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Simultaneous determination of HIV protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in human plasma by high-performance liquid chromatography-tandem mass spectrometry

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Abstract

We report a precise and accurate method for simultaneous quantification of protease inhibitors (PIs) amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in plasma. An internal standard was added to samples prior to protein precipitation with acetonitrile followed by addition of ammonium formate buffer. Analysis was by HPLC-MS/MS. Calibration curves were validated over concentration ranges encompassing both subtherapeutic and potentially 'toxic' drug concentrations. Inter- and intra-assay variation were below 11% and PI recovery was above 87%. The bioanalytical method described is successfully applied to measure PI concentrations obtained from clinical pharmacokinetic studies and routine therapeutic drug monitoring (TDM).

Keywords: Protease inhibitors; HPLC-MS/MS; Therapeutic drug monitoring

1. Introduction

Protease inhibitors (PIs) are a potent class of antiretroviral therapy used for long-term treatment of human immunodeficiency virus (HIV) [1] and are associated with improving morbidity and mortality of HIV infected individuals [2]. PIs exert their activity by prevention of post-translational processing of gag and gag-pol polyprotein precursors and results in the production of non-infectious, immature virions [1]. At present eight PIs have been licensed for use as part of combination therapy in Europe and the United States (amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir). PIs are associated with unfavourable pharmacokinetics (PK) and a range of drug related side effects including gastrointestinal disturbances and lipid abnormalities [3,4]. PIs are rapidly metabolised by cytochrome P450 enzymes, namely CYP3A4, and to a lesser extent CYP2D6 and CYP2C19 and are prone to extensive and complex drug–drug interactions when given in combination together or with other therapies [5–7]. All PIs are inhibitors of CYP3A with ritonavir used as a PK booster for other PIs [8,9]. Lopinavir and amprenavir have enzyme-inducing properties [6]. In addition to being substrates of P450 enzymes, they are also substrates and inhibitors of *p*-glycoprotein (P-gp), a transmembrane, ATP-dependent efflux pump for a wide variety of compounds [10].

PK studies investigating interactions between PIs and those assessing new dosing strategies require accurate and precise measurement of drug concentrations at a range of time points and studies investigating interactions between several drugs require quantification of the different medications involved. Moreover, since PI plasma concentrations correlate with clinical outcome [11–15], therapeutic drug monitoring (TDM) can be used in selected patients groups to optimise therapy. Bioanalytical methods to determine total drug in plasma for PK studies and routine TDM need to be accurate, precise and specific. Ideally, they should also be capable of analysing a large number of samples

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quickly and require a small volume of plasma. Evaluation of several drugs within an assay is essential to optimise assay capacity and turnaround time.

Methods used to quantify PIs in plasma are widely reported in the literature. Early work only needed to determine one or a small number of PIs [16–19] but due to an increase in the number of available PIs and the introduction of combination PI therapy bioanalytical assays capable of measuring multiple PIs were necessary. Recently, techniques to determine intracellular concentrations of PIs have also been described [20–22]. Several methods have been reported to concurrently determine a number HIV PIs in human plasma by liquid chromatography coupled to mass spectrometry [23–29]. Our laboratory required measurement of plasma concentrations of all licensed PIs for clinical PK studies and routine TDM and therefore developed and validated an assay that can be used to quantify plasma concentrations of seven PIs using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The method was adapted from a previous assay used within our laboratory that measured only four PIs [30]. This type of analysis was chosen in preference to HPLC with UV detection as it is generally faster and does not require complete resolution of drugs for detection and quantification. For example, Tribut and co-workers developed an HPLC-UV assay to measure seven PIs and two non-nucleoside reverse transcriptase inhibitors with a run time of approximately 30 min [31]. Whereas a LC-MS/MS method has been optimised to quantify 15 antiretrovirals in a total run time of 4.5 min [29]. Validation was conducted based on modified Westgard regulations and FDA international guidelines for bioanalytical assay validation [32–34] and guidelines acceptable for Clinical Pathology Accreditation (CPA) [35].

2. Experimental

2.1. Equipment

The HPLC system consisted of a Surveyor AS autosampler (200 vial capacity; set at a temperature of 15 °C), a Surveyor PDA detector and a Surveyor LC pump all purchased from Thermo Electron Corporation (Hemel Hempstead, UK). Two E2M30 rotary vacuum pumps (Aztech Trading, Loughborough, Leicestershire, UK), a NM30LA nitrogen generator (Peak Scientific, Inchinnan, Renfrewshire, UK) and a 10 l helium cylinder (size V; BOC Gases, Worsley, Manchester, UK) were also used. The HPLC system was interfaced with an ion trap LCQ Deca XP Plus mass spectrometer with an electrospray ionisation (ESI) source (Thermo Electron Corporation, Hemel Hempstead, UK) operated by Xcalibur software (Version 1.3, Thermo Electron Corporation, Hemel Hempstead, UK). The analytical column used to elute amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV) was a HyPURITY C_{18} column [5 μm: 100 mm × 2.1 mm (column oven set at a temperature of 26 °C); Thermo Electron Corporation, Runcorn, Cheshire, UK] protected by a precolumn guard (Si 60, 5 μm; Merck, Germany).

2.2. Chemicals

APV and ATV were kindly contributed by Glaxo Wellcome Research & Development (Middlesex, UK) and Bristol-Myers Squibb (Hounslow, UK), respectively. SQV and internal standard (IS; Ro31-9564) were kindly supplied by Roche Discovery (Welwyn, UK). LPV and RTV were a kind gift from Abbott Laboratories (Chicago, USA). IDV and NFV were donated by Merck Sharpe & Dohme (Hertfordshire, UK) and Agouron Pharmaceuticals (La Jolla, USA), respectively. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR Laboratory Supplies (Poole, UK) and ammonium formate obtained from Fisher Scientific (Loughborough, UK). Drug free plasma was obtained from the National Blood Transfusion Service (Liverpool, UK) and de-ionised water was used throughout (Option 4 water purifier, Elga LabWater, High Wycombe, UK).

2.3. Preparation of calibrators, quality controls and internal standard

Briefly, separate solutions of APV, ATV, IDV, LPV, NFV, RTV and SQV were dissolved in MeOH to obtain final drug concentrations of 1 mg/ml. These solutions were diluted with drug free plasma to yield two levels: level 5 (APV, NFV, SQV 500 ng/ml; ATV 300 ng/ml, IDV 1000 ng/ml; LPV 750 ng/ml; RTV 250 ng/ml) and level 9 (APV, NFV, RTV, SQV 5000 ng/ml; ATV 6000 ng/ml; IDV 10,000 ng/ml; LPV 15,000 ng/ml) of a nine point standard curve containing all seven drugs.

Preparation of quality control (QC) samples was identical to that of calibrator samples. However, three levels of QC (APV, ATV, IDV, NFV, SQV 150, 2000 and 4000 ng/ml; LPV 750, 3000 and 10,000 ng/ml; RTV 100, 1000 and 4000 ng/ml) were generated on dilution of separate 1 mg/ml solutions with drug free plasma. The three levels of QC will be referred to as low (LQC), medium (MQC) and high (HQC) throughout.

The IS compound (Ro31-9564) was dissolved in MeOH to a concentration of 100 μg/ml. An IS solution of 1 μg/ml was obtained by dilution of this 100 μg/ml stock solution in MeOH/water (50:50, v/v).

2.4. Sample pretreatment

Levels 5 and 9 calibrators were diluted in duplicate with appropriate volumes of drug free plasma to yield a nine point standard curve containing all seven PIs (100 μl per calibrator level). IS (20 μl) was added to duplicates of the standard curve and QC samples (100 μl) prior to the addition of ACN (1 ml). Samples were vortexed and left to stand at room temperature (15 min). All tubes were vortexed and centrifuged (1780 × *g*; 10 min; 4 °C) and the solvent phase decanted to clean glass tubes prior to addition of ammonium formate buffer (300 μl; 20 mM). Samples were vortexed and an aliquot (150 μl) from each tube transferred to autosampler vials for injection (10 μl) onto the column.

2.5. Chromatographic and mass spectrometric conditions

APV, ATV, IDV, LPV, NFV, RTV and SQV were eluted using a gradient mobile phase [ACN:ammonium formate buffer

Table 1
Mobile phase gradient program, consisting of acetonitrile (ACN) and ammonium formate buffer (20 mM), to elute amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and internal standard

Time (min)	ACN:ammonium formate (20 mM)	
	50:50 (%)	70:30 (%)
0	100	0
2	0	100
6.5	0	100
7.0	100	0
10	100	0

(20 mM); 50/50 and 70/30, v/v] maintained at a flow rate of 0.4 ml/min. An outline of the gradient program is described (Table 1). Analysis of all seven PIs involved the mass spectrometer operating under vacuum in positive ion mode. At the beginning (0.00–0.25 min) and at the end (7.99–9.00 min) of each run the elutant was diverted from the mass spectrometer to waste by an electronically controlled divert valve. Initially, gas phase charged molecular ions were produced in the ion source of the instrument and daughter ions of the parent molecules within the ion trap. Subsequently these daughter ions were separated according to their mass-to-charge (m/z) ratio. Finally, the concentration of APV, ATV, IDV, LPV, NFV, RTV and SQV was determined by measuring the intensity of separate PI:IS daughter ion ratios. A summary of parent and daughter ions of all seven PIs and relative collision energies used to produce these ions are shown (Table 2).

2.6. Validation of APV, ATV, IDV, LPV, NFV, RTV, SQV standard curve and quality control samples

A minimum of 12 standard curves were prepared along with QC samples (LQC, MQC and HQC) on separate days and analysed by HPLC-MS/MS to ascertain and assign a fixed mean target value to each calibrator level (levels 2–9) for all seven PIs, thus resulting in final standard curve concentrations. This is in accordance with CPA regulations. Mean target calibrator concentrations are determined in order to account for slight error or deviations in nominal concentrations when the stocks of calibrators are made up. All values are included, however anomalous values (such as those that may occur due to injection difficulties) are excluded and assays continued until at least 12 duplicate values for each concentration are obtained. These mean target concentrations are effectively the "nominal" concentrations, however they will be referred to throughout the manuscript as mean target values.

To reduce turn around time QC samples were run with calibrators before their mean target values were determined however, they were treated as unknown samples within each assay run. Once the final mean standard curves were established all assays were reprocessed with Xcalibur using these concentrations to determine final mean target QC values. After these concentrations were obtained subsequent assays assessing PI stability and evaluating patient samples had to be acceptable based on criteria adapted from CPA and Westgard regulations and FDA guidelines.

2.7. Stability and recovery

Stability at various conditions, of APV, ATV IDV, LPV, NFV, RTV and SQV has been described [16,17,19,23,28,36–38]. Samples arriving at our laboratory undergo heat inactivation (58 °C; 40 min) prior to sample pretreatment and may also go through a freeze/thaw cycle. Therefore drug free plasma was spiked with all seven drugs to obtain three separate concentrations (APV: 400, 4000 and 8000 ng/ml; ATV, SQV: 100, 3000 and 6000 ng/ml; IDV: 100, 5000 and 10,000 ng/ml; LPV: 2000, 7000 and 14,000 ng/ml; NFV: 800, 3000 and 6000 ng/ml; RTV: 100, 1500 and 3000 ng/ml) on the day of analysis. These concentrations (referred to as low, medium and high) approximate to the therapeutic ranges defined in the literature [39,40] or span the range of respective standard curves. Samples were prepared and analysed in triplicate on three separate occasions to determine plasma concentrations of each drug under the three different treatment/storage conditions (prepared fresh, heat inactivated and heat inactivated-freeze/thaw). These experiments were performed subsequent to calibrator and QC validation.

Recovery of PIs from plasma was assessed in six replicates by comparison of directly injected drug solution and drug extracted from plasma (protein precipitation) at three distinct concentrations (identical to those used in stability analyses) on three separate occasions. Mobile phase was spiked with all seven drugs to obtain the selected concentrations of drug solution and drug free plasma was spiked to yield identical concentrations

Table 2
Parent and daughter ions and relative collision energies (RCE) used to evaluate amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and internal standard by HPLC-MS/MS

Analyte	Parent ion (m/z)	Daughter ions (m/z)			RCE (%)	RT (min)
Indinavir	614.40	465.20	596.30		30	1.10
Amprenavir	506.20	245.00	417.90		30	1.38
Atazanavir	705.40	335.10	534.20	612.20	30	1.97
Ritonavir	721.40	267.90	295.90	426.10	45	2.32
Saquinavir	671.40	433.20	570.30	571.30	45	2.63
Lopinavir	629.40	447.10	611.20		45	2.74
Nelfinavir	568.30	331.10	467.20	468.30	40	5.03
Internal standard	674.40	388.20	436.10	573.20	35	6.14

Retention times (RT) are also displayed for each analyte.

for protein precipitation. Percentage recovery was calculated by evaluating resulting peak areas.

2.8. Data analysis

Data acquisition and processing was performed by Xcalibur software. Standard curves were constructed using 1/concentration^2 weighted quadratic regression of peak area:IS ratio versus target concentration and drug content of unknown samples were interpolated.

Final mean target calibrator (levels 2–9) and QC (LQC, MQC and HQC) concentrations were calculated using Microsoft Excel XP for Windows (Microsoft Corporation, USA). In addition coefficient of variation [CV%; (standard deviation/mean) × 100] and percentage test [% test; (2 × standard deviation/mean) × 100] values were also determined. Xcalibur software requires a % test value in order to assess QC failure within an assay run.

Stability of PIs under fresh, inactivated and inactivated-freeze/thaw conditions was assessed by one-way analysis of variance (ANOVA) with Bonferroni correction at three concentrations. A p value < 0.05 indicated a significant effect of the treatment/storage conditions on PI stability. Data were subjected to Shapiro–Wilk test for non-normality prior to ANOVA and statistical analyses performed by Arcus Quickstat (Version 1.1©1997, Biomedical Software, StatsDirect Ltd., Cheshire, UK). Percentage recovery of all seven drugs from plasma was calculated as follows: (peak area of drug extracted from plasma/peak area of drug from directly injected solution) × 100.

3. Results

3.1. Detection and chromatography

APV, ATV, IDV, LPV, NFV, RTV and SQV were detected and quantified over a total of 9 min, split into four separate segments. ATV, RTV, LPV and SQV all had similar elution times and therefore appeared in the same segment. To obtain optimal conditions for quantification with HPLC-MS/MS, scanning a maximum of three drugs per time segment was considered appropriate. Two Xcalibur instrument methods were therefore established, one determined APV, IDV, LPV, NFV, RTV and SQV (method 1) and the other APV, ATV, IDV, LPV, NFV and SQV (method 2). For method 1 APV and IDV were detected

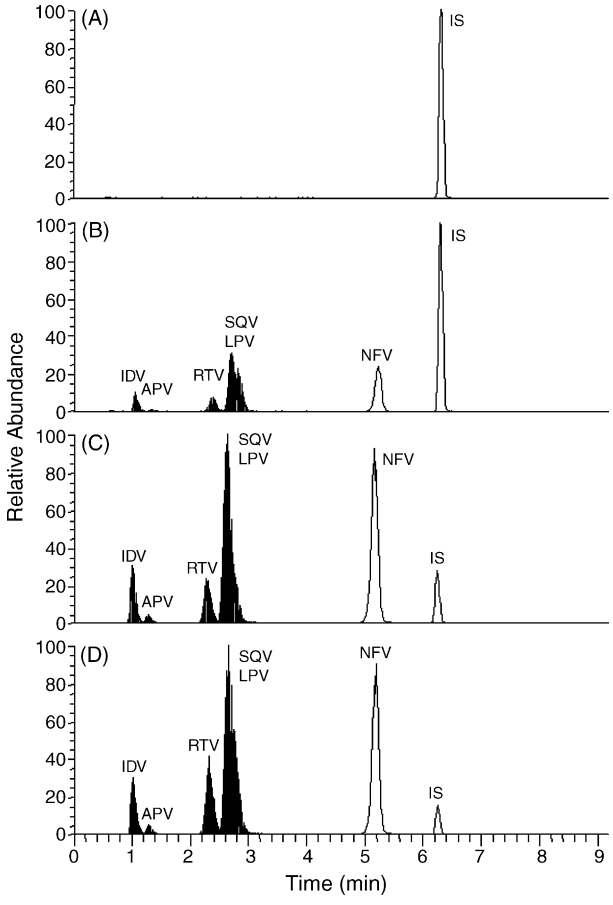

Fig. 1. Chromatograms corresponding to method 1 at total retention time (9 min) of (A) a blank plasma sample spiked with internal standard (IS) and (B) a low quality control, (C) a medium quality control and (D) a high quality control sample containing indinavir (IDV), amprenavir (APV), ritonavir (RTV), saquinavir (SQV), lopinavir (LPV), nelfinavir (NFV) and internal standard (IS).

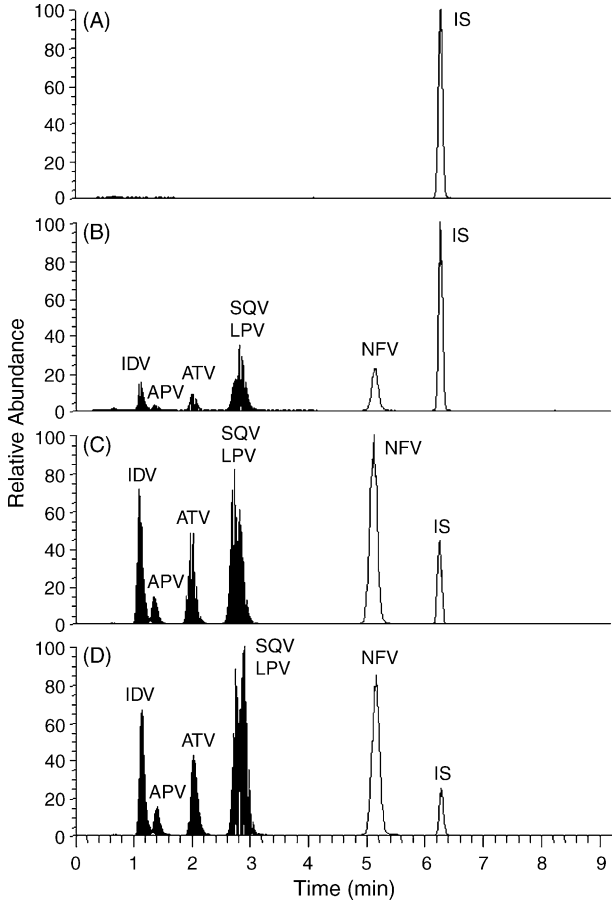

Fig. 2. Chromatograms corresponding to method 2 at total retention time (9 min) of (A) a blank plasma sample spiked with internal standard (IS) and (B) a low quality control, (C) a medium quality control and (D) a high quality control sample containing indinavir (IDV), amprenavir (APV), atazanavir (ATV), saquinavir (SQV), lopinavir (LPV), nelfinavir (NFV) and internal standard (IS).

in segment 1 (0.00–1.86 min), LPV, RTV and SQV in segment 2 (1.86–4.09 min), NFV in segment 3 (4.09–5.80 min) and IS in segment 4 (5.80–9.00 min). For method 2 APV and IDV were again detected in segment 1 (0.00–1.70 min), ATV, LPV and SQV in segment 2 (1.70–4.08 min), NFV in segment 3 (4.08–5.79 min) and IS in segment 4 (5.79–9.00 min). Scanning events can be used within each segment to produce unique daughter ions. Retention times of APV, ATV, IDV, LPV, NFV, RTV, SQV and IS are summarised (Table 2). The capillary temperature was maintained at 360 °C throughout assay runs. For segments 1 and 3 sheath and auxiliary gas flow (nitrogen at the source; helium at the ion trap), and source voltage were set at 50 units, 15 units and 4.5 kV, respectively. Sheath gas flow was 65 and 60 units in segments 2 and 4, respectively and auxiliary gas flow and source voltage was maintained at 10 units and 5 kV for these two segments. Capillary voltage in segments 1–4 was 27, 3, 36 and 31 V, respectively. Source current was maintained at 80 μA for all segments. Typical chromatograms of a blank sample (containing only IS) and a LQC, MQC and HQC for methods 1 and 2 are shown (Figs. 1 and 2, respectively).

3.2. Validation of APV, ATV, IDV, LPV, NFV, RTV and SQV standard curve and quality control samples

A minimum of 12 standard curves and QC samples at each level (LQC, MQC and HQC) were analysed by HPLC-MS/MS and processed. Mean target calibrator concentrations and hence the ranges of each standard curve were determined to be between 56 and 5136 ng/ml (APV), 47 and 6239 ng/ml (ATV), 102 and 9481 ng/ml (IDV), 95 and 15,584 ng/ml (LPV), 62 and 4670 ng/ml (NFV), 25 and 4941 ng/ml (RTV), 71 and 4958 ng/ml (SQV). Mean target LQC, MQC and HQC concentrations were calculated as 179, 2214 and 3920 ng/ml (APV); 173, 2136 and 3835 ng/ml (ATV); 184, 2174 and 4268 ng/ml (IDV); 790, 2949 and 10,460 ng/ml (LPV); 185, 2056 and 3669 ng/ml (NFV); 102, 947 and 3901 ng/ml (RTV); 186, 1952 and 3677 ng/ml (SQV). Variation was less than 14% at the LLQ and did not exceed 14 and 10% for all other calibrator levels and QC samples, respectively.

Assays measuring PI concentrations in human samples must be acceptable according to our assay acceptance criteria. Briefly, in order for an assay to be suitable the concentration at the lower limit of quantification should not deviate more than ±20% from the mean target concentration and no more than 15% at the other calibrator concentrations. Furthermore, at least 6 calibrators other than the zero calibrator must have both duplicates within the acceptable limits. Acceptability of QC samples is based on modified Westgard rules.

3.3. Linearity and lower and upper limits of quantification and limit of detection

All standard curves for simultaneous quantification of APV, ATV, IDV, LPV, NFV, RTV and SQV were adequately described by a 1/concentration^2 weighted quadratic regression equation.

Table 3
Accuracy and precision for the simultaneous determination of amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir by HPLC-MS/MS in human plasma

Drug	QC level	Target (ng/ml)	Accuracy (% bias)	Precision (%)	
				Inter-assay	Intra-assay
Amprenavir	Low	179	−2.8	9	4
	Medium	2214	−5.4	6	4
	High	3920	9.9	5	5
Atazanavir	Low	173	0.9	8	6
	Medium	2136	8.3	8	4
	High	3835	10.1	7	4
Indinavir	Low	184	−9.7	9	5
	Medium	2174	3.8	5	4
	High	4268	6.2	5	5
Lopinavir	Low	790	−0.2	6	5
	Medium	2949	8.8	6	4
	High	10460	5.4	7	4
Nelfinavir	Low	185	−7.0	6	5
	Medium	2056	5.9	5	3
	High	3669	6.8	4	4
Ritonavir	Low	102	−1.8	5	5
	Medium	947	0.5	9	8
	High	3901	7.8	8	7
Saquinavir	Low	186	−10.1	7	5
	Medium	1952	6.7	7	3
	High	3677	6.6	10	4

Accuracy = [(measured concentration − mean target concentration)/measured concentration] × 100.

Correlation co-efficient (r^2) for all validation standard curves was above 0.990.

Lower and upper limits of quantification (LLQ, ULQ) were arbitrarily set as the bottom (level 2) and top (level 9) points of the standard curve, respectively. Limit of detection (LOD) was defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). LLQ, ULQ and LOD were 56, 5136 and 29 ng/ml (APV); 47, 6239 and 4.6 ng/ml (ATV); 102, 9481 and 2.4 ng/ml (IDV); 95, 15,584 and 3.9 ng/ml (LPV); 62, 4670 and 2.4 ng/ml (NFV); 25, 4941 and 1.2 ng/ml (RTV); 71, 4958 and 3.5 ng/ml (SQV), respectively.

3.4. Accuracy and precision

Accuracy was evaluated by calculating percentage bias [% bias; [(measured concentration − mean target concentration)/measured concentration] × 100] of the mean target QC concentrations of six replicates of QC sample at each concentration within an assay. Assay precision was assessed by calculation of inter- and intra-assay variability of LQC, MQC and HQC samples. Inter-assay variability was determined from the calibrator and QC validation assays (minimum of 12). Intra-assay precision was calculated from six replicates of LQC, MQC and HQC samples within an assay (used to calculate accuracy). Accuracy (% bias) and precision (expressed as CV%) for all seven PIs are summarised (Table 3).

3.5. Stability and recovery

Assays assessing PI stability were acceptable according to our assay acceptance criteria as described previously (Section 3.2). Plasma concentrations did not differ significantly after heat inactivation or heat inactivation-freeze/thaw compared to fresh sample analysis (Table 4). Also, no significant difference between concentrations obtained after heat inactivation and those measured after heat inactivation-freeze/thaw was observed (data not shown).

Recovery of all seven PIs was above 87% at all three concentration levels. Percentage recovery for each individual drug is shown (Table 5).

3.6. Quantification of patient samples

The method described has been used to determine full patient profiles for combinations of PIs for a number of clinical studies, some of which have been recently published. The PIs evaluated included SQV/RTV [41,42], APV/SQV/RTV [43] and

Table 4
Protease inhibitor concentrations in plasma when analysed in triplicate fresh, after heat inactivation and after heat inactivation and one freeze/thaw cycle on three separate occasions

Treatment/storage condition	Low	*p* value	Medium	*p* value	High	*p* value
Amprenavir (ng/ml)						
Fresh	382 ± 26		3850 ± 355		8006 ± 767	
Heat inactivated	384 ± 34	0.45	3381 ± 592	0.41	7640 ± 437	0.13
Heat inactivated-freeze/thaw	387 ± 37	0.39	3831 ± 296	0.46	7599 ± 768	0.11
Atazanavir (ng/ml)						
Fresh	84 ± 13		2388 ± 347		4856 ± 251	
Heat inactivated	81 ± 11	0.25	2309 ± 312	0.32	4636 ± 375	0.08
Heat inactivated-freeze/thaw	88 ± 7	0.22	2449 ± 408	0.36	5082 ± 338	0.08
Indinavir (ng/ml)						
Fresh	82 ± 17		4959 ± 845		9438 ± 988	
Inactivated	86 ± 19	0.32	5062 ± 713	0.40	9014 ± 535	0.14
Inactivated-freeze/thaw	83 ± 16	0.45	4913 ± 895	0.45	9010 ± 878	0.14
Lopinavir (ng/ml)						
Fresh	2003 ± 168		6451 ± 427		14195 ± 1791	
Inactivated	2046 ± 244	0.32	6683 ± 849	0.14	13541 ± 1075	0.17
Inactivated-freeze/thaw	2029 ± 149	0.39	6484 ± 319	0.47	13429 ± 1378	0.14
Nelfinavir (ng/ml)						
Fresh	974 ± 43		3248 ± 316		6543 ± 602	
Inactivated	969 ± 57	0.43	3301 ± 282	0.15	6151 ± 400	0.07
Inactivated-freeze/thaw	950 ± 60	0.17	3223 ± 368	0.30	6294 ± 452	0.30
Ritonavir (ng/ml)						
Fresh	92 ± 8		1244 ± 74		2725 ± 214	
Inactivated	89 ± 8	0.19	1239 ± 117	0.45	2631 ± 193	0.14
Inactivated-freeze/thaw	90 ± 7	0.24	1230 ± 51	0.36	2595 ± 137	0.07
Saquinavir (ng/ml)						
Fresh	114 ± 4		2864 ± 327		6074 ± 657	
Inactivated	114 ± 8	0.48	2879 ± 401	0.46	5874 ± 394	0.25
Inactivated-freeze/thaw	118 ± 7	0.11	2816 ± 307	0.39	5781 ± 775	0.17

Data are expressed as mean (±S.D.) and *p* values refer to comparisons made with samples prepared fresh (one-way analysis of variance with Bonferroni correction).

Table 5
Percentage recovery from plasma at three concentrations (low, medium, high) of amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir calculated by comparing peak areas of directly injected drug solution and drug that underwent protein precipitation in plasma on three separate occasions ($n = 6$)

Drug	Percentage recovery (%)		
	Low	Medium	High
Amprenavir	100 (±13)	93 (±8.0)	96 (±10)
Atazanavir	93 (±15)	90 (±9.0)	106 (±10)
Indinavir	104 (±33)	94 (±24)	98 (±12)
Lopinavir	104 (±11)	95 (±7.0)	109 (±9.0)
Nelfinavir	95 (±8.9)	90 (±7.0)	99 (±10)
Ritonavir	89 (±15)	112 (±15)	114 (±15)
Saquinavir	91 (±20)	88 (±9.0)	89 (±7.0)

Data are expressed as mean (±S.D.). Percentage recovery = (peak area extracted from plasma/peak area from directly injected solution) × 100.

ATV/SQV/RTV [44]. Furthermore, the method is used daily to measure PI plasma concentrations of samples received by the Liverpool TDM Service. Chromatograms of TDM samples from four patients receiving PI-based therapy are shown (Fig. 3).

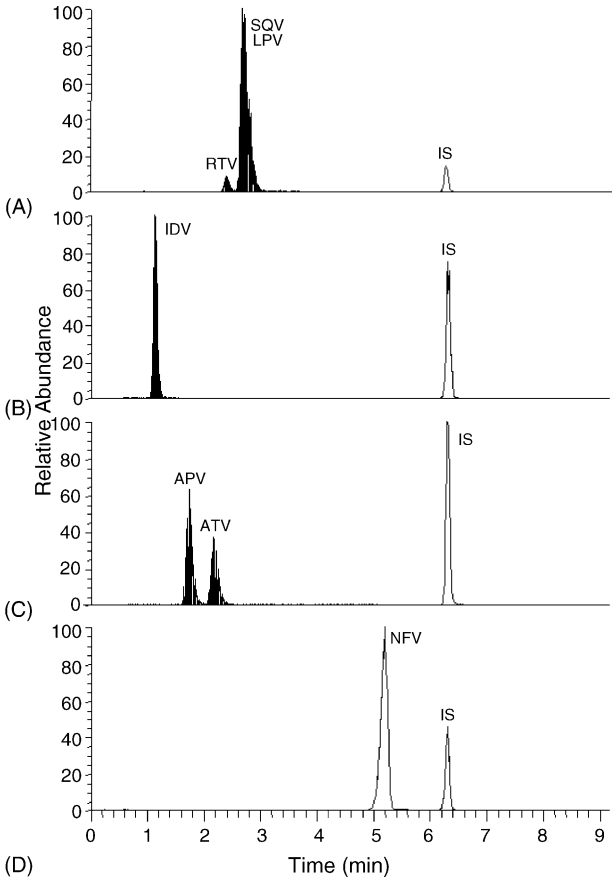

Fig. 3. Chromatograms representing TDM samples taken from four patients who were receiving (A) ritonavir (RTV), saquinavir (SQV) and lopinavir (LPV), (B) indinavir (IDV) alone, (C) amprenavir (APV) and atazanavir (ATV) and (D) nelfinavir (NFV) alone, as part of combination antiretroviral therapy.

4. Discussion and conclusion

Development and validation of an assay to simultaneously determine PIs APV, ATV, IDV, LPV, NFV, RTV and SQV by HPLC-MS/MS with accuracy, precision and specificity has been optimised and described. This assay has been used to successfully determine PK profiles of a number of HIV infected individuals participating in various clinical studies [41–44] as well as continual analysis of routine TDM samples for the Liverpool TDM Service.

PI plasma concentrations demonstrate high inter-patient variability; therefore ranges of standard curves were chosen to encompass the broad spectrum of drug concentrations likely to be encountered in PK studies. It is important that the assay detects and quantifies below and above defined minimum effective concentrations (MEC) and toxic levels of PIs, respectively. Therapeutic ranges of PIs have been reported in the literature [39,40], however these are only estimates and considerable debate remains regarding some PIs. The RTV standard curve was validated over a lower concentration range because generally RTV is administered at low doses to boost concomitant PI exposure [8,9], consequently lower plasma concentrations of RTV result. If analysed samples are determined above the ULQ it is possible to dilute the patient sample with drug free plasma and repeat the assay. When performing this procedure two high QC samples are diluted in duplicate by the same dilution factor to allow validation of the dilution process.

The assay requires a small volume of plasma for analysis (100 μl). This is advantageous when measuring PI concentrations as part of clinical studies as they often necessitate hourly sampling to generate complete PK profiles; therefore less blood can be drawn from the patient. This is identical to a number of assays measuring multiple PIs by LC-MS/MS [23,24,27] however, two relatively recent methods required 250 μl and 1 ml, respectively [26,27]. The method combines a short run time of 9 min per sample with a quick and simple extraction procedure allowing a large number of samples to be processed quickly and efficiently. Similarly it is beneficial that seven PIs can be quantified within one assay, as combination PI therapy is common, in particular among antiretroviral-experienced patients. It was not possible however, to assay all seven from a single preparation. ATV, LPV, RTV and SQV possess similar chemical properties and as such have a similar elution time. To ensure optimal conditions for quantification two Xcalibur instrument methods were developed. The first containing RTV without ATV and the other included ATV and not RTV. If determination of both ATV and RTV is required analysis can be carried out with both Xcalibur instrumentation methods and sample re-injection. As all drugs are contained in calibrator and QC samples no further sample preparation or apparatus are required to quantify all seven PIs.

HIV positive plasma intended for PK study or TDM is heat inactivated prior to analysis (58 °C; 40 min) to decrease risk of infection to the operator. Conditions used within our laboratory are similar to those published in the literature [45–47]. SQV and ATV have been found to be the PIs that are most unstable after heat treatment. Investigations by Hoetelmans and colleagues could not quantify SQV concentrations when heated to 60 °C

(1 h) due to repeated sample coagulation [16], signifying that SQV may not be stable when heat inactivated. Wiltshire et al. examined the stability of SQV when heated to 56, 60 and 65 °C. SQV was stable at 56 °C but there was a mean decrease in concentration of 18% at 60 °C. Analysis could not be carried out at 65 °C because the samples formed a gel [38]. Studies conducted at our laboratory have found SQV to be sufficiently stable when treated for 40 min at 58 °C. Schuster and colleagues evaluated the stability of ATV after heating (60 °C; 30 min) and discovered it to be unstable with ATV concentrations differing up to 40% from nominal values [37]. Worked conducted at our laboratory did not show any significant difference in ATV plasma concentrations compared to fresh samples when heated to 58 °C suggesting ATV is more stable at a slightly lower temperature. Moreover, the previous investigators did not use non-heated control samples for comparison. Furthermore, APV, IDV, LPV, NFV and RTV appear to be sufficiently stable under our inactivation conditions and after one freeze/thaw cycle in comparison to controls. Evaluation of more than one freeze/thaw cycle was not deemed necessary as it only on rare occasions that samples will be analysed more than once. Furthermore, investigations have shown that PIs are stable after more than one freeze/thaw cycle [36,37,48–51].

The HPLC-MS/MS method to quantify PIs APV, ATV, IDV, LPV, NFV, RTV and SQV is fully validated with accuracy (% bias) between −10.1 and 10.1% and inter- and intra-assay precision did not exceed 10 and 8%, respectively. Recovery was above 87% for all seven drugs at all three evaluated concentration levels. A minimum of 12 assays were prepared and analysed to obtain overall mean target values for calibrator and QC samples. This compensates for slight variation in stock solution preparation and deviation from nominal calibrator and QC concentrations. Assay performance is assessed by routinely monitoring internal QC samples. To further evaluate assay operation, the laboratory has participated in an external quality control program (International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring in HIV Infection, Nijmegen, The Netherlands) twice yearly since the scheme's initiation in December 1999. In the most recent round (June 2005) our assay received 21/21 acceptable results for PI quantification. Our laboratory also possesses CPA status.

In conclusion, the assay described is quick and easy with a simple and rapid extraction procedure requiring only 100 μl of plasma. It is successfully used to quantify total plasma concentrations of seven PIs (APV, ATV, IDV, LPV, NFV, RTV and SQV) in HIV infected individuals participating in PK studies. In addition it is used to analyse routine TDM plasma samples and by adaptations of extraction procedure, has the potential to determine PI concentrations in lymphocytes, semen and cerebrospinal fluid (CSF) and to quantify unbound drug concentrations obtained from ultrafiltration.

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