

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

3

4 **Abstract**

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

9 **Results:** The investigation of efavirenz distribution required the development of a
10 versatile and sensitive method. In addition to plasma, quantification was required in
11 brain tissue and phosphate buffered saline. The assay presented here presented here
12 was linear from 1.9ng/mL to 500ng/mL. Accuracy and precision ranged between
13 93.7% and 99.5%, 1.5% and 5.6%, respectively.

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

17

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

20

21

22

23 **1.1 Introduction**

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

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

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

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

66 To investigate CNS concentrations of efavirenz, multiple LC-MS/MS methods have
67 been developed to analyse efavirenz concentrations in CSF (cerebrospinal fluid) [14-
68 16]. Although assessing CSF is a step towards understanding efavirenz
69 concentrations in the CNS, CSF and brain tissue concentrations of drugs may vary
70 widely and may not represent the disposition of efavirenz in the CNS [17, 18].

71

72 **1.2 Methods and Materials**

73 **1.2.1 Materials**

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

77

78 **1.2.2 Tuning for Efavirenz and Internal Standard**

79 Detection of efavirenz and internal standard (IS) lopinavir was conducted using a
80 TSQ endura LC-MS/MS (Thermo scientific). Lopinavir was selected as IS due to
81 similar log P (efavirenz 3.89, lopinavir 3.9) and has been shown previously not to
82 interfere with efavirenz detection [4, 7, 19]. Tuning was performed using direct
83 infusion (20µl/min) of a 500ng/mL stock of efavirenz with 50% mobile phase A
84 (100% H₂O [LC-MS/MS grade] 5mM ammonium formate), 50% mobile phase B
85 (100% acetonitrile [ACN] 5mM ammonium formate) at a flow rate of 300µl/min.
86 Ionisation was achieved via heated electron spray ionization in negative mode.
87 Although positive mode is more commonly used efavirenz is poorly detectable in
88 positive mode [6, 8]. The following parameters were optimised to achieve the
89 highest signal intensity for efavirenz: spray voltage, sheath gas and auxiliary gas.
90 The IS was then directly injected (500ng/mL) to ensure detection using the optimised
91 efavirenz settings. Following optimization for the parent mass of efavirenz (315) and
92 IS (628), selected reaction monitoring (SRM) scan was utilised for detection of the
93 break down products.

94

95 **1.2.3 Chromatographic Separation**

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

99

100 **1.2.4 Extraction from Plasma and PBS**

101 100 µl of sample (PBS was spiked with 20µl of ACN to aid efavirenz dissolution)
102 was transferred to glass vials were 20µl of IS (2500ng/mL) were added to all
103 standards, QC's and samples. Samples were diluted with ACN (sample: ACN ratio
104 1:4) and thoroughly vortexed. Samples were then centrifuged at 4000g for 10
105 minutes at 4°C. The supernatant fraction was transferred to a fresh glass vial and
106 evaporated, samples were placed in a rotary vacuum centrifuge at 30°C and then
107 reconstituted in 140µl of H₂O:ACN (60:40). 100µl of the sample was then
108 transferred into 200µl chromatography vials. 5µl of each sample was injected for
109 analysis.

110

111 **1.2.5 Extraction from Brain Tissue**

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

115

116 **1.2.6 Assay Validation**

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

120

121 **Linearity**

122 A calibration curve of efavirenz was prepared in rat plasma via serial dilution,
123 ranging from 1.9ng/mL to 500ng/mL. Extraction was performed using protein
124 precipitation. Linearity was assessed by 3 independent preparations of the standard
125 curve. Maximum allowed deviation of standards was set at 15% of the stated value,
126 excluding the lower limit of quantification where deviation was set at no more than
127 20%.

128

129 **Recovery**

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

133

134 **Selectivity**

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

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

140

141 **Accuracy and Precision**

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

$$149 \quad \% \text{ variability of accuracy} = \frac{\text{error}}{\text{stated value}} \times 100$$

150 The precision of an analytical method describes the closeness of individual measures
151 of an analyte when the procedure is applied repeatedly to multiple aliquots of a
152 single volume of biological matrix. Precision of the assay was determined by
153 preparation of three concentrations (in the range of the standard curve 20ng/mL,
154 100ng/mL and 400ng/mL) with each preparation in triplicate. The mean value of
155 each concentration should be within 15% of the stated concentration (except the
156 lower concentration, where deviation should be less than 20%). Precision was
157 calculated using the following formula:

$$158 \quad \% \text{ variation of precision} = \frac{\text{standard deviation}}{\text{mean assay value}} \times 100$$

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

164

165 **Animals and treatment**

166 Male Wistar rats (Charles River UK) weighing 180 – 220 g on arrival were used for
167 PK analysis of efavirenz. Food and water were provided *ad libitum*. Treated and
168 untreated Wistar rats were sacrificed using an appropriate schedule 1 method (rising
169 concentration of CO₂). Following termination brain was extracted and stored at -
170 80°C. All animal work was conducted in accordance with the Animals (Scientific
171 Procedures) Act 1986 (ASPA), implemented by the United Kingdom Home Office.

172

173 **1.2.7 Determination of Fraction Unbound of Efavirenz in Brain Homogenate**

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

183
$$Undiluted\ fu = \frac{\left(\frac{1}{D}\right)}{\left[\frac{1}{fu(apparent)} - 1\right] + \left(\frac{1}{D}\right)}$$

184

185 **1.2.8 Efavirenz Penetration into Rat Brain Tissue**

186 Eight male Wistar rats (Charles River, UK) weighing 180-220g were dosed with
187 efavirenz (10 mg/kg, 2 mL/kg 0.5% methylcellulose in dH₂O) based on individual
188 weight taken prior to dosing. Dosing was administered once daily *via* oral gavage
189 over 5 weeks. The animals were terminated 2 hours after the final dose and blood
190 was collected *via* cardiac puncture. Blood samples were centrifuged at 2000g for 10
191 minutes at 4°C to separate plasma. Plasma was immediately frozen at -80°C and
192 stored for later analysis. Brain tissue was also collected and following washing in
193 phosphate buffered saline for 30 seconds 3 times, immediately stored at -30°C for
194 analysis. All animal work was conducted in accordance with the Animals (Scientific
195 Procedures) Act 1986 (ASPA), implemented by the United Kingdom Home Office.

196 **1.2.9 Statistics**

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

200

201 **1.3 Results**

202 **1.3.1 Tuning Settings**

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

206

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

213

214 **1.3.2 Extraction Efficiency from Plasma, Brain Tissue and PBS**

215 The recovery was measured at of the three QC concentrations (Figure 1). The mean
216 recovery (across all 3 QCs) from plasma, brain tissue and PBS were 93% (standard
217 deviation 2.9), 99% (standard deviation 4.49), and 95% (standard deviation 3.31),
218 respectively. When recovery from to plasma was compared to recovery from brain
219 tissue, there was a small but statistically significant difference in recovery at the low
220 (92% vs 101 %, P = 0.001) and high (97% vs 101%QCs P = 0.04). When recovery
221 from to plasma was compared to recovery from PBS, there was a small but
222 statistically significant difference in recovery at the low QC (92% vs 99%, P =
223 0.007). Recovery at all other levels showed no statistically difference.

224

225 **1.3.3 Assay Validation**

226

227 **Linearity**

228 Standards extracted from plasma showed good linearity ($R^2 = 0.9992$). The peak area
229 ratio (analyte to IS; variation of IS was less than 15% in each run) was proportional
230 to the stated concentrations over the range of 500ng/mL to 1.9ng/mL. Figure 2
231 shows a representative calibration curve. Calibration curve was generated using a
232 quadratic equation with a weighting of 1/X. Although a linear equation produced
233 an acceptable R^2 (>0.99) the quadratic equation better described the
234 relationship between signal response and standard concentration (R^2
235 >0.999). Both equations resulted in the assay passing (QC variability $<15\%$
236 at all levels).

237

238 **Selectivity**

239 The matrix effect of plasma was examined by comparing extracted blank plasma to
240 extracted plasma spiked with 1.9ng/mL of efavirenz. Figure 3a shows the
241 chromatogram produced by the extracted blank. There is a visible peak (area of 134)
242 at the retention time of efavirenz (3.7 minutes). FDA guidelines require the lower
243 limit of quantification produce a peak area of at least five fold greater than that
244 observed in the blank matrix. Figure 3b shows the peak produced from the lower
245 limit of quantification (1.9ng/mL). The peak area is 1491, which complies with FDA
246 guidelines. Figure 3c shows the peak produced by the highest standard (500ng/mL).
247 The bottom panels of figure 3 demonstrate the signal from the IS (retention time of
248 3.59 minutes) in extracted blank plasma (3a), the lower limit of quantification of

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

251

252 **Accuracy and Precision**

253 The accuracy and precision for each individual run at 3 QC levels (low (20ng/mL),
254 medium (100ng/mL) and high (400ng/mL) is shown in table 3. The percentage error
255 of accuracy fell below 15% for each of the 3 repeats (1 varied between -0.25% and -
256 11.45%, 2 varied between 0.01% and -6.32%, 3 varied between 0.78% and -4.66%).
257 The percentage error of precision also fell below 15% for each of the 3 repeats (1
258 varied between -5.52% and 11.05%, 2 varied between 2.93% and 5.66%, 3 varied
259 between 1.25% and 3.78%). The QC concentrations were selected based on the
260 anticipated concentrations in the study samples. FDA guidelines recommend QC the
261 low QC be within 3 fold of the lower limit of quantification, the medium QC near
262 the center of the linear range and a high QC near the upper limit of quantification.
263 Supplementary figure 1 shows accuracy and precision for each individual run with 3
264 QC levels conforming to FDA guidelines (low (5ng/mL), medium (200ng/mL) and
265 high (400ng/mL). The percentage error of accuracy and precision fell below 15% for
266 each of the 3 repeats.

267

268 **Inter-assay Variability**

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

272 repetitions of the assay. The percentage error in accuracy fell below 15% across all 3
273 repeats (range between -0.52% and -6.34). Percentage variance of precision also fell
274 below 15% across all 3 repeats (range between 1.48% and 5.61%). Supplementary
275 figure 2 shows the variance of accuracy and precision calculated from the mean
276 values of the 3 repetitions of the assay (for low QC 5ng/mL, medium QC 200 ng/mL
277 and high QC 400 ng/mL). Percentage variance of accuracy and precision fell below
278 15% between all 3 repeats.

279

280 1.3.4 Partial Validation of Brain Tissue Homogenate and PBS

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

287

288 **1.3.5 Determination of Fraction Unbound of Efavirenz in Brain Homogenate**

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

295 **1.3.6 Efavirenz Penetration Into Brain Tissue**

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

300

301 **1.4 Discussion**

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

308 Primary validation was conducted in plasma satisfying FDA bioanalytical method
309 development guidelines, demonstrating good accuracy, precision and linearity.
310 Although full validation for different matrices is not required, matrix effects must be
311 assessed for each matrix. The change in matrix may potentially affect the behaviour
312 of the assay significantly. Brain tissue homogenate and cell culture media both
313 contain different quantities of protein compared to plasma. As efavirenz is highly
314 protein bound (99% in plasma) and poorly water soluble (<10µg/mL), the change in
315 matrix has the potential to alter efavirenz recovery [21, 22]. As the change in matrix
316 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].

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

345

346 **1.5 Conclusion**

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

352

353 **1.6 Future Perspective**

354 The assay presented here has been developed and validated for the detection of
355 efavirenz in rat plasma, rat brain tissue homogenate and PBS. However, future
356 studies may be able to build upon the work presented here. Although our study
357 focused on samples taken from rats, there is the potential to further utilise the assay
358 to analyse samples from other species, such as mice and humans. Other matrices of
359 interest may also be investigated, in particular CSF in (rat and or human). To adapt
360 this assay would require partial validation, to investigate the potential effects of a
361 change in matrix. Recent publications have implicated the metabolites of efavirenz in
362 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

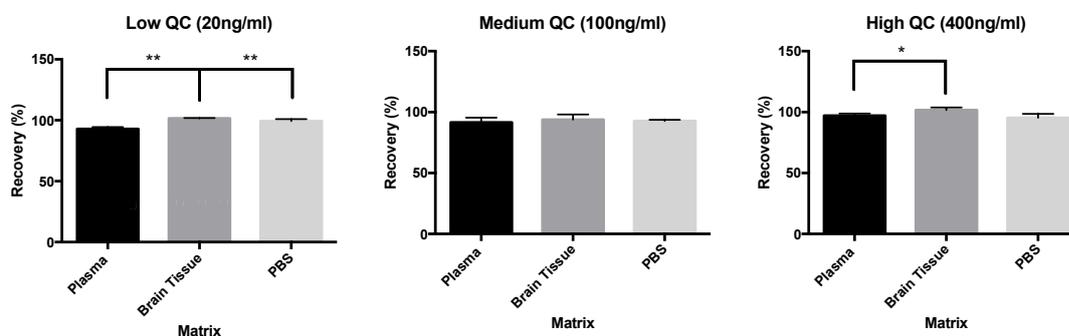
372 1.8 References

- 373 1. Cory TJ.Schacker TW.Stevenson M.Fletcher CV. Overcoming
374 pharmacologic sanctuaries. *Current opinion in HIV and AIDS* 8(3),
375 190-195 (2013).
- 376 2. Fletcher CV.Staskus K.Wietgreffe SW *et al.* Persistent HIV-1
377 replication is associated with lower antiretroviral drug concentrations
378 in lymphatic tissues. *Proceedings of the National Academy of*
379 *Sciences* 111(6), 2307-2312 (2014).
- 380 3. Ma Q.Vaida F.Wong J *et al.* Long-term efavirenz use is associated
381 with worse neurocognitive functioning in HIV-infected patients. *Journal*
382 *of neurovirology* 22(2), 170-178 (2016).
- 383 4. DrugBank - Efavirenz. 2016(05/07/81), (2016).
- 384 5. Food and Drug Administration F. Guidance for Industry Bioanalytical
385 Method Validation. 2015(16th June), (2013). ** This document details
386 the US FDA guidelines for development of bioanalytical methods.
- 387 6. Olagunju A.Bolaji OO.Amara A *et al.* Development, validation and
388 clinical application of a novel method for the quantification of efavirenz
389 in dried breast milk spots using LC-MS/MS. *The Journal of*
390 *antimicrobial chemotherapy* 70(2), 555-561 (2015).
- 391 7. Huang Y.Gandhi M.Greenblatt RM *et al.* Sensitive analysis of anti-HIV
392 drugs, efavirenz, lopinavir and ritonavir, in human hair by liquid
393 chromatography coupled with tandem mass spectrometry. *Rapid*
394 *communications in mass spectrometry : RCM* 22(21), 3401-3409
395 (2008).
- 396 8. Srivastava P.Moorthy GS.Gross R.Barrett JS. A sensitive and
397 selective liquid chromatography/tandem mass spectrometry method
398 for quantitative analysis of efavirenz in human plasma. *PloS one* 8(6),

- 399 e63305 (2013). * Details efavirenz stability at room temperature, -80°C
400 and in response to freeze thaw cycles.
- 401 9. Wyen C.Hendra H.Siccardi M *et al.* Cytochrome P450 2B6 (CYP2B6)
402 and constitutive androstane receptor (CAR) polymorphisms are
403 associated with early discontinuation of efavirenz-containing
404 regimens. *The Journal of antimicrobial chemotherapy* 66(9), 2092-
405 2098 (2011).
- 406 10. Marzolini C.Telenti A.Decosterd LA *et al.* Efavirenz plasma levels can
407 predict treatment failure and central nervous system side effects in
408 HIV-1-infected patients. *Aids* 15(1), 71-75 (2001).* Describes the
409 importance of efavirenz plasma concentrations and the association
410 with CNS toxicity.
- 411 11. Kailasa SK.Wu H-F. Rapid Quantification of Efavirenz in Human
412 Plasma by Electrospray Ionization Tandem Mass Spectrometry.
413 *Journal of the Chinese Chemical Society* 61(4), 437-441 (2014).
- 414 12. Olagunju A.Siccardi M.Amara A *et al.* CYP2B6 516G>T (rs3745274)
415 and smoking status are associated with efavirenz plasma
416 concentration in a Serbian cohort of HIV patients. *Therapeutic drug*
417 *monitoring* 36(6), 734-738 (2014).
- 418 13. Hewavitharana AK.Tan SK.Shaw PN. Strategies for the Detection and
419 Elimination of Matrix Effects in Quantitative LC-MS Analysis. *Lc Gc N*
420 *Am* 32(1), 54-+ (2014).
- 421 14. Best BM.Koopmans PP.Letendre SL *et al.* Efavirenz concentrations in
422 CSF exceed IC50 for wild-type HIV. *The Journal of antimicrobial*
423 *chemotherapy* 66(2), 354-357 (2011).** Efavirenz concentrations in
424 plasma and CSF in a cohort of 80 patients, summarising current
425 knowledge of efavirenz CNS penetration.
- 426 15. Tashima KT.Caliendo AM.Ahmad M *et al.* Cerebrospinal fluid human
427 immunodeficiency virus type 1 (HIV-1) suppression and efavirenz drug
428 concentrations in HIV-1-infected patients receiving combination
429 therapy. *The Journal of infectious diseases* 180(3), 862-864 (1999).
- 430 16. Yilmaz A.Price RW.Gisslen M. Antiretroviral drug treatment of CNS
431 HIV-1 infection. *The Journal of antimicrobial chemotherapy* 67(2),
432 299-311 (2012).
- 433 17. Shen DD.Artru AA.Adkison KK. Principles and applicability of CSF
434 sampling for the assessment of CNS drug delivery and
435 pharmacodynamics. *Advanced drug delivery reviews* 56(12), 1825-
436 1857 (2004).
- 437 18. Gibbs JE.Gaffen Z.Thomas SA. Nevirapine uptake into the central
438 nervous system of the Guinea pig: an in situ brain perfusion study.
439 *The Journal of pharmacology and experimental therapeutics* 317(2),
440 746-751 (2006).
- 441 19. DrugBank - Lopinavir. 2016(07/07/16), (2016).
- 442 20. Himmelsbach M. 10 years of MS instrumental developments--impact
443 on LC-MS/MS in clinical chemistry. *Journal of chromatography. B,*
444 *Analytical technologies in the biomedical and life sciences* 883-884 3-
445 17 (2012).
- 446 21. Siccardi M.Almond L.Schipani A *et al.* Pharmacokinetic and
447 pharmacodynamic analysis of efavirenz dose reduction using an in

- 448 vitro-in vivo extrapolation model. *Clinical pharmacology and*
 449 *therapeutics* 92(4), 494-502 (2012).
- 450 22. Mcdonald TO.Giardiello M.Martin P *et al.* Antiretroviral solid drug
 451 nanoparticles with enhanced oral bioavailability: production,
 452 characterization, and in vitro-in vivo correlation. *Advanced healthcare*
 453 *materials* 3(3), 400-411 (2014).
- 454 23. Ema EMA. Guideline on bioanalytical method validation. 2015(16th
 455 June), (2011).
- 456 24. Thompson CG.Bokhart MT.Sykes C *et al.* Mass spectrometry imaging
 457 reveals heterogeneous efavirenz distribution within putative HIV
 458 reservoirs. *Antimicrobial agents and chemotherapy* 59(5), 2944-2948
 459 (2015).
- 460 25. Almond LM.Hoggard PG.Edirisinghe D.Khoo SH.Back DJ. Intracellular
 461 and plasma pharmacokinetics of efavirenz in HIV-infected individuals.
 462 *The Journal of antimicrobial chemotherapy* 56(4), 738-744 (2005).
- 463 26. Avery LB.Sacktor N.Mcarthur JC.Hendrix CW. Protein-free efavirenz
 464 concentrations in cerebrospinal fluid and blood plasma are equivalent:
 465 applying the law of mass action to predict protein-free drug
 466 concentration. *Antimicrobial agents and chemotherapy* 57(3), 1409-
 467 1414 (2013).
- 468 27. Brandmann M.Nehls U.Dringen R. 8-Hydroxy-efavirenz, the primary
 469 metabolite of the antiretroviral drug Efavirenz, stimulates the glycolytic
 470 flux in cultured rat astrocytes. *Neurochemical research* 38(12), 2524-
 471 2534 (2013).
- 472 28. Tovar-Y-Romo LB.Bumpus NN.Pomerantz D *et al.* Dendritic spine
 473 injury induced by the 8-hydroxy metabolite of efavirenz. *The Journal of*
 474 *pharmacology and experimental therapeutics* 343(3), 696-703 (2012).
- 475 29. Winston A.Amin J.Clarke A *et al.* Cerebrospinal fluid exposure of
 476 efavirenz and its major metabolites when dosed at 400 mg and 600
 477 mg once daily: a randomized controlled trial. *Clinical infectious*
 478 *diseases : an official publication of the Infectious Diseases Society of*
 479 *America* 60(7), 1026-1032 (2015).

480 Figures and Tables



481

482 Figure 1

483 Efavirenz recovery.

484 Figure 1 shows the percentage recovery for the low (a), medium (b) and high (c)
485 QCs in extracted plasma, extracted brain tissue and PBS. Data is show percentage of
486 unextracted standards. Statistically significant differences are highlighted using * (*P
487 = <0.05), **P = <0.005)

488

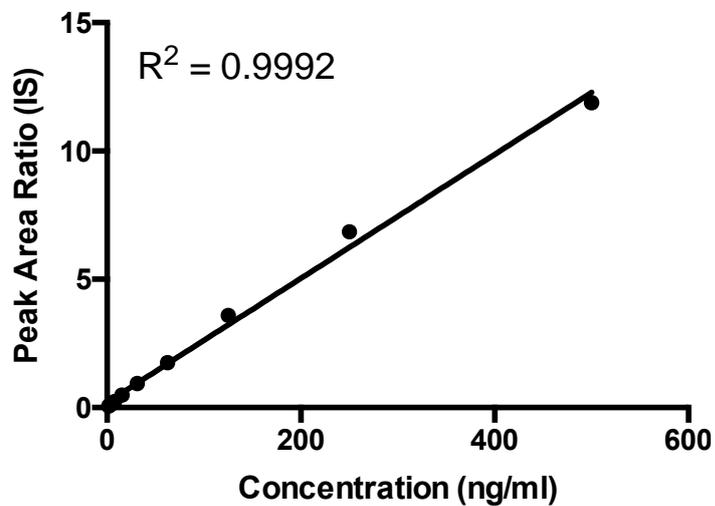
489

490

491

492

493



494

495 **Figure 2**

496 **Efavirenz Linearity**

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

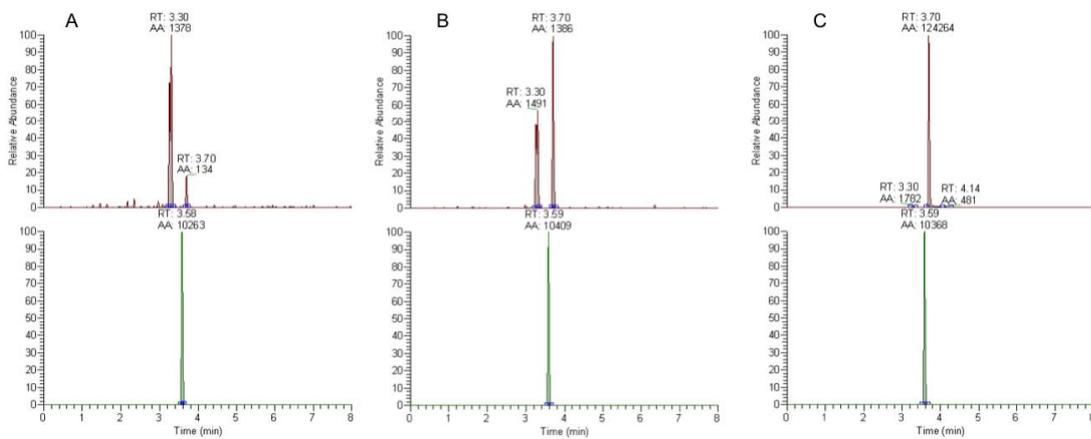
499

500

501

502

503



504

505 **Figure 3**

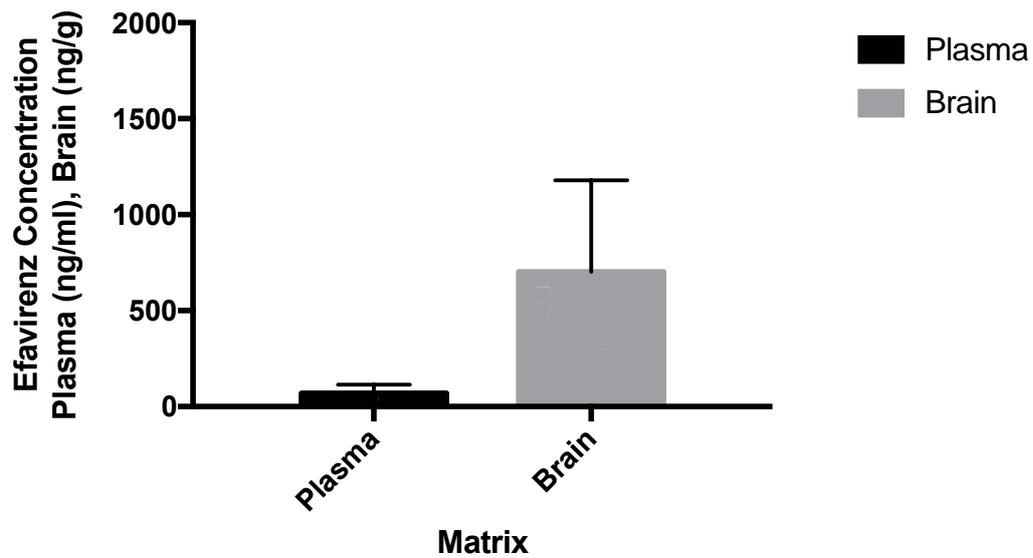
506 **Selectivity**

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

511

512

513



514

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).

520

521

522

523

524

525

526

527

528 **Chromatographic Conditions**

529

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

530

531 **Table 1** shows the chromatographic gradient of mobile phase A (100% H₂O, 5mM
532 ammonium formate) and mobile phase B (100% ACN, 5mM ammonium formate) over 8
533 minutes for the detection of efavirenz and IS.

534

535

536

537

538

539

540

541 **Product Ions Produced by SRM**

542

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

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

544 **Intraday Accuracy and Precision**

545

	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

546

547

548 **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
549 high (400ng/ml).

550

551

552 **Interday Accuracy and Precision**

553

	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

554

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]).

558

559

560

561

562

563

564

565

566 **Accuracy and Precision for Partial Validation in Brain Tissue and PBS**

567

		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

568

569 **Table 5** shows the results of the partial validation for brain tissue homogenate and PBS.

570 Accuracy and precision were assessed in triplicate of 3 QCs (low [20ng/mL], medium

571 [100ng/mL] and high [400ng/mL]).

572