Enantioseparation of Flobufen with Cyclodextrins Studied by Capillary Electrophoresis and NMR

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Purpose. The aim of this study was to obtain the resolution of flobufen enantiomers, an antiinflammatory active substance, by capillary electrophoresis with cyclodextrins. The mechanism of complexation and determination of the stoichiometry of the complexes were studied by NMR and the analytical method was developed and validated.

Methods. Zone capillary electrophoresis coupled to direct ultraviolet detection was selected. The interaction between flobufen and the chiral selector was studied by NMR. Optimization of the separation was performed using a Box-Wilson Central Composite Design for three factors related to the composition of the electrolyte.

Results. Heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) was found to be the most efficient selector via the formation of a 1:1 complex proved by NMR. Constants of complexation of flobufen enantiomers were determined by NMR and capillary electrophoresis. Optimal values for the critical factors of the analytical system were: pH (5.50), content in methanol (10% v/v), and TM-β-CD (30 mM). The ability of capillary electrophoresis to quantify as low as 0.1% (w/w) of *R* in *S*-flobufen or vice-versa was established.

Conclusions. Capillary electrophoresis was shown to be a valuable method to control the enantiocomposition of flobufen by use of a chiral selector whose interactions with the analytes could be explored by NMR.

KEY WORDS: capillary electrophoresis; central composite design; cyclodextrins; enantiomeric separation; flobufen; NMR.

INTRODUCTION

Optical active substances can be produced as racemate or specific enantiomer. In the case where a given antipode is selected, the other will be considered as an impurity. Thus, special attention must be given to the identity and the optical purity of active substances. At the present time, most monographs in the Pharmacopeias (1,2) control optical identity or purity by the measurement of optical rotation to confirm racemic nature (usual value between +0.1 and –0.1 degrees) or the determination of specific rotation in the case of a chiral drug. However, this test has a poor limit of detection bringing about the introduction of chiral techniques in Pharmacopeias using mainly high-performance liquid chromatography (HPLC).

Taking as an example flobufen (*R,S*)-4-(2′,4′-difluorobiphenyl-4-yl)-4-oxo-2-methylbutanoic acid, a chiral nonsteroidal anti-inflammatory drug (NSAID) belonging to the class of arylalkanoic acids, a capillary electrophoresis (CE) method was developed and validated to control raw material (racemate and optical isomers) and optic stability of the racemate in the finished product.

MATERIALS AND METHODS

Reagents

The reagents used included β-cyclodextrin (β-CD) from Aldrich (Milwaukee, WI, USA); heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) from Sigma (St Louis, MO, USA); 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) from Roquette (Lestrem, France); (*RS*)-flurbiprofen and *S*-naproxen from Sigma; racemic flobufen (flo), *R* and *S* enantiomers, and racemic flobufen formulations were all from Virbac Laboratories (Carros, France). Deuterium oxide (D_2O) was used as received from Eurisotope (Gif-sur-Yvette, France) and 40% (w/w) sodium deuteroxide (NaOD) was obtained from Aldrich. All other chemicals and solvents used were analytical grade.

Capillary Electrophoresis

All experiments were performed on a Beckman P/ACE 5500 model (Beckman, Fullerton, USA) equipped with a diode array detector, automatic injector and sampler. Capillary temperature was controlled by coolant over the range 15–50 ± 0.1°C. A Beckman untreated fused-silica capillary 75-μm I.D. with an overall length of 57 cm (effective length 50 cm) was used. The experimental set-up, data acquisition, and processing were governed using Beckman P/ACE Station software (Version 1.0 on Microsoft Windows 95).

During development, the background electrolyte (BGE) was prepared by dissolving the appropriate amount of cyclodextrin (CD) in the desired mixture of methanol–87.5 mM acetate buffer. Solutions of *R-, S-,* and *RS*-flobufen were prepared by dissolving each compound at the appropriate concentration in BGE, benzylic alcohol being used as neutral marker. All solutions were passed through a membrane filter of 0.45-μm pore size from Alltech (Templeuve, France) and degassed by sonication before used.

Electroosmotic Mobility

Electroosmotic mobility μ_{eo} was calculated from the following expression:

$$\mu_{eo} = lL/t_{eo}V \quad (1)$$

where l is the capillary length between the injection and the detector, L is the overall capillary length, t_{eo} the migration time of electroosmotic flow (neutral marker), and V the applied voltage.

The apparent mobilities μ_{ap} of the analytes were calculated from Eq. (1), using the migration time for each compound as t_{eo}.

The apparent mobility is related to the electrophoretic mobility for the ion and the electroosmotic mobility by the following expression:

$$\mu_{ap} = \mu_{ep} + \mu_{eo} \quad (2)$$

The values calculated for μ_{ep} were corrected by the factor I/I_{CD}, where I is the intensity of the current obtained without

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CD and I_{CD} the intensity of the current obtained in the presence of CD. This correction takes account of the influence of CD on the BGE viscosity (3,4).

Resolution

The resolution *R*s was calculated using the usual equation:

$$Rs = \frac{2(t_{m2} - t_{m1})}{W_1 + W_2} \quad (3)$$

where t_{m1} and t_{m2} are the migration times of the enantiomers and W_1 and W_2 are the corresponding widths at the bases of the peaks.

Apparent Constants of CD Complexes

The apparent constants of the complexes formed between each enantiomer and the CD were determined in the electrophoretic system consisting of 87.5 mM acetate buffer, pH 5.00, with CD concentration over the range 0–40 mM. They were calculated from the expressions developed by Wren and Rowe (4).

In a 1:1 selector-analyte equilibrium

$$S + A \overset{K}{\Leftrightarrow} SA$$

the equilibrium constant defined in terms of concentrations is

$$K = \frac{[SA]}{[A][S]} \quad (4)$$

The apparent electrophoretic mobility μ_{ap} of a given analyte is related to the proportion of time it is free and the proportion of time it is complexed with the selector:

$$\mu_{ap} = \frac{1}{1 + K[S]}\mu_f + \frac{K[S]}{1 + K[S]}\mu_c \quad (5)$$

where μ_f and μ_c are the electrophoretic mobilities of the free analyte and the complex respectively.

By referencing the measured electrophoretic mobilities (i.e., μ_{ap}, and μ_c) to that of the free enantiomer (μ_f), Equation (5) can be rearranged to

$$\frac{(\mu_{ap} - \mu_f)}{[S]} = -K(\mu_{ap} - \mu_f) + K(\mu_c - \mu_f) \quad (6)$$

Thus, the equilibrium constant *K* and the electrophoretic mobilities μ_c of the complexes can be determined by linear regression (μ_f being determined experimentally).

Validation

An optimized system was used: BGE consisted of 30 mM TM-β-CD in methanol–87.5 mM acetate buffer, pH 5.50 (10: 90, v/v), at a temperature of 25°C, a voltage of +20 kV, a hydrodynamic injection time of 3 s (injected volume ca. 16 nL), and ultraviolet detection at 265 nm. Before each experimental batch, the capillary was successively rinsed with 0.1 M sodium hydroxide (10 min), water (5 min), and then BGE (10 min). Finally, before each injection, the capillary was filled with BGE for 2 min. Peak areas were normalized to their migration times to compensate for their differential detector residence times.

Quantitative Applications

The determination of the enantiomeric ratio of *RS*-flobufen was performed in the raw material and in the medicinal product through three replicate analyses (final concentration 50 μg/mL).

NMR Spectroscopy

^{1}H-NMR spectra were recorded on a Bruker-DRX 400 spectrometer at 400 MHz at 25 ± 0.1°C (Service Commun de RMN, Faculty of Science, NANCY). The chemical shift at 4.7 ppm due to residual solvents was used as internal reference.

Determination of the Stoichiometry of the CD/Flobufen Complexes

Determination was performed using the continuous variation method developed by Job (5). The sums of the concentrations of TM-β-CD and *R*- or *S*-flobufen were kept constant (6 mM), the molar ratio varied from 0 to 1 by preparing 6 mM solutions of CD and flobufen enantiomers in D_2O with NaOD (pD ≈ 8) and mixing them to constant volume to obtain the desired molar ratio.

Apparent Constants of CD Complexes

When 1:1 complexes were observed, the Benesi-Hildebrand method (6) was used to determine the association constant *K:*

$$\frac{\Delta\delta_{obs}}{[S]} = K.\Delta\delta_c - K.\Delta\delta_{obs} \quad (7)$$

where $\Delta\delta_{obs}$ is the difference between the chemical shift of one proton of the flobufen enantiomer tested in the presence of TM-β-CD and in the free state, [*S*] is the concentration of free TM-β-CD, and $\Delta\delta_c$ is the difference between the chemical shift of one resonance of enantiomer in the complexed and in the free states. The enantiomer concentration was set at 1 mM and that of TM-β-CD varied between 3 and 10 mM. In this case, the widely used assumption $[S] = [S]_t$ (where $[S]_t$ is the total concentration of TM-β-CD) could not be used. Thus, [*S*] was resolved in terms of $[S]_t$ and used in Eq. (7) to obtain the estimated parameters (*K* and $\Delta\delta_c$). This process was repeated and iterated as required until parameter converge (7).

Central Composite Design

The Box–Wilson central composite design (CCD) (8) was used to optimize the enantioseparation. The three-factor Box–Wilson CCD required eight experimental points augmented by six extra star points and nine center-point experiments. This defined the geometric distance between the center point and each star point, *a.* The distance *a* was calculated from Eq. (8):

$$a^2 = \frac{\sqrt{(N_c + N_a + N_0)N_c} - N_c}{2} \quad (8)$$

where *a* is the axial spacing, N_c the number of the factor points, N_a the number of the axial star points, and N_0 the number of replicate experiments at the center point. The value of *a* then became 1.668 for $N_c = 8$, $N_a = 6$, and $N_0 = 9$.

The results from the CCD could be assessed by multilinear regression (Microsoft Excel, version 7.0 a, Microsoft Corp.), using an equation of the type:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (9)$$

where Y is the experimental response (e.g., resolution, *Rs* or migration time, *tm*) and X_1, X_2, and X_3 are the factors. The b coefficients represent the parameters of the model which were iteratively optimized.

Simplex Optimization

To optimize the mathematical model Y given by the experimental design, a simplex method was used. This way, the Y value was calculated for m sets of starting conditions where m was given by the number of factors to be optimized plus 1. In this case, therefore, m was 4. The point corresponding to the lowest value of Y was then reflected in relation to the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest Y was reflected and the process repeated sequentially until an apparent optimum was obtained.

RESULTS

Preliminary Studies in CE

The resolution of flobufen isomers was studied with various cyclodextrins used as chiral selector. No resolution was obtained with β-CD, HP-β-CD, and DM-β-CD. Values of apparent formation constants showed that flobufen had strong affinity with each of these CD, leading to undifferentiated affinity between the enantiomers ($K_{\beta\text{-CD}} = 1657 \pm 44$ M^{-1}; $K_{\text{HP-}\beta\text{-CD}} = 3100 \pm 534$ M^{-1}, and $K_{\text{DM-}\beta\text{-CD}} = 2558 \pm 409$ M^{-1}). Only TM-β-CD led to a satisfactory separation ($Rs = 1.32$ with 30 mM TM-β-CD in 87.5 mM acetate buffer pH = 5.00), with the respective values of K being 569 ± 15 M^{-1} for *R*-flobufen and 532 ± 18 M^{-1} for *S*-flobufen (Fig. 1). Moreover, addition of methanol in the BGE had a positive effect on the resolution whereas temperature (15 to 40°C) was found to be an uncritical parameter.

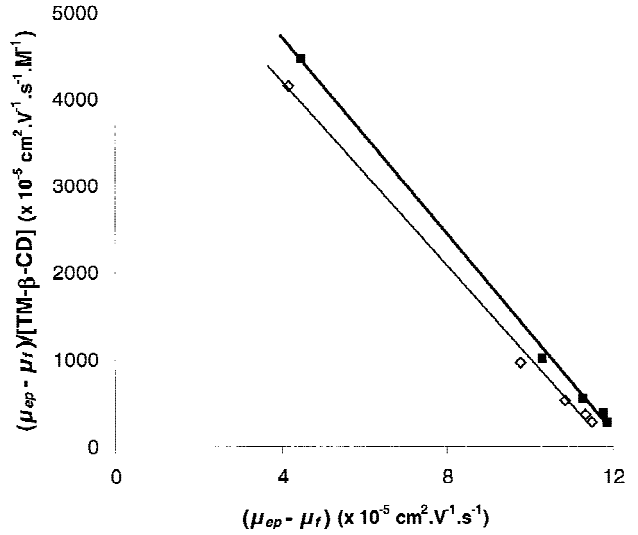
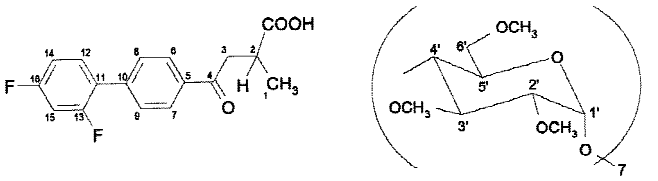


Fig. 1. Determination of apparent constants of TM-β-CD complexes with *R*- (■) and *S*-flobufen (◇) using equation (6). Slope yields $-K$ value, i.e., $K_R = 569 \pm 15$ M^{-1} and $K_S = 532 \pm 18$ M^{-1}.

Table I. Proton Assignment and Chemical Shift Changes for the Protons of *R*- and *S*-Flobufen and TM-β-CD in a 1:1 Molar Ratio



	Protons	$\Delta\delta_{obs}$ $(R)^a$	$\Delta\delta_{obs}$ $(S)^a$	$\Delta\Delta\delta_{obs}{}^b$
Flobufen	1	–0.2	1.6	1.8
	2	7.0	5.8	1.2
	3a	nd[c]	nd[c]	nd[c]
	3b	1.2	10.6	9.4
	6,7	6.4	7.8	1.4
	8,9	–8.0	0.4	8.4
	12	–9.6	2.0	11.6
	14	17.2	22.8	5.6
	15	16.0	21.2	5.2
TM-β-CD	1′	–27.4	–26.8	0.6
	2′	–29.8	–27.2	2.6
	3′	–71.2	–63.6	7.6
	4′	–24.4	–24.4	0.0
	5′	–54.4	–55.2	0.8
	6′a	–27.4	–29.6	2.2
	6′b	–18.2	–20.8	2.6
	2′-OCH_3	–24.4	–22.0	2.4
	3′-OCH_3	–12.4	–11.2	1.2
	6′-OCH_3	0	–1.2	1.2

[a] $\Delta\delta_{obs} = \delta_{complex} - \delta_{free}$, Hz.
[b] $\Delta\Delta\delta_{obs} = |\Delta\delta_{obs}(S) - \Delta\delta_{obs}(R)|$.
[c] nd = not determined.

NMR Spectroscopy

The inclusion of analytes in the cyclodextrins can be proved by observing the chemical shift of the protons from the host and the guest molecules.

Table I shows the change in chemical shift ($\Delta\delta$) observed on complexation of each enantiomer with TM-β-CD and the separation between signals ($\Delta\Delta\delta$) arising from the individual flobufen enantiomers. The internal protons H3′ and H5′ of

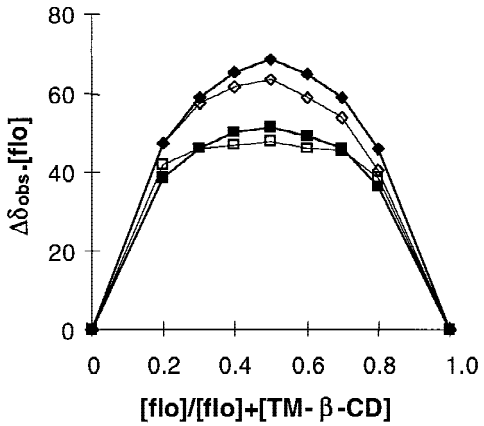


Fig. 2. Job's plot for the protons H14 (■), H15 (□) or *R*-flobufen, and H14 (♦), H15 (◇) of *S*-flobufen in TM-β-CD-complexes.

Table II. Central Composite Design for the Enantioseparation of *RS*-Flobufen

	Lower levels		Center point	Upper levels	
	–1.668	–1	0	+1	+1.668
pH	4.17	4.50	5.00	5.50	5.83
[TM-β-CD] (mM)	3.3	10	20	30	36.7
Percent methanol (v/v)	0	4	10	16	20

TM-β-CD underwent the greatest enantiomer-induced chemical shift changes. Upfield shifts indicated that the enantiomers created diamagnetic anisotropy effects in the interior of the cavity due to the inclusion of aromatic rings rich in π electrons. The displacements for the H14 and H15 protons of flobufen were the strongest for each enantiomer, confirming that flobufen interacted with the inside of the cavity and thus, that inclusion occurred. The H1 (shift in the opposite direction) and H3b ($|\Delta\Delta\delta| = 9.4$) protons near the chiral center showed the largest differences.

Job plots for H14 and H15 protons showed a symmetrical curve (Fig. 2), indicating that the complexes had 1:1 stoichiometry. The association constants were 998 ± 32 M^{-1} (H14) and 967 ± 34 (H15) for *R*-flobufen and 675 ± 32 M^{-1} (H14) and 670 ± 38 (H15) for *S*-flobufen.

Optimization Using CCD

After selecting the chiral selector, a CCD was used to optimize the enantioseparation of *RS*-flobufen investigating three factors: the pH of BGE (X_1), the TM-β-CD concentration (X_2), and the methanol concentration (X_3). The levels for these experimental parameters (Table II) were chosen based on preexperiments and knowledge of the system. As experimental response *Y*, a response factor (*RF*) was defined which was a combination of the resolution (*Rs*) and the migration time of the last peak (tm_2):

$$RF = \frac{Rs^2}{tm_2}$$

RF controls the time needed to reach a fast but well-separating system and in this present work, the highest value of *RF* was used as a criterion of separation. The estimated parameters generated by the regression model are given in Table III. The difference between the predicted and the actual values obtained experimentally was used as a criterion to evaluate the regression model. This generated model can be assessed statistically using the coefficient of multiple determination ($R^2 = 0.9844$) and the 95% confidence level (*P* value <0.05). At a 95% confidence level ($P < 0.05$) some terms were not significant, consequently the reduced model excluded the variable X_2 and the interaction X_1X_3.

Table III. Central Composite Design: Regression Results for the Model

Variable	Parameter	Coefficient	*P* value
	b_0	–12.8015	0.0000
X_1	b_1	4.9025	0.0000
X_2	b_2	0.0001	0.9843[a]
X_3	b_3	0.0545	0.0021
X_1^2	b_{11}	–0.4769	0.0000
X_2^2	b_{22}	–0.0011	0.0000
X_3^2	b_{33}	–0.0019	0.0000
X_1X_2	b_{12}	0.0104	0.0000
X_1X_3	b_{13}	–0.0034	0.2466[a]
X_2X_3	b_{23}	–0.0014	0.0474
$X_1X_2X_3$	b_{123}	0.0003	0.0380

[a] $P > 0.05$, non significant.

Using the simplex method the optimum conditions were:

$$X_1 = 4.50 \quad X_2 = 10 \quad X_3 = 4$$
$$X_1 = 4.50 \quad X_2 = 30 \quad X_3 = 16$$
$$X_1 = 5.87 \quad X_2 = 20 \quad X_3 = 10$$
$$X_1 = 5.00 \quad X_2 = 20 \quad X_3 = 10$$

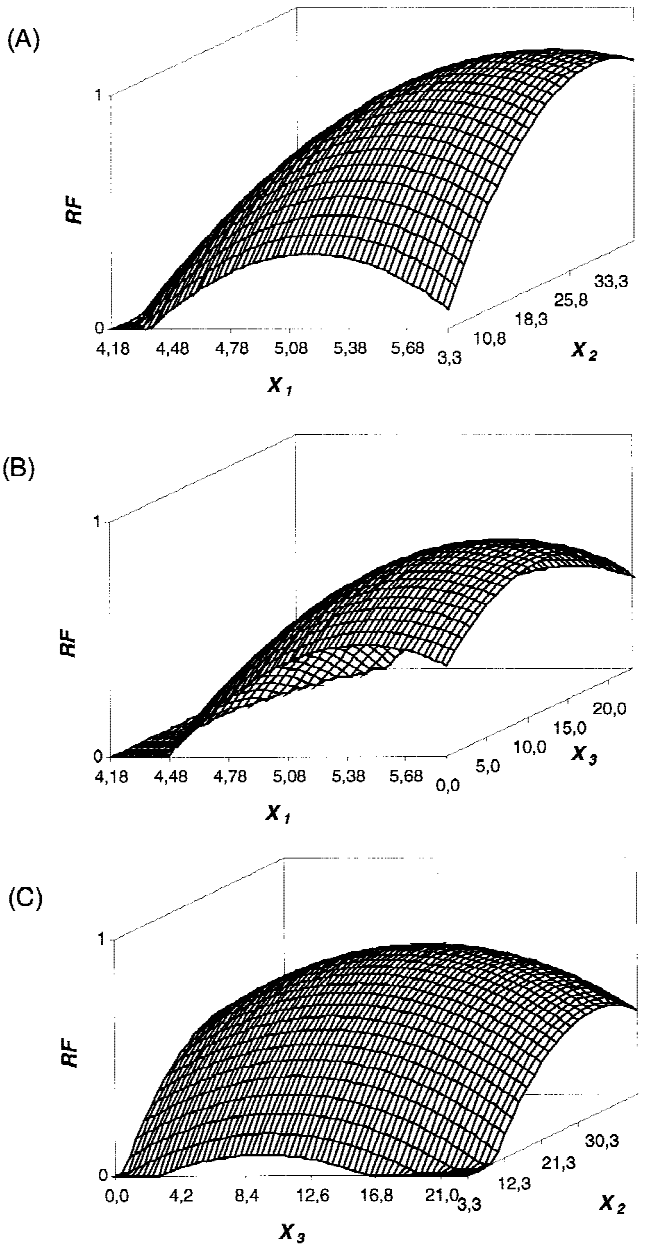


Fig. 3. Response surfaces for *RF* model of *R*- and *S*-flobufen. (A) X_1 (pH) vs. X_2 (TM-β-CD, mM); X_3 (methanol, v/v) is held constant at 10%. (B) X_1 (pH) vs. X_3 (methanol, % v/v); X_2 (TM-β-CD) is held constant at 30 mM. (C) X_2 (TM-β-CD, mM) vs. X_3 (methanol, % v/v); X_1 (pH) is held constant at 5.50.

The optimum conditions obtained after 32 iterative processes were: $X_1 = 5.50$, $X_2 = 29.1$, and $X_3 = 11.0$.

The response factor *RF* was plotted against two experimental factors whereas the third was held constant (Fig. 3) to give an idea of the ruggedness of the optimized method. These three-dimensional response surfaces can be examined to establish the optimum zone. The optimum conditions determined in this way ($X_1 = 5.50$, $X_2 = 28.8$, and $X_3 = 10.8$) were in good agreement with the results obtained with the simplex method.

The definitive conditions chosen on the basis of the optimum values and a convenient approach i.e., pH 5.50, 30 mM TM-β-CD, and methanol 10% (v/v) were tested experimentally. The observed separation (Fig. 4) corresponded to *Rs* value of 2.6 with a migration time of 11.93 min.

Validation of the Optimized Method

Validation consisted in studying the selectivity, linearity, repeatability, limit of detection, and response factors (1) of each enantiomer.

Selectivity

R-flobufen migrated faster than *S*-flobufen. The placebo did not present any peaks at the migration time of the enantiomers of *RS*-flobufen. Selectivity was also confirmed against *RS*-flurbiprofen, a structurally related NSAID, and *S*-naproxen, a potential internal standard.

Linearity

The linearity of the detector response (peak area) was assessed over the range 25.0 to 75.0 μg/ml (8.2×10^{-5} to 2.5×10^{-4} M) corresponding to 50–150% of the nominal content in the formulation after dilution (1/20, v/v). The correlation coefficients obtained were 0.9984 and 0.9997 for *R* and *S* enantiomers, respectively, and the *y*-axis intercept was not significantly different from 0 (at 95% level of confidence).

Repeatability

Repeatability was tested on solutions of *R* and *S* enantiomers (50 μg/ml). The precision estimated by RSD of peak areas was 1.71% and 0.39%, respectively (n = 6). The incorporation of an internal standard (*S*-naproxen) did not improve the precision of the method.

Limit of Detection

The determination of the detection limit was based on an analysis of samples of known concentration of each enantiomer producing a signal-to-noise ratio of 3. In the optimized conditions, the detection limit was around 0.15 μg/ml for each enantiomer (Fig. 5). Based on the initial concentration of the antipode, purity of each enantiomer was over 99.9% (w/w).

Molar Absorptivity of the Complexes

As the flobufen enantiomers were partially resolved under complexes with TM-β-CD, a synthetic racemic flobufen sample prepared from pure enantiomers (equimolar ratio of 1.000) was analyzed to examine the respective response of the enantiomers in the analytical system. The *R/S* normalized peak-area ratio obtained displayed a significant difference between the enantiomer responses: mean ratio of 1.015 (n = 6) with an interval of confidence from 1.012 to 1.019 ($P = 0.05$) excluding the 1.000 value.

Quantitative Application

Five batches of *RS*-flobufen were analyzed. The mean enantiomeric ratio after correction was 0.998 (CV = 0.72%). An analysis of two batches of specific formulations showed that the enantiomeric ratio was maintained in comparison with that of the starting material.

DISCUSSION

Chiral separation in CE using CD as a chiral selector is based on differences in mobilities between analyte and ana-

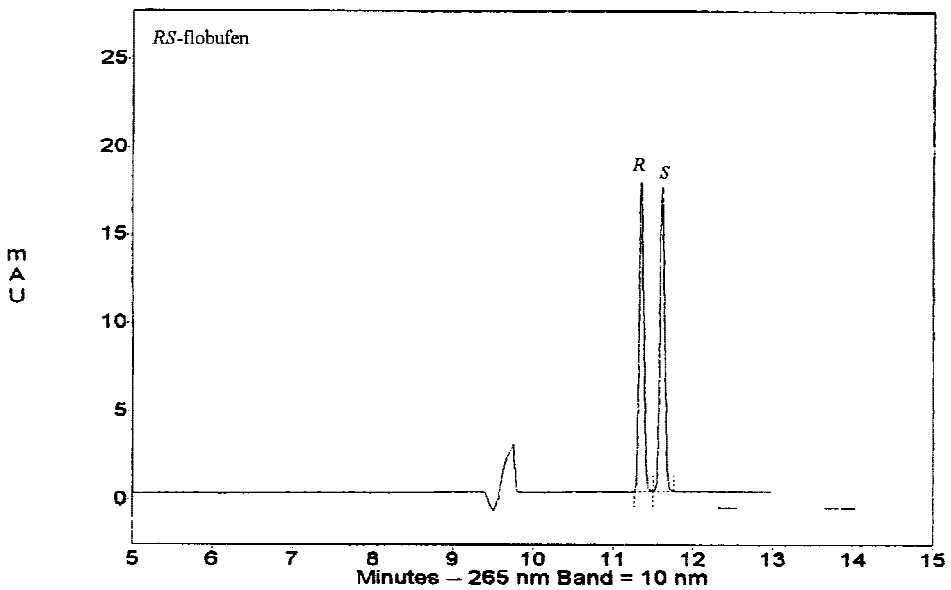


Fig. 4. Enantioselective separation of *RS*-flobufen in the optimized conditions. Methanol–87.5 mM acetate buffer pH 5.50 with 30 mM TM-β-CD (10:90, v/v).

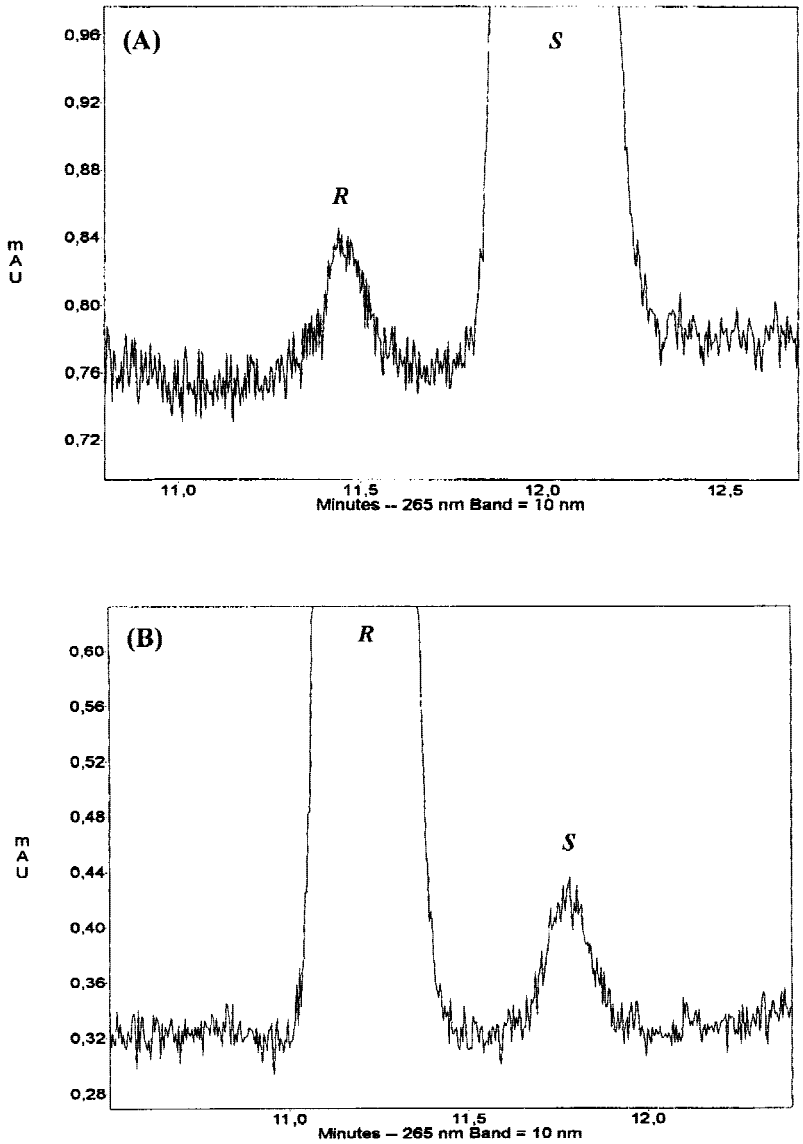


Fig. 5. Separation capacity and limit of detection of the optimized method. (A) *R*-flobufen (0.15 μg/mL) in the presence of *S*-flobufen (150 μg/mL). (B) *S*-flobufen (0.15 μg/mL) in the presence of *R*-flobufen (150 μg/mL).

lyte-CD complexes and depends on values of the equilibrium constants of the analyte-CD complexes. It is also related to the type and concentration of CD and pH of the BGE.

Natural cyclodextrins were first used as chiral selectors, but modified cyclodextrins were rapidly preferred (9). The calculated values of apparent formation constants, much larger for DM-β-CD than TM-β-CD, confirmed that better complexation does not involve better enantioselectivity since TM-β-CD was found to be the best chiral selector for the separation of flobufen enantiomers as was soon observed for other NSAIDs (9–12). The hydrophobic interactions within the CD cavity are not sufficient to achieve enantioseparation. Methylation at hydroxyl groups on the CD ring occurs in the selectivity by extending the intramolecular cavity which is no longer rigidified by intramolecular hydrogen bonds (13). This distorsion seems to promote flexibility and improves the fit between TM-β-CD and flobufen.

To obtain more detailed information on selector–selectant interactions of flobufen enantiomers with TM-β-CD, NMR studies were performed. The NMR spectroscopy confirmed that flobufen forms inclusion complexes with TM-β-CD in the host:guest molar ratios of 1:1. The data indicate some differences in the spatial interactions of TM-β-CD with the enantiomers of flobufen which lead to different values of the apparent constants of complexes, higher for *R*-flobufen than for *S*-flobufen. Higher values obtained by NMR than by CE could be explained by the fact that only the strongest interactions are considered in NMR and therefore only two protons are implied in the calculation. However, the association constants determined by NMR and CE were in the same range (500 to 1000 M^{-1}) indicating that flobufen enantiomers have weak affinity with TM-β-CD. NMR presents usefulness for investigating inclusion process used in separation techniques (14–16).

Flobufen is an ionizable analyte and according to the model described by Rawjee *et al.* (17), it belongs to a class of

weak acids in which complexation with cyclodextrin is only selective in their uncharged forms. Chiral selectivity increased with decreasing pH, but partial ionization of the carboxylic was needed since uncharged cyclodextrins migrate at the same velocity as the EOF. Consequently, the optimum value of the pH of the background electrolyte was found to be 5.5, corresponding to near 95% of the ionization of flobufen (pK_a = 4.2). The separation efficiency can also be drastically influenced by the addition of methanol. Wren and Rowe (18) explained the effect of organic solvent in terms of changes in the inclusion-complex formation constants. Solvent addition decreased the value of formation constants, affecting the optimum concentration of CD and enhancing the separation. These considerations were used as a basis for our experimental design.

CCD was shown to be a useful tool for method development (19–21). The shape of the three-dimensional surfaces (Fig. 3) and the existence of an optimum indicated that the control of the three parameters (pH, methanol content and TM-β-CD concentration) was predominant in the separation of flobufen enantiomers. These three-dimensional response surfaces established the region of optimal robustness for the method. The region appeared to be in the zone of optimal value of the response factor (*RF*) describing the resolution between the flobufen enantiomers.

Successful validation was achieved including suitable assessments of selectivity, linearity and repeatability. Optical purity of the enantiomers was verified after estimation of the limit of detection of the method. The developed and validated method was able to quantify 0.1% *R*-flobufen in *S*-flobufen and vice versa. As the enantiomers of the species being resolved are partially complexed into the cyclodextrin each enantiomer may have a different ultraviolet response (22,23). Although the ultraviolet response was found slightly different for each complex, no correction was needed since response factor is near 1. At the present time, few monographs in Pharmacopoeias set limits for content in antipode in a chiral drug. HPLC is often used and the limits are higher than 0.1% (for examples 0.5% for *S*-selegenine, 1% for *R*-timolol, 2% for *R*-dexchlorpheniramine, and 3% for *R*-methotrexate in the European Pharmacopeia). HPLC methods need chiral stationary phases and the separation efficiency measured by the number of theoretical plates is known to be poorer than by CE. Thus, the capillary electrophoresis method is an ideal method to control the enantiomeric purity of chiral drugs. Moreover, conservation of the enantiomeric purity or the enantiomeric ratio during the manufacturing process and the stability studies can be easily demonstrated.

In conclusion, the method developed in the present work is simple and robust. Contrary to the method initially described by Tesarvá *et al.* (24), the separation of flobufen enantiomers can be obtained without the addition of surfactant and resolution is better.

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