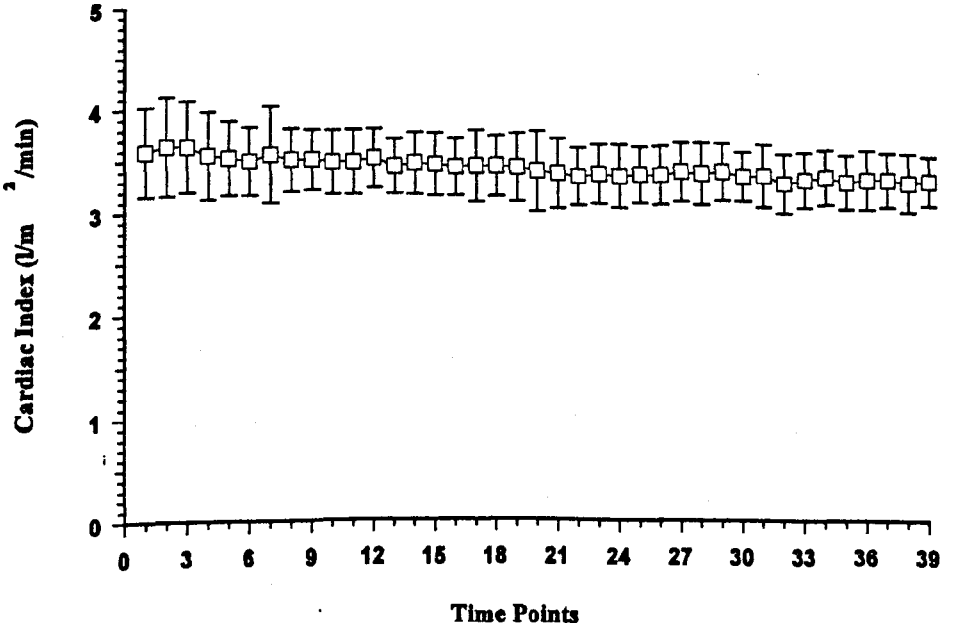
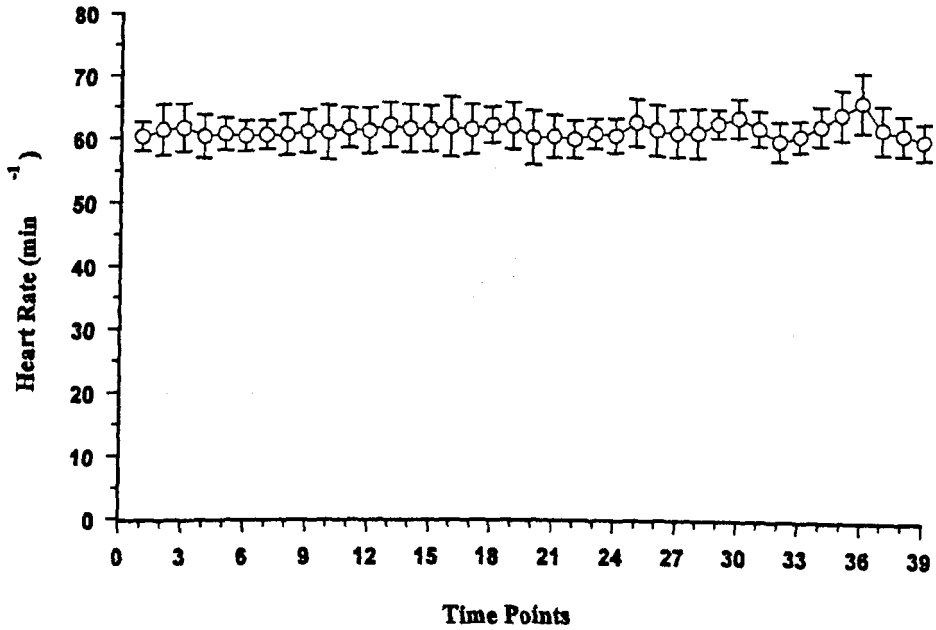


Figure 3.3 Time-profile of the heart rate during the protocol



The thoracic fluid index remained constant throughout the protocol showing no evidence of a systematic malfunction of the impedance cardiograph. There were slight trends during the time-course of the protocol in the variables displayed, hence comparisons could not be made over long periods of time. For this reason in table 3.3 I have used analysis of variance to compare time-point 18 (the fifth minute of ischaemia) with time point 21, the 3rd minute of recovery following 5 minutes of forearm ischaemia instead of the mean baseline reading as planned. Similarly I have compared time point 36 (the third minute of exercise) with time point 39 (the third minute of recovery). This will reduce the possibility of confounding by time-dependent changes but could mask small effects for which recovery is slow. Whilst prolonged forearm ischaemia has no effect on central haemodynamics, sustained single-forearm, isometric, exercise causes an increase in heart rate and a commensurate fall in SI such that CI is unchanged. With no change in CI during either maximal ischaemia or maximal exercise there is unlikely to be any effect on peripheral

blood flows due to reflex changes in central haemodynamics.

In table 3.4 it can be seen that over the 4 hours between the morning and afternoon sessions, there were significant changes in TEB indices. Both SI and HR increased and hence CI increased dramatically.

Table 3.2 Protocol time points

Protocol Stage	Time Point Number	Elapsed Time (minutes)
First Baseline	1-3	0-3
1 Minute Ischaemia	4	5-6
Recovery	5-7	6-9
3 Minutes Ischaemia	8-10	11-14
Recovery	11-13	14-17
5 Minutes Ischaemia	14-18	19-24
Recovery	19-21	24-27
Second Baseline	22-24	32-35
1 Minute Exercise	25	37-38
Recovery	26-28	38-41
2 Minutes Exercise	29-30	43-45
Recovery	31-33	45-48
3 Minutes Exercise	34-36	50-53
Recovery	37-39	53-56

Table 3.3 Effects of single-forearm ischaemia or exercise on TEB indices

Index		Ischaemia	Recovery	P	%CV	Exercise	Recovery	P	%CV
SI	Mean	55.2	55.5	0.70	9	49.7	54.9	0.0001	8
	SD	12.6	12.4			9.4	11.7		
CI	Mean	3.41	3.34	0.58	12	3.28	3.27	0.63	10
	SD	0.93	0.93			0.84	0.82		
HR	Mean	62.1	60.3	0.06	6	66.0	60.1	0.0001	8
	SD	9.8	9.5			10.3	10.3		

Table 3.4 Diurnal variation in mean baseline TEB indices

Index		Morning	Afternoon	P
SI	Mean	56.6	62.1	0.0001
	SD	11.4	16.0	
CI	Mean	3.27	3.95	0.0001
	SD	0.78	1.13	
HR	Mean	58.3	63.8	0.0001
	SD	8.9	10.4	

3.6.3 Blood pressure

Table 3.5 shows the mean blood pressures recorded at each of the 4 time points during the protocol and their standard deviations(SD). Each blood pressure was recorded twice for each volunteer, session and time point and corrected for arm circumference using the cuff correction factor. These two blood pressure values were meaned. Apart from a slightly lower initial diastolic blood pressure there is no evidence of systematic drift in the blood pressure during the protocol. Mean arterial pressure is calculated by adding a third of the difference between the systolic and diastolic pressures to the diastolic pressure.

Table 3.5 Corrected blood pressures - within-volunteer, between-session variability

	Systolic Blood Pressure (mmHg)		Diastolic Blood Pressure (mmHg)		Mean Arterial Pressure (mmHg)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Pre-Ischaemia	123.3	8	71.7	6.2	88.9	6
Post-Ischaemia	124.6	7.8	76.4	5.7	92.5	5.7
Pre-Exercise	123.1	8.9	76.7	6	92.1	6
Post-Exercise	126	8.8	77	6.3	93.3	6.4

From table 3.6 below, it is apparent that there is a small, significant fall in diastolic and mean arterial pressures between morning and afternoon sessions indicative of diurnal variation in the diastolic pressure.

Table 3.6 Changes in blood pressure between sessions

mmHg		Morning	Afternoon	P
Systolic Blood Pressure	**Mean**	122.4	124.1	0.27
	SD	8.8	9.1	
Diastolic Blood Pressure	**Mean**	75.6	67.7	0.0001
	SD	6.6	8.6	
Mean Arterial Pressure	**Mean**	91.2	86.5	0.0003
	SD	6.2	7.4	

Use of the 'Gaged Cuff™ caused no change in either diastolic or systolic blood pressures. To address the issue of a possible improvement in precision brought about by use of the correction factor, the absolute difference of systolic and diastolic blood pressure measurements before and after correction from their means were compared by nested analysis of variance (table 3.7). The

small reductions in variance observed did not achieve significance, however given the underlying variability of this measure more volunteers would be needed to determine whether this small effect is real. Further analysis will be carried out using data from subsequent studies. If confirmed by other studies it would be of importance in larger-scale population studies.

Table 3.7 Comparison of blood pressure variability before and after correction

	Mean Absolute Difference in Systolic BP (mmHg)	Mean Absolute Difference in Diastolic BP (mmHg)
Corrected Data	7.00	6.00
Uncorrected Data	7.85	6.91
Coefficient of Variation	45%	38%
P	0.22	0.07

3.6.4 Forearm blood flow

3.6.4.1 Baseline forearm blood flow

Table 3.8 shows the mean baseline blood flows obtained during the first study conducted at Zeneca. Using nested analysis of variance there was no difference between the first baseline FBFs and the second and third baselines either in the active forearm or the control forearm. The fourth baseline was not analysed as blood flow in the active forearm had clearly not recovered following the 3 minute period of exercise 8 minutes before (Appendix A). There is a small increase in baseline FBF during the protocol. This might be related to the ambient temperature which tended to be lower initially increasing throughout the protocol due to problems with air-conditioning. Even analysing the data for both forearms together there is no significant difference between the first and second baseline flows ie first baseline 3.03 (SD 1.01)

mls/100mls/min versus second baseline 3.18 (SD 1.05) mls/100mls/min P<0.31. This indicates that there is no advantage to be gained in waiting 10 minutes after setting-up. For this reason in subsequent uses of this protocol only one initial baseline was measured once the equipment was verified to be operational. As the post-exercise baseline was not helpful, it was also omitted from future protocols.

Table 3.8 also shows a consistent difference between the dominant and nondominant forearms. This difference is small, but significant comparing the two forearms using nested ANOVA of the first two baseline mean flows, ie nondominant 3.05 (SD 1.01) versus dominant forearm 3.17 (1.05) mls/100mls/min, P=0.034). The third baseline is omitted from this analysis as by then the two forearms have been treated differently. A similar analysis was carried out on baseline data from the second study, but instead analysing the means of the 12 readings for each forearm, I improved the power by putting all of the readings made into the analysis of variance, including only those readings in which flows in both forearms measured simultaneously were both successful. This is valid as the comparison is between the two forearms and simultaneous measurement of blood flow is clearly the best way to eliminate variation in vascular tone with time. Effectively the protocol yielded upto 12 repeated measures on 4 separate occasions on each of 12 volunteers. The result for the second study was highly significant: the baseline flow in the nondominant forearm was 3.44 (SD 1.27) mls/100mls/min compared to 3.71 (1.21) mls/100mls/min in the dominant forearm, P=0.0001. Baseline blood flow is 8% higher in the dominant forearm. The increased SD reflects the use of individual FBF measurements rather than the means of 12 readings.

Table 3.8 Baseline FBF by forearm and protocol point (first study)

BASELINES	Active/Nondominant Forearm as Mean (SD) in mls/100mls/min			Control/Dominant Forearm as Mean (SD) in mls/100mls/min		
		Comparison	P		Comparison	P
1st (Pre-ischaemia)	3.01 (1.00)	1st vs 2nd	0.55	3.05 (1.02)	1st vs 2nd	0.43
2nd (Pre-ischaemia)	3.09 (1.04)	2nd vs 3rd	0.31	3.29 (1.07)	2nd vs 3rd	0.46
3rd (Pre-Exercise)	3.26 (1.27)	1st vs 3rd	0.11	3.44 (1.18)	1st vs 3rd	0.13

Table 3.9 contains the mean blood flows for both the control and active forearms for the second study carried out in Liverpool, showing a small, but significant fall in baseline FBF during the protocol in the active arm only. It is difficult to know what to attribute this to, since baseline FBF values were re-attained within 3 minutes following the final period of ischaemia (figure 3.6) and in any case, incomplete recovery would be a cause of increased rather decreased FBF.

Baseline FBF is higher in the second study due to the computer selectively analysing the first 4 seconds of signal during each venous occlusion. As discussed in chapter 2 this leads the computer to estimate resting FBF to be an average of a 10% higher than estimated by eye.

Table 3.9 Comparison of within-session, baseline forearm blood flows(second study)

	Active/ Nondominant Forearm Blood Flow (mls/100mls/min)			Control/ Dominant Forearm Blood Flow(mls/100mls/min)		
	Mean	SD	P	Mean	SD	P
Baseline 1	3.50	1.12	<0.013	3.79	1.1	0.29
Baseline 2	3.19	1.02		3.62	1.22	

Nested analysis of variance of the two baseline flows (table 3.10) showed that there was no

significant difference in the variability whether measured between sessions, within a day or between sessions on different days. There was some advantage in combining data from both forearms either as a ratio (active/control) or as a mean. Log-transforming the data was not beneficial.

Table 3.10 Nested analysis of variance of baseline forearm blood flow

%CV	Active FBF	Control FBF	Mean of Both FBFs	Ratio of FBFs	Log of Ratio
Total (between volunteer)	33	32	30	26	27
Within volunteer, between day	25	27	24	23	23
Within day, between session	24	26	23	23	22
Within session, between baseline	14	12	12	13	13
Overall Mean Value	3.34	3.71	3.50	0.94	-0.11

From table 3.11 it can be seen that resting forearm blood flow is subject to diurnal variation. This will contribute to the within-day, between session variability, but not to the between-day variability. The first baseline FBF was recorded at the same time each day for each volunteer at either 09.30 or 11.30 hours and the second session ran 4 hours later. A four hour delay equates to a 20% increase in resting FBF. Data from both forearms were analysed together, but the same pattern emerged for both forearms when they were analysed separately. Only the first baseline was analysed to prevent confounding by the effects of the rest of the protocol.

Table 3.11 Comparison of first baseline forearm blood flows by session(second study)

	Forearm Blood Flow (mls/100mls/min)		
	Mean	**SD**	**P**
Session 1	3.31	1.08	0.0001
Session 2	3.98	1.05	

3.6.4.2 Hyperaemic forearm blood flow

Copies of the FBF recovery profiles following periods of exercise and ischaemia recorded during the earlier study on 10 volunteers at Zeneca can be found in appendix A. Forearm blood flow profiles expressed as excess blood flow above baseline obtained from 12 volunteers at Liverpool are shown in figures 3.4 to 3.9. There was a minor difference in the protocols used. Due to changes in the position of the forearm during exercise, very few readings were on scale immediately following exercise in the first study. To prevent this the plethysmograph was re-zeroed following exercise during the second study at Liverpool, causing a 2 second delay before measurements could be taken. This delay will have reduced the magnitude of the blood flows subsequently recorded, but allowed the first FBF measurement following exercise to be recorded on nearly all occasions. Both active forearm sets of results (indicated by the open circles) show the near exponential recovery following periods of exercise and ischaemia. The recovery following exercise is much slower than that following ischaemia, although the peak blood flow following ischaemia is generally greater. Each mean FBF value was obtained by meaning the FBFs obtained for that time-point and session for all of the volunteers and then meaning them again for all four sessions. In this way the standard deviations displayed represent within-volunteer, between session values, which are of relevance to the eventual study design, rather

than between-volunteer values, more commonly used.

Control forearm blood flow (indicated by open triangles) shows no change immediately following either ischaemia or exercise. In our first study (see Appendix), slight increases in control FBF were apparent immediately following longer periods of exercise, however some volunteers admitted that they might have tensed their control forearm towards the end of longer periods of isotonic exercise.

Figure 3.5

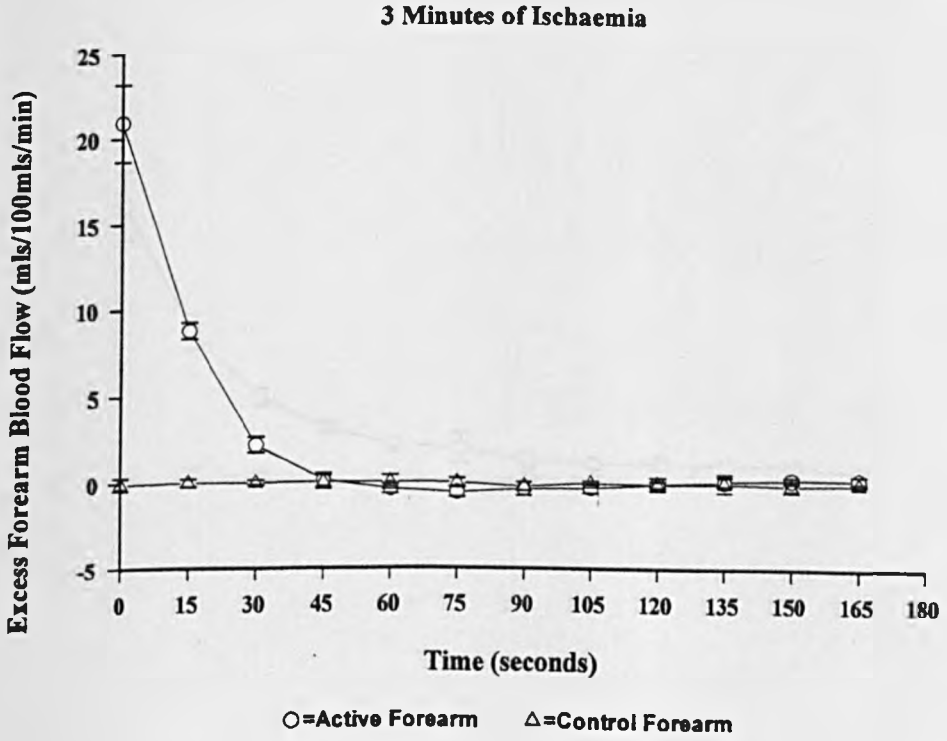

3 Minutes of Ischaemia
Excess Forearm Blood Flow (mls/100mls/min)
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○=Active Forearm
△=Control Forearm

Figure 3.4

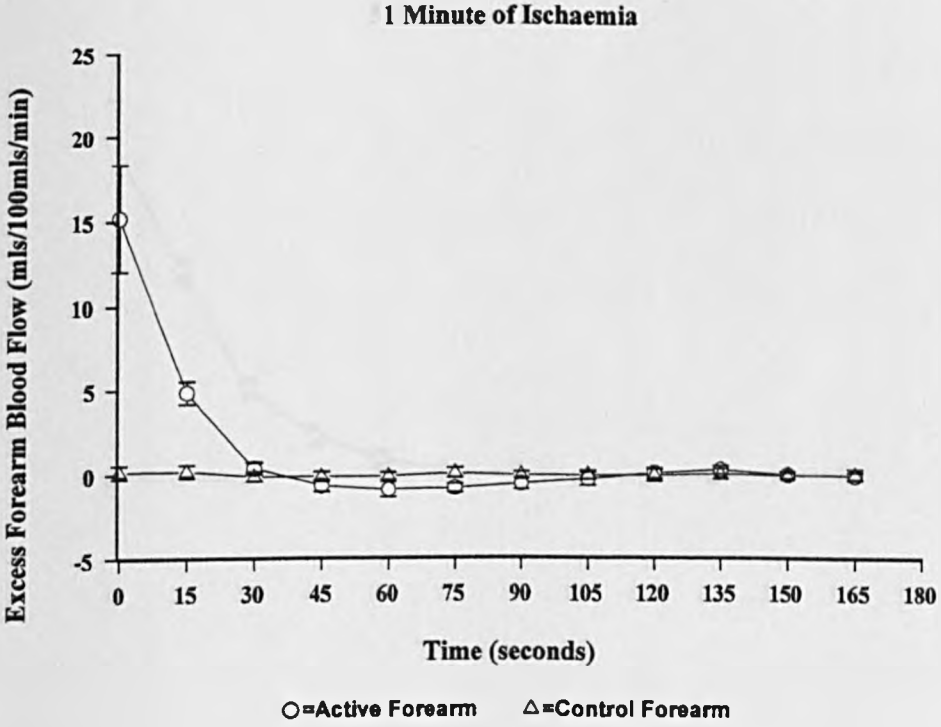

1 Minute of Ischaemia
Excess Forearm Blood Flow (mls/100mls/min)
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○=Active Forearm
△=Control Forearm

Figure 3.7

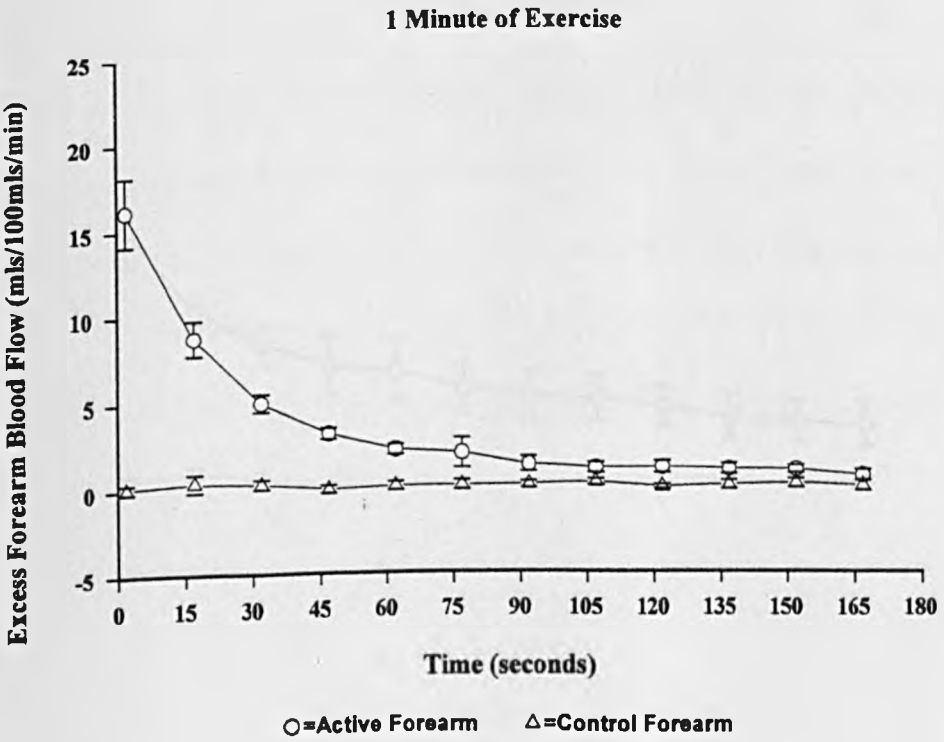

1 Minute of Exercise
Excess Forearm Blood Flow (mls/100mls/min)
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○=Active Forearm
△=Control Forearm

Figure 3.6

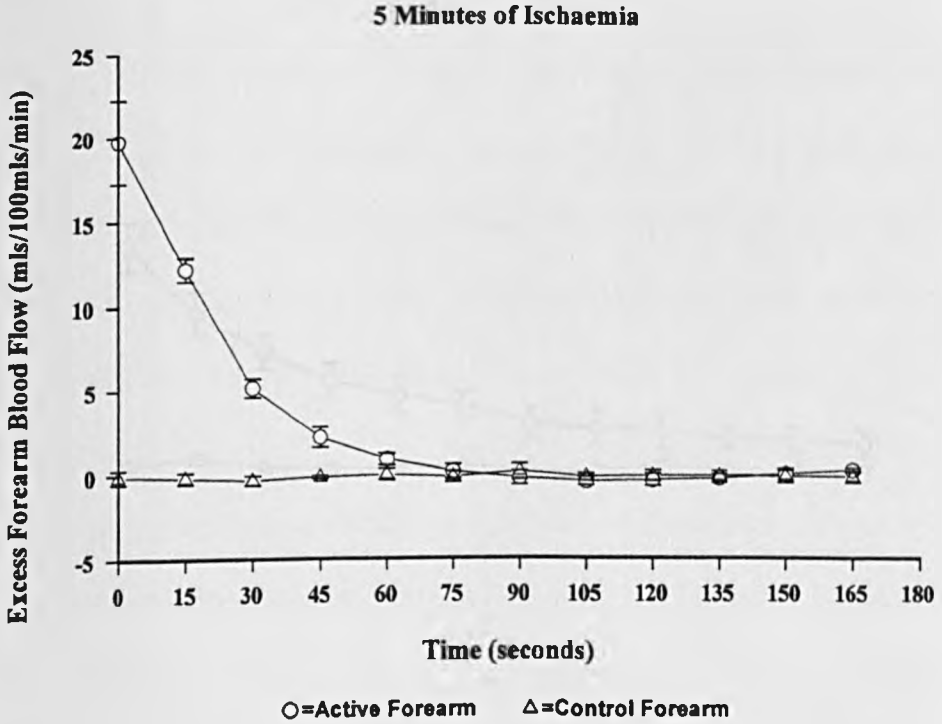

5 Minutes of Ischaemia
Excess Forearm Blood Flow (mls/100mls/min)
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○=Active Forearm
△=Control Forearm

Figure 3.8

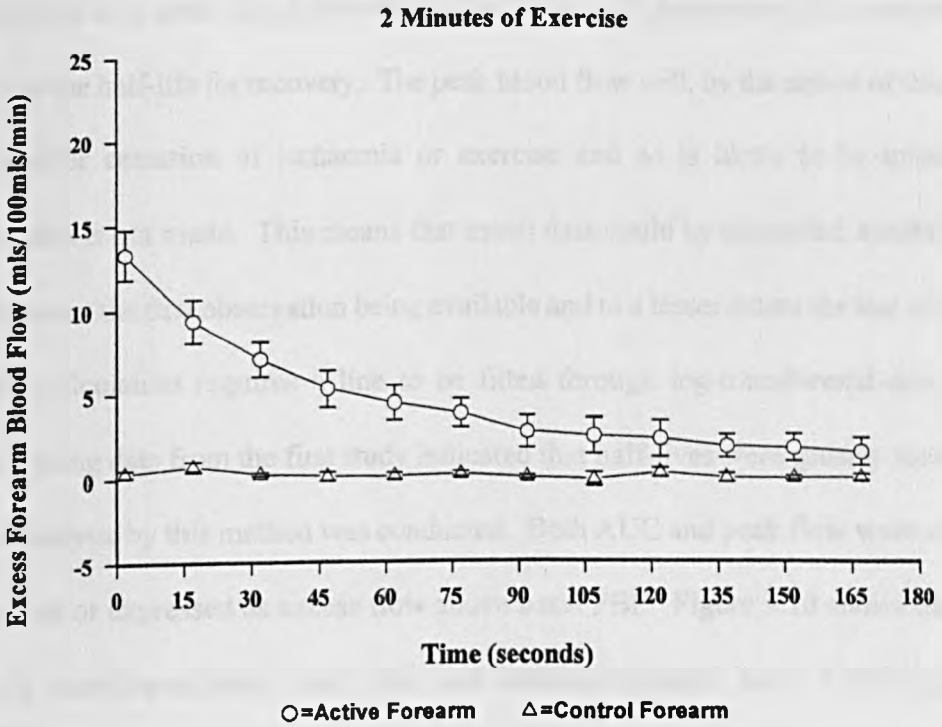
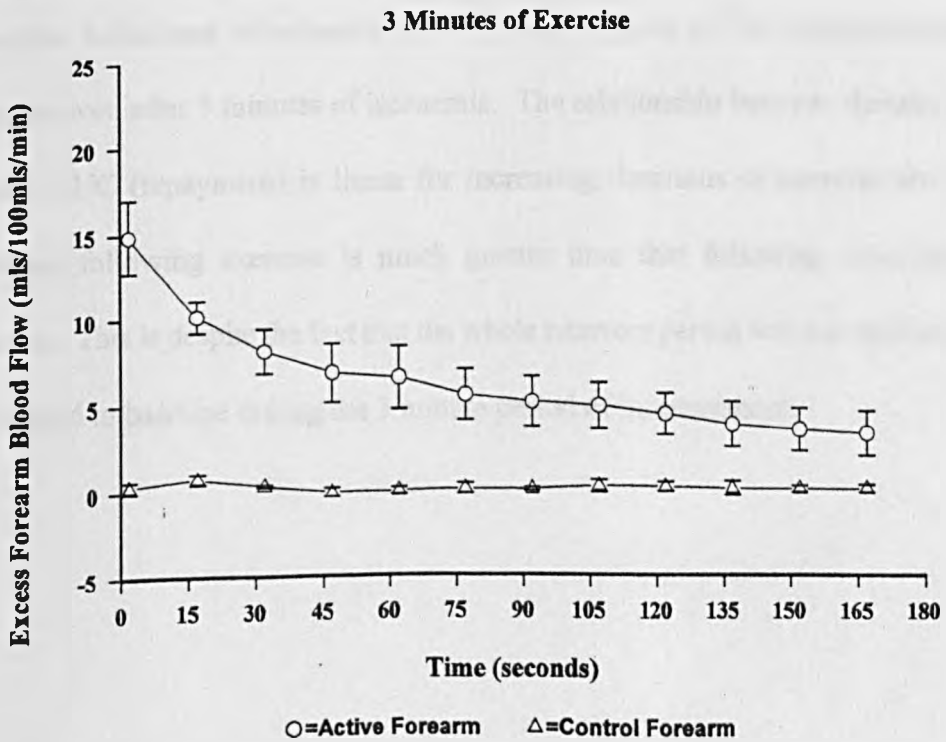
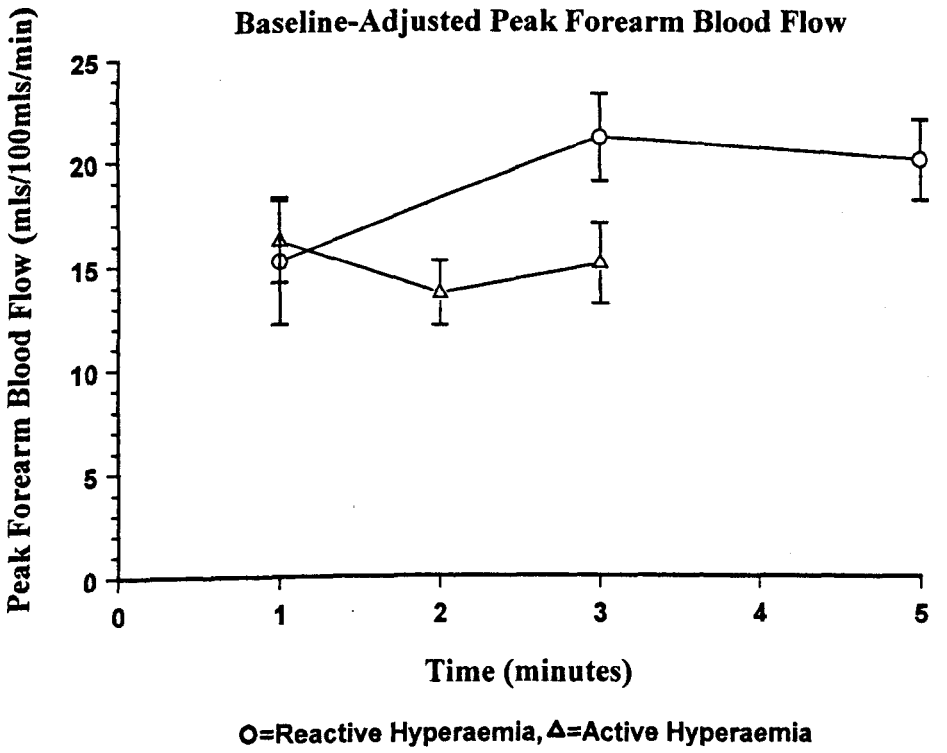


Figure 3.9



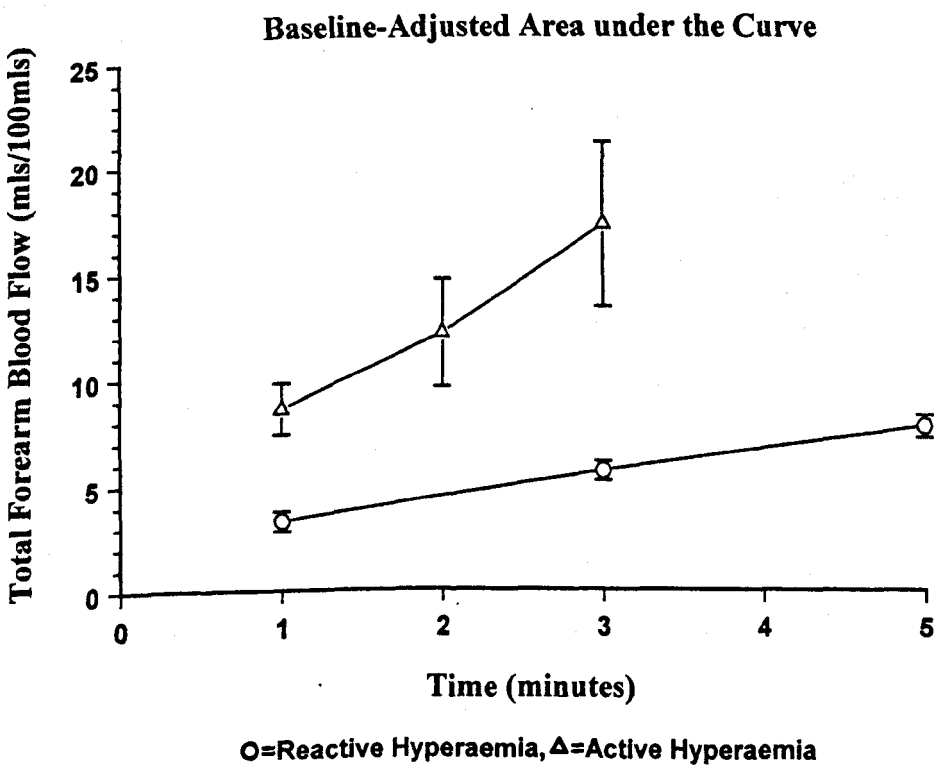
An exponential decline, such as that evinced by reactive and active hyperaemia can be summarised as a peak blood flow during the period of observation, the area under the curve (AUC) or the half-life for recovery. The peak blood flow will, by the nature of things, occur very shortly after cessation of ischaemia or exercise and so is likely to be missed if the first observation is not made. This means that much data could be discarded, similarly the AUC is dependent on the first observation being available and to a lesser extent the last of the series. The half-life calculation requires a line to be fitted through log-transformed data. Preliminary analysis using data from the first study indicated that half-lives were grossly variable, hence no further analysis by this method was conducted. Both AUC and peak flow were analysed either unchanged or expressed as excess flow above basal FBF. Figure 3.10 shows the relationships between baseline-adjusted peak flow and baseline-adjusted AUC following exercise and ischaemia. AUC is the product of FBF and time, but measured over a maximum of 2.75 minutes. Total flow or baseline adjusted AUC is obtained by subtracting basal FBF, which can be regarded as the total flow "repaid" following a period of reduction in flow, providing FBF has returned to baseline. In the case of ischaemia recovery was complete as FBF returned to baseline within 3 minutes even after 5 minutes of ischaemia. The relationship between duration and baseline-adjusted AUC (repayment) is linear for increasing durations of exercise also, but the total repayment following exercise is much greater than that following equivalent periods of ischaemia. This is despite the fact that the whole recovery period was not measured, as FBF had not returned to baseline during the 3 minute period of measurement.

Figure 3.10



O=Reactive Hyperaemia, Δ=Active Hyperaemia

Figure 3.11



O=Reactive Hyperaemia, Δ=Active Hyperaemia

Once the blood flow profile following each period of exercise or ischaemia has been summarised, a further summary figure is derived by calculating the mean or the AUC for all three of the levels of exercise and separately for all three periods of ischaemia, so that two figures were derived, one for 1 to 3 minutes exercise and one for 1 to 5 minutes ischaemia, for each of the 4 sessions for each volunteer Table 3.12 contains the mean values obtained for the different summary measures for exercise and ischaemia and the coefficient of variation. Tables 3.13, 3.14 and 3.15 show the coefficients of variation for summary endpoints using baseline as a covariate in the analysis when the data is normalised for baseline FBF (FBF/baseline FBF), expressed as forearm compliance (FBF/MAP) or log-transformed. None of these ways of treating the data offer any distinct advantage, although log-transformation does reduce the variability of data processed as AUCs but not when processed as peak FBFs. Normalisation for preceding baseline FBF is deleterious.

Table 3.12 Hyperaemic forearm blood flow variability by summary measure

mls/100mls/min		Baseline Covariate		Baseline Subtracted	
AUCs		%CV	Mean	%CV	Mean
AUC of AUCs	Ischaemia	20.8	15.5	33.4	5.8
	Exercise	31.5	21.9	44.3	12.8
Ave of AUCs	Ischaemia	11.0	15.1	26.3	5.64
	Exercise	23.1	18.0	28.3	14.8
Peaks					
AUC of Peaks	Ischaemia	12.8	23.1	15.1	19.4
	Exercise	23.1	18.0	28.3	14.8
Ave of Peaks	Ischaemia	12.3	22.3	14.4	18.9
	Exercise	22.8	18.3	27.7	15.1

Table 3.13 Variability of FBF:baseline FBF ratio by summary measure

		Baseline Covariate		Baseline Subtracted	
AUCs		**%CV**	**Mean**	**%CV**	**Mean**
AUC of AUCs	**Ischaemia**	19.7	4.29	44.3	1.59
	Exercise	35.3	7.44	54.7	4.59
Ave of AUCs	**Ischaemia**	11.3	4.18	31.4	1.47
	Exercise	33.4	7.30	55.2	4.55
Peaks					
AUC of Peaks	**Ischaemia**	19.5	6.67	28.7	5.67
	Exercise	32.9	6.15	44.4	5.15
Ave of Peaks	**Ischaemia**	17.9	6.49	27.6	5.49
	Exercise	33.4	6.26	45.4	5.26

Table 3.14 Forearm compliance (mls/100mls/min/mmHg)

		Baseline Covariate	
AUCs		**%CV**	**Mean**
AUC of AUCs	**Ischaemia**	22.6	0.178
	Exercise	33.1	0.242
Ave of AUCs	**Ischaemia**	11.6	0.173
	Exercise	25.2	0.235
Peaks			
AUC of Peaks	**Ischaemia**	13.6	0.264
	Exercise	23.4	0.197
Ave of Peaks	**Ischaemia**	13.5	0.258
	Exercise	23.0	0.201

Table 3.15 Log-transformed forearm blood flow data

		Baseline Covariate	
AUCs		**SD**	**Mean**
AUC of AUCs	**Ischaemia**	16.2	2.70
	Exercise	28.7	3.02
Ave of AUCs	**Ischaemia**	9.9	2.68
	Exercise	24.1	3.01
Peaks			
AUC of Peaks	**Ischaemia**	14.3	2.88
	Exercise	30.5	2.63
Ave of Peaks	**Ischaemia**	13.4	2.86
	Exercise	29.4	2.65

3.6.5 Maximum voluntary contraction

The three readings for both forearms for each session were averaged and then the mean session readings for each volunteer were meaned together to produce an overall mean for each forearm of each volunteer. These volunteer means were meaned together depending on whether volunteers were left or right handed to produce mean values for the MVC for the dominant and non-dominant forearm. The MVC for the dominant forearm was 1.22 Barr (range 0.89 to 1.47) and for the non-dominant forearm was 1.11 Barr (range 0.73 to 1.42). As 1 Barr is 760mmHg, 100mmHg maintained by the non-dominant forearm in these volunteers corresponds to 12% of MVC, range 9 to 18%.

3.6.6 Adverse events

Upto 40% of volunteers complained of a persistent, mild headache sometimes starting the day of the study, occasionally the day before. It was relieved by paracetamol and in all cases had resolved by the day following the study. Some volunteers complained of mild backache towards the end of each study period, purely related to being required to remain supine for upto 2 hours. This settled once they were free to move around again.

3.7 Summary

Many volunteers experienced minor, self-limiting adverse events during these studies. These were mostly headaches or difficulty concentrating and less frequently lower back pains related to prolonged recumbency.

Blood pressure, heart rate, stroke index, cardiac index and baseline forearm blood flow all showed marked diurnal variation. In the two studies described I have developed a protocol for measuring active and reactive hyperaemic responses non-invasively in the human forearm and determined the precision of the measures analysed in a variety of ways. From the first study I found that there was nothing to be gained from waiting longer for flows to settle. I also found that baseline FBF was 8% greater in the dominant compared to the nondominant forearm. No change was noted in control FBF during periods of ischaemia or exercise, neither was there any change in the cardiac index, as the increase in heart rate associated with exercise was compensated for by a commensurate fall in stroke index. In this study, 100mmHg of pressure maintained by the non-dominant forearm corresponds to 12% of MVC.

Reactive hyperaemia is more reproducible than exercise hyperaemia. There is a slight

advantage to expressing responses to exercise as a peak FBF compared to an AUC (or total hyperaemia). It is better to summarise the three responses to exercise or to ischaemia for each volunteer as an average rather than an AUC. Any systematic changes in basal flow are best accommodated, predictably by adding baseline flow measurements to the model used for analysis of variance. There is no advantage from log-transforming the data or expressing the results as forearm compliance rather than FBF. Assuming a difference due to treatment of 30% in excess FBF in exercise hyperaemia, with a power of 80% and assuming significance when $P<0.05$, the estimated number of volunteers needed to complete the protocol would be 12.

Chapter 4

Effects of theophylline on reactive and active hyperaemia in healthy male volunteers

4.1 Introduction

On the basis of a wealth of data from diverse animal models adenosine would be expected to contribute to active hyperaemia in man, though there are conflicting reports[125,129]. Theophylline has been shown to be a competitive, adenosine receptor antagonist[116,127] and intra-arterial theophylline has been shown to antagonise adenosine-induced vasodilatation in the forearm[121]. If adenosine has the same importance that it has in the cat[128] or dog[124], then around a 30% reduction in active hyperaemia would be predicted. Theophylline has been shown to reduce reactive hyperaemia in the forearm by upto 35%, though significant inhibition occurred only after 5 minutes of occlusion and not after 1 or 10 minutes[136].

It was hoped that this study would clarify the situation in the human forearm.

4.2 Aims

This study was designed to determine the effect of theophylline on hyperaemic responses in the human forearm at doses achieving plasma levels in the upper therapeutic range. To analyse these responses correctly it was also necessary to determine whether theophylline had any effect on basal forearm blood flow, mean arterial pressure or central haemodynamics.

4.3 Design

We used the protocol developed in the previous chapter to measure hyperaemic responses. Only

one initial basal forearm blood flow was measured and measurements were begun 18 minutes after the apparatus had been applied and tested, so effectively the first basal FBF measurements were omitted when compared to the very first protocol. The only other difference from the very first protocol was that measurements of exercise hyperaemia were initiated 2 seconds after exercise had stopped to allow the plethysmograph baseline voltage to be re-set. The protocol for the study is shown in Table 4.1.

This was a double-blind, placebo-controlled, trial in which the order of treatment was randomised in a balanced way to try and ensure that the numbers of volunteers given placebo first or given theophylline first were equal. Volunteers were asked to attend at the same time on 2 days a week apart. They ate the same light breakfast or brunch at home each week and attended the study site in time for dosing 2 hours after their meal.

4.4 Volunteers characteristics

We studied 12 healthy, male volunteers recruited from the volunteer panel at Zeneca aged 20-49 years (mean 33 years), weighing 62-90kg (mean 75kg) and height 1.66-1.88m (mean 1.77m). Two volunteers were regular smokers. They had normal blood counts, liver function, renal function, urinalysis, blood sugar and electrolytes, resting ECG and 24 hour ambulatory ECGs, with no significant abnormalities on clinical examination. Volunteers were only included if they were 18-62 years old, 60-89kg in weight, on no regular medications, had a sitting blood pressure of less than 140/90mmHg and consumed 21 units of alcohol a week or less.

Table 4.1 Timing of measurements and interventions

Elapsed time (minutes)	FBF/TEB	Supine pulse & BP	Duration of ischaemia (minutes)	Duration of exercise (minutes)
18-20		+		
20-23	+			
28-29			1	
29-32	+			
34-37			3	
37-40	+			
42-47			5	
47-50	+			
50-52		+		
57-59		+		
59-62	+			
67-68				1
68-71	+			
73-75				2
75-78	+			
80-83				3
83-86	+			
86-88		+		
88-91	+			
91-93		+		

4.5 Volunteer restrictions

These were similar to those for the previous study. Volunteers were asked to avoid medications and strenuous exercise for the day prior to the study, to avoid caffeine containing foods and drinks and stop smoking for 2 days prior to each study day and to abstain from alcohol for the previous 3 days.

4.6 Dosing

Both theophylline (Nuelin™ 125mg tablets, 3M Healthcare) and placebo were given packed inside gelatin capsules to conceal any differences in appearance or taste. Each dose was given with 150mls of distilled water. Pharmacy packed the capsules for each patient according to the randomisation scheme. The dose of theophylline was adjusted according to the weight of the volunteer: those weighing 70kg or over were given 5 capsules containing either placebo or a total of 625mg of theophylline, those under 70kg were given 4 capsules or 500mg of theophylline. This equates to a dose range of 7.0-8.9mg/kg. Given that oral microcrystalline theophylline is almost completely absorbed within 3 hours [235] and the volume of distribution is 0.47l/kg[227], this should give a maximum concentration of theophylline (C_{max}) of 15-19mg/L or 83-105μM (MW 180.17). The t_{max} for Nuelin has been measured to be 2.1 hours in healthy volunteers[227] and although the plasma theophylline concentration after oral falls steadily after this time, it should remain within the therapeutic range for upto 4 hours falling more rapidly thereafter[227,236].

4.7 Results

4.7.1 Room temperature

The initial room temperature was invariably the minimum room temperature. The mean, minimum room temperature was 22.7°C, range 22 to 24°C and the mean maximum room temperature was 23.6°C, ranging from 23 to 24°C. The maximum increase during any study day was 2°C, mean 0.8°C.

4.7.2 Transthoracic electrical bioimpedance (TEB)

Cardiac index, stroke index and heart rate were measured by TEB as previously described. Six baseline measurements were made each day, so these were meaned together to provide a single summary reading for each volunteer and day. Statistical comparison was carried out using ANOVA and allowing for variability contributed by volunteers, the treatment order and the day.

Table 4.2 Changes in baseline TEB indices caused by theophylline

	Placebo		Theophylline		
	Mean	**SD**	**Mean**	**SD**	**P**
Cardiac index	2.98	0.57	3.25	0.62	0.002
Stroke index	52.4	10.3	53.5	11.1	0.59
Heart rate	57.4	5.0	61.7	8.4	0.04

At rest theophylline causes a significant, 9% increase in the cardiac index, due to an increase in heart rate as the stroke index is unchanged (table 4.2).

Table 4.3 Changes in TEB indices post-exercise by treatment

		Placebo			Theophylline		
		Mean	SD	P	Mean	SD	P
Cardiac Index	**Baseline**	2.94	0.57	0.003	3.18	0.57	0.006
	Exercise	3.44	0.70		3.60	0.78	
Stroke Index	**Baseline**	51.4	10.9	0.15	51.2	10.7	0.17
	Exercise	53.9	10.3		48.6	9.4	
Heart Rate	**Baseline**	57.9	5.1	0.003	62.55	8.6	0.02
	Exercise	65.1	10.0		76.4	19.3	

TEB indices measured in the first minute after 3 minutes of handgrip exercise are shown in table 4.3, broken down according to treatment. As in the reproducibility study exercise is associated with a tachycardia, however on this occasion there is a significant increase in cardiac index whilst stroke index increased marginally in the presence of placebo and decreased slightly in the presence of theophylline. The effects of theophylline on systemic responses to handgrip exercise are shown in table 4.4. The reduction in stroke index brought about by theophylline does achieve significance, though as this was a *post-hoc* analysis, the result must be treated with caution.

Table 4.4 Effect of theophylline on exercise-induced changes in TEB indices

N=12	Placebo		Theophylline		
	Mean Increase	SD	Mean Increase	SD	P
Cardiac Index	0.50	0.45	0.42	0.43	0.59
Stroke Index	2.51	5.57	-3.20	7.59	0.24
Heart Rate	7.24	6.42	13.90	17.31	0.19

4.7.3 Pulse and blood pressure

Table 4.5 Changes in pulse and blood pressure

	Placebo		Theophylline		
	Mean	SD	Mean	SD	P
MAP	83.9	5.7	85.9	6.5	0.04
Systolic BP	112.0	9.1	117.5	12.1	0.014
Diastolic BP	69.7	5.4	70.0	5.6	0.70
Pulse	57.5	5.6	63.5	10.0	0.008

Table 4.5 shows the changes in the resting pulse rate and blood pressure brought about by theophylline. There is a 2.5% increase in mean arterial pressure which is statistically significant. This change is much too small to account for the change in baseline forearm blood flow noted. The increase in MAP is brought about solely by an increase in systolic BP, there is no change in diastolic blood pressure. The increase in pulse rate accords well with that measured by TEB.

4.7.4 Forearm blood flow

4.7.4.1 Baseline forearm blood flow

Comparison of the first or pre-ischaemia baseline with the second or pre-exercise baseline blood flow measurements in both active and control forearms and even the mean reading-by-reading average blood flow measurements from both forearms (combined FBF) by ANOVA allowing for volunteer, treatment, and study day effects revealed no difference between the two baselines (Table 4.6). There was no significant difference in baseline blood flow between the two forearms when the two mean baseline blood flow readings were averaged for each volunteer and session and compared by ANOVA: active FBF 4.07mls/100mls/min (SD 2.33) versus control 4.36 (2.33), P=0.48.

Table 4.6 Comparison of first and second baseline FBFs by forearm

Baseline Flows	Active FBF			Control FBF			Combined FBF		
	Mean	SD	P	Mean	SD	P	Mean	SD	P
First	4.12	1.98	0.85	4.22	1.91	0.51	4.15	1.81	0.69
Second	4.04	2.77		4.50	2.73		4.30	2.66	

In view of these findings an overall mean baseline FBF was derived for each volunteer using combined FBFs and meaning the two baseline FBF readings together producing one value of FBF for each volunteer and study day. These values were compared again using ANOVA and allowing for volunteer, treatment and study day effects. **Theophylline caused a significant, 49% increase in FBF from 3.39 (SD 1.12) to 5.05 mls/100mls/min (2.71), P<0.02.**

As theophylline caused a significant increase in mean arterial pressure, albeit by only 2.5%, the correct way to process flow data is to express it as an arterial compliance, the flow divided by MAP. Blood vessel compliance does not change when the MAP changes due to the way it is calculated. There was still a 44% increase in compliance in the presence of theophylline from 0.041 (0.015) to 0.059 (0.030) mls/100mls/min/mmHg, P<0.02, indicating that forearm vasodilatation by theophylline is more than just a consequence of the change in MAP.

4.7.4.2 Hyperaemic forearm blood flow

Figure 4.1

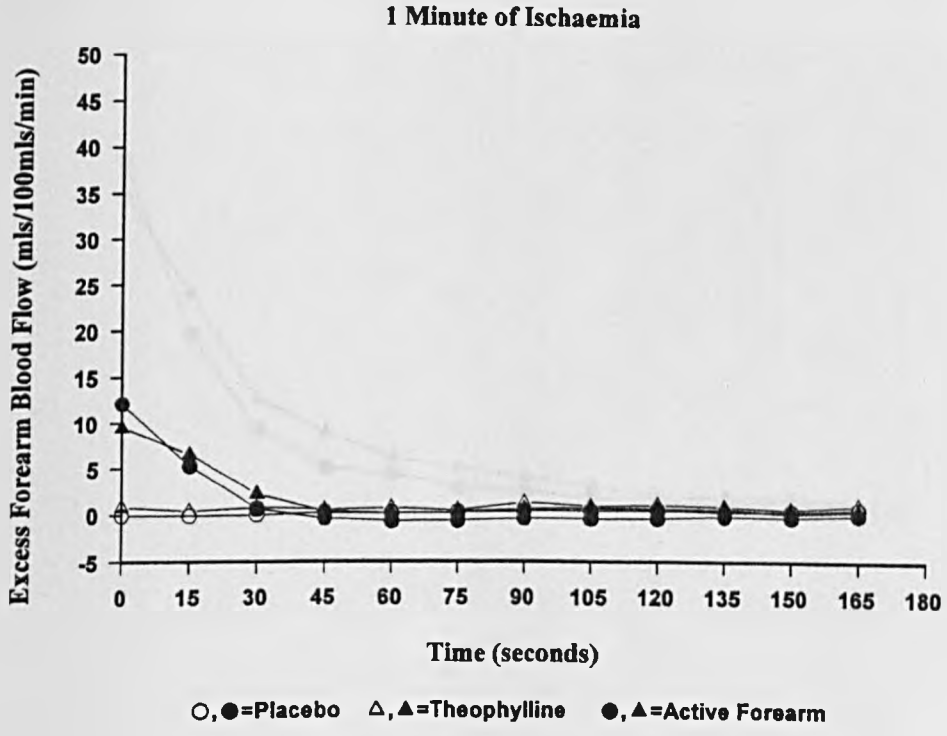


Figure 4.2

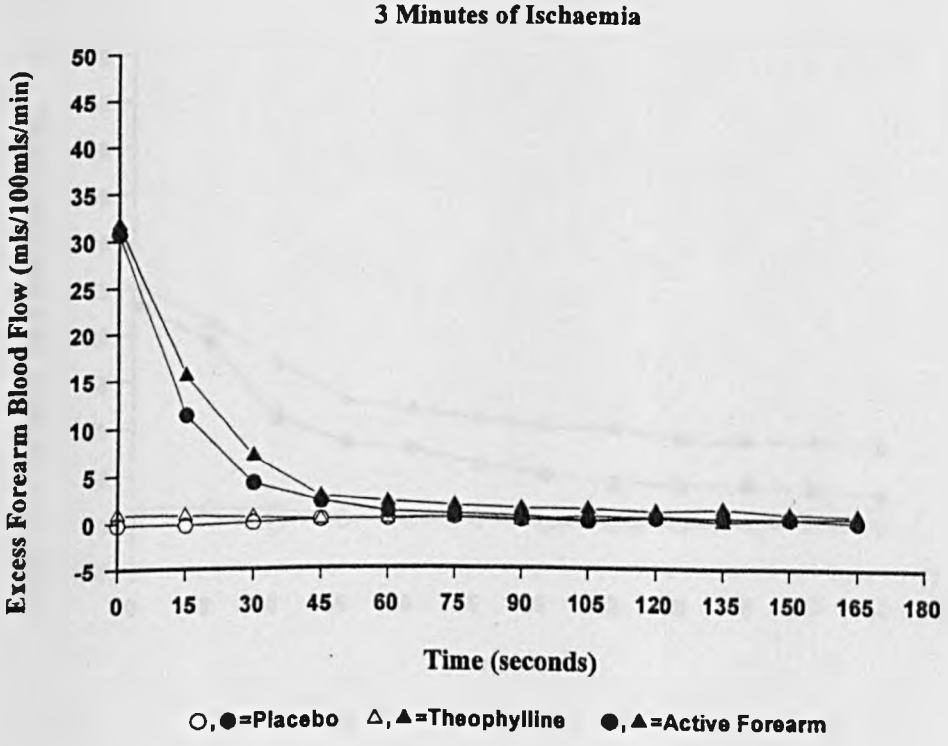


Figure 4.4

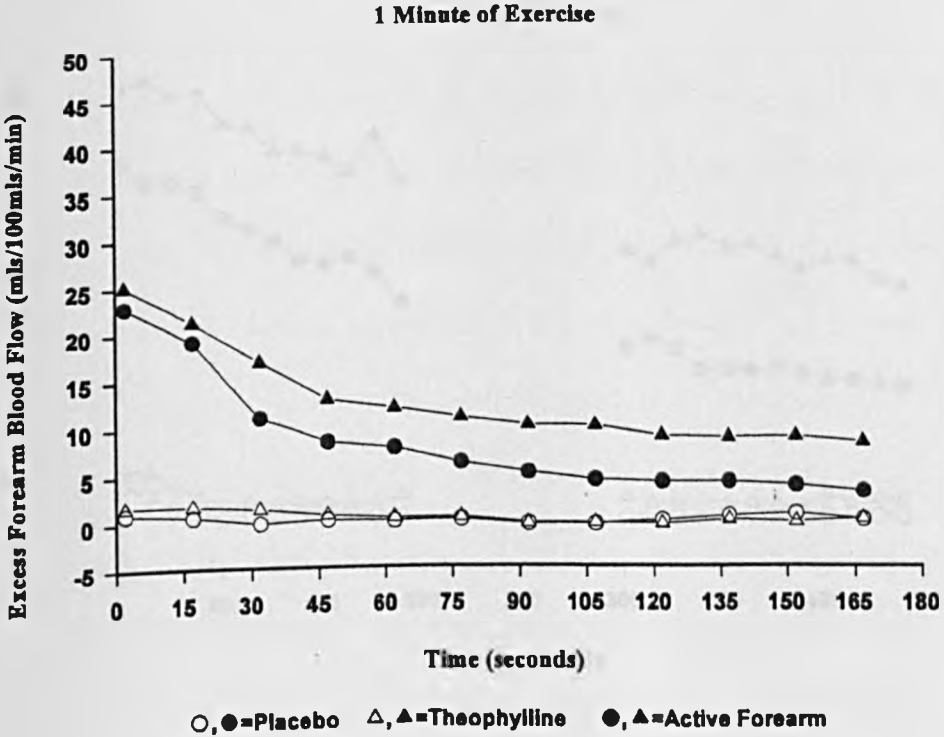

1 Minute of Exercise
Excess Forearm Blood Flow (mls/100mls/min)
50
45
40
35
30
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○, ●=Placebo
△, ▲=Theophylline
●, ▲=Active Forearm

Figure 4.3

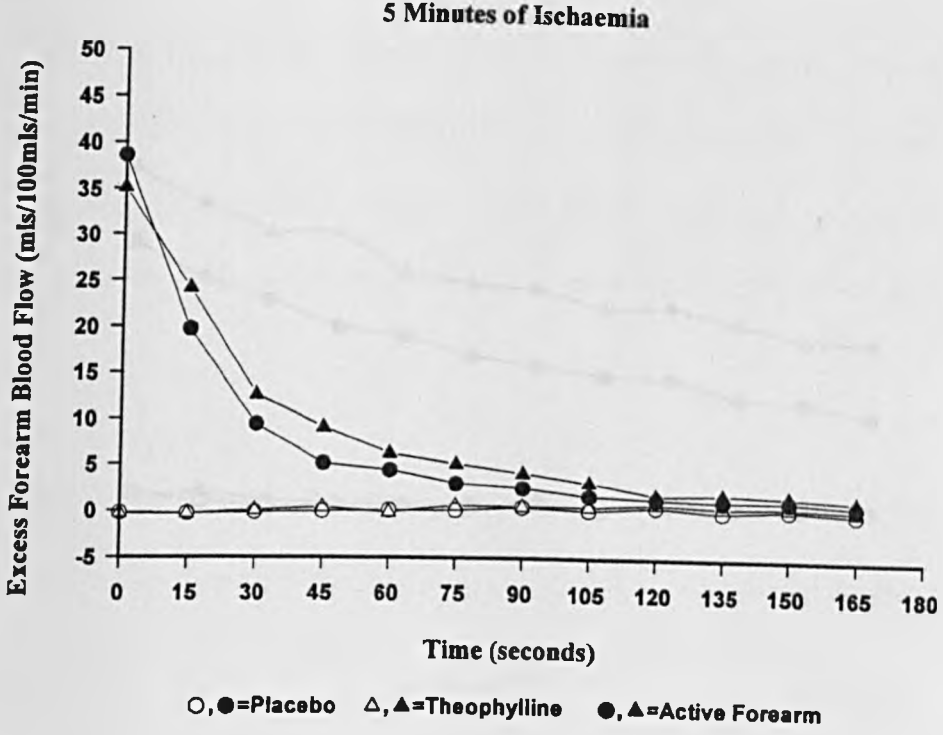

5 Minutes of Ischaemia
Excess Forearm Blood Flow (mls/100mls/min)
50
45
40
35
30
25
20
15
10
5
0
-5
0
15
30
45
60
75
90
105
120
135
150
165
180
Time (seconds)
○,●=Placebo
△,▲=Theophylline
●,▲=Active Forearm

Figure 4.5

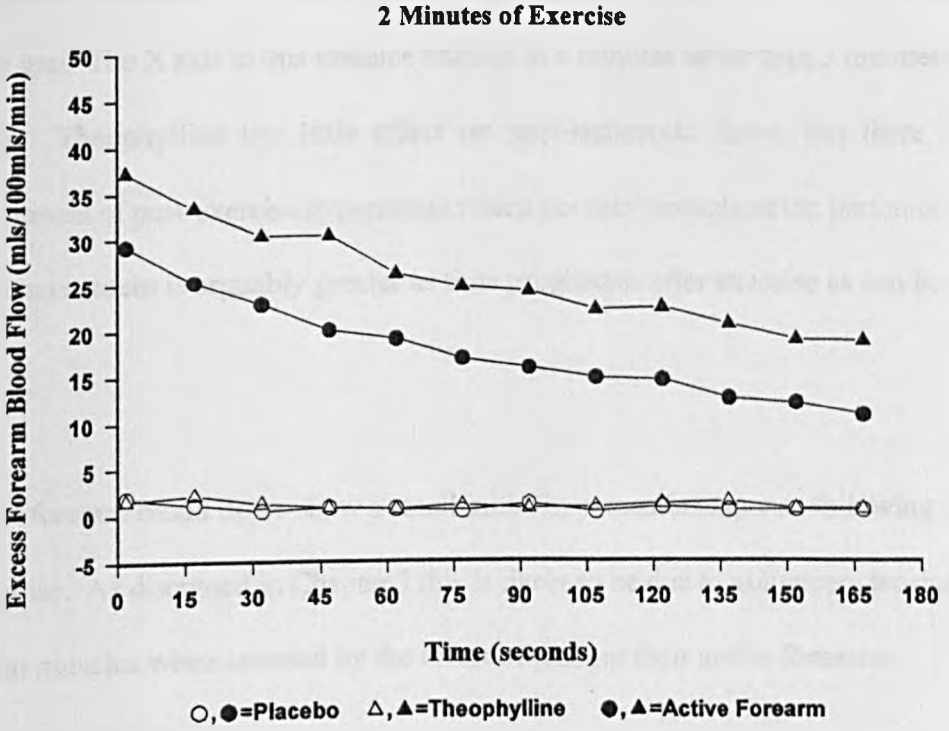
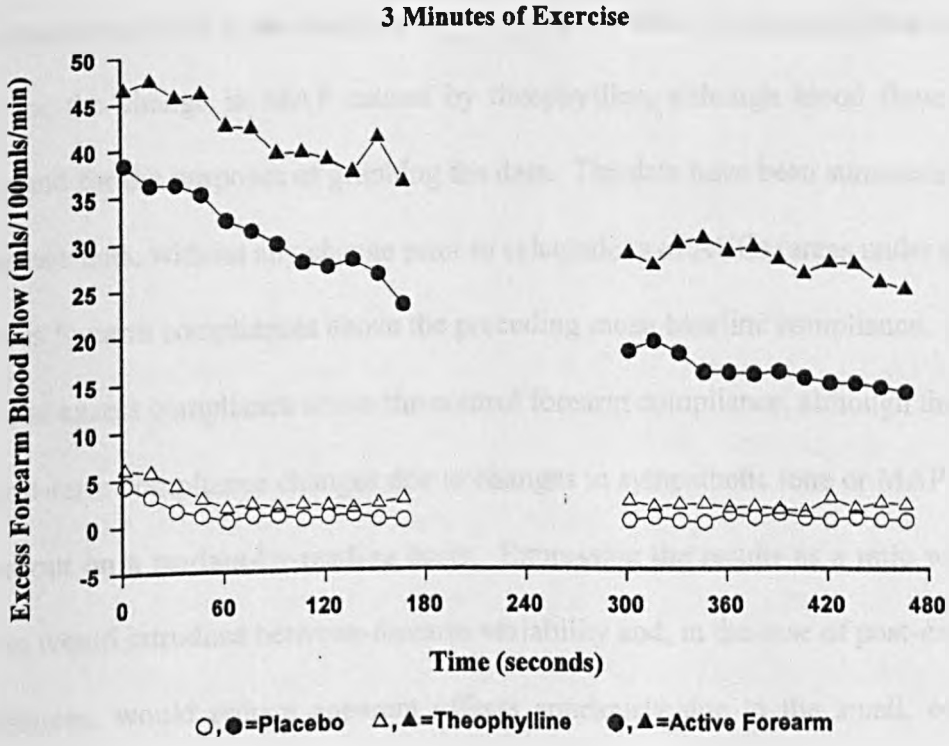


Figure 4.6



Figures 4.1 to 4.6 show excess forearm blood flow, ie the increase in flow over the preceding mean basal flow. Figure 4.6 shows not only the flows recorded during the first 3 minutes after 3 minutes of isotonic exercise, but also the subsequent 'baseline' flow results recorded after a 2 minute gap. The X axis in this instance extends to 8 minutes rather than 3 minutes as in the other figures. Theophylline has little effect on post-ischaemic flows, but there is an obvious enhancement of post-exercise hyperaemia which persists throughout the period of measurement. This enhancement is arguably greater as time progresses after exercise as can be seen in figure 4.6.

Control forearm blood flows show a small initial hyperaemic response following longer periods of exercise. As discussed in Chapter 3 this is likely to be due to volunteers tensing their control forearm muscles when stressed by the effort of tensing their active forearms.

4.7.4.3 Summary measures of hyperaemic forearm blood flow

As explained earlier it is necessary to use compliances when making statistical comparisons to allow for the change in MAP caused by theophylline, although blood flows are easier to understand for the purposes of graphing the data. The data have been summarised in table 4.7 both as raw data, without any change prior to calculations of AUCs (areas under the curves) and as excess forearm compliances above the preceding mean baseline compliance. It was decided not to use excess compliance above the control forearm compliance, although this would allow for short-term compliance changes due to changes in sympathetic tone or MAP, as it could be carried out on a reading-by-reading basis. Expressing the results as a ratio with the control forearm would introduce between-forearm variability and, in the case of post-exercise forearm compliances, would reduce apparent effects spuriously due to the small, control forearm

hyperaemic responses. As previously noted, baseline forearm compliance is increased by theophylline. To use baseline compliance as a covariate in ANOVA would be to extract the treatment effect from the analysis and is methodologically unsound.

Table 4.7 Summary measures of forearm compliance

mls/100mls/min /mmHg	PLACEBO			THEOPHYLLINE			
	Mean	SD	N	Mean	SD	N	P
ISCHAEMIA	**Average peak forearm compliance**						
Raw data	0.33	0.11	9	0.35	0.13	12	0.96
Excess FBF	0.29	0.11	9	0.29	0.14	12	0.87
EXERCISE							
Raw data	0.43	0.11	11	0.53	0.22	11	0.04
Excess FBF	0.39	0.11	11	0.47	0.21	11	0.054
ISCHAEMIA	**Average AUC of forearm compliances**						
Raw data	0.23	0.08	9	0.31	0.11	12	0.10
Excess FBF	0.10	0.06	9	0.15	0.10	12	0.27
EXERCISE							
Raw data	0.76	0.20	11	1.02	0.43	11	0.033
Excess FBF	0.65	0.20	11	0.86	0.38	11	0.042

The question addressed by analysis of the raw data is whether or not theophylline has any influence over exercise hyperaemia. There is a significant, 34% increase in the AUC and a significant, 25% increase in peak forearm compliance. The peak forearm compliance following ischaemia is unchanged, but the AUC is increased by 35%, though this is not significant. Variability is the major reason for the difficulty achieving significance, with a coefficient of

variation of 35%. Analysis of the reproducibility study suggested that changes of greater than 30% would have achieved significance.

Analysis of the data using excess compliance above the preceding baseline increases variability dramatically, as it did in the variability study. This type of analysis addresses the related question of whether theophylline increases hyperaemia even allowing for the pre-existing increase in basal flow. Figures 4.1 to 4.6 show excess forearm blood flow and strongly suggest a significant increase in exercise hyperaemia caused by theophylline. Despite the increase in variability the AUC of excess compliance following exercise achieves significance, though the peak excess compliance does not. There is strong likelihood that this is due to a type II error as the reproducibility study would predicts that greater numbers of volunteers would be required to achieve significance for the observed difference.

Figures 4.7 and 4.8 show how the scatter of AUCs in the presence of placebo translates to that in the presence of theophylline. Following ischaemia the AUCs in the presence of placebo are reasonably tight, in the presence of drug the distribution becomes bimodal and the spread is much increased. Theophylline seems either to cause no change or a slight reduction in the AUC or it causes a marked increase. This type of effect also seems to operate for post-exercise AUCs though it is less marked and the distribution does not become bimodal.

Figure 4.7

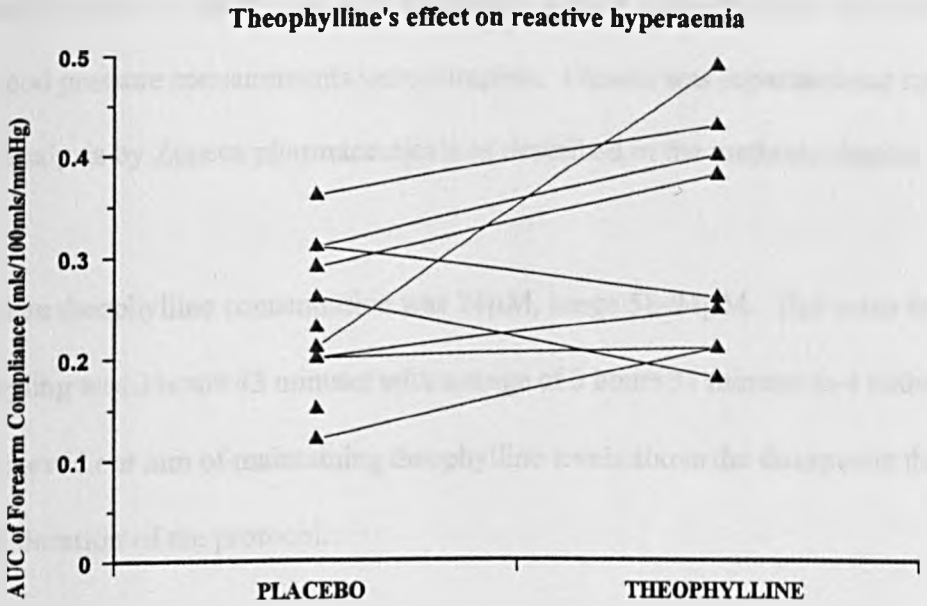
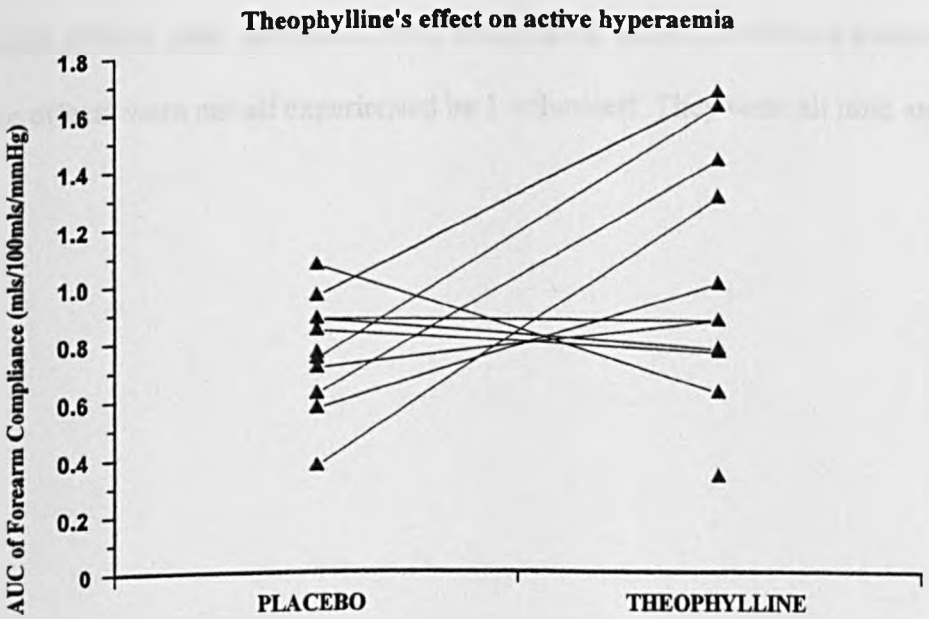


Figure 4.8



4.7.5 Theophylline levels

A blood sample was taken from each volunteer on each study day once the forearm blood flow and blood pressure measurements were complete. Plasma was separated and rapidly frozen for batch analysis by Zeneca pharmaceuticals as described in the methods chapter.

The mean theophylline concentration was 74μM, range 58-94μM. The mean time from dosing to sampling was 3 hours 43 minutes with a range of 3 hours 31 minutes to 4 hours and 5 minutes. We achieved our aim of maintaining theophylline levels above the therapeutic threshold (55μM) for the duration of the protocol.

4.7.6 Adverse events

3 volunteers complained on headache on the day they were given placebo and 2 volunteers admitted to having a headache on the day they were given theophylline. Whilst on theophylline 2 volunteers complained of tremor, whilst only 1 volunteer mentioned nausea. There was one complaint of back pain, faintness, fever, pharyngitis, rhinitis, fever and paraesthesiae. These adverse effects were not all experienced by 1 volunteer! They were all mild and self-limiting.

4.8 Summary

At levels comfortably within the therapeutic range, oral theophylline increased heart rate and consequently also increased cardiac index by 7.5%. Perhaps as a consequence it increases systolic blood pressure and to a much smaller extent MAP (2.5%). Overall the systemic vascular resistance will have fallen by nearly 5% (1.025/1.075). This change in systemic vascular resistance is attributable partly or even perhaps wholly to vasodilatation in skeletal muscle. Baseline forearm compliance increased significantly by 44%. Since the MAP was maintained or increased slightly, the tachycardia and increase in cardiac index observed must be other actions of theophylline rather than secondary to baroreceptor activation.

Theophylline also caused a paradoxical increase in exercise hyperaemia. The scattergrams suggest that theophylline either reduces active hyperaemia slightly or increases it markedly. The mechanism for this is uncertain.

Chapter 5

Adenosine arterial infusion methods development studies

5.1 Introduction

To help interpret the effects of theophylline on hyperaemic flows in the human forearm it is important to determine whether *oral* theophylline can inhibit adenosine-induced vasodilatation in the human forearm. Previous studies have shown that theophylline coinfused with adenosine into the brachial artery inhibits adenosine-induced vasodilatation[121,122,232]. Although care was taken in these studies to select a dose of theophylline low enough not to cause vasodilatation itself no attempt was made to measure plasma theophylline levels. It is not known whether oral theophylline achieving circulating levels within the recommended therapeutic range, would have significant effects as an adenosine receptor antagonist in the forearm circulation.

Two studies were conducted to develop the best adenosine arterial infusion protocol for my purposes. The first was a pilot study and the second was a more detailed, reproducibility study. The two studies are considered in turn, however to prevent reiteration they are discussed jointly.

5.2 Adenosine arterial infusion pilot study

5.2.1 Aims

The most important aim of the pilot study however was to establish the safety of this technique in my hands at Zeneca Pharmaceuticals. It was designed to find out whether the highest dose of adenosine used in other arterial infusion studies[121,122,232] had any effect on central haemodynamics, to find out how long it took for forearm blood flows to reach a new equilibrium when adenosine or sodium nitroprusside were infused and finally how long it took forearm blood flow to return to baseline levels once the adenosine infusion stopped.

5.2.2 Arterial infusion monitoring and training

For one week I attended the Clinical Research Centre at the Western General Hospital, Edinburgh which frequently uses the ultra-fine bore needle infusion technique to study human forearm blood flow responses. During the week I inserted 5 arterial needles under supervision and monitored them for difficulties during the subsequent studies. The head of the laboratory (Prof. D J Webb) attended Zeneca to supervise the use of arterial needles during the pilot study.

5.2.3 Pilot study design

The doses of sodium nitroprusside (SNP) and adenosine infused were chosen by reference to previous studies in which these drugs had been infused into the brachial artery in man[121,122,226,232]. Flow profiles during one of these previous adenosine infusion studies suggested that 5 minutes was likely to be sufficient for forearm blood flow to have achieved a steady state value[232]. Another study indicated that 3 minutes would be sufficient for FBF to reach equilibrium during infusion of SNP, although in their study a lower dose was used[237].

The needle was inserted into the brachial artery 45 minutes before first FBF measurements to

allow time for the equipment to be applied and tested and to allow some further undisturbed time for the volunteers to recover after preparation.

Adenosine was infused twice as in the reproducibility study I intended to assess whether an average of two FBF responses to adenosine would reduce variability. Twenty minutes was allowed between adenosine infusions for recovery as this was as much time as otherwise the protocol would be longer than 2 hours. Two hours was felt to be the maximum time for which volunteers could reasonably be required to keep the needle in. Baseline flow was measured at 4 points in the protocol: just prior to the first adenosine infusion, 7 and 15 minutes after the first adenosine infusion stopped and finally 7 minutes after the second adenosine infusion. In this way baseline flow was measured before each adenosine or SNP infusion, allowing a 2 minute rest prior to the measurements taken during each infusion. The additional measurement was taken starting 7 minutes after the first adenosine infusion to discover whether baseline forearm blood flow had been re-attained earlier than 15 minutes. The exact timing of measurements and infusions is shown in Table 5.1.

Twelve lead ECGs were taken immediately before and after each study and compared to previous ones and standard lead II was continuously monitored during the infusions. Pulse and blood pressure was recorded using a mercury sphygmomanometer before and after each infusion of adenosine or SNP. Any symptoms were recorded at the same time as the pulse and blood pressure were measured.

All drug solutions were made up so the final delivery rate could be attained by infusing the diluted drug at 1ml/min. When adenosine or SNP were not being infused then normal saline was

infused at 1ml/min as the infusion control and to prevent blood refluxing and clotting inside the arterial needle.

Once the needle had been taken out pressure was applied to the site by hand for 10 minutes. The volunteer was then kept under observation for 2 hours and allowed home once the site had been checked.

Table 5.1 Pilot study timing of measurements and interventions

Elapsed time (minutes)	FBF/TEB	Supine pulse & BP & Symptom Check	Infusion
0-45			Normal Saline
45-48	+		Normal Saline
48-50		+	Normal Saline
50-55	+		50μg/min Adenosine
55-57		+	Normal Saline
57-62			Normal Saline
62-65	+		Normal Saline
65-70			Normal Saline
70-73	+		Normal Saline
73-75		+	Normal Saline
75-80	+		50μg/min Adenosine
80-82		+	Normal Saline
82-87			Normal Saline
87-90	+		Normal Saline
90-92		+	Normal Saline
92-95	+		10μg/min SNP

5.2.4 Pilot study volunteer characteristics

Six healthy male volunteers aged 18-62, within 20% of their ideal weight (Metropolitan Life) and 60-100kg in weight were selected . They had no significant abnormalities on clinical examination, in particular their resting blood pressure was neither greater than 140/90mmHg nor less than 100/70mmHg and there was no evidence of atherosclerosis. Their urea, electrolytes, liver function tests, random lipid profiles, random blood sugar, blood count and urinalysis were within acceptable limits. Each had a normal 24 hour ECG carried out within the past year. They were excluded if they were on regular medication, had an acute illness within 2 weeks of the study day or took more than 21 units of alcohol per week.

The mean age of the volunteers was 32 years (range 24 to 42 years), mean height 1.74m (range 1.66 to 1.84m) and means weight 76kg (range 68 to 86kg). All volunteers were Caucasian.

5.2.5 Pilot study volunteer restrictions

Volunteers were required to abstain from smoking or caffeine containing food or drink for 40 hours prior to the study, from alcohol for 72 hours and from any medication (without specific approval) for 24 hours. They took the day off work and did not return after the study. They were asked not to undertake heavy exercise following the study.

5.2.6 Preparation of adenosine and sodium nitroprusside (SNP)

All solutions were prepared just prior to use. Adenosine is supplied as 2ml sealed glass ampoules containing 3mg/ml (Adenocor™: Sanofi-Winthrop). Normal saline supplied as 100ml bottles (Phoenix pharmaceuticals) was used to dilute the adenosine. The adenosine solution was first diluted 10 fold by mixing 1ml of the original solution with 9mls of normal saline. Two

millilitres of the resulting solution was further diluted by mixing with a further 10mls of normal saline to yield a solution containing 50ug/ml of adenosine which was drawn up into a 60ml, infusion pump syringe, labelled with the concentration, date and time, and stored at 4°C ready for use.

Sodium nitroprusside is supplied as powder in sealed ampoules containing 50mg (Nipride™: Roche). This was first diluted using 2mls of 5% dextrose and a further 3mls of normal saline to produce a stock solution of 10mg/ml. 0.5mls of this stock solution was diluted 1000 fold by addition to a 500ml bag of normal saline from which 0.5mls had already been removed. This produced a final solution concentration of SNP of 10mg/ml. Sixty millilitres of this was drawn up into a syringe suitable for the infusion pump, labelled, wrapped in foil (as SNP is light sensitive) and stored at 4°C until used.

5.2.7 Pilot study results

5.2.7.1 Missing data

The needle was noted to have occluded following the third baseline reading on volunteer 3, hence the study was abandoned at that stage. The needle came out of the artery following the first baseline measurement on volunteer 6 and so the study was abandoned at that stage.

5.2.7.2 Room temperature

The minimum room temperature ranged from 22-24°C and the maximum room temperature ranged from 23 to 24°C.

5.2.7.3 Transthoracic electrical bioimpedance (TEB)

Mean results were recorded every minute during forearm blood flow measurements. Stroke index (SI), cardiac index (CI) and heart rate (HR) have been analysed. There were 3 recordings during each of the 4 baseline measurements and these were meaned to produce just one result for each baseline period. As there may be trends in the recordings taken during adenosine and SNP infusions these results were not meaned together. Figures 5.1 to 5.3 summarise the results, showing the mean values for all volunteers and also the between-volunteer standard deviation as a measure of variability for the 4 TEB indices.

There is no discernible change in any of the indices either during intra-arterial infusion of 50μg/min of adenosine or intra-arterial infusion of 10μg/min of SNP.

Figure 5.1 Stroke index during intra-arterial infusions

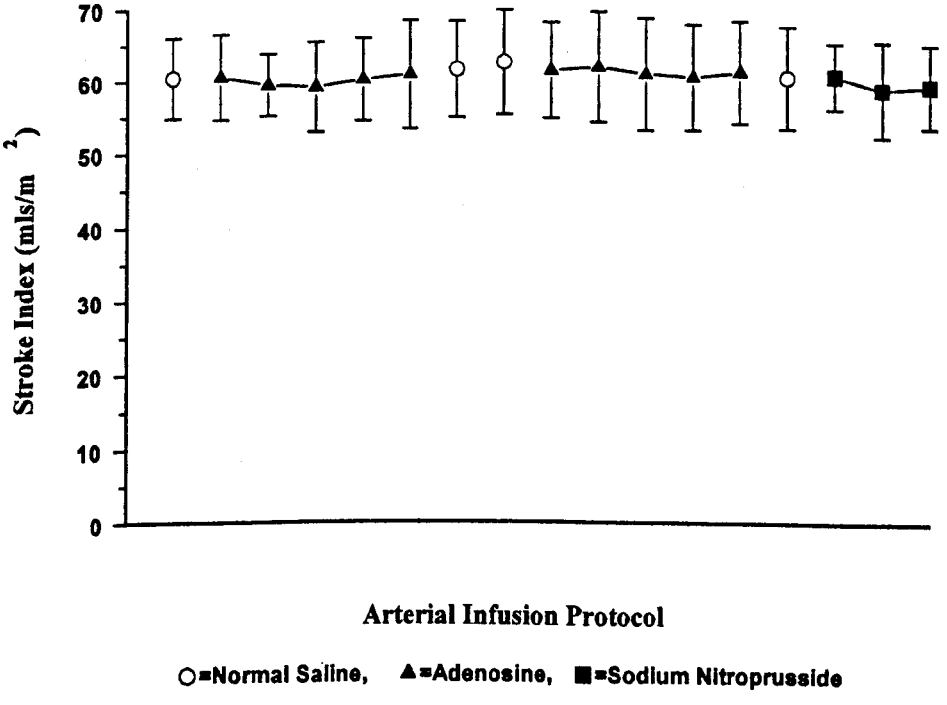


Figure 5.2 Cardiac index during intra-arterial infusions

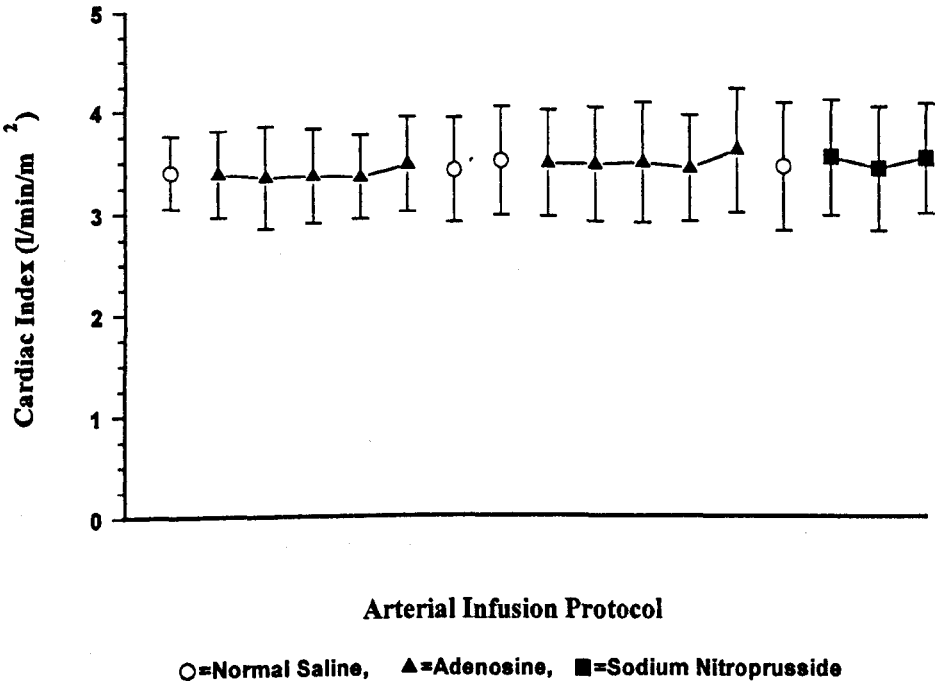
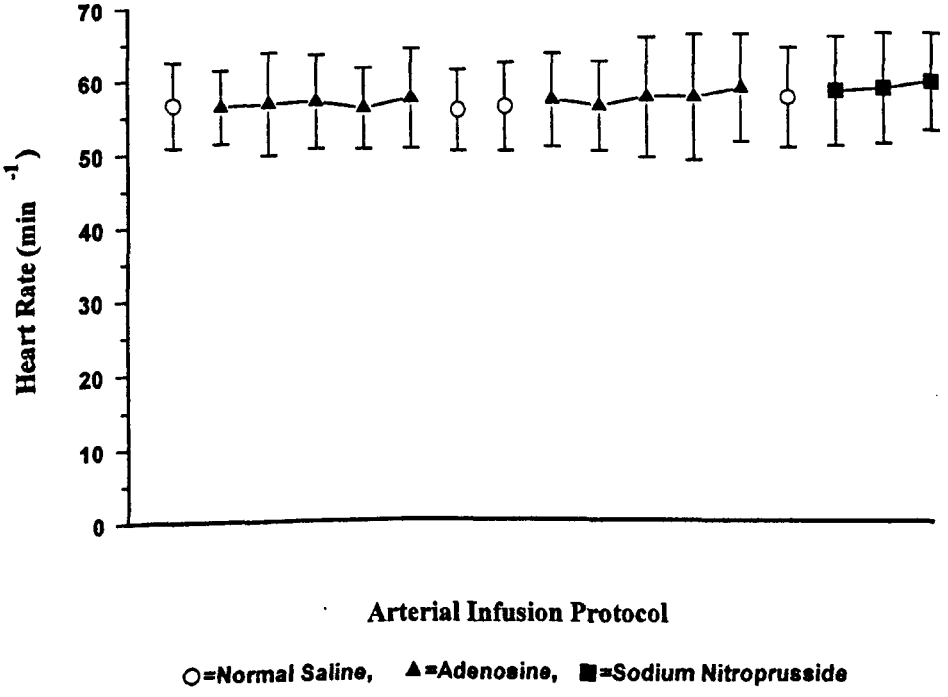


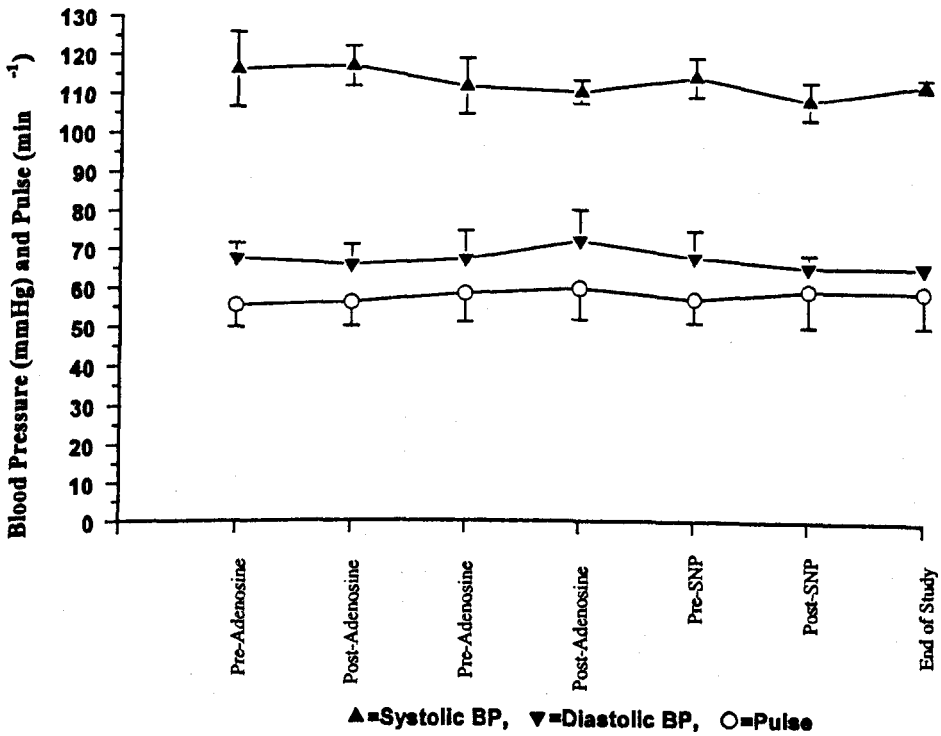
Figure 5.3 Heart rate during intra-arterial infusions



5.2.7.3 Pulse and blood pressure

Neither intra-arterial infusion of 50ug/min of adenosine nor infusion of 10mg/min of SNP has any discernible effect on pulse and blood pressure as can be seen in figure 5.4 below.

Figure 5.4 Time profile of pulse and blood pressure



5.2.7.5 Forearm blood flow

Table 5.2 lists both control and active forearm baseline blood flows for each volunteer and the overall means for each baseline. The four baselines were recorded before the first adenosine infusion, starting 7 minutes after the adenosine stopped, starting 15 minutes after the adenosine infusion ceased and starting 7 minutes after the second adenosine infusion stopped.

Table 5.2 Baseline forearm blood flows

mls/100mls/min	Active Forearm Baselines				Control Forearm Baselines			
	1	2	3	4	1	2	3	4
Volunteer 1	2.42	4.36	3.88	3.32	3.09	2.94	2.64	2.71
Volunteer 2	4.57	6.43	5.47	5.88	2.77	3.27	3.48	3.75
Volunteer 3	8.47	14.63	10.20		3.48	2.93	6.22	
Volunteer 4	3.20	3.85	3.41	2.72	3.13	2.79	2.76	2.74
Volunteer 5	4.42	5.36	5.48	7.88	2.51	2.11	2.67	2.52
Volunteer 6	4.41				3.43			
Mean	4.48	6.92	5.69	4.95	3.07	2.81	3.55	2.93

No statistical analysis was carried out on the baseline FBFs. Figures 5.5 to 5.9 show the FBF profiles during the first and second adenosine infusions and during infusion of SNP. FBFs are expressed as excess flows, ie with the preceding mean baseline FBF subtracted. For comparison the control FBFs are also shown, again expressed as excess FBF. Figure 5.10 shows active and control FBFs meaned for the 5 volunteers completing the study.

Figure 5.5 Volunteer 1

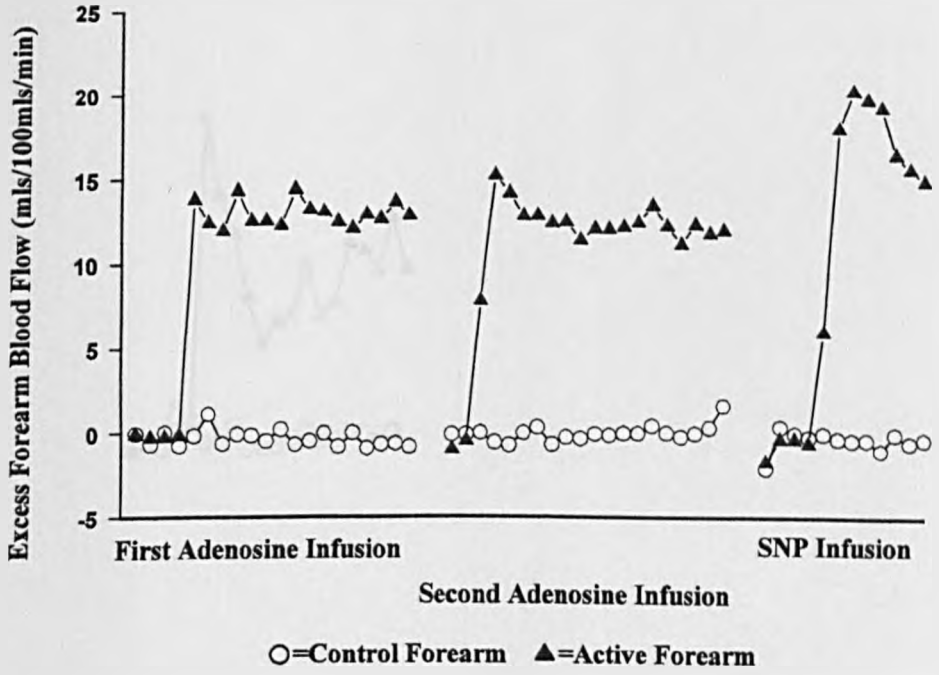


Figure 5.6 Volunteer 2

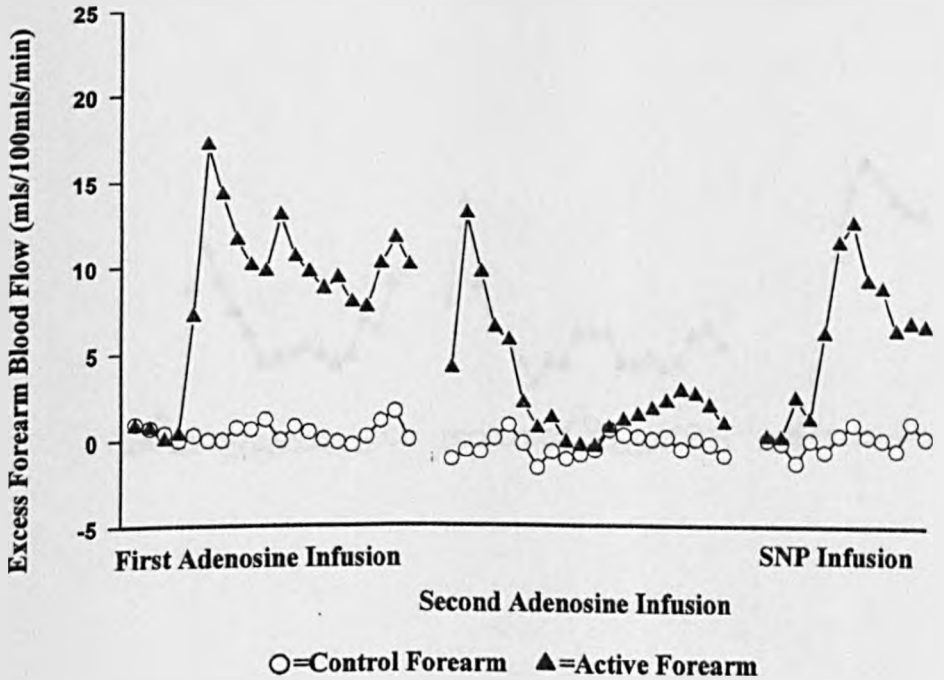


Figure 5.7 Volunteer 3

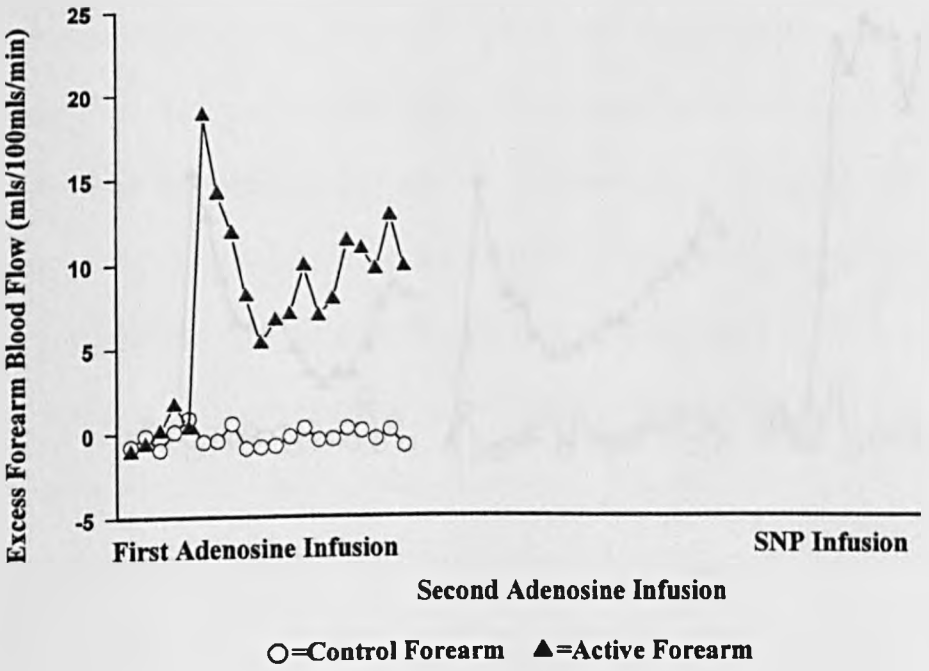


Figure 5.8 Volunteer 4

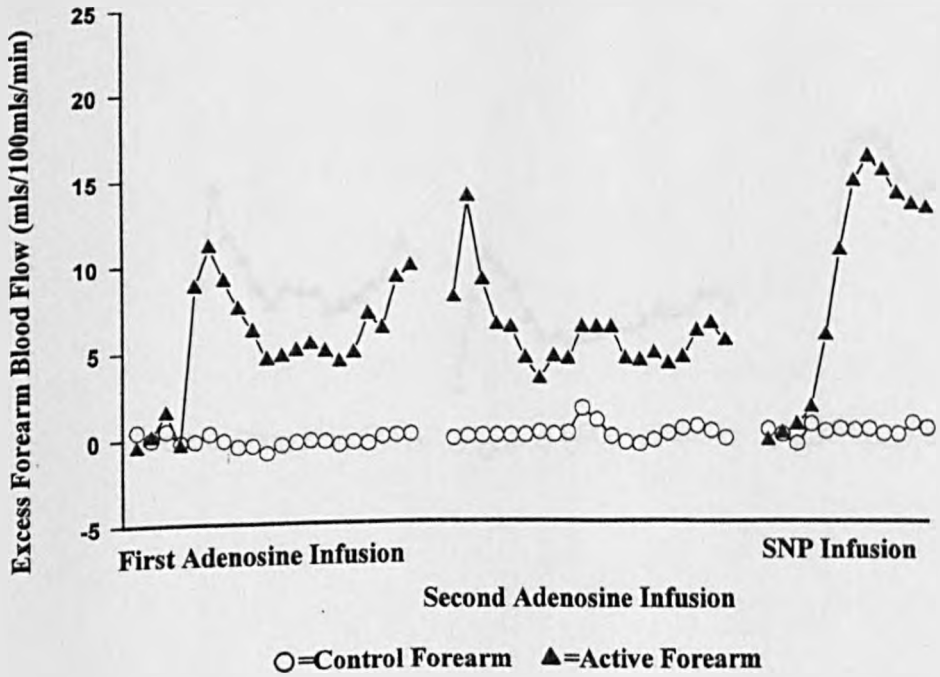


Figure 5.9 Volunteer 5

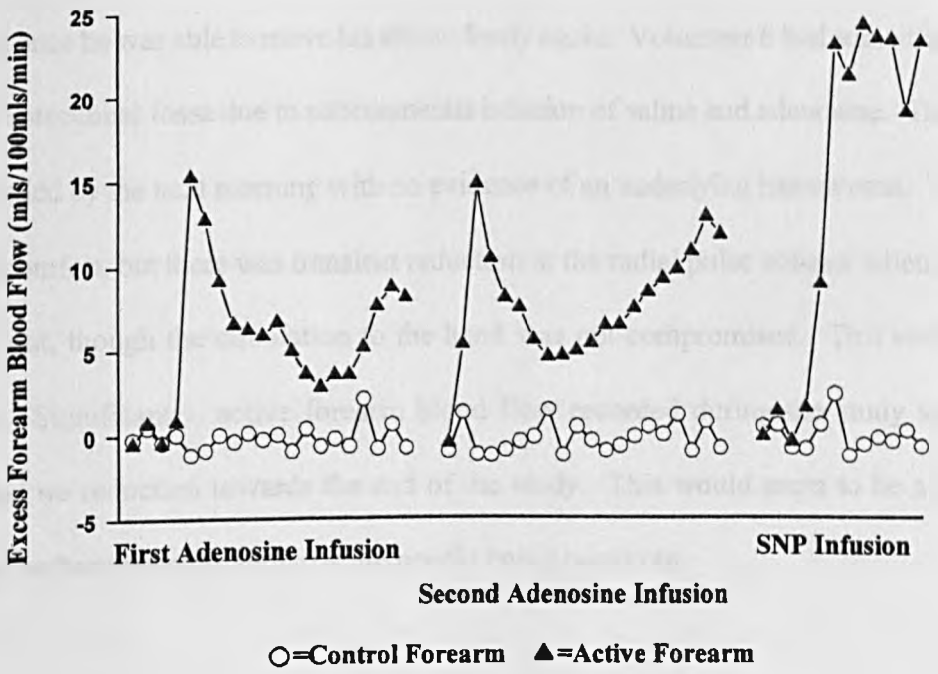
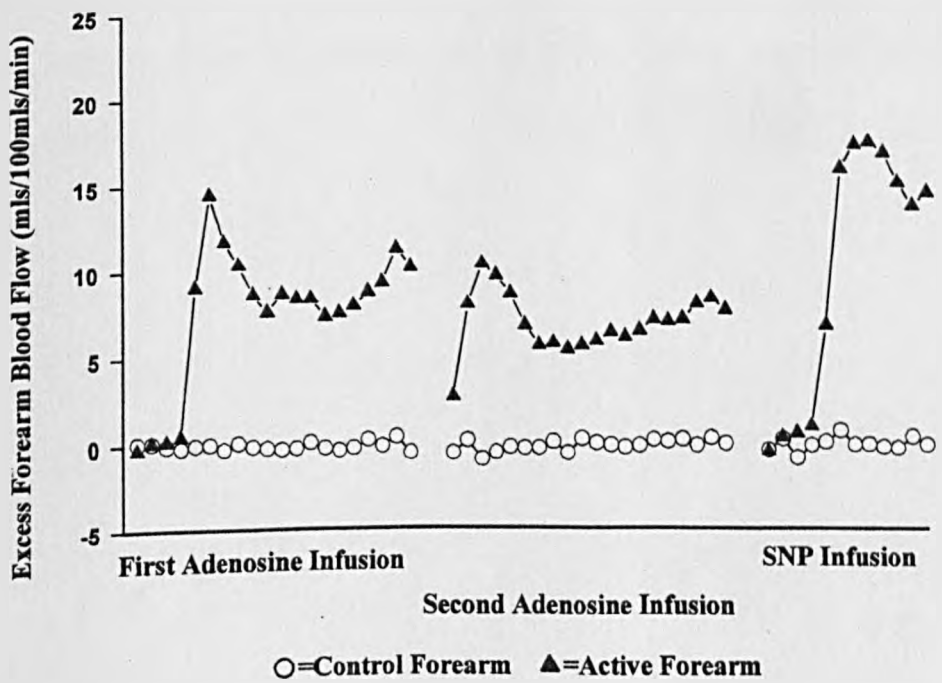


Figure 5.10 Mean forearm blood flow profiles



5.2.7.6 Adverse events

Three adverse events were recorded. One volunteer mentioned arthralgia of one elbow which settled once he was able to move his elbow freely again. Volunteer 6 had some transient swelling in the antecubital fossa due to subcutaneous infusion of saline and adenosine. This was painless and settled by the next morning with no evidence of an underlying haematoma. Volunteer 1 had no discomfort, but there was transient reduction in the radial pulse volume when the needle was taken out, though the circulation to the hand was not compromised. This settled within two hours. Significantly, active forearm blood flow recorded during the study was normal and showed no reduction towards the end of the study. This would seem to be a case of arterial spasm perhaps brought about by the needle being taken out.

5.2.8 Summary

Two volunteers experienced adverse effects following arterial infusions. Both of these were transient and resolved spontaneously. Despite my relative inexperience in arterial needling I was able to use the technique with reasonable safety.

There was no evidence of any systemic effect of the doses of adenosine and SNP employed: the TEB indices showed no measurable change during the infusions, neither did pulse and blood pressure and finally the control FBF was static throughout. There was evidence of residual vasodilatation after the first adenosine infusion as both second and third baseline flows were consistently higher than the first baseline flow. There is also a consistent difference in the first and second adenosine infusion time profiles in that there was a minute's delay before vasodilatation began when adenosine was first infused, whereas the second time vasodilatation was immediate. Adenosine and SNP infusions caused an initial peak vasodilatation followed by a plateau. FBF reaches a plateau by the third minute of infusion even during the first adenosine infusion. It is uncertain whether FBF has reached an equilibrium by the third minute of the SNP infusion, however since it is planned to use the SNP as a positive control this is unlikely to be of importance.

5.3 Main study - reproducibility of adenosine-induced forearm vasodilatation

5.3.1 Aims

It is worth re-stating the aims of this study. As before I needed to measure the reproducibility of adenosine-induced vasodilatation when adenosine is infused in increasing amounts so that the number of volunteers required to study adenosine antagonism by theophylline could be estimated. Additionally I wanted to determine whether it is better to increase the rate at which adenosine is infused by changing syringes in the infusion pump, using different concentration of adenosine (as many groups do) or by progressively increasing the infusion rate. To do this I needed to incorporate infusion rate controls to determine whether changes in infusion rate had any measurable effect on forearm blood flow. I also wanted to get some idea of how long it takes for forearm blood flow to settle to basal levels after insertion of the arterial needle. Finally I wanted to find out whether repeating the adenosine infusions could be used as a means of improving reproducibility.

5.3.2 Design

This was an open study to assess between-day reproducibility of adenosine-induced vasodilatation. Other groups have carried out arterial needling repeatedly on the same volunteer without adverse effect, but one week is regarded as a workable minimum to allow between needle insertions into the same brachial artery (personal communication Prof D J Webb). We carried out the same infusion protocol on 10 volunteers twice allowing one week between studies. Each time we used the same forearm if possible, preferably the non-dominant forearm and each volunteer attended at the same time on both weeks.

Previous studies indicated that the lowest dose of adenosine with any discernible effect when

infused into the brachial artery was 5μg/min. The pilot study confirmed that 50μg/min was a reasonable maximum dose. In earlier studies on reactive hyperaemia (Chapter 3) volunteers had experienced hand ischaemia for 9 minutes. Prof Webb advised that his group had measured FBF continuously in volunteers for upto 15 minutes without causing undue discomfort. From the pilot study it was apparent that the shortest period that adenosine could be infused for was 3 minutes. Measuring FBF during 3 incremental doses of adenosine, allowing for wrist cuff inflation for 1 minute prior to FBF measurements would only require hand ischaemia for 10 minutes, comfortably within the 15 minute guideline.

As the increasing infusion rate was going to be handled as a dose-response curve it was decided to increase the doses in half-logarithm increments. The doses chosen were 5, 15 and 50μg/min. The same dose of SNP (10mg/min) was chosen for this study, again infused over 3 minutes.

The period between adenosine infusions was increased to 30 minutes to allow longer for FBF to settle to basal levels and in the hope that the second infusion would not be perturbed by the first. Prior to the first series of adenosine infusions a series of 3 saline infusions were carried out. Saline was infused at exactly the same rate that the adenosine was subsequently given, so that any differences in FBF caused by the increases in infusion rate could be measured and if necessary these saline infusions could be used as the baseline FBF values when analysing the adenosine infusion FBF data. Baseline FBF with normal saline infused at 1ml/min was measured twice prior to the infusion rate controls starting 7 and 17 minutes after insertion of the arterial needle. Further baselines were measured starting 5 minutes before each of the adenosine infusions and the SNP infusion to check whether baseline FBF had been re-attained prior to the vasodilator infusions and if so to be used as a suitable baseline for the subsequent infusion to minimise

temporal effects. On the advice of Prof Webb further baseline measurements were carried out taken over the 2 minutes prior to the adenosine and SNP infusions with no rest in between. The period of continuous hand ischaemia would be increased now to 12 minutes. Whilst these baseline FBF measurements would not be as precise (see Chapter 3) as they incorporated only 8 rather 12 repeated measurements, it was hoped they would be a more accurate baseline as they were conducted immediately prior to the drug infusions. The protocol incorporating these features is summarised in Table 5.3.

Standard lead II was monitored continuously during the protocol and fresh 12 lead ECGs were checked at the beginning and end of the protocol. A maximum and minimum thermometer was read and reset at the beginning of the protocol and read at the end. Symptoms were recorded at the beginning and end of the protocol and volunteers were required to remain for 2 hours after the needle had been taken out so that they could be monitored for adverse events. Pulse and blood pressure were recorded twice a minute apart using a mercury sphygmomanometer and averaged together as in the pilot study. They were recorded immediately before and after infusions of adenosine or SNP. Unlike the pilot study no additional, end of protocol measurements were taken. As in previous studies, blood pressure was adjusted for the circumference of the arm using the Gaged™ cuff.

Table 5.3 Timing of measurements and interventions

Time(mins)	Infusate	Rate (mls/min)	FBF/TEB	Pulse/BP
0	Normal Saline	1		+
7-10	Normal Saline	1	+	
10-17	Normal Saline	1		
17-20	Normal Saline	1	+	
20-22	Normal Saline	1		+
22-25	Normal Saline	0.16	+	
25-28	Normal Saline	0.48	+	
28-31	Normal Saline	1.6	+	
31-33	Normal Saline	1		
33-36	Normal Saline	1	+	
36-38	Normal Saline	1		+
38-40	Normal Saline	1	+	
40-43	Adenosine	0.16	+	
43-46	Adenosine	0.48	+	
46-49	Adenosine	1.6	+	
49	Normal Saline	1		+
49-74	Normal Saline	1		
74-77	Normal Saline	1	+	
77-79	Normal Saline	1		+
79-81	Normal Saline	1	+	
81-84	Adenosine	0.16	+	
84-87	Adenosine	0.48	+	
87-90	Adenosine	1.6	+	
90	Normal Saline	1		+
90-95	Normal Saline	1		
95-98	Normal Saline	1	+	
98-100	Normal Saline	1		+
100-102	Normal Saline	1	+	
100-103	SNP	1	+	
103-104	SNP	0		+

5.3.3 Volunteer characteristics

The inclusion and exclusion criteria were identical to those of the pilot study (5.3.3) Ten healthy male volunteers were selected from the Zeneca volunteer panel mean age 32 years (range 21 to 43 years), mean height 1.76m (range 1.70 to 1.86m) and mean weight 73kg (range 61 to 84kg). All volunteers were Caucasian.

5.3.4 Volunteer restrictions

The volunteer restrictions were identical to those imposed for the pilot study (5.2.5), but additionally the volunteers were required to take a light breakfast at home 2 hours before their study was due to start and to eat and drink the same food and drink at the same time on both study days.

5.3.5 Preparation of adenosine and sodium nitroprusside infusions

To minimise the time taken for solutions to clear the deadspace a more dilute solution of adenosine was used than in the pilot study so that it could be infused at a greater rate. The infusion pump would only deliver upto 99mls/hour so a maximum infusion rate of 1.6mls/min (or 96mls/hour) was chosen. Therefore to deliver 50μg/min, the maximum adenosine concentration, a solution of 31.25mg/ml was needed. Adenosine is supplied as a solution containing 3mg/ml (AdenocorTM: Sanofi-Winthrop). As in the pilot study 1ml of this is diluted to produce a 300μg/ml solution. Five millilitres of this were further diluted with 43mls of normal saline producing the desired final solution strength of 31.25μg/ml. This was infused at 0.16, 0.48 and 1.6mls/min corresponding to adenosine infusion rates of 5, 15 and 50μg/min.

A solution of 10μg/ml sodium nitroprusside was made up as described for the pilot study (5.2.6).

As in the pilot study, solutions were made up fresh prior to each study, drawn up into 60ml luer-lock syringes suitable for the infusion pump and stored at 4°C until use. Care was taken to wrap the syringe containing the SNP solution in foil to protect it from light.

5.3.6 Results

5.3.6.1 Missing data

Blood refluxed into the steel needle and occluded it when changing from syringe during the second study day with volunteer 6. No further measurements were made after the 2 minute baseline immediately prior to the first series of adenosine infusions. Data from the 2 minute baseline FBF and TEB measurements were not processed. Because of the low initial infusion rate of the adenosine solution it was necessary to start the infusion earlier to allow clearance of the deadspace. Unfortunately, if blood refluxed into the needle when syringes were changed over, this low rate could not be relied upon to clear the blood within 30 seconds to prevent clotting within the needle. Under these circumstances it was necessary to flush the needle manually with resulting uncertainty about the exact time that adenosine entered the circulation casting doubt on the validity of the baseline readings. Similar problems were encountered with SNP, though less frequently.

5.3.6.2 Room temperature

The mean initial room temperature was 23°C, range 22 to 24°C and the minimum temperature was also 23°C maximum 26°C. The maximum increase in temperature during any session was 2°C. The mean temperatures recorded during day 1 and day2 were the same.

5.3.6.3 Transthoracic electrical bioimpedance (TEB)

Figures 5.12 to 5.14 show the trends in TEB indices during the intra-arterial infusion protocol, showing the first day (open symbols) and the second day separately. There were 5 baseline readings during the protocol, each lasting 3 minutes. These three readings have been averaged together for both days for each volunteer. These average baseline readings are shown as single points (circles). Also shown as circles are the readings taken during the saline infusion-rate control infusions. These are shown as 3 linked circles corresponding to infusion rates of 0.16, 0.48 and 1.6mls/min in that order.

Measurements during the adenosine infusions (triangles) and during the SNP infusion (squares) have not been averaged together as it is conceivable that there might be a trend over time during the 3 minutes that the infusion rate is held constant. For this reason there are 9 linked measurements during the 2 adenosine infusions comprising 3 readings taken during each of the three infusion rates of adenosine (5, 15 and 50μg/min) and similarly there are 3 readings taken during the SNP infusion.

Figure 5.11 Stroke index during intra-arterial infusions

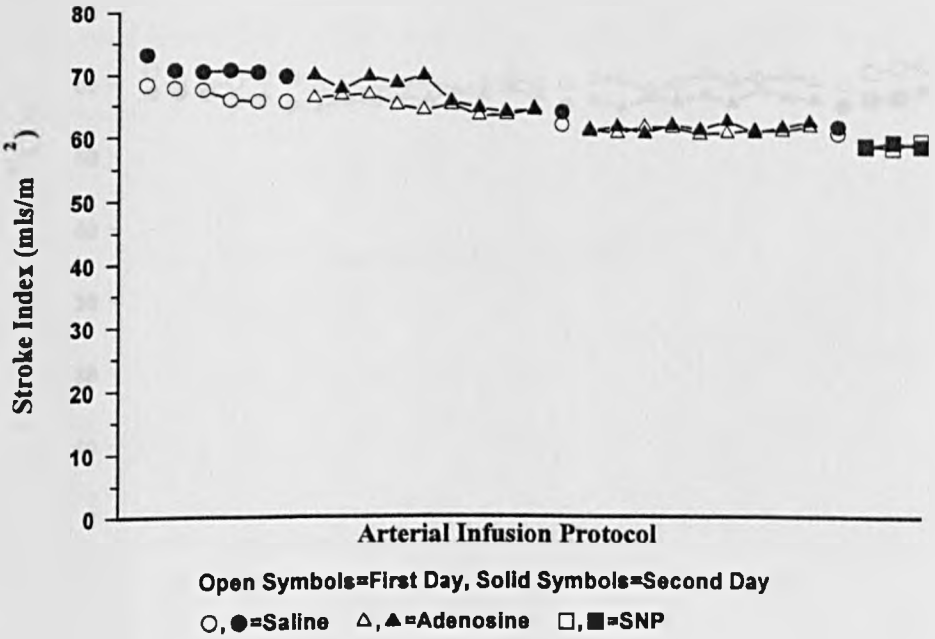


Figure 5.12 Cardiac index during intra-arterial infusions

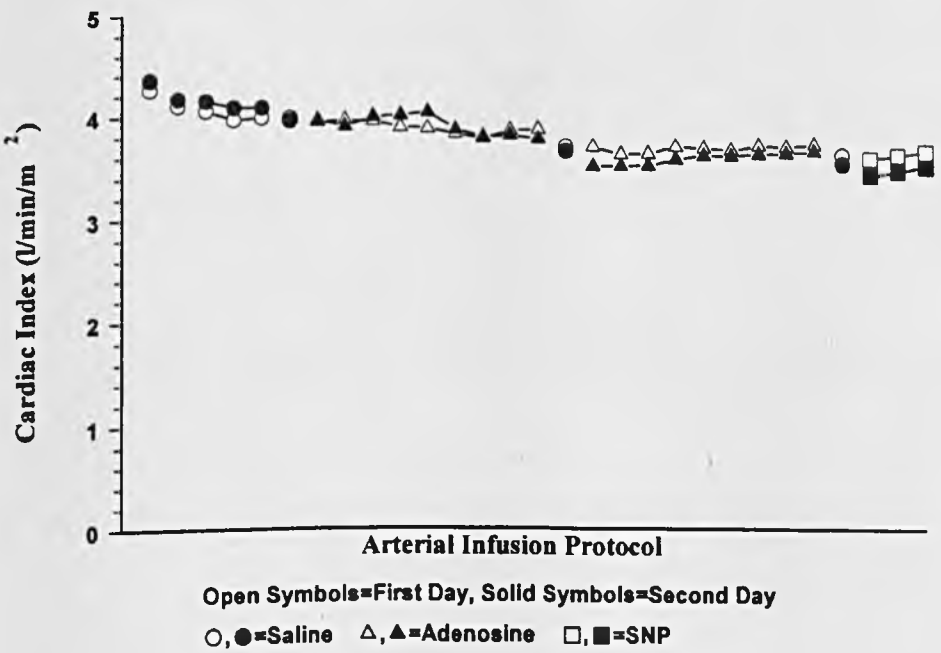
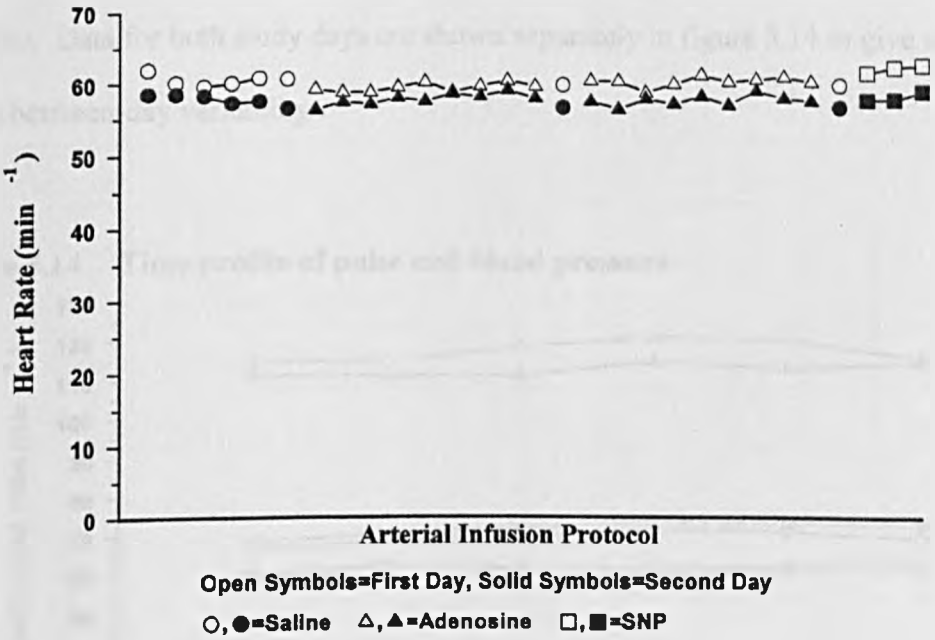


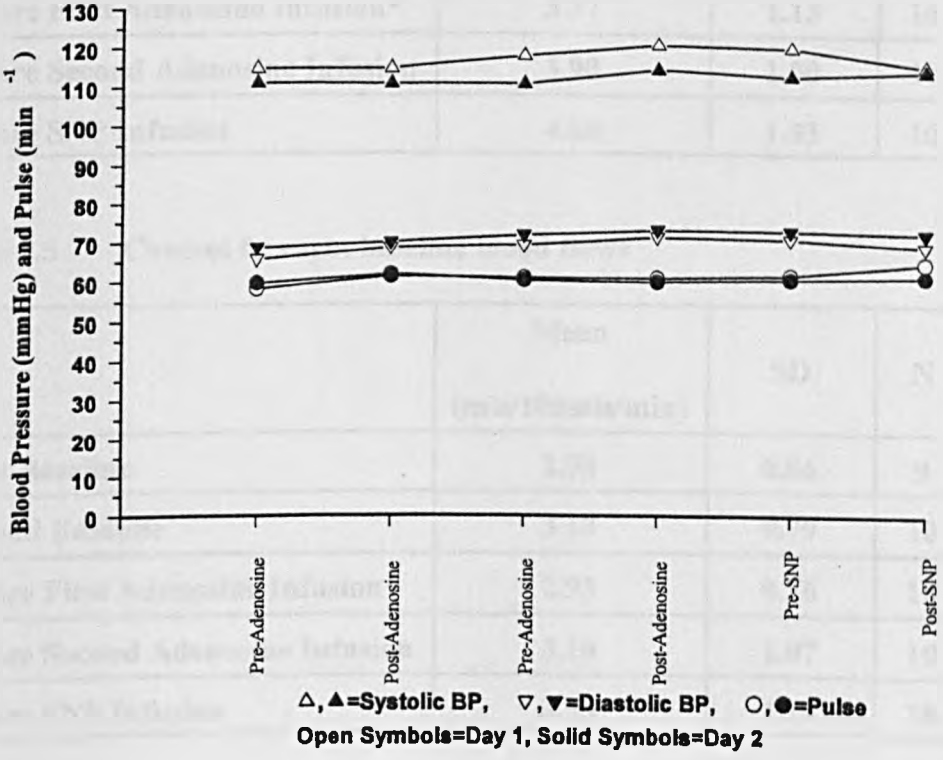
Figure 5.13 Heart rate during intra-arterial infusions



5.3.6.4 Pulse and blood pressure

As can be seen below, neither adenosine nor SNP had any discernible effect on pulse or blood pressure. Data for both study days are shown separately in figure 5.14 to give some indication of the between-day variability .

Figure 5.14 Time profile of pulse and blood pressure



5.3.6.5 Forearm blood flow

5.3.6.5.1 Baseline forearm blood flow

The mean baseline FBFs measured during the protocol in the infused and control forearms are shown in tables 5.4 and 5.5. Mean FBFs for each volunteer and protocol point were derived by averaging results for the two study days prior to statistical analysis. I calculated P values for differences between these baseline FBFs and the baseline FBF measured just prior to the first adenosine infusion ANOVA allowing for volunteer effects.

Table 5.4 Infused forearm baseline blood flows

	Mean (mls/100mls/min)	SD	N	P (vs *)
First Baseline	3.20	1.10	8	0.80
Second Baseline	3.31	1.01	10	0.86
Before First Adenosine Infusion*	3.37	1.13	10	*
Before Second Adenosine Infusion	3.90	1.50	10	0.17
Before SNP Infusion	4.88	1.93	10	0.0003

Table 5.5 Control forearm baseline blood flows

	Mean (mls/100mls/min)	SD	N	P (vs *)
First Baseline	2.95	0.86	9	0.93
Second Baseline	3.13	0.79	10	0.35
Before First Adenosine Infusion*	2.93	0.76	10	*
Before Second Adenosine Infusion	3.10	1.07	10	0.43
Before SNP Infusion	3.33	1.14	10	0.07

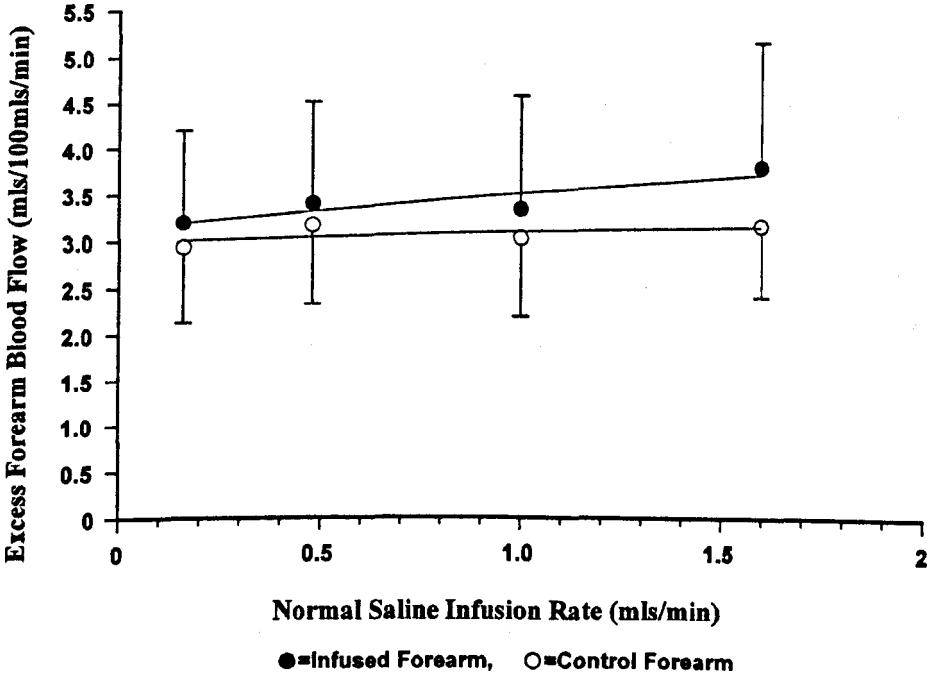
Table 5.6 Comparison of infused and control FBFs by study day

		Mean (mls/100mls/min)	SD	N	P
INFUSED FOREARM	Day 1	3.73	1.49	10	0.0001
	Day 2	2.87	0.80	10	
CONTROL FOREARM	Day 1	3.16	1.16	10	0.05
	Day 2	2.81	0.78	10	

There is a marked reduction in baseline FBF on study day 2 compared to study day 1 especially in the infused forearm (table 5.6).

5.3.6.5.2 Normal saline control data

Figure 5.15 Infusion rate control data



The gradient of the line fitted to the FBF measured in the infused forearm in response to increasing normal saline infusion rates is 0.37 per minute, which is not significantly different from zero (two-tailed P=0.11, r=0.89). The gradient of the line fitted to the control FBFs is smaller 0.10 per minute (two-tailed P=0.42, r=0.57). Another way of addressing the issue of whether there is a significant change in FBF in the infused forearm when the infusion rate changes is to compare FBF directly. As the issue is whether to use the first pre-adenosine baseline, when saline was infused at 1ml/min or to use the saline infusion-rate control baseline appropriate for each adenosine infusion rate the comparisons needed would be the mean of the

two 1ml/min baseline flows straddling the 3 saline infusion-rate controls in the protocol versus the mean flows recorded when saline was infused at 0.16, 0.48 and 1.6mls/min. As this entails 3 comparisons the level at which a P value can be held to be significant should be reduced to 0.017(Bonferroni)[238].

Table 5.7 Effect of saline infusion rate on infused forearm blood flow

Rate of Infusion mls/min	Mean (mls/100mls/min)	SD	N	P (vs *)
0.16	3.20	1.11	10	0.25
0.48	3.40	1.22	10	0.62
1.00*	3.34	0.99	10	*
1.6	3.81	1.36	10	0.0004

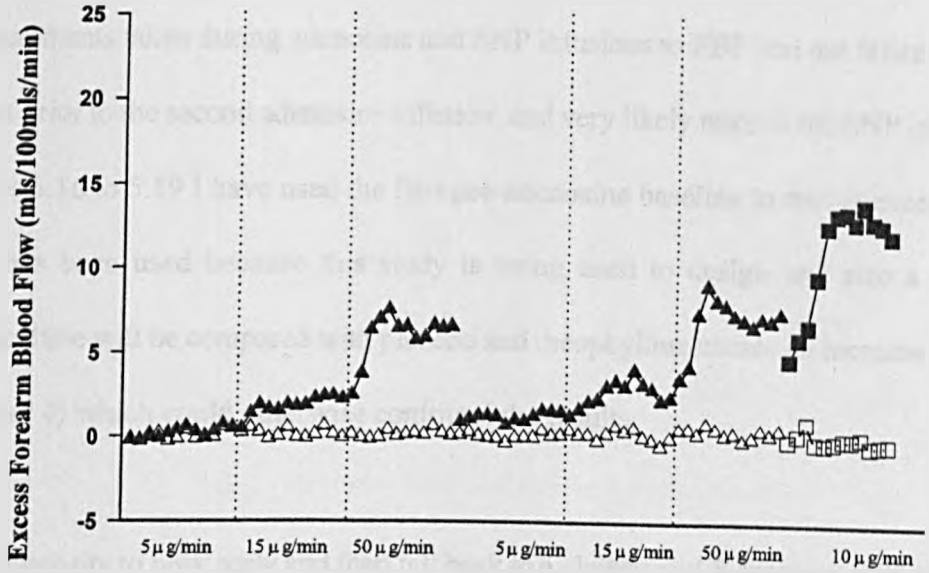
Table 5.8 Effect of saline infusion rate on control forearm blood flow

Rate of Infusion mls/min	Mean (mls/100mls/min)	SD	N	P (vs *)
0.16	2.94	0.81	10	0.19
0.48	3.17	0.84	10	0.06
1.00*	3.03	0.77	10	*
1.6	3.18	0.76	10	0.04

From table 5.7 it is apparent that there is a small, but significant change in flow when the normal saline infusion rate is increased from 1 to 1.6mls/min. Control forearm data are shown in table 5.8. Interestingly there is a small increase in control FBF detected when the infusion rate in the contralateral forearm is increased to 1.6mls/min. This does not reach significance under the modified criteria.

5.3.6.5.3 Adenosine and sodium nitroprusside (SNP) infusion data

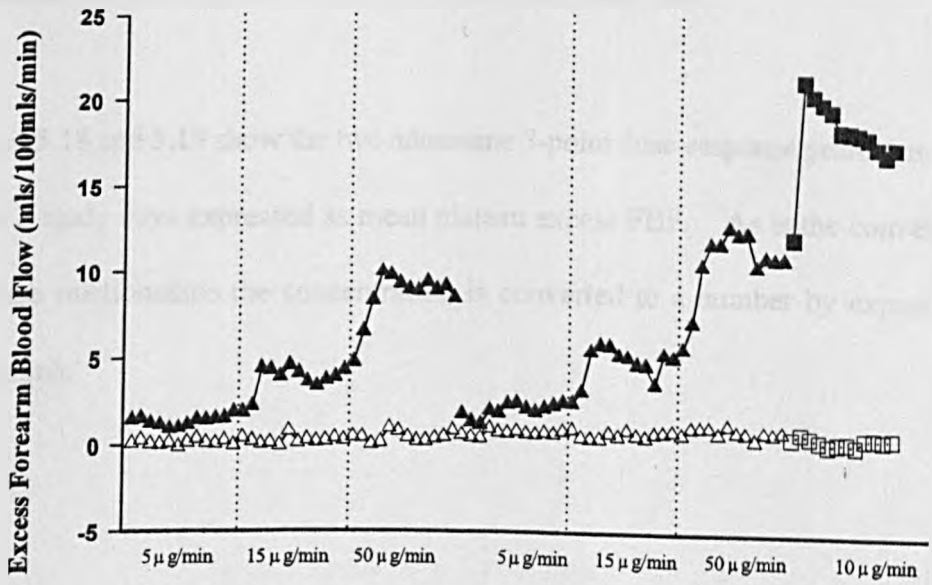
Figure 5.16 Adenosine and SNP infusions on the first study day



▲,△=Adenosine Infusions, ■,□=SNP Infusion

Solid Symbols=Infused Forearm, Open Symbols=Control Forearm

Figure 5.17 Adenosine and SNP infusions on the second study day



▲,△=Adenosine Infusions, ■,□=SNP Infusion

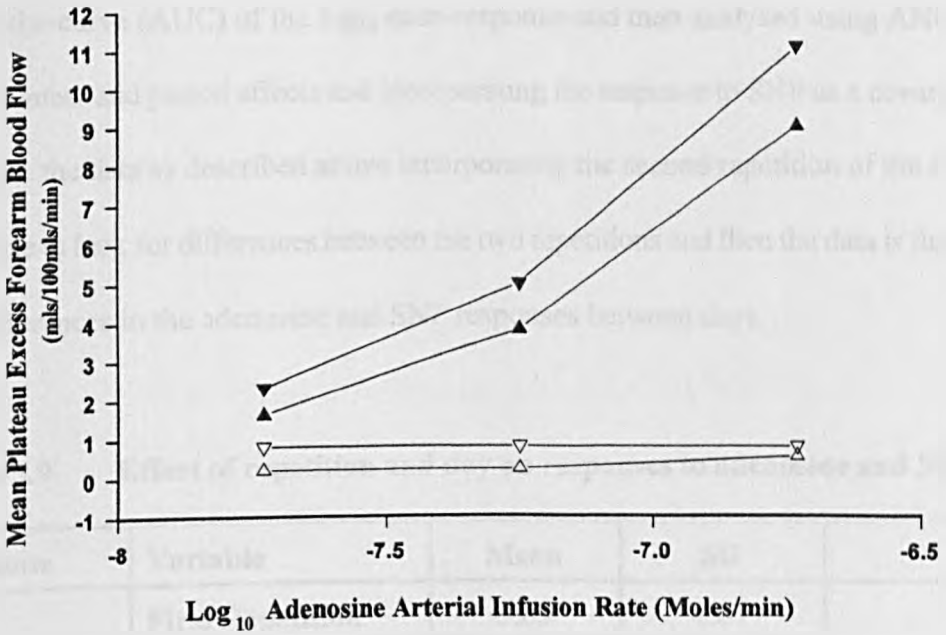
Solid Symbols=Infused Forearm, Open Symbols=Control Forearm

Figures 5.18 and 5.19 show the time profiles of excess forearm blood flow in response to the 3 dose rates of adenosine, given twice and to SNP. The 3 minute baseline measurement starting 5 minutes before the first adenosine infusion was used as the baseline for all subsequent FBF measurements taken during adenosine and SNP infusions as FBF had not fallen back to baseline values prior to the second adenosine infusion and very likely prior to the SNP infusion also. For figures 5.16 to 5.19 I have used the first pre-adenosine baseline to derive excess FBF. Excess FBF has been used because this study is being used to design and size a study in which theophylline will be compared with placebo and theophylline causes an increase in baseline FBF (chapter 4) which could otherwise confound the results.

The propensity to peak early and then fall back to a plateau is still apparent following the increase to 50μg/min of adenosine, but less marked than in the pilot study. Mean plateau readings were derived by meaning the final 4 readings during any three minute infusion of adenosine or SNP. As a result of this a plateau is reached earlier, by around the 7th reading. The responses to adenosine and SNP appear reduced on the second study day.

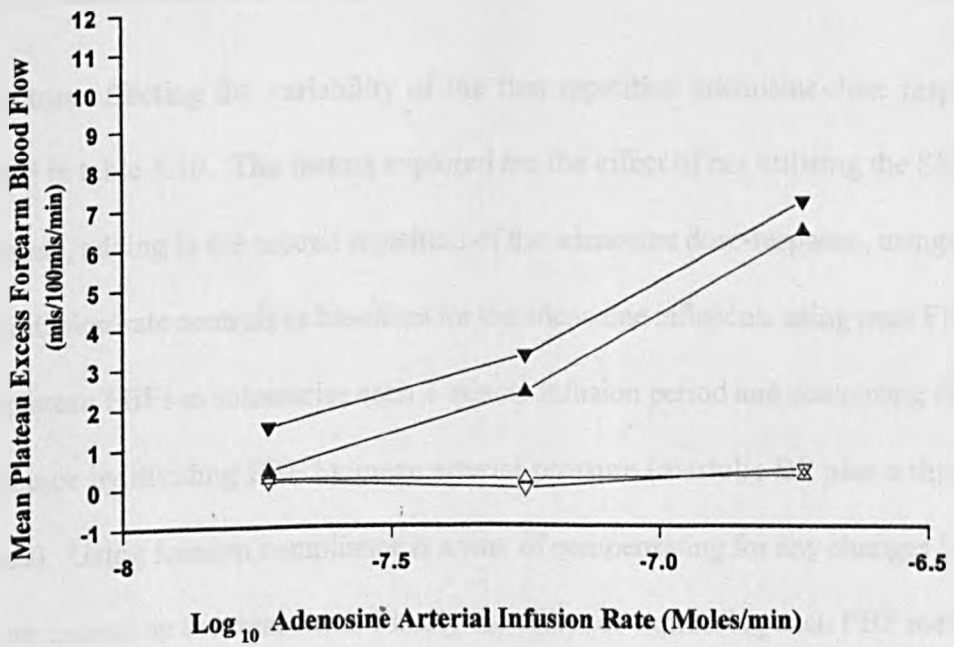
Figures 5.18 and 5.19 show the two adenosine 3-point dose-response profiles measured on each of the 2 study days expressed as mean plateau excess FBF. As is the convention with dose-response relationships the concentration is converted to a number by expressing in base 10 logarithms.

Figure 5.18 Log dose-response for adenosine infusions on study day 1



Solid Symbols=Infused Forearm, Open Symbols=Control Forearm
▲,△=First Infusion ▼,▽=Second Infusion

Figure 5.19 Log dose-response for adenosine infusions on study day 2



Solid Symbols=Infused Forearm, Open Symbols=Control Forearm
▲,△=First Infusion ▼,▽=Second Infusion

The response to each 3-dose adenosine dose-response relationship was summarised as the area-under-the-curve (AUC) of the $\log_{10}$ dose-response and then analysed using ANOVA allowing for volunteer and period effects and incorporating the response to SNP as a covariate. Table 5.9 analyses the data as described above incorporating the second repetition of the adenosine dose response to look for differences between the two repetitions and then the data is further examined for differences in the adenosine and SNP responses between days.

Table 5.9 Effect of repetition and day on responses to adenosine and SNP

Infusate	Variable	Mean	SD	P
Adenosine	First Repetition	3.63	1.87	0.12
	Second Repetition	4.78	3.00	
	First Study Day	5.13	2.57	0.03
	Second Study Day	3.21	2.08	
SNP	First Study Day	17.59	14.01	0.4
	Second Study Day	13.07	8.11	

The factors affecting the variability of the first repetition adenosine-dose response data are explored in table 5.10. The factors explored are the effect of not utilising the SNP response as a covariate, adding in the second repetition of the adenosine dose-response, using the individual saline infusion-rate controls as baselines for the adenosine infusions, using peak FBFs rather than mean plateau FBFs to summarise each 3 minute infusion period and converting FBF to forearm compliance by dividing FBF by mean arterial pressure (diastolic BP plus a third of the pulse pressure). Using forearm compliance is a way of compensating for any changes in mean arterial pressure caused by the treatment. Finally the effect of expressing each FBF measurement as a ratio to the control FBF measured simultaneously the effect of this on variability, otherwise

processed as the first line is shown in the penultimate line of the table. Many groups take the ratio of active:control FBF, subtract the baseline FBF ratio and, after appropriate summary measures have been used, divide by the baseline ratio to produce a result which is a percentage increase in the FBF ratio. The variability of this transformation is shown in the final line of the table.

Table 5.10 Variability of the adenosine dose-response AUC data

Notes	%CV	Mean	N for 30% change
Analysed as described (first repetition)	28	3.63	17
Using both repetitions	49	4.17	53
Without using SNP as a covariate	45	3.63	45
Using infusion rate controls as baseline	35	3.54	27
Using peak FBFs rather than mean plateaux	25	5.5	14
Using forearm compliance rather than FBF	30	0.043	20
Analysed as described, using FBF ratio	55	1.17	67
% increase over baseline in FBF ratio	47	1.07	49

$$N=20.(\frac{CV}{\Delta})^2$$

The formula above is used to the estimated number of volunteers required in a study (N) with a power of 90% to detect a certain proportional change in a measurement(Δ), in this case the AUC of the adenosine dose-response above baseline. CV is the coefficient of variation and a P of <5% is assumed to be significant. The final column of the table shows the value of N assuming a change in 30%.

Turning finally to the number of volunteers needed in the next study: assuming significance at less than 5% and using mean excess plateau FBFs then data from 17 volunteers would have a 90% chance of detecting a 30% reduction in adenosine-induced vasodilatation due to theophylline.

Studying previous work with theophylline, in which aminophylline (the EDTA chelate of theophylline) was coinfused with adenosine the reduction was well in excess of 30%[121,122,232]. Sixteen volunteers would be able to detect a 31% change and would have the advantage, that, being divisible by 4, a balanced randomisation scheme based on groups of 4 volunteers would be possible.

5.3.6.6 Adverse events

Five volunteers experienced adverse events which were all transient and mild. One complained of tingling and paraesthesiae in the fingers lasting 7 minutes. Two volunteers mentioned warmth in the infused forearm during infusions of vasodilators which passed off once the vasodilator infusion stopped. Two other volunteers complained of an ache in the arm or forearm during the procedure one of which lasted 70 minutes and one volunteer complained of headache and nausea on the study day, which did not coincide with the arterial infusions and was therefore unlikely to be related.

5.3.7 Summary

In this study there were no adverse events related to arterial puncture and symptoms related to the drugs infused were mild, transient and local. There was no evidence of any systemic effect of either adenosine or SNP. There was a significant increase in FBF when in intra-arterial infusion rate increased from1 to 1.6mls/min. Adenosine-induced vasodilatation and baseline FBF were reduced on the second study day compared to the first.

The summary data show that using the SNP as a covariate confers a definite advantage, whereas using data from the second adenosine infusion as a covariate was detrimental. Using the infusion rate controls as baselines for the adenosine infusion data also increased variability. Summarising the responses to each adenosine infusion rate as the peak excess FBF rather than the mean, excess plateau flow caused a small improvement in variability. Expressing FBF as a ratio of the infused to the control FBF measured simultaneously was markedly detrimental to the variability whether subsequently expressed only as an excess ratio above the mean baseline FBF ratio or whether further divided by the baseline ratio. The use of forearm compliance rather than FBF had little effect on variability.

Sixteen volunteers would have a 90% probability of detecting a 31% change in the adenosine dose-response (using the SNP response as a covariate) at the 5% significance level.

Chapter 6

Effects of theophylline on adenosine-induced vasodilatation and phosphodiesterases

6.1 Introduction

Previous studies have shown that theophylline is an adenosine-antagonist in the human forearm, but the clinical significance of this remains unclear. In all cases aminophylline, the EDTA chelate of theophylline, was coinfused with adenosine directly into the brachial artery, consequently there is uncertainty concerning the tissue levels likely to be achieved and whether this activity would be of importance following oral administration of theophylline achieving levels in the recommended therapeutic range.

It is crucial to the interpretation of this series of studies to establish whether theophylline does antagonise adenosine-induced vasodilatation in the human forearm when given orally. It would be helpful to establish, as far as practicable, whether oral theophylline has other activities also.

Theophylline has activity as a non-specific phosphodiesterase (PDE) inhibitor in-vitro. The levels required to achieve measurable PDE inhibition in human tissue tend to be above the therapeutic range in human tissue[119,120] (with one notable exception[239]). It has been suggested that the peripheral vasodilatation noted with theophylline is evidence of PDE inhibition[121]. The only support for this proposition is that other PDE inhibitors also cause vasodilatation[240].

A variety of studies have measured cAMP levels in plasma and urine and one study did find an increase in plasma cAMP levels caused by theophylline when a full day profile was measured[241].

Plasma cAMP could arise from almost any tissue in the body and its levels would increase, not only in response to PDE inhibition, but also in response to stimulation of adenylyl cyclase, hence increasing the plasma cAMP concentration is not a specific measure of PDE inhibition.

6.2 Aims

The main aim of this study was to determine whether oral theophylline reduced adenosine-induced vasodilatation in the human forearm. In addition I wanted to find out whether there was any evidence of PDE inhibition. To help in the interpretation of FBF results I looked for any effect of theophylline on central haemodynamics and on resting FBF.

6.3 Design

This was a randomised, double-blind, cross-over comparison of oral theophylline (7.0-8.9mg/kg) and placebo in 16 healthy, male volunteers using the same design as the cross-over comparison described in Chapter 4. Sixteen volunteers would be sufficient to detect a 30% change in the adenosine response given the within-volunteer, between-day variability measured during the last study (see Chapter 5). Volunteers were studied twice a week apart. A week was allowed for washout as it is inconceivable that any theophylline would be remaining after dosing using a formulation normally given 4 times daily. A week is also regarded as a reasonable period to allow complete recovery from arterial puncture and it will prevent confounding by any variation in physiological parameters by week-day.

Volunteers were asked to take exactly the same breakfast, at the same time on both days and attend ready for dosing with theophylline or placebo at the same time each day, 90 minutes before insertion of the arterial needle. A 12 lead ECG was carried out and examined prior to dosing and

was repeated on completion of the arterial infusions. Standard lead II was monitored continuously during the intra-arterial infusions. Volunteers were asked to empty their bladders 30 minutes after dosing and their urine was collected for a further 2 and a half hours.

The study day was contrived so that the arterial needle would be inserted 30 minutes prior to the adenosine infusions starting and so that a blood sample could be taken for measurement of the plasma theophylline level, monocyte PDE activity and the plasma cAMP level (for assays see chapter 2) and the adenosine infusion started 2 hours after dosing. Microcrystalline theophylline is rapidly absorbed achieving levels of 76 to 87μM between 2 and 6 hours after dosing, t_{max} 3.4 hours in one study on patients with bronchial asthma[236], however administration of a lower dose of theophylline (Nuelin™) yielded a t_{max} of 2.1 hours (range 1 to 3 hours) with the plasma theophylline level remaining fairly constant between 2 and 3 hours..

Symptoms were recorded before and after the cumulative adenosine and SNP infusions. A single, seated blood pressure, pulse rate and symptoms were recorded prior to dosing. Symptoms were again recorded when two recordings of blood pressure and pulse rate were made just prior and just after the cumulative adenosine and SNP infusions.

The infusion protocol was an amended version of the reproducibility study described in chapter 5. The cumulative adenosine infusion protocol was modified by the addition of a 3 minute 100mg/min adenosine infusion. This is because 50mg/min was still on the ascending part of the dose-response relationship and curve-fitting to the dose-response relationship can be carried out with much greater precision if the plateau is reached. Another group had tried infusing 150mg/min, but reported some change in blood pressure[123]. The infusion rate of SNP, normal

saline and adenosine was held kept constant at 1ml/min throughout to avoid the need for infusion rate controls. This meant that 4 different concentrations of adenosine were infused.

A separate 3 minute baseline was recorded prior to the cumulative adenosine infusion and two further 2 minute baselines were carried out, and, just as for the reproducibility study, these formed a continuous series of measurements with the cumulative adenosine infusion and with the SNP infusion. By recording baseline flow as close as possible to vasodilatation due to adenosine and SNP it was hoped to minimise fluctuation with time and improve the accuracy of the baseline measurement. Table 6.1 shows the time-course of events once the arterial needle was inserted.

As before, firm pressure was applied to the needle site for 10 minutes after the needle was removed and the site was monitored for a further 2 hours before the volunteer was allowed to leave.

6.4 Volunteer characteristics

Volunteers were selected on the same basis as they were for the reproducibility study (section 5.3.3), except that the maximum weight allowed was 90kg to keep within 7-9mg/kg of theophylline using the oral dosing schedule described later. Sixteen, healthy, male Caucasian volunteers were selected of which 5 smoked. Their average age was 34 years (range 23-52 years), weight 77kg (range 62-90kg) and their height was 1.75m (range 1.6-1.85m).

Table 6.1 Timing of measurements and interventions

Time(mins)	Infusate	Rate(μg/min)	FBF/TEB	Pulse/BP
0-20	Normal Saline	90		
20-23	Normal Saline	90	+	
23	Normal Saline	90		+
26 SAMPLE	Normal Saline	90		
28-30	Normal Saline	90	+	
30-33	Adenosine	5	+	
33-36	Adenosine	15	+	
36-39	Adenosine	50	+	
39-42	Adenosine	100	+	
42	Normal Saline	90		+
50	Normal Saline	90		+
52-54	Normal Saline	90	+	
54-57	SNP	10	+	
57	None			+

6.5 Volunteer restrictions

As in previous studies, volunteers were required to abstain from smoking or caffeine containing food or drink for 40 hours prior to the study, from alcohol for 72 hours and from any medication (without specific approval) for 24 hours. They took the day off work and did not return after the study. They were asked not to undertake heavy exercise following the study.

6.6 Preparation of adenosine and sodium nitroprusside

Sodium nitroprusside was diluted to achieve a final concentration of 10μg/ml as described in Chapter 5. Four different concentrations of adenosine were prepared: 5, 15, 50 and 100μg/ml.

As before a 300ug/ml solution was prepared first by mixing 2mls of the 3mg/ml solution of adenosine provided in the glass ampoule supplied (Adenocor™: Sanofi-Winthrop) with a further 18mls of normal saline. This solution was further diluted with normal saline to provide the final solutions as shown in the following table. These solutions were drawn up into a 60ml, infusion pump syringe, labelled with the concentration, date and time, and stored at 4°C ready for use.

Table 6.2 Preparation of the adenosine solutions

Final Concentration	Total Volume (mls)	Volume of 300μg/ml Solution (mls)	Volume of Normal Saline (mls)
5μg/ml	60	1	59
15μg/ml	20	1	19
50μg/ml	24	4	20
100μg/ml	15	5	10

6.7 Dosing

The same procedure was followed as detailed in Chapter 4. Both theophylline(Nuelin™ 125mg tablets, 3M Healthcare) and placebo were given packed inside gelatin capsules to conceal any differences in appearance or taste. Each dose was given with 150mls of distilled water. Pharmacy packed the capsules for each patient according to the randomisation scheme. The dose of theophylline was adjusted according to the weight of the volunteer: those weighing 70kg or over were given 5 capsules containing either placebo or a total of 625mg of theophylline, those under 70kg were given 4 capsules or 500mg of theophylline. This equates to a dose range of 7.0-8.9mg/kg. As explained before this would be predicted to produce drug levels at the higher end

of the therapeutic levels and in the previous study it did just this, with volunteers experiencing only very few, minor symptoms attributable to theophylline (Chapter 3). The t_{max} for Nuelin has been measured to be 2.1 hours in healthy volunteers[227] and the time profile for theophylline after oral dosing shows steady reduction in concentration from this time, but that levels are likely to remain within the therapeutic range for upto 4 hours with more rapidly falling levels thereafter[227,236].

6.8 Results

6.8.1 Missing data

Data was not recorded after the first baseline FBF recording and subsequent pulse and blood pressure measurements for volunteer 11on the second study day as the arterial needle clotted after changing the syringe. For the same reason, no further data was collected on the first study day for volunteer 12 after the final adenosine infusion.

6.8.2 Room temperature

The initial room temperature was invariably the minimum room temperature. The mean, minimum room temperature was 22.5°C, range 22 to 25°C and the mean maximum room temperature was 23.7°C, ranging from 23 to 25°C. The maximum increase during any study day was 2°C, mean 0.6°C.

6.8.3 Transthoracic electrical bioimpedance (TEB)

As figures 6.1 to 6.3 show, though there are progressive changes in three TEB indices with time, these are not attributable to adenosine or SNP since there is no apparent difference between measurements recorded during the last minute of the cumulative adenosine infusion and the two

baseline measurements taken between the adenosine and SNP infusions.

Theophylline does have an effect and this is illustrated in table 6.3. Heart rate and cardiac index both increased significantly, though stroke index was unchanged.

Table 6.3 Effects of theophylline on TEB indices

INDEX	Placebo		Theophylline		N	P
	Mean	SD	Mean	SD		
Cardiac Index (l/min/m^2)	3.11	0.59	3.42	0.59	16	0.02
Stroke Index (mls/m^2)	55.7	8.6	56.2	8.5	16	0.73
Heart Rate (min^{-1})	56.0	6.3	61.1	6.6	16	0.002

Figure 6.1 Stroke index time profile by treatment

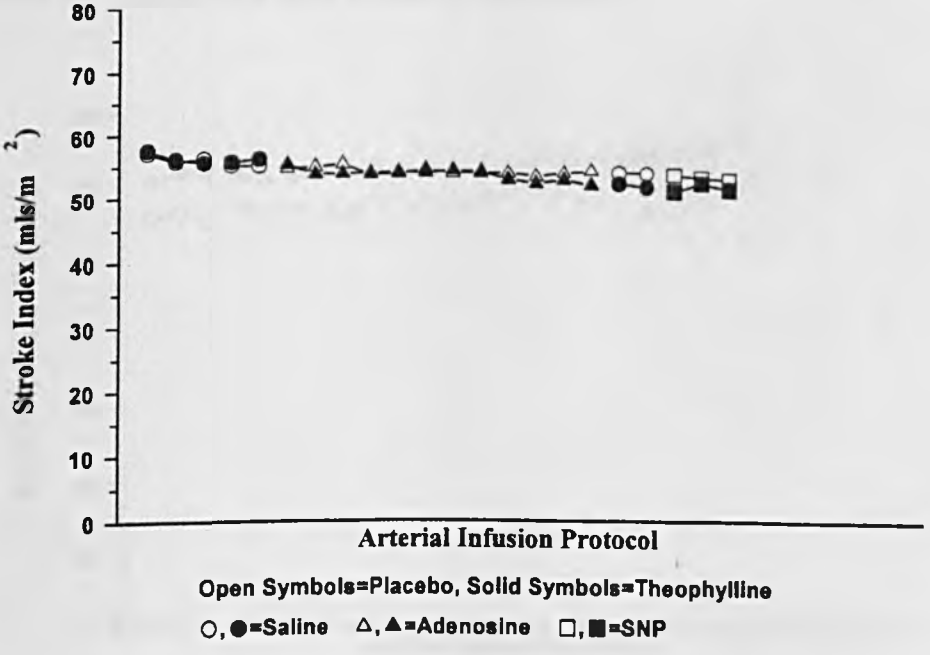


Figure 6.2 Cardiac index time profile by treatment

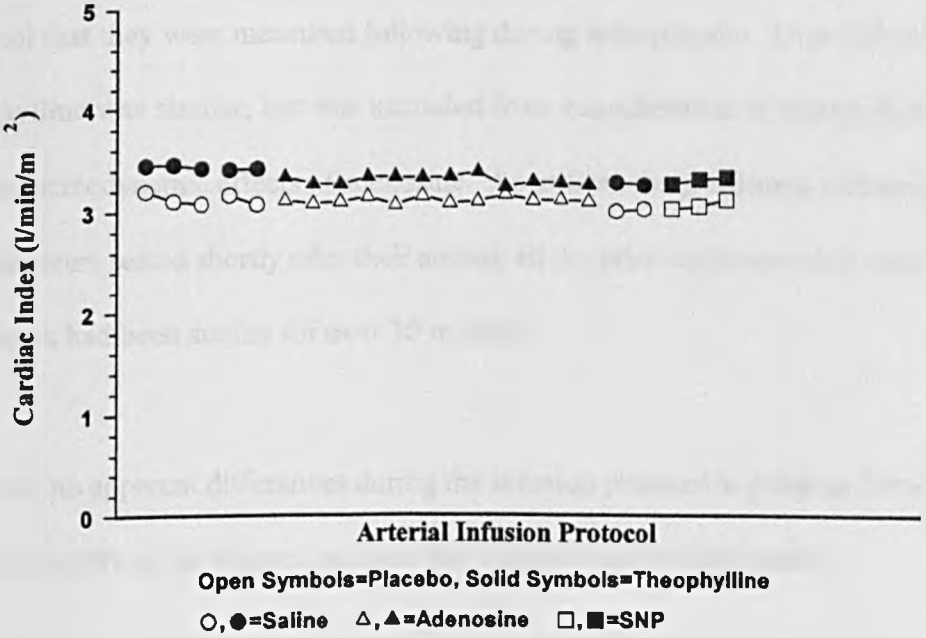
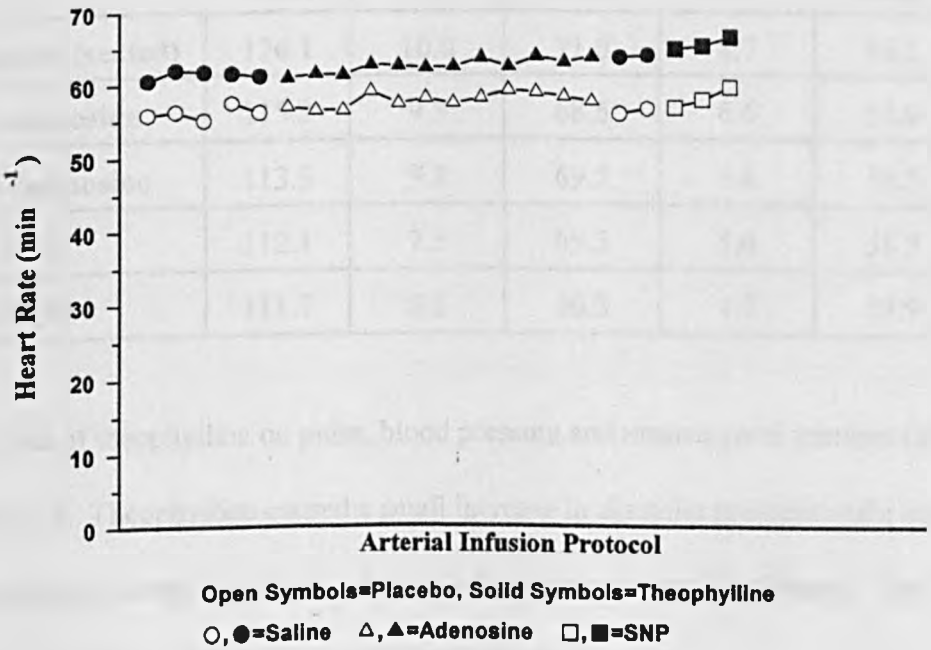


Figure 6.3 Heart rate time profile by treatment



6.8.4 Pulse and blood pressure

Table 6.4 shows the mean blood pressure and pulse measurements according to the point in the protocol that they were measured following dosing with placebo. Data following dosing with theophylline was similar, but was excluded from consideration as theophylline could obscure central haemodynamic effects of adenosine. Apart from the pre-dosing measurement taken with the volunteers seated shortly after their arrival, all the other measurements were taken when the volunteers had been supine for over 30 minutes.

There are no apparent differences during the infusion protocol in pulse or blood pressure. Due to the similarity of the figures no statistical comparison has been made.

Table 6.4 Trends in pulse and blood pressure during the placebo study day

Protocol Point	Systolic BP (mmHg)		Diastolic BP (mmHg)		Pulse (per minute)	
	mean	SD	mean	SD	mean	SD
Pre-dose (seated)	126.1	10.0	71.0	8.7	66.1	11.2
Pre-adenosine	115.3	9.3	68.6	6.6	55.9	7.9
Post-adenosine	113.3	9.3	69.5	5.6	56.5	9.2
Pre-SNP	112.1	7.5	69.3	5.6	58.5	9.7
Post-SNP	111.7	8.6	66.3	4.2	59.9	8.7

The effect of theophylline on pulse, blood pressure and mean arterial pressure (MAP) are shown in table 6.5. Theophylline caused a small increase in diastolic pressure and a somewhat greater proportional increase in pulse rate both of which just achieved significance. The resulting, small increase in MAP just failed to reach significance.

Table 6.5 The effects of theophylline and placebo on pulse and blood pressure

	Placebo		Theophylline		P
	Mean	SD	Mean	SD	
MAP (mmHg)	83.3	4.9	86.4	5.7	0.051
Systolic BP (mmHg)	113.1	7.9	116.9	8.3	0.14
Diastolic BP (mmHg)	68.4	4.4	71.1	6.1	0.043
Pulse (min^{-1})	57.7	8.5	63.4	8.9	0.042

6.8.5 Forearm blood flow

As before (Chapter 3), theophylline increased baseline FBF. As it acts systemically, FBF measurements taken simultaneously in both forearms were meaned together and the first 20 FBF measurements, taken before any drug was infused, were then meaned and compared by ANOVA allowing for volunteer, order and period effects. *Theophylline caused a 56% increase in FBF from 2.37 mls/100mls/min (SD 0.83) to 3.69mls/100mls/min (SD 0.98), P=0.004.* This 56% increase in FBF is almost the same as that (49%) recorded in chapter 4 (section 4.6.3.1).

Comparing the 3 minute baseline FBF measured upto 4 minutes prior to the adenosine infusion with the 2 minute baseline FBF measured immediately prior to the adenosine infusion (Table 6.6) there is a significant 15% increase in the control forearm in the presence of theophylline. In view of this difference, the 2 minute baseline taken immediately prior to the adenosine infusions was taken to be the baseline flow in subsequent analyses. The table also shows comparisons between the second, pre-adenosine baseline and the pre-SNP baseline and control FBF measured during the maximum rate of adenosine infusion, 100μg/min.

Table 6.6 Baseline FBF by treatment and by forearm

PLACEBO							
Baseline or Adenosine	**N**	**Infused Forearm**			**Control Forearm**		
		Mean	**SD**	**P vs ***	**Mean**	**SD**	**P vs ***
First Baseline (3 min)	16	2.44	0.87	0.96	2.26	0.91	0.21
***Second Baseline (2 min)**	16	2.40	0.99	-	2.39	0.84	-
Adenosine (100μg/min)	16	-	-	-	2.51	0.85	0.22
Pre-SNP Baseline (2 min)	16	2.76	1.17	0.60	2.42	0.89	0.40
THEOPHYLLINE							
First Baseline (3 min)	16	3.43	1.00	0.65	3.72	1.11	0.04
***Second Baseline (2 min)**	16	3.52	1.44	-	4.28	1.50	-
Adenosine (100μg/min)	16	-	-	-	4.80	2.03	0.10
Pre-SNP Baseline (2 min)	14	4.23	1.92	0.09	4.71	1.82	0.18

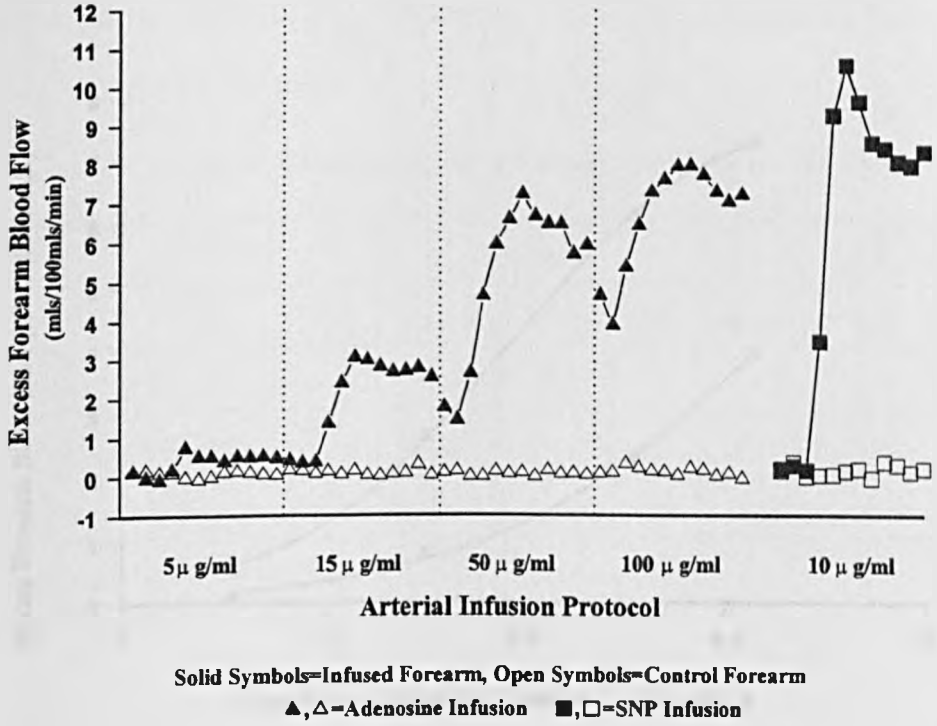
Figures 6.4 and 6.5 show excess FBF responses to the cumulative adenosine infusion and to SNP in the presence of placebo and theophylline. As described in the previous chapter excess FBF responses in the infused forearm at each dose level of adenosine were first summarised by meaning the 4 measurements taken during the final minute at that infusion rate. Figure 6.6 shows the mean values for all volunteers plotted according to treatment against the logarithm_{10} of the adenosine infusion rate expressed in moles per minute. Sigmoid curves were fitted to the responses measured in the presence of placebo and theophylline (Fig.P™ - Fig.P Software Corporation, Durham, NC., USA) yielding EC_{50}s of 1.1×10^{-7} M of adenosine/min (pA_2 -7.05) with placebo and 2.63×10^{-6} M of adenosine/min (pA_2 -6.42) in the presence of theophylline. *Theophylline causes a 4 fold rightward shift of the adenosine dose-response.* The AUC of the

log. dose-response curves were compared using ANOVA allowing for volunteer, period and order effects, incorporating the SNP response as a covariate. *The AUC fell from 5.03 mls/100mls/min (SD 3.09) with placebo to 1.92 mls/100mls/min (1.18) with theophylline, P=0.0025.*

There is a gradual increase in FBF during the protocol with theophylline which is apparent in figure 6.5 and also in table 6.6. Though there is no significant change in blood flow when the 2 minute pre-adenosine control baseline FBF is compared to control FBF measured during the 100mg/min adenosine infusion or the pre-SNP 2 minute baseline FBF, some would consider it advisable to allow for this possible temporal effect when considering the action of theophylline. The simplest way is to express measurements as a ratio of the blood flows measured in the infused and the control forearm simultaneously. Otherwise the analysis was carried out entirely as described above. *The AUC of the FBF ratio was 2.09 (SD 1.17) in the presence of placebo and 0.48 (0.35) in the presence of theophylline and this difference was still significant, P=0.0006.*

The mean, excess FBF during the last minute of the SNP infusion in the presence of placebo or theophylline were compared using ANOVA allowing for volunteer, period and order effects. There was no change: the mean, excess FBF was 8.11 mls/100mls/min (SD 4.26) with placebo and 7.87 mls/100mls/min (4.24) with theophylline, P=0.85.

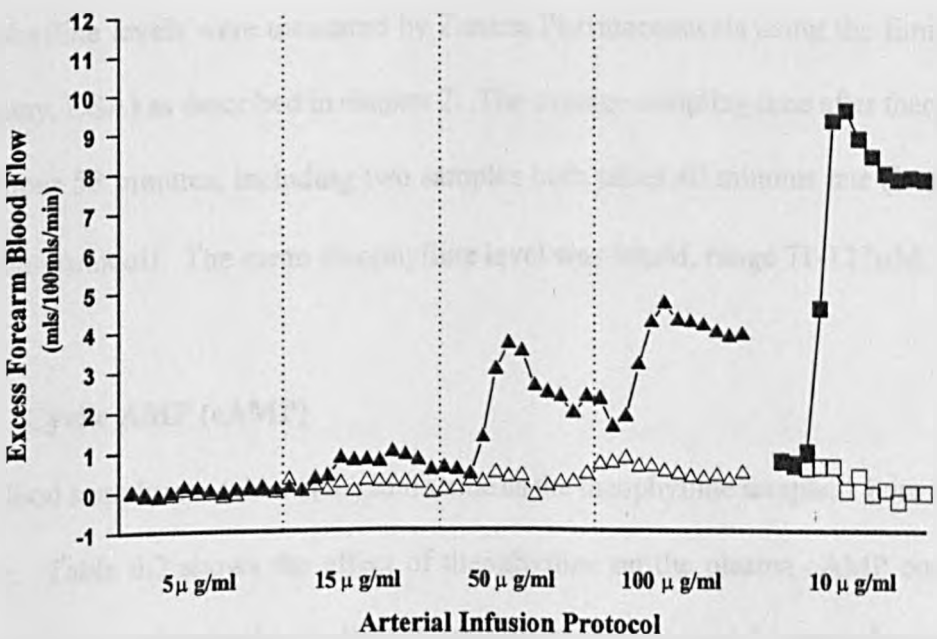
Figure 6.4 Response to adenosine and SNP in the presence of placebo



Solid Symbols=Infused Forearm, Open Symbols=Control Forearm

▲,△=Adenosine Infusion ■,□=SNP Infusion

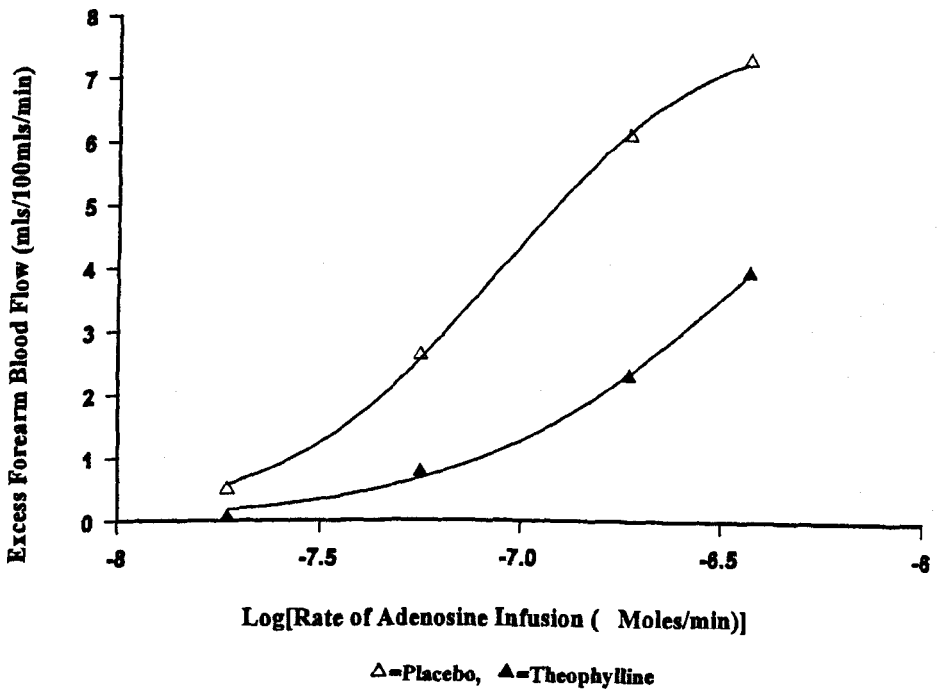
Figure 6.5 Response to adenosine and SNP in the presence of theophylline



Solid Symbols=Infused Forearm, Open Symbols=Control Forearm

▲,△=Adenosine Infusion ■,□=SNP Infusion

Figure 6.6 Effects of theophylline and placebo on the adenosine dose-response



6.8.6 Theophylline levels

Theophylline levels were measured by Zeneca Pharmaceuticals using the Emit® assay (Syva Company, USA) as described in chapter 2. The average sampling time after theophylline dosing was 1hour 58 minutes, including two samples both taken 40 minutes late (ie after the arterial infusion protocol). The mean theophylline level was 90μM, range 71-122μM.

6.8.7 Cyclic AMP (cAMP)

This blood sample was taken at the same time as the theophylline sample, 1 hour 58 minutes after dosing. Table 6.7 shows the effect of theophylline on the plasma cAMP concentration, the urinary plasma concentration (collected between 30 minutes and 3 hours after dosing), the total urinary volume and the total urinary excretion of cAMP. There is a significant increase in plasma

cAMP and a marked diuresis, but there is no change in the urinary cAMP concentration, though the total urinary excretion of cAMP is increased as a consequence of the diuresis.

Table 6.7 The effect of theophylline on urinary and plasma cAMP concentrations

Variable	Placebo			Theophylline			P
	mean	SD	N	mean	SD	N	
Plasma[cAMP](nM)	11.2	2.6	14	17.6	7.5	14	0.009
Urinary[cAMP](mM)	5.23	0.92	15	5.53	0.97	16	0.3
Urinary volume (l)	0.32	0.19	16	0.58	0.22	16	0.002
Total urinary cAMP (mmol)	1.61	1.05	15	3.1	1.1	16	0.004

6.8.8 Ex-vivo monocyte phosphodiesterase activity

As described in chapter 2, inhibition of the type IV PDE found in monocytes has been found to reduce TNF production by them in response to a lipopolysaccharide (LPS) challenge *in vitro*. This is the basis for this assay. The plasma TNF level was assayed prior to challenge with LPS and as the level is usually below the limits of detection by this assay method, any samples found to have detectable levels were excluded from analysis to prevent confounding. Data from 2 volunteers was excluded in this way.

There was some reduction in the response to the TNF challenge in the presence of theophylline, indicating some inhibition of monocyte type IV PDE. *The TNF level was reduced from 10.6 (SD 3.0) to 8.8 (SD 2.4)nM, but this did not achieve significance: P=0.056 (ANOVA).*

6.8.9 Adverse events

There were no adverse events specifically attributable to arterial puncture or the adenosine and sodium nitroprusside infused during the protocol. In common with the other studies several volunteers complained of headache and difficulty concentrating coming on the day of the study (before dosing) or commonly the day prior and presumably attributable to caffeine withdrawal. Complaints of nausea or tremor, possibly attributable to theophylline were infrequent, mild and self-limiting.

6.9 Summary

In this study I have demonstrated that oral theophylline, at plasma levels within the therapeutic range, inhibited adenosine-induced forearm vasodilatation. Evidence is also presented suggesting that theophylline acts as a PDE inhibitor at therapeutic levels, it increased plasma cAMP levels and also increased total urinary cAMP excretion, by virtue of a moderate diuresis.

Theophylline's haemodynamics effects have also been studied. It caused:

i. a marked increase in resting FBF

ii. a 10% increase in resting heart rate

iii. an increase in cardiac index, but no change in the stroke index

iv. a small increase in MAP which was not statistically significant.

Chapter 7

Effects of oxpentifylline on reactive and active hyperaemia in healthy male volunteers

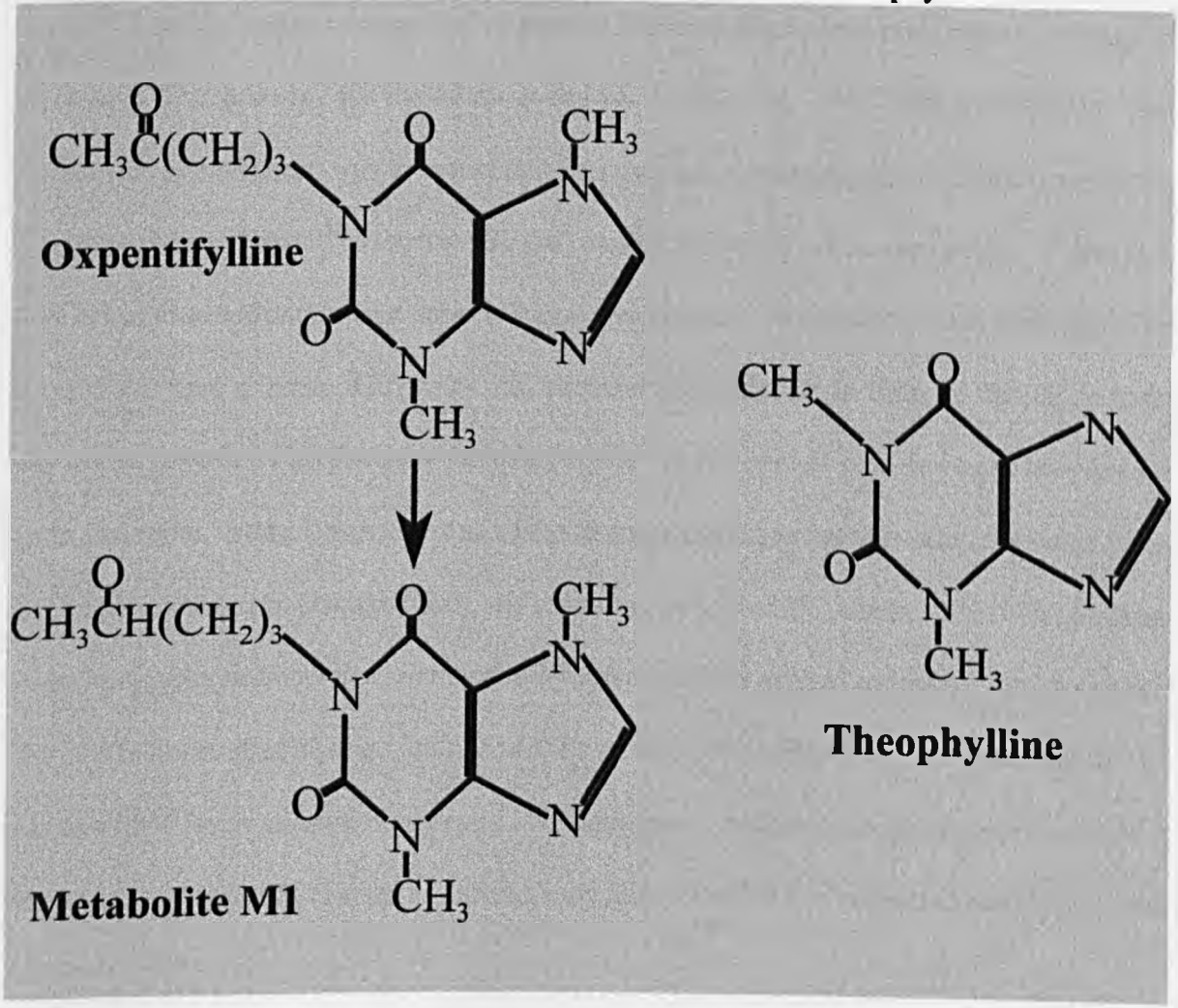
7.1 Introduction

In chapter 4 theophylline caused paradoxical enhancement of active hyperaemia. Since there is no other adenosine-antagonist licensed for use in man, it was necessary to take a different approach to defining how theophylline caused this effect. Oxpentifylline is a close structural analogue of theophylline (Figure 7.1) and like theophylline it is a phosphodiesterase inhibitor[127,242,243], but unlike theophylline, it is only a *very weak* adenosine antagonist[116,127,244]. If the enhancement of active hyperaemia seen using theophylline was due to phosphodiesterase inhibition, then oxpentifylline would be expected to have the same or arguably greater effect, since there would be no conflicting effect due to adenosine antagonism. I chose oxpentifylline, rather than other phosphodiesterase inhibitors, because I hoped it would have the same spectrum of inhibition of phosphodiesterases as theophylline. Oxpentifylline is already licensed for treatment of peripheral vascular disease, causing mild and readily reversible side-effects (Trental 400™ data sheet), obviating any ethical problems.

The principal metabolite of oxpentifylline formed by reduction of the keto group in the alkyl side-chain to the corresponding alcohol is known as metabolite M1[245,246], it is shown in Figure 7.1. Metabolite M1 is allegedly active[247] with equal potency to the parent drug[248]. Following oral administration of slow-release oxpentifylline (Trental) it achieves concentrations in excess of the parent drug by t_{max} for oxpentifylline. The t_{max} for the both oxpentifylline and metabolite M1 are similar (3.32hrs and 3.15hrs respectively)[228]. In view of this metabolite M1 might well be

responsible for a major part of the pharmacological activity of oxpentifylline.

Figure 7.1 Structures of oxpentifylline, metabolite M1 and theophylline



7.2 Aims

The primary aim of this study was to determine whether oral oxpentifylline enhanced active hyperaemia in the human forearm. In addition I wanted to find out whether there was any evidence of PDE inhibition. To help in the interpretation of FBF results I looked for an effect of oxpentifylline on central haemodynamics and on resting FBF.

7.3 Design

This was a randomized, placebo-controlled, double-blind trial using the protocol developed in chapter 4 to measure hyperaemic responses. As then, measurements of exercise hyperaemia were initiated 2 seconds after exercise had stopped to allow the plethysmograph baseline voltage to be re-set. The protocol for the study is shown in table 7.1. The order of treatment was randomized in a balanced way to ensure that the numbers of volunteers given placebo or 400mg of oxpentifylline (Trental™,Hoechst UK Ltd) on their first attendance were equal. Volunteers were asked to attend at the same time on 2 days a week apart. They ate the same light breakfast at the same time at home each week and attended the study site in time for dosing an hour afterwards. Two and a half hours after dosing volunteers were asked to lie down in a temperature controlled room. In the 3 previous studies the temperature has remained within the range 22 to 25°C with a maximum change during any recording period of 2°C, mean 0.6°C. The forearm plethysmography and impedance cardiography equipment was applied and tested. At 2 hours and 55 minutes hours after dosing 10mls of blood was taken from a hand vein for measurement of plasma cAMP levels and *ex-vivo* assay of TNF production by monocytes in response to antigenic challenge, as detailed in chapter 2. Immediately after this FBF was measured according to the protocol.

7.4 Volunteers characteristics

I studied 16 healthy, male volunteers recruited from the volunteer panel at Zeneca aged 18-55 years (mean 33 years), weighing 65.9-88.7kg (mean 76kg) and height 1.65-1.88m (mean 1.78m). Fifteen volunteers were Caucasian and one was Asian. Eleven volunteers were non-smokers and the remainder were ex-smokers for a year or more. All had normal blood counts, liver function, renal function, urinalysis, blood sugar and electrolytes, resting ECG and 24 hour ambulatory

ECGs, with no significant abnormalities on clinical examination. Volunteers were only included if they were 18-62 years old, 60-89kg in weight, on no regular medications, had a sitting blood pressure of less than 140/90mmHg and consumed 21 units of alcohol a week or less.

Table 7.1 Timing of measurements and interventions

Elapsed time	FBF/TEB	Supine pulse	Duration of	Duration of
0	Dose of drug or placebo given			
175	Blood sample taken			
180-182		+		
182-185	+			
187-188			1	
188-191	+			
193-196			3	
196-199	+			
201-206			5	
206-209	+			
209-211		+		
216-218		+		
218-221	+			
223-224				1
224-227	+			
229-231				2
231-234	+			
236-239				3
239-242	+			
242-244		+		
300	HOME			

7.5 Volunteer restrictions

As in previous studies, volunteers were required to abstain from caffeine containing food or drink for 40 hours prior to the study, from alcohol for 72 hours and from any medication (without specific approval) for 24 hours. They took the day off work and did not return after the study. They were asked not to undertake heavy exercise following the study.

7.6 Dosing

Both oxpentifylline (Trental™ 400mg tablets, Hoechst UK Ltd) and placebo were given packed inside identical, gelatin capsules to conceal any differences in appearance or taste. Each dose was given with 150mls of distilled water. The Royal Liverpool University Hospital pharmacy packed the capsules for each volunteer and study day according to a randomisation scheme supplied by Zeneca Pharmaceuticals. After one oral dose of Trental 400mg the t_{max} for both oxpentifylline and its principal, active metabolite have been measured to be just over 3 hours in healthy volunteers and both have a half-life in excess of 3 hours[228]. For this reason blood for assaying PDE inhibition by oxpentifylline was taken close to 3 hours after dosing and the FBF protocol for measuring active and reactive hyperaemic responses was started soon after.

7.7 Results

7.7.1 Missing data

Volunteer 3 experienced an adverse event prior to any measurements being taken on his first study day. His symptoms (described in section 7.7.7) precluded taking measurements and so he was withdrawn from further study. Fifteen volunteers were analysed although it was not always possible to analyse all FBF data due to recording artefacts.

One of volunteer 9's samples for assaying monocyte production of TNF was mislaid by the laboratory, so the remaining sample from the other study day was not assayed as there was nothing to compare it with.

7.7.2 Transthoracic electrical bioimpedance (TEB)

Mean values for the cardiac index, stroke index and heart rate were obtained for each volunteer in the presence of oxpentifylline and placebo by meaning the 6 one minute values recorded during the two periods of baseline forearm blood flow. These mean values were compared using ANOVA allowing for volunteer, period and order effects. Table 7.2 shows the results.

Table 7.2 The effects of oxpentifylline and placebo on TEB indices

INDEX	Placebo		Oxpentifylline		N	P
	Mean	SD	Mean	SD		
Cardiac Index ($l/min/m^2$)	3.14	0.60	3.25	0.80	15	0.58
Stroke Index (mls/m^2)	54.5	12.6	56.3	11.3	15	0.58
Heart Rate (min^{-1})	58.3	5.4	58.0	7.7	15	0.90

The mean of the 3 minute long TEB recordings made during the pre-exercise baseline period were compared with the recording made during the final minute of 3 minute handgrip exercise period. The 6% increase in cardiac index noted did not achieve significance, but heart rate increased by 18% and stroke index fell by 9%. Comparisons were made by the two-tailed Student's t test.

Table 7.3 The effects of exercise on TEB indices (placebo only)

INDEX	Baseline		3 mins exercise		N	P
	Mean	SD	Mean	SD		
Cardiac Index (l/min/m^2)	3.08	0.55	3.26	0.67	15	0.06
Stroke Index (mls/m^2)	53.4	12.1	48.7	14.4	15	0.01
Heart Rate (min^{-1})	58.3	5.6	68.6	10.0	15	0.0001

7.7.3 Pulse and blood pressure

The two pulse and blood pressure readings taken consecutively before the first period of forearm ischaemia were meaned together. Values obtained in the presence of oxpentifylline and placebo were compared using ANOVA allowing for volunteer, period and order effects. The results are shown in table 7.4.

Table 7.4 The effects of oxpentifylline and placebo on pulse and blood pressure

N=15	Placebo		Oxpentifylline		P
	Mean	SD	Mean	SD	
MAP (mmHg)	85.4	6.2	85.7	7.2	0.72
Systolic BP (mmHg)	115.2	8.4	113.4	8.7	0.20
Diastolic BP (mmHg)	70.6	6.2	71.9	8.0	0.37
Pulse (min^{-1})	57.5	6.5	57.1	7.2	0.95

7.7.4 Forearm blood flow

Mean baseline FBF was derived by first meaning the FBF measured simultaneously in both forearms. There were 12 such paired measurements before the first period of ischaemia and a further 12 measurements before the first period of exercise. These were meaned together to produce a mean pre-ischaemia and a pre-exercise for each volunteer. Mean excess peak FBFs following both exercise and ischaemia were derived as described in chapter 3. The AUCs following each period of exercise and ischaemia were calculated by the trapezoidal method (see chapter 3) and the AUCs were likewise summarised as mean excess AUCs for both exercise and ischaemia. These overall means derived for placebo and for oxpentifylline for all volunteers were compared using ANOVA allowing for volunteer, period and order effects. The results are shown in table 7.5 showing no evidence of any effect of oxpentifylline on either baseline FBF or any of the summary measures of forearm hyperaemia.

Table 7.5 The effects of oxpentifylline and placebo on FBF

	Placebo			Oxpentifylline			P
	Mean	SD	N	Mean	SD	N	
Baseline FBF	4.20	1.33	15	4.09	1.59	15	0.73
Post-Ischaemic FBF							
Mean AUCs	5.29	2.79	12	6.86	2.78	14	0.44
Mean Peaks	20.0	5.25	14	22.1	5.71	14	0.10
Post-Exercise FBF							
Mean AUCs	19.2	14.3	14	18.9	11.6	14	0.88
Mean Peaks	18.5	8.0	14	18.7	7.8	14	0.98

Figure 7.2 The effects of oxpentifylline and placebo on hyperaemic FBF

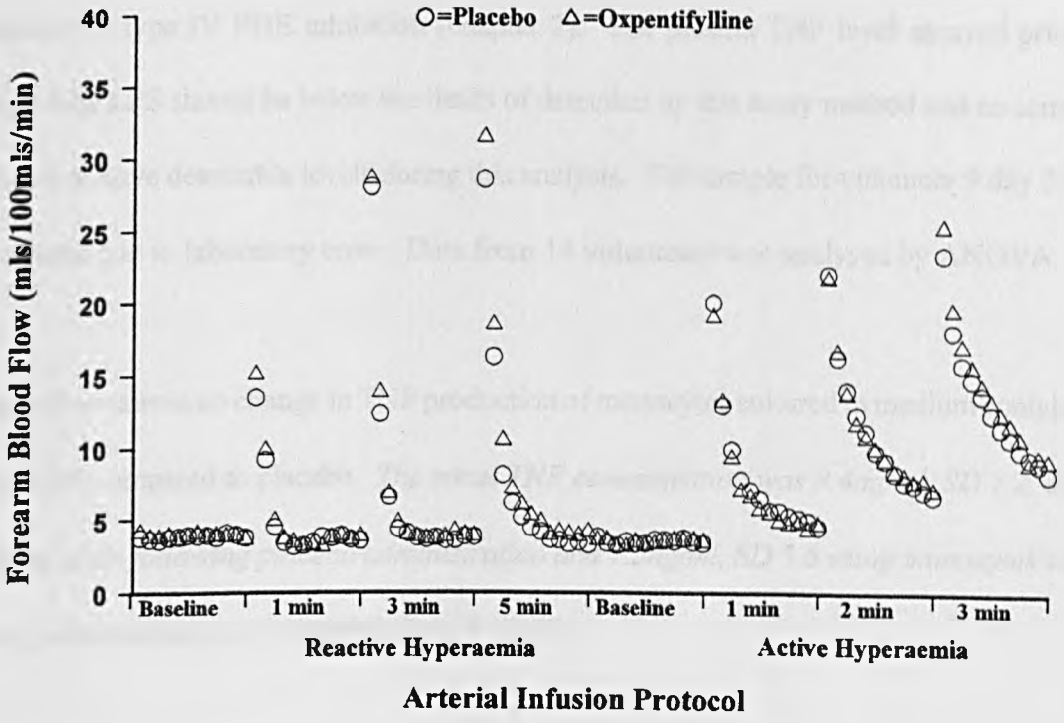


Figure 7.2 graphs the mean data by treatment, this confirms the lack of effect of oxpentifylline on baseline FBF, reactive hyperaemia and active hyperaemia.

7.7.5 Plasma cyclic adenosine monophosphate (cAMP) concentration

The plasma cAMP was measured on 2 days for all 15 volunteers using the assay described in chapter 2. *There was a significant decrease in the mean plasma cAMP level when oxpentifylline was given, falling from a mean value of 18.9nM (SD 4.0) with placebo to 15.7nM (SD 2.2) with oxpentifylline, P=0.011.*

7.7.6 Ex-vivo monocyte phosphodiesterase activity

This assay uses inhibition of LPS stimulated TNF production by monocytes cultured *ex-vivo* as an indicator of type IV PDE inhibition (chapter 2). The plasma TNF level assayed prior to challenge with LPS should be below the limits of detection by this assay method and no samples were found to have detectable levels during this analysis. The sample for volunteer 9 day 2 was not assayable due to laboratory error. Data from 14 volunteers was analysed by ANOVA.

Oxpentifylline caused no change in TNF production of monocytes cultured in medium containing 10μg/ml LPS compared to placebo. *The mean TNF concentration was 8.4ng/ml, SD 7.2, using monocytes taken following placebo administration and 7.5ng/ml, SD 5.6 using monocytes taken following administration of oxpentifylline; P=0.69.*

7.7.7 Adverse events

Volunteer 3 became nauseated and faint with mild headache two and a half hours after dosing on his first study day. His symptoms settled spontaneously over the following 2 hours with bed rest. Once the study was completed the code was broken and it was apparent that he had been given oxpentifylline. Given the known side-effects of oxpentifylline and the timing of the symptoms I felt it most likely that these symptoms were drug-related.

As in previous studies, several other volunteers mentioned a mild headache which came on most often the day prior to the study. Since this subsided once study restrictions on caffeine were lifted, this was attributed to caffeine-withdrawal.

7.8 Summary

A single, oral, 400mg dose of oxpentifylline had no apparent haemodynamic effects at the time both the parent drug and its principal active metabolite would be at their maximum plasma level. There was no change in stroke index, cardiac index, heart rate (whether detected by the impedance cardiograph or by taking the pulse rate). Similarly systolic and diastolic blood pressure and mean arterial pressure were unchanged. There was no change in baseline FBF nor in active or reactive hyperaemia brought about by oxpentifylline.

There was no change in monocyte TNF production in response to LPS challenge indicating no change in PDE type IV activity, however the coefficient of variation was 69%, so that the uncertainty about the treatment effect could encompass quite marked inhibition of PDE IV. The plasma cAMP concentration fell significantly by 17% in the presence of oxpentifylline. This lends support to the idea that this dose of oxpentifylline did not inhibit phosphodiesterases at the dose used, however the reason for the *fall* in plasma cAMP is uncertain.

During exercise cardiac index increased by 6%, but this was not significant. Heart rate increased markedly and stroke index fell by 9%. These findings corroborate the findings of the reproducibility study described in chapter 3.

Chapter 8

Reproducibility of reactive and active hyperaemic responses in patients with peripheral vascular disease

8.1 Introduction

I wanted to verify my findings in volunteers in patients with peripheral vascular disease. Before I could begin to study the effects of drugs on hyperaemic responses in patients with peripheral vascular disease (PVD) it was important to measure the reproducibility of these responses in this patient group. The reproducibility in patients is likely to be reduced compared to healthy male volunteers as I did not impose the same recruiting restrictions on admission blood pressure, age, obesity, biochemical indices, gender and use of medications that I did with volunteers. I wanted a relatively unselected patient group.

8.2 Aims

There were two primary aims: firstly to measure the reproducibility of the mean AUC of active and reactive hyperaemic responses using the protocol refined in chapter 3 and secondly I wanted to train a pool of volunteers in the ways of the protocol and enter them into the 3-way cross-over study described in the chapter 9. A subsidiary aim of this work was to produce forearm hyperaemia recovery profiles from patients with PVD and compare them with similar data obtained in the same laboratory from healthy male volunteers (see chapter 3) to determine whether such measurements could be a useful index of functional vascular impairment in vascular disease.

8.3 Design

This study used the active-reactive hyperaemia protocol refined in chapter 4. The exact timing of measurements is shown in table 8.1. Volunteers were asked to attend the temperature-controlled, vascular laboratory at the same time, either 9 or 11am, on two mornings a week apart. They were required to eat the same breakfast on both occasions. Two series of identical measurements were taken each day, the second set starting 4 hours after the first. Before measurements began, volunteers were required to lie supine for 30 minutes whilst the forearm plethysmography equipment was applied an tested after which 10mls of blood was taken for plasma cAMP estimation (detailed in chapter 2). Volunteers were asked to take the same lunch an hour prior to returning to the laboratory for the second time each day. They were allowed free access to fluids before and after each period of measurement, but were reminded to empty their bladders before each period of measurement. The protocol had been accepted by the hospital ethics committee prior to the study.

Table 8.1 Timing of measurements and interventions

Elapsed time (minutes)	FBF	Supine pulse & BP	Duration of ischaemia (minutes)	Duration of exercise (minutes)
0-2		+		
2-5	+			
7-8			1	
8-11	+			
13-16			3	
16-19	+			
21-26			5	
26-29	+			
29-31		+		
36-38		+		
38-41	+			
43-44				1
44-47	+			
49-51				2
51-54	+			
56-59				3
59-62	+			
62-64		+		

8.4 Volunteers characteristics

Thirteen volunteers were recruited from the peripheral vascular clinic at the Royal Liverpool University Hospital (Mr G McLoughlin) by scanning the outpatient clinic notes and contacting patients within reasonable travelling distance of the hospital. They had to meet these inclusion

criteria: aged 18-75, male or post-menopausal if female, weight 60-89kg. Twelve carried on to enter the cross-over study described in chapter 9. Patients were sent an information sheet prior to attendance for an interview. They were accepted, having given their informed, written, consent if they were not specifically excluded by any of the following criteria: they were taking regular medications for angina other than aspirin or GTN as required, if they were hypertensive and ever had a systolic blood pressure exceeding 200mmHg or a diastolic blood pressure exceeding 105mmHg, if they had a stroke or transient ischaemic attack within the preceding 6 months, if they ever had epilepsy or a grand mal seizure, if they had a myocardial infarct or unstable angina within the preceding 6 months, any history of cardiac dysrhythmias, hepatic or renal impairment, re-vascularisation procedures within 3 months requiring them to continue on aspirin or dipyridamole, treatment with oxpentifylline within the preceding 2 months or evidence of peripheral vascular disease in either arm (reduced pulse volume or a discrepancy in systolic or diastolic blood pressure between sides of over 10mmHg).

All volunteers were Caucasian. Eleven of the thirteen volunteers were men, 4 were smokers, 8 ex-smokers and 1 non-smoker. All patients had arteriographically proven PVD, 7 had previous surgery or balloon angioplasty. They were aged 53 to 75 years (mean 63 years), weighed 60 to 88kg (mean 69kg) and their heights ranged from 1.66 to 1.79m for the men (mean 1.72m) and the women were 1.58m and 1.62m in height.

8.5 Volunteer restrictions

Patients were required to stop all drugs (except glyceryl trinitrate used as required) for 2 weeks prior to the study and until the end of the second study day. For 48 hours prior to and including each study day they were asked to avoid alcohol and smoking (or nicotine) and for 40 hours prior

to and including each study day they were asked to avoid caffeine in food or drinks (including decaffeinated drinks). If they needed analgesia they were asked to use paracetamol and if they needed to transgress these restrictions they were asked to contact me during office hours and were given details of how to do so.

8.6 Results

8.6.1 Missing data

Six sessions of FBF data were not analysed from 4 volunteers. One was not analysed because the volunteer had smoked immediately prior to their first session and baseline FBF was grossly elevated. Two sessions were lost because a volunteer was sick on their second study day, a further session because another volunteer withdrew from the study after the first study day and finally one session was lost due to computer error.

No blood samples were taken from the first 4 volunteers for cAMP estimation as a cooled centrifuge had not been located for separation of plasma. Samples from the second day of 2 further volunteers were not taken due to sickness on one occasion and due to the volunteer withdrawing from the study on the second occasion.

8.6.2 Room temperature

The mean, initial room temperatures was 24.4°C, range 22 to 27°C and the mean, final room temperature was 24.3°C, range21 to 27°C. The maximum increase during any study day was 3°C, mean 0.8°C.

8.6.3 Pulse and blood pressure

The profile of pulse and blood pressure data recorded at various protocol points is shown in table 8.2. The value of N shown is the total number of sessions (upto 4 sessions per volunteer) analysed. Table 8.3 shows derived mean arterial pressures (MAPs) by protocol point. Analysis of variance was used for statistical comparisons allowing for volunteer and period effects. There were significant differences in MAP comparing values obtained before and after ischaemia and particularly after exercise. The pre-ischaemia and pre-exercise values also differed significantly. There were no significant differences in pulse, systolic, diastolic blood pressure or MAP between the first and second study days or between morning and afternoon sessions (values are not shown).

Table 8.2 Pulse and blood pressure by protocol point

	Systolic BP (mmHg)		Diastolic BP (mmHg)		Pulse (beats/minute)	
	Mean	SD	Mean	SD	Mean	SD
Pre-Ischaemia	142.1	17.2	72.5	9.4	72.6	10.4
Post-Ischaemia	144.5	19.3	74.1	9.3	71.8	10.6
Pre-Exercise	144.5	18.1	75.2	10.0	71.1	10.7
Post-Exercise	153.2	20.8	77.3	9.7	72.4	11.4

Table 8.3 Comparison of mean arterial pressures by protocol point

MAP(mmHg)	Mean	SD	N	Comparison	P
1. Pre-Ischaemia	95.7	10.6	37	1 with 2	0.017
2. Post-Ischaemia	97.6	11.0	39	1 with 3	0.021
3. Pre-Exercise	98.3	11.1	41	3 with 4	0.0005
4. Post-Exercise	101.9	11.5	41		

8.6.4 Forearm blood flow

8.6.4.1 Baseline forearm blood flows

Mean baseline FBFs were compared by analysis of variance to determine the effect of protocol position, time of day and study day on baseline flow. The results are shown in table 8.4.

Table 8.4 Comparisons of mean baseline blood flows by forearm (mls/100mls/min)

Factor		Active Forearm			Control Forearm		
	N	Mean	SD	P	Mean	SD	P
Pre-Ischaemia	46	4.36	1.42	0.057	4.08	1.44	0.071
Pre-Exercise	46	3.91	1.44		3.72	1.41	
Morning Session	46	3.71	1.41	0.001	3.54	1.28	0.001
Afternoon Session	46	4.55	1.37		4.25	1.49	
First Study Day	48	4.16	1.19	0.064	3.92	1.27	0.52
Second Study Day	44	4.10	1.69		3.87	1.59	

There is a significant increase in FBF in both forearms between the first and second sessions of the day, however there is no change in FBF between study days. Evidence of a decline in baseline FBF during the protocol can be seen, which does not achieve significance.

The nested analysis of variance of baseline FBF shows more variability in the active forearm than the control forearm, as would be expected. The variability is increased compared to that measured in healthy, male volunteers (table 3.2).

Table 8.5 Nested analysis of variance of baseline blood flows by forearm

%Coefficient of Variation	Active Forearm	Control Forearm
Total (between volunteer)	35	37
Within volunteer, between day	32	30
Within day, between session	27	22
Within session, between baseline	21	17
Overall mean (mls/100mls/min)	4.13	3.89

8.6.4.2 Hyperaemic forearm compliances compared to healthy volunteers

Table 8.6 shows the characteristics of the 12 healthy, male volunteers studied in chapter 3 compared to those of the 11 *male* volunteers studied in this study. As there is a clear difference in mean arterial pressure, FBFs have been converted to compliances by dividing by the MAP derived from the preceding BP reading. The profiles of the compliances measured in the two groups during the 6 periods of hyperaemia are displayed in figures 8.1 to 8.6.

Table 8.6 Volunteer characteristics by subject group

Mean:Range or SD	Peripheral Vascular Disease		Healthy Volunteers	
Gender	Male		Male	
Race	Caucasian		11 Caucasian, 1 Oriental	
Number of Subjects	11		12	
Current and Ex-Smokers	4 and 7		0 and 0	
Weight in Kg	73	60-88	81	62-100
Height in metres	1.72	1.66-1.79	1.75	1.56-1.86
Room Temperature in °C	24	21-27	24	21-27
Systolic BP in mmHg	142	11	123	5
Diastolic BP in mmHg	74	5	72	5
MAP in mmHg	97	6	89	4

Figure 8.1 **Excess forearm compliance time-profile by subject group**

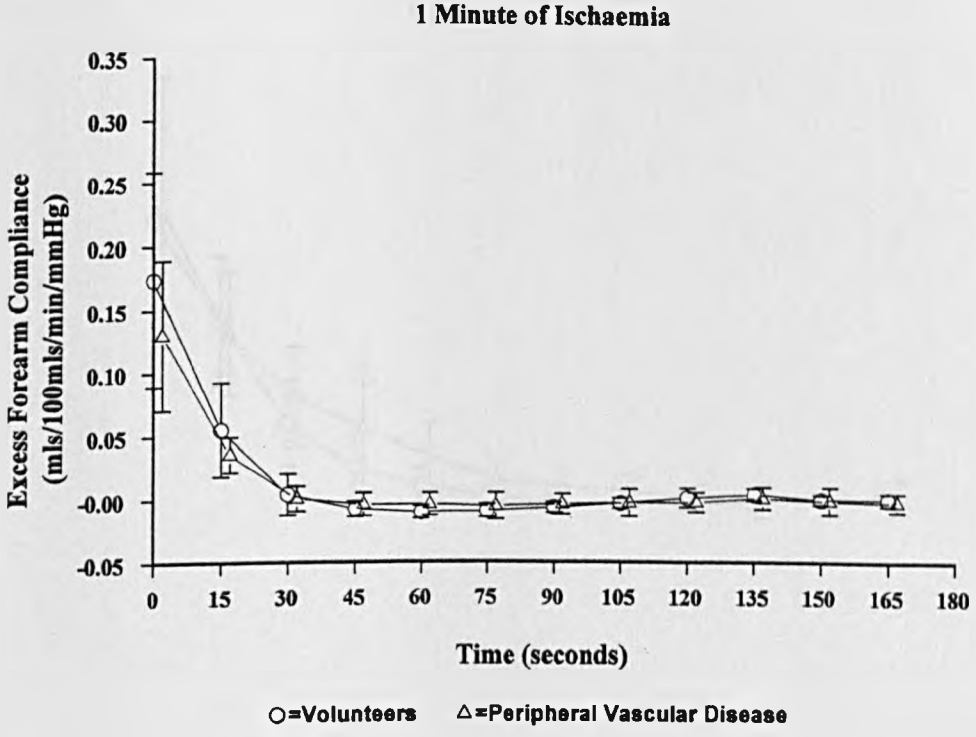


Figure 8.2 **Excess forearm compliance time-profile by subject group**

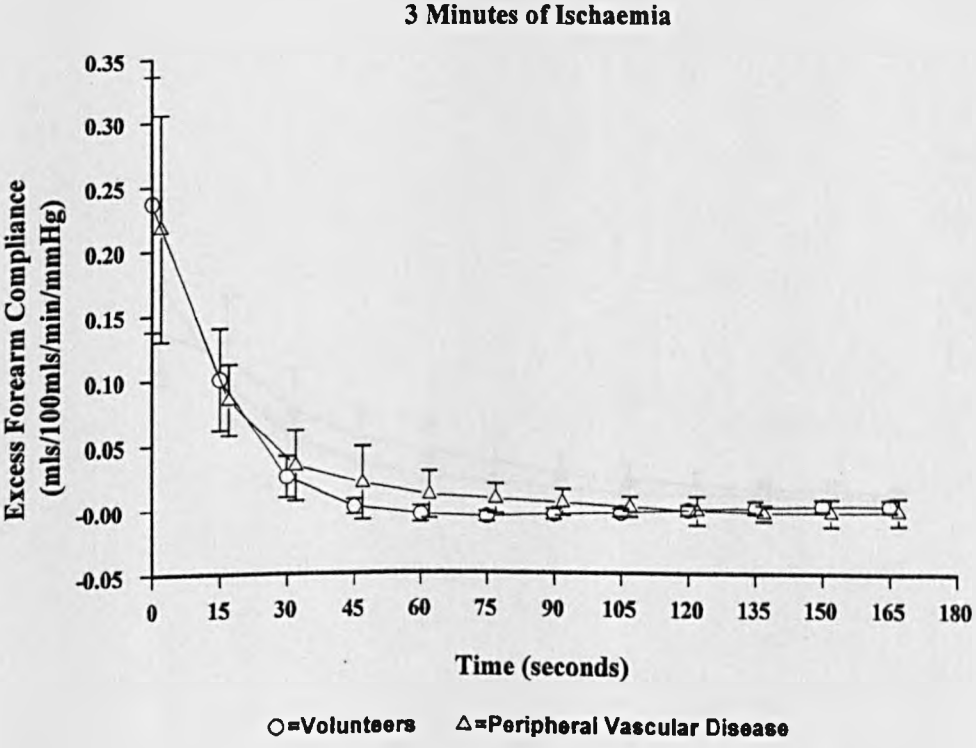


Figure 8.3 **Excess forearm compliance time-profile by subject group**

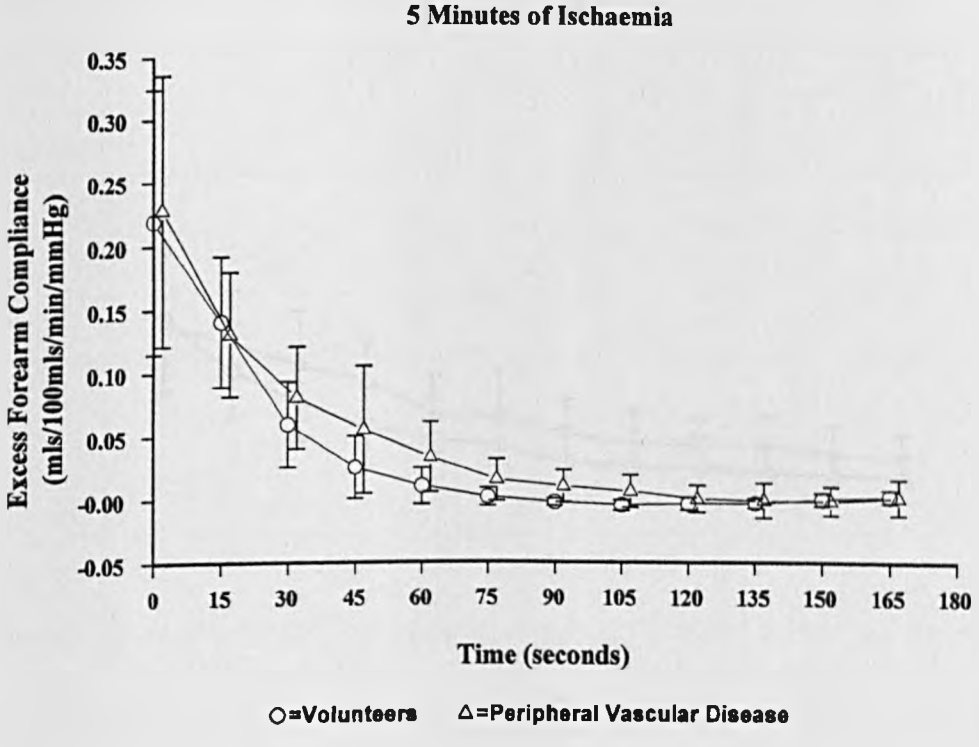


Figure 8.4 **Excess forearm compliance time-profile by subject group**

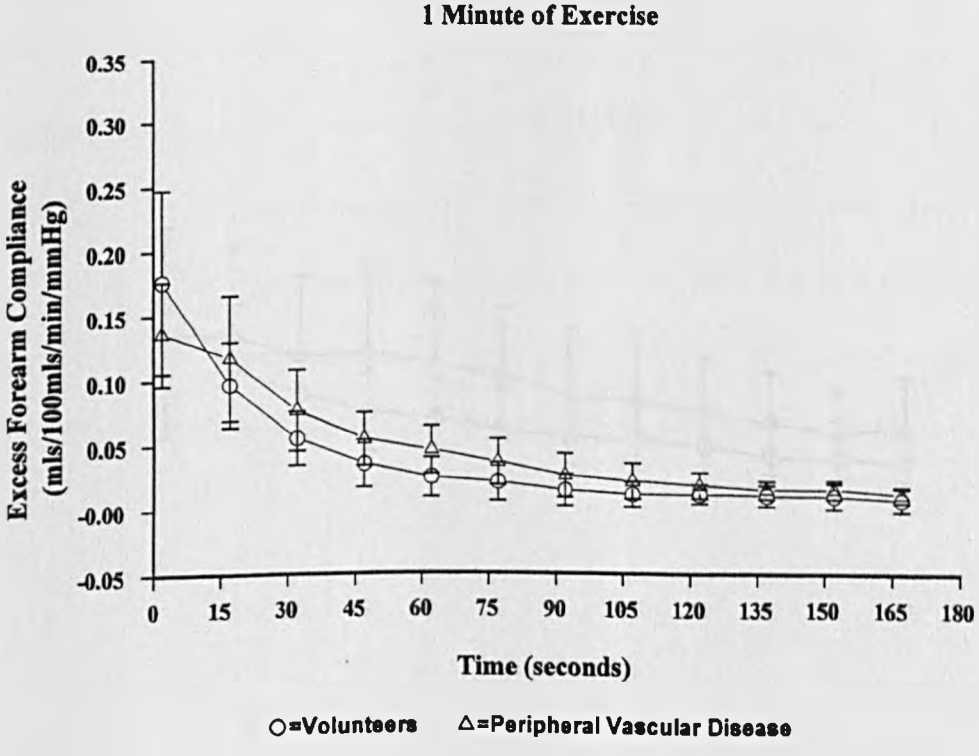


Figure 8.5 Excess forearm compliance time-profile by subject group

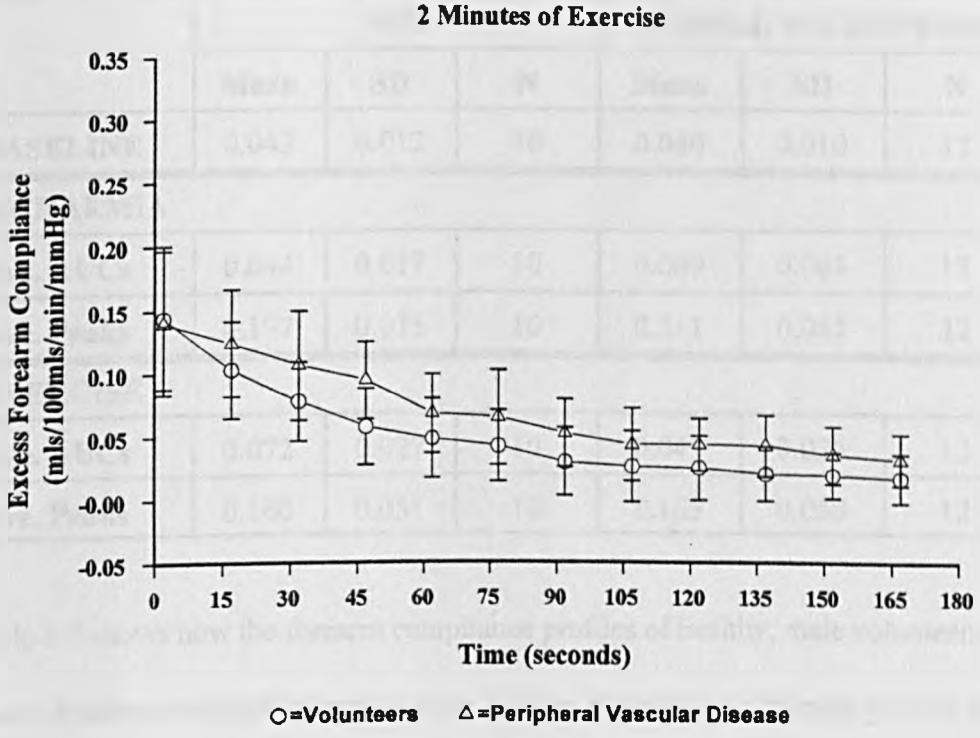


Figure 8.6 Excess forearm compliance time-profile by subject group

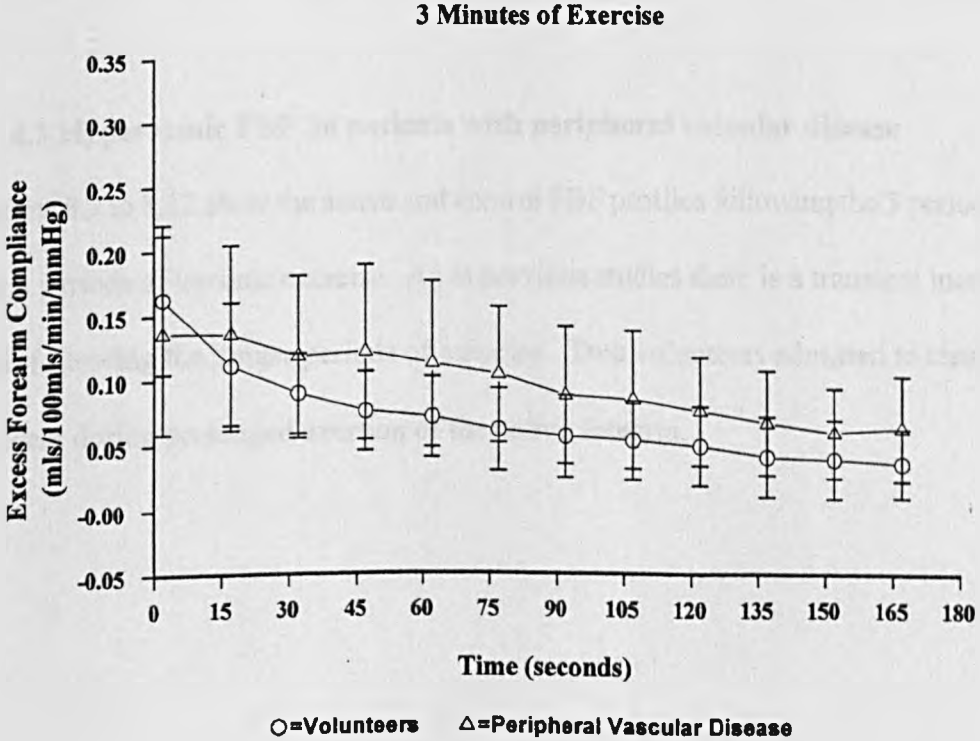


Table 8.7 Comparison of forearm compliance measures (mls/100mls/min/mmHg)

	PVD			NORMAL VOLUNTEERS			
	Mean	SD	N	Mean	SD	N	P
BASELINE	0.043	0.012	10	0.040	0.010	12	0.55
ISCHAEMIA							
Ave. AUCs	0.044	0.017	10	0.009	0.004	12	0.0001
Ave. Peaks	0.197	0.075	10	0.211	0.085	12	0.67
EXERCISE							
Ave. AUCs	0.072	0.027	10	0.049	0.026	12	0.0502
Ave. Peaks	0.160	0.051	10	0.163	0.050	12	0.90

Table 8.7 shows how the forearm compliance profiles of healthy, male volunteers compare with those of patients with peripheral vascular disease, but without clinically evident involvement of the brachial artery. The unpaired, two-way, Student's t test was used. The peak compliances are very similar, but there is significant prolongation of reactive hyperaemia in patients. Prolongation of active hyperaemia is not quite significant.

8.6.4.3 Hyperaemic FBF in patients with peripheral vascular disease

Figures 8.7 to 8.12 show the active and control FBF profiles following the 3 periods of ischaemia and 3 periods of isotonic exercise. As in previous studies there is a transient increase in control FBF following the longer periods of exercise. Two volunteers admitted to tensing the control forearm during prolonged exertion of the active forearm.

Figure 8.7 FBF time-profile in subjects with PVD

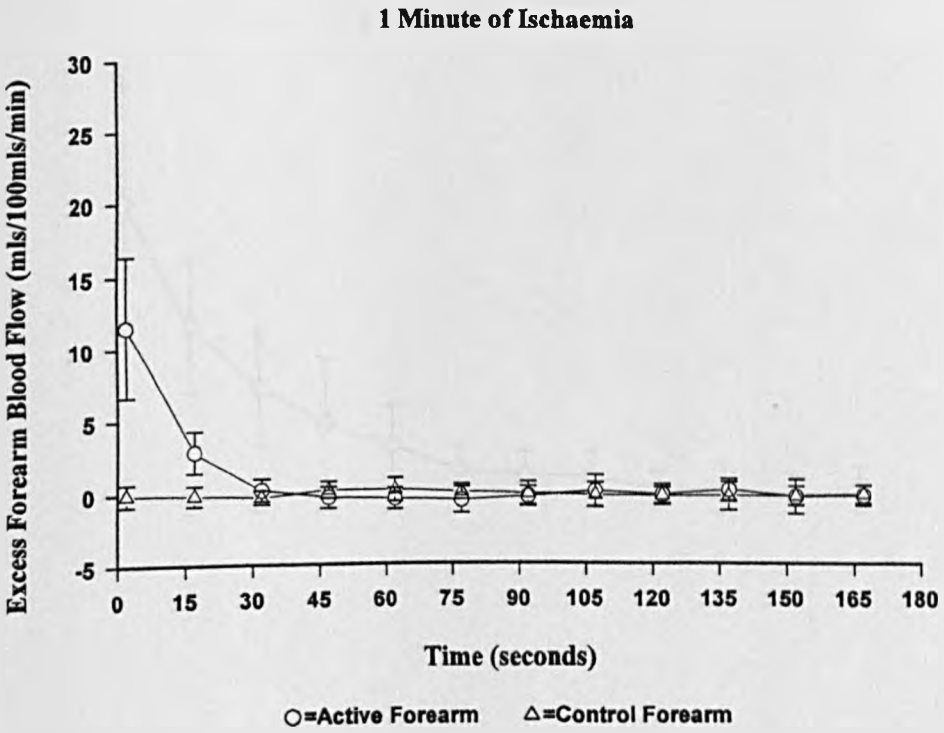


Figure 8.8 FBF time-profile in subjects with PVD

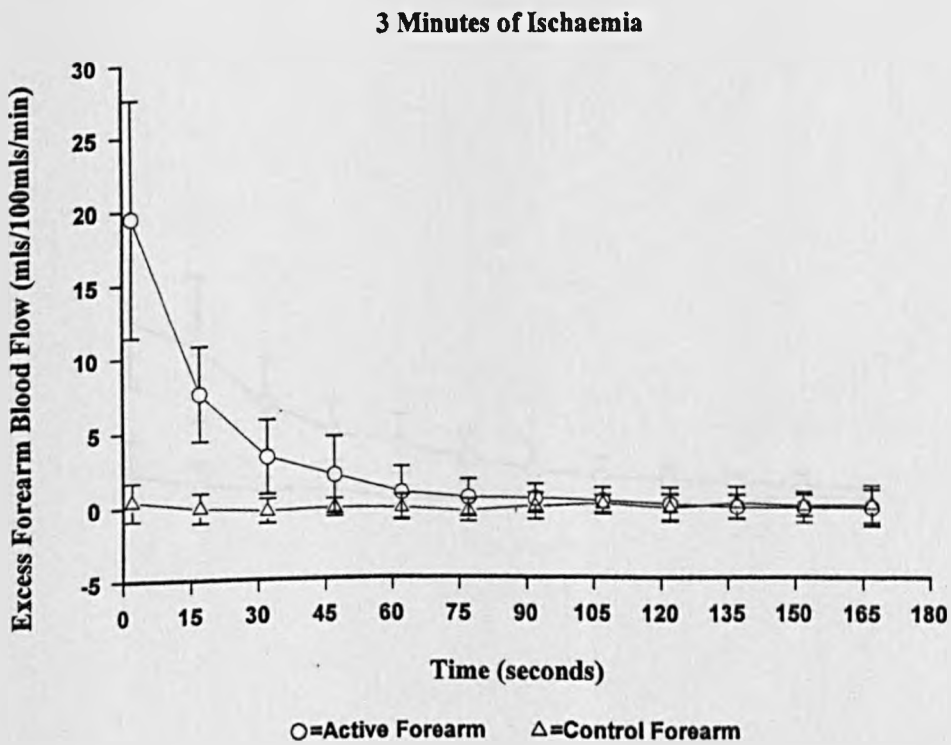


Figure 8.9 FBF time-profile in subjects with PVD

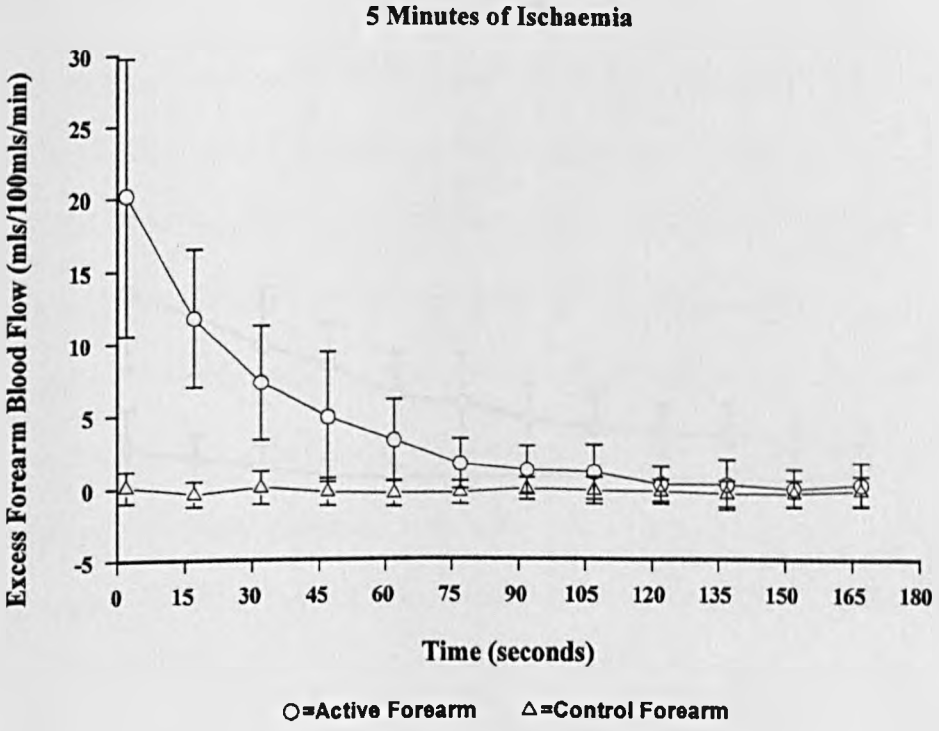


Figure 8.10 FBF time-profile in subjects with PVD

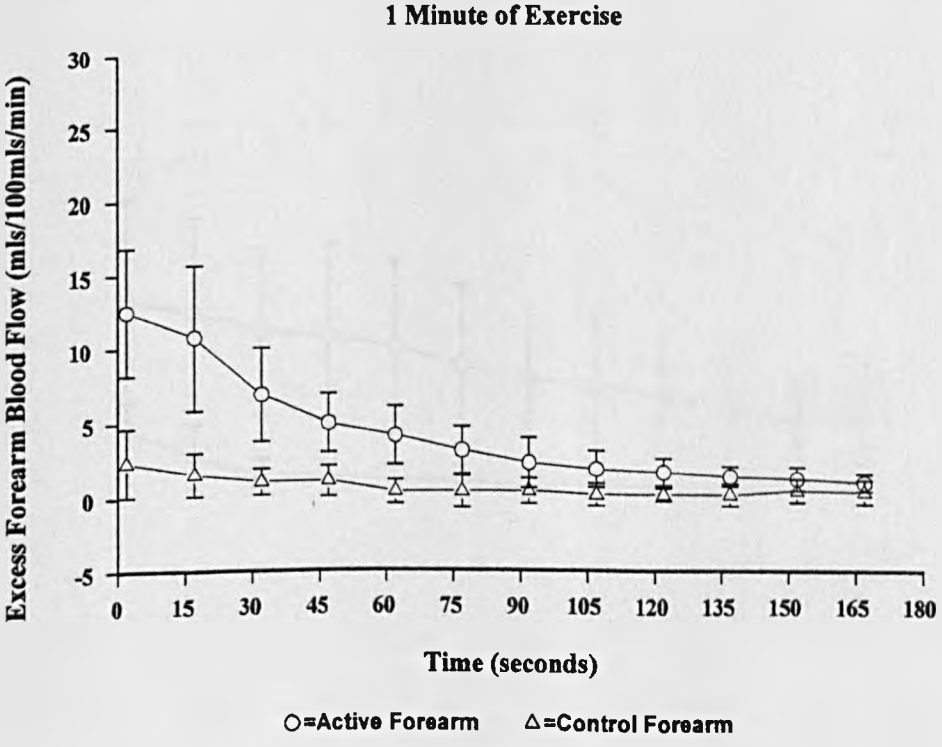


Figure 8.11 FBF time-profile in subjects with PVD

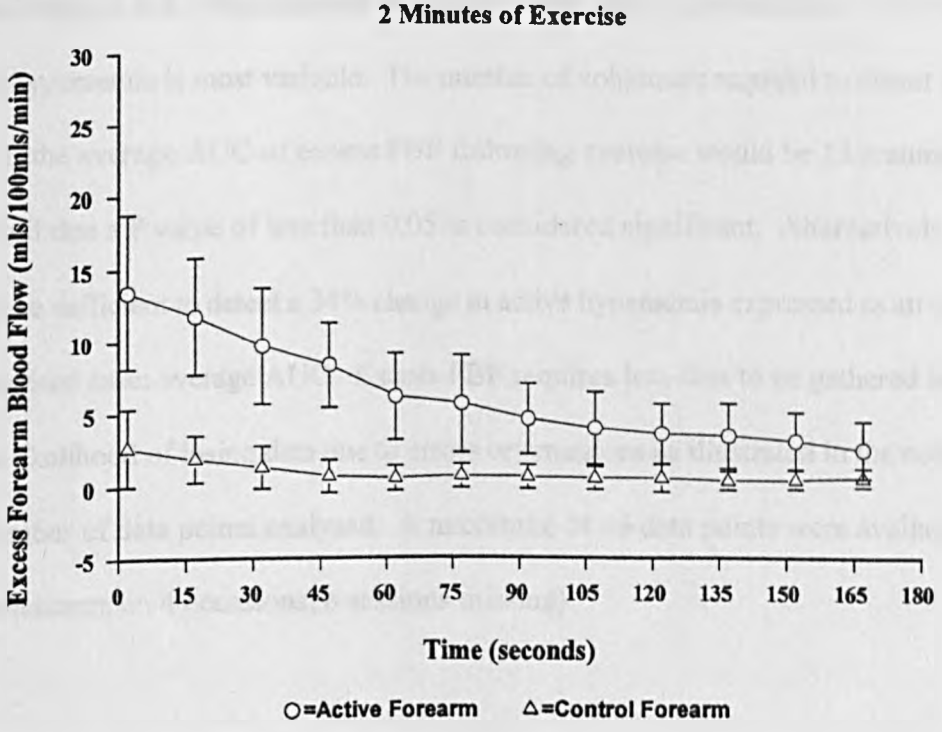


Figure 8.12 FBF time-profile in subjects with PVD

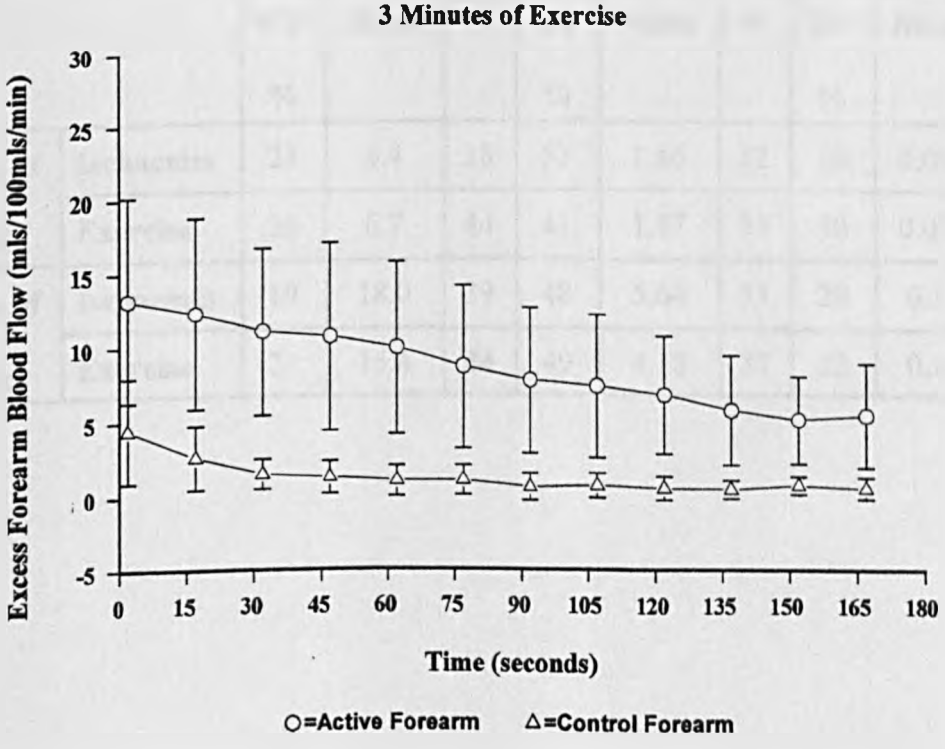


Table 8.8 shows the variability of the two summary measures adopted in chapter 3. Excess FBF is least variable and is the summary measure adopted in previous studies. As in previous studies active hyperaemia is most variable. The number of volunteers required to detect a difference of 30% in the average AUC of excess FBF following exercise would be 15 assuming a power of 90% and that a P value of less than 0.05 is considered significant. Alternatively 12 volunteers would be sufficient to detect a 34% change in active hyperaemia expressed as an excess FBF and summarised as an average AUC. Excess FBF requires less data to be gathered and hence there is less likelihood of losing data due to errors or omissions as illustrated in the columns showing the number of data points analysed. A maximum of 46 data points were available for analysis (13 volunteers on 4 occasions, 6 sessions missing).

Table 8.8 Variability by summary measure

		Excess FBF			Excess FBF Ratio			Excess Forearm Compliance		
		CV %	Mean	N	CV %	Mean	N	CV %	Mean	N
Ave. of AUCs	Ischaemia	23	4.4	38	53	1.46	32	26	0.046	31
	Exercise	26	6.7	44	41	1.47	35	30	0.072	38
Ave. of Peaks	Ischaemia	19	18.0	39	48	5.64	33	20	0.19	32
	Exercise	21	15.4	44	49	4.18	37	22	0.16	38

8.6.5 Plasma cyclic AMP (cAMP) concentration

The plasma cAMP assay is described in detail in chapter 2. Plasma was collected in a pre-cooled tube and promptly separated by centrifugation at 4°C. Plasma was stored for subsequent batch analysis at -20°C. The analysis was carried out using an assay kit: Biotrak™ (Amersham International PLC, UK), a competitive enzyme immunoassay.

The mean plasma cAMP level was 14.2nM, broadly in accord with the mean values recorded in healthy, male volunteers given placebo in previous studies (11.2nM in chapter 6 and 18.9nM in chapter 7). Paired plasma samples from 7 volunteers yielded an estimated within-volunteer, between-day coefficient of variation was 30%. In 12 volunteers this assay would have a 90% chance of detecting a 40% change in the plasma cAMP concentration due to treatment assuming significance P values of 0.05 or less.

8.6.6 Adverse events

Five volunteers found 3 minutes of isotonic exercise moderately uncomfortable, particularly on their first attempt. One volunteer volunteered that they had required paracetamol for a headache beginning the day prior to their attendance. Two volunteers were unable to give up smoking, both smoking on the study day prior to the study.

8.7 Summary

The coefficient of variation of the summary measures for active and reactive hyperaemia indicate that a 34% change in active hyperaemia (the most variable measure) would be detectable in 12 volunteers. Forearm compliance profiles during active and reactive hyperaemia are remarkably similar in patients with PVD to healthy controls despite major differences in age, smoking status and supine BP. Although there was no change in the peak compliances recorded following exercise or ischaemia, there was some prolongation of reactive hyperaemia. There was an increase in baseline FBF recorded during the second session of the day compared to baseline FBF recorded 4 hours earlier, during the first session.

There was a small increase in MAP after ischaemia and before exercise compared to the first, pre-ischaemia measurements. There was a greater increase in MAP recorded after exercise compared to pre-exercise levels. The plasma cAMP assay performed well in my hands and if this is maintained in the cross-over study it should be capable of detecting a 40% change in plasma cAMP.

Chapter 9

The effects of low-dose and standard-dose theophylline on reactive and active hyperaemic responses in patients with peripheral vascular disease

9.1 Introduction

In chapter 4 I showed that theophylline enhanced active hyperaemia at doses achieving plasma levels in the upper therapeutic range. In chapter 6 I showed that these levels of theophylline did inhibit adenosine-induced vasodilatation in the forearm, but that there was evidence that theophylline was inhibiting phosphodiesterase also. The IC_{50} for theophylline for a variety of phosphodiesterases tends to be at the upper limit of the therapeutic range[239] or more often above it[119,120], whereas adenosine antagonism has been demonstrated in man using doses of theophylline unlikely to achieve plasma levels above the lower limit of the range[249]. By pitching the dose of theophylline at a lower level it should be possible to separate these two activities, maintaining nearly all of the adenosine antagonism whilst reducing the phosphodiesterase inhibition to negligible levels.

9.2 Aims

I wanted to establish whether the enhancement of active hyperaemia by standard-dose theophylline in healthy volunteers could be reproduced in patients with peripheral vascular disease, whether selective inhibition of active hyperaemia was demonstrable with low-dose theophylline and whether there was any difference between active hyperaemic responses in the presence of standard-dose and low-dose theophylline as predicted by the pharmacology already outlined.

9.3 Design

This was a double-blind, 3-way cross-over, randomised comparison of the effect of single doses of placebo, standard-dose theophylline and low-dose theophylline. Volunteers were asked to attend the vascular laboratory on 3 days a week apart at the same time each day (8.30am, 10.30am or 12.30pm). Each time they were asked to take the same meal (breakfast or lunch) immediately prior to their attendance. They were asked about adverse events and providing they were well, they were given their individually bottled dose of medication and the time recorded. Following this they were allowed to leave with free access to fluids, to return 90 minutes later.

On their return they were asked to empty their bladders, asked about any symptoms and, if well, the plethysmography equipment was applied and tested. Two hours after dosing 15mls of blood was taken from a hand vein for plasma cAMP and serum theophylline levels. FBF measurements were then carried-out in accordance with the active-reactive hyperaemia protocol detailed in table 8.1. This protocol had been accepted by the hospital ethics committee prior to the study.

9.4 Volunteer characteristics and restrictions

Volunteers were recruited first for the reproducibility study as described in section 8.4. Of the 13 originally recruited, 12 agreed to enter the 3-way cross-over study. Volunteer restrictions are described in section 8.5.

Their were marginal changes in demography compared to the reproducibility study due to one volunteer leaving the group. As before all volunteers were Caucasian, 10 were men, 3 were smokers, 8 were ex-smokers and 1 had never smoked. All had angiographically proven PVD, 6 had previous surgery or balloon angioplasty. Their ages ranged from 58 to 76 years (mean 66

years), their weights ranged from 60 to 88kg (mean 73) and the men's heights were from 1.66 to 1.78m (mean 1.72m). The 2 women were 1.58 and 1.62m tall.

9.5 Dosing

Both theophylline (Nuelin™ 125mg tablets, 3M Healthcare) and placebo were given packed inside gelatin capsules to conceal any differences in appearance or taste. Each dose was given with 150mls of tap water. The Royal Liverpool Hospital Pharmacy packed the capsules for each patient according to the randomisation scheme. As in chapter 4 the standard-dose of theophylline was adjusted according to the weight of the volunteer: those weighing 70kg or over were given 5 capsules containing either placebo or a total of 625mg of theophylline, those under 70kg were given 4 capsules or 500mg of theophylline. This equates to a dose range of 7.0-8.9mg/kg. In chapter 4 the plasma theophylline level taken between 3hours 31minutes and 4 hours and 5 minutes after dosing was 58 to 94μM and in chapter 6 the plasma theophylline level 2 hours after dosing ranged from 71 to 122μM. This indicates that the theophylline level can be expected to remain in the therapeutic range throughout the protocol as predicted by the pharmacokinetics of oral, microcrystalline theophylline described in chapter 4.

In the study mentioned in the introduction, the dose of theophylline which reversed dipyridamole-induced myocardial ischaemia in patients with angina was 3mg/kg[249]. Dipyridamole is thought to act by enhancing the actions of endogenous adenosine by inhibiting cellular re-uptake. As Nuelin™ contains 125mg of theophylline, the only way of achieving a dosing level of 3mg/kg was to give 250mg to all volunteers irrespective of their weight in the range 60-89kg, which equates to a dose range of 2.8 to 4.2mg/kg.

9.6 Results

9.6.1 Missing data

Volunteer 2 was admitted to hospital with an infected, ischaemic leg ulcer and moderate hypertension before his final study day.

9.6.2 Room temperature

The mean, initial and final room temperatures were both 24.5°C and they both ranged from 22 to 26°C. The maximum increase during any study day was 3°C, mean 0.6°C.

9.6.3 Pulse and blood pressure

In this study single measurements of pulse and blood pressure were taken before and after the 3 periods of ischaemia and before and after the 3 periods of exercise. To obtain a single value for each study-day for each volunteer the pre-ischaemia and pre-exercise recordings of pulse and blood pressure were meaned. As can be seen in table 9.1, theophylline had little effect on systolic, diastolic or MAP, whether at low or standard dose. There was a small, but significant, increase in resting pulse rate at standard-dose as detected in earlier studies.

Table 9.1 The effects of low-dose and standard-dose theophylline on pulse and BP

MAP (mmHg)	Mean	SD	N	P vs Placebo	P Std. vs Low-Dose
Placebo	101.0	17.6	12	-	
Low-Dose Theophylline	100.6	13.8	11	0.67	0.81
Standard-Dose Theophylline	102.6	15.4	12	0.84	
SYSTOLIC BP (mmHg)					
Placebo	146.8	27.7	12	-	
Low-Dose Theophylline	146.2	20.0	11	0.59	0.77
Standard-Dose Theophylline	149.5	24.2	12	0.79	
DIASTOLIC BP (mmHg)					
Placebo	78.2	14.0	12	-	
Low-Dose Theophylline	77.8	12.7	11	0.78	0.86
Standard-Dose Theophylline	79.1	11.9	12	0.91	
PULSE (beats per minute)					
Placebo	72.8	10.3	12	-	
Low-Dose Theophylline	74.6	14.2	11	0.59	0.77
Standard-Dose Theophylline	77.6	14.2	12	0.04	

9.6.4 Forearm blood flow

A mean baseline flow was obtained for each attendance of each volunteer by meaning each simultaneous active and control FBF measurement together and then meaning the 12 pre-ischaemia measurements together and the 12 pre-exercise measurements together. The 2 figures derived were then meaned together to produce an overall mean for each attendance. These were compared by treatment using ANOVA allowing for volunteer, period and order effects. As table 9.2 shows, neither low-dose nor standard-dose theophylline had any significant effect on baseline

FBF. The difference between baseline FBF after low-dose and standard-dose theophylline was not significant either (P=0.11).

Table 9.2 Comparison of baseline forearm blood flow by treatment

mls/100mls/min	Mean	SD	N	P vs Placebo
Placebo	4.38	1.55	12	-
Low-Dose Theophylline	4.30	1.05	11	0.5
Standard-Dose Theophylline	5.09	1.47	12	0.3

Figures 9.1 to 9.6 show reactive and active hyperaemic response profiles in the presence of placebo, low-dose theophylline and standard-dose theophylline. Error bars have not been drawn for the sake of clarity. There was little difference between the three and this was confirmed when summary measures were compared using ANOVA. The results are shown in table 9.3. As in previous studies excess FBF above baseline was used throughout, although in this study there no significant difference in baseline FBF caused by the treatments. The two summary measures are the same as those used before. Each hyperaemia profile was expressed as both a time-weighted, mean positive FBF (a derivative of the AUC) and a peak, excess FBF. The three values derived by each of these two methods for each of the 3 durations of exercise and of ischaemia were then meaned together to produce single values. In this way two summary values were produced for reactive hyperaemia and two values for active hyperaemia for each treatment for each volunteer. In table 9.3 mean PFBF refers to the summary figure derived from the time-weighted, mean, positive FBF.

Table 9.3 Comparison of hyperaemic FBF summary measures by treatment

mls/100mls/min		Mean	SD	N	P vs Placebo	P Low-Dose vs Std.-Dose
ISCHAEMIA	mean PFBF					
	Placebo	4.78	2.47	11	-	
	Low-Dose	4.11	0.80	10	0.12	0.18
	Std.-Dose	4.45	1.36	12	0.76	
	Peak FBF					
	Placebo	20.98	10.55	11	-	
	Low-Dose	19.83	8.40	10	0.25	0.89
	Std.-Dose	19.78	8.51	12	0.28	
EXERCISE	mean PFBF					
	Placebo	6.62	3.36	12	-	
	Low-Dose	6.20	2.02	10	0.46	0.27
	Std.-Dose	7.99	3.55	11	0.67	
	Peak FBF					
	Placebo	14.55	6.65	12	-	
	Low-Dose	14.07	5.10	10	0.68	0.28
	Std.-Dose	16.13	8.38	12	0.46	

The results reflect the profiles seen in the figures. There are no significant differences in active or reactive hyperaemia in the forearm of patients with PVD whether the result is expressed as the time-weighted mean, positive FBF or as the peak, excess FBF.

Figure 9.1

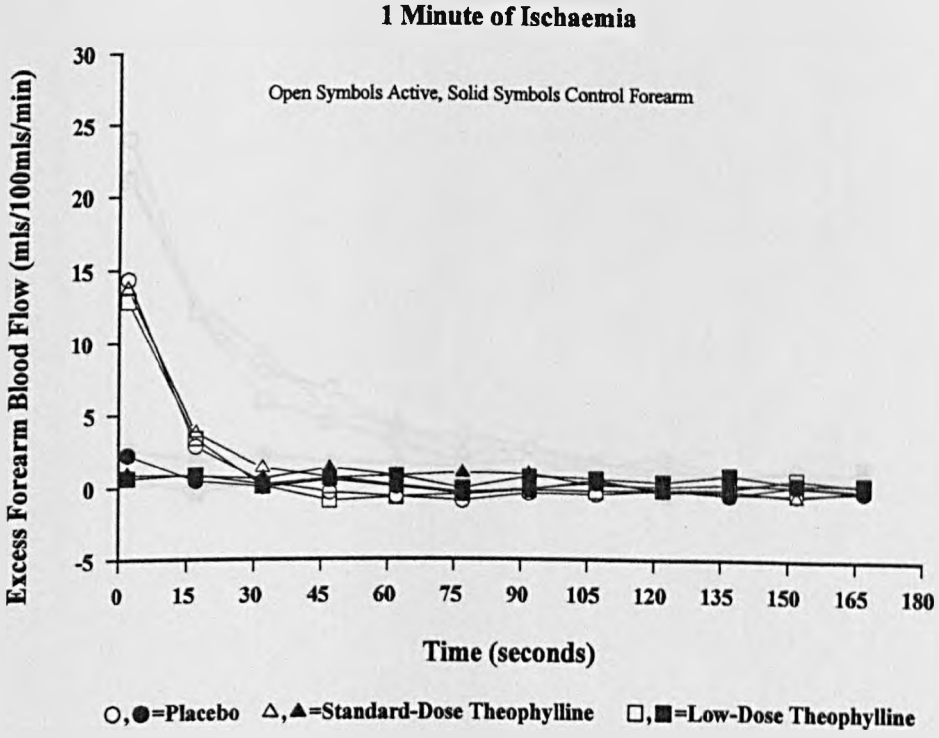


Figure 9.2

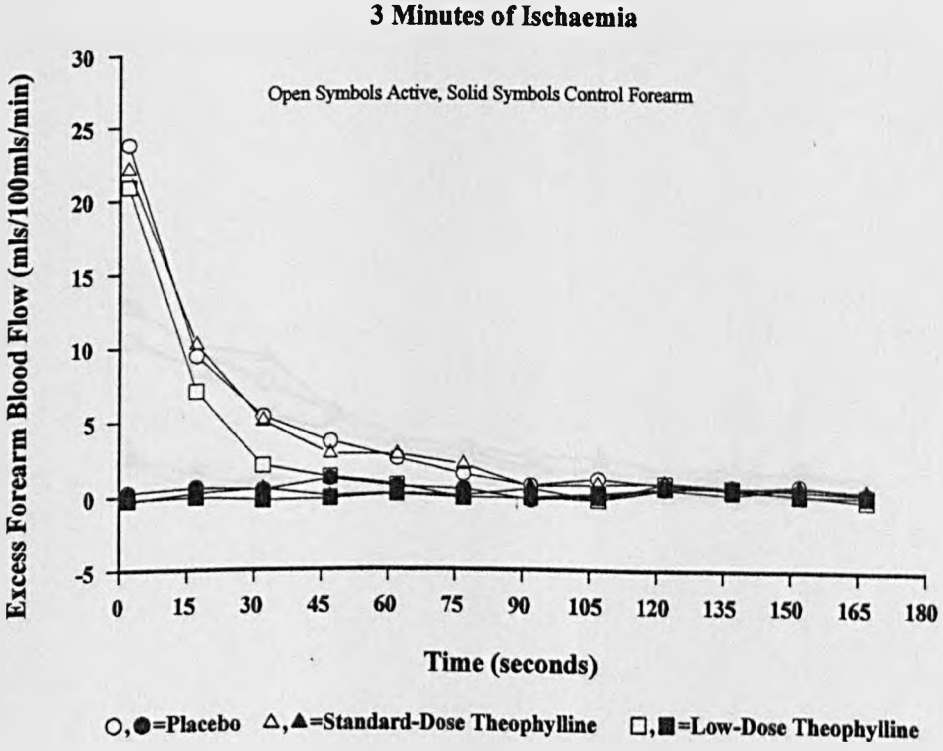


Figure 9.3

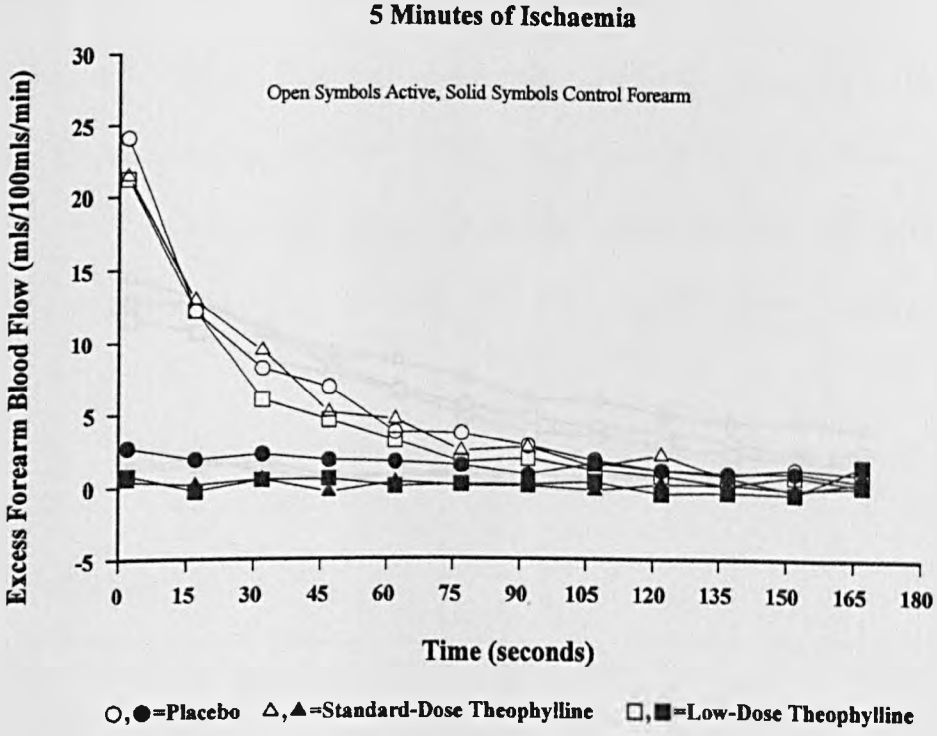


Figure 9.4

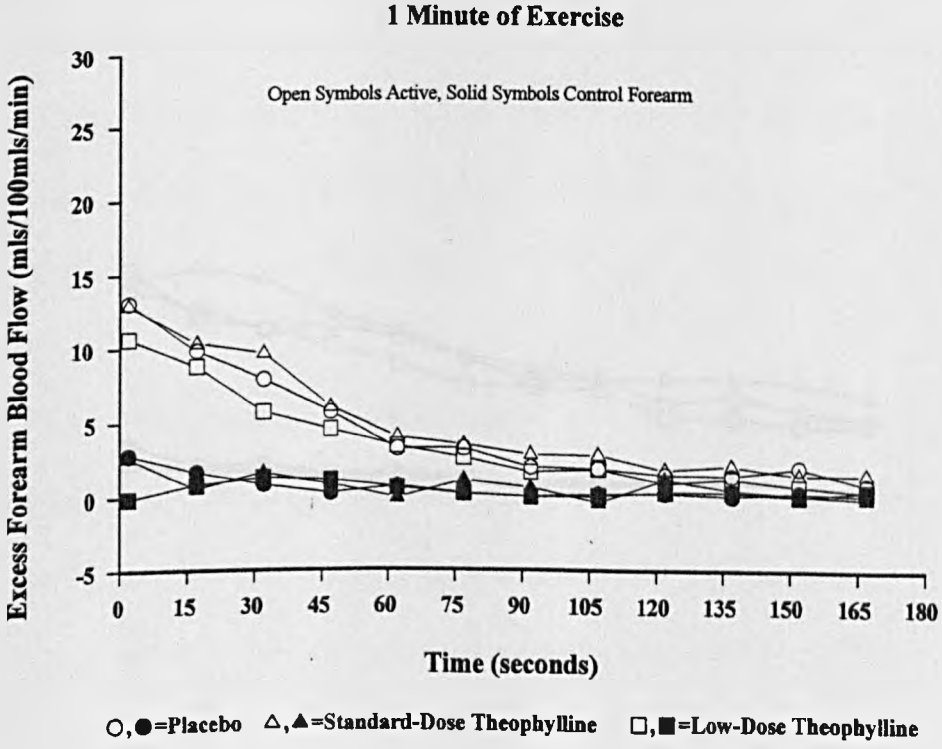


Figure 9.5

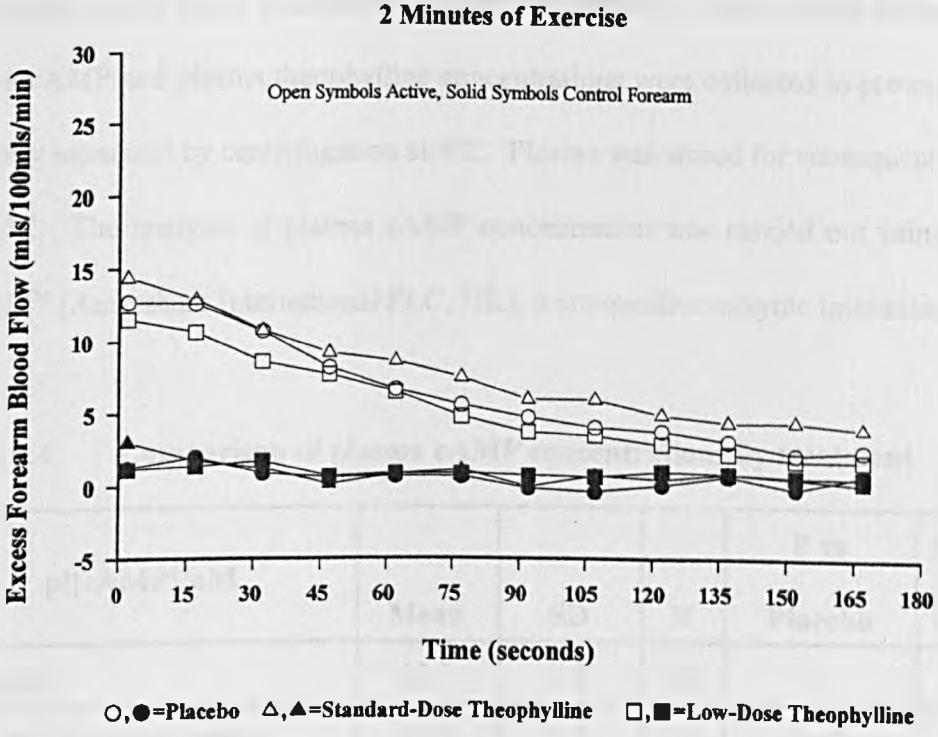
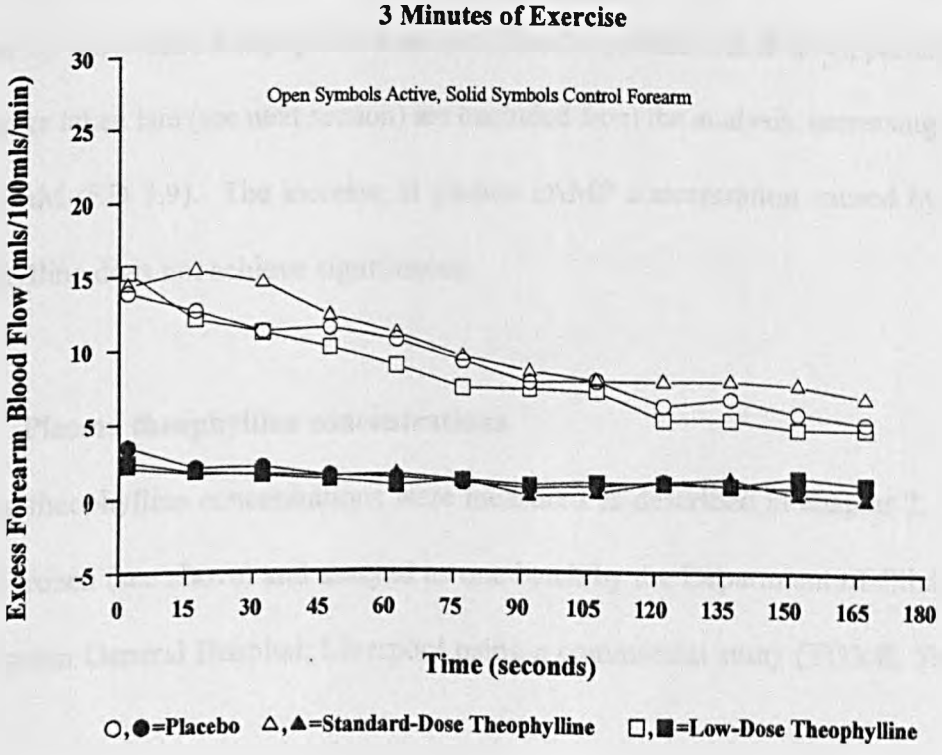


Figure 9.6



9.6.5 Plasma cyclic adenosine monophosphate (cAMP) concentrations

The plasma cAMP assay is described in detail in chapter 2. The samples for measurement of plasma cAMP and plasma theophylline concentrations were collected in pre-cooled tubes and promptly separated by centrifugation at 4°C. Plasma was stored for subsequent batch analysis at -20°C. The analysis of plasma cAMP concentration was carried out using an assay kit: Biotrak™ (Amersham International PLC, UK), a competitive enzyme immunoassay.

Table 9.4 Comparison of plasma cAMP concentrations by treatment

pl[cAMP]/nM	Mean	SD	N	P vs Placebo	P Low-Dose vs Std.-Dose
Placebo	12.7	6.1	12	-	
Low-Dose Theophylline	11.8	5.3	10	0.82	0.09
Standard-Dose Theophylline	15.3	7.4	12	0.10	

Table 9.4 contains the plasma cAMP concentrations measured by me using a kit from Sigma (see chapter 2). Low-dose theophylline does not effect the plasma cAMP level, particularly when the 2 samples taken late (see next section) are excluded from the analysis, increasing the mean level to 12.3nM (SD 5.9). The increase in plasma cAMP concentration caused by standard-dose theophylline does not achieve significance.

9.6.6 Plasma theophylline concentrations

Plasma theophylline concentrations were measured as described in chapter 2. Samples were stored frozen (see above) and assayed as one batch by the Department of Clinical Chemistry, Broadgreen General Hospital, Liverpool using a commercial assay (TDX®, Sigma Chemical Co.).

9.7 Summary

These results contrast with those found in healthy volunteers. Neither standard-dose nor low-dose theophylline had any significant effect on active or reactive hyperaemia in volunteers with PVD. Hence I have been unable to reproduce the enhanced active hyperaemia caused by standard-dose theophylline in healthy volunteers. Neither have I been able to demonstrate a significant difference between the effects of standard and low-dose theophylline on active or reactive hyperaemia.

Baseline FBF was unchanged by low-dose theophylline, but only increased insignificantly by 16% following standard-dose theophylline.

The plasma concentration measured at 2 hours was 85μm following standard-dose theophylline. This is similar to that recorded in a healthy volunteers in chapter 6. The increase in plasma cAMP concentration following standard-dose theophylline did not achieve significance and is smaller than that recorded in healthy volunteers. As predicted, low-dose theophylline had little effect on the plasma cAMP concentration.

Apart from an increase in pulse rate following standard-dose theophylline, neither dose of theophylline had any significant effect on central haemodynamics.

No theophylline was detected following placebo. Following low-dose theophylline the mean theophylline level was 35μM, range 23 to 47μM. The two lowest values were from samples taken late: 23μM at 3 hours 10 minutes and 25μM at 2 hours 50 minutes. Excluding these samples the average sample time was 2 hours 1 minute, range 1 hour 58 minutes to 2 hours 10 minutes and the mean theophylline level was 37μM, range 29 to 47μM.

The mean level following standard-dose theophylline was 84μM, range 72 to 95μM. The mean sample time was 2 hours 4 minutes, range 1 hour 55 minutes to 2 hours 15 minutes.

9.6.7 Adverse events

There were no side-effects related to theophylline, though several volunteers continued to experience moderate discomfort whilst maintaining isotonic pressure for 3 minutes. Around a third of the volunteers admitted to mild headache or 'muzziness' commencing one or two days after caffeine withdrawal.

Chapter 10

Discussion

10.1 Introduction

The aim of this work was to delineate the role of adenosine in reactive and active hyperaemia in human skeletal muscle in both healthy volunteers and in patients with PVD. My stratagem has been to use theophylline, which has been shown to antagonize adenosine-induced vasodilatation in the human forearm to try and define the adenosine-dependent component of active and reactive hyperaemia firstly in healthy volunteers and then in patients with PVD. As all of the previous work establishing antagonism of adenosine-induced vasodilatation by theophylline in man has infused theophylline as the EDTA salt (aminophylline) directly into the brachial artery and no-one has quantified the blood or tissue levels of theophylline in the infused forearm I needed to establish whether oral theophylline obtaining peak blood concentrations in the upper therapeutic range measurably inhibited forearm vasodilatation due to locally infused adenosine. To reliably establish the effects of theophylline on reactive and active hyperaemia and of theophylline on adenosine-induced vasodilatation in the human forearm I needed to: establish reproducible methods for measuring these in healthy volunteers and to use an appropriate number of volunteers knowing the reproducibility of these methods in volunteers and when applicable in patients with PVD.

10.2 Reproducibility of baseline FBF

10.2.1 Diurnal variation

In the reproducibility studies on healthy volunteers (chapter 3) a number of factors contributing to variability in baseline forearm blood flow and to reactive and active hyperaemia were

identified. There was diurnal variation in baseline vascular tone and heart rate in both healthy volunteers and patients with PVD (chapter 8) and marked diurnal variation in central haemodynamics in healthy volunteers, though MAP did not change in patients. In healthy volunteers the 4 hour difference between sessions within each day was associated with a significant 20% increase in baseline FBF, a 5% fall in mean arterial pressure due to a fall in diastolic blood pressure, a 9% increase in resting heart rate, a 21% increase in CI and a 10% increase in SI. Several studies have shown diurnal variation in baseline FBF with an increase as the day progresses quite similar to the increase I noted[186,225,226]. One study showed that vasodilatation following the α-adrenergic antagonist phentolamine abolished diurnal variation whereas similar vasodilatation by sodium nitroprusside was still subject to diurnal variation[226]. Hence the increase in baseline FBF later in the day relates to a reduction in α-adrenergic tone in the forearm. Ambulatory monitoring of blood pressure showed a progressive fall in diastolic, systolic and mean arterial pressure from a high point around 9am[250], in accord with my findings. A unifying hypothesis to account for all of the findings would be a gradual reduction in sympathetic tone in the peripheral vasculature with increased peripheral blood flow starting 2 hours or so after waking causing a reduction in blood pressure. This in turn triggers a baroreflex with a reduction in central parasympathetic tone, consequent tachycardia and increased stroke index and cardiac index.

Care must be taken when measuring FBF responses or central haemodynamic indices to account for diurnal variation best achieved by conducting experiments at the same time each day.

10.2.2 Forearm dominance

There was evidence that baseline FBF is higher in the dominant forearm, perhaps because it contains a larger proportion of muscle compared to skin and connective tissue. In both reproducibility studies on healthy volunteers baseline flows in the dominant forearm significantly exceed those of the nondominant forearm, by 4 and 8%. Studying 2 groups of 6 subjects another group failed to find a significant difference in baseline forearm blood flow by dominance of the forearm[223], however the power of this study would be too low to delineate such a small effect reliably. The circumference of the dominant forearm has been measured to be 2% greater than the non-dominant forearm[223] equating to a 4% increase in cross-sectional area. Assuming that the extra tissue is mostly muscle, that the volume of bone increases only marginally and given that blood flow in bone is relatively low[176], it is possible that the increased proportion of muscle compared to bone in dominant forearms accounts for the 4 to 8% increase in baseline FBF noted.

10.2.3 Variability between sessions

Much of the variability in baseline FBF measurements accrues between sessions, with little extra variability contributed between days. This is doubtless in part because of the diurnal variation discussed, but also I suspect because the plethysmography equipment used to measure FBF is taken off and re-applied between sessions. In my hands the within-volunteer, within-day, between-session coefficient of variation was 24% in the active forearm and 26% in the control forearm. Within-volunteer, within-day, between-session reproducibility of baseline FBF measured by other investigators was 25%[251]. Comparing mean FBF measured during consecutive minutes yielded a CV of 12% when FBF was measured by an ECG triggered strain-gauge plethysmograph over 3 cardiac cycles upto 12 times each minute[225]. This is quite similar to my results of 12% in the nondominant forearm and 14% in the dominant forearm. Another group

measured 9-11% within-session variability and a mean, between-day CV of just 17% (range 2-23%)[252]. They used a direct, in-situ calibration method briefly infusing saline at progressively greater rates via an intra-arterial catheter during venous occlusion and deriving the overall gradient by linear regression applied to the points obtained. The between-day variability of the calibration coefficient was 17% and within-session variability was 7%. Unfortunately no comparison was made between the in-situ calibration readings and those obtained either by mechanically stretching the gauge or electronically, so the contribution of the novel calibration method to the good between-day reproducibility cannot be assessed. In this paper mercury-in-Silastic™ gauges were used which spanned the whole circumference of the arm, whereas Medasonic™ gauges are of fixed length, cover perhaps 75% of the forearm circumference and presume equal expansion of all segments of forearm circumference. This is clearly not so and would vary according to the exact position along the forearm and according to the exact portion of the circumference to which the gauge was applied. For example during a second application the inextensible part of the gauge could include more of the extensor aspect of the forearm where the ulna is close to the surface. FBF readings would be higher therefore as the inextensible part of the gauge would overlie more of the part of the forearm which would be expected to expand less and conversely more of the extensible part of the gauge would overlie the segment of the forearm which would be expected to expand more. My predecessor in Liverpool studying 6 healthy, trained volunteers weekly for 6 weeks and also recorded an impressively low between-day CV of 10.5%[253]. He calibrated his gauges mechanically, off the limb, but he used gauges which completely encircled the forearm. Direct comparison of the between-session reproducibility of Medasonic™ and strain-gauges encircling the forearm would clearly help to determine whether complete encirclement of the forearm does improve between-session reproducibility.

10.2.4 Variability of the active: control FBF ratios

Since both forearms show simultaneous increases in blood flow in response to sudden stresses such as a sudden loud noise, it has been suggested that much variation in FBF can be eliminated by expressing FBF as a ratio of the experimental forearm to the control forearm[134]. There was some questionable advantage in expressing baseline FBF as a ratio (table 3.10) in that the between volunteer variability fell from 32% to 26% and the within-volunteer, between-day and between-day variabilities fell marginally, however the within-session variability for the FBF ratio was much the same at 13% compared to 12 and 14% in the individual forearms. This does not accord well with the idea that the ratio will eliminate the effect of transient changes in systemic sympathetic tone, but is more consistent with amelioration of the effects of longer term changes in vascular tone, such as the diurnal variation noted earlier.

I did not find any significant change in mean arterial pressure 3 minutes after 3 minutes of single-forearm handgrip exercise or 3 minutes following 5 minutes of forearm occlusion. Neither did I find any change in control forearm blood flow during periods of ischaemia or handgrip exercise in the active forearm. Occasional, transient minor increases in control forearm blood flow, when they did occur were related to tensing of the control forearm during prolonged contractions. Small hyperaemic responses in the contralateral forearm during handgrip exercise can be eliminated by training volunteers to keep their contralateral forearm relaxed by monitoring it electromyographically[254]. My data does not rule out significant changes in blood pressure during interventions as recorded by other investigators during single-forearm exercise, particularly as the increase in mean arterial pressure they noted resolved within 30 seconds of cessation even following prolonged exercise[254,255]. However the increase in MAP they noted was not associated with changes in baseline, contralateral forearm blood flow (which accords with my findings) nor

did it increase active hyperaemic responses triggered by brief contralateral handgrip contractions[254].

10.3 Total (reactive) hyperaemia

Earlier studies by other investigators recorded a wide range of estimates for total hyperaemia from 0.2 mls/100mls after 1 minute of occlusion [136], 2.7 and 3mls/100mls after 3 minutes of occlusion[136,256] and 4.0, 12.6, 13.4 and 31.8 mls/100mls after 5 minutes of occlusion[136,143,144,256]. I recorded values for total hyperaemia of 3.3, 5.6 and 7.6 mls in my study. These seem low, but could be accounted for by variability between laboratories and the fact that I used the nondominant forearm and total hyperaemia in the dominant forearm has been found to be significantly greater in the right forearms of volunteers, presuming that the majority were right handed[136]. Elevation of the forearm above the level of the left ventricle has been shown to reduce the initial inflation artefact, making initial, high-flow signals easier to interpret, but it also reduces FBF measurements at rest and during reactive hyperaemia[257]. This is another factor which could account for some between-session variability and account for the wide spread of values encountered in the literature. Variation could be reduced by keeping the elbow level fixed at (say) the mid-axillary line and fixing the angle of the forearm to the horizontal.

Following even 5 minutes of ischaemia, recovery is complete within 2 minutes so it is fair to say that the total hyperaemia recorded over 3 minutes is the complete hyperaemic response to the periods of arterial occlusion studied. There is a linear relationship between duration of ischaemia and total hyperaemia. It is interesting to note that 1 minute of ischaemia would accrue a debt of approximately 3.5mls/100mls of tissue and the repayment is 3.3mls/100mls, whereas after 3 minutes, a debt of 10.5mls/100mls is repaid by 5.6mls/100mls and 5 minutes or 17.5mls/100mls

equates to 7.6mls/100mls repayment.

10.4 Peak FBF during reactive hyperaemia

Peak FBF during reactive hyperaemia compares well with literature precedents. A study which like mine measured peak forearm blood flow after 1, 3 and 5 minutes of ischaemia recorded peak FBFs of 8, 17 and 19mls/100mls/min[136] compared to my baseline-adjusted results of 15, 20 and 20mls/100mls/min, although it is not clear how soon after release of occlusion these authors made their first measurements. After 1 and 3 minutes of occlusion peak FBFs of 17 and 35mls/100mls/min, adjusted for baseline FBF[258] have been recorded and after 5 minutes of arterial occlusion other authors recorded a peak flow of 21.9mls and 23.3mls/100mls/min[143,144]. It is possible that peak FBF was slightly underestimated in my studies as reactive hyperaemia subsides so quickly, that it is possible to miss the peak FBF if for some reason it is delayed. Two studies recording higher flows measured FBF every 3 to 5 seconds to minimise this possibility[143,144] and the other measured FBF at 5 and 15s[258]. At high FBFs and especially if the veins are already distended, flow is only accurately represented for the first few seconds before the veins fill, compliance falls and FBF is underestimated. Peak FBF would only have to be deferred by a 2 or 3 seconds to be underestimated. With measurements every 15 seconds flow would have largely subsided by the second post occlusion measurement if the first measurement is aberrant. There were instances when the first measurement was lower than the second, but this was thankfully rare. Others have studied this using a computerised system measuring FBF on average every 4.8 seconds. The first 2 readings after a 10 minute occlusion were lower than the third. Furthermore, they also noted that at high flows, following arterial occlusion, FBF could only be reliably estimated during the first few seconds of venous occlusion, as the signal began to plateau. Significantly the highest estimates of peak FBF mentioned earlier were from groups

measuring FBF every 3 to 5 seconds[143,144]. Despite this, my between-session CV for (say) the average peak flow of 12% is comparable to the between-day CV of 13% obtained measuring FBF every 5 seconds after 10 minutes arterial occlusion[252].

10.5 Forearm volume during arterial occlusion

I did notice that the forearm expanded progressively during periods of occlusion, most of the expansion occurring during the first minute. I was unable to abolish this by increasing the occlusion pressure. This manifested in progressive movement of the baseline on the chart recorder and necessitated re-zeroing of the plethysmograph prior to measurements. The baseline returned to normal during the first minute of measurements after the occlusion. It did not seem to be possible accelerate the return to baseline by elevation of the forearm. I presumed initially that this 'leak' was due to blood shunting through the humerus via nutrient arteries and veins above and below the cuff. An increase in forearm volume of nearly 2% during 10 minutes of arterial occlusion has been measured[258]. Others have noted the plateauing of the signal attributable probably to rapid venous filling after periods of occlusion[259]. Lowering the pressure around the forearm causes an increase in forearm volume which takes 30 seconds to develop and a minute to clear[134]. Near infrared spectrophotometry can measure blood volume and the oxidation status of haemoproteins in the forearm noninvasively. During 8 minutes of tourniquet ischaemia the blood volume (in fact the total amount of haemoglobin) remained constant[137]. This suggests that the expansion in forearm volume is due to accumulating tissue fluid rather than blood. Increased tissue pressure will reduce venous compliance and so would account for the early plateau phase noted. It would also account for the gradual reduction in forearm volume (downward drift in strain-gauge resistance), noticeable between the cycles of venous occlusion for measurement of FBF immediately following release of arterial occlusion.

10.6 Active Hyperaemia

The recovery following exercise is much slower than that following ischaemia, although the peak blood flow following ischaemia is generally greater. This is in accord with published work[21,47]. The relationship between duration and AUC (repayment) is almost linear for increasing durations of exercise, but the total repayment following exercise is much greater than that following equivalent periods of ischaemia. This is despite the fact that the whole recovery period was not measured (FBF had not returned to baseline during the period of measurement) and only around 20% of forearm muscle is actively contracting during handgrip exercise, viz: extensor carpi profundus, flexor digitorum superficialis and to a lesser extent flexor digitorum profundus[255].

I could find only one other study in which the reproducibility of active hyperaemia was studied. Peak forearm blood flows were measured each minute during 5 minute periods of exercise at three exercise loads. This was repeated after 2 hours and the difference calculated between the results obtained during the first and second exercise periods. It is not clear how the authors summarised their results, but using a simple power calculation for estimating sample size[260] it is possible to deduce that the between-session, within-day, within-volunteer coefficient of variation must have been 16%. My value was 23%. It is interesting to note that they used strain-gauges which completely encircled the forearm.

10.7 Summary measures of hyperaemia

A systematic study of the effect of preceding baseline resistance on the magnitude of subsequent vasodilator responses showed that there was a close, positive correlation between them in the canine hindlimb[261]. Similarly, when baseline flow was increased by heating the forearm the absolute magnitude of subsequent flow responses during reactive hyperaemia were increased.

The AUC or total hyperaemia did increase when the temperature was increased from 14 to 32°C, but showed no further increase when the temperature was increased to 43°C, though baseline FBF increased 5 fold[256]. Incorporating baseline FBF as a covariate reduced variability of most summary endpoints for both ischaemia and exercise, however the overall mean was dramatically decreased by subtracting baseline FBF in some instances, so that the proportional change in hyperaemia might be commensurately enhanced enabling an effect to be detected even if underlying variability is increased. The work in the canine hindlimb suggests that expressing FBF responses as a ratio with the preceding baseline FBF would reduce variability, however I found that the variability of both active and reactive hyperaemia summary endpoints was increased. Incorporating baseline as a covariate in analysis of variance having already converted the FBF data to logarithms is a means of expressing the results as a ratio as analysis of variance effectively subtracts the covariate. There was some reduction in variability if log data is used when processed as total hyperaemia (or AUC), but the variability of peak log flows was no better than that of untransformed data.

10.8 Central haemodynamic effects

Even during the 5th minute of arterial occlusion, I found no change in central haemodynamics, but exercise was associated with an increase in heart rate and an equivalent reduction in SI, such that CI was unchanged. Other investigators have measured efferent muscle sympathetic nerve activity in the peroneal nerve during 6 minutes of forearm ischaemia to discover whether forearm ischaemia could have any effect on central haemodynamics. They also found no change, though they were able to demonstrate increased activity during isometric, handgrip exercise[262].

The central haemodynamic effects of single forearm exercise, such as the isometric handgrip

have been extensively studied. There is prompt elevation of blood pressure, known as the exercise pressor effect, also an increase in heart rate[254,263,264] and an associated increase in efferent sympathetic tone detectable in other limbs[262]. Whilst occlusion of the forearm circulation has no effect on central indices *per se* (c.v.), occlusion of the circulation in an exercising forearm prolonged the pressor effect and maintained the increased level of sympathetic nervous activity in afferents supplying calf muscles, though heart rate settled to normal as usual despite arterial occlusion. Following exercise without occlusion blood pressure and heart rate settled to pre-exercise levels within a minute. Voluntary biceps contraction increased both heart rate and blood pressure, but an equivalent involuntary contraction due to electrical stimulation had a similar pressor effect, but had no effect on heart rate[262]. An increase in calf, vascular resistance has been measured during forearm exercise which could be maintained after exercise by arresting the circulation in the exercising forearm[265]. There are two components to the exercise pressor effect therefore, one initiated centrally, causing an increase in heart rate and the other caused by accumulation of metabolites in the muscle during contraction. Fine, nonmyelinated or thinly myelinated (predominantly group IV) afferent nerve fibres identified by their characteristically slow conduction velocity have been found in skeletal muscle. They are activated by a variety of metabolic stimuli via metaboreceptors or chemoreceptors which return to the spinal cord by the dorsal root. They are believed to be the link between the metabolic status of the exercising limb and activation of sympathetic afferents elsewhere[266].

In my studies on volunteers I studied the effects of 1, 2 and 3 minutes of exercise at 12% of MVC on average. My findings varied slightly, but were broadly compatible with the mechanisms outlined above. In chapter 3 I studied 12 volunteers on 4 occasions and measured cardiac index, stroke index and heart rate by TEB throughout exercise. In chapter 7 I also monitored TEB

indices throughout exercise and this time 15 volunteers were studied, but I can only analyse the data on the occasion they were given placebo, to prevent possible confounding by drug effects. The data from chapter 4 and from the earlier reproducibility study, held in the appendix, is not strictly comparable, because TEB data is only available for the period following exercise, not during exercise. The impedance cardiograph was not available during the studies on patients with PVD.

Heart rate increased by upto 18% during the last minute of 3 minutes of handgrip exercise, cardiac index stayed the same or increased only insignificantly, whereas stroke index fell by 9% in both studies. Reduced vascular resistance in the exercising forearm could be more than compensated for by an increase in vascular resistance elsewhere, hence MAP could increase during exercise even with CI staying constant or increasing only slightly. The increase in heart rate can then be offset by a commensurate fall in SI.

Since I measured blood flow in the control forearm, it was not practical to measure blood pressure until 3 minutes after exercise and given that the exercise pressor effect offsets within a minute, it is no surprise that the blood pressure recordings in my studies were hardly changed throughout. However 10-20mmHg rises in diastolic and systolic BP have been measured in healthy volunteers during isometric handgrip exercise at 10% MVC[255]. In two previous studies on volunteers the cardiac output was found to be increased by 22% in both studies and stroke volume was constant and the calculated peripheral vascular resistance was unchanged[263,264]. These studies used sustained handgrip exercise at MVCs of 25 and 30% whereas the MVC in my study was only 12%, range 9-18%. In support of this contention an early study measured cardiac output and stroke volume at MVCs of 10, 20 and 50%. Although cardiac output did increase

at 10% of MVC, it was only a 10% increase. Stroke volume responses were variable at 10 and 20% of MVC, but it decreased markedly at 50% of MVC[254].

10.9 Caffeine elimination and withdrawal symptoms

Many volunteers in my studies admitted to mild headache and loss of concentration, usually commencing the day before the study after their first full day abstention from caffeine. Caffeine is capable of causing a degree of dependence in a high proportion of people at daily doses typically consumed in tea and coffee[267]. One study found that 9 out of 11 volunteers identified by questionnaire as likely to be dependent developed headache and in 7 it was classified as severe. Headache is the most commonly reported symptom of caffeine withdrawal[67-269], though fatigue and lack of vigour are also common[269].

I based my decision to ask volunteers to abstain from caffeine for 40 hours on the maximum half-life of 8 hours measured in volunteers[233]. Five half-lives is conventionally regarded as sufficient to ensure complete elimination of the drug. It is debatable however whether 40 hours is long enough to abstain from caffeine as when volunteers were given 750mg/day in divided doses, at 36 hours after the last dose there were still detectable amounts of caffeine and its metabolite paraxanthine, also an adenosine antagonist[270]. Its fair to say that 750mg of caffeine is probably more than the average daily consumption by tea and coffee drinkers as each cup contains between 75 and 150mg of caffeine[271], hence 750mg equates to 5 to 10 cups of tea and coffee daily.

10.10 Intra-arterial infusion of drugs

Providing investigators are suitably trained, the intra-arterial infusion of drugs into volunteers via ultra-fine bore needles can be carried out safely. Further, both adenosine and SNP were safe

at the doses used, with no evidence of systemic activity. A period of upto 15 minutes hand ischaemia was well tolerated by the volunteers as was an arterial infusion protocol lasting 104 minutes.

Using arterial infusion rates as low as 0.16mls/min created difficulties keeping the needle free of blood following a change of syringe. Infusate flow rates in excess of 1ml/min cause vasodilatation. It is tempting to assume that this is just due to supplementation of the pre-existing brachial artery flow with the infusate. This is not so. The baseline FBF was 3.34mls/100ml/min in the infused forearm at a saline infusion rate of 1ml/min. Since the average male forearm is approximately 1L in volume, this equates to a total flow of 33.4mls/min. Increasing the infusate flow rate to 1.6mls/min would increase this to 34mls/min, an increase of less than 2%. However the measured FBF increased by 14% to 3.81mls/100mls/min. Experiments using 27G needles placed directly in blood have demonstrated haemolysis at higher saline infusion rates, presumed to be due infusate forming a jet and inducing turbulence[150,272]. Haemolysis causes release of vasodilators such as ATP and other adenosine phosphates[272] possibly accounting for the vasodilatation observed.

Caution is necessary when interpreting results of arterial infusion studies using fine-bore needles (27G or finer) if the infusate flow rate exceeds 1ml/min, particularly if the infusate flow rate is varied without incorporation of infusion rate controls. Infusate flow rates even less than 1ml/min could induce turbulent flow and associated haemolysis if needles of finer gauge than 27G are used.

When there is a significant change in baseline FBF with time, for whatever reason, it makes sense

to reduce the time elapsing between baseline FBF measurement and FBF responses to intra-arterial infusions. There is an advantage therefore of measuring baseline FBF immediately beforehand without the need to deflate the wrist cuffs and rest the forearms. Studying the variability, there is some reduction of variability in the presence of theophylline when baseline FBF is measured over 3 rather than just 2 minutes as employed by some.

Recovery following adenosine infusion is slow: in the pilot studies baseline blood flows in the active forearm recorded 18 minutes after cessation of adenosine infusion are consistently higher than the pre-adenosine baseline (chapter 5), in the reproducibility study, the baseline flow had not fully returned to pre-adenosine levels even 25 minutes after adenosine infusion, though this did not achieve significance. The response to adenosine was arguably greater during the second series of infusions each day than the first though this did not achieve significance largely due to the variability of the second adenosine dose-response.

The reason for this slow offset in adenosine-induced vasodilatation and possible enhancement of the effect of subsequently infused adenosine is a matter of speculation. A study similar to the pilot study noted that the time taken to reach peak FBF was reduced during a second adenosine infusion started 30 minutes after the first one ceased[232]. In my pilot study the deadspace in the plastic tubing between syringe and needle took 24 seconds to clear at an infusion rate of 1ml/min, so persistent vasodilatation beyond one or two minutes is unlikely to be related to adenosine retained in the tubing. Neither is it likely to be due to free adenosine persisting in tissue since cellular uptake is so avid and adenosine is rapidly degraded in circulation. Other possible mechanisms would include persistent binding of adenosine to its receptors or prolonged activation of intracellular second messenger systems.

Both adenosine and SNP cause an initial peak vasodilatation following which the FBF settles back to a lower, plateau level. The initial increase in FBF when the adenosine infusion rate changes is less spectacular in the reproducibility study perhaps because the individual increases in adenosine infusion rate are less. It is tempting to speculate that this is due to a relatively larger concentration of vasodilator being delivered to the tissue initially, before vasodilatation has taken place. As vasodilatation gets under way, the vasodilator is diluted by a greater volume of blood before it can reach the tissues. This type of profile has been recorded before[232,273].

There is a difference between the first and second study day in baseline FBF, adenosine-induced vasodilatation and also in SNP-induced vasodilatation. In the case of SNP this does not achieve significance, however that is not surprising given the variability of the data (coefficient of variation 74%). A reduction in stress levels could easily account for this 'learning' effect at least when considering reductions in baseline FBF. Adrenaline has been shown to increase exercise and ischaemic hyperaemia[14], so by analogy it might also increase responses to adenosine and SNP. Some of the dramatic reduction in blood flow in the infused forearm could be due to reduced trauma to the artery or possibly due to reduced amounts of lignocaine being used on the second study day as more needles will have successfully entered the brachial artery at the first site chosen. If lignocaine impinges on the artery it can cause vasodilatation due to temporary anaesthesia of the sympathetic plexus in the adventitia. There was persistent vasodilatation in the infused forearm of volunteer 5 in the pilot study (chapter 5) following a deep sub-cutaneous injection of lignocaine prior to insertion of the arterial needle.

Studying the adenosine log-dose response relationship from the reproducibility study (chapter

5) it was clear that the full extent of the relationship has not been explored. Consequently, when studying the action of theophylline (chapter 6) I used a fourth dose level of adenosine, 100μg/min. Because of this I summarised the adenosine responses as an AUC of the log[dose]-response relationship rather than more simply meaning them which assumes a linear relationship. The greater rate of infusion of adenosine had no measurable effects on control FBF or TEB indices to indicate systemic effects.

Many groups express each of their FBF measurements as a ratio of the infused FBF to control FBF measured simultaneously[51], to allow for momentary variations in sympathetic tone and hopefully reduce variability[134]. All raw data is converted to FBF ratios by dividing infused FBF by the control FBF measured simultaneously, then this is further divided by the mean, baseline FBF ratio measured just prior to drug infusion, multiplied by 100 and finally the mean, baseline FBF ratio is subtracted yielding the percentage increase in FBF ratio. Unfortunately the use of the FBF ratio increases the variability of the data, however it is subsequently processed. Dividing by the baseline FBF ratio is not beneficial either and it introduces the possibility that effects could be obscured by changes in baseline, if as in my studies systemically administered drug influences FBF as well effecting the activity of drugs infused into the forearm. The most straightforward way to handle the data is to convert it to an excess FBF by subtracting the appropriate baseline. Whether responses to each dose-level of adenosine are best summarised as the mean excess plateau FBFs (as conventionally) or as the peak excess FBFs is debatable, though there may be a small advantage in using peak flows.

Repeating the adenosine dose-response is not of any benefit and in fact increases variability. Interestingly there is a major benefit from using the SNP response as a covariate in the analysis.

It is tempting to speculate that incorporating the response to a dose of SNP causing submaximal vasodilatation in the model used in analysis of variance compensates for daily variations in autonomic tone in the infused forearm. The SNP response was a useful covariate as theophylline did not effect SNP-induced forearm vasodilatation.

10.11 Effects of theophylline

Oral dosing with theophylline produced levels towards the upper limit of the therapeutic range at 2 hours after dosing in volunteers (90μM) and patients with PVD (85μM). Even when measured at over 3 and a half hours the theophylline level was still within the therapeutic range (74μM). One or two volunteers experienced symptoms of mild theophylline toxicity, which resolved quickly and spontaneously. Pharmacokinetic studies on volunteers have indicated that 2 hours is approximately the time when maximal theophylline levels would be expected using this microcrystalline formulation[227].

Theophylline increased pulse rate by 10% in healthy volunteers and by 7% in patients with PVD. TEB was not measured during studies on patients with PVD, but intriguingly in healthy volunteers there was also a 10% increase in CI, whilst SI was unchanged. Hence the rise in CI is brought about purely by the increase in heart rate. MAP pressure rose by 2 to 4% in volunteers, which attained bare significance, whilst there was an insignificant rise in patients with PVD. As CI rose by 10% in volunteers, this implies that systemic vascular resistance fell by 6% or more. Some of the fall in systemic vascular resistance is contributed by skeletal muscle vasodilatation as there were 49 and 56% increases in baseline FBF measured in the 2 studies on healthy volunteers. In patients with PVD, standard-dose theophylline caused a 16% increase baseline FBF which was not statistically significant.

One study (6 healthy men) demonstrated a significant 89% increase in FBF after an intravenous theophylline infusion; theophylline level 64μM. There was an insignificant 32% increase in the cardiac index and small changes only in heart rate and systolic and diastolic blood pressure[274]. Infused theophylline achieving a mean plasma level of 108μM increased forearm blood flow by 41% in 5 healthy, male volunteers[275], but in 4 volunteers, had variable effects on cardiac output and stroke volume. At lower infusion rates (mean theophylline level 56μM) heart rate, stroke volume and cardiac output actually fell. Systolic and diastolic pressure were unchanged at both theophylline levels. A further study of the effects of infused theophylline (mean plasma theophylline 58μM) in 6 healthy volunteers found significant increases in both systolic and diastolic blood pressure of 10 and 23% (17% increase in MAP), with an 11% increase in pulse rate[276]. Infusion of 1g of aminophylline over 30 minutes into 10 patients with chronic obstructive airways disease (but no heart failure) caused a 10% fall in MAP, a 30% increase in heart rate, a 27% increase in cardiac index and a 26% fall in systemic vascular resistance[277]. Clearly different subject populations might account for some of the discrepancies observed, as might the small numbers studied, but it is tempting to speculate that theophylline has contrasting effects at plasma levels at the extremes of the therapeutic range.

Several studies have demonstrated adenosine antagonism by theophylline in the human forearm, but each time they studied the effects of continuously infused intra-arterial theophylline (aminophylline)[121-123,232]. It is difficult to know what sort of tissue levels of theophylline are reached during intra-arterial infusions so it was difficult to be sure whether oral theophylline would share the same activity at doses commonly encountered. In chapter 6 I established that oral theophylline achieving levels (mean level 90μM) within the recommended therapeutic range for theophylline significantly reduced forearm vasodilatation in response to adenosine. This

equated to a four-fold rightward shift of the adenosine dose-response curve. In rat arterioles *in vitro* in which a level of aminophylline equivalent to a plasma level of 200mM also caused a 4-fold rightward shift in the adenosine EC_{50} from 1.9μM to 9μM.

In chapter 4 theophylline also caused a paradoxical increase in active hyperaemia. On the basis that adenosine is a vasodilator in the human forearm and I subsequently demonstrated that this oral dosing regime with theophylline significantly inhibited adenosine-induced vasodilation in the forearm, I expected a reduction in active hyperaemia. It is difficult to believe that adenosine could account for this in any way. Only one other study has considered the effect of theophylline on active hyperaemia in man. In this case theophylline was infused in a placebo-controlled, double-blind, cross-over trial on 5 healthy, volunteers achieving plasma levels of 75μM. Ischaemic, standardised, handgrip exercise was performed to exhaustion. There was no change in forearm blood flows measured at 30s, and then every 10 minutes for 40 minutes[278]. Since theophylline improved exercise tolerance, the subjects exercised for 12% longer when given theophylline, so this study did not test the hypothesis and it is possible to say that a reduction in blood flow has been obscured by the greater amount of ischaemic exercise performed in the presence of theophylline. Measurements at 30s are likely to reflect reactive hyperaemia(since arterial inflow was prevented during exercise), whilst a single measurement at 10 minutes is unlikely to be helpful on its own given the small number of subjects.

Did our subjects perform more work as a result of decreased perception of ischaemic-work pain due to theophylline? This seems improbable as subjects were monitored maintaining their handgrip pressure at 100mmHg and the level of exercise was short and at only 9-18% of MVC. If this were the case the vast majority of enhancement of exercise hyperaemia would be seen after

the 3 minute period of exercise and not consistently across all durations as is the case.

Adenosine has been shown to cause dose-dependent inhibition of noradrenaline release in the isolated rabbit heart during electrical stimulation of the sympathetic nerves and this was prevented by theophylline[279]. Intravenous theophylline has been shown to increase plasma adrenaline and noradrenaline in a dose related way, i.e. at a plasma level of 110μM theophylline increased plasma adrenaline by 262% and plasma noradrenaline by 64%[280]. Could adrenaline-induced vasodilatation account for enhanced active hyperaemia? Infusing adrenaline certainly increases FBF, yet subsequent hyperaemic responses were only slightly increased commensurate with the difference in baseline flow[14]. This suggests that the responses noted in my study were not related to increased levels of adrenaline. Responses to vasodilators in the canine hindlimb were found to correlate with the preceding baseline flow[261]. The result obtained with infused adrenaline would indicate that drug-induced vasodilatation was purely additive with active hyperaemia and therefore that responses beyond that would indicate specific enhancement of active hyperaemia. Similarly prior vasodilatation by papaverine had only an additive effect on adenosine-induced vasodilatation in the forearm[121].

In addition to inhibiting release of noradrenaline from sympathetic efferents (above) adenosine has been shown to activate sympathetic afferent nerves when given by intra-arterial infusion in the forearm[281]. Equivalent vasodilatation by SNP had no effect. In a similar study adenosine infused into the brachial artery increased sympathetic efferent tone in the peroneal nerve, increased MAP and at high doses also increased heart rate. Infusion of theophylline into one brachial artery (systemic level only 5μM) prevented the increase in peroneal nerve sympathetic efferent tone and increased MAP caused by isometric handgrip for that forearm only, but had no

effect on the associated tachycardia[282]. Further experiments on the forearm have shown that intra-arterial infusion of theophylline at doses below those causing vasodilatation, enhanced vasoconstriction caused by activation of the sympathetic nervous system by lower body negative pressure[123]. It seems that adenosine released locally serves both to inhibit local sympathetic efferent vasoconstriction whilst activating sympathetic afferents to cause skeletal muscle vasoconstriction in resting muscle elsewhere. Whilst I did not measure MAP *during* isometric handgrip exercise in the double-blind, cross-over trial of theophylline (chapter 4), I did measure heart rate, SI and CI in the minute after exercise. Heart rate increased by 14 beats/min in the presence of theophylline and 7 beats/min with placebo (not significant) compared to pre-exercise baseline levels. Cardiac index increased similarly with placebo and theophylline and theophylline converted a small increase in SI to a small decrease and this was of borderline significance.

Forearm vasodilation has been supposed to be related to phosphodiesterase inhibition by theophylline[121], presumably on the basis that some phosphodiesterase inhibitors cause forearm vasodilatation[121,240] and theophylline, like other methylxanthines, has been shown to be a phosphodiesterase inhibitor. Unfortunately the levels of theophylline required to achieve measurable PDE inhibition in human tissue tend to be above the therapeutic range in human tissue[119,120]. However one PDE isoform, substrate specific for cAMP, has been identified in human platelets with a Ki of 45μM for theophylline, corresponding to a plasma level of 90μM, allowing that half of plasma theophylline is protein bound in man[239]. It is possible therefore that there are PDE isoforms in vascular smooth muscle, perivascular nerves or the vascular endothelium which can be inhibited by theophylline at therapeutic levels.

The increased variability in active hyperaemia and central haemodynamic measures detected in the presence of theophylline would be explained if it were postulated that two or more pharmacological activities of theophylline had conflicting actions. The net response in an individual would depend on the balance of these actions on exercise hyperaemia. I suggest that theophylline *has* two conflicting actions on active hyperaemia. One is adenosine antagonism, the effect sought, and the other phosphodiesterase inhibition. Phosphodiesterase inhibitors eg papaverine and milrinone are forearm, skeletal muscle vasodilators[121,240], but their effect on active hyperaemia is unknown. I contend that theophylline enhances active hyperaemia by some action other than adenosine antagonism, most likely by phosphodiesterase inhibition. It is possible of course that adenosine somehow opposes active hyperaemia in skeletal muscle in man in direct contradistinction to its role in various animal models.

A placebo-controlled study in anaesthetized dogs which showed potentiation of SNP-induced hypotension by theophylline with leftward displacement of the dose-response curve. Theophylline pre-treatment achieving an initial plasma level of 43μM significantly increased the plasma cGMP concentration at higher SNP infusion rates. The authors postulated that both these effects were due to inhibition of PDE-mediated cGMP degradation by theophylline[283]. Since SNP, like other organic nitrates, is thought to act by releasing nitric oxide (NO) in the vascular smooth muscle cell, which in turn activates soluble guanylate cyclase, increasing intracellular levels of cGMP (5' cyclic guanosine monophosphate), non-specific PDE inhibition might have been expected to enhance vasodilatation by SNP. A cGMP-specific PDE has been identified in vascular smooth muscle. It has been classified as a type V PDE on the basis of its sequence and its substrate specificity[284]. Dipyridamole is conventionally regarded as an adenosine-reuptake inhibitor, but it is also a potent and fairly specific inhibitor of type V PDE[239]. It has been shown

to enhance pulmonary vasodilatation by inhaled NO[285]. Whilst theophylline preferentially inhibits cAMP specific PDEs, it has a metabolite, 3-methylxanthine, which preferentially inhibits type V PDE[286]. This metabolite has been shown to be equally as potent as theophylline at relaxing guinea-pig tracheal smooth muscle[286]. In a chronic dosing study the plasma concentration of the metabolite was found to be 40% of theophylline's[287]. In my study theophylline had no effect on SNP-induced vasodilatation. This was useful as otherwise the response to SNP would not have been appropriate as a covariate in ANOVA. This suggests either that theophylline has little effect on vascular smooth muscle type V PDE in man, the metabolite does not reach sufficient levels acutely to have significant effects or, at the dose-level of SNP infused, the rate of degradation of cGMP is not critical.

To try to determine whether theophylline was also inhibiting PDE, we measured cAMP in plasma and urine and also used ex-vivo monocyte lipopolysaccharide-stimulated TNF-a production as a measure of PDE IV inhibition. The plasma cAMP level did increase significantly. Theophylline caused a 260ml increase in urinary output compared to placebo between a half and 3 hours after dosing and though the urinary cAMP concentration was unchanged there was a net increase in total urinary cAMP production as a result of the diuresis. Theophylline is known to have mild diuretic properties in man[288], perhaps by countering adenosine's actions on the kidney: post-glomerular vasodilatation (and hence decreased glomerular filtration) and inhibition of renin release[289]. Since cAMP is a second messenger common to other receptors it is possible to advance other explanations for an increase brought about by theophylline. Stimulation of the A_1 receptor inhibits its production, hence adenosine antagonism at the A_1 receptor is one possible explanation. Theophylline has been shown to cause catecholamine release, so adrenaline could increase circulating cAMP concentrations by stimulation of the β-receptor which in turn

stimulates adenylyl cyclase. Unfortunately, ex-vivo production of TNF-α by monocytes, the measure of PDE inhibition I used, is too variable, so more volunteers would have been needed to determine the significance or not of the reduction in production noted with theophylline.

10.12 Effects of oxpentifylline

Oxpentifylline is a close structural analogue of theophylline and like theophylline it is a phosphodiesterase inhibitor[127,242,243], but unlike theophylline, it is only a *very weak* adenosine antagonist[116,127,244]. If the enhancement of active hyperaemia seen using theophylline was due to phosphodiesterase inhibition, then oxpentifylline would be expected to have the same or arguably greater effect, since there would be no conflicting effect due to adenosine antagonism. I chose oxpentifylline, rather than a non-xanthine phosphodiesterase inhibitor, because I hoped it would have the same spectrum of inhibition of phosphodiesterases as theophylline. Oxpentifylline is already licensed for treatment of peripheral vascular disease, causing mild and readily reversible side-effects (Trental 400™ data sheet), obviating any ethical problems.

Others have compared enprofylline (3-propylxanthine) with theophylline in the same way[274,288,290,291]. Although enprofylline has comparatively little adenosine A_1 receptor antagonism compared to theophylline[116,127], but is a more potent PDE inhibitor[292], it has been shown to be possibly more potent than theophylline as an adenosine A_2 receptor antagonist[116]. Additionally enprofylline is not licensed for use in this country. For these reasons I preferred to use oxpentifylline.

My principal aim was to try and reproduce the enhancement of active hyperaemia already seen with theophylline. Based on the literature I had expected oxpentifylline to increase baseline

forearm blood flow and to increase hyperaemic flows also. Two papers have considered the effect of oxpentifylline on peak, post-ischaemic flow[293,294]. In neither paper was it clear whether the peak flow was adjusted for any change in baseline calf flow and both studies measured calf blood flows in the legs of patients with PVD and impaired circulation in the leg concerned. In 10 patients with PVD intravenous infusion of 500mg of oxpentifylline caused a significant rise in resting calf blood flow and peak calf blood flow after 3 minutes of ischaemia, despite falls in both systemic systolic and diastolic blood pressures with an associated tachycardia. There was also some reduction in the time taken for the limb to recover to pre-ischaemic baseline flows[294]. In the same paper the effects of thirty days treatment with 400mg oxpentifylline (Trental™) 3 times daily were also presented. Resting calf blood flow and peak post-ischaemic calf blood flow were again increased, though systemic blood pressure and heart rate were this time unaffected. These results were confirmed in a 90-day, placebo-controlled, cross-over study using 400mg of oxpentifylline (Trental™) twice daily studying 18 patients with stable PVD. As in the previous study, baseline calf blood flow and peak, post-ischaemic calf blood flow were both significantly increased, but there were small, significant, increases in these indices in patients given placebo first indicating some confounding, training effect.[293]. Since chronic dosing studies have not demonstrated any evidence of accumulation of oxpentifylline or metabolite M1 over 9 days using 400mg (Trental™) 3 times daily[228], it would be reasonable to expect that a single oral dose of oxpentifylline, as used in this study, would cause measurable vasodilatation and would increase post-ischaemic hyperaemia.

There are several possible explanations for the lack of activity of oxpentifylline measured in chapter 7: insufficient drug levels might have been were attained in circulation, the drug might have failed to penetrate monocytes and vascular tissue sufficiently, the drug might inhibit PDE

subtypes whose activity was not measured in this study or the drug might act differently in healthy volunteers than it does in patients with PVD. The maximum blood concentrations of oxpentifylline and metabolite M1 in volunteers after a single oral dose of 400mg of oxpentifylline (Trental™) have been measured and were 1.1μM and 1.2μM respectively[228]. Consideration of which explanation is likely is to some extent compromised because the activity of metabolite M1 has been studied so little. The original reviews alleging that metabolite M1 is active[247,248] both referred to one paper[245]. This paper studied the pharmacokinetics of oxpentifylline and its major metabolites. The author asserted that metabolite M1 accounted for some activity of the drug, both in the English summary and in the original German, however no evidence was presented to support this. More recently activity of oxpentifylline and metabolite M1 were compared in the isolated, perfused porcine ear. Secretion of plasminogen activator by the vascular endothelium is known to be stimulated by raised intracellular levels of cAMP in this model[295]. Metabolite M1 caused concentration-dependent release of plasminogen activator detectable at concentrations as low as 1μM, yet even 100μM oxpentifylline was inactive[296]. If this is the case for some or all phosphodiesterases in man then the *in vitro* data on oxpentifylline will be misleading or irrelevant.

Putting this reservation aside, what evidence is there for PDE inhibition by oxpentifylline in the micromolar range? Oxpentifylline at 185μM caused a 25% reduction in PDE activity in homogenized human umbilical cord arteries and a 49% reduction in PDE activity in homogenized human umbilical veins. When intact arteries were perfused for 30 minutes with the same concentration of drug there was a 32% fall in PDE activity[243], indicating good tissue penetration as would be expected for a drug which is readily soluble in both water and benzene (Merck Index) and more soluble in water and lipid than theophylline[297]. This work takes no account of

the importance of the vascular endothelium nor the subsequent discovery of many PDE isoenzymes. It is conceivable that oxpentifylline profoundly inhibited one PDE isoenzyme in the vascular endothelium or in vascular smooth muscle, but that a greater quantity of enzyme in other cell types or even elsewhere in the same cell, more resistant to inhibition by oxpentifylline, diluted the effect. This argument is supported by more recent research in which TNF-induced suppression of cell-surface expression of thrombomodulin by human umbilical vein endothelial cells was partially alleviated by oxpentifylline. This effect was dose-dependent and was significant at oxpentifylline concentrations as low as 3.6μM[298]. It seems likely that tissue levels of oxpentifylline were too low after oral dosing to cause vasodilatation by acting directly on vascular smooth muscle, though more subtle effects mediated by the vascular endothelium would still be possible.

Having established that micromolar concentrations of oxpentifylline and metabolite M1 were probably in circulation when observations were made and that tissue penetration is likely to be good, what levels of oxpentifylline have been observed to work on mononuclear cells? Production of TNF by peripheral blood mononuclear cells from healthy volunteers in response to antigen challenge was reduced by 50% in the presence of 10μM oxpentifylline[299] and a 10% reduction in TNF production was observed by another group in the presence of 8μM oxpentifylline with no measurable effect on the intracellular concentration of cAMP[216]. In further in vitro studies 3.6μM oxpentifylline caused a significant reduction in phagocytosis by peripheral blood mononuclear cells from healthy volunteers, yet 75μM oxpentifylline was required to increase the intracellular concentration of cAMP 1.75 fold[300]. Incubation with oxpentifylline caused concentration-dependent inhibition of TNF production by peripheral blood mononuclear from patients with active Crohn's disease, patients with ulcerative colitis and from healthy

controls. The IC_{50} was 90μM, but there was a significant, 10-20% reduction in TNF production at 3.6μM. Production of TNF by mucosal biopsy specimens in culture from the same patients with Crohn's disease was reduced by approximately 50% by all concentrations of oxpentifylline tested including 3.6μM[301]. In volunteers, prior infusion of oxpentifylline abolished the rise in serum TNF in response to endotoxin otherwise observed[302] and 400mg of oral oxpentifylline (Trental™) 4 times daily for 2 days suppressed TNF production by peripheral blood mononuclear cells *ex vivo* in 6 healthy volunteers and in 5 out of 6 patients with active Crohn's disease after 4 weeks on treatment[303]. Similar results were recorded in 2 patients with cancer[304]. The *in vitro* results indicate that 1μM oxpentifylline would be barely enough to have an effect, though concentrations below 3.6μM were not studied. The *ex vivo* results tend to suggest that an effect should have been measurable, but monocytes were studied at least 2 days after starting treatment, allowing the possibility of subtle conditioning effects of oxpentifylline or metabolite M1 on the immune system.

In this study I found that oral oxpentifylline had no effect on baseline FBF, blood pressure, heart rate, stroke volume or cardiac output. Intra-arterial infusion of oxpentifylline does cause dose-dependent vasodilatation, but at doses (30mg over 30 minutes) likely to produce tissue levels 15 times those attained by the standard oral regimen[305]. Another group has shown a significant increase in systolic blood pressure in 10 healthy volunteers following 2 days of oral oxpentifylline, whereas in the same study there was if anything a small fall in systolic blood pressure in 12 patients with PVD[306]. Further studies tend to confirm the idea that oxpentifylline has variable haemodynamic effects depending on the initial haemodynamic status of the patients studied. Intravenous oxpentifylline had no effect on stroke index or cardiac index in 11 patients with coronary artery disease undergoing cardiac catheterisation. There was a slight, but

statistically significant fall in systolic blood pressure, but no change in diastolic blood pressure following infusion of the drug[307]. Ten patients with severe heart failure were studied following heart valve replacement; infusion of oxpentifylline caused a mild, transient tachycardia, a marked increase in cardiac index and stroke index with a concomitant 20% fall in systemic vascular resistance[308]. Finally in patients with PVD, but no evidence of impaired left ventricular function, 100mg of oxpentifylline given intravenously induced a 10% increase in cardiac output and an 8% fall in systemic vascular resistance lasting 20 minutes with no change in blood pressure or pulse[309]. Obviously, intravenous infusion of oxpentifylline will achieve higher circulating drug levels than the same dose given orally, so it is not surprising that my results differ from such studies. In addition most of these studies were carried out on patients with cardiovascular disease and the acute haemodynamic effects of oxpentifylline seem to depend on the health of a subject's cardiovascular system.

I am uncertain about the importance of the small reduction in plasma cAMP level caused by oxpentifylline. The plasma cAMP concentration is high in the placebo arm of the study compared to the level measured following placebo during the preceding study (chapter 6). Since the cell or even the tissue of origin is unclear, it is difficult to develop a hypothesis to explain why oxpentifylline did not cause an increase in circulating cAMP levels like theophylline did. An increase in circulating cAMP would have lent support to the idea that oxpentifylline was acting as a PDE inhibitor at the dose used, but no change in the level, or even the small fall observed, does not imply that oxpentifylline was not acting as a PDE inhibitor in tissues not contributing to the plasma pool.

Further work to follow these observations would include studies on intravenous oxpentifylline,

using a dose which would increase baseline FBF by an average of 50%.

10.13 Responses in subjects with peripheral vascular disease

The reproducibility of the summary measures of forearm hyperaemia was not as good in volunteers with PVD (chapter 8) as that obtained in disease-free volunteers (chapter 3). This is not surprising as the volunteers with PVD are more heterogenous with current and ex-smokers, even two who admitted they could not stop smoking on the day of a study. One volunteer had benign, essential tremor reducing precision further. Despite this the coefficient of variation of the most variable measure (mean AUC of active hyperaemia) is still low enough to allow reasonable confidence that a change of 34% due to treatment would achieve significance providing 12 volunteers completed the study and assuming a power of 90% and a significance level of 0.05.

Despite marked differences in age, smoking habits and resting blood pressure, the forearm compliance profiles during reactive and active hyperaemia are remarkably similar in patients with PVD compared to healthy controls. Although there is little or no change in the initial peak forearm compliance, compliance remains elevated for longer in patients. Reactive hyperaemia has been studied in the affected legs of patients with PVD. There was a reduced peak flow and elevated subsequent flows compared to the unaffected limb[310,311] or compared to a group of normal volunteers[311,312]. This was attributed to a reduced initial flow, limited by proximal stenosis, prolonging repayment of the 'flow debt'. An early study using healthy volunteers showed that limitation of flow by compression of the brachial artery throughout both reactive and active hyperaemia for varying periods reduced the total hyperaemia and also delayed the hyperaemia. When pressure on the brachial artery was released, hyperaemia would occur even

when hyperaemia in the control, free-flow forearm had already stopped[313]. My data do not support the idea that reduced peak flow is the cause for increased blood flow later during the recovery period casts doubt on debt repayment being the driving force for this.

The MAP increased slightly, but significantly from the pre-ischaemic level to the post-ischaemic and pre-exercise readings. There was a marked increase noted when measured 3 minutes after exercise ceased. Control FBF did increase transiently initially but had returned to baseline levels before the end of the measurement period and before the post-exercise blood pressure could be measured. This suggests that this increase in blood pressure could not account for the late increase in FBF seen in patients with PVD in this study unless a complex interaction of systemic pressure and exercise hyperaemia is proposed. In healthy volunteers blood pressure does increase *during* isometric, handgrip exercise, but it returns to pre-exercise levels within a minute of stopping exercise[263]. Hence the MAP would have been greater still immediately following exercise in our patients. Another group recorded FBF during sustained, submaximal, *contralateral*, handgrip exercise in normal volunteers. They found no change in baseline flow and no change in responses to intermittent isometric contractions of the forearm studied compared to those obtained when the contralateral forearm was at rest, despite a 50% increase in MAP[27]. Unless patients with PVD behave very differently, changes in MAP seem unlikely to be the cause of this effect.

In contrast to the findings in healthy volunteers, theophylline had no significant effect on active or reactive hyperaemia at either dose level in volunteers with PVD. I had hoped to demonstrate some enhancement of active hyperaemia by standard-dose theophylline and ideally some reduction in active hyperaemia by low-dose theophylline. Standard-dose theophylline is

associated with an insignificant, 20% enhancement of active hyperaemia and likewise low-dose theophylline is associated with a very slight reduction in active hyperaemia.

Standard-dose theophylline did achieve similar plasma theophylline concentrations in patients with PVD to those measured at 2 hours in healthy volunteers, i.e. 85μM versus 90μM in chapter 6. The plasma cAMP concentrations measured in the presence of placebo and standard-dose theophylline were broadly similar to those measured in normal volunteers in chapter 6, but the plasma cAMP concentration only increased by 20% compared to 57%. Baseline FBF only increased by 16% using standard-dose theophylline , so it is arguable that there was less PDE inhibition in patients with PVD than in healthy volunteers.

Perhaps the sensitivity of PDE to theophylline or tissue penetration by theophylline is reduced in subjects with PVD. An alternative explanation is that their skeletal muscle arterioles are less able to relax than those in healthy volunteers. Low-dose theophylline had no effect on either the plasma cAMP level nor on resting FBF. So by these criteria at least it had no discernible activity as a PDE inhibitor.

The pattern of responses to standard-dose theophylline is similar to that noted in volunteers (tables 4.2 and 6.5), but reduced. Standard-dose theophylline did increase resting pulse rate significantly, though to a lesser degree than in healthy volunteers. There was a slight, but insignificant increase in pulse rate associated with low-dose theophylline. Standard-dose theophylline was associated with small and insignificant increases in systolic BP and MAP. Low-dose theophylline had no apparent effect on central haemodynamics.

Though this study is inconclusive, the results are still compatible with the idea that low-dose theophylline might reduce active hyperaemia by adenosine inhibition, whilst standard-dose theophylline might enhance active hyperaemia by virtue of its additional activity as a PDE inhibitor. Even if the hypothesis is true, it is difficult to pick the right dose levels of theophylline to discriminate between these two activities. The watershed dose at which enhancement of active hyperaemia by theophylline begins to predominate over the effect of adenosine antagonism will vary between individuals and there will be a middle dose range in which the two effects cancel out. The effect measured by a study such as this using fixed dose levels will be diluted. It may be that there is only a very narrow dose-range in which adenosine antagonism is sufficient to be detectable and PDE inhibition is negligible.

This study illustrates the difficulties inherent in predicting the behaviour of drugs in patients from their behaviour in healthy volunteers. Care must be taken when extrapolating from one population to another.

10.14 Future developments

I have been able to establish that oral theophylline achieving well-tolerated plasma levels does inhibit adenosine-induced forearm vasodilatation. However theophylline is a complex drug with conflicting actions on blood flow in the human forearm and so was not ideally suited to my purpose. I was not able establish whether adenosine has a role in active hyperaemia or not. The paradoxical enhancement of active hyperaemia in healthy volunteers deserves further investigation. I would not use oxpentifylline again, but would choose a relatively non-specific phosphodiesterase inhibitor such as papaverine, which I would infuse into the brachial artery at a dose adjusted in each volunteer to double basal flow.

Selective adenosine A_{2a} receptor antagonists are currently under development by pharmaceutical companies. One has been shown to reduce active hyperaemia in the feline hindlimb by 30% when flow is unrestricted, but had no effect on the increased conductance associated with exercise when flow was limited[132,314]. This type of profile might be highly beneficial if verified in patients with PVD, as such a drug would have the potential to selectively inhibit active hyperaemia in the healthy circulation whilst leaving active 'hyperaemia' in the diseased circulation unaffected - as originally hoped. This would represent a major breakthrough. Further studies like mine using these agents would be a logical next step.

There are two basic reservations with regard to this approach. Non-specific adenosine antagonism by caffeine exacerbated hypertension in cats with clip-induced unilateral renal artery stenosis[315]. Adenosine A_{2a} receptor knockout mice are viable, but they had increased platelet aggregation, had a 20% increase in resting mean arterial pressure and a 50% increase in resting heart rate. Administration of a specific adenosine A_{2a} receptor agonist had no effect on the

knockout mice, but induced a tachycardia in the wild type mice and reduced their blood pressure[316]. Hopefully increased platelet aggregation will be prevented by aspirin, which patients with PVD should ideally be taking. However, upto 60% of patients with PVD have radiographically detectable renal artery stenosis[317] and many are hypertensive and so there is a real risk that an adenosine antagonist might precipitate a hypertensive crisis. Adenosine-induced inhibition of renin release has been attributed to the adenosine A_1 receptor[318], so it is possible that an adenosine A_{2a} receptor antagonist will not share the exacerbating effect of caffeine in renal hypertension.

It is probably the adenosine A_{2a} receptor that is responsible for hypotensive and bradycardic effects of adenosine when microinjected into the nucleus tractus solitarius in the brain stem in the rat[319]. It seems likely that this forms part of a reflex arc for regulation of blood pressure via the baroreceptors for example. If an adenosine A_{2a} receptor antagonist does penetrate the CNS it could reset the barostat at a higher level, which accords well with the findings in adenosine A_{2a} knockout mice.

Finally, if adenosine is the means whereby metabolic demand in tissues is met by increasing blood flow, there is a real danger that tissue ischaemia will be enhanced during adenosine A_{2a} receptor blockade. Whilst this might be acceptable in skeletal muscles, similar mechanisms operate in the myocardium and possibly also in the brain. Many patients with PVD die of ischaemic heart disease and they often have concomitant cerebrovascular disease. If the link between metabolic need and supply is broken in the brain or the heart the consequences could be severe.

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Appendix

An open study to assess the reproducibility of forearm blood flow measurements during post-ischaemic and post-exercise hyperaemia in healthy volunteers

1. Introduction

This was an open, non-randomised study in 10 healthy volunteers. Volunteers were studied on each of two study days one week apart. On each day volunteers had their forearm blood flow measured post-exercise and post-ischaemic challenge. The ischaemic/exercise challenge was performed twice on each day, once in the morning and once in the afternoon.

2. Data description

Ischaemia was induced by inflation of a standard 35cm blood pressure cuff to 200mmHg. The cuff was inflated for periods of 1, 3 and 5 minutes in succession. Forearm blood flow measurements were taken every 15 seconds for 3 minutes after each ischaemic challenge, i.e. 12 measurements in all.

The volunteers performed a tonic exercise involving compression of the bulb of a manual sphygmomanometer to maintain a pressure of 100mmHg for periods of 1, 2 and 3 minutes. Forearm blood flow measurements were taken every 15 seconds for 3 minutes after each exercise challenge, i.e. 12 measurements in all.

Two baseline forearm blood flow readings (12 measurements each) were recorded prior to each ischaemic challenge. These have been called baseline 1 and baseline 2. One baseline forearm blood flow reading session, baseline 3, was conducted prior to the exercise challenge. A final baseline forearm blood flow reading session, baseline 4, was recorded immediately after the final 3 minute exercise challenge.

3. Statistical methods

3.1 Data description

The mean forearm blood flow profiles, adjusted for baseline, measured over the 3 minutes of recording time (12 values) were plotted at each exercise/ischaemic challenge occasion.

3.2 Investigation of baseline data

The change in baseline measurements from baseline 1 to baseline 2 and from baseline 2 to baseline 3 were calculated as follows:

(1) The mean of the last 8 forearm blood flow recordings collected at each baseline for each volunteer on each study day and session.

(2) The resulting meaned values at each baseline were themselves meaned for each volunteer over study days and sessions. This then gave a single value for each session for each baseline.

(3) The change between each baseline was calculated within each volunteer by subtracting his mean value at baseline 2 from the baseline 1 mean and by subtracting his mean value at baseline 2 from the baseline 3 mean.

A single paired t-test was used, post hoc, to test whether the three baselines differed.

The variability of these baseline measurements was further investigated by using the nested nature of the study design to break the total variability of the data into its various components. The use of the mean of the last eight values and the maximum baseline flow were investigated in this way for baselines 1, 2 and 3.

3.3 Reproducibility

The primary aim of the analysis was to estimate the within subject variability of various endpoints of forearm blood flow following occlusion and following exercise. These endpoints under investigation were:

1) Peak flow

In the case of ischaemia, this was the maximum forearm blood flow attained from all twelve recorded measurements after each period of ischaemia and exercise. In the case of exercise, because so few recordings were obtained for the first recorded measurement, this was the maximum forearm blood flow attained excluding the first blood flow reading.

2) Peak flow adjusted for baseline

These were calculated as 1) above, except that the forearm blood flow readings had the basal flow subtracted. The basal flow was calculated from the baseline recordings taken over the 3 minutes immediately prior to the occlusion or exercise period. The baseline was calculated as the mean of the last 2 minutes of recorded data. These baseline values for each volunteer/study day/session were then used to adjust the forearm blood flow readings.

3) Peak flow normalised for baseline

These were calculated as 1) above, except that the forearm blood flow readings had been normalised for basal flow. The basal flow was calculated from the baseline recordings taken over the 3 minutes immediately prior to the occlusion or exercise period. The baseline was calculated as the mean of the last 2 minutes of recorded data. These baseline values for each volunteer/study day/session were then used to adjust the forearm blood flow readings subsequently taken in the following way:

$$\frac{\text{fabf value} - \text{baseline}}{\text{baseline}} * 100$$

Appendix

4) AUC

The AUCs for ischaemia were calculated using the trapezium rule. The 2nd, 3rd, 4th and 5th forearm blood flow values adjusted for baseline were used to calculate the AUC. Areas were determined following 1, 3 and 5 minutes of occlusion for each volunteer on each study day and at each session.

The AUCs for exercise were calculated using the trapezium rule. The first and last measurement after each exercise period were excluded and the area was calculated using the remaining values having subtracted the baseline flow for that volunteer/visit/session. As for occlusion, areas were determined following 1, 2 and 3 minutes exercise for each volunteer, on each study day and at each session.

5) AUC normalised for baseline

The AUC calculated as described in part 3) using forearm blood flow readings was normalised for basal flow. The normalisation was carried out as described in part 3).

To allow easier comparison of the different models to be fitted and their subsequent estimates of variability, only those volunteers who had both the morning and afternoon endpoints calculated on each study day were included in the analyses. These data were analysed in 3 ways:

(a) Covariate analysis

The afternoon response was analysed by analysis of covariance allowing for the effects of subject and visit with the morning response being the covariate. The residual mean square for this model is the appropriate estimate of within subject standard deviation that would occur in a crossover study analysis of covariance.

(b) Change from morning to afternoon FBF session

The change in response, afternoon peak flow/AUC minus morning peak flow/AUC, was calculated for each subject for both study days. This change was analysed by analysis of variance allowing for the effects of subject and visit. The within subject standard deviation obtained in this way is an estimate of the within subject standard deviation that would occur in a crossover study analysis of change from pretreatment.

(c) Analysis of variance - afternoon FBF session only

The afternoon response was analysed by analysis of variance allowing for the effects of subject and visit. This approach allows for estimation of the within subject standard deviation that would occur in a crossover study in which only one forearm blood flow measurement occurred on each study day.

Using the above methods, decisions concerning the design and sizing of similar future studies

can be made.

6) Half-life

It had been intended to investigate the within-subject variability of the half-life of the forearm blood flow response after each ischaemic/exercise challenge. On investigation of the data, the data did not easily lend themselves to the calculation of such an endpoint. Hence this endpoint has not been analysed.

3.4 Temperature

It was intended to investigate any apparent trends in the minimum temperature recorded at each session and study day. Because of the expected limited range of values of this data, the initial intention was to briefly 'eyeball' the data.

3.5 Change in BOMED on ischaemic and exercise challenges

The change in BOMED measurements from immediately prior to the ischaemia and exercise challenge sessions to each stage of the ischaemia and exercise challenges was calculated as follows:

(1) The first of the 3 BOMED recordings collected at each stage of the forearm blood flow measurement challenges was taken within each volunteer on each study day and session.

The first of the 3 BOMED measurements collected at the ischaemia and exercise baseline recording sessions were also taken within each volunteer on each study day and session.

(2) The resulting values at each stage of the exercise and ischaemia challenges and at baseline were meaned within volunteer over study days and sessions. This then gave a single value for each volunteer for each stage of the challenges and for each baseline representing an average measure of that volunteer's response.

(3) The change at each level of the ischaemia and exercise challenges was calculated within each volunteer by subtracting his response value on the challenge from his appropriate baseline response value.

Having calculated the change at each stage of the ischaemic/exercise challenge within each volunteer, a simple paired t-test was used, post hoc, to assess the statistical significance of mean changes in the BOMED data.

As there were over sixty t-tests to be conducted, a Bonferroni correction was used to ensure an overall significance level of 5%.

Appendix

3.6 Control forearm data

The comparison of the baseline measurements for the control and active arm for baselines 1, 2 and 3 was undertaken as follows:

(1) The mean of the last 8 forearm blood flow recordings collected at each baseline was taken within each volunteer on each study day, session and arm.

(2) The resulting meaned values at each baseline were themselves meaned within volunteer over study days and sessions for each arm. This then gave a two values for each volunteer for each baseline, one for the control forearm and one for the active forearm.

(3) The difference between arms for each baseline was calculated within each volunteer by subtracting his mean value at baseline of the control forearm from the baseline mean from the active forearm.

A simple paired t-test was used, post hoc to test whether the three baselines differed between the control and active forearms.

4 Results

4.1 Data description

Figures 1 and 2 show the forearm blood flow profiles after each ischaemic and exercise challenge. In both cases, there is an obvious effect of increasing the length of the challenge on the peak flow achieved. In the case of ischaemia the forearm blood flow readings very rapidly, within the first 5 readings, fall back down to baseline levels. The return to baseline levels after the exercise challenges is much slower.

It should be noted when considering the exercise challenge data that very few readings were obtained at the first time point, thus the means presented at this time point should be treated with caution.

Figures 3 and 4 show the overall mean forearm blood flow profiles and their appropriate standard errors.

Appendix

4.2 Investigation of baseline data

Comparing baseline 1 with baseline 2, there was no evidence, P=0.3, that the means were significantly different.

There was no evidence, P=0.11, that baseline 2 was statistically significantly different from baseline 3.

The variability of baselines 1 and 2 and the appropriate coefficients of variation (CV) are presented below:

Variability	**Mean of last 8 values**	**Peak Flow**
	CV	**CV**
Within session	12%	14%
Between sessions	19%	20%
Between days	30%	26%
Between subjects	34%	35%
Overall Mean	3.2	3.8

There is very little difference in coefficients of variation between the two methods of presenting the baseline data. Thus, the standard method of presenting the mean of the last eight values will be adopted.

The components of variation for baseline 3 are presented below:

Variability	**Mean of last 8 values**	**Peak Flow**
	CV	**CV**
Between sessions	30%	26%
Between days	32%	30%
Between subjects	43%	39%
Overall Mean	3.5	4.2

The standard approach to presenting the baseline data will be adopted. It should be noted that variability of this data, even at baseline, is relatively high.

Appendix

4.3 Reproducibility
1) Peak flow

Figures 5 and 6 illustrate the mean peak flows achieved after each ischaemic/exercise challenge and the between subject variability of these estimates. It should be noted that the baseline values plotted in Figures 5 onwards have all been based on the means of the last 2 minutes of baseline data.

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the unadjusted peak flow.

		Analysis Covariate	PM FAFB	PM session
1 min	Mean	15		
	SD	5	5	5
	CV	32	37	31
3 min	Mean	35		
	SD	15	21	15
	CV	43	61	44
5 min	Mean	37		
	SD	10	12	10
	CV	28	33	27
1 min Exercise	Mean	18		
	SD	7	7	6
	CV	39	41	35
2 min Exercise	Mean	24		
	SD	8	7	8
	CV	35	31	34
3 min Exercise	Mean	30		
	SD	12	11	11
	CV	39	37	36

KEY: SD Within subject standard deviation
CV Coefficient of variation
Units: ml/min/100ml of forearm tissue

From the above results it would appear that correcting for an initial ischaemic or exercise challenge is of little worth in providing extra precision as opposed to using a single ischaemic/exercise challenge on each study day.

Appendix

2) Peak flow - increase from baseline

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the unadjusted peak flow.

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the unadjusted peak flow.

		Analysis		
		Covariate	PM FAFB	PM session
1 min	Mean	11		
	SD	4	5	4
	CV	39	45	36
3 min	Mean	33		
	SD	14	21	15
	CV	44	64	46
5 min	Mean	35		
	SD	10	12	10
	CV	29	34	27
1 min Exercise	Mean	15		
	SD	6	8	8
	CV	44	51	41
2 min Exercise	Mean	20		
	SD	8	8	8
	CV	42	37	41
3 min Exercise	Mean	26		
	SD	12	11	11
	CV	44	42	41

KEY: SD Within subject standard deviation
CV Coefficient of variation
Units: ml/min/100ml of forearm tissue

From the above results it would appear that correcting for an initial ischaemic or exercise challenge is of little worth in providing extra precision as opposed to using a single ischaemic/exercise challenge on each study day.

Appendix

3) Peak flow normalised for baseline

Figures 7 and 8 illustrate the mean normalised peak flows achieved after each ischaemic/exercise challenge and the between subject variability of these estimates.

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the normalised peak flow.

		Analysis		
		Covariate	PM FAFB	PM session
1 min	Mean	382		
	SD	87	177	101
	CV	27	46	27
3 min	Mean	1056		
	SD	467	768	462
	CV	44	73	44
5 min	Mean	1177		
	SD	351	548	376
	CV	30	47	32
1 min Exercise	Mean	419		
	SD	116	118	163
	CV	28	28	39
2 min Exercise	Mean	545		
	SD	255	380	228
	CV	47	70	42
3 min Exercise	Mean	755		
	SD	340	584	308
	CV	45	77	41

KEY: SD Within subject standard deviation
CV Coefficient of variation
Units: *ml/min/100ml* of forearm tissue

An observed in 1), there is little worth in correcting for an initial ischemic/exercise challenge in terms of increasing precision. Hence one ischaemic/exercise challenge per day would appear to be optimal.

Similarly, there is little difference between the magnitude of the CV from the ANOVA models for the normalised and untransformed data.

Appendix

4) AUC adjusted for baseline

Figures 9 and 10 illustrate the mean AUCs achieved after each ischaemic/exercise challenge and the between subject variability of these estimates.

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the untransformed AUC.

		Analysis		
		Covariate	PM FAFB	PM session
1 min	Mean	0.84		
	SD	0.41	0.77	0.41
	CV	49	92	49
3 min	Mean	3.09		
	SD	1.54	3.2	1.61
	CV	50	104	52
5 min	Mean	8.32		
	SD	3.4	2.41	2.5
	CV	41	29	30
1 min Exercise	Mean	4.28		
	SD	3.02	2.64	2.81
	CV	71	62	66
2 min Exercise	Mean	8.54		
	SD	4.53	4.40	3.99
	CV	53	51	47
3 min Exercise	Mean	15.39		
	SD	6.76	5.86	7.19
	CV	44	38	47

KEY: SD Within subject standard deviation
CV Coefficient of variation
Units: ml/min/100ml of forearm tissue

There is little difference between the CV produced by either the covariate analysis and the PM session only analysis.

Appendix

5) AUC normalised for baseline

Figures 11 and 12 illustrate the mean normalised AUCs achieved after each ischaemic/exercise challenge and the between subject variability of these estimates.

The results corresponding to (a)-(c) are given below. In all cases the variable being analysed is the normalised AUC.

		Analysis		
		Covariate	PM FAFB	PM session
1 min	Mean	30		
	SD	14	22	13
	CV	46	73	42
3 min	Mean	101		
	SD	41	76	36
	CV	40	76	35
5 min	Mean	243		
	SD	87	70	90
	CV	36	29	37
1 min Exercise	Mean	118		
	SD	44	41	72
	CV	38	35	62
2 min Exercise	Mean	238		
	SD	131	191	115
	CV	55	80	48
3 min Exercise	Mean	450		
	SD	136	189	139
	CV	30	42	31

KEY: SD Within subject standard deviation
CV Coefficient of variation
Units: ml/min/100ml of forearm tissue

As for 3), there is little advantage in performing the ischaemic/exercise challenge twice on each study day. The precision of the untransformed AUC for the PM session only tends to be better than that for the normalised AUC.

Appendix

6) Volunteer number estimates

In the light of the above results, volunteer number estimates have been produced for the PM session only estimates of within subject variability. These calculations have assumed that we wish to detect a 30% difference between treatments in a crossover study, with 80% power at the 5% significance level. These calculations have only been produced for the exercise endpoints, as it is these which will be of importance in subsequent drug studies.

Endpoint	**30% Difference**	**Within subject SD**	**Volunteer No.**
Peak Flow			
1 min Exercise	5.52	6.4	24
2 min Exercise	7.20	8.1	22
3 min Exercise	9.00	10.8	25
Peak Flow (change from baseline)			
1 min Exercise	4.43	6.1	32
2 min Exercise	6.10	8.3	31
3 min Exercise	7.91	10.8	32
Peak Flow (normalised)			
1 min Exercise	125.7	163	29
2 min Exercise	163.5	228	33
3 min Exercise	226.5	308	32
AUC (change from baseline)			
1 min Exercise	1.28	2.81	78
2 min Exercise	2.56	3.99	41
3 min Exercise	4.62	7.19	41
AUC (normalised)			
1 min Exercise	35.3	72.4	69
2 min Exercise	71.3	114.8	43
3 min Exercise	134.9	138.5	19

Appendix

4.4 Temperature

There is very little spread of minimum temperatures (tabulated on file) recorded during the study. No formal statistical analysis has been undertaken. By looking at the data, there is no evidence of any trends either from session 1 to session 2 on a study or from study day 1 to study 2.

4.5 Change in BOMED on ischaemic and exercise challenges

In the case of te ischaemic challenge, there were four statistically significant differences (overall P-value <0.05) observed. They all represented decreases in the BOMED indices from baseline.

BOMED Variable	Challenge Level	Difference from Baseline	Standard Deviation
End Diastolic Index	5 mins	-5.9	2.9
Peak Flow Index	5 mins	-25.6	12.2
Index of Contractility	5 mins	-3.8	1.6
Acceleration Index	5 mins	-8.9	3.8

For the exercise challenge, there were two statistically significant differences (overall P-value <0.05) observed. They both represented increases in the BOMED indices baseline.

BOMED Variable	Challenge Level	Difference from Baseline	Standard Deviation
Heart Rate	3 mins	4.4	1.9
Ejection Fraction	3 mins	2.5	1.2

4.6 Control forearm data

Comparing baseline 1 from the active forearm with baseline 1 from the control forearm, there was no evidence, P=0.7, that the means were statistically significantly different.

There was no evidence that baseline 2 or baseline 3 from the active forearm was statistically significant different from their respective baselines from the control forearm, P=0.58 and P=0.7 respectively.

Figures 13 and 14 show the overall mean forearm flood flow in the control forearm data during the ischaemic and exercise challenges. At the latter exercise challenges, there would appear to be increases in control forearm blood flow.

Appendix

5. Conclusions

Looking at all the endpoints investigated in this study, it would appear that correcting for an initial forearm blood flow measurement session does not provide any extra precision. The high variability of this data has resulted in relatively large estimates of volunteer numbers for future studies. The use of peak flow as the primary endpoint generally requires fewer volunteers than AUC.

Rebecca Sheale (Biometrician, ICI Pharmaceuticals PLC)
31/03/93

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Figure 1 Forearm blood flow minus baseline for ischaemia data - by session

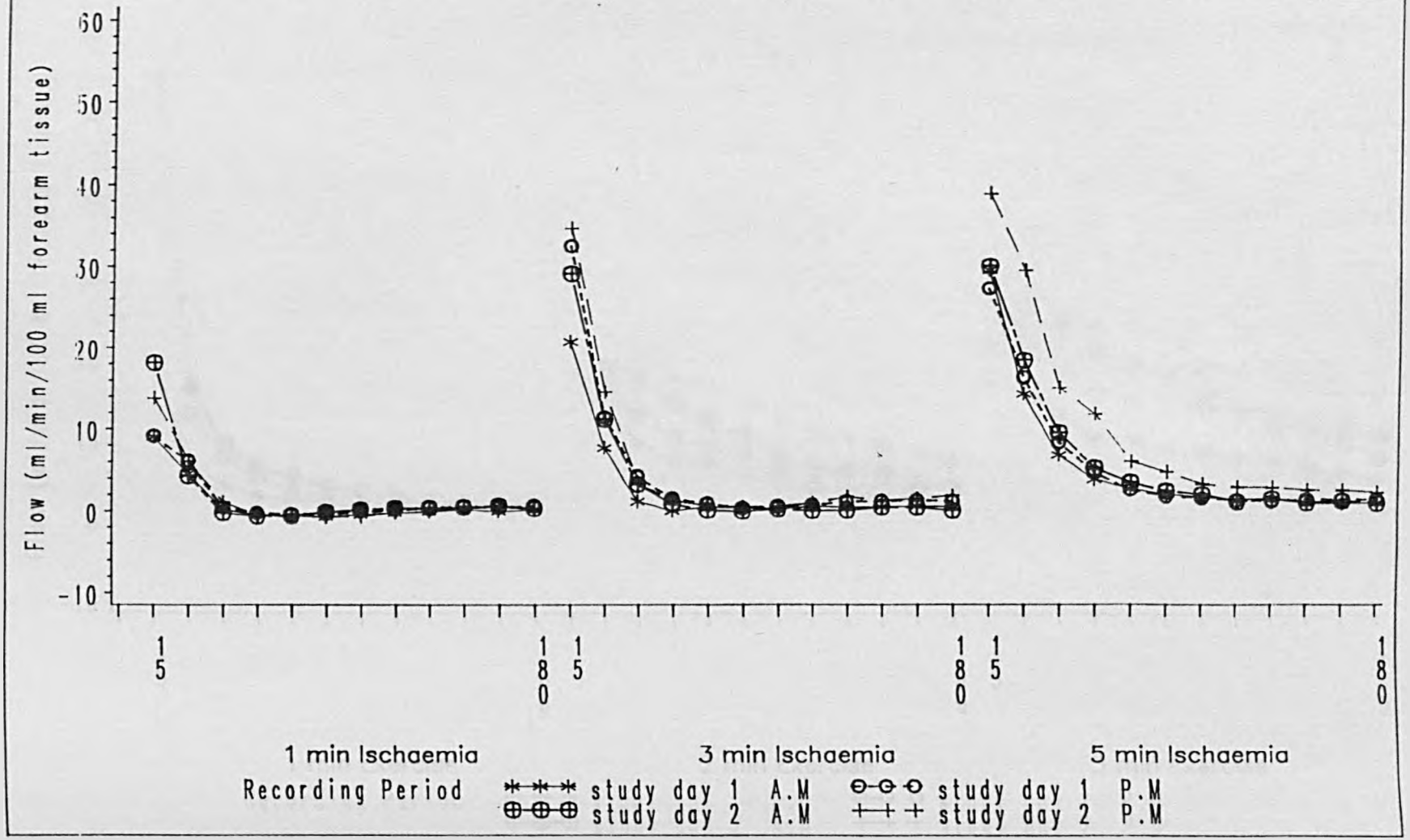

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Figure 2 Forearm blood flow minus baseline for exercise data - by session

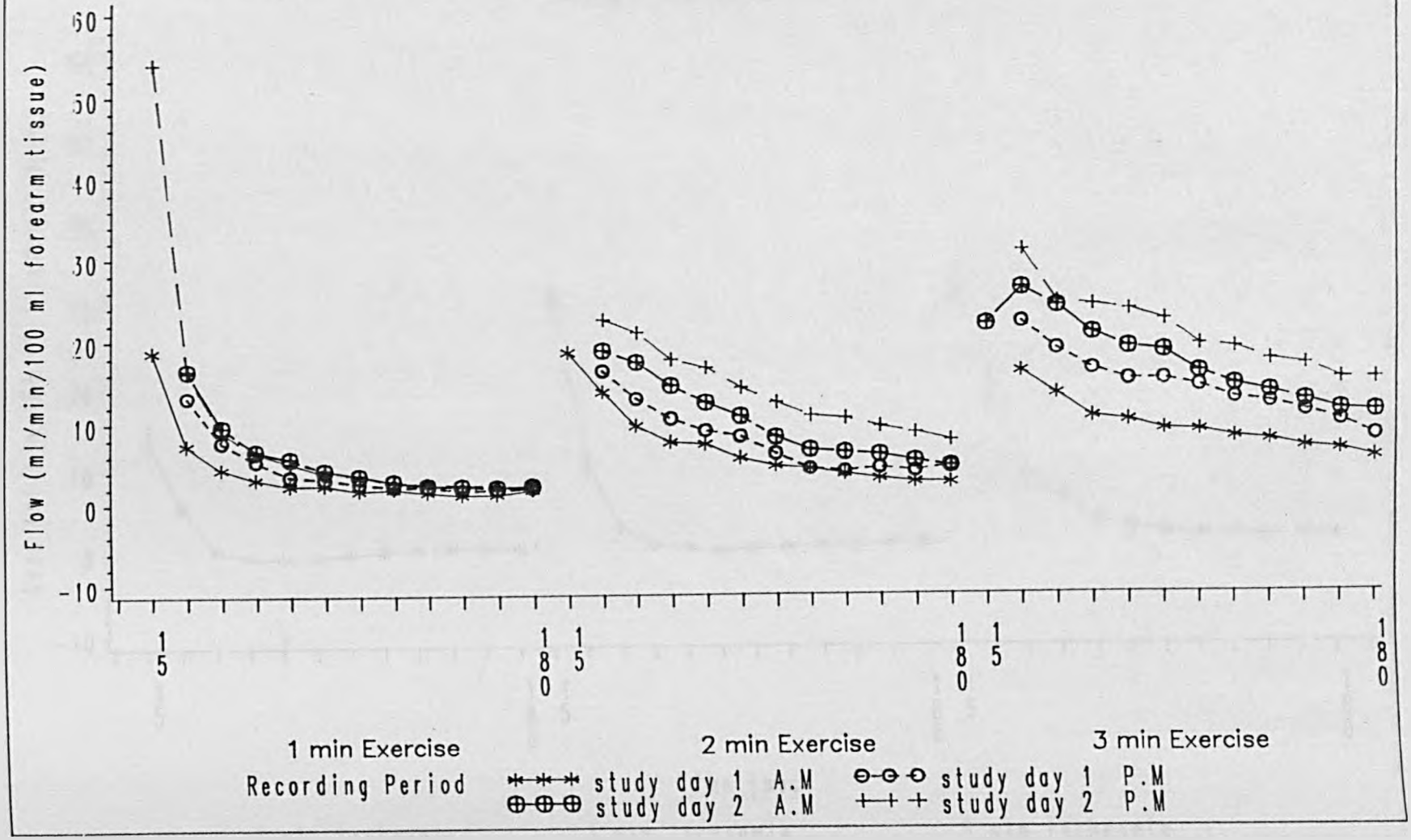

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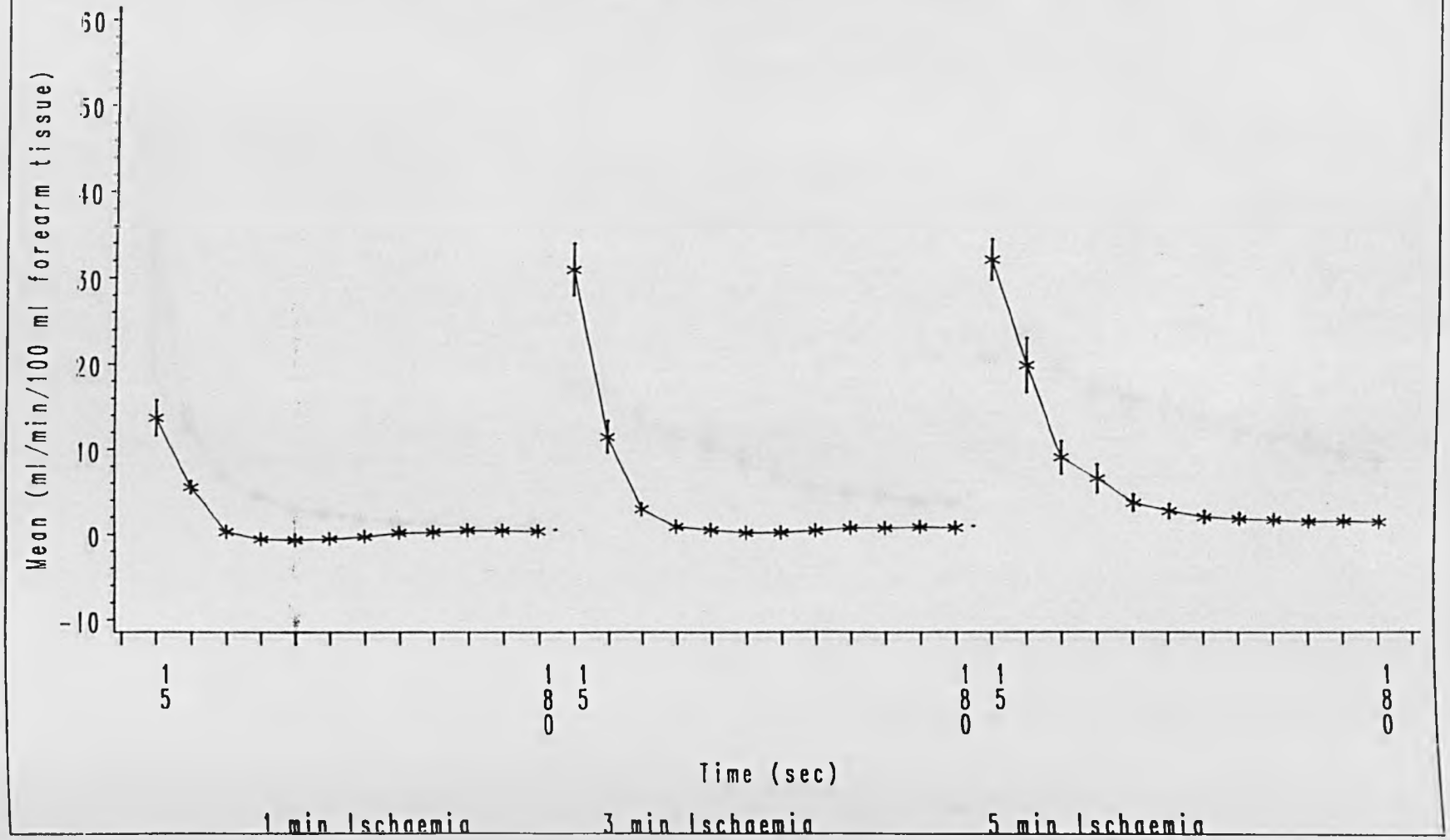
Figure 3 Forearm blood flow overall mean (+/- se)
Change from baseline



CP00HQ/0008

Figure 4 Forearm blood flow overall mean (+/- se)
Change from baseline

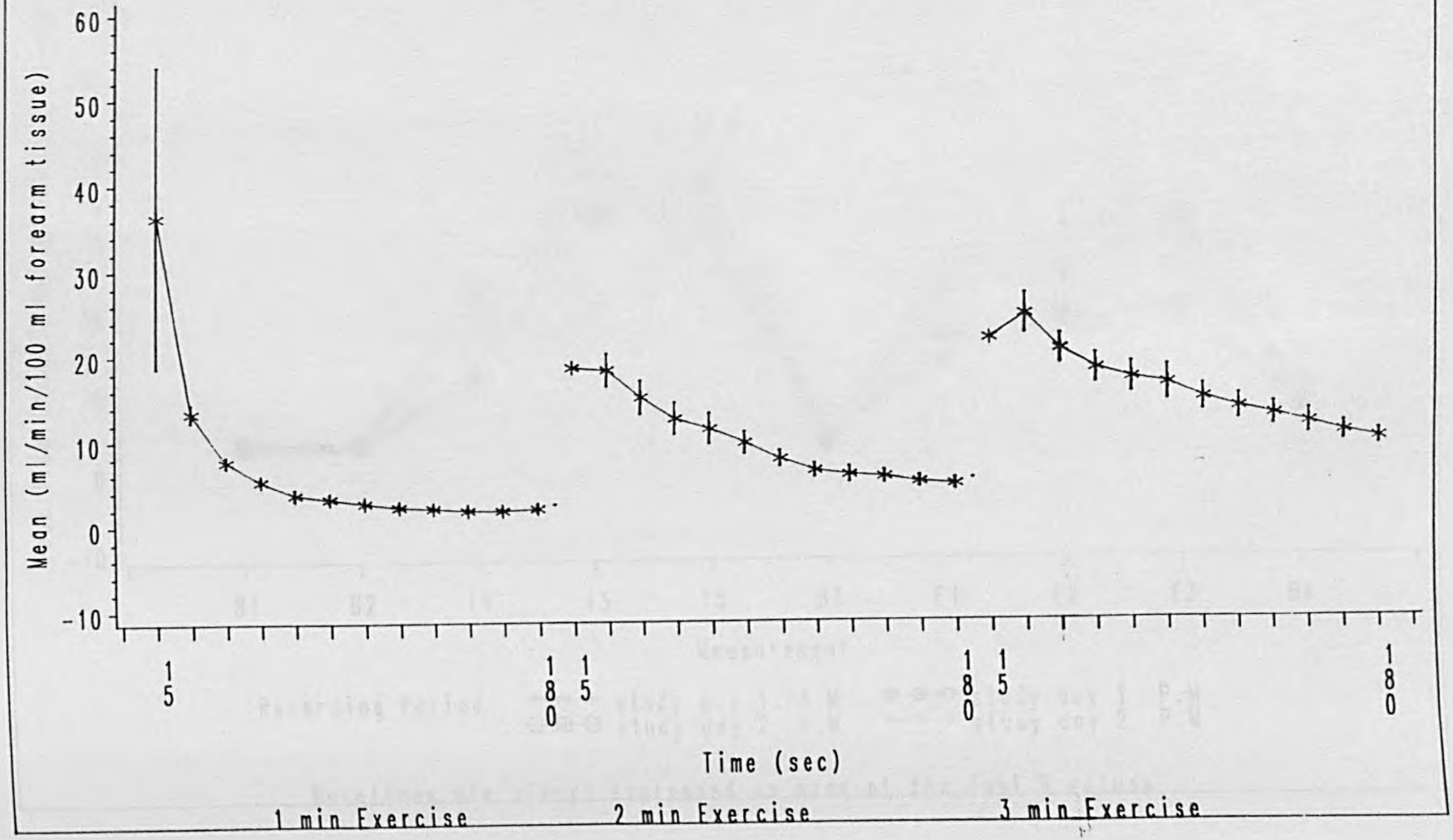


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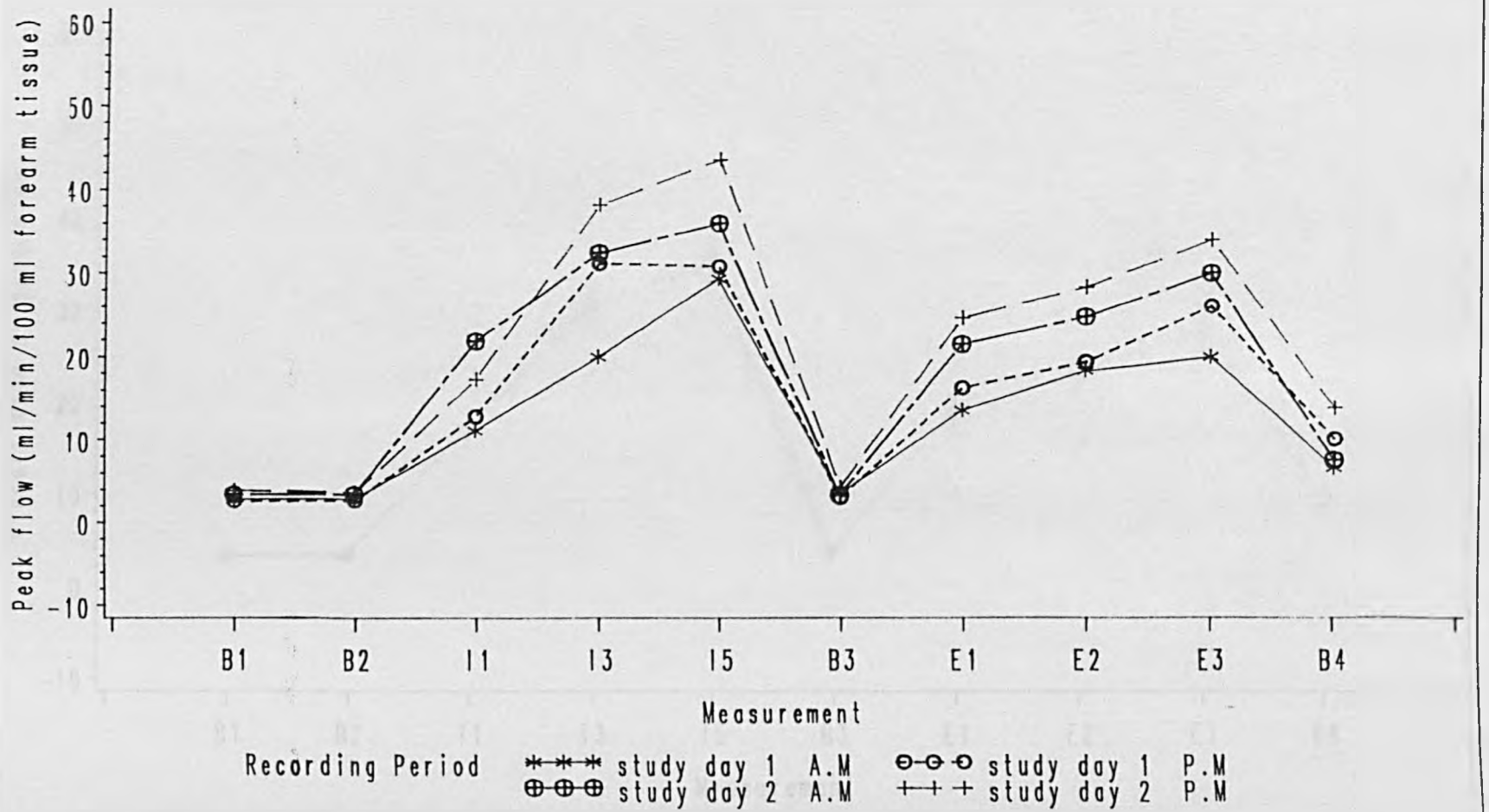
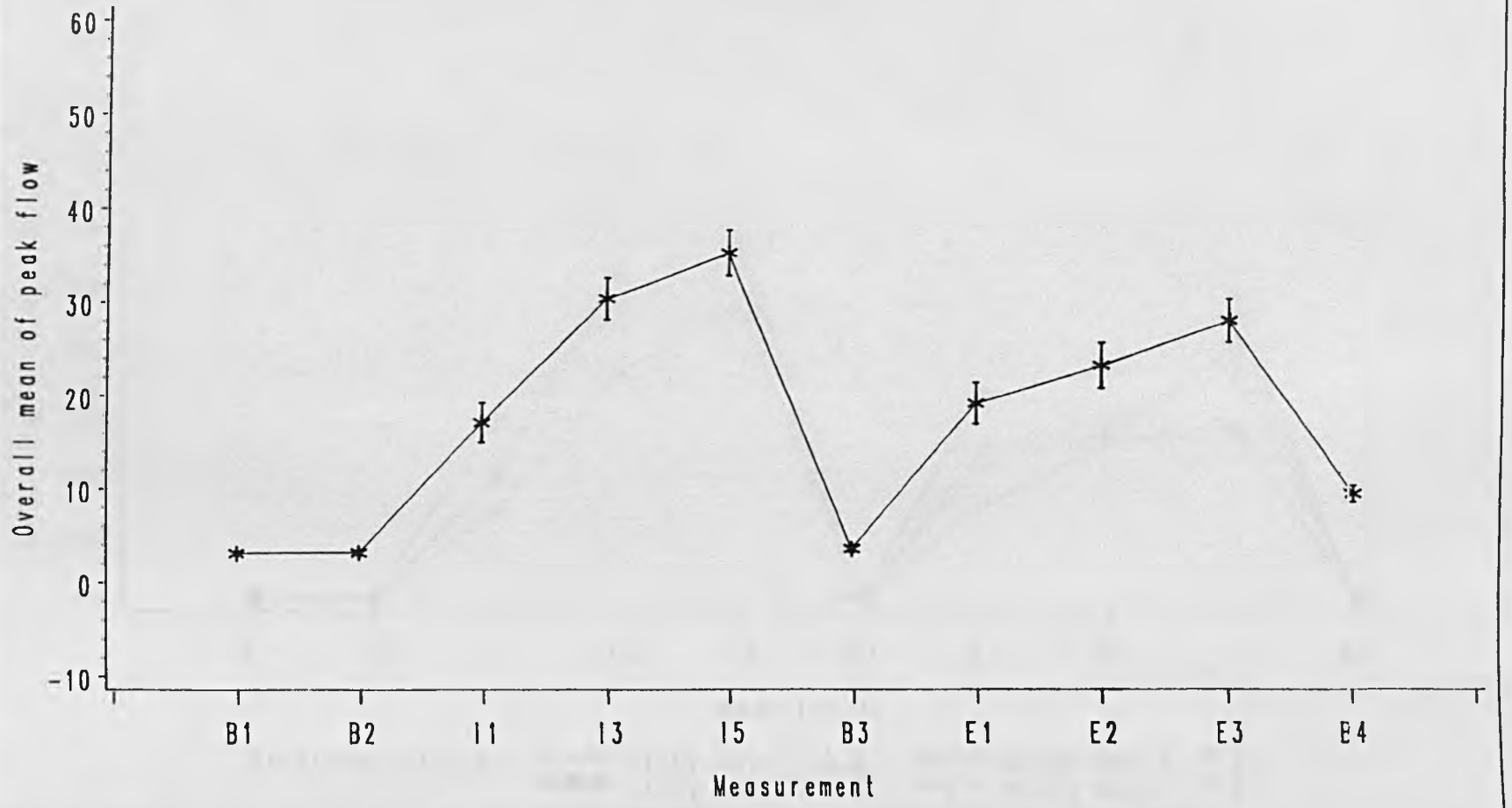
Figure 5 Forearm blood flow mean of peak flow - by session
Unadjusted data



Baselines are always expressed as mean of the last 8 values

CP00HQ/0008

Figure 6 Forearm blood flow mean of peak flow (+/- se)
Unadjusted data



Baselines are always expressed as mean of the last 8 values
Peak flow measured in ml/min/100 ml forearm tissue

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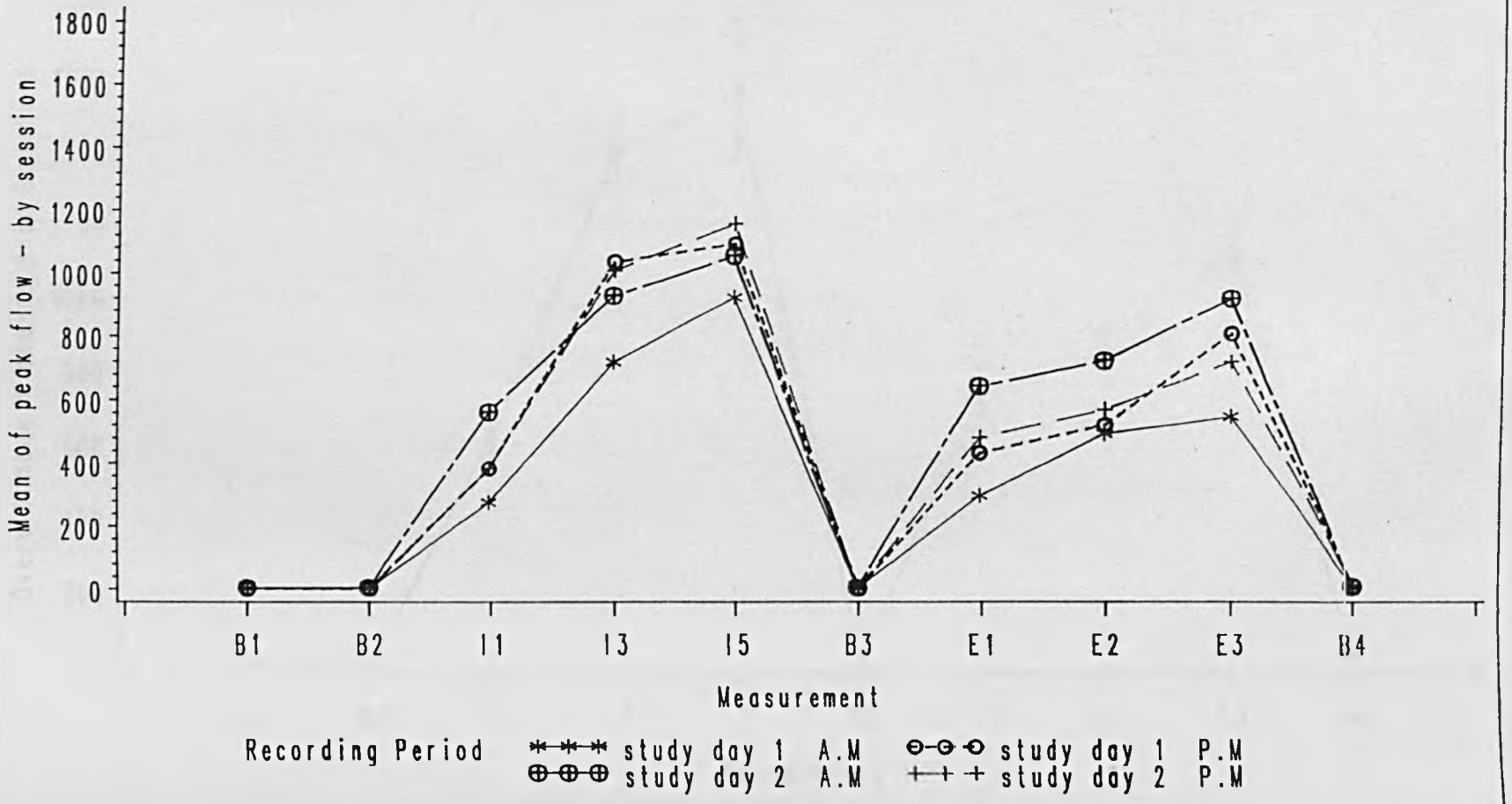
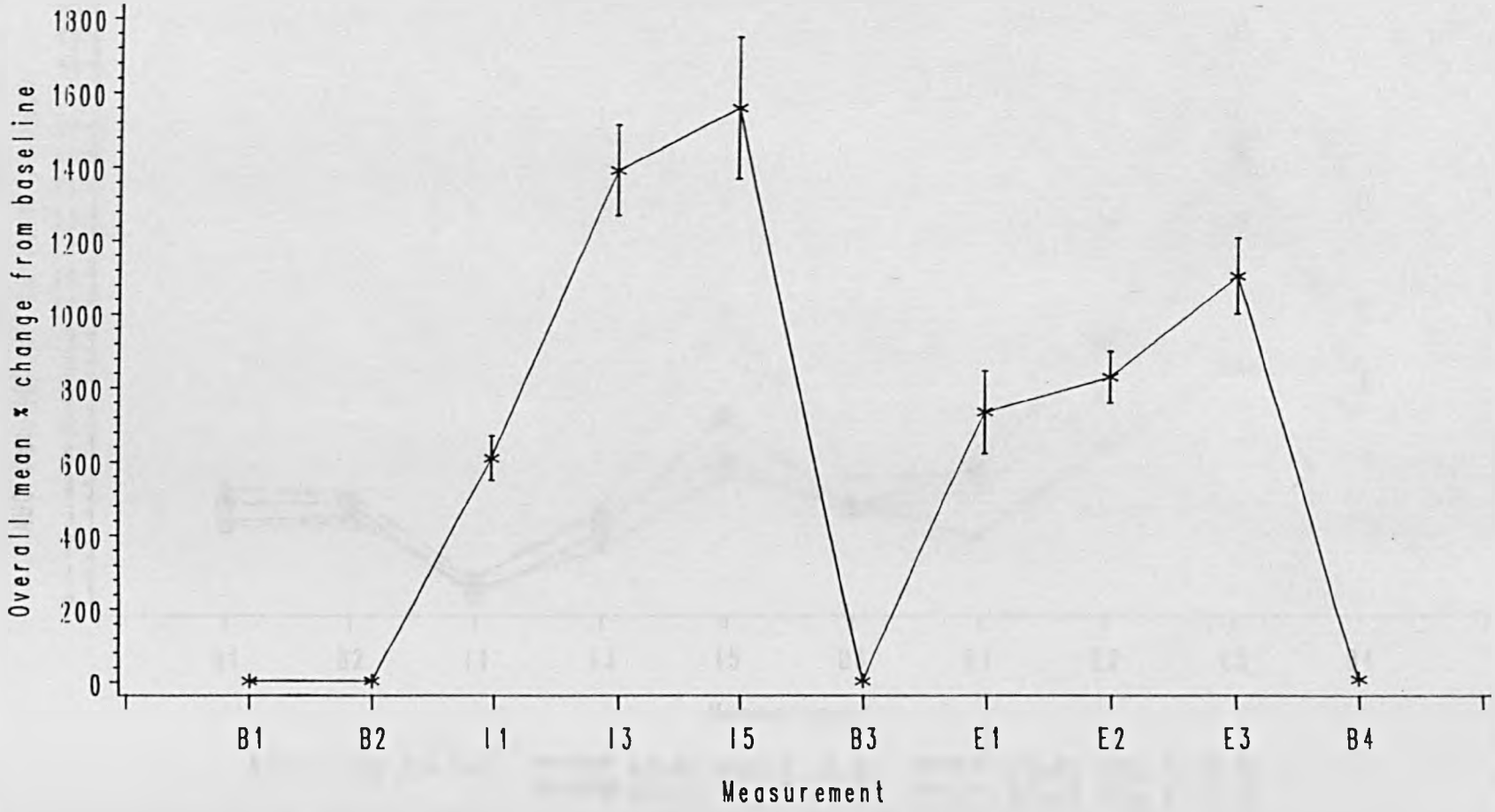
Figure 7 Forearm blood flow Mean of peak flow - by session
Normalised data



Baselines are always expressed as mean of the last 8 values
Peak flow measured in ml/min/100 ml forearm tissue

CP00HQ/0008

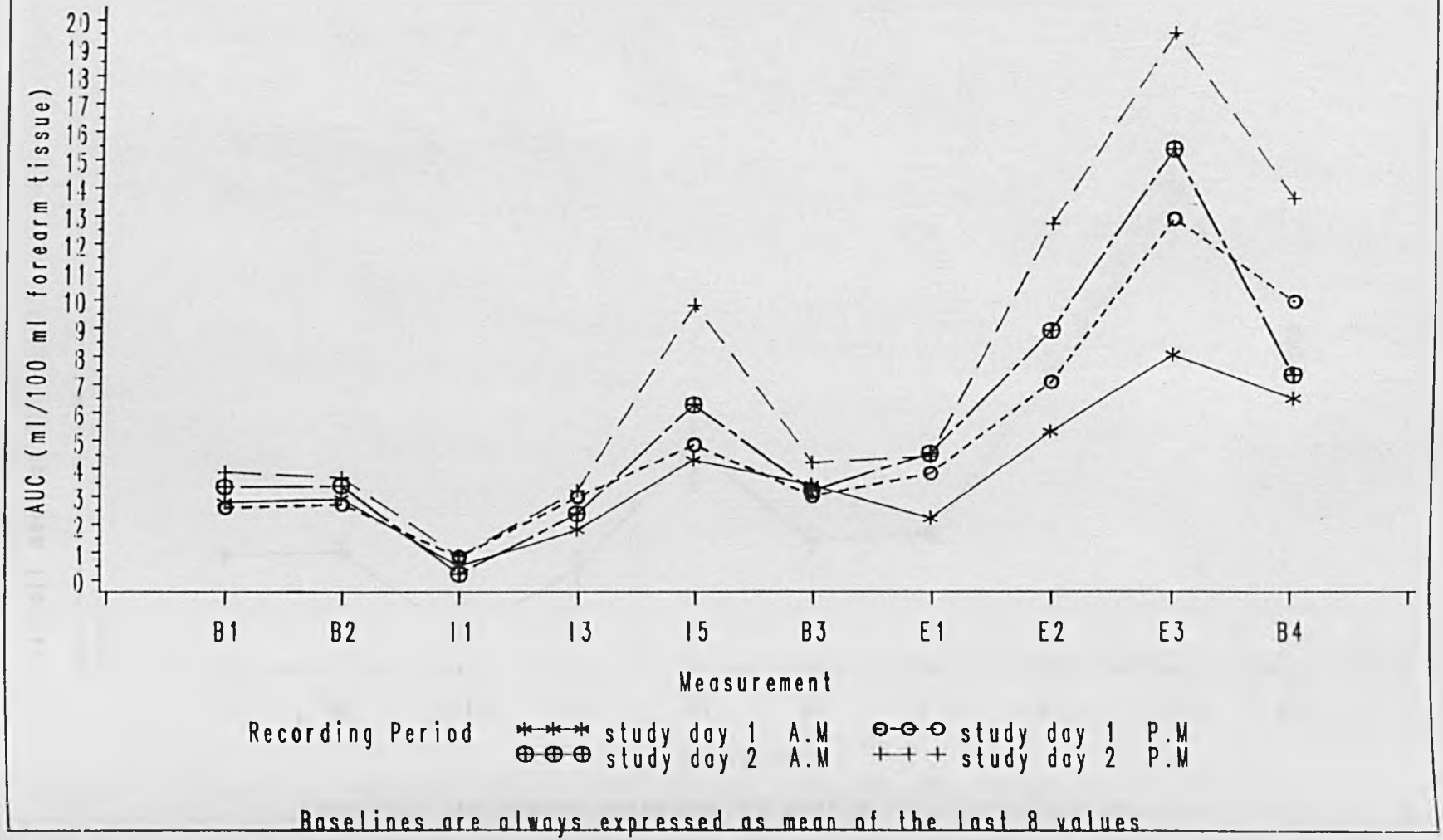
Figure 8 Forearm blood flow mean of peak flow (+/- se)
Normalised data



Baselines are always expressed as mean of the last 8 values
Peak flow measured in ml/min/100 ml forearm tissue

CP00HQ/0008

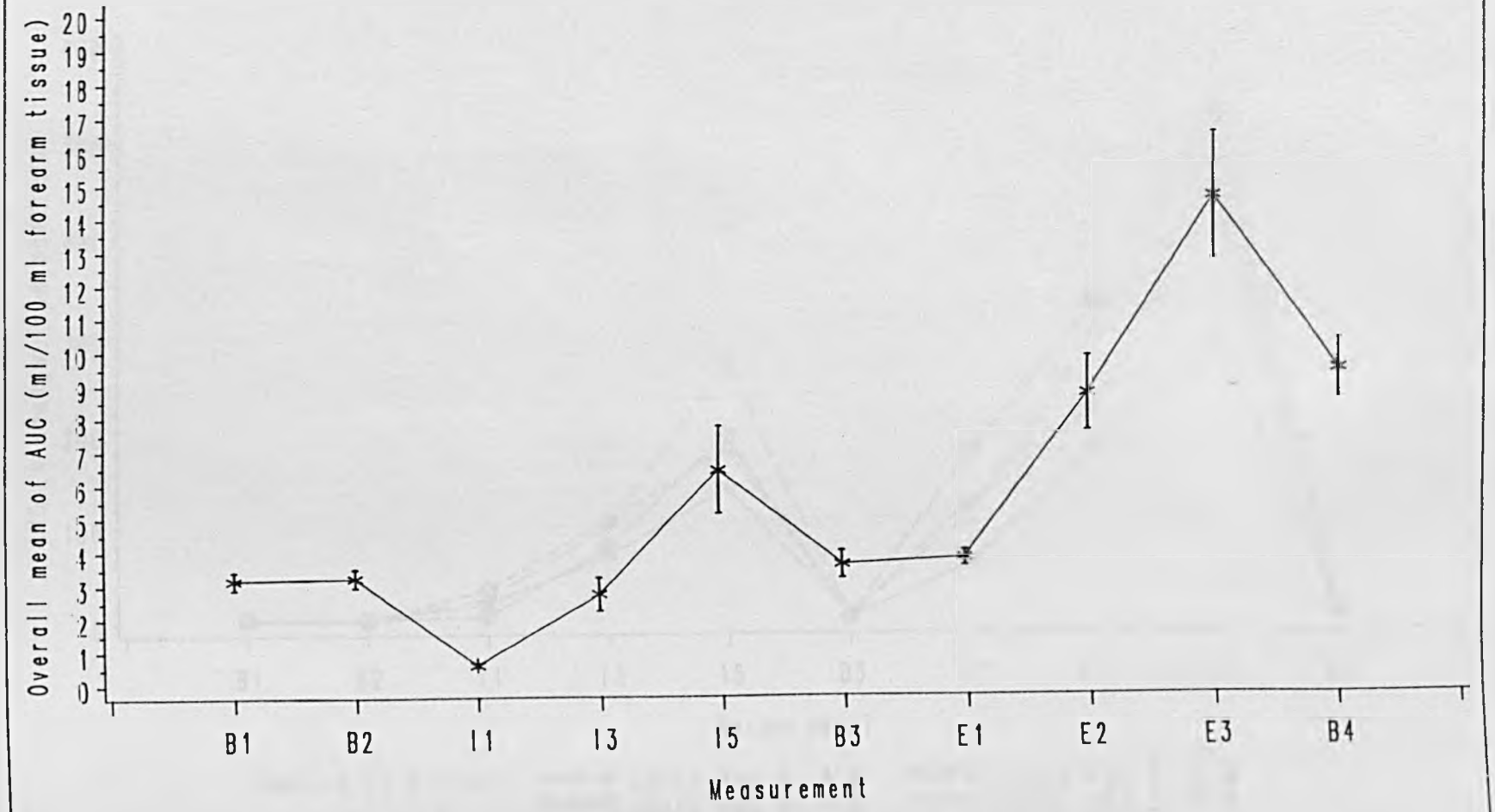
Figure 9 Forearm blood flow mean AUC (ml/100 ml forearm tissue) - by session
Change from baseline



Baselines are always expressed as mean of the last 8 values

CP00HQ/0008

Figure 10 Forearm blood flow mean of AUC (+/- se)
Change from baseline



Baselines are always expressed as mean of the last 8 values

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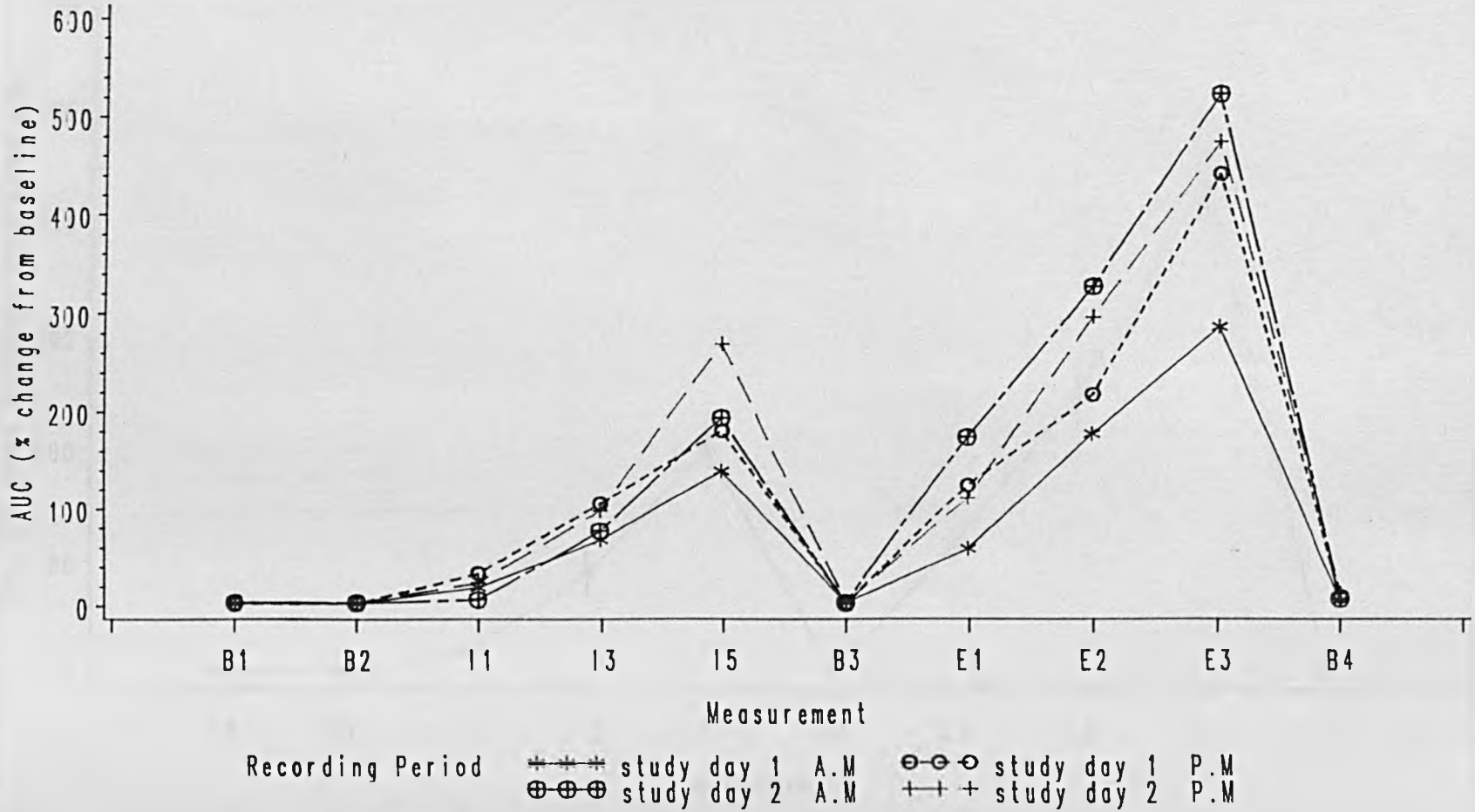
Figure 11 Forearm blood flow mean AUC (% change from baseline) - by session
Normalised data



Baselines are always expressed as mean of the last 8 values

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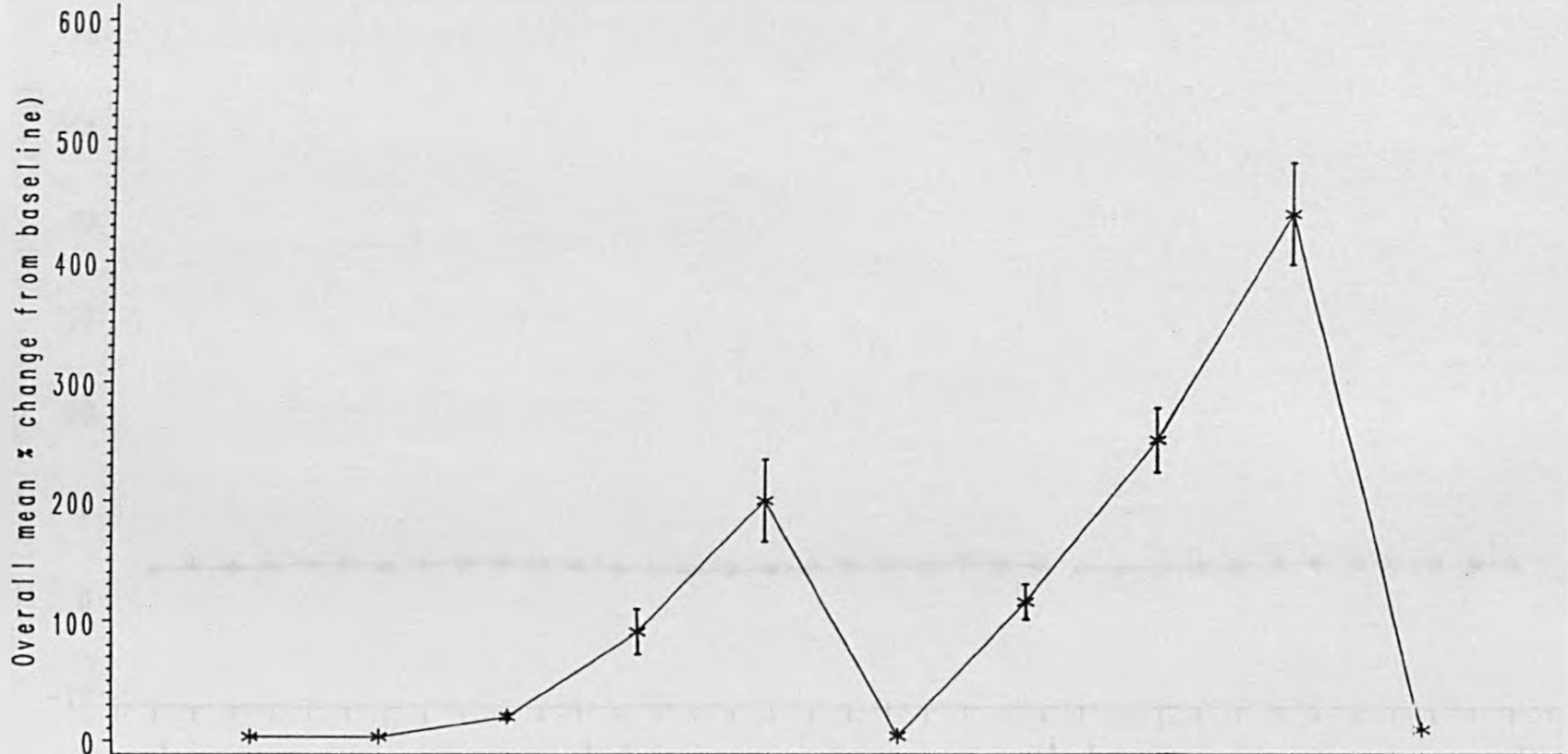
Figure 12 Forearm blood flow mean of AUC (+/- se)
Normalised data



Baselines are always expressed as mean of the last 8 values

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FIGURE 13 Forearm blood flow overall mean (+/- se)
Control arm data

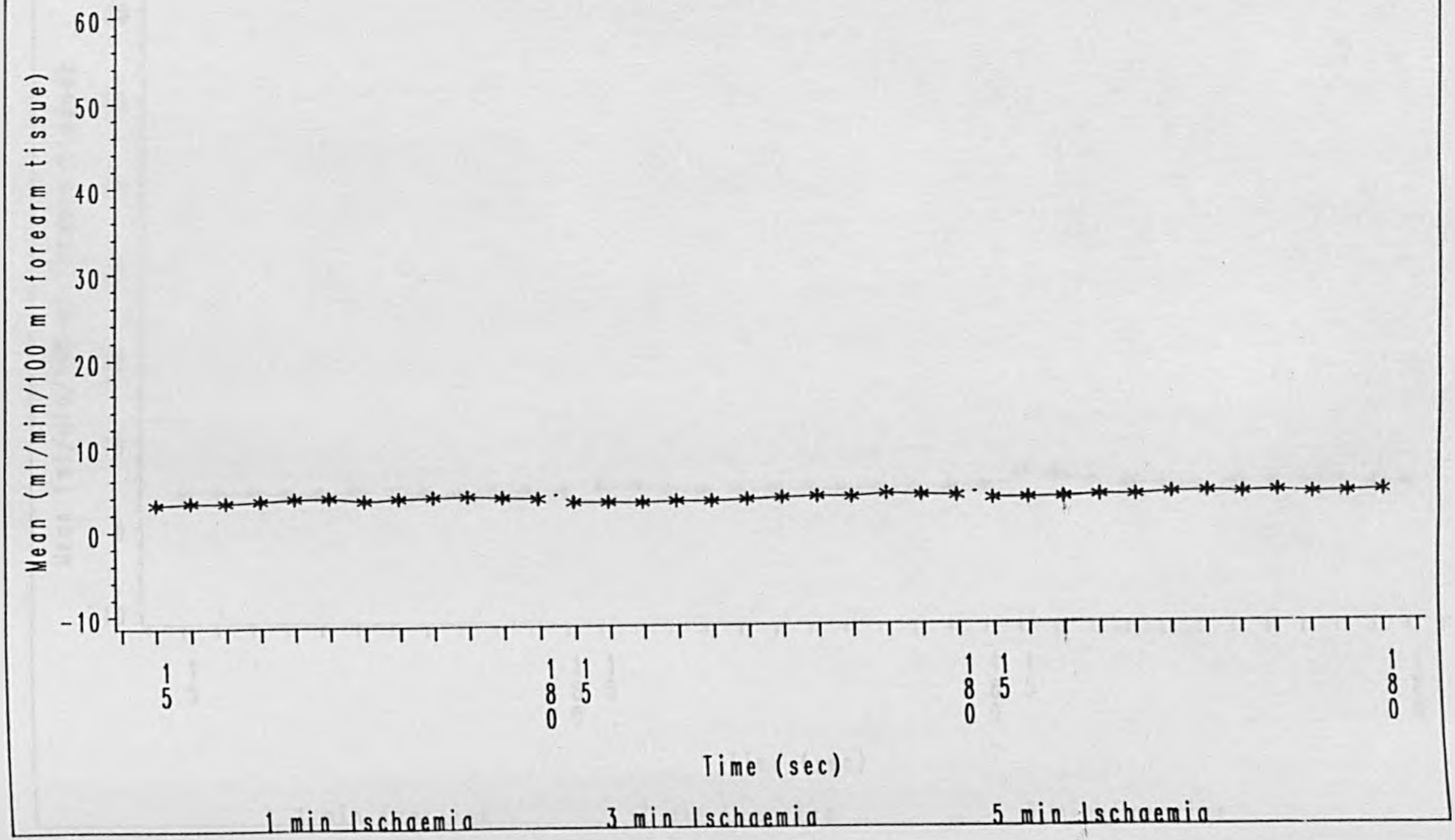

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Figure 14 Forearm blood flow overall mean (+/- se)
Control arm data

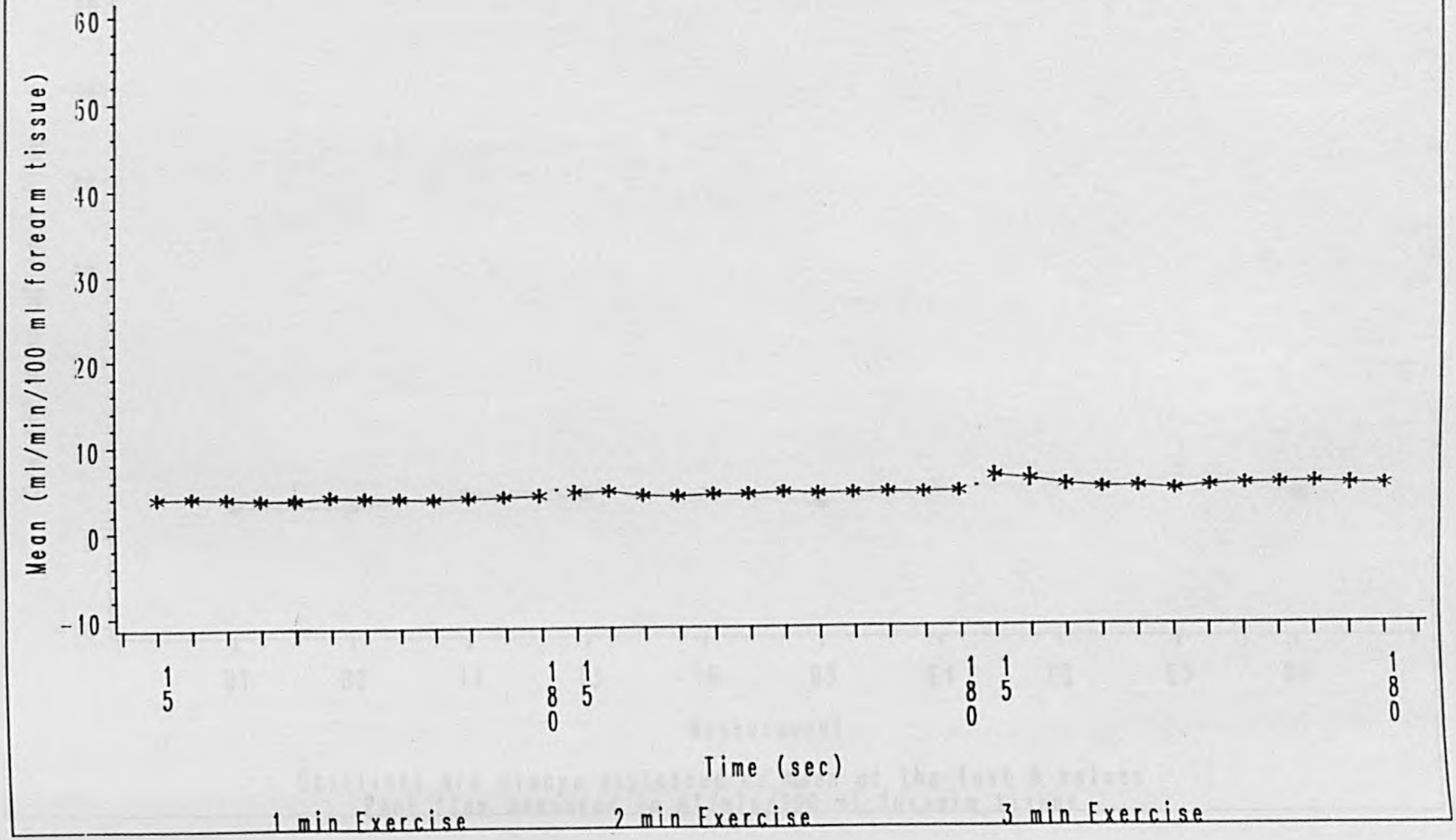

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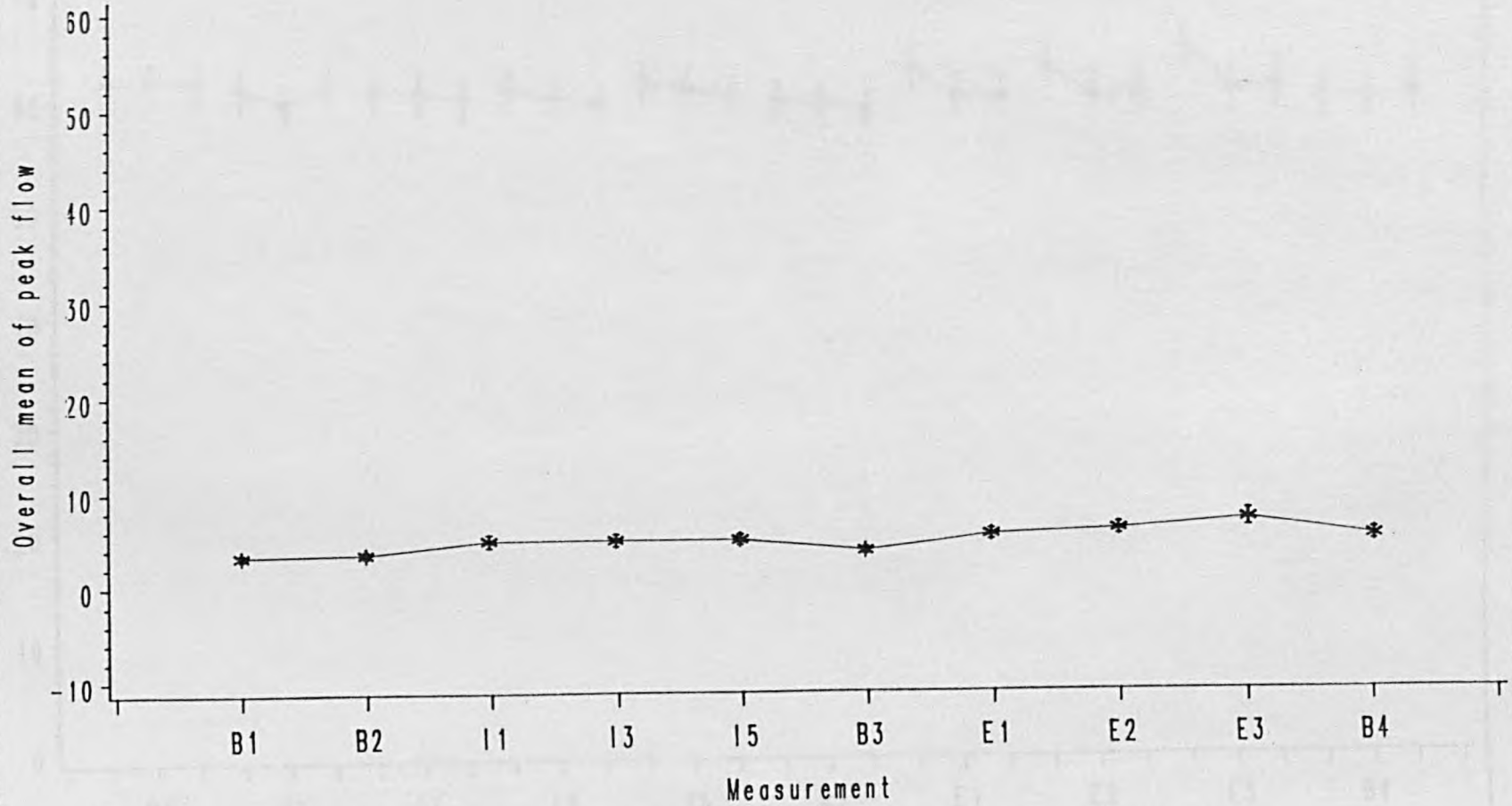
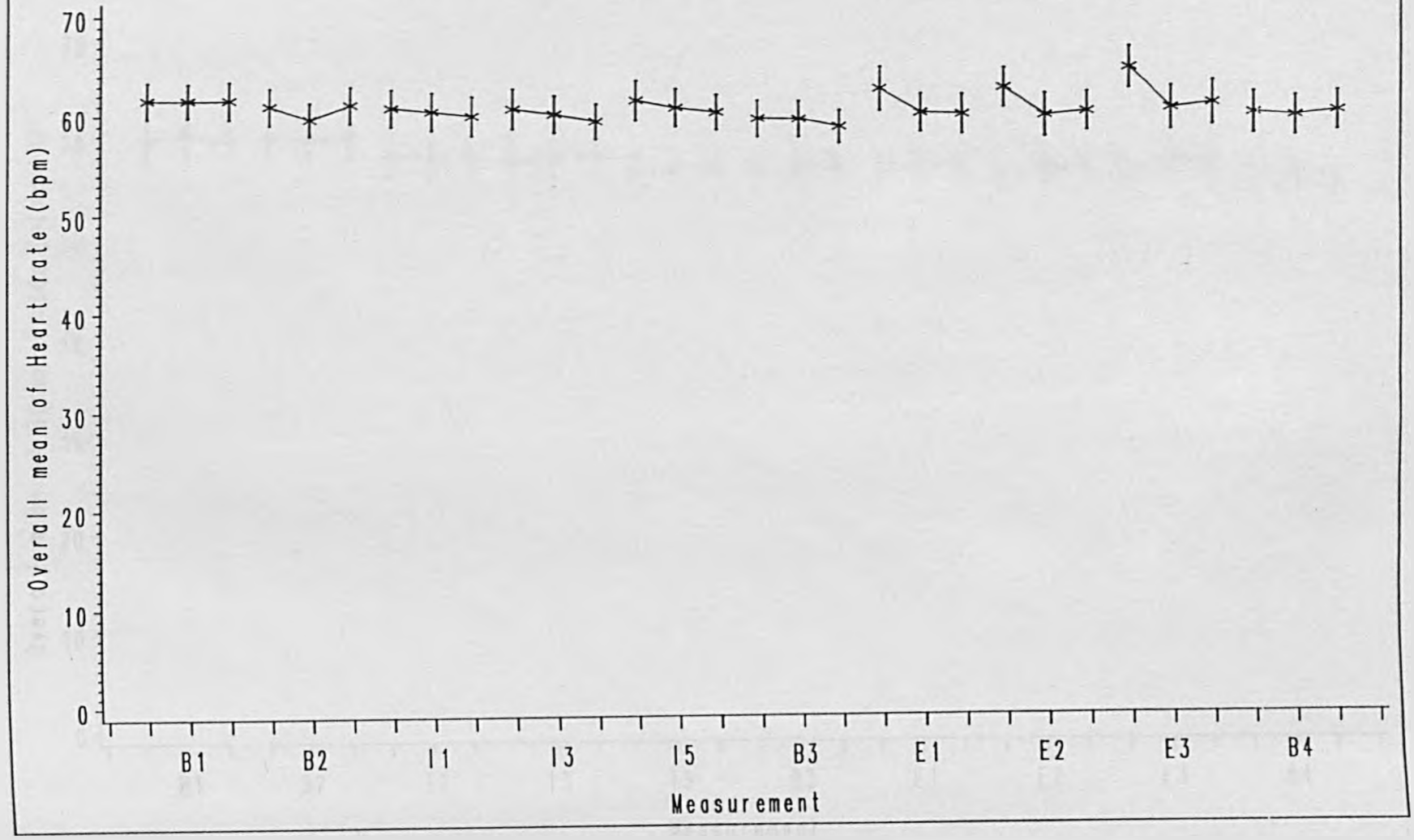
Figure 15 Forearm blood flow mean of peak flow (+/- se)
Control arm data

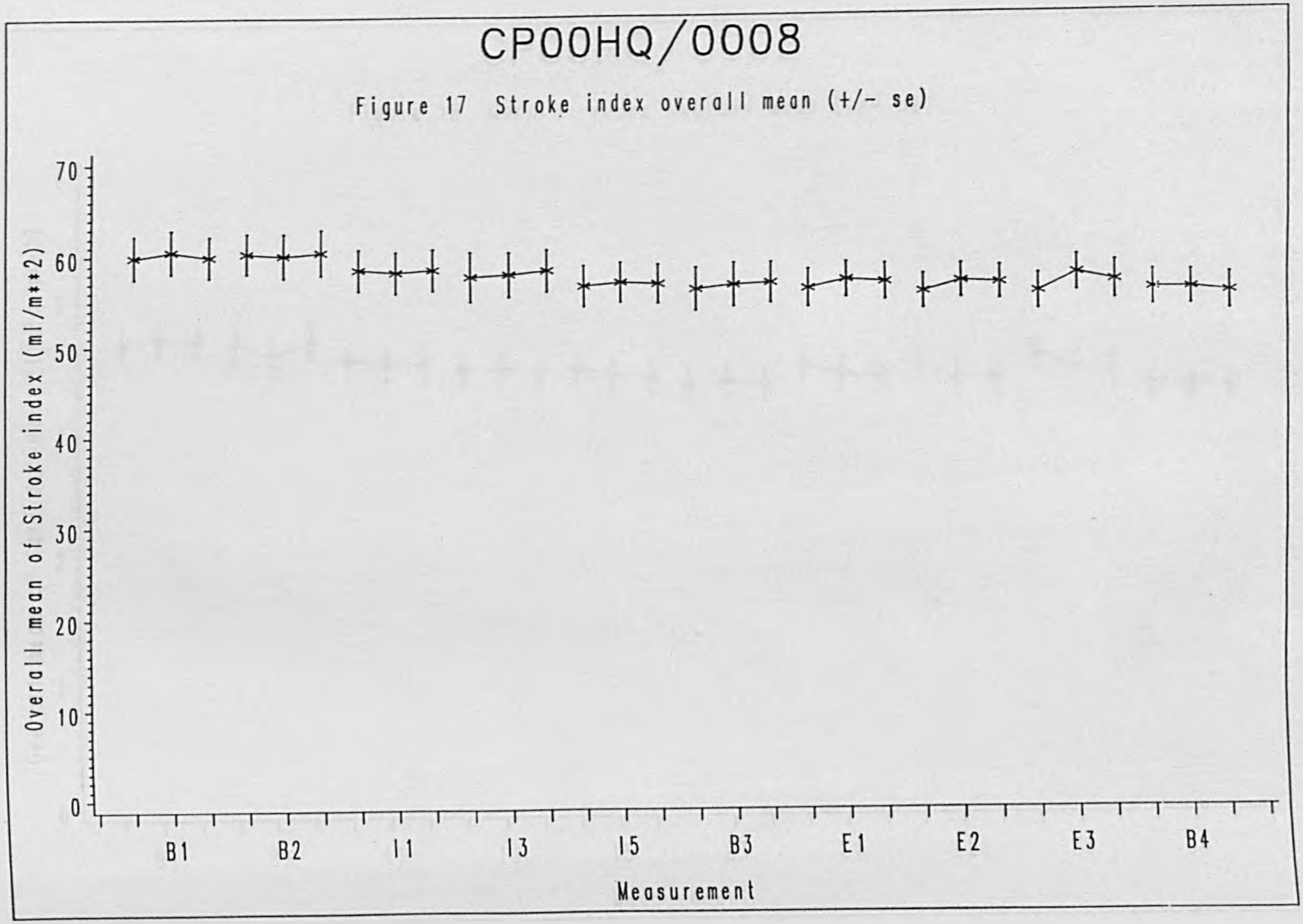


Baselines are always expressed as mean of the last 8 values
Peak flow measured in ml/min/100 ml forearm tissue

CP00HQ/0008

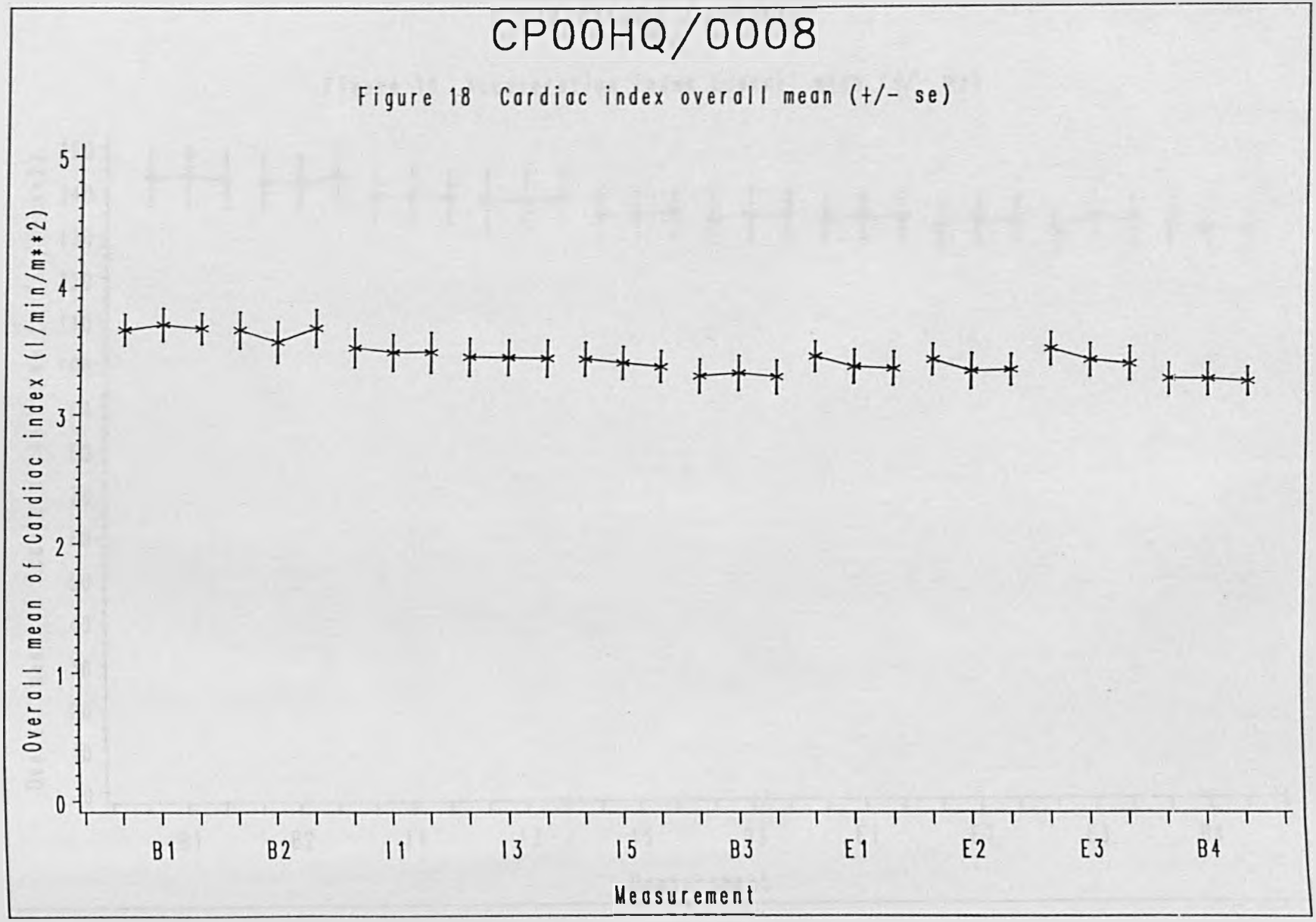
Figure 16 Heart rate overall mean (+/- se)




CP00HQ/0008
Figure 17 Stroke index overall mean (+/- se)
Overall mean of Stroke index (ml/m**2)
70
60
50
40
30
20
10
0
B1
B2
I1
I3
I5
B3
E1
E2
E3
B4
Measurement

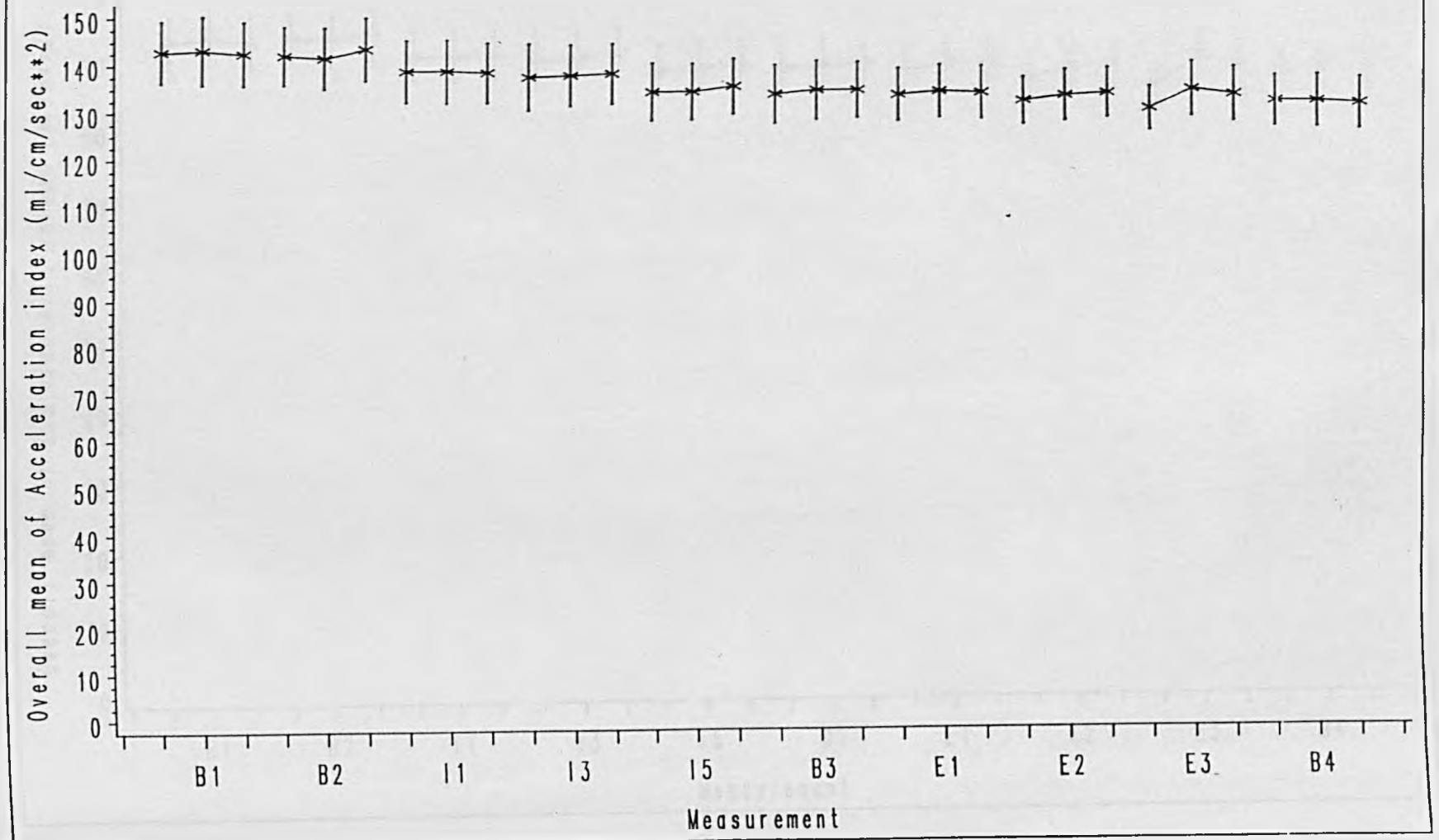
CP00HQ/0008

Figure 18 Cardiac index overall mean (+/- se)



CP00HQ/0008

Figure 19 Acceleration index overall mean (+/- se)



CP00HQ/0008

Figure 20 End diastolic volume overall mean (+/- se)

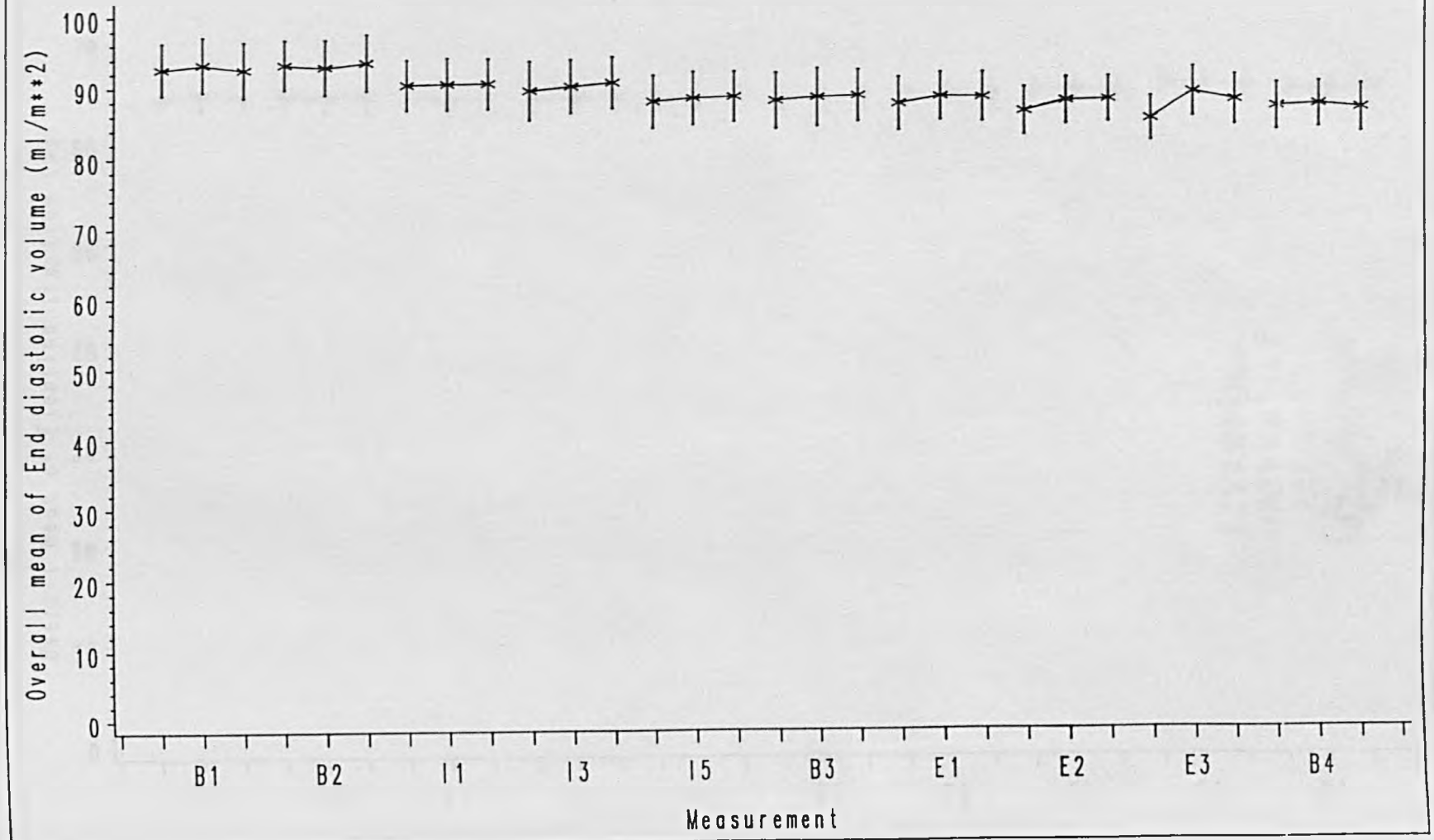


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Figure 21 Ejection fraction overall mean (+/- se)