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High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma

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Abstract

An accurate, sensitive, and specific reverse-phase high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of HIV-protease inhibitors (PIs) (indinavir, IDV; amprenavir, APV; saquinavir, SQV; nelfinavir, NFV; ritonavir, RTV; and lopinavir, LPV) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine, NVP; delavirdine, DLV; and efavirenz, EFV) in human blood plasma is described. The method provides excellent resolution and peak shape for nine analytes through a linear gradient (36–86%) of 25% phosphate buffer (pH 4.5), 60% acetonitrile, 15% methanol, and 0.75 ml TFA, with a gradient mobile phase flow rate (0.9–1.1 ml) over 30 min run time. The optimized solid phase extraction (SPE) extraction method using (1.0 ml, 100 mg BOND ELUT-C18 Varian) column provides a clean base line and high extraction efficiency using a 550 μl plasma sample. The method was validated over the range of 10–10,000 ng/ml for NVP, IDV, and SQV; 10–5000 ng/ml for EFV; 25–10000 ng/ml for APV; and 25–5000 ng/ml for DLV, NFV, RTV, and LPV. This method is accurate (average accuracies of three different concentrations ranged from 91 to 112%), and precise (within- and between-day precision measures ranged from 0.2 to 5.7% and 0.1 to 5.4%, respectively). This method is suitable for use in clinical pharmacokinetic studies as well as in therapeutic drug monitoring (TDM).

Keywords: HIV protease inhibitors; Non-nucleoside reverse transcriptase inhibitors

1. Introduction

Drug therapy for HIV infection has become increasingly sophisticated and complex [1,2]. Currently there are seven protease inhibitors (PIs) and three non-nucleoside reverse transcriptase inhibitors (NNRTIs) in use for the treatment of HIV-infected patients. The specific choice of drugs depends upon a number of factors, such as drug resistance, tolerability, drug interactions, and effectiveness of the treatment [3–5]. A number of reports have suggested that therapeutic drug monitoring (TDM) of antiretrovirals may be useful for PIs and NNRTIs [6–8]. TDM has been proposed so that practitioners may better maintain appropriate plasma concentrations of drugs in their patients by identifying interactions with other medications and assessing medication adherence. A number of assays for the simultaneous quantification of PIs [9–12,20] or NNRTIs [13] have been reported in the literature. To date, no published method is available for simultaneously measuring all PIs and NNRTIs in one patient sample with high-performance liquid chromatography (HPLC) and UV-detection.

In this paper, we describe a validated assay for the simultaneous determination of six protease inhibitors and three non-nucleoside reverse transcriptase inhibitors in a single sample of human plasma using an optimized solid phase extraction (SPE) procedure followed by an optimized RP-HPLC method with UV-diode array detection (DAD).

2. Experimental

2.1. Chemicals

Midazolam was purchased from Sigma Chemical Company (St. Louis, MO, USA). Nevirapine, NVP; indinavir,

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IDV; nelfinavir, NFV; efavirenz, EFV; and ritonavir, RTV were obtained from the NIH AIDS Research & Reference Reagent Program (McKesson HBOC BioServices, Rockville, MD, USA). Saquinavir (SQV) was obtained from Roche Discovery (Welwyn Gaeden City, Herts, UK), lopinavir (LPV) from Abbott Laboratories (Abbott Park, IL, USA) and amprenavir (APV) from GlaxoSmithKline (Research Triangle Park, NC, USA). Delavirdine (DLV) was extracted from 100 mg Rescriptor® tablets. HPLC-grade chemicals were purchased from Fisher Scientific (Norcross, GA, USA). Purified compressed nitrogen gas was obtained from National Welders Supply (Charlotte, NC, USA).

2.2. Equipment

A high-performance liquid chromatography system consisting of an Agilent Technologies (Wilmington, DE, USA) model HP1100 binary pump, an HP1100 degasser, an HP1100 auto sampler, an HP1100 UV-DAD-detector, and HP ChemStation software (Version A.08.03) run on a Compaq Evo computer (operated by Windows 2000 professional), was used for this method.

2.3. Preparation of standards

2.3.1. Extraction of delavirdine from Rescriptor® 100 mg tablets

Ten Rescriptor® (the commercial USA formulation) tablets were powdered then well mixed. For a 1 mg/ml delavirdine preparation, an accurate weight of the white powder equivalent to 10 mg was transferred to a 10 ml volumetric flask. The powder was dissolved in 3.0 ml methanol. To ensure the complete extraction of the drug, this solution was sonicated for 15 min, and the volume subsequently made up to 10 ml with methanol and HPLC-grade water (final methanol content equal to 50%). The delavirdine extract concentration was confirmed through three different batches: each batch was made from a combination of 10 tablets. The concentration of delavirdine in each batch was constant (within $100 \pm 0.5\%$). Our primary standard was the combination of equal volumes of each batch. The final solution was filtered and used as a primary standard.

2.3.2. Preparing the standard master stock solution

Individual clear stock solutions of NFV, IDV, RTV, LPV, SQV, APV, EFV, and NVP were prepared as a 1 mg/ml concentration. Five milligram of each analyte were accurately weighed and dissolved with 50% methanol in water for RTV, IDV, APV, SQV, EFV, and NVP; 70% methanol in water for LPV; and 80% methanol in water for NFV. The master stock solution was prepared as a composite of all nine analytes (1.0 ml each), adjusted to a final concentration of 100 μg/ml using 1.0 ml of HPLC-grade water. The methanol content of this stock solution was 50%. This 100 μg/ml standard was used to prepare eight working solutions in methanol/HPLC-grade water (1:1) at concentrations of 0.1, 0.25, 0.5, 1, 5, 10, 50, and 100 μg/ml. Plasma calibration samples at 0.01, 0.025, 0.05, 0.1, 0.5, 1, 5, and 10 μg/ml of all nine drugs combined were prepared by using a 1:10 dilution of the respective working solutions to blank plasma. From another 100 μg/ml of working stock solution, concentrations of 1.2, 6, and 30 μg/ml were prepared in methanol/HPLC-grade water (1:1). Plasma quality control (QC) samples at 0.12, 0.6, and 3 μg/ml were prepared using a 1:10 dilution of their respective working solutions to blank plasma. This plasma was devoid of any drugs, and obtained from whole blood anticoagulated with sodium EDTA (Biological Specialty Corporation, Colmar, PA, USA). The final methanol content of all calibrators and quality controls was 5%.

Solutions of potential drugs of interference (primarily nucleoside analogue reverse transcriptase inhibitors and other drugs that may be co-administered with antiretrovirals) were prepared from pure standard or clear filtered extracts of the pharmaceutical formulation. These solutions were prepared in 100% methanol to a final concentration of 1.0 mg/ml, and diluted with mobile phase before injection onto the HPLC system at concentrations of 10 μg/ml.

2.4. Internal standard (IS) preparation

Midazolam (10 mg) was weighed and dissolved in methanol to achieve a final concentration of 1.0 mg/ml (stock solution). On the day of analysis, an aliquot from this solution was diluted in 100 mM ammonium acetate buffer (pH adjusted using ammonium hydroxide to 7.6) to a final concentration of 1.0 μg/ml.

2.5. Sample pre-treatment

Blood samples were collected in sodium EDTA tubes and centrifuged (2800 rpm for 15 min at 4 °C) within 15 min of collection. Plasma was carefully transferred to clean cryovials and stored at −70 °C. Prior to extraction, all plasma samples were heated for 45 min at 58 °C to inactivate the HIV virus.

On the day of analysis, 550 μl of the internal standard solution was placed into 1.5 ml conical plastic Eppendorf tubes. Then, 550 μl of patient plasma, QC solutions or calibrator solutions was transferred to the tubes. The solutions were vortexed for 30 s and centrifuged at 13000 rpm for 10 min. One milliliter of the resultant supernatant was transferred to a SPE extraction cartridge.

2.6. SPE extraction method

Solid phase extraction columns (1.0 ml, 100 mg BOND ELUT-C18 Varian, Harbor City, CA, USA) were placed on a vacuum elution manifold (20-SPE system, Waters, Milford, MA, USA). The cartridges were conditioned with 1.0 ml of methanol and equilibrated with 1.0 ml of 100 mM-ammonium acetate (pH 7.6). Subsequently, 1.0 ml

of the plasma/internal standard mixture supernatant was allowed to pass through the column bed with minimal suction (1–3 mmHg). The column was further washed with 1.0 ml of 90% 10 mM ammonium acetate buffer (pH 7.0) and 10% methanol, and the bed suctioned dry for 1 min. The drugs were then eluted with 800 μl of a 50/50 mixture of methanol and acetonitrile. The eluent was evaporated to dryness under a nitrogen stream at 40 °C, and the residue was reconstituted with 100 μl of mobile phase. The resulting solutions were carefully vortexed for 30 s and centrifuged at 13000 rpm for 10 min. The supernatants were transferred to 200 μl HPLC microvials (Agilent Technologies) and 80 μl was injected onto the column.

2.7. High-performance liquid chromatographic conditions

The chromatographic separation of analytes was performed with a gradient elution. A Zorbax®C-18 (150 mm × 4.6 mm, 3.5 μm particle size, Agilent (Wilmington, DE, USA) analytical column, with a Zorbax®C-18 (12.5 mm × 4.6 mm, 3.5 μm, Agilent) guard column were used for separation. During elution, the absorbance wavelength was set at 220 nm (0–9 min) and 210 nm (9–30 min).

The two mobile phase components were as follows: *mobile phase (A)*: 50 mM phosphate monobasic (pH 4.5 adjusted with diluted phosphoric acid). This buffer solution was filtered through a 0.45 μm membrane filter (Millipore, Milford, MA, USA) then mixed as 850 ml buffer to 150 ml methanol. *Mobile phase (B)*: 250 ml of mobile phase (A) (pH 4.5) was mixed with 600 ml of acetonitrile, 150 ml of methanol and 0.75 ml TFA.

A linear gradient was programmed from 36 to 86% mobile phase (B) over 30 min, with a 5 min re-equilibration time. The analysis was performed at 30 °C, with a gradient mobile phase flow rate starting at 0.9 ml/min and ending at 1.1 ml/min over the 30 min run time.

2.8. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of six male and female blank plasma samples. Interference from 13 commonly used medications was also investigated. These included atorvastatin, propranolol, gemfibrazil, chlorhexadine, nystatin, acyclovir, acetylsalicylic acid, and the nucleoside reverse transcriptase inhibitors zidovudine, stavudine, abacavir, lamivudine, didanosine, and zalcitabine.

2.9. Limit of detection and limits of quantification

The detection limit was defined as signal to nose ratio of 3:1. The lower limit of quantification (LLQ) was defined as the concentration for which both the relative standard deviation (R.S.D.) and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) was defined as the concentration for which both the relative standard deviation and the percent deviation from the nominal concentration were less than 15% [14].

2.10. Stability

HIV-infected patient samples are routinely heated at 58 °C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of all the drugs in plasma under these conditions. An additional stability test was performed to verify the stability of the drugs in the autosampler tubes while waiting for HPLC analysis. The samples were left at room temperature for 24 h prior to analysis. The stability during sample handling was also verified by subjecting samples to three freeze-thaw cycles, and storage for 7 days in the refrigerator at 4 °C prior to analyses. Quality control samples at three concentrations (0.12, 0.6, and 3 μg/ml) were utilized in this stability test.

3. Results

3.1. Linearity

The peak area ratio values of the calibration standards were proportional to the concentration of each drug in plasma over the range tested. The weighted linear regression data for the calibration curves ($n = 3$) are shown in Table 1. The mean ± S.D. of three standard curve slopes and intercepts, and the linear range for each analyte is also shown in Table 1. The linear range was 0.01–10 μg/ml for NVP, IDV, and SQV; 0.01–5 μg/ml for EFV; 0.01–5 μg/ml for APV; and 0.025–5 μg/ml for DLV, NFV, RTV, and LPV. The regression coefficient (r^2) of all calibration curves was greater than 0.997.

3.2. Selectivity

A representative chromatogram of a blank plasma sample is shown in Fig. 1. The approximate retention times for all nine analytes and internal standard are listed in Table 1. No

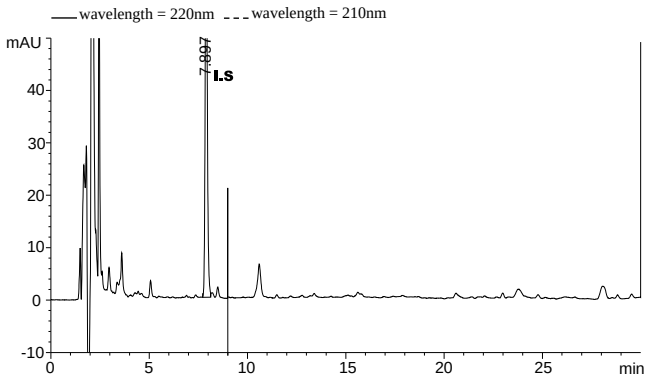

Fig. 1. Sample chromatogram of blank pooled human plasma, extracted through the method with internal standard.

Table 1
Summary of analyte retention time, linearity range, slope, r^2, and intercept values

HIV-drug	Retention time (min)	Range (μg/ml)	r^2	Slope ± S.D.	Intercept ± S.D.
NVP	4.4	0.01–10	0.999	1.21 ± 0.04	14.68 ± 3.85
IDV	10.3	0.01–10	0.999	0.53 ± 0.01	2.15 ± 0.07
DLV	11.9	0.025–5	0.999	0.61 ± 0.02	42.69 ± 30.11
APV	15.6	0.025–10	0.998	0.61 ± 0.02	14.24 ± 8.14
NFV	17.2	0.025–5	0.998	0.50 ± 0.00	34.68 ± 19.18
SQV	18.9	0.01–10	0.999	0.53 ± 0.02	14.35 ± 10.13
EFV	24.9	0.01–5	0.999	0.50 ± 0.07	27.55 ± 14.77
RTV	27.1	0.025–5	0.997	0.30 ± 0.03	11.96 ± 15.9
LPV	28.5	0.025–5	0.998	0.37 ± 0.08	6.30 ± 5.52

endogenous substances interfered with any of the analytes in blank plasma extracts. The retention times for potential drugs of interference (Table 2) were very different from the compounds of interest. Additionally, we have used this method to evaluate patient drug concentrations from four different pharmacokinetic studies (1450 patients, total), and have not seen any interference from other compounds these patients may have been taking.

3.3. The limit of quantification

The low limit of quantification for NVP, IDV, EFV, and SQV was 10 ng/ml and for all other analytes was 0.025 μg/ml. The upper limit of quantification was 10 μg/ml for all analytes except DLV, NFV, RTV, and LPV which was 5 μg/ml. Chromatograms from the standard curves at concentrations of 0.01 and 5 μg/ml are shown in Fig. 2.

3.4. Accuracy, precision

Results from the validation of this method in human plasma were acceptable. The accuracy of all analytes ranged from 94.8 to 109% with a mean of 103%. Precision and accuracy throughout the concentration range of the control samples for the three levels of validation; lower, medium, and high are presented in Table 3. The mean intra-assay precision was always lower than 5.8%. The mean inter-day precision for each of the nine analytes was similar, with mean R.S.D. ranging from 2.9 to 5.2%. The inter-assay deviation from the nominal concentration was always <14.0% and the range of inter-day deviation of the nine analytes were between 0.8 and 5.8%.

3.5. Recovery

The absolute recovery of the protease inhibitors, non-nucleoside protease inhibitors, NRTIs, and internal standard (midazolam) from plasma extracted with solid phase SPE columns was calculated by comparing chromatogram peak areas. This was calculated from the quality control samples as the ratio of the analyte concentration in human blood plasma undergoing solid phase extraction to that of the identical concentrations of the analytes prepared in mobile phase without extraction. This extraction method reliably eliminated interfering material from plasma. All analytes and internal standard recoveries were >90%, with the exception of RTV, LPV, and NFV, which were >74%.

3.6. Stability

The six protease inhibitors, three non-nucleoside RT inhibitors and internal standard were stable under all conditions tested, with all results falling within the ac-

Table 2
Retention times for drugs that may be used concomitantly in HIV-infected patients, to test for analytical method interference

Drug name	Retention time (min)
Atorvastatin	23.73
Propranolol	6.78
Gemfibrazil	28.84
Chlorhexadine	10.11
Nystatin	16.15 and 17.53
Acyclovir	1.79
Acetylsalicylic Acid	4.2
Zidovudine	2.78
Stavudine	2.11
Abacavir	2.13
Lamivudine	2.12
Didanosine	2.14
Zalcitabine	1.80

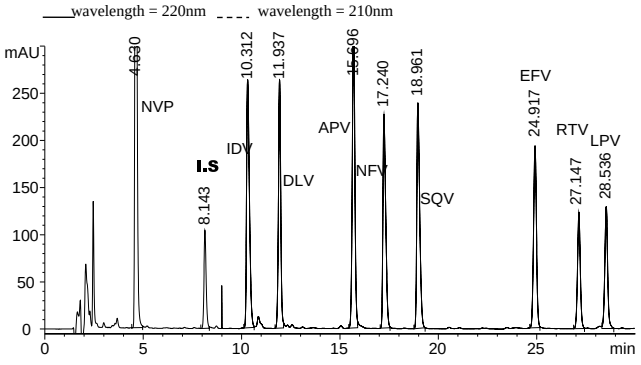

Fig. 2. Chromatogram of the assay analytes (NVP, IDV, DLV, APV, NFV, SQV, EFV, RTV, and LPV) at 5000 ng/ml and the internal standard (IS) midazolam.

Table 3
Summary of accuracy and precision (%) of method validation at three levels: low, medium, and high

HIV-drug	Concentration (ng/ml)	Accuracy (%)	Precision (%)	
			Within-day C.V. (%) $N = 3$	Between-day C.V. (%) $N = 9$
NVP	120	107	1.1	0.5
	600	104	5.0	2.6
	3000	102	2.7	1.3
IDV	120	104	2.3	1.2
	600	105	1.1	0.6
	3000	101	2.2	1.1
DLV	120	103	2.6	1.3
	600	107	0.2	0.1
	3000	100	2.1	1.0
APV	120	99	5.1	2.6
	600	110	5.7	4.5
	3000	111	0.8	0.4
NFV	120	91	4.6	2.3
	600	112	0.8	0.4
	3000	102	5.6	4.5
SQV	120	91	1.5	0.7
	600	112	1.0	0.5
	3000	102	3.2	1.6
EFV	120	106	2.1	1.1
	600	112	1.7	5.4
	3000	101	2.7	3.2
RTV	120	109	2.3	1.2
	600	111	5.4	3.3
	3000	104	2.6	1.3
LPV	120	97	5.7	3.7
	600	114	2.6	4.0
	3000	111	5.8	3.8

ceptance criteria of ±15% deviation from the nominal concentration.

3.7. Analysis of patient samples

We examined the applicability of the described method by analyzing plasma samples collected from HIV-infected patients. Patients in this study were using different combinations of antiretrovirals. For patient samples and QC calculations, calibration curves were obtained by weighted (1/concentration^2) least squares linear regression analysis. Fig. 3 shows chromatograms from two of these patients. Fig. 3a is a chromatogram of a plasma sample containing IDV and RTV and Fig. 3b contains NVP and NFV.

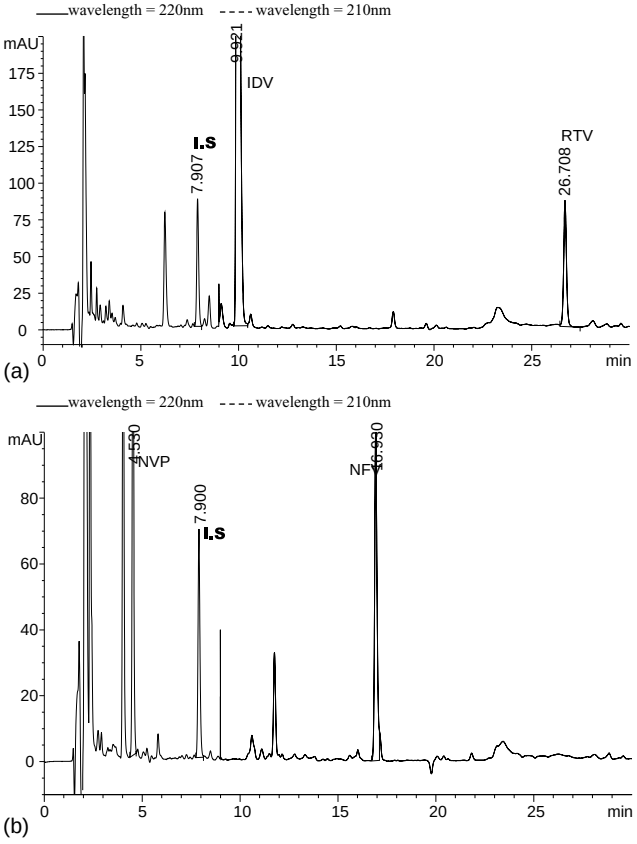

Fig. 3. (a) Chromatogram of a patient sample containing IDV and RTV. (b) Chromatogram of a patient sample containing NVP and NFV.

4. Discussion

To date, there has been no published HPLC–UV method developed for simultaneously measuring all PIs and NNRTIs. However, since PI or PI–NNRTI combinations are becoming more common, a single assay to measure all of these antiretroviral agents would be most useful. Recently, Turner et al. [15] introduced a method for measuring eight analytes after liquid–liquid extraction. This method did not include nevirapine or an internal standard. Many other methods have been published for analyzing only protease inhibitors using a liquid–liquid extraction procedure [16,17] or only non-nucleoside reverse transcriptase inhibitors [13]. This paper describes the development, validation, and use of an HPLC method for the simultaneous quantitative determination of all three non-nucleosides reverse transcriptase inhibitors and six protease inhibitors in one HPLC run after solid phase extraction.

Our method relies on excellent chromatographic resolution for all nine analyte peaks and internal standard peak, selection of a proper internal standard, and optimization of an SPE extraction procedure. The column originally used for developing this method was a Zorbax SB-C18 (150 mm × 4.6 mm, with a 5 μm particle size). Using this column we were unable to achieve the appropriate separation between most of the analytes. However, changing to a column with a 3.5 μm particle size increased the theoretical plate number from 13,000 to 25,000. Using this column, we were able to separate efavirenz from ritonavir, and indinavir from delavirdine. Adding 0.75 ml of TFA improve the separation

between amprenavir, nelfinavir, and saquinavir. Using 15% methanol in the mobile phase assisted in resolving indinavir from an endogenous interfering peak.

The gradient flow rate from 0.9 to 1.1 ml over 30 min maintained a steady pump pressure during the shift from mobile phase (A) (less organic) to (B) (highly organic). This flow rate gradient improved the peak shape for ritonavir and lopinavir and shortened the run time.

The wavelength of 220 nm was most suitable for both nevirapine and midazolam (IS) while 210 nm was best for all other analytes. Overall, these chromatographic conditions produced excellent separation with sharp and symmetrical peaks (Fig. 2) in 30 min. For internal standard selection, we tried three compounds: flurazepam, hexobarbital, and midazolam. Of these three, midazolam behaved optimally: its peak was best separated from indinavir, and its extraction efficiency was steady throughout the different calibration levels of the curve. The function of an internal standard is to compensate for losses of the analytes, especially with extraction procedures in which the analytes of interest are transferred from one container to another. The internal standard should be extracted with high extraction efficiency under the conditions of extraction, and it should have strong absorbance and λmax close to the range of the analytes, as well as to be in good resolution from other analytes. We believe midazolam has all of these characteristics. Additionally, midazolam is not used as a routine outpatient prescription; it has a very short half-life, and is used only for outpatient medical procedures (such as endoscopy and colonoscopy) to decrease anxiety and minimize memory of the event and acutely in hospitalized patients. Other investigators have used benzodiazepines as internal standards in their assays. For example, in analyzing HIV-drugs, Morazoloni et al. [18] used clozapine as an internal standard, and Merry et al. [19] used bromazepam as an internal standard.

Midazolam 1 μg/ml preparations in 100 mM ammonium acetate (pH 7.6) was important for sample dilution, in order to lower the methanol content to 2.5%. Also, adding plasma to the internal standard solution 1:1 reduced variability during sample preparations and reduced the methanol content of the QC and calibrator samples to 2.5%. Furthermore, this allowed accurate transfer of 1.0 ml supernatant from all patient samples, calibrators, and QC's to the conditioned SPE cartridge and smooth movement through the SPE column with minimal external pressure (1–3 mmHg).

Our SPE method proved to be rugged. We found the C-18 cartridge to yield the highest extraction efficiency for most analytes. We also compared four C-18 columns from different manufacturers; Sep-Pack Vac-C18 from Waters, AccuBond-C18 from Agilent, PerpSep-C18 from Fisher and Bond Elute-C18 from Varian. The BOND ELUT-C18 from Varian provided the cleanest base line and highest extraction ratios.

The optimization process also included testing a wide pH range (6.0–8.5) for the internal standard working solution. At pH <6.5, SQV was not retained on the column. At pH <7.0 SQV, RTV, NFV, and IDV were poorly extracted. At pH 8.0, SQV, NVP, DLV, and midazolam were also poorly extracted. However, pH 7.6 was found to be suitable for all analytes.

The current manuscript offers several other advantages over currently published methods: the assay analyzes two HIV-drug classes simultaneously. Both classes contain drugs that have shown concentration–effect relationships and lend themselves to therapeutic drug monitoring. Since early eluting compounds may be used in combination with late eluting compounds, it is much simpler to use one assay that two for these patients. Our method is more sensitive, having a LLD of 0.01–0.025 μg/ml, while others have a LLD of 0.05 μg/ml. Optimized chromatographic conditions (mobile phase composition, gradient, and gradient flow) improve the method's selectivity by improving the baseline and increasing the resolution. Using an internal standard improves accuracy and reproducibility of the method. Additionally, the state of the art optimized SPE method is the first extraction method to handle all of these compounds from a single sample. Additionally, the SPE method in this manuscript is more controllable than a liquid–liquid extraction method.

5. Conclusion

Our optimized HPLC method, after a rugged SPE extraction procedure, provides a sensitive and accurate means for simultaneously determining six PI's and three NNRTI's in the plasma of HIV-infected patients. The solid phase extraction method provides excellent sample clean up and high recoveries. The HPLC method demonstrates good linearity, precision, and accuracy within a wide concentration range for each drug. This method can be used to analyze samples of patients treated with combination therapy regimens, for therapeutic drug monitoring.

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