Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1049 (2004) 195–203
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Chiral capillary electrophoretic method for quantification of apomorphine

Pham Thi Thanh Ha[a], Ann Van Schepdael[a,*], Laurence Van Vaeck[b], Patrick Augustijns[b], Jos Hoogmartens[a]

[a] *Laboratory of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmaceutical Sciences, 4 Van Evenstraat, B-3000 Leuven, Belgium*
[b] *Laboratory of Pharmacotechnology and Biopharmacy, Faculty of Pharmaceutical Sciences, K.U. Leuven, Leuven, Belgium*

Received 1 December 2003; received in revised form 30 July 2004; accepted 11 August 2004

Abstract

A new method for chiral determination of apomorphine enantiomers was developed and validated. Seven different neutral and charged cyclodextrins were tested for enantioselectivity on *R,S*-apomorphine. Sulfobutylether-β-cyclodextrin was found to offer the best resolution, but with this system, four peaks were detected from a solution of the two enantiomers, which was suggested to be the result of different forms of the complex between the selector and apomorphine. A complexation constant was estimated for a complex of 1:1 ratio for the second and the fourth peak, whereas the other two peaks were fitted to a model ratio of 1:2 (analyte–selector). To avoid this phenomenon, hydroxypropyl-β-cyclodextrin was then chosen as the chiral selector. An optimisation study was performed on three factors: concentration of the chiral selector, pH of the buffer, and applied voltage. Optimum conditions were: 14 mM of hydroxypropyl-β-cyclodextrin, pH 3.0, and 16 kV. UV detection was at 200 nm. The method was validated at the chosen conditions, offering a limit of detection of 0.2 μM and a limit of quantification of 0.5 μM. The validated method was applied for the determination of *R,S*-apomorphine in a transport study with an in vitro cell culture model of the intestinal mucosa (Caco-2).

Keywords: Apomorphine; Capillary electrophoresis; Chiral; Cyclodextrins; Complexation constant

1. Introduction

R-Apomorphine (R-APO), a potent dopamine agonist evenly responding to D_1 and D_2 receptors, was the first dopamine agonist given to patients with Parkinson's disease [1,2]. It gained renewed attention later on thanks to the effectiveness in the management of Parkinsonism, by reducing the "off" period by at least 50% [3]. A considerable number of studies have been done on the pharmacokinetic and pharmacodynamic behaviour of R-APO by several routes, intravenously [4], subcutaneously [5], intranasally [6], sublingually [7], or iontophoretically [8]. The analysis of apomorphine in almost all studies, however, was performed with liquid chromatographic methods with UV detection [9,10], LIF detection [4], and especially electrochemical detection [5,11–14]. No method was found using capillary electrophoresis. Although *R*-apomorphine is administered as a single enantiomer, it is important to study the stereoselectivity of the uptake, distribution, and metabolism of apomorphine in the body. Therefore, several chiral LC methods have been developed for the determination of both enantiomers [11,12]. In this study, a chiral capillary electrophoretic method was developed and validated for the analysis of apomorphine enantiomers. As an application, the method was used in the determination of apomorphine enantiomers in a transport study using an in vitro cell culture model of the

* Corresponding author. Tel.: +32 16 323443; fax: +32 16 323448.
E-mail address: ann.vanschepdael@pharm.kuleuven.ac.be (A. Van Schepdael).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.019

intestinal mucosa, Caco-2. This cell culture system was used just as a surrogate for a monolayer system to explore the feasibility of the approach in exploring the transepithelial transport characteristics of apomorphine. More applications can be carried out afterwards on nasal mucosa cell culture system to study the intranasal administration of apomorphine.

2. Experimental

2.1. Chemicals

R-Apomorphine hydrochloride hemihydrate 99% was purchased from Sigma Aldrich Chemie (Steinheim, Germany). *S*-Apomorphine hydrochloride 99% was from Aldrich (Milwaukee, WI).

β-Cyclodextrin hydrate (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Acros Organics (Geel, Belgium). Methyl-β-cyclodextrin (Me-β-CD) and sulfated-β-cyclodextrin (S-β-CD) sodium salt were provided by Aldrich. Heptakis-(2,3-diacetyl-6-sulfato)-β-cyclodextrin (HDAS) was purchased from Regis Technologies (Morton Grove, IL, USA). Advasep 4 (sulfobutylether-β-cyclodextrin sodium salt, SBE-β-CD) was from CyDex (Overland Park, KS, USA). Carboxyethyl-β-cyclodextrin (CE-β-CD) was obtained from Cyclolab (Budapest, Hungary).

Hanks' Balanced Salt Solution (HBSS) without Phenol Red was from Cambrex Bio Science (Verviers, Belgium). Caco-2 cells for the in vitro uptake study were purchased from Invitrogen (Merelbeke, Belgium). Mesityl oxide [used as electro-osmotic flow (EOF) marker], sodium dihydrogen phosphate and sodium tetraborate decahydrate originated from Acros Organics. Tris 99.9% was purchased from AppliChem (Darmstadt, Germany). *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) 99% and 2-[*N*-morpholino]-ethanesulfonic acid (MES) 99.5% were obtained from Aldrich. Sodium hydroxide and *ortho*-phosphoric acid 85% p.a. were obtained from Riedel-de Haën (Seelze, Germany) and hydrochloric acid 37% and methanol were purchased from Fisher Chemicals (Leicestershire, UK). All aqueous solutions were prepared with water purified by a Milli-Q 50 ultra purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

All capillary electrophoretic experiments were performed on a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA) equipped with a photodiode-array detection (DAD) system, controlled by 32 Karat software, version 5.0 (Beckman Coulter). Uncoated fused-silica capillaries of 50 and 75 μm i.d. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The pH of the solutions was adjusted with a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland).

2.3. Primary conditions

A 50 μm i.d. uncoated fused-silica capillary was used in the beginning, but to enhance sensitivity, a 75 μm i.d. was finally chosen, with a total length (L_T) of 50 cm and an effective length (L_E) of 40 cm. The temperature of the capillary was always maintained at 25 °C with the liquid-cooling system. A voltage of 20 kV was applied. Detection was performed at 270 nm, which is one of the two maxima (200 and 270 nm) in the UV spectrum of APO.

Several buffer systems were tested for choosing the appropriate background electrolyte (BGE). Since a 75 μm i.d. was used, two biological buffers were also included to avoid too high a current.

(1) 50 and 100 mM sodium phosphate buffer, pH 7.0 and 3.0
(2) 50 and 100 mM Tris, adjusted to pH 7.0 and 3.0 with 100 mM H_3PO_4
(3) 100 mM BES, adjusted with 100 mM NaOH to pH 7.0
(4) 100 mM MES, adjusted with 100 mM NaOH to pH 6.0 and 5.5

A buffer pH lower than 7.0 was selected to obtain higher stability of APO [15]. The instability is believed to result from the auto-oxidation of APO during exposure to light and air [15,16]. For the same reason, 0.005% (w/v) mercaptoethanol was added to APO solution as an anti-oxidation reagent [13].

Among all, Tris–phosphate buffer gave the best results. Sodium phosphate buffer generated too high a current (up to 150 μA). MES and BES buffers could overcome this drawback, but the peak shape was very poor, resulting in low efficiency. Moreover, the results suggested that lower pH gave much better result, while MES and BES buffers are not suitable for the acid pH range. The 100 mM Tris–phosphate buffer pH 3.0 was therefore chosen as background electrolyte (I = 75 μA).

3. Results and discussion

3.1. Cyclodextrin screening

Solutions of 100 μM R-APO and 100 μM S-APO were prepared in 0.005% (m/v) mercaptoethanol solution, and were injected by pressure [3447.4 Pa (0.5 psi), 5 s]. Seven cyclodextrins (CDs) were introduced in the screening, including three neutral CDs: β-CD, Me-β-CD, and HP-β-CD; one ionisable: CE-β-CD, which acts more like a neutral CD at pH 3.0; and three negatively charged ones: SBE-β-CD, S-β-CD, and HDAS. With the anionic cyclodextrins, no peaks were detected at normal polarity. They were therefore also tested at reversed polarity, with the same field strength. Results are shown in Table 1.

SBE-β-CD at reversed polarity gave the best resolution among all types.

Surprisingly, with a concentration of 5 and 10 mM of SBE-β-CD (but not with 20 mM), each enantiomer of APO was

Table 1
Resolution of *R,S*-apomorphine with different cyclodextrins

CD type	CD concentration (mM)	Selectivity	Resolution	Mig. order
β-CD	5	1.03	1.69	R,S
Me-β-CD	10	1.06	4.46	R,S
HP-β-CD	5	1.06	4.39	R,S
	10	1.07	4.90	R,S
	20	1.07	5.73	R,S
CE-β-CD	5	1.05	3.39	R,S
SBE-β-CD[a]	5[b]	1.12	6.11	S,R
	10[b]	1.08	5.34	S,R
	20	1.14	9.79	S,R
S-β-CD[a]	5	–	–	–
HDAS[a]	5	–	–	–

[a] Obtained at reversed polarity.
[b] Selectivity and resolution were calculated on peaks 2 and 3 in Table 2.

giving two similar peaks, even with a freshly prepared solution (Fig. 1a). The picture obtained with a one-day-old solution was more complicated, as shown in Fig. 1b, where peaks 1 and 2 are the main peaks from S-APO and 3, 4 are from R-APO. However, the double peaking phenomenon did not occur when apomorphine solution was made in HBSS (also with 0.005% mercaptoethanol): only two peaks were detected from the racemic mixture. To find out the reason for the change in peak profile, every component of HBSS was added one by one to the APO solution. It was realised that sodium chloride (at a concentration as high as 0.2 M) was the reason. On the other hand, after three to five runs with the buffer containing 5 mM SBE-β-CD, these two peaks from each enantiomer also merged, and the peak shape deteriorated. This change was supposed to result from a very fast buffer depletion, because a significant portion of SBE-β-CD could already have migrated into the outlet vial. The screening was then continued with more efforts to interpret this phenomenon.

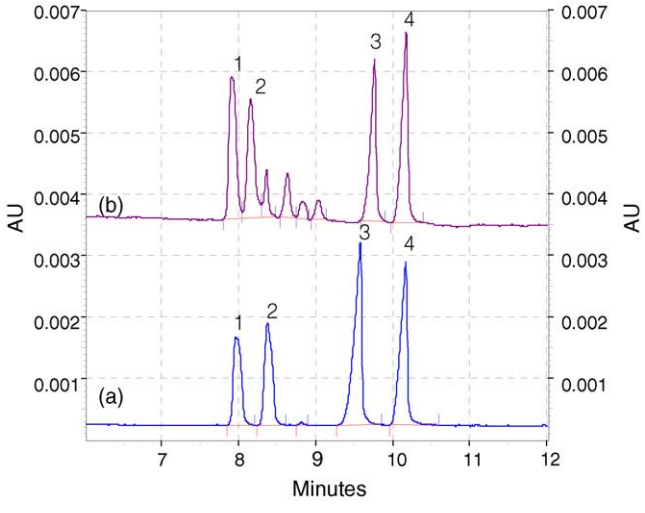

Fig. 1. Electropherograms of R,S-APO, with BGE of 100 mM Tris–phosphate buffer pH 3.0 containing SBE 5 mM, 75 μm i.d., 50 cm L_T capillary at 25 °C, applied voltage 20 kV, detection at 270 nm, in (a) a fresh solution and (b) a one-day-old solution.

3.2. Studying the complexes with SBE-β-CD

There were at that time two assumptions to explain for this '4-peak-phenomenon':

- First assumption: Only two out of the four main peaks are R,S-APO. The other two are two main degradation products. Because of the fast oxidation of APO, it is quite possible that the amount of anti-oxidant was not adequate, and decomposition already took place in solution after 10–15 min.
- Second assumption: All four peaks are peaks from apomorphine, formed by different complexation ratios with SBE-β-CD. If this assumption is correct, different concentrations of SBE-β-CD would be able to change the ratio of the areas of the two peaks corresponding to one enantiomer.

To test the first assumption, we tried various anti-oxidants that have been suggested in literature for APO stabilisation: sodium metabisulfite [10,11,14], sodium thiosulfate [12,15] and ascorbic acid [15,17], all at 10 mM, and mercaptoethanol at 0.1%. With sodium thiosulfate and mercaptoethanol, the solutions were colored after one or two days only. Sodium metabisulfite could delay the coloration of the solution longer, but the best anti-oxidant was ascorbic acid. With ascorbic acid, the solution only turned slightly greenish after one week. Therefore, ascorbic acid was chosen to replace mercaptoethanol as the anti-oxidant. However, with all the tested anti-oxidants, the same double peaking phenomenon was still observed, which suggested that this assumption could be excluded.

For the second assumption, buffers containing different concentrations of SBE-β-CD were used to analyse APO dissolved in an aqueous solution of 10 mM ascorbic acid. At all nine investigated concentrations from 2 to 10 mM, four peaks were found. Ratios between the corrected areas of peaks 2/1 and peaks 4/3 (Fig. 1) and selectivity of each pair are reported in Table 2. The variation of the ratio of corrected peak areas

Table 2
Influence of SBE-β-CD concentration on the '4-peak-phenomenon'

SBE-β-CD concentration (mM)	S-APO (peaks 2/1)		R-APO (peaks 4/3)	
	Selectivity	Ratio corr. area	Selectivity	Ratio corr. area
2	1.10	0.50	1.13	0.12
3	1.09	0.65	1.11	0.32
4	1.08	0.96	1.11	0.44
5	1.09	1.17	1.11	0.50
6	1.08	1.07	1.10	0.67
7	1.08	0.77	1.11	0.76
8	1.08	0.83	1.11	0.99
9	1.07	0.26	1.10	1.20
10	1.07	0.32	1.10	1.38

suggests that the formation of more than two peaks was due to different forms of the complex.

With this assumption, it is presumed that for each enantiomer, one peak is due to the 1:1 ratio complex, and the other is another ratio complex (1:2 or 2:1 analyte–selector).

For the 1:1 complex:

$$A + S \overset{K_1}{\rightleftharpoons} AS$$

the experimentally measured electrophoretic mobility, μ, is the weighted average of the mobilities of the analyte in the free and complexed forms:

$$\mu = \alpha\mu_A + (1-\alpha)\mu_{AS} \quad (1)$$

where μ_A and μ_{AS} are the electrophoretic mobilities of the free analyte and the analyte–selector complex, respectively, and α is the ratio of free analyte in the mixture:

$$\alpha = \frac{[A]}{[A]+[AS]} \quad (2)$$

On the other hand, the equilibrium constant of the analyte–selector complex can be defined in Eq. (3):

$$K_1 = \frac{[AS]}{[A][S]} \quad (3)$$

The substitution of Eqs. (2) and (3) into Eq. (1), after rearrangement and consolidation gives the following relationship:

$$\frac{1}{\mu-\mu_A} = \frac{1}{K_1} \times \frac{1}{\mu_{AS}-\mu_A} \times \frac{1}{[S]} + \frac{1}{\mu_{AS}-\mu_A} \quad (4)$$

The electrophoretic mobility of the chiral compound in the free state μ_A can be experimentally measured in the absence of chiral selector, and $1/(\mu - \mu_A)$ can therefore be plotted over 1/[S] in a linear relationship. From this linear regression, usually called double reciprocal method [18,19], the mobility of the fully complexed form μ_{AS} as well as the formation constant of the complex K_1 can be determined.

The experimental data of all four peaks were fitted to this equation. Only peaks number 2 and 4 (Fig. 1) showed a linear relationship, with correlation coefficients of r_2 = 0.9967 and r_4 = 0.9993 for S-APO and R-APO, respectively. The calculated results are presented in Table 3.

The other two peaks, with higher mobilities, would consequently represent 1 analyte–2 selector complexes. The formation constant of this complex, however, could not be determined as easily as the previous one, since the mixture eluting within this one peak can be of diverse composition. The calculations are different depending upon the equilibrium mode. We propose two different modes: mode 1 when the 1:2 complex is formed through the formation of 1:1 complex, and mode 2 when 1:1 and 1:2 complexes are formed independently, or in other words, the analyte is engaged and dissociated concurrently from the two molecules of cyclodextrin.

Mode 1:

$$A + 2S \overset{K_1}{\rightleftharpoons} AS + S \overset{K_2}{\rightleftharpoons} AS_2$$

In this second-order equilibrium, the total mobility μ would be dependent on the mobilities of three different forms: μ_A of the free analyte, μ_{AS} of the 1:1 complex, and μ_{AS_2} of the 1:2 complex.

Eq. (1) becomes:

$$\mu = \alpha\mu_A + \beta\mu_{AS} + (1-\alpha-\beta)\mu_{AS_2} \quad (5)$$

where

$$\alpha = \frac{[A]}{[A]+[AS]+[AS_2]}$$

and

$$\beta = \frac{[AS]}{[A]+[AS]+[AS_2]}$$

With

$$K_1 = \frac{[AS]}{[A][S]}$$

and

$$K_2 = \frac{[AS_2]}{[AS][S]}$$

rearrangement and replacement of α, β, K_1, and K_2 into Eq. (5) gave the following equation:

$$\mu - \mu_A = \frac{(\mu_{AS}-\mu_A)K_1[S] + (\mu_{AS_2}-\mu_A)K_1K_2[S]^2}{1 + K_1[S] + K_1K_2[S]^2} \quad (6)$$

After dividing both numerator and denominator on the right side of Eq. (6) by $K_1K_2[S]^2$, rearrangement gives us Eq. (7):

$$\mu - \mu_A = \frac{(\mu_{AS_2}-\mu_A) + ((\mu_{AS}-\mu_A)/K_2) \times (1/[S])}{1 + (1/K_2) \times (1/[S]) + (1/K_1K_2) \times (1/[S]^2)} \quad (7)$$

If 1/[S] and $(\mu - \mu_A)$ are taken as variables x and y, Eq. (7) will be in the form of the following function:

$$y = \frac{a+bx}{1+cx+dx^2} \quad (8)$$

Table 3
Calculation of complexation constant

Complex ratio	S-APO		R-APO	
	1:2 (peak 1)	1:1 (peak 2)	1:2 (peak 3)	1:1 (peak 4)
Correlation coefficient	0.9984	0.9967	0.9893	0.9993
Formation constant K (M^{-1})	4.02	3.75	1.56	1.63
μ_{AS} or μ_{AS_2} ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	8.35	7.82	8.13	7.37

where a, b, c, and d are unknown parameters to be estimated by data fittings, and

$$a = \mu_{AS_2} - \mu_A \qquad b = \frac{\mu_{AS} - \mu_A}{K_2}$$

$$c = \frac{1}{K_2} \qquad d = \frac{1}{K_1 K_2}$$

Experimental data were fitted by non-linear regression according to Eq. (8). The non-linear regression was performed with the support of two different softwares, Statistica 6.0 and Sigmaplot 2001. Unfortunately, results of the fittings showed a very poor fit of experimental data to this equation, indicating that mode 1 did not provide a reliable explanation of the observed phenomenon.

Mode 2 is when 1:2 complex is formed independently:

$$A + 2S \overset{K_2}{\rightleftharpoons} AS_2$$

The formation constant of the second-order complex K_2 would depend only on mobilities of the free analyte and the 1:2 complex, and Eq. (4) becomes Eq. (9):

$$\frac{1}{\mu - \mu_A} = \frac{1}{K_2} \times \frac{1}{\mu_{AS_2} - \mu_A} \times \frac{1}{[S]^2} + \frac{1}{\mu_{AS_2} - \mu_A} \qquad (9)$$

Data from peaks 1 and 3 were fitted to this equation again by linear regression, with $1/[S]^2$ and $1/(\mu - \mu_A)$ as variables x and y. Correlation coefficients were r_1 = 0.9984 and r_3 = 0.9893 for S-APO and R-APO (see Table 3). The formation constants of the complexes and the theoretical mobilities of the fully complexed form of 1:2 ratio μ_{AS_2} were also calculated.

Each enantiomer was proposed to form two complexes with similar equilibrium constants, but the estimated constants of complexes with S-APO were higher than those with R-APO. This might be the reason why the two peaks of R-APO were migrating slower than S-APO peaks, although calculated μ_{AS} and μ_{AS_2} of R-APO were not much lower than those of S-APO. It might be suggested that the phenomenon of four peaks results from the formation of different complex ratios between analyte and selector. The two early migrating peaks (no. 1 and 3) were 1:2 complexes whereas the two later peaks (no. 2 and 4) contained 1:1 complexes.

The screening of SBE-β-CD concentration was also performed with a high ionic strength solution of APO, containing 0.2 M NaCl. Fig. 2 presents the change with different concentrations of SBE-β-CD, from 1 to 10 mM. In consistence with the observation in Section 3.1, only two peaks were found. A possible explanation for the differences observed with the absence and presence of NaCl in the sample solution could be the role of ionic strength of the sample solution. The analyte–cyclodextrin interactions in the solution were mainly inclusion and electrostatic interactions (since apomorphine and SBE-β-CD were oppositely charged). When NaCl was present in the sample at a very high concentration (0.2 M), the high ionic strength could minimize the electrostatic interaction of the weakly charged apomorphine with the cyclodextrin, and this might be the reason why the formation of different complexes of analyte–selector was not possible. To test the influence of ionic strength, sodium chloride was replaced by another salt. Potassium phosphate was added to the sample solution at the same concentration as NaCl, and the sample was analysed at different concentrations of SBE-β-CD (2, 4, 6, 8, and 10 mM). As expected, the phenomenon of four peaks was indeed not observed. However, the actual mechanism of the influence of ionic strength on the stoichiometry of the complexes could not be easily described.

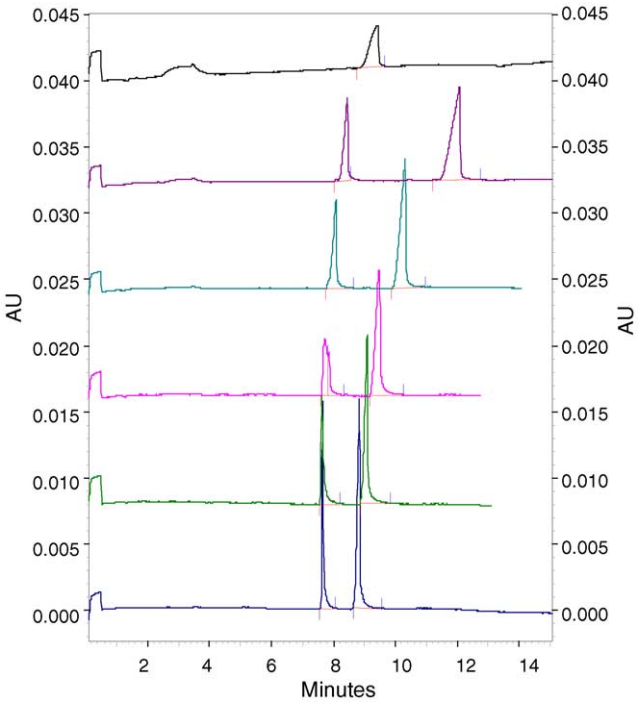

Fig. 2. R,S-APO in 0.2 M NaCl, with BGEs containing 1, 2, 4, 6, 8, and 10 mM SBE-β-CD, respectively, from top to bottom.

Although the presence of NaCl in the sample solution could avoid the formation of different complexes with SBE-β-CD, it was still decided to choose HP-β-CD for the next step of method development. This type of CD presented best stereoselectivity among the remaining cyclodextrins (Table 1).

3.3. Method development with HP-β-CD

3.3.1. Choosing conditions for optimisation

To shorten the analysis time, the capillary total length was decreased to 40 cm. A shorter length would no longer maintain reasonable resolution. Normal polarity was used, resulting in a reversed migration order, with R-APO being the first peak. The degradation products migrated later than the two main peaks. The use of a lower temperature (20 °C) was also explored, but the peak shape improvement could not compensate for the longer analysis time. Therefore, it was decided to keep the capillary temperature at 25 °C.

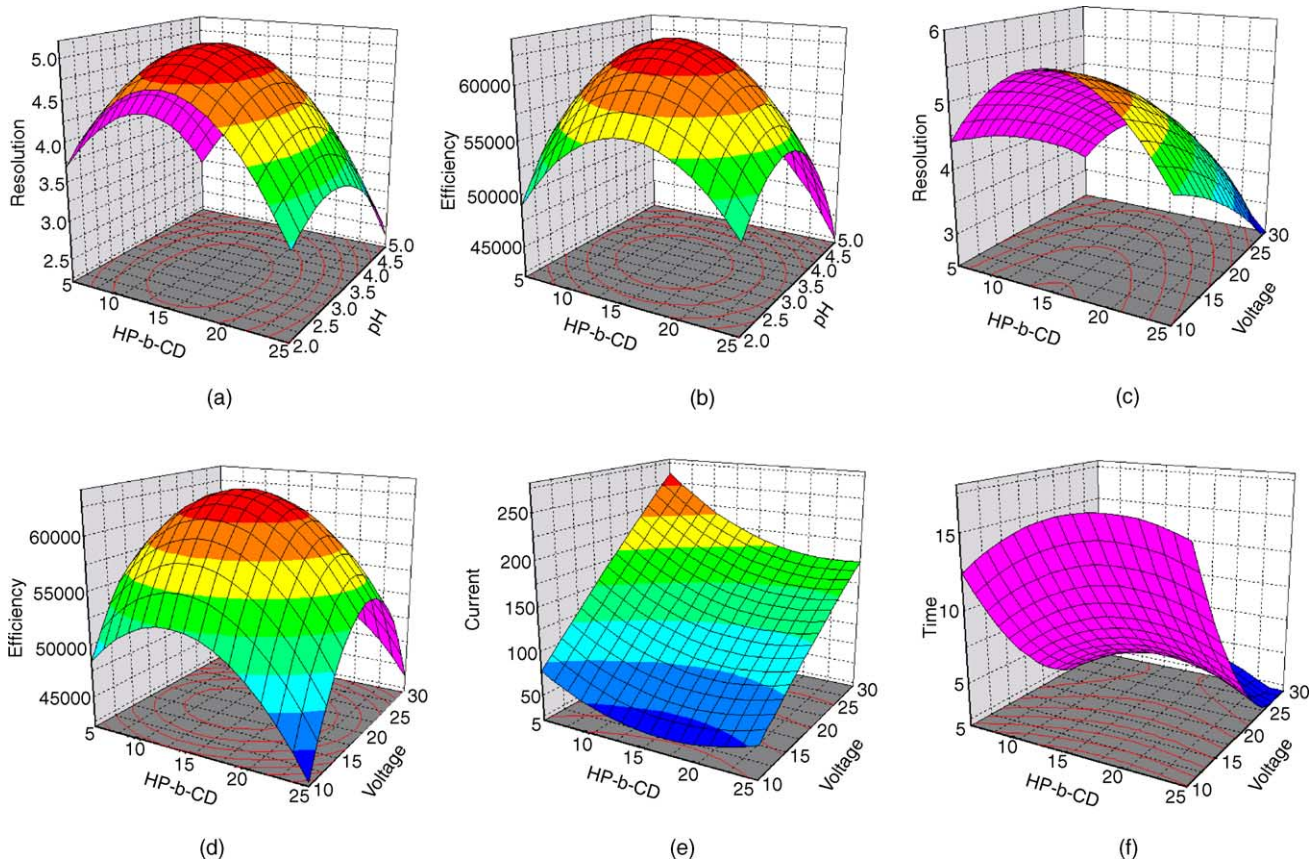

Fig. 3. Optimisation response surfaces of: (a) resolution and (b) efficiency (theoretical plates) with HP-β-CD concentration and buffer pH as variables; (c) resolution, (d) efficiency, (e) current, and (f) analysis time with HP-β-CD concentration and voltage as variables.

Three critical parameters were chosen as factors for optimisation: concentration of HP-β-CD, pH of the Tris–phosphate buffer, and voltage applied. More experiments were also performed to define the investigation range of each parameter, which were finally set at: 5.0–25.0 mM HP-β-CD, pH 2.0–5.0, 10–30 kV.

Responses to be considered were: resolution between the two enantiomer peaks, total analysis time, generated current, number of theoretical plates, and symmetry factor calculated on the S-APO peak.

3.3.2. Optimisation results

Results of the optimisation study showed that resolution would be maintained higher than 4.5 within the range of 12.5–17.5 mM HP-β-CD and at pH 2.5–3.5 (Fig. 3a). The efficiency of the system (expressed in number of theoretical plates), fortunately, had the same optimum area in function of pH and HP-β-CD concentration (Fig. 3b). The influence of voltage on these two parameters, however, was slightly different. The best resolution was achieved at 10–20 kV while the best efficiency is observed at 15–25 kV (Fig. 3c and d). On the other hand, the generated current was below 100 μA only in the region of 10–15 kV (Fig. 3e), whereas only a voltage of higher than 15 kV could offer a reasonable analysis time (Fig. 3f).

Finally, the optimum point was chosen at pH 3.0, 14 mM of HP-β-CD and 16 kV. A typical electropherogram is presented in Fig. 4.

For the best sensitivity, injection time was increased up to 10 s at 3447.4 Pa (0.5 psi), the highest possible limit with

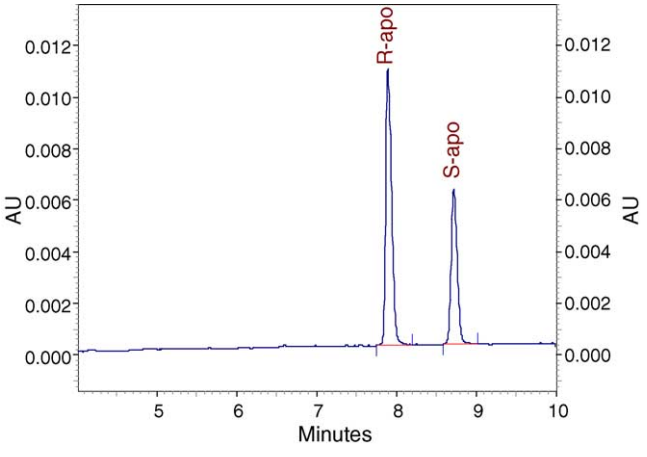

Fig. 4. Electropherogram at optimum point with BGE of 100 mM Tris–phosphate buffer pH 3.0 containing 14 mM HP-β-CD, 16 kV at normal polarity (I = 98 μA), 75 μm i.d. fused silica capillary, 40 cm L_T, 30 cm L_E at 25 °C.

which sufficient resolution and good peak shape was still maintained. A longer sample plug could lead to peak splitting.

3.4. Method validation

The final conditions chosen for method validation are:

- Capillary: uncoated fused-silica capillary 75 μm i.d., 40 cm L_T (30 cm L_E), kept at 25 °C.
- BGE: 100 mM Tris solution, adjusted by 100 mM H_3PO_4 to pH 3.0.
- Chiral selector: 14 mM HP-β-CD dissolved in the above BGE.
- Voltage: 16 kV, at normal polarity.
- Detection: UV at 270 nm.
- Washing: daily wash with 5 min 0.1 M NaOH, then 5 min H_2O. Between-run wash: 0.5 min 0.1 M NaOH, 0.5 min H_2O and 1.0 min running buffer. All set at 137.9 kPa (20 psi).
- Sample preparation: apomorphine solutions were prepared in 0.2 M NaCl with 10 mM ascorbic acid as anti-oxidant. Injection 10 s, at 3447.4 Pa (0.5 psi).

3.4.1. Sensitivity

At 270 nm, the limit of detection (LOD) was 0.4 μM (with signal-to-noise (*S*/*N*) ratio of 3) and the limit of quantitation (LOQ) (*S*/*N* = 10) was 1.0 μM, all measured with R-APO. Since with this method background noise was not very high at the other maximum absorbance wavelength of 200 nm, the LOD and LOQ were determined again at this wavelength for both enantiomers. Sensitivity was actually improved at 200 nm, with an LOD and LOQ of 0.2 and 0.5 μM for R-APO, and 0.3 and 0.8 μM for S-APO, respectively. All subsequent experiments were performed at 200 nm.

3.4.2. Repeatability

A solution of 100 μM R-APO was injected 20 times consecutively. The R.S.D. was 4.1% on migration time, and 3.8% on corrected area of apomorphine. However, it was observed that the migration time was gradually increasing with time (Fig. 5, series a). This increase was due to the change of the capillary surface, but not to buffer depletion as with SBE-β-CD, because the use of new buffer did not influence the tendency. To avoid the change of capillary surface, different between-run washing procedures were tried. The best result was found with a wash only with the running buffer for 1 min. However, the migration time kept changing. The daily wash used before was therefore adapted by adding 2 min rinsing with running buffer and a 20 min run under a voltage of 15–20 kV to achieve an equilibration of the capillary surface. The repeatability test was done again with this washing procedure, and an R.S.D. of 0.63% (n = 20) was found on migration time, and 2.6% on corrected peak area (Fig. 5, series b).

The repeatability study was applied with this washing procedure on both enantiomers, either alone or in a mixture.

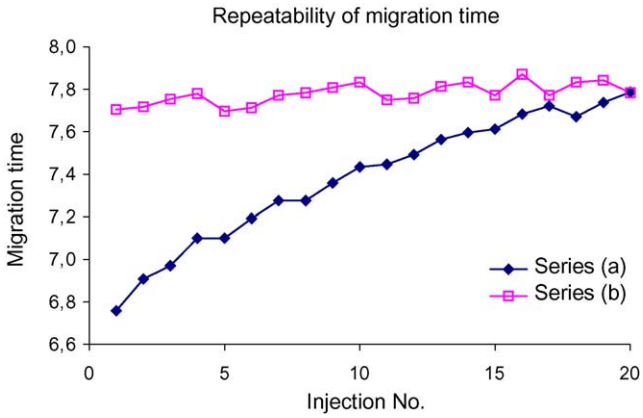

Fig. 5. Variation of migration time during 20 consecutive injections. Between-run wash with (a) NaOH, water, and buffer, and (b) only buffer.

Six injections of 100 μM R-APO either from a single-isomer solution or from an enantiomers mixture offered the same R.S.D. of 1.7% on corrected peak area. The R.S.D. (n = 6) of 100 μM S-APO in a single-isomer solution, or from an enantiomers mixture was 1.2 and 1.8%, respectively.

3.4.3. Linearity

Linearity was checked on solutions containing both enantiomers, with nine concentrations of each enantiomer ranging from 1.0 to 100 μM. Each of the nine concentration points (1.0, 2.0, 5.0, 10, 20, 40, 60, 80, and 100 μM) was injected three times. The correlation coefficients were r_R = 0.9997 and r_S = 0.9996 within the investigated range. Obtained linear equations were $y_R = 414.9x - 66.4$ and $y_S = 253.8x - 36.9$ where x is APO concentration (μM) and y_R, y_S are the corresponding corrected peak areas of R- and S-APO, respectively.

3.5. Example of sample analysis in an in vitro transport study

3.5.1. Transport experiments

Transport of apomorphine enantiomers was studied using an in vitro cell culture system of the intestinal mucosa, i.e. the human colon adenocarcinoma cell line (Caco-2). Caco-2 cells were grown in 75 cm^2 culture flasks at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were cultured according to the standard conditions described before [20].

Transport medium (TM) consisted of HBSS containing 25 mM D-(+)-glucose and 10 mM MES (pH was adjusted to 6.5 with HCl 1 M). The TM used for solutions of APO also contained 10 mM ascorbic acid for improved stability of APO (pH adjusted with sodium hydroxide 2 M to 6.5).

Caco-2 cells were plated on Costar® Transwell membrane inserts (3 μm pore diameter, 12 mm diameter; Corning Inc., NY) at a density of 40,000 cells/cm^2, and the monolayers were used 21–25 days post-seeding. A constant volume of 0.5 and 1.5 ml was used at the apical side and the basolateral side of the monolayer, respectively.

Table 4
R-APO concentration (μM) in each compartment after 60 min of apical-basolateral transport

	Replicates	Rep 1	Rep 2	Rep 3	Rep 4	Average	R.S.D. %
R-APO	Apical side	81	82	76	80	80	3.2
Concentration (μM)	Basolateral side	8.0	6.0	6.2	7.4	6.9	13.7

Before initiating the transport process, the monolayers were washed three times with TM, and a pre-incubation step of 30 min was used also in TM. The transport study was performed in both directions: from the apical to the basolateral side ($n = 4$) and from the basolateral to the apical side ($n = 3$). For each transport direction, three conditions were tested: R-APO 100 μM, S-APO 100 μM, and R,S-APO each 100 μM. After 60 min of incubation, samples were collected from both donor and acceptor compartments for sample analysis.

3.5.2. Sample analysis

Although TM contains a large amount of different salts, the samples could be analysed directly without any sample preparation step. The concentration of APO in the samples was determined immediately after the transport study. Table 4 gives an idea of the variability on R-APO concentration measured.

Results of the transport experiments were expressed as permeability coefficients P_{app} ($cm\,s^{-1}$):

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{AC_0} \quad (10)$$

where $\Delta Q/\Delta t$ is the amount of drug appearing in the acceptor compartment in function of time (nmol/s), C_0 is the initial concentration in the donor compartment (μM), and A is the surface area (cm^2) across which the transport occurred. Fig. 6 presents the average permeability coefficients (±S.D.) of R- and S-APO, alone and in mixture, in both directions.

No polarity or stereoselectivity in transport could be observed with these experiments. This means that at the concentration of apomorphine used, no active carrier systems affect the transepithelial transport of apomorphine.

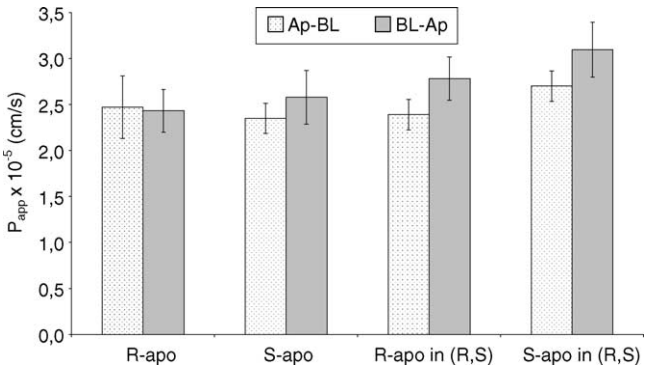

Fig. 6. Apparent permeability coefficient (P_{app}) for R- and S-APO in the Caco-2 model after application of 100 μM of R-, S- or R,S-APO to the donor compartment. Results are expressed as P_{app} ($\times 10^{-5}\,cm\,s^{-1}$ + S.D.). (Ap-BL: transport from the apical to the basolateral side, absorptive transport; BL-Ap: transport from the basolateral to the apical compartment, secretory transport) (Ap-BL: $n = 4$; BL-Ap: $n = 3$).

4. Conclusions

The developed method was proved to be fast and efficient. The detection limit in the μM range facilitates the use of this method for the in vitro transport experiment of apomorphine enantiomers. During method development, it was realised that the use of certain chiral selectors at a certain concentration could yield different peaks corresponding to different complexed forms. The formation constants of the complexes between 1 apomorphine and 1 or 2 SBE-β-CD molecules were proposed. It is interesting to describe this phenomenon, which has not been reported so far.

The results of the analysis during transport experiments showed the feasibility of the developed method with samples from transport studies, where apomorphine has to be determined in a solution of high ionic strength. Despite the presence of a lot of different salts, no special sample preparation was required.

References

[1] C. Neef, T. van Laar, Clin. Pharmacokinet. 37 (1999) 257.
[2] D. Deleu, M.G. Northway, Y. Hanssens, Clin. Pharmacokinet. 41 (2002) 216.
[3] J.P. Frankel, A.J. Lees, P.A. Kempster, G.M. Stern, J. Neurol. Neurosurg. Psychiatry 53 (1990) 96.
[4] A.J. Manson, H. Hanagasi, K. Turner, P.N. Patsalos, P. Carey, N. Ratnaraj, A.J. Lees, Brain 124 (2001) 331.
[5] E. Sam, S. Sarre, Y. Michotte, N. Verbeke, Eur. J. Pharmacol. 329 (1997) 9.
[6] M.I. Ugwoke, E. Sam, G. Van den Mooter, N. Verbeke, R. Kinget, Eur. J. Pharm. Sci. 9 (1999) 213.
[7] J.L. Montastruc, O. Rascol, J.M. Senard, V. Gualano, H. Bagheri, G. Houin, A. Lees, A. Rascol, Clin. Neuropharmacol. 14 (1991) 432.
[8] H.E. Junginger, Adv. Drug Deliver. Rev. 54 (Suppl. 1) (2002) 57.
[9] E. Ameyibor, J.T. Stewart, J. Chromatogr. B 686 (1996) 297.
[10] N.M.K.N.Y. Kin, S. Lal, J.X. Thavundayil, Prog. Neuro-Psychopharmacol. Biol. Psychiat. 25 (2001) 1461.
[11] R. van der Geest, P. Kruger, J.M. Gubbens-Stibbe, T. van Laar, H.E. Bodde, M. Danhof, J. Chromatogr. B 702 (1997) 131.
[12] R. van der Geest, T. van Laar, P. Kruger, J.M. Gubbens-Stibbe, H.E. Bodde, R.A.C. Roos, M. Danhof, Clin. Neuropharmacol. 21 (1998) 159.
[13] E. Sam, P. Augustijns, N. Verbeke, J. Chromatogr. B 658 (1994) 311.
[14] A. Bolner, L. Barbato, F. Tagliaro, A. Monge, F. Stocchi, G. Nordera, Forensic Sci. Int. 89 (1997) 81.

[15] K. Florey, , Analytical Profiles of Drug Substances, vol. 20, Academic Press Inc., London, 1991, p. 121.
[16] M.J. O' Neil, The Merck Index, 19th ed., Merck & Co., New Jersey, USA, 2001, p. 654.
[17] M.J. Priston, G.J. Sewell, J. Chromatogr. B 681 (1996) 161.
[18] K. Rundlett, D. Armstrong, Electrophoresis 18 (1997) 2194.
[19] K. Rundlett, D. Armstrong, J. Chromatogr. A 721 (1996) 173.
[20] P. Augustijns, P. Annaert, P. Heylen, G. Van den Mooter, R. Kinget, Int. J. Pharm. 166 (1998) 45.