Journal of Chromatography A, 1217 (2010) 7949–7955

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a nonaqueous capillary electrophoretic method for the enantiomeric purity determination of a synthetic intermediate of new 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyrans using a single-isomer anionic cyclodextrin derivative and an ionic liquid

Anne Rousseau[a], Xavier Florence[b], Bernard Pirotte[b], Anne Varenne[c], Pierre Gareil[c], Didier Villemin[d], Patrice Chiap[e], Jacques Crommen[a], Marianne Fillet[a], Anne-Catherine Servais[a,*]

[a] *Laboratory of Analytical Pharmaceutical Chemistry, Department of Pharmaceutical Sciences, CIRM, University of Liege, CHU, B36, B-4000 Liège, Belgium*
[b] *Laboratory of Medicinal Chemistry, Dept. of Pharmaceutical Sciences, CIRM, University of Liège, CHU, B36, B-4000 Liège, Belgium*
[c] *Laboratory of Physicochemistry of Electrolytes, Colloids and Analytical Sciences, UMR CNRS 7195, Chimie-ParisTech, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France*
[d] *Laboratory of Molecular and Thio-organic Chemistry, UMR CNRS 6507, ENSI Caen, 6, Boulevard du Maréchal Juin, 14050 Caen Cedex, France*
[e] *Advanced Technology Corporation (ATC), CHU, B36, B-4000 Liège, Belgium*

ARTICLE INFO

Article history:
Available online 30 July 2010

Keywords:
Nonaqueous capillary electrophoresis
Single-isomer anionic cyclodextrins
Ionic liquid
Benzopyran derivatives
Enantiomeric purity

ABSTRACT

The enantiomeric purity determination of a synthetic intermediate of new 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyrans, i.e. 4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran, was successfully carried out using an anionic cyclodextrin (CD) derivative combined with a chiral ionic liquid (IL). In order to obtain high resolution and efficiency values, the addition of a chiral IL, i.e. ethylcholine bis(trifluoromethylsulfonyl)imide (EtChol NTf_2), to the background electrolyte containing heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD) was found to be essential. A simultaneous increase in separation selectivity and enantioresolution seems to indicate a synergistic effect of HDMS-β-CD and EtChol NTf_2. The best enantioseparation of the key intermediate was achieved using a methanolic solution of 0.75 M formic acid, 10 mM ammonium formate, 1.5 mM HDMS-β-CD and 5 mM EtChol NTf_2. Levamisole was selected as internal standard. The optimized conditions allowed the determination of 0.1% of each enantiomer in the presence of its stereoisomer using the method of standard additions. The NACE method was then fully validated with respect to selectivity, response function, trueness, precision, accuracy, linearity and limits of detection and quantification.

1. Introduction

Chirality has gained a lot of attention in the pharmaceutical industry since the Food and Drug Administration and the European Medicines Agency have issued guidelines requiring information on the enantiomeric purity of the optically active compounds prior to their marketing [1,2]. To date, many drugs are racemic compounds and usually the desired pharmacological activity resides in one enantiomer while the other enantiomer may be toxic, less active and/or possesses undesirable side effects [3]. Therefore, the development of a chiral drug in its single-isomer form and the control of its purity are of great importance not only to avoid unwanted pharmaceutical and/or toxicological side effects but also to guarantee its therapeutic efficacy and safety.

Over the last decades, CE has been found to be an efficient alternative to the classical chromatographic techniques for enantiomeric separations [4]. CE can be considered as an ideal technique to separate enantiomers as well as to determine their purity, due to its high efficiency, rapidity and high resolving capability, combined with low consumption of samples, solvents and reagents [5–9]. The possibility of using not only aqueous but also nonaqueous solvents increases the versatility of method development in CE. Moreover, nonaqueous media have extended the application range of CE to the analysis of compounds with poor solubility in water [10]. In CE, chiral separation is simply achieved by adding to the background electrolyte (BGE) appropriate chiral selectors, mainly cyclodextrins (CDs) and their derivatives. Besides neutral

Abbreviations: BGE, background electrolyte; camphorSO_3^-, camphorsulfonate; CMCD, carboxymethyl-β-CD; EtChol NTf_2, ethylcholine bis(trifluoromethylsulfonyl)imide; HDAS-β-CD, heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD; HDMS-β-CD, heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD; ILs, ionic liquids; MeOH, methanol; NACE, nonaqueous CE; PhChol NTf_2, phenylcholine bis(trifluoromethylsulfonyl)imide; R_s, resolution.

* Corresponding author. Fax: +32 4 366 4347.
E-mail address: acservais@ulg.ac.be (A.-C. Servais).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.07.005

CDs, various chemically modified CDs have been used for enantioseparations in nonaqueous CE (NACE) [11,12]. Single-isomer sulfated CDs, especially heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD) and heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD), were successfully applied to the enantioseparation of a series of basic drugs in NACE [13–16]. Ionic liquids (ILs) have unique chemical and physical properties, such as negligible vapour pressure and interesting solvation ability [17–19]. Rizvi and Shamsi published the first CE enantioseparation of acidic analytes using two chiral ILs surfactants, undecenoxycarbonyl-L-pyrrolidinol bromide and undecenoxycarbonyl-L-leucinol bromide in monomeric and polymeric forms [20]. A dual system, made up of *N*-undecenoxyl-carbonyl-L-leucinol bromide and trimethyl-β-CD, was applied for the simultaneous enantioseparation of five profens in micellar electrokinetic chromatography [21]. Tran and Mejac synthesized and used a chiral IL, S-[3-(chloro-2-hydroxypropyl)trimethylammonium][bis((trifluoromethylsulfonyl)amide)], to separate the enantiomers of various pharmaceutical compounds in CE [22]. François et al. have studied the effect of two chiral ILs, namely ethyl- and phenylcholine of bis(trifluoromethylsulfonyl)imide (EtChol- and PhChol NTf_2), on the enantioseparation of 2-arylpropionic acids [23]. These chiral ILs did not exhibit enantioselectivity towards the studied model compounds. However, a simultaneous increase in enantioresolution and separation selectivity was observed with a BGE containing both a chiral IL and a neutral CD derivative (di- or trimethyl-β-CD) in water and water-methanol (MeOH) mixtures, suggesting synergistic effects.

ATP-sensitive potassium channels represent an important class of potassium channels. The activity of these channels is mainly regulated by changes in the intracellular concentration of adenosine triphosphate, implying that these channels couple cell metabolism to membrane excitability [24–26]. These channels are distributed in many tissues where they are involved in major physiological processes such as the insulin releasing process [27–29], cardioprotection [30], the control of the vascular tone [31], neuroprotection, and the control of neurotransmitter release [32,33]. (±)-Cromakalim is the prototype of the benzopyran potassium channel opener. This drug has shown to exert a marked vasorelaxant activity [34] but is also known, in contrast to diazoxide, to be only slightly active as an inhibitor of insulin secretion [35,36]. A series of diversely 4-arylthiourea-substituted 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyrans structurally related to (±)-cromakalim has been recently synthesized [37]. These new compounds were examined as putative potassium channel openers on rat pancreatic islets as well as on rat aorta rings. These compounds were synthesized as pure enantiomers from a racemic synthetic intermediate, i.e. *R*/*S*-4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran, whose enantiomers were separated by preparative liquid chromatography using a chiral column.

In the present study, a NACE method for the enantiomeric purity determination of the synthetic key intermediate of new benzopyran derivatives, i.e. 4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran, was developed. The enantiomeric purity determination of this intermediate is of utmost importance for the synthesis of the final compounds as pure enantiomers for pharmacological testing.

2. Materials and methods

2.1. Instrumentation

All experiments were carried out on a $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, an on-column diode-array detector and a temperature control system (15–60 °C ± 0.1 °C). A CE Chemstation (Hewlett-Packard, Palo Alto, CA, USA) was used for instrument control, data acquisition and data handling. Fused-silica capillaries were provided by ThermoSeparation Products (San Jose, CA, USA).

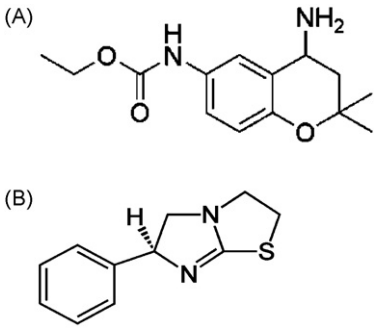

Fig. 1. Chemical structures of (A) the synthetic intermediate, i.e. 4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran, and (B) levamisole.

The statistical calculations for validation were performed by means of e.noval version 2.0 software (Arlenda, Liège, Belgium).

2.2. Chemicals and reagents

The synthetic intermediate of new 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyrans, i.e. *R*/*S*-4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran, and its *R*- and *S*-enantiomers were kindly provided by the Laboratory of Medicinal Chemistry (Liège, Belgium). Levamisole was supplied by Sigma–Aldrich (Saint Louis, MO, USA). Their chemical structures are presented in Fig. 1. EtChol NTf_2 (*R*(−)-enantiomer) and PhChol NTf_2 (*R*(−)-enantiomer) were synthesized and donated by Villemin's group (Caen, France) [23]. Single-isomers HDMS-β-CD and HDAS-β-CD were obtained from Analytical Controls (Rotterdam, The Netherlands).

Carboxymethyl-β-CD (CMCD) was kindly provided by Cyclolab (Budapest, Hungary). Ammonium formate and ammonium camphorsulfonate (camphorSO_3^-) were from Sigma–Aldrich. Trifluoroacetic acid and 98–100% formic acid were supplied by Acros-Organics (Geel, Belgium). All reagents were of analytical grade. MeOH (Merck, Darmstadt, Germany) was of HPLC grade. The BGE and samples solutions were filtered through a Polypure polypropylene membrane filter (0.2 μm) from Alltech (Laarne, Belgium) before use.

2.3. Electrophoretic technique

Electrophoretic separations were carried out with uncoated fused-silica capillaries having 50 μm internal diameter and 48.5 cm length (40 cm to the detector). At the beginning of each working day, the capillary was washed with MeOH, 1 M TFA in MeOH and the BGE (each washing step performed for 15 min). Before each injection, the capillary was washed successively with 1 M TFA in MeOH for 4 min, with the BGE without CD for 2 min and then equilibrated with the BGE-CD for 3 min. At the end of each working day, the capillary was rinsed for 30 min with 1 M TFA in MeOH, 30 min with the BGE without CD and 15 min with MeOH. Capillary wash cycles were performed at a pressure of approximately 1 bar. The applied voltage was 30 kV (unless otherwise stated) and UV detection was set at 250 nm. Injections were made by applying a pressure of 50 mbar for a period of 3 s (corresponding to 8.8 nL, i.e. 0.9% of the total volume of the capillary) and the capillary was thermostated at 15 °C. Resolution (R_s) was calculated according to the standard expression based on peak width at half height [38]. The effective electrophoretic selectivity (α_{eff}) was calculated according

Table 1
Preparation of standard solutions related to *S*- and *R*-enantiomers for validation.

Concentration level (% relative to 1.0 mg/mL of *R*- or *S*-enantiomer)	Concentration of *S*- and *R*-enantiomers (μg/mL)	
	Calibration standards	Validation standards
0.10	1.0	1.0
0.20	–	2.0
0.60	6.0	6.0
1.20	12.0	12.0
Total	6 samples/day	12 samples/day

to $\alpha_{eff} = \mu_{ep1}/\mu_{ep2}$ where μ_{ep1} and μ_{ep2} are the effective mobilities for enantiomers *S* and *R*.

2.3.1. Solutions used for method development

A stock solution of the racemic test compound was prepared by dissolving an accurately weighed amount of approximately 2.4 mg in 10 mL of MeOH. A stock solution of levamisole was also prepared by dissolving 12.5 mg in 10 mL of MeOH. These stock solutions were then diluted 10-fold and a mixed methanolic solution of the racemic test compound and levamisole was then obtained at 24 μg/mL and 125 μg/mL, respectively.

R- or *S*-enantiomer of the test compound was also added to the diluted stock solution in order to obtain two reference solutions representing 0.2% (2 μg/mL) of impurity level and containing 1 mg/mL of *S*- or *R*-form.

2.3.2. Solutions used for method validation

For the determination of *R*-enantiomer purity, a stock solution of *S*-enantiomer was prepared by dissolving, in a 5 mL volumetric flask, an accurately weighed amount of approximately 2.4 mg of the racemic synthetic intermediate. Then, subsequent dilutions were carried out in order to obtain three calibration curves ($k = 3$) ranging from 1.0 to 12 μg/mL ($m = 3$) (cf. Table 1) and containing 1 mg/mL of *R*-enantiomer and 125 μg/mL of levamisole, the internal standard. Two replicates ($n = 2$) were prepared per concentration level. Each solution was injected two times. The means were considered. The number of concentration levels (i.e. 3) is sufficient to generate different regression models.

Three independent series of validation standards were also prepared, in the same way, with final concentrations of 1, 2, 6 and 12 μg/mL (cf. Table 1). Three replicates ($n = 3$) were prepared per concentration level ($m = 4$). Each validation standard was injected two times for three different days.

For the evaluation of method selectivity, a solution containing a mixture of levamisole (125 μg/mL), *S*-enantiomer (2 μg/mL) and *R*-enantiomer (1 mg/mL) and a solution without *S*-enantiomer were prepared.

It is worth noting that for the determination of *S*-enantiomer purity, the same solutions were prepared by using *S*-enantiomer instead of the *R*-form and vice versa.

3. Results and discussion

3.1. Optimization of the NACE conditions

In a previous work dealing with the chiral separation of several β-blockers in NACE, the usefulness of two single-isomer anionic CD derivatives, namely HDMS-β-CD and HDAS-β-CD, was studied [16]. Except for two analytes, HDAS-β-CD was found to give higher enantioresolution values than HDMS-β-CD. Moreover, a high CD concentration was favourable to the enantioseparation of these compounds (40 mM). Accordingly, a BGE made up of 40 mM HDAS-β-CD and 10 mM ammonium formate in MeOH acidified with 0.75 M formic acid was first evaluated to separate the enantiomers of *R*/*S*-4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2*H*-1-benzopyran. Under these conditions, a baseline enantioseparation was observed (R_s value: 1.9) which is not sufficient for an enantiomeric purity determination. It is worth noting that the concentration of HDAS-β-CD was limited to 40 mM due to solubility limitation. Lower HDAS-β-CD concentrations were found to give rise to poor enantiomeric resolution and below 15 mM, no enantioseparation was observed. Therefore, the other anionic CD derivative, HDMS-β-CD, was evaluated. By contrast to what was observed with HDAS-β-CD, no peak was detected after 60 min using 40 mM HDMS-β-CD. It can be concluded that this analyte has a high affinity for this CD derivative. Therefore, the effect of HDMS-β-CD concentrations on the enantioresolution was studied over the 0.5–5 mM range. The enantiomeric resolution increased with increasing HDMS-β-CD concentration, reached an optimum at 1.5 mM (R_s value: 6.5) and then decreased until 5 mM (R_s value: 3.9). Fig. 2A illustrates the electropherogram obtained using 1.5 mM HDMS-β-CD concentration. Nevertheless, as can be seen in Fig. 2B, these experimental conditions could not be successfully applied to the enantiomeric purity determination of our compound. Indeed, in the presence of a very high concentration of *R*-enantiomer (1 mg/mL), it was not

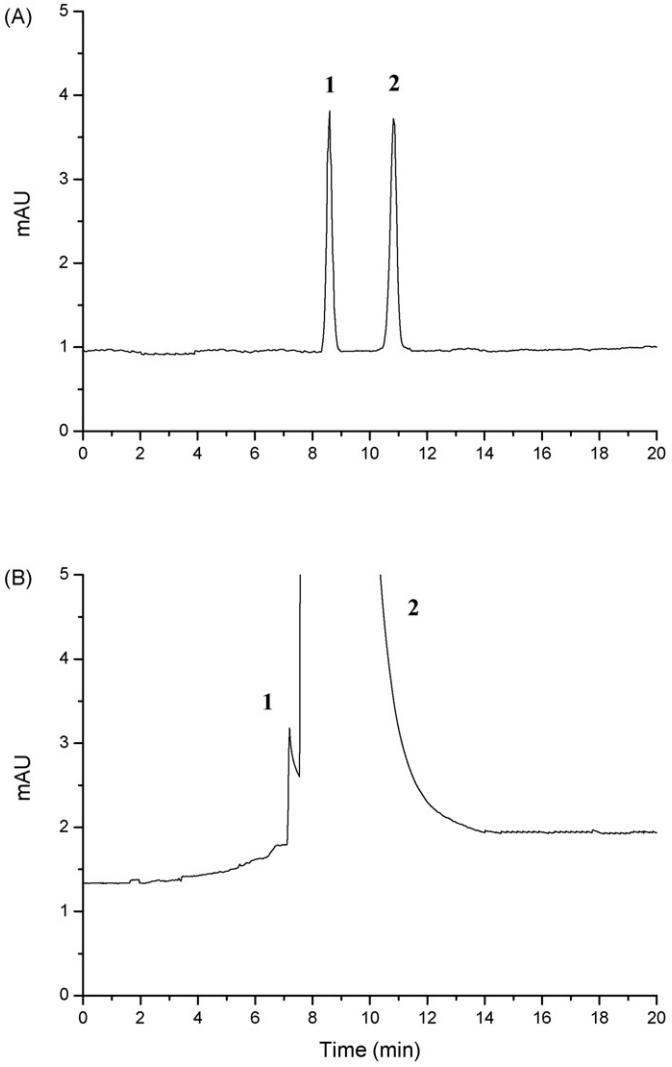

Fig. 2. Typical electropherograms of a methanolic solution of racemic compound (24 μg/mL) (A) and a methanolic solution of *R*-enantiomer (1 mg/mL) containing S-enantiomer (2 μg/mL). Peaks: 1, *S*-enantiomer; 2, *R*-enantiomer. BGE: 1.5 HDMS-β-CD and 10 mM ammonium formate in MeOH acidified with 0.75 M formic acid. Voltage: 25 kV. Other CE conditions are as described in Section 2.

possible to quantify very small amounts of *S*-enantiomer even if the latter migrates before the large *R*-enantiomer peak. In a previous paper, the use of only one anionic CD could not lead to a complete resolution of oxfendazole enantiomers and the combination of HDMS-β-CD and HDAS-β-CD was found to be necessary [39]. In order to improve the peak efficiency and the enantioseparation, combinations of two CD derivatives (HDMS-β-CD with HDAS-β-CD or CMCD) were then studied but these dual CD systems were unsuccessful. Our group has also carried out the enantiomeric purity determination of *R*-flurbiprofen for which the addition of ammonium camphorsulfonate was found to be necessary to avoid loss of peak efficiency and enantioresolution [40]. With this aim in view, ammonium camphorSO_3^- was then added but no favourable effect was obtained.

Finally, EtChol NTf_2, a chiral IL, at a concentration of 1.5 mM, was used in our nonaqueous system. The presence of EtChol NTf_2 led to a much higher enantiomeric resolution (R_s value: 22.6) compared to that observed in the presence of HDMS-β-CD alone (R_s value: 6.5). The effect on enantioresolution of the IL, over the 1–10 mM range, was investigated in the presence of 1.5 mM HDMS-β-CD. The combination of 5 mM EtChol NTf_2 and 1.5 mM HDMS-β-CD gave rise to the best enantioresolution (R_s value: 24.5). The increase in enantioresolution may be related to a reversal of the electroosmotic flow, resulting from the adsorption of the IL cation on the capillary wall (μ_{EOF}: -4.95×10^{-5} cm^2/V s) as compared to the experiment without IL (μ_{EOF}: $+3.68 \times 10^{-5}$ cm^2/V s). PhChol NTf_2, another chiral IL, was also studied but lower resolutions were obtained with this IL (R_s value: 19.7). The same experiment was carried out with Li NTf_2 salt instead of the chiral IL in order to differentiate a salt effect from a specific effect due to the chiral nature of the IL cations, as reported by François et al. [23]. Under the addition to HDMS-β-CD of 5 mM Li NTf_2, the enantioresolution was higher than in the experiment without IL (R_s value: 10.9; μ_{EOF}: -4.24×10^{-5} cm^2/V s) but lower compared to the experiment with a chiral IL. The effective electrophoretic selectivity (α_{eff}), a parameter independent of the electroosmotic mobility, was then calculated in order to highlight possible synergistic effect of the chiral IL and the CD derivative, as already reported by François et al. [23]. The highest α_{eff} value (1.70) was observed in the presence of EtChol NTf_2, compared to the experiments with Li NTf_2 and without any IL (1.25 and 1.17,

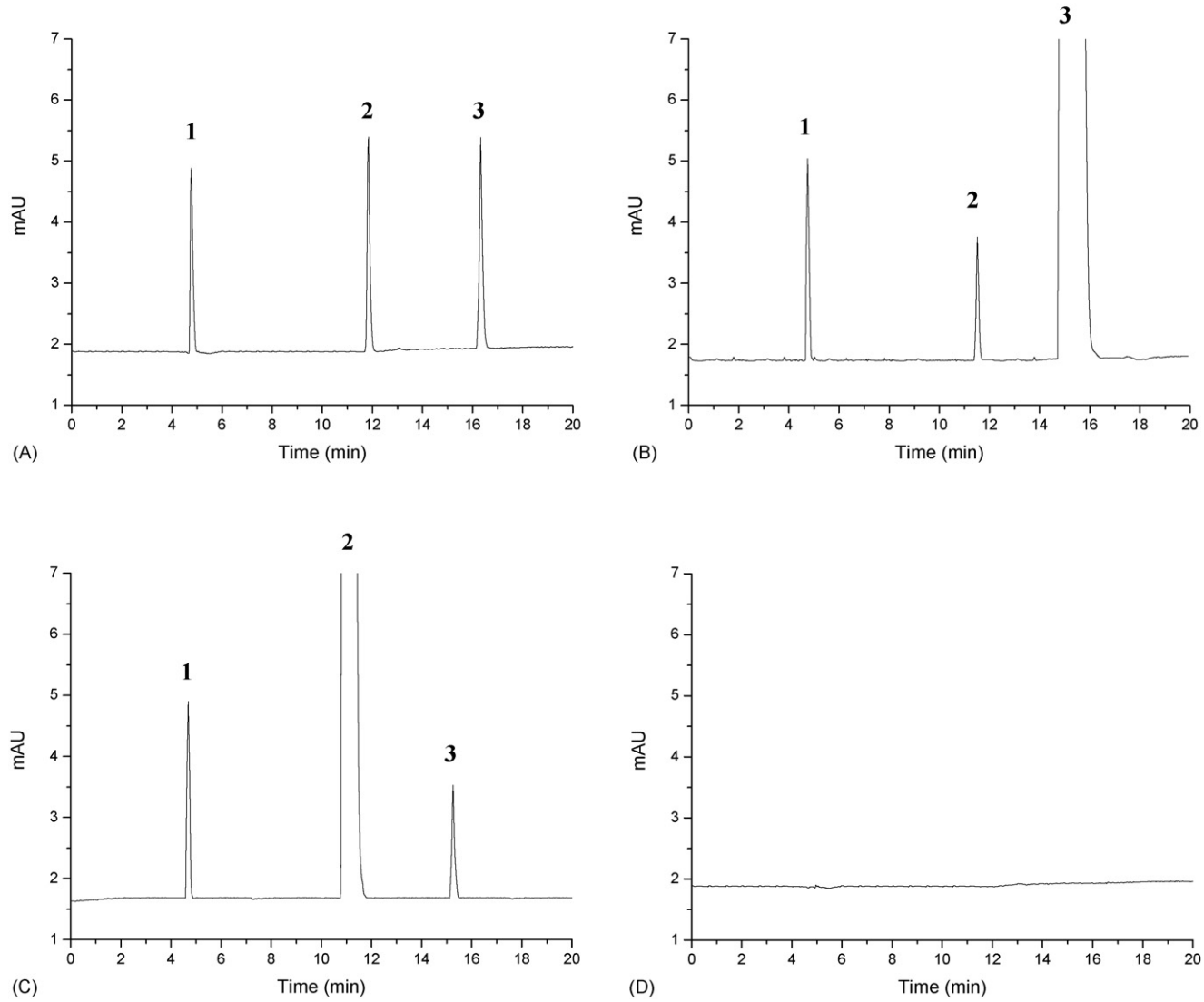

Fig. 3. Typical electropherograms of a methanolic solution of levamisole (125 μg/mL) and racemic compound (24 μg/mL) (A), a methanolic solution of *R*-enantiomer (1 mg/mL) containing *S*-enantiomer (2 μg/mL) and levamisole (125 μg/mL) (B), a methanolic solution of *S*-enantiomer (1 mg/mL) containing *R*-enantiomer (2 μg/mL) and levamisole (125 μg/mL) (C), methanol (D). Peaks: 1, levamisole; 2, *S*-enantiomer; 3, *R*-enantiomer. BGE: 1.5 HDMS-β-CD, 5 mM EtChol NTf_2 and 10 mM ammonium formate in MeOH acidified with 0.75 M formic acid. Voltage: 30 kV. CE conditions are as described in Section 2.

respectively). Therefore, the simultaneous increase in α_{eff} and R_s upon adding a chiral IL seems to indicate that there is a synergistic effect of HDMS-β-CD and EtChol NTf_2. It is worth noting that no enantioseparation is observed with the IL alone. A higher peak efficiency as well as shorter migration times were obtained upon increasing the applied voltage from 25 kV to 30 kV.

The internal standard should preferably be an achiral basic compound and migrate before the peaks of the synthetic intermediate to avoid an increase of the analysis time. Therefore, levamisole (Fig. 1B) at a concentration of 125 μg/mL was selected as internal standard. Following this optimization, the BGE composition giving the best enantioresolution (R_s value observed by applying a voltage of 30 kV: 20.9) was found to be 1.5 mM HDMS-β-CD, 5 mM EtChol NTf_2 and 10 mM ammonium formate in MeOH acidified with 0.75 M formic acid. Fig. 3A shows the electropherogram obtained in these conditions. Moreover, under the selected experimental conditions, the determination of 0.1% of one enantiomer in the other form could be performed, as can be seen in Fig. 3B and C.

3.2. Method validation

3.2.1. Selection of the calibration model

The first step of a validation method is the assessment of the relationship between the response and concentration in order to avoid serious difficulties in the estimation of the other validation criteria. In order to select the most appropriate calibration model, the original SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) approach, using accuracy profiles, has been applied. It is based on β-expectation tolerance intervals for the total measurement error which includes trueness (bias) and intermediate precision (standard deviation). Once the validation experiments have been carried out, several regression models were fitted to the calibration standards. From each regression line obtained, the concentrations of the validation standards were back-calculated in order to determine, at each concentration level, the mean relative bias, the upper and lower β-expectation tolerance limits as well as the relative standard deviation for intermediate precision [41].

Then, different accuracy profiles were plotted from these data to select the most appropriate regression model for the intended use of the analytical method [42]. Since the present analytical method deals with impurities, the acceptance limits were set at ±10% while the maximum risk (1-β) to obtain results outside these acceptance limits was set at 10%. In this study, regression analysis should be performed using a linear regression model, as expected considering the UV detection. This is the model that adequately demonstrates the capability of the method over the concentration range considered, since the tolerance intervals were totally included inside the acceptance limits (±10%) for both enantiomers (cf. Fig. 4). The obtained equations for the 3 days are presented in Table 2. The determination coefficients (r^2) obtained for both enantiomers demonstrate the good relationship between the normalized peak area ratio and the analyte concentration.

3.2.2. Selectivity

Typical electropherograms obtained by injecting the solution containing levamisole, *S*- and *R*-enantiomers (cf. Fig. 3A) and methanol (cf. Fig. 3D) were compared and, as can be seen from these figures, no peak was observed at the migration times of the three analytes. The selectivity was also demonstrated by evaluating the ability to easily integrate and therefore quantify a low amount of *S*- or *R*-enantiomer in the presence of the large peak corresponding to the other enantiomer (1.0 mg/mL). Fig. 3B and C shows that the peaks corresponding to *S*- and *R*-enantiomers can be well integrated and quantified even at a low concentration level around the targeted limit of quantification that is 0.1%.

Solutions of 1.0 mg/mL of *R*- and *S*-enantiomers were also injected. A small peak, identified as *S*- and *R*-enantiomers by

Table 2
Validation results referred to *S*- and *R*-enantiomers using the linear regression model.

Validation criteria	*S*-enantiomer			R-enantiomer		
Response function ($k=3$; $m=3$; $n=2$) (1–12 μg/mL)						
Slope	0.019	0.019	0.018	0.016	0.016	0.018
Intercept	0.083	0.089	0.089	0.064	0.069	0.075
r^2	0.9997	0.9997	0.9996	0.9976	0.9979	0.9901
Trueness ($k=3$; $n=3$)						
Relative bias (%)				−0.22		
1 μg/mL	−0.92			1.0		
2 μg/mL	−4.3			<0.1		
6 μg/mL	1.9			0.49		
12 μg/mL	1.6					
Linearity ($k=3$; $m=4$; $n=3$) (1–12 μg/mL)				1.005		
Slope	1.023			−0.005		
Intercept	−0.067			0.9983		
r^2	0.9995					

Precision ($k=3$; $n=3$)	Repeatability RSD (%)	Intermediate precision RSD (%)	Repeatability RSD (%)	Intermediate precision RSD (%)
1 μg/mL	0.091	1.7	3.0	3.0
2 μg/mL	0.45	1.2	3.1	3.9
6 μg/mL	1.8	2.1	2.7	3.1
12 μg/mL	1.1	1.1	1.5	3.0
Accuracy ($k=3$; $n=3$)				
β-Expectation tolerance limits in %				
1 μg/mL	−6.64/4.81		−6.05/5.62	
2 μg/mL	−7.83/0.73		−7.47/9.49	
6 μg/mL	−2.68/6.47		−6.64/6.69	
12 μg/mL	−0.56/3.76		−7.56/8.55	
LOD (μg/mL)	0.3		0.3	
LOQ (μg/mL)	1.0		1.0	

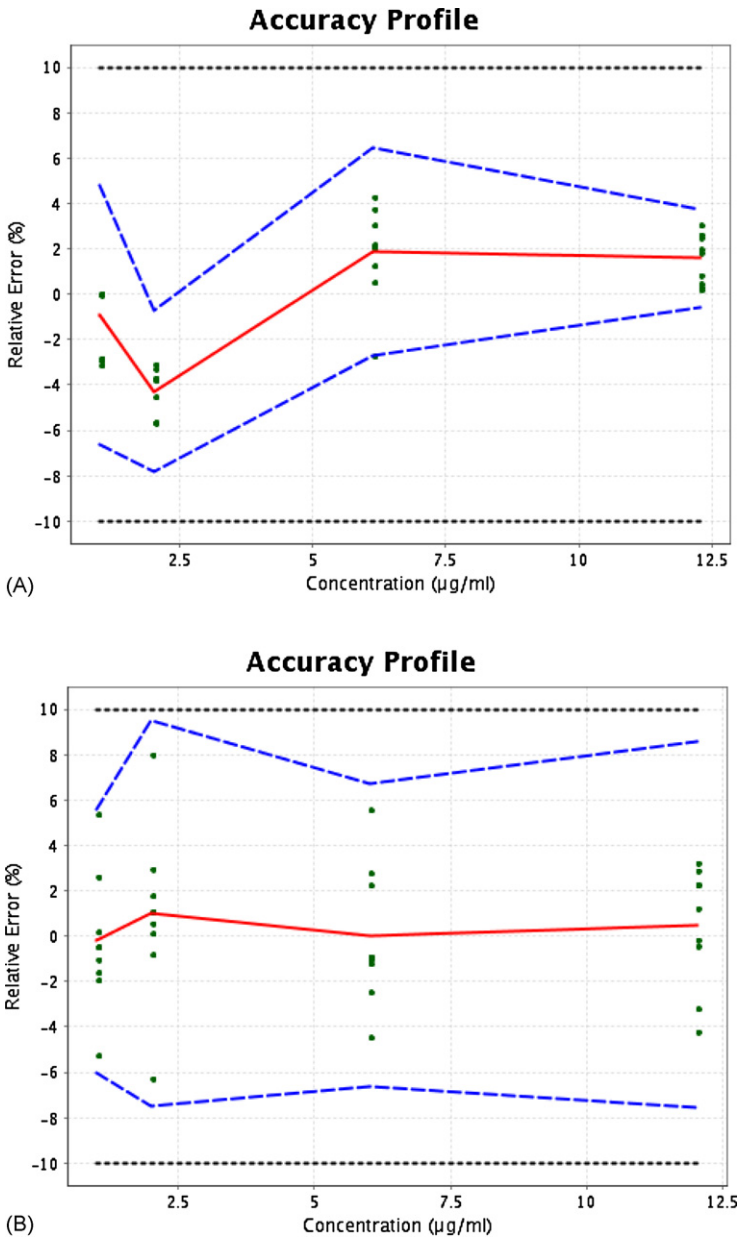

Fig. 4. Accuracy profiles of *S*- (A) and *R*-enantiomers (B) of the synthetic intermediate (concentration in µg/mL) using the linear regression model. The dashed lines correspond the β-expectation tolerance limits expressed in relative error. The dotted lines represent the acceptance limits at ±10% and the solid line the relative error (%).

comparison of the migration times, was observed in each electropherogram (data not shown). The obtained regression equations (cf. Table 2) were used to determine the enantiomeric purity (method of standard additions). Taking into account the mean over the 3 days, it can be concluded that the used batches of *R*- and *S*-enantiomer contain 0.46% and 0.41% of the other stereoisomer, respectively.

3.2.3. Other validation criteria

The validation results for *S*- and *R*-enantiomers are presented in Table 2.

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one. As shown in Table 2, trueness, expressed in term of relative bias (%), was assessed from the validation standards at four concentration levels, ranging from 1 to 12 µg/mL ($k = 3$, $n = 3$). Compared to the regulatory requirements, the results show that the relative biases of the analytical procedure were found acceptable since they are largely below the maximum value of 10%, irrespective of the concentration level. The biases of the analytical procedure were found to be particularly acceptable at the lowest concentration level as they were largely lower than the maximum value.

The linearity of an analytical method is its ability, within a definite range, to obtain results directly proportional to the concentrations of the analyte in the sample. For all series, a regression line was fitted on the back-calculated concentrations of the validation standards as a function of the introduced concentrations by applying the linear regression model based on the least squares method. The results attesting the method linearity, namely the regression equation corresponding to that relationship with the coefficient of determination, are presented in Table 2.

The precision of the proposed method was then estimated by measuring repeatability and intermediate precision at the four concentration levels used in the validation protocol. The variance of repeatability and time-dependent intermediate precision, as well as their corresponding relative standard deviation (RSD) values,

were calculated from the estimated concentrations. As can be seen in Table 2, the RSD values for both repeatability and intermediate precision were below the acceptable maximum value of 10%, illustrating the good precision of the developed method within the range of concentration examined (0.1–1.2%).

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value. The accuracy of the analytical method takes into account the total error which is a combination of systematic and random errors, related to the test results. The upper and lower β-expectation tolerance limits expressed in relative bias (%) as a function of the introduced concentrations are presented in Table 2.

As can be seen from the results in Table 2 and in Fig. 4, the proposed method can be considered as accurate over the concentration range investigated, since the different tolerance limits of the mean relative bias did not exceed the acceptance limits settled at 10% for each concentration level, including the lowest one (1 μg/mL).

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. In the present work, the LOD was estimated using the mean intercept of the calibration model and the residual variance of the regression. By applying this computation method, the LOD of the developed method was found to be equal to 0.3 μg/mL for both *S*- and *R*-enantiomers. The lower limit of quantification (LOQ) of an analytical procedure is the lowest quantity of the targeted substance in the sample that can be assayed under the experimental conditions prescribed with a well defined accuracy, i.e. taking into account the systematic and random errors. The LOQ was obtained by calculating the smallest concentration beyond which the β-expectation tolerance limits fall outside the acceptance limits. Since both accuracy profiles were comprised inside the acceptance limits over the whole concentration range investigated (Fig. 4), the LOQ for both enantiomers corresponded to 1 μg/mL, i.e. the first concentration level investigated. Indeed, precision and trueness were demonstrated at this concentration level.

4. Concluding remarks

NACE using an anionic CD and a chiral IL can be considered as a powerful technique to determine low levels of enantiomeric impurities. In order to obtain high efficiency and resolution values, the addition of a chiral ionic liquid to the background electrolyte (BGE) containing the single-isomer anionic CD was found to be essential. It was assessed that the optimized method permitted the determination of 0.1% of *R*-enantiomer in *S*-form and vice versa. The method showed suitable performance in terms of selectivity, trueness, precision, accuracy and linearity.

Acknowledgements

Research grants from the Belgium National Fund for Scientific Research (FNRS) to two of us (A.-C.S. and M.F.) are gratefully acknowledged. Many thanks are also due to FNRS and to the Léon Fredericq foundation for their financial support. The authors would like to thank Sébastien Mathu for his technical support.

References

[1] FDA's policy statement for the development of new stereoisomeric drugs, 1992. http://www.fda.gov/cder/guidance/stereo.htm.
[2] H.Y. Aboul-Enein, I. Ali, Chiral Separation by Liquid Chromatography and Related Technologies, Marcel Dekker Inc., New York, USA, 2003.
[3] E. Francotte, W. Lindner (Eds.), Chirality in Drug Research, Methods and Principles in Medicinal Chemistry 33, Wiley-VCH, Weinheim, 2006.
[4] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, 1997.
[5] G. Gübitz, M.G. Schmid, Electrophoresis 28 (2007) 114.
[6] B. Preinerstorfer, M. Lämmerhofer, W. Lindner, Electrophoresis 30 (2008) 100.
[7] T. Ward, Anal. Chem. 74 (2002) 2863.
[8] G. Blaschke, B. Chankvetadze, J. Chromatogr. A 875 (2000) 3.
[9] S. Lee, S. Jung, Carbohyd. Res. 338 (2003) 1143.
[10] R.S. Sahota, M.G. Khaledi, Anal. Chem. 66 (1994) 1141.
[11] F. Wang, M.G. Khaledi, Anal. Chem. 68 (1996) 3460.
[12] F. Wang, M.G. Khaledi, J. Chromatogr. B 731 (1999) 187.
[13] A.-C. Servais, M. Fillet, A.M. Abushoffa, P. Hubert, J. Crommen, Electrophoresis 24 (2003) 363.
[14] A.-C. Servais, M. Fillet, P. Chiap, W. Dewé, P. Hubert, J. Crommen, Electrophoresis 25 (2004) 2701.
[15] A.-C. Servais, M. Fillet, P. Chiap, W. Dewé, P. Hubert, J. Crommen, J. Chromatogr. A 1068 (2005) 143.
[16] A. Rousseau, P. Chiap, R. Oprean, J. Crommen, M. Fillet, A.-C. Servais, Electrophoresis 30 (2009) 2862.
[17] M. Vaher, M. Koel, M. Kaljurand, Chromatographia 53 (2001) S302.
[18] Y. François, A. Varenne, E. Juillerat, A.-C. Servais, P. Chiap, P. Gareil, J. Chromatogr. A 1138 (2007) 268.
[19] A. Seiman, M. Vaher, M. Kaljurand, J. Chromatogr. A 1189 (2008) 266.
[20] S.A.A. Rizvi, S.A. Shamsi, Anal. Chem. 78 (2006) 7061.
[21] B. Wang, J. He, V. Bianchi, S.A. Shamsi, Electrophoresis 30 (2009) 2812.
[22] C.D. Tran, I. Mejac, J. Chromatogr. A 1204 (2008) 204.
[23] Y. François, A. Varenne, E. Juillerat, D. Villemin, P. Gareil, J. Chromatogr. A 1155 (2007) 134.
[24] F.M. Ashcroft, J. Clin. Invest. 115 (2005) 2047.
[25] C.G. Nichols, Nature 440 (2006) 470.
[26] S. Seino, Annu. Rev. Physiol. 61 (1999) 337.
[27] F.M. Ashcroft, Am. J. Physiol. 293 (2007) E880.
[28] T. Miki, S. Seino, J. Mol. Cell. Cardiol. 38 (2005) 917.
[29] S. Sattiraju, S. Reyes, G.C. Kane, A. Terzic, Clin. Pharmacol. Ther. 83 (2008) 354.
[30] G.C. Kane, X.K. Liu, S. Yamada, T.M. Olson, A. Terzic, J. Mol. Cell. Cardiol. 38 (2005) 937.
[31] N.B. Standen, J.M. Quayle, N.W. Davies, J.E. Brayden, Y. Huang, M.T. Nelson, Science 245 (1989) 177.
[32] M.M. Soundarapandian, X. Zhong, L. Peng, D. Wu, Y. Lu, J. Neurochem. 103 (2007) 1721.
[33] K. Yamada, N. Inagaki, J. Mol. Cell. Cardiol. 38 (2005) 945.
[34] T.C. Hamilton, S.W. Weir, A.H. Weston, Br. J. Pharmacol. 88 (1986) 103.
[35] P. Lebrun, M.H. Antoine, V. Devreux, M. Hermann, A. Herchuelz, J. Pharmacol. Exp. Ther. 255 (1990) 948.
[36] P. Lebrun, P. Arkhammar, M.H. Antoine, Q.A. Nguyen, J.B. Hansen, B. Pirotte, Diabetologia 43 (2000) 723.
[37] X. Florence, Ph.D. Thesis, University of Liege, 2009.
[38] Council of Europe. The European Pharmacopoeia, 6th ed., Council of Europe, Strasbourg, France, 2008.
[39] A. Rousseau, F. Gillotin, P. Chiap, J. Crommen, M. Fillet, A.-C. Servais, Electrophoresis 31 (2010) 1482.
[40] A. Rousseau, P. Chiap, R. Ivanyi, J. Crommen, M. Fillet, A.-C. Servais, J. Chromatogr. A 1204 (2008) 219.
[41] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma Pratiques 13 (2003) 101.
[42] B. Boulanger, P. Chiap, W. Dewe, J. Crommen, P. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753.