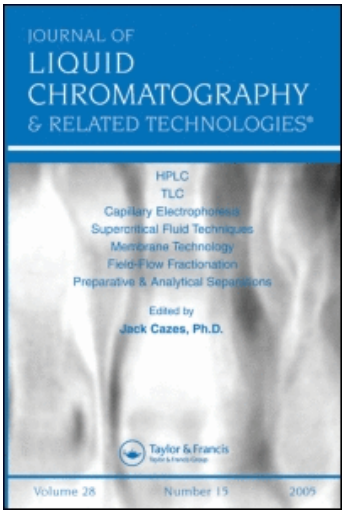

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Chiral Capillary Electrophoretic Determination of 2′,3′-Dideoxy-5-fluoro-3′-thiacytidine in Rat Plasma

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ABSTRACT

Chiral drugs have the potential for differential pharmacokinetics and metabolism of individual enantiomers. Analytical methods are needed for the separation and quantitation of the enantiomers. Here, we present a method for the separation and quantitation of two enantiomers of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) from rat blood plasma using organic protein precipitation with liquid–liquid extraction and capillary electrophoresis (CE). Lamivudine (3TC) was used as an internal standard. The CE system consisted of a 75 μm I.D., 37 cm length fused silica capillary, and a UV detector monitoring a wavelength of 280 nm. The run buffer was aqueous containing 90 mM hydroxypropyl-β-cyclodextrin in 50 mM phosphate at pH 2.5. The system was maintained at 25°C, and the

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separation voltage was 25 kV with a runtime of 15 min. The method was linear over the range from 0.5 to 100 μg/mL. The method had baseline resolution of the enantiomers and showed high precision and accuracy both within and between runs at three different concentrations, including the lower limit of quantitation (0.5 μg/mL).

Key Words: FTC; RCV; Dideoxyfluorothiacytidine; Chiral capillary electrophoresis; Cyclodextrin.

INTRODUCTION

Currently, much research is ongoing for the discovery of new antiviral compounds. Of these, 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) is a relatively new chiral antiviral, currently in phase III clinical trials, with potent activity against the hepatitis B virus (HBV) and human immunodeficiency virus (HIV).[1–4] For many antiviral drugs, tests have shown that the *S*(−) enantiomers generally have greater activity than the corresponding *R*(+) enantiomers.[2,4–9]

Biological systems have a tendency to be chiral, an example being that most higher organisms preferentially utilizing *L*-amino acids for protein synthesis. Due to this chiral environment, differential metabolism or other bio-interactions may occur between different enantiomers of a single chiral compound. This may include different potency between enantiomers, chiral inversion, and enantiomer–enantiomer interactions.[5–9] If an achiral assay is used, data may be skewed if the dispositions of the individual enantiomers are different. In order to protect against misleading information, chiral assays should by used whenever possible.[10] Chiral assays continue to be developed using common analytical techniques including high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE).

Capillary electrophoresis is particularly adept for chiral separations due to its very high separation efficiency. Chiral CE most often employs the use of modified cyclodextrins.[11,12] Other methods for chiral CE include the use of Crown ethers, polysaccharides, macrocyclic antibiotics, and electrokinetic chromatography with optically active micelles and/or proteins.[13–15] Cyclodextrins are used for chiral separation in order to take advantage of very slight differences in the inclusion rate constants between the individual enantiomers and the cyclodextrin. Changing the number of subunits or the modification to the cyclodextrin can greatly alter the size and shape of the pocket for analyte inclusion.[15]

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EXPERIMENTAL

Reagents and Chemicals

Racivir, and the individual enantiomers of Racivir ((+)FTC and (−)FTC), were supplied by Pharmasset Inc. (Tucker, GA). The internal standard, 3TC, was provided by Dr. Raymond Schinazi (Emory University, Atlanta, GA). Hydroxypropyl-β-cyclodextrin was obtained from Cerestar (Hammond, IN). HPLC grade acetonitrile, phosphoric acid, and granular ammonium sulfate were purchased from J. T. Baker, Inc. (Philipsburg, NJ). Monobasic sodium phosphate was acquired from EM Science (Gibbstown, NJ). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN).

Preparation of Stock and Standard Solutions

Stock solutions of (+)FTC and (−)FTC and the internal standard, 3TC, were prepared in deionized water. Appropriate volumes of stock solutions (between 10 and 20 μL) were added to microcentrifuge tubes with enough blank rat plasma to bring the total volume up to 100 μL. Concentrations of the two enantiomers were 100, 75, 25, 10, 2.5, and 0.5 μg/mL for the calibration curves and 90, 5, and 0.5 μg/mL for validation points.

Sample Preparation

All samples were prepared by organic protein precipitation followed by liquid–liquid extraction. To 100 μL plasma, 50 μL 3TC (60 μg/mL) was added and mixed thoroughly. Cold acetonitrile of 600 μL was then added while gently mixing. The mixture was vigorously mixed for 30 sec. Excess ammonium sulfate was added and vigorously mixed for an additional 45 sec and centrifuged for 3 min at $9000 \times g$. The mixture separated into two phases with an organic layer on top of the saturated ammonium sulfate aqueous layer. The organic layer was removed and placed in a clean tube and evaporated to dryness under vacuum. The dried samples were reconstituted in 75 μL methanol/water (30% v/v).

Electrophoretic System

All CE experiments were performed using a P/ACE System 5000 (Beckman Coulter Inc., Fullerton, CA) equipped with a UV detector. An uncoated fused silica capillary with a total length of 42 cm, an effective length

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of 37 cm, and a 75 μm ID (Polymicron Technologies, Phoenix, AZ) was used for analysis. The capillary was thermostated at 25°C and the voltage applied was 25 kV. A 0.5 cm detection window was created by stripping the polyamide coating off the capillary. The detection window was 5 cm from the cathode end of the capillary. The run buffer consisted of an aqueous solution of 50 mM phosphate buffer at pH 2.5 (adjusted with concentrated phosphoric acid) and 150 mM hydroxypropyl-β-cyclodextrin. The analytes were monitored at a wavelength of 280 nm.

Sample introduction was performed using a 5 sec pressure injection (0.5 PSI). The separation voltage was ramped to 25 kV over 30 sec. Before each analysis, the capillary was rinsed for 2 min first with 0.1 M sodium hydroxide and 2 min with the run buffer. New capillaries were conditioned by rinsing with 1 M sodium hydroxide for 5 min followed by 5 min each with 1 M hydrochloric acid, water, and run buffer solutions.

RESULTS AND DISCUSSION

Baseline resolution was achieved for the two enantiomers of FTC with the use of 90 mM hydroxypropyl-β-clyclodextrin in phosphate buffer. Figure 1(A)

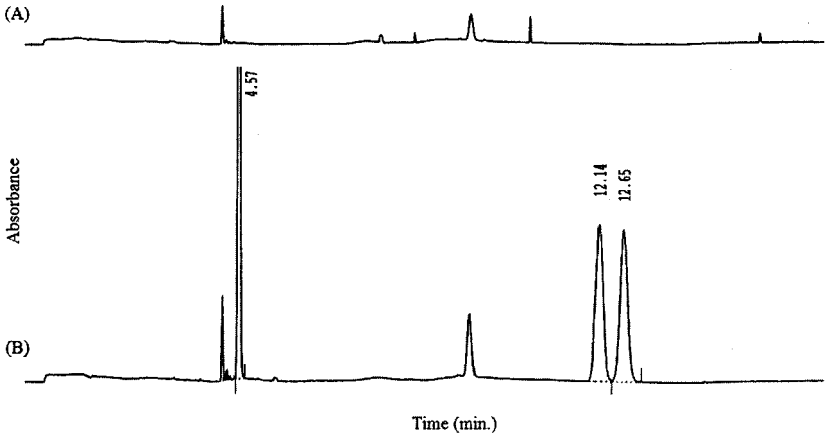

Figure 1. Typical electropherogram of (A) blank plasma and (B) plasma spiked with (+)FTC (12.14 min), (−)FTC (12.65 min), and the internal standard 3TC (4.57 min) on a 37 cm, 75 μm fused silica capillary. The run buffer contained 90 mM hydroxypropyl-β-cyclodextrin in 50 mM phosphate buffer (pH 2.5) with detection at 280 nm. The capillary was thermostated at 25°C and the run voltage was 25 kV.

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shows an electropherogram of extracted blank plasma. Figure 1(B) shows a typical electropherogram of the two enantiomers of FTC and the internal standard spiked into blank plasma. Figure 2 shows the structures of (−)FTC, (+)FTC, and the internal standard, 3TC.

Analyte stacking was used to increase sensitivity by reducing band broadening, thereby sharpening peaks. In order to do this, the sample was prepared in a lower conductivity solvent (methanol/water) than the run buffer. When high voltage is applied across the capillary, a greater electric field develops across the sample plug than the rest of the capillary filled with buffer. The higher electric field causes sample ions to move faster until they move out of the sample plug. This results in analyte stacking within a narrow zone of the capillary.[16,17]

Several cyclodextrins of various sizes and modifications were tested against FTC using a long capillary and long separation time. Hydroxypropyl-β-cyclodextrin gave the best resolution of the two enantiomers of FTC. The capillary length was then optimized to give baseline resolution with the shortest runtime. The system was able to recognize the difference between FTC and 3TC, resulting in a very large difference in migration times. 3TC had no, or very little, affinity for the cyclodextrin and, therefore, had a short migration time (ca. 4.5 min). FTC, on the other hand, was able to form inclusion complexes with the cyclodextrin. The affinity for the cyclodextrin resulted in broadening the analyte peaks, which is due to the added partitioning in and out of the cyclodextrin. When the compound enters a cyclodextrin molecule, it slows considerably. The slight difference between the affinities of the enantiomers provided a difference in migration times between the two enantiomers of FTC.

The calibration curve showed good linearity over the range from 0.5 to 100 μg/mL for both enantiomers. The method was validated over the

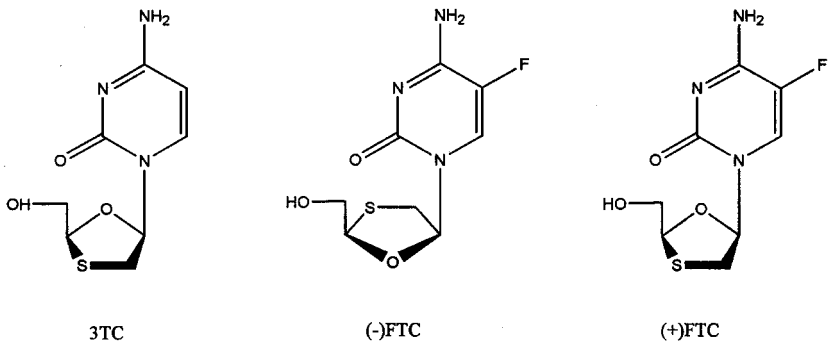

Figure 2. Structures of (−)FTC; (+)FTC, and the internal standard, 3TC.

Table 1. Within-run precision and accuracy data, $n = 6$.

Spike concentration (μg/mL)	Average concentration found		Percentage error		Percentage RSD	
	(+)FTC	(−)FTC	(+)FTC	(−)FTC	(+)FTC	(−)FTC
0.5	0.510	0.545	8.9	11.3	13.0	11.1
5	5.28	5.26	5.5	5.3	2.4	2.8
90	91.0	91.3	1.1	1.5	0.5	0.6

calibration range using replicate extractions at three concentrations: 90, 5, and 0.5 μg/mL [the lower limit of quantitation (LLOQ)]. The coefficients of determination were 0.999 or better ($n = 3$). The within run precision and accuracy ($n = 6$), expressed as percent relative standard deviation (%RSD) and percent error, were less than 2.9% and 5.6%, respectively, for concentrations above the LLOQ. At the LLOQ, within run precision and accuracy was 13.0% RSD and 11.3% error or better. Detailed within run validation data is listed in Table 1. Between run precision and accuracy ($n = 18$) were less than 3.2% RSD and 4.8% error at concentrations above the LLOQ, and were 15.5% RSD and 11.2% error or better at the LLOQ. Detailed between run precision and accuracy data is listed in Table 2.

CONCLUSIONS

The HPCE assay as described, is sensitive and selective enough for the separation and quantitation of (+)FTC and (−)FTC from plasma. The organic precipitation and liquid–liquid extraction provides good sample cleanup with

Table 2. Between-run precision and accuracy data, $n = 18$.

Spike concentration (μg/mL)	Average concentration found		Percentage error		Percentage RSD	
	(+)FTC	(−)FTC	(+)FTC	(−)FTC	(+)FTC	(−)FTC
0.5	0.503	0.527	9.9	11.2	15.5	13.6
5	5.23	5.21	4.7	4.4	2.7	3.1
90	91.6	92.1	2.0	2.6	1.6	1.8

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no endogenous interferences. This method has very good within-run and between-run precision and accuracy over the range of 0.5–100 μg/mL. This method is reliable for the separation and quantitation of FTC enantiomers.

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