Chimia 53 (1999) 501–505

ISSN 0009–4293

On-Line Capillary Electrophoresis-Electrospray Mass Spectrometry for the Analysis of Pharmaceuticals

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Abstract. The on-line coupling of capillary electrophoresis (CE) with mass spectrometry using the electrospray-ionization mode (ESI-MS) is a promising combination of two analytical techniques. While CE provides high separation efficiency per unit of time, mass spectrometry affords high sensitivity and selectivity as well as molecular structural information. Three different projects are under way in our laboratory and are presented to illustrate the potential of CE-ESI-MS in pharmaceutical analysis. First, the determination of Ecstasy and other amphetamine derivatives in urine samples is reported. Second, the analysis of secondary metabolites, present in plant extracts such as tropane alkaloids, is depicted. Furthermore, the enantioselective analysis of pharmaceutical drugs and metabolites in human plasma is also described.

1. Introduction

Since its introduction in the 80's [1][2], capillary electrophoresis (CE) has rapidly become a powerful separation technique and has found applications in a number of different fields, such as environmental analysis, clinical chemistry, and biochemistry [3]. In particular, CE has revealed great potential for the analysis of pharmaceutical compounds. The high efficiency, short analysis time, rapid method development, simple instrumentation, and low sample requirement of CE are the main reasons for this success. In addition, exotic and expensive background-electrolyte solutions (BGE) can be used, causing only small economical and disposal problems, because flow rates in CE are low compared to those in liquid chromatography.

UV-VIS spectrophotometry is often chosen for the on-line detection of compounds separated by CE. However, the CE-UV bottleneck is its relatively low sensitivity, due to the short optical path-length afforded by the small internal diameters of the capillaries. Additionally, many interesting pharmaceutical compounds do not possess a chromophore and, therefore, their UV detection requires a derivatization procedure. In order to enhance sensitivity, laser-induced fluorescence detection has been developed, but it is limited to compounds carrying strongly fluorescent functions [4][5]. Although non-fluorescent compounds can be tagged with fluorophores, such procedures are tedious and generally compromise the time gain and the small volume capabilities of CE. Moreover, with spectroscopic detectors, peak identity is generally confirmed by using migration times only. However, this information is often insufficient to identify compounds of interest unequivocally because, for example, the electroosmotic flow in CE can vary.

The on-line coupling of capillary electrophoresis with mass spectrometry (MS) is a promising combination of two analytical techniques. Among the available ionization techniques, electrospray ionization (ESI) is most widely used for on-line coupling of CE with MS [6][7]. The electrospray-ionization process produces singly- and multiply-charged molecules and, thus, is well suited for the analysis of moderately polar compounds possessing a mass range from 10^2 to 10^5 Da. Excellent reviews on CE and CE-MS have been published and can be consulted for a more systematic coverage of the field [8–13].

In this paper, the potential of CE-ESI-MS in pharmaceutical analysis will be illustrated by selecting some applications performed recently in our laboratory. The applicability of this technique will be demonstrated for the analysis of Ecstasy and its derivatives in urine samples. The usefulness of the on-line information obtained both by CE-MS and CE-UV will be emphasized for the analysis of natural compounds with pharmaceutical interest, such as tropane alkaloids in plant extracts. In-source collision-induced dissociation (CID) will be highlighted concerning the differentiation of pharmaceutical compounds which possess the same molecular mass, such as positional isomers. Finally, the potential of CE-ESI-MS, combined with the partial filling technique, will be discussed for the stereoselective analysis of chiral drugs in biological matrices.

2. Practical Considerations

For successful coupling of CE with MS, mainly three different interfaces ensuring electrical continuity have been developed and described in the literature: the liquid-junction [14], sheathless [15], and sheath-flow [16] interface. However, because of its instrumental simplicity and also because of its possibility to enhance the ionization process by chemical reaction, the coaxial sheath-flow interface is by far the most popular and suitable configuration for CE-ESI-MS [17]. For the latter, it is necessary to add a make-up

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flow to the electroosmotic flow (*ca.* 100 nl/min) in order to attain the optimal conditions of electrospray ionization (1–10 µl/min).

However, this CE-ESI-MS coupling induces some limitations concerning the choice of the background-electrolyte solution. Indeed, the non-volatile buffers commonly used in capillary zone electrophoresis (CZE), such as phosphate, borate, and citrate are not compatible with CE-MS, although the use of a make-up flow containing 50–80% organic solvent may encompass this incompatibility. These non-volatile buffers have detrimental effects on the performance of the mass spectrometer, since they can enhance the contamination risk of the ionization chamber and suppress the analyte signal. In addition, additives such as surfactants, cyclodextrins as well as ion-pairing agents, commonly used to improve selectivity in CZE, are not suitable for ESI-MS. Therefore, volatile buffers such as formic acid, acetic acid, ammonium acetate, ammonium formate, and ammonium carbonate are often recommended for CE-ESI-MS [18–20].

Finally, it can be noted that CZE offers better efficiencies in the presence of high-ionic-strength buffers, whereas the ionization process in electrospray is impaired by a substantial ion strength. Such opposite requirements have to be considered in order to achieve a good CE separation and to obtain high sensitivity with ESI-MS. All mass-spectrometric measurements presented here were carried out in the positive-ion mode and were performed on a single-quadrupole *HP Series 1100 MSD* (*Hewlett Packard*, Palo Alto, CA, USA) (*Fig. 1*).

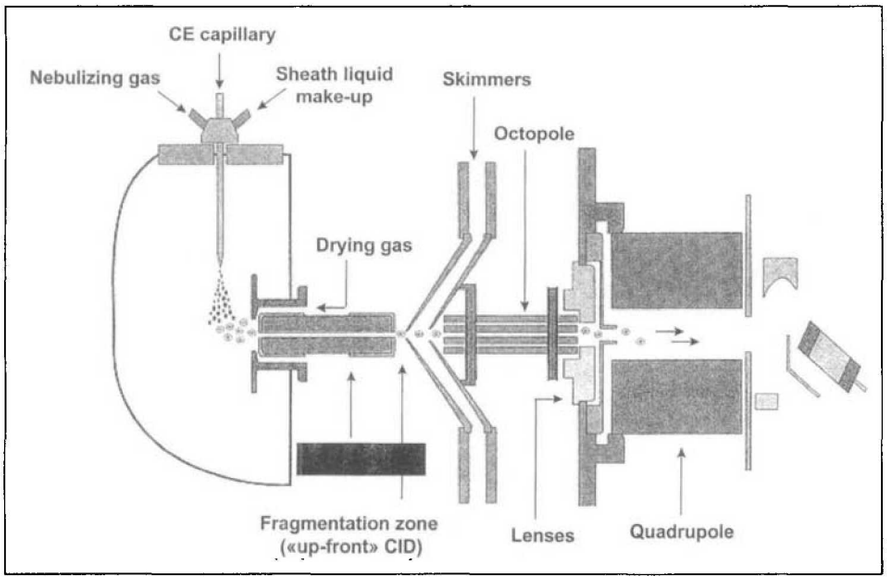

Fig. 1. *Schematic drawing of the orthogonal flow sprayer for CE-MS used for pharmaceutical analysis* [21]

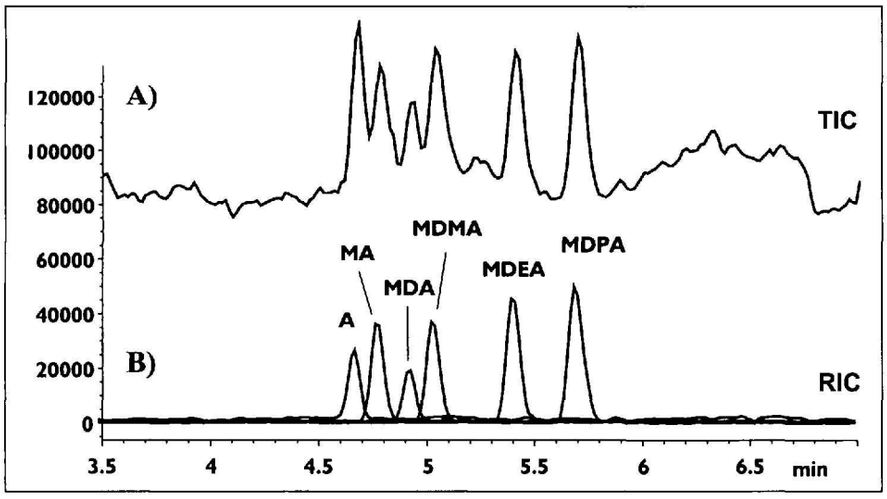

Fig. 2. *A) Total ion current of a spiked urine sample after a liquid/liquid extraction procedure. B)* Overlaid reconstructed ion current (RIC) of each protonated molecular ion (MH^+). Experimental conditions, see [19]. Peak identification: A, amphetamine; MA, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDEA, 3,4-methylenedioxyethamphetamine; MDPA, 3,4-methylenedioxypropamphetamine.

3. Applications

3.1. *Ecstasy and Its Derivatives in Urine*

Amphetamines and other related derivatives are powerful stimulants of the central nervous system and are often misused by recreational users. A chronic abuse of amphetamines often leads to hallucinations and psychosis, as well as dysphoria and depression upon withdrawal [22]. In our laboratory, several chromatographic [23][24] and electrophoretic [25] methods, relying on spectrophotometric detectors, were developed and validated for the separation of amphetamine and related compounds. In particular, capillary zone electrophoresis [26] coupled with a diode-array detector was successfully applied to the analysis of amphetamines sold on the black market as tablets of various composition. However, for the analysis of Ecstasy and derivatives in urine, it was necessary to develop a CE-ESI-MS method in order to enhance sensitivity [19]. After having optimized both CE and ESI-MS experimental parameters, the selected conditions were applied to the analysis of urine samples. *Fig. 2* shows a CE-ESI mass spectrum using a poly(vinyl alcohol) (PVA)-coated capillary and 100 mM formic acid as buffer electrolyte solution. Before injection, the spiked urine sample was pretreated by liquid-liquid extraction. MS-Data acquisition was performed in the selected-ion-monitoring mode (SIM) by choosing each protonated molecular ion. Under these conditions, sensitivity was sufficient to determine Ecstasy and related amphetamines in urine, and no interference occurred from endogenous compounds.

3.2. *Tropane Alkaloids in Plant Extract*

Tropane alkaloids are commonly found in *Solanaceae* and related families [27]. The principal alkaloids of medicinal interest in this group are (–)-hyoscyamine, atropine, and scopolamine which have widespread activities, such as spasmolytic and ophthalmic effects. Therefore, there is a need to develop rapid, sensitive, and accurate analytical methods for an unequivocal identification and quantification

of these alkaloids, both in pharmaceutical preparations and in plant extracts. For such purposes, several methods, such as capillary zone electrophoresis (CZE) [28][29], micellar electrokinetic capillary chromatography (MEKC) [30][31], as well as nonaqueous CE [32], were developed in our research group. However, as tropane alkaloids only possess a weak chromophore, CE-UV analysis is severely hampered when these compounds are present at low concentrations in complex matrices. Thus, a sensitive and selective detection technique, such as MS, is required.

A CZE method, coupled to a diode-array detector and interfaced with electrospray mass spectrometry was developed for the simultaneous analysis of hyoscyamine and scopolamine in plant extracts [33]. *Fig. 3A* depicts the electropherogram of hairy-root extract under optimized CE-ESI-MS conditions. It is noteworthy that tropine, which does not possess a chromophore, was not detected by CE-UV, while the MS trace reveals the presence of this compound. The display of ion traces for masses corresponding to their respective protonated molecular ions allowed efficient identification and quantification of all tropane alkaloids. Thus, beside tropine, other alkaloids corresponding to hyoscyamine, scopolamine, and 6β-hydroxyhyoscyamine were identified (*Fig. 3B*). Furthermore, compared to CE-UV, the selected-ion-monitoring mode significantly improved the method's sensitivity by a factor of 10^3–10^4. The good sensitivity obtained by CE-ESI-MS for these alkaloids can be explained by their high proton affinity.

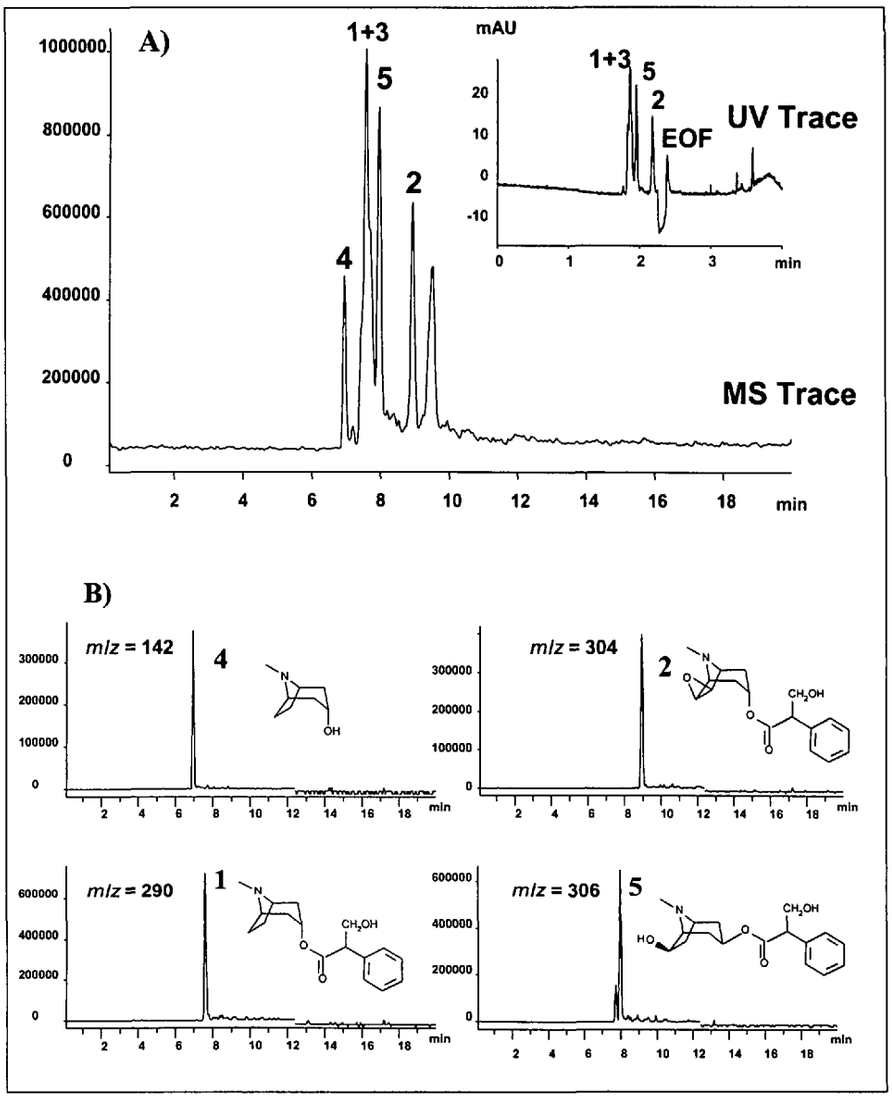

Fig. 3. *CE-MS Analysis of a* Datura candida x D. *aurea hairy-root extract. A*) Total ion current (TIC) and UV signals. *B*) The individual mass traces of the protonated alkaloids. For CE-MS conditions, see [33]. Peak identification: 1) hyoscyamine, 2) scopolamine, 3) littorine, 4) tropanol, 5) 6β-hydroxyhyoscyamine.

3.3. *Isomer Differentiation*

The use of a soft ionization technique, such as electrospray, gives rise to protonated molecules and only little fragmentation. However, fragmentation can be desirable to differentiate compounds possessing similar molecular weight, such as positional isomers which cannot be separated by CZE. Indeed, some isomers exhibit a different fragmentation pathway.

In the case of amphetamine analysis, MDEA and MBDB which are structural isomers, are not baseline-resolved under optimized CE conditions. Thus, a selective fragmentation is necessary to identify these compounds by ESI-MS. It is noteworthy that fragmentation is possible with a single-quadrupole instrument by increasing the fragmentor voltage within the MS ion-focusing region. As shown in *Fig. 4*, at a high fragmentor voltage (90 V), MBDB and MDEA could be differentiated by their fragment ions, *m/z* 177 and 163 for MBDB and MDEA, respectively. They correspond to the charge-neutral loss of the amine group. Furthermore, the ion *m/z* 147 found for MBDB corresponds to the loss of CH_2O.

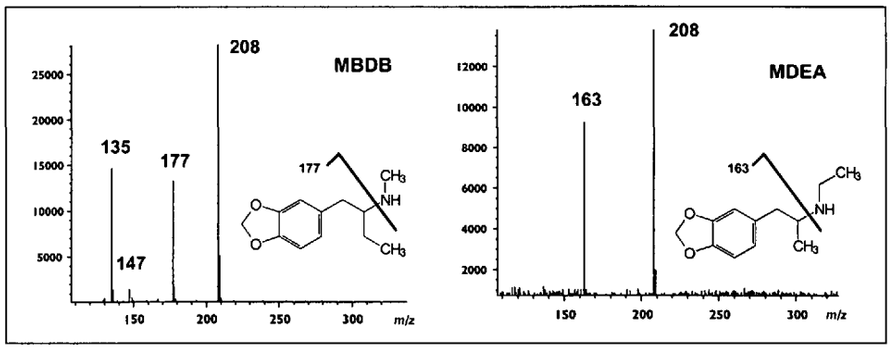

Fig. 4. *MS Spectra of MBDB (N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine*) and MDEA after collision-induced dissociation* (skimmer voltage at 90 V). The fragment ions, *m/z* 177 (MBDB) and 163 (MDEA), correspond to the charge-neutral loss of the amine group [19].

Littorine, which is a positional isomer of hyoscyamine, has been reported to be a

constituent of some *Solanaceae*, and the determination of this compound is particularly important in hairy-root cultures. Indeed, several published methods do not separate littorine from hyoscyamine, and thus overestimate the content of the latter. Since the two positional isomers were not separated by CE, fragmentation was induced by setting the fragmentor voltage to 200 V. As shown in *Fig. 5*, beside the protonated molecular ion *m/z* 290, both mass spectra showed a peak at *m/z* 124, which corresponds to the loss of tropic acid and phenyllactic acid for hyoscyamine and littorine, respectively. However, littorine showed an additional peak at *m/z* 142. After protonation, the intermediate complex of littorine is less stable at a high voltage than hyoscyamine, and a different fragmentation pathway is induced [33]. These results were confirmed by tandem mass spectrometry (data not shown).

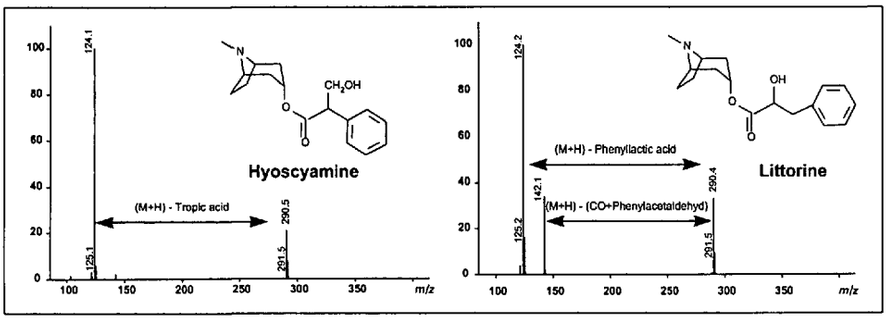

Fig. 5. *MS Spectra of hyoscyamine and littorine after collision-induced dissociation* (skimmer voltage at 200 V). The daughter ion at *m/z* 124 corresponds to the loss of tropic acid and phenyllactic acid for hyoscyamine and littorine, respectively. The *m/z* 142 signal is caused by the loss of phenylacetaldehydey and carbon monoxide from littorine [33].

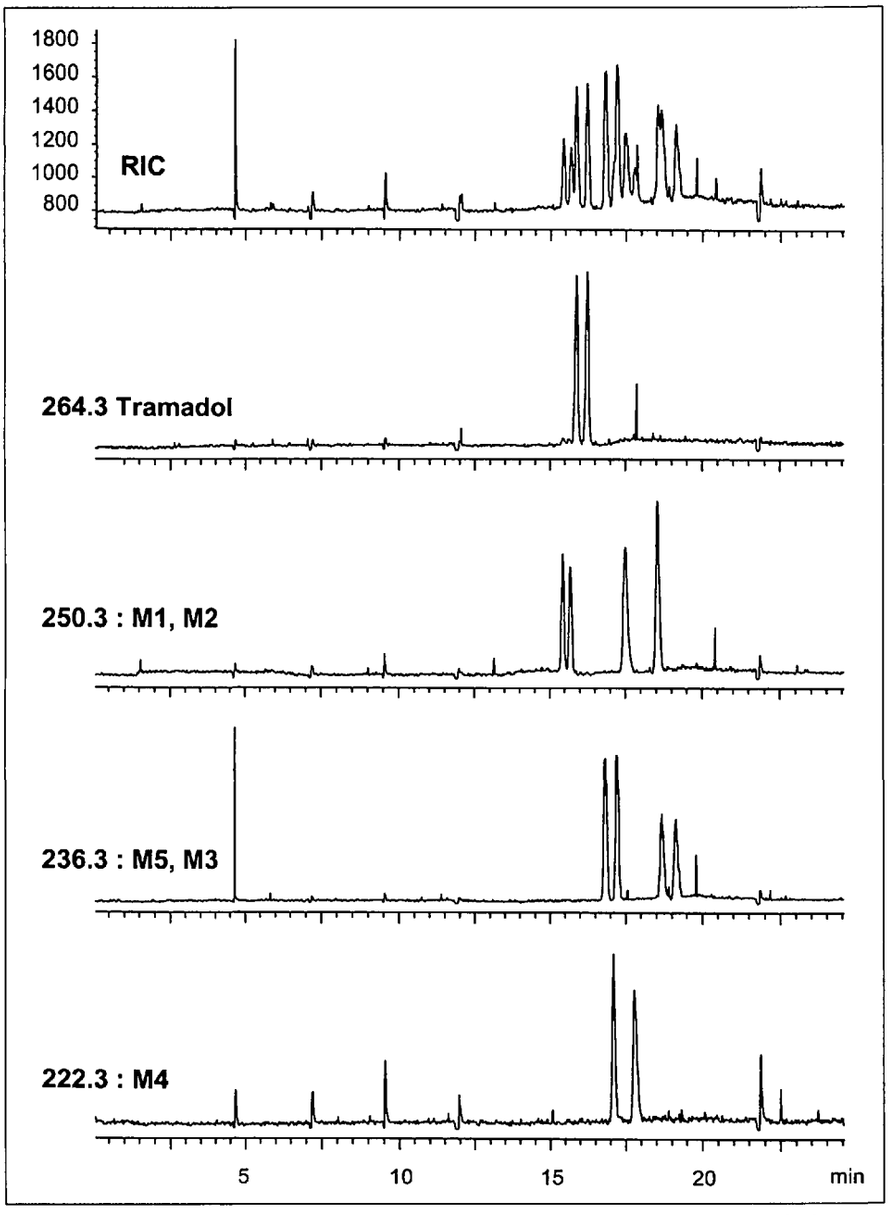

Fig. 6. *CE-ESI-MS enantiomeric separation of tramadol and its metabolites M1–M5* [42]

3.4. *Stereoselective Analysis of Tramadol and Its Metabolites*

It is well recognized that a large number of pharmaceutical compounds possess an asymmetric carbon atom and can form two enantiomers. They may have different activities and must be considered as two distinct species. Recently, a regulatory policy [34] has been edited to address the development of new chiral compounds. All tests have to be done on both forms and even on the racemic mixture. Therefore, the growing awareness of drug stereochemistry has initiated a tremendous development of enantioselective analytical methods. In this context, CE has gained wide acceptance, and its usefulness in resolving chiral compounds is abundantly documented [35][36].

The enantiomeric separation by CE requires the addition of a chiral selector to the BGE. Among the available selectors reported in the literature, cyclodextrins (CDs) are most widely used in CE. Neutral and charged CD derivatives with various functional groups have been recently developed to induce different interactions and enhance enantioselectivity. However, as already mentioned, the main problem encountered for the coupling of CE with MS is the contamination risk of the MS source by non-volatile additives such as CDs.

To avoid any presence of the chiral selector in the ion source of the MS detector, the partial filling technique is generally used [37–41]. This involves filling a discrete portion of the capillary with a background electrolyte containing a suitable amount of chiral selector to achieve enantiomeric separation. Generally, a coated capillary is employed to avoid any electroosmotic flow. In the case of basic compounds, negatively charged CDs are used; the application of the electric field results in a counter-current process in which the chiral selector and the enantiomers migrate in opposite directions.

Tramadol hydrochloride is a centrally acting analgesic agent used in the treat-

ment of chronic pain. This drug is marketed as a racemic mixture, and each enantiomer displays different opioid and monoaminergic properties, as well as differences in the metabolic pathway. The (+)-enantiomer was reported to exhibit a ten-fold higher analgesic potency than the (–)-enantiomer. Tramadol is metabolized by two main pathways to form five *N*- and *O*-demethylated compounds (M1–M5). *O*- and *N*-demethylation of tramadol was found to be highly stereoselective, and all metabolites possessed an asymmetric carbon. Therefore, sensitive and stereoselective analytical methods are necessary for an accurate determination of tramadol and its metabolites in clinical studies.

Different negatively charged cyclodextrins, including sulfated-, sulfobutylether- (SBE), and carboxymethylated-β-CD as well as phosphated-γ-CD were investigated for the purpose of enantiomer separation of tramadol and its metabolites. The best separation was achieved in the presence of SBE-β-CD as chiral selector. Its concentration, as well as the plug length (part of the capillary effectively filled with the chiral selector), were systematically studied. As illustrated in *Fig. 6*, the best enantiomeric separation of this complex mixture was achieved by filling 90% of the capillary with a 50 mM ammonium acetate buffer solution set at pH 4.0 containing 2.5 mg/ml of SBE-β-CD [42]. The use of a PVA-coated capillary was necessary to suppress the electroosmotic flow. Under optimized conditions, (+)-tramadol migrated first.

In spite of peak overlapping observed in the reconstructed ion electropherogram, the recording of selected masses allowed an unambiguous determination of each analyte, which demonstrates the high selectivity of MS in comparison to conventional detectors. Acquisition in the SIM mode allowed to enhance both sensitivity and selectivity. As shown in *Fig. 6*, all compounds were baseline-resolved with a reasonable analysis time. It is noteworthy that the use of charged CDs not only allowed enantiomeric separation of the investigated drugs, but also improved selectivity between metabolites with the same molecular mass, such as M1 and M2, as well as M3 and M5.

4. Conclusions and Outlook

CE is recognized as a very attractive separation method, but it suffers from the lack of sensitivity when UV detection is applied. The coupling of capillary electrophoresis with mass spectrometry is a powerful technique which opens new development perspectives in pharmaceutical analysis.

The commercialization of CE-ESI-MS instrumentation at a reasonable price will certainly increase the number of analyses of pharmaceutical compounds performed by CE. This technique allows to attain the low detection limits required for the analysis of drugs and metabolites in biological matrices. Furthermore, data acquisition in the selected-ion-monitoring mode gives high selectivity and sensitivity which reduce the complexity of the sample-preparation procedure. Finally, beside the molecular-mass determination of migrating peaks, ESI-MS allows to confirm a compound's identity by using collision-induced dissociation (CID). However, a complete identification of unknown compounds necessitates the use of tandem mass spectrometry.

It can be noted that CE-ESI-MS is still in its infancy and presents some limitations, especially for the determination of compounds at trace levels (concentration inferior to ppb) or for compounds which cannot be easily ionized. Moreover, work is in progress in our laboratory to evaluate whether this technique fulfills the validation criteria recommended by official guidelines and required for the quantification of drugs and metabolites.

Received: June 29, 1999

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