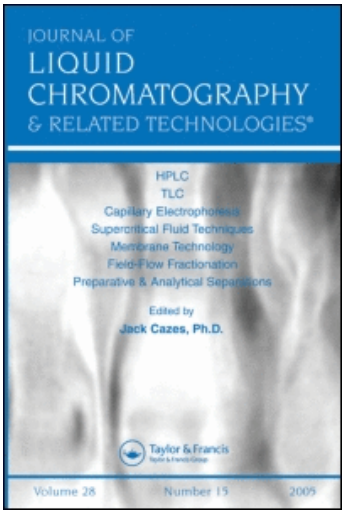

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APPLICATION OF COLUMN SWITCHING IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLORTHALIDONE ENANTIOMERS IN UNTREATED URINE

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ABSTRACT

A liquid chromatographic method incorporating column-switching for the separation and determination of chlorthalidone enantiomers in urine is described. Untreated urine samples (50 μL) were directly introduced into a 20 mm x 2.1 mm I. D. precolumn packed with a Hypersil ODS-C_{18}, 30 μm, stationary phase. Polar urinary compounds were removed by flushing the precolumn with 4 mL of water (pumped at a flow rate of 1 mL/min), and the racemic analyte was then transferred to a LiChroCART ChiraDex, 5 μm, 250 mm x 4 mm I. D. column, where the enantiomers were separated by means of a methanol/0.05 M acetate buffer adjusted to pH 4 mobile-phase (40:60, v/v), and quantified at 230 nm. The system shows good linearity and reproducibility in the 0.25 - 5.0 μg/mL, thus covering therapeutic levels of chlorthalidone in urine. The usefulness of the described procedure has been tested by analyzing urine samples obtained after drug administration.

INTRODUCTION

In drug analysis, stereochemical of separation is currently of great interest, because the separation and determination optical isomers is essential to understand the mechanism of drug action. Chiral separations can be also of interest in the development of new drugs.

Owing to their potential for separation, High Performance Liquid Chromatography (HPLC) is presently the most widely used technique for enantiomeric analysis. In this respect, different approaches have been reported: (i) indirect methods based on formation of the diastereomeric derivatives and subsequent separation in a conventional (achiral) stationary phase, and direct methods based on (ii) separation in a conventional stationary phase by means of a chiral mobile-phase, or (iii) separation in a chiral stationary phase.

Although HPLC is an increasingly popular technique for direct separation of enantiomers (specially through the development of new chemically bonded chiral stationary phases), some complications are encountered when analyzing biological samples. For example, most applications described are based on normal phase chromatography. This is a major difficulty in the analysis of biological samples because the analyte must be transferred from an aqueous to an organic phase before injection. Otherwise, chiral selectivity rapidly decreases.[1] Important progress has been made since the introduction of cylclodextrin bonded phases, which can be used in a conventional reverse phase mode.

On the other hand, a very selective clean-up is necessary because chiral separations increase the number of peaks to be resolved (every endogenous compounds can lead to a pair of peaks).[1,2] Moreover, low efficiencies associated with chiral columns limit the sensitivity. This means that some kind of enrichment and/or derivatization of the analytes are often required.[3]

Switching chromatography has become especially important in the context of chiral separation because highly selective separations can be achieved by coupling chiral columns to conventional reverse phase systems. Several successful applications of this technique in drug analysis have been reported. However, most of the described assays have not been applied to real samples,[4,5] or involve off-line clean-up or preconcentration steps before injection into the chromatographic systems.[2,6] In addition, relatively sophisticated set-ups are sometimes required to obtain adequate resolution and sensitivity.[7-9] In such cases, several (chiral and a chiral) columns, pumping systems and/or detectors must be combined within the same network to effect enantiomeric resolution and peak compression; peak compression is necessary to overcome band broadening due to chiral separation and

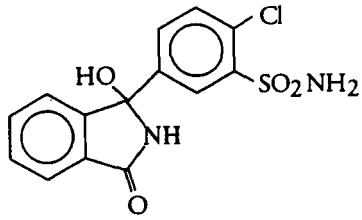

Figure 1. Chemical structure of chlorthalidone.

analyte transfer from one column to another.

In this work we have evaluated the usefulness of column-switching for the on-line sample conditioning, analyte enrichment and enantiomeric separation using a cylclodextrin bonded phase column. The diuretic chlorthalidone (Figure 1), has been used as a model of substance because we previously evaluated the potential of switching techniques for sample pretreatment in the analysis of some diuretics (including chlorthalidone) in urine,[10] and also because the recent increase in the consumption of chlorthalidone in combination with other antihypertensive agents have resulted in very low doses of drug; therefore, very sensitive and highly specific methods to measure chlorthalidone levels in biological samples are required. On the basis of these studies, a procedure for the determination of chlorthalidone enantiomers in urine has been developed.

MATERIALS

Apparatus

The chromatographic system used consisted of two quaternary pumps (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 Series) with a sample loop injector of 100 μL, and a high pressure six port valve (Rheodyne model 7000). A diode array detector (Hewlett Packard, 1040 series) linked to a data system (Hewlett Packard HPLC Chem Station) was used for data acquisition and storage. The detector was set to collect a spectrum every 640 ms over the range 200-400 nm, and the chromatographic signal was monitored at 230 nm. All the assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical grade. Methanol was of HPLC grade

(Scharlau, Barcelona, Spain). Water was distilled, deionized and filtered in nylon membranes, 0.45 μm (Teknokroma, Barcelona, Spain). Racemic chlorthalidone was obtained from ICI-Pharma (Pontevedra, Spain). Propylamine hydrochloride (Fluka, Buchs, Switzerland), sodium acetate (Panreac, Barcelona, Spain) and acetic acid (Probus, Badalona, Spain) were also used.

METHODS

Standard Solutions

Standard solutions of chlorthalidone were prepared by dissolving 50 mg of the racemic compound in 25 mL of methanol; these solutions were stored in the dark at 2^0C. Under such conditions, the standard solutions are stable at least for a month. Working solutions were prepared daily by dilution of the stock solutions with the appropriate volumes of purified water.

Columns and Mobile-Phases

The pre-column (20 mm x 2.1 mm I.D.) was dry-packed with a Hypersil ODS-C_{18}, 30 μm, stationary-phase (Merck, Darmstadt, Germany). Purified water was used as washing solvent to eliminate the biological matrix from the precolumn. The analytical column was a LiChroCART ChiraDex, 250 mm x 4 mm I.D., 5 μm, column (Merck). A methanol/0.05 M acetate buffer (pH = 4) mixture was used for the enantiomeric separation. The acetate buffer was prepared by dissolving 2 g of sodium acetate in 500 mL of purified water, after the addition of 0.5 mL of propylamine hydrochloride. The pH was adjusted to 4 by adding concentrated acetic acid.

The mobile-phases were prepared daily, filtered with a nylon membrane, 0.45 μm, (Teknokroma) and degassed with helium before use.

Column Switching Operation

The system used for the on-line sample pretreatment and enantiomeric separation is shown in Figure 2. At the beginning of each assay 50 μL of sample were injected from the sample injector to the precolumn. Polar components of the matrix were directly washed-out with water by means of Pump 1 (at a flow rate of 1 mL/min). At the same time, the analytical column was being reequilibrated with an

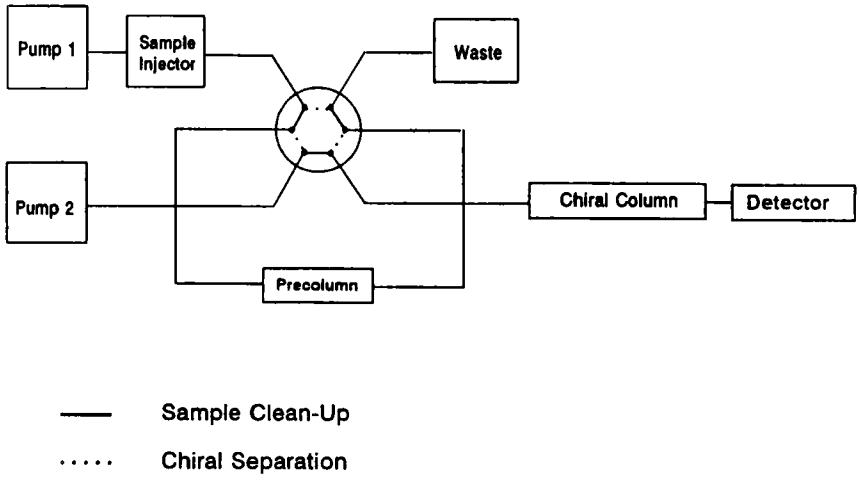

Figure 2. Schematic representation of the switching system used for the determination of chlorthalidone enantiomers in urine.

acetonitrile/acetate buffer (pH = 4) eluent (40:60, v/v) delivered by Pump 2 at a flow rate of 1 mL/min. At t = 4 min, the switching valve was rotated, so the trapped analyte was transferred from the precolumn to the chiral column, where chlorthalidone enantiomers were separated. At t = 9 min the switching valve was turned back to the original position to regenerate and reequilibrate both the precolumn and the analytical column.

Recovery Studies

Blank urine samples were spiked with chlorthalidone standard solutions reproducing different concentrations for each enantiomer in the 0.25 - 5.0 μg/mL range. The percentage of drug recovered for a particular injection was calculated by comparing the peak areas obtained for each isomer in the spiked samples, with the values obtained for a direct injection of 50 μL of an aqueous solution containing the same concentration of analyte. Each concentration was assayed in triplicate.

Preparation of Standards for Calibration

Standards for calibration were prepared by spiking urine samples with the appropriate volumes of the chlorthalidone standard solutions reproducing different

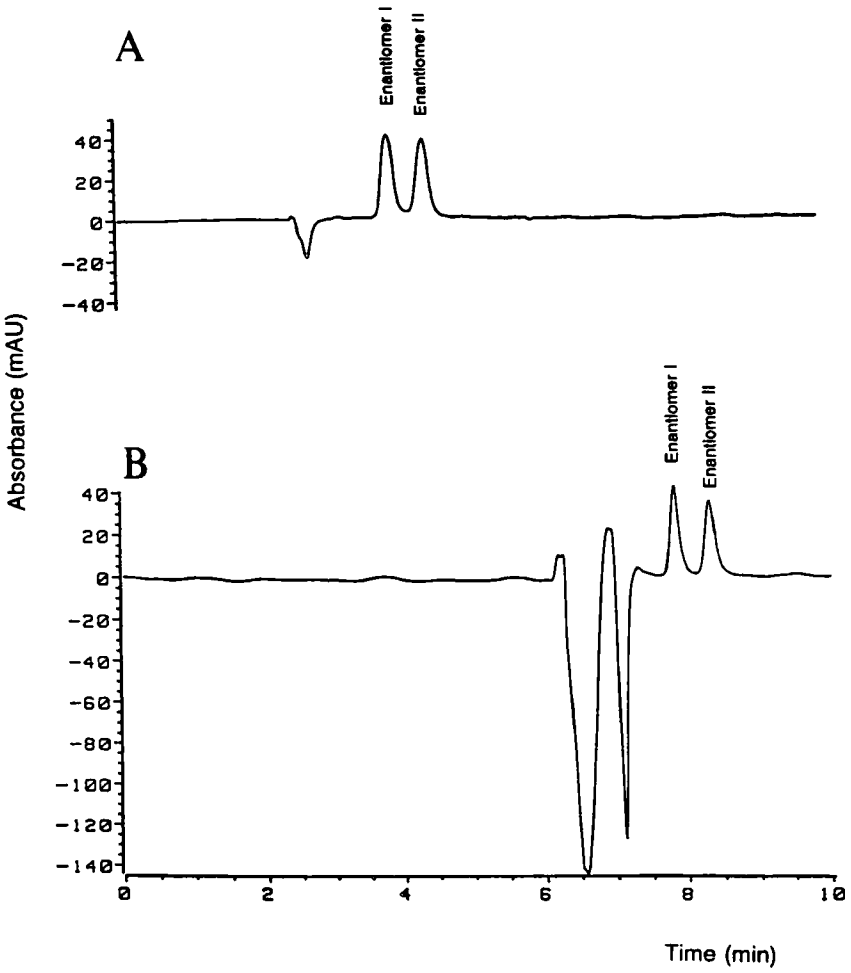

Figure 3. Chromatograms obtained for an aqueous solution of racemic chlorthalidone by (A) direct injection into the chiral column and (B) injection in the switching system (duration of the flushing stage, 4 min). Concentration of each enantiomer, 2.5 μg/mL. For experimental details, see text.

concentrations for each enantiomer in the 0.25 - 5.0 μg/mL range. These samples were processed as described above. Peak areas at 230 nm were plotted versus the analyte concentration, and the resulting calibration curves were used to calculate the concentration of each enantiomer in the unknown samples. Each concentration was assayed in triplicate.

Table 1

Analytical Data for the On-Line Determination of Chlorthalidone Enantomers in Urine

Enantiomer	Recovery[a] (n = 3) (%)	Linearity				Intra-day Precision[a] (n = 6) (%)	Inter-day Precision[a] (n = 15) (%)	Limit of Detection (ng/mL)
		$y = a + bk$	S_{xy}	n				
I	92 ± 2	a = 1 ± 4 b = 88.9 ± 1.1	8.83	18	4		7	20
II	92 ± 3	a = 2 ± 4 b = 89.0 ± 1.5	10.7	18	4		8	20

[a] Determined at half of highest concentration in tested range.xxxx

Human Studies

Urinary excretion studies were performed with a human healthy volunteer after a single dose administration of racemic chlorthalidone (25 mg). Urine samples were collected at appropriate time intervals post-dose, and analyzed as described above.

RESULTS AND DISCUSSION

Chiral Separation

Conditions for the resolution of chlorthalidone enantiomers were optimized by direct injection of aqueous standard solutions into the chiral column. Initially, different water/methanol and water/acetonitrile eluents were tested as mobile phase for separation. However, in all instances, every enantiomer leaded to a pair of peaks, which were partially overlapped. The relative intensities of the peaks observed for each enantiomer were found to be highly dependent on the pH of the aqueous component of the mobile phase, as well as on the eluent strength. This effect was not observed when chlorthalidone was chromatographed with conventional (achiral)

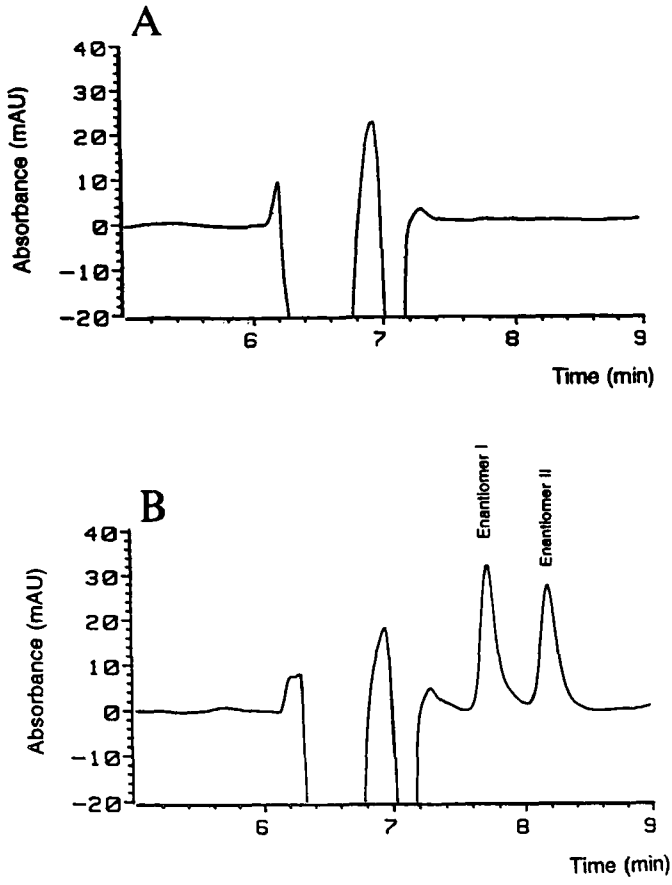

Figure 4. Chromatograms obtained for (A) blank urine and (B) urine spiked with racemic chlorthalidone. Concentration of each enantiomer, 2.5 μg/mL. For experimental details, see text.

reverse phase columns,[10,11] and it is most probably due to the partial ionization of the drug.[1] Since the pH is a critical variable, we tested acetate and phosphate buffers adjusted to different pH in the 3 - 7 interval. Best results were obtained with a mixture methanol/acetate buffer adjusted to pH 4, (60:40, v:v), at a flow rate of 1 mL/min. Under these conditions, each enantiomer was eluted as a single peak, and both enantiomers were base-line resolved (Figure 3A).

Table 2

Accuracy for Chlorthalidone in Urine (n = 3)

Added Concentration (μg/mL)	Subject Number	Isomer I		Isomer II	
		Determined Concentration (μg/mL)	E_r (%)	Determined Concentration (μg/mL)	E_r (%)
0.375	1	0.36 ± 0.01	- 4.0	0.38 ± 0.01	+ 1.3
	2	0.38 ± 0.02	+ 1.3	0.38 ± 0.01	+ 1.3
	3	0.39 ± 0.01	+ 3.0	0.375 ± 0.001	0.0
1.25	1	1.28 ± 0.09	+ 2.4	1.28 ± 0.03	+ 2.4
	2	1.35 ± 0.09	+ 8.0	1.37 ± 0.07	+ 9.6
	3	1.36 ± 0.06	+ 8.8	1.29 ± 0.06	+ 3.2
2.5	1	2.49 ± 0.01	- 0.4	2.54 ± 0.06	+ 1.6
	2	2.56 ± 0.09	+ 2.4	2.60 ± 0.06	+ 4.0
	3	2.48 ± 0.02	- 0.8	2.48 ± 0.04	- 0.8
3.75	1	3.79 ± 0.06	+ 1.1	3.80 ± 0.09	+ 1.3
	2	3.80 ± 0.05	+ 1.3	3.7 ± 0.1	- 1.3
	3	3.78 ± 0.04	+ 0.8	3.70 ± 0.04	- 1.3

Column-Switching System

The described configuration (Figure 2) allows the quantification of the total amount of each enantiomer present in the samples. On the basis of previous experiences, we selected a Hypersil stationary phase for packing the precolumn and water was used as mobile-phase for washing-out the urinary endogenous components.[10] The breakthrough volume of chlorthalidone in this precolumn was found to be higher than 15 mL of water, which means that large volumes of water can be used to remove matrix components from the precolumn.

As can be seen by comparing figures 3A and 3B, with the set-up used we did not observe additional peak dispersion compared with direct injection of the analytes

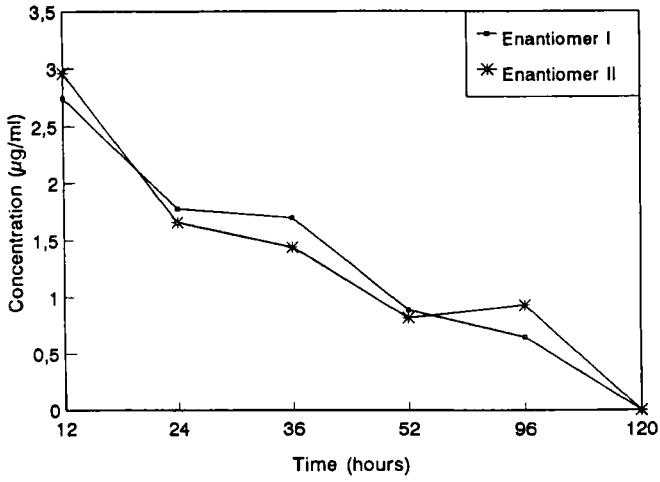

Figure 5. Urinary excretion-time profile of chlorthalidone enantiomers. Dose administered, 25 mg. For experimental details, see text.

into the chiral column. Negative peaks observed can be explained by the absorption properties of the acetate buffer used for elution.[12] Indeed, these peaks were much more intense when using the switching approach because the volume of water introduced in the flow scheme of Pump 2 is larger. However, these system peaks do not interfere with the quantitation of chlorthalidone enantiomers.

Analysis of Urine Samples

A vast majority of the urinary endogenous compounds are eliminated from the precolumn with the first 1.5 mL fraction of water used in the clean-up step. However, in order to prevent the injection of medium polarity matrix components into the chiral column, the flushing stage was extended to 4 min, so the precolumn was flushed with 4 mL of water. As a result, excellent selectivity is achieved. This is illustrated in Figure 4, which shows typical chromatograms obtained for blank urine and urine spiked with racemic chlorthalidone.

Some relevant analytical parameters obtained with the described procedure are summarized in Table 1. Linearity and reproducibility were satisfactory over the studied interval, analyte recoveries being also reasonable. No significant differences between enantiomers were observed.

Accuracy of the method was evaluated by determining the concentration of chlorthalidone enantiomers in spiked urine samples. The results of these studies are shown in Table 2. As can be seen from this table, the method provides concentrations close to the real ones in all cases tested, with relative errors ranging from -4 % (for the enantiomer I at a concentration 0.375 μg/mL) to +9.6 % (for enantiomer II at a concentration 1.25 μg/mL). The limit of detection (calculated as the concentration required to generate a signal-to-noise ratio of 3) was 20 ng/mL for both enantiomers (Table 1). This sensitivity is about 5 times lower than that obtained for a racemic mixture of chlorthalidone when an achiral C_{18} column (of the same length) and a similar switching device were used.[10]

Utility

Since chlorthalidone is clinically used as a racemate, the utility of the described approach was tested by measuring the concentration chlorthalidone enantiomers levels in urine after a single dose administration of the racemic drug. The results obtained are shown in Figure 5. Both enantiomers can be detected at least 96 hours after drug administration. Therefore, the sensitivity of the described approach can be considered satisfactory for most applications concerning the determination of chlorthalidone enantiomers in urine, taking into account the pharmacological properties of this diuretic.[12]

The total analysis time, including precolumn reequilibration, takes about 10 min, and the system can be repeatedly used with satisfactory stability for several injections. However, occasional precolumn replacement (every 100 injections) is recommended to ensure suitable performance of the chiral column.

CONCLUSIONS

The described approach illustrates the potential of column-switching for the on-line determination of chlorthalidone enantiomers in urine. The described system does not introduce peak dispersion, which means that additional columns to effect peak compression are not required. Therefore, determination of the total amount of both isomers can be achieved using a very simple set-up. In addition, since sample clean up and enrichment are on-line performed, no manipulations of the samples are involved, so, the system is well suited for fully automation. Compared with conventional columns, the employment of a chiral phase diminishes the sensitivity by a factor of about 5. However, chlorthalidone enantiomers can be determined with suitable accuracy and precision, the sensitivity being also satisfactory for most applications.

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