Journal of Chromatography B, 867 (2008) 205–212

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of protease inhibitors and non-nucleoside reverse transcriptase inhibitors in dried blood spots by liquid chromatography–triple quadrupole mass spectrometry

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ARTICLE INFO

Article history:
Received 20 February 2008
Accepted 2 April 2008
Available online 8 April 2008

Keywords:
Antiretroviral
HIV
Therapeutic drug monitoring
TDM
LC–MS
Chromatography
Mass spectrometry
Dried blood spots
Dried blood spot
Bioanalysis
Pharmacokinetics
Protease inhibitors
Non-nucleoside reverse transcriptase inhibitors
Atazanavir
Darunavir
Lopinavir
Ritonavir
Efavirenz
Nevirapine

ABSTRACT

A bioanalytical method for the determination of most commonly prescribed protease inhibitors (atazanavir, darunavir, lopinavir and ritonavir) and non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine) was developed and validated according to FDA guidelines. In brief, dried blood spots were punched out of a collection paper with a 0.25 in. diameter punch. The analytes were extracted from the punched-out disc using a mixture of acetonitrile, methanol and 0.2M zinc sulphate in water (1:1:2, v/v/v) containing the internal standards dibenzepine, 13C6-efavirenz and D5-saquinavir. 20 μL of the extract was injected onto the reversed-phase C18 column (150 mm × 2.0 mm) for separation from endogenous compounds and the analytes were quantified using a triple quadrupole mass spectrometer. The analytical run time was only 10 min. Validated concentration ranges covered the ranges encountered in routine clinical practice. The assay was linear over the concentration ranges tested (0.1–20 mg/L for atazanavir, lopinavir, nevirapine and efavirenz and 0.05–10 mg/L for darunavir and ritonavir). Accuracies and inter- and intra-run precisions at all levels ranged from 96.2 to 113.9% and 3.1 to 13.3%, respectively. Analytes in dried blood spots were stable for at least 7 days at 30 °C. The method enabled patient-friendly sample collection, easy and cheap sample shipment and non-hospital based sampling for therapeutic drug monitoring and pharmacokinetic studies.

1. Introduction

Routinely, drug concentrations for therapeutic drug monitoring (TDM) or pharmacokinetic studies are measured in plasma that is obtained by venipuncture at the clinic. However, sampling blood for TDM on specimen collection cards has several advantages over venous blood sampling. First of all, sampling of blood on collection cards allows sampling outside the hospital, for example, in resource-limited settings or for self-sampling by patients at home. Moreover, a drop of blood on a collection card can be obtained with a simple fingerprick or heelprick. This allows easy sample collection in populations where intensive sampling by means of venipuncture may be unethical or difficult, for example, in children, neonates and intravenous drug users who are suffering from phlebitis or abscesses at injection sites. Lastly, dried blood spots can be easily stored or transported without the requirements of special storage [1]. This allows easy and cheap shipment of samples throughout the world.

Bioanalytical methods describing the quantitative analysis of drugs in dried blood spots are rare [2–4]. Only one method describing the analysis of antiretroviral drugs has been previously published. The latter method described the analysis of antiretro-

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doi:10.1016/j.jchromb.2008.04.003

viral drugs in dried blood spots. However, two different ionization methods were used to quantify the antiretroviral drugs and the protease inhibitor darunavir was not yet available and included [5]. In this study, we describe the validation of a bioanalytical method by means of high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) for the simultaneous quantification of the most commonly used protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) including the novel protease inhibitor darunavir in dried blood spots using a one ionization method in a single run.

2. Experimental

2.1. Chemicals and materials

Efavirenz originated from Merck Sharp & Dohme (Haarlem, The Netherlands), atazanavir sulphate and 13C6-efavirenz from Bristol-Myers Squibb (Princeton, NJ, USA), dibenzepine hydrochloride from TEBU-BIO (Heerhugowaard, The Netherlands), lopinavir and ritonavir from Abbott Laboratories (Abbott Park, IL, USA), nevirapine from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA) and D5-saquinavir-mesylate from Roche Products, Research and Development (Welwyn Garden City, UK). Acetonitrile and methanol were HPLC-grade and obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, dimethylsulfoxide (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 blood with EDTA as anticoagulant was obtained from healthy human volunteers. Whatman 903 protein saver cards® for sample collection, 2 mL Eppendorf reaction vials, 1.5 mL autosampler vials and autosampler vial inserts 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 stock solutions, calibration standards and validation samples

Approximately 10 mg of each analyte were accurately weighted (after correction for the weight of counter ions and impurities) and dissolved in 10 mL of methanol (DMSO for nevirapine) in a volumetric flask to give a 1 mg/mL stock solution. Stock solutions of the internal standards D5-saquinavir, 13C6-efavirenz and dibenzepine were prepared in methanol in concentrations of approximately 0.4 mg/mL for D5-saquinavir and 1 mg/mL for 13C6-efavirenz and 1.5 mg/mL for dibenzepine.

The dried blood spot extraction solution containing the internal standards was prepared by adding 100 μL of the D5-saquinavir and dibenzepine and 200 μL of the 13C6-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). For the preparation of the highest calibration standard, 100 μL of the efavirenz, nevirapine, lopinavir, and atazanavir and 50 μL of the darunavir and ritonavir stock solutions were transferred to a 5 mL volumetric flask. The methanol was evaporated at 40 °C under a gentle stream of nitrogen gas. 0.2 mL of 50% methanol in water was used to dissolve the analytes again after evaporation. Thereafter, EDTA-anticoagulated human blood was added up to 5 mL to prepare the highest calibration standard. Serial dilutions of the highest calibration standard with the same drug-free blood followed to obtain a calibration range of 0.05–10 μg/mL for ritonavir and darunavir and a calibration range of 0.1–20 μg/mL for efavirenz, nevirapine, lopinavir, and atazanavir. Validation samples were similarly prepared at the lower limit of quantitation (LLQ) and at low, mid and high concentrations covering the chosen concentration ranges.

Calibration samples and validation samples were spotted on the filter cards by transferring 40 μL onto the card with a volumetric pipette. Thereafter, the blood spots were left to dry overnight at ambient temperature. Eluent A 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.

2.3. Chromatographic and mass spectrometric conditions

The method was based on a method previously developed by us, describing the simultaneous analysis of NNRTIs and PIs in plasma [6]. The concentration ranges were chosen based on the levels expected in routine clinical practice. The validation of the method was executed based on the most recent FDA guidelines for bioanalytical validation [8].

An Agilent (Agilent technologies, Palo Alto, CA, USA) HPLC system was used consisting of an 1100 series pump and cooled autosampler (4 °C). Separation was carried out on a Phenomenex Gemini C18 column (150 mm × 2.0 mm) with a Phenomenex Securityguard Gemini C18 precolumn (4.0 mm × 2.0 mm, particle size 5 μm) (Torrence, Ca, USA). The column outlet was connected to the electrospray 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 2.5 min of the eluent flow to waste to prevent the introduction of endogenous compounds into the mass spectrometer. A stepwise gradient was used to elute the compounds from the column. At time zero 20 μL of the dried blood spot 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 conditioned for 3 min with 85% eluent A mixed with 15% B before the next injection. The flow rate was 250 μL/min. The 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 nebulizer (1.8 L/min) and turbo (7.0 L/min) gasses were zero air, while curtain (1.3 mL/min) and collision activated dissociation gas (240×10^{12} molecules/cm^2) consisted of nitrogen (grade 5.0). The electrospray voltage was +4 kV and the dwell time was 50 ms with a 5 ms pause between scans. Q1 and Q3 were operating at unit mass resolution. Precursor ions of all analytes and internal standards were determined from spectra obtained during the infusion of standard solutions using an infusion pump connected directly to the electrospray source inlet. The precursor ions were subjected to collision induced dissociation to determine the product ions. Multiple reaction monitoring (MRM) in positive mode was used for drug quantification. Data were processed by Analyst software (version 1.2, Sciex).

2.4. Patient sampling

For patient sampling, a drop of blood was obtained with a finger prick made in the top of the finger with a disposable lancet. After puncture of the skin, the finger was gently massaged from palm to finger and the drop of blood was collected on a filter card. The one-time determination of drug concentrations in dried blood spots, by means of a blood spot sampling was considered exempt from review by our institutional review board.

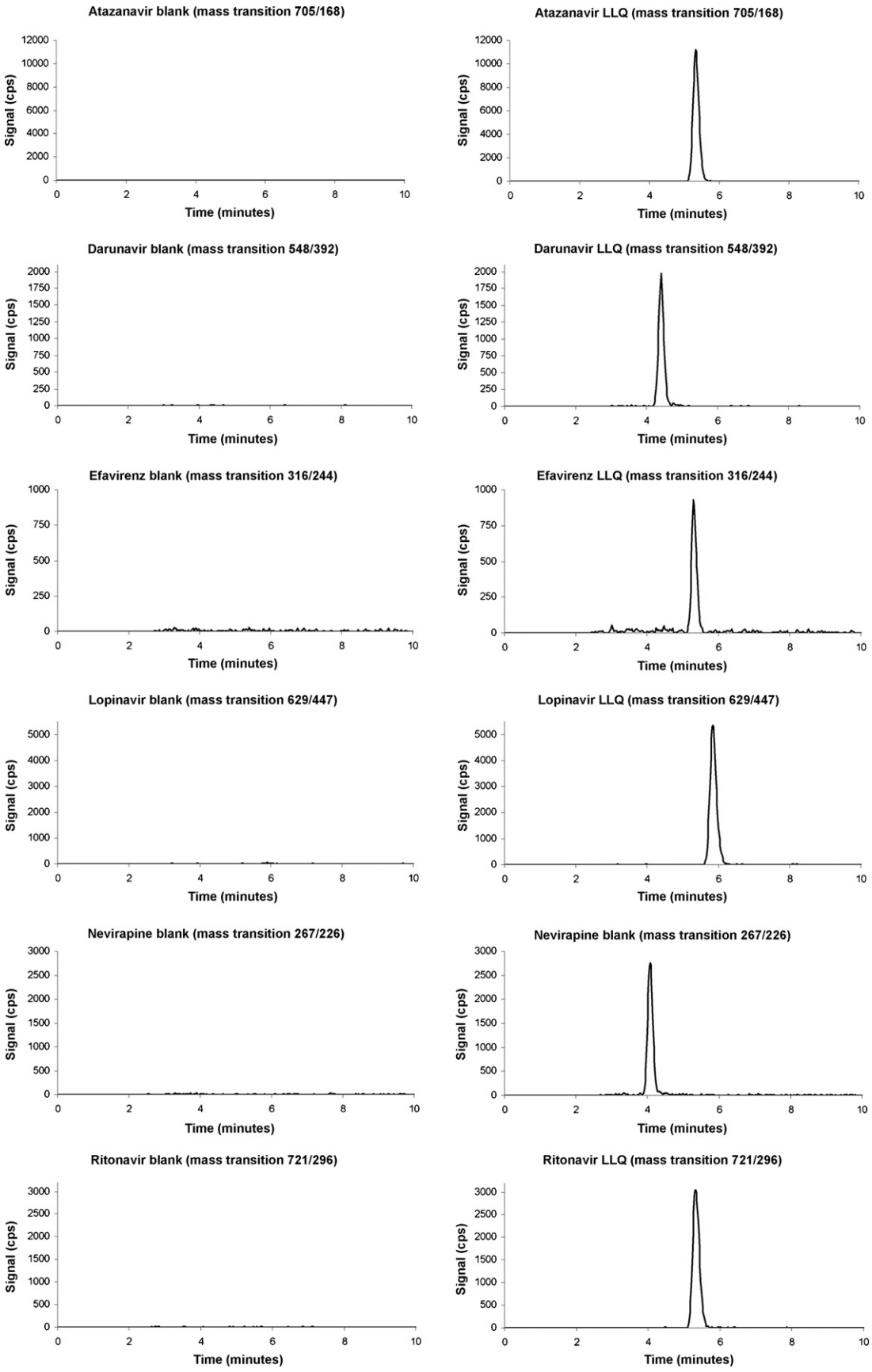
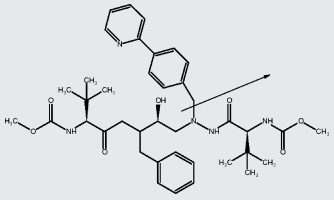
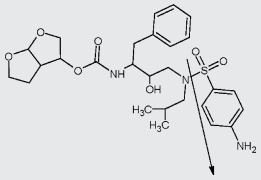
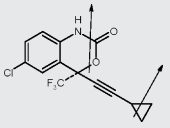
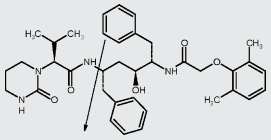
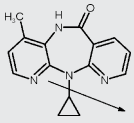
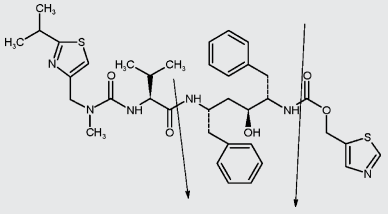
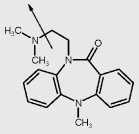
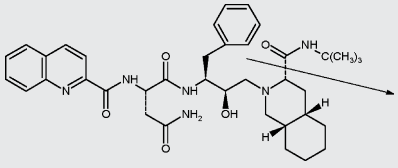


Fig. 1. Blank and LLQ chromatograms of all analytes.

Table 1
Selected transitions, proposed fragmentation pathways and retention times of all analytes and internal standards

Compound	Mass transition (*m*/*z*)	Proposed fragmentation pathway	Retention time (min)	Internal standard used
Atazanavir	705 → 168		5.2	D5-Saquinavir
Darunavir	548 → 392		4.5	D5-Saquinavir
Efavirenz 13C6-Efavirenz	316 → 244 322 → 250		5.2	13C6-Efavirenz
Lopinavir	629 → 447		5.7	D5-Saquinavir
Nevirapine	267 → 226		4.1	Dibenzepine
Ritonavir	721 → 296		5.2	D5-Saquinavir
Dibenzepine	296 → 251		3.8	–
D5-Saquinavir	676 → 575		5.5	–

2.5. Sample processing

A 0.25 in. diameter disc was punched out of the dried blood spot, 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 extraction solution was added. The vial was subsequently sonicated for 60 min and the extract was directly transferred to an autosampler vial with insert.

2.6. Validation procedures

All validations were performed according to the most recent published FDA guidelines for validation of bioanalytical assays [8].

2.6.1. Linearity

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

Table 2
Intra- and inter-assay performance data of all analytes at four concentration levels, $n = 5$

Analyte	Nominal concentration (mg/L)	Mean accuracy (%)	Mean intra-assay precision (%)	Inter-assay precision (%)
Atazanavir				
LLQ	0.0985	97.4	5.2	9.7
LOW	0.197	97.7	4.6	5.6
MID	4.93	100.2	4.1	3.3
HIGH	14.8	102.5	3.3	3.1
Darunavir				
LLQ	0.0500	105.3	6.8	12.7
LOW	0.100	96.2	4.9	8.1
MID	2.50	102.2	4.6	4.4
HIGH	7.50	113.9	4.0	4.7
Efavirenz				
LLQ	0.102	102.9	7.4	12.3
LOW	0.204	99.2	6.1	8.9
MID	5.11	104.0	5.5	4.7
HIGH	15.3	109.9	6.0	6.1
Lopinavir				
LLQ	0.107	103.5	5.4	10.0
LOW	0.213	97.8	4.4	6.0
MID	5.33	101.6	4.4	3.9
HIGH	16.0	109.2	4.0	3.9
Nevirapine				
LLQ	0.101	98.3	6.1	8.1
LOW	0.203	99.0	5.4	6.2
MID	5.07	104.2	4.1	4.4
HIGH	15.2	104.2	3.4	5.2
Ritonavir				
LLQ	0.0546	100.6	4.9	13.3
LOW	0.109	100.6	5.2	8.2
MID	2.73	102.5	3.9	3.6
HIGH	8.19	106.6	4.0	3.9

(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 LLQ level a deviation of 20% was permitted.

2.6.2. Accuracy and precision

Accuracy, 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 precision (relative standard deviation) should not exceed 15%, except for the LLQ, where 20% deviation was allowed.

2.6.3. Recovery

Extraction recoveries were determined at two concentration levels in triplicate. A dried blood spot containing 20 μL of blood was not punched out, but completely extracted in 0.4 mL of extraction solution. The recovery was calculated as the fraction of the extracted amount from the dried blood spot compared with the same amount of analyte spiked to the extraction solution.

2.6.4. Dried blood spot size

The relationship between dried blood spot area and the amount of blood spotted on the paper was examined by spotting increasing volumes of blood (10–70 μL) in duplicate on a collection paper and by measuring the area of the obtained spots. The relationship between dried blood spot surface area and the amount of blood spotted on the paper should be linear, representing equal distribution of blood on the collection paper.

The influence of dried blood spot size on recovery was assessed at two concentrations in triplicate by spotting two different volumes (20 and 60 μL) of blood on the collection cards. When all concentrations of punched-out dried blood spots of 20 and 60 μL were calculated on a calibration curve made with 40 μL punched-out blood spots were within 85–115%, spot size was supposed not to influence the recovery.

Table 3
Extraction recovery of all analytes at two concentration levels, $n = 3$

Analyte	Nominal concentration (mg/L)	Recovery (%)	Standard deviation (%)
Atazanavir			
LOW	0.197	103.6	15.9
HIGH	14.8	106.8	8.7
Darunavir			
LOW	0.100	94.4	8.7
HIGH	7.50	99.6	6.7
Efavirenz			
LOW	0.204	104.4	17.5
HIGH	15.3	101.8	11.8
Lopinavir			
LOW	0.213	108.5	21.3
HIGH	16.0	97.9	6.28
Nevirapine			
LOW	0.203	103.6	15.3
HIGH	15.2	100.1	4.8
Ritonavir			
LOW	0.109	100.6	12.8
HIGH	8.19	101.17	5.18

Table 4
Influence of amount on recovery of blood at two concentration levels, $n = 3$

Analyte	Amount of blood spotted on paper (μL)	Nominal concentration (mg/L)	Back calculated accuracy (%)	Standard deviation (%)
Atazanavir				
LOW	20	0.197	105.0	4.0
	60		99.4	1.5
HIGH	20	14.8	87.8	3.2
	60		89.9	5.9
Darunavir				
LOW	20	0.100	93.9	5.2
	60		94.3	3.9
HIGH	20	7.50	107.7	4.9
	60		111.3	8.1
Efavirenz				
LOW	20	0.204	95.4	5.7
	60		95.5	3.3
HIGH	20	15.3	97.7	4.9
	60		96.5	3.2
Lopinavir				
LOW	20	0.213	93.9	5.2
	60		94.3	3.9
HIGH	20	16.0	107.7	4.9
	60		111.3	8.1
Nevirapine				
LOW	20	0.203	103.3	6.2
	60		103.1	6.4
HIGH	20	15.2	90.5	3.8
	60		89.4	2.7
Ritonavir				
LOW	20	0.109	104.0	3.5
	60		99.9	3.8
HIGH	20	8.19	90.2	3.3
	60		93.3	6.0

2.6.5. Selectivity, specificity and ion suppression

Out of six different batches of blank human blood, dried blood spots were prepared and subsequently processed. The ion suppression was assessed by the simultaneous post-column infusion of a solution containing all analytes and internal standards at a concentration of 1 μg/mL into the detector during the chromatographic analysis of extracts of the six different batches of dried blood and the subsequent analysis of eluent injections. Ion suppression was supposed to be acceptable when in the observed signal of the blank extracts and the eluent were comparable in the elution window of the analyte or internal standard.

Moreover, analysis of the blank extracts of the six different batches of dried blood without post-column infusion was performed to ensure that no interfering peaks were found in the elution windows of the analytes and internal standards.

2.6.6. Stability

Stability of the analytes was investigated at various concentrations during all steps of the analysis. This included stability of the analytes in the dried blood spots and in the final extract. Stability of all analytes in stock solutions has been investigated before [9–14].

Stability of the analytes in dried blood spots were assessed at two concentrations in five-fold for 7 days of storage in a stove at 30 °C. The processed sample stability of all analytes in the final extract was studied at three concentrations in triplicate by comparing a set of validation samples made 3 days earlier and kept

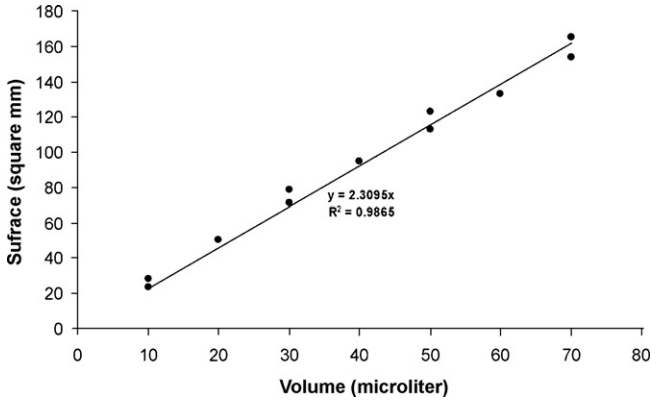


Fig. 2. Relation between amount of blood and spot size.

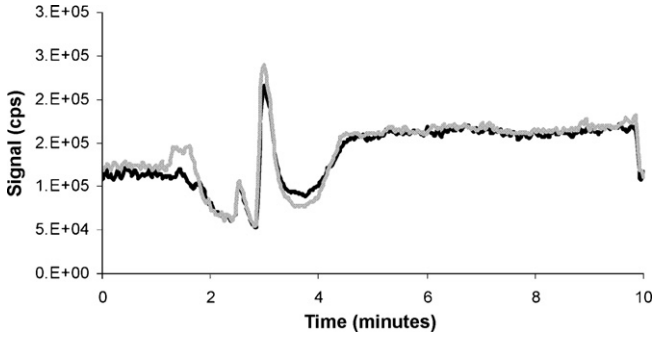


Fig. 3. Typical overlay of the observed signals of injection of a blank dried blood spot extract and eluent in the mass transition of darunavir during post-column infusion of the analytes and internal standards. The grey line shows the signal when a blank dried blood spot extract was injected, the black line represents the signal when eluent A was injected.

Table 5
Stability of all analytes in dried blood spots during 7 days storage at 30 °C

Analyte	Nominal concentration (mg/L)	Recovered concentration in dried blood spots after 7 days storage at 30 °C (%)	Standard deviation (%)
Atazanavir			
LOW	0.197	99.4	3.0
HIGH	14.8	103.9	7.6
Darunavir			
LOW	0.100	99.7	3.2
HIGH	7.50	102.2	6.1
Efavirenz			
LOW	0.204	100.9	6.0
HIGH	15.3	112.3	7.2
Lopinavir			
LOW	0.213	95.6	8.5
HIGH	16.0	99.1	6.9
Nevirapine			
LOW	0.203	93.6	6.4
HIGH	15.2	101.4	4.9
Ritonavir			
LOW	0.109	92.2	6.0
HIGH	8.19	99.8	10.1

refrigerated at 4 °C with new calibration standards. Long-term stability studies of all analytes in dried blood spots is currently ongoing.

The analytes were considered to be stable in the biological matrix and in the final extract when 85–115% of the initial concentration was found.

3. Results and discussion

3.1. Method development

Molecular formulas, proposed fragmentation pathways, retention times and monitored mass transitions of all analytes and internal standards have been previously described by us and are summarized in Table 1 [6,9,15].

For extraction of the analytes from dried blood spots different methods were initially tested. Analytes could be successfully extracted from dried blood spots using an organic solvent like methanol or acetonitrile. To prevent solvent effects during chromatography, an extra dilution or evaporation step subsequently followed by reconstitution in eluent was necessary. Koal et al. [5] previously described the one step extraction of analytes from dried blood spots by using a mixture of 50% methanol in a 0.2M zinc sulphate solution in water. This method allowed direct extraction and subsequent injection of the analytes. This method was slightly adapted and a final mixture of methanol, acetonitrile and 0.2M zinc sulphate in water (25:25:50, v/v/v) containing the internal standards was used.

When the signal of a validation sample at the LLQ level was compared with blank dried blood spot control sample an excellent signal-to-noise ratio was observed, as can be observed in the representative multiple reaction monitoring (MRM) chromatograms of all analytes at the LLQ together with their respective blanks in Fig. 1.

3.2. Validation results

3.2.1. Linearity

The assay was linear over the tested concentration ranges. Accuracies and inter- and intra-run precisions at all levels ranged from 96 to 114% and 3.1 to 13.3%, respectively. The intra- and inter-assay performance data are presented in Table 2.

Table 6
Stability of all analytes in the final extract

Analyte	Nominal concentration (mg/L)	Mean recovered concentration after 3 days storage at 4 °C (%)	Standard deviation (%)
Atazanavir			
LOW	0.197	97.3	4.7
MID	4.93	102.5	5.9
HIGH	14.8	103.0	1.0
Darunavir			
LOW	0.100	92.4	5.6
MID	2.50	104.7	7.6
HIGH	7.50	115.0	1.0
Efavirenz			
LOW	0.204	105.3	7.6
MID	5.11	108.3	7.8
HIGH	15.3	111.3	1.5
Lopinavir			
LOW	0.213	96.4	3.2
MID	5.33	101.9	5.7
HIGH	16.0	106.7	0.6
Nevirapine			
LOW	0.203	95.4	6.0
MID	5.07	98.4	5.9
HIGH	15.2	99.9	1.1
Ritonavir			
LOW	0.109	100.8	7.3
MID	2.73	104.3	5.9
HIGH	8.19	106.7	1.2

3.2.2. Recovery

Table 3 shows the calculated recoveries of all analytes. All recoveries were within the range of 94–109% and reproducible.

3.2.3. Dried blood spot size

At least 15 μL of blood was necessary to obtain a blood spot with a diameter of 0.25 in. on both sides of the collection paper. 70 μL of blood was necessary to fill the pre-drawn circle on the collection paper. A linear relationship between dried blood spot area in mm^2 versus the volume of blood in μL applied to the collection paper was observed in the range of 10–70 μL (Fig. 2). This could be described with the formula $y = 2.3095x$ with a regression coefficient of 0.987, where y is the blood spot area in mm^2 and x is the amount of blood in μL.

As observed in Table 4, back calculated accuracies from 20 and 60 μL blood spots at two concentration levels were all within 85–115% limits, indicating that the amount of blood spotted on the filter paper did not influence the amount of analyte present in the punched-out disc.

3.2.4. Selectivity, specificity and ion suppression

Fig. 3 shows a typical overlay of the observed signals of injection of a blank dried blood spot extract and eluent in the mass transition of darunavir during post-column infusion of the analytes and internal standards. The grey line shows the signal when a blank dried blood spot extract was injected, the black line represents the signal when eluent A was injected. Similar overlays could be observed for all analytes and internal standards (not shown). Usually, some ion suppression was observed 2–4 min after injection, depending on the analyte. No difference in signal was observed at the time of elution of the analytes, indicating that there was no significant ion-enhancement or suppression at the elution time. Furthermore no

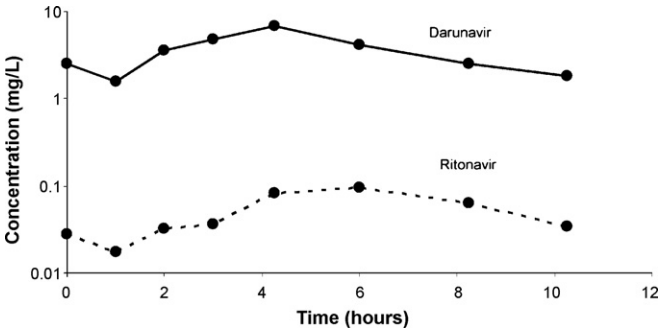


Fig. 4. Full pharmacokinetic curve of darunavir and ritonavir in dried blood spots in a treatment-experienced HIV-1 infected patient on a regime containing tenofovirdisoproxilfumarate 245 mg once daily, lamivudine 300 mg once daily, ritonavir 100 mg twice daily, darunavir 300 mg twice daily and raltegravir 400 mg twice daily.

interfering peaks, interfering ion-suppression or ion-enhancement were found in 6 different batches of blood.

3.2.5. *Stability*

Stability of the analytes in dried blood spots is depicted in Table 5. All analytes were stable in dried blood spots for at least 7 days at 30 °C. Moreover, the processed sample stability of all analytes in the final extract was assured for 3 days at 4 °C, as can be observed in Table 6.

3.3. *Clinical application*

Fig. 4 shows the pharmacokinetic curves of darunavir and ritonavir co-administered in a HIV-infected patient during a 12 h dosing interval determined from dried blood spots.

4. Conclusion

A simple and rapid assay was developed and validated for the simultaneous determination of the antiretroviral drugs atazanavir, darunavir, efavirenz, lopinavir, nevirapine and ritonavir in dried blood spots by means of LC–MS/MS. The validation was based on the most recent FDA guidelines for bioanalytical validation. The method has shown to be accurate, precise and robust. Stability of all the compounds in dried blood spots for a week at 30 °C indicated that collection of dried blood spots is feasible in resource-limited settings where refrigeration is not possible. Moreover, the equal distribution of blood on the collection paper enables sampling without accurate pipetting on filter cards. Sampling by means of a fingerprick or heelprick is, therefore, possible. The chromatographic system using a quick stepwise gradient provides inclusion of a wide range of other analytes. Thus far, analysis of antiretroviral drugs in dried blood spots has been validated. However, the method opens up future possibilities for the analysis of dried plasma spots, enabling cheap shipment of plasma samples as well.

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