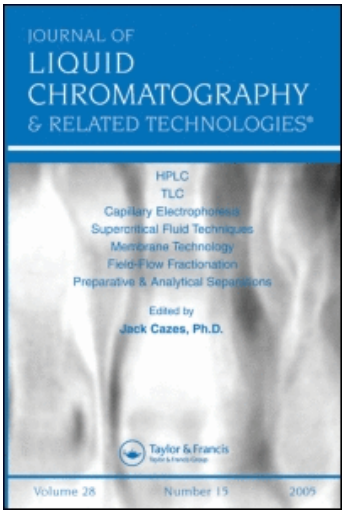

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DETERMINATION OF ZIDOVUDINE/ ZALCITABINE/NEVIRAPINE IN HUMAN PLASMA BY ION-PAIR HPLC

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ABSTRACT

A novel high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine (AZT)/zalcitabine (ddC)/nevirapine in human plasma. Plasma samples were treated using a solid-phase extraction procedure. The analytes were separated using a mobile phase containing 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 internal standard. The method was validated over the range of 57.6-2880 ng/mL for AZT, 20.2-2020 ng/mL for ddC and 53.2-13300 ng/mL for nevirapine. Intra-day and inter-day accuracy were less than 10.7% and intra-day and inter-day precision were less than 13.7%. Extraction recoveries of all analytes from plasma were higher than 88.5%. The assay should be applic-

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able for pharmacokinetic studies and routine monitoring of these drugs in plasma.

INTRODUCTION

Over the past several years, the use of multidrug therapy has greatly enhanced the success of acquired immunodeficiency syndrome (AIDS) treatment.(1-3) Combination therapy of two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) is one of the preferred treatment options.(4,5) Nevirapine was the first NNRTI, which is notorious for rapidly eliciting resistance due to mutations of the amino acids surrounding the NNRTI binding site.(6) In association with two NRTI, nevirapine significantly reduces the viral load and increases CD4 cell counts, particularly in treatment-naive patients(7,8) 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. The combination of AZT with ddC is a well-proven, highly effective regimen.(5)

Therapeutic monitoring of these drugs is recommended in order to avoid or delay resistance from the virus, to avoid the usual underestimated non-adherence, and to manage drug interaction. Analytical methods have been described to quantify individual drugs in biological media,(9-11) but few methods have been reported for combined anti-HIV drugs. We have developed and validated a high-performance liquid chromatography (HPLC) assay with UV detection for the simultaneous determination of AZT, ddC, and nevirapine in human plasma. Because this combination includes drugs from both NRTI and NNRTI categories, they have quite different physiochemical characteristics, such as polarity and solubility, and their assay was a challenge for method development. The method could also be applicable for drug monitoring and determination of pharmacokinetic profiles for this drug combination.

EXPERIMENTAL

Chemicals

Zidovudine (AZT), zalcitabine (ddC), 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). Monobasic sodium phosphate, phosphoric acid, and HPLC grade methanol and acetonitrile were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Water was purified by a cartridge system

(Continental Water System, Roswell, GA, USA). Drug free human plasma was obtained from Bioreclamation Inc., Lot # BRH01495 (Hicksville, NY 11801).

Instrumentation

The high performance liquid chromatographic system was equipped with a Beckman model 110 B pump (Fullerton, CA), an Alcott 738 model autosampler (Norcross, GA), a Lambda-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, Milford, MA) protected by an Applied Biosystems RP-8 guard column (Foster City, CA).

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 into the HPLC system. The UV detector was operated at a wavelength of 265 nm.

Preparation of Standard Stock Solutions

Stock solutions of zidovudine (AZT), zalcitabine (ddC), 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, 202, 266, and 1430 μg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with 20 mM sodium phosphate buffer solution.

Sample Preparation Procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted stock solutions and 50 μL of the internal standard solution were added to blank plasma to obtain a final volume of 1 mL. Extraction cartridges (Waters Oasis™ HLB 1cc 30mg Extraction Cartridges) were placed on a vacuum elution manifold (VAC-ELUT™, Varian Sample

Preparation Products, Harbor City, CA 90710) and rinsed with 1 mL of absolute methanol followed by 1 mL of purified water. Care was taken such that the cartridges did not run dry. 1 mL of each spiked plasma sample was then loaded onto a SPE cartridge and a 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. Then 1 mL of water-acetonitrile (70:30, v/v) was used to elute the analytes, followed by a 20 μL injection into the liquid chromatograph.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

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

Linearity of Method

Calibration plots for the analytes in human plasma were prepared by adding standard stock solutions to drug-free plasma, yielding concentrations of 57.6-2880 ng/mL (57.6, 144, 288, 1440, and 2880 ng/mL) for AZT, 20.2-2020 ng/mL (20.2, 40.4, 101, 202, and 2020 ng/mL) for ddC, and 53.2-13300 ng/mL (53.2, 133, 266, 2660, and 13300 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, which were then used to calculate analyte concentration in each spiked sample.

Precision and Accuracy of Method

The intra-day accuracy and precision of the assay in human plasma were determined by assaying two quality control samples in triplicate at low and high concentrations for each compound (144 and 288 ng/mL for AZT, 40.4 and 202 ng/mL for ddC, 133 and 2660 ng/mL for nevirapine) within the same day (n=6). The inter-day accuracy and precision for the samples were analyzed on three different days (n=18). Accuracy was calculated by comparing concentration of spiked samples to the known concentration. Precision was reported as percent relative standard deviation (%RSD).

Recovery of Analytes from Plasma

The recoveries of each drug and internal standard from plasma were determined by comparing the peak area of each compound after extraction with the respective non-extracted standard solution at the same concentration. Both low and high concentrations for each compound were checked, (144 and 288 ng/mL for AZT, 40.4 and 202 ng/mL for ddC, 133 and 2660 ng/mL for nevirapine). The concentration of the aprobarbital internal standard in the samples was 71.5 μg/mL.

RESULTS AND DISCUSSION

Because of different physiochemical properties among these drugs, it was difficult to separate them under isocratic HPLC conditions. A series of HPLC columns were investigated from silica to C_{18}, but none of them gave a satisfactory chromatographic separation. With a highly polar column such as silica, ddC and AZT eluted near the solvent front, and nevirapine eluted at about 5 min, even at a low percent organic solvent in the mobile phase. With a nonpolar column such as C_{18}, ddC and AZT were baseline separated at a low percent organic solvent in the mobile phase, but nevirapine would not elute within 30 min. Thus, a reverse phase ion pair HPLC method was developed to alter the chromatographic selectivity of ddC and nevirapine, and separate them from AZT under isocratic conditions. This ion pair method offered a reliable solution for the chromatography of analytes that would be difficult to separate by other means.

Acetonitrile was chosen over methanol as organic modifier in the mobile phase because of its solvent strength. The chemical structures of AZT, ddC, nevirapine, and aprobarbital (internal standard) are shown in Figure 1. Under mobile phase acidic conditions, ddC and nevirapine are ionized, and AZT is unionized. 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 for use as the ion pair reagent. After the addition of the ion pair reagent into the acidic mobile phase and the use of an octylsilane column, the retention times of ddC and nevirapine increased as predicted by ion pair theory. The order of elution switched from ddC, AZT, and nevirapine to AZT, ddC, and nevirapine.

The hydrophobicity of the ion pair reagent was important to the optimization of the separation, since the higher the hydrophobicity of the ion pair, the larger the retention factors of ddC and nevirapine. Concentrations from 0 to 50 mM of 1-octanesulfonic acid sodium salt were investigated to optimize the separation of ddC and nevirapine from AZT. It was found that the octanesulfonic acid compound achieved suitable retention times for all three analytes on the octylsi-

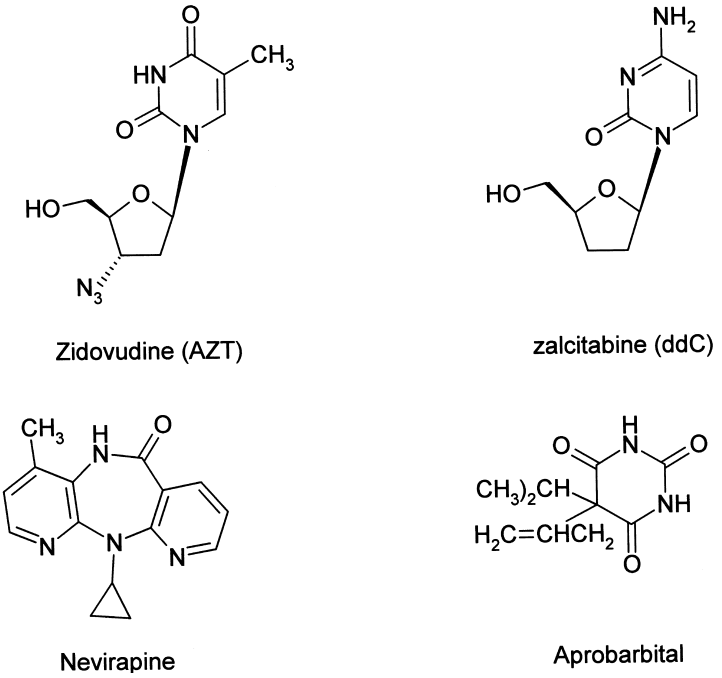

Figure 1. The chemical structures of analytes.

lane column. The retention of ddC increased while the retention for AZT slightly decreased, as the concentration of the ion pair reagent increased. Above 50 mM ion pair reagent, the retention of all analytes decreased because micelles of the ion pair molecules began to be formed, and this led to competing partition processes. It was found that 8 mM 1-octanesulfonic acid sodium salt gave the best separation for all analytes. It was observed that the chromatographic selectivity of ddC and AZT exhibited a pH dependency, as the pH of the phosphate buffer was varied from 2.5 to 4.5. Elution times and resolution of ddC and AZT increased and then decreased as buffer pH increased. The best separation of ddC and nevirapine from AZT was achieved at pH 3.2.

Baseline separations of AZT, ddC, and nivirapine were achieved with retention times of 3.1, 5.2, and 15.0 min, respectively. The internal standard aprobarbital gave a retention time of 11.0 min. Aprobarbital was chosen because of its structural similarity to the NRTI drugs and its good recovery from plasma using

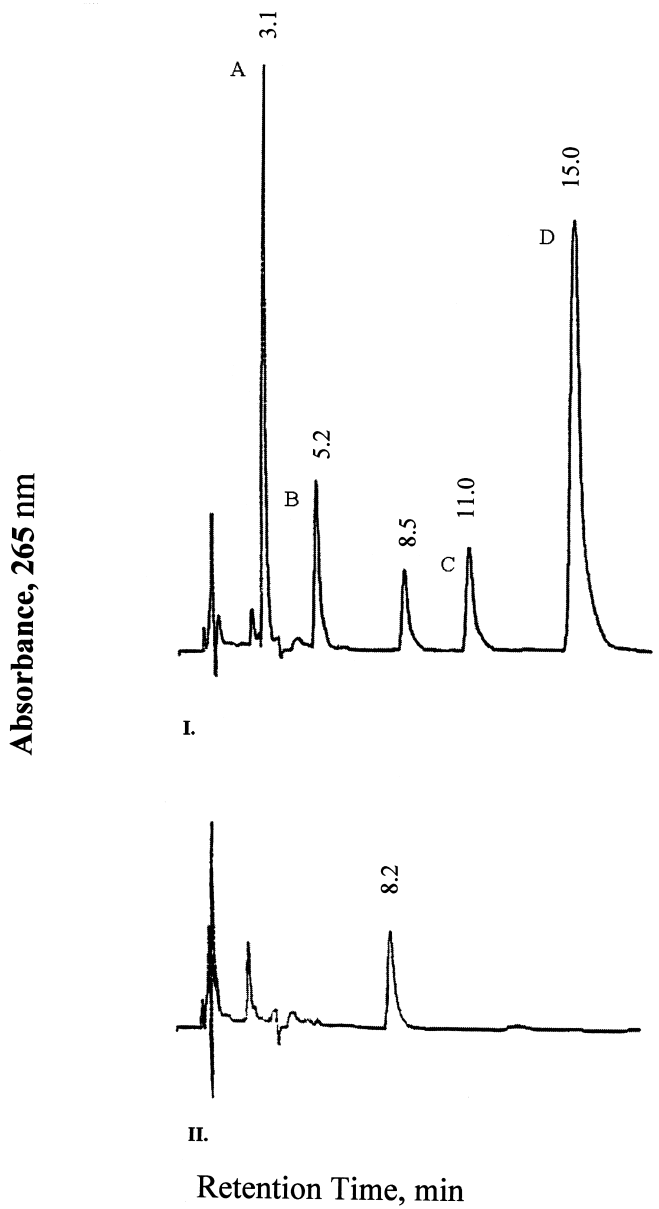

Figure 2. Chromatogram of I. blank human plasma and II. human plasma spiked with (A) AZT, (B) ddC, (C) internal standard and (D) nevirapine. The peak at 8.2-8.5 min is an unknown plasma component.

the SPE method. Figures 2 I and II, show typical chromatograms obtained from drug-free human plasma and a spiked plasma sample, respectively. The drugs show a good separation from endogenous plasma interferences. The peak present in both blank and spiked plasma eluting around 8.2-8.5 min is an unknown endogenous component of plasma.

During the 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 plasma recoveries using discs were less than 30% for AZT and ddC. Cartridges gave much higher recoveries, particularly, the C_{18} and Oasis™. Oasis™ provided the highest recoveries of the drugs as well as relatively cleaner assay samples. 25mM ammonium acetate (pH 7.0) was used to wash cartridges after loading spiked plasma to help retain the hydrophilic analytes AZT and ddC. Higher recoveries were observed using ammonium acetate than water washes. Because no organic solvent was present in the wash solution, it was not strong enough to clean up all the endogenous interferences. The eluent including 30% acetonitrile was strong enough to elute the analytes and leave most of the highly hydrophobic interferences of plasma in the SPE cartridges. Absolute recoveries greater than 88.5% were obtained for all three analytes. The detailed data is listed in Table 1. The recovery of the internal standard was 96.2%.

The calibration curves showed good linearity in the range of 57.6-2880 ng/mL for AZT, 20.2-2020 ng/mL for ddC and 53.2-13300 ng/mL for nevirapine. 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, ddC, and nevirapine were 28.8, 20.2, and

Table 1. Inter-Day and Intra-Day Accuracy, Precision and Recovery for the Analysis of AZT, ddC 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
ddC	202	1.1	4.2	3.9	1.7	88.5±3.8
	40.4	7.0	9.1	6.2	1.8	91.1±4.3
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, ddC 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
ddC	20.2-2020	20.2	20.2
Nevirapine	53.2-13300	13.3	53.2

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

13.3 ng/mL, respectively. The LOQ for AZT, ddC and nevirapine were 57.6, 20.2, 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 (percent error at high and low concentration varied from 0.1 to 10.7% for intra-day and 1.7 to 9.7% for inter-day) and precise (intra-day precision ranged from 0.4 to 5.4% and inter-day precision ranged from 4.1 to 13.7%).

CONCLUSION

A sensitive and specific HPLC isocratic ion pair assay was developed and validated for the simultaneous analysis of AZT/ddC/nevirapine in plasma. The HPLC method could be used for monitoring drug concentrations in human plasma, and for pharmacokinetic studies in HIV-infected patients.

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