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Chromatographic separation of chlorthalidone enantiomers using β-cyclodextrins as chiral additives

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Abstract

Different β-cyclodextrins have been tested as chiral additives in the mobile phase for the chromatographic analysis of chlorthalidone enantiomers in a C_{18} LiChrospher (125×4 mm I.D.) column. The effect on enantioresolution of different parameters was studied: composition of the mobile phase (percentage of organic solvent, type of buffer and pH), mobile phase flow-rate, and type and concentration of β-cyclodextrin. A 25:75 mixture of methanol and 0.1 *M* phosphate buffer, pH 4, containing 2% triethylamine (v/v), and 12.5 m*M* β-cyclodextrin, at a flow-rate of 0.8 ml/min, was found to be the best option for the resolution of chlorthalidone enantiomers. Under such conditions, linear calibration curves were obtained in the 0.5–20-μg/ml interval using UV detection at 230 nm. The limit of detection for both isomers was 50 ng/ml. The utility of the described assay has been tested by analyzing chlorthalidone in different pharmaceutical preparations. Examples of application to biological samples are also given.

Keywords: Enantiomer separation; Chlorthalidone

1. Introduction

Chlorthalidone is a diuretic widely used in the treatment of hypertension and edema. The increasing consumption of chlorthalidone, alone and in combination with other therapeutic agents, has resulted in a great interest in analytical methods allowing the detection and quantification of this compound in different types of samples. For this purpose, many analytical procedures have been developed, including UV spectroscopy, gas chromatography (GC) and high-performance liquid chromatography (HPLC) [1]. In the last years the analysis of chlorthalidone has been extended to the separation and determination of enantiomers (see Fig. 1), as the two isomers can show different biological activities [2]. HPLC has proved to be an effective technique for the enantioseparation of chlorthalidone, and different methods using chiral stationary phases have been described [3–7].

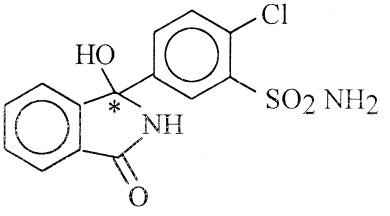

Fig. 1. Chemical structure of chlorthalidone (the asterisk indicates the chiral centre).

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In chiral analysis, the addition of a chiral component to the mobile phase is a technique undergoing rapid development. In particular, the addition of cyclodextrins seems to be a useful alternative for the enantioresolution of several drugs. Cyclodextrins are cyclic oligosaccharides consisting of six, seven or eight glucopyranose units (commonly referred to as α-, β- and γ-cyclodextrins, respectively) with the ability to form inclusion complexes. Cavity size is the major determinant as to which cyclodextrin is used in complexation. The cavity size of β-cyclodextrin is well suited for a variety of compounds of interest in the biomedical field. Moreover, the structure of the β-cyclodextrin can be modified by the selective substitution of some hydroxyl groups. Partial substitution is more likely to occur at the hydroxyl groups at C-2 and C-6 positions of the glucopyranose units [8]. The apparent cavity size can be modified with the introduction of a bulky group by substitution on some of the C-2 hydroxyl groups. Substitution can also introduce additional sites for interaction [8,9]. Derivatization has also been proposed to increase solubility, as β-cyclodextrin shows an anomalous low solubility in aqueous–organic solvents (chiral separations often require relatively high concentrations of the selector to move complexation towards its maximum). For these reasons, the employment of β-cyclodextrins (β-cyclodextrin and its derivatives) in the mobile phase offers the advantage of flexibility, a wide range of possible additives and often a lower cost compared with the equivalent chiral stationary phase [8]. Cyclodextrins are also extensively used as chiral selectors in capillary zone electrophoresis.

Chlorthalidone has been used as a model compound in different theoretical studies on the resolution of optical isomers using β-cyclodextrin additives in HPLC [8,10]. Immobilized β-cyclodextrins have also been used for the separation of chlorthalidone enantiomers [3,5–7]. In the last years, the enantioresolution of this diuretic has also been reported using β-cyclodextrins as chiral selectors in capillary electrochromatography [11] and in capillary zone electrophoresis [12]. However, most of these assays deal with fundamental aspects of the separations, and they have not been applied to the quantification of chlorthalidone enantiomers in real samples.

In this paper, we have studied the utility of different β-cyclodextrins as chiral additives in the chromatographic separation of chlorthalidone enantiomers. A final procedure for the analysis of chlorthalidone enantiomers is presented. The proposed conditions have been applied to measure chlorthalidone in different types of samples.

2. Experimental

2.1. Apparatus

The chromatographic system used consisted of a quaternary pump (Hewlett-Packard 1050 Series, Palo Alto, CA, USA), and an automatic sample injector (Hewlett-Packard 1050 Series) with a sample loop injector of 100 μl. For detection, a UV detector (Hewlett-Packard 1100 Series) was used. The detector was linked to a data system (Hewlett-Packard HPLC Chem Station) for data acquisition and storage. The chromatographic signal was monitored at 230 nm. All the assays were carried out at ambient temperature.

2.2. Reagents

All the reagents were of analytical grade. Racemic chlorthalidone was obtained from ICI-Pharma (Pontevedra, Spain). β-Cyclodextrin and (2-hydroxypropyl)-β-cyclodextrin were purchased from Fluka (Buchs, Switzerland), and triethylamine (TEA) and methyl-β-cyclodextrin were obtained from Aldrich (Steinheim, Germany). Methanol was of HPLC grade (Scharlau, Barcelona, Spain). Phosphoric acid, acetic acid and sodium acetate were obtained from Probus (Badalona, Spain). Sodium dihydrogen phosphate monohydrate (Merck, Darmstadt, Germany) was also used.

2.3. Preparation of solutions

Stock standard solutions of chlorthalidone (1000 μg/ml) were prepared in water. Working solutions of the analyte were prepared by dilution of the stock solutions with water. Water was distilled, deionized and filtered in 0.45-μm nylon membranes (Teknok-

roma, Barcelona, Spain). All solutions were stored in the dark at 2°C.

2.4. Column and mobile phases

A LiChrospher 100 RP_{18}, 5 μm, 125 mm×4 mm I.D. (Merck) column was used for the separation of chlorthalidone enantiomers. The mobile phase consisted of different mixtures of acetonitrile and a buffer (phosphate or acetate) containing the chiral additive. The phosphate and acetate buffers were prepared by dissolving sodium dihydrogen phosphate monohydrate or sodium acetate, respectively, in water; TEA (2%, v/v) was also added. The β-cyclodextrin was added to the resulting solution, and finally, the pH was adjusted to the appropriate value by adding phosphoric acid or acetic acid. All solutions were filtered with 0.45-μm nylon membranes, (Teknokroma, Barcelona, Spain) and degassed with helium before use.

2.5. Analysis of pharmaceuticals

Normapresil tablets (Laboratorio Semar, Barcelona, Spain) and Transitensin tablets (Ciba-Geigy, Barcelona, Spain) labelled to contain 25 and 10 mg of chlorthalidone, respectively, were analyzed according with the proposed conditions. The tables were weighed and powdered, and the required amount was dissolved in the minimum amount of methanol. The solution was further diluted with water to the appropriate volume, and 20 μl were then analyzed according with the proposed conditions. Each sample was assayed in triplicate.

2.6. Analysis of urine and plasma samples

For conditioning of the urine or plasma samples, C_{18} solid-phase extraction cartridges Bond Elut, 100 mg/ml (Varian, Harbor City, CA, USA) were employed. Sample conditioning was carried out as follows: the cartridges were conditioned by drawing with 1 ml of methanol followed by 1 ml of water; next, 1 ml of the samples was drawn through the cartridges, and endogenous compounds were flushed out with 2 ml of water; the cartridges were then dried with air, and the analyte was eluted from the cartridges with 1 ml of methanol. Finally, 20-μl aliquots of the extracts were injected into the chromatograph. Each sample was assayed in triplicate.

3. Results and discussion

3.1. Resolution of chlorthalidone enantiomers

3.1.1. Chromatographic conditions

Initially, we tested the influence of the mobile phase composition on resolution. In this study, underivatized β-cyclodextrin was added to the mobile phase, as successful enantioresolution had been previously obtained when using this chiral selector in the stationary phase [7]. Methanol was the organic component of the mobile-phase, whereas a phosphate or an acetate buffer (at different pH values) was the aqueous component of the eluent. According with published results, TEA (2%, v/v) was also added to the mobile phase [7,8].

Preliminary experiments showed that the type of buffer does not significantly affect resolution. However, lower background noise was observed when using the phosphate buffer, probably because under such conditions the mobile phase presents a negligible absorbance at 230 nm. In contrast, solvents containing acetate buffers exhibit a significant absorbance at wavelengths lower than 250 nm [13]. Since chlorthalidone response increases as the wavelength decreases, a methanol–phosphate buffer mixture was preferred.

As illustrated in Fig. 2, for a given mobile-phase flow-rate the percentage of methanol in the mobile phase significantly affects the separation of the isomers. For a fixed concentration of β-cyclodextrin (10 m*M*) and a mobile phase flow-rate of 0.8 ml/min, the minimum percentage of methanol that provided near baseline enantioresolution was 25%. Lower methanol contents resulted in better resolution, but under such conditions the retention times drastically increased. For example, 30% of methanol led to retention times of 9.7 and 10.6 min for chlorthalidone enantiomers, whereas 20% of methanol led to retention times of 29.8 and 34.5 min for chlorthalidone enantiomers. It should be noted that the percentage of methanol in the mobile phase also determines the solubility of the chiral selector.

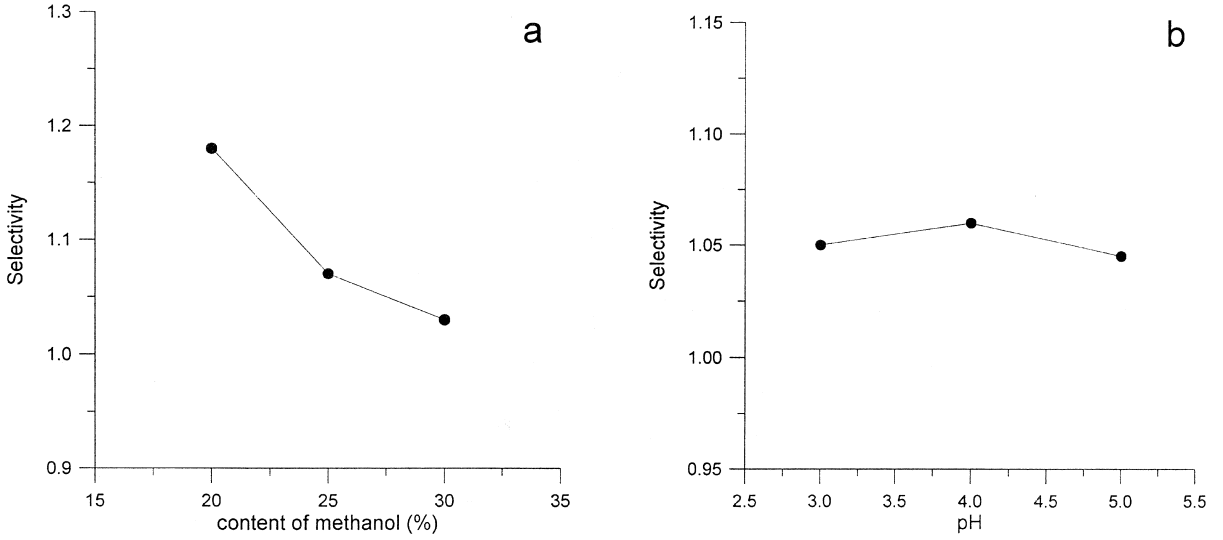

Fig. 2. (a) Effect of the concentration of methanol in the mobile-phase on the separation selectivity. Composition of the aqueous component of the mobile phase, 0.1 *M* phosphate buffer, pH 4, containing 2% TEA (v/v) and 10 m*M* β-cyclodextrin; mobile phase flow-rate, 0.8 ml/min. (b) Effect of pH of the buffer on the separation selectivity. Mobile phase, methanol–0.1 *M* phosphate buffer (25:75) containing 2% TEA and 10 m*M* β-cyclodextrin; flow-rate, 0.8 ml/min. For other experimental details, see text.

On the other hand, the pH has been reported to be a parameter affecting the separation of chlorthalidone enantiomers when using β-cyclodextrins [7,12]. Consequently, the effect of the pH of the aqueous component of the mobile phase on the separation was investigated. The results obtained (for a phosphate buffer) are shown in Fig. 2b. Although the retention times increased as the pH was increased, the separation was not significantly affected by the pH. However, the separation selectivity was slightly better at pH 4. For subsequent work the pH of the buffer was adjusted at 4.

Finally, the effect on resolution of the mobile-phase flow-rate was examined for a mobile phase consisting of methanol–phosphate buffer (25:75) (data not shown). A mobile-phase flow-rate of 0.8 ml/min was selected as a compromise between resolution and time of analysis.

3.1.2. Effect of the type and concentration of β-cyclodextrin

Different cyclodextrins, native β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin and methyl-β-cyclodextrin, were compared as chiral additives for the enantioresolution of chlorthalidone. In all instances, a methanol–phosphate buffer, pH 4 (25:75, v/v), at a flow-rate of 0.8 ml/min was used for elution. The results obtained are depicted in Fig. 3. Best separation was achieved with underivatized β-cyclodextrin. Although the resolution could be improved by increasing the concentration of selector when using (2-hydroxypropyl)-β-cyclodextrin or methyl-β-cyclodextrin, underivatized β-cyclodextrin was the only selector that provided (partial) enantioresolution at tested concentration (6.46 m*M*).

On the other hand, the concentration of chiral additive has a significant effect on resolution. Fig. 4 depicts the effect of the concentration of native β-cyclodextrin on the separation (for a mobile phase containing 25% of methanol). This figure shows that the separation is improved as the concentration of the chiral agent is increased. As expected, the retention time decreases as the concentration of chiral additive in mobile-phase increases, due to the formation of the analyte–cyclodextrin complexes in the mobile-phase. A concentration of 12.5 m*M* of underivatized β-cyclodextrin was selected as the best option for the separation of chlorthalidone enantiomers, with reasonable retention times. It should be noted that the optimum selector concentration depends on the percentage of organic solvent in the mobile phase.

On the basis of the results presented in the above

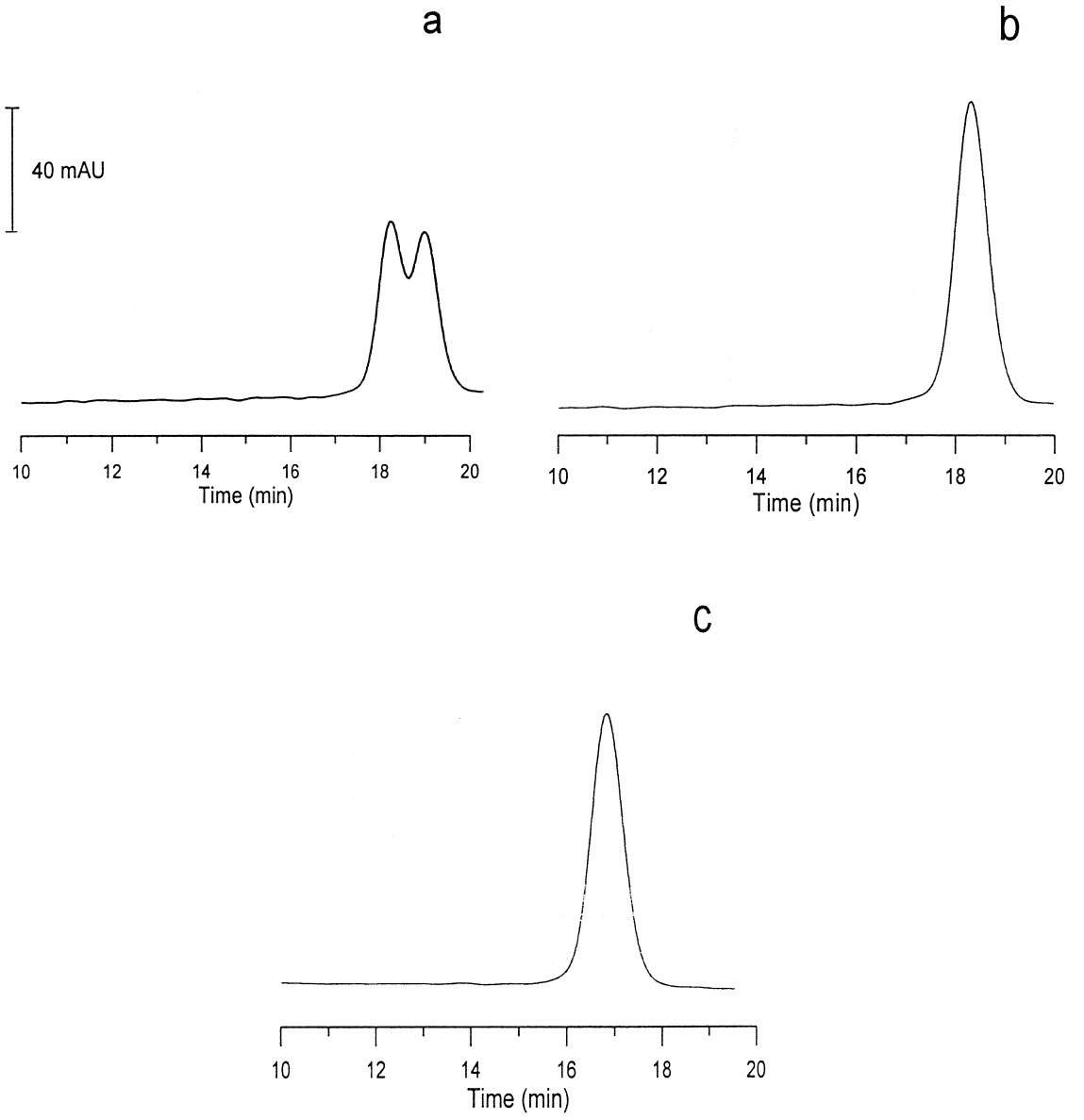

Fig. 3. Chromatograms obtained for chlorthalidone enantiomers (5 μg/ml, each isomer) with: (a) native β-cyclodextrin, (b) (2-hydroxypropyl)-β-cyclodextrin and (c) methyl-β-cyclodextrin. Mobile phase, methanol–0.1 *M* phosphate buffer, pH 4 (25:75), containing 2% TEA (v/v), and 6.5 m*M* of the chiral additive; flow-rate, 0.8 ml/min. For other experimental details, see text.

sections, the conditions finally selected for the analysis of chlorthalidone enantiomers were as follows: methanol–0.1 *M* phosphate buffer (25:75, v/v) containing 12.5 m*M* β-cyclodextrin and 2% TEA (v/v), pH 4, at a flow-rate of 0.8 ml/min. Under such conditions near baseline separation was achieved (separation selectivity, $\alpha=1.12$), the number of theoretical plates being $n=4751\pm231$ ($n=6$). As an example, Fig. 5 shows the chromatogram obtained for a solution of racemic chlorthalidone (5 μg/ml, each isomer). Although the enantiomers were not processed separately, previously published re-

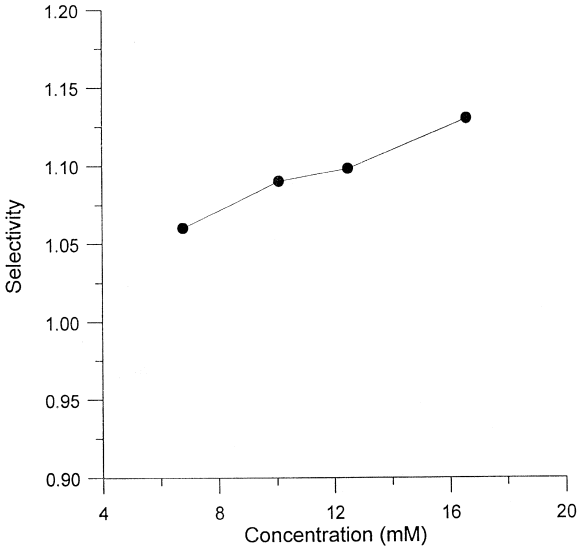

Fig. 4. Effect of the concentration of native β-cyclodextrin on the separation selectivity. Mobile phase, methanol–0.1 *M* phosphate buffer, pH 4 (25:75), containing 2% TEA (v/v), and the chiral additive; flow-rate, 0.8 ml/min. For other experimental details, see text.

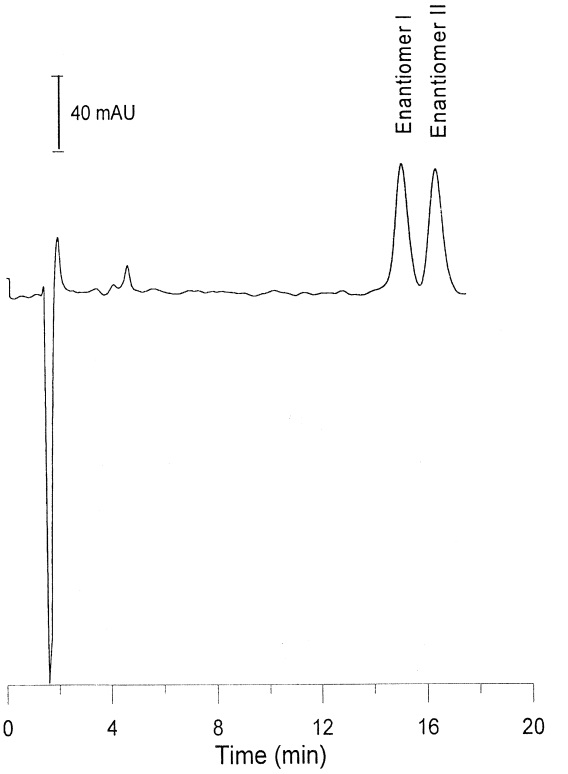

Fig. 5. Chromatogram obtained for an standard solution of chlorthalidone (at a concentration of 5 μg/ml, each enantiomer) under the proposed conditions. Mobile phase, methanol–0.1 *M* phosphate buffer, pH 4 (25:75), containing 2% of TEA (v/v), and 12.5 m*M* of the chiral additive; flow-rate, 0.8 ml/min. For other experimental details, see text.

sults suggest that the first eluting peak most probably corresponds to (+)-chlorthalidone [8].

3.2. Quantification of chlorthalidone enantiomers

The reliability of the described method was tested by analyzing aqueous solutions containing chlorthalidone in the 0.5–20.0-μg/ml interval (each isomer). The linearity was evaluated by plotting the peak area against the concentration of analyte. Table 1 lists the results obtained. As can be deduced from this table, the calibration curves were linear. Moreover, no significant differences in the calibration curves between both enantiomers were observed. On the other hand, the resolution between both enantiomers was found to be approximately constant for the different concentrations assayed within the interval tested. The intra- and inter-day reproducibilities were also satisfactory, with coefficient of variation ranging from 1 to 7% and 2 to 9%, respectively (Table 1). In principle, suitable results can be expected in the quantification of non-racemic mixtures of chlorthalidone containing similar concentrations of both enantiomers. However, a large amount of the first-eluting enantiomer could disturb the measurement of the second-eluting enantiomer. In such a case, quantification by using peak heights instead of peak areas is preferable.

The accuracy of the method was evaluated by analyzing samples with different concentrations of the analyte within the interval tested. As can be seen in Table 2, the proposed assay provides satisfactory results, with relative errors ranging from +1 to −7% (the later value was obtained at the lowest concentration assayed).

The limits of detection were 50 ng/ml for both isomers. This value is similar to that found by the equivalent procedure using immobilized β-cyclodextrin in the stationary phase [7]. However, the proposed method was found to be less sensitive that

Table 1
Linearity and reproducibility data

Analyte	Linearity[a] ($y = a + bx$)			Reproducibility		
	$a \pm S_a$	$b \pm S_b$	$t_{calc.}$[b]	Conc. (μg/ml)	Intra-day (%) ($n=3$)	Inter-day (%) ($n=8$)
Enantiomer I	23±21	82±2	41	5	5	5
				10	3	9
				15	4	3
Enantiomer II	27±24	82±2	41	5	7	9
				10	1	8
				15	4	2

[a] $n=15$.
[b] $t_{calc.} = b/S_b$; $t_{tabulated} = 3.01$ for a confidence level of 99% and 13 degrees of freedom.

Table 2
Accuracy data ($n=3$)

Analyte	Concentration (μg/ml)		E_r
	Added	Determined	(%)
Enantiomer I	2.5	2.4±0.2	−4
	7.5	7.6±0.1	1
	16	15.52±0.09	−3
Enantiomer II	2.5	2.32±0.05	−7
	7.5	7.7±0.3	3
	16	15±1	−6

recent achiral HPLC methods for chlorthalidone. In such methods, the limits of detection are typically a few ng/ml [2,14].

The proposed procedure was also evaluated for possible interferences of compounds commonly administered with chlorthalidone. The tested compounds were the β-adrenoceptor antagonists, atenolol and oxprenolol, and the diuretic, spironolactone. Under the proposed conditions no interferences were observed with chlorthalidone enantiomers.

3.3. Application to pharmaceutical and biological samples

The applicability of the described method in chiral analysis was evaluated by determining chlorthalidone enantiomers in pharmaceutical and biological samples. Table 3 summarizes the results obtained in the analysis of the two formulations assayed, Trasitensin and Normopresil tablets (the amount of chlorthalidone being 10 and 25 mg per tablet, respectively). Compared with the declared amount of drugs, the method provided differences ranging from −9 to −4%. It should be noted that in both formulations,

Table 3
Results obtained in the analysis of pharmaceutical and urine samples

Pharmaceuticals		Amount (mg)		E_r
		Declared	Determined	(%)
Normopresil	Enantiomer I	12.5	11.5±0.4	−8
	Enantiomer II	12.5	11.4±0.6	−9
Transitesin	Enantiomer I	5	4.8±0.1	−4
	Enantiomer II	5	4.8±0.1	−4
Urine		Concentration (μg/ml)		
		Added	Determined	
	Enantiomer I	5	4.8±0.3	−4
	Enantiomer II	5	5.01±0.09	+0.2

chlorthalidone was in combination with other therapeutic agents (oxprenolol and atenol for Trasitensin and Normopresil, respectively).

For analysis of chlorthalidone in biological fluids, sample cleanup was performed in C_{18} solid-phase extraction cartridges. As an example, Fig. 6 shows the chromatograms obtained for blank urine and urine spiked with chlorthalidone at a concentration of 2.5 μg/ml, each isomer. As can be observed, the sample treatment provided satisfactory selectivity, with most matrix components eluting at retention times much lower than the analytes. Therefore, similar values of the minimum detectable amounts can be expected for both aqueous and biological samples. The selectivity was also satisfactory for plasma samples.

The practicability of the proposed method for the analysis of chlorthalidone in biological samples was evaluated by analyzing urine spiked with racemic chlorthalidone at a concentration of 5 μg/ml (2.5 μg/ml) each isomer. According with previous results, these concentrations correspond to the concentrations present in real samples 12–24 h after the administration of chlorthalidone at therapeutic concentrations [7]. The concentration of chlorthalidone enantiomers was calculated from the calibration curves obtained for the aqueous solutions, and taking into account the percent of analyte recovered after sample cleanup. Preliminary assays demonstrated that the percent of drug recovered under the described conditions was 102±4% (n=3), which is in concordance with the results presented in Ref. [15]. The levels of chlorthalidone enantiomers found are also indicated in Table 3. The values found are close to the real ones, which indicates that the present method can also be adapted to the determination of chlorthalidone in biological samples.

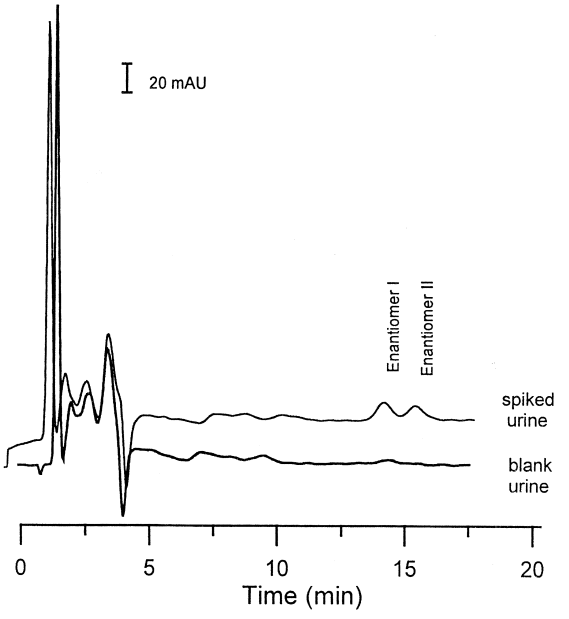

Fig. 6. Chromatograms obtained for blank urine and urine spiked with chlorthalidone enantiomers (at a concentration of 2.5 μg/ml, each enantiomer). Mobile phase, methanol–0.1 *M* phosphate buffer, pH 4 (25:75), containing 2% TEA (v/v), and 12.5 m*M* of the chiral additive; flow-rate, 0.8 ml/min. For other experimental details, see text.

4. Conclusions

This work illustrates the potential of underivatized β-cyclodextrin as a chiral additive in the mobile-phase for enantioresolution of chlorthalidone enantiomers. Other cyclodextrins such as (2-hydroxypropyl)-β-cyclodextrin or methyl-β-cyclodextrin have also been tested, but no improvement in resolution was observed compared to the nonderivatized selector. Under optimized conditions, β-cyclodextrin allows the quantification of chlorthalidone enantiomers with satisfactory linearity and reproducibility. The described assay provides limits of detection comparable to those reported by methods using immobilized β-cyclodextrin as chiral stationary phase. As regards the application to real samples, the proposed method provides satisfactory selectivity to be applied to a wide variety of samples, such as pharmaceutical preparations or biological fluids. In this latter instance, a simple solid-phase extraction with (relatively non-selective) C_{18} solid-phase extraction cartridges provided appropriate selectivity in both urine and plasma samples.

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