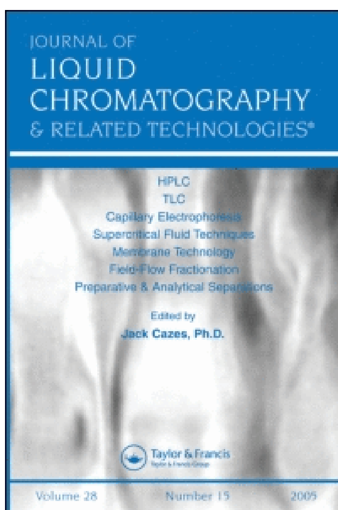
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DETERMINATION OF STAVUDINE/ DIDANOSINE/SAQUINAVIR AND STAVUDINE/DIDANOSINE/EFAVIRENZ IN HUMAN SERUM BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Bin Fan[a]; James. T. Stewart[a]
[a] Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA, U.S.A.

Online publication date: 05 September 2002

To cite this Article Fan, Bin and Stewart, James. T.(2002) 'DETERMINATION OF STAVUDINE/ DIDANOSINE/SAQUINAVIR AND STAVUDINE/DIDANOSINE/EFAVIRENZ IN HUMAN SERUM BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)', Journal of Liquid Chromatography & Related Technologies, 25: 6, 937 — 947

To link to this Article: DOI: 10.1081/JLC-120003271
URL: http://dx.doi.org/10.1081/JLC-120003271

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 25(6), 937–947 (2002)

Downloaded At: 09:07 24 January 2011

DETERMINATION OF STAVUDINE/ DIDANOSINE/SAQUINAVIR AND STAVUDINE/DIDANOSINE/EFAVIRENZ IN HUMAN SERUM BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Bin Fan and James T. Stewart*

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA

ABSTRACT

Anti-HIV drug mixtures A and B containing stavudine (d4T)/didanosine (ddI)/saquinavir and stavudine (d4T)/didano-didanosine (ddI)/efavirenz, respectively, were separated and quantitated in human serum using micellar electrokinetic chromatography (MEKC). Serum samples were treated using a solid-phase extraction procedure. The effects of various factors such as buffer type, buffer and surfactant concentration, and pH on the separation of the analytes were investigated. The optimized resolution of both mixtures was achieved with a run buffer containing 18 mM sodium dodecylsulfate (SDS) in 15 mM phosphate and borate buffer (pH 9.0). An uncoated 52 cm (effective length

*Corresponding author. E-mail: jstewart@merc.rx.uga.edu

Copyright © 2002 by Marcel Dekker, Inc. www.dekker.com

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 15 min with a voltage of +15 kV and a current around 30 μA.

The methods were validated over the range of 0.7–35.3 μg/mL for d4T, 0.8–18.5 μg/mL for ddI, 0.5–12.2 μg/mL for saquinavir in mixture A, and 0.7–35.3 μg/mL for d4T, 0.8–18.5 μg/mL for ddI, 0.6–31.9 μg/mL for efavirenz in mixture B. Intra-day and inter-day accuracy were less than 13.7% and intra-day and inter-day precision were less than 14.5% for both mixtures. Extraction recoveries of all analytes from serum were higher than 77.3%.

The assay should be applicable for pharmacokinetic studies and routine monitoring of these drugs in serum.

Downloaded At: 09:07 24 January 2011

INTRODUCTION

Today an estimated 30.6 million people are living with HIV infections or AIDS (1). Early in the epidemic, survival rates of less than 1 year were observed. However, the situation has changed significantly over the past 2 to 3 years following a number of advances, including the availability of newer antiretroviral agents and the demonstration that combination drug therapy is more effective than monotherapy (2,3). Highly active antiretroviral therapy (HAART) and the use of aggressive combination antiretroviral regimens consisting of reverse transcriptase inhibitors and protease inhibitors, have become the standard of patient care (4,5). The rationale for combining one or more anti-HIV agents is to provide more complete viral suppression, to limit the emergence of drug resistance during chronic viral replication, and to provide more effective antiretroviral treatment, even when mixtures of drug-resistant and drug-sensitive strains are present (6,7).

Separation and quantitative analysis 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. Reported methods for the determination of antiretroviral drugs in patient sera have been based on HPLC methodology (8–10). Recently, capillary electrophoresis (CE) has also been shown to be a powerful alternative to HPLC (11,12). Conventional capillary zone electrophoresis (CZE) is most commonly used, but it fails to resolve uncharged anti-HIV drugs, such as AZT and d4T. Micellar electrokinetic chromatography (MEKC) is a mode of CE that is capable of separating uncharged compounds (13,14). It is viewed as a chromatographic technique in which migrating charged

micelles act as a pseudostationary phase. MEKC offers analyte partitioning between micelles formed by a surfactant in the run buffer, such that mixtures of charged and uncharged anti-HIV drugs can be satisfactorily separated.

The aim of this study was to develop and validate a MEKC method for the determination of serum concentrations of d4T/ddI/saquinavir (mixture A) and d4T/ddI/efavirenz (mixture B). These assays employ a solid-phase extraction protocol and could be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug-drug interactions.

EXPERIMENTAL

Chemical and Reagents

Stavudine (d4T), didanosine (ddI), aprobarbital (internal standard), dibasic sodium phosphate, and sodium dodecylsulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Saquinavir was kindly provided by Roche Pharmaceuticals (Hertfordshire, UK). Efavirenz was provided by DuPont Pharmaceuticals Company (Wilmington, DE 19805). 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™ HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA, USA). Drug free serum was obtained from Biological Specialty (Colmar, PA, USA).

Downloaded At: 09:07 24 January 2011

Instrumentation

MEKC was performed with an Applied Biosystems 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA). Separation was carried out on an uncoated 52 cm (effective length 30 cm) × 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary temperature was maintained at 30°C with air coolant. The capillary was conditioned with 1 N sodium hydroxide for 1 hour 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 +15 kV for the separation and the detection wavelength was 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.

Preparation of Standard Solutions

Stock solutions of stavudine (d4T), didanosine (ddI), saquinavir, and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 1414, 826, 912, and 1600 μg/mL in mixture A, respectively. Stock solutions of stavudine (d4T), didanosine (ddI), efavirenz, and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 1414, 826, 1275, and 1600 μg/mL in mixture B, respectively. Calibration standards were prepared by diluting these stock solutions with drug-free serum, and 10 μL of the stock aprobarbital internal standard solution was added to obtain a final volume of 1 mL. The concentrations in mixture A were 0.7–35.3 μg/mL (0.7, 2.8, 7.1, 14.1, and 35.3 μg/mL) for d4T, 0.8–18.5 μg/mL (0.8, 1.9, 4.1, 8.3, and 18.5 μg/mL) for ddI, 0.5–12.2 μg/mL (0.5, 2.4, 4.9, 9.8, and 12.2 μg/mL) for saquinavir. The concentrations in mixture B were 0.7–35.3 μg/mL (0.7, 2.8, 7.1, 14.1, and 35.3 μg/mL) for d4T, 0.8–18.5 μg/mL (0.8, 1.9, 4.1, 8.3, and 18.5 μg/mL) for ddI, 0.6–31.9 μg/mL (0.6, 2.6, 6.4, 12.8, and 31.9 μg/mL) for efavirenz. Samples were stored at 4°C.

Downloaded At: 09:07 24 January 2011

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 quality control samples at low, medium, and high concentrations for d4T (1.4, 7.0, and 28.0 μg/mL), ddI (1.6, 8.0, and 16.0 μg/mL), saquinavir (1.0, 5.0, and 10.0 μg/mL) in mixture A in three analytical runs within the same day; and for d4T (1.4, 7.0, and 28.0 μg/mL), ddI (1.6, 8.0, and 16.0 μg/mL), efavirenz (1.2, 7.5, and 27.5 μg/mL) in mixture B in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on three 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 samples to unextracted stock solutions.

Sample Preparation Procedure

Waters Oasis™ HLB 1cc cartridges were conditioned with 1 mL methanol followed by 1 mL deionized water. One mL 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 mL 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.

RESULTS AND DISCUSSION

Two procedures were evaluated for preparation of analytical samples of spiked analytes in 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. The recoveries for all analytes were much lower compared to the SPE method. Solid-phase extraction using a series of different extraction cartridges, such as C_{18}, C_8 and Oasis™ cartridges, were investigated. Larger than 20% loss of d4T was observed with the C_8 cartridges during the loading step. Oasis™ gave the highest recoveries of the analytes, as well as cleaner assay samples, using a cartridge wash solution containing 10% methanol.

In MEKC, the choice of run buffer composition was crucial. The effects of buffer type, concentration, and pH on the separation were investigated. A sodium phosphate-sodium borate run buffer provided sharp, symmetric peaks, and also gave good reproducibility in migration times and buffer capacity over a large pH range. Besides running current, buffer concentrations affected buffering capacity and electroosmosis flow (EOF). Generally, the higher the buffer concentration, the higher the electrical current, and the greater the buffering capacity. This will prevent buffer depletion and improve assay reproducibility. Longer migration times and sharper peaks for the analytes were obtained with higher buffer concentrations. When the run buffer concentration was optimized to 15 mM, the best separations of both mixtures A and B were achieved with a migration time <15 min. The pH of the buffer was an important factor to manipulate selectivity of the ionizable analytes except d4T. Typically a pH between 7 and 9 was employed to assure proper MEKC flow characteristics. The effects of buffer pH on the separations were studied from pH 6.5 to 10.5.

Electroosmotic flow had an important effect on resolution and analysis time in the MEKC separations. At pH <6.5, the EOF was relatively small, and it was difficult to get symmetric and reproducible peaks. EOF increased with increasing pH and the migration times decreased, but with an adverse impact on peak efficiency. pH 9.0 was found to be the optimum pH for both mixtures A and B, since the best separations of the analyte mixtures were achieved.

Surfactant type and concentration also influenced the electrophoretic migration in MEKC. Sodium dodecyl sulfate (SDS) in MEKC served two functions: it reduced the capillary surface interactions and it formed charged micelles. At a low critical micellar concentration (CMC), SDS reduced the

Downloaded At: 09:07 24 January 2011

conductivity of the buffer and joule heating. The primary role of the surfactant concentration was to adjust the retention factor within the optimum range to achieve better resolution of the analytes. The concentration of SDS was set at 18 mM for both mixtures A and B.

The MEKC separations of mixture A and B were performed using an untreated fused-silica capillary. The capillary exhibited rapid electroosmosis flow, and consequently, resulted in a short elution range. The implementation of MEKC entailed washing the capillary with sodium hydroxide solution. The frequency of rinsing the capillary and the wash solutions had the greatest effect on migration reproducibility. In addition, the migration behavior of the analytes that interacted with micelles was not repeatable unless the proper rinse protocol was applied.

Downloaded At: 09:07 24 January 2011

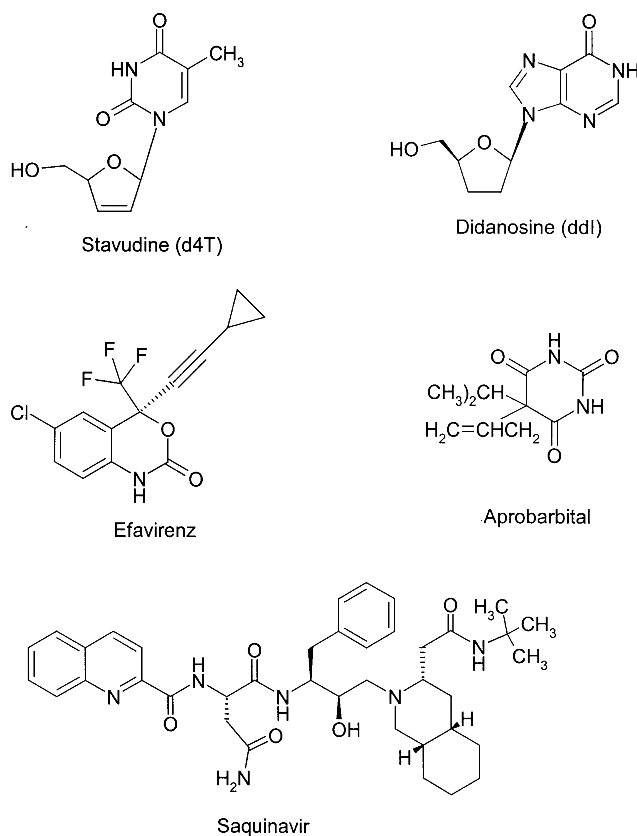


Figure 1. The chemical structures of analytes in mixtures A and B.

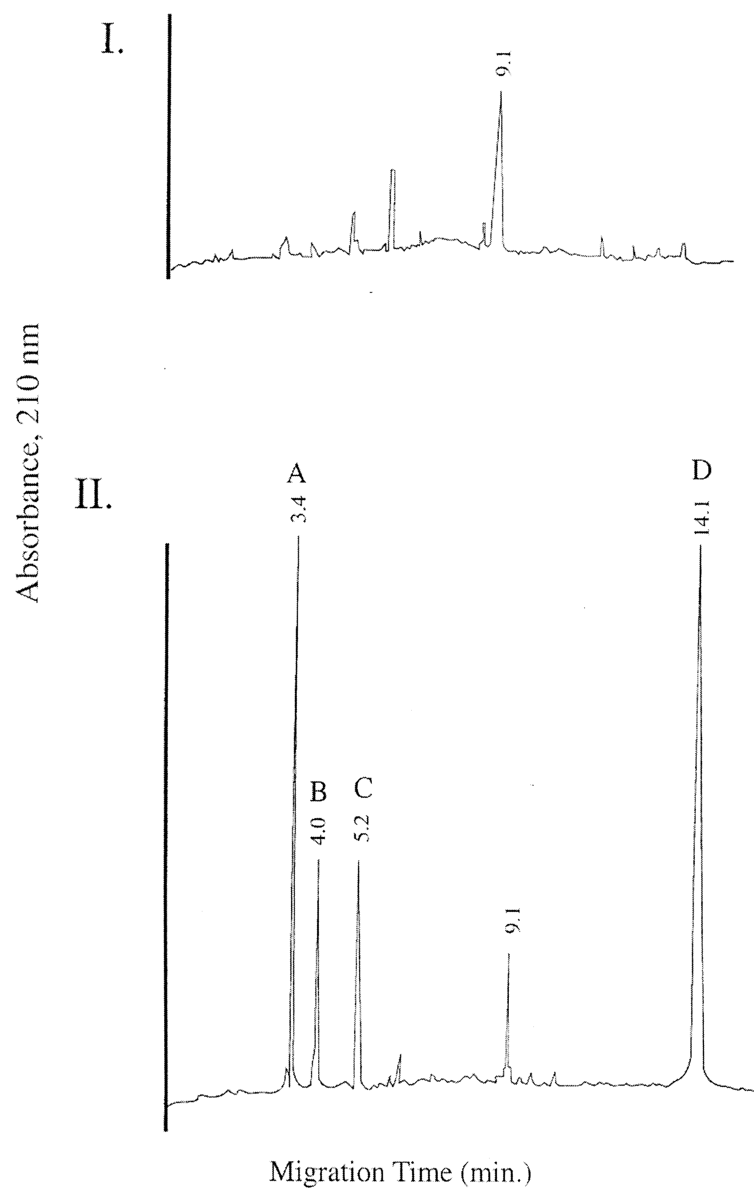


Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 14.1 μg/mL d4T, (B) 8.0 μg/mL ddI, (C) 16.0 μg/mL internal standard, and (D) 9.8 μg/mL saquinavir in mixture A. The peak at 9.1 min is an unknown serum component.

Downloaded At: 09:07 24 January 2011

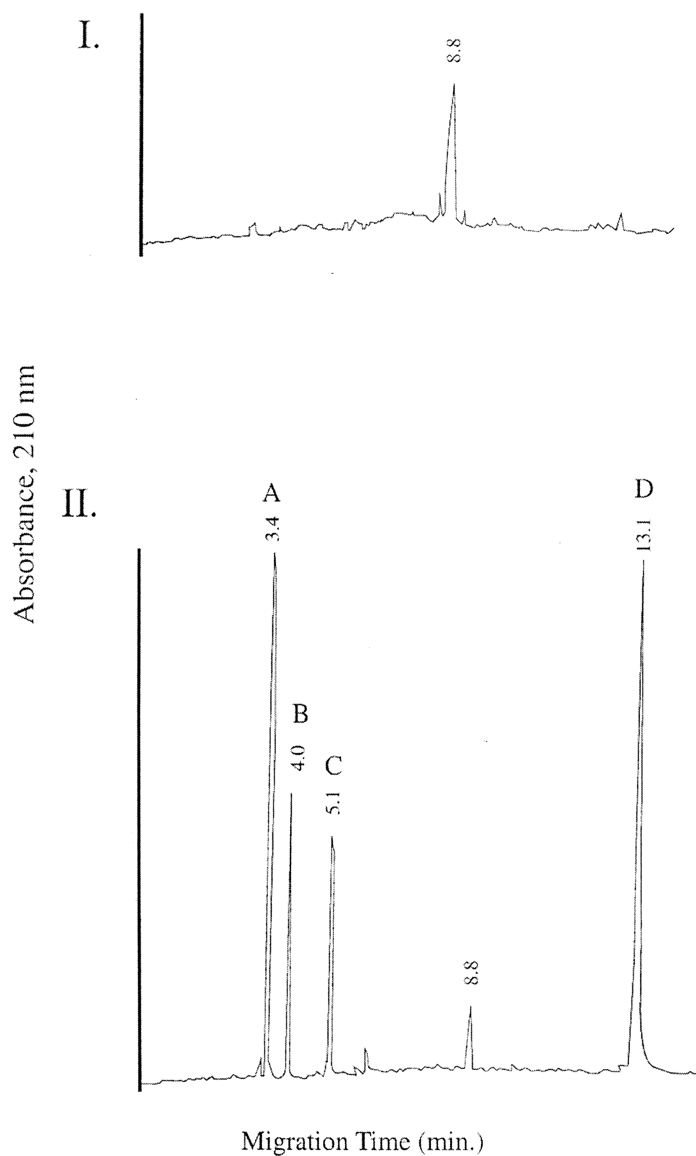


Figure 3. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 14.1 μg/mL d4T, (B) 8.3 μg/mL ddI, (C) 16.0 μg/mL internal standard, and (D) 12.8 μg/mL efavirenz in mixture B. The peak at 8.8 min is an unknown serum component.

Downloaded At: 09:07 24 January 2011

A correlation between inconsistent migration behavior and fluctuation in electric current was observed, which might indicate the existence of nonequilibrium conditions between the run buffer and the capillary wall. The washing procedures used decreased the deleterious effects of capillary "aging," which can result in changes in sample-wall interactions and electroosmotic flow.

Figure 1 lists the chemical structures of the analytes in the mixtures A and B. In mixture A, d4T, ddI and saquinavir were baseline separated with migration times of 3.4, 4.0, and 14.1 min, respectively. The migration time for the internal standard aprobarbital was 5.2 min.

Figure 2 shows the electropherograms of blank serum and spiked serum samples for mixture A. In mixture B, baseline separation of the d4T, ddI, and efavirenz mixture was achieved with migration times of 3.4, 4.0, and 13.1 min, respectively. The internal standard aprobarbital gave a migration time of 5.1 min in this mixture.

Figure 3 shows the electropherograms of blank serum and spiked serum samples with mixture B. The calibration curves for mixture A showed good linearity in the concentration range of 0.7–35.3 μg/mL for d4T, 0.8–18.5 μg/mL for ddI, and 0.5–12.2 μg/mL for saquinavir. The calibration curves for mixture B showed good linearity in the concentration range of 0.7–35.3 μg/mL for d4T, 0.8–18.5 μg/mL for ddI, and 0.6–31.9 μg/mL for efavirenz.

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 methods proved to be accurate and precise. Extraction recoveries of all analytes from serum were higher than 77.3%. The data from validation of the methods in human serum are listed in Table 2.

Downloaded At: 09:07 24 January 2011

Table 1. Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantification (LOQ) of d4T, ddI, Saquinavir (Mixture A) and d4T, ddI, Efavirenz (Mixture B) in Spiked Human Serum

Drug	Range of Calibration Curves (μg/mL)	Limits of Detection (LOD) (μg/mL)[a]	Limits of Quantification (LOQ) (μg/mL)[b]
d4T	0.7–35.3	0.3	0.7
ddI	0.8–18.5	0.4	0.8
Saquinavir[c]	0.5–12.2	0.3	0.5
Efavirenz[d]	0.6–31.9	0.3	0.6

[a]S/N = 3.
[b]S/N = 10.
[c]Present in mixture A with d4T and ddI.
[d]Present in mixture B with d4T and ddI.

Downloaded At: 09:07 24 January 2011

Table 2. Inter-Day and Intra-Day Accuracy, Precision, and Recovery for the Analysis of d4T, ddI, Saquinavir (Mixture A) and d4T, ddI, Efavirenz (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]	
d4T	28.0	1.5	1.8	3.7	1.7	95.4 ± 3.0
	7.0	3.6	8.2	3.2	10.6	88.3 ± 4.5
	1.4	7.8	1.5	11.4	6.4	84.6 ± 4.8
ddI	16.0	4.8	10.8	4.2	12.4	83.1 ± 5.1
	8.0	10.2	5.7	4.7	9.7	77.3 ± 3.3
	1.6	12.2	14.5	9.8	2.8	82.0 ± 6.5
Saquinavir[d]	10.0	1.7	4.7	6.4	5.6	99.1 ± 2.2
	5.0	4.1	13.9	2.8	11.0	100.7 ± 1.2
	1.0	8.2	12.8	7.2	9.8	95.6 ± 4.4
Efavirenz[e]	27.5	2.1	6.0	5.2	5.7	103.2 ± 2.8
	7.5	6.6	6.1	8.1	11.8	86.0 ± 1.9
	1.2	5.0	7.0	13.7	8.6	89.7 ± 2.0

[a]Based on $n = 3$.
[b]Based on $n = 9$.
[c]Mean ± SD based on $n = 9$.
[d]Present in mixture A with d4T and ddI.
[e]Present in mixture B with d4T and ddI.

CONCLUSION

The MEKC separation and quantitation of anti-HIV drug mixtures containing d4T/ddI/saquinavir (mixture A) and d4T/ddI/efavirenz (mixture B) using solid phase extraction provided fast, sensitive, and selective procedures for these mixtures in human serum.

REFERENCES

1. Wise, J. Brit. Med. J. **1997**, *315*, 1486.
2. Hammer, S.B.; Katzenstein, D.A.; Hughes, M.D.; Gundacker, H.; Schooley, R.T.; Haubrich, R.H.; Henry, W.K.; Lederman, M.M.; Phair, J.P.; Niu, M.; Hirsch, M.S.; Merigan, T.C.; Blaschke, T.F.; Simpson, D.; McLaren, C.; Rooney, J.; Salgo, J. N. Engl. J. Med. **1996**, *335* (15), 1081–1090.
3. Delta Coordinating Committee. Lancet **1996**, *348*, 283–91.
4. Centers for Disease Control and Prevention. Morb. Mortal. Wkly. Rept. **1998**, *47*, 42–82.
5. St Clair, M.H.; Pennington, K.N.; Rooney, J.; Barry, D.W. J. Acquir. Immune Defic. Syndr. Hum. Retroviral. **1995**, *10*, S83–91.
6. Johnson, V.A. AIDS Res. Hum. Retrovir. **1994**, *10*, 907–1012.
7. Seifert, R.D.; Stewart, M.B.; Sramet, J.J.; Conrad, J.; Kaul, S.; Cutler, N.R. Br. J. Clin. Pharmacol. **1994**, *38*, 405–410.
8. Ha, H.R.; Follah, F.; Blomenhard, Y.; Krahenbul, S. J. Chromatogr. B. **1997**, *694*, 427.
9. Wu, E.Y.; Wilkinson, I.I.J.M.; Naret, D.G.; Daniels, V.L.; Williams, L.J.; Khalil, D.A.; Shetty, B.H. J. Chromatogr. B **1997**, *695*, 373.
10. Hoetelmans, R.M.W.; Essenberg, M.; Profijt, M.; Meenhorst, P.L.; Mulder, J.W.; Beijnen, J.H. J. Chromatogr. B **1998**, *695*, 119.
11. Jenkins, M.A.; Guerin, M.D. J. Chromatgr. B **1996**, *682*, 23.
12. Altria, K.D. *Capillary Electrophoresis Guidebook*; Humana Press: Totowa, N.J., 1996; 265 p.
13. Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. Anal. Chem. **1984**, *56*, 111.
14. Terabe, S.; Otsuka, K.; Ando, T. Anal. Chem. **1985**, *57*, 834.

Received November 12, 2001
Accepted December 10, 2001
Manuscript 5683

Downloaded At: 09:07 24 January 2011