**Comparison of dried blood spots versus conventional plasma collection for the characterisation of efavirenz pharmacokinetics in a large-scale global clinical trial - The ENCORE1 study**

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**Short title: efavirenz pharmacokinetics in DBS**

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**Abstract**

**Background:** The aim of this study was to determine the utility of dried blood spots (DBS) compared with conventional plasma collection methods for characterisation of efavirenz pharmacokinetics, in the setting of a large-scale, global clinical trial (ENCORE1).

**Methods:** 630 patients were recruited from 38 sites and had single matched whole blood DBS and plasma samples (mid-dose interval) taken at weeks 4 and 12 of treatment. In addition, a sub-group of patients underwent intensive DBS and plasma sampling (0-24 hours) to provide full profile data for pharmacokinetic parameters. Efavirenz concentrations were determined by validated HPLC-MS/MS methods. A DBS-predicted plasma concentration was derived and linear regression and Bland-Altman plots were used to compare DBS-predicted plasma concentrations with that of measured plasma concentrations.

**Results:** Efavirenz DBS and plasma concentrations were significantly correlated (R2=0.904, P<0.001; n=1094), and DBS concentrations were, on average, 53% ±9.5% lower than plasma. In the main study the DBS-predicted plasma values significantly underestimated the true measured concentration of efavirenz in plasma; the mean difference (95% confidence interval; CI) between efavirenz DBS-predicted concentrations and measured plasma concentrations was -0.451 mg/L (-0.504 to -0.398) at week 4 (n=561). However, in the intensive study the mean difference was only 0.086 mg/L (-0.006 to 0.178) at 12 hours post-dose (n=46), and was not statistically significant.

**Conclusions:** Our data show a high correlation between measurements of efavirenz concentrations in plasma and in DBS. However, DBS concentrations significantly underestimated the true measured plasma concentrations in the sparse samples taken in this large multinational ENCORE1 trial.

**Keywords: dried blood spots, efavirenz, pharmacokinetics, ENCORE1**

**Introduction**

Analysis of plasma concentrations continues to be the method of choice for the characterisation of antiretroviral pharmacokinetics (PK). However, plasma collection requires specialised training and laboratory facilities, including cold storage and transport, which incur significant costs when implementing large scale, multi-centre clinical trials, particularly those conducted in resource-limited environments.

Dried blood spots (DBS) offer a number of practical and cost-saving benefits as compared with conventional sampling; they are easier to collect, with no need for centrifugation and separation of samples, and can be shipped and stored at ambient temperatures. This is especially important, doing away with maintaining costly cold-storage during the transportation of samples. DBS also require minimal blood volumes and pose less of an infection risk to clinical and laboratory personnel.

Quantification of antiretrovirals from DBS is by no means a novel approach; a number of analytical methods using liquid chromatography with mass spectrometry (LC-MS) or UV detection have been reported [[1-5](#_ENREF_1)]. Furthermore, small-scale clinical studies have investigated the suitability of DBS sampling for therapeutic drug monitoring and PK studies [1, 6].

However, DBS sampling is less frequently applied as a quantitative measure in prospective randomised clinical trials (RCT) [[7](#_ENREF_6)]. The Food and Drug Administration (FDA) and European Medicines Agency (EMA) regulatory bodies have not issued definitive guidance on the application of DBS as a standalone method in RCT, and their extensive guidance on liquid-matrices, such as plasma or serum, cannot be applied interchangeably to dried matrices. The 2013 updated guidance by the FDA stated that, whilst they encourage the development of new technologies, for the purpose of new drug applications and regulatory submissions, data supplied from “new technologies” should be supported by established methods [[8](#_ENREF_7)].

Our objective was to determine the suitability of DBS versus conventional plasma collection methods for measuring efavirenz concentrations and thereby characterising the PK of the drug, in the setting of a large-scale, multinational clinical trial. ENCORE1 was a multi-centre, randomised, double-blind, placebo-controlled trial comparing the safety and efficacy of a reduced 400 mg once daily dose of efavirenz in combination with tenofovir/emtricitabine (Truvada, 300/200 mg), with the standard 600 mg once daily dose (with tenofovir/emtricitabine) in antiretroviral-naïve patients [[9](#_ENREF_8), 10].

**Materials and Methods**

Patients were recruited at 38 sites in 13 countries (Argentina, Australia, Chile, Germany, Hong Kong, Israel, Malaysia, Mexico, Nigeria, Singapore, South Africa, Thailand and United Kingdom - ClinicalTrials.gov number NCT01011413) [[9](#_ENREF_8)]. Ethical and regulatory approval and written informed consent were obtained.

Steady-state efavirenz concentrations were measured in plasma and DBS samples taken from the same blood tube at various time points over the study. All patients had single mid-dose interval (between 8 and 16 hours post-dose) blood samples taken at week 4 and 12 of treatment. A sub-group of patients at 4 sites underwent additional intensive PK sampling at pre-dose and 2, 4, 8, 12, 16 and 24 hours post‐dose, between weeks 4 and 8 of the study.

Whole venous blood was collected in EDTA tubes and five 50 µL spots were pipetted onto Whatman 903 Protein Saver cards (GE Healthcare, Little Chalfont, Buckinghamshire, UK). DBS were air dried at room temperature and stored with desiccant sachets and a humidity indicator in individual gas impermeable ziplock bags at 4°C until shipping by post (main study) and at ambient temperature by courier (Intensive PK sub-study) to a central laboratory. The remaining blood was used for preparation of plasma and stored at -20°C until shipping to a central laboratory on dry ice.

Efavirenz concentrations in plasma and DBS were determined by validated LC-MS methods. The lower and upper limits of quantification (LLQ, ULQ) were 0.025 and 10 mg/L in plasma, and 0.025 and 5 mg/L in DBS, respectively. Plasma samples were extracted using protein precipitation; and for DBS, the whole spot was punched and extracted using a liquid-liquid extraction procedure [[11](#_ENREF_9)].

The relationship between efavirenz concentrations in DBS and plasma was explored, both in the main study population and the cohort undergoing intensive PK sampling. A predicted plasma concentration (PlasmaPRED) was derived using the formula (DBS**M**/(1-HCT))×0.995, where DBS**M** is the measured efavirenz concentration in DBS, HCT is a standardised haematocrit of 0.45 L/L and 0.995 is the fraction of efavirenz bound to plasma proteins [[12](#_ENREF_10)]. PK parameters, area under the concentration-time curve over 24 hours (AUC24), maximum concentrations (Cmax) and trough concentrations at 24 hours (C24) were calculated using non-compartmental methods (WinNonlin Phoenix, version 6.1; Pharsight, Mountain View, CA, USA). Differences between DBS, DBS-predicted plasma, measured plasma concentrations and PK parameters (intensive sub-study only) were assessed by use of a Wilcoxon signed rank test (Statsdirect version 3.0; StatsDirect Ltd, Cheshire, UK). Linear regression and Bland-Altman plots (SigmaPlot 12, Systat Software Inc) were used to compare DBS predicted plasma concentrations with that of measured plasma concentrations. Bias between the two sampling approaches is indicated by the Bland Altman plots if the 95% confidence intervals of the mean difference do not include 0.

**Results**

A total of 1094 paired mid-dose interval plasma and DBS samples were collected at weeks 4 (n=561) and 12 (n=533), from 38 sites in the main study. Of these, 579 samples were from patients receiving the 400 mg dose and 515 from those receiving the 600 mg dose; 348 (32%) samples were collected from females. An additional 320 paired plasma and DBS samples were available from 46 patients (28 on 400mg, 18 on 600mg; 15 female) who underwent intensive PK sampling at 4 of the participating sites.

DBS efavirenz concentrations showed a significant correlation with that of plasma (R2=0.904, P<0.001; n=1094), with DBS concentrations on average (±SD), 53% ±9.5% lower (P < 0.0001). In the intensive cohort, efavirenz concentrations in DBS were on average 41% ±12% lower than corresponding plasma concentrations during the mid-dosing interval (12-16 hours post-dose) and 42% ±9% lower at 24 hours post-dose (R2= 0.953, P < 0.0001; n=320). Furthermore, efavirenz DBS AUC24, Cmax and C24 were significantly (42%, 44% and 43%; P<0.0001) lower than the PK parameters derived from plasma; however DBS-predicted PK values were not significantly different from that of measured plasma with the exception of the AUC24 P=0.004 (as shown in Table 1).

The linear relationship between the DBS-predicted efavirenz plasma concentrations and measured plasma concentrations, for both the main study (mid-dose interval samples at week 4, n=561 and week 12, n=533) and intensive sub-study (12 hours post-dose; n=46) are shown in Figure 1. In the main study, there was little deviation between the DBS-predicted and measured plasma values at week 4 (R2=0.895; y = 0.851x – 0.0243; n=561) and week 12 (R2=0.913; y = 0.894x – 0.1209; n=533). In the intensive study, the linear relationship between DBS predicted and measured plasma values was improved and closer to the line of true identity at 12 hours (R2=0.954; y = 0.932x + 0.2229; n=46) and 16 hours (R2=0.960; y = 0.991x + 0.0916; n=46) post-dose.

Figure 2 shows the Bland Altman plots for the mid-dose and intensive PK samples. The mean difference (95% confidence interval; CI) between efavirenz DBS-predicted plasma concentrations and measured plasma concentrations was -0.451 mg/L (-0.504 to -0.398) and -0.431 mg/L (-0.484 to -0.379) at week 4 (Figure 2a) and week 12 (Figure 2b), respectively. Hence, in the main study cohort the DBS-predicted values significantly underestimated the true measured concentration of efavirenz in plasma. The intensive study samples showed no bias (95% CI included 0). The mean difference was not statistically significant; only 0.086 mg/L (-0.006 to 0.178) at 12 hours post-dose (n=46) and 0.043 mg/L (-0.004 to 0.081) for all time points (n=320) over 24 hours post-dose (Figure 2c).

**Discussion**

DBS represent a valuable sampling tool for use in well-controlled PK studies and we present here paired DBS/plasma data from the largest HIV antiretroviral DBS study. However, the amplification of this methodology from a relatively small scale (4 sites) to a larger scale (38 sites) multicentre trial across diverse health resource settings resulted in significant underestimation of efavirenz concentrations in the sparse population samples.

At the initiation of the study, laboratory staff were trained regarding the standard procedures around preparation, storage and shipping of DBS using a manual and video reference. The current findings may result from inter-operator differences in pipetting accuracy and spotting technique at the various sites. If the blood sample is not thoroughly mixed prior to spotting, or if the whole spot is processed and inconsistent volumes are applied to the filter card, the drug concentration will be affected. Such limitations must be addressed before widespread utilisation of DBS can be recommended in a global field setting.

DBS sub-punches of a pre-defined diameter (3-6 mm) may help to normalise sample-to-sample variations in DBS volume [[11](#_ENREF_9)]. Taking a sub-punch is advantageous as there is less need for study personnel to apply an accurate volume to the card so the technique is less reliant on operator skill. This technique has already been implemented successfully in PK studies investigating antiretroviral exposures in DBS [[13](#_ENREF_11)] and in dried breast milk spots [[14](#_ENREF_12)].

The duration of the samples in transit may be important, given that the main study DBS samples were sent by regular mail, whereas the intensive DBS samples were packaged and dispatched via courier, both at ambient temperature. However, ongoing stability studies from our laboratory have shown that efavirenz remains stable in DBS when stored at room temperature (20°C) for up to 18 months [[11](#_ENREF_9)]. Furthermore, efavirenz stability (up to 1 month) has been demonstrated following exposure of DBS to the extreme temperatures (37°C and 45°C) likely to be encountered in the various sites of a global clinical trial and during the shipping process [[1](#_ENREF_1)].

A limitation of this study is that we did not correct for patient specific haematocrits or investigate differences in binding of the drug to plasma proteins and erythrocytes. Nonetheless, the data from the intensive PK study, conducted in 46 patients, are comparable with that already presented in the literature. Kromdijk *et al* reported a mean difference between the DBS-predicted efavirenz plasma concentration and measured plasma concentration of 0.080 mg/L when adjusted using a patient-specific haematocrit; and 0.170 mg/L when adjusted using a standardised haematocrit for males [[15](#_ENREF_13)].

**Conclusion**

In conclusion, measurements of efavirenz concentrations in plasma and DBS were highly correlated. However, DBS concentrations (after correction for haematocrit and plasma protein binding) significantly underestimated the true measured plasma concentrations in the sparse samples taken in this large multinational ENCORE1 trial. Hence, widespread utilisation of DBS as a stand-alone method in large-scale clinical trials may have to be judged on a case-by-case basis, considering factors such sample integrity, provision of on-site resources, and staff training and expertise.

**Transparency Declarations**

Saye Khoo and David Back have received research grants and travel bursaries from Gilead, Viiv, Merck, Bristol Myers Squibb, Boehringer Ingelheim and Janssen Pharmaceuticals. David Back has board membership and consultancy with Viiv, Merck and Janssen Pharmaceuticals. Sean Emery has received research grants from the Bill & Melinda Gates Foundation. Laura Else has received travel bursaries from Janssen Pharmaceuticals. All other authors: none to declare.

**References**

1. Hoffman JT, Rossi SS, Espina-Quinto R et al. Determination of efavirenz in human dried blood spots by reversed-phase high-performance liquid chromatography with UV detection. *Ther Drug Monit.* 2013; 35: 203-208.

2. Ter Heine R, Hillebrand MJ, Rosing H et al. Quantification of the HIV-integrase inhibitor raltegravir and detection of its main metabolite in human plasma, dried blood spots and peripheral blood mononuclear cell lysate by means of high-performance liquid chromatography tandem mass spectrometry. *J Pharm Biomed Anal.* 2009; 49: 451-458.

3. Meesters RJ, van Kampen JJ, Reedijk ML et al. Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. *Anal Bioanal Chem.* 2010; 398: 319-328.

4. Koal T, Burhenne H, Romling R et al. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom.* 2005; 19: 2995-3001.

5. Calcagno A, Motta I, Milia MG et al. Dried plasma/blood spots for monitoring antiretroviral treatment efficacy and pharmacokinetics: a cross-sectional study in rural Burundi. *B Journ Clin Pharm.* 2015; 79: 801-808.

6. Ter Heine R, Mulder JW, van Gorp EC et al. Clinical evaluation of the determination of plasma concentrations of darunavir, etravirine, raltegravir and ritonavir in dried blood spot samples. *Bioanalysis* 2011; 3: 1093-1097.

7. Spooner N, Lad R, Barfield M. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. *Anal Chem.* 2009; 81: 1557-1563.

8. US Department of Health and Human Services FDA, Center for Drug Evaluation and Research (CDER), Center for Veterinary medicine (CVM*). Guidance for Industry: Bioanalytical Method Validation*. Draft Guidance.September 2013. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf (Last accessed 11 August 2017).

9. Group ES, Puls R, Amin J et al. Efficacy of 400 mg efavirenz versus standard 600 mg dose in HIV-infected, antiretroviral-naive adults (ENCORE1): a randomised, double-blind, placebo-controlled, non-inferiority trial. *Lancet.* 2014; 383: 1474-1482.

10. Dickinson L, Amin J, Else L, et al. Pharmacokinetic and pharmacodynamic comparison of once-daily efavirenz (400 mg vs. 600 mg) in treatment-naïve HIV-infected patients: Results of the ENCORE1 study. *Clin Pharmacol Ther*. 2015; 98(4): 406-416.

11. Amara A, Else LJ, Tjia J et al. A validated method for quantification of efavirenz in dried blood spots (DBS) using HPLC-MS/MS. *Ther Drug Monit.* 2015; 37(2):220-228.

12. Boffito M, Back DJ, Blaschke TF et al. Protein binding in antiretroviral therapies. *AIDS Res Hum Retroviruses.* 2003; 19: 825-835.

13. Olagunju A, Bolaji O, Amara A et al. Pharmacogenetics of pregnancy-induced changes in efavirenz pharmacokinetics. *Clin Pharmacol Ther*. 2015; 97(3):298-306

14. Olagunju A, Bolaji OO, Amara A et al. Development, validation and clinical application of a novel method for the quantification of efavirenz in dried breast milk spots using LC-MS/MS. *J Antimicrob Chemother*. 2015; 70: 555-561.

15. Kromdijk W, Mulder JW, Rosing H et al. Use of dried blood spots for the determination of plasma concentrations of nevirapine and efavirenz. *J Antimicrob Chemother.* 2012; 67: 1211-1216.

**Figure legends**

**Figure 1.**

Dried blood spots (DBS)-predicted efavirenz concentrations plotted against measured plasma concentrations for mid-dosing interval samples from **a)** week 4 (R2=0.895; y = 0.851x – 0.0243; n=561 samples) and **b)** week 12 (R2=0.913; y = 0.894x – 0.1209; n=533 samples), and **c)** the 12 hour samples from the intensive cohort (R2=0.954; y = 0.932x + 0.2229; n=46 samples)

The solid black line represents the regression line and the dashed lines indicate the 95% confidence intervals. The dotted line indicates the line of true identity

**Figure 2.**

Bland Altman plots of the mid-dose samples at a) week 4 (n=561 samples) and b) week 12 (n=533 samples) of the main study, and c) the intensive pharmacokinetic samples over a 24 hour dosing interval (n=320 samples; 46 subjects)

The solid line indicates the mean difference and the dashed lines the 95% limits of agreement (mean difference ± standard deviation)

DBS = dried blood spots

**Table 1. Efavirenz pharmacokinetic parameters from measured plasma, dried blood spots (DBS) and DBS-predicted plasma. Data are presented as geometric mean with 95% CI.**

|  |  |  |  |
| --- | --- | --- | --- |
| **N=46** | **PlasmaM** | **DBSM** | **PlasmaPRED** |
| AUC24(ng.h/mL) | 43666(41245, 60913) | 25344a (19352,38732) | 45849b(43220, 61859) |
| Cmax(ng/mL) | 3141(3003, 4049) | 1770a(1471, 2411) | 3201(3060, 3964) |
| C24(ng/mL) | 1263 (1204, 1946) | 720a(512, 1250) | 1303(1238, 1948) |

**PlasmaM =** measured plasma concentrations, **DBSM =** measured DBS concentrations, **PlasmaPRED =** DBS-predicted plasma (derived using formula DBS**M**/(1-HCT)x0.995).

**AUC24** = area under the concentration-time curve over 24 hours

**Cmax** = maximum analyte concentrations

**C24** = trough concentrations at 24 hours post-dose

**a** P<0.0001 compared with plasmaM

**b** P=0.004 compared with plasmaM