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Efficient applications of capillary electrophoresis–tandem mass spectrometry to the analysis of adrenoreceptor antagonist enantiomers using a partial filling technique

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Abstract

Throughout the separation of chiral basic drugs by capillary electrophoresis (CE) with neutral hydroxypropyl-β-cyclodextrin (HP-β-CD) as chiral selector, the sensitivity of detection can be improved by using tandem mass spectrometric (MS–MS) detection with a partial filling technique rather than with UV spectrometric detection. Prior to sample injection, the capillary was partly filled with HP-β-CD dissolved in volatile ammonium formate buffer (pH 4, ionic strength 50 m*M*). The effects of modifying the HP-β-CD concentration in the selector zone and the length of the separation zone on the enantioresolution and the signal-to-noise ratio of the pseudo-molecular MH^+ ion were investigated. For a given selector zone length, as the concentration of the neutral cyclodextrin increases, the resolution between enantiomers becomes higher (the opposite of the behavior of the signal-to-noise ratio) and then reaches an optimum value. The decrease of the selector zone length lowered the resolution between the enantiomers but increased peak efficiencies and signal-to-noise ratio values. Accordingly, partial capillary filling at 80% (v/v) and 10 m*M* concentration of HP-β-CD was selected as a suitable compromise between resolution and sensitivity of MS detection. Limits of detection for each adrenoreceptor antagonist enantiomer were 5 ng/ml (0.02 μ*M*) in CE–MS–MS instead of 150 ng/ml (0.60 μ*M*) in CE–UV, which enhances sensitivity by a factor of 30.

Keywords: Partial-filling capillary electrophoresis; Enantiomer separation; Mass spectrometry; Cyclodextrins

1. Introduction

Chiral analysis of pharmaceutical compounds is a significant field of application in the pharmaceutical industry, because in many cases enantiomers possess distinct pharmacological properties. Determination of enantiomeric purity is of primary importance during the different stages of drug development and manufacture. Hence, the distomer usually needs to be quantitated at low level (0.1%) in the drug substance [1].

In the last few years, capillary electrophoresis (CE) has been widely developed for the analysis of drugs due to many attractive features such as high peak efficiencies, great resolution, low sample volume and reduced analysis times. As reported in recent reviews [2,3], CE is obviously established as one of the major techniques in the field of analytical chiral separations of pharmaceutical drugs. The most common approach for enantiomeric CE separation involves the addition of neutral or anionic cyclodextrins (CDs) to the running buffer, such as car-

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boxymethylated [4,5], sulfoalkyl ethers [6–11], sulfated [12,13] and single-isomer sulfated cyclodextrins [14–16]. The enantioselectivity of CDs stems from the inclusion of the hydrophobic moiety of drug molecule into the CD cavity and also from hydrogen bonding between solute and secondary hydroxyl groups at the entrance to the CD cavity. However, the most commonly used UV detector lacks sensitivity and the coupling of CE with mass spectrometry (MS) seems to be a promising analytical tool [17–22]. Additionally, MS detection provides the capacity to determine the molecular mass as well as to obtain structure-related fragmentation information on the analytes. In our laboratory, we recently reported non-chiral applications of CE–MS–MS for ppb-level determination of anti-human immunodeficiency virus nucleoside drugs [20] and phosphonic acids [21,22].

The main limitation of CE–MS concerns the compatibility of electrophoretic buffers with the electrospray MS source. Indeed, common CE buffers (phosphate, borate) or additives (CDs) are non-volatile compounds and not suitable for on-line CE–MS, they contaminate the ionization chamber of the mass spectrometer during the experiment and cause a degree of analyte signal suppression. In order to avoid the introduction of non-volatile cyclodextrins into the MS source, the partial filling technique, in which the capillary is partially filled with the cyclodextrin-based buffer [23–31], can be carried out. In the present work, enantiomers of an adrenoreceptor antagonist are separated using hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral selector. Prior to sample injection, the capillary was partially filled with cyclodextrin dissolved in ammonium formate buffer. When the positive voltage was applied, the positively charged enantiomers migrated towards the mass spectrometer first into the zone containing the neutral selector (called CD zone) and then into the non-CD zone. This procedure is simple, easy to automate and can be performed without modification of either CE or MS equipment.

As part of our ongoing research upon chiral drug separation, we report preliminary studies concerning the enantioselective separation and quantitation at ppb level of a potent adrenoreceptor antagonist by CE–MS–MS using an ionspray ionization mode and employing the partial filling technique. We specially focused on the effect of several operating parameters such as the concentration of HP-β-CD in the buffer, length of CD zone and composition of the sheath liquid upon enantioresolution and sensitivity of MS detection.

2. Experimental

2.1. Chemicals

(+)-2-(4,5-Dihydro-1*H*-imidazo-2-yl)-2′-methoxy-5-fluorobenzo-[1,4]-dioxan hydrochloride (Fig. 1) is a potent adrenoreceptor antagonist obtained from Centre de Recherche Pierre Fabre (Castres, France). This molecule (M_r 252) has one chiral center on the benzodioxan ring and exhibits its activity as the *S*-form. The synthesized racemic drug was analyzed by high-performance liquid chromatography (HPLC) using a Chiralcel OD column and a hexane–ethanol (90:10, v/v) mixture as mobile phase.

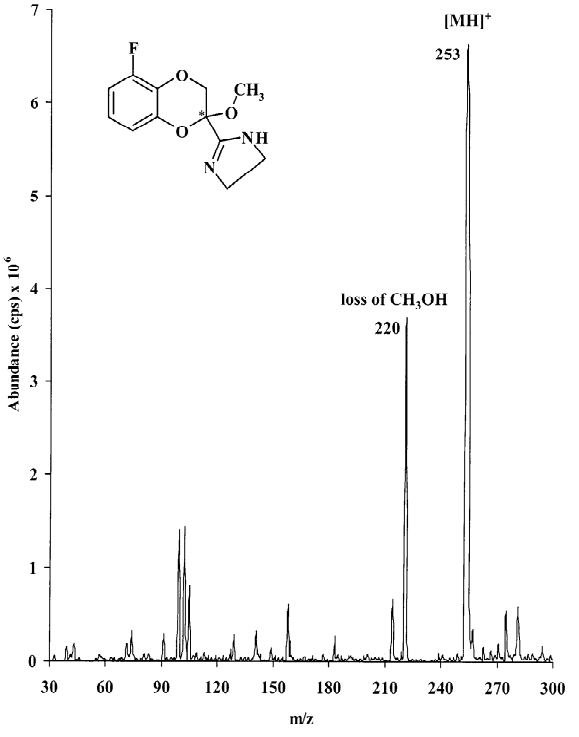

Fig. 1. Ionspray mass spectrum of the adrenoreceptor antagonist in 30–300 mass range. Experimental conditions: infusion of the racemic drug (10 μl, 100 μg/ml), ionspray voltage: +5.6 kV.

All chemicals and reagents were of analytical grade. An ELGA apparatus (Villeurbanne, France) supplied ultra-pure water used for dilutions or buffer preparations. HPLC-grade methanol, ethanol, propanol and isopropanol were purchased from J.T. Baker (Noisy le Sec, France). Formic acid, acetic acid, trifluoroacetic acid and ammonia were obtained from Fluka (St. Quentin-Fallavier, France). HP-β-CD was provided by Wacker (Munich, Germany).

Ammonium formate was selected as volatile running buffer for CE–MS separations. The electrophoretic buffer was prepared at pH 4 and fixed ionic strength (50 m*M*) with the help of Phoebus software [32] (Beckman, Villepinte, France); its composition was 72.9 m*M* formic acid and 49.9 m*M* ammonia. The buffer capacity, calculated with this software, was high enough (37 m*M*/pH unity) to avoid any variation of buffer pH in the capillary during the experiment. HP-β-CD was dissolved in the buffer at the required final concentration. Subsequently, buffer solutions were filtered through a polypropylene filter membrane with a 0.22 μm porosity (Prolabo, Paris, France).

The pK_a value (10.4) of the adrenoreceptor antagonist was determined by an automatic potentiometric titrator (Mettler DL 25, Viroflay, France) at ambient temperature using an internal procedure.

2.2. Capillary electrophoresis system

All open-tube CE separations were carried out on a P/ACE MDQ apparatus (Beckman Coulter, San Jose, CA, USA) using a fused-silica capillary (70 cm×50 μm I.D.×150 μm O.D.). The CE capillary length was 70 cm because of the relevant dimensions of the CE apparatus and the MS instrument. Electrophoretic separations were performed at a positive voltage (+20 kV) and at 25°C. Analytes were injected in the hydrodynamic mode using 0.5 p.s.i. for 10 s (1 p.s.i.=6894.76 Pa). Between two consecutive experiments, the capillary was rinsed sequentially with 1 *M* sodium hydroxide (2 min), water (3 min) and then buffer (3 min). These conditioning steps improve the repeatability of the electroosmotic flow (EOF) [33]. Data were collected with an electrophoresis data calculation program (System MDQ software, version 2.2, Beckman). The consumption of selectors is extremely low since the inlet vial did contain only pure buffer without selector.

2.3. Mass spectrometry

All CE–MS measurements were performed on a Perkin-Elmer Sciex API 300 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Toronto, Canada) equipped with an ionspray source. The mass spectrometer was operated in the positive ion mode with an ionspray voltage set at +5.6 kV, orifice voltage +30 V and focusing ring voltage at +250 V. Dry air was used as nebulizing gas while nitrogen was used as curtain gas at a flow-rate of 1.25 l/min. The sheath-flow interface was provided by Perkin-Elmer Sciex and directly used. The coaxial sheath liquid enables the contact between the electrospray needle and the high-voltage supply of the CE. A Harvard Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) delivered the sheath liquid to the interface at a flow-rate of 5 μl/min. The optimization of MS sensitivity was made by modification of the sheath liquid composition. An ionspray source voltage of +5.6 kV was necessary to create charged ions in the liquid and applied to the detection side of the CE electrical circuit. A voltage drop of +14.4 kV remains as the driving force for electromigration when +20 kV are applied on the injection side by the CE power supply.

In the full scan mode, mass peaks were scanned from 30 to 300 u (step size: 0.042 u, dwell time: 1 ms). In the selected ion monitoring (SIM) mode, the mass spectrometer was operated with a dwell time of 1000 ms on each mass. A Macintosh computer was used for instrument control, data acquisition and data processing using LC_2 Tune software.

3. Results and discussion

Preliminary MS experiments were performed to determine the fragmentation of the adrenoreceptor antagonist molecule. A methanolic solution (100 μg/ml) of racemic drug was injected by infusion and MS data were acquired in full scan mode to investigate its fragmentation in the 30–300 mass range

(Fig. 1). Protonated molecules $(MH)^+$ were detected by electrospray MS at m/z 253 while the minor ion at m/z 220 was due to the loss of a molecule of methanol ($M-CH_3OH^+$). In further experiments, MS data acquisition was performed in the SIM mode at m/z 253.

3.1. Selection of the sheath liquid

The extremely low flow-rates (nl/min1) of CE were inappropriate to guarantee stable ionspray MS conditions. Therefore, a sheath liquid was added to the electrophoretic buffer to obtain a stable electrospray ionization. In the positive mode, the sheath liquid should contain H^+ ions to protonate basic drug molecules. The racemic drug (100 μg/ml) was firstly injected by hydrodynamic mode (5 p.s.i. for 10 s) through the CE capillary. The two enantiomers moved towards MS instrument by applying 20 p.s.i. pressure. Under this condition, no electrophoresis was performed.

Several volatile water–solvent mixtures, differing by the nature of the organic acid (acetic, formic or trifluoroacetic acids) and of the solvent (methanol, ethanol, propanol, isopropanol) were evaluated as sheath liquids. The selected response was the height of drug peak based on total ion current (TIC). First, experimental data indicated that the ion abundance signal was higher with acetic acid than with trifluoroacetic acid, as already observed in liquid chromatography [34]. Otherwise, the intensity of the drug signal at m/z 253 decreased by a factor of five when the amount of acetic acid added to a water–methanol (5:95, v/v) mixture increased from 0.25 to 1% (44 to 174 m*M*).

The addition of an organic solvent to a purely aqueous system provided higher detection sensitivities in CE–MS. This result may be explained by a decrease of droplet surface tension and an easier solvent evaporation. Consequently, an improved desorption efficiency of the protonated molecule ion occurs [21,35]. In the present study, no significant difference in sensitivity was observed between various investigated protic solvents. A water–methanol mixture was selected as sheath liquid. Accordingly, subsequent CE–MS experiments were performed with the following sheath liquid: 44 m*M* acetic acid in a water–methanol (5:95, v/v) mixture.

3.2. Effect of HP-β-CD concentration in the CD zone

The separation of cationic enantiomers was carried out by CE–MS on a bare silica capillary with HP-β-CD as chiral selector. Both the resolution and the signal-to-noise ratio depend, simultaneously, on the concentration of the cyclodextrin and the length of the filled zone. A systematic method development approach was conducted by modifying the HP-β-CD concentration in the CD zone, and subsequently, the length of the CD zone. This combination was selected for this separation, but is not necessarily the optimized setting.

The concentration of HP-β-CD varied from 3.5 to 30.0 m*M*. The selected volatile CE–MS buffer contained 44.3 m*M* formic acid and 29.9 m*M* ammonia (pH 4, ionic strength 50 m*M*). Low pH is preferable since it will further decrease the EOF. Higher ionic strengths of the buffer would probably induce excessive currents (50 μA) and reduce the yield of ionization process. The sample was injected at the anodic inlet and the positively charged drug ($pK_a=10.4$) migrated towards the cathodic outlet. The filling time of the total capillary was determined by monitoring the MS signal of a HP-β-CD solution injected in the hydrodynamic mode (5 p.s.i., 10 s).

The capillary was first filled with an effective HP-β-CD zone length of 90% (v/v). The change in resolution and in MH^+ ion abundance (at m/z 253) of the enantiomers with increasing HP-β-CD concentration were investigated (Fig. 2). In the 3.5–20 m*M* HP-β-CD concentration range, the resolution was greater when the concentration of the chiral agent increased due an enhanced complexation of each enantiomer with HP-β-CD (resolutions of 1 and 2.8 for 3.5 and 10 m*M*,, respectively). Optimum resolution (3.2) was apparent with 20 m*M* HP-β-CD and then decreased for higher cyclodextrin concentrations. In contrast, the signal-to-noise ratio at m/z 253 decreased by a factor of three when the HP-β-CD concentration varied from 3.5 m*M* up to 20 m*M*. Therefore, 10 m*M* HP-β-CD appeared to be a suitable compromise between resolution (2.8) and signal-to-noise ratio.

3.3. Influence of the selector zone length

To examine the length of capillary that had to be

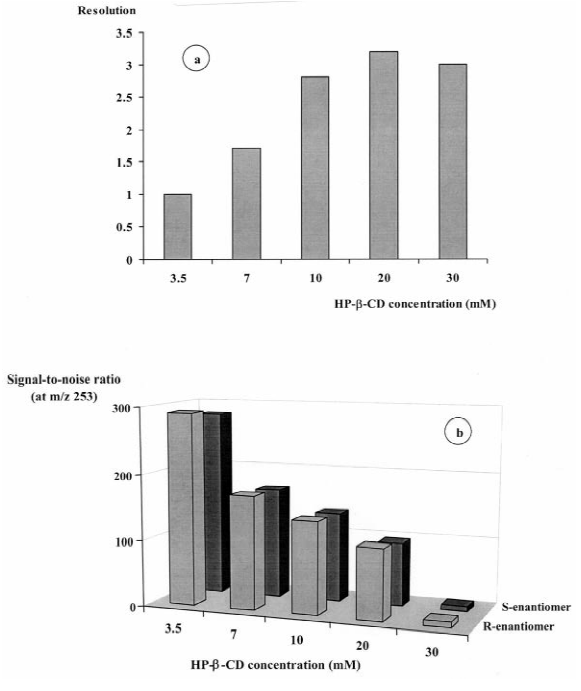

Fig. 2. Influence of HP-β-CD concentration on enantioresolution (a) and signal-to-noise ratio of m/z 253 ion (b). Capillary electrophoresis: capillary: 70 cm×50 μm I.D.; applied voltage: +20 kV; temperature: 25°C; buffer: formic acid–ammonia (pH 4, ionic strength 50 m*M*)+HP-β-CD; hydrodynamic injection: 10 s, 0.5 p.s.i.; analyte: 100 μg/ml; percentage of filled capillary: 90% (v/v). Mass spectrometry: ionspray voltage: +5.6 kV, sheath liquid: water–methanol–acetic acid (5:95:0.25, v/v/v), flow-rate: 5 μl/min.

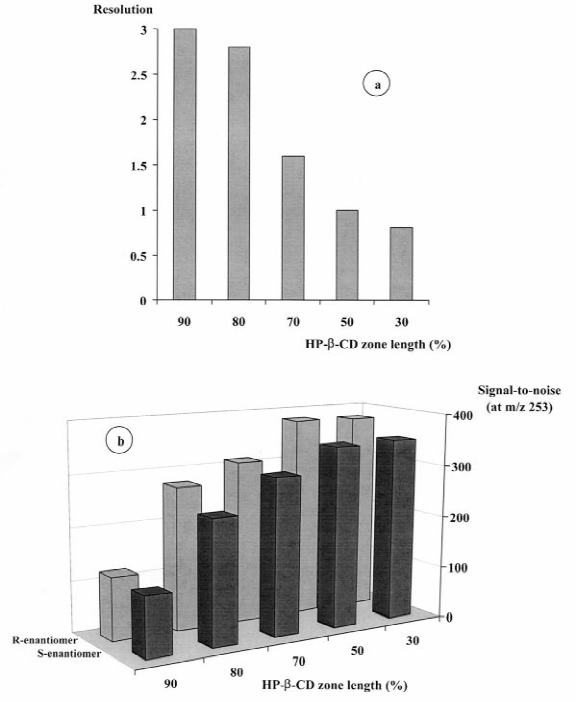

Fig. 3. Effect of HP-β-CD zone length on the enantioresolution (a) and signal-to-noise ratio of m/z 253 ion (b). Experimental conditions as in Fig. 2 except buffer composition: formic acid–ammonia (pH 4, ionic strength 50 m*M*)+10 m*M* HP-β-CD.

filled with the chiral selector, different injection times of the cyclodextrin-based buffer were investigated. Drug enantiomers were resolved using 10 m*M* HP-β-CD as a chiral selector at different selector zone lengths (30–90%). The effects of CD zone length upon several parameters, such as resolution between enantiomers, peak efficiency and signal-to-noise ratio of MH^+ ion, were investigated (Table 1). The enantioresolution decreased when the CD zone was narrower as shown in Fig. 3a (resolutions of 3.0 and 1.0 for 90 and 50%, v/v, partial filling, respectively). However, the 70–30% (v/v) range led to unsatisfactory resolution values (1.5 to 0.8, respectively). Indeed, resolution decreased when a lower amount of chiral agent was present in the capillary

Table 1
Influence of HP-β-CD zone length (from 30 to 90%) on the resolution, signal-to-noise ratio and peak efficiency

HP-β-CD zone length (%, v/v)	Resolution	Signal-to-noise ratio of MH^+ ion		Peak efficiency (theoretical plates)	
		Enantiomer *R*	Enantiomer *S*	Enantiomer *R*	Enantiomer *S*
30	0.8	380	350	150 000	140 000
50	1.0	383	347	145 000	130 000
70	1.3	311	300	135 000	120 000
80	2.8	275	238	130 000	110 000
90	3.0	120	115	90 000	75 000

Experimental conditions as in Fig. 2 except for buffer composition: formic acid–ammonia (pH 4, ionic strength 50 m*M*)+10 m*M* HP-β-CD.

due to a smaller time period for the enantiomers to complex. Otherwise, decreasing CD zone length induced shorter migration times of the enantiomers but higher peak efficiencies and signal-to-noise ratio values (Fig. 3b). Thus, peak efficiency of the *S*-enantiomer increased from 90 000 to 150 000 theo-

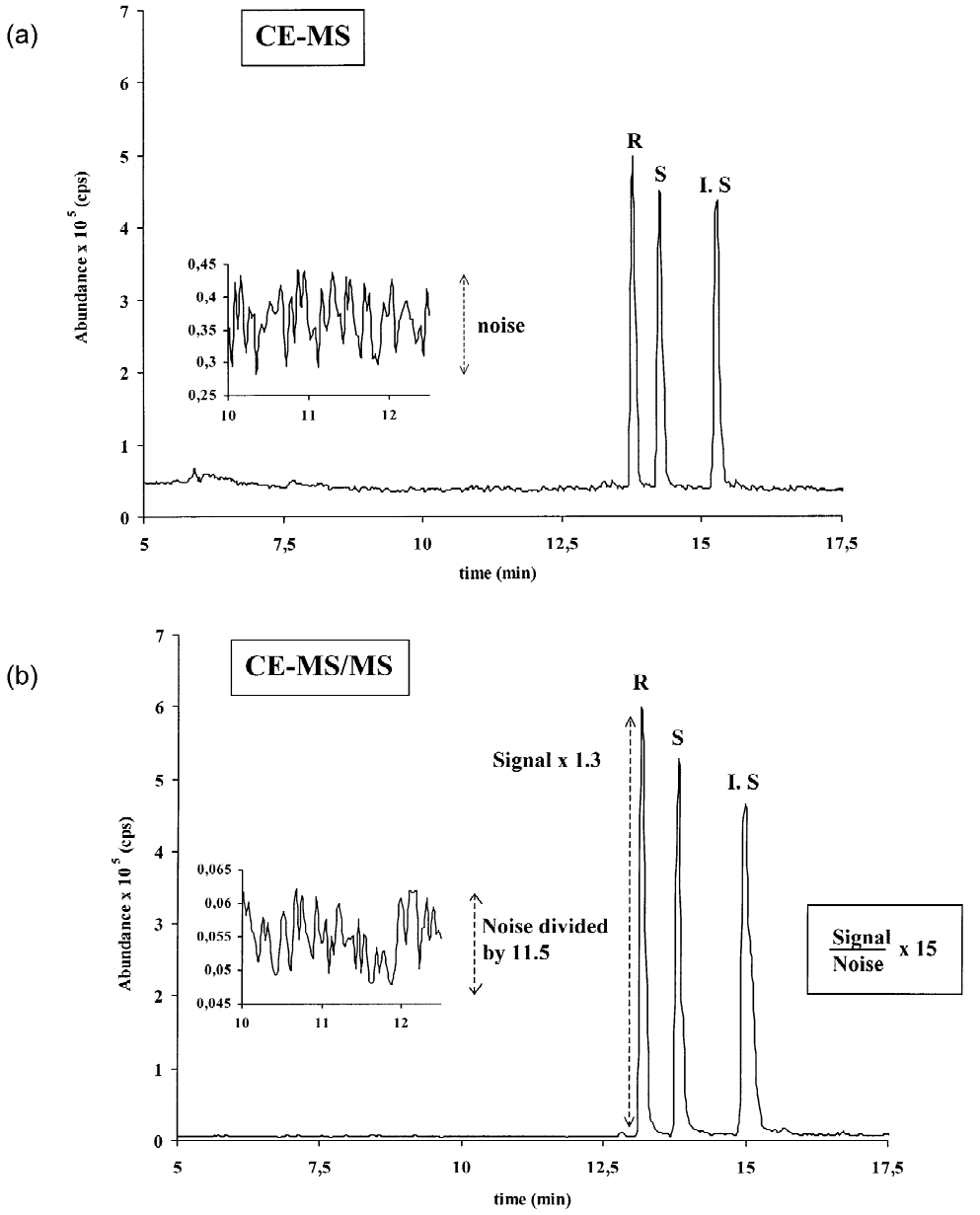

Fig. 4. Improvement of sensitivity betweeen MS (a) and MS–MS (b) mode detection during the CE separation of enantiomers employing a partial filling technique. Capillary electrophoresis: capillary: 70 cm×50 μm I.D.; voltage: +20 kV; temperature: 25°C; running buffer: formic acid–ammonia (pH 4, ionic strength 50 m*M*)+10 m*M* HP-β-CD; hydrodynamic injection: 10 s, 0.5 p.s.i.; analyte: 5 μg/ml, internal standard: 2.5 μg/ml. Mass spectrometry: (a) and (b): ionspray voltage: +5.6 kV, sheath liquid: water–methanol–acetic acid (5:95:0.25, v/v/v), flow-rate: 5 μl/min. Percentage of filled capillary: 80% (v/v); (b) collision energy: −30 V.

retical plates and the corresponding signal-to-noise ratio of m/z 253 ion was three times higher when the filling percentage decreased from 90 to 30%. Nevertheless, lower peak efficiencies were always observed by using the partial filling technique than when the capillary was completely filled with the chiral selector. Such trends were attributed to the zone broadening occurring at the interface between the CD and non-CD zones [28].

In a final set, a partial capillary filling of 80% (v/v) was selected as a compromise between resolution and sensitivity. Enantiomers of the adrenoreceptor anatagonist were successfully resolved with formic acid–ammonia (pH 4, 50 m*M* ionic strength) buffer containing 10 m*M* HP-β-CD (resolution 2.8; run time: 16 min; peak efficiency of *R*-enantiomer: 130 000 theoretical plates).

3.4. Quantification of the enantiomers by CE-MS

The quantification of enantiomers was performed by following the MH^+ ions (m/z 253) of the enantiomers and of the internal standard (m/z 260). Calibration curves of enantiomers were determined by CE-MS in the 0.25–10 μg/ml (1–39.7 μ*M*) concentration range. The concentration of the internal standard was 2.5 μg/ml. Regression analysis yielded straight lines with $r^2=0.9988$ for the *R*-form and 0.9980 for the *S*-form. From these results, the detection limits (S/N 3) were found to be 75 ng/ml (0.3 μ*M*) for each enantiomer.

In order to improve the sensitivity of the method, MS-MS detection was performed. After collision with nitrogen gas, intense fragmentation ions, which occur at m/z 220 for the enantiomers and at m/z 155 for propranolol, are related to $(M-CH_3OH)^+$ and $(M-C_{10}H_7OH)^+$ ions, respectively. Analysis of the racemic drug was carried out by CE–MS (Fig. 4a) and then by CE–MS–MS (Fig. 4b). The comparison of MS and MS–MS modes indicated an increase of the response factors by a factor fifteen. Limits of detection were equal to 5 ng/ml (0.02 μ*M*) in CE–MS–MS compared to 150 ng/ml (0.6 μ*M*) in CE–UV for each enantiomer of the adrenoreceptor antagonist. Finally, CE–MS–MS with a partial filling method was a convenient and sensitive analytical technique to detect ng/ml levels of drug enantiomers in biological fluids for pharmacokinetic studies.

4. Conclusion

The use of CE–MS–MS combined with a partial filling technique has been successfully applied to the separation and quantification of adrenoreceptor antagonist enantiomers using neutral HP-β-CD as a chiral selector. No decrease in sensitivity and increase in noise level were observed in the present study. Furthermore, no traces of CD were seen when scanning occurred in the high mass region. The chiral separation can be easily regulated by changing the length of the selector zone and the selector concentration. An effective CD zone length of 80% and 10 m*M* HP-β-CD concentration seems an acceptable compromise between resolution and sensitivity. Tandem MS mode provides a sensitivity enhancement of a factor of 15 compared to MS mode and thirty compared to UV detection. Limits of detection were equal to 150 ng/ml (0.60 μ*M*) in CE–UV, 75 ng/ml (0.30 μ*M*) in CE–MS and only 5 ng/ml (0.02 μ*M*) in CE–MS–MS for each enantiomer of the adrenoreceptor antagonist.

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