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Quantification of etravirine (TMC125) in plasma, dried blood spots and peripheral blood mononuclear cell lysate by liquid chromatography tandem mass spectrometry

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ABSTRACT

For the quantification of the novel non-nucleoside reverse transcriptase inhibitor etravirine in human plasma, dried blood spots and peripheral blood mononuclear cell (PBMC) lysate, an assay was developed and validated, using liquid chromatography coupled with tandem mass spectrometry.

Etravirine was extracted from plasma by means of protein precipitation with a mixture of methanol and acetonitrile using only 50 μL plasma. Extraction from dried blood spots was performed with a one-step extraction with a mixture of methanol, acetonitrile and 0.2 M zinc sulphate in water (1:1:2, v/v/v) and extraction from cell lysate was performed in 50% methanol in water. Chromatographic separation was performed on a reversed phase C18 column (150 mm × 2.0 mm, particle size 5 μm) with a quick stepwise gradient using an acetate buffer (pH 5) and methanol, at a flow rate of 0.25 mL/min. $^{13}C_6$-efavirenz was used as an internal standard. The analytical run time was only 10 min. The triple quadrupole mass spectrometer was operated in the positive ion-mode and multiple reaction monitoring was used for drug quantification. The method was validated over a range of 25–5000 ng/mL in plasma, 50–10,000 ng/mL in dried blood spots and a range of 5–2500 ng/mL in PBMC lysate. Accuracies ranged from 89% to 106% in plasma, from 94% to 109% in dried blood spots and from 91% to 105% in PBMC lysate. Precisions at the all concentration levels ranged from 1.9% to 14% in plasma, 4.7% to 20% in dried blood spots and from 3.1% to 11% in PBMC lysate. The bioanalytical assay was successfully incorporated with previously developed assays for the determination of all currently approved PIs and NNRTIs in plasma and dried blood spots and it is now applied for therapeutic drug monitoring and pharmacological research in HIV-infected patients treated with etravirine.

1. Introduction

Etravirine (TMC125) is a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) of the human immunodeficiency virus type 1 (HIV-1), which shows high activity against both wild type and drug-resistant strains of HIV-1, including strains resistant for currently approved NNRTIs. Etravirine is currently approved for the treatment of HIV-1 infection in antiretroviral treatment-experienced adult patients and is dosed 200 mg twice daily in combination with other antiretroviral agents [1,2].

Etravirine is metabolized by cytochrome P450 (CYP) 3A4, CYP2C9 and CYP2C19 with subsequent glucuronidation of the metabolites. The main etravirine metabolites M12 and M8 are mono- and dihydroxylated products and circulate in plasma [3].

Determination of drug concentrations in plasma is the gold standard for purposes of therapeutic drug monitoring (TDM) or pharmacokinetic studies [4]. However, quantification of drug levels in dried blood spots obtained with a simple fingerprick provides a patient-friendly alternative for sample collection in patient populations where intensive venous sampling is unethical or impossible and it allows non-hospital based sampling. Moreover, when using dried blood spots for drug quantification there is no need for the use of anticoagulant containing sampling tubes, plasma separation or the necessity of cold sample storage. Lastly, dried blood spots can be easily stored or transported without the requirements of special storage, allowing easy and affordable shipment [5].

The site of action of etravirine is within the infected cell. Cell-associated drug levels of etravirine provide information on drug disposition in a compartment where HIV replicates and may therefore be useful in understanding its clinical pharmacology. The intracellular accumulation of etravirine may be a result of passive diffusion and active transport and may therefore vary from person to person. Hence, measurement of cell-associated concentrations

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etravirine may more adequately reflect treatment effectiveness than measurements of plasma concentrations.

Our main goal was to include etravirine in an already existing assay, allowing the simultaneous determination of all currently approved antiretroviral drugs (the human immunodeficiency protease inhibitors amprenavir, atazanavir, darunavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir, the active nelfinavir metabolite M8 and the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine) by means of liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) in a run time of only 10 min [6,7]. We here present the inclusion and validation of etravirine in the existing assay for the determination of etravirine in plasma, dried blood spots and peripheral blood mononuclear cell (PBMC) lysate.

2. Experimental

2.1. Chemicals and materials

Etravirine was kindly provided by Tibotec Pharmaceuticals (Co Cork, Ireland) and the internal standard (IS) $^{13}C_6$-efavirenz was provided by Bristol-Myers Squibb (Princeton, NJ, USA). Acetonitrile and methanol were HPLC-grade and obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, dimethyl sulfoxide (DMSO), glacial acetic acid and zinc sulphate heptahydrate were obtained from Merck (Amsterdam, The Netherlands). Distilled water originated from Aqua B. Braun (Melsungen, Germany). Drug-free plasma, blood and buffy coat were obtained from healthy human volunteers. Whatman 903 protein saver cards® for sample collection, 2 mL Eppendorf reaction vials, 1.5 mL autosampler vials, autosampler vial inserts, BD Vacutainer® CPT™ cell preparation tubes, BD Vacutainer® EDTA-anticoagulated blood collection tubes, phosphate buffered saline (PBS), 14 mL sterile polypropylene tubes and Ficoll® were obtained from VWR International B.V. (Amsterdam, The Netherlands). A 0.25-in. diameter punch was obtained from Fiskars (Madison, WI, USA). Haemolance plus® high flow lancets for single use for patient sampling were obtained from HaeMedic AB (Munka Ljungby, Sweden).

2.2. Preparation of solutions, calibration standards, validation samples and blank PBMC pellets

Approximately 5 mg etravirine was accurately weighted (after correction for the weight of counter ions and impurities) and dissolved in 5 mL of DMSO in a volumetric flask to give a 1 mg/mL stock solution. A stock solution of the IS ($^{13}C_6$-efavirenz) was similarly prepared at a concentration of 1 mg/mL in methanol.

The protein precipitation solution for plasma was prepared by mixing 148 mL methanol, 150 mL acetonitrile and 2 mL of $^{13}C_6$-efavirenz stock solution. The dried blood spot extraction solution was prepared by adding 200 μL of the $^{13}C_6$-efavirenz stock solutions to a 200 mL mixture of acetonitrile, methanol and 0.2 M zinc sulphate in water (1:1:2, v/v/v). The cell extraction solution was prepared by adding 13 μL of the $^{13}C_6$-efavirenz stock solution to 100 mL of a 1:1 (v/v) methanol–water mixture. Eluent A for HPLC was prepared by mixing 700 mL of methanol with 440 mL of 10 mM acetic acid and 860 mL of 10 mM ammonium acetate solution in water. Eluent B consisted of 100% methanol. 50 mM ammonium acetate buffer was prepared by mixing 200 mL of 50 mM ammonium acetate solution in water with 100 mL of 50 mM acetic acid solution in water.

For the preparation of the highest calibration standard in plasma, 50 μL of the etravirine stock solution was added to a 10 mL volumetric flask. Thereafter, EDTA-anticoagulated human plasma was added up to 10 mL and its contents were vortex-mixed. Serial dilutions of the highest calibration standard with the same drug-free plasma followed to obtain a calibration range of 25–5000 ng/mL. Validation samples were similarly prepared at four concentration levels in plasma. For the preparation of the highest calibration standard in EDTA-anticoagulated blood, 50 μL of the etravirine stock solution was added to a 5 mL volumetric flask. EDTA-anticoagulated blood was added up to 5 mL and thereafter the contents were vortex-mixed. Serial dilutions with EDTA-anticoagulated blood followed to obtain a calibration range of 50–10,000 ng/mL. The calibration and validation samples in blood were spotted on the filter cards by transferring 25 μL onto the card with a volumetric pipette. Thereafter, the blood spots were left to dry overnight at ambient temperature.

Calibration standards and validation samples for the determination of etravirine in cell lysate were prepared in the cell extraction solution in a concentration range of 5–2500 ng/mL. For quantification of etravirine in PBMC lysate from clinical samples, the exact amount of etravirine in the sample can therefore be calculated and the cell-associated concentration can then be calculated using a cellular volume of 0.4 pL [8].

Drug-free PBMCs were isolated from buffy coat as described before [9]. After isolation the cells were washed twice with 10 mL of ice-cold PBS, the amount of cells were counted using a hematology analyzer and aliquots of 5×10^6 cells were centrifuged in Eppendorf reaction vials for 10 min at $650 \times g$. The supernatant was aspirated and the cell pellet was stored at −20 °C.

2.3. Equipment

An Agilent (Agilent technologies, Palo Alto, CA, USA) HPLC system was used consisting of an 1100 series pump and cooled autosampler (4 °C). Attached to this system was a pre-column (Gemini C18 pre-column, 4.0 mm × 2.0 mm I.D.; Phenomenex, Torrance, CA, USA) and an analytical column (Gemini C18, 150 mm × 2.0 mm I.D., 5 μm particle size; Phenomenex). The column outlet was connected to the turbo ionspray sample inlet (Sciex, Thornhill, ON, Canada) through a post-column splitter (1:4) (ICP-04-20-CR, LC Packings, Amsterdam, The Netherlands) and a divert valve. The divert valve was used to direct the first 3 min of the eluent flow to waste to prevent the introduction of endogenous compounds into the mass spectrometer. Cell count was performed using a Cell-dyn® hematology analyzer (Cell-dyn® 4000, Abbott Diagnostics, Hoofddorp, The Netherlands).

2.4. LC–MS/MS conditions

A stepwise gradient was used to elute the compounds from the column. These chromatographic conditions allow a wide range of different analytes to be separated from endogeneous compounds and to be detected using mass spectrometry, as previously described by us [6,7]. Briefly, at time zero either 10 μL of the plasma extract, 20 μL of the dried blood spot extract or 30 μL of the PBMC lysate extract was introduced into the flow consisting of 85% eluent A mixed with 15% eluent B. After 0.1 min 15% of eluent A was mixed with 85% of eluent B and this composition was maintained for 6.9 min. Thereafter the column was reconditioned for 3 min with 85% eluent A mixed with 15% B before the next injection. The flow rate was 250 μL/min.

The turbo ionspray source temperature was held constant at 350 °C. Ions were created at atmospheric pressure and transferred to an API 3000 triple quadrupole mass spectrometer (Sciex). The ionisation source parameters were: nebulizer gas, 12 arbitrary units (a.u.); curtain gas, 6 a.u.; ionspray voltage, 4000 V; heater gas, 350 °C; turbo gas, 7 L/min. Nitrogen was used as curtain gas, nebulizer gas, collision-activated dissociation (CAD) gas and turbo

gas. Multiple reaction monitoring (MRM) in positive mode was used for drug quantification and mass transitions were obtained from collision-induced dissociation. Used mass transitions of the protonated precursor/product ion pairs are listed in Table 1. Data were processed by Analyst software (version 1.2, Sciex).

2.5. Sample preparation

To 50 μL of plasma, 100 μL of the protein precipitation solution was added and after vortex-mixing, samples were centrifuged for 10 min at 23,100 × *g* for 10 min. Thereafter, a volume of 100 μL of the clear supernatant was transferred to a vial with insert. The plasma extract was diluted by adding 100 μL of the 50 mM ammonium actetate buffer and subsequent vortex-mixing of the contents of the capped vial. From dried blood spots, a 0.25-in. diameter disc was punched out of the filter card, ensuring that an area completely filled with blood was obtained. The punched-out disc was transferred to a 2 mL Eppendorf reaction vial and 200 μL of the dried blood spot extraction solution was added. The vial was then sonicated for 60 min. at room temperature and 180 μL of the extract was directly transferred to an autosampler vial with insert. Drug-free PBMC pellets were resuspended with 200 μL of the cell extraction solution containing the calibration standards or validation samples. PBMCs from patient samples were resuspended with 200 μL lysing solution containing only the IS. After sonication of the suspension for 60 min, the suspension was centrifuged for 10 min at 23,100 × *g* and 180 μL of the supernatant was then transferred to an autosampler vial with insert.

2.6. Patient sampling

Plasma samples were obtained by venipuncture and collection of blood in EDTA-anticoagulated collection tubes. After collection, the tubes were centrifuged at 600 × *g* for 10 min for separation of plasma and erythrocytes and the plasma fraction was stored at −20 °C. For dried blood spot sampling, a drop of blood was obtained with a fingerprick made in the fingertip with a disposable lancet. After puncture of the skin, the finger was gently massaged from palm to finger. The first drop of blood was discarded and the second drop of blood was collected on a filter card and left to dry overnight.

PBMCs from patients were isolated using Vacutainer CPT tubes. The CPT tube contains citrate as anticoagulant, a gel layer, and a Ficoll solution for separation of mononuclear cells from other blood components. After collection, the tube was centrifuged at 1650 × *g* for 30 min at ambient temperature, within 2 h after collection. The thin mononuclear cell layer was then resuspended with the plasma and transferred to a 14 mL polypropylene tube on an ice bath. The PBMC suspension in plasma was centrifuged for 10 min at 600 × *g* at 4 °C for 10 min. Thereafter, the supernatant was removed and the cells were washed twice in 14 mL ice-cold PBS and subsequently centrifuged for 10 min at 600 × *g* at 4 °C to remove extracellular etravirine. Before the last centrifugation step a 300 μL aliquot of the 14 mL cell suspension was used for cell counting and ultimately the cell pellet was stored at −20 °C.

2.7. Validation procedures

Validation of the method was performed according to the FDA guidelines for Bioanalytical Method Validation [10].

2.7.1. Linearity

Calibration standards were prepared in duplicate for each run and analysed in three independent runs. Calibration curves

Table 1
Intra- and inter-assay performance.

Nominal concentration in plasma (ng/mL)	Accuracy in plasma (%)	Mean intra-assay precision in plasma (%)	Mean inter-assay precision in plasma (%)	Nominal concentration in dried blood spots (ng/mL)	Accuracy in dried blood spots (%)	Mean intra-assay precision in dried blood spots (%)	Mean inter-assay precision in dried blood spots (%)	Nominal concentration in PBMC lysate (ng/mL)	Accuracy in PBMC lysate (%)	Mean intra-assay precision in PBMC lysate (%)	Mean inter-assay precision in PBMC lysate (%)
25.0	89.2	8.9	9.6	50	108.2	15	20	5	91.9	7.4	8.3
62.5	106	5.6	5.6	100	96.7	10	12	15	105	4.5	7.3
1875	101	4.0	5.3	2500	94.7	6.8	9.7	1250	100	3.1	11
3750	96.3	1.9	14	7500	97.9	4.7	9.1	2000	99.1	3.7	11

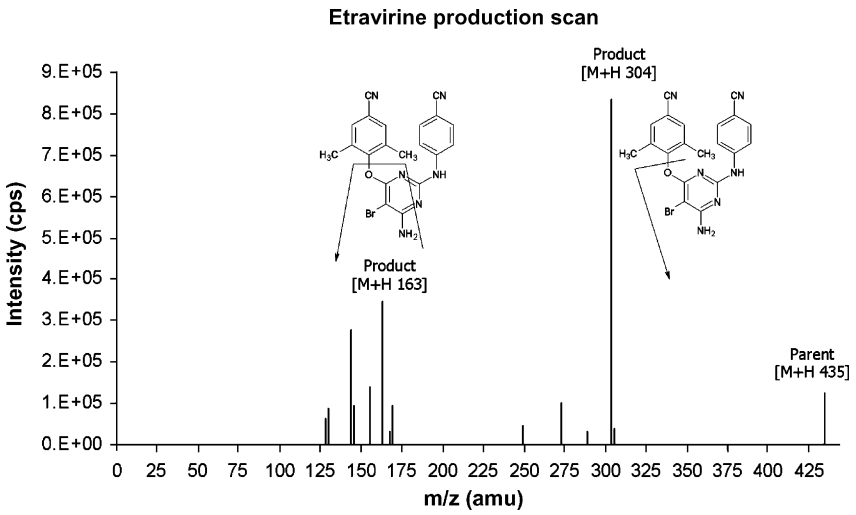

Fig. 1. Q3 product ion-scan of etravirine and proposed fragmentation routes.

(area ratio with the internal standard versus nominal concentration) were fitted by least-squares linear regression using 1/concentration^2 as weighting factor. To assess linearity, deviations of the mean calculated concentrations over three runs should be within 85–115% of nominal concentrations for the non-zero calibration standards. At the lower limit of quantitation (LLQ) level a deviation of 20% was permitted.

2.7.2. Accuracy and precision

Accuracies, intra- and inter-assay precision of the method were determined by assaying five replicates of each of the validation samples at the LLQ, low, mid and high concentration range in three separate runs. Accuracy was measured as the percentage of the concentration found as calculated with the calibration standards. The intra- and inter-assay precisions (relative standard deviation) should not exceed 15%, except for the LLQ, where 20% deviation was allowed.

2.7.3. Extraction recovery

Extraction recoveries were determined at three concentration levels in triplicate. The protein precipitation recoveries from plasma were calculated by comparing the analytical results for extracted samples with those in academic solutions in triplicate. For the determination of the extraction efficacy from dried blood spots, a dried blood spot containing 25 μL of blood was not punched out, but completely extracted in 400 μL of extraction solution. The recovery from dried blood spots was calculated as the fraction of the extracted amount from the dried blood spot compared with the same amount of analyte spiked to 400 μL of extraction solution. No real recovery could be determined for etravirine from PBMCs, because spiking of an exact amount to an individual PBMC is impossible and because in vitro accumulation not necessarily reflects in vivo accumulation.

2.7.4. PBMC matrix effect

The amount of cells in clinical PBMC samples varies from sample to sample due to natural variation in the number of circulating PBMCs. A clinical cell pellet may therefore not always contain the same amount of cells as during validation conditions. We therefore investigated the matrix effect of PBMC extract on analytical results in triplicate at three concentration levels in three different amounts of cells. Cell pellets containing either 0.5×10^6 or 20×10^6 cells were prepared, a range covering the amount of cells that can be recovered from 8 mL of whole blood. When at all concentration levels in every cell pellet the amount of recovered analyte was within 85–115% of nominal concentrations, the amount of cells in the pellets was supposed not to influence the analytical results in the range of $(0–20) \times 10^6$ cells.

2.7.5. Specificity and selectivity

For the determination of selectivity and specificity six different batches of blank plasma, dried blood spots or PBMC pellets as well as six batches of all three different matrices spiked at the LLQ level were investigated. Areas of peaks co-eluting with the analytes should not exceed 20% of the area at the LLQ level. The deviation of the nominal concentration for the LLQ samples should be within ±20%.

2.7.6. Ion-suppression

The ion-suppression of the three matrices was examined by the simultaneous post-column infusion of etravirine and the $^{13}C_6$-efavirenz. A solution containing 1 μg/mL etravirine and $^{13}C_6$-efavirenz was infused at a flow rate of 10 μL/min during the chromatographic analysis of double blank extracts. The chromatographic signals of injections of eluent were compared with the chromatographic signals of injections of plasma extracts for each MS/MS transition.

2.7.7. Stability

Stability of etravirine was investigated during various steps of the analysis. This included the stability of etravirine in stock solutions. The stability of the stock solution after 1 month of storage at −20 °C was investigated in triplicate. The stock solution was considered stable when 95–105% of the nominal concentration was found when compared with a freshly prepared stock solution. Also, the stability of etravirine in plasma was assessed in triplicate at three concentration levels at room temperature, 40 min at 60 °C (for HIV deactivation) and after three freeze–thaw cycles. The stability of etravirine in dried blood spots was assessed in fivefold after 1 week of storage at 30 °C. As no PBMCs could be spiked with an exact amount of etravirine, no stability experiments in PBMCs could be performed.

The reinjection reproducibility was assessed as well as the stability the final extracts in all matrices after storage at 4 °C at three concentration levels in triplicate. Re-injection was found to be reproducible when 85–115% of the nominal concentration was recovered. Etravirine in the final extract was considered to be stable

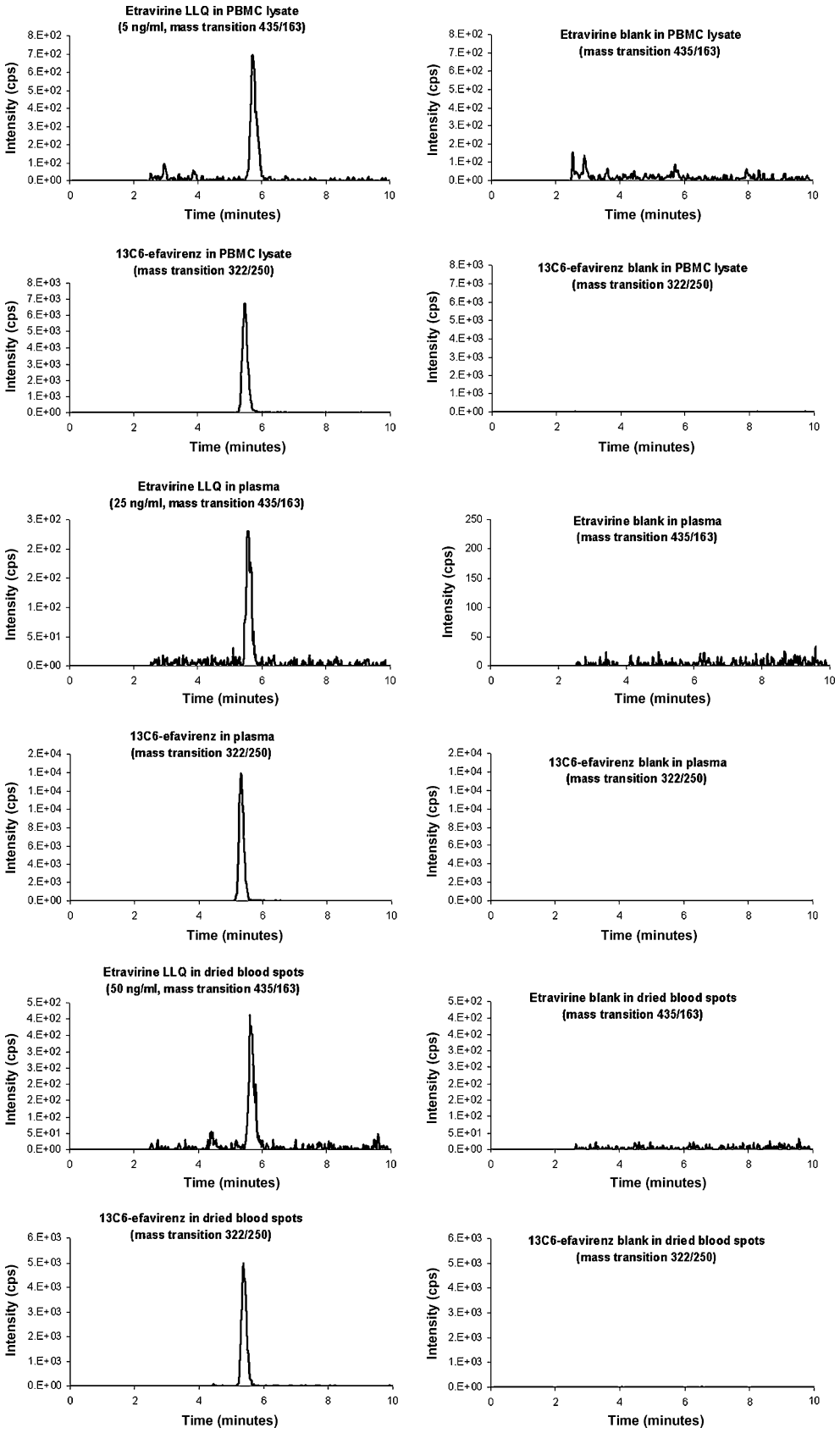

Fig. 2. Chromatograms of etravirine and $^{13}C_6$-efavirenz in plasma, dried blood spots and PBMCs.

Table 2
Extraction recovery for etravirine analysis in plasma and dried blood spots.

Nominal concentration in plasma (ng/mL)	Recovery from plasma (%) ($n=3$)	S.D. (%)	Nominal concentration in dried blood spots (ng/mL)	Recovery from dried blood spots (%) ($n=3$)	S.D. (%)
62.5	88.1	17	100	73.8	6.0
1875	93.6	3.2	2500	73.4	8.5
3750	87.4	1.5	7500	71.3	1.4

when 85–115% of the nominal concentrations was recovered when compared with freshly prepared calibration standards.

3. Results and discussion

3.1. MS/MS optimisation

When optimising MS and MS/MS conditions for etravirine, in the Q1 spectrum two fragments of etravirine were observed at m/z of 435 and 437, caused by the two naturally occurring bromo-isotopes. Fig. 1 shows the Q3 spectrum when etravirine ($[M+H]^+$ m/z 435) was subjected to collision-induced dissociation and the proposed fragmentation pathways for the two major fragments. For multiple reaction monitoring (MRM), the mass transitions m/z of 435 → 304, 435 → 163, 437 → 306 and 437 → 165 were monitored. The isotope of etravirine with a mass m/z of 435 gave ion products with the highest intensity in MRM. Although both mass transitions of m/z 435 → 304 and 435 → 163 produced chromatograms in MRM with similar signal-to-noise ratios, the mass transition of m/z 435 → 163 was found to give a more robust signal and was ultimately chosen for drug quantification. The chosen mass transition for $^{13}C_6$-efavirenz was m/z 322 → 250, whose fragmentation pathway of has been previously described by us [6]. Detection of the two major etravirine metabolites, the mono- and dimethyl hydroxylation products of the dimethyl benzonitrile moiety of etravirine, was previously described by Scholler-Gyure et al. [3] using the mass transitions m/z 451 → 353 and m/z 469 → 369 for the mono- and dihydroxylated products of etravirine, respectively. These mass transitions were included in the MRM-method for metabolite screening.

3.2. Chromatography

Etravirine could successfully be included in an assay previously developed by us, for the quantification of the NNRTIs and PIs in plasma and dried blood spots. In this system, etravirine eluted from the column 5.7 min after injection. In the previously developed LC–MS/MS method, four internal standards were already incorporated, including $^{13}C_6$-efavirenz. Best assay performance was observed with $^{13}C_6$-efavirenz, and this internal standard was used for further validation. $^{13}C_6$-efavirenz eluted at 5.4 min after injection. Fig. 2 shows the chromatograms of etravirine at the LLQ level (25 ng/mL in plasma, 50 ng/mL in dried blood spots and 5 ng/mL in PBMC lysate) the internal standard and chromatograms of their respective blanks in the different matrices. The chromatographic carry-over of etravirine from a highest calibration standard was less than 10% of the response of a sample at the LLQ level and therefore not considered to influence the analytical results.

Furthermore, in patient plasma samples in the mass transitions for the etravirine metabolites, the mono- and dihydroxylated etravirine products eluted at approximately 4.8 and 4.2 min, respectively. Although we did not have reference substance of the metabolites available for quantification or for confirmation of identity, it shows that the developed method may be useful for metabolic profiling of etravirine as well.

3.3. Method validation results

3.3.1. Linearity

The assay was linear over the tested concentration ranges in the different matrices (25–5000 ng/mL in plasma, 50–10,000 ng/mL in dried blood spots and 5–2500 ng/mL in cell lysate). The intra- and inter-assay performance data are presented in Table 1.

3.3.2. Accuracy and precision

Assay performance data for etravirine in the different matrices are summarised in Table 1. Intra- and inter-assay accuracies are defined in terms of relative error with a precision in terms of relative standard deviation, all determined at four concentration levels. The intra-assay accuracy was within all predefined limits at all concentration levels in all matrices.

3.3.3. Extraction recovery

Extraction recoveries from plasma and dried blood spots are shown in Table 2. As observed, the extraction from plasma and dried blood spots was high and reproducible at all concentrations.

3.3.4. PBMC matrix effect

Table 3 shows the recovered concentrations at three levels in triplicate in three different batches containing different amount of cells. As shown, all recovered concentrations were within 85–115% of nominal concentrations. Therefore, in the range of $(0–20) \times 10^6$ cells, all clinical samples, calibration standards and quality control samples can be treated with an equal amount of cell extraction solution. The similar response in the validation samples containing 0 cells (absence of matrix) compared with the response in PBMC lysate containing matrix also indicated that ion-suppression did not influence the analytical results.

3.3.5. Specificity and selectivity

The analytical method was shown to be specific and selective. No interfering peaks co-eluting with the analyte were observed in the six different batches of plasma, blood and PBMCs, neither did the recovered concentrations of the samples spiked at the LLQ level in the different batches of all matrices exceed the 80–120% accuracy limits.

Table 3
PBMC matrix effect. Recovered concentrations at three concentration levels with three different amounts of cells in the PBMC pellet.

Nominal concentration (ng/mL)	Cell amount in pellet (10^6 cells)	Recovered concentration compared to nominal (%) $n=3$	S.D. (%)
15	0	105	4.0
	5	103	2.0
	20	111	3.1
1250	0	97.7	2.0
	5	98.7	1.3
	20	102	3.3
2000	0	93.9	4.3
	5	95.7	1.2
	20	101	2.8

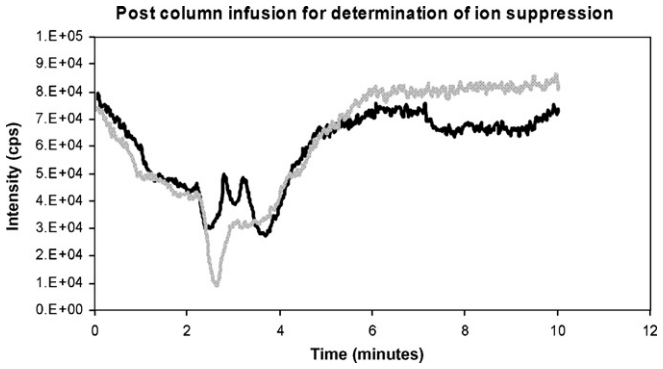

Fig. 3. Chromatograms typical overlay of the observed signals of injection of a blank PBMC extract and after injection of eluent at the mass transition of etravirine. The black line shows signal when eluent was injected and the grey line represents the signal of a PBMC extract injection.

3.3.6. Ion-suppression

Fig. 3 shows a typical overlay of the observed signals of injection of a blank plasma extract and after injection of eluent at the mass transition of etravirine. The black line shows the signal when eluent was injected and the grey line represents the signal of a PBMC extract injection. As observed, most ion-suppression is seen 2–4 min after injection. In the elution window of etravirine (5.5–6.5 min) no difference is observed between the two signals, indicating that endogenous interferences do not influence the analysis of etravirine. Similar signals were observed $^{13}C_6$-efavirenz as well as after injections of blank dried blood spot and plasma extracts.

3.3.7. Stability

Etravirine was proven to be stable in DMSO for at least 1 month when stored at −20 °C. Long-term stability studies of etravirine in stock solution are currently ongoing.

Stability of etravirine in plasma at different storage conditions is shown in Table 4A. As observed, etravirine was stable in plasma after three freeze–thaw cycles, 4 days storage at ambient temperature and after HIV deactivation for 40 min at 60 °C. Also, the stability etravirine of in dried blood spots when subjected to storage for 7 days at 30 °C was sufficient, as shown in Table 4B.

Furthermore, reinjection reproducibility and stability in the final extracts of plasma, dried blood spots and PBMC lysate were assured for at least 2 days when stored at 4 °C.

3.3.8. Application of the method

A pharmacokinetic curve of etravirine in plasma and dried blood spots from one patient, during a 12-h dosing interval is depicted in Fig. 4. Etravirine was dosed 200 mg twice daily. The obtained drug concentration versus time curves shows the utility of the method for application in pharmacokinetic studies and the adequacy of the selected calibration ranges.

As observed in Fig. 4, dried blood spot concentrations do not necessarily have to be the same as plasma concentrations. This may imply that etravirine might accumulate in red blood cells, or that capillary blood concentrations do not have to be the same as venous blood concentrations. Using the developed quantitative assay for etravirine in plasma, dried blood spots and PBMC lysate, the relationship between plasma, dried blood spot and cell-associated drug concentrations can now be determined.

Table 4B
Stability of etravirine in dried blood spots.

Nominal concentration (ng/mL)	Mean recovery after 7 days storage at 30 °C (%) (n = 5)	R.S.D. (%)
100	97.6	9.1
2500	89.7	3.8
7500	98.8	6.6

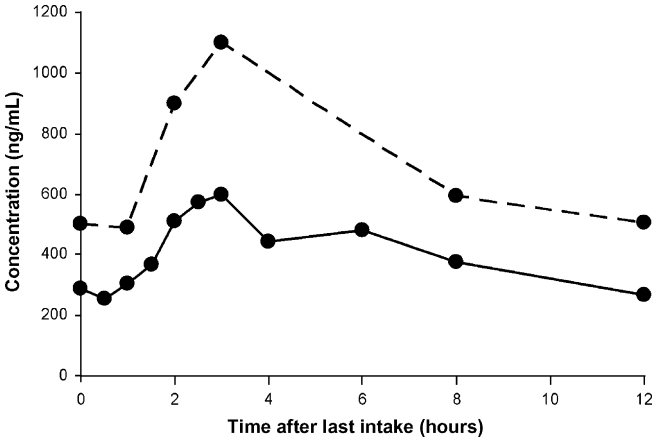

Fig. 4. Steady-state drug concentration versus time curve in plasma (solid line) and dried blood spots (dashed line) in a HIV-infected patient taking 200 mg etravirine twice daily.

4. Conclusion

A sensitive and fast assay for the quantification of etravirine in plasma, dried blood spots and PBMC lysate by means of LC–MS/MS was successfully developed and validated. The method was proven to be robust, precise and accurate. The low sample volume of 50 μL used for the plasma determination of etravirine allows quantification from plasma samples that are already collected for routine clinical care, without the need for collection of extra blood.

Because etravirine could be successfully included in a previously developed assay, the method allowed simultaneous quantification of etravirine with the PIs amprenavir, atazanavir, darunavir, indinavir, lopinavir, nelfinavir, the active nelfinavir metabolite M8, ritonavir, saquinavir and tipranavir as well as the NNRTIs efavirenz and nevirapine from the same sample using the same setup, thereby reducing analysis time and costs [6,7]. Furthermore, the method could be used for detection of the main etravirine metabolites and the used chromatographic conditions allow inclusion of a wide range of different analytes into the same method.

Table 4A
Stability of etravirine in plasma.

Nominal concentration (ng/mL)	Mean recovery after 3 freeze–thaw cycles (%) (n = 3)	R.S.D. (%)	Mean recovery after 4 days storage at ambient temperature (%) (n = 3)	R.S.D. (%)	Mean recovery after HIV inactivation (40 min at 60 °C) (%) (n = 3)	R.S.D. (%)
62.5	96.9	7.3	102	3.6	105	1.6
1875	96.7	4.9	106	0.5	99.4	1.9
3750	86.2	0.6	87.8	2.8	87.7	4.6

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