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Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC

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Abstract

A new high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine (AZT)/lamivudine (3TC)/nevirapine in human plasma. Plasma samples were treated using a solid-phase extraction procedure. The compounds were separated using a mobile phase of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt)–acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid on an octylsilane column (150 × 3.9 mm i.d.) with UV detection at 265 nm. Aprobarbital was chosen as the internal standard (IS). The method was validated over the range of 57.6–2880 ng/ml for AZT, 59.0–17 650 ng/ml for 3TC and 53.2–13 300 ng/ml for nevirapine. The method was shown to be accurate, with intra-day and inter-day accuracy from 0.1 to 11% and precise, with intra-day and inter-day precision from 0.4 to 14%. Extraction recoveries of the analytes and IS from plasma were higher than 92%. The assay should be suitable for use in pharmacokinetic studies and routine plasma monitoring of this triple-drug therapy in AIDS patients.

Keywords: Zidovudine; Lamivudine; Nevirapine; Aprobarbital; Solid-phase extraction; Ion pair; Human plasma

1. Introduction

Over the last couple of years, combination therapy has proven to be the most effective approach to treat HIV disease [1–3]. The preferred options for patients who have not been previously treated include a three-drug regimen with at least two nucleoside reverse transcriptase inhibitors (NRTI) such as zidovudine (AZT) and lamivudine (3TC) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), such as nevirapine [4]. The profound and sustained viral suppression achievable with combinations such as indinavir (IDV), 3TC and AZT has resulted in a dramatic shift in HIV treatment paradigms [2,5]. There are several combinations of NRTI to choose from in the initial three-drug regimen and, until very recently, the primary treatment combinations were based on AZT [4]. AZT and 3TC are front line therapies

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for the treatment of HIV infection and recently have become available in a combination tablet dosage form (Cimbivir™) [6].

Numerous analytical methods, such as high performance liquid chromatography (HPLC) with UV detection [7,8], mass spectrometry detection [6] and immunoassay [9,10], have been reported for either AZT or 3TC. However, no methods have been reported for the simultaneous determination of an AZT, 3TC and nevirapine mixture in human plasma. We have developed and validated an isocratic ion pair HPLC assay with UV detection for the simultaneous determination of AZT, 3TC and nevirapine in human plasma. The method is potentially suitable for drug monitoring and determination of pharmacokinetic profiles.

2. Material and methods

2.1. Chemicals and reagents

AZT, aprobarbital (internal standard) and 1-octanesulfonic acid sodium salt were purchased from Sigma Chemical Company (St. Louis, MO 63178). Nevirapine was kindly provided by Abbott Laboratories (North Chicago, IL 60064). 3TC was provided by Dr Chung K. Chu (University of Georgia, Athens, GA, USA). Monobasic sodium phosphate, phosphoric acid, HPLC grade methanol and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Water was purified by a cartridge system (Continental Water System, Roswell, GA, USA).

2.2. Instrumentation

The HPLC system consisted of a Beckman model 110 B pump (Fullerton, CA), an Alcott model 738 autosampler (Alcott Chromatography Inc., Norcross, GA), a Lamdba-Max model 481 LC spectrophotometer (Waters, Milford, MA), and a HP 3394A integrator (Hewlett Packard, Avondale, PA). Separation was performed on a Waters Nova-Pak C_8 column (150 × 3.9 mm i.d., 5 μm particle size, Waters, Millford, MA) protected by an Applied Biosystems RP-8 guard column (Foster City, CA).

2.3. Chromatographic conditions

The chromatographic analysis was performed at ambient temperature with isocratic elution. The mobile phase consisted of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt)–acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid. The pump was set at a flow rate of 1.0 ml/min. A sample volume of 20 μl was injected in triplicate onto the HPLC column. The chromatogram was monitored with UV detection at 265 nm.

2.4. Preparation of standard stock solutions

Standard stock solutions of AZT, 3TC, nevirapine and the internal standard (aprobarbital) were prepared by dissolving appropriate amounts of each drug in absolute methanol to obtain final drug concentrations of 288, 295, 266 and 1430 μg/ml, respectively. Working solutions were prepared by further diluting these stock solutions with 20 mM sodium phosphate buffer solution.

2.5. Sample preparation procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted standard solutions and 50 μl of the internal standard solution were added to blank plasma to a final volume of 1 ml. Extraction cartridges (Waters Oasis™ HLB 1 cc 30 mg Extraction Cartridge) were placed on a vacuum elution manifold (VAC-ELUT™, Varian Sample Preparation Products, Harbor City, CA 90710) 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 plasma sample was then loaded onto the SPE cartridges and the vacuum applied. The cartridges were then washed with 1 ml 25 mM ammonium acetate buffer (pH 7.0) followed by vacuum suction for 1 min. Water–acetonitrile (1 ml, 70:30, v/v) was used to elute the analytes and 20 μl was injected into the HPLC system.

2.6. Limit of detection (LOD) and limit of quantification (LOQ)

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

2.7. Linearity of method

Calibration plots for the analytes in plasma were prepared by spiking drug-free plasma with standard stock solutions to yield concentrations of 57.6–2880 ng/ml (57.6, 144, 288, 1440 and 2880 ng/ml) for AZT, 59–17 650 ng/ml (59, 147.5, 295, 2950 and 17 650 ng/ml) for 3TC and 53.2–13 300 ng/ml (53.2, 133, 266, 2660 and 13 300 ng/ml) for nevirapine. Triplicate injections of each concentration were performed. 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 coefficient data which were then used to calculate analyte concentration in each sample.

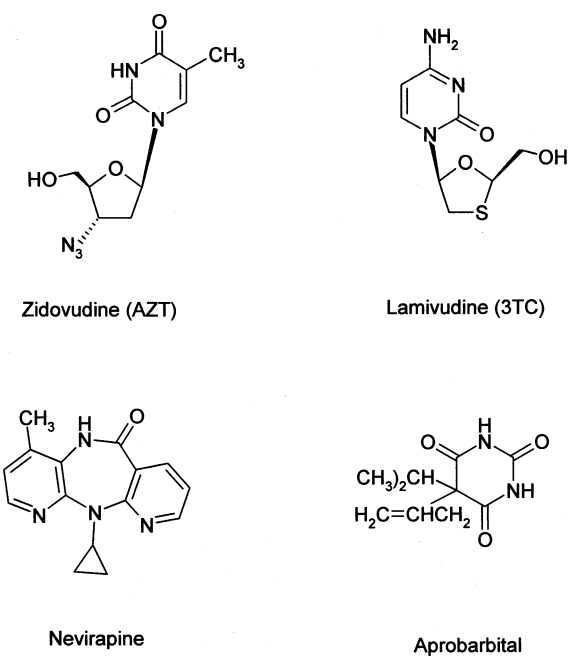

Fig. 1. The chemical structures of analytes.

2.8. Precision and accuracy of method

The intra-day accuracy and precision of the assay in plasma were determined by assaying two quality control samples in triplicate at low and high concentrations for each compound (144 ng/ml and 288 ng/ml for AZT, 295 ng/ml and 2950 ng/ml for 3TC, 133 ng/ml and 2660 ng/ml for nevirapine) within the same day ($n = 6$). The inter-day accuracy and precision of the samples were analyzed on three different days ($n = 18$). Percent accuracy was determined as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as percent relative standard deviation (%RSD).

2.9. Recovery of analytes from plasma

The recoveries of each drug and internal standard from plasma were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration. Both low and high concentrations for each compound were checked (144 and 288 ng/ml for AZT, 295 and 2950 ng/ml for 3TC, 133 and 2660 ng/ml for nevirapine). The concentration of the internal standard aprobarbital was 71.5 μg/ml.

3. Results and discussion

Because of different physiochemical properties among AZT, 3TC and nevirapine, it was difficult to separate them simultaneously under isocratic conditions. The chemical structures of AZT, 3TC, nevirapine and aprobarbital (internal standard) are shown in Fig. 1. A series of HPLC columns from silica to C_{18} were investigated, but none of them gave satisfactory chromatographic separations. With polar columns like silica, 3TC and AZT were eluted near the solvent front, and nevirapine was eluted at about 5 min even at a low percent organic solvent in the mobile phase.

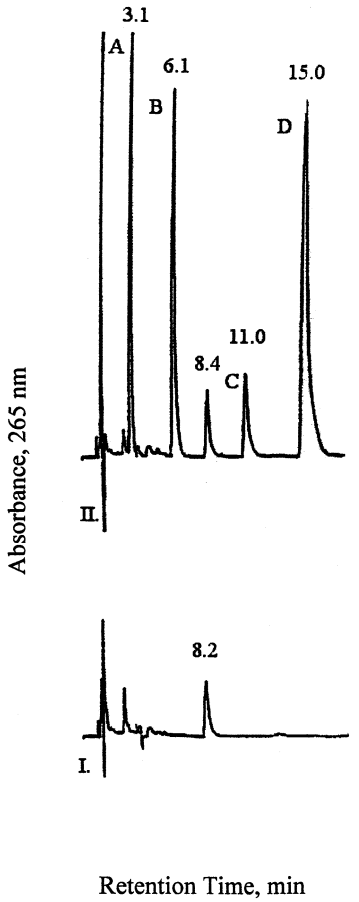

Fig. 2. Chromatogram of: (I) blank human plasma; and (II) human plasma spiked with (A) AZT, (B) 3TC, (C) internal standard and (D) nevirapine. The peak at 8.2–8.4 min is an unknown plasma component.

With nonpolar columns such as C_{18}, 3TC and AZT were baseline separated at a low percent organic solvent in the mobile phase, but nevirapine did not elute within 30 min. A gradient method was considered, but was not successful due to baseline drift problem that could not be overcome. Thus a reverse phase ion pair HPLC method was developed to alter the chromatographic selectivity of 3TC and nevirapine, and separate them from AZT under isocratic conditions. This ion pair method offered a reliable solution to the chromatography of analytes that were difficult to separate. It would also be a good technique for separating a mixture of 3TC and AZT.

In order to study an ion pair method for separating 3TC and nevirapine from AZT, factors such as organic solvent, choice of ion pair reagent and pH of mobile phase were investigated to manipulate chromatographic retention. Acetonitrile was chosen over methanol as organic modifier in the mobile phase because of its solvent strength. Negatively charged sulfonate ions (SO_3^-) with different lengths of alkyl chain, such as 1-pentanesulfonic acid, 1-hexanesulfonic acid, 1-heptanesulfonic acid, 1-octanesulfonic acid and lauryl sulfate were investigated as a suitable ion pair reagent. The ion pairs formed can enhance selectivity because of hydrogen bonding capacity and also offer electrostatic interaction with analytes of opposite charges. With the addition of the ion pair reagent into the mobile phase and the use of an octylsilane column, the retention times of 3TC and nevirapine increased as predicted. The order of elution switched from 3TC, AZT and nevirapine to AZT, 3TC and nevirapine. The hydrophobicity of the ion pair was important to the optimizing of the separation; the higher the hydrophobicity of the ion pair, the larger the retention factors of 3TC and nevirapine. Concentrations from 0 to 50 mM of 1-octanesulfonic acid sodium salt were investigated to optimize the separation. It was found that 1-octanesulfonic acid sodium salt added to the mobile phase achieved suitable retention times for all three analytes with baseline resolution. The retention of 3TC increased and the retention of AZT slightly decreased as the concentration of the ion pair reagent was increased. Above 50 mM ion pair concentration, analyte retention decreased because micelles of the ion pair molecules began to be formed, and their formation led to competing partition processes. Eight millimolar 1-octanesulfonic acid was the optimum ion pair concentration that gave the best separation of AZT, 3TC and nevirapine.

It was observed that the chromatographic selectivity of 3TC and AZT under these ion-pair conditions exhibited a profound pH dependency. As the pH of the phosphate buffer increased from 2.5 to 4.5, elution time and resolution initially increased and then decreased as buffer pH increased. The best separation for 3TC and

nevirapine from AZT as ion pairs was achieved at pH 3.2.

Baseline separations of AZT, 3TC and nevirapine were achieved with retention times of 3.1, 6.1 and 15.0 min, respectively. The internal standard aprobarbital gave a retention time of 11.0 min. Aprobarbital was chosen because it is structurally similar to NRTI drugs and gave good recoveries from plasma using the SPE method. Fig. 2 I and II show the chromatograms obtained from drug-free human plasma and a spiked plasma sample, respectively. All the drugs showed good separation from endogenous plasma interferences. The only significant plasma peak present in both blank and sample eluted at 8.2–8.4 min and did not interfere with the separations.

During development of the solid-phase extraction method, a series of different extraction cartridges and discs were investigated, such as C_{18}, C_8, phenyl, Oasis™ cartridges and C_{18}, C_{18}AR, and mixed-mode Discs. The recoveries using discs were less than 30% for AZT and 3TC. Cartridges gave much higher recoveries, particular for C_{18} and Oasis™. Oasis™ provided the highest recoveries of all of these drugs as well as a much cleaner sample. Twenty-five millimolar ammonium acetate (pH 7.0) was used to wash the cartridges after loading spiked plasma to help retain the highly hydrophilic analytes AZT and 3TC. Higher recoveries were observed using ammonium acetate than water washes. Because there was no organic solvent in the wash solution, not all of the endogenous interferences could be removed. An endogenous non-interfering plasma component was observed around 8 min. A 30% acetonitrile solution was strong enough to elute

Table 1
Inter-day and intra-day accuracy, precision and recovery for the analysis of AZT, 3TC and nevirapine in human plasma

	Concentration (ng/ml)	Precision (%)		Accuracy (%)		Plasma recovery[c]
		Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	(%)
AZT	288	5.4	6.2	2.6	5.4	102.3 ± 3.3
	144	3.5	13.8	3.8	9.7	103.0 ± 2.6
3TC	2950	4.0	0.4	2.2	0.2	97.9 ± 1.9
	295	4.8	2.7	5.4	6.9	100.6 ± 4.1
Nevirapine	2660	0.4	4.1	0.1	2.0	91.8 ± 2.1
	133	4.5	12.1	10.7	4.2	106.3 ± 3.8

[a] Based on $n = 3$.
[b] Based on $n = 9$.
[c] Mean ± SD based on $n = 6$.

Table 2
Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of AZT, 3TC and nevirapine in spiked human plasma

Drug	Range of calibration curves (ng/ml)	Limit of detection (LOD) (ng/ml)[a]	Limit of quantification (LOQ) (ng/ml)[b]
AZT	57.6–2880	28.8	57.6
3TC	59.0–17 650	14.8	59.0
Nevirapine	53.2–13 300	13.3	53.2

[a] S/N = 3.
[b] S/N = 10.

all of the analytes including nevirapine and leave most of the highly hydrophobic plasma interferences on the SPE cartridges. Absolute plasma recoveries greater than 92% were obtained for all three analytes and internal standard. The detailed data is listed in Table 1. The recovery of the internal standard from plasma was 96%.

The calibration curves showed good linearity in the range of 57.6–2880 ng/ml for AZT, 59.0–17 650 ng/ml for 3TC and 53.2–13 300 ng/ml for nevirapine. Typical anti-HIV drug concentrations in patients are variable depending on the dose administrated, but they ranged from 50 to 1000 ng/ml for AZT, 10–5000 ng/ml for 3TC and 100–10 000 ng/ml for nevirapine. We further expanded the concentration ranges in order to make the HPLC method applicable to the myriad of other studies for which anti-HIV agents would need to be measured in biological samples. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.999 as determined by least-squares analysis. LOD and LOQ data are shown in Table 2. The LOD for AZT, 3TC and nevirapine were 28.8, 14.8 and 13.3 ng/ml, respectively. The LOQ for AZT, 3TC and nevirapine were 57.6, 59.0 and 53.2 ng/ml, respectively. The results from the validation of the method in human plasma are listed in Table 1. The method proved to be accurate (relative error at high and low concentration from 0.1 to 10.7% for intra-day and from 0.2 to 9.7% for inter-day) and precise (intra-day precision ranged from 0.4 to 5.4% and inter-day precision ranged from 0.4 to 13.8%).

4. Conclusions

A sensitive, specific and validated HPLC isocratic assay was developed for the simultaneous analysis of AZT/3TC/nevirapine in human plasma. The HPLC method should be useful for monitoring plasma drug concentrations, and pharmacokinetic studies in HIV-infected patients, and for any other studies where it is desirable to measure concentrations of these anti-HIV agents in biological samples.

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