Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1007 (2003) 165–177
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Robustness testing of chiral separations by capillary electrophoresis using highly-sulfated cyclodextrins

Catherine Perrin[a,b], Huguette Fabre[b], Mohamed Maftouh[c], D. Luc Massart[a], Yvan Vander Heyden[a,*]

[a]*Department Pharmaceutical and Biomedical Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 (VUB), Brussel, Belgium*
[b]*Laboratoire de Chimie Analytique, Faculté de Pharmacie, Montpellier, France*
[c]*Sanofi-Synthelabo Recherche, Analytical Research Department, Toulouse, France*

Received 14 April 2003; received in revised form 19 May 2003; accepted 23 May 2003

Abstract

The robustness of a generic method for chiral separation in capillary electrophoresis using highly-sulfated cyclodextrins in a low pH phosphate buffer and the ''short-end injection technique'' was studied. In this study, we focused on the robustness of the separations and not of the quantitative analysis of the enantiomers. The robustness was evaluated for the enantiomeric separation of a basic (propranolol), a neutral (praziquantel) and an acidic (warfarin) compound. The influence of eight factors which were believed to affect significantly the separations was studied using a 11-factor, 12-experiment Plackett–Burman design. Statistical interpretation of the factor effects on different analytical responses (selectivity and resolution) was performed. The separations of the three compounds could be considered as rather robust as the factor effects were generally not significant ($\alpha = 0.05$) and small.

Keywords: Chemometrics; Robustness; Enantiomer separation; Experimental design; Sulfated cyclodextrins; Propranolol; Praziquantel; Warfarin

1. Introduction

Capillary electrophoresis has been established as a very efficient technique for the separation of drug enantiomers. The main advantages of the technique are its high efficiency, short analysis times, versatility due to the great variety of chiral selectors that can be added to the background electrolyte (BGE), short equilibration times required when changing the chiral selector and low consumption of selector [1–6]. Among all possible selectors, cyclodextrins (CDs) are by far the most popular [1–7]. A wide array of charged and uncharged CD derivatives with different enantiorecognition abilities has been synthesized.

The main drawback in chiral separation methods using derivatized CDs is that these selectors are mainly available as complex mixtures which contain a large number of isomers differing in their degree of substitution, which may result in batch to batch selectivity differences [8–10]. The use of pure single

*Corresponding author. Tel.: +32-2-477-4737; fax: +32-2-477-4735.
E-mail address: yvanvdh@fabi.vub.ac.be (Y. Vander Heyden).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00965-8

enantiomers or very reproducible mixtures is required to obtain reproducible and robust methods. However, many efficient selectors are not available as pure single enantiomers or as chemically pure compounds. This is the case for several highly-sulfated cyclodextrins (HS-CDs) which have recently been synthesized and have shown to be very efficient and versatile selectors [11–16]. The selectors used in this study are commercially available in three forms α, β and γ HS-CD with an average degree of sulfation of 11, 12 and 13, respectively [14]. With these selectors, enantiomeric separations can usually be achieved with a single set of generic analytical conditions, i.e. a CD concentration of 5% (w/v) in a phosphate buffer of pH 2.5, which makes these selectors suitable for the rapid screening of large series of molecules [12–14]. Furthermore, due to their high selectivity, the short-end injection technique [17] can be used to reduce dramatically the analysis time, lower the applied voltage [12,13,18] and hence reduce the Joule effect.

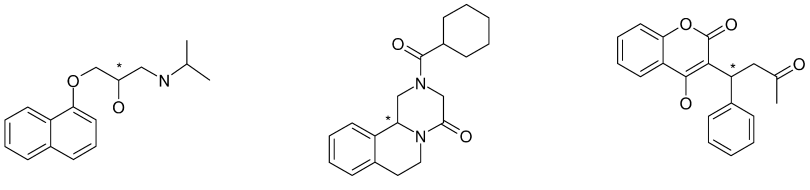
The robustness of a method can be defined as its ability to remain unaffected by small but deliberate variations in the experimental factors [19,20]. Robustness testing is part of method validation in which the variations in method conditions occurring between different laboratories are simulated. It is now well accepted that it should be performed at the beginning of the validation process or even at the end of the method development [19,21,22]. The introduced variations are intended to represent the variations that may occur when the method is performed under different conditions (different analysts, different instruments, different laboratories). The use of experimental designs, which allows investigating a large number of variables simultaneously, is recommended for testing the robustness of a method. Screening designs such as Plackett–Burman and fractional factorial designs are well suited to minimize the number of experiments [22–24].

The aim of this study was to evaluate whether reproducible and robust separations of charged and neutral chiral compounds might be expected when using HS-CDs as chiral selectors with the generic method described above [12]. The robustness of the separation was evaluated for a basic (propranolol), a neutral (praziquantel) and an acidic (warfarin) drug (Fig. 1). The evaluation of the obtained separations was made in the context of developing a generic CE separation strategy for chiral compounds. Such strategy was presented earlier [12]. However, it is important that the proposed strategy, in general, leads to robust separations or that one knows which factors are to be controlled strictly.

2. Materials and methods

2.1. Chemicals

R-Propranolol, *S*-propranolol, rac-warfarin and rac-praziquantel were purchased from Sigma (Steinheim, Germany). Highly-sulfated cyclodextrins (α, β and γ forms) were purchased from Beckman (Fullerton, CA, USA). Phosphoric acid 85% (w/v) and methanol were from Merck (Darmstadt, Germany), and triethanolamine from Fluka (Buchs, Switzerland). Doubly-distilled water, produced in



Propranolol
pK_A= 9.5

Praziquantel

Warfarin
pK_A= 5.0

Fig. 1. Hydrogen-depleted structure of the three chiral drugs whose separation was evaluated in the robustness study.

house (from a quartz apparatus), was used throughout.

2.2. Reagents

2.2.1. Electrolyte solutions

The BGE consisted of a solution of HS-CD dissolved to the required concentration in an acidic phosphate buffer prepared from phosphoric acid. The pH of the buffer was adjusted to the required value with triethanolamine (TEA). The CD and buffer concentrations, as well as the pH values of the electrolyte were adjusted according to the experimental set-up (see Results and discussion). The solutions were stored at 4 °C.

2.2.2. Sample solution

Stock solutions of *R*- and *S*-propranolol (500 μg/ml) in water were prepared. The *S*-propranolol solution was spiked with *R*-propranolol at a 0.4% (w/w) level.

Solutions of rac-praziquantel (200 μg/ml) in water and rac-warfarin (50 μg/ml) in water–methanol (70:30, v/v) were prepared. The solutions were stored at 4 °C.

2.3. Apparatus—separation protocol at nominal conditions

All experiments were performed on a P/ACE MDQ instrument (Beckman, Fullerton, CA, USA) equipped with a photodiode array detection system. A fused-silica capillary (TSP, Composite Metal Services, Hallow, UK), 31.2 cm total length (21 cm to the detector window), 50 μm I.D., housed in a cartridge with a detection window (100×800 μm) was used for all experiments. Prior to its first use, the capillary was preconditioned by washing for 20 min with 0.1 *M* sodium hydroxide and then for 3 min with water. Analytes were injected from the end of the capillary closest to the detection window (i.e. short-end injection). The effective separation length was 10 cm. The operating conditions for the generic method under investigation are given in Table 1.

2.4. Calculations

The separation selectivity, *S*, was calculated using the mobility difference between the enantiomers [25]:

$$S = \frac{\Delta\mu_{\text{eff}}}{\bar{\mu} + \mu_{\text{eof}}} = \frac{\mu_1 - \mu_2}{\bar{\mu} + \mu_{\text{eof}}} \quad (1)$$

where $\Delta\mu_{\text{eff}} = \mu_1 - \mu_2$ is the difference in effective mobilities of the two enantiomers, $\bar{\mu} = 1/2(\mu_1 + \mu_2)$ the average effective mobility of the two enantiomers, and μ_{eof} the mobility of the electroosmotic flow.

The resolution was calculated as follows [26]:

$$R_s = 2\left(\frac{t_2 - t_1}{w_2 + w_1}\right) \quad (2)$$

where t_1, t_2 are the migration times and w_1, w_2 the peak widths at baseline of the first and second enantiomers, respectively.

2.5. Software

The statistical analysis of the data was done with the software RTS (ruggedness testing strategy, software of the Vrije Universiteit Brussel, version 1.1.11) [27].

Table 1
Separation protocol at nominal levels

Step of the analysis protocol	Conditions
1 Rinse	60 s, 0.1 *M* NaOH, 20 °C
2 Rinse	120 s, 50 m*M* phosphate buffer, pH 2.5, 20 °C
3 Rinse	60 s, separation buffer, 20 °C
4 Sample injection	3 s, 0.5 p.s.i., cathodic side, 20 °C
5 Separation	+ 9.4 kV, 20 °C
6 Detection	214 nm

3. Results and discussion

The generic method which is proposed uses an electrolyte consisting of 5% HS-CDs (w/v) in a 50 m*M* solution of phosphoric acid adjusted at pH 2.5 with triethanolamine to prevent analyte adsorption to the capillary wall [12,28]. Preliminary studies showed that the rinsing steps are of a great importance to obtain a good repeatability of migration times (MTs) and peak areas. Indeed when the capillary was only rinsed with the running buffer, a progressive increase of the MTs was observed resulting in nonrepeatable experiments. After some investigation, it was found necessary to first rinse the capillary with a diluted solution of sodium hydroxide to achieve repeatable MTs, although this is quite unusual when using an acidic running buffer. Afterwards, the capillary is rinsed for 2 min with a phosphate buffer, pH 2.5, to equilibrate the capillary and prevent hysteresis effects due to sodium hydroxide. Finally, the capillary is rinsed with the separation buffer. The sample is hydrodynamically injected at the end of the capillary situated on the detector side (''short-end injection technique''), and the separation is performed in the conventional polarity mode. The short separation length (10 cm) gives a fast separation with a rather low voltage, which results in a current of approximately 110 μA. Although the current value is rather high due to the presence of the HS-CD, the current appears to be very stable and the experiments are repeatable. The electrolyte solution has to be changed after 20 runs due to the depletion in HS-CDs occurring in the electrolyte vial situated at the cathodic side as the HS-CDs migrate progressively to the anode.

The chiral separation of 0.4% (w/w) *R*-enantiomer in *S*-propranolol, of rac-praziquantel and of rac-warfarin was achieved with the HS-α-CD, the HS-γ-CD and the HS-β-CD, respectively [10]. Electropherograms obtained under nominal conditions are shown in Fig. 2. It might be remarked that under the conditions applied the acidic compound, warfarin, is

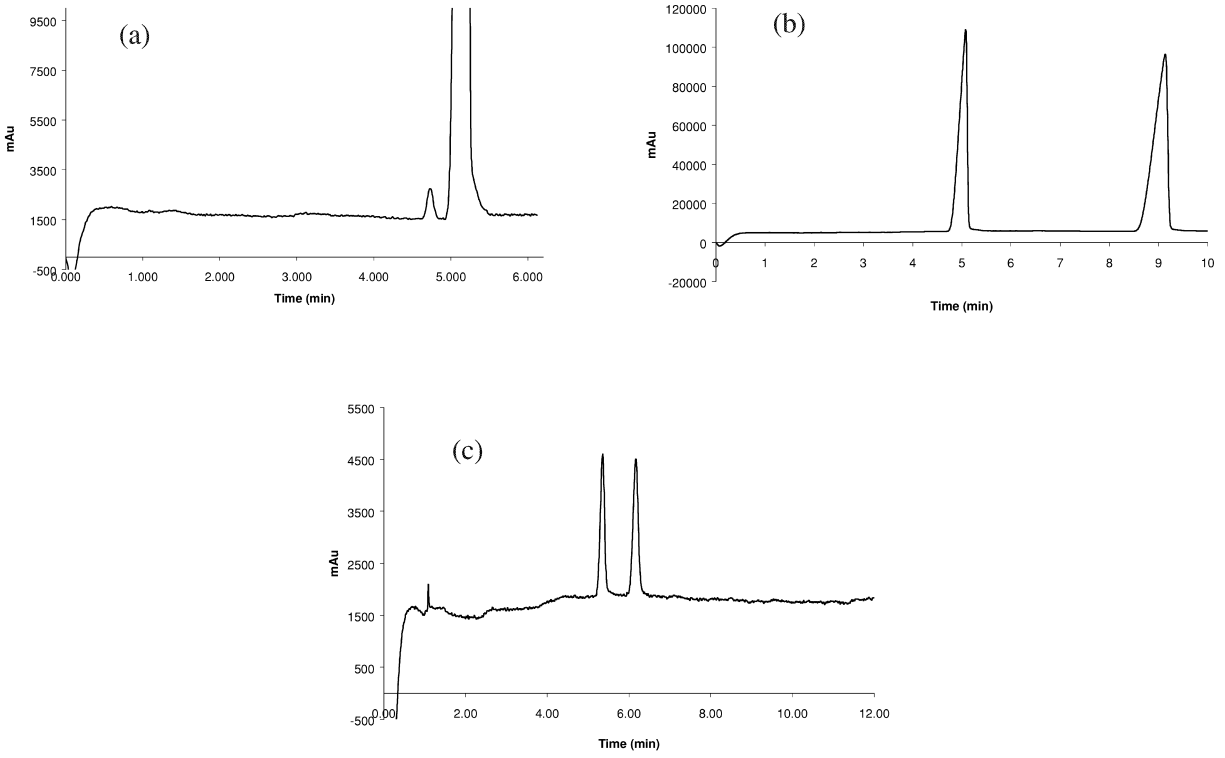


Fig. 2. Electropherograms of the chiral separation of propranolol with HS-α-CD (a), praziquantel with HS-γ-CD (b) and warfarin with HS-β-CD (c), under nominal conditions (see Tables 1 and 4).

largely uncharged. However, for most acidic drugs this will be the case at pH 2.5 and thus warfarin might be considered a representative acidic drug molecule.

3.1. Repeatability

Good repeatability under nominal conditions is a prerequisite for conducting a robustness test. The repeatability of the separations under nominal conditions was investigated by injecting each analyte solution ten times successively (Table 2). Very good repeatability was achieved with $<2\%$ RSD for selectivity and resolution, and peak area variations within 4%. Excellent MTs repeatability was obtained ($<0.5\%$).

3.2. Robustness

3.2.1. Selection of factors, factor levels and experimental design

Eight factors which potentially might affect the enantiomeric separation were selected, based on theoretical considerations, to be tested for robustness

(i) According to the theoretical model of Wren [29] selectivity and resolution are mainly affected by changes in the difference of apparent mobilities between the two enantiomers ($\Delta\mu$) which depends on the equilibrium constants of the two enantiomer–CD complexes (K_1, K_2), on the electrophoretic mobilities of the uncomplexed and complexed analyte, and on the CD concentration, following the equation:

$$\Delta\mu = \frac{(\mu_f - \mu_c)(K_2 - K_1)C}{1 + (K_1 + K_2)C + K_1K_2C^2} \quad (3)$$

where μ_f is the electrophoretic mobility of the free analyte, μ_c the electrophoretic mobility of the analyte–chiral selector complex and C the concentration of the chiral selector.

(ii) Williams and Vigh [30,31] have proposed an extended mathematical model which takes into account the acid–base equilibrium for weak analytes and considers the pH of the BGE as another important factor for selectivity. The pH affects the mobility of the complexed and uncomplexed analytes and the level of the EOF, and therefore may significantly alter the separation.

(iii) The separation temperature influences the kinetics and the thermodynamics of the inclusion-complexation process. It also affects the mobility of the species and of the EOF, by changing the viscosity of the electrolyte.

(iv) The buffer concentration may modify the inclusion–complexation equilibrium by a salting out effect. It has also an impact on the EOF, on the analyte mobility and on the plate number by a stacking or destacking effect.

(v) The variations of the electric field affect peak efficiency, EOF velocity and thus resolution.

(vi) The injection volume (injection time×applied pressure) determines the plug length and may alter efficiency and resolution.

(vii) The rinse volumes may affect the level of the EOF and the capillary equilibrium.

Table 2
Repeatability estimates of the chiral separation of propranolol, praziquantel and warfarin under nominal conditions ($n=10$)

	Responses						
	S	R_s	Peak area 1[a]	Peak area 2[a]	MT_1	MT_2	MT_2/MT_1
Propranolol							
Mean	0.026	2.65	254	36033	4.738	5.228	1.103
RSD (%)	0.91	1.00	1.79	1.40	0.49	0.37	0.29
Praziquantel							
Mean	0.147	9.05	31983	16282	5.178	9.487	1.832
RSD (%)	0.41	0.80	1.98	3.12	0.20	0.32	0.26
Warfarin							
Mean	0.034	4.07	542	478	5.401	6.186	1.145
RSD (%)	0.71	1.70	3.90	3.68	0.28	0.30	0.10

[a] 1 and 2 are related to the first and the last migrating enantiomer, respectively.

From the above considerations, the factors tested were: the HS-CD concentration, the pH of the electrolyte, the phosphate concentration of the buffer, the separation temperature, the separation voltage, the rinse volume with sodium hydroxide, the rinse volume with the separation electrolyte and the injection time at fixed injection pressure. A 11-factor 12-experiment Plackett–Burman design (Table 3) at two levels (low, −1 and high, +1) which requires 12 experiments was used. The design was selected based on the robustness testing strategies described in Ref. [22]. For more theoretical background about Plackett–Burman designs and their construction, we refer to Refs. [22,23]. Plackett–Burman designs are frequently used in robustness testing [32–37].

The design selected can examine maximally 11 factors. If the number to be examined is less, e.g. eight factors in this study, the remaining factor columns represent imaginary variables, called dummy factors. These dummies do not represent anything physical or chemical and thus their occurrence at low and high level has no impact on the execution of the analysis protocol. However, the effects calculated for these dummies, which are due to experimental error, can be used in the statistical interpretation of the factor effects on the different experimental responses (see Section 3.2.2).

The factor levels were chosen below and above the nominal values, specified in the analytical procedure (Table 4). The extreme levels were determined based on the uncertainty with which the factor can be set and reset at the nominal level. This uncertainty was estimated for each factor, based on the precision of the glassware, balance, pH meter . . . used for the preparation of the solutions, or on the precision of the instrumentation used for the separation. In order to simulate the possible inter- and

Table 4
Factors and their levels as studied in the robustness test; level 0 corresponds to the nominal value

Factors	Level	Value
1 [HS-CD]	−1	4.5% (w/v)
	0	5.0% (w/v)
	+1	5.5% (w/v)
2 pH of BGE	−1	2.2
	0	2.5
	+1	2.8
3 [BGE]	−1	45 m*M*
	0	50 m*M*
	+1	55 m*M*
4 Capillary temperature	−1	17 °C
	0	20 °C
	+1	23 °C
5 Separation voltage	−1	8.5 kV
	0	9.4 kV
	+1	10.3 kV
6 Rinse volume with NaOH	−1	4.27 μl
	0	5.12 μl
	+1	5.98 μl
7 Rinse volume with BGE	−1	4.27 μl
	0	5.12 μl
	+1	5.98 μl
8 Injection time	−1	2.7 s
	0	3.0 s
	+1	3.3 s

Table 3
The 11-factor 12-experiment Plackett–Burman design used to screen eight factors in the robustness study

Exp. no.	Factors								Dummy factors		
	1	2	3	4	5	6	7	8	D1	D2	D3
1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1
2	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1
3	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1
4	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1
5	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1
6	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1
7	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1
8	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1
9	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1
10	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1
11	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

intra-laboratory variations, the extreme levels of the factors were calculated by enlarging the uncertainty intervals by a factor three, as suggested by Vander Heyden et al. [22].

The responses analyzed were the selectivity factor, S, and the resolution, R_s, which characterize the separation. Experiments of the design were performed in a random sequence. In order to check for possible drift (i.e. time effects) [22], an experiment at nominal levels was performed at the beginning, in the middle and at the end of the experimental design.

3.2.2. Calculation of effects and statistical interpretation

The effect of each factor, E_X, on the different experimental responses was calculated as follows:

$$E_{x[+1,-1]} = \frac{\sum Y(+1)}{n} - \frac{\sum Y(-1)}{n} \quad (4)$$

where $\Sigma Y(-1)$ and $\Sigma Y(+1)$ represent the sum of the responses when the factor is at low or high level, respectively, and n is the number of experiments in the design where the factor is at a low or high level, respectively.

In order to be more easily interpretable from an analyst point of view, the effects of the factors were normalized to the average nominal response ($\bar{Y}$):

$$\%\, E_X = \frac{E_X}{\bar{Y}} \cdot 100 \quad (5)$$

Statistical significance of the effects was determined by comparing each calculated effect with a critical value ($E_{critical}$) derived from a t-test [22,23]:

$$|E_X| \Leftrightarrow E_{critical} = t_{critical} SE_e \quad (6)$$

or when normalized

$$|\%E_X| \Leftrightarrow \%E_{critical} = \frac{E_{critical} \cdot 100}{\bar{Y}} \quad (7)$$

where SE_e represents the standard error on an effect and $t_{critical}$, the tabulated t-value at a significance level α and with an appropriate number of degrees of freedom.

An estimate of the experimental error SE_e was calculated for each response from the three dummy factors available in the Plackett–Burman design [22]:

$$SE_e = \sqrt{\frac{\sum E^2_{dummy}}{n_{dummy}}} \quad (8)$$

where ΣE^2_{dummy} is the sum of squared dummy effects and n_{dummy} is the number of dummy factors (three in this study). Several error estimates can be used in the statistical interpretation, but the use of dummy factor effects from Plackett–Burman designs was found appropriate in robustness tests [38].

An effect is considered to be significant at the significance level α, if $|E_X| \geq E_{critical}$. The significance levels $\alpha = 0.05$ and $\alpha = 0.01$ were considered in this study. The number of degrees of freedom to determine $t_{critical}$ is n_{dummy} [22,23].

The calculated effect of a factor represents the change in the response that can be expected when this factor is varied from the low to the high level. Therefore, the effect from an extreme level to the nominal level is the calculated effect divided by two. The latter estimates are only valid when: (i) a linear behavior of the response in the interval [−,+] is assumed; (ii) the factors are quantitative, and (iii) the nominal levels are situated in the middle of the [−,+] intervals [23].

3.2.3. Results of the robustness tests

The CDs used during the robustness tests are those with which chiral separation was achieved earlier, i.e. HS-α-CD for propranolol, HS-γ-CD for praziquantel and HS-β-CD for warfarin, respectively. The experiments performed at the beginning, in the middle and at the end of the experimental design showed that no drift in the considered responses occurred (Table 5).

The calculated effects of the factors and the $E_{critical}$ values at $\alpha = 0.05$ and 0.01 are given in Tables 6–8 and visualized in Figs. 3–5 for propranolol, praziquantel and warfarin respectively. A negative sign of the effect means there is a decrease of the response when the factor is changed from the low to the high level.

3.2.3.1. Propranolol enantiomers (basic analytes)

None of the factors had a significant effect either on selectivity or resolution (Table 6, Fig. 3) notwithstanding the fact that the MT for each enantiomer (results not presented) was significantly influenced

Table 5
Selectivity and resolution obtained for propranolol, praziquantel and warfarin enantiomers in the Plackett–Burman design

Exp. no.	Propranolol		Praziquantel		Warfarin	
	S	R_s	S	R_s	S	R_s
0	0.025	2.64	0.149	9.02	0.036	4.11
1	0.021	2.30	0.125	9.07	0.037	4.15
2	0.023	2.63	0.144	10.12	0.037	4.45
3	0.021	2.42	0.132	8.27	0.036	3.38
4	0.022	2.37	0.134	8.86	0.038	4.24
5	0.021	2.41	0.139	9.17	0.037	3.42
6	0.025	2.55	0.134	8.75	0.037	3.65
0	0.027	2.63	0.143	8.97	0.036	4.08
7	0.023	2.42	0.135	9.43	0.036	3.58
8	0.021	2.56	0.144	9.05	0.036	4.43
9	0.023	2.71	0.146	11.08	0.035	4.07
10	0.023	2.63	0.140	8.04	0.038	4.30
11	0.021	2.19	0.136	9.99	0.037	3.28
12	0.022	2.60	0.143	9.62	0.036	3.99
0	0.027	2.68	0.143	9.11	0.036	4.05

S, selectivity; R_s, resolution.

by several factors, among which the pH of the buffer and the separation voltage had the largest effects. Since CDs and propranolol are fully ionised in the pH range investigated, the pH change does not influence the stability of the inclusion complexes (which is confirmed by the results obtained for selectivity) and the increase of the MTs with pH can be attributed to the increase of the EOF, which is in the opposite direction. The MTs were also significantly influenced by variations of the separation voltage which decreased with an increasing voltage. However, as selectivity and resolution were not affected, the separation of propranolol enantiomers can be considered robust.

3.2.3.2. Praziquantel enantiomers (neutral analytes)

A significant effect ($\alpha = 0.05$) of the capillary temperature on both selectivity and resolution was observed (Table 7; Fig. 4). The temperature has a negative effect which means that an increase in the temperature results in a decrease in selectivity and resolution. The temperature can affect the stability of the inclusion complexes. However, the effect on the selectivity was small and only at the border of the significance value. The effect of the injection time was also significant for resolution ($\alpha = 0.05$), which was due to a decrease in peak efficiencies with increasing injection volume. The maximal decrease in resolution in the examined domain which could be expected if the factors temperature and injection volumes are varied simultaneously at the worst-case

Table 6
Calculated effects of the factors on the separation of propranolol enantiomers

Factor	Response			
	Selectivity		Resolution	
	Effect	% Effect	Effect	% Effect
1 [HS-CD]	−0.00067	−2.67	−0.027	−1.01
2 pH of BGE	0.00033	1.33	0.107	4.04
3 [BGE]	0.00000	0.00	0.027	1.01
4 Capillary temperature	−0.00033	−1.33	−0.073	−2.78
5 Separation voltage	−0.00033	−1.33	−0.120	−4.55
6 Rinse volume with NaOH	−0.00067	−2.67	−0.003	−0.13
7 Rinse volume with BGE	0.00100	4.00	−0.003	−0.13
8 Injection time	−0.00033	−1.33	−0.100	−3.79
D1	−0.00033	−1.33	−0.040	−1.52
D2	0.00067	2.67	0.047	1.77
D3	0.00067	2.67	0.023	0.88
Critical value 5% (absolute value)	0.00194	7.35	0.121	4.58
Critical value 1% (absolute value)	0.00337	13.49	0.222	8.40

Table 7
Calculated effects of the factors on the separation of praziquantel enantiomers

Factor	Response			
	Selectivity		Resolution	
	Effect	% Effect	Effect	% Effect
1 [HS − CD]	−0.00283	−1.90	0.388	4.31
2 pH of BGE	0.00250	1.68	0.165	1.83
3 [BGE]	0.00383	2.57	0.315	3.49
4 Capillary temperature	−0.00850	−5.71*	−0.915	−10.15*
5 Separation voltage	−0.00417	−2.80	0.305	3.38
6 Rinse volume with NaOH	−0.00250	−1.68	−0.432	−4.79
7 Rinse volume with BGE	0.00017	−0.11	0.262	2.90
8 Injection time	−0.00083	−0.56	−0.968	−10.74*
D1	0.00217	1.45	0.392	4.35
D2	−0.00250	−1.68	−0.062	−0.68
D3	0.00250	1.68	−0.115	−1.28
Critical value 5% (absolute value)	0.00761	5.11	0.758	8.41
Critical value 1% (absolute value)	0.01398	9.39	1.392	15.44

*, Significant at $\alpha = 0.05$.

level (i.e. high level) is around 10% when compared to the nominal level. The result remains acceptable given the very good nominal separation. Thus, the robustness test taught us that for the separation of praziquantel, the factors temperature and injection time are best controlled within the interval examined.

Although an increased level of several factors (CD concentration, BGE concentration, separation voltage

Table 8
Calculated effects of the factors on the separation of warfarin enantiomers

Factor	Response			
	Selectivity		Resolution	
	Effect	% Effect	Effect	% Effect
1 [HS-CD]	−0.00117	−2.78	−0.193	−4.68
2 pH of BGE	0.00033	0.93	0.723	17.51**
3 [BGE]	0.00033	0.93	−0.190	−4.60
4 Capillary temperature	0.00067	1.85	−0.157	−3.79
5 Separation voltage	0.00067	1.85	−0.117	−2.83
6 Rinse volume with NaOH	0.00033	0.93	0.033	0.81
7 Rinse volume with BGE	−0.00033	−0.93	−0.033	−0.81
8 Injection time	0.00000	0.00	−0.040	−0.97
D1	−0.00033	−0.93	−0.127	−3.07
D2	0.00000	0.00	−0.100	−2.42
D3	0.00067	1.85	0.073	1.78
Critical value 5% (absolute value)	0.00102	3.80	0.326	7.89
Critical value 1% (absolute value)	0.00186	6.98	0.598	14.47

**, Significant at $\alpha = 0.01$.

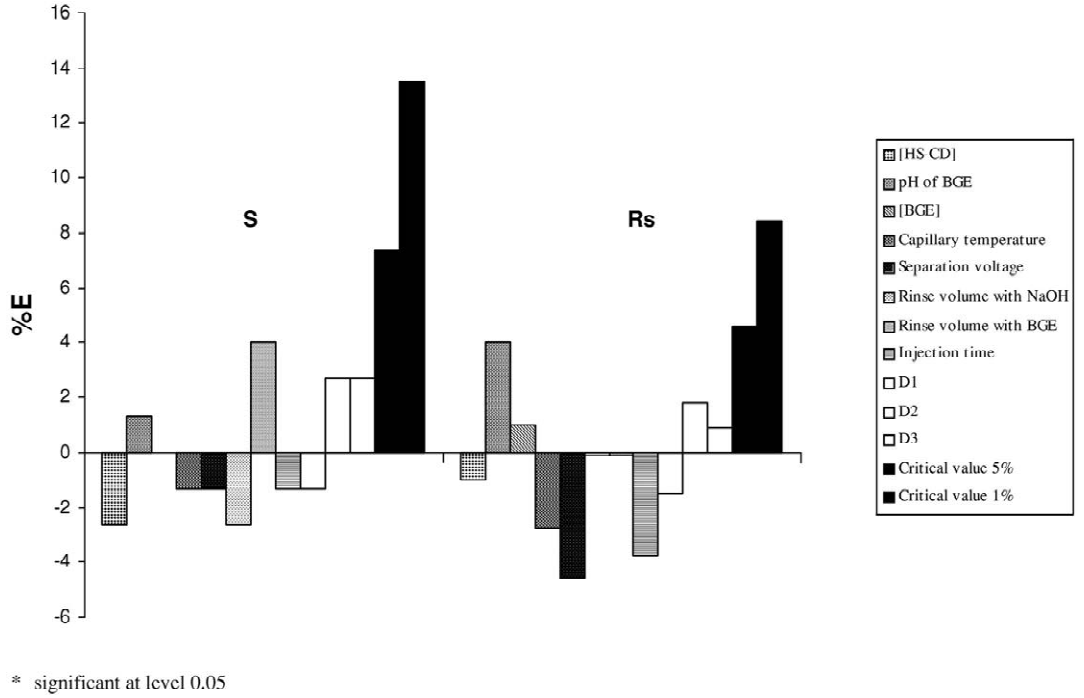


Fig. 3. Effect of the factors on the separation of propranolol enantiomers. Dummy factor effects (D1, D2, D3) and critical effects are also shown.

and temperature) resulted in a significant negative decrease in the MTs, the relative MTs (i.e. MT_2/MT_1) remained unaffected by any of the factors, which is also an indication for the rather robust behavior of the separation.

3.2.3.3. Warfarin enantiomers (acidic analytes)

Selectivity was unaffected by any changes of the factors. The pH of the electrolyte turned out to be a critical factor for resolution (Table 8; Fig. 5). Calculation of the peak efficiency showed that the increase observed in resolution when the pH was changed from 2.2 to 2.8 was due to a dramatic increase (30%) of the peak efficiency for both enantiomers. The variation in resolution with respect to the nominal level is acceptable ($<10\%$) but to maintain the resolution similar to that at nominal conditions, the pH is preferably controlled to as close as possible around the nominal level.

As observed for propranolol and praziquantel, several factors (CD concentration, BGE concentration, separation voltage and temperature) were found to be statistically significant on the MTs, mainly the separation voltage and the separation temperature. However, the decrease of MTs is lower than 9% (from the extreme level to the nominal level) for each enantiomer and the relative MTs were not affected by changes in the factor levels.

3.2.4. Batch-to-batch precision

The batch-to-batch variations can be a weak point of chiral separations using derivatized CDs, due to the large number of possible isomers, yielding different selectivity. The HS-CDs used in this study are produced as mixtures with an average degree of substitution and the synthesis process should ensure very reproducible mixtures. The batch-to-batch precision was evaluated using four different lots of each HS-CD. Freshly prepared phosphate buffers were used to prepare each electrolyte solution. Each solution was injected twice. Very good precision was found between batches with $<5\%$ variation for

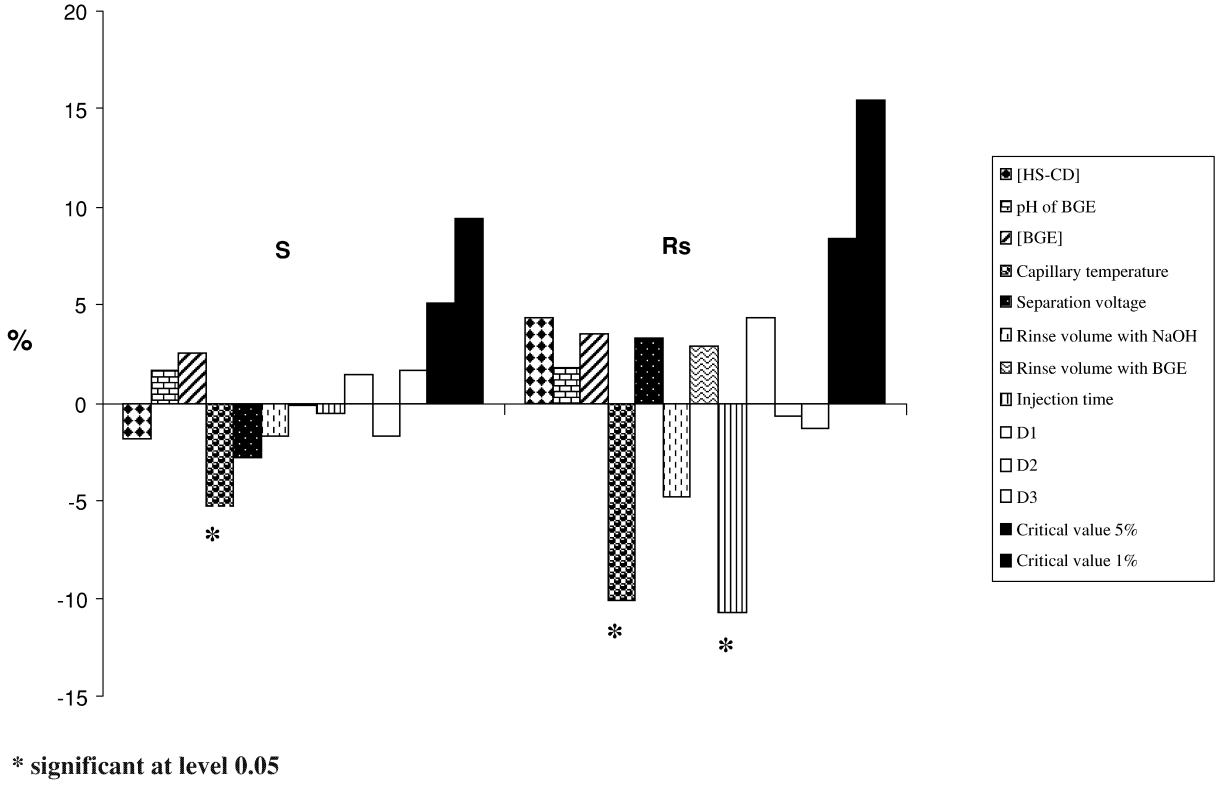


Fig. 4. Effect of the factors on the separation of praziquantel enantiomers. Dummy factor effects (D1, D2, D3) and critical effects are also shown.

selectivity, resolution, migration times and peak areas (Table 9). The use of the relative MTs between the two enantiomers improves appreciably the repeatability (<1% variation).

4. Conclusions

The robustness of the enantiomeric separation of a basic (propranolol), a neutral (praziquantel) and an acidic (warfarin) compound with a generic CE method using highly-sulfated cyclodextrins in a low pH buffer and using the ''short end injection technique'' was evaluated. The Plackett–Burman design used for robustness testing yielded the conclusion that the separations could be considered as rather robust with regards to eight potential critical factors. However, the robustness test showed that the pH is best controlled strictly, especially when separating the enantiomers of an acidic compound (warfarin in this case). For this latter analyte an increase in the pH resulted in an increase of resolution. Another recommendation from the robustness test is to pay some attention to the standardization of the separation temperature since it might have an effect on some separations. It is also interesting to notice that the cyclodextrin concentration, which is usually known to play a key role on enantiomeric separation, was not found to be a critical factor in this robustness study. However, the robustness test has shown that the generic approach from which the nominal conditions were defined led to separations that were not dramatically disturbed by small changes in the method parameters. This confirms that the generic method which is proposed is a good candidate for

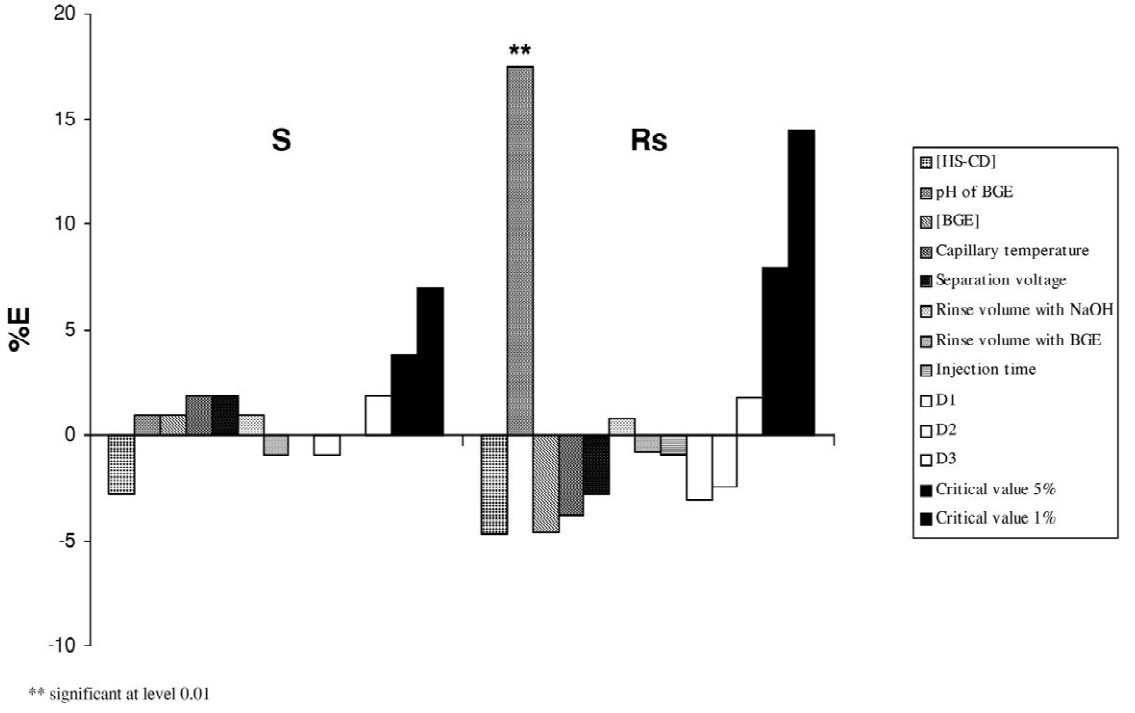


Fig. 5. Effect of the factors on the separation of warfarin enantiomers. Dummy factor effects (D1, D2, D3) and critical effects are also shown.

further validation and for routine use. Furthermore, in addition to their high selectivity, the very good repeatability of separations (MTs, resolution, peak areas) which were achieved using different batches of HS-CD is also an important point in favor of these selectors.

Table 9
Repeatability of the chiral separation of propranolol, praziquantel and warfarin under nominal conditions using different HS-CD batches ($n=4$)

	Responses						
	S	R_s	Peak area 1[a]	Peak area 2[a]	MT 1	MT 2	MT_2/MT_1
Propranolol							
Mean	0.028	2.71	251	35 809	4.687	5.182	1.107
RSD (%)	1.32	3.80	3.05	2.86	3.21	2.98	0.80
Praziquantel							
Mean	0.144	9.03	35975	16 580	5.218	9.444	1.809
RSD (%)	4.89	1.54	4.83	3.58	2.26	4.31	1.50
Warfarin							
Mean	0.036	3.98	541	476	5.425	6.261	1.154
RSD (%)	1.15	3.28	3.85	3.85	2.73	2.83	0.17

MT, migration time.
[a] 1 and 2 are related to the first and the last migrating enantiomer, respectively.

References

[1] G. Gübitz, M.G. Schmid, J. Chromatogr. A 792 (1997) 179.
[2] R. Vespalec, P. Boček, Chem. Rev. 100 (2000) 3715.
[3] K. Verleysen, P. Sandra, Electrophoresis 19 (1998) 2798.
[4] B. Chankvetadze, J. Chromatogr. A 792 (1997) 269.
[5] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (2001) 309.
[6] A. Amini, Electrophoresis 22 (2001) 3107.
[7] S. Fanali, J. Chromatogr. A 875 (2000) 89.
[8] E.C. Rickard, R.J. Bopp, J. Chromatogr. 680 (1994) 609.
[9] I.E. Valko, H.A.H. Billiet, J. Frank, K.C.A.M. Luyben, J. Chromatogr. 678 (1994) 139.
[10] L. Liu, L.M. Osborne, M.A. Nussbaum, J. Chromatogr. A 745 (1996) 45.
[11] F.-T.A. Chen, PACE Setter 3 (1999) 9.
[12] C. Perrin, Y. Vander Heyden, M. Maftouh, D.L. Massart, Electrophoresis 22 (2001) 3203.
[13] J. Chapman, F.-T.A. Chen, LC·GC Eur. 14 (2001) 33.
[14] F.-T.A. Chen, G. Shen, R.A. Evangelista, J. Chromatogr. A 924 (2001) 523.
[15] K. Verleysen, T. Van den Bosch, P. Sandra, Electrophoresis 20 (1999) 2650.
[16] N. Matthijs, C. Perrin, M. Maftouh, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 27 (2002) 515.
[17] K.D. Altria, M.A. Kelly, B.J. Clark, Chromatographia 700 (1996) 179.
[18] A.B. Bergholdt, S.V. Lehmann, Chirality 10 (1998) 699.
[19] ICH Harmonised Tripartite Guideline, Validation of analytical procedures: methodology. Recommended for adoption at step 4 of the ICH process on 6 by the ICH Steering committee on 6 November 1996 (http://www.ifpma.org/pdfifpma/q2b.pdf).
[20] The United States Pharmacopeia, 26th ed., United States Pharmacopeia Convention, Rockville, 2003.
[21] F.J. Van de Vaart, Het Pharmaceutisch Weekblad 127 (1992) 1229.
[22] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.
[23] Y. Vander Heyden, D.L. Massart, in: A. Smilde, J. De Boer, M. Hendriks (Eds.), Robustness of Analytical Methods and Pharmaceutical Technological Products, Elsevier, Amsterdam, 1996, p. 79.
[24] E. Morgan, Chemometrics: Experimental Design, Analytical Chemistry by Open Learning, Wiley, Chichester, 1991.
[25] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, 1997.
[26] J.C. Giddings, Sep. Purif. Sci. 4 (1969) 181.
[27] F. Questier, Y. Vander Heyden, D.L. Massart, J. Pharm. Biomed. Anal. 18 (1998) 287.
[28] M. Fillet, J. Crommen, Séparation énantiomérique de medicaments par électrophorèse capillaire à l'aide de cyclodextrines, Ph.D. Thesis in Pharmaceutical Science, Université de Liège, 1998.
[29] S.A. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[30] R.L. Williams, G. Vigh, J. Chromatogr. A 716 (1995) 197.
[31] B.A. Williams, G. Vigh, J. Chromatogr. A 777 (1997) 295.
[32] A. Nguyen Minh Nguyet, L. Tallieu, J. Plaizier-Vercammen, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 32 (2003) 1.
[33] S.-W. Sun, H.-T. Su, J. Pharm. Biomed. Anal. 29 (2002) 881.
[34] S. Furlanetto, S. Orlandini, E. La Porta, S. Coran, S. Pinzauti, J. Pharm. Biomed. Anal. 28 (2002) 1161.
[35] M.B. Sanz, L.A. Sarabia, A. Herrero, M.C. Ortiz, Talanta 56 (2002) 1039.
[36] H. Fabre, N. Mesplet, J. Chromatogr. A 897 (2000) 329.
[37] M. Jimidar, N. Niemeijer, R. Peeters, J. Hoogmartens, J. Pharm. Biomed. Anal. 18 (1998) 479.
[38] Y. Vander Heyden, C. Hartmann, D.L. Massart, L. Michel, P. Kiechle, F. Anni, Anal. Chim. Acta 3169 (1995) 15.