On-line coupling of cyclodextrin mediated nonaqueous capillary electrophoresis to mass spectrometry for the determination of salbutamol enantiomers in urine

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Abstract

The usefulness of the on-line coupling of nonaqueous capillary electrophoresis (NACE) with electrospray ionization (ESI) mass spectrometry (MS) using heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS-β-CD) was demonstrated for the enantioselective determination of low concentrations of salbutamol in human urine. After optimization of several parameters, such as sheath-liquid composition and flow rate, nebulizing gas pressure, CE counter-pressure and position of the CE capillary outlet, a limit of quantification of 18 and 20 ng/ml was obtained for salbutamol enantiomers. Moreover, the relative standard deviation values for repeatability at a concentration of 30 ng/ml were below 7% for both enantiomers. Typical regression lines obtained after application of a simple linear regression model revealed a good relationship between peak area and analyte concentration (with 0.9988 and 0.9966 as coefficients of determination). This paper proposes an easy to use and sensitive NACE-MS method to determine enantiomers of a basic chiral drug in biological fluids preceded by solid-phase extraction as sample cleanup.
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1. Introduction

Analytical separation methods are still needed to control the enantiomeric purity of drugs. The regulatory authorities (i.e. FDA) require quantitation of chiral drugs in pharmacological, toxicological and clinical settings. Thus, sensitive and robust methods able to separate and detect low concentrations of enantiomers could play a key role in the determination of the biological function of chiral drugs, their metabolism or their rate in biological fluids.

Capillary electrophoresis (CE) is known to be one of the most powerful analytical techniques for chiral separations, principally due to its high separation efficiency [1–5]. Over the past few years, nonaqueous (NA) media have been introduced with success to obtain and improve selectivity in CE [6–9]. However, the

drawback of CE with UV detection is the low suitability for the determination of trace levels of pharmaceuticals and metabolites in pharmacokinetic studies. For those applications, more sensitive detectors are needed, specially in NACE-UV due to UV-absorption of many organic solvents. On-line coupling of chiral CE with electrospray ionization mass spectrometry (ESI-MS) may solve the problem, since MS is a sensitive and selective detector. In literature, many applications illustrate the gain of sensitivity in chiral CE by using MS instead of UV detection [10–12]. In the field of chiral NACE with MS detection, the enantiomers of basic drugs were separated using an ion-pairing reagent as chiral selector [13].

With the recent developments of a range of ionization sources and the improvement of mass analyzers, sensitivity, repeatability as well as ruggedness of CE-MS for pharmaceutical analysis have considerably improved [14–17].

Despite CE-MS is widely used for qualitative analysis, few quantitative applications have been published so far [18,19] and, to the best of our knowledge, chiral separations using

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cyclodextrins in NA media coupled with MS have never been undertaken.

Salbutamol is a sympathomimetic drug with potent β_2-adrenoceptor stimulating properties widely used in the treatment of respiratory diseases. Because this drug is usually administrated as the racemate, each enantiomer needs to be monitored separately, since metabolism, excretion or clearance might be radically different for both enantiomers [20]. The therapeutic activity of salbutamol has been shown to be associated with the R(−) enantiomer with little or no adrenoceptor stimulation attributed to the S(+) enantiomer [21].

In this paper, the potential of cyclodextrin-NACE coupled with ESI-MS was investigated as an enantioselective bioanalytical tool for detecting and quantifying enantiomers of drugs in vivo. This approach was applied to the quantitative determination of salbutamol enantiomers in human urine after a solid-phase extraction (SPE) procedure, as a model case. In a previous paper [22], a SPE-NACE-UV method for the determination of the enantiomers of this drug was validated and LOD of 125 ng/ml was obtained. In the present study, the transferability of this method to a MS detector was demonstrated and a significant improvement of the sensitivity was observed.

2. Experimental

2.1. Instrumentation

CE-UV 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 ($15–60 \pm 0.1\,^{\circ}$C). A CE Chemstation (Agilent Technologies) was used for instrument control, data acquisition and data handling. Fused-silica capillaries were provided by Beckman Coulter (Fullerton, CA, USA). Capillary dimension was 50 and 375 μm for internal and external diameters, respectively, with a total length of 48.5 cm (40 cm to the detector). UV detection was performed at 230 nm.

CE-MS experiments were performed using a Prince CE system (Prince Technologies, Emmen, The Netherlands) equipped with an Agilent 1100 Series LC/MSD SL ion-trap mass spectrometer from Agilent Technologies. The separation was performed in a fused-silica capillary of 48.5 cm total length, and an internal diameter of 50 μm. The ESI-MS measurements were carried out in the positive ionization mode. CE was coupled to MS using a coaxial CE-MS sprayer (Agilent Technologies) which provides both a coaxial sheath liquid make-up flow and a nebulization gas to assist droplet formation. The flow-rate of the sheath liquid was 2.5 μl/min and the nebulizing gas rate was 4 l/h. Drying and nebulization gases were both nitrogen. During rinsing of the CE capillary with the BGE and during CE injection, the nebulizer gas flow and the high voltage of the CE-MS sprayer were switched off. To minimize negative effects (e.g., peak broadening and loss of resolution) due to a suction effect caused by the nebulizing gas, a counter-pressure of approximately—16 mbar was applied (9 s after the injection) on the inlet vial during CE-MS analysis.

The electrospray voltage was set at 5.0 kV, the nebulizing gas pressure was adjusted to 6 p.s.i and the drying gas flow-rate as well as its temperature were set at 4 l/min and 150 °C, respectively. MS detection was carried out in the full scan mode in the 205–245 m/z mass range. The ion-charge-control (ICC) mode of the MS was used to prevent overloading of the trap. In order to obtain good sensitivity (reduced noise levels), the ion accumulation time was adjusted to 100 ms.

The automatic sample preparation with extraction cartridges (ASPEC) system from Gilson (Villers-le-Bel, France) consisted of a sample processor equipped with an XYZ-motion robotic arm, a Model 401 diluter/pipetter connected to a needle attached to the robotic arm and a set of racks and accessories for handling disposable extraction cartridges (DECs), urine samples and solvents. An IBM-compatible computer (CPU type 80486) equipped with GME-715 version 1.1 (HPLC System Controller) software from Gilson was used to control the ASPEC system.

The e.noval® software (Arlenda, Liège, Belgium) was used to determine the accuracy profiles as well as the quantitative results.

2.2. Chemicals and reagents

(*R, S*)-salbutamol was supplied by Cambrex Profarmaco (Milano, Italy). Heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS-β-CD) was obtained from Antek Instruments (Houston, TX, USA). Their chemical structures are presented in Fig. 1. Ammonium formate was from Sigma-Aldrich (St. Louis, MO, USA). Formic acid 98–100% and ammonia solution 25% were obtained from Merck (Darmstadt, Germany). Sodium formate was purchased from U.C.B. (Leuven, Belgium). Ascorbic acid was from Certa (Braine-l'Alleud, Belgium). All reagents were of analytical grade. Water used in all experiments was of Milli-Q quality (Millipore, Bedford, MA, USA). Isolute HCX-3 (130 mg/1 ml) DECs were obtained from International Sorbent Technology (Mid Glamorgan, UK). Methanol from Merck was of HPLC grade. The background electrolyte (BGE) and samples solutions were filtered through a Polypure polypropylene membrane filter (0.2 μm) from Alltech (Laarne, Belgium) before use.
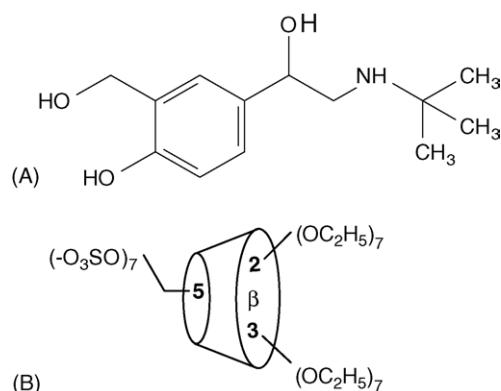

Fig. 1. Structures of (A) salbutamol and (B) heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS-β-CD).

2.3. Electrophoretic technique

A new capillary was conditioned with methanol for 15 min. At the beginning of each working day, the capillary was washed with methanol for 15 min and with the BGE for 15 min. Before each injection, the capillary was washed successively with methanol for 2 min and then equilibrated with the BGE for 2 min. The applied voltage was 25 kV. The selected BGE was composed of 10 mM ammonium formate and 15 mM HDAS-β-CD in methanol acidified with 0.75 M formic acid. Injections were made by pressure for a period of 30 s (corresponding to 88 nl, i.e. 9% of the total volume of the capillary) and the capillary was thermostated at 20 °C. The resolution (R_s) was calculated according to the standard expression based on peak width at half height [23].

2.4. Standard solutions

The solution used for method development was prepared by dissolving salbutamol at a concentration of ca. 100 ng/ml in methanol. To evaluate quantitative CE performances, a stock solution was prepared by dissolving 48 mg of salbutamol in 50 ml of methanol containing 1% ascorbic acid (which was added in order to prevent oxidation). The stock solution of salbutamol was diluted in sodium formate solution (pH 6.0) to obtain various concentrations. 800 µl of drug free urine was spiked with 200 µl of these diluted solutions to obtain a calibration curve ranging from 15 to 150 ng/ml for each enantiomer. Each calibration sample was treated in duplicate.

2.5. Urine sample preparation

The urine samples were treated according to a previously published SPE procedure [22], which uses Isolute HCX-3 DECs containing a sorbent providing a dual retention mechanism (SO_3^-/C18). The eluate was collected and gently evaporated to dryness under a nitrogen stream. The dried extracts were then reconstituted in an appropriate volume of methanol (250 µl) and the methanolic solutions were sonicated for 10 min, filtered through a polypropylene membrane filter and injected into the CE system.

3. Results and discussion

3.1. Parameters for CE-ESI-MS

The aim of the study was to prove that the NACE method developed for the determination of salbutamol enantiomers in CE-UV [22] is directly applicable in CE-MS. The selected BGE, which was composed of 10 mM ammonium formate and 15 mM HDAS-β-CD in methanol acidified with 0.75 M formic acid, offers good possibilities to be directly applied for MS coupling since the BGE contains volatile solvents and salts and the CD migrates towards the capillary inlet away from the MS detector.

Stable CE-ESI-MS conditions are depending of some critical electrospray parameters, i.e. the sheath liquid composition and flow rate, the nebulizing gas pressure, the electrospray volt-age and the capillary outlet position [12,18,24]. Sheath liquid is used to connect electrically the CE outlet to ground potential at the sprayer and to supply sufficient liquid for forming a stable electropsray. In order to determine the effect of the sheath liquid composition on the MS signal abundance and stability, mixtures of water and acetonitrile, methanol or isopropanol in the presence of 0.1% formic or acetic acid were tested. An acetonitrile-water mixture (75/25; v/v) containing 0.1% formic acid gave the highest ion abundance for salbutamol as well as the most stable conditions.

The sheath liquid flow rate was also investigated. As expected, high flow rates (i.e. 5 µl/min) resulted in lower signal-to-noise ratio, due to the dilution of the enantiomers, whereas very low flow rates gave rise to an unstable spray. Finally, a flow rate of 2.5 µl/min was selected.

It is well known that the nebulizing gas pressure, applied in the ESI source in order to assist droplet formation and stabilize the spray, may influence sensitivity [25]. The effect of the nebulizing gas pressure over the 2–20 psi range was investigated. A value of 6 psi was found to give the best results in terms of sensitivity and stability. This corresponds to a relatively low gas pressure but it is sufficient to have a good performance, probably due to the high volatility of the BGE and the low sheath liquid flow rate. On the other hand, the application of the nebulizing gas generates a reduced pressure at the capillary outlet and, thus, a hydrodynamic flow may occur in the capillary. For this reason, air may be entering the capillary inlet during the injection process causing current instabilities. Therefore, the nebulizer gas flow was switched off during injection. Moreover, the hydrodynamic flow (which is laminar) causes reduced CE performance (efficiency and resolution) and decreased migration times. Consequently, it was necessary to compensate the suction effect by applying a counter-pressure at the CE inlet. In order to determine the appropriate pressure to be applied, a NACE-UV analysis was performed as reference electropherogram in terms of migration times and separation performances, by substituting the outlet BGE vial by a vial containing the selected sheath liquid (Fig. 2A). A resolution of 6.1 was observed in these conditions. As can be seen in Fig. 2B, a counter-pressure of −16 mbar was found to preserve migration times and gave the best peak efficiency and resolution in NACE-MS (R_s value: 3.1).

In this study, electrospray voltage was kept constant at 5 kV, while the position of the capillary outlet was optimized. The best sensitivity and stability were obtained with a capillary outlet carefully adjusted 0.5 mm inside the needle (1 mark on the Agilent CE sprayer).

It is important to notice that the selected BGE, even if it contains CDs, i.e. a nonvolatile component, did not suppress the MS signal. Indeed, the peak area of salbutamol, obtained after CE injection in the capillary with a BGE without CD, is equal to the peak area of the enantiomers in the chiral electrolyte. Furthermore, no background signal at the *m/z* of salbutamol was observed when the BGE containing the CD was used. A more extensive discussion of the parameters affecting the coupling of CD-NACE with MS will be given in a separate paper.

Before evaluating the quantitative performance of the developed NACE-ESI-MS method, the ion mode monitoring was
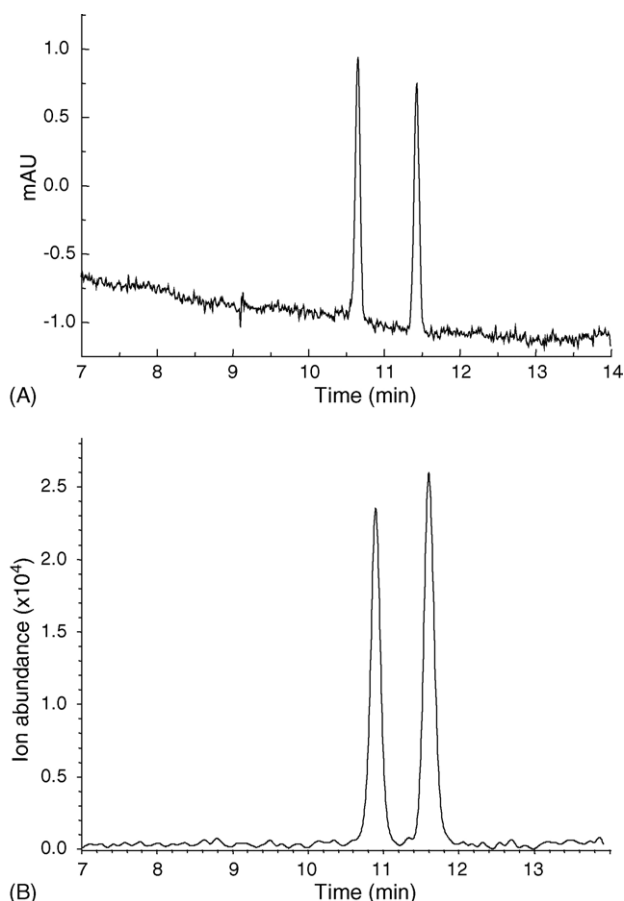
Fig. 2. Enantioseparation of salbutamol using a BGE made up of 10 mM ammonium formate and 15 mM HDAS-β-CD in methanol acidified with 0.75 M formic acid. (A) NACE-UV at 230 nm, 1.5 μg/ml of each enantiomer. (B) SPE-NACE-ESI-MS (extracted ion current, EIC at 240.4 *m/z*), 30 ng/ml of each enantiomer. Other conditions: see Section 2.

studied in terms of sensitivity. Single ion mode (SIM) and full-scan monitoring were compared, but SIM did not significantly improve the sensitivity compared to full-scan mode in the 200–250 *m/z* mass range. As long as in ion-trap MS in full-scan mode, a relatively small mass range and a relatively long accumulation time are used, no significant reduction of the noise level and, thus, improvement of the sensitivity is obtained compared to SIM.

3.2. Quantitative data and applicability

In order to evaluate the applicability of this NACE-MS method for quantitative purposes in bioanalysis, the response function was determined and the concentration range, the LOQ as well as the method repeatability were estimated. The SPE technique was selected for urine sample cleanup prior to CE-MS analysis according to the previously published method [22] in order to eliminate inorganic ions and other endogenous compounds that can interfere in the quantification, and to concentrate the analyte.

On the basis of the response function and repeatability results, it has been noticed that isoprenaline, the internal standard of the NACE-UV method, did not improve the NACE-MS quantita-

tion. MS parameters were optimised in order to efficiently ionize salbutamol but these conditions were not optimal for isoprenaline. Nevertheless, we found that our method did not require an internal standard to compensate for CE injection variations that are minimized in this case since the injection time is quite long (30 s). However, if an internal standard would be absolutely necessary, one could use a deuterated compound.

Concerning the analytical response, it is important to remember that the peak area has not to be normalized by the migration time since detection is performed outside the capillary and therefore is no longer dependent of analyte velocity [18].

The first step consisted in the assessment of the relationship between response (peak area) and concentration in order to avoid serious difficulties in the estimation of other validation criteria [26]. In order to select the most appropriate response function, the SFSTP approach based on the accuracy profile has been used [27,28]. The response function is considered as adequate when the accuracy profile is within the acceptance limits—fixed a priori at ±20%—on all the dosing range of interest. Once the experiments have been performed, the response function can be determined by applying different regression models and selecting the most appropriate accuracy profile. The linear regression model based on the least-squares method was selected since it represents the simplest model adequately describing the concentration-response relationship for both enantiomers (Fig. 3). The following equations were obtained (15, 30, 75, and 150 ng/ml):

$$Y = -1.722.10^4 + 8811X \rightarrow r^2 = 0.9988 \quad \text{(Enantiomer 1)}$$

$$Y = -2.306.10^4 + 8153X \rightarrow r^2 = 0.9966 \quad \text{(Enantiomer 2)}$$

The determination coefficients (r^2) obtained for both regression lines demonstrate the good relationship between peak area and analyte concentration.

In addition, the accuracy profiles allowed the estimation of the LOQ. Indeed, this one corresponds to the concentration at which the tolerance limits are no longer included within the acceptance limits (±20%), i.e. 18 ng/ml for the first enantiomer and 20 ng/ml for the second one. Fig. 4 presents a typical electropherogram of salbutamol enantiomers at a concentration close to LOQ obtained by using SPE-NACE-ESI-MS. It is noted that, compared with the LOQ of the NACE-UV method (i.e. 375 ng/ml for an injection time of 15 s), the use of an MS detector (with an injection time of 30 s) gives rise to a tenfold increase of sensitivity. As for the LOD, it was estimated using the intercept and the residual variance of the regression line. By applying this computation method, the LOD of the developed method was equal to 8 ng/ml for the first enantiomer and to 14 ng/ml for the second one.

Regardless of the calibration line, the analysis of six urine samples spiked with salbutamol at a concentration of 30 ng/ml of each enantiomer (i.e. a concentration close the LOQ) has been performed. Relative standard deviation values exhibit good results for a SPE-NACE-MS method (5.5% for the first enantiomer and 6.9% for the second one) illustrating the good repeatability of the proposed method considering all the steps involved.
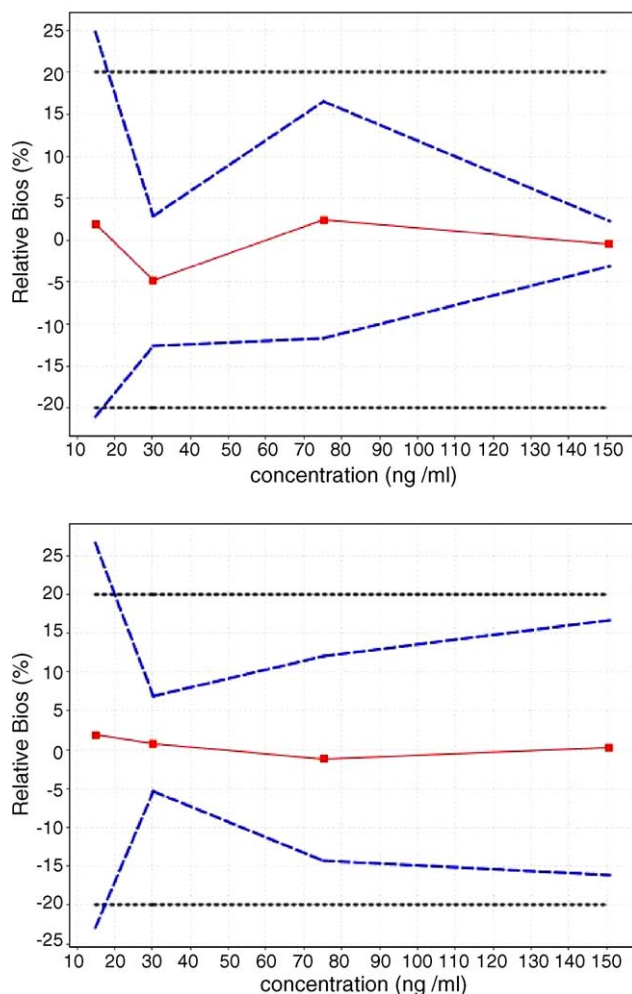
Fig. 3. Accuracy profiles for enantiomer 1 (1) and enantiomer 2 (2) of salbutamol (concentration ng/ml) using linear regression model. The dashed lines correspond to the β-expectation tolerance limits expressed in relative bias. The dotted lines represent the acceptance limits at ±20% and the solid line with the symbol (■) the relative bias (%).

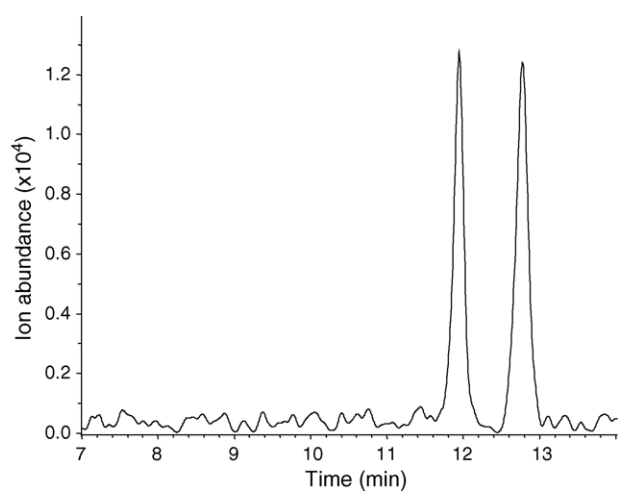Fig. 4. Typical electropherogram of salbutamol enantiomers at a concentration close to LOQ obtained using SPE-NACE-ESI-MS (EIC at 240.4 *m/z*) using a BGE of 10 mM ammonium formate and 15 mM HDAS-β-CD in methanol acidified with 0.75 M formic acid. Other conditions: see Section 2.

4. Conclusions

The on-line coupling of NACE and MS can be considered as a powerful technique to determine low levels of the enantiomers of a basic chiral drug in biological samples. Several ESI parameters, including sheath-liquid composition and flow rate, nebulizing gas pressure and CE counter-pressure, were optimized in order to maximize sensitivity and to obtain a stable ionization process.

The quantitative performance of the developed method was evaluated. After having selected a linear regression model as response function on the basis of the accuracy profiles, the LOQ were found to be about 20 ng/ml for both enantiomers. The applicability of the proposed SPE-NACE-MS method for quantitative determination in human urine has now been demonstrated, but a full validation should still be undertaken before applying this procedure for routine analysis.

Acknowledgements

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