Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 08 July 2010

To cite this Article Aboul-Enein, Hassan Y. and Ali, Imran(2005) 'HPLC and Solid-Phase Extraction of Cromakalim Enantiomers in Human Plasma by Using Reversed-Phase Polysaccharide Chiral Stationary Phases', Separation Science and Technology, 39: 10, 2389 — 2400
To link to this Article: DOI: 10.1081/SS-120039344
URL: http://dx.doi.org/10.1081/SS-120039344

SEPARATION SCIENCE AND TECHNOLOGY
Vol. 39, No. 10, pp. 2389–2400, 2004

HPLC and Solid-Phase Extraction of Cromakalim Enantiomers in Human Plasma by Using Reversed-Phase Polysaccharide Chiral Stationary Phases

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ABSTRACT

Cromakalim is an antihypertensive drug (potassium channel activator) that acts as potent vasodilator functioning by hyperpolarization of vascular smooth muscle membranes and opening of potassium channels. Attempts have been made to resolve the enantiomers of cromakalim (3S,4S and 3R,4R) on polysaccharide chiral stationary phases (CSPs) under reversed-phase mode. The columns used were Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R. The mobile phases used were

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DOI: 10.1081/SS-120039344 0149-6395 (Print); 1520-5754 (Online)

water–acetonitrile (70 : 30, v/v) for the Chiralpak AD-R and the Chiralcel OD-R columns, and water–acetonitrile (80 : 20, v/v) for the Chiralcel OJ-R column. The flow rate of the mobile phases was 0.5 mL/min. The detection was carried out at 220 nm. The α-values of the resolved enantiomers were 1.26, 1.23, and 1.13 on Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R columns, respectively, while the values of R_s were 1.20, 0.40, and 0.30 on Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R columns, respectively. Concentration of cromakalim in human plasma was determined by using solid-phase extraction method.

Key Words: Chiral resolution; Cromakalim; Chiralpak AD-R; Chiralcel OD-R; Chiralcel OJ-R; Soild-phase extraction; Human plasma.

INTRODUCTION

Cromakalim is an antihypertensive drug (potassium channel activator) that acts as a potent vasodilator functioning by hyperpolarization of vascular smooth muscle membranes and opening of potassium channels.[1] Cromakalim is administered as a racemic mixture, because it has two chiral centers (Fig. 1). A search of the literature indicates that enantiomers may differ in their pharmacological actions.[2–4] Therefore, the determination of enantiomeric purity is of high importance in pharmaceutical and pharmacological activities. The US Food and Drug Administration also has issued guidelines to pharmaceutical and agrochemical industries to specify the enantiomeric purity of the optically active compounds before their marketing.[5] It has been reported that the enantiomers of cromakalim also differ in their biological activities.[6–8] Polysaccharides-based chiral stationary phases (CSPs) have been used successfully for the resolution of a wide variety of racemates.[3,9–18]

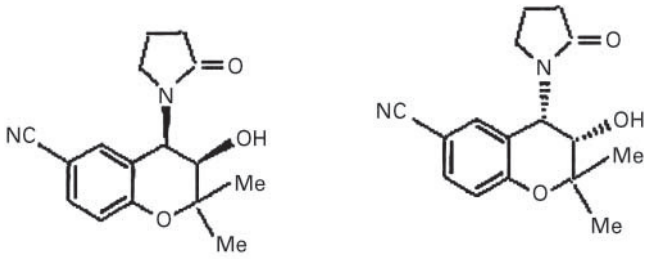

Figure 1. The chemical structure of cromakalim enantiomers

Therefore, attempts have been made to resolve (−)-3*S*,4*S* and (+)-3*R*,4*R* cromakalim enantiomers on polysaccharide CSPs under reversed-phase mode, and the results of these findings are presented herein.

EXPERIMENTAL

Chemicals and Reagents

The racemic mixtures and the optically pure forms of cromakalim were obtained from SmithKline Beecham Frythe, Welwyn, UK. The solutions of this drug (1 μg/mL) were prepared in methanol. Acetonitrile, methanol, acetic acid, trifluoroacetic acid of HPLC grade, phosphoric acid, and sodium phosphate (Na_2HPO_4) were purchased from Fisher Scientific (Fairlawn, NJ). Hydrochloric acid used was obtained from J. T. Baker Inc., Phillipsburg, NJ. Phosphate buffers were prepared as per the standard procedure.

Instruments Used

The HPLC system used in this study was purchased from Waters (Milford, MA) and consisted of Waters solvent delivery pump (model 510), Waters injector (model WISP 710B), Waters tunable absorbance detector (model 484), and Waters integrator (model 740). The order of elution of the enantiomers was confirmed by using a polarimeteric detector (Shodex OR-1, J. M. Sciences Inc., Buffalo, NY). The columns used were Chiralpak AD-R (15 cm × 4.6 mm) [amylose *tris* 3,5-dimethylphenylcarbamate], Chiralcel OD-R (15 cm × 4.6 mm) [cellulose *tris* 3,5-dimethylphenylcarbamate], and Chiralcel OJ-R (15 cm × 20 mm) [cellulose *tris* 4-methylbenzoate]. These columns were obtained from Daicel Chemical Industries, Tokyo, Japan. Purified water was prepared by using a Millipore Milli-Q (Bedford, MA) water purification system. A C_{18} Sep-Pak Classic (short body) cartridge was obtained from Waters (Milford, MA). pH was recorded with the pH meter (model 611, Orion Research Inc., USA).

Chromatographic Conditions

An aliquot of 20 μL of each of the solutions was injected onto a HPLC system described above. The mobile phases used were water–acetonitrile (70 : 30, v/v) on Chiralpak AD-R and Chiralcel OD-R columns, and water–acetonitrile (80 : 20, v/v) on Chiralcel OJ-R column. The mobile phases

were filtered and degassed before use. The flow rate of the mobile phases was 0.5 mL/min. The chart speed was kept constant at 0.1 cm/min. All the experiments were carried out at 23°C ± 1°C. The detection was carried out at 220 nm. The chromatographic parameters such as capacity factor (k), separation factor (α), and resolution factor (R_s) were calculated.

Solid-Phase Extraction

A 10 μL solution (10 μg/mL) of cromakalim enantiomers was mixed with 990 μL of human plasma. The spiked plasma was mixed with 3 mL of acetone, vortexed for 5 min, and centrifuged for 5 min at 3000 rpm. The clear supernatant was separated and evaporated to dryness under nitrogen atmosphere. The residue was dissolved in 1 mL of phosphate buffer (50 mM, pH 3.0) and this was loaded onto a C_{18} cartridge, preconditioned. Cartridge was washed with 1 mL of deionized water, and cromakalim was eluted with pure methanol (0.5 mL) twice, and the two eluents were combined. The combined eluent was used for the analysis of cromakalim enantiomers by HPLC.

RESULTS AND DISCUSSION

The capacity (k), separation (α), and resolution (R_s) factors for the resolved enantiomers of cromakalim are given in Table 1. Table 1 shows that the best resolution of cromakalim was achieved with the Chiralpak AD-R column. The typical chromatograms of the resolved cromakalim enantiomers are shown in Figs. 2 and 3 for standard solution and human plasma,

Table 1. The capacity (k), separation (α), and resolution (R_s) factors for the enantiomeric resolution of cromakalim on Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R columns by using different ratios of water–acetonitrile as the mobile phases at 0.5 mL/min flow rate.

k_1 (+)-(3*S*,4*S*)	k_2 (−)-(3*R*,4*R*)	α	R_s
Chiralpak AD-R with water–acetonitrile (70 : 30, v/v)			
22.55	28.36	1.26	1.20
Chiralcel OD-R with water–acetonitrile (70 : 30, v/v)			
22.93	28.29	1.23	0.40
Chiralcel OJ-R with water–acetonitrile (80 : 20, v/v)			
9.30	10.00	1.08	0.30

Note: For details see Experimental section.

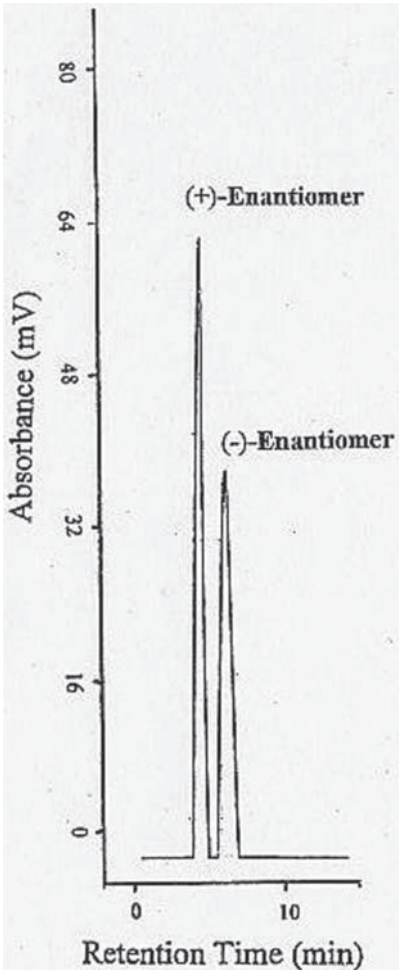

Figure 2. Chromatogram showing the enantiomeric resolution of cromakalim (standard solution) on Chiralpak AD-R column by using water–acetonitrile (70 : 30, v/v) at 0.5 mL/min flow rate.

respectively. It has been observed that the (+)-enantiomer eluted first followed by the (−)-enantiomer on all the chiral columns used in this study.

A variation in the chromatographic parameters was carried out to obtain the best resolution. To optimize the chromatographic conditions, various ratios of water, alcohols, and acetonitrile were tested, but no good resolution could be achieved. As a result of extensive experiments, the optimized

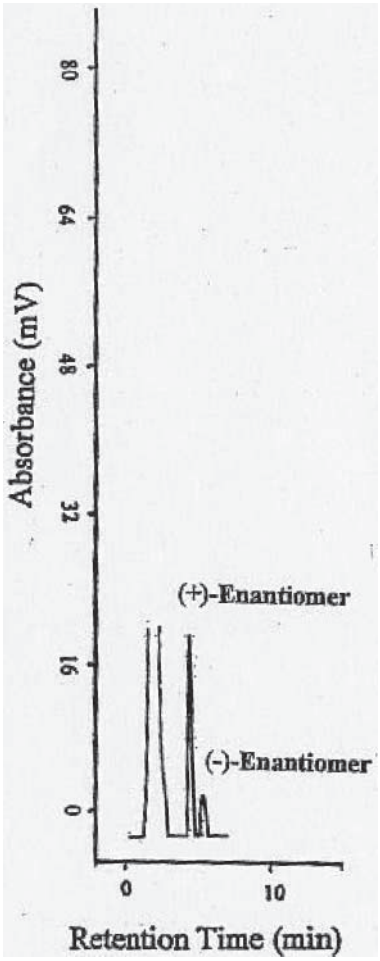

Figure 3. Chromatogram showing the enantiomeric resolution of cromakalim (in human plasma) on Chiralpak AD-R column by using water–acetonitrile (70 : 30, v/v) at 0.5 mL/min flow rate.

chromatographic conditions were developed and are reported herein. Similarly, solid-phase extraction (SPE) conditions also were optimized by using other C_{18} cartridges. In addition, phosphate buffers of different concentrations and pHs were tested in SPE. The elution of cromakalim enantiomers was carried out by using pure methanol and methanol-containing hydrochloric acid, acetic acid, trifluoroacetic acid, the best results are presented herein.

Enantiomers of cromakalim have successfully been resolved on the Chiralpak AD-R column, whereas a partial resolution of this drug was observed on Chiralcel OD-R and Chiralcel OJ-R columns (Table 1). The α-values of the resolved enantiomers were 1.26, 1.23, and 1.13 on Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R columns, respectively. The values of R_s were 1.20, 0.40, and 0.30 on Chiralpak AD-R, Chiralcel OD-R, and Chiralcel OJ-R columns, respectively. From these findings, it is clear that the resolution efficiency of these column for the enantiomeric resolution of cromakalim is in the order of Chiralpak AD-R > Chiralcel OD-R > Chiralcel OJ-R. The partial resolution of cromakalim on Chiralcel OD-R and Chiralcel OJ-R columns may be due to the poor resolving power of these two columns in comparison to the Chiralpak AD-R column. The better resolution capacity of amylose may be attributed to the fact that the amylose CSPs are more helical in nature and possess well-defined grooves, making it different from the corresponding cellulose analogs, which appeared to be more linear and rigid in nature[19] and, hence, amylose provides better chiral environment to these racemates.

Polysaccharides, such as cellulose and amylose, are the most readily available optically active polymers and are known to show chiral discrimination capability. However, native cellulose and amylose do not offer any practically useful CSP because of insufficient optical-resolving power. On the other hand, polysaccharides are easily converted to a variety of derivatives such as *tris*-esters and *tris*-carbamates by the reaction of active hydroxyl groups with appropriate reagents. The chiral recognition mechanism at a molecular level on the cellulose-based CSPs is still unclear, although it has been reported that the chiral resolution by these CSPs is achieved through the different hydrogen, $\pi-\pi$ and dipole–dipole induced interactions between the chiral stationary phase and the enantiomers.[20–22] The structure of this drug (Fig. 1) contains electronegative atoms namely nitrogen and oxygen along with one aromatic ring. Therefore, the resolution of the enantiomers of cromakalim was achieved through hydrogen bonding, dipole–dipole induced interactions and $\pi-\pi$ interactions of different magnitudes between the CSPs and the enantiomers of cromakalim. In addition, the contribution of steric effect in chiral resolution also has been reported.[23,24] Accordingly, the enantiomers of the reported drug fit stereogenically in the different fashion into the chiral grooves of the stationary phases that are stabilized by these interactions of different magnitudes and, hence, the resolution of enantiomers occurred.

It is very interesting to note that the resolution on the Chiralcel OD-R was better than the resolution on the Chiralcel OJ-R column. It may be due to the stronger bondings in the Chiralcel OD-R column in comparison with the Chiralcel OJ-R column. The structures of polysaccharides CSPs are presented by Aboul-Enein,[25] and it shows that Chiralcel OD-R is the carbamate derivative, while Chiralcel OJ-R is the ester derivative of cellulose. The difference

between these carbamate and ester derivatives lies in the presence of one extra methyl and amide group in the carbamate derivative. The presence of one extra methyl group in carbamate derivative increases the density of π electrons on the phenyl ring of carbamate derivative through its positive inductive effect and, hence, the carbamate derivative provides the stronger $\pi-\pi$ interactions than the $\pi-\pi$ interactions provided by the ester derivative. In addition, the presence of an amide group in carbamate derivatives increases the magnitude of the hydrogen bonding between the analyte and chiral selector.

During optimization of the mobile-phase composition, attempts have been made to use the different ratios of water and acetonitrile. The different percentages of acetonitrile used were 10, 20, 30, 40, and 50. The maximum resolution of the drug obtained is reported herewith. However, it is very interesting to note that the peaks were very sharp at high concentrations of acetonitrile (40–50%), with low retention times, while peaks were broad at low concentrations of acetonitrile (10–20%), with higher retention times. It may be concluded that the difference of the magnitude of bondings of the two enantiomers was not enough for their resolution under both the low (10–20%) and the high (40–50%) concentrations of acetonitrile.

Various cartridges, phosphate buffers, and eluting solvents were tried in SPE, but a C_{18} cartridge with phosphate buffer (50 mM, pH 3.0) and methanol as the eluting solvent gave the best SPE conditions for the extraction of cromakalim enantiomers from human plasma. It is interesting to note that percentage recoveries of cromakalim enantiomers were 90 and 40 for (+)- and (−)- antipodes, respectively. This indicates that (−)-cromakalim, the pharmacologically active enantiomer, binds strongly with plasma protein, in comparison with (+)-enantiomer.[26–28]

VALIDATION OF THE METHOD

Validation of the reported method was carried out by running five sets ($n = 5$) of the chromatographic and extraction procedures under identical conditions. The regression analysis was carried out by using the Microsoft Excel program, and the results are given in Table 2, which indicates that the standard deviation (SD) was ± 0.10 for both enantiomeric resolution and peak areas. The correlation coefficient (R^2) and confidence levels for CE method were 0.9999% and 99.0%, respectively. Similarly, the SD for SPE experiment was ± 0.11, while the values of correlation coefficient and confidence levels were 0.9999 and 99%, respectively. The linearity range was from 10 to 50 ng/mL, and the correlation coefficients for calibration curves were higher than 0.999, as determined by least-square analysis. The detection limit for the developed method was 10 ng for both enantiomers.

Table 2. Regression analysis data for analysis of cromakalim enantiomers.

	R_s			PA			SPE (% recovery)		
	SD	CC	CL (%)	SD	CC	CL (%)	SD	CC	CL (%)
					$n = 5$				
(+)-Enantiomer	±0.10	0.9999	99	±0.11	0.9999	99	±0.11	0.9999	99
(−)-Enantiomer	±0.10	0.9999	99	±0.10	0.9999	99	±0.11	0.9999	99

Note: CC, Correlation coefficient; CL, confidence level; PA, peak area; SD, standard deviation, and SPE, solid-phase extraction.

The inter- and intra-days (7 days) assays analysis also were carried out, which indicated no deviation from the reported results, indicating the stability of this drug under the reported chromatographic conditions.

CONCLUSION

In this study, the chiral resolution of cromakalim on polysaccharide-based CSPs was achieved under reversed-phase mode. The resolution was in the order of Chiralpak AD-R > Chiralcel OD-R > Chiralcel OJ-R. By taking into consideration the results obtained, one can conclude that the enantiomeric resolution of cromakalim on polysaccharide CSPs is governed by dipole-induced dipole interactions, hydrogen bondings, $\pi-\pi$ interactions and a steric effect, as discussed above. The percentage recoveries of (+)- and (−)-enantiomers in SPE by using C_{18} cartridge were 90 and 40, respectively. The reported HPLC system is simple, fast, and reproducible, and can be used for the enantiomeric resolution of cromakalim on a semipreparative scale for further pharmacological investigations of the individual enantiomers.

ACKNOWLEDGMENT

The authors (I.A. and H.Y.A-E.) would like to thank the King Faisal Specialist Hospital and Research Center, administration for their support for the Pharmaceutical Analysis Laboratory Research Program.

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