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Determinations of zidovudine/didanosine/nevirapine and zidovudine/didanosine/ritonavir in human serum by micellar electrokinetic chromatography

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Abstract

Micellar electrokinetic chromatography methods were developed and validated to separate and quantitate anti-HIV drug mixtures containing zidovudine(AZT)/didanosine(ddI)/nevirapine (mixture A) and AZT/ddI/ritonavir (mixture B) in human serum. Serum samples were prepared using a solid-phase extraction procedure. The effects of various factors such as buffer type, buffer and surfactant concentrations, and pH on the separations were investigated. The optimized resolution was achieved with a run buffer containing 18 mM sodium dodecylsulfate in 15 mM phosphate and borate buffer (pH 9.0). 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. Aprobarbital was chosen as the internal standard. All analytes were separated within 14 min with a voltage of +15 kV and a current around 30 μA. The methods were validated over the range of 0.5–25.0 μg/ml for AZT, 0.8–18.5 μg/ml for ddI, 0.5–22.8 μg/ml for nevirapine in mixture A and the range of 0.5–25.0 μg/ml for AZT, 0.8–18.5 μg/ml for ddI, 1.2–28.8 μg/ml for ritonavir in mixture B. Intra-day and inter-day accuracy was less than 12.4% and intra-day and inter-day precision was less than 13.9% for both mixtures. Extraction recoveries of all analytes from serum were higher than 75.9%. The assay should be applicable to pharmacokinetic studies and routine monitoring of these drugs in serum.

Keywords: Zidovudine; Didanosine; Nevirapine; Ritonavir; Micellar electrokinetic chromatography; Human serum

1. Introduction

The current standard of health care for patients with HIV disease is a triple therapy regimen, usually consisting of two nucleoside analogues in combination with a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor [1–3]. Current therapeutic guidelines are based on the

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use of zidovudine (AZT), the first nucleoside analogue approved by FDA, as first-line therapy, and most combination therapy in clinical trials includes AZT [4]. Another nucleoside analogue, didanosine (ddI), is a good candidate for combination anti-retroviral therapy along with AZT, because of its synergistic anti-HIV effect in vitro and different patterns of adverse effects [5,6]. As more anti-HIV drugs are approved for use, various drug combinations are being used. Nevirapine, the first non-nucleoside reverse transcriptase inhibitor, and ritonavir, a protease inhibitor, are also included in other therapeutic options along with AZT.

The separation and determination of antiviral drugs are needed to study their pharmacokinetics in biological fluids as well as to monitor antiretroviral therapy in the treatment of HIV infection. Determination of antiretroviral drugs has been achieved using high-performance liquid chromatography [7–9]. Recently, capillary electrophoresis (CE) has been shown to be a powerful alternative to HPLC [10–12] since CE is generally faster, more efficient, requires less sample and solvent waste is negligible. Micellar electrokinetic chromatography (MEKC), a mode of CE, has been used for the separation of neutral molecules, which can not be analyzed by conventional capillary zone electrophoresis (CZE).

In these studies, MEKC assays to determine AZT/ddI/nevirapine (mixture A) and AZT/ddI/ritonavir (mixture B) in human serum were developed and validated. The assays employ solid-phase extraction and a common CE run buffer, and should be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug–drug interactions.

2. Experimental

2.1. Chemical and reagents

AZT, ddI, aprobarbital (internal standard), dibasic sodium phosphate and sodium dodecylsulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Ritonavir and nevirapine were kindly provided by Abbott Laboratories (North Chicago, IL 60064). Concentrated phosphoric acid, sodium hydroxide and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Sodium tetraborate was purchased from Fisher Scientific, Inc. (Fair Lawn, NJ 07410). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Water Oasis^{TM} HLB 1 cm^3 30 mg cartridges were purchased from Waters Corp (Milford, MA). Drug-free human serum was obtained from Biological Specialty (Colmar, PA).

2.2. Instrumentation

MEKC was performed with an Applied Biosystems 270A CE system (Applied Biosystems, Foster City, CA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA). Separations were carried out at 30 °C with an uncoated 52 cm (effective length 30 cm) × 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ). The capillary was conditioned with 1 N sodium hydroxide for 1 h followed by 30 min of the run buffer before each day's run. Before each run, the capillary was rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. The applied voltage was optimized at +15 kV for the separation and the detection wavelength was set at 210 nm. The detection window was created by stripping the polyimide coating of the capillary in a length of 5 mm. The sample was injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 3 s.

2.3. Preparation of standard solutions

Stock solutions of AZT, ddI, nevirapine, ritonavir and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 998, 413, 912, 1152 and 1600 μg/ml, respectively. Calibration standards were prepared by spiking drug-free serum with aliquots of the stock solutions, followed by the addition of 10 μl of the stock internal standard solution and serum to obtain a final volume of 1 ml. The concentrations of calibration standards in mixture A were 0.5–25.0 μg/ml (0.5, 2.0, 5.0, 10.0 and 25.0 μg/ml) for

AZT, 0.8–18.5 μg/ml (0.8, 1.9, 4.1, 8.3, and 18.5 μg/ml) for ddI, 0.2–22.8 μg/ml (0.2, 0.5, 1.8, 9.1 and 22.8 μg/ml) for nevirapine. The concentrations in mixture B were 0.5–25.0 μg/ml (0.5, 2.0, 5.0, 10.0 and 25.0 μg/ml) for AZT, 0.8–18.5 μg/ml (0.8, 1.9, 4.1, 8.3, and 18.5 μg/ml) for ddI, 1.2–28.8 μg/ml (1.2, 2.3, 5.8, 11.5, and 28.8 μg/ml) for ritonavir. Samples were stored at 4 °C until assay.

2.4. Sample preparation procedure

Waters Oasis™ HLB 1 cm^3 cartridges were conditioned with 1 ml methanol followed by 1 ml deionized water. One milliliter of the spiked serum samples was loaded onto the cartridges and drawn 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 to dryness and reconstituted in 1 ml deionized water. Three second hydrodynamic injections of samples were made at the anodic end of the capillary.

2.5. 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. 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 in mixture A (1.0, 5.0 and 20.0 μg/ml for AZT, 1.6, 8.0 and 16.0 μg/ml for ddI, 1.0, 4.5 and 18.0 μg/ml for nevirapine); and in mixture B (1.0, 5.0 and 20.0 μg/ml for AZT, 1.6, 8.0 and 16.0 μg/ml for ddI, 2.0, 10.0 and 25.0 μg/ml for ritonavir) in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on 3 different days. Three sets of samples at each analyte concentration were performed. The absolute recoveries of each drug and internal standard were obtained by comparing the extracted serum analytes to unextracted stock solutions.

3. Results and discussion

Two procedures were initially evaluated for preparation of analytical samples from spiked human serum prior to analysis by MEKC. Protein precipitation using acetonitrile resulted in large numbers of endogenous peaks, which interfered with the compounds being analyzed. Solid-phase extraction using a series of different extraction cartridges, such as C_{18}, C_8, Oasis™ cartridges was investigated. Significant loss of AZT ($>20\%$) was observed in the C_8 cartridges during the loading step. Oasis™ gave the highest recoveries of the drugs as well as cleaner assay samples when a cartridge wash solution of 10% methanol was used.

It was difficult to determine AZT by CZE since it is an uncharged analyte. Uncharged analytes can be potentially determined by CZE at pH >7, but only one at a time. In our lab, a poor peak shape was obtained for AZT using CZE. A mixture of charged and uncharged analytes can be better assayed by MEKC which is a hybrid of reverse-phase liquid chromatography and CZE. The MEKC separation process incorporates hydrophobic and polar interactions, a partitioning mechanism, and electromigration. Because of its special mechanism for separation, MEKC was used in this paper for the separation and quantitation of uncharged AZT in the presence of the charged analytes ddI, nevirapine and ritonavir.

Using MEKC, the effects of buffer type and concentration, surfactant concentration and pH on the separation were investigated. A sodium phosphate–sodium borate buffer provided sharp, symmetric peaks, and gave good reproducibility in migration times and buffer capacity over a broad pH range. Besides run current, buffer concentrations affected buffering capacity and electroosmosis flow (EOF). Generally, the larger the buffer concentration, the higher the electrical current, and the greater the buffering capacity. This prevented buffer depletion and improved assay reproducibility. Longer migration times and sharper peaks for the analytes were obtained with buffer concentrations in the 10–20 mM range. The best separation of the analytes in both mixtures A and B was obtained with migration times of <14 min

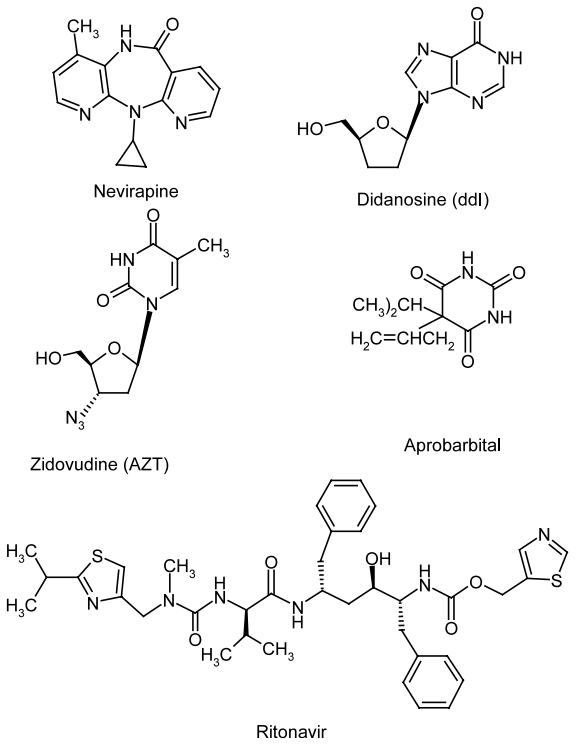

Fig. 1. The chemical structures of analytes in mixtures A and B.

using a 15 mM buffer. The pH of the run buffer was also an important factor in manipulating the selectivity of the charged analytes (except for AZT). Typically a pH between 7 and 9 is employed to assure proper MEKC flow characteristics. The effect of buffer pH on the separations was studied from pH 6.5 to 10.5. Electroosmotic flow had an important effect on resolution and analysis time in this MEKC separation. At pH <6.5, the EOF is relatively small, and it was difficult to get symmetric and reproducible peaks. EOF increased with increasing pH, and migration times decreased but with an adverse impact on peak efficiency. pH 9.0 was found to be the optimum pH for separation of both mixtures A and B.

Surfactant type and concentration are also important variables that influence the electrophoretic migration in MEKC. SDS was selected as surfactant because it permitted the separation of low to moderate hydrophobic compounds and provided selectivity similar to that of reverse-phase LC. With a low critical micellar concentration, SDS reduces the conductivity of the buffer and joule heating. The primary role of the surfactant concentration in MEKC is to adjust the retention factor within the optimum range to achieve the best resolution. The concentrations of SDS studied were in the 10–20 mM range. Eighteen millimole SDS was found to provide the best separation of both mixtures A and B.

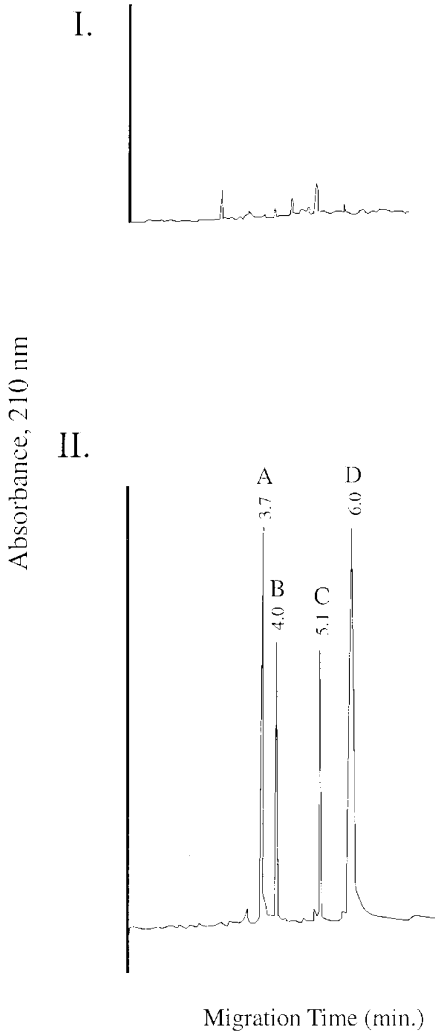

Fig. 2. Representative electropherograms of (I) blank human serum and (II) human serum spiked with (A) 10.0 μg/ml AZT, (B) 8.0 μg/ml ddI (C) 16.0 μg/ml internal standard and (D) 9.1 μg/ml nevirapine in mixture A.

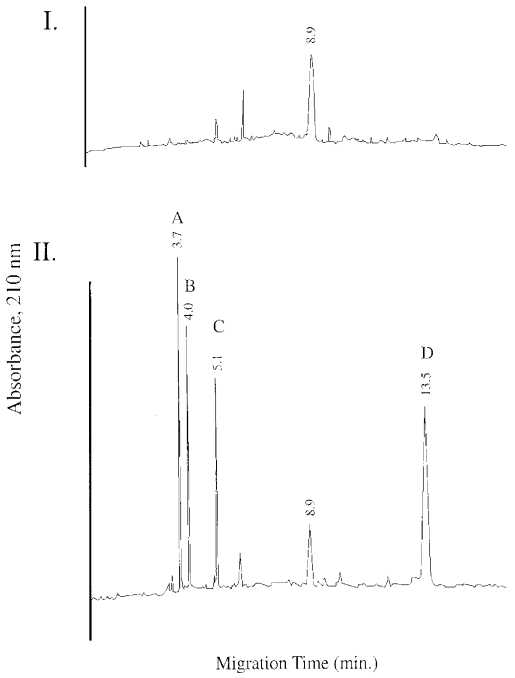

Fig. 3. Representative electropherograms of (I) blank human serum and (II) human serum spiked with (A) 10.0 μg/ml AZT, (B) 8.3 μg/ml ddI (C) 16.0 μg/ml internal standard and (D) 11.5 μg/ml ritonavir in mixture B. The peak at 8.9 min is an unknown serum component.

The MEKC separations of mixtures A and B were performed using an uncoated fused-silica capillary. The capillary exhibited rapid EOF, and this resulted in a short elution range for the analytes. The implementation of MEKC often entails an initial washing of the capillary with 1 N sodium hydroxide solution. The frequency of rinsing the capillary and the solutions used for rinsing had the greatest effect on migration reproducibility. In addition, the migration behavior of solutes that interact with micelles is not repeatable unless the proper rinse protocol is applied. A correlation between inconsistent migration behavior and fluctuation in electric current was observed, which might indicate the existence of non-equilibrium conditions between the run buffer and the capillary wall. The washing procedure we utilized decreased the deleterious effects of capillary 'aging', which resulted from changes in sample-wall interactions and electroosmotic flow.

The structures of the compounds found in mixtures A and B are shown in Fig. 1. In mixture A, baseline separation of the AZT, ddI and nevirapine mixture was achieved with migration times of 3.7, 4.0 and 6.0 min, respectively. The internal standard, aprobarbital, gave a migration time of 5.1 min. Fig. 2 shows the electropherograms of blank serum and spiked serum samples in mixture A. In mixture B, the AZT, ddI and ritonavir mixture was baseline separated with migration times of 3.7, 4.0 and 13.5 min, respectively. The migration time for the internal standard was again at 5.1 min. Fig. 3 shows the electropherograms of blank serum and spiked samples in mixture B. The calibration curves for mixture A showed good linearity in the concentration range of 0.5–25.0 μg/ml for AZT, 0.8–18.5 μg/ml for ddI, 0.5–22.4 μg/ml for nevirapine. The calibration curves for mixture B showed good linearity in the concentration range of 0.5–25.0 μg/ml for AZT, 0.8–18.5 μg/ml for ddI, and 1.2–28.8

Table 1
Range of calibration curves, LOD and LOQ of AZT, ddI, nevirapine (mixture A) and AZT, ddI, ritonavir (mixture B) in spiked human serum

Drug	Range of calibration curves (μg/ml)	LOD (μg/ml)[a]	LOQ (μg/ml)[b]
AZT	0.5–25.0	0.2	0.5
ddI	0.8–18.5	0.4	0.8
Nevirapine[c]	0.2–22.8	0.1	0.2
Ritonavir[d]	1.2–28.8	0.6	1.2

[a] S/N = 3.
[b] S/N = 10.
[c] Present in mixture A along with AZT and ddI.
[d] Present in mixture B along with AZT and ddI.

Table 2
Inter-day and intra-day accuracy, precision and recovery for the analysis of AZT, ddI, nevirapine (mixture A) and AZT, ddI, ritonavir (mixture B) in human serum

	Concentration (μg/ml)	Precision (%)		Accuracy (%)		Plasma recovery[c] (%)
		Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	
AZT	20.0	4.4	2.1	3.2	1.5	95.2±1.7
	5.0	9.5	5.7	3.1	0.2	79.3±3.6
	1.0	9.8	2.0	8.2	5.8	76.1±5.4
DdI	16.0	13.9	12.3	2.3	0.8	80.8±3.0
	8.0	12.6	7.2	10.8	0.5	77.9±1.2
	1.6	10.8	10.7	9.4	7.1	75.9±5.2
Nevirapine[d]	18.0	3.1	0.4	0.7	0.4	102.1±2.3
	4.5	6.8	4.8	11.2	10.4	95.3±5.5
	1.0	5.1	7.3	4.4	11.9	101.1±2.0
Ritonavir[e]	25.0	5.4	8.5	3.7	6.2	94.4±1.8
	10.0	7.5	6.2	12.4	8.3	92.3±1.1
	2.0	11.4	10.6	11.3	10.1	92.4±7.1

[a] Based on $n = 3$.
[b] Based on $n = 9$.
[c] Mean±SD based on $n = 9$.
[d] Present in mixture A along with AZT and ddI.
[e] Present in mixture B along with AZT and ddI.

μg/ml for ritonavir. The linear regression coefficients (r^2) of calibration curves of each drug were higher than 0.99. limits of detection (LOD) and limits of quantification (LOQ) data are shown in Table 1. The methods proved to be accurate and precise. Extraction recoveries of all analytes from serum were higher than 75.9%. The results from method validation in human serum are listed in Table 2.

4. Conclusions

Solid-phase extraction and MEKC methods provided fast, sensitive and selective procedures for the simultaneous determination of AZT/ddI/nevirapine (mixture A) and AZT/ddI/ritonavir (mixture B) in human serum.

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