Journal of Chromatography B, 878 (2010) 1455–1465

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Validation of a rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds

Laura Else[a,*], Victoria Watson[a,b], John Tjia[a], Andrew Hughes[b], Marco Siccardi[a], Saye Khoo[a], David Back[a]

[a] Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, UK
[b] NIHR Biomedical Research Centre, Royal Liverpool Hospital Trust, Liverpool, UK

ARTICLE INFO

Article history:
Received 10 November 2009
Accepted 17 March 2010
Available online 9 April 2010

Keywords:
HPLC–MS/MS
Protein precipitation
Protease inhibitors
Etravirine
Rilpivirine
Maraviroc

ABSTRACT

Clinical pharmacokinetic studies of antiretrovirals require accurate and precise measurement of plasma drug concentrations. Here we describe a simple, fast and sensitive HPLC–MS/MS method for determination of the commonly used protease inhibitors (PI) amprenavir, atazanavir, darunavir, lopinavir, ritonavir, saquinavir and the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine, as well as the more recent antiretrovirals, the CCR5 antagonist maraviroc and the "second generation" NNRTI etravirine and rilpivirine. An internal standard (quinoxalone; QX) was added to plasma aliquots (100 μl) prior to protein precipitation with acetonitrile (500 μl) followed by centrifugation and addition of 0.05% formic acid (200 μl) to the supernatant. Chromatographic separation was achieved using a gradient (acetonitrile and 0.05% formic acid) mobile phase on a reverse-phase C_{18} column. Detection was via selective reaction monitoring (SRM) operating in positive ionization mode on a triple-quadrupole mass spectrometer. All compounds eluted within a 5 min run time. Calibration curves were validated over concentration ranges reflecting therapeutic concentrations observed in HIV-infected patients from pharmacokinetic data reported in the literature. Correlation coefficients (r^2) exceeded 0.998. Inter- and intra-assay variation ranged between 1% and 10% and % recovery exceeded 90% for all analytes. The method described is being successfully applied to measure plasma antiretroviral concentrations from samples obtained from clinical pharmacokinetic studies.

1. Introduction

Highly active antiretroviral therapy (HAART) has dramatically reduced HIV-1-associated mortality and morbidity [1] and currently comprises 25 drugs from five different classes; the nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI), protease inhibitors (PI), and most recently entry inhibitors and integrase inhibitors. Current 2008 British HIV Association guidelines recommend for treatment naive patients, a combination of three or more antiretroviral agents; an NNRTI or a ritonavir boosted PI in combination with a dual NRTI backbone [2]. However, despite the long-term benefits of HAART, an estimated 8% of treatment naive and 33% of experienced patients do not achieve viral suppression or experience viral rebound within 12 months of initiating HAART [3].

For patients harbouring resistant virus and those failing multiple regimens, antiretroviral drug combinations have become increasingly complex and in recent years new and more potent agents have been introduced which possess activity against both wild-type and resistant viral strains. The chemokine receptor (CCR5) antagonist maraviroc was approved by the FDA and EMEA in 2007. Maraviroc acts specifically against CCR5-trophic (R5) HIV-1 and prevents R5 virus engaging with the CCR5 co-receptor located on the host CD4 cell membrane, but is not effective against CXCR4-trophic (X4) and dual/mixed trophic strains which become increasingly dominant in the later stages of HIV-1 infection [4]. The integrase inhibitor raltegravir (also licensed in 2007) inhibits the integration of pro-viral DNA into the host genome and has demonstrated potent antiviral activity in multi-drug experienced patients [5,6]. Also, in view of increasing resistance, new NNRTI were also urgently needed. The second generation NNRTI, etravirine (TMC125) and

* Corresponding author at: Department of Pharmacology and Therapeutics, University of Liverpool, Pharmacology Research Laboratories, Block H, First Floor, 70 Pembroke Place, Liverpool L69 3GF, UK. Tel.: +44 151 794 5553; fax: +44 151 794 5656.
E-mail addresses: l.j.else@liv.ac.uk, lauraelse@hotmail.co.uk (L. Else), vwatson@liv.ac.uk (V. Watson), j.f.tjia@liv.ac.uk (J. Tjia), Athughes@liv.ac.uk (A. Hughes), siccardi@liv.ac.uk (M. Siccardi), Khoo@liv.ac.uk (S. Khoo), d.j.back@liv.ac.uk (D. Back).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.03.036

rilpivirine (TMC278) are diarylpyrimidine compounds which possess favorable binding interactions toward reverse transcriptase of both mutant HIV-1 strains as well as wild-type virus, including the common K103N mutation [7]. In 2008, the FDA and EMEA granted accelerated approval of etravirine based on data from the phase III DUET_1/2 studies [8,9]. Rilpivirine, shown in initial phase IIb studies to be equivalent to the standard-of-care efavirenz [10], is not yet licensed and is currently undergoing non-inferiority phase III trials (ECHO and THRIVE) for use in treatment naive and experienced patients.

The PI and NNRTI undergo cytochrome P450 mediated metabolism via CYP3A4 and to a lesser extent by CYP2B6, CYP2D6 and CYP2C19 which renders them prone to variable pharmacokinetics and extensive drug–drug interactions when given in combination or with other concomitant medications [11]. Moreover, they can variably affect their own metabolism through the induction and inhibition of these enzymes. All PI inhibit CYP3A4, with ritonavir being the most potent and is used exclusively at subtherapeutic doses to "boost" other PI [12,13]. In addition, ritonavir, lopinavir and amprenavir have CYP enzyme inducing properties [14–16]. The first generation NNRTI nevirapine and efavirenz are substrates and inducers of CYP3A4 and CYP2B6 (the major enzyme involved in the metabolism of efavirenz) [17]. The second generation NNRTI rilpivirine is metabolized primarily by CYP3A4, and etravirine by CYP3A4, CYP2C9, CYP2C19 [18]. Maraviroc is a substrate for both CYP3A4 and the efflux transporter P-gycloprotein, and has shown clinically significant interactions with both PI and NNRTI, rendering mandatory maraviroc dosage adjustments with some associations [19,20].

The quantification of antiretrovirals from plasma is a valuable pharmacological tool since PI and NNRTI demonstrate pharmacokinetic/pharmacodynamic (PK/PD) [21–24] and pharmacokinetic/toxicity relationships [25–27]. Thus characterisation of the relationship between antiretroviral pharmacokinetics (systemic exposure or a single concentration) and drug response (beneficial or adverse) is key to the selection of an optimal dose for a drug, understanding inter- and intra-subject variability, and to design strategies to optimize response and tolerability while avoiding unwanted toxicity. For this reason, comprehensive pharmacokinetic studies investigating drug interactions, as well as those assessing new dosing strategies require accurate and precise measurement of drug concentrations to ensure that correct and meaningful data are fed back into clinical care. Indeed, routine therapeutic drug monitoring (TDM) and pharmacokinetic drug interaction studies between existing and new antiretrovirals, and with concomitant medications are essential for the optimization and management of antiretroviral therapy, in order to maintain efficacy and prevent drug toxicity and resistance. Also important are clinical studies investigating pharmacokinetics in specific patient groups including pregnant women and children, who are in need of tailored antiretroviral dosage regimens.

Several methodologies have been reported in the literature which simultaneously determine PI and NNRTI plasma concentrations using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) [28–33]. However, some methods are limited by relatively long analytical run times (10–25 min), which reduce turnover when analysing multiple samples and others have only moderate sensitivity. As new drugs emerge it is important that assay methodology incorporates both new (maraviroc, raltegravir, etravirine and rilpivirine) and existing antiretroviral classes.

A number of previously published HPLC–UV and LC–MS/MS assays have quantified darunavir from human plasma either alone [34] or with other antiretroviral drugs [30,35–37]. Raltegravir has been quantified separately using HPLC with fluorescent detection [38] and LC–MS/MS methods [39,40]. Also, LC–MS/MS assays for maraviroc [19,41,42] and etravirine [18,43,44] have been briefly described as part of *in vivo* pharmacokinetic and drug interaction studies but not in the form of a comprehensive method development and validation. A more recent LC–MS/MS method quantified raltegravir, maraviroc, darunavir and etravirine together in a separate assay, but existing agents (with exception of ritonavir) were not included [45], thereby, limiting the assay's utility in a routine setting. Only one bio-analytical method, using solid phase extraction and HPLC coupled to photodiode array detection, has simultaneously quantified raltegravir and etravirine alongside existing PI and NNRTI, however, the chromatographic separation did not allow for the simultaneous quantification of amprenavir and darunavir, which due to similarities in their chemical structure, co-eluted as a single peak [36]. LC–MS/MS methods have been described for the quantification of rilpivirine for pre-clinical studies in dog and rat plasma [46,47]; however, to date, no bio-analytical assay has measured rilpivirine alongside current antiretrovirals.

Here we describe a simple, fast and sensitive HPLC–MS/MS method for the determination of the commonly used PI [amprenavir (APV), atazanavir (ATV), darunavir (DRV), lopinavir (LPV), ritonavir (RTV), saquinavir (SQV)] and NNRTI [nevirapine (NVP)], as well as recently licensed CCR5 antagonist maraviroc (MVC) and the second generation NNRTI etravirine (ETV) and rilpivirine (RPV). The method was adapted from a previous assay used within our laboratory to measure 7 PI [48]. Validation was conducted based on modified Westgard regulations and FDA international guidelines for bio-analytical assay validation [49,50]. In particular, we emphasise that the novelty of this analytical methodology is that it includes RPV, which has not been incorporated into past LC–MS/MS methods. As RPV is not yet licensed, determination of RPV plasma concentrations as part of intensive pharmacokinetic and drug interaction studies will be crucial for the future safe and effective use of this agent in the wider HIV-infected population.

2. Materials and methods

2.1.1. Chemicals

APV was kindly donated by Glaxo Wellcome Research and Development (Middlesex, UK), ATV (atazanavir sulphate) by Bristol-Myers Squibb (Hounslow, UK), SQV by Roche Discovery (Welwyn, UK), LPV and RTV by Abbott Laboratories (Chicago, IL, USA) and NVP by Boehringer Ingelheim Pharmaceuticals, Inc. (Berkshire, UK). DRV (darunavir ethanolate), ETV and RPV (rilpivirine hydrochloride) were kindly contributed by Tibotec (Mechelen, Belgium) and MVC was donated by Pfizer (Sandwich, Kent, UK). The internal standard, quinoxaline (6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline) and formic acid (minimum 95% pure) were obtained from Sigma–Aldrich, UK.

LC–MS grade acetonitrile (ACN) was obtained from Fisher Scientific (Loughborough, UK) and methanol (MeOH) from VMR Laboratory supplies (Poole, UK). HPLC grade de-ionized water was produced from an Elga Option 4 water purifier (Elga LabWater, High Wycombe, UK). The water was further purified to 18.2 MΩ with a Purelab Classic UVF (Elga LabWater, High Wycombe, UK). Drug free plasma was obtained from the National blood service (Liverpool, UK).

2.1.2. Equipment

The HPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at a temperature of 15 °C) and an Accela LC pump (Thermo Electron Corporation, Hemel Hempstead, UK). A reverse-phase Ascentis^{TM} C_{18} column (3 μm: 100 mm × 2.1 mm) set at an oven temperature of 26 °C (Supelco, Dorset, UK) was used

Table 1
Step-wise gradient program consisting of acetonitrile (ACN) and water (0.05% formic acid).

Time (min)	ACN: water (0.05% formic acid)	
	5:95 (%)	80:20 (%)
0	100	0
0.1	100	0
1.0	0	100
2.0	0	100
2.1	100	0
5.0	100	0

to elute all analytes and internal standard was interfaced with a 2 μm guard column, C18 Quest column saver (Thermo Electron Corporation, Hemel Hepstead, UK). The HPLC system was interfaced with a triple-quadrupole TSQ Quantum Ultra mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, UK) with a heated-electrospray ionization (H-ESI) source. Two E2M30 rotary vacuum pumps (Edwards High Vacuum International, West Sussex, UK), a NM30LA nitrogen generator (Peak Scientific, Renfrewshire, Scotland, UK) and 99% pure argon gas (10L SIZE V, BOC Gases, Worsely, Manchester, UK) were used. TSQ Tune Software (Thermo Electron Corporation, Hemel Hepstead, UK) was used for the optimization of tuning parameters. LC Quan™ software (Version 2.5.6, Thermo Electron Corporation, Hemel Hepstead, UK) was used for data acquisition and processing.

2.1.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a rapid step-wise gradient [ACN:water (0.05% formic acid) 5:95 and 80:20, v/v] mobile phase at a flow rate of 400 μl/min over a total run time of 5 min. An outline of the mobile phase gradient program is summarized in Table 1. Initial conditions consisted of A: 100% ACN:water (0.05% formic acid) 5:95, v/v from 0 to 0.1 min, increasing in organic content to B: 100% ACN:water (0.05% formic acid) 80:20 v/v from 0.1 to 1.0 min and held over 1.1 min. The column was then equilibrated to the initial conditions over a total run time of 5 min. Samples were injected (10 μl) on to the column via a full loop injection system and the needle was washed with 2 ml of ACN:water (0.1% formic acid), 80:20, v/v, between injections. The triple-quadrupole mass spectrometer was operated in positive ionization mode and detection and quantification was performed using selective reaction monitoring (SRM).

2.1.4. Preparation of calibrators, quality controls and internal standard

Stock solutions were prepared by dissolving separate solutions of analyte in MeOH to obtain final drug concentrations of 1 mg/ml (as base). Stock solutions were then further diluted with drug free plasma to yield working solutions which were dispensed (500 μl) into labeled screw cap tubes and stored at −20 °C until use. On the day of analysis, working solutions were diluted in duplicate with appropriate volumes of drug free plasma (100 μl per calibrator level) to yield concentrations ranging from 10 to 10,000 ng/ml (APV, ATV, NVP, RPV and SQV), 5 to 5000 ng/ml (ETV and RTV), 5 to 1000 ng/ml (MVC) and 15 to 15,000 ng/ml (DRV and LPV).

Internal quality control samples (QC) were prepared similarly by the dilution of drug free plasma to yield four QC levels: an LLQC within three times the assay's lower limit of quantification (APV, ATV, NVP, RPV, SQV 25 ng/ml; ETV, RTV, MVC 12.5 ng/ml; DRV, LPV 40 ng/ml), LQC (APV, ATV, NVP, RPV, SQV 150 ng/ml; ETV, RTV 100 ng/ml; MVC 50 ng/ml; DRV, LPV 250 ng/ml), MQC (APV, ATV, NVP, RPV, SQV 1500 ng/ml; ETV, RTV 800 ng/ml; MVC 400 ng/ml; DRV, LPV 3500 ng/ml) and HQC (APV, ATV, NVP, RPV, SQV 8000 ng/ml; ETV, RTV 4000 ng/ml; MVC 700 ng/ml; DRV, LPV 12,000 ng/ml). Aliquots of QC (250 μl) were dispensed into an equivalent number of labeled 2 ml screw cap tubes and stored at −20 °C until use.

A 1 mg/ml stock solution of internal standard (QX; quinoxaline) was prepared by dissolving 10 mg QX in 10 ml of MeOH. This was stored for up to 6 months at 4 °C. A working 1 μg/ml QX solution was then prepared through dilution of the 1 mg/ml stock solution in MeOH:water (50:50, v/v). Formic acid (0.05%) was prepared by the addition of 500 μl of formic acid to 1 L of de-ionized water.

2.1.5. Sample pre-treatment

Calibrators and QC samples were pipetted in duplicate (100 μl). QX (20 μl and 1 μg/ml) was added to all plasma aliquots prior to protein precipitation with ACN (500 μl). Samples were vortexed and left to stand at room temperature (15 min). After, samples were re-vortexed and centrifuged and the supernatant decanted into correspondingly labeled 5 ml glass tubes followed by the addition of 0.05% formic acid (200 μl). Samples were then re-vortexed and transferred to autosampler vials ready for injection (10 μl) onto the HPLC column.

2.1.6. Validation of calibrators and quality controls

A minimum of 10 calibration curves were prepared on separate days in order to ascertain the concentration at each calibrator level for all 10 drugs. All data acquisition and processing were performed using LC Quan™ software (Version 2.5.6, Thermo Electron Corporation, Hemel Hempstead, UK). Calibrator curves were constructed using a 1/concentration weighted quadratic regression equation of analyte:internal standard peak area ratios versus target concentration, from which unknown drug concentrations were interpolated.

In addition, a minimum of 10 QC samples (at LLQC, LQC, MQC and HQC) were treated as unknown values and run in duplicate on separate days alongside a validated calibration curve in order to determine final QC concentrations and inter-assay precision and accuracy. Intra-assay variation was ascertained by running six LLQC, LQC, MQC and HQC samples within a single analytical run. After the validation procedure, subsequent assay acceptance criteria for evaluating clinical samples are based on Westgard regulations and FDA research guidelines.

2.1.7. Recovery and matrix effects

Percentage (%) recovery from plasma for all 10 compounds was determined by comparing the chromatographic peak areas from a given concentration of analyte spiked in blank plasma and extracted via protein precipitation with peak areas obtained for the same concentration of analyte in mobile phase. Analyte recovery was tested at low, medium and high QC levels in triplicate and on four separate occasions ($n = 12$). The response data for analyte in mobile phase provide a relative 100% response value and the corresponding response data for extracted samples containing the analyte highlight whether any loss in signal is attributable to the extraction process and to the sample matrix. Percentage recovery = (peak area extracted from plasma/peak area of directly injected solution) × 100. Co-eluting matrix components can suppress or even enhance the ion intensity of analytes and hence compromise the reproducibility and accuracy when quantifying drugs from multiple batches of human plasma. Therefore, an absolute matrix effect was determined for all analytes by comparing the chromatographic peak areas of analytes spiked into blank plasma extracts (i.e. after protein precipitation) to peak areas obtained from the same concentration of analyte in mobile phase. % matrix

effect = (peak area of analyte spiked in blank plasma extract/peak area of analyte in mobile phase) × 100. The relative contribution (%) of the extraction process (extraction yield) could also be derived by dividing the peak areas of analyte spiked in blank plasma (before protein precipitation) with equivalent concentrations of analyte spiked into blank plasma extracts (after protein precipitation).

Matrix effects over an entire chromatographic run were performed using a post-column infusion to ensure that no interfering peaks were found in the elution windows of the analytes and QX. Six different batches of blank plasma, extracted via protein precipitation, and eluent (control) were injected (10 μl, full loop) onto the HPLC column while analyte (100 ng/ml) or QX (1 μg/ml) was infused post-column directly into the H-ESI source at a flow rate of 5 μl/min. As a result, any endogenous component that elutes from the column and causes a variation in ESI response of the infused analytes will be seen as a change in the response of the infused analyte.

2.1.8. Stability

Stability experiments should reflect the conditions likely to be encountered during sample transfer, handling and analysis. In our laboratory, clinical samples from both patient and healthy volunteers are heat inactivated (58 °C and 40 min), stored at −20 °C and then thawed prior to analysis. Stability studies under heat inactivated and inactivated freeze/thaw conditions have been performed previously in our laboratory for NVP, APV, ATV, LPV, RTV and SQV [48]. Stability data for the more recently licensed compounds; DRV, ETV, MVC and RPV were therefore generated.

Blank plasma was spiked with DRV, ETV, MVC and RPV at low, medium and high concentrations: LQC (RPV 150 ng/ml; ETV 100 ng/ml; MVC 50 ng/ml; DRV 250 ng/ml), MQC (RPV 1500 ng/ml; ETV 800 ng/ml; MVC 400 ng/ml; DRV 3500 ng/ml) and HQC (RPV 8000 ng/ml; ETV 4000 ng/ml; MVC 700 ng/ml; DRV 12,000 ng/ml), designed to encompass the therapeutic ranges defined in the literature. Samples were prepared and analysed in triplicate and on four separate occasions ($n = 12$) under three different treatment conditions: freshly prepared (serving as a control), heat inactivated, and heat inactivated followed by three freeze/thaw cycles in which samples were frozen overnight and allowed to thaw for 1 h the following morning and then refrozen for 24 h. The peak areas obtained from samples undergoing heat inactivation and heat inactivation/freeze–thaw cycles were then compared to corresponding peak areas for each drug obtained under control conditions. All short-term stability and recovery experiments were performed prior to the validation of calibrators and QC, respectively. Long-term stability studies were also performed. Batches of non-heat inactivated plasma QC samples (LQC, MQC and HQC) were analysed after storage at −20 °C for 4, 7, 14 days, and 1 month, and compared to the mean back calculated values for QC at equivalent concentrations prepared on the first day of the long-term stability testing.

2.1.9. Data analysis

Inter- and intra-assay variation for LLQC, LQC, MQC and HQC samples were expressed in terms of a coefficient of variation [CV% = (standard deviation/mean) × 100]. Bio-analytical assay validation was in accordance with FDA and modified Westgard regulations in which mean target concentrations at lower limit of quantification (LLQ) should be within ±20% of their nominal (target) value and the CV% should not exceed 20%. For all other calibrators and QC samples, mean target concentrations should be within ±15% of their nominal level and the %CV should not be exceeded 15%. All calculations were performed using Microsoft Excel 2007 for windows XP (Microsoft Corporation, USA).

Stability of DRV, ETV, MVC and RPV under fresh, heat inactivated and heat inactivated freeze/thaw conditions was assessed using an one-way ANOVA with a Bonferroni correction over three (LQC, MQC and HQC) concentrations, or if data were non-normally distributed using the non-parametric equivalent (Kruskal–Wallis test). Normality of data was assessed using a Shapiro–Wilk test. All statistical calculations were performed and analysed using Arcus Quickstat (Version 1.1©1997, Biomedical Software, Statsdirect Ltd., Cheshire, UK). *P* values were two-sided at the 0.05 significance level.

3. Results

3.1.1. Detection and chromatography

Compound specific parameters including the tube lens (V) and the relative collision energy (V) were optimized for a maximum of six transitions per analyte using TSQ Tune Software (Thermo Electron Corporation, Hemel Hempstead, UK) and the two fragment ions with the highest signal-to-noise ratio were selected for quantification. The parent-to-fragment [*m/z*] transitions, tube lens and relative collision energies used are summarized in Table 2. APV, ATV, DRV, LPV, RTV, SQV, NVP, MVC, ETV and RPV eluted over a total run time of 5 min split into three segments. The retention times for all drugs are summarized in Table 2. MVC, SQV, RPV and NVP eluted within segment 1 (0.00–1.77 min); APV, DRV, ATV and QX within segment 2 (1.78–2.21 min) and RTV, LPV and ETV within segment 3 (2.22–5.00 min).

The triple-quadrupole mass spectrometer was operating in positive SRM mode set to a narrow scan width (0.01 *m/z*) and scan time (0.01 s) for all transitions. Data were collected in centroid mode. The sheath and auxiliary gas flow (nitrogen and argon) and spray voltage were 60 units, 40 units and 4.0 kV, respectively. The capillary temperature and vapourising temperature within the H-ESI source were maintained at 300 and 350 °C throughout an assay

Table 2
Parent-to-fragment (daughter) mass transitions, tube lens, relative collision energy (RCE) and retention times (RT) for all analytes and internal standard analysed using HPLC–MS/MS.

Drug	Drug class	Parent ion (*m/z*)	Fragment ions (*m/z*)	Tube lens (V)	RCE (V)	RT (min)
Maraviroc	CCR5 antagonist	514.3	279.9, 389.0	90	29, 20	1.47
Saquinavir	PI	671.4	570.2, 224.8	130	31, 52	1.59
Rilpivirine	NNRTI	367.2	194.9, 191.9	116	36, 40	1.62
Nevirapine	NNRTI	267.1	226.9, 196.9	85	29, 42	1.65
Amprenavir	PI	506.3	155.9, 244.9	86	29, 17	2.03
Darunavir	PI	548.3	392.0, 155.9	96	14, 33	2.03
Atazanavir	PI	705.4	167.9, 334.9	119	41, 28	2.05
Quinoxalone	IS	313.2	284.0, 246.0	109	45, 42	2.12
Ritonavir	PI	721.4	295.9, 267.9	95	18, 27	2.31
Lopinavir	PI	629.4	154.9, 429.1	77	37, 21	2.41
Etravirine	NNRTI	435.0	303.8, 143.9	131	38, 38	2.70

PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

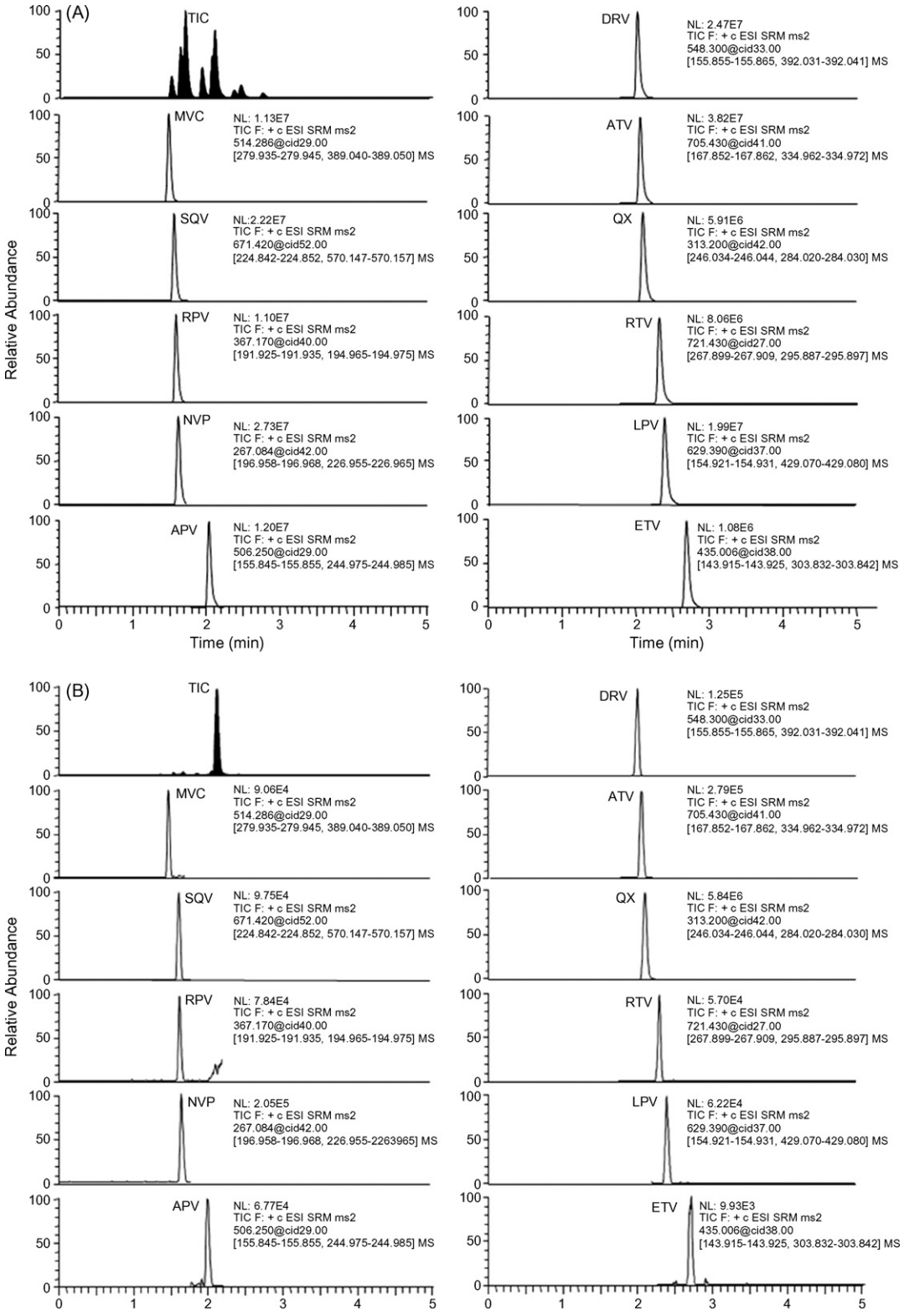

Fig. 1. Chromatograms corresponding to the total ion count (TIC; shown in solid black) and individual retention times of 10 antiretroviral drugs and internal standard (QX) from extracted plasma samples at (A) the MQC and (B) the LLQ level, over a total run time of 5 min split into three segments. Segment 1: 0.00–1.77 min containing maraviroc (MVC), saquinavir (SQV), rilpivirine (RPV) and nevirapine (NVP). Segment 2: 1.78–2.21 min containing amprenavir (APV), darunavir (DRV), atazanavir (ATV) and quinoxalone (QX). Segment 3: 2.22–5.00 min containing ritonavir (RTV), lopinavir (LPV) and etravirine (ETV).

run. A low pass chromatography filter [Chrom Filter; LC Quan™ software (Version 2.5.6, Thermo Electron Corporation, Hemel Hempstead, UK)] was applied to reduce background interference and improve peak smoothing by removing high frequency noise from the baseline and the peak profile. The filter is applied to the ion signal in real time as the data is acquired, and unlike post-acquisition smoothing algorithms it improves the signal-to-noise ratio while retaining chromatographic peak shape and does not induce

artificial peak tailing. A single parameter is specified for the filter based on the expected baseline width of the chromatographic peaks in the assay, the system then calculates the appropriate bandwidths and applies them to the data [51]. Fig. 1 shows a typical chromatogram depicting the total ion count and individual chromatographic peaks for all 11 mass transitions obtained from extracted plasma samples at (A) the MQC and (B) the LLQ level.

3.1.2. Validation of calibrators and quality controls

Ten standard curves and QC samples were run in order to ascertain mean target calibrator and QC concentrations. Standard curves ranged between 11 and 10,063 ng/ml (APV), 11 and 10,017 ng/ml (ATV), 16 and 15,062 ng/ml (DRV), 5 and 5000 ng/ml (ETV), 16 and 15,083 ng/ml (LPV), 5 and 1009 ng/ml (MVC), 11 and 10,056 ng/ml (NVP), 11 and 10,045 ng/ml (RPV), 5 and 5018 ng/ml (RTV), 10 and 10,087 ng/ml (SQV). Mean target LLQC, LQC, MQC and HQC concentrations are presented in Table 3. Variation was less than 14% at the lower limit of quantification and ranged between 2% and 11% at all other calibrator levels. All standard curves were adequately described using a 1/concentration weighted quadratic regression equation. The correlation coefficient (r^2) for all drug calibration curves exceeded 0.998.

3.1.3. Assay lower and upper limits of quantification and limit of detection

The lower and upper limits of quantification (LLQ and ULQ) are defined as the bottom and top points of the standard curve. The assay limit of detection (LOD) is defined as lowest concentration of analyte that produces a chromatographic peak distinguishable from the background noise (minimum ratio 3:1). The LOD, LLQ and ULQ were 1.3, 11 and 10,063 ng/ml (APV), 0.2, 11 and 10,017 ng/ml (ATV), 1.0, 16 and 15,062 ng/ml (DRV), 2.7, 5 and 5000 ng/ml (ETV), 2.0, 16 and 15,083 ng/ml (LPV), 0.7, 5 and 1009 ng/ml (MVC), 0.3, 11 and 10,056 ng/ml (NVP), 2.7, 11 and 10,045 ng/ml (RPV), 1.3, 5 and 5018 ng/ml (RTV), 0.3, 10 and 10,087 ng/ml (SQV), respectively. Under FDA research guidelines, the LLQ should be at least five times the response of a blank plasma sample (signal-to-noise ratio $\geq$5.0). Mean signal-to-noise ratios at the LLQ were determined from 10 standard curves by the following equation: (signal-to-noise = peak area of an LLQ extract/peak area of a blank plasma extract) and were 170 (APV), 751 (ATV), 621 (DRV), 10 (ETV), 178 (LPV), 99 (MVC), 65 (NVP), 35 (RPV), 91 (RTV) and 90 (SQV).

3.1.4. Accuracy and precision

Assay precision was assessed by the calculation of inter- and intra-assay variability of LLQC, LQC, MQC and HQC samples and expressed in terms of a CV%. Inter-assay variation was determined from the validation of 10 standard curves and QC samples, while intra-assay variation was ascertained by running six replicates of QC samples within a single assay run. Accuracy was evaluated by calculating the % bias from six replicates of LLQC, LQC, MQC and HQC in relation to target QC concentrations, respectively. Accuracy (% bias) was between −4.9% and 13.5% and inter- and intra-assay precision (CV%) did not exceed 10%, for all compounds (Table 3).

3.1.5. Recovery and matrix effects

The mean (±CV%) percentage (%) recovery, and contribution of the sample matrix and of the extraction yield are summarized in Table 4. Matrix effects were examined qualitatively by simultaneously infusing antiretroviral drugs and IS directly into the H-ESI source during a chromatographic analysis of six batches of blank plasma extracts. Fig. 2 shows a typical chromatographic overlay of signals following injection of six separate blank plasma extracts and an injection of eluent (control) for the mass transitions of all compounds and QX. Ion suppression (SQV, APV, ATV, RPV, NVP) or enhancement (RTV, LPV, ETV) was evident within the first 0.5–1.5 min of the analytical run. Crucially, however, during the period of elution for these compounds, signal intensity was relatively constant. In addition, an absolute matrix effect was assessed by comparing the peak area response of analyte spiked in blank plasma extracts to the same concentration of analyte in mobile phase. A value above or below 100% was indicative of ion enhancement or suppression. The results from these quantitative analyses indicated no effect of the sample matrix on the ionization of all 10 analytes studied (Table 4). Interestingly, in a post-column infusion a slight suppression in ionization was noted at the retention time of DRV for 5/6 plasma extracts; however, this effect was not apparent in a quantitative analysis (% matrix effect = 100 ± 10.1).

Table 4
Percentage (%) recovery, matrix effect and extraction yield of all antiretroviral drugs.

Analtye	% recovery	% matrix effect	% extraction yield
Maraviroc	113 (3.9)	114 (9.5)	101 (7.4)
Saquinavir	112 (4.5)	113 (6.0)	100 (4.4)
Rilpivirine	99 (7.1)	105 (8.7)	95 (2.5)
Nevirapine	98 (8.7)	100 (8.4)	100 (5.4)
Amprenavir	94 (10.5)	102 (11.8)	95 (7.6)
Darunavir	98 (7.7)	100 (10.1)	100 (4.9)
Atazanavir	114 (8.6)	111 (12.5)	100 (6.9)
Ritonavir	98 (9.4)	93 (9.2)	104 (10.0)
Lopinavir	114 (9.7)	108 (14.2)	104 (5.0)
Etravirine	91 (8.8)	89 (11.1)	97 (11.8)

Data expressed as the mean (±CV%). Samples were spiked and analysed in triplicate on four separate occasions (n = 12).
% recovery = (peak area of analyte extracted from plasma/peak area of directly injected solution) × 100.
% matrix effect = (peak area of analyte spiked in blank plasma extract/peak area of directly injected solution) × 100.
% extraction yield = (peak area of analyte extracted from plasma/peak area of analyte spiked in blank plasma extract) × 100.

As some of the analytes co-eluted, the presence of "cross-talk" between agents with overlapping retention times [SQV, RPV, NVP (1.59–1.65 min) and APV, DRV, ATV (2.03–2.05 min)] was evaluated by injecting (10 μl, full loop) co-eluting agents (at their respective ULQ) onto the HPLC column, while infusing individual analytes (at 100 ng/ml) post-column. In addition, quantitative experiments were performed whereby plasma spiked (at the LQC only; n = 12) with all three co-eluting drugs (SQV, RPV, NVP, and APV, DRV, ATV) and plasma spiked with the individual agents alone were read off a validated calibration curve (containing all 10 ART) and compared. From both the infusion experiments (data not shown) and the spiking experiments, there was no evidence that co-eluting analytes disturb the ionization efficiency, as concentrations in plasma spiked with all co-eluting analytes (SQV, RPV, NVP and APV, DRV, ATV) were within 2–6% of the concentrations obtained from plasma spiked with the individual analytes alone.

3.1.6. Stability

Stability data for DRV, ETV, MVC and RPV are shown in Table 5. Mean concentrations (expressed as a percentage of control) of the heat inactivated samples and samples undergoing heat inactivation and three freeze/thaw cycles were within 10% and 12% of the freshly prepared controls, respectively ($P \geq 0.07$). For long-term stability testing, QC samples (LQC, MQC and HQC) were stored in the freezer (−20 °C) and compared to freshly prepared QC samples on days 4, 7, 14 and at 1 month. The analysis revealed slight

Table 3
The accuracy (% bias) and precision (CV%) for the simultaneous determination of 10 antiretroviral compounds by HPLC–MS/MS at all QC concentrations.

Analyte	QC	Mean (ng/ml)	Accuracy (% bias)	Precision (CV%)	
				Inter-assay	Intra-assay
Maraviroc	LLQC	13.2	5.1	7.6	7.1
	LQC	48	5.6	7.0	6.0
	MQC	392	4.7	8.3	2.3
	HQC	691	−2.3	8.1	4.2
Saquinavir	LLQC	25.2	0.7	5.4	6.8
	LQC	152	4.7	6.1	3.6
	MQC	1543	0.1	6.2	3.3
	HQC	7075	−3.5	5.2	1.2
Rilpivirine	LLQC	24.2	−3.1	6.2	6.7
	LQC	140	2.6	6.2	4.3
	MQC	1522	1.8	6.4	4.0
	HQC	7222	−2.3	4.9	2.2
Nevirapine	LLQC	25.6	2.5	8.0	7.6
	LQC	140	2.8	6.6	4.6
	MQC	1499	−0.4	5.8	3.7
	HQC	7372	−4.9	5.0	1.7
Amprenavir	LLQC	25.9	3.6	9.0	6.2
	LQC	154	7.1	5.0	5.0
	MQC	1537	6.6	6.5	2.8
	HQC	7795	2.0	4.9	2.2
Darunavir	LLQC	43.9	8.9	8.3	8.0
	LQC	230	3.6	6.2	5.1
	MQC	3948	4.5	6.0	3.0
	HQC	11887	1.6	5.0	2.1
Atazanavir	LLQC	25.5	1.9	7.4	5.6
	LQC	132	2.1	5.7	3.9
	MQC	1518	0.1	6.0	3.2
	HQC	7433	−0.8	4.6	1.7
Ritonavir	LLQC	14.6	13.5	9.7	8.7
	LQC	87	9.4	5.8	3.0
	MQC	797	5.5	6.2	3.1
	HQC	3848	1.4	5.1	2.5
Lopinavir	LLQC	46.6	13.1	6.6	3.4
	LQC	247	12.0	5.5	5.6
	MQC	3978	12.4	4.9	4.0
	HQC	11802	8.8	4.4	2.0
Etravirine	LLQC	13.1	4.5	8.7	8.8
	LQC	79	1.0	6.3	10.0
	MQC	589	3.1	6.3	3.0
	HQC	3777	−2.9	5.8	1.6

CV% = (standard deviation/mean) × 100.
% bias = [(measured concentration/mean target concentration)/measured concentration] × 100.

fluctuations in analytical response over this period, however, crucially drug concentrations did not deviate by ±15% from the QC samples analysed on day 1 of the long-term study. As a result, there was no indication that decomposition had occurred for any antiretroviral agent, suggesting that calibrators and QC remain stable under our current storage conditions for at least 1 month.

4. Discussion

The development and validation of an ultra-sensitive assay to simultaneously quantify 10 antiretroviral compounds, including both existing and new classes, with accuracy and precision, has been described. This bio-analytical method is now being successfully applied to measure antiretroviral plasma concentrations obtained from clinical pharmacokinetic studies.

The calibration curves for all compounds were constructed to reflect therapeutic concentrations observed in HIV-infected patients from pharmacokinetic data reported in the literature. RTV was validated over a lower concentration range relevant to its use in boosting the systemic exposure of other PI. When analysing clinical samples, if concentrations exceed the ULQ, the assay can be repeated specifying an appropriate dilution factor. As a means of validating this procedure, two HQC samples are also diluted by the same dilution factor. The chromatograms in Fig. 3 show extracted plasma samples that were obtained from HIV-infected patients receiving (A) NVP [200 mg twice daily (BD)] and LPV/RTV (400/100 mg BD) and (B) DRV (600 mg BD), ETV (200 mg BD) and RTV (100 mg BD). Blood samples were obtained 12 h after drug intake and plasma had been heat inactivated (58 °C and 40 min) prior to analysis. Plasma concentrations were (A) 4510, 5024 and 190 ng/ml for NVP, LPV and RTV, and (B) 3430, 270 and 435 ng/ml for DRV, ETV and RTV, respectively.

The HPLC–MS/MS method was fully validated with accuracy (% bias) and precision (CV%), which did not exceed 13% and 10% for all compounds. The correlation coefficients (r^2) for all calibration curves were ≥0.998, respectively. Percentage recovery was greater than 90% for all analytes. Assay performance is assessed routinely by monitoring internal QC samples by use of Levy-Jenning plots (based on modified Westgard rules) as a way of detecting

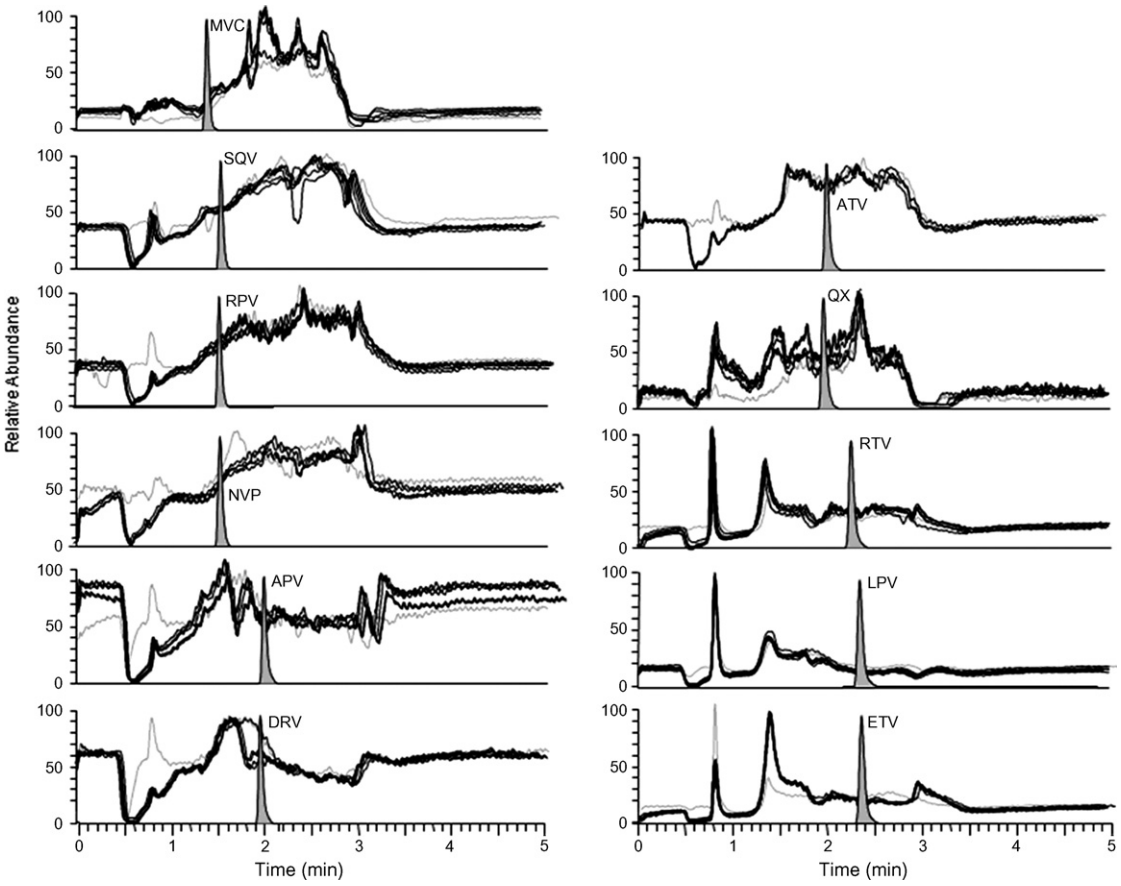

Fig. 2. Post-column infusion chromatograms depicting the matrix (ion suppression) effects from six different blank plasma samples extracted by protein precipitation, following a direct infusion of analyte (100 ng/ml) or internal standard (1 μg/ml). The infusion chromatograms following injections of eluent (light grey lines) and blank plasma extracts (black lines) are overlaid with the chromatographic peaks of each compound.

random versus systematic errors, and any drifts in assay conditions that may occur over time [52]. For this the LC Quan™ software requires a percentage (%) test value [to either (2 × standard deviation/mean) × 100 or (3 × standard deviation/mean) × 100] in order to ascertain QC failure within an assay run. Furthermore, to ensure accuracy when measuring clinical samples, our laboratory participates twice yearly in an external quality assurance programme (KKGT, Radboud University Medical Centre, Nijmegen, The Netherlands) [53].

HIV positive plasma samples are heat inactivated (58 °C and 40 min) prior to analysis in order to protect laboratory employees. Conditions used within our laboratory are similar to those published in the literature [54,55]. Recent studies have shown DRV, ETV and MVC to be stable in plasma after HIV inactivation and up to

Table 5
Stability data for DRV, ETV, MVC and RPV analysed under control conditions (freshly prepared), heat inactivation and heat inactivation with three freeze/thaw cycles.

Drug	Low	*P* value	Medium	*P* value	High	*P* value
Darunavir						
Control	100		100		100	
Heat inactivated	107 (7)	0.072	106 (7)	0.137	106 (15)	0.299
Heat–freeze/thaw	106 (6)	0.088	104 (8)	0.288	100 (7)	0.986
Etravirine						
Control	100		100		100	
Heat inactivated	108 (15)	0.543	110 (10)	0.473	108 (14)	0.624
Heat–freeze/thaw	105 (11)	0.654	102 (7)	0.889	100 (14)	0.994
						Maraviroc
Control	100		100		100	
Heat inactivated	99 (7)	0.913	97 (9)	0.602	97 (7)	0.532
Heat–freeze/thaw	93 (3)	0.220	88 (3)	0.073	93 (2)	0.177
						Rilpivirine
Control	100		100		100	
Heat inactivated	108 (12)	0.253	106 (9)	0.070	108 (11)	0.100
Heat–freeze/thaw	107 (15)	0.449	104 (5)	0.136	104 (6)	0.193

Samples were prepared and analysed in triplicate, on four separate occasions ($n = 12$).
Peak areas of analytes obtained under heat and heat/freeze thaw conditions are expressed as a percentage (±SD) of peak areas obtained under control conditions.
P values refer to comparisons made with freshly prepared samples (control) and ascertained using an one-way ANOVA with Bonferroni correction.

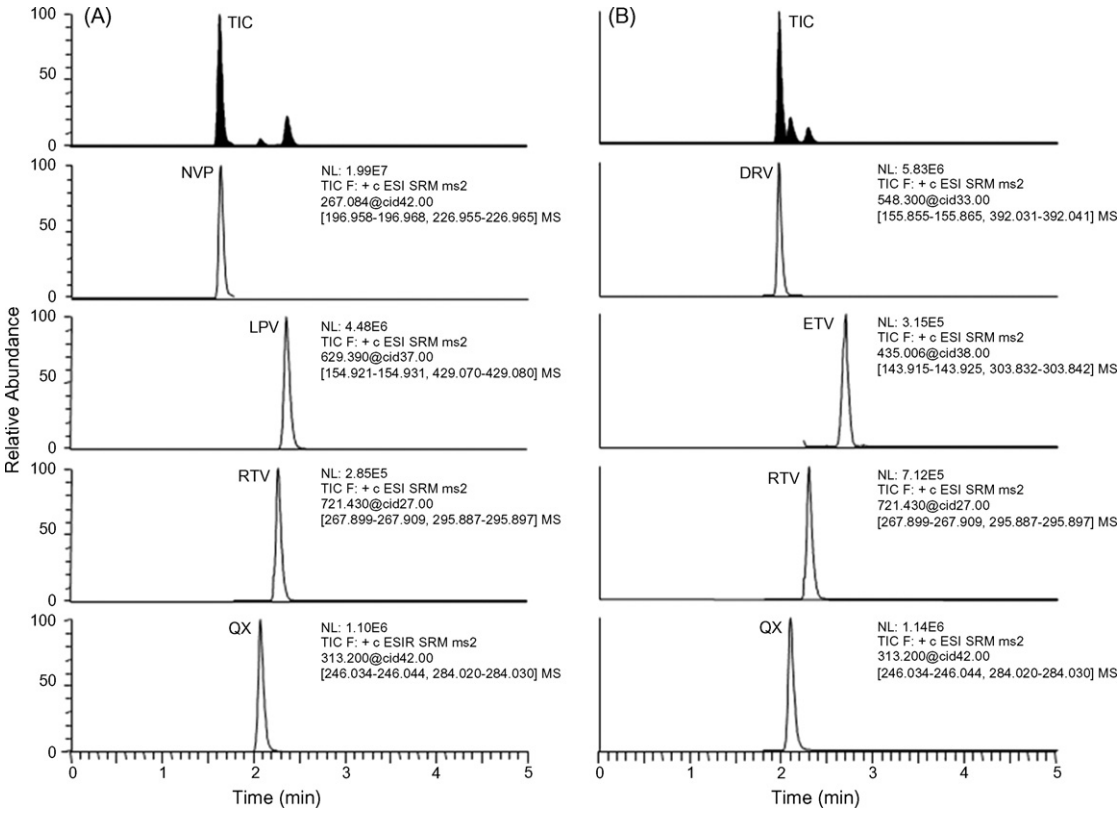

Fig. 3. Chromatograms of extracted plasma samples from HIV-infected patients receiving (A) NVP, LPV and RTV (4510, 5024 and 190 ng/ml) and (B) DRV, ETV and RTV (3430, 270 and 435 ng/ml). Blood samples were taken at 12 h post-dose.

three freeze/thaw cycles, and in storage (−20 °C) for up to 6 weeks [45,56]. Fundamentally, under our laboratory conditions, DRV, ETV, MVC and RPV appear to be stable when subjected to heat inactivation and up to three freeze/thaw cycles, in comparison to freshly prepared controls. Moreover, long-term stability data suggest that all analytes remained sufficiently stable under our current storage conditions (−20 °C) for up to 1 month. The stability of agents at room temperature (24 °C) or within the autosampler (which is maintained at 15 °C) was not evaluated. Previous publications show DRV, ETV, MVC and RAL to be stable at room temperature, 4 °C and when frozen [36,40,45]. Furthermore, the stability of NNRTI and PI under different conditions has been assessed and reported by other articles [30,32,33,35].

The assay described combines a very short run time of 5 min per sample with a quick and simple sample pre-treatment procedure and is therefore, suitable for high-throughput TDM purposes whereby large numbers of samples are processed quickly and efficiently. In addition, the method requires only a relatively small volume of plasma for analysis (100 μl). This is advantageous when quantifying drug in patients such as children and neonates, or from alternative matrices (e.g. CSF) from which only limited sample can be drawn or is not easily obtained. Intensive pharmacokinetic trials also necessitate frequent sampling as a means to ascertain total drug exposure over time; therefore, less blood needs to be drawn from the patient. Similarly, quantification of multiple agents within a single assay run is beneficial since antiretrovirals are given in combination, particularly in treatment experienced patients.

The current assay offers high sensitivity for all compounds (LLQ = 5–16 ng/ml) over a dynamic concentration range, and therefore has potential application for numerous pharmacokinetic analyses. High sensitivity in the current method was achieved and optimized by screening up to 6 mass transitions per compound, from which two daughter (fragment) ions were selected for quantification, based on their relative intensity and contribution to background interference. It is important to note that mass transitions of high intensity are not always optimal and may in fact produce a higher background and hence compromise sensitivity and chromatographic resolution, particularly in the LLQ region. For instance, we found the ETV fragment ion 162.90 *m/z* to be associated with high background interference which affected peaks at low concentrations. The ion was therefore substituted for 143.92 *m/z*, which improved the signal-to-noise ratio at the LLQ to an acceptable level (≥5.0). However, simultaneous quantification of multiple antiretrovirals via HPLC–MS/MS can pose a number of problems on the basis that the compounds possess different chemical properties [lipophilicity (log *P*/log *D*) and pKa]. Therefore, it is often impossible to achieve both optimum chromatographic resolution and sensitivity for all analytes, as would be possible if compounds were analysed separately.

Due to the high specify achieved in HPLC–MS/MS, inferences from the sample matrix and co-eluting compounds which are apparent with UV detection, often go unnoticed in SRM mass spectrometry and can have a deleterious impact on assay performance. This phenomenon, often observed as a reduction in response, is generally referred to as ion suppression and caused by the presence of non-volatile or less volatile solutes (e.g. salts, endogenous compounds and metabolites) in the ESI source which change the efficiency of droplet formation, impacting on the amount of charged ion in the gas phase ultimately reaching the MS detector [57]. Ion suppression can be evaluated by the use of post-column continuous infusions [58]. The FDA encourages that tests for matrix effects are performed as part of an assay validation procedure for all compounds and at therapeutically relevant concentrations in up to

six different batches of plasma, since ion suppression effects vary depending on the compound, its concentration (which relates to the matrix:analyte ratio) and the sample matrix itself (both plasma and precipitant). During post-column infusions of the current assay (Fig. 2), we observed no significant fluctuations in signal in the mass transition window of all analytes (with exception of DRV, where a slight suppression was observed in 5/6 plasma extracts) and QX at their respective retention times following injection of blank plasma. The most significant ionization effects, both suppression (SQV, APV, ATV, RPV and NVP) and enhancement (RTV, LPV and ETV), occurred within the first 1.5 min of the analytical run; the latter also occurred under control conditions and more likely corresponded to a rapid shift in the mobile phase gradient from low to high organic conditions [59]. Since plasma components tend to elute early, it is essential analytes elute beyond this high noise period and mobile phases should be adjusted accordingly to ensure adequate separation from endogenous interferences. In a quantitative analysis there was a slight suppression in the absolute response for ETV (−11%) and slight enhancement in ATV (+11%), SQV (+13%) and MVC (+14%) signal intensity. Although, as these effects remained constant across low, medium and high concentrations, plasma sample matrices are unlikely to compromise overall assay quantification and sensitivity. Despite an apparent suppression in the ionization of DRV in the infusion experiment, there was no evidence of any matrix effect in the spiking experiments (Table 4). These data highlight the importance of performing both time-dependent (infusion) and quantitative experiments on multiple batches of plasma in order to comprehensively evaluate the effect of the sample matrix upon overall analytical recovery, and that wherever possible, calibrator and QC samples should most accurately reflect the composition of the clinical samples to be analysed.

One limitation of the current method is that by the use of a quick rinsing gradient to ensure a rapid analytical run time, limited chromatographic separation was achieved which meant some analytes co-eluted. However, given the specificity of SRM (scan width 0.01 *m/z*) the presence of "cross-talk" between the mass transitions is highly unlikely, which was verified by a series of post-infusion and spiking experiments (Section 3.1.5). It is important to highlight that the integrase inhibitor raltegravir (RAL) was initially included during the early stages of method development. However, recently published methods for RAL have observed in patient samples an additional peak corresponding to the same *m/z* transitions as the parent drug (RAL = 445.1 → 361.0) which elutes earlier in the analytical run [45,60]. It was postulated that this secondary peak had originated from in-source degradation of the primary RAL metabolite, the RAL-glucuronide [61]. Based on these recent findings, plasma samples from patients receiving RAL were screened under our assay mobile phase conditions; but due to the rapid elution phase, the two peaks co-eluted. Therefore, quantification of clinical samples using the current method could lead to significant overestimations of active RAL concentrations from plasma and invalidate the pharmacokinetic data. Our laboratory therefore measures RAL via a separate assay using a modified gradient containing a lower organic (ACN) content to improve chromatographic separation. Under these conditions the additional peak elutes at 1.66 min followed by the parent compound (RAL) at 2.15 min, respectively. The secondary peak was identified as the RAL-glucuronide (MW 620) in a separate analysis by single ion monitoring in negative mode at $[M-H]^-$ = 619, which confirmed that the RAL-glucuronide and the early secondary peak co-elute. The NNRTI efavirenz (EFV) was also not included in the current method as optimum scanning for EFV is in negative mode, with capillary and vapourising temperatures operating at approximately 320 and 250 °C, respectively. A rapid shift to these parameters from positive ionization mode was found to compromise EFV sensitivity in the negative mode.

The majority of PI and NNRTI have a defined minimum effective concentration (MEC), although these serve only as guides and can differ depending on whether patients are treatment naive or experienced and harbouring wild-type or multi-drug resistant virus. Moreover, high inter-subject variability in PI and NNRTI pharmacokinetics is well documented among HIV-infected subjects [62,63] and clinical pharmacokinetic studies performed in diverse patient groups, as well as those investigating new antiretroviral combinations can result in unpredictable drug concentrations. It is therefore essential bio-analytical methods detect and quantify drug below the MEC and at toxic concentrations as a means of identifying non-adherence and patients at risk of treatment failure or adverse events.

Many HIV-infected patients receive concomitant medications for co-existing medical conditions alongside antiretroviral treatment. Interactions between the PI and agents including proton pump inhibitors, HMG-CoA reductase inhibitors and antituberculous and antifungal agents are common and may result in sub-therapeutic antiretroviral concentrations or an increased risk of toxicity. Also with the introduction of new antiretroviral drugs which possess efficacy against resistant viral strains and improved safety profiles, it is likely in the future that patients will be switched to these newer agents following the development of resistance or toxicity to current first-line regimens. Therefore, further pharmacokinetic studies investigating drug interactions between existing and new antiretroviral classes and including potential co-medications are vital to ensure that clinicians and patients are well informed of key disposition issues. MVC exposure is affected by compounds that modulate the activity of CYP3A4, including co-administered PI and NNRTI and a number of antibacterials and antifungals, which may in some instances necessitate an adjustment in MVC dosage [64]. There is a role for monitoring MVC plasma concentrations in such circumstances.

ETV steady-state plasma concentrations were reduced (21–37%) in healthy volunteers switching from efavirenz (after a 14 day intake) to ETV. Although this interaction was deemed not clinically significant since ETV concentrations were in excess of the drug's protein binding corrected EC_{50}, further studies are required in HIV-infected subjects [65]. Equally important are pharmacokinetic data on ETV in specific patient populations, particularly pregnant women and patients with psychiatric disorders, where the first generation NNRTI has limited application due to concerns over teratogenicity/CNS disorders (efavirenz) and hepatotoxicity (NVP).

Initial data show that RPV displays some unfavorable interactions with rifabutin (RPV AUC decreased 46%) and ketoconazole (RPV AUC increased 49%) [66,67]. There is also concern over the effect of increasing pH on RPV absorption and thus the potential for interactions involving gastric acid reducing agents [68]. Clarification of such interactions will be particularly important following marketing approval when RPV becomes more widely-used in the HIV-infected population.

In conclusion, a simple and rapid assay for the quantification of all currently approved PI (APV, ATV, DRV, LPV, RTV and SQV) and NNRTI (NVP), as well as recently licensed antiretroviral classes (MRC) and the second generation NNRTI (ETV and RPV) in human plasma has been developed and validated. The method was proven to be specific, accurate, precise and robust. Furthermore, the assay demonstrates a high sensitivity for all analytes and the step-wise gradient potentially allows for the addition of new analytes into the same analytical run. The method is now successfully applied to measure antiretroviral concentrations obtained from clinical pharmacokinetic studies, and due to the high signal-to-noise ratio at the LLQ level, could be adapted to measure low antiretroviral concentrations found in sanctuary sites such as the genital tract and in cerebrospinal fluid.

Funding

The authors thank the National Institute of Health Research (NIHR–Department of Health) for infrastructural and project support.

Conflict of interest

S.K. and D.B. have received research grants and travel bursaries from Merck, Bristol-Myers Squibb, GlaxoSmithKline, Pfizer, Abbott, Boehringer Ingelheim and Tibotec.

L.E., V.W., J.T., A.H. and M.S.: none to declare.

References

[1] F.J. Palella Jr., R.K. Baker, A.C. Moorman, J.S. Chmiel, K.C. Wood, J.T. Brooks, S.D. Holmberg, J. Acquir. Immune Defic. Syndr. 43 (2006) 27.
[2] B.G. Gazzard, HIV Med. 9 (2008) 563.
[3] A.A. Benzie, L.K. Bansi, C.A. Sabin, S. Portsmouth, T. Hill, M. Johnson, R. Gilson, P. Easterbrook, B. Gazzard, M. Fisher, C. Orkin, D. Dunn, V. Delpech, G.P. Taylor, J.C. Walsh, A.N. Phillips, AIDS 21 (2007) 1423.
[4] P. Dorr, M. Westby, S. Dobbs, P. Griffin, B. Irvine, M. Macartney, J. Mori, G. Rickett, C. Smith-Burchnell, C. Napier, R. Webster, D. Armour, D. Price, B. Stammen, A. Wood, M. Perros, Antimicrob. Agents Chemother. 49 (2005) 4721.
[5] D. Cooper, J. Gatell, J. Rockstroh, C. Katlama, P. Yeni, A. Lazzarin, X. Xu, R. Isaacs, H. Teppler, B. Nguyen, B.-S. Group, Proceedings of the 15th Conference on Retroviruses and Opportunistic Infections, Boston, USA, 2008 (abstract 788).
[6] R. Steigbigel, P. Kumar, J. Eron, Proceedings of the 15th Conference on Retroviruses and Opportunistic Infections, Boston, USA, 2008 (abstract 789).
[7] K. Das, A.D. Clark Jr., P.J. Lewi, J. Heeres, M.R. De Jonge, L.M. Koymans, H.M. Vinkers, F. Daeyaert, D.W. Ludovici, M.J. Kukla, B. De Corte, R.W. Kavash, C.Y. Ho, H. Ye, M.A. Lichtenstein, K. Andries, R. Pauwels, M.P. De Bethune, P.L. Boyer, P. Clark, S.H. Hughes, P.A. Janssen, E. Arnold, J. Med. Chem. 47 (2004) 2550.
[8] R. Haubrich, P. Cahn, B. Grinsztejn, J. Lalezari, J. Madruga, A. Mills, M. Peeters, J. Vingerhoets, K. Iveson, G. De Smedt, Proceedings of the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2008 (abstract 790).
[9] M. Johnson, T. Campbell, B. Clotet, C. Katlama, A. Lazzarin, W. Towner, M. Peeters, J. Vingerhoets, S. Bollen, G. De Smedt, Proceedings of the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2008 (abstract 791).
[10] M. Santoscoy, P. Cahn, C. Gonsalez, W. Hao, A. Pozniak, P. Shalit, S. Vanveggel, K. Boven, Proceedings of the XVII International AIDS Conference, Mexico City, Mexico, 2008 (abstract TUAB0103).
[11] M.M. de Maat, G.C. Ekhart, A.D. Huitema, C.H. Koks, J.W. Mulder, J.H. Beijnen, Clin. Pharmacokinet. 42 (2003) 223.
[12] C.S. Ernest 2nd, S.D. Hall, D.R. Jones, J. Pharmacol. Exp. Ther. 312 (2005) 583.
[13] G. Moyle, AIDS Read. 11 (2001) 87.
[14] M.M. Foisy, E.M. Yakiwchuk, C.A. Hughes, Ann. Pharmacother. 42 (2008) 1048.
[15] R.F. Yeh, V.E. Gaver, K.B. Patterson, N.L. Rezk, F. Baxter-Meheux, M.J. Blake, J.J. Eron Jr., C.E. Klein, J.C. Rublein, A.D. Kashuba, J. Acquir. Immune Defic. Syndr. 42 (2006) 52.
[16] M.B. Wire, M.J. Shelton, S. Studenberg, Clin. Pharmacokinet. 45 (2006) 137.
[17] P.F. Smith, R. DiCenzo, G.D. Morse, Clin. Pharmacokinet. 40 (2001) 893.
[18] M. Scholler-Gyure, T.N. Kakuda, G. De Smedt, H. Vanaken, M.P. Bouche, M. Peeters, B. Woodfall, R.M. Hoetelmans, Br. J. Clin. Pharmacol. 66 (2008) 508.
[19] S. Abel, T.M. Jenkins, L.A. Whitlock, C.E. Ridgway, G.J. Muirhead, Br. J. Clin. Pharmacol. 65 (Suppl. 1) (2008) 38.
[20] S. Abel, D. Russell, R.J. Taylor-Worth, C.E. Ridgway, G.J. Muirhead, Br. J. Clin. Pharmacol. 65 (Suppl. 1) (2008) 27.
[21] D.M. Burger, P.W. Hugen, R.E. Aarnoutse, R.M. Hoetelmans, M. Jambroes, P.T. Nieuwkerk, G. Schreij, M.M. Schneider, M.E. van der Ende, J.M. Lange, Ther. Drug Monit. 25 (2003) 73.
[22] T.E. de Vries-Sluijs, J.P. Dieleman, D. Arts, A.D. Huitema, J.H. Beijnen, M. Schutten, M.E. van der Ende, Clin. Pharmacokinet. 42 (2003) 599.
[23] B. Masquelier, D. Breilh, D. Neau, S. Lawson-Ayayi, V. Lavignolle, J.M. Ragnaud, M. Dupon, P. Morlat, F. Dabis, H. Fleury, Antimicrob. Agents Chemother. 46 (2002) 2926.
[24] X. Duval, F. Mentre, C. Lamotte, G. Chene, B. Spire, P. Dellamonica, X. Panhard, D. Salmon, F. Raffi, G. Peytavin, C. Leport, Ther. Drug Monit. 27 (2005) 63.
[25] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, AIDS 15 (2001) 71.
[26] J.E. Ray, D. Marriott, M.T. Bloch, A.J. McLachlan, Br. J. Clin. Pharmacol. 60 (2005) 291.
[27] E. Seminari, G. Gentilini, L. Galli, H. Hasson, A. Danise, E. Carini, F. Dorigatti, A. Soldarini, A. Lazzarin, A. Castagna, J. Antimicrob. Chemother. 56 (2005) 790.
[28] A. D'Avolio, M. Sciandra, M. Siccardi, L. Baietto, D.G. de Requena, S. Bonora, G. Di Perri, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 848 (2007) 374.
[29] B.H. Jung, N.L. Rezk, A.S. Bridges, A.H. Corbett, A.D. Kashuba, Biomed. Chromatogr. 21 (2007) 1095.
[30] 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.
[31] P. Villani, M. Feroggio, L. Gianelli, A. Bartoli, M. Montagna, R. Maserati, M.B. Regazzi, Ther. Drug Monit. 23 (2001) 380.
[32] K.M. Crommentuyn, H. Rosing, M.J. Hillebrand, A.D. Huitema, J.H. Beijnen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 804 (2004) 359.
[33] M.L. Turner, K. Reed-Walker, J.R. King, E.P. Acosta, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 784 (2003) 331.
[34] L. Goldwirt, S. Chhun, E. Rey, O. Launay, J.P. Viard, G. Pons, V. Jullien, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 857 (2007) 327.
[35] A. D'Avolio, M. Siccardi, M. Sciandra, L. Baietto, S. Bonora, L. Trentini, G. Di Perri, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 859 (2007) 234.
[36] A. D'Avolio, L. Baietto, M. Siccardi, M. Sciandra, M. Simiele, V. Oddone, S. Bonora, G. Di Perri, Ther. Drug Monit. 30 (2008) 662.
[37] 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: Analyt. Technol. Biomed. Life Sci. 867 (2008) 205.
[38] J.M. Poirier, P. Robidou, P. Jaillon, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 867 (2008) 277.
[39] M.C. Long, C. Bennetto-Hood, E.P. Acosta, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 867 (2008) 165.
[40] S.A. Merschman, P.T. Vallano, L.A. Wenning, B.K. Matuszewski, E.J. Woolf, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 857 (2007) 15.
[41] S. Abel, D. Russell, L.A. Whitlock, C.E. Ridgway, G.J. Muirhead, Br. J. Clin. Pharmacol. 65 (Suppl. 1) (2008) 47.
[42] A.L. Pozniak, M. Boffito, D. Russell, C.E. Ridgway, G.J. Muirhead, Br. J. Clin. Pharmacol. 65 (Suppl. 1) (2008) 54.
[43] M. Boffito, A. Winston, A. Jackson, C. Fletcher, A. Pozniak, M. Nelson, G. Moyle, I. Tolowinska, R. Hoetelmans, D. Miralles, B. Gazzard, AIDS 21 (2007) 1449.
[44] M. Scholler-Gyure, T.N. Kakuda, V. Sekar, B. Woodfall, G. De Smedt, E. Lefebvre, M. Peeters, R.M. Hoetelmans, Antivir. Ther. 12 (2007) 789.
[45] A. Fayet, A. Beguin, B. Zanolari, S. Cruchon, N. Guignard, A. Telenti, M. Cavassini, H.F. Gunthard, T. Buclin, J. Biollaz, B. Rochat, L.A. Decosterd, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1057.
[46] E. Van Gyseghem, M. Pendela, L. Baert, J. Rosier, G. Van't Klooster, H. De Man, M.P. Bouche, L. Schueller, P. Van Remoortere, P. Wigerinck, E. Adams, J. Hoogmartens, G. Van den Mooter, Eur. J. Pharm. Biopharm. 70 (2008) 853.
[47] H.E. van't Klooster, G. Borghys, H. Looszova, A. Bouche, M.P. van Velsen, F.L. Baert, Antimicrob. Agents Chemother. (2010).
[48] L. Dickinson, L. Robinson, J. Tjia, S. Khoo, D. Back, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 829 (2005) 82.
[49] J.O. Westgard, P.L. Barry, M.R. Hunt, T. Groth, Clin. Chem. 27 (1981) 493.
[50] FDA, 2001. Available from: <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf/>.
[51] I.M.C. Campbell, M. Wakefield, K. Halm, R. Zhang, A. Schoen, Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs, California, USA, 1997.
[52] Health Protection Agency. Quality assurance in the diagnostic virology and serology laboratory. National Standard Method QSOP 27 Issue 5.1, 2008. Available from: <http://www.hpa-standardmethods.org.uk/pdf/>.
[53] J.A. Droste, R.E. Aarnoutse, P.P. Koopmans, Y.A. Hekster, D.M. Burger, J. Acquir. Immune Defic. Syndr. 32 (2003) 287.
[54] B. Spire, D. Dormont, F. Barre-Sinoussi, L. Montagnier, J.C. Chermann, Lancet 1 (1985) 188.
[55] A. Dasgupta, D. Bard, Ther. Drug Monit. 16 (1994) 612.
[56] R. Ter Heine, H. Rosing, E.C. van Gorp, J.W. Mulder, J.H. Beijnen, A.D. Huitema, J. Pharm. Biomed. Anal. (2008).
[57] T.M. Annesley, Clin. Chem. 49 (2003) 1041.
[58] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[59] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[60] R. Ter Heine, M.J. Hillebrand, H. Rosing, E.C. van Gorp, J.W. Mulder, J.H. Beijnen, A.D. Huitema, J. Pharm. Biomed. Anal. (2008).
[61] 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.
[62] R.E. Nettles, T.L. Kieffer, T. Parsons, J. Johnson, J. Cofrancesco Jr., J.E. Gallant, K.A. Carson, R.F. Siliciano, C. Flexner, Clin. Infect. Dis. 42 (2006) 1189.
[63] M.R. van der Leur, D.M. Burger, C.J. la Porte, P.P. Koopmans, Ther. Drug Monit. 28 (2006) 650.
[64] S. Abel, D.J. Back, M. Vourvahis, Antivir. Ther. 14 (2009) 607.
[65] M. Boffito, A. Jackson, M. Lamorde, D. Back, V. Watson, J. Taylor, L. Waters, D. Asboe, B. Gazzard, A. Pozniak, J. Acquir. Immune Defic. Syndr. 52 (2009) 222.
[66] H.M. Crauwels, R.P.G. van Heeswijk, D. Kestens, M. Stevens, Proceedings of the 42nd Aids Conference, Mexico City, Mexico, 2008 (abstract TUPE0080).
[67] R.P.G. van Heeswijk, R.M. Hoetelmans, D. Kestens, Proceedings of the 41st International AIDS Conference, Toronto, Canada, 2006.
[68] R.P.G. van Heeswijk, R. Hoetelmans, D. Kestens, Proceedings of the 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Sydney, Australia, 2007 (abstract TUPDB01).