Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 451–458

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Quantification of the HIV-integrase inhibitor raltegravir and detection of its main metabolite in human plasma, dried blood spots and peripheral blood mononuclear cell lysate by means of high-performance liquid chromatography tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 25 September 2008
Received in revised form
19 November 2008
Accepted 20 November 2008
Available online 27 November 2008

Keywords:
Raltegravir
LC–MS/MS
HIV
Pharmacology
Dried blood spots

ABSTRACT

For the quantification of the HIV-integrase inhibitor raltegravir 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. The assay also allowed detection, but no quantification due to absence of reference substance, of the main metabolite, raltegravir–glucuronide.

Raltegravir 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 simple one-step extraction with a mixture of methanol, acetonitrile and 0.2 M zincsulphate 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. The analytical run time was 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 50–10,000 ng/mL in plasma and dried blood spots and a range of 1–500 ng/mL in PBMC lysate. Dibenzepine was used as the internal standard. The method was proven to be specific, accurate, precise and robust. Accuracies ranged from 104% to 105% in plasma, from 93% to 105% in dried blood spots and from 82% to 113% in PBMC lysate. Precision over the complete concentration range was less than 6%, 11% and 13% in plasma, dried blood spots and PBMC lysate, respectively. The method is now applied for therapeutic drug monitoring and pharmacological research in HIV-infected patients treated with raltegravir.

1. Introduction

Until now antiretroviral drugs have targeted the viral enzymes reverse transcriptase, protease or interfered with viral entry. Raltegravir (Fig. 1A) is a drug from a new class of antiretroviral drugs called integrase inhibitors. These agents target the viral enzyme that catalyzes insertion of viral DNA into the host genome [1]. Raltegravir has shown potent antiretroviral effect in treatment-experienced human immunodeficiency virus 1 (HIV-1) infected patients [2,3]. Based on promising results, the FDA and EMEA granted approval of raltegravir for the use of HIV-1 treatment in treatment-experienced adults [4,5].

Currently, determination of drug concentrations in plasma is the gold standard for purposes of therapeutic drug monitoring (TDM) or pharmacokinetic studies. 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 cheap shipment.

The site of action of raltegravir is within the infected cell. Cell-associated drug levels of raltegravir provide information on drug disposition in a compartment where HIV replicates and may therefore be useful in understanding its clinical pharmacology.

We here present the development and validation of a sensitve and fast assay for the determination of raltegravir in plasma, dried blood spots and peripheral blood mononuclear cell (PBMC) lysate by means of liquid chromatography coupled with electrospray tandem mass spectrometry (LC–MS/MS). Previously, 2 different assays

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doi:10.1016/j.jpba.2008.11.025

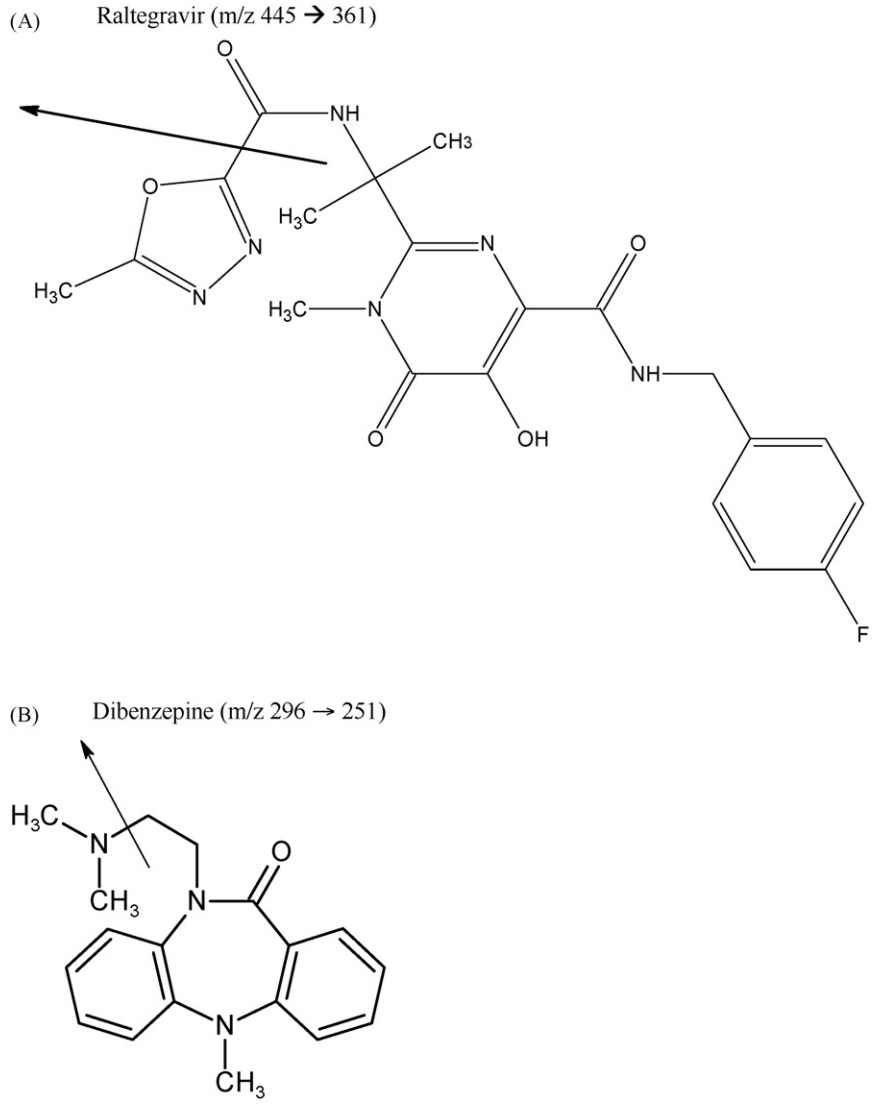

Fig. 1. Proposed fragmentation pathways of raltegravir (A) and the internal standard dibenzepine (B). (A) Raltegravir (m/z 445 → 361) and (B) dibenzepine (m/z 296 → 251).

for the determination of raltegravir in human plasma by means of LC–MS/MS have been described [6,7]. To the best of our knowledge, no assay for raltegravir has been previously published for the quantification of raltegravir in PBMC lysate or dried blood spots. Furthermore, contrary to the previous developed methods, our developed method uses the same sample pretreatment, chromatographic setup and detection as in methods previously described by us for the quantification of protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) from dried blood spots and plasma [8,9]. This allowed simultaneous quantification of raltegravir, PIs and NNRTIs from a single plasma or dried blood spot sample using the same setup, thereby reducing analysis time and costs when concentrations of multiple antiretroviral drugs have to be quantificated.

2. Experimental

2.1. Chemicals

Raltegravir potassium salt originated from Merck Sharp & Dohme (Haarlem, The Netherlands), dibenzepine hydrochloride was obtained from TEBU-BIO (Heerhugowaard, The Netherlands). 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 plasma, blood and buffy coat was 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. Chromatographic and mass spectrometric conditions

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) (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. The source temperature was maintained 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 300 ms with a 5 ms pause between scans. Q1 and Q3 were operating at unit mass resolution. Precursor ions of raltegravir and dibenzepine were determined from spectra obtained during the infusion of standard solutions using an infusion pump connected directly to the electrospray source inlet. The precusor ions were subjected to collision induced dissociation to determine the product ions. The transitions of the protonated precursor/product ions used for quantitation were from m/z 445 to 361 for raltegravir and m/z 296 to 251 for the internal standard dibenzepine. Multiple reaction monitoring (MRM) in positive mode was used for drug quantification. Data were processed by Analyst software (version 1.2, Sciex).

2.3. Preparations of standards and solutions

Stock solutions of raltegravir were prepared from independent weighting; one for calibration standards (CAL) and one for validation samples (VS). Approximately 10 mg of the analyte were accurately weighted (compound weighing was corrected for purity) and dissolved in 10 mL of methanol in a volumetric flask to give a 1 mg/mL stock solution. A stock solution of the internal standard dibenzepine hydrochloride was made in methanol in a concentration of approximately 1.5 mg/ml.

The plasma protein precipitation reagent containing the internal standard was prepared by mixing 149 mL methanol, 150 mL acetonitrile and 1 mL of dibenzepine stock solution. The dried blood spot extraction solution containing the internal standard was prepared by adding 100 μL of the dibenzepine stock solution 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 20 μL of the dibenzepine stock solution to 1000 mL of a 1:1 (v/v) methanol–water mixture. For the preparation of the highest calibration standards for dried blood spots and plasma determination, 50 μL of the raltegravir CAL stock solution, was added to a 5 mL volumetric flask. Human plasma or blood with EDTA as anticoagulant was added up to 5 mL in the same volumetric flask. Thereafter the contents of the volumetric flask were vortex mixed during 60 s. Serial dilutions with either plasma or blood of the highest calibration standard followed to obtain a calibration range of 50–10,000 ng raltegravir per mL. The choice for this 200-fold range was based on concentrations achieved in patients in pharmacokinetic studies of raltegravir and the ability for preparation of calibration standards from the highest calibration standard by serial dilutions with blank plasma or blood. The calibration and validation samples in blood were spotted on the filter cards by transferring 25 μL onto the card with a pipette. Thereafter, the blood spots were left to dry overnight at ambient temperature.

Calibration standards and validation samples for the determination of raltegravir in cell lysate were prepared in the cell extraction solution in a concentration range of 1–500 ng/mL.

Drug-free PBMCs were isolated from buffy coat as described before [10]. After isolation the cells were washed twice with 10 mL of icecold PBS, the number of cells was counted and aliquots of 5×10^6 cells were centrifuged in eppendorf reaction vials for 10 min at 650 × g. The supernatant was aspirated and the cell pellets were stored at −20 °C.

Eluent A was prepared by mixing 700 mL of methanol with 440 mL of 10 mM acetic acid and 860 mL of 10 mM ammoniumacetate solution in water. Eluent B consisted of 100% methanol.

2.4. Sample processing

2.4.1. Plasma sample processing

Analyte extraction was performed as previously described [8]. To 50 μL of plasma, 100 μL of protein precipitation reagent were added. After vortex mixing, samples were centrifuged at 23,100 × g for 10 min. A volume of 100 μL of the supernatant was then transferred to an autosampler vial with a 250 μL insert. The extracts were diluted by adding 100 μL of the 50 mM ammonium acetate buffer set at pH 5. After dilution, the vial was capped and its contents were vortex-mixed for 10 s.

2.4.2. Dried blood spot sample processing

Raltegravir was extracted using the same single step extraction method as previously used by us for extraction of PIs and NNRTIs from dried blood spots [9]. A 0.25-in. diameter disc was punched out of the dried blood spot, ensuring that an area completely filled with blood was obtained, corresponding to approximately 15 μL of blood [9]. 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 subsequently sonicated for 60 min and the extract was directly transferred to an autosampler vial with insert.

2.4.3. PBMC pellet sample processing

Drug-free PBMC pellets were resuspended with 200 μL of the cell extraction solution containing the calibration standards or validation samples. PBMC 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 transferred to an autosampler vial with insert.

2.5. Validation procedures

The validation of the assay was based on the FDA guidelines for bioanalytical method validation [11].

2.5.1. Response functions

Calibration curves were constructed by least-squares linear regression analysis without weighting and by using $1/x$ and $1/x^2$ (where x = concentration) as weighting factors. In order to establish the best weighting factor, back-calculated calibration concentrations were determined. The model with the lowest total bias and the optimal consistency of bias across the concentration range was used for further analysis and quantification.

2.5.2. Accuracy and precision

Accuracy and precision were determined by quantification of validation samples with analyte concentrations at the lower limit of quantification (LLQ) and in the low, mid and high concentration ranges of the calibration curves. Each validation sample was analysed in a minimum of 5 replicates in 3 separate analytical runs. The accuracy was defined as the percentage of the calculated concentration compared to the known concentration. The coefficient of variation was used to report the precisions. The intra- and inter-assay accuracies as well as the precisions should be within ±20% for the LLQ and ±15% for all other concentrations.

2.5.3. Specificity, selectivity and ion suppression

For plasma and PBMCs, six different batches of control drug-free plasma were prepared as double blanks and spiked at the LLQ level to determine whether endogenous compounds from plasma interfered with the detection of the analytes or the internal standards. 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%.

For dried blood spots, out of six different batches of blank human blood, blank spots were prepared and subsequently processed. The ion suppression was assessed by the simultaneous post-column infusion of a solution containing the analyte and internal standard at a concentration of 1000 ng/mL into the detector during the chromatographic analysis of extracts of the six different dried batches of dried blood spots and plasma and the subsequent analysis of eluent injections. Ion suppression was supposed to be negligible 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 analyte and internal standard.

2.5.4. Recovery

The protein precipitation recovery of raltegravir from plasma was determined in triplicate at three concentrations by comparing the analytical results for extracted samples with those of the spiked blank plasma extracts. Extraction recoveries from dried blood spots were determined at two concentration levels in triplicate. The recovery was calculated as the fraction of the extracted amount from a full dried blood spot of 20 μL compared with a similar amount of analyte spiked to the extraction solution. The influence of dried blood spot size on analyte recovery from the collection card was previously assessed. We have shown that in the range of 10–70 μL blood, the relationship between amount of blood and are of the dried blood spot is linear and that the amount of blood transferred to the collection card did not influence the analysis [9].

Total recovery could not be determined from PBMCs, since individual PBMCs cannot be spiked to an absolute amount in an in vitro environment. Therefore, no recovery experiments could be performed in PBMCs.

2.5.5. PBMC matrix effect

The amount of cells in clinical PBMC samples vary from sample to sample due to natural variation in the number of systemic circulating PBMCs and variation in cell recovery during isolation. We therefore investigated the matrix effect of PBMC extraction 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.

2.5.6. Stability

The stability of raltegravir in plasma has been previously thoroughly investigated by spiking blank plasma with raltegravir. Raltegravir was proven to be stable in plasma up to 23 months stored at −20 °C. Furthermore, raltegravir spiked to plasma was proven to be stable in plasma after three freeze-thaw cycles, HIV heat deactivation (90 min storage at 56 °C) and to be stable after storage at room temperature up to 5 h [6,7]. During a dosing interval, the amount of raltegravir–glucuronide is approximately 30% of to the total amount of raltegravir [12]. The stability of the raltegravir–glucuronide may influence the determination of raltegravir, as a result of hydrolysis of the glucuronide bond and formation of raltegravir. However, no stability tests have been performed with clinical material, also containing the raltegravir metabolite. We therefore tested the stability of a clinical patient sample in dried blood spots and plasma at different conditions in triplicate. Clinical dried blood spot samples were subjected to storage at ambient temperature and 30 °C for 1 week. Clinical plasma samples were subjected to storage at 4 °C and storage at ambient temperature during 7 days. The clinical samples were considered to be stable when after 1 week of storage at different conditions, 85–115% of the initial concentration was found. Currently, long-term stability studies are ongoing.

The processed sample stability of all analytes in the final plasma extract was studied at three concentrations in triplicate by comparing validation samples made 2 days earlier with new calibration standards. Moreover, the re-injection reproducibility was studied over a period of 3 days. The processed sample stability of raltegravir in the final extract of the dried blood spots was studied at three concentrations in triplicate by comparing a set of validation samples made 3 days earlier and kept refrigerated at 4 °C with new calibration standards. The analyte was considered to be stable in the final extracts when 85–115% of the initial concentration was found.

The stability of raltegravir stock solution in methanol, was investigated after 251 days of storage at −20 °C by comparing the stock solution with a freshly prepared stock solution. Raltegravir was considered to be stable at −20 °C in methanol when 95–105% of the initial amount was found.

3. Results and discussion

3.1. Method development

A previous method for the simultaneous determination of all currently approved non-nucleoside reverse transcriptase inhibitors and protease inhibitors was used as a starting point for the development for the raltegravir assay [8]. Dibenzepine was already used as an internal standard in this assay and performed well as an internal standard for raltegravir. Therefore no addition of a new internal standard was considered necessary. Molecular formulas and proposed fragmentation pathways of raltegravir and dibenzepine are shown in Fig. 1. Fragmentation and mass spectrometric conditions for dibenzepine have been previsouly described by us [8]. The positive-ion electrospray Q1 mass spectrum and the MS/MS product-ion spectrum of raltegravir is depicted in Fig. 2.

The most intense signal in the Q1 spectrum was seen at mass-to-charge (m/z) ratio 445.3, corresponding with the $[M+H]^+$ ion. In the product ion mass spectrum the most intense fragment was observed at m/z 361.0. This fragment is proposed to originate from the parent raltegravir molecule by cleavage of the oxadiazole moiety from the molecule as shown in Fig. 1.

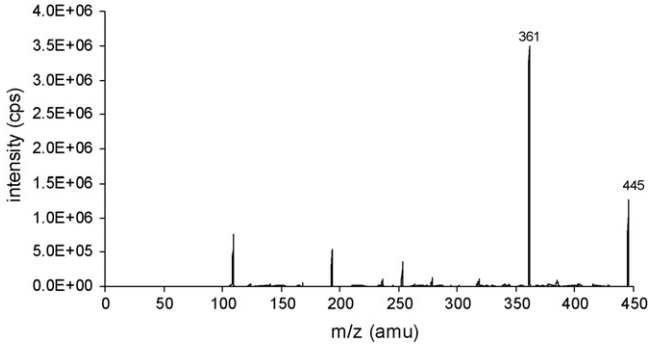

Fig. 2. Positive MS/MS product-ion spectrum of m/z 445 for raltegravir.

Raltegravir spiked to blank plasma could be succesfully quantitated when included in the previously developed method [8]. However, when measuring a patient sample, a secondary peak overlapping the peak corresponding to raltegravir was observed in the same mass transition as raltegravir (445 → 361 *m*/*z*). This peak was traced back to originate from in-source degradation of the main raltegravir metabolite, raltegravir–glucuronide, to raltegravir. Glucuronidated raltegravir circulates in plasma and is excreted in urine and feces [12,13]. When the in-source declustering potential was reduced, the main ion entering the mass spectrometer at time 4.2 min had a *m*/*z* ratio of 621, corresponding with the [M+H] of raltegravir–glucuronide. After collision-induced dissociation, the main product had a *m*/*z* of 445, corresponding to raltegravir. With the declustering potential optimized for raltegravir, raltegravir–glucuronide dissociated in the source and its identity was confirmed with a signal in the mass transition window of raltegravir. The in-source degradation could be reduced but not be totally diminished by decreasing the in-source declustering

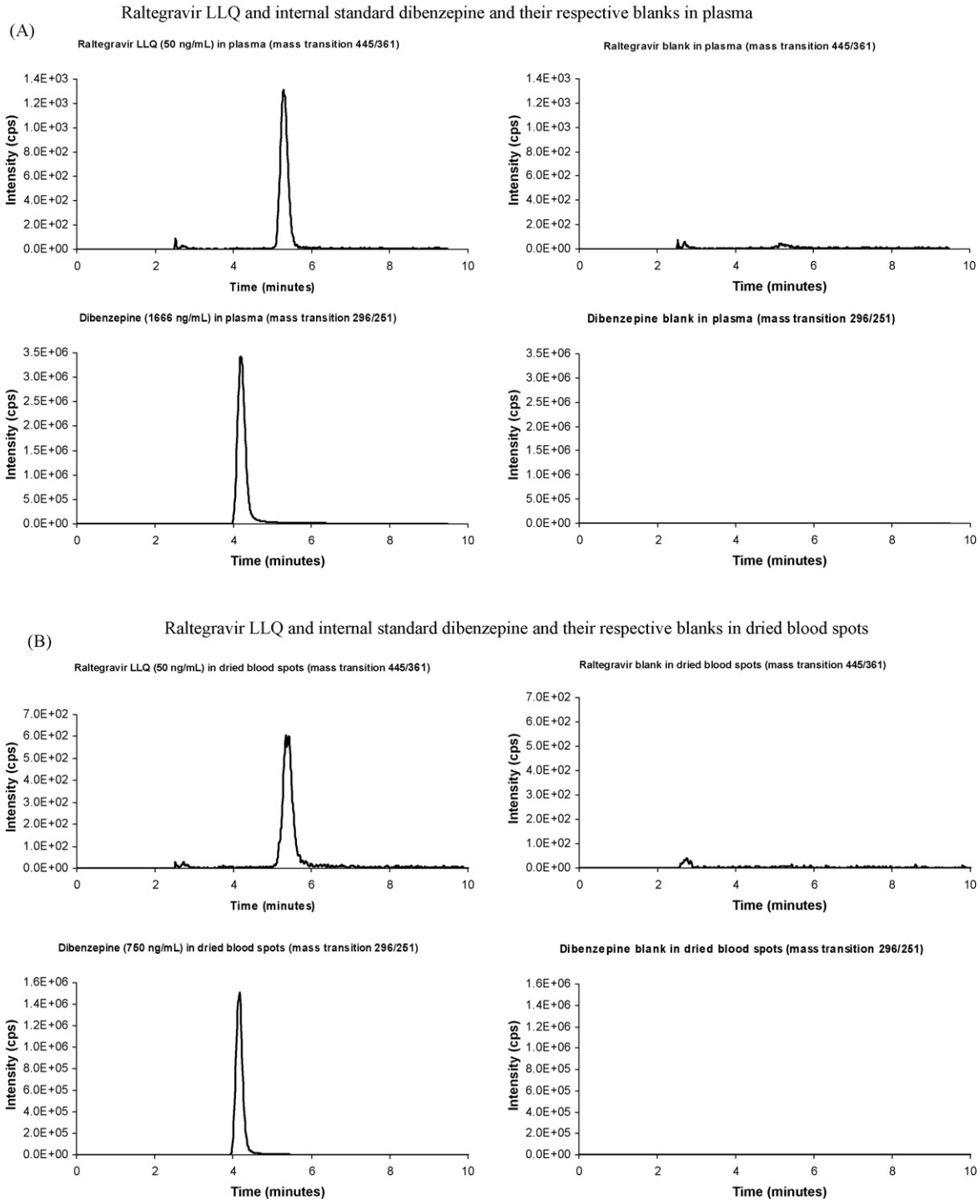

Fig. 3. Chromatograms. (A) Raltegravir LLQ and internal standard dibenzepine and their respective blanks in plasma, (B) raltegravir LLQ and internal standard dibenzepine and their respective blanks in dried blood spots, (C) Raltegravir LLQ and internal standard dibenzepine and their respective blanks in PBMC lysate, and (D) representative chromatogram of patient sample in dried blood spots.

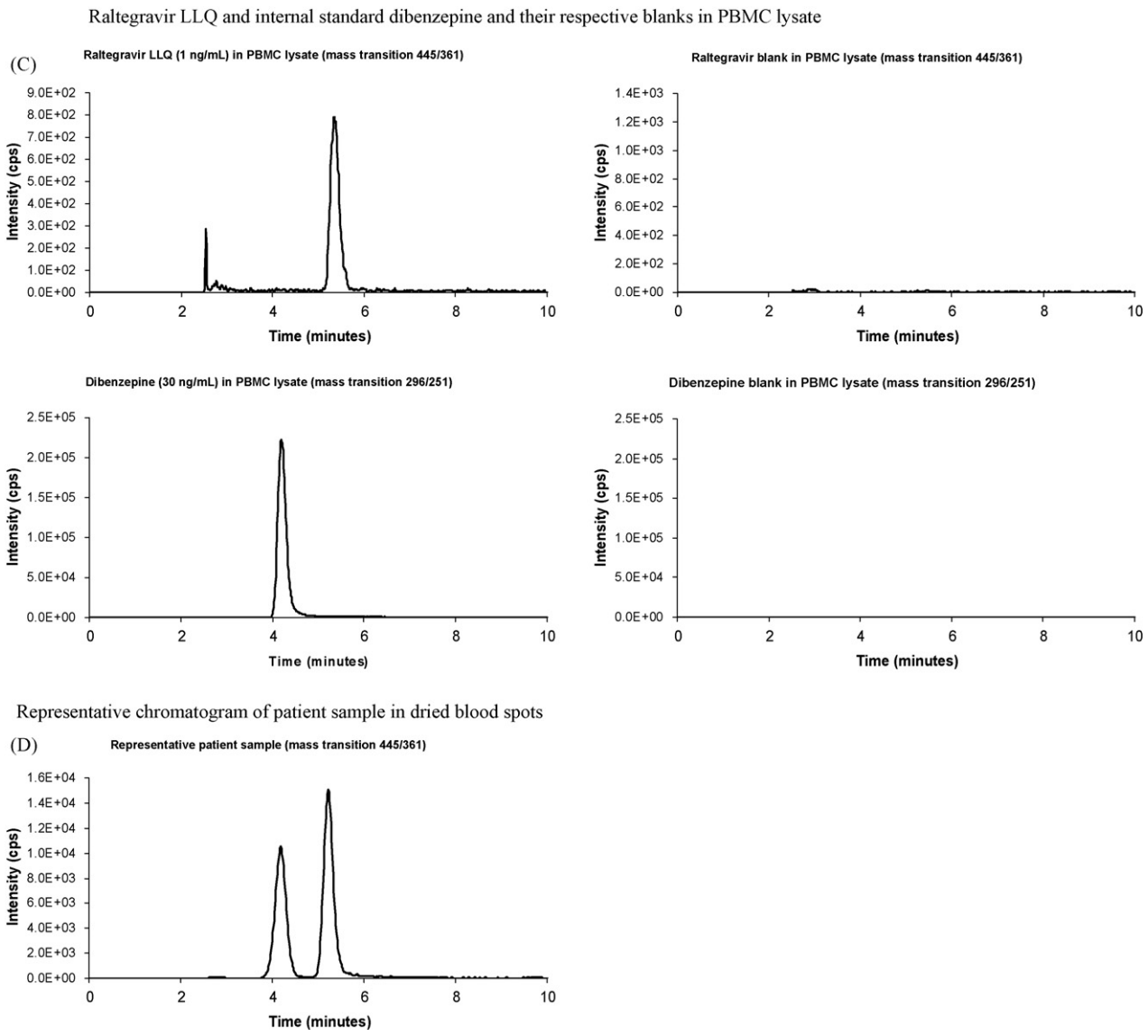

Fig. 3. (*Continued*).

potential. However, raltegravir–glucuronide could be separated from raltegravir on the column by changing the chromatographic conditions. The amount of modifier in the isocratic elution phase of the initial method was reduced to separate both compounds. Final gradient composition was as follows: at time zero 10 μL of the plasma extract or 20 μL of the dried blood spot extract or 30 μL of the PBMC lysate were introduced in the flow consisting of 85% eluent A mixed with 15% methanol. After 0.1 min 40% of eluent A was mixed with 60% of methanol and this composition was maintained for 6.9 min. Thereafter the column was conditioned for 3 min with 85% eluent A mixed with 15% methanol before the next injection. The flow rate was 250 μL/min. Chromatographic separation of raltegravir and its metabolite allowed simultaneous detection of the metabolite and quantification of raltegravir. We were not able to quantificate the raltegravir–glucuronide, due to absence of reference substance. Representative multiple reaction monitoring (MRM) chromatograms and of raltegravir at the LLQ level and dibenzepine spiked to plasma, dried blood spots and PBMC lysate their respective blanks are shown in Fig. 3 in panel A, B and C respectively. A chromatogram of a representative patient sample extracted from a dried blood spot showing the peak from the raltegravir–glucuronide as well is depicted in panel D.

3.2. Validation results

The assay was linear over the tested concentration ranges (50–10,000 ng/mL in plasma and dried blood spots and 1–500 ng/ml in PBMC lysate). Best assay performance was obtained by using a weighting factor of $1/x^2$ for the calibration curves. The intra- and inter-assay performance data in plasma, dried blood spots and PBMC lysate are presented in Tables 1A, 1B and 1C respectively. Accuracies ranged from 104% to 105% in plasma, from 93% to 105% in dried blood spots and from 82% to 113% in PBMC

Table 1A
Intra- and inter-assay performance data of the analytes at four concentration levels in plasma.

Nominal concentration (ng/mL)	Accuracy (%)	Mean intra-assay precision (CV[a] %)	Inter-assay precision (CV[a] %)
50	104.4	3.6	5.9
125	104.1	1.9	2.5
3750	104.9	1.2	1.4
7500	104.6	1.8	2.0

[a] CV: coefficient of variation.

Table 1B
Intra- and inter-assay performance data of the analytes at four concentration levels in dried blood spots.

Nominal concentration (μg/mL)	Accuracy (%)	Intra-assay precision (CV[a] %)	Inter-assay precision (CV[a] %)
50	93.1	6.4	10.8
100	93.5	6.2	6.5
2500	98.4	5.0	6.6
7500	105.0	3.9	4.9

[a] CV: coefficient of variation.

Table 1C
Intra- and inter-assay performance data of the analytes at four concentration levels in PBMC lysate.

Nominal concentration (μg/mL)	Accuracy (%)	Intra-assay precision (CV[a] %)	Inter-assay precision (CV[a] %)
1	87.9	4.1	12.2
3	94.3	1.1	6.9
250	103.0	1.1	5.9
500	105.6	1.5	5.9

[a] CV: coefficient of variation.

Table 2A
Protein precipitation recovery from plasma.

Analyte nominal concentration (μg/mL)	Mean recovery (%)	S.D. (%)
Raltegravir		
0.125	90.2	3.6
3.75	98.9	2.2
7.5	100.7	5.4

lysate. All precisions were less than 6%, 11% and 13% in plasma, dried blood spots and PBMC lysate, respectively.

3.2.1. Specificity, selectivity and ion suppression

MRM chromatograms of six batches of control human plasma, dried blood spots and PBMC lysate contained no endogenous peaks co-eluting with any of the analytes. LLQ samples, prepared in these six batches could be quantified within the required 20% deviation from the nominal concentration.

During post-column infusion experiments no difference in signal in the mass transition window of either raltegravir or dibenzepine was observed at their respective retention times when a plasma, dried blood spot or PBMC extract was injected when compared to the signal of an eluent injection. Therefore, ion suppression was considered not to influence the analysis. Furthermore no interfering peaks, interfering ion-suppression or ion-enhancement were found in 6 different batches of blood.

3.2.2. Recovery

The calculated recovery of raltegravir from plasma and dried blood spots is shown in Tables 2A and 2B. The recovery of raltegravir from plasma and dried blood spots was complete and reproducible.

3.2.3. PBMC matrix effect

As shown in Table 3, the amount of cells influences the quantification of raltegravir, indicating that clinical PBMC pellets cannot all be treated equally using the same amount of lysing solution. The amount of lysing solution should therefore be adjusted to the amount of cells in the clinical sample: the amount of cells per volume in the clinical PBMC lysate should resemble the amount of cells per volume in the quality control and calibrator PBMC lysates (5 million cells in 200 μL of cell extraction solution equals 25×10^3 cells/μL).

Table 2B
Extraction recovery from dried blood spots.

Analyte nominal concentration (μg/mL)	Mean recovery (%)	S.D. (%)
Raltegravir		
0.100	111.3	16.2
7.5	105.5	9.2

Table 3
PBMC matrix effect.

Nominal concentration (ng/ml)	Cell amount in pellet (10^6 cells)	Recovered concentration compared to nominal (%) $n = 3$	CV (%)
3	0	28.1	10.0
	5	96.9	1.6
	20	117.3	0.5
250	0	76.7	5.1
	5	105	0.6
	20	128	0.8
400	0	81.0	2.8
	5	106.7	1.4
	20	130.0	0.8

Table 4A
Stability data for raltegravir in clinical plasma samples after 7 days of storage ($n = 3$).

Plasma storage condition (7 days storage)	Recovered amount compared to initial amount (%)	CV (%)
4 °C	89.3	3.7
Ambient temperature	83.3	4.9

3.2.4. Stability

Table 4A shows the stability data of raltegravir in a clinical plasma sample. Raltegravir in plasma was proven to be stable for at least 1 week stored at 4 °C, after HIV inactivation at 60 °C for 40 min or after 3 freeze-thaw cycles. However, some degradation is in plasma is observed when stored for 1 week at ambient temperature, indicating that clinical samples should be stored preferably at −20 °C when transported to the laboratory. No influence of the presence of raltegravir–glucuronide was observed. Stability of raltegravir in clinical dried blood spot samples is shown in Table 4B. Raltegravir showed excellent stability in dried blood spots when stored for 7 days at ambient temperature or at 30 °C, indicating that dried blood spot sampling of raltegravir can be performed in an non-hospital based environment were refridgeration is not possible or in, for example, resource-limited settings. Furthermore, reinjection reproducibility and stability in the final extracts of plasma, dried blood spots and PBMC lysate was assured for at least 3 days when stored at 4 °C and the raltegravir stock solution in methanol was stable for at least 251 days when stored at −20 °C.

3.2.5. Application of the method: analysis of clinical dried blood spot samples

Fig. 4 shows a pharmacokinetic curve during a 12-h dosing interval of raltegravir in dried blood spots from a heavily pretreated patient on a salvage regime containing darunavir/ritonavir 600/100 mg BID, raltegravir 400 mg BID, lamivudine 300 mg QD and tenofovir 245 mg QD. The volume of collected blood was always sufficient to fill at least a 0.25-in. diameter circle on the collection

Table 4B
Stability data for raltegravir in clinical dried blood samples after 7 days of storage ($n = 3$).

Dried blood spot storage condition (7 days storage)	Recovered amount compared to initial amount (%)	CV (%)
Ambient temperature	100.4	2.2
30 °C	106.2	2.3

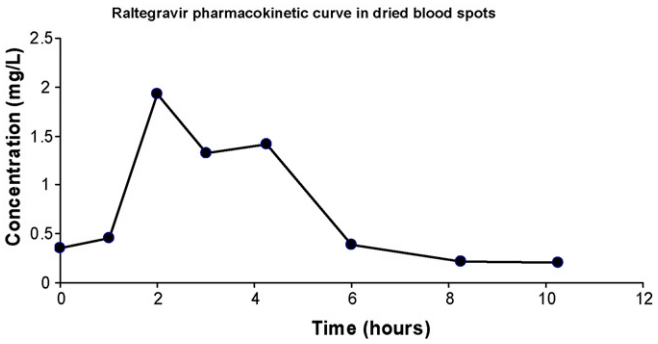

Fig. 4. Full pharmacokinetic curve of raltegravir in dried blood spots in a treatment experienced HIV-1 infected patient on a regime containing tenofovirdisoproxulfumarate 245 mg once daily, lamivudine 300 mg once daily, ritonavir 100 mg twice daily, darunavir 600 mg twice daily and raltegravir 400 mg twice daily.

paper. Venous sampling could not be performed due to phlebitis. The obtained drug concentration versus time curve shows the utility of dried blood spot sampling for pharmacokinetic analysis of raltegravir.

4. Conclusion

A simple and rapid assay was developed and validated for the quantification of the integrase inhibitor raltegravir and detection of its main metabolite in plasma as well as dried blood spots and PBMC lysate by means of LC–MS/MS. The developed assay was proven to be sensitive, accurate, precise and robust. The presence of the raltegravir–glucuronide did not influence the stability of raltegravir in plasma and dried blood. The method allows simultaneous quantification of raltegravir with PIs and NNRTIs from the same sample using the same chromatographic and detection setup. Furthermore, the developed quantitative assay for raltegravir in dried blood spots opens up opportunities for pharmacokinetic studies in children and neonates and enables self-sampling for purposes of therapeutic drug monitoring. The assay is now successfully applied for pharmacological studies and therapeutic drug monitoring of raltegravir.

References

[1] L. Palmisano, Expert. Rev. Anti. Infect. Ther. 5 (2007) 67–75.
[2] B. Grinsztejn, B.Y. Nguyen, C. Katlama, J.M. Gatell, A. Lazzarin, D. Vittecoq, C.J. Gonzalez, J. Chen, C.M. Harvey, R.D. Isaacs, Lancet 369 (2007) 1261–1269.
[3] M. Markowitz, J.O. Morales-Ramirez, B.Y. Nguyen, C.M. Kovacs, R.T. Steigbigel, D.A. Cooper, R. Liporace, R. Schwartz, R. Isaacs, L.R. Gilde, L. Wenning, J. Zhao, H. Teppler, J. Acquir. Immune. Defic. Syndr. 43 (2006) 509–515.
[4] D.A. Cooper, J.M. Gatell, J. Rockstroh, C. Katlama, P. Yeni, A. Lazzarin, J. Chen, R. Isaacs, H. Teppler, B.Y. Nguyen, Results of BENCHMRK-1, a phase III study evaluating the efficacy and safety of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus, 2007.
[5] R. T. Steigbigel, P. Kumar, J. Eron, M. Schechter, M. Markowitz, M. Loufty, J. Zhao, R. Isaacs, B.Y. Nguyen, H. Teppler. Results of BENCHMRK-2, a phase III study evaluating the efficacy and safety of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus, 2007.
[6] M.C. Long, C. Bennetto-Hood, E.P. Acosta, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 867 (2008) 165–171.
[7] S.A. Merschman, P.T. Vallano, L.A. Wenning, B.K. Matuszewski, E.J. Woolf, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 857 (2007) 15–24.
[8] R. ter Heine, C.G. Alderden-Los, H. Rosing, M.J. Hillebrand, E.C. van Gorp, A.D. Huitema, J.H. Beijnen, Rapid Commun. Mass Spectrom. 21 (2007) 2505–2514.
[9] R. ter Heine, H. Rosing, E.C. van Gorp, J.W. Mulder, W.A. van der Steeg, J.H. Beijnen, A.D. Huitema, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 867 (2008) 205–212.
[10] S. Colombo, A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, L.A. Decosterd, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 819 (2005) 259–276.
[11] U.S.Department of Health and Human Services Food and Drug Administration, Guidance for Industry Bioanalytical Method Validation, http://www.fda.gov/CDER/GUIDANCE/4252fnl.htm, 27 February 2007.
[12] E. Monteagudo, S. Pesci, M. Taliani, F. Fiore, A. Petrocchi, E. Nizi, M. Rowley, R. Laufer, V. Summa, Xenobiotica 37 (2007) 1000–1012.
[13] K. Kassahun, I. McIntosh, D. Cui, D. Hreniuk, S. Merschman, K. Lasseter, N. Azrolan, M. Iwamoto, J.A. Wagner, L.A. Wenning, Drug Metab. Dispos. 35 (2007) 1657–1663.