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Confirmation testing of amphetamines and designer drugs in human urine by capillary electrophoresis-ion trap mass spectrometry

Monitoring of amphetamines and designer drugs in human urine is a timely topic in clinical toxicology, surveillance of drug substitution, forensic science, drug testing at the workplace, and doping control. Confirmation testing of urinary amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) and 3,4-methylenedioxyamphetamine (MDA) by capillary electrophoresis (CE) combined with atmospheric pressure electrospray ionization and ion trap mass spectrometry (MS) is described. Using an aqueous pH 4.6 buffer composed of ammonium acetate/acetic acid, CE-MS and CE-MS^2 provided data that permitted the unambiguous confirmation of these drugs in external quality control urines. Furthermore, other drugs of abuse present in alkaline urinary extracts, including methadone and morphine, could also be monitored. The data presented illustrate that the sensitivity achieved with the benchtop MS is comparable to that observed by CE with UV absorption detection. CE-MS^2 is further shown to be capable of identifying comigrating compounds, including the comigration of amphetamine with nicotine.

Keywords: Capillary electrophoresis / Amphetamine / Methamphetamine / Designer drugs / Methadone / Confirmation testing / Multiwavelength detection / Capillary electrophoresis - mass spectrometry
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1 Introduction

Many immunoassays and chromatographic methods have been developed as instrumental approaches for the analysis of drugs of abuse in body fluids. Immunological techniques are attractive because of their ease of performance and speed of analysis. Thus, they are typically employed for screening purposes. Chromatographic methods are based upon solute separation and are thus typically used for confirmatory testing [1–4]. During the past decade, instrumentation for electrokinetic separations in fused-silica capillaries of very small ID (25–75 μm) has become available and has been found suitable for drug monitoring in body fluids [5–7]. A comprehensive concept for toxicological drug screening and confirmation by capillary electrophoresis (CE) has been developed and successfully applied to the monitoring of drugs of abuse in patient urines [8–10]. In that approach, confirmation testing is based upon on-column, fast scanning UV absorption detection of the solutes and comparison of extracted normalized spectra with those of standards.

In analogy to the use of gas chromatography - mass spectrometry (GC-MS; [11]) and liquid chromatography - mass spectrometry (LC-MS; [12, 13]) in instrumental analysis, hyphenation of CE with MS (CE-MS) has been shown to be an attractive approach to gather structural information of compounds [14–16]. Not surprisingly, first applications dealing with drug monitoring appeared in the literature. CE-MS determination of urinary *N*-1-hydroxyethylflurazepam (major metabolite of flurazepam) [17], haloperidol [18], β-agonists [19], nonsteroidal anti-inflammatory drugs and some of their metabolites [20], paracetamol and metabolites [21–23], nonopioid analgesics [23] and methylphenidate [24] has been reported, work that was mainly geared towards the elucidation and confirmation of drug metabolism. Furthermore, application of CE-MS to urinary confirmation testing of methadone (MET) and its major urinary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), has been described [25].

Monitoring of amphetamines and designer drugs, including amphetamine (A), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA, Exstasy) and 3,4-methylenedioxyamphetamine (MDA), in human urine is a timely topic in clinical toxicology, surveillance of drug sub-

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Abbreviations: A, amphetamine; **EDDP**, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; **MA**, methamphetamine; **MDA**, 3,4-methylenedioxyamphetamine; **MDMA**, 3,4-methylenedioxymethamphetamine; **MET**, methadone; **MO**, morphine; **MWD**, multiwavelength detection; **NIC**, nicotine; **SRM**, selected reaction monitoring

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stitution programs, forensic science, drug testing at the workplace, and doping control. In previous communications from our laboratory, analysis of these substances in urine by various capillary electrophoretic techniques with UV or fluorescence detection was described [26–28]. Furthermore, separation and identification of model mixtures of designer drugs by CE-MS [29] and identification of amphetamine and related drugs in fortified, diluted urine by CE-MS [30] have been reported.

A benchtop ion trap MS featuring MS^n ($n \leq 9$) capability is now commercially available. Recently, this instrument was demonstrated to be suitable for urinary drug analysis [22–24]. To our knowledge, the application of CE-MS to urinary confirmation testing of amphetamines and designer drugs has been proposed repeatedly but has not yet been explored. In this paper, analysis of urinary amphetamine and analogs by CE-ion trap MS (in the CE-MS and CE-MS^2 formats) is described and compared to CE with on-column multiwavelength absorption detection (CE-MWD). CE-MS^2 is also shown to permit the confirmation of other basic compounds, including MET, EDDP, morphine (MO) and nicotine (NIC), and the distinction between comigrating substances.

2 Materials and methods

2.1 Chemicals, standard solutions, origin of urine samples, and urine storage

All chemicals used were of analytical or research grade. D-(+)-amphetamine, racemic MA (as sulfate and hydrochloride, respectively), MO and MET (as base and hydrochloride, respectively) were from the University Hospital Pharmacy (Bern, Switzerland). MDMA, MDA and EDDP were from Alltech (State College, PA, USA). Aqueous stock solutions of about 1 mg/mL of each base were prepared, stored at –18°C, and mixed and diluted with water to appropriate concentrations before use. External quality control urines were purchased from Cardiff Bioanalytical Services (UKNEQAS for drugs of abuse, Cardiff, UK). The samples were stored at –18°C until analysis. Our own urines were used as blank matrices. Before analysis, the urines were centrifuged at about 1500 × *g* for 10 min or filtered through disposable 0.45 μm filters (model FP 030/2; Schleicher & Schuell, Keene, NH, USA).

2.2 Preparation of urines for CE analysis

Urine samples (2 mL) were extracted at pH 9 using Toxi-Tubes A (Analytical Systems, Laguna Hills, CA, USA). This commercial liquid/liquid extraction system comprises an organic solvent mixture composed of CH_2Cl_2 and $C_2H_4Cl_2$. After vigorously shaking for about 30 s and centrifugation (1500 × *g*, 10 min), the organic phase was transferred into a clean glass tube, two drops of 2 M acetic acid in ethylacetate were added, and the organic solvent was evaporated under a gentle stream of nitrogen in a water bath at 35°C. The residue was reconstituted in 100 μL water. Extraction recoveries for A, MA, MDA, MDMA, EDDP and MET were determined to be above 70% [26].

2.3 Electrophoretic instrumentation and running conditions for CE-MWD

A BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) was employed. If not otherwise stated, it was equipped with an untreated 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 60 cm total length (55.4 cm to the detector) which was mounted in a user-assembled cartridge (Bio-Rad), sample was injected by applying positive pressure of 4 psi × s, a constant voltage of 20 kV (current about 15 μA) was applied, the temperature of cartridge and carousel were maintained at 20°C, and detection was effected *via* fast scanning at 5 nm resolution in the range between 195 and 320 nm. The single wavelength electropherograms depicted are those registered for 195 nm. Before each experiment the capillary was rinsed with 0.1 M NaOH for 2 min, water for 2 min, and running buffer for 4 min. BioFocus Integration software (Version 5.2; Bio-Rad) was employed for data conversion and evaluation. The buffer employed was composed of ammonium acetate/acetic acid (20 mM each, pH 4.6). The outlet buffer vial comprised 1% acetic acid in a mixture of methanol and water (60:40 v/v).

2.4 Electrophoretic instrumentation and running conditions for CE-MS

For mass spectrometric detection a Finnigan LCQ ion trap MS was used (Finnigan MAT, San Jose, CA, USA). The standard coaxial interface was replaced against the commercial CE interface (Finnigan MAT) using an additional sheath liquid flow of 3 μL/min to stabilize the electrospray. The sheath liquid was composed of methanol, water, and acetic acid 60/39/1 v/v/v. The N_2 sheathflow was set to 20 units. The electrophoretic separation was done on a Cristal CE system Model 310 (Prince Technologies, Emmen, NL) that was equipped with an untreated 75 μm ID fused-silica capillary of 70 or 80 cm length (360 μm OD; SGE, Weiterstadt, Germany). Sample was introduced by applying a positive pressure of 50 or 100 mbar for 6 or 12 s. A separation voltage of 30 kV was used. Due to the electrospray voltage of 4 kV, the effective separation voltage was 26 kV. After each run the fused-silica capillary was rinsed with separation buffer for 2 min. The separation

buffer for CE-MS was the same as that employed above for CE-MWD. Full-scan mass spectra were acquired in the mass ranges 100–300 or 100–500 Th using automatic gain control (AGC) with three microscans and a maximum injection time of 200 ms. The capillary temperature was 200°C. For MS^2, in some cases data-dependent scans with dynamic exclusion were performed with a relative collision energy of 18% and an isolation width of 2 Th. In these experiments the instrument automatically switches to MS^2 as soon as a mass peak exceeds a predefined threshold. Using this technique, identification of a compound by its mass and confirmation of its structure is possible in one run without prior knowledge of masses or migration times. Protonated ions are identified by *m/z* values of ± 0.1 Th.

3 Results and discussion

3.1 CE-MWD

The single wavelength data presented in Fig. 1A represent those obtained with a model mixture comprising A, MA, MDA, MDMA, EDDP, and MET (about 100 μg/mL each, dissolved in water) in a CE-MS compatible buffer composed of ammonium acetate/acetic acid (20 mM each, pH 4.6) [25]. Separation of the six compounds of interest is shown to be complete in less than 10 min. Injection of extracts of urine blank and fortified urine blank (about 1 μg/mL of each drug) provided the electropherograms depicted in Figs. 1B and 1C. Complete three-dimensional data obtained for the extract of the fortified urine are presented in Fig. 2. Analysis of urine blank revealed one major endogenous peak only, a peak that is monitored between MDMA and EDDP. Furthermore, a small peak detected after about 7.6 min of current flow appears within the same time interval as A. Direct injection of urine (compare insets in Fig. 1, B and C) can allow the detection of A, MA, MDA, and MDMA if ppm concentrations are present. However, analysis of EDDP and MET *via* direct urine injection was found to be impossible using this buffer (inset in Fig. 1C; [25]). Commencing with 2 mL of urine and with extract reconstitution in 100 μL water (*cf.* Section 2.2), spectral identification limits for A, MA, MDA, MDMA, EDDP, and MET were found to be between 100 and 200 ng/mL. Detection limits (S/N = 3) were three to five times lower.

The data presented in Fig. 3 were produced from the extract of quality control urine 122. This specimen stemmed from a patient undergoing treatment in a locked drug-abuse ward. The urine is reported to contain cannabinoids, MET and EDDP, diazepam, temazepam and carbamazepine and to have been spiked with phencyclidine (40 ng/mnL), MDMA (3000 ng/mL) and MDA (300 ng/

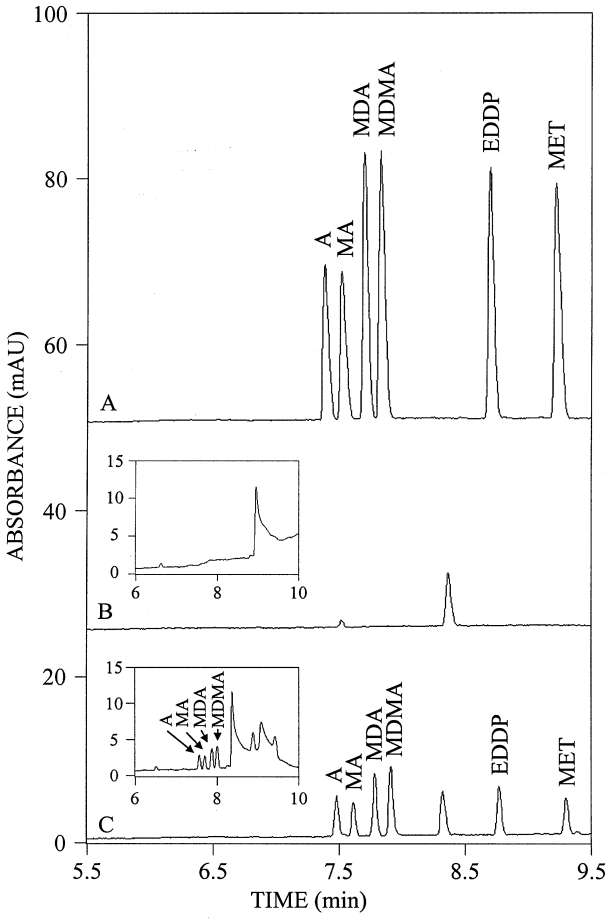

Figure 1. Single wavelength (195 nm) electropherogram of (A) a model mixture composed of A, MA, MDA, MDMA, EDDP, and MET; (B) an extract of urine blank; and (C) an extract of urine blank that was fortified with the six compounds. The inserts in (B) and (C) represent data obtained after direct injections of urine. Other conditions as in Sections 2.2 and 2.3.

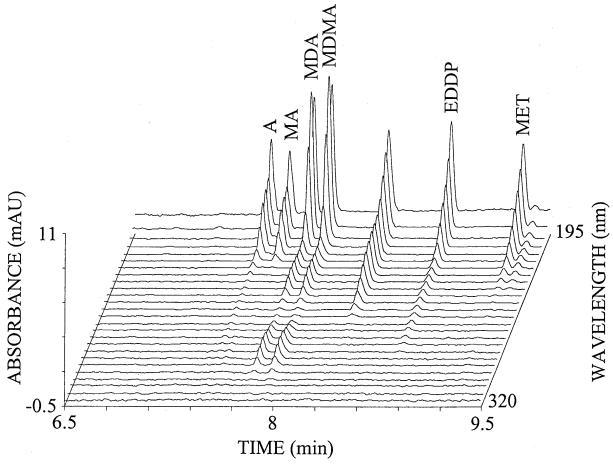

Figure 2. CE-MWD data of an extract of urine blank that was fortified with A, MA, MDA, MDMA, EDDP, and MET. Other conditions as in Fig. 1C.

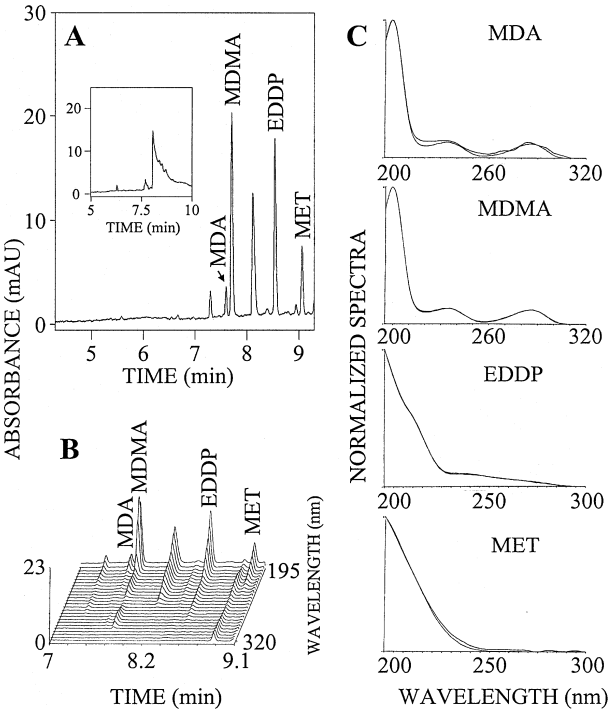

Figure 3. CE data of quality control urine 122 in (A) the single wavelength (195 nm) and (B) the multiwavelength (between 195 and 320 nm) format. (C) Depicts the normalized absorption spectra in comparison with those of standards. The inset in (A) represents data obtained by direct injection of the urine. Other conditions as in Sections 2.2 and 2.3.

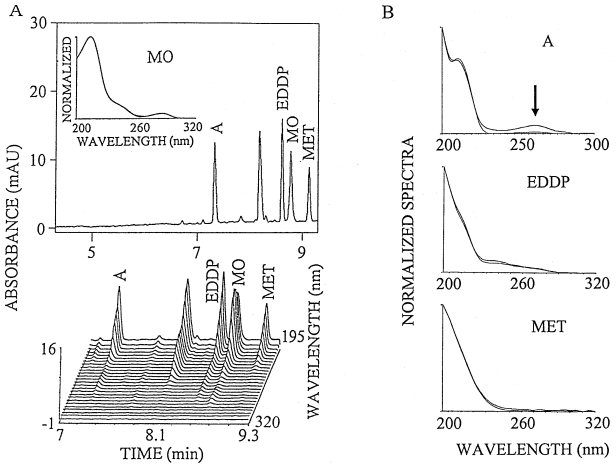

Figure 4. CE data of quality control urine 106. Other conditions as for Fig. 3.

mL). Analysis by CE-MWD revealed the presence of MDA, MDMA, EDDP, and MET. The endogenous peaks (one before MDA and one between MDMA and EDDP; Figs. 3A, 3B) were also monitored (see above). MWD is shown to permit identification of the relevant peaks *via* spectral identity proofs (Fig. 3C). In that approach, normalized absorption spectra are compared to those obtained *via* injection of standards (*e.g.*, those of Fig. 2). For all four compounds, an almost perfect overlay of the normalized spectra was obtained. This is similar to the data produced previously using an alkaline buffer [26], a buffer that cannot be used for CE-MS. Direct injection of the control urine (data shown as inset in Fig. 3A) did provide a small peak at 7.7 min. This peak was found to include MDMA. Direct proof *via* spectral analysis, however, was not possible.

CE-MWD data obtained with the quality control sample No. 106 that tested positive for amphetamines, MET, opiates, *etc*, using immunological screening methods [26] are presented in Fig. 4. The electropherogram of the urine extract revealed five major peaks (panel A), peaks that could be assigned to A, EDDP, MET, MO and the unidentified endogenous substance (see above). Peak identification was again possible *via* spectral identity proofs (panels A and B). For MET, EDDP, and MO, spectral agreement was found to be excellent. This was, however, not the case for A (top graph in panel B). For that drug, good agreement was noted for wavelengths < 220 nm, whereas at higher wavelengths the data revealed the presence of another substance (see peak at about 260 nm, marked with arrow). Thus, in contrast to the alkaline buffer system employed previously [26], A appears not to migrate as a single component in the ammonium acetate/acetic acid buffer. According to the spectrum, comigration with NIC cannot be excluded. Due to lack of a standard of NIC, however, this notion could not be evaluated. This quality control urine was reported to have been prepared from urine of two polydrug abusers and to contain significant amounts of A (2.38 μg/mL), MET (2.29 μg/mL), EDDP (1.86 μg/mL), and free MO (5.64 μg/mL). Furthermore, it also comprised a number of other drugs, including benzoylecgonine (2.46 μg/mL), cannabinoid metabolites, and various benzodiazepines, substances that appear not to interfere with the employed assay.

3.2 CE-MS

All CE-MS data presented in this work were obtained employing the ammonium acetate/acetic acid buffer described above. Graphs in Fig. 5 represent CE-MS data monitored after injection of a model mixture composed of A, MA, MDA and MDMA (20 μg/mL each, dissolved in water). Mass traces of all four $[M+H]^+$ ions, namely at *m/z* values of 136.1, 150.1, 180.1, and 194.1, respectively, obtained in the full scan mode (*m/z* range: 100–300 Th) are depicted. Peak intensities of all four compounds were found to be comparable. Furthermore, the detection sequence is shown to be identical to that obtained with

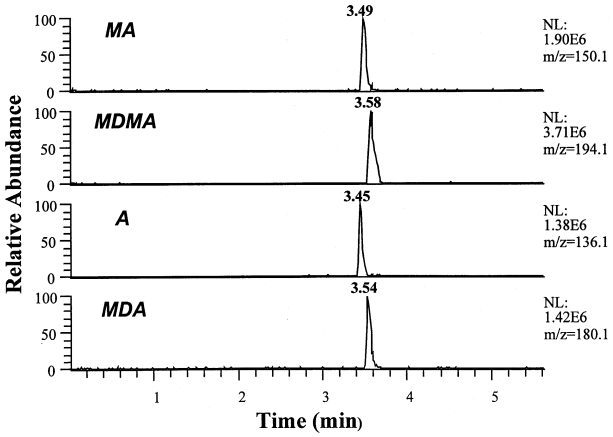

Figure 5. CE-MS data of a model mixture composed of MA, MDMA, A, and MDA represented by single mass traces. CE capillary length and current were 70 cm and 35 μA, respectively. Sample was injected *via* application of 100 mbar for 12 s.

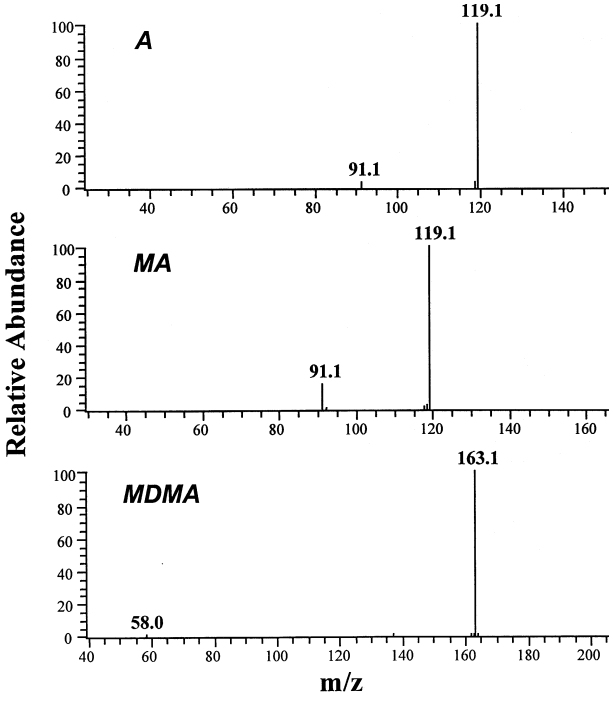

Figure 6. MS^2 data of A, MA, and MDMA that were gathered with the same sample as in Fig. 5 (separate run).

UV absorption detection (compare with Fig. 1). Although the effective capillary length employed in the CE-MS setup was longer, detection time intervals were noted to be significantly shorter and, consequently, resolution was observed to be lower. The presence of a hydrodynamic coflow (siphoning) towards the cathode is presumed to be responsible for that effect. This co-flow reduces the likelihood of ion penetration from the sheath liquid into the capillary [25]. MS/MS data of A, MA, and MDMA are presented in Fig. 6. In all cases, major fragments are formed due to the loss of NH_2R. Not surprisingly, the major fragments of A and MA were found to be identical. The same was found to be true for MDA and MDMA (data not shown). Furthermore, ion trap fragmentation of MDMA and MDA to *m/z* 163.1 is identical to that observed by Gaus *et al.* [29] using CE-MS with an ionspray interface and a quadrupole MS.

CE-MS data obtained from the extract of quality control urine 122 are presented in Fig. 7. Selected mass traces are depicted in the order of decreasing peak intensities. A different, longer capillary as that used to generate the data of Fig. 5 was employed and measurements were performed on a separate occasion with a different instrumental setup. Detection time intervals were found to be significantly larger compared to the data presented in Fig. 5, a change which is mainly attributed to a smaller cathodic co-flow (see above). The data of Fig. 7 show mass traces for MDA (*m/z* 180.1) and MDMA (*m/z* 194.1). Furthermore, the full scan data also revealed the presence of EDDP (*m/z* 278.3) and MET (*m/z* 310.2). Not surprisingly, the presence of MDA and MDMA could be confirmed *via* MS/MS and the same was found to be true for EDDP and MET (data not shown). Using MS^2, two characteristic fragments of EDDP (*m/z* 249.2 and 234.3) and one of methadone (*m/z* 265.1) were noted (see below). The fragmentation pattern for MET and EDDP obtained in an ion trap MS is identical to that observed previously on a triple quadrupole MS [25].

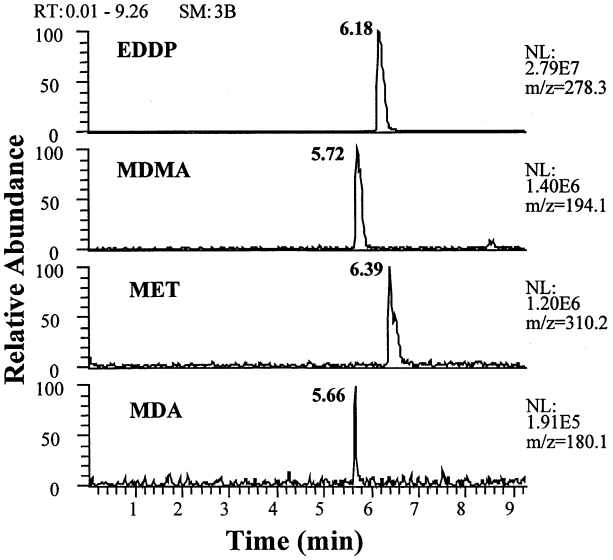

Figure 7. CE-MS mass traces obtained for the analysis of the extract of quality control urine 122. CE capillary length and current were 80 cm and 39 μA, respectively. Sample was injected *via* application of 50 mbar for 12 s.

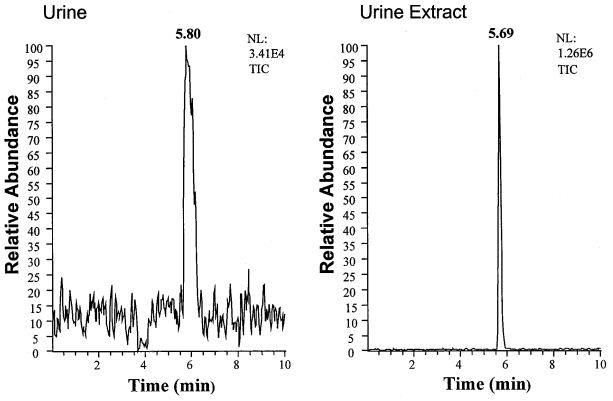

Figure 8. SRM data for MDMA (*m/z* 194.1 → *m/z* 163.1) obtained *via* injection of plain urine 122 (left panel) and after extraction (right panel). Sample was injected *via* application of 50 mbar for 6 s. Other conditions as in Fig. 7.

Operation of the MS under selected reaction monitoring (SRM) conditions [24] represents another approach for confirmation of the presence of a solute. Using SRM, the precursor-product ion transition between a precursor ion and a product ion is monitored. The presence of MDMA in the quality control urine 122 and its extract could be confirmed *via* SRM (Fig. 8). The protonated MDMA ion (*m/z* 194.1) was isolated with a 2 Th width and subjected to fragmentation with a collision energy 18% maximum. The fragment ion with *m/z* 163.1 (Fig. 6) was monitored. The total ion electropherograms presented in Fig. 8 are those obtained for analysis of the untreated urine (left graph) and the urine extract (right graph). The MDMA concentration in the urine is 3 μg/mL, a concentration that was found to be close to the detection limit when urine is directly injected. This is comparable to the detection limit in a setup with UV absorption detection (Figs. 1 and 2). With liquid/liquid extraction as employed in this work (*cf.* Section 2.2), the recovery of MDMA is about 80% [26]. Thus, the MDMA concentration in the extract should be about 16-fold higher compared to its concentration in plain urine. Not surprisingly, the SRM peak intensity obtained for the extract was found to be significantly higher (37-fold compared to the response obtained after injection of plain urine).

The CE-MS data obtained with the extract of quality control urine 106 are presented in Figs. 9 and 10. Mass traces depicted in Fig. 9 could be identified as A (*m/z* 136.1), EDDP (*m/z* 278.4), MET (*m/z* 310.3), NIC (*m/z* 163.3) and MO (*m/z* 286.3). The detection sequence of these compounds is essentially in agreement with that monitored in Fig. 4. Resolution, however, is much decreased, this being best seen by the comigration of EDDP and MO. Shorter analysis time intervals and sam-

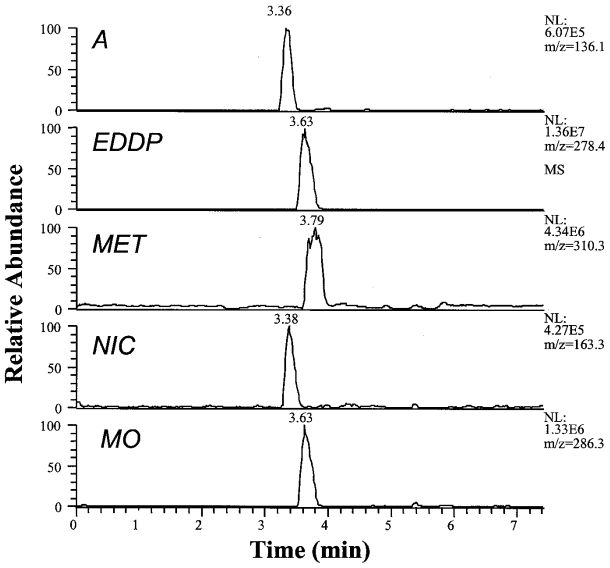

Figure 9. CE-MS mass traces obtained for the analysis of the extract of quality control urine 106. Experimental conditions were the same as in Fig. 5, except the current was about 42 μA.

ple overload (broad peaks in Fig. 9) are presumed to be responsible for that fact. MS detection, however, is capable of selectively and unambiguously detecting comigrating substances (see the data presented in Fig. 10). MS data for the time interval of EDDP detection were found to contain $[M+H]^+$ ions of EDDP (*m/z* 278.4) and MO (*m/z* 286.3), whereas the MS^2 data for the *m/z* 278.4 fragment provided the expected fragmentation pattern of EDDP only. Similarly, for the time interval of A detection, $[M+H]^+$ ions of A (*m/z* 136.1) and NIC (*m/z* 163.3) were monitored. The MS^2 data for *m/z* 136.1, however, do reveal the fragmentation pattern of A only. Finally, the MS data of MET indicate that this zone is not contaminated with any other major compound.

4 Concluding remarks

CE-MS and CE-MS^2 data of urinary extracts produced in the CE-ion trap MS configuration with electrospray ionization used in this work are shown to permit unambiguous confirmation of the presence of amphetamines and designer drugs in human urine. In that approach, MS^n is capable of distinguishing comigrating substances provided that they produce different fragments in the collision cell. Although substantial differences in detection times were noted between different capillaries (compare data of Figs. 5 and 7), changes that were mainly based upon the difference in the magnitude of cathodic buffer flow (siphoning) and not on the difference in capillary length, intraday reproducibility of detection times was found to be < 1.5% (n = 4) (compare data of Figs. 5 and 9). Without

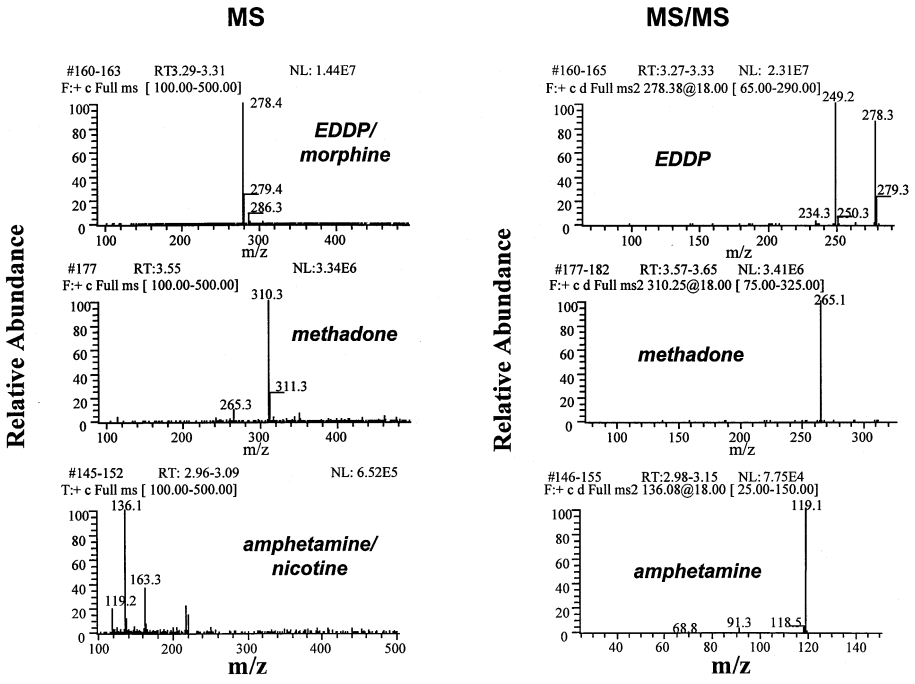

Figure 10. Full scan (*m/z* range: 100–500 Th) MS data of the peaks comprising EDDP/MO, MET, and A/NIC (left panel, from top to bottom) and MS^2 data for EDDP, MET, and A (right panel, from top to bottom) obtained for the analysis of the extract of quality control urine 106.

extraction, urinary concentrations on the ppm level are required (SRM mode, Fig. 8) for unambiguous solute identification. After sample extraction as described in Section 2.2 and hydrodynamic sample injection, sub ppm urinary drug concentrations in the order of 50–200 ng/mL can be detected. This sensitivity is comparable to that observed by CE with UV detection and is sufficient for confirmatory testing of most urinary drugs of abuse. Thus, CE-MS has the potential of becoming an attractive method for analytical toxicology and thereby an alternative to the widely used GC-MS confirmation approach [11] and to the LC-MS techniques that gained popularity in recent years [12, 13].

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