1. **Dose-linearity of the pharmacokinetics of an intravenous [14C]midazolam microdose in children**
2. [14C]midazolam microdose in children
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Key words:

* Drug metabolism
* Paediatrics
* Cytochrome P450

# Main text

# Abstract

**Aims:** Drug disposition in children may vary from adults due to age-related variation in drug metabolism. Microdose studies present an innovation to study pharmacokinetics (PK) in paediatrics, however, it should be used only when the PK is dose linear. We aimed to assess dose-linearity of a [14C]midazolam microdose, by comparing the PK of an intravenous (IV) microtracer (a microdose given simultaneously with a therapeutic midazolam dose), with the PK of a single isolated microdose.

**Methods:** Preterm to two-year-old infants admitted to the intensive care unit received [14C]midazolam IV as a microtracer or microdose, followed by dense blood sampling up to 36 hours. Plasma-concentrations of [14C]midazolam and [14C]1-hydroxy-midazolam were determined by accelerator mass spectrometry. Non-compartmental PK analysis (NCA) was performed and a population PK model was developed.

**Results:** Of 15 infants (median gestational age 39.4 [range 23.9-41.4] weeks, postnatal age 11.4 [0.6-49.1] weeks), six received a microtracer and nine a microdose [14C]midazolam (111 Bq kg-1; 37.6 ng kg-1). In a two-compartment PK model, bodyweight was the most significant covariate for volume of distribution. There was no statistically significant difference in any PK parameter between the microdose and microtracer, nor in the AUC ratio [14C]1-OH-midazolam/[14C]midazolam, showing the PK of midazolam to be linear within the range of the therapeutic and microdoses.

**Conclusion:** Our data supports the dose-linearity of the PK of an IV [14C]midazolam microdose in children. Hence, a [14C]midazolam microdosing approach may be used as an alternative to a therapeutic dose of midazolam to study developmental changes in hepatic CYP3A activity.

# Statement 1

What is already known about this subject:

* Microdose studies with radioactive labelled ([14C]) substrates present an innovation to study pharmacokinetics of drugs in paediatrics
* Dose-linearity between the pharmacokinetics of a microdose and therapeutic dose is an important prerequisite
* Dose-linearity of the pharmacokinetics of a microdose of [14C]midazolam has not been established in children

# Statement 2

What this study adds:

* The pharmacokinetics of a microdose of [14C]midazolam are dose linear to therapeutic doses in children
* As midazolam is a well-established marker for the developmentally regulated CYP3A enzyme, a [14C]midazolam microdosing approach is a minimal risk strategy to study hepatic CYP3A activity variability in children

# Introduction

Drug disposition in children may vary from adults due to age-related variation in the processes governing absorption, distribution, metabolism and excretion.[1,2] This variation is largest in the first years of life and is not directly proportionate to size.[3,4] However, in daily clinical practice drug dosing in paediatrics is often based on bodyweight based corrections, which because of variation arising from development, can result in sub-therapeutic or toxic drug exposure in certain subgroups.[2] Hence, doses used for children cannot simply be extrapolated from adults using a simple bodyweight-based correction.

Phenotyping studies, in which model drugs representative for a certain pathway are studied across the paediatric age range, can be used to elucidate the age-related variation in drug disposition pathways in vivo.[5] However, these studies are faced with ethical, practical and scientific challenges. Children are vulnerable, and so exposing them to (almost) therapeutic doses of drugs for a non-therapeutic reason, as in a phenotyping study, may not be ethically acceptable. Moreover, blood sampling for pharmacokinetic (PK) analyses in children is challenging because of the burden for the individual child, the smaller blood volume that can be taken, as well as the technical difficulties associated with sampling.

Microdosing studies present an attractive alternative to overcome the ethical and analytical challenges of phenotyping studies.[6] A microdose is a very small, sub-therapeutic dose of a drug (<1/100th of the therapeutic dose or <100 µg), that is unlikely to result in pharmacological effects or adverse events.[7,8] A radioactive label [14C] allows ultra-sensitive quantification of extremely low plasma-concentrations by accelerator mass spectrometry (AMS) for which only 10-15 µl plasma is required.[9,10] The radiation dose associated with a [14C]microdose is safe as it is below 1 μSievert. This is much lower than yearly background exposure (2.5 mSv year-1 in The Netherlands), a computed tomography (CT)-scan of the head (1200 μSievert), or chest x-ray (12 μSievert).[6]

Microdosing studies can provide unique information of the PK of drugs in children, and with that valuable information on developmental changes in drug metabolism pathways, as shown successfully before.[6,11-13] Importantly, a prerequisite is that the PK of a microdose are linear to the PK of a therapeutic dose.[14,15] Lack of linearity may occur for example, when a therapeutic dose saturates drug metabolism pathways, plasma protein binding and/or active transporters, which may result in altered PK when studying a microdose.[15] A very elegant approach to study dose-linearity is by comparing the PK parameters of an isolated [14C]microdose with the PK parameters of a [14C]microtracer, where the labeled microdose is administered concurrently or even mixed with a therapeutic drug dose.[12].

Cytochrome P450 (CYP) 3A is a developmentally regulated drug metabolizing enzyme that is abundant in the liver and accounts for nearly 46% of the oxidative metabolism of clinically relevant drugs.[1,2,16-21] As midazolam is a well-established model substrate for CYP3A activity, this drug may be used for phenotyping studies using a microdosing approach to elucidate developmental changes in CYP3A.[5,22-25] To the best of our knowledge, dose-linearity of the PK of a microdose to those of a therapeutic dose of midazolam has been established in adults[14,26,27], but not in children. Yet, the results in adults cannot simply be extrapolated to children due to the development of drug metabolism, hepatic blood flow, protein binding and drug transport.

We therefore aimed to study the dose-linearity of the PK of a [14C]midazolam microdose in children, by studying the PK parameters of midazolam when given as an intravenous (IV) [14C]microdose, and as a [14C]microtracer given simultaneously with a therapeutic midazolam dose.

# Methods

## Study design

This study was part of the ERA-NET PRIOMEDCHILD project ‘Paediatric Accelerator Mass Spectrometry Evaluation Research Study (PAMPER)'. The two units participating in this study were the Alder Hey Children’s NHS Foundation Trust, Liverpool, UK and the Liverpool Women’s NHS Foundation Trust, Liverpool, UK. Children were recruited on the pediatric intensive care units (PICUs) of these units. Ethical approval was obtained from the Research Ethics Committees for the hospitals where patients were enrolled. All parents or an adult who carried parental responsibility provided written informed consent for their child to be included prior to any study-specific procedures. No radioactive substance administration approval was required as the administered radioactive dose was below 1 μSievert, the UK Administration of Radioactive Substances Advisory Committee (ARSAC) exemption level.

## Subjects

Children were eligible to be included in this study from birth up to two years of age, when they had intravenous lines in placefor intravenous administration, and had suitable vascular access for blood sampling. Exclusion criteria were serious hepatic impairment (defined by aspartate-aminotransferase [ASAT] and alanine-aminotransferase [ALAT] > 200 U L-1) or renal impairment (defined by plasma creatinine > 150 µmol), hemofiltration, peritoneal/hemodialysis or extracorporeal membrane oxygenation (ECMO).

## Study procedures

A single [14C]midazolam (111 Bq kg-1; 37.6 ng kg-1) dose was administered IV either as a microtracer during therapeutic midazolam infusion or as an isolated microdose (Figure 1). The microtracer was mixed with the first therapeutic loading dose of midazolam given by the treating physician for sedation, and was administered over 30 min. The microdose was administered with a similar infusion rate to ensure similar exposure to [14C]levels. The IV therapeutic midazolam dose was prescribed by the treating physician for clinical purposes according to British National Formulary for Children dosing guidelines. Blood samples were taken before and up to 36 hours after administration of the [14C]midazolam microtracer or microdose. The time points for blood sampling were based on the PK of midazolam in paediatric ICU patients where a median half-life of 5.5 hours was found [28]. To ensure complete sampling of a single dose, at least 5 times the half-life was taken. Moreover, to capture the distribution, metabolism and elimination phase, the sampling times were set on pre-dose, and 0.17, 0.5, 1, 2, 4, 6, 10, 24 and 36 hours post-IV dose. The maximum number of study specific blood samples was limited to 6 per subject. The specific time points for each patient were decided based on discussion between the research team, clinical team and parents to ensure cares were coordinated at this time and with minimal disruption to the patients’ routine. The maximum amount of blood could not exceed the guidelines by European Medicines Agency (up to 1% of calculated circulating blood volume).[29] The blood samples were centrifuged and plasma was stored at -80ºC until analysed.

## Radiopharmaceutical Preparation

[14C]midazolam was synthesized by Selcia Ltd, United Kingdom at a specific activity of 1072 MBq mmol-1 (equal to 2.95 MBq mg-1). The chemical name is 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-[1-14C] imidazo[l,5-a][l,4]benzodiazepine hydrochloride. In the Radiopharmacy Department, Addenbrookes Hospital, Cambridge, United Kingdom under aseptic conditions [14C]midazolam was brought in ethanol 96% solution, the activity was measured and the solution was further diluted 10 000 fold in 5% w/v dextrose solution to the required concentration. The final solution was filter sterilised (pore size 0.2 µm) and batched for intravenous injection. The final [14C]midazolam concentration was 500Bq mL-1.

## [14C]midazolam and [14C]1-hydroxy-midazolam plasma concentration analysis

### Plasma sample extraction and Ultra Performance Liquid Chromatography (UPLC) Separation

Methanol (10 µL) was added to plasma samples in order precipitate proteins and to extract the test substance using protein precipitation plates. Each run consisted of samples measured once and eight calibrator levels in duplicate plus three different QC levels in duplicate. The extract was evaporated to dryness, re-dissolved and analysed using UPLC. The fraction where midazolam and 1-hydroxy-midazolam eluted from the column was collected for each sample, evaporated to dryness and subsequently analysed using Combustion-CO2-AMS. Fractions were transferred to a tin foil cup and evaporated to dryness prior to Accelerator Mass Spectrometry (AMS) analysis.

### Accelerator Mass Spectrometry analysis

[14C]levels were quantified as described before.[13,30] The UPLC and AMS qualification was performed in accordance with the recommendation of the European Bioanalytical Forum.[31] The tin foil cups (see 5.5.1) were combusted on an elemental analyser (Vario Micro; Elementar, Langenselbold, Germany). Generated CO2 was transferred to a home-built gas interface, composed of a zeolite trap and syringe.[30] CO2 was adsorbed to the trap on the interface; and after heating of the trap, the CO2 was transferred to a vacuum syringe using helium. A final CO2/helium mixture of 6% was directed to the AMS ion source, at a pressure of 1 bar and a flow of 60 µL min-1. A 1-MV Tandetron AMS (High Voltage Engineering Europe B.V., Amersfoort, The Netherlands)[32] was used. The lower limit of quantification (LLOQ) was 0.31 mBq mL-1.

## Patient characteristics

Patient characteristics (age, weight) and patient lab values (creatinine, total bilirubin, ASAT, ALAT) were described using standard statistics, and data was presented as median (range). Microtracer and microdosing groups were compared using Mann-Whitney test, as data were not distributed normally.

## Pharmacokinetic Analysis

### Exploration of the data

The data was first explored by visualization of time-concentration profiles of [14C]midazolam and [14C]1-hydroxy-midazolam (GraphPad Prism 5). Next, their area under the curve (AUC) and the ratio AUC [14C]1-hydroxy-midazolam/[14C]midazolam was estimated using a log-linear non-compartmental model (Excel PKSolver add-in software[33]) and compared between microdose and microtracer administration using Mann-Whitney U test.

### Nonlinear mixed effects modelling

[14C]midazolam concentration–time data were analysed using the nonlinear mixed effects modelling software NONMEM version 7.4 (ICON; Globomax LLC, Ellicott, MD). Model development was in four steps: (1) selection of a structural model, (2) selection of an error model, (3) covariate analysis, and (4) internal validation of the model.

For model selection, we used the objective function value (OFV) and standard goodness of fit plots. For the OFV, a drop of more than 3.84 points between nested models was considered statistically significant, which corresponds to p<0.05 assuming a chi-square distribution.[34,35] For the structural and error models, a decrease in OFV of 3.84 points was considered statistically significant (P<0.05). For the structural model, one, two and three compartment models were tested. Inclusion of log-normally distributed inter-individual variability (IIV) was tested on all model parameters. For the residual unexplained variability additive, proportional and a combination of additive and proportional error model were tested. The continuous covariates evaluated were postnatal age, postmenstrual age, bodyweight, creatinine, ALAT, ASAT, and total bilirubin. Categorical covariates included treatment arm (i.e. microdosing or microtracer administration) only. All covariates were tested on all model parameters. Potential covariates were evaluated using forward inclusion and backward elimination with a level of significance of less than 0.005 (ΔOFV< -7.9 points) and less than 0.001 (ΔOFV >10.8 points), respectively. In addition, inclusion of a covariate in the model had to result in a decline in unexplained IIV and/or improved goodness of fit plots before it was included in the final model.[36,37] Next, the model was internally validated using bootstrap analysis in Perl-speaks-NONMEM (PsN).

# Results

## Subjects and data

Fifteen infants (gestational age 39.4 [23.9-41.4] weeks, postnatal age 11.4 [0.6-49.1 weeks]) were included in the study of which nine received a microdose and six a microtracer [14C]midazolam. See Table 1 for the patient characteristics. There were no significant differences found between characteristics of the microdose and microtracer group.

The complete dataset included data on 67 blood samples. Eight measurements had [14C]midazolam concentrations under the AMS detection limit and were not included in the analysis.[38]

## Exploration of the data

The time-concentration profiles of [14C]midazolam and [14C]1-hydroxy-midazolam of the individual subjects are depicted in Figure 2 and 3. In Table 2 the individual AUCs and ratio AUC0-t [14C]1-hydroxy-midazolam/[14C]midazolam of the microdose and microtracer are presented. There were no significant differences found between the two groups.

## Nonlinear mixed effects modeling

A two-compartment model described the PK of [14C]midazolam best. Inclusion of IIV for clearance improved the model statistically significantly. A combined error model was superior over a proportional error model or an additive error model.

Bodyweight was a significant predictor for the central volume of distribution and was therefore included in the model. After inclusion of bodyweight, age and other tested covariates were not found to be statistically significant. There was a trend for a relation between bodyweight and clearance, but this did not reach statistical significance (∆OFV -4.38).

Inclusion of the covariate 'treatment' (e.g. microtracer or microdose) upon inclusion on any of the PK parameters was found to not statistically significantly influence the model fit (∆OFV >0.01).

The PK parameter estimates of the final model and the bootstrap results are presented in Table 3. Most RSE values of the parameter estimates are well below 50%, suggesting that the estimates are precise. Mean bootstrap values are close to model estimates and 0 is not in the 95% bootstrap interval, meaning the model is robust. Figure 4 shows the diagnostic plots for the final model and illustrates the predictive value of the model for both the microtracer and microdose group. The figure shows no bias, suggesting that concentrations for both the microdose and the microtracer are accurately predicted by this model, supporting dose-linearity of the microdose.

# Discussion

Our study shows dose-linearity of the PK of a [14C]midazolam microdose to the therapeutic dose in children, by the finding that none of the PK parameters of midazolam were influenced by the treatment group, i.e. microdose or microtracer [14C]midazolam. A lack of difference in AUC values for [14C]midazolam and [14C]1-hydroxy-midazolam further supports that there is no difference between the PK of a microtracer and microdose.

These results are in line with the findings in adults (n=6), where dose-linearity of a 100 µg [14C]midazolam microdose was assessed in a cross-over design with 3 treatment regimens.[14] The subjects were administered (1) an oral microdose, (2) an IV microdose and (3) a simultaneous dose of an IV microtracer with a therapeutic nonradiolabeled oral dose. Like our results, no difference in IV disposition of midazolam was found when given as a microdose alone or in presence of a therapeutic dose in children.

Previously, studies have reported the midazolam PK in paediatrics after a single IV administration.[39-41] Clearance in our study was found to be 2.06 L h-1 for an infant of 4 kg (equal to 8.6 ml kg-1 min-1). In preterm infants the clearance was reported to be lower (median 1.8 [range 0.7-6.7] ml kg-1 min-1)[39] reflecting that CYP3A activity is less mature in preterm infants than in an infant of 4 kg. A study with critically ill children reported a clearance of 1.11 L h-1 for an infant of 5 kg (equal to 3.7 ml kg-1 min-1)[42], which is lower than in our population. This paper concludes that inflammation (reflected by high C-reactive protein concentrations) and/or number of failing organs influenced midazolam clearance, possibly as a result of reduced CYP3A activity.[42] The lower clearance can likely be explained by the fact that this study included patients with a higher inflammation-state and/or more failing organs, as subjects in the current study were only eligible when renal- or hepatic failure was absent. This is further evidenced by two studies investigating a 0.15 mg kg-1 dose in healthy children, where clearance was found to be similar (3-10 year old, clearance mean±SD 9.11±1.21 ml kg-1 min-1 [41]) as or slightly higher (0.5-2 year, clearance 11.3±6.3 ml kg-1 min-1 [40]) than in our population.

Regulatory authorities have indicated that microdose studies with radioactive labelled compounds are an acceptable component of drug development.[7,43] Yet, to the best of our knowledge this approach has not been used during paediatric drug development, despite this study and previous other studies illustrating feasibility and ethical acceptance in that population.[11-13] For paracetamol the dose-linearity of an oral and IV microdose was successfully assessed in paediatrics.[12] A slightly different approach was taken to study developmental changes in oral disposition of paracetamol and metabolites when an oral microtracer of [14C]paracetamol was administered together with a therapeutic dose of IV paracetamol.[11,13] The known developmental change from mainly sulfation to glucuronidation was confirmed, and data were added on intestinal and hepatic metabolism of paracetamol in a large paediatric age range. Together with our study, these studies pave the way for microdose studies to be incorporated into paediatric drug development plans to explore PK in this vulnerable population.

This study is limited by the lack of information on the severity of disease and inflammation in these patients and by the wide age range in which extensive development in drug metabolism and transport occurs. The effect of age and disease on CYP3A activity increased the variability in PK of midazolam, possibly obscuring a difference between the PK of a microtracer and a microdose. However, we showed the age range was comparable in both treatment groups, and we assumed the disease severity was similar in the two groups. Another limitation is that the sample size is relatively small. Nevertheless, PK parameters between a microdose and a microtracer were similar and compared with literature values. Moreover, in adults low sample sizes were used to show dose-linearity of midazolam.[14]

A future perspective more specific to this particular study, is that the results indicate that a [14C]midazolam microdose can be used as an alternative to a midazolam therapeutic dose to study CYP3A activity in children. In the case of taking that approach, an attempt can be made in extrapolating the results to other CYP3A-substrates and predict their disposition using a physiology based pharmacokinetic (PBPK) modelling approach. Importantly, whether this may be possible will depend on the characteristics of these substrates, as described by Calvier et al.[44] As a substantial number of clinically relevant drugs used in children are metabolized by CYP3A[16], this has the potential to impact the efficacy and safety of drug dosing in paediatrics through more informed adaptations of dosing regimens to this population.

We conclude that the PK parameters of [14C]midazolam administered as a microdose did not differ significantly in infants from that of a microtracer. This supports the dose-linearity of an IV [14C]midazolam microdose in children, thus a [14C]midazolam microdosing approach as an alternative to a therapeutic midazolam dose can be used to study developmental changes in hepatic CYP3A activity.

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# Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**viii. References**

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**ix. Tables**

Table 1: Characteristics of patients that participated in the study and received a microdose or microtracer [14C]midazolam. Data is presented as median (range).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Total** | **Microdose** | **Microtracer** | **Mann Whitney U** **microdose vs microtracer group (p-value)** |
| Number of patients | 15 | 9 | 6 | - |
| Number of samples | 67 | 37 | 30 | - |
| Samples per patient (n) | 5 (2-5) | 5 (2-5) | 5 (5-5) | - |
| Gestational age (weeks) | 39.4 (23.9-41.4) | 39.4 (23.9-41.4) | 38.4 (26.7-41.0) | 0.15 |
| Postnatal age (weeks) | 11.4 (0.6-49.1) | 11.4 (0.6-49.1) | 13.4 (2.6-42.3) | 0.39 |
| Weight (kg) | 3.6 (2.6-8.9) | 3.5 (2.7-8.9) | 3.8 (2.6-6.0) | 1.00 |
| Plasma creatinine (µmol L-1) | 35 (20-51) | 41 (29-51) | 33 (20-36) | 0.07 |
| Total bilirubin (µmol L-1) | 9 (2-274) | 9 (5-274) | 9 (2-146) | 0.46 |
| ASAT (U L-1) | 42 (12-93) | 41 (12-93) | 57 (25-85) | 0.39 |
| ALAT (U L-1) | 17 (7-68) | 15 (7-43) | 23 (16-68) | 0.09 |

Table 2: Area under the curve (AUC) of [14C]midazolam and [14C]1-hydroxy-midazolam after administration of a microdose or microtracer [14C]midazolam presented as median (range). afor one subject this parameter could not be established as there were only 2 plasma samples available. bAUC0-t  ratio=[14C]1-hydroxy-midazolam AUC0-t/[14C]midazolam AUC0-t

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Total (n=15)** | **Microdose (n=9)** | **Microtracer (n=6)** | **Mann Whitney U microdose vs microtracer group(p-value)** |
| **[14C]midazolam** |
| AUC0-t (ng L-1 \*h) | 46.77 (32.42 – 196.77) | 46.77 (32.42 – 196.77) | 48.28 (39.17 – 81.40)  | 0.86 |
| AUC0-∞ (ng L-1 \*h) | 48.90 (34.15 – 218.80)(n=14a) | 48.90 (34.15 – 218.80)(n=8a) | 49.11 (39.75 – 82.45) | 0.66 |
| **[14C]1-hydroxy-midazolam** |
| AUC0-t (ng L-1 \*h) | 10.89 (5.28 – 24.21) | 10.19 (5.28 – 24.21) | 11.20 (5.84 – 19.93) | 0.86 |
| AUC0-∞ (ng L-1 \*h) | 12.39 (5.99 – 26.41)(n=14a) | 13.14 (7.40 – 26.41)(n=8†) | 12.39 (5.99 – 26.27)  | 0.95 |
| **[14C]1-hydroxy-midazolam / [14C]midazolam** |
| AUC0-t ratiob | 0.23 (0.11-0.51) | 0.23 (0.11-0.49) | 0.21 (0.13-0.51) | 0.69 |

Table 3: Parameter estimates of the pharmacokinetic model for IV [14C]midazolam.

|  |  |  |
| --- | --- | --- |
| **Parameter** | **Estimate (RSE%)** | **Bootstrap median (2.5th to 97.5th bootstrap percentile)** |
| **Clearance** |
| CL (L h-1) | 2.06 (24) | 2.23 (1.57-3.23) |
| **Inter-compartmental clearance** |
| Q (L h-1) | 0.79 (44) | 0.90 (0.60-2.45) |
| **Volume of distribution** |
| V1i = V14kg \* (WT/4)k1 |  |
| * V14kg (L)
 | 3.81 (8) | 3.75 (3.07-4.66) |
| * k1
 | 1.36 (10) | 1.34 (0.68-1.68) |
| V2 (L) | 3.19 (18) | 3.30 (2.64-6.41) |
| **Inter-individual variability** |
| ω2 CL | 0.73 (42) | 0.62 (0.13-1.41) |
| **Residual error** |
| Proportional error | 0.09 (24) | 0.08 (0.05-0.14) |
| Additional error | 0.08 (50) | 0.07 (0.01-0.20) |

*Definition of abbreviations:* CL= population predicted clearance; Q= intercompartmental clearance; V1i = individual predicted volume of distribution in the central compartment for individual i; V14kg = population value for volume of distribution in the central compartment at 4 kg; WT= body weight; k1 = exponent to relate body weight to volume of distribution; V2 = volume of distribution in the peripheral compartment; ω2 = variance for the inter-individual variability of the parameter mentioned. The bootstrap was based on 50 resampled datasets.

**x. Figure legends**

Figure 1: Explanation of the terms IV ‘microdose’ and ‘microtracer’ midazolam

Figure 2: Individual (n=9) semilog plasma concentration-time profiles of [14C]midazolam and [14C]1-hydroxy-midazolam after administration of a [14C]midazolam microdose

Figure 3: Individual (n=6) semilog plasma concentration-time profiles of [14C]midazolam and [14C]1-hydroxy-midazolam after administration of a [14C]midazolam microtracer

Figure 4: Diagnostic plots for [14C]midazolam pharmacokinetic model, using different symbols for the different treatments. (A) Observed versus population predicted [14C]midazolam concentrations. (B) Observed versus individually predicted [14C]midazolam concentrations. (C) Weighted residuals versus population predicted [14C]midazolam concentration. (D) Weighted residuals versus time. Solid lines represent the line of unity in A and B, and a value of 0 in C and D. Dotted line represent ±1.96 standard deviation, representing the interval in which 95% of the CWRES values are expected.

**xi. Word count**

|  |  |
| --- | --- |
| **Part of manuscript** | **Wordcount** |
| Introduction | 580 |
| Methods | 1179 |
| Results | 361 |
| Discussion | 832 |
| *Total* | *2952* |