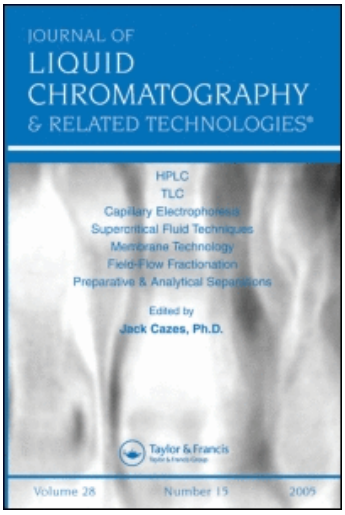

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DETERMINATION OF LAMIVUDINE/DIDANOSINE/SAQUINAVIR IN HUMAN SERUM USING CAPILLARY ZONE ELECTROPHORESIS

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DETERMINATION OF LAMIVUDINE/DIDANOSINE/SAQUINAVIR IN HUMAN SERUM USING CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

The anti-HIV drug mixture of lamivudine (3TC), didanosine (ddI), and saquinavir was separated and quantitated in human serum with capillary zone electrophoresis. Serum samples were treated using a solid-phase extraction procedure. The effects of various factors, such as run buffer type, buffer concentration, and pH on the separation were investigated. The optimized resolution was achieved with a run buffer containing 100 nM *N*,*N*-dimethyloctylamine in 80 mM phosphate buffer (pH 2.5). An uncoated 52 cm (effective length 30 cm) × 50 μm ID fused-silica capillary operated at 30°C was used in the analysis with UV detection at 210 nm. Diltiazem was chosen as an internal standard. All analytes were separated within 10 min with a voltage of +20 kV and a current around 30 μA.

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The method was validated over the range of 0.4–37.8 μg/mL for 3TC, 1.4–34 μg/mL for ddI and 0.5–24.4 μg/mL for saquinavir. Intra-day and inter-day accuracy was less than 13.7% and intra-day and inter-day precision was less than 13.3%. Extraction recoveries of all analytes from plasma were higher than 79%. The assay should be applicable for pharmacokinetic studies and routine monitoring of these drugs in serum.

INTRODUCTION

The availability of new and potent drugs and progress in understanding the pathogenesis of HIV-1 infection has led to the establishment of new treatment paradigms. The use of multidrug therapy has greatly enhanced the success of AIDS treatment (1–3). Highly active antiretroviral therapy (HAART) and the use of aggressive combination antiretroviral regimens consisting of reverse transcriptase inhibitor and protease inhibitors, have become the standard of care (4–5). These combination regimens achieved near-complete suppression of HIV-RNA concentrations and lead to considerable improvements in life expectancy of infected individuals (6–8).

The concentrations of antiretroviral drugs in human serum have become a useful parameter in the clinical management of HIV disease. The serum levels of these drugs seem to be connected with virologic efficacy (9–11). It is important to have a simple and routine assay method for monitoring these drug levels in order to prevent the emergence of drug resistance and to identify problems with compliance (12).

Reported methods for the determination of antiretroviral drugs in patients sera have largely been based on HPLC methodology (13–15). HPLC has the disadvantages of requiring rather large sample volumes, high cost of consumable supplies, complicated system operation and maintenance, and the technique generates substantial quantities of hazardous organic solvents with high disposal costs. The technique of capillary zone electrophoresis (CZE) overcomes many of the drawbacks of HPLC and has emerged in recent years as a proven clinical tool for the pursuit of pharmacological studies (16,17). CZE will allow the simultaneous measurement of several drugs, has low operating and consumable costs, and uses primarily aqueous run buffers.

The CZE method described herein was developed and validated to evaluate serum concentrations of 3TC, ddI, and saquinavir in a single assay run. The assay employs a solid-phase extraction protocol and a common phosphate run buffer and could be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug-drug interactions.

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EXPERIMENTAL

Chemicals and Reagents

Didanosine (ddI) and internal standard diltiazem were purchased from Sigma Chemical Company (St. Louis, MO 63178). Lamivudine (3TC) was kindly provided by Dr. Chung K. Chu (The University of Georgia, Athens, GA, USA). Saquinavir was provided by Roche Pharmaceuticals (Hertfordshire, UK). Monobasic sodium phosphate, concentrated phosphoric acid, sodium hydroxide, and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). *N*,*N*-Dimethyloctylamine was purchased from Aldrich Chemical Company, Inc (Milwaukee, WI 53233). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Waters Oasis^{TM} HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA, USA). Drug free human serum was obtained from Bioreclamation Inc (East Meadow, NJ 11554).

Instrumentation

The CZE experiments were performed on an Applied Biosystems 270A electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA). Separations were carried out in an uncoated 52 cm (effective length 30 cm) × 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary was thermostated at 30°C with air coolant. The detection window was created by stripping the polyimide coating of the capillary in a length of 5 mm. The capillary was conditioned with 1 N sodium hydroxide for 1 h followed by 30 min of deionized water before each day's run. Before each run, the capillary was also rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. The applied voltage was +20 kV for the separation and the detection wavelength was set at 210 nm. All the samples were injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 3 s.

Preparation of Standard Stock Solution

Stock solutions of lamivudine (3TC), didanosine (ddI), saquinavir, and diltiazem (internal standard) were prepared in deionized water to give concentrations of 1020, 540, 488, and 820 μg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with deionized water. Calibration plots for the analytes in serum were prepared by spiking drug-free serum with standard stock solution to yield concentrations of 0.4–37.8 μg/mL (0.4, 1.8, 7.6, 18.0, and 37.8 μg/mL) for 3TC, 1.4–34.0 μg/mL (1.4, 4.1, 6.8,

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10.2, and 34.0 μg/mL) for ddI and 0.5–24.4 μg/mL (0.5, 2.4, 6.1, 12.2, and 24.4 μg/mL) for saquinavir. Samples were stored at 4°C until use.

Sample Preparation Procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various solutions and 10 μL of an 820 μg/mL internal standard solution were added to drug-free serum to obtain a final volume of 1 mL. Waters Oasis™ HLB 1 cc cartridges were conditioned with 1 mL of methanol followed by 1 mL deionized water. One milliliter of the spiked serum samples was loaded onto the cartridges and drawn through by applying a vacuum. The cartridges were then washed with 1 mL methanol : water (10 : 90, v/v). One milliliter methanol was used to elute the adsorbed analytes. The eluting solvent was evaporated and reconstituted in 1 mL of deionized water. 3 s hydrodynamic injections of samples were made at the anodic end of the capillary.

Assay Validation

The method accuracy was obtained by comparing the concentrations calculated from the calibration curves versus concentrations added. Precision was calculated as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay were determined by assaying three quality control samples at low, medium, and high concentrations for each compound (1.4, 7.2, and 15.1 μg/mL for 3TC; 3.4, 8.7, and 13.6 μg/mL for ddI; 1.0, 5.0, and 10.0 μg/mL for saquinavir) in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on three different days. Triplicate injections of each analyte concentration were performed. The absolute recoveries of each drug and internal standard clean-up procedure were obtained by comparing the extracted serum analytes to unextracted stock solutions.

RESULTS AND DISCUSSION

Two methods were evaluated for sample preparation of spiked human serum prior to analysis by CZE. Protein precipitation using acetonitrile resulted in a number of endogenous peaks, which interfered with the compounds analyzed. The recoveries for all analytes using protein precipitation were lower ($< 40\%$) compared to the solid-phase extraction method ($>79\%$). Solid-phase extraction using a series of different extraction cartridges, such as C_{18}, C_8, Oasis™ cartridges, was investigated. Oasis™ cartridges gave the highest recoveries of the drugs, as well as cleaner assay samples.

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The effects of phosphate run buffer concentration (20–100 mM) and pH (2–9) on the separation were investigated. Besides run current, buffer concentrations affect buffering capacity and EOF. Generally, the higher the buffer concentration, the higher the current and the greater the buffering capacity. This prevents buffer depletion and improves assay reproducibility. Higher run buffer concentration also decreased the effect of charges on the capillary wall and, consequently, reduced EOF. Long migration times and sharper peaks for the analytes were obtained with higher run buffer concentrations. At pH < 3.0, the EOF was relatively small and peak efficiency was high. EOF increased with increasing pH, and the migration times decreased, but with an adverse impact on peak efficiency. The optimized resolution of the mixture was achieved with 80 mM phosphate run buffer with the pH adjusted to 2.5 with concentrated phosphoric acid.

Another reason to use a high concentration run buffer is to apply the sample stacking technique to increase sensitivity. Deionized water was used to dissolve and dilute the analytes. Thus, the electrical conductance of the sample zone was lower than that of the run buffer. An overall applied voltage of +20 kV was held constant, and a higher electrical field was developed across the sample zone. The samples migrated faster until they reached the boundary of the sample zone, where they

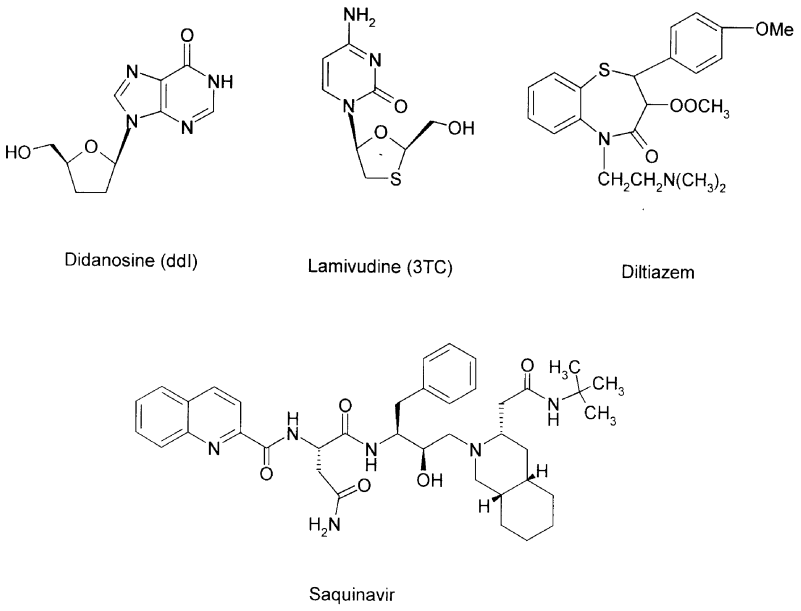

Figure 1. The chemical structures of analytes.

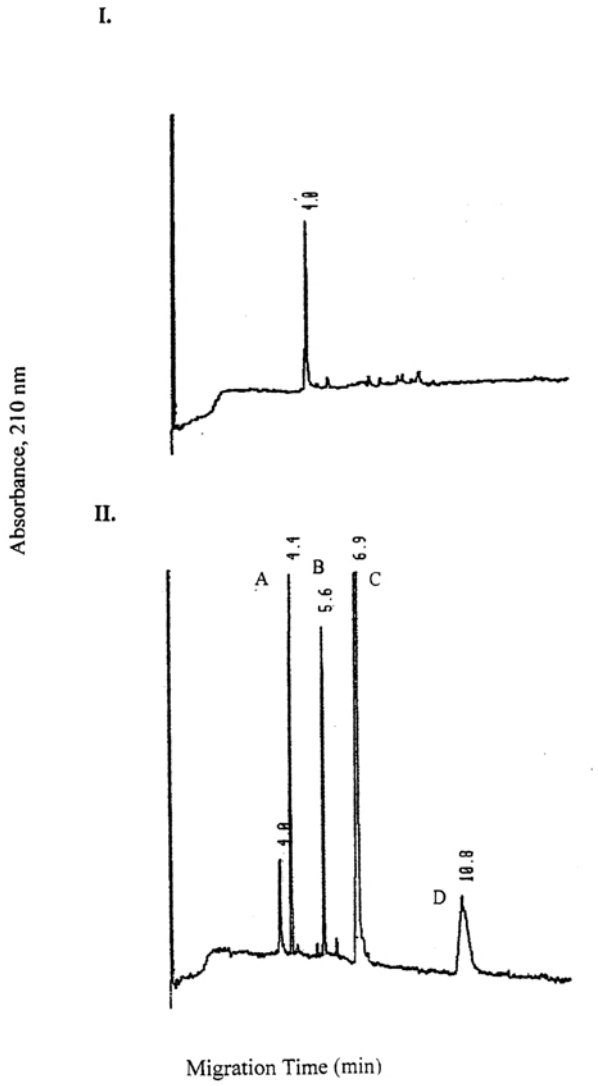

Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 3TC (18.0 µg/mL), (B) internal standard (C) saquinavir (12.2 µg/mL) and (D) ddI (10.2 µg/mL). The peak at 4.0 min is an unknown serum component.

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were stacked at the interface with the run buffer. This approach made it possible to load a larger volume of sample to improve the limits of detection of the analytes.

Our initial studies indicated that an asymmetric, split peak was observed for ddI in the electropherogram. Adsorption onto, or interaction with the capillary wall, could possibly be the main cause. A variety of additives to the run buffer, such as diethylamine, triethylamine, and *N*,*N*-dimethyloctylamine were investigated to coat the capillary wall in order to reduce wall interactions. It was found that 100 nM *N*,*N*-dimethyloctylamine added to the phosphate run buffer eliminated the adsorption effect, and a sharp and symmetric peak was obtained for ddI.

Table 1. Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantification (LOQ) of 3TC, ddI, and Saquinavir in Spiked Human Serum

Drug	Range of Calibration Curves (μg/mL)	Limit of Detection (LOD) (μg/mL)[a]	Limit of Quantification (LOQ) (μg/mL)[b]
3TC	0.4–37.8	0.2	0.4
ddI	1.4–34.0	0.9	1.4
Saquinavir	0.5–24.4	0.2	0.5

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

Table 2. Inter-Day and Intra-Day Accuracy, Precision and Recovery for the Analysis of 3TC, ddI, and Saquinavir in Human Serum

Drug	Concentration (μg/mL)	Precision[a] (%)		Accuracy[a] (%)		Serum Recovery[b] (%)
		Intra-Day	Inter-Day	Intra-Day	Inter-Day	
3TC	15.1	0.5	1.7	8.1	6.6	91.9 ± 3.5
	7.2	0.7	0.9	13.7	13.2	96.4 ± 4.7
	1.4	13.3	7.7	4.3	7.1	88.3 ± 1.5
ddI	13.6	9.7	2.6	3.4	1.8	82.3 ± 3.1
	8.7	1.1	8.1	14.2	9.8	85.7 ± 2.3
	3.4	7.1	7.6	13.7	8.8	78.5 ± 1.6
Saquinavir	10.0	0.2	11.7	0.5	7.7	96.7 ± 3.6
	5.0	10.6	8.3	3.0	8.0	85.6 ± 2.4
	1.0	4.6	5.7	7.7	2.5	93.2 ± 4.1

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

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Baseline separation of 3TC, ddI, and saquinavir was achieved with retention times of 4.4, 10.8, and 6.9 min, respectively. The internal standard diltiazem gave a migration time of 5.6 min. The structures of these compounds are shown in Figure 1. Figure 2 shows electropherograms of blank and spiked serum samples. The calibration curves showed good linearity in the concentration ranges of 0.4–37.8 μg/mL for 3TC, 0.5–24.4 μg/mL for saquinavir, and 1.4–34.0 μg/mL for ddI. The regression coefficients (r^2) of calibration curves of each drug were higher than 0.99. LOD and LOQ data are shown in Table 1. The results from the validation of the method in human serum are shown in Table 2.

CONCLUSION

A solid phase extraction procedure coupled with a capillary zone electrophoresis method provided a fast, sensitive, and selective procedure for the simultaneous determination of a 3TC/ddI/saquinavir mixture in human serum.

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