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Capillary electrophoresis–mass spectrometry coupling versus micro-high-performance liquid chromatography–mass spectrometry coupling: a case study

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Abstract

Capillary zone electrophoresis (CZE) and micro-high-performance liquid chromatography (μ-HPLC) coupled to electrospray ionisation (ESI) mass spectrometry were compared with respect to their applicability to problems arising in pharmaceutical drug research and development. Both techniques, which are similar with regard to their operational parameters, were coupled to an API III plus triple quadrupole mass spectrometer using laboratory-built interfaces. The results achieved with the two combinations were compared for sensitivity and general applicability to the quantitative analysis of pharmaceuticals in biological fluids. Midazolam, the 8-chloro-6-(2-fluoro-phenyl)-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine, and three of its metabolites were used as test compounds, either as standard solution or after sample clean-up from human plasma. Following different sample preparation routes, liquid–liquid extraction or solid-phase extraction, differences in detection limits as well as robustness in CZE or μ-HPLC coupled with ion spray mass spectrometry (IS-MS) were investigated. Detection limits of about 500 pg/ml for the drug and 2 ng/ml for the metabolites were achieved, using 1 ml of human plasma, only when liquid–liquid extraction was used for sample preparation. Sample preparation using the simpler and faster solid-phase extraction route resulted in deterioration of the separation or clogging of the columns. In all cases, when standard solutions or sample extracts were used, CZE–ESI-MS provided both different selectivity and greater sensitivity.

Keywords: Midazolam

1. Introduction

The complexity of the problems encountered in the life sciences demands powerful analytical techniques. Often, one technique is unable to provide the right answer or describe a particular situation completely. When a combination of a separation technique with a specific spectrometric detection technique is used, the degree of complexity is reduced by the introduction of a time dimension. The detection limits that have to be achieved in the analysis of drugs and their metabolites in current drug research and development are falling as more potent drugs are developed and, consequently, lower doses are administered to patients or animals. A powerful approach to achieving the highest possible sensitivity

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lies in a combination of a separation technique like liquid chromatography with a sensitive and universal detector such as a mass spectrometer (LC–MS). Atmospheric pressure ionization (API) mass spectrometry, which has become established over the last decade as a new mass spectrometric tool, used in combination with standard or narrow bore HPLC-columns, has brought LC–MS to the forefront of analytical techniques.

Since it is known [1] that the pneumatically-assisted electrospray (ion spray, IS) ion source acts as a concentration-sensitive detector, as is the case for the ultraviolet (UV) detector, it is apparent that the use of micro separation techniques such as capillary zone electrophoresis (CZE) or micro-high-performance liquid chromatography (μ-HPLC) will increase sensitivity in comparison with standard-bore HPLC. During the last decade, electrospray ionisation mass spectrometry (ESI) in combination with HPLC or CZE has become, by virtue of its superior sensitivity and selectivity in comparison to e.g. HPLC–UV detection, a standard tool for the quantification or structural elucidation of analytes from complex mixtures [2,3]. Another important point for the coupling of CZE to a mass spectrometer is the path length of only a few micrometers, which is insufficient for sensitive on-column UV detection.

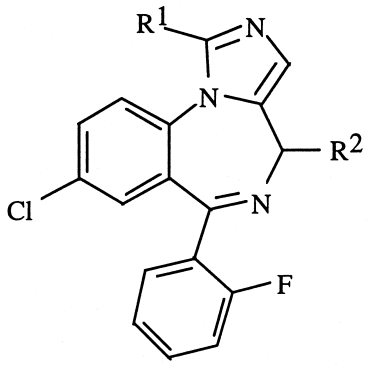
The combination of HPLC either with electrospray ionisation or, for its greater flow compatibility, with ion spray is well established in many laboratories, but the coupling of CZE to ESI or IS is still a challenging task. During the development of the CZE–ESI coupling, different interfaces have been designed and have recently been reviewed [4–9]. However, a universal and robust working interface, like the liquid chromatography interfaces, is still not available.

In addition to the concentration-sensitive behaviour of the ESI or ion spray ion source, the flow-rate generated by the sheath flow interface for CZE–MS and the flow-rate from a 300 μm I.D. μ-HPLC column are also similar. This reduces the number of variables to be considered in the comparison of the two combinations.

A literature search revealed only few studies that compare the two separation techniques with ESI or IS. Mück and Henion reported in 1989 [10] on the separation of leucine enkephalin and methionine enkephalin in equine cerebrospinal fluid. Using the same sample clean-up and enrichment procedure, and application of the samples to either μ-HPLC or CE coupled to tandem MS, they found that the required detection limit of 1–5 ng/ml could not be achieved with CZE–MS–MS. A general approach to overcoming the limited sample loadability of CZE was proposed by Tinke et al. [11]. They used isotachophoresis (ITP) prior to CZE to increase the loadability in CZE–MS. In 1992, Pleasance et al. [12] compared the performance of both separation techniques, as well as flow injection analysis (FIA), for monitoring paralytic shellfish toxins. CZE–MS and CZE–MS–MS appeared to be interesting but challenging alternatives to HPLC for the separation of the toxins. The comparison of nanoscale packed capillary (75 μm I.D.) HPLC and CZE coupled to MS and in-source MS–MS for the monitoring of macrolide antibiotics was reported by Parker et al. [13] and Deterding et al. [14]. Nanoscale capillary liquid chromatography (nCLC) was found to be superior to CZE coupled to ESI-MS regarding loadability, when mass spectrometric sensitivity was limited. On the other hand, CZE was found to be more sensitive due to narrower peak width. For the coupling of both nCLC and CZE to MS the sheath-flow interface was used to increase the overall flow for stable ionization conditions. A fully validated quantitative assay for the determination of ethylene-diaminetetraacetic acid (EDTA) from human plasma was recently reported by Sheppard and Henion [15]. A detection limit of 15 μg/l was achieved using field amplification injection to increase the sample loading.

This paper evaluates the merits and drawbacks of CZE or μ-HPLC coupled to ion spray MS, with the focus on applicability in drug metabolism and pharmacokinetics, using a test mixture of a benzodiazepine (midazolam, 8-chloro-6-(2-fluoro-phenyl)-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine and three of its metabolites (for structures see Fig. 1). This mixture was selected for its capacity for separation by both CZE and μ-HPLC. Biological samples were prepared from spiked human plasma using either liquid–liquid extraction or solid-phase extraction. Detection limits, selectivity and robustness achieved with both sample preparation routes and applied to both combinations, CZE–IS-MS and μ-HPLC–IS-MS, are compared.

The test drug, midazolam (I), is a pharmacologi-



Compound	R^1	R^2	Formula	MW
I	$-CH_3$	-H	$C_{18}H_{13}ClFN_3$	325.77
II	$-CH_2OH$	-H	$C_{18}H_{13}ClFN_3O$	341.77
III	$-CH_3$	-OH	$C_{18}H_{13}ClFN_3O$	341.77
IV	-Gluc	-H	$C_{24}H_{21}CLFN_3O_7$	517.90

Fig. 1. Structure and molecular mass (M_r) of the components of the test mixture of midazolam (I) and its metabolites (II–IV).

cally active compound which is well established in clinical practice for induction of anesthesia and for sedation of artificially ventilated patients in intensive care units. The drug is extensively metabolized in the human liver by cytochrome P450 IIIA4 to the 1-hydroxy derivative (II), (8-chloro-6-(2-fluoro-phenyl)-4H-imidazo-[1,5-a][1,4]benzodiazepin-1-yl)-methanol, and to the 4-hydroxy derivative (III), 8-chloro-6-(2-fluoro-phenyl)-4-hydroxy-1-methyl-4H-imidazo-[1,5-a][1,4]benzodiazepine) [16]. The main excretion pathway of the drug is via the glucuronide conjugate (IV), [8-chloro-6-(2-fluoro-phenyl)-4H-imidazo-[1,5-a][1,4]benzodiazepin-1-ylmethyl] β-D-glucopyranosiduronic acid. Quantification of the parent drug, I, and the main metabolite, II, was done by gas chromatography (GC) [17,18] with detection limits between 2 and 4 ng/ml, and HPLC [19,20] with detection limits between 2 and 5 ng/ml. Using GC, the 1-hydroxy metabolite II has to be converted to the trimethylsilyl derivative, because it is insufficiently volatile, whereas the drug and its metabolites can be analyzed in the unchanged form by the HPLC assay. Quantification of benzodiazepines is of general interest, and recently an LC–MS assay for the quantification of different 1,4-diazepines such as diazepam (7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-[1,4]benzodiazpin-2-one), nitrazepam (7-nitro-5-phenyl-1,3-dihydro-2H-[1,4]benzodiazpin-2-one, flunitrazepam (5-(2-fluoro-phenyl)-1-methyl-7-nitro-1,3-dihydro-2H-[1,4]benzodiazpine-2-one) and medazepam (7-chloro-1-methyl-5-phenyl-2,3-dihydro-2H-[1,4]benzodiazepin-2-one) using solid-phase extraction (SPE), was reported [21]. By using 1 ml of serum or urine, a quantification limit of 1 ng/ml was reached.

2. Experimental

2.1. CZE device

For CZE–MS experiments, an HPE™ 100 high-performance electrophoresis device from Bio-Rad was used, modified as follows. The original device was limited to a maximum delivered voltage of 12 kV for separation. For ion spray or electrospray ion sources, where the spray capillaries are on high voltage, 3 to 6 kV are necessary for charging and atomization of the liquid and are applied to the ground or detection side of the CZE circuit. Therefore, a net voltage drop of only 9 to 6 kV remains as the driving force for electromigration, when 12 kV are applied on the injection side by the CE high voltage power supply. Because of the physical size

of the CZE device and of the ion source, the length of the CZE capillary cannot be shorter than 50 cm. This results in a voltage drop of at most 180 V/cm, assuming that a net voltage of 9 kV can be applied for a 50-cm capillary. In most cases, a 1-m capillary and a voltage of 6 kV were used for electrospray. Then, the voltage drop per centimetre employed for separation would be about of 60 V/cm. This situation prolongs the separation time and decreases separation power. To improve the separation speed, the control unit of the CZE device was modified to deliver a maximum voltage of 18 kV, resulting in a voltage drop of 120 V/cm for a 1-m capillary. The original separation assembly was removed and replaced by a sampling device made from a cube of plastic, which allows positive pressure injection. The laboratory-built assembly was constructed as follows. Two different holes, separated by only a few millimeters, were drilled on the top of the plastic cube for inserting the electrode and the CZE capillary. The hole for the CZE capillary has a diameter of 0.3 mm and the hole for the electrode has a diameter of 1.1 mm. A thread was inserted through the bottom of the cube to fit the sample vial or buffer vial. Sample vials, 6×32 mm with 300 μl volume, and buffer vials, 15×45 mm with 4 ml volume, were used. Using an adapter made from polytetrafluoroethylene (PTFE), the sample vials were inserted into the buffer vials. A seal made from PTFE and silicone gum was inserted on top of the thread to achieve a tight fit between cube and vial. A third hole was drilled on one side of the cube and joined to the hole for the electrode. A PTFE coil of 1.6 mm O.D. and 0.5 mm I.D. was threaded through this hole, through which pressure was applied for injection or for rinsing the CZE capillary with buffer, sodium hydroxide or water. The PTFE coil was connected at the other end to an electrical motor-driven, time-controlled three-way vent to select the pressure and time for rinsing the capillary or for sampling. The entry of this vent was connected to a nitrogen cylinder. For positive pressure sampling, a pressure of 1 p.s.i. (1 p.s.i.=6894.76 Pa) for 5 s used in most cases, whereas a pressure of 20 p.s.i. was used for an appropriate time to rinse the capillary or to replace the background buffer.

Later, a CZE-device HP3D (Hewlett-Packard, Waldbronn, Germany) with a commercial interface cartridge for CE–MS was used. This device was in use particularly for overnight operation. By contrast with the older modified Bio-Rad system, the HP device is able to deliver up to 30 kV, which results in a separation voltage of at least 24 kV, when a maximum electrospray voltage of 6 kV is applied. This leads to a decrease of the migration time for the separation of the analytes as well as to an increase in separation power.

2.2. CZE–ESI-MS interface

For the coupling of the CZE devices to an API III^{plus} triple quadrupole mass spectrometer (PE SCIEX, Concord, Canada), a sheath-liquid type interface as described by Smith et al. [8] was used, appropriately modified to fulfil the requirements.

A laboratory-made three-layer sprayer was used consisting of an assembly of concentric hypodermic needles arranged as described below. A 0.8-mm diameter hole was drilled through a low dead-volume T-piece from Valco (Houston, TX, USA). One port of the T-piece was connected to a stainless steel capillary 30 mm×0.8 mm I.D. and 1.6 mm O.D., with a swagelok ferrule and a 1/8 inch screw. The end of this capillary was shrunk to 0.6 mm and directed towards the orifice of the mass spectrometer. The capillary was used for delivering nebulizing gas. A stainless steel capillary 120 mm×0.25 mm I.D. and 0.5 mm O.D. was placed inside the capillary. This capillary was fixed on the opposing side of the T-piece with a graphite ferrule and a 1/8 inch screw. The thin inner stainless steel capillary protruded approximately 1 mm from the nebulizer capillary. The screw and the graphite ferrule were used for charging the make-up liquid, delivered in the gap between the inner stainless steel capillary and the CZE capillary, by connecting it to the high voltage power supply of the mass spectrometer. The third port of the T-piece was connected to the nebulizer gas line. The opposite end of the inner stainless steel capillary was connected to a second Valco low dead-volume T-piece using a polyether ether ketone (PEEK) sleeve, 30 mm×0.5 mm I.D. and 1.6 mm O.D., a 1/16 inch ferrule, and a 1/8 inch screw. Through the opposite end of this T-piece the CZE capillary, 50–100 μm I.D. and 170–200 μm O.D., was guided through the inner stainless steel capillary to the opposite end of the assembly towards the mass spectrometer. The CZE capillary was fixed using a

PTFE sleeve (100 mm×200 μm I.D., 1.6 mm O.D.) with a 1/16 inch plastic ferrule and 1/8 inch screw. The position of the fused-silica capillary was fixed in such a way that it protruded less then 1 mm from the thin stainless steel capillary. A few millimeters of the coating of the fused-silica capillary were burned off from both ends to improve wetting and electrical contact on the MS side, and to prevent carry-over on the CZE side. The third port of the second T-piece was used for delivering the make-up liquid via a PTFE coil, (50 cm×200 μm I.D., and 1.6 mm O.D.). This interface was robust in routine use for several months.

The sheath-flow device was chosen because, by contrast with the liquid junction interface, it allows faster method development by quick replacement of the make-up liquid and optimisation of MS conditions.

2.3. μ-HPLC system

The μ-HPLC–MS system consisted of an HPLC pump operating, typically, at a flow-rate of 200 μl/min. At this flow-rate, good gradients could be obtained either with an L 6200A HPLC pump from Merck–Hitachi (Merck, Darmstadt, Germany), operating in low pressure gradient mode, or two Shimadzu HPLC pumps, type LC-10AD, from Burkhard Instumente (Geroldswil, Switzerland) connected to a dynamic high pressure mixer with 75 μl internal volume, operating in high pressure gradient mode. Separations were achieved on either a 250 mm×300 μm I.D. micro column supplied by LC-Packings (Amsterdam, The Netherlands) or on a fused-silica column, 250 mm×300 μm I.D., supplied by Micro Tech Scientific (Saratoga, CA, USA), both filled with Kromasil RP-18, 5 μm particle size (Eka-Nobel, Sweden). To operate at flow-rates of approximately 5–10 μl/min as required for micro-columns, the effluent was split by insertion of a T-piece in front of the injector. The flow-rate was adjusted to the desired level by coupling one or two 2 mm I.D. columns to the third port of the T-piece. The injector used was a type CI4W.06 (Valco, Houston, TX, USA) equipped with either a 60 or 200 nl internal loop. For overnight operation an autoinjector CMA 200 (Carnegy Medical, Stockholm, Sweden) with a 2 μl external loop was coupled to the micro-column. In this case, only 500 nl were injected applying the partial filled loop option.

2.4. Mass spectrometric equipment

For detection, an API III^{plus} triple quadrupole mass spectrometer (PE SCIEX, Concord, Canada) equipped with an articulated ion spray source was coupled to the CE or to the μ-HPLC systems. The ion spray interface was chosen to improve mixing of the CZE buffer and the sheath liquid on the tip of the liquid sheath interface and to guarantee stable spray conditions. Nitrogen or air was applied as nebulizing gas, while only nitrogen was used as curtain gas at a flow-rate of 1.2 l/min. The orifice voltage was set (45 to 60 V) to prevent fragmentation in the high-pressure region of the mass spectrometer on the one hand and to minimize solvent clusters which can enter the mass spectrometer on the other. When tandem mass spectrometric experiments with collision-induced dissociation (CID) were carried out, argon was introduced as the collision gas into quadrupole 2 (q2) at a collision gas thickness of approximately 300×10^{13} atoms/cm^2.

Selected reaction electropherograms or selected reaction ion chromatograms were recorded by selecting the protonated molecular ion $[M+H]^+$ in quadrupole 1 (Q1), which then underwent collision-induced fragmentation in quadrupole 2 (q2).

The most abundant fragment ions were then detected in the ion detector after they were selected in quadrupole 3 (Q3). Calibration of the mass axis of the mass spectrometer was carried out on a daily basis by infusion of a solution of seven quaternary alkylammonium cations, each at 200 nMol, with a flow-rate between 10 and 20 μl/min. The mixture covers a mass range from 200 to 580 a.m.u. and has proved to be sufficient for tuning the mass spectrometer at unit mass resolution in the mass range used for the experiments.

2.5. Reagents

Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and were gradient grade. Ammonium acetate and acetic acid were also from Merck, analytical reagent grade. Dichloromethane was obtained from Fluka (Buchs, Switzerland), analytical reagent grade. 1-Chlorobutane, high purity

grade, was from Fisons (Loughborough, UK). Water was double distilled or obtained from Merck, gradient grade. Buffer solution at pH 11 and pH 9 was Titrisol standard buffer solution from Merck.

8-Chloro-6-(2-fluoro-phenyl)-1-methyl-4H-imidazo-[1, 5-a] [1, 4]-benzodiazepine, midazolam (I), (8-chloro-6-(2-fluoro-phenyl)-4H-imidazo-[1,5-a]-[1, 4]-benzodiazepin-1-yl)-methanol, the 1-hydroxy derivative (II), 8-chloro-6-(2-fluoro-phenyl)-4-hydroxy-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine, the 4-hydroxy derivative (III), and [8-chloro-6-(2-fluoro-phenyl)-4H-imidazo-[1,5-a][1,4]-benzodiazepin-1-yl-methyl] β-D-glucopyranosiduronic acid, the 1-glucuronide derivative (IV), were from Hoffmann-La Roche (Basel, Switzerland). 8-Chloro-6-(2-chloro-phenyl)-1-($^2H_3$)methyl-4H-(4,4-$^2H_2$)-imidazo-[1,5-a][1,4]-benzodiazepine, pentadeuterated midazolam, was from Hoffmann-La Roche (Nutley, USA).

2.6. Sample preparation

2.6.1. Liquid–liquid extraction

Liquid–liquid extraction was carried out as stated below via a procedure similar to that described by Zell and Timm [22] for the determination of a benzodiazepine antagonist by GC.

To a 1 ml aliquot of human plasma, 20 μl internal standard (I.S.) solution containing 100 ng pentadeuterated midazolam was added and shaken for a few seconds. Then, 1 ml buffer at pH 11 was added and the sample was extracted with 5 ml 1-chlorobutane–dichloromethane (96:4, v/v) by gentle shaking for 20 min. After this, the samples were centrifuged at 1500 *g* for 5 min for phase separation, and the organic layer was transferred to another tube. After evaporation under a stream of nitrogen at 50°C, samples were reconstituted in 200 μl methanol–5 m*M* ammonium acetate–acetic acid (80:20:1, v/v/v) by sonication for 10 min. The samples were then transferred to 200 μl conical microvials, evaporated to dryness in a vacuum centrifuge and reconstituted in 20 μl water.

2.6.2. Solid-phase extraction

For SPE, 10 μl of internal standard (I.S.) solution (containing 100 ng of I.S.) were added to 250 μl of human plasma and mixed for a few seconds. Then 250 μl 0.5 *M* $HClO_4$ were added for protein precipitation. The samples were centrifuged and the supernatant was transferred to another tube. A 750 μl volume of 0.5 *M* Na_2HPO_4 was added to the supernatant to adjust the pH to approximately 7. For SPE, Bond Elute C_{18} (Varian, Harbor City, USA) cartridges containing 100 mg of stationary phase were used, and were first flushed with 1 ml methanol and then conditioned with 1 ml water. After the sample was applied, the cartridge was washed with 1 ml water before the analyte was eluted with 1 ml of a mixture of acetonitrile–5 m*M* ammonium acetate–acetic acid (60:40:1, v/v/v). The samples were then evaporated to dryness and reconstructed with 20 μl water. Attempts to use solvents with different amounts of organic modifier for washing the sample failed because of poor recoveries, especially for the metabolites. The sample volume used here, in contrast to e.g. liquid–liquid extraction, was limited to 0.250 ml, because the capacity of the SPE cartridge was limited to 1 ml liquid.

3. Results and discussion

3.1. Selection of the CZE interface

The coupling of CZE to API-MS has been a challenge since the very early development of the technique by Smith and co-workers in 1987. It has been reviewed recently [23]. Several different interfaces have been designed and can be assigned to three classes. The first approach reported was the sheathless interface, which uses the CZE capillary terminus as an electrospray device. Electrical contact was made through different coatings of silver or gold. The sheathless interface was used in the beginning with pure electrospray, and more recently in combination with micro-electrospray or nano-electrospray [24]. This interface prevents the dilution of the eluting analytes that occurs with the two other interface types [25], the liquid junction and the sheathflow interface. On the other hand, with the addition of a make-up solvent at the CZE capillary terminus or via a liquid gap, post-column modulation of (i) the pH, (ii) the flow-rate out of the capillary and (iii) the composition of the eluent out of the capillary is feasible and can better meet needs in

terms of electrospray response. For all variants of sheathless interfaces, a good, long-acting and stable electrical contact on the CZE capillary terminus remains the main challenge. The production of these, in most cases gold-coated, tips is still time consuming, needs a lot of expertise, and is only a one-way solution i.e., each capillary has to be newly coated after several hours of operation. The flow from the CZE capillary, which is the basis for the electrospray process, is controlled and compromised by the electroosmotic flow, the I.D. of the sprayer tip, and the electric field strength, which is related to the voltage drop and the distance between interface and capillary terminus.

Taking all the above mentioned points into account, the sheath-liquid and the liquid junction interface appear the more universally applicable interface type, even though a dilution of the analyte occurs.

3.2. Performance of CZE–ESI-MS with standard solutions

CE is often considered as a powerful separation technique which has the ability to provide a separation power of more then one million theoretical plates per meter. This is illustrated in Fig. 2, where a standard solution of midazolam (I) and three of its metabolites (II to IV) are separated on a 100 cm×75 μm I.D. uncoated fused-silica capillary using a buffer of 50 m*M* ammonium acetate, pH 4.8. A sheathflow of 20 μl/min of methanol–water–acetic acid (80:20:1, v/v/v) was added to improve stability of the spray conditions. The amount injected was approximately 10 nl. The injection was carried out by applying a pressure of 0.5 p.s.i. for 2 s using the modified Bio-Rad system. Voltages of 18 kV on the injection side and 5 kV on the electrospray side were used for separation. More than one million plates per meter could be calculated for metabolite II, whereas for the drug I and for the metabolites III and IV, figures in the range of 70 000 to 184 000 plates only could be achieved. Calculation of the numbers of plates was carried out using Eq. (1) [26], where V_R and t_R express the retention time either in volumetric or time dimensions and w_h is the peak width at half height in the corresponding dimension, and the ratio in the brackets is dimensionless.

$$N = 5.545(V_R/w_h)2 = 5.545(t_R/w_h)^2 \quad (1)$$

As can be seen from Fig. 2, separation efficiency and sensitivity are compound dependent. Differences in sensitivity can be considered as the sum of parameters depending on the detector (MS) and on separation. Differences depending on detection can be

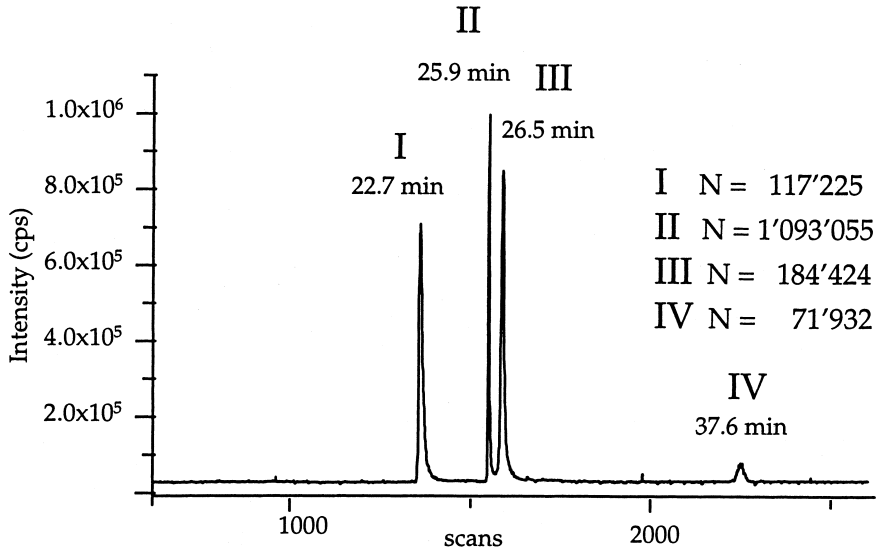


Fig. 2. Total selected ion electropherogram of a test mixture of midazolam (I) and its metabolites (II–IV).

explained as differences in the evaporation rate as well as differences in the intensities of the most abundant fragment ion selected for MS detection in selected reaction monitoring mode (SRM). Differences depending on separation originate mainly from differences in peak dispersion. The fundamental equation that describes the dependence of the plate numbers and, inversely, peak dispersion on operational parameters in CZE (see Eq. (2)) [27] shows that only the sum of electromigration and electroosmotic flow, the applied voltage and the diffusion coefficient influence the numbers of plates.

$$N = (\mu_{eo} + \mu_{ep})V/2D \quad (2)$$

where μ_{eo} is the coefficient for electroosmotic flow, μ_{ep} is the coefficient for electrophoretic mobility of the analyte, V the voltage applied and D the diffusion coefficient. The result was that high separation power could be achieved only for analytes with low diffusion coefficients where a high electroosmotic flow was present and a high voltage was applied during separation. Since the electroosmotic flow is much higher than the velocity of each analyte driven by electromigration alone, and the electroosmotic flow is increased at elevated pH, cations will yield high plate numbers at basic pH, but will be poorly detected by IS or ESI at the same pH. Anions, on the other hand, will be separated best by CZE because the electromigration is inversely directed to the electroosmotic flow, but will show low separation power because they migrate more slowly and undergo longer longitudinal diffusion. MS detection of anions at elevated pH will be better then cations under the same conditions.

3.3. Comparison of CZE–ESI-MS versus μ-HPLC–ESI-MS with standard solutions

3.3.1. Sensitivity

The differences in the separation and sensitivity achieved by applying the identical test solution of midazolam and its metabolites either to CZE–MS–MS or to μ-HPLC–MS–MS are shown in Figs. 3 and 4. Fig. 3 shows the extracted selected reaction ion electropherogram obtained using a 100 cm×75 μm I.D. uncoated fused-silica capillary. The running buffer used consisted of 30 m*M* ammonium acetate adjusted to pH 4.9 with acetic acid. Separation was

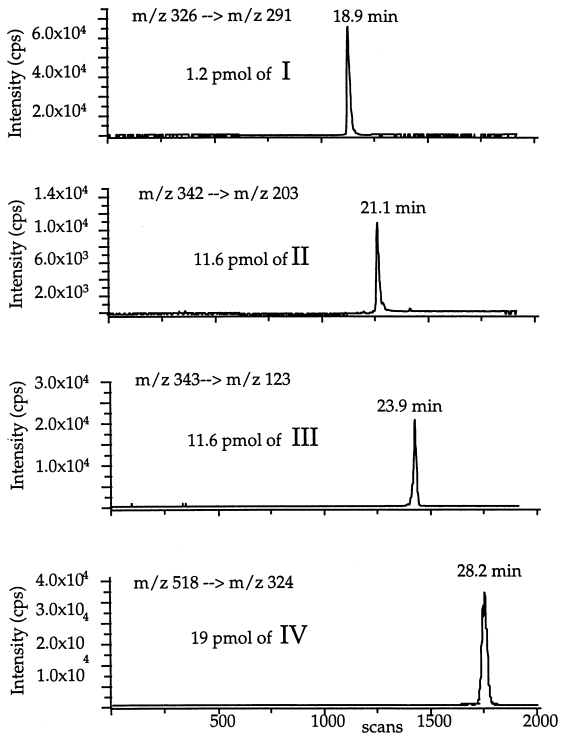


Fig. 3. Extracted selected reaction ion electropherogram of the test mixture of midazolam (I) and its metabolites (II–IV).

achieved by applying 18 kV on the cathode side reduced by 3.5 kV on the anode side of the mass spectrometer. A sheath flow of methanol–water–acetic acid (80:20:1, v/v/v) was delivered at 20 μl/min. Injection was carried out by applying a pressure of 1 p.s.i. for 5 s, which corresponded to approximately 40 nl sample solution. The sample amounts applied were calculated to 1.2 pmol of I, 11.6 pmol of II and III, and 19 pmol of IV.

The extracted selected reaction ion chromatogram of 200 nl of the same test mixture applied to the μ-HPLC–MS system is shown in Fig. 4. The separation was performed on a 250×0.32 mm micro-column filled with Superspher RP-18, particle size 5 μm. A gradient was formed from solvent A: acetonitrile and solvent B: 5 m*M* ammonium acetate adjusted to pH 4.8 with acetic acid. The amount of solvent A was raised from 0 to 100% over 20 min. The amounts injected onto the column, using a manual injector equipped with a 200 nl internal loop, were 6 pmol of I, 58 pmol of II and III, and 95 pmol

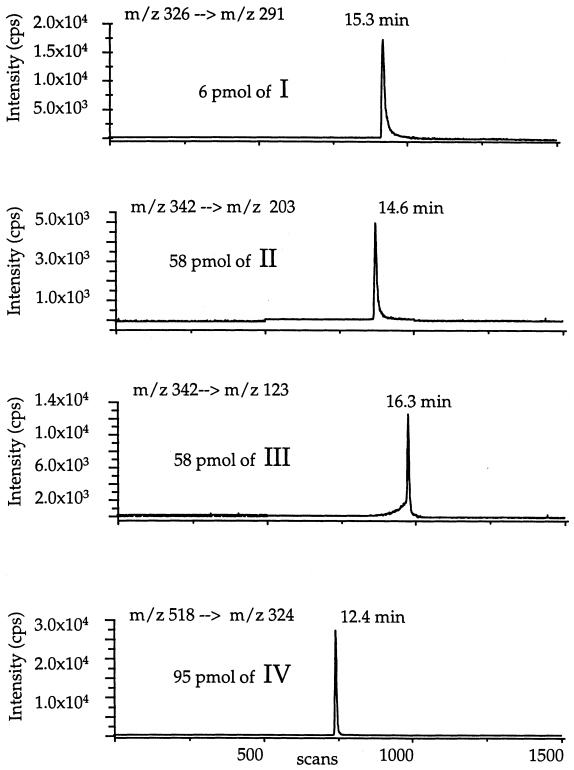


Fig. 4. Extracted selected reaction ion chromatogram of the test mixture of midazolam (I) and its metabolites (II–IV).

of IV. The amounts injected for both separation techniques, normalized to the peak heights measured and the elution volumes calculated, are summarized in Tables 1 and 2, respectively. As can be seen from Table 1, CZE–MS–MS was approximately 9 times more sensitive for II to 17 times more sensitive for I than μ-HPLC–MS–MS. Better response is often related to the narrower peak width which is achievable in CZE and consequently the higher analyte concentration. If the results shown in Table 2 are set in relation to those shown in Table 1, it is obvious that this argument cannot be employed to provide an explanation in this case. Table 2 shows that the peaks in μ-HPLC–MS–MS are much narrower than in CZE–MS–MS. To explain this unexpected behaviour, additional factors should be considered. First, it may be possible that the mixing of the buffer from the CZE capillary with the sheath-liquid at the tip of the interface is not complete, or the mixing takes place only in a very limited zone. Therefore, the calculated elution volumes of the peak do not reflect the real situation and may result in much higher analyte concentration than expected. Second, the composition of the eluent at the CZE–MS interface remains constant during the whole elution. This is because the flow-rate from the capillary is much lower than the sheath-liquid flow-rate and is therefore negligible in comparison. Therefore the ionization efficiency depends mainly on the composition of the sheathflow liquid. This is in contrast to the situation regarding the μ-HPLC column, which shows an increase of the organic content of the eluent linked to a higher ion evaporation rate along gradient elution, and may actually be lower when the peaks elute in comparison to CZE. However the difference in organic content of the eluent and the sheathflow liquid did not exceed 30% and cannot explain the big difference which was observed. Third, the current produced by the drop of the electric field along the CE capillary may result in the production of protons, which may also contribute to the increased sensitivity of the CE–MS approach.

3.3.2. Selectivity

If the order of elution of the components in Figs. 3 and 4 is compared, a reversed elution order for the

Table 1
Comparison of amount injected, peak areas, ratio of area and amount and ratio of increase in sensitivity CZE–MS–MS to μ-HPLC–MS–MS after injection of a standard solution

	CZE–MS–MS			μ-HPLC–MS–MS			Ratio CZE–μ-HPLC
	Amount (pmol)	Peak areas	Ratio area/amount	Amount (pmol)	Peak areas	Ratio area/amount	
I	1.2	55 926	46 605	6	15 863	2643	17.6
II	11.6	42 259	3643	58	23 986	413	8.8
III	11.6	11 305	974	58	4433	76	12.8
IV	19	20 643	1086	95	10 679	112	9.7

Table 2
Comparison of amount injected, peak areas and elution volume achieved with either CZE–MS–MS or μ-HPLC–MS–MS after injection of a standard solution

	CZE–MS–MS			μ-HPLC–MS–MS		
	Amount (pmol)	Peak areas	Vpeak (μl)	Amount (pmol)	Peak areas	Vpeak (μl)
I	1.2	55 926	11.4	6	15 863	5.69
II	11.6	42 259	9.72	58	23 986	4.62
III	11.6	11 305	14.08	58	4433	3.10
IV	19	20 643	18.44	95	10 679	2.67

glucuronide-conjugate IV in particular is observed. In LC, where retention is related to the partition ratio of the analyte between the mobile and stationary phases, this component appears as the most hydrophilic, and therefore elution will occur faster than for the other metabolites and the drug itself. In CZE, conjugate IV appears last in the electropherogram, this being closely related to the size/charge ratio of the molecule. Due to conjugation, compound IV has the greatest size/charge ratio in comparison with the others, and for this reason migrates more slowly than the other compounds. In CZE, the elution order started with the drug followed by the two hydroxy-metabolites, and finished with the glucuronide-conjugate. In HPLC, the glucuronide-conjugate elutes first, followed by the a hydroxy-metabolites and the drug, and the elution order concludes with the other hydroxy-metabolite, which represents the most hydrophobic compound of the mixture. Together with higher separation efficiency and different selectivity, CZE–MS may be very useful when phase II metabolites, which can be difficult to retain on the HPLC column, have to be monitored.

3.4. Application to biological samples

3.4.1. Liquid–liquid extraction

Figs. 5 and 6 represent an extracted selected reaction ion electropherogram and extracted selected reaction ion chromatogram respectively, of a 2 ng/ml (I to III) spiked human plasma sample prepared by liquid–liquid extraction. As internal standard, 100 ng of the pentadeuterated analogue of I were added before extraction. The samples were reconstituted with 20 μl water, which represents a concentrating factor of 50. An aliquot of 500 nl was injected by means of an autoinjector using the partial fill loop option onto the μ-HPLC system. Samples were eluted in isocratic mode with a mixture consisting of acetonitrile–methanol–5 m*M* ammonium acetate–acetic acid (37.5:37.5:25:1, v/v/v/v) at a flow-rate of 10 μl/min. The CZE–MS–MS separation was performed on a 100 cm×100 μm I.D. uncoated fused-silica capillary. The background buffer consisted of 30 m*M* ammonium acetate, pH 4.8, adjusted with acetic acid. The sheath-flow liquid, delivered by a syringe pump at a flow-rate of 10 μl/min was a mixture of methanol–5 m*M* ammonium acetate–

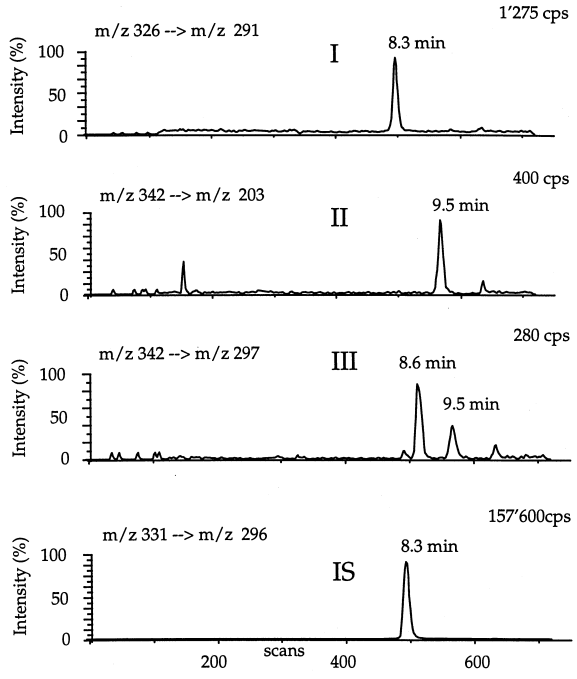


Fig. 5. Extracted selected reaction ion electropherogram of an extracted human plasma sample spiked with 2 ng/ml of midazolam (I) and its metabolites (II and III).

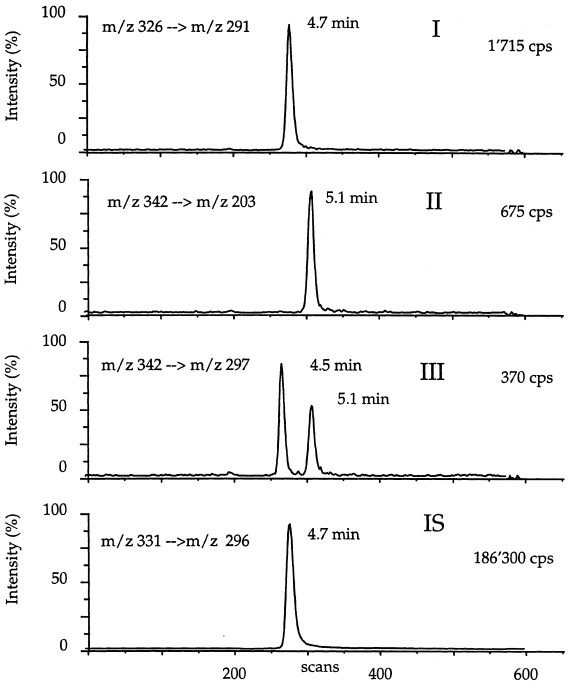


Fig. 6. Extracted selected reaction ion chromatogram of a extracted human plasma sample spiked with 2 ng/ml of midazolam (I) and metabolites (II and III).

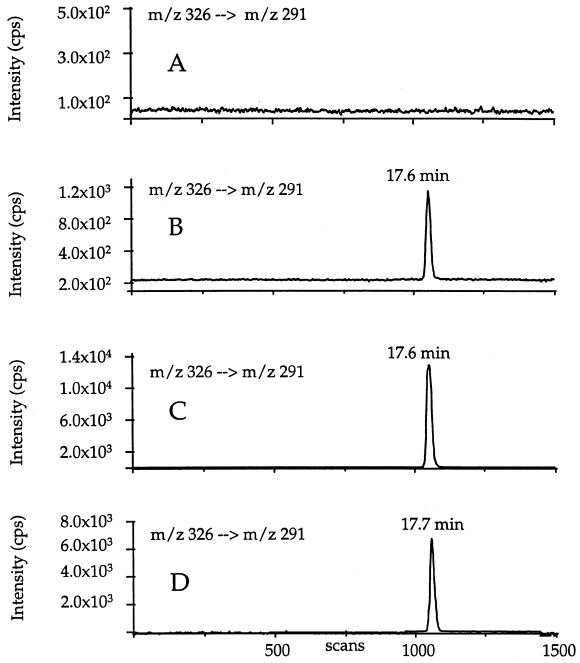


Fig. 7. Extracted selected reaction ion electropherograms of extracted plasma samples taken before (A), and 3 min (B), 47 min (C), and 2 h (D) after intravenous administration of midazolam (I) to a human volunteer.

acetic acid (80:20:1, v/v/v). Approximately 100 nl were injected by applying a pressure of 50 mbar to the injection vial for 5 s. A voltage of 25 kV was applied as separation power by means of the HP-CZE device described above. The comparison of the two traces shows that the peak heights achieved for both methods appear approximately constant (Figs. 5 and 6). Only 1/5 of the identical sample applied to the μ-HPLC–MS–MS was injected into the CZE–MS–MS system, showing a five-fold increase in response for the CZE–MS system. This is in line with the findings with standard solutions. The less pronounced effect for human plasma sample extracts could be related to ion suppression originating from the sample matrix. Nevertheless, calibration curves could be generated in the range 500 pg/ml to 200 ng/ml for I and 2 ng/ml to 1000 ng/ml for II and III for both applications, CZE–MS–MS and μ-HPLC–MS–MS.

To demonstrate the applicability of this approach to real samples, with the focus on sensitivity and selectivity, human plasma samples from a clinical trial taken after i.v. administration of midazolam were analyzed with CZE–MS–MS as illustrated in Fig. 7, using the modified Bio-Rad system with a separation power of approximately 13 kV. The pre-dose trace at the top shows no trace of the drug or any endogenous interference, whereas the traces below show midazolam levels corresponding to 58, 337 and 108 ng/ml.

3.4.2. Solid-phase extraction

Since liquid–liquid extraction is time consuming and needs a lot of manual handling, SPE has become an increasingly attractive alternative for fast and easy sample preparation, and it can be automated. Figs. 8 and 9 represent an extracted selected reaction ion electropherogram and extracted selected reaction ion chromatogram, respectively, of a 2 ng/ml (I to III) spiked human plasma sample prepared by solid-phase extraction. As internal standard, 100 ng of the pentadeuterated analogue of I were added before extraction. A sample volume of 1 μl was applied to the μ-HPLC system, whereas only 100 nl were

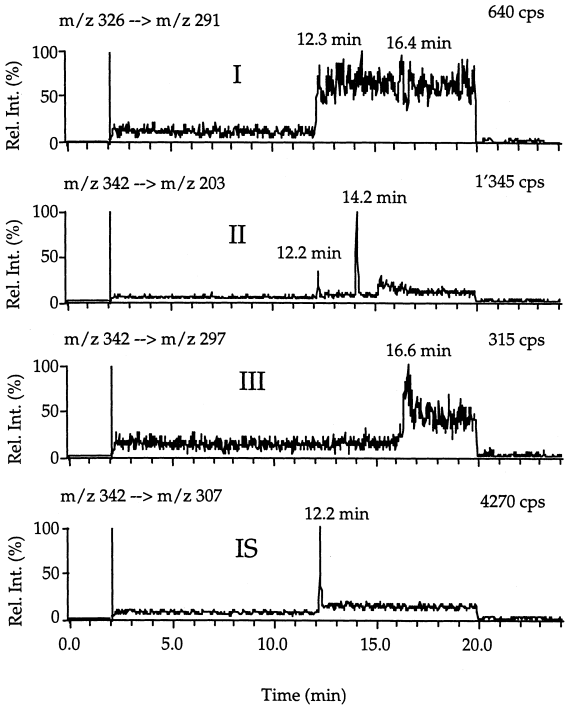


Fig. 8. Extracted selected reaction ion electropherogram obtained from human spiked plasma at 2 ng/ml of (I) to (III) after solid-phase extraction.

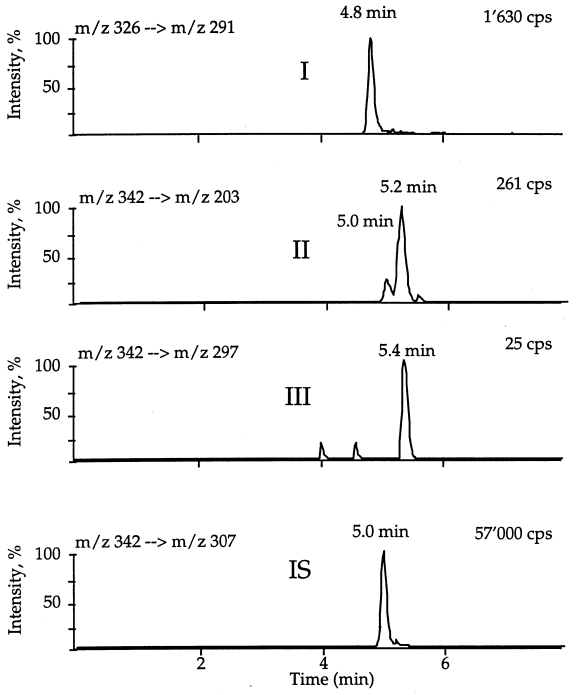


Fig. 9. Extracted selected reaction ion chromatogram obtained from human spiked plasma at 2 ng/ml of (I) to (III) after solid-phase extraction (1 μl injected).

injected onto the CZE capillary. In both cases the intensities observed were dramatically lower than for the samples generated by liquid–liquid extraction, in particular for CE–MS–MS (Figs. 5 and 6). After several injections, the back pressure of the μ-HPLC system exceeded the limit and it was not possible to generate a calibration curve. This was more marked for the CE–MS–MS system, where clogging and peak distortion were observed even after extensive flushing of the CE capillary following each run. It can be related to matrix components which are still present after the less selective solid-phase extraction. It should also be noted that only one quarter of the plasma volume employed in the liquid–liquid procedure was used due to of the limited capacity of the SPE cartridges as mentioned above. To overcome problems with matrix constituents in CZE combined with UV or fluorescence detection, micellar electrokinetic chromatography (MEKC) is most frequently used. But in CZE–MS the presence of surfactants will suppress the electrospray process and is therefore not applicable. Attempts to improve the SPE by introducing more selective washing steps failed due to the polarity range of the analytes to be covered.

Higher sensitivity could be achieved either with CZE or LC by using adequate preconcentration techniques before separation [28]. It was recently demonstrated that by using column-switching from narrow-bore column to μ-HPLC it was possible to inject almost the whole extract from a 1 ml plasma sample to achieve a detection limit of 1 pg/ml for a similar compound [29].

4. Conclusions

During the investigation of the merits and limitations of μ-HPLC–MS–MS and CZE–MS–MS for the separation and detection of a drug and its metabolites, the combination of CZE–MS–MS was found to be superior to that of μ-HPLC–MS–MS with regard to detection limits. The higher degree of sensitivity, achieved with the sheathflow interface,

could not be related to the high separation power of CZE. This was observed for standard a solution as well as for sample extracts from biological matrices. Different sample preparation routes were evaluated and liquid–liquid extraction was found to be the more appropriate mode for the coupling of micro separation techniques to MS. This underlines the importance of the sample preparation procedure. The detection limits achieved for the analytes were in the low ng/ml range, even with only nanolitre injection volumes and the use of microseparation techniques combined with atmospheric pressure ionization tandem mass spectrometry.

Sample concentration before application was found to be necessary for achieving the detection limits needed for pharmacokinetics, assuming an appropriate sample clean-up.

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