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Cationic β-cyclodextrin: a new versatile chiral additive for separation of drug enantiomers by high-performance liquid chromatography

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Abstract

The chromatographic separations of the enantiomers of a series of eight phenylhydantoin and methylhydantoin derivatives of amino acids are described, using reversed-phase HPLC with a chiral additive in the mobile phase: native β-cycodextrin (β-CD), carboxymethylated β-CD and a cationic β-CD derivative. Retentions time and selectivities obtained with these three chiral additives are compared. As the cationic β-CD gave promising results, its usefulness in the resolution of several drugs of particular interest (chlorthalidone, terbutaline, mephobarbital and hexobarbital) is demonstrated.

1. Introduction

The examination of drug enantiomers is currently accepted as one of the most important steps when studying the pharmacokinetic and pharmacodynamic properties of drugs with chiral centres [1]. However, in order to study these properties, the initial step involves the development of a suitable method for their separation. High-performance liquid chromatography (HPLC) has played a large part in this work, for which a number of chiral selectors, chemically bound to the stationary phase [chiral stationary phase (CSP)] or added to the mobile phase, have been investigated.

When a chiral selector is introduced into the mobile phase used with an achiral column, it offers the advantage of flexibility, a wide range of possible additives and often lower cost compared with the equivalent CSP [2]. One of the widely used chiral mobile phase additives is the cyclodextrin group (CDs). CDs are cyclic, non-reducing oligosaccharides consisting of D-glucose units bonded through α-1,4-linkages. According to the number of glucose units forming the cyclodextrin-ring (six, seven or eight), one differentiates between α-, β- and γ-cyclodextrins. β-Cyclodextrin, the most readily available of the cyclodextrins and generally the best sized complex former, shows an anomalously low solubility [3,4] in aqueous–organic solvents (1.85 g per 100 ml water). In this respect, chiral chromatograph-

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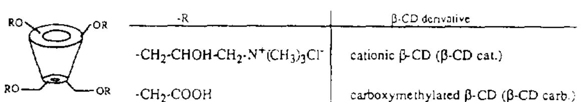


Fig. 1. β-CD derivatives used in the study.

ic separations require relatively high concentrations of CD to move complexation toward its maximum [5]. The use of urea in the eluent to enhance solubility, as recommended by Pharr et al. [6], is not a viable option in most experimental protocols as it leads to problems with baseline stability and high viscosity [7]. An alternative route to improve the solubility of CDs is available through derivatization of the CD structure at the hydroxyl groups. Several CD derivatives, such as methylated, hydroxyethyl, hydroxypropyl and acetylated β-CD, have already been produced and utilized industrially [8,9]; others have been prepared only on a laboratory scale and used for analytical or research purposes, with promising results.

In this work, carboxymethylated β-CD and a new cationic β-CD derivative (Fig. 1) were examined as chiral additives in the separation of the enantiomers of a group of eight amino acid derivatives in the form of phenylthiohydantoins (PTH) and methylthiohydantoins (MTH) and of several chiral drugs of current interest.

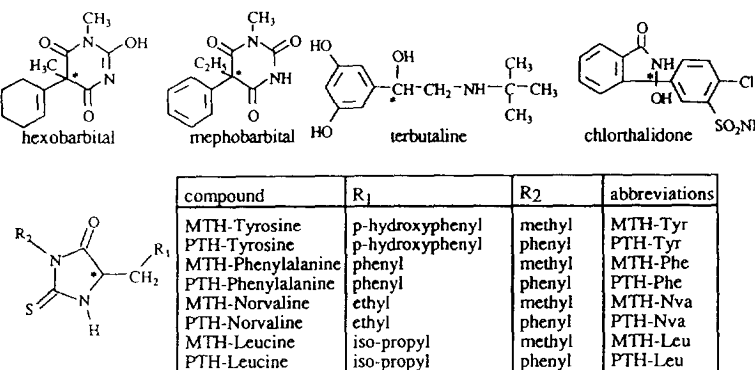
2. Experimental

2.1. Chemicals

PTH- and MTH-amino acids, chlorthalidone, terbutaline, hexobarbital and mephobarbital were obtained from Sigma (St. Louis, MO, USA) and were used without further purification (Fig. 2).

Acetic acid and triethylamine (TEA) were purchased from Carlo Erba (Rueil-Malmaison, France) and Janssen Chimica (Geel, Belgium), respectively. Ethanol (95%) was purified by distillation.

CDs (Fig. 1) were obtained from Roquette Frères (Lestrem, France). Cationic β-CD is characterized by an average molar degree of substitution (MS), i.e., number of substituents per glucose units, of 0.45. The sample contains less than 2% of free β-CD. It was used as an aqueous solution of pH 8.4 at a concentration of $2.80 \cdot 10^{-4}$ torus/g (i.e. number of cyclodextrin torus equivalents per gram of solution). Carboxymethylated β-CD is characterized by an MS value of 0.38. The sample contains less than 3% of native β-CD and is used as an aqueous solution of pH 5.18 at a concentration of



compound	R_1	R_2	abbreviations
MTH-Tyrosine	p-hydroxyphenyl	methyl	MTH-Tyr
PTH-Tyrosine	p-hydroxyphenyl	phenyl	PTH-Tyr
MTH-Phenylalanine	phenyl	methyl	MTH-Phe
PTH-Phenylalanine	phenyl	phenyl	PTH-Phe
MTH-Norvaline	ethyl	methyl	MTH-Nva
PTH-Norvaline	ethyl	phenyl	PTH-Nva
MTH-Leucine	iso-propyl	methyl	MTH-Leu
PTH-Leucine	iso-propyl	phenyl	PTH-Leu

Fig. 2. Structures of solutes studied.

$2.93 \cdot 10^{-4}$ torus/g. These MS values only indicate the average degree of substitution, without a precise localization on the torus.

2.2. Equipment

HPLC was performed with a Merck Hitachi LiChroGRAPH L-6000 HPLC pump, a Merck Hitachi LiChroGRAPH L-4000 UV detector and a Merck D2000 recorder. Separations were carried out with a Merck LiChroCART 250-4 Superspher 100 RP-18 column (4 μm, 250 × 4 mm I.D.).

Acetate buffer used in the mobile phases is an aqueous solution of 0.8% (v/v) of TEA, the pH of which is adjusted with acetic acid. Phosphate buffer is obtained by dissolution of 1.776 g/l of NaH_2PO_4 in water and the pH is adjusted with orthophosphoric acid. All the mobile phases were filtered through a Millipore HV membrane filter (0.45 μm) and were degassed prior to use by a vacuum ultrasonic method. Sample solutions were prepared so as to give a concentration of 8 mg/l for each solute. The amount of the sample injected was 20 μl. All chromatograms were obtained at 22.5°C. UV detection was performed at 264 nm for the amino acid derivatives, at 254 nm for chlorthalidone and barbiturates and at 275 nm for terbutaline. The dead volume was determined by injection of sodium nitrate.

2.3. Determination of the elution orders

The elution orders were determined by injection of partially resolved mixtures obtained by preparative chromatography on a microcrystalline cellulose triacetate column [10]. The elution order of the enantiomers of terbutaline, which is not resolved with this chromatographic system, was not determined.

2.4. Calculation of the stability constants

The determination of the stability constants, K_s, using the HPLC method with addition of cyclodextrin to an aqueous mobile phase has already been described [11]. For a neutral solute, Eq. 1, which expresses the stability constant K_s of a complex from chromatographic data, has been established theoretically [12,13]:

$$\frac{1}{k'} = \frac{1}{k'_0} + \frac{K_s[\mathrm{CD}]^n_\mathrm{T}}{k'_0} \quad (1)$$

where k'_0 is the capacity factor obtained in the absence of CD, k' is the capacity factor of the sample solute with a concentration $[\mathrm{CD}]_\mathrm{T}$ of cyclodextrin in the mobile phase and n is the stoichiometry of the complex. It is clear from Eq. 1 that k' shows a hyperbolic dependence on $[\mathrm{CD}]_\mathrm{T}$ and a plot of $1/k'$ vs. $[\mathrm{CD}]^n_\mathrm{T}$ gives a straight line whose slope is equal to K_s/k'_0. For the determination of the stoichiometries of CD complexes, several chromatographic runs with different CD concentrations are needed. When the stoichiometry of the complexes is known or assumed, only two chromatographic analyses are useful: one to determine k'_0 and the other to determine k' at a known CD concentration.

3. Results and discussion

Chromatographic separations, using CDs as mobile phase modifiers, are largely the result of selectivity in the formation of inclusion complexes. The elution time of a given solute is a function of the strength of these complexes. These two driving forces act synergistically and are directly related to the properties of the guest and to the interactions that may be engaged with the CD [14–16]. Hence, it is important to consider that in the derivatized cationic or carboxymethylated β-CD, the apparent diameter of the toroidal cone rim can be modified with the introduction of a more bulky group, by the selective substitution of some of the hydroxyl groups. Moreover, the effect of substitution would be expected to affect the extent of interaction with the side-chain of the analyte. These interaction modifications with the derivatization of the CD rim may be evaluated through the determination of the stability constants of the complexes.

The stability constants K_s of the complexes formed with the eight MTH- and PTH-amino acids were determined with β-CD, cationic β-CD and carboxymethylated β-CD, and are reported with the corresponding chromatographic data in Table 1. K_s values were calculated according to Eq. 1 on the basis of the chromatographic results from one concentration in the case of carboxymethylated β-CD (25 mM) and from two different concentrations in the case of cationic β-CD (25 and 40 mM). In the latter instance, the stoichiometry of the complexes was controlled by inspection of the plots $1/k'$ vs. cationic β-CD concentration for compounds that have maximum steric hindrance either in position R_1 (MTH-phenylalanine) or R_2 (PTH-norvaline), or in both of these positions (PTH-tyrosine). Obtaining a good linear correlation demonstrates the 1.1 stoichiometry of the complexes, as the occurrence of higher order of complexation would give a non-linear correlation between $1/k'$ and cationic β-CD concentration.

According to the results in Table 1, the use of carboxymethylated β-CD does not lead to a change compared with the use of native β-CD: the retention times are similar. The separations with carboxymethylated β-CD of compounds that are baseline separated with β-CD (MTH-tyrosine and MTH-phenylalanine) are not improved. All the other analyte separations are simply maintained or become worse. This may be explained by very weak stability constants: the inclusion is hindered by the carboxymethylation of the CD. However, a significant linear correlation (Fig. 3) is found between the complexing capacities of β-CD and carboxymethylated β-CD, if we do not consider the singular K_s value obtained with the laevorotatory enantiomer of MTH-tyrosine. This demonstrates that when inclusion may occur, it is governed by the same factors with the two chiral additives.

The K_s values obtained in presence of cationic β-CD (Table 1), if we do not consider the (−)-enantiomer of MTH-Tyr, are also well correlated with those determined with β-CD (Fig. 4, $R = 0.958$). The slope value ($s = 0.759$) and the intercept, which deviate significantly from unity and zero, respectively, indicate a weak complex-forming ability of cationic β-CD compared with native β-CD. The cationic groups modify the accessibility of the CD cavity and hinder the inclusion of the solute, which in turn results in decreased complex stability. Nevertheless, when inclusion occurs, new and more selective interactions take place, which result in a sufficiently large difference between the stability constants of the complexes formed with each enantiomer. This results in either an improved selectivity (MTH-Tyr) or emergence of selectivity (PTH-Phe and MTH-Leu).

Further, according to the high solubility of cationic β-CD in reversed mobile phases, a selectivity decrease may be compensated for with an increase in chiral additive concentration; for instance, with MTH-Phe enantiomers, the selectivities obtained with 25 and 40 mM cationic β-CD concentrations ($\alpha = 1.149$ and 1.188) straddle the value obtained with β-CD (25 mM; $\alpha = 1.177$).

Following the promising results obtained in the previous chiral chromatographic separations, it was of interest to establish the usefulness of cationic β-CD in the resolution of different drugs. The resolutions of the diuretic and hypertensive chlorthalidone and the bronchodilator terbutaline were reproduced according to Walhagen and Edholm [17] in the presence of

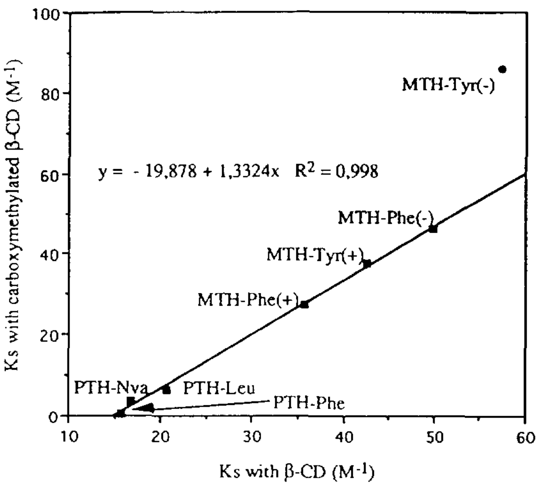


Fig. 3. Correlation between K_s values obtained with β-CD and carboxymethylated β-CD.

Table 1
Study of the influence of β-CD, cationic β-CD and carboxymethylated β-CD

Compound	Parameter	Without CD	25 mM β-CD	Cationic β-CD		25 mM carboxymethylated β-CD	K_s (l/mol)
				25 mM	40 mM		
MTH-Tyrosine	t_r	13.12	6.38(−) 7.07(+)	6.68(−) 7.88(+)	5.52(−) 6.56(+)	6.06(−) 7.78(+)	β-CD: 57.4(−); 42.6(+)
	k'	3.46	1.50(−) 1.77(+)	1.61 2.08	1.16 1.57	1.24 1.88	β-CD cat.: 50.9(−); 30.3(+)
	α	1.000	1.180	1.291	1.356	1.517	β-CD carb.: 86.1(−); 37.8(+)
PTH-Tyrosine	t_r	24.71	18.32	20.51	18.42	24.27	β-CD: 5.4
	k'	7.41	6.18	7.01	6.22	7.99	β-CD cat.: 0.1
	α	1.000	1.000	1.000	1.000	1.000	β-CD carb.):—
PTH-Phenylalanine	t_r	164.64	90.29	106.64(−) 111.18(+)	88.19 93.04	130.00 131.87	β-CD: 15.7
	k'	55.00	34.41	40.66 42.43	33.58 35.49	47.15 47.84	β-CD cat.: 7.1(−); 5.2(+)
	α	1.000	1.000	1.044	1.057	1.015	β-CD carb.: 0.6; 0.05
MTH-Phenylalanine	t_r	60.42	23.51(−) 27.21(+)	31.31(−) 35.58(+)	24.36 28.46	26.66(−) 33.26(+)	β-CD: 49.9(−); 35.7(+)
	k'	19.55	8.22(−) 9.67(+)	11.23(−) 12.90(+)	8.55 10.16	8.87 11.32	β-CD cat.: 28.0(−); 19.2(+)
	α	1.000	1.177	1.149	1.188	1.276	β-CD carb: 46.1(−)27.5(+)
PTH-Norvaline	t_r	75.74	45.91(−) 47.10(+)	58.30	50.94	58.24	β-CD: 16.8(−); 15.6(+)
	k'	24.76	17.00(−) 17.47(+)	21.77	18.98	20.57	β-CD cat.: 1.3
	α	1.000	1.027	1.000	1.000	1.000	β-CD carb.: 3.7
MTH-Norvaline	t_r	24.84	18.56	24.79	24.46	24.60	β-CD: 7.0
	k'	7.45	6.28	8.68	8.59	8.11	β-CD cat.:—
	α	1.000	1.000	1.000	1.000	1.000	β-CD carb.:—
MTH-Leucine	t_r	46.88	30.44	42.43(+) 43.79(−)	39.20(+) 41.23(−)	41.79	β-CD: 11.4
	k'	14.95	10.94	15.57(+) 16.11(−)	14.37(+) 15.17(−)	14.48	β-CD cat.:—
	α	1.000	1.000	1.034	1.056	1.000	β-CD carb.:—
PTH-Leucine	t_r	151.70	79.12(−) 81.25(+)	105.47(−) 107.66(+)	88.19(−) 91.02(+)	108.35	β-CD: 20.6(−); 19.0(+)
	k'	50.60	30.03(−) 30.86(+)	40.20(−) 41.05(+)	33.58(−) 34.69(+)	39.13	β-CD cat.: 5.3(−); 4.4(+)
	α	1.000	1.028	1.021	1.033	1.000	β-CD carb.: 6.6

Chromatographic conditions: LiChroCART 250-4 Superspher 100 RP-18 column (4 μm, 250 × 4 mm I.D.); mobile phase, ethanol–acetate buffer (pH 4.1) (20:80, v/v) + 25 mM torus CDX; flow rate, 0.8 ml/min; temperature, 22.5°C.

native β-CD and attempted with cationic β-CD. The chromatographic data and the chromatograms are reported in Table 2 and Fig. 5, respectively. The two compounds are well resolved with each chiral additive. However, the elution of chlorthalidone enantiomers, which is similar in the two cases, results in a decrease in resolution with cationic β-CD. This decrease is not significant for the terbutaline enantiomers, in spite of a great difference in elution time.

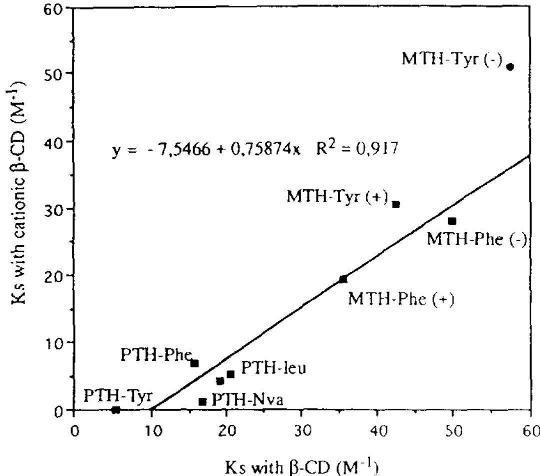


Fig. 4. Correlation between K_s values obtained with β-CD and cationic β-CD.

Barbiturates, which are widely used as anaesthetics, sedatives, hypnotics, anticonvulsants, etc., may be resolved into enantiomers of different pharmacological activity. Differences in the pharmacological effectiveness of these compounds have been widely investigated [18–23] since the synthesis and resolutions of optically active barbiturates were reviewed. However, chromatographic resolution in the presence of β-CD [14,24,25] requires a concentration near the solubility limit, whereas the use of methylated β-CD [26,27] needs a long time to reach the equilibrium of a dynamic coating of the stationary phase. These drawbacks do not occur when using cationic β-CD: (i) it is far more soluble in water than free β-CD and (ii) no dynamic coating is required. Moreover, the chromatographic data reported in Table 2 for hexobarbital and mephobarbital show a decrease in retention with an increase in resolution and selectivity, leading to shorter and better analysis conditions. These two compounds are baseline resolved in presence of cationic β-CD (Fig. 5).

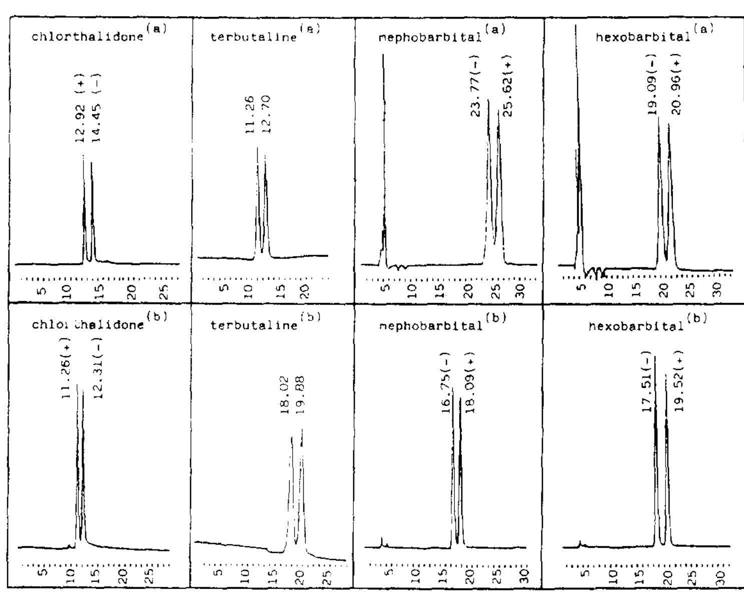


Fig. 5. Chromatograms of drugs obtained with (a) native β-CD and (b) cationic β-CD. Chromatographic conditions as described in Table 2.

Table 2
Influence of native β-CD and cationic β-CD on the enantiomeric separation of different drugs

Compound	Parameter	With β-CD	With cationic β-CD
Chlorthalidone[a]	t_r	12.92 min (+); 14.45 min (−)	11.26 min (+); 12.31 min (−)
	k'	3.857 (+); 4.432 (−)	3.504 (+); 3.924 (−)
	α	1.149	1.120
	R_s	2.5	1.5
Terbutaline[b]	t_r	11.26 min; 12.70 min	18.02 min; 19.88 min
	k'	2.657; 3.123	3.551; 4.020
	α	1.176	1.132
	R_s	1.6	1.4
Mephobarbital[c]	t_r	23.77 min (−); 25.62 min (+)	16.75 min (−); 18.09 min (+)
	k'	6.053 (−); 6.602 (+)	4.030; 4.432
	α	1.091	1.100
	R_s	1.4	1.7
Hexobarbital[c]	t_r	19.09 min (−); 20.96 min (+)	17.51 min (−); 19.52 min (+)
	k'	4.665 (−); 5.220 (+)	4.258 (−); 4.862 (+)
	α	1.119	1.142
	R_s	1.6	2.5

[a] EtOH–acetate buffer (pH 4.1) (20:80, v/v) + [CD] = 25 m*M*; flow-rate, 0.8 ml/min; temperature, 22.5°C; detection at 254 nm.
[b] Acetate buffer (pH 5.9) + 10 m*M* CD; flow-rate, 0.8 ml/min; temperature, 22.5°C; detection at 275 nm.
[c] EtOH–phosphate buffer (pH 2.5) (20:80, v/v) + 25 m*M* CD; flow-rate, 0.6 ml/min; temperature, 22.5°C; detection at 254 nm.

4. Conclusion

The use of cationic β-CD as a chiral additive in the mobile phase presents definite advantages over the use of other, commonly used, cyclodextrin chiral additives such as native β-CD or methylated cyclodextrin. The retention time and resolution may be controlled owing to the wide range of solubility of the cationic β-CD. In general, the enantioselectivities observed are at least as good as those for native β-CD under the same conditions and are potentially improved by using higher concentrations. In this work we have not taken advantage of the presence of charged nitrogen, which we believe might be used for chiral ion-pair chromatography in the future.

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