Development And Validation Of A LC-MS/MS Assay For The Quantification Of Efavirenz In Different Biological Matrices

Abstract

Background: The non-nucleoside reverse transcriptase inhibitor efavirenz is one of the most prescribed antiretroviral therapeutics. Efavirenz containing therapy has become associated with the occurrence of central nervous system side effects, including sleep disturbances, depression and even psychosis.

Results: The investigation of efavirenz distribution required the development of a versatile and sensitive method. In addition to plasma, quantification was required in brain tissue and phosphate buffered saline. The assay presented here presented here was linear from 1.9ng/mL to 500ng/mL. Accuracy and precision ranged between 93.7% and 99.5%, 1.5% and 5.6%, respectively.

Discussion: The method developed here represents a versatile, sensitive and easy to use assay. The assay has been applied to *in vitro* and *in vivo* samples demonstrating reliable efavirenz quantification in multiple matrices.

Key words: efavirenz, LC-MS/MS, plasma, brain tissue, protein binding, brain tissue

1.1 Introduction

Drug penetration into the central nervous system is an important factor influencing therapy efficacy and side effects in numerous diseases areas. This is a particularly relevant for the treatment of HIV, where the CNS represents a sanctuary site for the viral replication as well as a potential site for toxicity [1-3]. Efavirenz is a key antiretroviral and displays many desirable pharmacokinetic properties such as a long half-life allowing once daily dosing and potency against HIV [4]. Despite these favourable properties efavirenz-containing therapy is associated with the development of central nervous system (CNS) toxicities. There is a paucity of information describing the distribution and characterisation of drugs in the CNS [3]. We have validated a rapid, versatile and sensitive liquid chromatography tandem mass spectrometer (LC-MS/MS) for the detection of efavirenz in different matrices to investigate efavirenz distribution in to the CNS.

The assay presented here was developed and validated in accordance with Food and Drug Administration (FDA) guidelines, assessing fundamental parameters including accuracy, precision and sensitivity [5]. Criteria such as linearity, accuracy (the degree of variation from known value, assessed by controls [QCs]), precision (the degree of variation within repeated measurements), selectivity (ensuring detection of the analyte and not an endogenous compound within the sample matrix) and recovery (determining the percentage of recovery and more importantly the reproducibility of the extraction process) were all assessed. The FDA guidelines also recommend a stability study be carried out. However or the purposes of this study a stability was deemed unnecessary as efavirenz has been in use for over 15 years and has been the subject of multiple studies. The stability of efavirenz at a variety of temperatures and matrices has previously been demonstrated [6-8].

Efavirenz was first licensed for the treatment of HIV in 1998, since then multiple methods for detection in plasma have been developed for LC-MS/MS. Many of the methods developed have been utilised to assess association with efavirenz plasma concentrations and CNS toxicity or polymorphisms in key proteins influencing efavirenz disposition [9, 10]. Some recently published methods show linearity with lower limit of quantification ranging from 20ng/mL to 300pg/mL [11, 12].

Despite the sensitivity and specificity of analysis LC-MS/MS, matrix effect is a well documented source of major concern [13]. Matrix effect may impact on various stages of the analytical process, such as ionisation of the analyte (either suppression or enhancement of ionisation) and extraction efficiency [5, 13]. Given the influence of the matrix on the quantification of an analyte, a change in matrix may have detrimental effects on the reliability of the assay. The method presented here was developed for robust quantification of efavirenz in multiple matrices (plasma, brain tissue homogenate and phosphate buffered saline [PBS]). The majority of published methods describe quantification of efavirenz in a single matrix [6, 8]. The greatest advantage of the assay developed here is robust quantification of efavirenz in multiple matrices, with minimal impact of matrix effect. The versatility demonstrated here will allow assessment of efavirenz in *in vitro* and i*n vivo* samples.

To investigate CNS concentrations of efavirenz, multiple LC-MS/MS methods have been developed to analyse efavirenz concentrations in CSF (cerebrospinal fluid) [14-16]. Although assessing CSF is a step towards understanding efavirenz concentrations in the CNS, CSF and brain tissue concentrations of drugs may vary widely and may not represent the disposition of efavirenz in the CNS [17, 18].

1.2 Methods and Materials

1.2.1 Materials

Efavirenz powder (>98% pure) was purchased from LGM Pharma inc (Boca Raton, USA). Lopinavir powder (>98% pure) was purchased from LGC Pharma (London, UK). All other consumables were purchased from Sigma Aldrich (Dorset, UK).

1.2.2 Tuning for Efavirenz and Internal Standard

Detection of efavirenz and internal standard (IS) lopinavir was conducted using a TSQ endura LC-MS/MS (Thermo scientific). Lopinavir was selected as IS due to similar log P (efavirenz 3.89, lopinavir 3.9) and has been shown previously not to interfere with efavirenz detection [4, 7, 19]. Tuning was performed using direct infusion (20µl/min) of a 500ng/mL stock of efavirenz with 50% mobile phase A (100% H_2O [LC-MS/MS grade] 5mM ammonium formate), 50% mobile phase B (100% acetonitrile [ACN] 5mM ammonium formate) at a flow rate of 300µl/min. Ionisation was achieved via heated electron spray ionization in negative mode. Although positive mode is more commonly used efavirenz is poorly detectable in positive mode [6, 8]. The following parameters were optimised to achieve the highest signal intensity for efavirenz: spray voltage, sheath gas and auxiliary gas. The IS was then directly injected (500ng/mL) to ensure detection using the optimised efavirenz settings. Following optimization for the parent mass of efavirenz (315) and IS (628), selected reaction monitoring (SRM) scan was utilised for detection of the break down products.

1.2.3 Chromatographic Separation

The chromatographic separation was achieved using a multi step gradient with a Hypersil gold C-18 column (Thermo scientific) (Table 1). The assay was conducted over 8 minutes at a flow rate of 300µl/min.

1.2.4 Extraction from Plasma and PBS

100 µl of sample (PBS was spiked with 20µl of ACN to aid efavirenz dissolution) was transferred to glass vials were 20µl of IS (2500ng/mL) were added to all standards, QC's and samples. Samples were diluted with ACN (sample: ACN ratio 1:4) and thoroughly vortexed. Samples were then centrifuged at 4000g for 10 minutes at 4°C. The supernatant fraction was transferred to a fresh glass vial and evaporated, samples were placed in a rotary vacuum centrifuge at 30°C and then reconstituted in 140µl of H_2O:ACN (60:40). 100µl of the sample was then transferred into 200µl chromatography vials. 5µl of each sample was injected for analysis.

1.2.5 Extraction from Brain Tissue

Rat brain tissue was homogenised in 3 volumes (W:V) of plasma. 100µl of brain tissue homogenate was then treated with ACN as detailed in the protein precipitation method detailed in the previous section.

1.2.6 Assay Validation

The assay was validated according to the most recent FDA guidelines [5]. The following criteria were assessed: linearity, recovery, specificity, accuracy, precision, inter-assay and intra-assay variability.

Linearity

A calibration curve of efavirenz was prepared in rat plasma via serial dilution, ranging from 1.9ng/mL to 500ng/mL. Extraction was performed using protein precipitation. Linearity was assessed by 3 independent preparations of the standard curve. Maximum allowed deviation of standards was set at 15% of the stated value, excluding the lower limit of quantification where deviation was set at no more than 20%.

Recovery

Recovery experiments were performed by comparing the results for extracted samples of efavirenz at three concentrations (20ng/mL, 100ng/mL and 400ng/mL) with non-extracted standards that were taken to represent 100% recovery.

Selectivity

The degree of interference from the matrix (due to potential interfering substances including endogenous matrix components, metabolites and decomposition products) was assessed via comparison of extracted blank samples with the lowest point of the

standard curve (lower limit of quantification). The lower limit of quantification was a minimum of 5 times greater than the background signal.

Accuracy and Precision

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte. Accuracy was assessed by preparation of three concentrations (in the range of the standard curve 20ng/mL, 100ng/mL and 400ng/mL) with each preparation in triplicate. The mean value of each concentration should be within 15% of the stated concentration (except the lower concentration, where deviation should be less than 20%**)** [5]. Accuracy was calculated using the following formula:

$$\%\ variability\ of accuracy = \frac{error}{stated\ value}\ x\ 100$$

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single volume of biological matrix. Precision of the assay was determined by preparation of three concentrations (in the range of the standard curve 20ng/mL, 100ng/mL and 400ng/mL) with each preparation in triplicate. The mean value of each concentration should be within 15% of the stated concentration (except the lower concentration, where deviation should be less than 20%**)**. Precision was calculated using the following formula:

$$\%\ variation\ of\ precision = \frac{standard\ deviation}{mean\ assay\ value}\ x\ 100$$

Accuracy and precision were assessed for intra and inter assay variability. The standard curve and QCs were prepared in triplicate and analysed 3 times. Variance in accuracy and precision should not vary within 15% of the stated concentration (except the lower concentration, where deviation should be less than 20%) within a single assay or between repetitions of the assay [5].

Animals and treatment

Male Wistar rats (Charles River UK) weighing 180 – 220 g on arrival were used for PK analysis of efavirenz. Food and water were provided *ad libitum*. Treated and untreated Wistar rats were sacrificed using an appropriate schedule 1 method (rising concentration of CO_2). Following termination brain was extracted and stored at -80˚C. All animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA), implemented by the United Kingdom Home Office.

1.2.7 Determination of Fraction Unbound of Efavirenz in Brain Homogenate

Rat brain tissue (obtained from untreated wistar rats) was homogenised in 2 volumes (W:V) of 1% saline solution. Since efavirenz is highly protein bound, a dilution of brain tissue (10% and 20% brain tissue were prepared with 1% PBS) was used. 200 µl of brain homogenate was spiked with 5000 ng mL^{-1} efavirenz and added to the donor chamber. The receiver chamber contained 350 µl of Sorensons buffer. The rapid equilibrium dialysis (RED) plate (Thermo, UK) was then placed in a shaking incubator for 4 hours at 37°C at 100 rpm. 250 µl were removed from the receiver chamber and frozen at -80°C for analysis. The fraction of drug unbound (fu) in brain tissue was then calculated from the diluted brain tissue using the following formula:

$$Undiluted\ fu = \frac{\left(\frac{1}{D}\right)}{\left[\frac{1}{fu(apparent)} - 1\right] + \left(\frac{1}{D}\right)}$$

1.2.8 Efavirenz Penetration into Rat Brain Tissue

Eight male Wistar rats (Charles River, UK) weighing 180-220g were dosed with efavirenz (10 mg/kg, 2 mL/kg 0.5% methylcellulose in dH_2O) based on individual weight taken prior to dosing. Dosing was administered once daily *via* oral gavage over 5 weeks. The animals were terminated 2 hours after the final dose and blood was collected *via* cardiac puncture. Blood samples were centrifuged at 2000g for 10 minutes at 4°C to separate plasma. Plasma was immediately frozen at -80°C and stored for later analysis. Brain tissue was also collected and following washing in phosphate buffered saline for 30 seconds 3 times, immediately stored at -30°C for analysis. All animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA), implemented by the United Kingdom Home Office.

1.2.9 Statistics

Data were assessed for normality using the Shapiro Wilk test. Statistical analysis was performed by Mann-Whitney U test and significance was defined as $P < 0.05$. All data are given as mean with standard deviation.

1.3 Results

1.3.1 Tuning Settings

The aim of optimising the tuning settings was firstly to maximise the detection of efavirenz and secondly to ensure detection of the IS. The optimised global settings were negative ion 2700 V, sheath gas 35, aux gas 15 and sweep gas 0.

In addition to detecting the parent molecule, the detection of the product ions of each compound was also optimised. By searching for both the parent and product ions, sensitivity and specificity are increased. This is particularly advantageous when analytes are contained in complex matrices such as plasma [20]. Table 2 shows the product ions produced during the selected reaction monitoring scan for efavirenz and IS.

1.3.2 Extraction Efficiency from Plasma, Brain Tissue and PBS

The recovery was measured at of the three QC concentrations (Figure 1). The mean recovery (across all 3 QCs) from plasma, brain tissue and PBS were 93% (standard deviation 2.9), 99% (standard deviation 4.49), and 95% (standard deviation 3.31), respectively. When recovery from to plasma was compared to recovery from brain tissue, there was a small but statistically significant difference in recovery at the low (92% vs 101 %, P = 0.001) and high (97% vs 101%QCs P = 0.04). When recovery from to plasma was compared to recovery from PBS, there was a small but statistically significant difference in recovery at the low QC (92% vs 99%, P = 0.007). Recovery at all other levels showed no statistically difference.

1.3.3 Assay Validation

Linearity

Standards extracted from plasma showed good linearity (R^2 = 0.9992). The peak area ratio (analyte to IS; variation of IS was less than 15% in each run) was proportional to the stated concentrations over the range of 500ng/mL to 1.9ng/mL. Figure 2 shows a representative calibration curve. Calibration curve was generated using a quadratic equation with a weighting of 1/X. Although a linear equation produced an acceptable R^2 (>0.99) the quadratic equation better described the relationship between signal response and standard concentration (R^2 >0.999). Both equations resulted in the assay passing (QC variability <15% at all levels).

Selectivity

The matrix effect of plasma was examined by comparing extracted blank plasma to extracted plasma spiked with 1.9ng/mL of efavirenz. Figure 3a shows the chromatogram produced by the extracted blank. There is a visible peak (area of 134) at the retention time of efavirenz (3.7 minutes). FDA guidelines require the lower limit of quantification produce a peak area of at least five fold greater than that observed in the blank matrix. Figure 3b shows the peak produced from the lower limit of quantification (1.9ng/mL). The peak area is 1491, which complies with FDA guidelines. Figure 3c shows the peak produced by the highest standard (500ng/mL). The bottom panels of figure 3 demonstrate the signal from the IS (retention time of 3.59 minutes) in extracted blank plasma (3a), the lower limit of quantification of

efavirenz (3b) and highest standard of efavirenz (3c). The signal produced by IS shows no interference with efavirenz.

Accuracy and Precision

The accuracy and precision for each individual run at 3 QC levels (low (20ng/mL), medium (100ng/mL) and high (400ng/mL) is shown in table 3. The percentage error of accuracy fell below 15% for each of the 3 repeats (1 varied between -0.25% and -11.45%, 2 varied between 0.01% and -6.32%, 3 varied between 0.78% and -4.66%). The percentage error of precision also fell below 15% for each of the 3 repeats (1 varied between -5.52% and 11.05%, 2 varied between 2.93% and 5.66%, 3 varied between 1.25% and 3.78%). The QC concentrations were selected based on the anticipated concentrations in the study samples. FDA guidelines recommend QC the low QC be within 3 fold of the lower limit of quantification, the medium QC near the center of the linear range and a high QC near the upper limit of quantification. Supplementary figure 1 shows accuracy and precision for each individual run with 3 QC levels conforming to FDA guidelines (low (5ng/mL), medium (200ng/mL) and high (400ng/mL). The percentage error of accuracy and precision fell below 15% for each of the 3 repeats.

Inter-assay Variability

The variability between assays was calculated to demonstrate that the assay maintained accuracy and precision across repetitions of the assay. Table 4 shows the variance of accuracy and precision calculated from the mean values of the 3

repetitions of the assay. The percentage error in accuracy fell below 15% across all 3 repeats (range between -0.52% and -6.34). Percentage variance of precision also fell below 15% across all 3 repeats (range between 1.48% and 5.61%). Supplementary figure 2 shows the variance of accuracy and precision calculated from the mean values of the 3 repetitions of the assay (for low QC 5ng/mL, medium QC 200 ng/mL and high QC 400 ng/mL). Percentage variance of accuracy and precision fell below 15% between all 3 repeats.

1.3.4 Partial Validation of Brain Tissue Homogenate and PBS

In order to assess the effect of changing matrix, QC's were prepared and extracted from brain tissue homogenate and PBS. The extracted samples were then quantified using a plasma standard curve. The accuracy and precision for each matrix was assessed at 3 QC levels (low (20ng/mL), medium (100ng/mL) and high (400ng/mL) is shown in table 5. The percentage error of accuracy fell below 15% for each matrix demonstrating reliable quantification.

1.3.5 Determination of Fraction Unbound of Efavirenz in Brain Homogenate

The data generated from the rapid equilibrium dialysis experiments demonstrated a high degree of protein binding in brain tissue. The mean (± standard deviation) concentration of free efavirenz detected in was 209.7 ± 33.4 ng/mL, and 165 ± 22.0 ng/mL, in 10% and 20% brain homogenate respectively. The protein binding in brain tissue homogenate was determined as 99.8% (10% homogenate) and 99.8% (20% homogenate). The average protein binding was 99.8%.

1.3.6 Efavirenz Penetration Into Brain Tissue

The median plasma and brain tissue concentrations of efavirenz are shown in figure 4. The median plasma concentration of efavirenz was 69.7 ng/mL (interquartile range [IQR] 44.9-130.6). The median concentration of efavirenz in brain tissue was approximately 10 fold higher, 702.9 ng/g (IQR 475.5-1018.0).

1.4 Discussion

The assay presented here represents a simple, robust and sensitive LC-MS/MS assay. In addition to accurate and precise quantification in plasma this assay has been shown to be versatile allowing quantification in brain tissue homogenate and PBS. The assay was fully validated in plasma. As the change in matrix represents a minor change to the assay only partial validation for the change of matrices was required, in accordance with guidelines [5].

Primary validation was conducted in plasma satisfying FDA bioanalytical method development guidelines, demonstrating good accuracy, precision and linearity. Although full validation for different matrices is not required, matrix effects must be assessed for each matrix. The change in matrix may potentially affect the behaviour of the assay significantly. Brain tissue homogenate and cell culture media both contain different quantities of protein compared to plasma. As efavirenz is highly protein bound (99% in plasma) and poorly water soluble (<10µg/mL), the change in matrix has the potential to alter efavirenz recovery [21, 22]. As the change in matrix is considered a minor change, partial validation was acceptable. Partial validation

317 required the determination of intra assay variability in accuracy and precision [23].
318 These data demonstrate the versatility and reliability of the assay presented here.

319 The sensitivity of the assay developed here is of a comparable standard to recent
320 publications. Some of the newer assays surpass the sensitivity here, 200pg/mL in
321 brain tissue and 300ng/mL in plasma [11, 24]. The greatest advantage of the assay
322 developed here is the ability to assess efavirenz in plasma, brain tissue and PBS. The
323 versatility of this assay demonstrates its suitability for application in the analysis of
324 *in vitro* and *in vivo* samples. The assay may be further adapted to analyse efavirenz
325 in additional matrices. It should also be noted that the assay was developed to assess
326 a range of concentrations not predicted to be lower than 10ng/mL. As the lower limit
327 of quantification (defined as >5x background) gave suitable sensitivity for the
328 anticipated concentrations in the study samples the limit of detection was not
329 established. The true limit of the assay is potentially much lower than the range
330 validated here.

331 The versatility of this assay has allowed for the quantification of efavirenz in
332 multiple matrices. The data generated show the protein binding of efavirenz to be
333 higher in brain tissue (99.8%) than either CSF (76%) or even plasma (99%) [25, 26].
334 The data generated *in vivo* shows efavirenz concentrations in brain tissue were
335 approximately 10 fold higher than those in plasma. These data indicate CSF
336 concentrations of efavirenz may underestimate exposure in the brain and warrant
337 further investigation.

338 One significant improvement would be to include the major metabolites of efavirenz,
339 8OH efavirenz and 7OH efavirenz. Recent publications have demonstrated, *in vitro*,
340 a higher cytotoxicity of 8OH efavirenz compared to the parent compound [27, 28].

LC-MS/MS methods have been developed to examine efavirenz and its metabolites in CSF [29]. The authors investigated dose reduction of efavirenz (600mg once daily to 400mg once daily) and demonstrated 8OH efavirenz concentrations in CSF did not appear to be dependant on plasma concentrations of efavirenz.

1.5 Conclusion

This assay detailed here describes the optimisation of a robust, simple and sensitive LC-MS/MS assay. The final assay conformed to FDA bioanalytical development guidelines and was capable of assessing efavirenz in multiple matrices. The application of this assay has been applied to investigate efavirenz distribution in the CNS.

1.6 Future Perspective

The assay presented here has been developed and validated for the detection of efavirenz in rat plasma, rat brain tissue homogenate and PBS. However, future studies may be able to build upon the work presented here. Although our study focused on samples taken from rats, there is the potential to further utilise the assay to analyse samples from other species, such as mice and humans. Other matrices of interest may also be investigated, in particular CSF in (rat and or human). To adapt this assay would require partial validation, to investigate the potential effects of a change in matrix. Recent publications have implicated the metabolites of efavirenz in the development of CNS toxicity. The assay presented here could be further

363 modified to quantify not only efavirenz but also its major metabolites. This would
364 allow future investigations to fully explore efavirenz penetration into the CNS.

365

366 **1.7 Author disclosure**

367 Andrew Owen has received research funding from Merck, Pfizer and AstraZeneca,
368 consultancy from Merck and Norgine, and is a co-inventor of patents relating to HIV
369 nanomedicines. Marco Siccardi has received research funding from ViiV and
370 Janssen.

371

372 **1.8 References**

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Figures and Tables

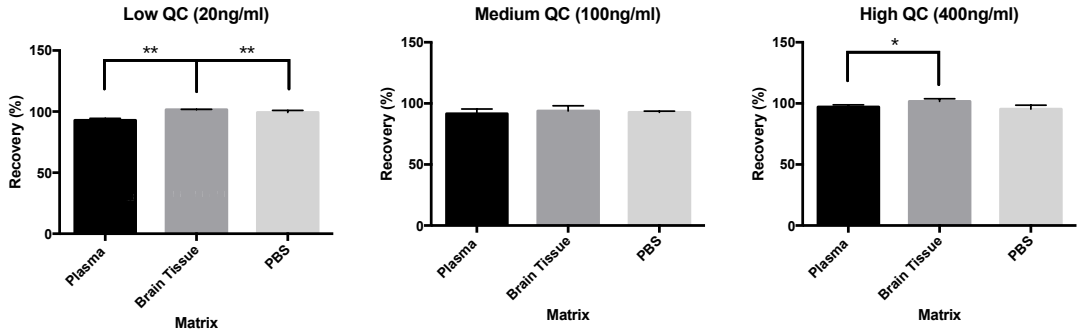


Figure 1

Efavirenz recovery.

Figure 1 shows the percentage recovery for the low (a), medium (b) and high (c) QCs in extracted plasma, extracted brain tissue and PBS. Data is show percentage of unextracted standards. Statistically significant differences are highlighted using * (*P = <0.05), **P = <0.005)

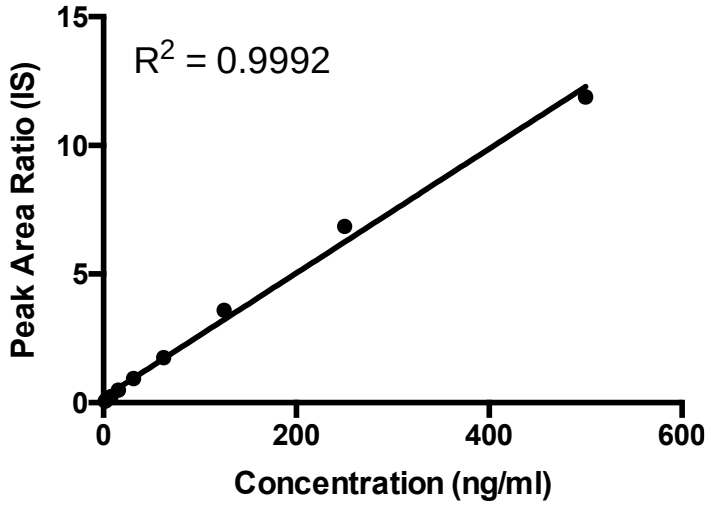


Figure 2

Efavirenz Linearity

Figure 2 shows the standard curve generated from extracted plasma standards of efavirenz over the range of 500ng/mL to 1.9ng/mL.

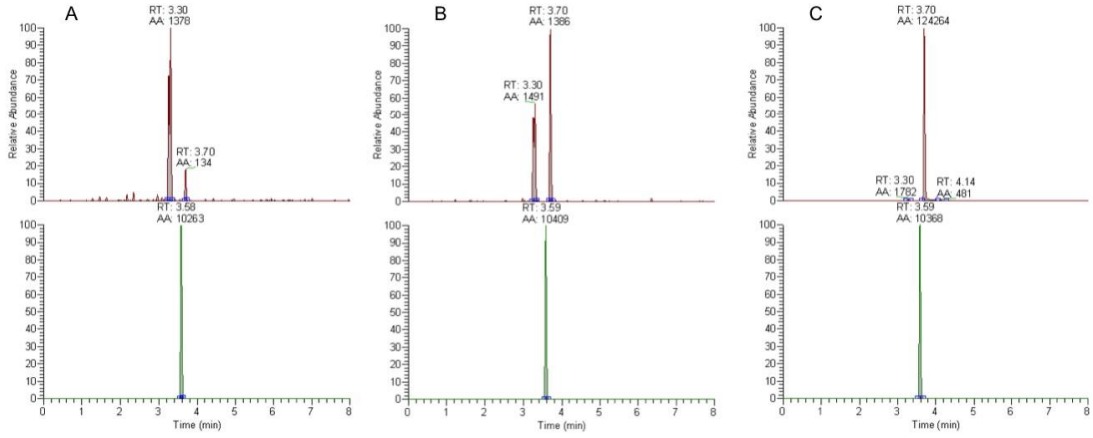
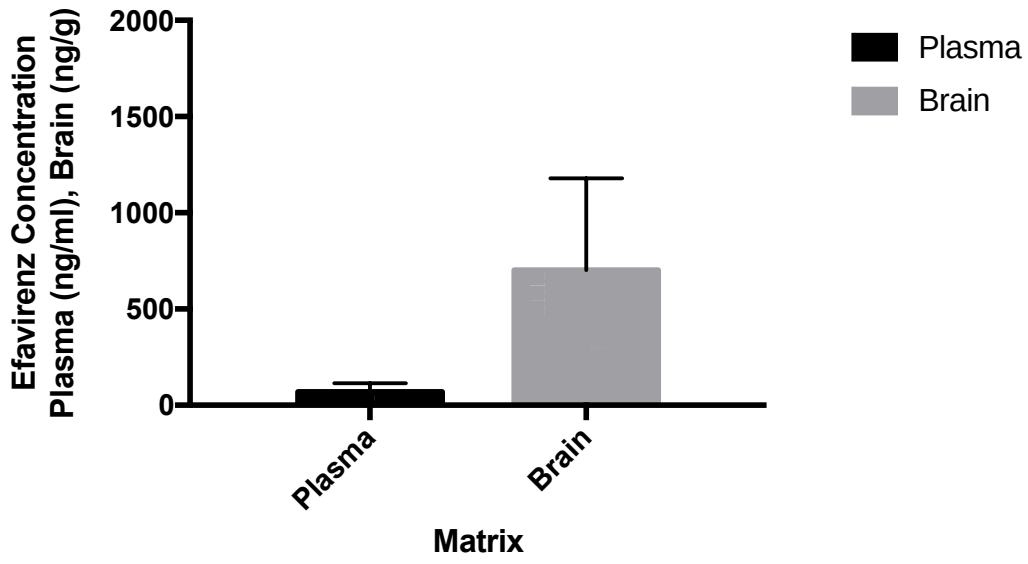


Figure 3

Selectivity

Figure 3 shows a representative chromatogram from blank plasma (A), lower limit of quantification (1.9ng/mL) (B) and the highest standard (500ng/mL) (C). The upper panel of each figure shows the peak produced by efavirenz (retention time 3.7). The lower panel show the peak produced by the IS (lopinavir) (retention time 3.58).



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515 **Figure 4**

516 **Distribution of efavirenz in brain tissue and plasma**

517 Figure 4 shows the concentration of efavirenz in plasma and brain tissue determined
518 following oral administration of efavirenz (10mg/kg) to male wistar rats over 5
519 weeks. Data points represent median (plus IQR).

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Chromatographic Conditions

Time (mins)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	90	10
0.1	90	10
0.5	14	86
5.0	8	92
5.1	3	97
6.0	3	97
6.0	90	10
8.0	90	10

Table 1 shows the chromatographic gradient of mobile phase A (100% H_2O, 5mM ammonium formate) and mobile phase B (100% ACN, 5mM ammonium formate) over 8 minutes for the detection of efavirenz and IS.

Product Ions Produced by SRM

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Efavirenz	315	242.1	16.5
		244.0	17.0
		250.0	17.0
Lopinavir	627	121.2	33.5
		178.1	26.5
		198.1	22.5

Table 2 shows the parent mass, product ion and the collision energy for efavirenz and IS.

Intraday Accuracy and Precision

	Assay 1	Variance of accuracy (%)	Variance of precision (%)	Assay 2	Variance of accuracy (%)	Variance of precision (%)	Assay 3	Variance of accuracy (%)	Variance of precision (%)
Low (20ng/ml)	19.95	-0.25	11.05	19.58	-0.42	5.66	20.16	0.78	2.74
Medium (100ng/ml)	89.38	-10.62	5.52	100.01	0.01	3.88	95.34	-4.66	3.78
High (400ng/ml)	354.21	-11.45	6.63	374.70	-6.32	2.93	394.97	-1.26	1.25

Table 3 shows the accuracy and precision of 3 repetitions of the assay. Accuracy and precision were assessed in triplicate at 3 levels (low (20ng/ml), medium (100ng/ml) and high (400ng/ml).

552 **Interday Accuracy and Precision**

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	Average (ng/mL)	Standard Deviation	Accuracy (%)	Precision (%)
Low (20ng/mL)	19.90	0.29	-0.52	1.48
Medium (100ng/mL)	94.91	5.33	-5.09	5.61
High (400ng/mL)	374.63	20.38	-6.34	5.44

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555 **Table 4** shows the accuracy and precision of 3 repetitions of the assay (inter-assay variability).
556 Accuracy and precision were assessed in triplicate of 3 QCs (low [20ng/mL], medium
557 [100ng/mL] and high [400ng/mL]).

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Accuracy and Precision for Partial Validation in Brain Tissue and PBS

		Average (ng/mL)	Standard Deviation	Accuracy (%)	Precision (%)
Low (20ng/mL)	Brain Tissue	22.9	0.4	14.8	1.6
	PBS	22.5	0.6	12.3	2.6
Medium (100ng/mL)	Brain Tissue	100	3.7	0.0	3.7
	PBS	98.8	0.7	-1.2	0.7
High (400ng/mL)	Brain Tissue	387.9	2.4	-3.0	2.4
	PBS	363.4	2.0	-9.2	2

Table 5 shows the results of the partial validation for brain tissue homogenate and PBS. Accuracy and precision were assessed in triplicate of 3 QCs (low [20ng/mL], medium [100ng/mL] and high [400ng/mL]).