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Analysis of Benzodiazepines in Dynamically Coated Capillaries by CE-DAD, CE-MS and CE-MS^2

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Abstract

The applicability of a low pH volatile electrolyte for fast analysis of benzodiazepines with CE-MS was investigated. The electrolyte is based on a commercially available CEofix buffer system that produces a substantial and highly reproducible electroosmotic flow through a dynamic double coating principle. The system was first evaluated with a mixture of benzodiazepine standards in CE-DAD and the electrolyte composition was further optimized for CE-MS. The LOD for the six selected benzodiazepines with CE-MS was ca. 100 ppb, except for diazepam, for which the LOD was lower than 50 ppb. RSDs varied from 0.51 to 1.02% ($n = 7$) for migration times and from 4.75 to 11.80% ($n = 7$) for peak areas. The method was successfully applied to the analysis of a spiked urine sample after solid-phase extraction (SPE). CE-MS^2 was performed on a standard mixture.

Keywords: Benzodiazepines; Capillary electrophoresis; Dynamic coating; Diode array detection; Mass spectroscopy

1. Introduction

Capillary electrophoresis (CE) is an alternative or complementary technique for HPLC separations. Due to its speed of analysis, high efficiency and low solvent and sample consumption, the technique has gained momentum in pharmaceutical and forensic research laboratories. MS detection has many advantages over other CE detection methods like DAD and fluorescence. MS is more sensitive than most other detectors for quantitation of target compounds, and moreover, is highly selective providing information on the identity of compounds.

The hyphenation of CE to MS combines the high speed and efficiency of CE with the selectivity and sensitivity inherent to MS. The combination of both techniques, however, is still cumbersome. Main problems are the increased analysis time, the lack of suitable volatile buffer systems and the poor repeatability, reproducibility and sensitivity. The prolonged analysis time is a consequence of the long capillary lengths that are needed to couple a CE instrument to

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the MS. This is not a problem as such, but counteracts one of the main advantages of CE, namely its speed. Another problem often encountered in CE-MS is the lack of suitable "volatile" buffer systems compatible with MS. Many of the electrolytes used in CE analyses with UV or DAD detection are inorganic and non-volatile in nature. From a separation point of view these electrolytes perform excellent, but are usually detrimental for CE-MS analyses. On the other hand, the separation performance of volatile electrolytes is often disappointing regarding efficiency and selectivity. The last and maybe the most important drawback of many CE-MS methods is the lack of repeatability and sensitivity.

Applying a buffer system that dynamically coats the inner wall of fused silica capillaries with a double layer in order to obtain fast and reproducible analyses can counteract most of these problems. The patented double coating principle of CEofix solutions is based on flushing two solutions through the capillary (Patent ref. No. 5,611,903). The CEofix principle is as follows. The buffer containing the polycation (called initiator) is flushed through the capillary. The polycations stick strongly to the capillary wall due to charge interactions. The capillary is then flushed with the running buffer containing the polyanion (called accelerator). These polyanions stick to the first layer of polycations forming a double layer. The polyanion layer contains sulphate groups and is rather insensitive to pH variations. By this procedure, a large number of negative charges are present at the capillary wall and consequently a high and reproducible electroosmotic flow (EOF) is generated when an electric field is applied across the capillary, even at low pH. After each analysis, the coating is stripped from the wall by a short rinse with NaOH followed by water. The dynamic coating is re-applied using the above procedure before the next analysis is started. The coating and rinsing procedures take about 2 min. Since the coating is dynamic and is replaced between every run, no memory effects occur.

The use of dynamic coating to create a high and pH-independent EOF has already been applied in the past. Bendahl et al. [1] used a polybrene/poly(styrenesulfonate) double coating system for the analysis of basic compounds by CE-DAD, MEKC and CE-MS. Graul and Schlenoff analyzed basic proteins by CE using a poly(diallyldimethylammonium)/poly(styrenesulfonate) coating [2].

In this contribution, the possibilities of CE-MS for the analysis of benzodiazepines applying the double coating principle are evaluated. The electrolyte composition is based on patented commercially available CEofix or CElixir solutions. The involatile phosphate present in the original buffer composition is replaced by formic acid. This modification, together with the use of some buffer additives, results in fast, reproducible and sensitive CE-MS analyses of the selected compounds. Benzodiazepines were chosen as test compounds for two reasons. The pK_a values for these compounds are low (ca. 1.3–4.0), so they are difficult to ionize. This means that CE analyses at low pH would lead to long analysis time when EOF is not present. Introducing EOF to the analysis will thus significantly speed up the analysis. However, the low pK_a value of some of the investigated compounds will inevitably lead to decreased efficiency and resolution in CE. A second reason for the choice of benzodiazepines as test compounds originates from their widespread use as pharmaceuticals against anxiety and sleep disturbances. Also, they are frequently abused and their analysis is of great interest from a toxicological point-of-view. Due to the extensive use and misuse of benzodiazepines, a lot of research has been performed on their analysis. Reviews on the analysis of benzodiazepines [3] or drugs in general [4] were published. Most of the work includes SPE to extract the target compounds and dispose of the matrix of the sample (usually blood, plasma, bile or urine). The extraction step is then followed by a separation step. HPLC is the most common technique for the determination of benzodiazepines [5–7] and, when combined with MS, becomes a powerful tool for quantitative analysis as well as confirmation of the identity of the benzodiazepines present. The sensitivity and selectivity of LC-MS has made the technique very popular [8–17] and reviews on this hyphenated technique have been published [18,19]. In the search for alternatives for the analysis of benzodiazepines for toxicological screening purposes, CE methods have also been developed. Most analyses were performed with MEKC [20–27], others used CEC [28,29] or CE [14,25,30]. The use of CE in forensic toxicology was reviewed by Tagliaro et al. [31] and the application to benzodiazepines by Smyth and McClean

[32]. Hudson et al. [33] developed a comprehensive CE-DAD method to monitor over 400 basic drugs in whole blood, amongst which some benzodiazepines. In a later publication, the list was updated to over 550 basic and 100 acidic drugs [34]. The hyphenation of CE with MS is not as developed and exploited yet as LC-MS. Although much effort has already been put in the development of instrumentation and methods for CE-MS, LC-MS still performs significantly better regarding reproducibility and robustness. Initial results look very promising and show the potential of CE-MS for the analysis of benzodiazepines [14,29,35,36].

2. Experimental

2.1. Chemicals and standards

The benzodiazepine standards (Fig. 1) were dissolved separately in methanol (1000 ppm, stock solution). The solutions were diluted with water (unless stated otherwise) and mixed prior to injection. The water used for sample dilution and make-up liquid was LC-grade from Merck (Darmstadt, Germany). Other solvents were all LC-grade and purchased from Riedel-de Haën (Seelze, Germany). The standard electrolytes and the volatile and non-volatile CE-ofix buffer solutions were from Analis S.A. (Namur, Belgium). Trifluoroacetic acid (TFA) and ammonia (25%) were from Sigma (Bornem, Belgium).

2.2. CE-DAD

CE was carried out on a P/ACE MDQ capillary electrophoresis instrument equipped with DAD detector (Beckman Coulter, Fullerton, CA, USA). All separations were carried out in 75 μm i.d. bare fused silica capillaries (Composite Metal Services, Worcester, UK). The total length of the capillary was 60.2 cm (50.2 cm to detector) and the applied voltage was 18 kV. Injections were performed hydrodynamically at 1 psi for 5 s. The capillary temperature was set at 25 °C and detection was performed at 200 and 240 nm. The capillary rinsing steps were performed at 20 psi. When a new capillary was installed, it was rinsed with NaOH (1 M, 10 min) and water (5 min) prior to the first analysis. When a buffer without accelerator was used, the capillary was first rinsed with NaOH (0.1 M, 1 min), water (1 min) and buffer (2 min) before the first analysis. Between analyses, the capillary was rinsed with the running buffer (2 min). For an electrolyte with accelerator, the capillary was first rinsed with NaOH (0.1 M, 0.5 min), water (0.5 min), initiator solution (0.2 min) and accelerator (0.5 min) before

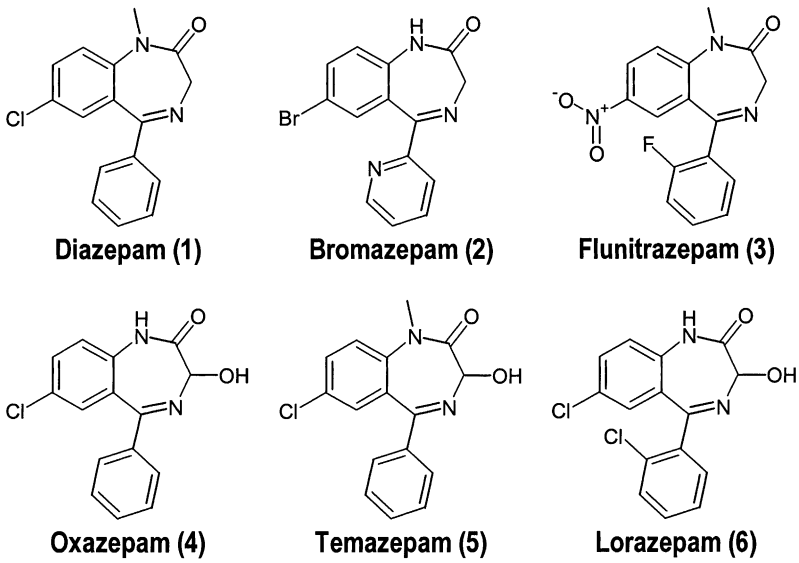

Fig. 1. Structures and peak numbers of the selected benzodiazepines.

the first analysis with this buffer. Between runs, the capillary was only rinsed with accelerator (0.5 min).

2.3. CE-MS

For the CE-MS experiments, the standard capillary cartridge was replaced with an external detector adaptor (EDA) cartridge from Beckman Coulter. This enables the outlet of the CE capillary to be inserted into the mass spectrometer. MS was performed on a LCQ ion trap mass spectrometer equipped with an ESI source (ThermoFinnigan, San Jose, CA, USA). The ionization source was adapted for CE-MS with a special ESI needle and a micrometer to fit standard CE capillaries and to enable precise positioning of the capillary outlet, respectively (ThermoFinnigan). A syringe pump installed on the MS instrument delivered the make-up liquid. The CE instrument was placed on a platform adjustable in height and position to avoid siphoning effects.

Analyses were carried out in 75 μm i.d. bare fused silica capillaries (Composite Metal Services) with a length of 93.5 cm. Injections were performed hydrodynamically at 2 psi for 5 s. DAD detection was bypassed. The applied CE voltage was 30 kV. The capillary temperature was set at 25 °C inside the CE instrument (ca. 40 cm). The section between the CE instrument and the MS (ca. 50 cm) was not thermostatted. Between analyses, the capillary was rinsed with the running buffer (2 min) when a buffer without the accelerator was applied. If an electrolyte with accelerator was used, the capillary was first rinsed, with the ionization source open, with NaOH (0.1 M, 1 min), water (1 min), initiator solution (0.5 min) and accelerator (0.5 min) before the first analysis. Then the source was closed and the first analysis started. Before each run, the capillary was only rinsed with accelerator (0.7 min) with the source closed.

MS detection was performed in the ESI positive ionization mode. The scan range was 100–400 atomic mass units (amu). The outlet of the capillary was precisely positioned equal with the ESI spray needle set at 5 kV (net voltage over the CE capillary is therefore 25 kV). During injection and CE voltage build-up this voltage was set to 0 kV. The heated capillary temperature was 160 °C. Nitrogen was used as sheath gas at 20 units (0.3 l/min) and no drying gas was used. The make-up flow was composed of methanol-water (80:20, v/v) containing formic acid (0.5%, v/v) and was delivered at a flow rate of 2 μl/min. The make-up liquid was degassed daily in an ultrasonic bath. For CE-MS^2 experiments, the trap collision induced dissociation (CID) voltage was set at 25% (1.25 V). The mass spectrometer was set to perform MS^2 on the molecular ion for the selected compounds.

2.4. Solid-phase extraction (SPE)

Benzodiazepines were added to a blank urine sample (10 ml) at a concentration of 0.5 ppm each. The pH of the sample was adjusted with ammonia to pH 10. Ultra-clean C18 cartridges (500 mg, 8 ml) from Alltech (Lokeren, Belgium) were conditioned with methanol (2 × 3 ml) and water (pH 10 with ammonia, 2 × 3 ml) consecutively. The sample was loaded on the cartridge at ca. 1 ml/min, the cartridge was rinsed with water (pH 10 with ammonia, 2 × 2 ml) and was left to dry for 3 min. The compounds were eluted with methanol (3 ml). The collected solvent was evaporated under nitrogen and the residue was redissolved in 1 ml LC-grade water.

3. Results and discussion

3.1. CE-DAD

Initial experiments were carried out with DAD detection to investigate the performance of various electrolytes for the analysis of the selected benzodiazepines and to evaluate various rinsing procedures. Analysis of these compounds is mostly carried out with low pH buffers. At the applied pH, the EOF is minimized leading to long analysis times and low signal-to-noise ratios.

These drawbacks could be overcome using the double coating procedure (CEofix) involving a rinse of the capillary with a buffered polycation followed by a rinse with a buffered polyanion. The double coating generates the same number of negative charges at the wall whatever pH, ensuring reproducible migration times. Several CEofix buffers were developed covering a pH-range from 2.5 to 9.2. These buffers are composed of phosphate and malic acid based electrolytes to which a polycation and a polyanion are added. A CEofix buffer system thus contains two

solutions, namely a solution containing the electrolyte and the polycation (initiator solution) and a solution containing the electrolyte and the polyanion (accelerator solution). All buffer solutions provide a high and stable EOF, independent of the pH. Optimization of the buffer can easily be done by fine-tuning the pH and by adding organic modifiers and/or surfactants or cyclodextrins.

The commercially available CEofix solutions are all composed of non-volatile electrolytes at high concentrations (typically 50–150 mM). They are therefore not suited for the hyphenation of CE to MS. It was the aim of this work to develop an MS compatible, i.e. volatile, CEofix solution. An electrolyte based on formic acid was chosen for this purpose. The influence of the accelerator in a formic acid solution (100 mM) on the analysis of a standard solution of three benzodiazepines was investigated. In a first series of experiments, the capillary was rinsed with NaOH (0.1 M), water and the formic acid solution as such (pH 2.4) and the standards were analyzed with the same electrolyte. The same analysis was performed using a formic acid solution that was modified for dynamic coating. Prior to analysis, the capillary was rinsed consecutively with NaOH (0.1 M), water and a formic acid solution containing the polycation (initiator). Then the capillary was flushed with a formic acid solution containing the polyanion (accelerator) and the benzodiazepine standards were analyzed with this electrolyte solution. The analysis time decreased ca. 4 times using the electrolyte solution containing the accelerator and trimethylamine. For a 60.2 cm capillary and an applied voltage of 18 kV, the analysis time using the accelerator solution was ca. 5.5 min compared to ca. 22 min when the formic acid as such was used (Fig. 2). The migration time decrease is largest for lorazepam because this is the compound with the lowest mobility. The peak shape for diazepam and bromazepam is satisfactory. Lorazepam elutes close to the EOF because it is hardly protonated ($pK_a = 1.3$) under the applied conditions. This also adversely affects the efficiency for this peak. The detection wavelength was set at 240 nm for the analysis with accelerator in the formic acid solution because the baseline at 200 nm was unstable with this buffer.

It is known that benzodiazepines are difficult to analyze with electrodriven separation methods carried out in free solution. The low pK_a value of most benzodiazepines [14,24,25] leads to poor ionization at pH 2.4 and, consequently, a lack of efficiency and electrophoretically based selectivity. The addition of micelles to the electrolyte causes zone sharpening resulting in an increase in efficiency as compared to CE [25]. MEKC has successfully been applied to the analysis of benzodiazepines [20–27]. However, the addition of surfactants such as sodium dodecylsulphate, bile salts or quaternary ammonium salts to form micelles renders the electrolyte unsuitable for on-line MS detection. The surfactant molecules drastically decrease the sensitivity and contaminate the MS during prolonged operation. The main goal of this study was to demonstrate the gain in analysis time produced

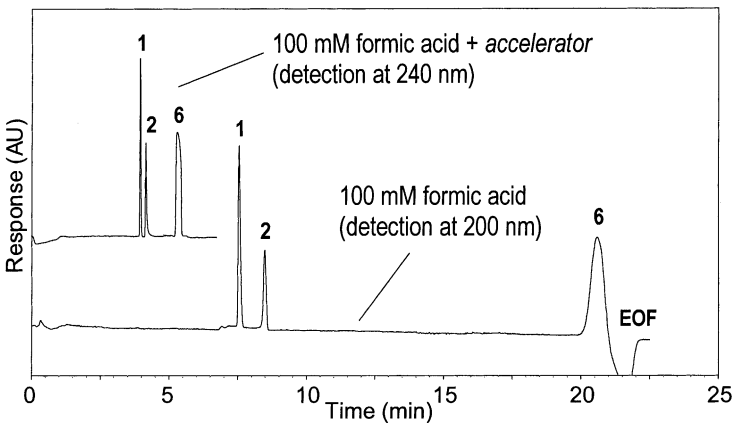

Fig. 2. CE-DAD analysis of a mixture of three benzodiazepines (20 ppm each). Operating parameters: see Section 2. Peak identity: see Fig. 1.

by the double coating principle and its applicability to MS detection. No attempts were made to further improve the separation of the benzodiazepines.

The standard rinsing procedure for analysis with CEofix solutions involves a rinsing step with NaOH (0.1 M), water, initiator solution (polycation), and accelerator solution (polyanion). Since it was the aim to couple the CE method with MS, an alternative rinsing procedure was developed. The frequent introduction of NaOH and polycation into the MS is detrimental for MS detection. If the complete rinsing procedure would be performed between runs, this would imply that the MS source would have to be opened during this rinsing procedure, or that a flow diverting system would have to be introduced between the CE capillary and the MS.

The experiments were performed on a standard mixture of 5 basic drugs using CE-DAD (results not shown) [37]. The stability of migration time of the last eluting drug in the mixture (trazodone) was the investigated factor for each rinsing procedure. Before the start of a sequence, the complete rinsing procedure for a CEofix buffer solution was carried out (see Section 2). Between analyses, the alternative rinsing procedure was performed. In a first test, the NaOH rinsing step was replaced by a rinsing step with NH_4OH (0.1 M, 0.5 min). A second experiment was performed in which the NaOH rinsing step was left out and the rinsing step with water was prolonged (1 min instead of 0.5 min). In a third test, the capillary was only rinsed with the accelerator solution (0.5 min) between runs. The results are summarized in Table 1. With the last procedure, migration time and peak area were stable for at least 28 consecutive injections. RSDs on migration times and peak areas were 0.25% ($n = 28$) or less and below 2.40% ($n = 28$), respectively, for the selected drugs.

3.2. CE-MS

With the volatile accelerator solution and the adapted rinsing procedure, sequences of samples could be analyzed with CE-MS without opening the MS source in between runs. In summary, before the first injection the complete rinsing procedure is performed with the MS source opened. Between runs, the capillary is only rinsed with the accelerator solution for 1 min without opening the MS source.

A standard solution containing six benzodiazepines was analyzed by CE-MS using a formic acid solution (100 mM) after flushing the capillary with this electrolyte solution. The same mixture was analyzed using the formic acid solution to which the accelerator was added. For the latter, the capillary was first flushed with the source open with NaOH, water and the electrolyte containing initiator. The ion source was then closed and the capillary was rinsed with the run buffer containing accelerator prior to injection. An example of an analysis with and without accelerator of a standard mixture of benzodiazepines is depicted in Fig. 3. The gain in analysis time as already observed in the CE-DAD experiments is obvious. This leads to an increased signal-to-noise ratio and consequently improved sensitivity compared to the analysis with the electrolyte without accelerator. Again, the efficiency and resolution of compounds with low pK_a values

Table 1
Stability of migration time for basic drugs in CE-DAD with different rinsing procedures

Rinsing procedure	t_m 1st injection (min)	t_m nth injection (min)	Δt_m (%)
Complete procedure	2.717	2.701 ($n = 10$)	−0.59
0.1 M NH_4OH (0.5 min) instead of 0.1 M NaOH (0.5 min)	2.721	2.943 ($n = 10$) 3.038 ($n = 20$)	+8.12 +11.65
No NaOH, water (1 min) instead of (0.5 min)	2.654	3.275 ($n = 5$)	+23.40
Only accelerator (0.5 min)	2.678	2.646 ($n = 10$) 2.650 ($n = 28$)	−1.19 −1.05

The migration time of a trazodone (15 ppm) was monitored. Electrolyte: formic acid (100 mM) + trimethylamine (pH 3.7) + accelerator, capillary: 75 μm i.d. × 40.2 cm L_{tot}, voltage: 12 kV, detection: UV at 200 nm. Other operating parameters: see Section 2.

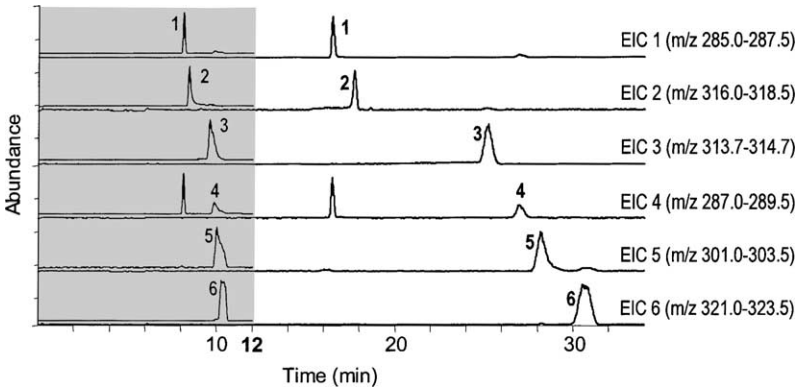

Fig. 3. CE-MS analysis of a mixture of six benzodiazepines (5 ppm each). Electrolyte: gray area: formic acid–TFA (100–1 mM) + accelerator, blank area: formic acid–TFA (100–1 mM). Other operating parameters: see Section 2. EIC = extracted ion chromatogram. Peak identity: see Fig. 1.

(compounds 3–6, pK_a = 1.3–1.7) is significantly lower than for compounds 1 and 2 that have larger pK_a values. Although not all peaks were resolved under the applied analytical conditions, the molecular ions could be extracted from the electropherogram to separate the compounds post-analysis.

A small amount of trifluoroacetic acid (TFA) was added to the accelerator solution to improve MS sensitivity. A TFA concentration of 1 mM was chosen because this amount resulted in good sensitivity and acceptably low CE current. The CE current has to be kept as low as possible (preferably below 20 μA) because high currents can lead to problems at the ESI interface and the ESI needle voltage. The addition of TFA significantly enhances the sensitivity for the selected benzodiazepines (Fig. 4). The effect is more pronounced for the compounds that are more difficult to ionize, i.e. the compounds with a longer migration time.

With the MS detector used in this work, the voltage in the ESI interface is applied on the spray needle while the heated capillary is grounded. Since the CE capillary is inserted into this needle, electrical contact is established between the CE inlet electrode and the MS spray needle. When no voltage is applied across the CE capillary, an electric field is generated across the capillary due to the voltage present on the spray needle. This leads to the generation of an EOF and the

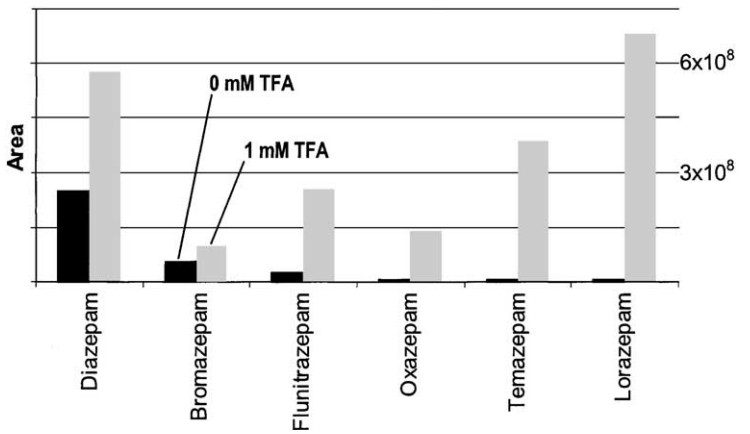

Fig. 4. Influence of the addition of TFA to the electrolyte solution on peak area for CE-MS analysis of benzodiazepines (5 ppm each). Operating parameters: see Section 2.

migration of analytes towards the injector. Care has to be taken to minimize this effect in order to maintain sensitivity and repeatability. Therefore, during injection and CE voltage build-up, the spray needle voltage was set at 0 kV. If this is not done, the benzodiazepines are hardly detected, even at the 5 ppm level. The effect is so drastic because of the high EOF generated by the double coating. In the case of the benzodiazepines, this effect is more pronounced for the compounds that are more protonated under the analytical conditions (diazepam and bromazepam) than for the less mobile benzodiazepines (lorazepam).

The performance of the volatile CEofix solution and analytical method was tested by 10 consecutive injections of a 5 ppm standard mixture with the formic acid–TFA (100–1 mM) solution containing accelerator and the adapted rinsing procedure. Migration times stabilize after 3 analyses. From then on, the system is stable for at least 7 more analyses. The RSDs varied from 0.51 to 1.02% ($n = 7$) for the migration time and from 4.75 to 11.80% ($n = 7$) for the peak area. The LOD (3 times signal-to-noise) is ca. 100 ppb for the selected benzodiazepines, except for diazepam, for which the LOD is less than 50 ppb. A calibration curve was set up in the range of 0–5 ppm at 4 concentration levels. Each concentration level was analyzed 2 times. Correlation coefficients were above 0.999 for all compounds. These results give an indication on the quantitative possibilities of the method.

SPE was carried out on a urine sample (10 ml) spiked with 0.5 ppm of each benzodiazepine. After SPE, the residue was redissolved in 1 ml water. The recovery of the extraction procedure was calculated by comparing the peak area of the basic drugs in the SPE extract with the peak area of the benzodiazepines in a standard solution of 5 ppm. The recoveries for the selected benzodiazepines varied between 29% (flunitrazepam) and 83% (oxazepam). All benzodiazepines were detected and identified based on the mass spectra (Fig. 5).

Commonly, samples are dissolved in buffer solution for analysis with CE. A problem was observed with a diazepam standard solution that was dissolved in an acidic solvent. An earlier analysis of the standard with CE-DAD using the commercial CEofix phosphate solution at pH 2.5 revealed 2 distinct peaks. The UV spectra for these closely eluting compounds were very similar but not completely identical. The main peak was assigned to diazepam based on the migration time and UV spectrum. The other peak, eluting in front of diazepam, could not be identified. This peak was not detected when MEKC or LC was performed on the sample. The question was raised if the additional peak originated from degradation/modification

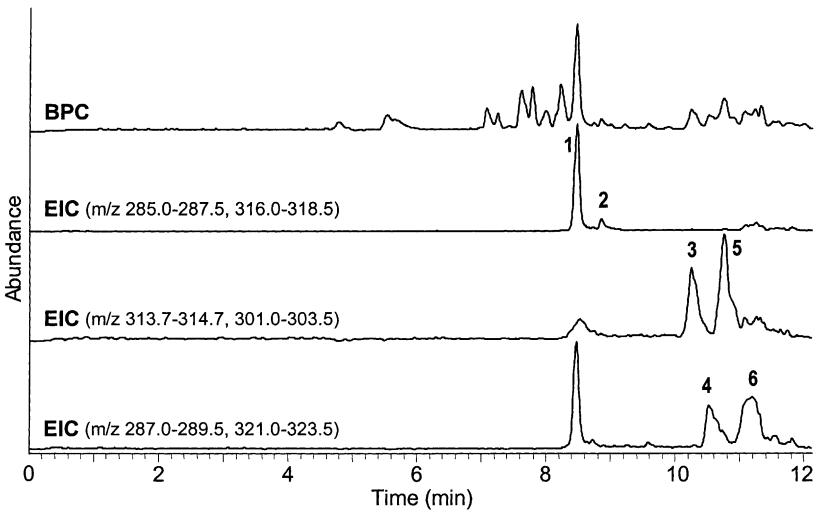

Fig. 5. CE-MS analysis of a urine extract spiked with a mixture of six benzodiazepines (0.5 ppm each). Electrolyte: formic acid–TFA (100–1 mM) + accelerator. Other operating parameters: see Section 2. BPC = base peak chromatogram, EIC = extracted ion chromatogram. Peak identity: see Fig. 1.

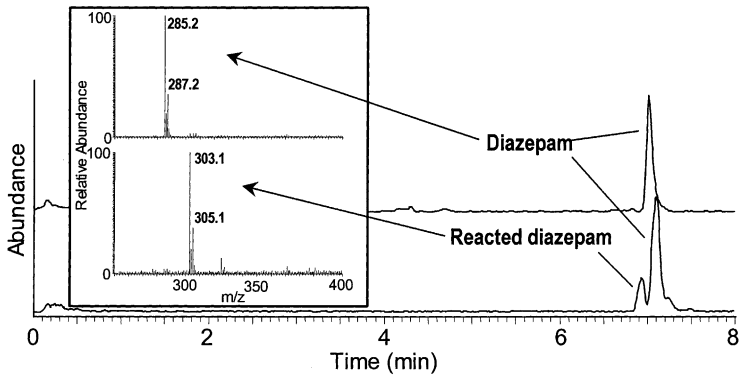

Fig. 6. CE-MS analysis of a diazepani standard (4 ppm) dissolved for 3 h in water (upper trace) or formic acid (50 mM, lower trace). Inserts: mass spectra of diazepam and reacted diazepam. Electrolyte: formic acid–TFA (100–1 mM) + accelerator. Other operating parameters: see Section 2.

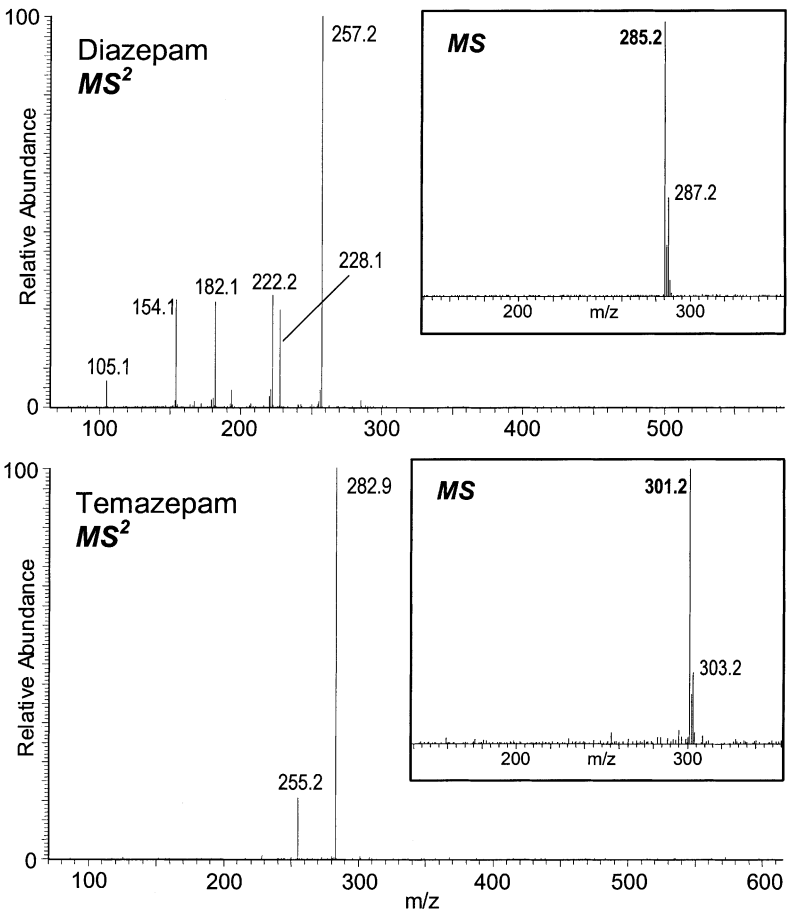

Fig. 7. Examples of MS and MS^2 spectra from CE-MS and CE-MS^2 analysis, respectively (MS^2 on the molecular ion of the compound, 5 ppm). Electrolyte: formic acid–TFA (100–1 mM) + accelerator. Other operating parameters: see Section 2.

of the compound in solution or if it was an impurity present in the diazepam standard. Benzodiazepines are known to hydrolyze in acidic solutions to form a benzophenone [3]. For diazepam, the corresponding benzophenone is 2-methylamino-5-chloro-benzophenone (MACB). The sample was analyzed with CE-MS using the formic acid solution with accelerator. Two peaks were observed. Comparison of the MS spectra showed that the molecular weight of the additional peak is 18 amu higher than the molecular weight of diazepam. The impurity thus is not MACB. Instead, the increase in molecular weight is indicative for the addition of water to the molecule. The influence of the acid in the sample solution was investigated by dissolving a pure diazepam standard in formic acid (50 mM) and in water. These solutions were analyzed immediately and after 1 and 3 h. The results clearly show that diazepam is modified in time when it is dissolved in an acidic medium. No modification was observed when the compound was dissolved in water (Fig. 6).

3.3. CE-MS^2

CE-MS^2 was performed on the standard mixture of benzodiazepines. This allows unequivocal confirmation of the presence of the substance in a sample based on the migration time, molecular weight and fragment ions. The CID voltage was optimized for optimal fragmentation of the molecular ion. This was done by infusing a 10 ppm standard solution at 2 μl/min into the mass spectrometer, and ramping the CID voltage during this operation. A voltage of 1.25 V produced the necessary ions with the best sensitivity. After CID optimization, CE-MS^2 was performed on a 5 ppm standard mixture using the formic acid–TFA (100–1 mM) electrolyte with accelerator and the analytical conditions described above. The mass spectrometer was set

Table 2
MS and MS^2 data collected with CE-MS for the selected benzodiazepines (5 ppm each)

Compound (mass)	MS-ions (rel. intensity)[a]	MS^2-ions (rel. intensity)	Identity MS^2-ions
Diazepam (284.1)	**285.2 (100)**	154.1 (27)	
	287.2 (36)	182.1(27)	
		222.2 (29)	
		228.1 (25)	$[M+H-CO-CH_3N]^+$
		257.2 (100)	$[M+H-CO]^+$
Bromazepam (315.0)	**316.2 (98)**	182.2 (7)	
	318.2 (100)	209.4 (14)	
		242.0 (9)	
		260.9 (15)	
		288.0 (100)	$[M+H-CO]^+$
Flunitrazepam (313.1)	**314.3 (100)**	240.1 (14)	$[M+H-CO-NO_2]^+$
		257.2 (9)	$[M+H-CO-CH_3N]^+$
		268.1 (100)	$[M+H-NO_2]^+$
		286.1 (48)	$[M+H-CO]^+$
		314.2 (11)	$[M+H]^+$
Oxazepam (286.1)	**287.2 (100)**	241.3 (7)	$[M+H-CO-H_2O]^+$
	289.2 (35)	269.0 (100)	$[M+H-H_2O]^+$
Temazepam (300.1)	**301.2 (100)**	255.2 (15)	$[M+H-CO-H_2O]^+$
	303.2 (27)	282.9 (100)	$[M+H-H_2O]^+$
Lorazepam (320.0)	303.2 (9)	275.2 (19)	$[M+H-CO-H_2O]^+$
	321.1 (100)	302.9 (100)	$[M+H-H_2O]^+$
	323.1 (63)		
	325.1 (12)		

Electrolyte: formic acid–TFA (100–1 mM) + accelerator. Other operating parameters: see Section 2.
[a] Ion in bold (molecular ion) was taken for fragmentation in MS^2.

to perform MS^2 on any of the molecular ions originating from the compounds in the test mixture. For this reason, each scan is divided into 2 subscans. In a first stage, MS is performed. If any of the predetermined molecular ions is detected at a certain level, MS^2 is performed on this ion in the trap. A drawback of this operation is the significant loss of signal intensity. However, since the MS spectra originate from a single ion, the background in these spectra is very low.

Examples of recorded mass spectra are shown in Fig. 7 and details on the fragments [35] are given in Table 2. The obtained spectra were in good agreement with the spectra obtained in the infusion experiments and can thus be used for confirmation of the identity of the benzodiazepines in real samples.

4. Conclusions

The low pH volatile formic acid electrolyte with accelerator results in fast and reproducible CE analysis of benzodiazepines. With an alternative rinsing procedure for dynamic double coating of the capillary wall, the method could be transferred to CE-MS. Sequences can be run fully automated without opening the ionization source during rinsing steps. Addition of TFA led to significantly increased sensitivity with LODs of 100 ppb or less for the selected benzodiazepines. Migration times of the benzodiazepines were highly reproducible with RSDs of 1.02% or lower ($n = 7$). The results clearly show the potential of the method for analyzing real samples. The method was successfully applied to the analysis of a spiked urine sample after SPE. On-line CE-MS^2 was performed on the standard mixture. The resulting MS^2 spectra were in good agreement with the infusion based and published data. This allows the unequivocal confirmation of the presence of a certain benzodiazepine in a sample and is useful for toxicological and forensic purposes.

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