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Short communication

HPLC–MS method for the quantification of nine anti-HIV drugs from dry plasma spot on glass filter and their long term stability in different conditions

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ABSTRACT

A bioanalytical method for the determination of most commonly prescribed protease inhibitors (saquinavir, atazanavir, amprenavir, darunavir, lopinavir and ritonavir) and non-nucleoside reverse transcriptase inhibitors (etravirine, efavirenz and nevirapine) was developed, modifying our previous HPLC–MS chromatographic run, validated and a complete short and long term stability evaluation was carried out. One hundred microlitres of plasma were distributed on a collection glass paper filter (Glass-Microfibre from Sartorius), then the filter underwent thermal treatment, both for drying and for HIV inactivation, and stored at room temperature, 4 °C and −20 °C. The analytes were extracted from the filter disc using *tert*-butylmethylether with basic pH, after the addition of the internal standards quinoxaline. The extract was dried, reconstituted and the chromatographic separation was performed on a reversed-phase C-18 column (150 mm × 2.0 mm) and the analytes were quantified using a single quadrupole mass spectrometer.

The method was validated considering the concentration ranges encountered in clinical trials and the routine clinical practice. The assay was linear over the concentration ranges tested. Accuracies ranged from 92.1% to 111.9% and intra-day and inter-day relative standard deviation for all quality control levels ranged from 0.2 to 12.9 and 3.1 to 14.4, respectively.

Analytes in dried plasma spots were stable for longer time when dried/inactivation step was carried out before storage compared to samples not dried/inactivated before the analysis. The dried/inactivation step allows shipment of samples at room temperature without any risks, therefore the developed and validated method enables an easy and cheap sample shipment for therapeutic drug monitoring and pharmacokinetic studies.

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1. Introduction

Since the introduction of zidovudine (AZT) in the 1980s as the first compound demonstrated to be effective against the human immunodeficiency virus (HIV), an intense research has led to the development of a wide arsenal of antiretroviral agents. Efforts for expanding the variety therapeutic agents continue and new drugs are constantly approved to improve the efficiency of the treatments. Five years ago, the HIV drugs were included in three therapeutic families, namely: nucleoside or nucleotide transcriptase inhibitor (NRTIs-NtRTI), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Recently a new NNRTI (etravirine) and two new PIs (tipranavir and darunavir) have been commercialized. Moreover, new therapeutic targets, and new therapeutic families have been investigated and, a fusion inhibitor (enfuvirtide), an entry inhibitor (maraviroc) and an integrase inhibitor (raltegravir) have been approved in the treatment of the HIV infection [1]. Treatment of HIV infection currently relies on the so-called "highly active antiretroviral therapy" (HAART) which proposes the combination of several drugs in a daily regimen. The complexity of HAART brings clinical issues to be solved such as minimizing the occurrence of viral resistances and preventing adverse effects.

Accurate measurement of PIs and NNRTIs plasma concentrations is crucial for pharmacokinetic/pharmacodynamic analyses, drug–drug interaction studies, and therapeutic drug monitoring (TDM). The latter is currently considered a useful tool for the optimization of antiretroviral therapy in most international guidelines [2–5]. Measurement of antiretroviral drugs plasma concentrations is a subject of raising interest, due to the use of TDM as a clinical tool and to wide distribution of pharmacokinetic (PK) studies of new and old compounds [3]. TDM can be performed by few laboratories but

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samples storage and dry-ice shipping to such reference laboratories are the most important technical and cost limitations to widespread availability of TDM. Dried blood spot has been described as a tool for PK measurement of other class of drugs [6–8] (e.g. antimalarial drugs) [9,10], however it did not find a broad application for antiretrovirals [11–14], mainly because the pharmacokinetic variable that has been evaluated in the great majority of PK/PD studies is plasma concentrations [2–6,15].

Primary aim of our study was to develop and validate a new analytical method, modifying our previous HPLC–MS chromatographic run [16], for simultaneous quantification of nine different antiretroviral drugs (six protease inhibitors [PIs]: saquinavir, atazanavir, amprenavir, darunavir, lopinavir, ritonavir; three non-nucleoside reverse transcriptase inhibitors [NNRTIs]: etravirine, efavirenz and nevirapine) in dried plasma spot, and secondarily to investigate drugs short and long term stability on glass paper filters at different storage conditions.

2. Experimental

2.1. Chemicals

The compounds were kindly obtained from the following pharmaceutical companies: nevirapine (NVP) from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, USA); efavirenz (EFV) and atazanavir (ATV) from Bristol Myers Squibb Company (Princeton, NJ, USA); amprenavir (APV) from GlaxoSmithKline (Brentford, UK); darunavir (DRV) and etravirine (ETV) from Tibotec (Mechelen, Belgium); saquinavir (SQV) from Roche (Mannheim, Germany); lopinavir (LPV) and ritonavir (RTV) from Abbott Laboratories (IL, USA). All the full formulae for all antiretroviral drugs were pictured in Fig. 1. Acetonitrile HPLC grade and methanol HPLC grade were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Quinoxaline (QX), formic acid and *tert*-butylmethylether (TBME) were obtained from Sigma–Aldrich (Milan, Italy). Glass paper filters (Glass-Microfibre Discs Grade MGC Dia 50 mm) were purchased from Sartorius Stedim (Florence, Italy). Ammonia solution 30% was obtain from Carlo Erba (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria Hospital (Turin, Italy).

2.2. Stock solutions, standards (STD) and quality controls (QC)

DRV, NVP, APV, SQV, ATV and stock solutions were made in a solution of methanol and HPLC grade water (90:10), EFV, RTV and LPV were made in a solution of methanol and HPLC grade water (95:5), and ETV were made in a pure solution of methanol (100%) to obtain a final concentration of 1 mg/ml; all stock solutions were then refrigerated at 4 °C until use, within 1 month. Working solution of internal standard (IS) was made with QX (0.625 μg/ml) in methanol and HPLC grade water (50:50, v/v) and stored at 4 °C until use. The highest calibration standard (STD 9) and three quality controls (QCs) were prepared by adding a determined volume of stock solutions to blank plasma; the other STDs were prepared by serial dilution from STD 9 to STD 1 with blank plasma, to obtain nine different spiked concentrations plus a blank sample (STD 0). Calibration range, from STD 9 to STD 1, for ETV was 3000–11.7 ng/ml, and QC concentrations were 2000 ng/ml (QC H), 500 ng/ml (QC M) and 50 ng/ml (QC L), respectively. For all other drugs calibration ranges and QC concentrations were the same as our previous paper [16]. STDs and QCs were stored at −20 °C until the use, avoiding more than three freeze–thaw cycles, and not more than 3 months.

2.3. STD, QC and samples preparation

STDs, QCs and patient samples were thawed at room temperature and 100 μl of plasma were carefully spotted in half a disc. STDs were performed in double and QCs in quadruple. Filters were dried for 15 min at room temperature and then underwent thermal treatment to inactivate HIV in a sealed envelope for 35 min at 58 °C. Filters were rolled up in a tube (falcon 15 ml) and 40 μl of internal standard working solution and 4.5 ml of an extraction solution (4 ml TBME + 500 μl NH3 15%) were added. Tubes were tumbled for 10 min at 35 rpm and then vortexed for 10 s. Organic phase of each sample was transferred in glass shot and dried in a vacuum centrifuge system at 60 °C, then recovered with 1 ml of water and acetonitrile solution (60:40, v/v) and 30 μl were injected in the HPLC–MS system for the analyses.

2.4. Chromatographic and MS conditions

The chromatographic conditions were based on the previous published paper [16]. The HPLC–MS instrument used was a

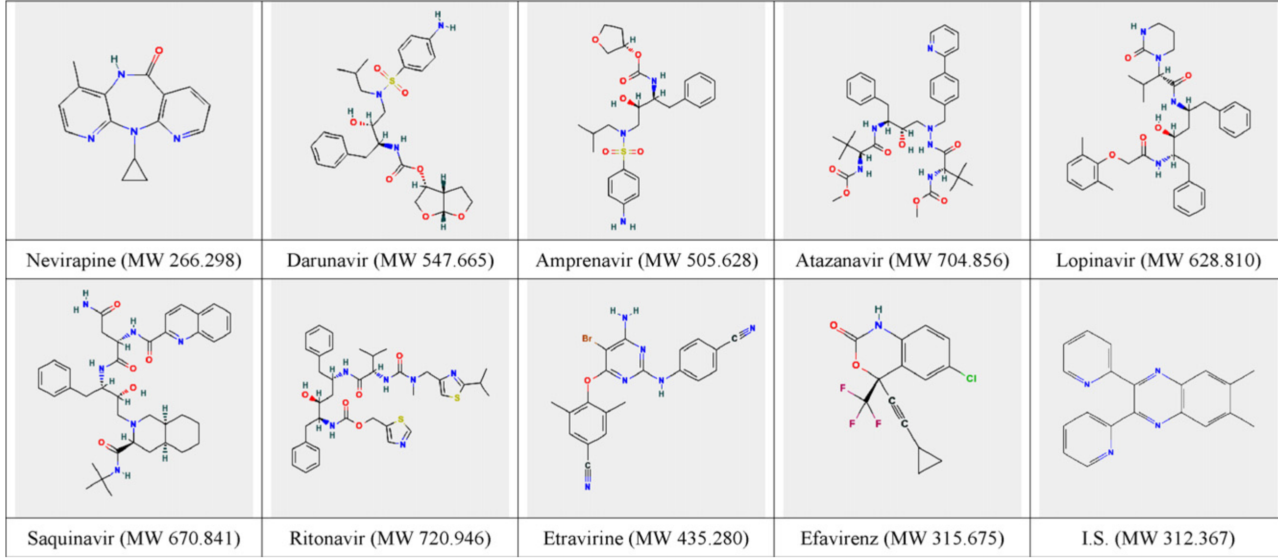

Fig. 1. The full formulae for all antiretroviral drugs and I.S. In the bracket each molecular weight (MW).

Waters system (Milan, Italy), with binary pump model 1525, AF degasser, 717-plus autosampler, and Micromass ZQ mass detector. LC–MS Empower 2 Pro software (version year 2005, Waters, Milan, Italy) was used. Chromatographic separation was performed at 35 °C using a column oven, on Atlantis T3 C-18 3 μm column (150 mm × 2.1 mm i.d.) (Waters, Milan, Italy), protected by a Security Guard with C-18 (4.0 mm × 3.0 mm i.d.) pre-column (Phenomenex, CA, USA). Chromatographic run was performed with a gradient [16]: the mobile phase A was composed by HPLC grade water (+0.05% formic acid) and mobile phase B by HPLC grade acetonitrile (+0.05% formic acid). Including the column, the "dead volume" of our system was around 2 ml. Detector settings were ESI, positive polarity ionization (except for EFV which was detected by negative polarity ionization, in the same run simultaneously using instantaneous switching, from 16.0 to 17.75 min); capillary voltage 3.5 kV; source temperature 110 °C; desolvation temperature 350 °C; nitrogen desolvation flow 800 l/h; nitrogen cone flow 100 l/h. Ion detection, cone voltages and ionization for each drug were the same of our previous paper [16], excluding ETV detected to 434.8 m/z, with 60 V (cone voltage) in ESI+ mode.

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of six different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated, and tested around their highest expected plasmatic concentrations. These included: zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABV), tenofovir (TDF), emtricitabine (FTC), tested at 5000 ng/ml, and enfuvirtide (T-20), evaluated at 10,000 ng/ml. An "interfering drug" has been considered as a molecule which exhibits a retention time close to 0.3 min from the analytes, and with the potential capability to cause ion suppression.

2.6. Matrix effect

Matrix effect was investigated using six different blank plasma and comparing peak areas obtained from standard solutions of solution of water and acetonitrile (60:40, v/v), containing all analytes at three different concentrations, and peak areas obtained from blanks post-extraction solution with the same amount of analytes, as described by Taylor [17]. Possible "matrix effect" was calculated, as deviation %, comparing the peak area obtained from the plasma extract with the peak area obtained from the standard solution.

2.7. Accuracy, precision, calibration and limit of quantification

Intra-day and inter-day accuracy and precision were determined by assaying eight spiked dried plasma spot (DPS) on filter at three different concentrations (QCs) for each drug. Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration. Each calibration curve was obtained using nine calibration points, in duplicate in the validation assays as requested by FDA guidelines [18]. Calibration curves were created by plotting the peak area ratios of each drugs relative to the IS against the various drug concentrations in the spiked plasma standards. A linear regression forced trough zero (STD 0) was used for all curves, and no weighting was applied. The limit of detection (LOD) in DPS was defined as the concentration that yields a signal-to-noise ratio of 3/1. Percent deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be <20%, and it was considered the lowest calibration standard, as requested by FDA [18].

2.8. Recovery

Recovery from DPS, using the extraction procedures, was assessed by comparing the peak area obtained from multiple analyses of spiked samples (QCs) with the peak area from standard solution of all analytes in a solution of water and acetonitrile (60:40, v/v) at the same concentrations.

2.9. Stability

The stability of NNRTIs and PIs on DPS at different conditions has never been investigated, and data from dried blood spot (DBS) stability are not useful for our study. We have investigated the influence of HIV thermal inactivation before and post-storage. Some QCs spotted on filters were dried with the thermal inactivation step (in oven, 35 min at 58 °C) and then stored in three different conditions (room temperature, 4 °C and −20 °C) and extracted and analyzed after different lengths of time: 3 h, 1 day, 2 days, 1 week, 1 month, and 3 months. Other QCs spotted on filters were immediately stored in the storage condition described above for the same length of time, and were inactivated only before the extraction procedure. All the QCs were analyzed in double for the validation and evaluation of the stability in different conditions. Drugs pre-assay stabilities were not evaluated, by relying on previously published papers [16,19]. Therefore, stability studies were performed also in post-extraction condition. Stability of all drugs and IS in DPS extracts at room temperature (20–25 °C, autosampler temperature) was evaluated: processed QCs, at three different concentrations, were analysed immediately after preparation and after being for 24 h at room temperature by comparing the peak areas of drugs in the two sample injections.

3. Results

Representative chromatograms of a blank plasma extracted, as STD 0, and STD 1 are shown in Fig. 2 and a chromatogram of QC H extracted is shown in Fig. 3. Mean regression coefficient (r^2) of all calibration curves was more than 0.995, and for all curves was chosen a linear regression forced through zero. As clearly indicated in FDA guidelines, the simplest model that adequately describes the concentration–response relationship was used, and the forcing through zero gives us an higher curve reliability.

The single ion monitored (SIM) for each drug was based on a previous published paper and the retention times of our analytes were the same [16], excluding ETV that elutes in 17.7 (±0.1) min. Due to their similar chemical structure and physiochemical properties, APV and DRV, eluted at the same retention time. Similarly LPV and EFV have a very similar retention time. These co-elutions did not affect quantification, as has been fully proven for APV-DRV in our previous study [16], and for LPV-EFV, in Section 3.5.

3.1. Specificity and selectivity

The assay did not show any significant interferences with antiretrovirals or other concomitant drugs taken at therapeutic dosage by patients (see Section 2.5), excluding the overlapping drugs mentioned above. The tested six blank plasma did not show any endogenous interferences, taking in account the analytes retention time windows (Fig. 2).

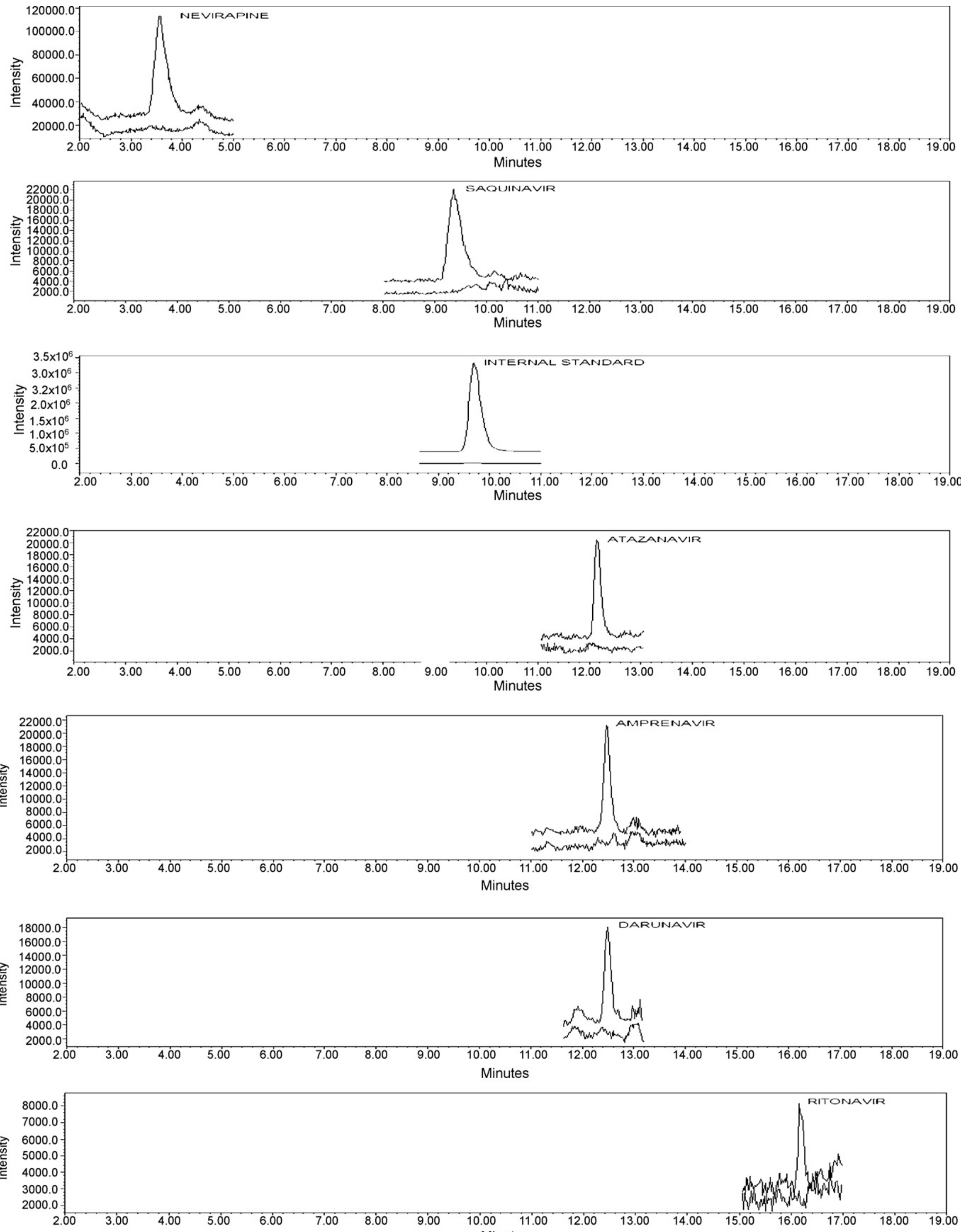

Fig. 2. Overlapping of SDT 1 and STD 0 (blank plasma extracted).

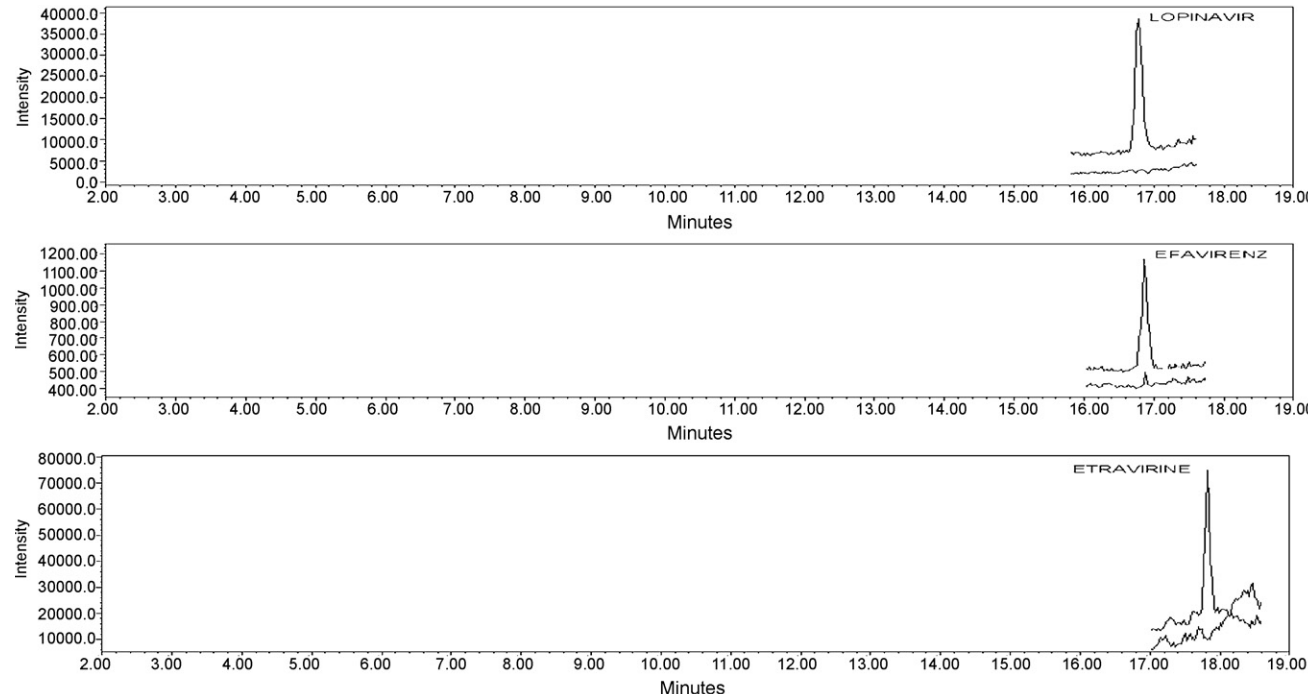

Fig. 2. (*Continued*).

3.2. Accuracy, precision, limit of quantification

Results of validation are listed in Table 1 for all analytes. All observed data (intra-day and inter-day precision [R.S.D. %]) were below 15.0%, according to FDA guidelines [18]. Co-elution of DRV and APV did not alter quantification of two drugs [16].

LOQ, that also represents the low limit of quantification (LLOQ), and LOD were the same as previously reported [16]. LOQ (LLOQ) and LOD for ETV were 11.7 and 5.8 ng/ml, respectively.

3.3. Recovery

Multiple aliquots ($n = 6$) at each of the three QC concentrations were assayed and mean recovery for all antiretroviral was above 85% except for saquinavir (80%) and etravirine (64%).

3.4. Analysis of plasma samples from treated patients

Our method was applied for assaying 30 clinical samples, corresponding to C_{trough} and C_{max} time, obtained from patients administered with at least one of the drug included in the assay, with a minimum of four samples for each drugs. Samples were also quantified using a method already published [19], which is considered as our reference and it is used for the quantification of International Quality Controls (KKGT, Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology) [20]. The results obtained with our new method were within 15% of variation if compared to the results obtained with our reference method.

3.5. Matrix effect

No matrix effect was observed for all antiretrovirals. The ranging of deviation % of the peak area was from −8.8% and 6.6%.

3.6. Stability

A linear regression was used to calculate the length of time to reach a 5%, 10% and 15% decrease of antiretrovirals concentrations, in different storage conditions and times. All extrapolated data were reported in Table 2. All the analytes were more stable if filters undergo thermal inactivation (58 °C for 35 min) before the storage in all three different conditions. SQV and ATV have showed (Table 2) a higher benefit from pre-thermal inactivation, clearly improving their stability (from few days to over 3 months) while amprenavir and darunavir are the drugs that have the less improve-

Table 1
Accuracy (%), intra-day and inter-day precision (R.S.D. %) assayed for all drugs ($n = 8$).

Drugs	QC high			QC medium			QC low		
	Accuracy %	Precision (R.S.D. %)		Accuracy %	Precision (R.S.D. %)		Accuracy %	Precision (R.S.D. %)	
		Intra-day	Inter-day		Intra-day	Inter-day		Intra-day	Inter-day
NVP	−1.2	0.4	3.9	−4.2	1.8	3.1	−2.3	1.5	9.4
SQV	−0.9	1.9	6.8	−1.7	4.5	8.7	9.2	12.9	9.9
ATV	1.9	6.3	9.3	−4.0	2.5	7.0	5.2	1.0	10.0
APV	−1.7	5.8	7.4	6.4	0.2	5.1	−5.6	1.7	5.1
DRV	−0.2	8.1	5.0	4.5	4.0	5.9	2.1	1.5	6.9
RTV	2.3	1.7	8.4	−4.4	8.4	10.9	−3.7	4.4	11.9
LPV	2.7	2.9	5.5	1.3	11.9	9.4	−7.9	6.1	14.4
EFV	3.0	3.8	8.0	3.3	6.5	11.1	7.4	11.2	14.8
ETV	−0.1	2.4	5.6	6.1	3.1	8.2	11.9	7.8	8.4

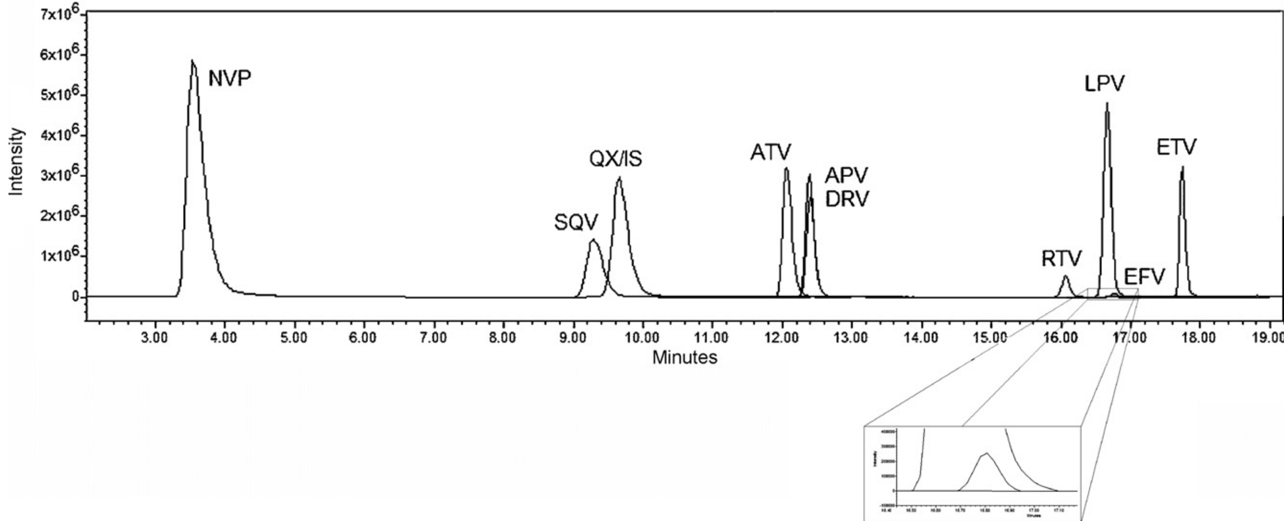

Fig. 3. Chromatogram of high quality control.

Table 2
Results about drugs stability, expressed in number of day, on filter stored in different conditions. Title "not dry" indicates the filters thermal inactivated only after storage and "dry" filters thermal inactivated before storage.

Drugs	Room temp. degradation						4 °C degradation						−20 °C degradation					
	5%		10%		15%		5%		10%		15%		5%		10%		15%	
	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry	Not dry	Dry
Nevirapine	71	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90
Saquinavir	8	71	16	>90	23	>90	14	71	28	>90	42	>90	42	>90	83	>90	>90	>90
Atazanavir	9	>90	18	>90	27	>90	11	>90	22	>90	33	>90	13	>90	26	>90	39	>90
Amprenavir	8	12	17	23	25	35	7	12	13	24	20	37	7	20	15	40	22	60
Darunavir	9	12	17	24	26	36	7	13	14	25	21	38	8	19	15	37	23	56
Ritonavir	22	>90	43	>90	65	>90	45	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90
Lopinavir	29	38	59	77	88	>90	63	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90
Efavirenz	50	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90
Etravirine	10	12	20	23	30	35	31	>90	62	>90	>90	>90	>90	>90	>90	>90	>90	>90

ment from the thermal inactivation step. In general terms, filter storage at −20 °C is the best way to keep antiretrovirals for long time (Table 2).

Moreover, the stability study of all drugs and IS in DPS extracts, kept for 24 h in the autosampler at room temperature, showed a variation less than 7% for all analytes at all concentrations. Taking into account the analytical variability, the processed samples are thus stable throughout the HPLC–MS analysis, always completed within 24 h.

4. Discussion and conclusion

The chromatographic settings of this method are based on one of our previously published assay [16], here updated with etravirine. This method could also be used to separate and detect other drugs as indinavir, nelfinavir, M-8 (nelfinavir metabolite), tipranavir and raltegravir; unfortunately, we could not correctly quantify these drugs with the described assay, probably due to the extraction procedure with a basic solution.

The selection of QX as internal standard was based on our previously experiences. It was used and fully validated in our published method [16,19,21]. It is not a prescribed drug, it is cheap, easy to purchase, and it has intermediate chemical characteristics useful for the wide range of antiretroviral drugs. Recovery seems to be adequate also for etravirine (64%), despite its value is relatively low. The data on accuracy, precision (Table 1) and LOQ/LOD support this statement. A possible cause of poor etravirine recovery could be produced by basic condition extraction coupled to the organic percentage (40%) of reconstitution solution. As indicated before, the "dead volume" of our system was around 2 ml. This volume has influenced the choice of gradient [16], giving a delay of 8 min to elution drugs respect the starting gradient of mobile phase.

The developed HPLC–MS method for the quantification of nine antiretroviral drugs (NVP, SQV, ATV, APV, DRV, RTV, LPV, EFV, ETV) from plasma spotted on glass filter papers, here described, was completely and fully validate following the FDA guidelines. Moreover, we have assessed short and long term stability in several storage conditions. For a routinely and practicable use of this method, a limit of drugs degradation of 5% can be considered acceptable. The data showed in Table 3 were considered for the evaluation of the acceptable storage condition and times (degradation <5%) for all antiretrovirals considered. An increase of stability could be described when filters undergo to thermal inactivation at 58 °C for 35 min (drug dehydration) before the storage, and the best storage condition was −20 °C. All drugs spotted on filter, in all conditions, showed to be stable at room temperature at least 7 days, a length of time long enough to send the filter, by normal mail, to a laboratory.

Actually, one of the major limitations of antiretroviral TDM wide diffusion is that only a few laboratories are able to correctly quantify this class of drugs. In Italy, at the moment, there are only two laboratories which participate in International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring in HIV Infection [data from KKGT, 2008] [20], and there is only one ISO 9001:2000 certified laboratory [22]. The other Infectious Diseases Centers are forced to send their samples to the reliable laboratories,

Table 3
Timing (day) to loss around 5% of drugs spotted on filter, in different storage conditions.

Drugs	Degradation 5%					
	Room temp.		4 °C		−20 °C	
	Not dry	Dry	Not dry	Dry	Not dry	Dry
Nevirapine	71	>90	>90	>90	>90	>90
Saquinavir	8	71	14	71	42	>90
Atazanavir	9	>90	11	>90	13	>90
Amprenavir	8	12	7	12	7	20
Darunavir	9	12	7	13	8	19
Ritonavir	22	>90	45	>90	>90	>90
Lopinavir	29	38	63	>90	>90	>90
Efavirenz	50	>90	>90	>90	>90	>90
Etravirine	10	12	31	>90	>90	>90

using expensive and cumbersome transport systems (package with dry ice). Therefore, we develop and validate a simpler and cheaper method using glass filter papers, which can be safely sent by-mail, promoting the practice of TDM.

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