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Determination of small drug molecules by capillary electrophoresis–atmospheric pressure ionization mass spectrometry

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ABSTRACT

Capillary electrophoresis (CE) separations are reported for sulfonamides and benzodiazepines in an uncoated fused-silica capillary. The capillary column exit was connected to a liquid junction–ion spray interface combination coupled to an atmospheric pressure ionization (API) triple quadrupole mass spectrometric (MS) system. On-line UV detection occurred 20 cm from the inlet of the capillary and with the API mass spectrometer (CE–API-MS) after the entire length of the capillary (100 cm). The separations were made using volatile buffers composed of ammonium acetate (15–20 m*M*) with 15–20% of methanol to facilitate ionization under electrospray conditions. This study showed that the major metabolite of flurazepam in man, N-1-hydroxyethylflurazepam, could be detected and characterized in human urine by CE–UV–MS following the administration of a single oral dose of 30 mg of flurazepam dihydrochloride. The presence of additional flurazepam metabolites in human urine was observed by using the system, suggesting that a combination of UV with MS detection should be useful for metabolic studies. In addition to molecular weight determination of compounds, structural information may be obtained by utilizing on-line tandem mass spectrometry (CE–UV–MS–MS). This was demonstrated for sulfamethazine where the protonated molecule species was transmitted into the collision cell of the tandem triple quadrupole mass spectrometer. Collision-induced dissociation of the protonated sulfamethazine molecule yielded structural information characteristic of the sulfa drug following the on-column injection of 2 pmol of sulfamethazine.

INTRODUCTION

The development and use of capillary electrophoresis (CE) as a separation technique has expanded in recent years [1]. In CE, charged compounds are separated under the influence of a high electrical field in small-diameter capillary tubes filled with buffer. Efficient separations, short analysis times and low sample consumption make CE a very attractive separation technique. The most common detectors for CE include UV absorbance or fluorescence detectors, but an increasing number of applications utilizing mass spectrometric (MS) detection have been reported [2–14].

Developments in CE–MS interfacing have been based either on electrospray or

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ion spray ionization at atmospheric pressure [2–11] or on continuous-flow fast atom bombardment (CF-FAB) [12–14]. The CE–MS inferface is needed to facilitate coupling of the low CE buffer flow to the mass spectrometer or to introduce the matrix required for the CF-FAB process. The make-up liquid is introduced either by a liquid junction [3,7–9,11,13,14] or as a sheath flow arrangement [4–6,10,12]. Here we have used a pneumatically assisted electrospray (ion spray) interface with the liquid junction coupling described previously [9]. The buffer in the liquid junction is typically the same as the separation buffer for CE and provides a suitable make-up buffer flow to sustain a stable spray to the API mass spectrometer. The advantage of using a mass spectrometer as the detector is that molecular weight information can be obtained in addition to the migration time for a compound. It is also possible to obtaine structural information from the analytes by tandem mass spectrometry (MS–MS).

The coupling of CE with electrospray ionization requires some compromise to be made with the electrophoretic buffer in order to obtain a high ion current response. Involatile buffers such as sodium citrate, phosphate and borate commonly used in CE separations are not generally suitable in combination with MS. The ion evaporation mechanism which produces gas-phase ions under these conditions operates best with volatile buffers at low concentration [15]. In addition, a high content of an organic solvent such as methanol or acetonitrile in the running buffer is desirable to facilitate the ion spray process [16]. A review of atmospheric pressure ionization mass spectrometry (API-MS) has been published recently [17].

In this work, we used a commercially available CE instrument interfaced to the API-MS system. The instrumental set-up and the use of the system for characterizing tryptic digests has been presented in a previous paper [11]. This paper describes an investigation using the CE–API-MS system for analyzing human urine extracts containing benzodiazepines and for characterizing sulfonamides. The convenience of using a UV detector in-line with the API-MS system is discussed and the utility of the technique is demonstrated for the detection of flurazepam metabolites in human urine.

EXPERIMENTAL

Chemicals

Fused-silica capillary tubing with dimensions of 75 μm I.D. and 375 μm O.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). The benzodiazepines chlordiazepoxide, flurazepam, diazepam, prazepam and oxazepam were obtained as Quik-Chek Drug Solutions (Alltech, Deerfield, IL, USA). The metabolite of flurazepam, N-1-hydroxyethylflurazepam, was kindly supplied by Hoffman-La Roche (Nutley, NJ, USA). β-Glucuronidase and the sulfonamides sulfanilamide, sulfamethazine, sulfathiazole, sulfamerazine, sulfadimethoxine and sulfamethoxazole were obtained from Sigma (St. Louis, MO, USA). Sequencing-grade trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Optima grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified in-house with a Barnstead Nanopure system. All other chemicals were of analytical-reagent or reagent grade and were used without further purification.

Capillary electrophoresis instrumentation

A Beckman (Palo Alto, CA, USA) P/ACE System 2000 capillary electrophoresis system was used. The total length of the capillary was 100 cm with the UV detector operated at 254 nm and positioned 20 cm from the inlet. Two modifications were made to the commercial CE instrumentation. The first was the installation of a simple switch to by-pass the safety lock between the capillary ends. No measurement of the current by the P/ACE 2000 could be made during CE–MS operation with this modification. The current through the system was measured by an external ammeter before or after the CE–MS separation. The second modification was that the capillary cartridge temperature control system was bypassed during CE–MS operation [11]. The CE instrument was equipped with a 30-kV high-voltage power supply. The standard pressure inejction feature of this system was preferred for sample injection in this work.

Mass spectrometry

A Sciex (Thornhill, Ontario, Canada) TAGA 6000E triple quadrupole mass spectrometer equipped with an API source was used. The capillary end from the CE system was coupled via a liquid junction to a pneumatically assisted electrospray (ion spray) interface [16] and the API-MS system. The same interface was used by Lee and co-workers [3,7–9]. The only modification in this work was that the inner capillary in the ion spray interface consisted of uncoated fused silica (75 μm I.D.) instead of stainless steel [11]. During CE–MS operation the liquid junction and the ion spray interface were floated at +4 kV and the mass spectrometer was operated in the positive-ion mode of detection. When the high voltage was maintained at 30 kV at the capillary inlet, the 4-kV potential maintained on the ion spray interface produced a potential difference across the separation capillary of 26 kV.

Gas-phase ions generated from the ion spray interface via the ion evaporation mechanism [15] were sampled into the mass spectrometer by a potential difference of about 3 kV set between the ion spray interface and the ion sampling orifice. The sampling orifice was a 100-μm diameter hole in the end of a conical skimmer extended toward the atmosphere. To minimize solvent cluster formation, this system utilizes a gas curtain of ultrapure nitrogen applied to the atmospheric side of the skimmer. For full-scan CE–MS work, the first quadrupole (Q1) was scanned from either m/z 250 or 300 to m/z 400 in about 5 s in steps of 1 u. For CE–MS–MS work, ultrapure argon (AIRCO, BOC Group, Murray Hill, NJ, USA) was used as the collision gas and was introduced into the collision cell (Q_2) at a target gas thickness of 2×10^{14} atoms/cm^2. The collision gas energy used for the MS–MS experiments was 70 eV. Both quadrupole mass analyzers were maintained at unit mass resolution in this work.

Infusion of the sulfonamides dissolved in the CE buffer [20 m*M* ammonium acetate (pH 6.8) containing 20% of methanol] were made with a Harvard (South Natick, MA, USA) syringe pump. During infusion, at a flow-rate of 4 μl/min, the first quadrupole was scanned from m/z 100 to 450.

Methods

Rinsing of the CE capillary was performed by pressurizing the inlet at 138 kPa. New capillaries were flushed with 9 column volumes of 1 *M* sodium hydroxide, 3 column volumes of distilled water, 3 column volumes of 0.1 *M* hydrochloric acid, 3

column volumes of distilled water and 9 column volumes of electrophoretic buffer. Between analyses the capillary was rinsed with 1.5 column volumes of 0.1 *M* sodium hydroxide followed by 9 column volumes of buffer.

The low-pH buffers were prepared from a solution of ammonium acetate to which trifluoroacetic acid (TFA) was added to give the desired pH. Methanol was then added to the buffer. The buffer concentrations reported in this work are the total concentration of ammonium acetate in the buffer. The electrophoretic buffers were filtered through a 0.45-μm Miller-HV filter unit prior to use (Millipore, Bedford, MA, USA).

The electroosmotic flow, v_{eo}, was calculated by measuring the migration time, $t_o = V/v_{eo}$ and V is the volume of the capillary. The calculation for column efficiency was based on $N = 5.54\,(t/w_{0.5})^2$, where N is the number of theoretical plates, t is the migration time for the compound and $w_{0.5}$ is the peak width at half-height.

The ion spray interface was positioned within 1 cm of the ion sampling orifice of the API-MS system. The position of the ion spray interface was adjusted while infusing flurazepam ($5 \cdot 10^{-6}$ *M*) or sulfamethazine ($2 \cdot 10^{-4}$ *M*) dissolved in the electrophoretic buffer through the capillary. Flurazepam was infused using the 138-kPa pressure (rinse feature) of the CE instrument and sulfamethazine was infused using the 3.45-kPa pressure typically used for injection. The singly charged ion at m/z 388 (flurazepam) or m/z 279 (sulfamethazine) was monitored and the mass spectrometric tuning parameters were adjusted to give the optimum ion current signal form the API-MS system.

Urine extraction and sample introduction

Urine samples were treated with β-glucuronidase prior to extraction. Two ml of 1.0 *M* potassium acetate solution (pH 5.0) containing $2 \cdot 10^6$ units/l of β-glucuronidase were added to 5 ml of urine in a 16 × 125 mm screw-capped test-tube and the treated urine was incubated at 65°C for 2 h. After incubation, the urine was cooled to ambient temperature and the pH adjusted to 9.5 with concentrated ammoniua solution. The urine was then extracted with 3.0 ml of 1-chlorobutane on a mixer at slow rotation for 15 min. The liquid phases were separated by centrifugation at 2500 *g* for 10 min. The upper organic layer was transferred to silanized, conical-bottomed centrifuge tubes and evaporated at 65°C under a gentle stream of nitrogen. Each extract was dissolved in 100 μl of electrophoretic buffer containing ammonium acetate (0.2 m*M*) adjusted to pH 1.3 with TFA in 15% (v/v) methanol. The extracts and standards were introduced into the capillary by pressurizing the inlet end of the capillary to 3.45 kPa. Injection volumes typically ranged from 5-to 28 nl.

RESULTS AND DISCUSSION

Benzodiazepines and sulfonamides are drugs that are often present at high concentrations in human urine after therapeutic doses. They are therefore suitable candidates for analysis by capillary electrophoresis with the present system. Although the amount of sample needed for CE separations and detection is very small, the sample concentration in the injection vial must be fairly high because only a few nanoliters are introduced into the capillary. A benefit of CE, however, is that the chemical derivatization of these drug classes often required for gas chromatographic (GC)–MS analyses [18–20] is not needed in CE–MS.

Sulfonamides

Sulfa drugs are used in the treatment for bacterial infections. There are, for example, veterinary preparations sold in the USA, for the treatment of large animals, that contain a mixture of three sulfa drugs, sulfamethazine and sulfathiazole together with either sulfamerazine or sulfanilamide. In the doping control of racehorses there is considerable interest in separating and detecting these and related substances in equine urine [21].

Fig. 1 shows the electropherograms obtained with (A) UV and (B) mass spectrometric detection for the separation of a synthetic mixture of six sulfonamides using an electrophoretic buffer consisting of 20 m*M* ammonium acetate (pH 6.8) containing 20% (v/v) of methanol. Minor structural variations between the sulfonamides result in different dissociation constants, pK_a = 5.6–10.4 [22,23]. The sulfonamides are separable by CE because they are negatively charged to different extents in the electrophoretic buffer. Changes in selectivity with pH in ion-pair high-performance liquid chromatographic (HPLC) separations of sulfonamides have been studied. It was found that dramatic changes in retention order may occur due to different selectivities

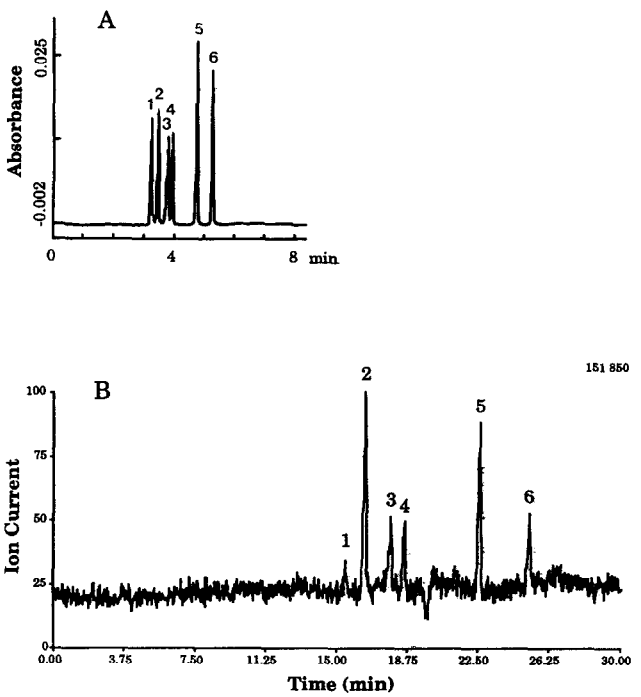

Fig. 1. (A) CE–UV and (B) CE–SIM–MS total selected ion current electropherograms for the separation of six sulfonamides. Capillary, uncoated fused silica, 100 cm × 75 μm I.D.; voltage, 26 kV; buffer, 20 m*M* ammonium acetate (pH 6.8) containing 20% (v/v) of methanol; injection, 5.5 nl of a $4 \cdot 10^{-4}$ *M* solution (2 pmol) of each drug. Peaks: 1 = sulfanilamide, *m/z* 190 $[M + NH_4]^+$ (pK_a = 10.4); 2 = sulfamethazine, *m/z* 279 $[M + H]^+$ (pK_a = 7.4); 3 = sulfathiazole, *m/z* 256 $[M + H]^+$ (pK_a = 7.27); 4 = sulfamerazine, *m/z* 265 $[M + H]^+$ (pK_a = 6.90); 5 = sulfadimethoxine, *m/z* 311 $[M + H]^+$ (pK_a = 5.9); 6 = sulfamethoxazole, *m/z* 254 $[M + H]^+$ (pK_a = 5.6). (A) CE–UV electropherogram at 254 nm; detection after 20-cm capillary. (B) CE–SIM–MS total selected ion current electropherogram; detection after 100-cm capillary.

in acid and ion-pair partition of the sulfonamides to the hydrophobic silica support coated with either 1-pentanol or butyronitrile [24].

The first peak in Fig. 1, sulfanilamide (pK_a = 10.4), is neutral in the electrophoretic buffer used and migrates with the electroosmotic flow. All the other sulfonamides are negatively charged under these CE conditions and migrate towards the positively charged anode. They are all carried towards the detector due to the electroosmotic flow (0.27 μl/min). The same migration order for the sulfonamides was obtained at pH 7.0 with 30 m*M* sodium dihydrogenphosphate–10 m*M* sodium tetraborate buffer [25]. The ammonium acetate–methanol buffer used in this study was chosen because volatile buffers benefit detection by API-MS. Fig. 1A shows the separation of 2 pmol (380–680 pg) of each sulfonamide within a 20-cm capillary length with UV detection at 254 nm and Fig. 1B shows the sulfonamides following elution from the 100-cm capillary length with API-MS detection under selected ion monitoring (SIM) conditions. The ammoniated molecular ion $[M + NH_4]^+$ for sulfanilamide and the protonated molecular ion $[M + H]^+$ for the other sulfonamides were monitored with the mass spectrometer operated in the positive-ion mode of detection. One advantage of using UV on-line with the API-MS system was that only after 4 min an indication was given as to whether the CE separation was taking place satisfactorily.

With the exception of sulfanilamide, the sulfonamides are partly negatively charged in the electrophoretic buffer and hence also partly negatively charged when they reach the ion spray interface. The sulfonamides contain two or more nitrogen atoms and a higher response was obtained when the API-MS system was operated in the positive-ion detection mode compared with the negative-ion detection mode. The relative response for positive- and negative-ion modes of detection is shown in Fig. 2.

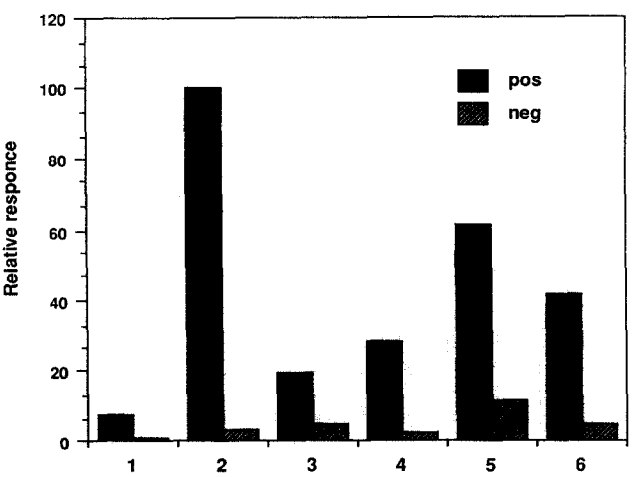

Fig. 2. Relative response of the sulfonamides by ion spray-MS operated under positive- and negative-ion modes of detection. The sulfonamides were dissolved in 20 m*M* ammonium acetate buffer (pH 6.8) containing 20% (v/v) of methanol to give a concentration of $2 \cdot 10^{-4}$ *M* and infused at a flow-rate of 4 μl/min. 1 = Sulfanilamide, *m/z* 190 $[M + NH_4]^+$, *m/z* 170 $[M - H]^-$; 2 = sulfamethazine, *m/z* 279 $[M + H]^+$, *m/z* 277 $[M - H]^-$; 3 = sulfathiazole, *m/z* 256 $[M + H]^+$, *m/z* 254 $[M - H]^-$; 4 = sulfamerazine, *m/z* 265 $[M + H]^+$, *m/z* 263 $[M - H]^-$; 5 = sulfadimethoxine, *m/z* 311 $[M + H]^+$, *m/z* 309 $[M - H]^-$; 6 = sulfamethoxazole, *m/z* 254 $[M + H]^+$, *m/z* 252 $[M - H]^-$.

These data were obtained from infusion of the individual sulfonamides into the API-MS system. The sulfonamides were dissolved in the electrophoretic buffer, 20 m*M* ammonium acetate (pH 6.8) containing 20% (v/v) of methanol, to give an infusion concentration of $2 \cdot 10^{-4}$ *M*. The position of the ion spray interface relative to the ion sampling orifice of the mass spectrometer and the applied potential was carefully adjusted to determine the optimum ion current response for the sulfonamides in the positive and negative-ion modes of detection, respectively. Sulfamethazine gave the highest response of the sulfonamides studied and was normalized to 100%.

One advantage provided by MS–MS is that structural information may be obtained for the analyte of interest. This is illustrated in Fig. 3 for sulfamethazine where the same synthetic mixture containing six sulfonamides was re-injected. The $[M+H]^+$ ion at *m/z* 279 for sulfamethazine was monitored with the first quadrupole (Q1), collision-induced dissociation (CID) was performed in the second quadrupole (Q2), and the third quadrupole (Q3) was scanned from *m/z* 10 to 350 in 6 s. Both mass-analyzing quadrupoles were maintained at unit mass resolution. The full-scan total ion electropherogram and the daughter-ion mass spectrum for 2 pmol (600 pg) of sulfamethazine was acquired to produce the data shown in Fig. 3. The migration time for sulfamethazine in Fig. 3A (16.5 min) agrees with peak 2 (sulfamethazine)

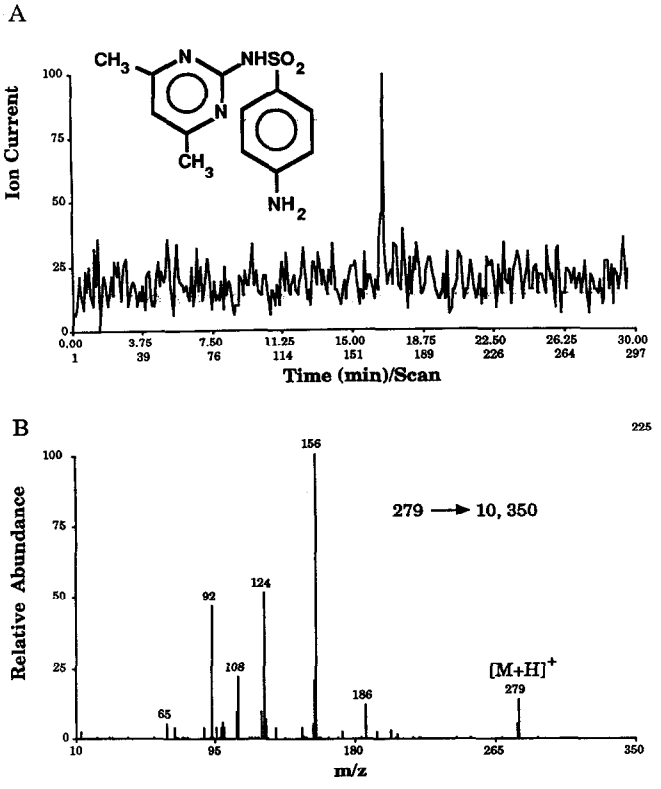

Fig. 3. (A) CE–MS–MS total ion electropherogram and (B) daughter-ion mass spectrum for sulfamethazine from a synthetic mixture of sulfonamides containing 600 pg each (2 pmol). Sample, injection volume and conditions as in Fig. 1.

observed in Fig. 1B. The CE–MS–MS trace shown in Fig. 3B displays structurally important fragment ions at *m/z* 186, 156, 124, 108, 92 and 65, which are characteristic of many sulfa drugs. The most abundant ion observed at *m/z* 156 is common to the mass spectra of sulfa drugs and is formed by cleavage of the sulfonamide–nitrogen bond [21,26]. All the expected MS–MS fragments for sulfamethazine are present compared with previous analyses by LC–MS–MS [21]. It should therefore be possible to use CE–UV–MS–MS for the determination of sulfa drugs in equine urine in the same way as was demonstrated for sulfadimethoxine in racehorse plasma and urine extracts by LC–MS–MS.

Benzodiazepines

Four benzodiazepines were separated using a 15 m*M* ammonium acetate buffer adjusted with TFA to pH 2.5 and containing 15% (v/v) of methanol. The separation of 1.4-2.0 pmol (554 pg each) of four benzodiazepines is shown in Fig. 4A with UV detection at 254 nm after a 20-cm capillary length and in Fig. 4B with API-MS from an experiment in which the mass spectrometer was operated under SIM conditions utilizing a 100-cm length of the capillary. A high separation efficiency was obtained.

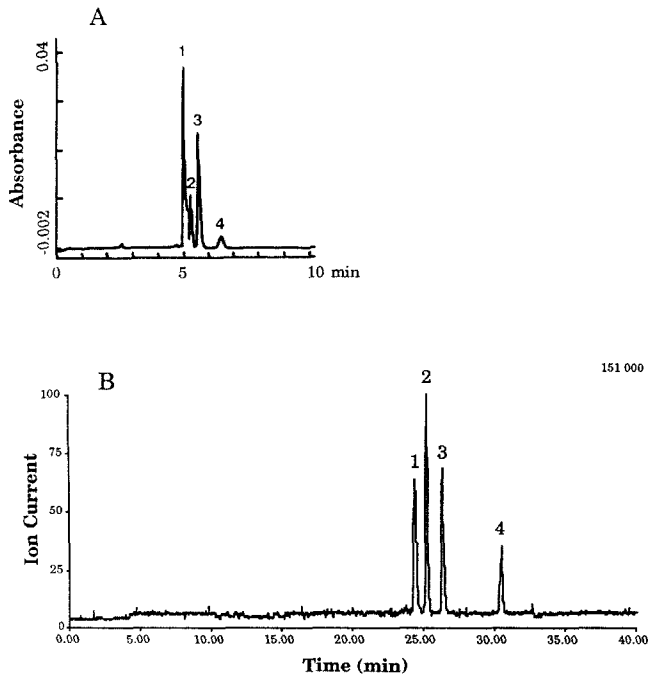

Fig. 4. (A) CE–UV and (B) CE–SIM-MS total selected ion current electropherograms from the separation of four benzodiazepines. Capillary uncoated fused silica, 100 cm × 75 μm I.D.; voltage, 26 kV; buffer, 15 m*M* ammonium acetate adjusted to pH 2.5 with TFA and containing 15% (v/v) of methanol; injection, 28 nl of a 20 μg/ml solution of each drug (1.4–2.0 pmol). Peaks: 1 = chlordiazepoxide, *m/z* 300 $[M+H]^+$; 2 = flurazepam, *m/z* 388 $[M+H]^+$; 3 = diazepam, *m/z* 286 $[M+H]^+$; 4 = prazepam, *m/z* 325 $[M+H]^+$. (A) CE–UV electropherogram at 254 nm, detection after 20-cm capillary; (B) CE–SIM-MS total selected ion electropherogram, detection after 100-cm capillary.

as evidenced from the 70 000–140 000 theoretical plates calculated from the CE–MS selected ion current electropherogram. The improved separation observed in Fig. 4B results from the longer residence time of the analytes in the capillary prior to MS detection.

Chlordiazepoxide, diazepam and prazepam have pK_a values between 4.6 and 2.7, and flurazepam has two pK_a values, 1.9 and 8.2 [22]. They exhibit varying degrees of positive charges in the electrophoretic buffer and migrated towards the negatively charged cathode under the experimental conditions. They were all detected as the $[M+H]^+$ ions by API-MS, with flurazepam giving the highest ion current response.

All benzodiazepines are extensively metabolized in humans. Chlordiazepoxide, diazepam and prazepam are excreted in urine mainly as glucuronide conjugates of oxazepam [22]. For flurazepam the main metabolite found in urine was the glucuronide conjugate of N-1-hydroxyethylflurazepam [22,27–29]. Oxazepam and N-1-hydroxyethylflurazepam could be analyzed with CE using a low pH in the electrophoretic buffer. The migration time in an electrophoretic buffer composed of 0.2 m*M* ammonium acetate adjusted with TFA to pH 1.3 and containing 15% (v/v) of methanol was 13 min for oxazepam and 11 min for N-1-hydroxyethylflurazepam at 20 cm with an applied voltage of 260 V/cm capillary. The capillary length for MS detection was 100 cm in the present instrumental set-up and the migration time for the compounds is thus about five times longer for API-MS detection. N-1-Hydroxyethylflurazepam was chosen for further studies owing to its predominance as a major metabolite of flurazepam and its shorter migration time. It should also be possible to detect oxazepam in human urine with the same approach as decribed below, but a shorter capillary would be preferred to shorten the overall analysis time.

A CE–MS total ion electropherogram for a synthetic mixture containing flurazepam and N-1-hydroxyethylflurazepam is shown in Fig. 5A. The amount injected was 2.2 ng for each compound and the electrophoretic buffer was 0.2 m*M* ammonium acetate adjusted with TFA to pH 1.3 and containing 15% (v/v) of methanol. The mass spectrometer was scanned from *m/z* 300 to 400. The mass spectra for peak 1 (Fig. 5B) and peak 2 (Fig. 5C) show *m/z* 388 and 333, respectively, as the most abundant ions, which correspond to the singly charged molecular ion $[M+H]^+$ for flurazepam and its metabolite, N-1-hydroxyethylflurazepam, respectively. For both compounds the isotope distribution expected for a compound containing one chlorine atom was observed.

Detection of N-1-hydroxyethylflurazepam in human urine

Urine samples were collected before and 2,4 and 7 h after the oral administration of 30 mg flurazepam dihydrochloride (one tablet of Dalmane) to a healthy volunteer. The major urinary metabolite of flurazepam, N-1-hydroxyethylflurazepam, accounts for 30–55% of an orally administered dose of flurazepam [27]. N-1-Hydroxyethylflurazepam is present mainly as the glucuronide conjugate, so the urine samples were first treated with β-glucuronidase, then extracted as described under Experimental and the extract was analyzed by CE–UV–MS.

The CE–UV and CE–MS electropherograms for the analysis of a human urine extract are shown in Fig. 6A and B, respectively. These data were obtained from the injection of the 4-h urine extract with an electrophoretic buffer of 0.2 m*M* ammonium acetate adjusted with TFA to pH 1.3 and containing 15% (v/v) of methanol. The

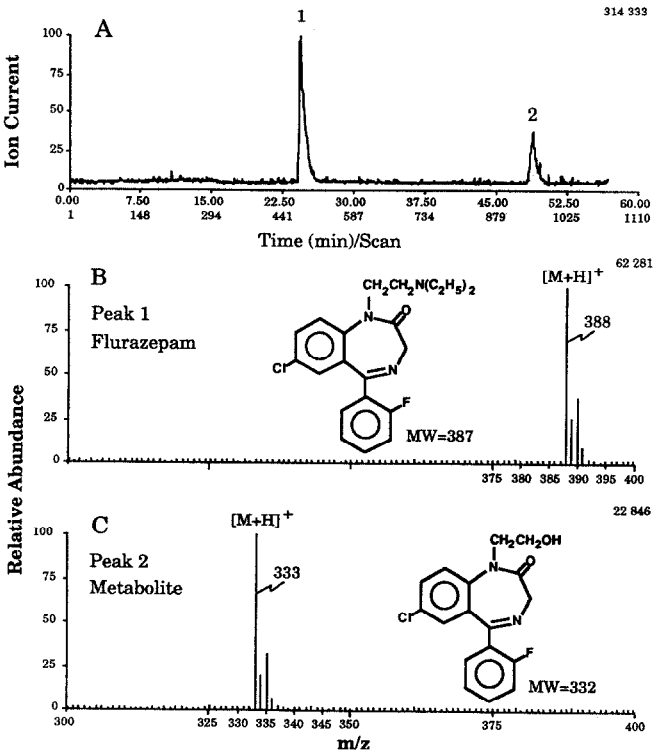

Fig. 5. (A) Full-scan CE–MS total ion current profile and mass spectra of (B) flurazepam and (C) metabolite. Capillary, uncoated fused silica 100 cm × 75 μm I.D. buffer, 0.2 m*M* ammonium acetate adjusted to pH 1.3 with TFA and containing 15% (v/v) of methanol; voltage, 26 kV; current, 8 μA; injection, 11 nl of a 200 μg/ml solution of each compound. Peaks: 1 = flurazepam (2.2 ng, 8 pmol); 2 = N-1-hydroxyethylflurazepam (2.2 ng, 7 pmol). MW = Molecular weight.

mass spectrometer was operated in the SIM mode, while the ions at m/z 388 for flurazepam and *m/z* 333 for N-1-hydroxyethylflurazepam were monitored. Only one component, corresponding to N-1-hydroxyethylflurazepam, was observed in the MS electropherogram (Fig. 6B). The concentration of N-1-hydroxyethylflurazepam in the 4-h urine was estimated to be 4 μg/ml from extracts of blank urine to which flurazepam and N-1-hydroxyethylflurazepam had been added prior to treatment with β-glucuronidase and extraction. Aliquots of 5.0 ml of urine were extracted with 1-chlorobutane and, after evaporation of the solvent, the residues were dissolved in 100 μl of electrophoretic buffer. Only 5 nl were injected, corresponding to about 1 ng of N-1-hydroxyethylflurazepam assuming a 100% recovery of N-1-hydroxyethylflurazepam via extraction by 1-chlorobutane. 1-Chlorobutane has been used in the screening procedure for basic drugs in blood or tissues prior to GC–MS analysis and has been shown to give clean extracts with up to 96.7% recovery of flurazepam in blood [30].

The third peak in the UV electropherogram observed at 254 nm (Fig. 6A) was N-1-hydroxyethylflurazepam. The first two peaks in Fig. 6A appear to be other metabolites of flurazepam present in the urine, as the extract of the urine collected before

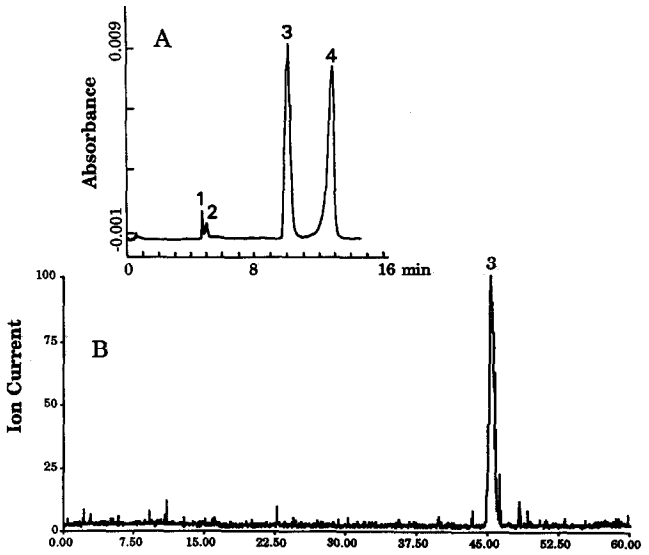

Fig. 6. (A) CE–UV and (B) CE–SIM-MS total selected ion current electropherogram from the analysis of an extract of human urine collected 4 h after the administration of a 30-mg oral dose of flurazepam dihydrochloride (one tablet of Dalmane). Injection volume, 5 nl; electrophoretic conditions as in Fig. 5. Peaks as in Fig. 7. (A) CE–UV electropherogram at 254 nm, detection after 20-cm capillary; (B) CE–SIM-MS total selected ion electropherogram, detection after 100 cm capillary.

administration of flurazepam (blank urine) did not show any peaks in this region. The blank urine extract was very clean, showing one peak corresponding to peak 4 in Fig. 6A. Peak 4 has the same migration time as a neutral compound (benzyl alcohol) and from this peak the electroosmotic flow and the electrophoretic mobility of individual components in the urine may be calculated. Peaks with the same migration times were found in the 7-h urine extract. The concentration of N-1-hydroxyethylflurazepam in the 7-h urine was estimated to be 0.5 μg/ml from extracts of blank urine to which 1-hydroxyethylflurazepam had been added. The *m/z* 333 peak observed in the MS electropherogram of the 7-h urine extract had a 3:1 signal-to-noise ratio and thus represents the practical detection limit for N-1-hydroxyethylflurazepam using the CE–MS system described. A detection limit of 0.5 μg/ml in urine has previously been reported for N-1-hydroxyethylflurazepam in a study of urinary metabolites of flurazepam in man by HPLC [29]. In CE–SIM-API-MS it would be possible to decrease the detection limit about tenfold by dissolving the extract residue in a smaller volume and introducing more than 5 nl of sample into the capillary column.

A full-scan CE–MS total ion current electropherogram of the 2-h urine extract was obtained (Fig. 7B). The on-line UV detector (Fig. 7A) indicated the presence of the same four components as observed in the 4- and 7-h urine extracts. The same electrophoretic buffer, 0.2 m*M* ammonium acetate adjusted with TFA to pH 1.3 and containing 15% (v/v) of methanol, was used and the mass spectrum was scanned from *m/z* 250 to 400. The peak at a migration time of 52 min corresponded to N-1-hydroxyethylflurazepam. Extracted ion current profiles of the $[M + H]^+$ ions for N-1-

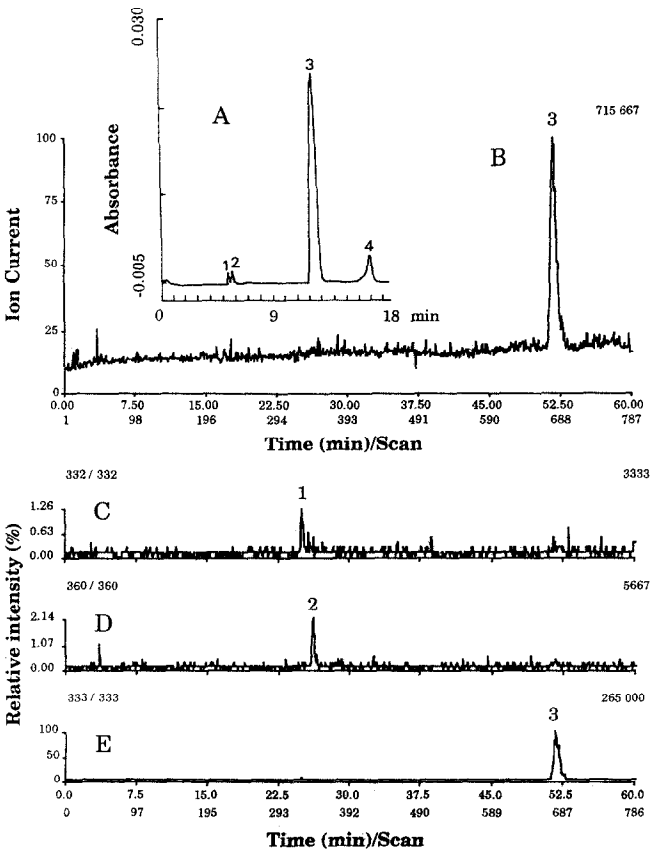

Fig. 7. (A) CE–UV and (B) full-scan CE–MS total ion current and (C–E) extracted ion current profiles from the analysis of an extract of human urine collected 2 h after the administration of a 30-mg oral dose of flurazepam dihydrochloride. (A) CE–UV electropherogram at 254 nm, detection after 20-cm capillary (B) full-scan acquisition from m/z 250 to 400, 5 s per scan, detection after 100-cm capillary. Peaks: 1 = didesethylflurazepam, m/z 332 $[M+H]^+$ (C); 2 = monodesethylflurazepam, m/z 360 $[M+H]^+$ (D); 3 = N-1-hydroxyethylflurazepam, m/z 333 $[M+H]^+$ (E); 4 = "neutral fraction" in the urine extract. No peak corresponding to flurazepam (m/z 388) was observed. Injection volume, 5 nl; electrophoretic conditions as in Fig. 5.

hydroxyethylflurazepam (Fig. 7E) and two possible metabolites of flurazepam, didesethylflurazepam at m/z 332 (Fig. 7C) and monodesethylflurazepam at m/z 360 (Fig. 7D), were observed. The flurazepam metabolites didesethylflurazepam and monodesethylflurazepam account for 0.5–13% of the dose in human urine [27]. No indication of the $[M+H]^+$ ion for flurazepam at m/z 388 was observed. The combination of UV and API-MS detection gave more information than could be obtained with only one of these detection methods. The UV electropherogram indicated the presence of two flurazepam metabolites (peaks 1 and 2 in Figs. 6A and 7A) and with API-MS detection the molecular weight of the metabolites could be determined and their identity proposed.

A change in migration time for N-1-hydroxyethylflurazepam was observed during the course of the day. This instability appeared to be due to column temperature variation. The first analytical determination in the morning produced longer migration times than those observed later in the day. We conclude that heat generated inside the unthermostated capillary resulted in decreased migration times during the course of the day owing to the decrease in viscosity of the buffer. The standard CE instrument used in this work normally thermostatically controls the entire electrophoretic capillary. For the CE–MS experiments described in this work, the thermostat control could not be utilized owing to the need to extend the capillary to the mass spectrometer. The electroosmotic flow-rate was 0.051–0.077 μl/min, calculated from the "neutral fraction" observed in the UV electropherograms. When MS is used for CE detection, small changes in migration times do not matter because the $[M + H]^+$ ion facilitates monitoring of the identity of the sample components. For unknown compounds MS–MS can facilitate the structural characterization of unknown compounds.

CONCLUSIONS

This study has shown that benzodiazepines and sulfonamides may be separated by CE with buffer systems compatible with the ion spray-API mass spectrometer system. The major metabolite of flurazepam in man, N-1-hydroxyethylflurazepam, can be monitored in human urine extracts after a single 30-mg oral dose of flurazepam dihydrochloride. The structural characterization of sulfamethazine was also demonstrated by CE–UV–MS–MS experiments.

It is an advantage to use UV detection on-line with API-MS detection for the analysis of human urine extracts containing flurazepam metabolites. The metabolites provide a good UV response at 254 nm, and the presence of metabolites other than N-1-hydroxyethylflurazepam was indicated in the UV electropherograms. With API-MS the identification of mixture component molecular weights was possible for the determination of these additional metabolites. Another advantage of the present CE–UV–API-MS system was the pressure injection system of the CE system, which allowed optimization of the position of the interface and MS tuning parameters while delivering a solution of a test analyte through the entire CE–UV–MS system. Rinsing of the capillary between analyses and sample injection could then be done without changing the position of the interface because the pressure injection system utilizes only the inlet end of the capillary.

In this study, small sample volumes (5–28 nl) were introduced into the CE capillary. The small injection volumes demand fairly concentrated samples to permit MS of dectection. For sulfonamides and the major metabolite of flurazepam, N-1-hydroxyethylflurazepam, their concentration in urine should be sufficient to allow analysis conditions does not appear to be practical at present unless the capillary column capacity or MS sensitivity is increased. It appears that the latter could be addressed by using an ion trap mass spectrometer equipped for ion spray sample introduction, and we are investigating this possibility [31].

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