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Simultaneous separation of different enantiomeric pairs in capillary electrophoresis by mixing different hemispherodextrins, a very versatile class of receptors

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Abstract

Six different racemates of the profen family were used as analytes in order to test the chiral selector properties of three members of a new class of cyclodextrin derivatives, hemispherodextrins (HMs), in capillary electrophoresis. In addition to experiments carried out to separate each enantiomeric pair one by one, other experiments were carried out on samples containing all six enantiomeric pairs. Electropherograms were obtained either by adding a single HM to the background electrolyte (BGE), or a binary mixture of HMs. The results obtained confirm the excellent chiral selector properties of the HMs, and furthermore show that these compounds can also be used for achiral selection. When mixing different HMs, a complementary effect in chiral selectivity is observed, which, in our opinion, deserves further study.

Keywords: Enantiomer separation; Background electrolyte composition; Hemispherodextrins; Profens; Cyclodextrins

1. Introduction

In the field of chiral separations, the class of cyclic oligosaccharides called cyclodextrins (CDs) and their derivatives is a well-established and very efficient tool [1]. Their use in HPLC dates back several years and they are used both in the stationary phase and as an additive to the mobile phase. Also in CE, CDs are extensively used as an additive to the background electrolyte (BGE) [2–13]. Since the mechanism of action of CDs is similar to that observed in micellar electrokinetic chromatography (MEKC), as the CDs function as a pseudostationary phase, the technique is called, appropriately in our opinion, CD-EKC. Although several different molecules are also used in CE as chiral selectors, CDs remain by far the most used.

Unfortunately, the parent CDs are neutral compounds and thus their use with neutral guests is obviously impossible. However, also with charged guests, since there is no variation in the electric charge of the complex with respect to the free ligand, the difference in mobility between two different guest species, which permits us to exploit the different affinities of the two enantiomers for the CD and thus to obtain a chiral separation, is based on secondary effects such as dimension and shape. Thus, only in the presence of large differences in the degree of formation of the complex between the two enantiomers is satisfactory selectivity achieved. If

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the affinity between host and guest is low, high concentrations of CD are necessary, and therefore, generally, the CD concentration used is near their solubility.

Derivatization of CDs often increases their solubility, and thus the usable concentration range of the selectors, while it is the straightforward way to obtain charged CDs by introducing suitable groups. However, owing to the difficulty in obtaining pure derivatives, commercially available substituted CDs are generally mixtures of substances, differing in the degree of substitution, or in the substitution position. Obviously, being mixtures does not imply that these hosts do not work as chiral selectors, but, as stated by Tanaka et al. [14], ''the supply of a pure single CD derivative is strongly recommended in order to provide good reproducibility . . . ''.

In our laboratory, we have synthesised a series of mono- and di-substituted CDs [15–19]. More recently, a new class of cyclodextrin derivatives was designed and synthesised: they are called hemispherodextrins (HMs) [20–23]. These compounds are characterised by the presence of a trehalose-containing bridge between the primary positions of two different glucopyranosinic rings of β-cyclodextrin, thus being members of the more general class of the so-called ''capped CDs''. The novelty of HMs with respect to the other capped CDs is due to the saccharidic nature of the ''cap'' that makes it similar to the CD cavity. The hemispherical shape thus obtained is therefore saccharidic, and the characteristic behaviour of the CD cavity now can be shown by this three-dimensional structure, a sort of cage which can very efficiently separate molecules from aqueous solution. Each HM differs from the others in the moiety that binds the trehalose to the primary positions of the A and D glucopyranosinic rings of the CD cavity (Fig. 1). As can be seen, while in THAMH the bridge consists of a NH group only, in THCMH we have a four-atom group and in THALAH a five-atom group. Thus, the most relevant difference between the individual HMs is in the dimension of the ''cap'' and, consequently, of the ''cage'' that can host the analyte. In previous investigations, these CD derivatives were tested as chiral selectors for enantiomeric pairs of phenoxy-

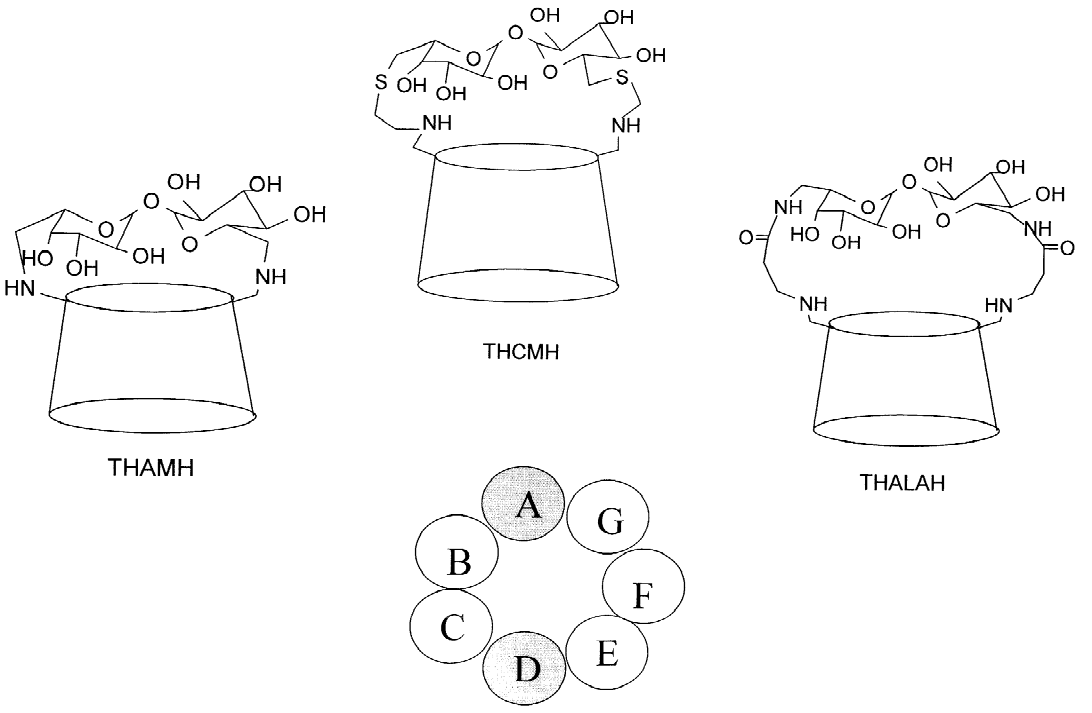

Fig. 1. Schematic structures of hemispherodextrins.

acids, and very good results were obtained [24,25]. The quality of the results can be seen by considering the low concentrations that were necessary to achieve satisfactory selectivity, as low as 0.10 m*M*, compared with the concentrations reported in the literature of up to hundreds of times this value!

The profen family of anti-inflammatory drugs are often used as analytes in CE in order to test the chiral selection ability of a receptor [26–28]. In order to obtain further information about the chiral selectivity properties shown by HMs in comparison with the receptors already reported in the literature, measurements were carried out for the separation of the enantiomeric pairs of six different profen racemates (Fig. 2). All measurements were carried out at pH 6.9, a value that ensures that the acid is present as anionic species and that the aminic hosts are mostly di-protonated, although in the presence of non-negligible amounts of mono-protonated species. The opposite charge between host and guest favours the formation of a complex, but the most important reason for using this pH value is that we have an anionic free ligand in the presence of a cationic complex. This permits us to have very different electrophoretic mobilities between these two species. Consequently, in our systems the difference in stability between the complexes formed by the two enantiomers can be fully exploited for the separation: it is sufficient for only a slight difference in degree of formation between them to obtain the necessary selectivity. Furthermore, in order to improve the resolution, the efficiency was optimised by using electrokinetic introduction of the sample.

Other measurements were carried out to try to contemporaneously separate all six pairs of enantiomers, thus testing the non-chiral selectivity of each HM. Finally, in order to test the complementarity between the different HMs, other measurements were carried out by adding binary mixtures of HMs to the BGE and this medium was used to separate a sample containing all six enantiomeric pairs.

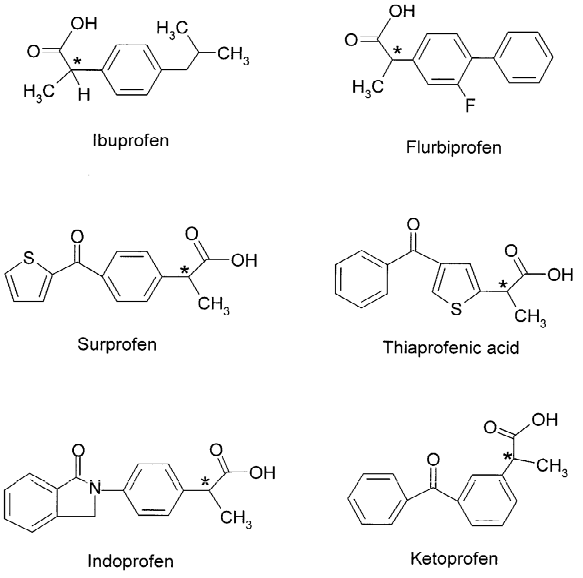

Fig. 2. Schematic structures of the profen racemates.

2. Experimental

2.1. Materials

The racemic mixtures of arylpropionic acids (flurbiprofen, ibuprofen, indoprofen, ketoprofen, suprofen and thiaprofenic acid, as shown in Fig. 2) were purchased from Aldrich. Hydrochloric acid, sodium hydroxide, sodium acetate and boric acid were obtained from Merck. THALAH, THCMH and THAMH (see Fig. 1) were synthesised in our laboratory [20–22]. Double-distilled water was used for solution preparation.

2.2. CE measurements

CD-EKC measurements were carried out on a Beckman P\ACE MDQ equipped with a diode-array detector. An uncoated fused-silica capillary (Beckman; 69 cm total length, 59.2 cm effective length, 75 μm I.D.) was held at a constant temperature of 20 °C. The system operated at a constant voltage of 25 kV.

BGEs for the chiral separation experiments were prepared by dissolving hemispherodextrins (0.30–3.0 m*M*) in an equimolar solution of 33 m*M* sodium acetate and boric acid buffer (pH 6.9). The sample solution (0.10 m*M* in racemate) was obtained by dissolving the analyte in the same buffer when the separation was carried out for one enantiomeric pair at a time. The sample solution for the simultaneous separation of all the investigated enantiomeric pairs

of profen was prepared by dissolving each racemate in the same solution, to achieve a final concentration of 0.10 m*M*. Experiments were also carried out with binary mixtures of HMs. In these cases, BGEs were prepared by mixing the chiral selectors (dissolved in the same buffer) in three different ratios, i.e. 3:1, 1:1, and 1:3, to achieve, in each case, an overall concentration of 1.0 m*M*.

The sample was injected electrokinetically (10 kV for 12 s). Before each experiment, the capillary was flushed successively (pressure $2.0 \cdot 10^6$ Pa) with 20 m*M* borate buffer, 0.1 *M* NaOH and the BGE used for the separation. The capillary was rinsed daily with 0.1 *M* HCl, water, 0.1 *M* NaOH and finally the BGE used.

The enantiomeric resolution (*R*) and selectivity coefficient (*S*) were calculated using Eqs. (1) and (2), respectively:

$$R = 2 \cdot (t_2 - t_1)/(w_1 + w_2) \quad (1)$$

$$S = 2 \cdot 10^3 \cdot (\mu_2 - \mu_1)/(\mu_1 + \mu_2) \quad (2)$$

where *t*, *w* and μ are, respectively, the migration time, width at baseline and the electrophoretic mobility. The *S* values were multiplied by 1000 in order to report fewer decimal places for the corresponding values in the tables. Resolution values of less than 1 are reported in the tables as an estimation of the potential separation capability. However, in our opinion, any value lower than unity indicates that quantitative separation was not attained, no matter how lower than unity the value is. Furthermore, it is not always clear how this value is calculated. In many cases the usual way of measuring the peak width, by determining the intersection point between the baseline and the tangent of the peak inflection point, can give erroneous results, owing to the necessary extrapolation of both the peak and the baseline.

3. Results and discussion

Table 1 summarises the results obtained for the measurements carried out for the single HM–single enantiomeric pair systems. Fig. 3 shows electropherograms for the THCMH–ibuprofen system for different HM concentrations.

Our hosts, at the pH of the buffer, are partly mono- and partly di-cationic. Owing to their positive charge, they have a tendency to interact with the capillary wall, in some way modifying the behaviour of the wall. This was readily verified by measuring

Table 1
Results of measurements carried out for single enantiomeric pairs. Values of the retention times of the slower enantiomer are reported (t_2), together with the *R* and *S* values. These were multiplied by 1000 in order to reduce the number of decimal places (see text)

C_{host} (m*M*)	Flurbiprofen (a)			Ibuprofen (b)			Indoprofen (c)			Ketoprofen (d)			Suprofen (e)			Thiaprofenic acid (f)		
	t_2	*S*	*R*	t_2	*S*	*R*	t_2	*S*	*R*	t_2	*S*	*R*	t_2	*S*	*R*	t_2	*S*	*R*
THALAH																		
3	5.99	0	0	4.69	0	0	7.47	18	1	8.82	15	1.18	7.11	0	0	6.35	11	1.08
2	6.25	0	0	4.70	0	0	7.08	10	1	8.24	11	0.53	7.03	10	0.39	6.31	12	0.67
0.8	5.70	0	0	4.87	8	0.30	6.73	20	1.44	7.47	12	0.50	6.95	16	0.60	5.93	19	0.90
0.3	5.24	0	0	4.15	29	0.36	5.87	19	0.31	6.14	0	0	6.58	22	0.41	5.60	29	0.36
THAMH																		
3[a]																		
2	8.95	39	1.94	5.42	0	0	10.89	28	1.82	10.60	55	2.33	8.06	0	0	5.21	0	0
0.8	8.26	25	1.29	4.63	0	0	9.66	0	0	8.30	0	0	6.70	0	0	8.57	12	1.35
0.3	7.64	0	0	6.60	0	0	10.97	18	1.11	6.69	0	0	6.70	0	0	8.71	39	0.86
THCMH																		
3	5.10	10	1	5.05	18	1.38	10.32	0	0	6.86	0	0	8.70	0	0	8.35	8	0.74
2	5.00	10	0.91	6.67	21	0.50	10.59	45	1.24	5.61	0	0	7.31	0	0	8.79	7	052
0.8	4.65	4	0.40	4.36	7	0.46	7.42	53	2.45	6.71	0	0	6.36	0	0	5.85	0	0
0.3	4.51	0	0	4.36	12	0.33	6.15	33	1.33	6.98	0	0	5.5	13	1.17	5.13	0	0

[a] No data available.

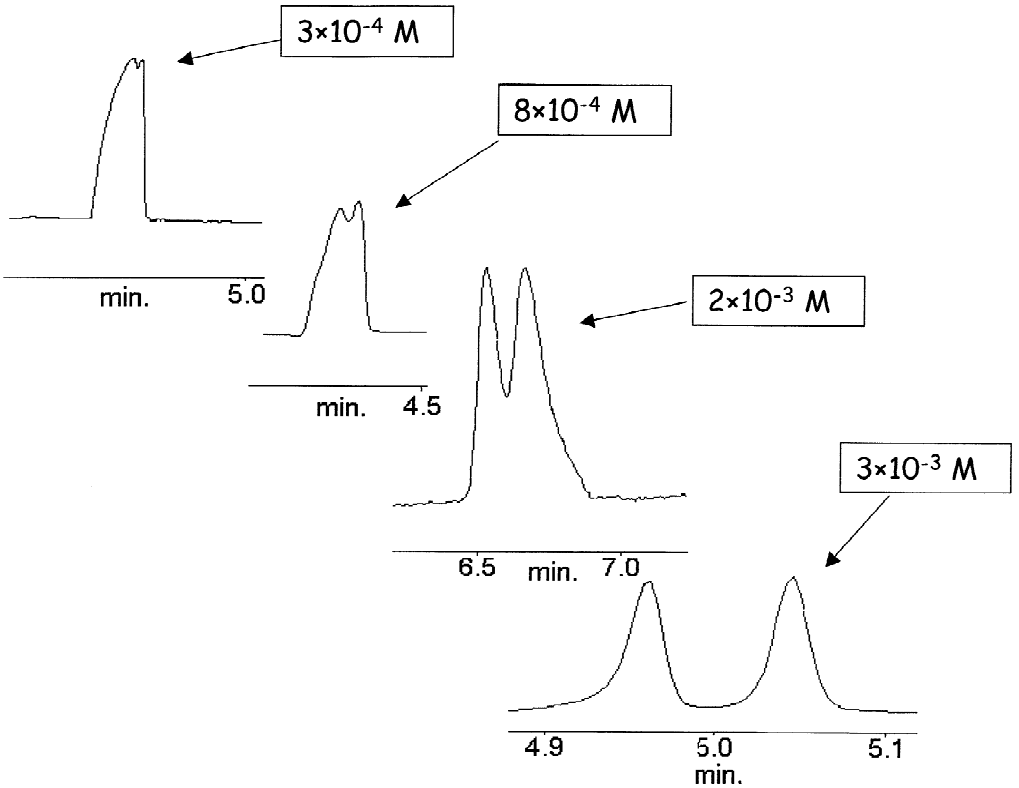

Fig. 3. Electropherograms for the THCMH–ibuprofen system at different THCMH concentrations.

the electroosmotic flow (EOF) before and after the separation run: a remarkable increase in the *t*(EOF) was observed. The usual procedure of rinsing the capillary appears to be unsatisfactory when trying to remove these molecules, and the wall still appears to be influenced by the flow of the host. In order to obtain reproducibility in the separation results it was necessary to carry out a supplementary rinse with a borate buffer (pH 9.5). It is likely that this rinse, by causing, due to the high pH of the buffer, deprotonation of the host molecules stacked on the capillary wall, helps to remove them. It is not fully clear why a NaOH rinse is insufficient to obtain the same result, but both the pH values and the analytical concentrations of these two rinsing solutions were very different and their different behaviour is not surprising.

As can be seen, HM concentrations as low as 0.30 m*M* were used, while the highest concentration examined was 3.0 m*M*. This range appears satisfactory in order to obtain chiral separation. Comparison of the results obtained for the same system by varying the HM concentration shows that there is no univocal behaviour: the concentration that gives the best resolution is not necessarily the highest, sometimes it is the lowest, in other cases it can be an intermediate concentration. This is not surprising if we consider that what determines the selectivity is not the degree of formation of the inclusion complex, but the difference in the degree of formation between the complexes of each enantiomer with respect to the complex of the other enantiomer. This requires that the degree of formation should be neither too low ($<$10%), nor too high ($>$90%). The degree of formation, in turn, depends both on the stability of the species and on the analytical concentration of the components. Thus, to obtain a medium value of the degree of formation we must adjust the analytical concentrations in order to correct values of the stability constants that are too low or too high. In

this context, carrying out measurements at selector concentrations as high as possible, as often done in the literature, may not always be appropriate. The possibility of obtaining a satisfactory selectivity at low HM concentration indicates that the HM–guest complex has a large stability constant. The lowest limit in selector concentration is probably due to the necessity of having a sufficient excess with respect to the guest concentration, which, in turn, cannot be too low in order to be optically detected.

Another observation is suggested by a comparison of the behaviour of the different hemispherodextrins. It appears that we cannot identify a chiral selector that can be considered the best: on varying the enantiomeric pair to be separated, the HM giving the best selectivity also varies. It is the system as a whole that determines the result of the CE separation. The specificity of the interactions that give rise to complex formation results in stability values differing in a complex way from one system to another. Recently, Lloyd et al. applied the QSER (quantitative structure–enantioselectivity relationship) concept to chiral separation [29], a procedure originally developed in the pharmaceutical sciences and already applied to the development of chiral stationary phases for liquid chromatography. The approach of these authors appears promising when considering the huge number of experimental parameters that can be changed in CE and particularly in CD-EKC, and the need to find a screening procedure for designing the separation experiment.

The retention times obtained for each system when varying the host concentration show a different trend for the different systems, sometimes showing a minimum or a maximum. This behaviour can easily be explained by considering that there are two different influences on the retention time that vary oppositely on varying the analytical concentration of the host. On the one hand, when there is an increase in the concentration, the degree of formation of the complex also increases. Considering that the free guest at that pH is anionic, while the complex is neutral or cationic, an increase in complex concentration should give rise to a decrease in retention time. On the other hand, increasing the host concentration gives rise to an increase in viscosity, together with a likely decrease of the dielectric constant. Thus, we will observe a decrease of the EOF, which, in turn, should increase the retention time. The combination of these two factors gives rise to the observed, complex behaviour, different from one system to another.

When considering the systems reported here, it is apparent from the data in Table 1 that each HM exhibits a chiral resolution ability which is a function of the specific analytes. This suggests a possible complementarity among the HMs. Thus, we tested the possibility of contemporaneously separating all six racemates in one run using binary HM mixtures. The results obtained are reported in Table 2. As can be seen, the results obtained for the separation of the six racemates together in the presence of one HM at a time are reported.

These experiments with systems formed by a single HM and all six racemates permit verification of the resolution of our hosts, not only the chiral resolution between enantiomers, but also the achiral resolution between one racemate and another. The ability of HMs to achieve chiral resolution should not negate the possibility of using these compounds as additives for any kind of separation. The electropherogram reported as an example in Fig. 4, in the presence of THALAH (0.50 m*M*), shows excellent separation of the different racemates. This can be better appreciated when comparing it with the electropherogram obtained in the absence of HMs: not only can no chiral resolution be obtained in the absence of a chiral additive, as expected, but also the peaks due to the different racemates all overlap and no discrimination is possible. This comparison also shows, by looking at the retention times obtained, that, in the absence of HMs, the guests are present as anionic species, as expected, and a time decrease due to the interaction with THALAH is clearly observed, giving a qualitative estimation of the strong host–guest interaction occurring. As expected, not all the racemates could be resolved into their enantiomers in a single run. The chronological order of the peaks corresponding to the different analytes gives the order of stability of the complexes that each of them forms with the HM. However, the same criterion cannot be considered when comparing peaks in different systems, particularly owing to the large effect of the type and concentration of chiral selector which we observed on the EOF, probably due to the change in both the dielectric constant and viscosity

Table 2
Results of separations carried out for all six racemates together in the presence of either a single or binary HM mixture. Values of the retention time of the slower enantiomer are reported (t_2), together with R and S values. These were multiplied by 1000 in order to reduce the number of decimal places (see text)

HM	C_1/C_2 (mM)	Flurbiprofen (a)			Ibuprofen (b)			Indoprofen (c)			Ketoprofen (d)			Suprofen (e)			Thiaprofenic acid (f)		
		t_2	S	R	t_2	S	R	t_2	S	R	t_2	S	R	t_2	S	R	t_2	S	R
A/–	1.0/–	4.35	0	0	4.14	0	0	5.04	18	1.38	5.44	13	1	4.73	11	1.25	4.44	14	1.50
A/–	0.50/–	5.42	0	0	5.08	0	0	6.58	18	1.71	7.68	11	1.14	6.25	13	1	5.69	5	0.83
B/–	1.0/–	7.81	0	0	7.33	0	0	10.22	0	0	10.44	0	0	9.03	0	0	8.38	13	0.71
C/–	1.0/–	5.67	4	0.33	5.38	6	0.67	6.63	0	0	7.07	3	0.67	5.97	19	1.05	5.55	18	0.90
A/C	0.50/0.50	4.61	0	0	4.40	14	1.09	5.54	0	0	5.78	0	0	5.05	18	1.13	4.72	24	2.20
A/C	0.25/0.75	4.68	0	0	4.46	11	0.91	5.66	0	0	5.94	0	0	5.09	20	1.18	4.74	23	1.38
A/C	0.75/0.25	4.59	0	0	4.35	14	0.80	5.46	9	0.67	5.64	0	0	5.02	18	1.13	4.71	26	1.50
A/B	0.50/0.50	5.83	0	0	5.47	9	0.71	7.42	16	1.14	7.92	10	0.73	6.71	14	1.06	6.08	15	1.28
A/B	0.25/0.75	6.34	0	0	5.98	7	0.57	8.02	0	0	8.45	0	0	7.27	0	0	6.66	6	0.40
A/B	0.75/0.25	5.21	0	0	4.93	8	1	6.31	16	1.33	6.67	9	1	5.83	14	1.23	5.38	13	1.27
C/B	0.50/0.50	5.60	13	0.67	5.33	19	1.82	6.98	0	0	7.37	0	0	6.06	25	2	5.57	31	2.43
C/B	0.25/075	5.57	11	1.09	5.35	27	1.87	7.04	0	0	7.27	0	0	6.13	21	1.37	5.66	32	2
C/B	0.75/0.25	6.73	15	1.05	6.36	14	1.20	8.17	0	0	8.92	0	0	7.21	24	1.79	6.59	23	1.76

A, THALAH; B, THAMH; C, THCMH.

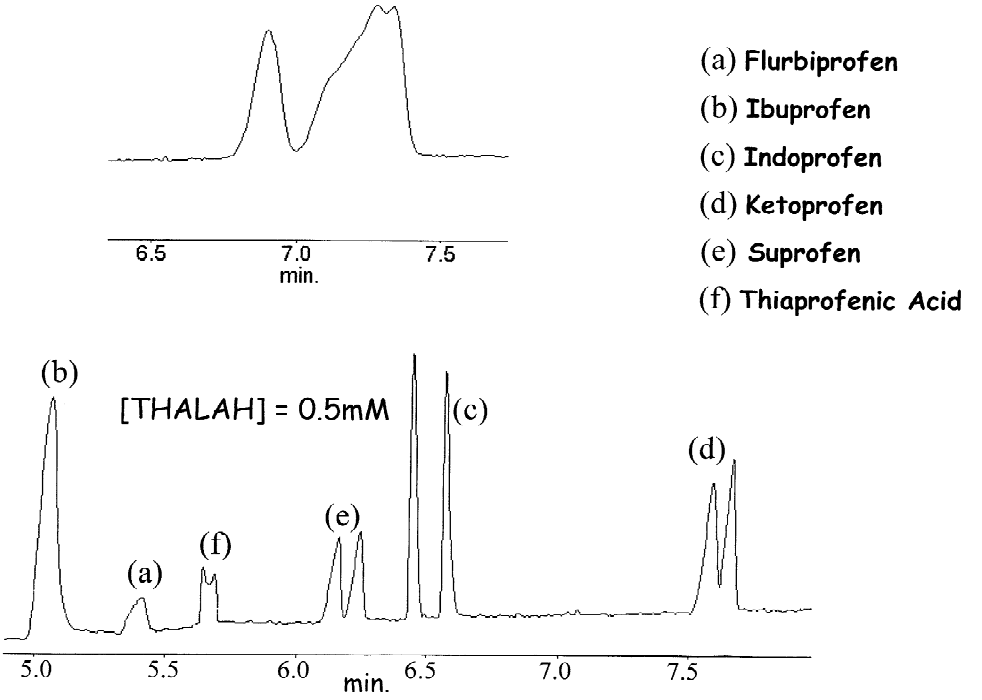

Fig. 4. Electropherograms for the simultaneous separation of all six profen racemates in the presence of THALAH, compared with the separation of the six racemates in the absence of a host (upper electropherogram).

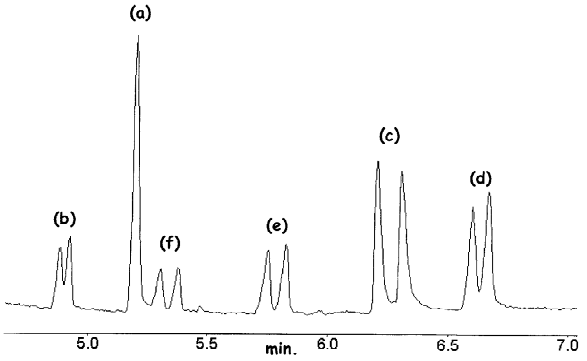

Fig. 5. Electropherogram of all six profen racemates in the presence of THALAH/THAMH (3:1 analytical concentration ratio), obtained at $\lambda = 198$ nm.

of the BGE. The observed order is the same for all these systems, with the only exception being the thiaprofenic acid racemate, which has a retention time shorter than that of flurbiprofen when the BGE contains THAMH, opposite to the case of all the other systems investigated. As stated in the Introduction, THAMH has a much smaller ''cage'' than the other two HMs and thus the described inversion in the retention order is not surprising.

When comparing the results of this group of experiments with those run with binary mixtures of HMs, also included in Table 2, it is soon apparent that, at least for three racemates (ibuprofen, thiaprofenic acid, suprofen), chiral selectivity increases with respect to the single HM run. In the case of thiaprofenic acid and ibuprofen, when analysing all the racemates at once, only the use of a HM binary mixture achieves a quantitative separation, obtaining resolutions as high as 2.43 for the system THCMH/THAMH with thiaprofenic acid, but the effect is large and evident for the systems with ibuprofen. Also in the case of suprofen, an improvement in resolution is clear, although satisfactory results were also obtained with a single HM. Analysis of the data reported in Table 2 also suggests that the concentration ratio in the binary mixture, while certainly affecting the separation, generally has no dramatic effect. The differences observed when varying the components of the mixtures are far greater than when varying the ratio only.

Electropherograms for two different systems are shown in Figs. 5 and 6. In the case of THALAH/THAMH (Fig. 5), the electropherogram obtained at $\lambda = 198$ nm, by maintaining a sufficient separation between each enantiomeric pair, straightforwardly shows the obtained separation. On the contrary, the case of THCMH/THAMH (Fig. 6) shows how, by increasing the chiral resolution, the resolution between two consecutive racemates may be insufficient to separate the peaks due to two enantiomeric pairs, thus obtaining overlapping of the peaks due to the slower enantiomer of the faster racemate overlapping the peak due to the faster enantiomer of the slower racemate. As seen from the figure, only by examining the electropherograms obtained at two different

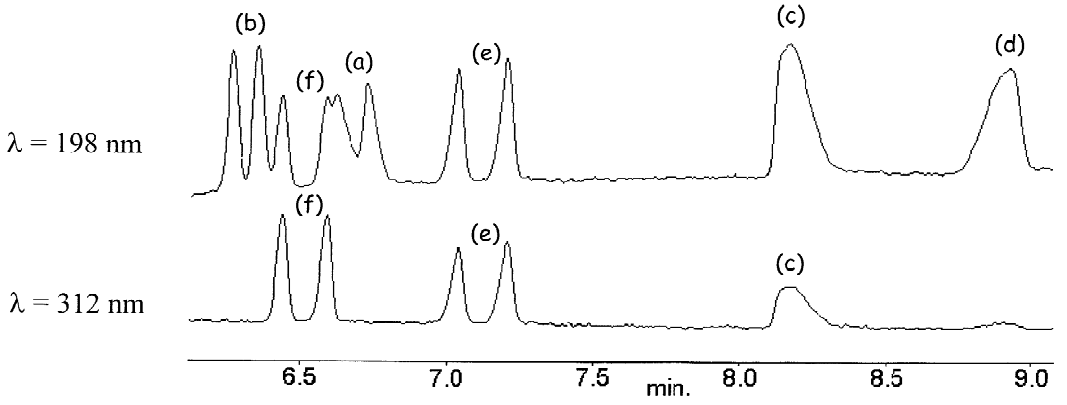

Fig. 6. Electropherogram of all six profen racemates in the presence of THCMH/THAMH (3:1 analytical concentration ratio), obtained at two different wavelengths.

wavelengths it is possible to observe these peaks separately.

4. Concluding remarks

This study confirms the very good chiral selector properties of this new class of CD derivatives, hemispherodextrins, which we reported in previous papers [24,25]. Once more, it is important to design specific hosts for the type of analytes to be separated, and QSER studies should be carried out. The design of appropriate hosts opens up to CE chiral separation a new range of analytical concentrations, much lower than those commonly used. The advantage of the use of pure compounds instead of commercially available mixtures of different derivatives is reinforced. This is recommended for reproducibility reasons.

The measurements carried out on more than one racemate at a time also verified the versatility of HMs with regard to achiral selectivity, and we hope in the near future to exploit these characteristics of HMs.

The complementarity among single HMs was confirmed by the experimental results, at least for specific systems, giving rise to a sort of complementary chiral selectivity, which, in our opinion, deserves further study with respect to other kinds of receptors to improve the performance of CE for samples of practical interest.

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