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Determination of the enantiomeric purity of dexfenfluramine by capillary electrophoresis: use of a Plackett–Burman design for the optimization of the separation

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Abstract

For the investigation of the stereochemical purity of the anorectic drug dexfenfluramine, a capillary electrophoretic method is presented using the chiral selector dimethyl β-cyclodextrin (DMCD). A Plackett–Burman experimental design was used as a multivariate strategy for the evaluation of the effects of varying several operating conditions at once. The impact of concentration of DMCD, concentration of methanol added to the buffer, pH of the background electrolytes, temperature of the capillary and applied voltage, has been investigated on the resolution of the enantiomers, the analysis time and the peak symmetry. From these results, optimal values of the variables were selected for the development of a method capable of determining the levo-rotatory enantiomeric impurity of dexfenfluramine.

Keywords: Optimization; Plackett–Burman design; Experimental design; Dexfenfluramine

1. Introduction

In the different domains of any pharmaceutical activity such as e.g. pharmacology, drug quality control, toxicology, etc., powerful analytical techniques are needed to attain adequate resolution of molecular structures as closely related as optical isomers or enantiomers. For these purposes rather complex and sophisticated chromatographic techniques are still being developed but in the last few years capillary electrophoresis (CE) has proved to be very promising in this field, mainly because of the high peak efficiencies that can be achieved [1–3]. Chiral separation of racemic drugs performed by CE techniques has been recently reviewed by Nishi and Terabe [4]. Among several approaches, the addition of cyclodextrins as chiral selectors to the background electrolyte (BGE) in capillary electrophoresis (CD-CE) seems the method of choice for the chiral resolution of basic drugs. Chiral separation is possible because different degrees in complexation between the two enantiomers and the chiral selector result in effective mobility differences [3,5–8]. However, the choice of the selector and the prediction of the separation conditions are not yet straightforward. The development of a chiral separation method requires several steps to optimize operating conditions, such as the type and concentration of cyclo-

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dextrin; the type, concentration and pH of the BGE; the amount of buffer additives; the temperature of the capillary and the applied voltage which were already investigated by several authors in different case studies [3,7–14]. Although valuable conclusions can already be drawn from monovariate investigations, optimum operating conditions would only be attained by investigating all possible combinations of factors. This means for example that for the evaluation of n factors at only two levels by a full factorial design the number of experiments would be 2^n. However, to avoid such tedious and time-consuming work, different approaches of experimental factorial designs can be applied to achieve an optimum method development. For example the Plackett–Burman design [15] which only focuses on the main effects of the factors, allows the number of experiments to be drastically reduced. For the evaluation of 7 factors at 2 levels a Plackett–Burmann design with only 8 experiments is described instead of the 128 required for a full factorial design. In separation science this kind of experimental design showed already its usefulness in several LC applications [16–18]. In the field of CE, Vindevogel and Sandra [19] have used a Plackett–Burman design to improve the separation of testosterone esters while recently Rogan et al. [20] have investigated the potential use of the Plackett–Burman design for the chiral separation of the clenbuterol enantiomers.

The aim of this study is to establish adequate experimental conditions for the resolution of the fenfluramine enantiomers to be achieved in a relatively short analysis time in order to provide a suitable method for the assessment of the stereochemical purity of dexfenfluramine.

Dexfenfluramine, the dextro-rotatory *S*-(+)-enantiomer of racemic fenfluramine has proved to exhibit a more potent anorectic activity than the parent racemic compound. Recently a chiral LC method for the quality control monitoring of dexfenfluramine was proposed by Dou et al. [21]. This method is accomplished by a precolumn derivatization of the analytes followed by separation on different chiral stationary phases. In a new monograph of the French Pharmacopoeia X, the stereochemical purity of dexfenfluramine is tested by a gas chromatographic (GC) method [22]. For solid raw materials the limit of *R*-(−)-fenfluramine is 2%. Bechet et al. [14] have proposed a CD-CE approach for the chiral separation of a set of basic racemic drugs, including fenfluramine. The separation conditions proposed by these authors will be used in the experimental design as the nominal values for fenfluramine.

2. Experimental

The drug substance (*RS*)-fenfluramine–HCl (B.P.1973) was purchased from Federa (Brussels, Belgium), and dexfenfluramine–HCl high purity grade [<2% *R*-(−)-enantiomer] was obtained from Office Chimique (Waterloo, Belgium). Heptakis (2,6-di-O-methyl)-β-cyclodextrin (DMCD) was purchased from Sigma (St. Louis, MO, USA). Buffer solutions of 100 m*M* of orthophosphoric acid (85%) (Merck, Darmstadt, Germany) were prepared by adjusting the pH with Tris(hydroxymethyl aminomethane) (Merck) before adjusting the volume. The buffer solutions were filtered through a 0.2-μm membrane filter and methanol was then added to the buffer at the concentration indicated. DMCD was dissolved in the methanolic buffer at the required concentration.

CE separation was performed using a P/ACE 2100 (Beckman, Palo Alto, CA, USA). A fused-silica capillary (Beckman) had 75 μm internal diameter, 375 μm external diameter and was 57 cm long, with the detection window at 7 cm from the capillary outlet. The capillary was temperature controlled by a liquid coolant at the temperatures indicated.

Sample solutions for the experimental designs were 50 μg/ml of (*RS*)-fenfluramine–HCl dissolved in water. Samples were introduced by pressure for 1 s, analysed with an applied voltage as specified and detected at 214 nm. In between runs, the capillary was flushed for 2 min with buffer and left equilibrating for 5 min.

The nominal levels of the operating conditions as described by Bechet et al. [14] were a buffer of pH 3 containing 15 m*M* DMCD, 30% methanol and operated at 15°C and 25 kV. These values were compared with the lower levels and higher levels in design I and II, respectively, except for the temperature which was for practical reasons always higher than the nominal values.

Table 1
Eight-experiment Plackett–Burman design for seven factors and its two implementations

Exp	Factors						
	F1	F2	F3	F4	F5	D1	D2
1	+1	+1	+1	−1	+1	−1	−1
2	−1	+1	+1	+1	−1	+1	−1
3	−1	−1	+1	+1	+1	−1	+1
4	+1	−1	−1	+1	+1	+1	−1
5	-1	+1	−1	−1	+1	+1	+1
6	+1	−1	+1	−1	−1	+1	+1
7	+1	+1	−1	+1	−1	−1	+1
8	−1	−1	−1	−1	−1	−1	−1

Factors	Levels:	Design I		Design II	
		−1	+1	−1	+1
F1 DMCD concentration (m*M*)		10	15	15	20
F2 Amount of MeOH added (%, v/v)		20	30	30	40
F3 pH of the buffer		2.5	3.0	3.0	3.5
F4 Temperature of the capillary (°C)		20	15	15	25
F5 Voltage (kV)		20	25	25	30
D1		–	–	–	–
D2		–	–	–	–

3. Theory

3.1. The Plackett–Burman design

In Table 1 the Plackett–Burman design is presented as a seven-variable array for five selected factors (F1–F5) and two dummy factors (D1 and D2). Dummy factors are imaginary variables for which the change from one level to another does not cause any physical change.

The factors investigated were DMCD concentration, amount of methanol added to the buffer, pH of the buffer, temperature of the capillary and the applied voltage. Two complementary designs were constructed around the experimental conditions proposed by Bechet et al. [14] for the separation of fenfluramine enantiomers. These conditions were considered as the nominal values and used as (+1) level in design I and (−1) level in design II. Table 1 also shows the values of the factors to be implemented for designs I and II, respectively.

The effect of changing a factor from a low to a high level value was examined on some selected quality responses such as the resolution (R_s) of the enantiomers, the analysis time (t_m), considered as the migration time of the last eluting peak, and also the tailing factor (A_s) of the last peak.

Resolution and tailing factor were calculated according to the following equations:

$$R_s = \frac{1.18(t_2 - t_1)}{W_{0.5(1)} + W_{0.5(2)}}$$

$$A_s = \frac{W_{0.1}}{2f}$$

where t_1 and t_2 are the migration times of the first and second enantiomer, $W_{0.5(1)}$ and $W_{0.5(2)}$ are the peak widths measured at half height, $W_{0.1}$ is the width at 10% of the peak height and f is the distance measured from the leading edge of the peak to the perpendicular of the peak maximum at 10% of the peak height.

A composite quality response, Q^*, was introduced which reflects the desirability to obtain sufficient resolution within a short analysis time. Therefore the experimental data of either migration time and resolution, as shown in Table 2 and Table 3 for design I and II, respectively, were scaled between 0

Table 2
Results of Design I

Exp	Response (Y)					
	t_m (min)	R_s	A_s	t_m^*	R_s^*	Q^*
Response						
1	20.71	1.43	1.00	0.59	1.00	0.86
2	16.83	0.97	1.15	1.00	0.23	0.49
3	18.21	0.96	1.00	0.85	0.22	0.43
4	20.36	0.83	1.01	0.62	0.00	0.21
5	18.01	1.32	1.06	0.87	0.82	0.84
6	26.23	1.14	0.99	0.00	0.52	0.34
7	25.75	1.21	1.03	0.05	0.63	0.44
8	22.63	1.17	0.99	0.38	0.57	0.51
$\bar{Y}$ (average)	21.09	1.13	1.03			0.51
Effects						
E(CD)	4.34	0.05	−0.043			−0.101
E(MeOH)	−1.53	0.21	0.063			0.285
E(pH)	−1.19	−0.01	0.012			0.034
E(T)	−1.61	−0.27	0.038			−0.246
E(V)	−3.54	0.01	−0.023			0.139
E(D1)	−1.47	−0.13	0.048			−0.090
E(D2)	1.92	0.06	−0.018			−0.005
2.SE	3.41	0.20	0.07			0.130
Normalized effects						
%E(CD)	20.59[a]	4.21	−4.13			−19.68
%E(MeOH)	−7.27	18.38[a]	6.08			55.41[a]
%E(pH)	−5.65	−0.66	1.22			6.60
%E(T)	−7.62	−24.14[a]	3.65			−47.80[a]
%E(V)	−16.77[a]	1.11	−2.19			27.10[a]
%E(D1)	−6.96	−11.30	4.62			−17.64
%E(D2)	9.09	5.09	−1.70			−50.10
%2·SE	16.19	17.52	6.96			21.95

[a] denotes significant effects.

and 1. For the migration time, the shortest one of the eight experiments of a design was given the value 1 and the longest, zero, whereas for the resolution the largest was given the value 1 and the smallest, the value zero. The transformed data t_m^* and R_s^* were obtained by linear interpolation. The new response (Q^*) was defined as:

$$Q^* = \frac{t_m^* + 2R_s^*}{3}$$

so that $0 \leq Q^* \leq 1$. The higher the Q^* value, the better the compromise between resolution and migration time. In this definition the resolution was attributed arbitrarily two times more weight than the analysis time since the former response was considered more important from the analytical point of view.

Within each design the effect (E_x) of a particular factor X was calculated from the difference between the average result at the (+1)* level ($\Sigma Y_{x(+1)}/4$) and the average result at the (−1) level ($\Sigma Y_{x(-1)}/4$):

$$E_x = \frac{\Sigma Y_{x(+1)}}{4} - \frac{\Sigma Y_{x(-1)}}{4}$$

To facilitate comparison of the effects E_x of the five factors on different responses a normalized effect (%E_x) was calculated as follows:

$$\%E_x = \frac{E_x}{\bar{Y}} \cdot 100$$

Table 3
Results of Design II

Exp	Response (Y)					
	t_m (min)	R_s	A_s	t_m^*	R_s^*	Q^*
Response						
1	21.14	1.47	0.99	0.42	1.00	0.81
2	16.88	1.43	1.16	0.75	0.91	0.85
3	13.66	1.05	1.17	1.00	0.02	0.35
4	15.79	1.04	1.09	0.83	0.00	0.28
5	19.54	1.41	0.99	0.54	0.86	0.75
6	26.48	1.09	1.02	0.00	0.12	0.08
7	18.24	1.41	1.32	0.64	0.86	0.79
8	25.50	1.35	1.07	0.08	0.72	0.51
$\bar{Y}$ (average)	19.65	1.28	1.10			0.55
Effects						
E(CD)	1.52	−0.06	0.008			−0.129
E(MeOH)	−1.41	0.30	0.028			0.498
E(pH)	−0.23	−0.04	−0.033			−0.060
E(T)	−7.02	−0.10	0.168			0.031
E(V)	−4.24	−0.08	−0.083			−0.010
E(D1)	0.04	−0.08	−0.073			−0.120
E(D2)	−0.35	−0.08	0.047			0.130
2·SE	0.49	0.16	0.12			0.250
Normalized effects						
%E(CD)	7.74[a]	−4.49	0.68			−23.32
%E(MeOH)	−7.18[a]	23.22[a]	2.50			90.27[a]
%E(pH)	−1.17	−3.32	−2.95			−10.87
%E(T)	−35.73[a]	−7.61	15.21[a]			5.70
%E(V)	−21.58[a]	−6.05	−7.49			−1.79
%E(D1)	0.19	−6.05	−6.58			21.82
%E(D2)	−1.77	−6.44	4.31			23.64
%2·SE	2.52	12.49	11.13			42.49

[a] Denotes significant effects.

where $\bar{Y}$ is the average of all results for a particular response

$$\bar{Y} = \frac{\Sigma Y_x}{8}$$

The effects of the dummy factors (D1 and D2) were used to estimate the variability of the experiments. Therefore, the standard error (S.E.) was calculated as:

$$\text{S.E.} = \sqrt{\frac{\Sigma E_{(Di)}^2}{n_i}}$$

where $E_{(Di)}$ is the effect of a dummy factor and n_i is the number of dummies involved. The effect of a factor X was considered significant if $|\%E_x| > \%2\cdot\text{SE}$ [16,23]

4. Results

The results of design I and II are shown in Table 2 and Table 3, respectively and visualized by effect-plots in Fig. 1.

4.1. Influence of pH

pH was the only factor that did not produce any significant effects. This is in accordance with the fact that fenfluramine is completely ionized ($pK_a = 9$) and

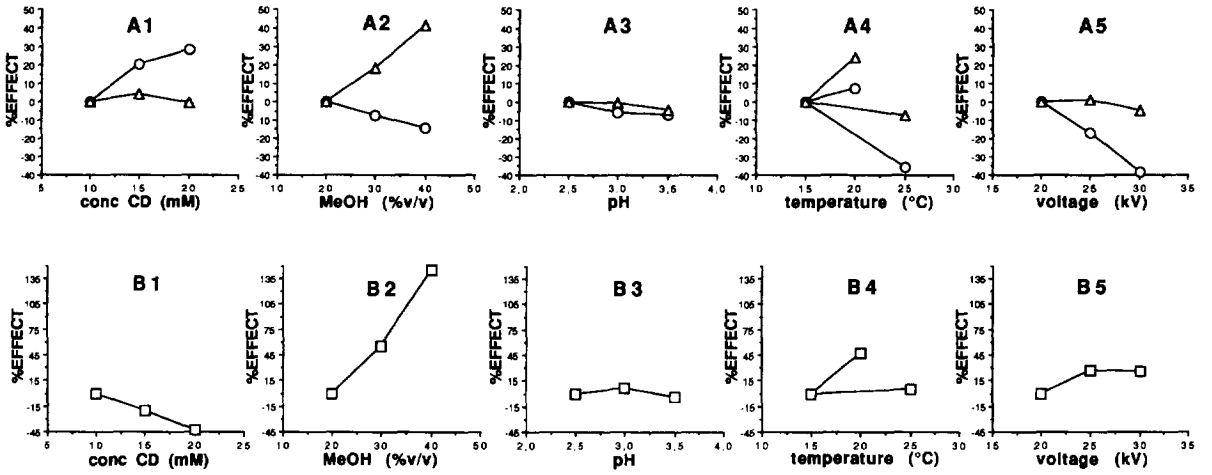

Fig. 1. Effect plots of the five factors on (A) migration time (○) and resolution (△); (B) composite response Q^* (□). 1, DMCD; 2, methanol; 3, pH; 4, temperature; 5, voltage.

that the electroosmotic flow is already extremely low in the pH range considered.

4.2. Influence of DMCD concentration

From the results of the design I as well as design II it is clearly seen that a high concentration of DMCD provides a significantly longer migration time than the lower one. This effect of cyclodextrin concentration on migration time also has been observed and commented by other authors [1,5,9,24,25]. As the concentration of DMCD is increased a larger fraction of complexed species (DMCD-enantiomer) is formed which has a smaller charge-to-mass ratio than the free enantiomer so that the mobility becomes lower. The resolution and the asymmetric factor were not significantly affected by variation of the DMCD concentration. The effect on the migration time is also visible in the small negative effect on Q^*, although it is statistically not significant.

4.3. Influence of methanol

Addition of methanol to the separating buffer showed a highly significant effect on the resolution of the fenfluramine enantiomers. This is in agreement with the observations by Bechet et al. [14] and the theory developed by Wren [5,6]. For enantiomers which are characterized by a high affinity for the chiral selector, such as fenfluramine, improved resolution can be obtained when working in conditions of decreased formation constants of the complexes, e.g. by adding methanol to the background electrolytes.

The effect of methanol on the migration time was only in the high concentration range (30–40%) statistically significant, whereas in the low concentration range (20–30%) a similar but not statistically significant effect was observed. In spite of the higher vicosity, the higher methanol concentration provided shorter migration time. This might be the result of the reduction in stability of the host–guest complex. In this situation the total mobilities of the fenfluramine enantiomers were more dominated by the increased fraction of free solutes which move faster due to their higher charge-to-mass ratio compared to the complex.

The favourable effects of methanol on both resolution and analysis time are also clearly visible in the significant effects on the composite response Q^*.

4.4. Influence of temperature

Because of the experimental difficulties in lowering the temperature below the nominal value of 15°C, the values for the temperature in the two designs do not follow the same trends as for the

other factors. This is also reflected in the slightly different nature of the effect plots. In the first design, the high value (20°C) was actually the lower level, therefore the trends are more obviously seen in the effect plots than in the table values.

For both resolution and migration time, maximum values are observed at 20°C. Only the high resolution at 20°C and the low migration time at 25°C are significant. The fact that a maximum is obtained at an intermediate factor value is very likely caused by opposing effects. The reduction of the viscosity, and thus increase in mobility, with temperature (apparently predominant and visible in the significant migration time decrease at 25°C) is an obvious factor. On the other hand, increasing the temperature should reduce complex formation constants and increase in resolution (as already explained in the discussion of the effects of methanol). This could explain the increase in resolution at 20°C. However, at 20°C, both effects, viscosity and formation constant reduction, should result in reduced migration time. Instead, a small increase is found at 20°C but this is statistically not significant.

The predominant effect on resolution also leads to a significant effect for the response factor Q^* at 20°C.

The tailing at 25°C was the only significant effect for this response in the two designs. It was not further considered or investigated.

4.5. Influence of the applied voltage

In the range of 20–30 kV the migration time is reduced at higher voltage values. This observation is obvious since the velocity of any charged compound is proportional to the electric field ($v=\mu E$).

An increase in resolution with voltage, expected from the fundamental CE-resolution equation [26], was not observed. However, it is well known that in-column heating effects at higher field strengths can easily compensate the expected positive effects from voltage on peak efficiency and resolution.

Although it was shown that a voltage of 30 kV produced a significantly shorter analysis time, the associated small loss in resolution resulted in the lack of significant effects on the composite response Q^* when changing from 25 to 30 kV. Therefore, the optimal voltage was determined from a separate experiment during the final optimization step.

5. Final optimization and analysis

From the results of the experimental design, the following conditions for the separation of the fenfluramine enantiomers were selected: low DMCD concentration (10 m*M*), high methanol content (40%) and intermediate temperature (20°C). The pH variations remain indifferent; therefore, the nominal pH of 3.0 (100 m*M* phosphate, adjusted with Tris) was retained. With these conditions the separation of the enantiomers performed at 25 kV and 30 kV was compared and resolution was found to be decreased from 1.7 at 25 kV to 1.3 at 30 kV. Taking into account the better resolution value, an applied voltage of 25 kV was preferred. Fig. 2 shows the typical electropherograms obtained at the nominal conditions (Fig. 2A) which gave a migration time for the second peak, the *R*-(−)-enantiomer, of 25.4 min and a resolution of 1.4, and at the optimized conditions (Fig. 2B) which gave a migration time for the second peak of 17.5 min and a resolution of 1.7. In the final result both the migration time and the resolution were improved.

The repeatability was evaluated on an aqueous solution of 400 μg/ml of dexfenfluramine–HCl in

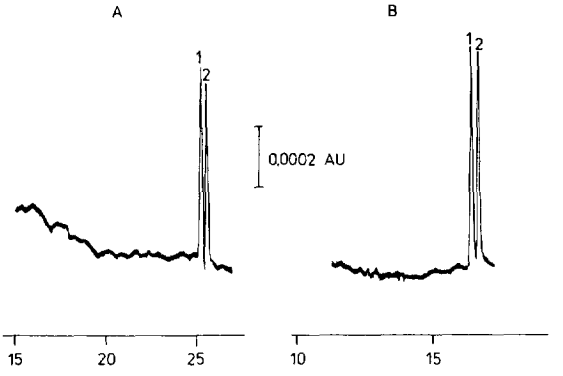

Fig. 2. Typical electropherograms of 50 μg/ml (*RS*)-fenfluramine solution obtained at (A) nominal conditions [15 m*M* DMCD in 100 m*M* phosphate buffer pH 3 containing 30% (v/v) MeOH, 25 kV, 15°C]; (B) optimized conditions [10 m*M* DMCD in 100 m*M* phosphate buffer pH 3 containing 40% (v/v) MeOH, 25 kV, 20°C]; peak 1, *S*-(+)-fenfluramine; peak 2, *R*-(−)-fenfluramine.

which a part was replaced by an appropriate quantity of (*RS*)-fenfluramine–HCl in order to get a final concentration of the *R*-(−)- enantiomer of 20 μg/ml corresponding to 5% of total (*RS*)-fenfluramine. Dexchlorfeniramine maleate was added as an internal standard at a concentration of 50 μg/ml. This solution was introduced six times for 1 s into the CE system. The relative standard deviation of the migration time and the area ratio to that of the internal standard were for dexfenfluramine (the first peak) 1.52% and 1.36%, respectively, whereas for the *R*-(−)-enantiomer (the second peak) the respective values were 1.54% and 5.06%.

A calibration line was constructed from a set of four aqueous standard solutions prepared by mixing appropriate volumes of 800 μg/ml of dexfenfluramine–HCl with appropriate volumes of 320 μg/ml of (*RS*)-fenfluramine–HCl in order to cover a concentration range of 8–32 μg/ml of *R*-(−)-fenfluramine. This range corresponds to 2–8% of 400 μg/ml of total (*RS*)-fenfluramine concentration of the standard solutions. Dexchlorfeniramine maleate was used as an internal standard at a concentration of 25 μg/ml. A linear regression line of the ratio of the peak area of *R*-(−)-fenfluramine to that of the internal standard as a function of the concentration was calculated using the least-squares method. Good linearity was obtained in the range studied (r= 0.999).

Using the optimized separation conditions and supported by the data of repeatability and linearity, the method could be applied for the assessment of the *R*-(−)-fenfluramine enantiomer as impurity present in dexfenfluramine–HCl raw material as well as pharmaceutical formulations. Therefore, sample solutions were prepared in water at a final concentration of 400 μg/ml dexfenfluramine–HCl to which the internal standard was added at a concentration of 25 μg/ml. The concentrations of *R*-(−)-fenfluramine were derived from the calibration line and the amounts of *R*-(−)-fenfluramine in the samples were calculated. In Fig. 3 the electropherograms obtained for two commercial samples of dexfenfluramine–HCl are shown, (A) a sample labelled <2% of *R*-(−)-enantiomer (undetected) and (B) a sample which was found to contain 3.8% of *R*-(−)-fenfluramine.

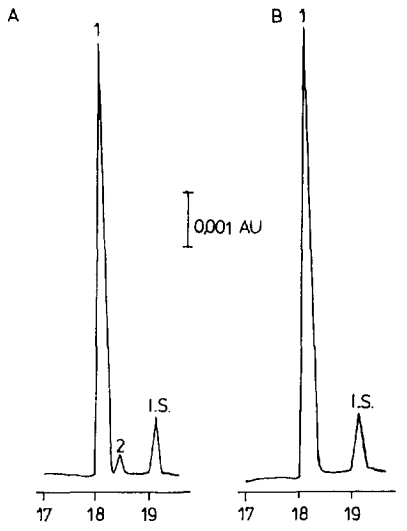

Fig. 3. Typical electropherograms of two commercial dexfenfluramine raw materials [sample 1 (A) and sample 2 (B)] obtained at optimized conditions (as in Fig. 2B). Peak 1, *S*-(+)-fenfluramine; peak 2, *R*-(−)-fenfluramine; I.S. (internal standard), dexchlorfeniramine maleate.

6. Conclusion

The use of a Plackett–Burman design has proved to be effective for the optimization of a CE chiral separation of the fenfluramine enantiomers. The effects of the concentration of the chiral selector DMCD, the concentration of methanol added to the separating buffer and the temperature of the capillary on the resolution and/or the migration time could be clearly observed from a statistical evaluation. The method developed and optimized by this design was tested with acceptable repeatability and linearity for the *R*-(−)-enantiomer impurity in the samples of dexfenfluramine.

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