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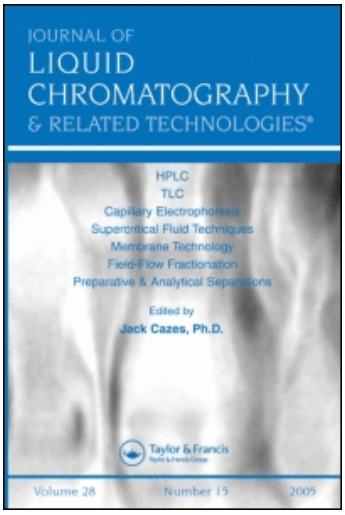

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ABSTRACT

A capillary electrophoresis (CE) method for the quantitative determination of R(+) and S(-) rogletimide in serum was developed. Stereoselective resolution of the rogletimide enantiomers was achieved using 60 mM alpha-cyclodextrin (α-CD) in 10 mM sodium dihydrogen phosphate/5 mM sodium tetraborate buffer of pH 2.5. A 72 cm uncoated fused-silica capillary at a constant voltage 15kV was used for the analysis and 0.03 mM hexadecyltrimethyl ammonium bromide (HTAB) was added to the buffer to decrease the adsorption of endogenous substances onto the silica wall. The analytes of interest were extracted from serum using a solid phase extraction (SPE) procedure. An octadecyl cartridge gave good recoveries in excess of 85% for both R(+) and S(-) rogletimide without any interference.

The detection limits were 50 ng/mL using 1 mL serum and the limit of quantitation were 100 ng/mL for each enantiomers. The calibration curves were linear over the range of 100-1200 ng/mL with S(-) aminoglutethimide as the internal standard and the coefficient of determination was greater than 0.999 (n=3). Precision

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and accuracy of the method were in the range 2.3-6.7% and 2.2-7.4%, respectively, for R(+) rogletimide and 1.7-6.4 % and 1.8-6.6%, respectively, for S(-) rogletimide. The CE method was compared to an existing HPLC method in terms of sensitivity and selectivity for the routine analysis of the drugs.

INTRODUCTION

The difference in the pharmacodynamic activities of enantiomers has created a need to study the pharmacological and toxicological properties of optically active compounds including drugs, agrochemicals, pesticides, herbicides, halogenated hydrocarbons, and/or their stereo-isomeric metabolic products.[1-3] It is widely believed that, in the near future, the pharmaceutical and agrochemical industries will be required by regulatory agencies, such as the Food and Drug Administration, to provide detailed information regarding the enantiomeric purity of drugs and therapeutic or toxic effects of individual enantiomers.[4-6] Hence, the development of rapid and accurate methods for stereochemical resolution of drugs is expected to remain an important issue for some time.

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Capillary electrophoresis (CE) has become a highly effective tool in separating ionic and neutral compounds.[7-11] It has been applied to analyze many different compounds, from small ions to large molecules such as proteins and DNA. CE offers the advantages of high efficiency, low sample consumption, small volume, of organic modifier (if any is used), and ease of operation.[12-17] Resolution of enantiomers in CE has been performed using various chiral selectors. Native and derivatized cyclodextrins, crown ethers, chiral ligand exchange, chiral micelles, proteins, carbohydrates, and macrocyclic antibiotics are some of the chiral selectors that have been used.[18-31]

The most commonly used chiral selectors are native and derivatized cyclodextrins (CDs). CDs are cyclic oligosaccharide molecules built up of D-(+)-glucopyranose units via α-(1,4)-linkages. Each glucose unit contributes five chiral centers to the molecule. The structure of a CD moiety is unique in that it resembles a truncated cone with both ends open. The primary hydroxyl groups (on C-6) on the glucopyranose units lie on the narrow end of the cone. These secondary hydroxyl groups (on C-2 and C-3) are located on the wider end and have a clockwise direction of rotation. This arrangement makes the interior hydrophobic and the rims hydrophilic. The most commonly used CDs are α-, β-, and γ-CD which have six, seven and eight glucopyranose units, respectively, in their structures.[31-36]

Cyclodextins have the advantage of being commercially available, transparent to UV light, stable over a wide pH range, non-toxic, and they are inexpensive.[33, 37]

A detailed overview of the experiments described in the literature revealed that α-CD is far less frequently used than β-CD; the application ratio α-:β- is 1:2 for native CD and 1:3 for derivatives.[38,39] For this reason, we started an extensive screening approach to establish the relative suitability of different CDs for the separation of drug enantiomers in CE. Bingcheng et al.,[40] report the resolution of 59 chiral drugs with native α-CD as chiral selector additive.

Rogletimide, 3-ethyl-3-(4-pyridyl)-piperidine-2,6-dione is an anti-tumor agent used in the treatment of postmenopausal women with hormone dependent metastatic breast carcinoma and is considered to be a more effective and selective chemotherapeutic agent in this respect.[41] Rogletimide is a chiral compound and is clinically administered as a racemic mixture, although it is reported that the R(+) enantiomer is more active than S(-) enantiomer.[41] HPLC resolution of R(+) and S(-) rogletimide has been reported by this laboratory and others using a chiral stationary phase along with the quantitation of enantiomers in serum.[42-44] Analysis of biological fluids using CE is less common and very few reports have been published.[45] No study has yet determined rogletimide enantiomers in serum using CE.

In this study, a CE method for the quantification of R(+) and S(-) enantiomers of rogletimide in serum is reported using α-CD as the chiral selector. The method is linear in the range of 100-1200 ng/mL and provides the required selectivity and sensitivity for monitoring the blood levels of R(+) and S(-) rogletimide.

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EXPERIMENTAL

Reagents and Chemicals

R(+) and S(-) rogletimide and the internal standard S(-) aminoglutethimide were gifts from Dr. M. Stogniew (U. S. Bioscience, Inc., West Conshohocken, PA, USA).

Hexadecyltrimethyl ammonium bromide (HTAB) was obtained from Sigma Chemical Co., St. Louis, MO. USA. Sodium dihydrogen phosphate, sodium tetraborate, phosphoric acid (85%) and methanol were obtained from J. T. Baker, Phillipsburg, NJ, USA. Native α-, β-, and γ-cyclodextrins, heptakis-(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) were also obtained from Sigma. Hydroxypropyl β-cyclodextrin (HP-β-CD), hydroxypropyl α-cyclodextrin (HP-α-CD), and hydroxypropyl γ-cyclodextrin (HP-γ-CD) were obtained from Aldrich Chemical Company, Milwaukee, WI, USA. Hydroxyethyl-β-cyclodextrin (HE-β-CD), carboxymethyl-β-cyclodextrin (M-β-CD) and amino-β-cyclodextrin were obtained from Advanced Separation Technologies,

Whippany, NJ, USA. Sulfated-β-cyclodextrin (S-β-CD) was kindly supplied by Cerestar USA, Inc., Hammond, IN, USA. Blank serum (Cat # 3160-34) was purchased from Instrumentation Lab., Lexington, MA, USA. The ethylsilane, octasilane, octadecylsilane, cyanopropyl, and phenyl solid phase extraction columns (100 mg/1 cc) were obtained from Varian Sample Preparation Products, Harbor City, CA, USA. All solutions were filtered through a 0.2 μm nylon filter (Acrodise 13, Gelman Science, Ann Arbor, MI, USA).

Instrumentation

All capillary electrophoresis experiments were performed using an ABI model 270A Capillary Electrophoretic System (Applied Biosystems, Foster City, CA, USA) equipped with a UV detector. An uncoated fused-silica capillary (total length, 72 cm; effective length, 50 cm; 50 μm i.d., (Polymicro Technologies, Phoenix, AZ, USA) was used for the analysis. The capillary temperature was kept at 30°C and the applied voltage was 15 kV. The polyimide coating of the capillary was stripped to create a 0.5 cm detection window. The detection was towards the cathodic end.

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Electrophoretic Conditions

The background electrolyte consisted of an aqueous solution of 10 mM sodium dihydrogen phosphate/5 mM sodium tetraborate, pH 2.5 (adjusted with 100 mM phosphoric acid) containing 60 mM α-CD and 0.03 mM HTAB. The analytes were monitored at 220 nm. New capillaries were conditioned by rinsing with 1 M sodium hydroxide for 10 min., followed by 10 min. each with water and running buffer solution. The sample was introduced using a vacuum injection for 20 sec. Before each analysis, the capillary was rinsed for 2 min. with 0.1 M NaOH and 3 min. with background electrolyte solution.

Preparation of Stock and Standard Solution

Individual stock solutions of R(+) and S(-) rogletimide and the internal standard, S(-) aminoglutethimide, were prepared in deionized water to give a concentration of 100 μg/mL, stored protected from light at 4°C, and were stable for at least two weeks. Appropriate dilutions of the individual rogletimide stock solutions were made to provide 5 μg/mL standard solutions which were used for spiking blank serum.

Preparation of Spiked Serum

Accurately measured aliquots (30, 50, and 90 μL of the individual 5 μg/mL standard R(+) and S(-) rogletimide solutions) were pipetted into indi-

vidual 1 mL volumetric tubes and evaporated to dryness with a nitrogen stream. Then 50 µL of the internal standard solution were added to each tube and drug-free serum was added to volume and mixed well to give final concentrations of 150, 300, and 900 ng/mL of each rogletimide enantiomer.

Assay Method

Bond-Elut octadecylsilane solid phase extraction (SPE) cartridges were attached to a vacuum manifold (Vac-Elut, Harbor City, CA, USA) and conditioned with 2 column volumes of absolute methanol followed by 2 column volumes of distilled water. (The sorbent must not be permitted to dry). Into the cartridges were transferred blank and spiked serum samples and the vacuum was applied.

After the entire serum sample had been aspirated through the cartridge, the cartridge was washed with 4x500 µL of distilled water and then dried under full vacuum for 3 min. The rogletimide enantiomers and the internal standard were eluted with 4x250 µL of absolute methanol. The eluates were filtered through a 0.2 µm syringe disc filter followed by evaporation under a slow nitrogen stream. The residue was reconstituted into 50 µL of deionized water, after which it was vacuum injected into the capillary for 20s. Absolute recovery of each analyte was calculated by comparing drug peak-area of the spiked analyte samples to un-extracted analyte of stock solution that has been injected directly into the electrophoretic system.

Calibration curves were constructed in the range of 100-1200 ng/mL of each rogletimide enantiomer. Linear regression analysis of normalized D/IS peak area ratios versus concentration gave slope and intercept data for each analyte which were used to calculate the concentration of each analyte in the serum sample.

RESULTS AND DISCUSSION

The chemical structures of R(+) and S(-) rogletimide and S(-) amino glutethimide are shown in Figure 1. To develop a reliable and sensitive CE method for the quantification of R(+) and S(-) rogletimide in serum, an analytical method was developed and validated with respect to the resolution of R(+) and S(-) rogletimide. Solid phase extraction was used to remove endogenous interferences and for concentrating the analytes, optimization of loading capacity, and, finally, accuracy and precision of the CE method.

A pair of enantiomers normally has the same electrophoretic mobility in free solution. Both interact with a chiral selector dissolved in the electrolyte solution to form inclusion complexes, which are assumed to have the same

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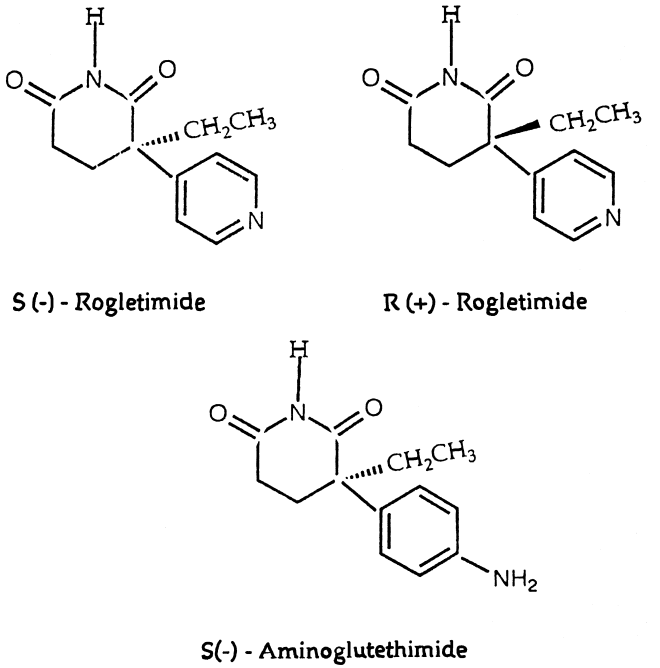

Figure 1. Chemical structures of rogletimide enantiomers and S(-)-aminoglutethimide (I. S.).

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electrophoretic mobilities. However, if the two enantiomers have different affinities for the chiral selector and the electrophoretic mobilities of the free and complexed enantiomers are different, chiral resolution becomes possible.[46,47] Cyclodextrins separate enantiomers utilizing the phenomenon of host-guest complexation.

The affinity of the analyte for the CD is due to the hydrophobic interactions between the analyte and the CD cavity and the hydrogen bonding of the analyte to the hydroxyl groups or introduced functional groups on the CD ring.[48] Various native and derivatized cyclodextrins were investigated for their capacity to separate the rogletimide enantiomers. The neutral cyclodextrins were α, β, γ CDs, MD-β-CD, TM-β-CD,HP-β-CD, HE-β-CD, M-β-CD, HP-γ-CD, HP-γ-CD. The charged CDs investigated were CM-β-CD, amino-β-CD, and S-β-CD.

No separations were obtained using any of the cyclodextrins except for α-CD in 10 mM phosphate/5 mM borate buffer at pH 2.5. The use of α-CD

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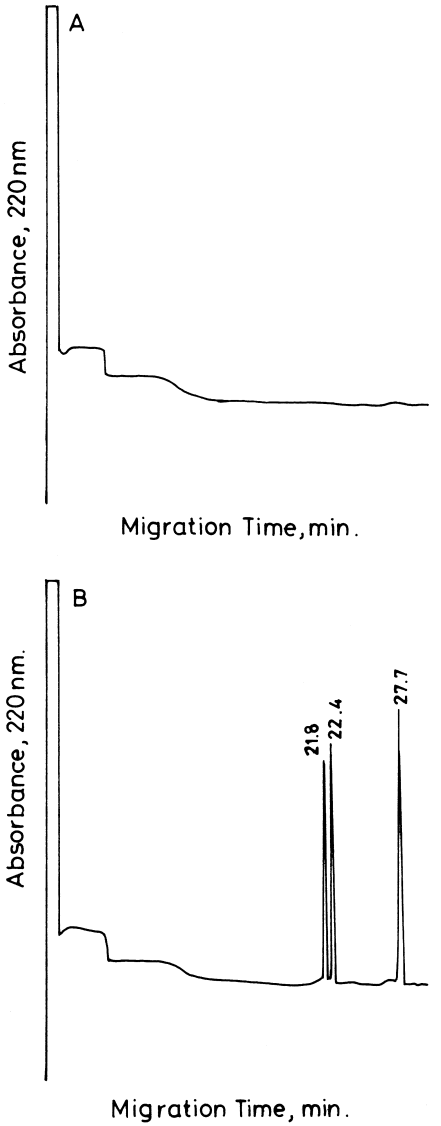

Figure 2. Typical electropherograms of (A) blank serum and (B) serum spiked with R(+) rogletimide (21.8 min), S(-) rogletimide (22.4 min) and S(-) aminoglutethimide (27.7 min.).

improved the separation dramatically as shown in Fig. 2B. Perhaps the size of the cavity of α-CD provides the best fit for the lipid moiety of rogletimide. We do not have any direct evidence through the measurement of light scattering. Optimization of the run buffer was performed by studying the effect of α-CD, phosphate/borate buffer and pH.

As the concentration of α-CD increased, the resolution of peaks and the separations of R(+) and S(-) rogletimide were improved (Fig. 2B). Furthermore, at higher concentration of α-CD, the general tendency of solutes to be solubilized in the cavity would be increased. Therefore, as the concentration of α-CD increases, the inclusion complexation increases and, consequently, the migration speeds of the solutes would be increased.

Our results are in agreement with theoretical expectations and findings of Yik et al.,[49] and Yoo et al.[50] However, significant broadening of the peaks was observed at concentrations above 70 mM.

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The effect of buffer concentration was investigated at 5, 10, 20, 30 mM phosphate/borate buffer and it was found that the separation of R(+) and S(-) forms were improved by using a mixture 10mM sodium dihydrogen phasphate/ 5 mM sodium tetraborate.[51] As the pH increased above 5.5, there was a complete loss of resolution with a concurrent decrease in migration time. As the voltage increased, there was a decrease in migration time and loss of resolution at voltages higher than 20 kV, due to the deleterious effects of joule heating; the efficiency decrease and at voltage < 15 kV, broad peaks were obtained with long migration times.

The effect of cationic detergents such as HTAB in reducing the adsorption of endogenous substances in serum by the silanol groups on the wall of the capillary has been documented.[52] The cationic detergents cover the negatively charged silica wall of a capillary, forming a net positive charge, thereby reducing the protein adsorption on the wall, while the cationic rogletimide is repelled. Under the acidic condition used in the experiment, and employing the cationic detergent, the electromigration of positively charged species toward the cathode was the principal force in effect, and the electroosmotic flow became negligible.[53]

The addition of HTAB to the background electrolyte system at a concentration of 30 nM was shown to provide good peak shapes and stable migration times, suggesting a role in minimizing the amount of irreversible adsorption. Migration time reproducibility was very high, with less than 0.5% RSD for all three peaks (n=9). Based upon these studies, the optimized conditions for the assay were a voltage of 15 kV, and a background electrolyte of 10 mM sodium dihydrogen phosphate/5 mM sodium tetraborate, pH 2.5, containing 60 mM α-CD and 30 nM HTAB. Fig. 2A and 2B show typical electropherograms of

blank serum and serum spiked with R(+) and S(-) rogletimide and IS S(-) aminoglutethimide.

For the extraction of the analytes from a serum sample, five solid phase extraction cartridges (ethylsilane, octylsilane, octadecylsilane, cyanopropyl, and phenyl) were investigated for serum sample clean-up prior to the CE assay. All the cartridges except octadecylsilane gave recoveries of about 50% or less for both rogletimide enantiomers. The octadecylsilane was selected for use since it provided the best data in terms of sample clean-up and recoveries of both rogletimide enantiomers. Absolute methanol was used as the eluent and mean recoveries using octadecylsilane cartridge were 89.1±2.86 for R(+) rogletimide, 87.3±2.16 for S(-) rogletimide and 86.3±2.62 for the internal standard S(-) aminoglutethimide (n=5).

To decrease the detection limit, on-capillary sample concentration was employed. The sample was prepared in a lower conductivity solution (water) than the electrolyte solution. Upon application of the voltages, a greater field will develop across the sample zone, causing the ions to migrate faster. When the ions reach the running buffer, the field decreases and they migrate slower. This process occurs until the analytes are compressed into a smaller zone.[54,55] Thus, the sample was reconstituted in water.

To obtain excellent reproducibility, the effects of different modes of injection and different procedures were investigated for capillary washing and their influence on reproducibility. It was found that vacuum injection was more reliable than electrokinetic injection, in terms of reproducibility, and washing with 0.1 M NaOH and buffer before each run was an absolute necessity. Normalized peak area values were obtained by dividing the corresponding peak areas by the migration times.[56] These normalized values were used in calculating the unknown concentrations in serum samples.

The validation of the CE assay was performed by evaluating limit of detection, limit of quantitation, linearity of detector response, method precision, and accuracy. The limits of detection (LOD) were 50 ng/mL and the limits of quantitation (LOQ) were 100 ng/mL for each enantiomer (n=3). The linear calibration curves showed good linearity in the range of 100-1200 ng/mL for R(+) and S(-) rogletimide. The coefficients of determination were greater than 0.999 (n=3). Representative linear regression equations obtained for R(+) and S(-) rogletimide were $Y = 0.001795x - 0.00698$ and $Y = 0.001964x - 0.00296$, respectively, where Y and x are the normalized drug to internal standard peak area ratios and concentrations of each analyte, respectively.

The intra-day precision and accuracy (n=3), as expressed by % RSD and % error were 2.5-6.1 and 2.2-7.4%, respectively, for R(+) rogletimide and 1.7-5.6 and 1.8-6.0% for S(-) rogletimide, respectively.

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Table 1

Accuracy and Precision Data for Rogletimide Enantiomers in Spiked Serum Samples

Analyte	Conc. Added (ng/mL)	Conc. Found (ng/mL)	RSD (%)	Error (%)
		Intra-Day		
R(+)rogletimide	150	146.75 ± 3.69	2.5	2.2
	300	311.34 ± 13.64	4.4	3.8
	900	966.52 ± 59.16	6.1	7.4
S(-)rogletimide	150	147.22 ± 2.48	1.7	1.8
	300	316.57 ± 12.36	3.9	5.5
	900	954.12 ± 53.21	5.6	6.0
		Inter-Day		
R(+)rogletimide	150	145.82 ± 3.42	2.3	2.8
	300	313.61 ± 14.84	4.7	4.5
	900	958.33 ± 64.24	6.7	6.5
R(-)rogletimide	150	144.36 ± 3.12	2.2	3.8
	300	319.87 ± 13.46	4.2	6.6
	900	958.50 ± 61.93	6.4	6.5

[a] Mean ± SD, based on n = 3. [b] Mean ± SD, based on n = 9.

The inter-day precision and accuracy (n=9, over three days), expressed by % RSD and % error were 2.3-6.7 and 2.8-6.5% respectively, for R(+) rogletimide and 2.2-6.4 and 3.6-6.5%, respectively, for S(-) rogletimide. The detailed analytical data are shown in Table 1.

In comparison to existing chiral HPLC methods for these drugs, the CE method provides good sensitivity and selectivity. Table 2 shows a comparison of this CE method with an HPLC assay for rogletimide previously reported by our laboratory.[44] Even though selectivity is lower, complete baseline resolution is obtained with the CE method because the high plate numbers are ten orders of magnitude greater than those obtained with the HPLC method. The CE migration times are longer than HPLC retention times, but they can be decreased by shortening the length of the capillary. The CE detection limits

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Table 2

Comparison of CE and HPLC Assays for R(+) and S(-)rogletimide

	CE		HPLC[a]	
Rogletimide	**R**	**S**	**R**	**S**
Selectivity (α)	1.02		1.70	
Resolution (Rs)	6.91		4.08	
Theoretical Plates (N)	41932	43882	1296	1112
Migration Times (t_m.min)	21.8	22.4	5.9	7.5
Retention Times (t_r.min)				
Linear Conc. Range (ng/mL)	100-1200		100-1500	
r^2	> 0.999		> 0.999	
Detection Limit (ng/mL)[b]	50	50	50	50
Quantitation Limit (ng/mL)	100	100	100	100

[a] See reference 44 for method. [b] S/N = 2.

could be further improved by using a Z-shaped capillary or by using a bubble-shaped detection window.

In conclusion, a sensitive and selective chiral CE assay has been developed for the analysis of R (+) and S(-) rogletimide in serum. The method is sensitive to 50 ng/mL of each enantiomer, with a total run time of 28 min. The method is comparable to an existing chiral HPLC method and could also be used for routine analysis of rogletimide enantiomers.

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