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ORIGINAL RESEARCH

Determination of lamivudine/stavudine/efavirenz in human serum using liquid chromatography/electrospray tandem mass spectrometry with ionization polarity switch

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ABSTRACT: A high-performance liquid chromatography/tandem mass spectrometry (LC-MS-MS) method with ionization polarity switch was developed and validated in human serum for the determination of a lamivudine (3TC)/stavudine (d4T)/efavirenz combination HIV therapy. Solid phase extraction (SPE) was used to extract these anti-HIV drugs and internal standard aprobarbital. A gradient mobile phase consisting of acetonitrile and 20 mM ammonium acetate buffer with pH adjusted to 4.5 using glacial acetic acid was utilized to separate these drugs on a hexylsilane column (150 × 2.0 mm i.d.). The total run time between injections was 18 min. The precursor and major product ions of these drugs were monitored on a triple quadrupole mass spectrometer in the multiple reactions monitoring (MRM) mode. Ionization polarity was switched in the middle of the LC run allowing these anti-HIV drugs with different physicochemical properties to be detected simultaneously. The effect of ion suppression from human serum was studied and no interference with the analysis was noted. The method was validated over the range of 1.1–540 ng/mL for 3TC, 12.5–6228 ng/mL for d4T and 1.0–519 ng/mL for efavirenz. The method was shown to be accurate, with intra-day and inter-day accuracy less than 14.0% and precise, with intra-day and inter-day precision less than 13.1%. The extraction recoveries of all analytes were higher than 90%.

INTRODUCTION

Combination therapies have proven to be the most effective approach for the treatment of HIV (Carpenter *et al.*, 1997; Gulick *et al.*, 1997; Cavert *et al.*, 1997), and the use of a three-drug regimen with two nucleoside reverse transcriptase inhibitors (NRTI) and a highly active protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) is the preferred option (Anonymous, 1998; International AIDS Society, 1997). NRTIs are the cornerstone of the majority of antiretroviral regimens. The current trend, widely utilized in clinical practice, is to substitute stavudine (d4T) for zidovudine (AZT) in combination regimens using AZT and lamivudine (3TC). The combination of d4T and 3TC is safe and effective, and it reduces the viral load in the cerebral spinal fluid as confirmed by clinical trials (Katlama *et al.*, 1998; Rouleau *et al.*, 1997; Foudraine *et al.*, 1998). Efavirenz is a new NNRTI. It appears to be particularly useful since it is potent, extremely well tolerated, and has a long half-life (Ruiz and Dupont, 1997).

Therapeutic monitoring of anti-HIV drugs is recommended in order to avoid or delay resistance from the virus and to avoid the usual underestimated non-adherence in order to manage drug interactions. Analytical methods have been reported to quantify individual HIV drugs in biological media (Burger *et al.*, 1992; Veldkamp *et al.*, 1999). LC-MS methods have also been described for the simultaneous analysis of multiple HIV drugs, such as those in the NRTI (Kenney *et al.*, 2000) or NNRTI and PI (Villani *et al.*, 2001) categories. However, no methods have been reported for the simultaneous determination of important antiretroviral combinations in both NRTI and NNRTI categories.

We have developed and validated a gradient high-performance liquid chromatography (HPLC) method utilizing triple quadrupole mass spectrometry (MS) detection with electrospray ionization (ESI) and polarity switching for the simultaneous determination of 3TC, d4T and efavirenz in human serum. Because this combination includes drugs from NRTI and NNRTI categories, they have quite different physiochemical characteristics, such as polarity and solubility. The chemical structures of 3TC, d4T and efavirenz are shown in Fig. 1. Apobarbital was chosen as the internal standard because of its similar structure to the NRTI drugs.

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Abbreviations used: AZT, zidovudine; d4T, stavudine; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; 3TC, lamivudine.

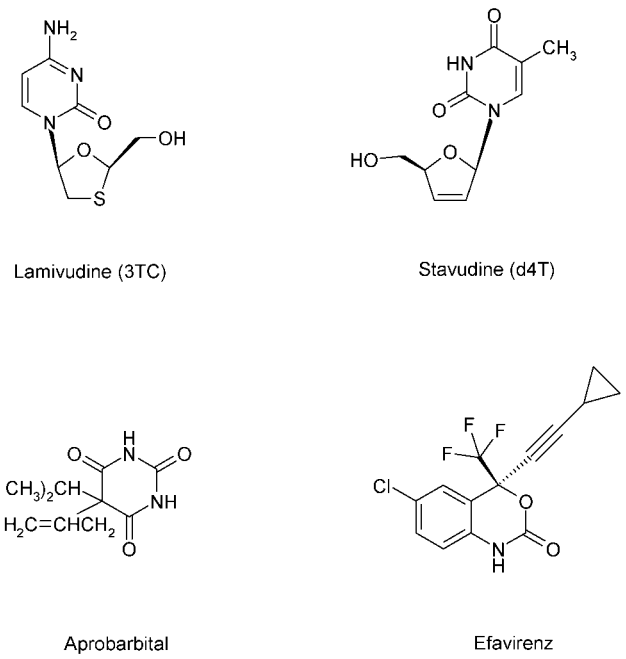


Figure 1. Chemical structures of 3TC, d4T, aprobarbital (I.S.) and efavirenz.

An HPLC-MS method involving an ionization polarity switch was first reported in 1997 by Barnes and co-workers for the analysis of pesticides (Barnes *et al.*, 1997). The ionization polarity switch was operated during each acquisition to determine the presence of eight compounds in the positive ion mode and two compounds in the negative ion mode. ESI has good ion current stability, which when coupled to the lower applied accelerating voltages in the quadrupole mass spectrometer makes it possible to monitor both positive and negative ions in the same LC-MS-MS run. The instrument used by Wang *et al.* switched between ion polarities every 0.05 s while maintaining a stable baseline (Wang *et al.*, 1998). The positive ion ESI mode was selected for 3TC and d4T because of improved sensitivity due to the presence of amino groups, which are easily protonated under the acidic mobile phase conditions (pH 4.5). For efavirenz, even during flow injection at a high concentration (10 μg/mL), the signal of the protonated molecular ion was weak. However, the deprotonated molecular signal, $(M\text{-}H)^-$, was abundant during the negative mode. Similarly, aprobarbital was readily deprotonated due to its chemical structure.

In this study, positive and negative ion modes were initially monitored simultaneously using the same instrument as Wang *et al.* However, at low concentration levels, it was difficult to acquire enough data points to adequately define the peaks for d4T and aprobarbital. In order to improve the peak shape, the detection polarity was switched from positive to negative in the middle of the LC run (at 5.3 min) because aprobarbital and efavirenz have longer retention times on the C_6 column than 3TC and d4T.

EXPERIMENTAL

Chemicals and reagents. d4T and aprobarbital (internal standard) were purchased from Sigma Chemical Company (St Louis, MO, USA). Efavirenz was kindly provided by DuPont Pharmaceuticals Company (Wilmington, DE, USA). 3TC was provided by Dr Chung K. Chu (University of Georgia, Athens, GA, USA). Ammonium acetate, glacial acetic acid, HPLC-grade methanol and acetonitrile were obtained from J. T. Baker Inc. (Phillipsburg, NJ, USA). Deionized water was purified by a cartridge system (Continental Water System, Rosewell, CA, USA). Oasis HLB solid-phase cartridges were obtained from Waters Corporation (Milford, MA, USA). Drug-free human serum was obtained from Bioreclamation Inc. (Hicksville, NY, USA).

Instrumentation and LC-MS-MS conditions. The HPLC system consisted of an Agilent Technologies 1100 HPLC system equipped with a vacuum degasser, quaternary pump and an autosampler (Palo Alto, CA, USA). The column utilized was a Phenomenex Sphereclone hexylsilane (150 × 2.0 mm i.d., 3 μm particle size, Torrance, CA, USA) protected by an ADVCART™ Advantage 100 octadecylsilane guard cartridge (15 × 3.2 mm, 5 μm, SCI-CON OF FLORIDA, Winter Park, FL, USA).

A gradient elution method was applied at ambient temperature using (A) 20 mM ammonium acetate buffer with pH adjusted to 4.5 with glacial acetic acid and (B) acetonitrile. The gradient conditions were as follow: from 0 min, 93% A and 7% B, flow rate 0.2 mL/min; ramp over 1 min to 10% A and 90% B, flow rate 0.3 mL/min; hold for 6 min; ramp over 1 min to 93% A and 7% B, flow rate 0.2 mL/min; hold for 10 min to re-equilibrate the system. A sample volume of 10 μL was injected in triplicate onto the column.

Positive and negative ESI-MS-MS was performed on a Micromass Quattro II triple quadrupole mass spectrometer (Beverly, MA, USA) interfaced to the 1100 HPLC system using a megaflow ESI probe. High purity nitrogen was used as ESI nebulizing gas and drying gas. MS control and spectral processing were carried out using Masslynx software, version 2.22 (Micromass, Beverly, MA, USA). The abundant precursor ion of each analyte produced by positive and negative ESI was selected for fragmentation in the collision cell containing argon gas (99.999% purity) maintained at approximately 1.2×10^{-4} Torr. The precursor and collision-induced fragment ions were monitored by the post-collision quadrupole analyzer, 0–5.3 min for 3TC and d4T, and 5.3–9.0 min for aprobarbital and efavirenz. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes is presented in Table 1. The source temperature and needle voltage were set at 150°C and 3.0 kV, respectively.

Preparation of standards. Stock solutions of 3TC, d4T, efavirenz and the internal standard (aprobarbital) were prepared by dissolving appropriate amounts of drug in absolute methanol to obtain final drug concentrations of 540, 1038, 173 and 1600 μg/mL, respectively. Working solutions were prepared by further diluting these stock solutions in 20 mM ammonium acetate buffer solution (pH 4.5). Calibration standards for the analytes in serum

Table 1. Optimized ESI (+) and ESI (−) mass spectrometric conditions for multiple reaction monitoring (MRM)

Compound	ESI mode	Parent ion (*m/z*)	Daughter ion (*m/z*)	Collision energy (eV)	Cone voltage (V)
3TC	+	230	112	15	30
d4T	+	225	127	10	15
Aprobarbital (IS)	−	209	166	22	30
Efavirenz	−	314	244	19	35

were prepared by spiking drug-free serum with the standard stock solution to yield concentrations of 1.1–540 ng/mL (1.1, 5.4, 10.8, 54, 270 and 540 ng/mL) for 3TC, 12.5–6228 ng/mL (12.5, 62.8, 124.6, 622.8, 3114 and 6228 ng/mL) for d4T and 1.0–519 ng/mL (1.0, 5.2, 10.4, 51.9, 260 and 519 ng/mL) for efavirenz.

Sample preparation procedure. To prepare calibration standards and quality control samples, appropriate quantities of the various diluted solutions and 10 μL of a 1600 μg/mL internal standard aprobarbital solution were added to blank serum to a final volume of 1 mL. Solid-phase extraction (SPE) was used to extract anti-HIV drugs and the internal standard from human serum. Extraction cartridges (Waters Oasis™ HLB 1 cm^3 30 mg) were placed on a vacuum elution manifold (VAC-ELUT™, Varian Sample Preparation Products, Harbor City, CA, USA) and rinsed with 1 mL of methanol followed by 1 mL of purified water. Care was taken that the cartridges did not run dry. One milliliter of the spiked serum samples was loaded onto the cartridges and drawn by applying vacuum. The cartridges were then washed with 1 mL of 20 mM ammonium acetate buffer (pH 7.0) followed by vacuum suction for 1 min. One milliliter of methanol was used to elute the adsorbed analytes, which were then concentrated in a vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY, USA). Extracts were reconstituted in 50 μL of mobile phase and injected into the LC-MS-MS system.

Ion suppression testing. A postcolumn infusion of the individual analytes was performed during injections of extracted blank serum samples using the same LC-MS-MS conditions described previously (King *et al.*, 2000) 3TC, d4T, efavirenz and the internal standard (aprobarbital) solutions were prepared by diluting the stock solutions in ammonium acetate buffer to obtain final drug concentrations of 5.40, 10.38, 17.30 and 16.00 μg/mL, respectively. Each analyte was infused individually through a zero dead volume tee at a flow rate of 30 μL/min. Eluate from the HPLC column combined with the infused analytes and entered the mass spectrometer through the electrospray interface.

Limit of detection (LOD) and limit of quantification (LOQ). Serum samples were spiked with decreasing concentrations of the analytes. The limit of detection (LOD) was defined by the concentration of analyte that yields a signal-to-noise ratio of 3. The limit of quantification (LOQ) was considered to be the lowest concentration that could be measured with a signal-to-noise ratio of 10.

Accuracy, precision, linearity and recovery. Accuracy was calculated by comparing the concentration of spiked samples to each nominal concentration. Precision was reported as percentage relative standard deviation (% RSD). The intra-day accuracy and precision of the assay in serum were determined by assaying three quality control samples at low, medium and high concentrations of each compound (28, 110 and 540 ng/mL for 3TC, 310, 1245 and 5000 ng/mL for d4T, 20, 75 and 400 ng/mL for efavirenz) in three analytical runs within the same day. The inter-day accuracy and precision of samples were analyzed on three different days. Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficients, which were then used to determine the concentration of each analyte in the quality control samples. The recoveries of each drug and the internal standard were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration.

RESULTS AND DISCUSSION

3TC and d4T are highly hydrophilic compounds, and thus are different from the highly hydrophobic efavirenz. It was difficult to separate them simultaneously under isocratic conditions, even using ion pair chromatography. A series of HPLC columns were investigated from silica to C_{18}, but none gave satisfactory chromatographic separations. With highly polar columns like silica, 3TC and d4T co-eluted at the solvent front. With a nonpolar column such as C_{18}, efavirenz did not elute within 60 min. Thus, a gradient HPLC method was developed and validated to separate 3TC, d4T and efavirenz by changing the ratio of organic modifier to buffer and flow rate. It offered a reliable solution for the chromatography of these analytes that were difficult to analyze by other means.

The gradient elution profile was optimized to improve the spacing of peaks in the chromatogram while maintaining the required resolution and to approximate the ideal separation. It began with the selection of the concave gradient shape, which was preferred for the chromatographic separation of the early eluted polar analytes 3TC and d4T. The steepness of the gradient was then optimized in order to achieve the required resolution of the critical pair of analytes 3TC and d4T as well as shorten the retention time for all the chromatographed compounds. Finally, the initial ratio of acetonitrile in the mobile phase was conveniently adjusted with the aim of

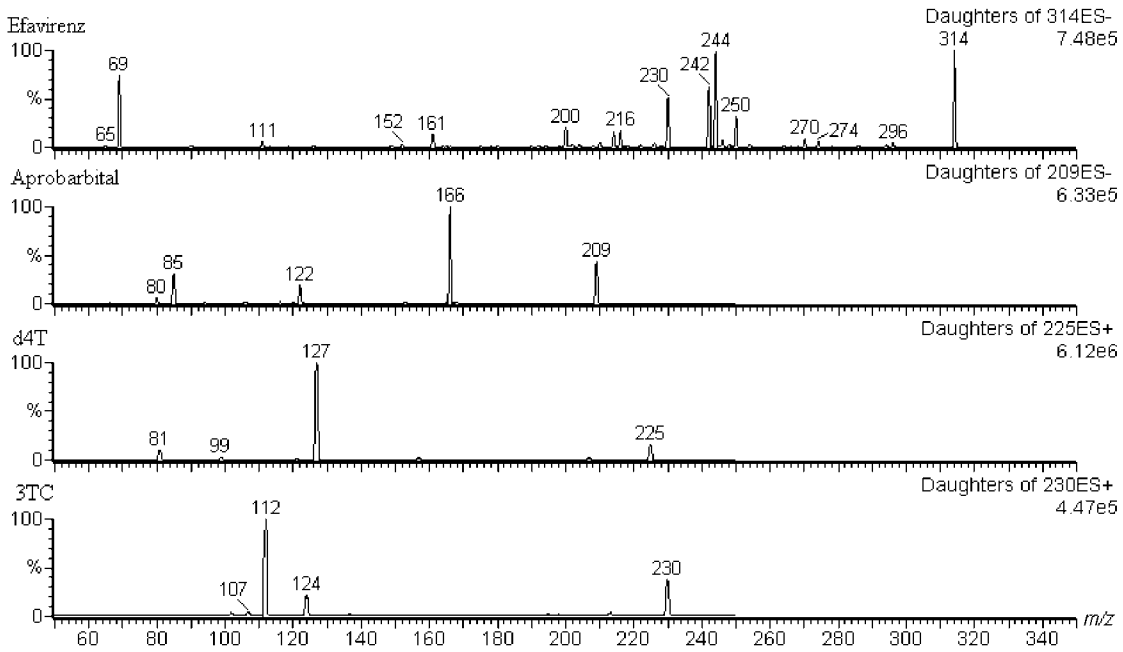
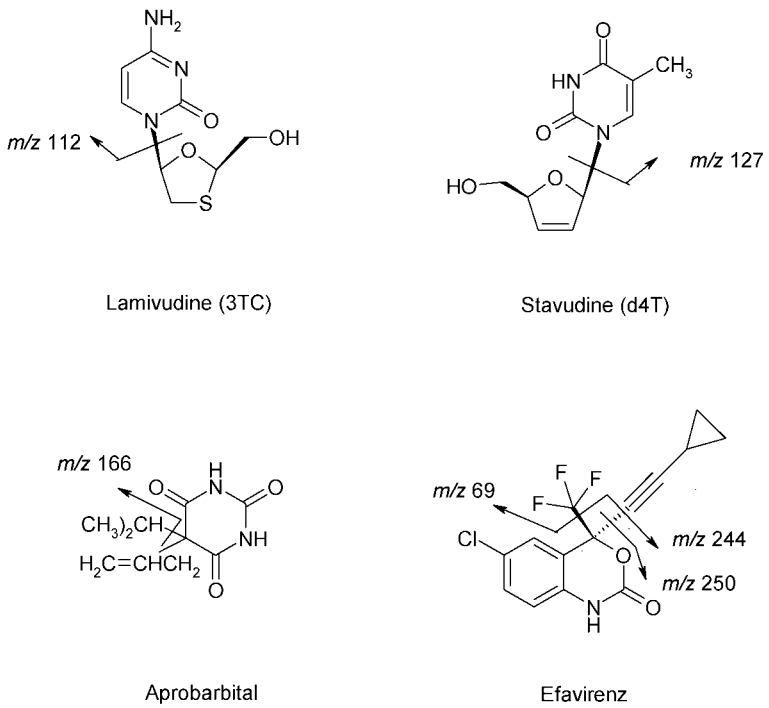


Figure 2. Mass spectra of efavirenz, aprobarbital (internal standard), d4T and 3TC.

keeping the retention times as short as possible. Acetonitrile was chosen as the organic modifier in the mobile phase because of its solvent strength. Efavirenz was not eluted quickly when using methanol.

Initially, a two-step elution with the first isocratic step (gradient delay) followed by gradient elution in the second step was tried. A good separation of 3TC and d4T was achieved under near optimum isocratic conditions in the first step, while the retention of the more strongly retained efavirenz was accelerated in the second gradient



Scheme 1. Proposed fragmentation pathways for 3TC, d4T, aprobarbital (I.S.), and efavirenz.

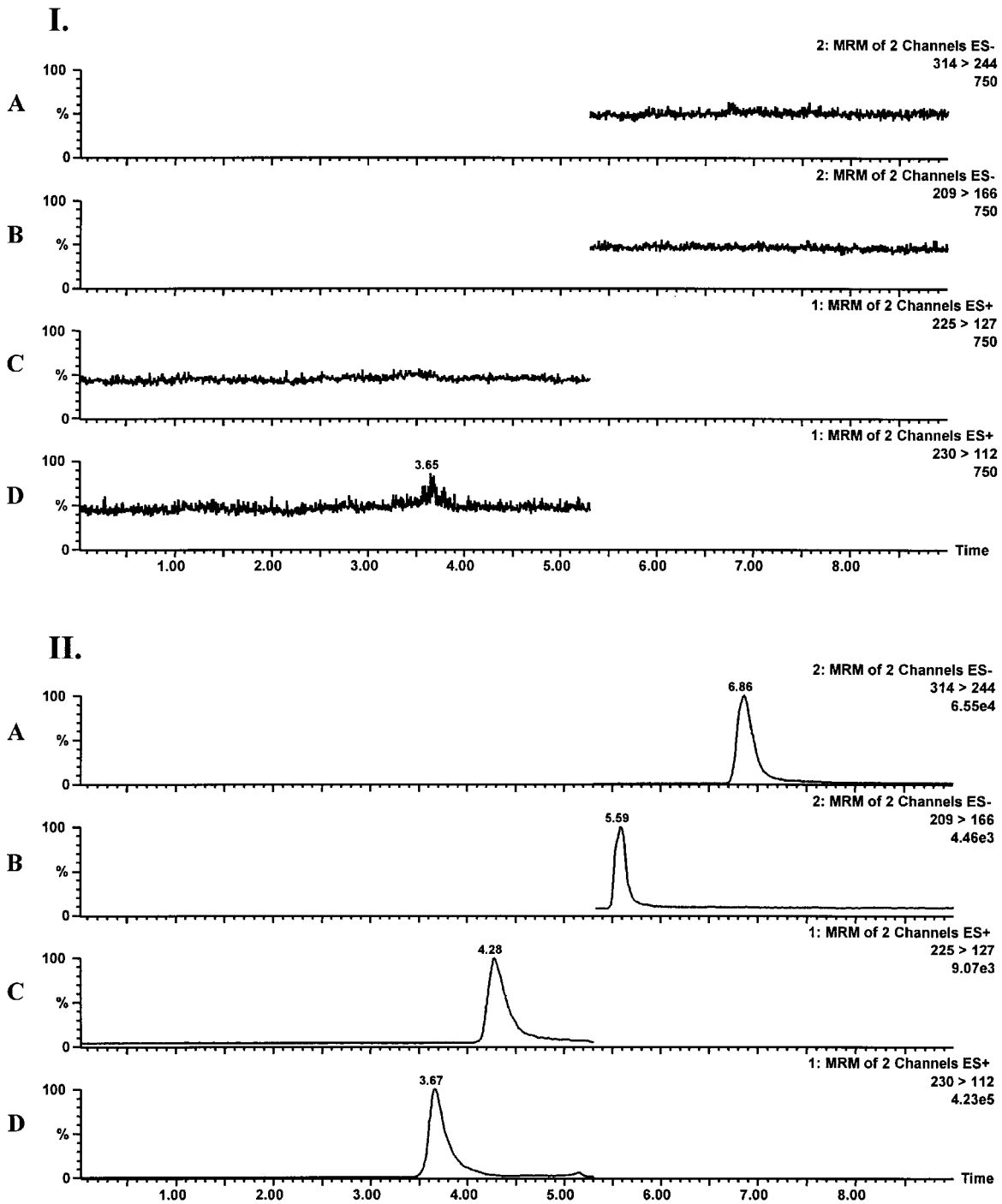


Figure 3. Chromatograms of (I) blank human serum and (II) human serum spiked with (A) efavirenz (B) internal standard, (C) d4T and (D) 3TC.

step, but was still longer than 15 min. In gradient elution chromatography, a certain minimum gradient delay existed, which was determined by the inner volume of the instrumentation between the point of mixing of the mobile phase components and the top of the column, usually 1–5 ml. This gradient delay was large for the 2.1 mm i.d. nanobore column used, and produced a longer retention time for efavirenz. In order to keep the separation time short, the gradient program was started at the time of sample injection. A baseline separation of 3TC and d4T was achieved as well as a shorter retention time for efavirenz. Furthermore, flow programming was combined with gradient elution to decrease the retention time of the last 'over-resolved' compound efavirenz.

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of 3TC, d4T and efavirenz in human serum

	Concentration (ng/mL)	Precision[a] (%) Intra-day	Inter-day	Accuracy[a] (% error) Intra-day	Inter-day	Recovery[b] (%)
3TC	500	2.4	7.4	2.1	3.3	97.9 ± 1.9
	108	2.2	12.8	11.8	11.4	100.6 ± 4.1
	30	0.2	6.4	3.1	9.8	91.9 ± 3.5
d4T	5000	5.8	11.9	12.2	14.0	94.3 ± 3.5
	1245	2.3	8.6	12.7	0.5	91.7 ± 2.3
	310	3.9	2.7	2.3	3.4	89.6 ± 1.7
Efavirenz	400	13.1	5.2	5.9	13.9	100.2 ± 1.2
	75	4.3	3.8	4.8	11.3	93.0 ± 2.9
	20	5.5	5.9	2.8	13.9	92.8 ± 3.0

[a] Based on $n = 9$.
[b] Mean ± s.d. based on $n = 6$.

When the flow rate was increased from 0.2 to 0.3 mL/min, the retention time of efavirenz was shortened from 14 to 7 min, while column pressure increased from 150 to 210 bar.

For the NRTI drugs 3TC and d4T, the precursor ions $[M + H]^+$ were formed as a result of the addition of a proton to form the positively charged molecular ion. For aprobarbital and efavirenz, $[M\text{-}H]^-$ ions were observed following loss of a proton. The base peaks in the CID mass spectra were *m/z* 112 for 3TC, m/z 127 for d4T, *m/z* 244 for efavirenz and *m/z* 166 for aprobarbital (Fig. 2). The precursor and major product ions of the analytes were monitored in the multiple reaction monitoring modes (Table 1). In the case of 3TC, protonation of the nitrogen on the purine base initiates glycosidic bond cleavage and results in the loss of the sugar moiety via alpha cleavage to give the ion at *m/z* 112. For d4T, the fragmentation is similar to 3TC, because of its structural similarity, resulting in the formation of the ion at *m/z* 127 by an alpha cleavage of the glycosidic bond. Complex tandem mass spectra were generated from the parent ion of efavirenz ($[M\text{-}H]^-$ at *m/z* 314) with the formation of multiple fragment ions. Major fragment ions at *m/z* 250, 230, 69 and a base peak at *m/z* 244 were observed following the loss of functional groups attached to the C-4 position of the efavirenz ring system. The proposed fragmentation pathways for all analytes are illustrated in Scheme 1.

Baseline separations of 3TC, d4T and efavirenz were achieved with retention times of 3.67, 4.28 and 6.86 min, respectively. The internal standard aprobarbital had a retention time of 5.59 min. Figure 3 shows the chromatograms obtained from drug-free human serum and a spiked serum sample. Since the precursor and major product ions of the compounds were monitored in the MRM mode, the method is highly selective and specific. There were no peaks from endogenous components observed from blank serum. Total run time between injections was 18 min.

During development of the solid-phase extraction method, a series of different extraction cartridges were investigated, such as C_{18}, C_8, phenyl and Oasis™ cartridges. C_{18} and Oasis™ cartridges did not show any loss of analytes when loading spiked serum samples. The Oasis™ SPE cartridges provided the highest recoveries for all drugs analyzed in this study. Twenty millimolar ammonium acetate (pH 7.0) was used to wash the cartridges after loading spiked serum to help retain the highly hydrophilic analytes 3TC and d4T and thus obtain a clean sample. Methanol was strong enough to elute all of the analytes and was also easily evaporated. Absolute recoveries greater than 89.6% were obtained for all three analytes and the internal standard. The detailed data is listed in Table 2. The recovery for the internal standard was 96.2%.

As a part of the method development, the effect of ion suppression from the matrix on the quantitative LC-MS-MS analysis of the four analytes was studied. In related chromatograms for each analyte including the internal standard, ion suppression was observed around 2.0 min. A second region of ion suppression occurred around 5.0 min. No ion suppression was observed at the retention times of the analytes or the internal standard. The current explanation for ion suppression in electrospray ionization is that a combination of solution and gas-phase reactions involving analyte ions and other components from the biological sample result in the loss of signal from the analyte ion (King *et al.*, 2000). The matrix effect is especially dependent on the degree of sample cleanup, the degree of chromatographic separation, and the retention of analytes on the analytical column (Matuszewski *et al.*, 1998). In this method, solid-phase extraction was used to isolate the samples and the analytes were retained in the column long enough to avoid ion suppression. Careful assessment of matrix effects constitutes an integral and important part of any quantitative LC-MS-MS assay validation procedure in biological fluids.

The calibration curves showed good linearity in the range of 1.1–540 ng/mL for 3TC, 12.5–6228 ng/mL for

d4T and 1.0–519 ng/mL for efavirenz. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.99 as determined by least-squares analysis. The LOD for 3TC, d4T and efavirenz were 0.5, 6.2 and 0.5 ng/mL, respectively. The LOQ for 3TC, d4T and efavirenz were 1.1, 12.5 and 1.0 ng/mL, respectively, and were equivalent to the low calibration standards. The results from the validation of the method in human serum are listed in Table 2. The method proved to be accurate (relative error at high, medium and low concentrations was less than 14.0% for inter-day and less than 12.7% for intra-day) and precise (inter-day precision was less than 12.8% and intra-day precision was less than 13.1%).

CONCLUSION

Solid phase extraction and LC-ESI-MS-MS methods with ionization polarity switch provide a fast, sensitive and selective procedure for the simultaneous determination of a 3TC/d4T/efavirenz mixture in human serum. The analytes in this method were shown to be free from ion suppression effects generated by the matrix. This method is a demonstration of a quantitative LC-MS-MS assay employing ionization polarity switching and should be useful in the routine monitoring of serum drug concentrations and in pharmacokinetic studies of HIV-infected patients.

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