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Capillary electrophoresis–electrospray ionization ion trap mass spectrometry for analysis and confirmation testing of morphine and related compounds in urine

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Abstract

Using an aqueous background electrolyte containing 25 m*M* ammonium acetate and NH_3 (pH 9), CE–tandem MS and CE–triple MS with atmospheric pressure electrospray ionization in the positive ion mode are shown to represent attractive approaches for analysis and confirmation testing of morphine (MOR) and related opioids in human urine. Injection of plain or diluted urine permits monitoring of solutes at concentrations above 2–5 μg/ml. For the recognition of lower concentrations, solute extraction and concentration is required. Liquid–liquid extraction at alkaline pH is shown to be suitable for analysis of free opioids only whereas solid-phase extraction using a mixed-mode polymer phase is demonstrated to permit analysis of both free and glucuronidated opioids. The former sample preparation approach, however, requires about half of the time only. Commencing with 2 ml of urine, reconstitution to provide a sample volume of 0.2 ml and hydrodynamic sample injection, detection limits for free opioids are shown to be on the 100–200 ng/ml drug level. Much improved (ppb) sensitivity is obtained by infusing the extract directly into the source of the MS system. However, solutes that produce equal fragments (such as the two glucuronides of MOR) can thereby not be distinguished. CE–tandem MS and CE–triple MS are demonstrated to be suitable to confirm the presence of MOR, MOR-3-glucuronide, 6-monoacetylmorphine, codeine, codeine-6-glucuronide, dihydrocodeine, methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine in a toxicological quality control urine. The same is shown for selected metabolites of codeine and dihydrocodeine in urines collected after administration of pharmaceutical preparations.

Keywords: Capillary electrophoresis–mass spectrometry; Morphine; Codeine; Glucuronides; Opioids

1. Introduction

Morphine (MOR), codeine (COD) and dihydrocodeine (DHC) (for structures see Fig. 1) are opioids that are widely used therapeutically and consumed illicitly and their determination in biological samples is important in clinical and forensic toxicology, in pharmacodynamic, pharmacokinetic and pharmacogenetic research, and for the assessment of patient compliance. Furthermore, the most abused opioid, diacetylmorphine (heroin), is metabolized to 6-monoacetylmorphine (6-MAM, specific metabolite) and MOR (non-specific metabolite). Thus, monitoring of 6-MAM in urine is used to reveal heroin consumption. Many immunological and chromatographic methods have been developed and are widely used for screening of the presence of opioids in body fluids and for their specific determination together with their metabolites in bio-

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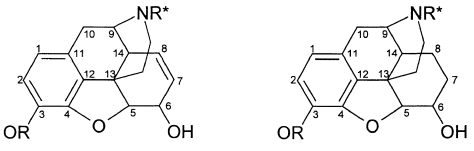

opioid	abbreviation	structure	R	R*
codeine	COD	I	CH_3	CH_3
dihydrocodeine	DHC	II	CH_3	CH_3
dihydromorphine	DHM	II	H	CH_3
morphine	MOR	I	H	CH_3
norcodeine	NCOD	I	CH_3	H
nordihydrocodeine	NDHC	II	CH_3	H
nordihydromorphine	NDHM	II	H	H
normorphine	NMOR	I	H	H

Fig. 1. Chemical structures of free opioids studied in this work.

logical specimens, respectively [1–3]. Due to the appealing advantages of employing capillary electrophoresis (CE) instead of a chromatographic approach, namely high separation efficiency, small sample size, small amounts of organic solvents and chemicals and inexpensive capillary columns [4,5], a number of reports discussing the use of CE for analysis and determination of urinary opiates have been published in the past few years [6–16].

The coupling of a separation technique and mass spectrometry (MS) is applied to gather structural information of separated compounds. In analogy to the widespread use of gas chromatography–mass spectrometry (GC–MS [1]) and liquid chromatography–mass spectrometry (LC–MS [2,3]) hyphenation of CE with MS (CE–MS) has been shown to be an attractive approach for the identification of urinary drugs, including *N*-1-hydroxyethylflurazepam (major metabolite of flurazepam) [17], haloperidol [18], paracetamol and metabolites [19,20], non-opioid analgesics and metabolites [21], methylphenidate [22], methadone [23–25] and amphetamines [24,25]. Recently, CE–MS has also been employed for the characterization of various alkaloid classes [26], the determination of MOR, COD, thebaine, papaverine and narcotine in crude extracts from opium [27,28] and the analysis of tramadol enantiomers in plasma [29]. Furthermore, in a previous communication from our laboratory, the feasibility of performing CE–MS based confirmation analysis of COD, DHC, codeine-6 glucuronide (COD-6-G) and dihydrocodeine-6-glucuronide (DHC-6-G) in urine extracts prepared by solid-phase extraction has been reported [30]. That work was based upon hyphenation of CE with a benchtop ion trap MS comprising electrospray ionization (ESI). In the meantime, our CE–MS determination and identification efforts were widened to the analysis of MOR, COD, DHC and their major unconjugated metabolites (Fig. 1), as well as to the excreted glucuronic acid conjugates of MOR, COD and DHC. For that purpose, optimized separation conditions in volatile buffers were elucidated by CE with UV detection. A urine spiked with a standard mixture comprising eight compounds, namely DHC, nordihydrocodeine (NDHC), dihydromorphine (DHM), nordihydromorphine (NDHM), COD, normorphine (NMOR), norcodeine (NCOD) and MOR, was analyzed by CE–MS (i) without any sample preparation step (direct urine injection), (ii) after liquid–liquid extraction at alkaline pH and (iii) after solid-phase extraction using a mixed-mode polymer phase. The three developed methods were applied to urines collected after selfadministration of COD and DHC containing pharmaceutical preparations and an external quality control urine that was prepared from the urines of two polydrug abusers. Data obtained were compared to those obtained by MS without hyphenation to CE.

2. Experimental

2.1. Chemicals, urine samples, blank matrices and standard solutions

DHC and its metabolites NDHC, DHM, NDHM and DHC-6-G were received from Mundipharma (Basel, Switzerland). NCOD, NMOR, 6-MAM and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) were purchased as methanolic solutions (1.0 mg/ml base) from Alltech (State College, PA, USA). MOR, COD, COD-6-G, MOR-3-G, MOR-6-G and naloxon-6-glucuronide were kindly received from Professor R. Brenneisen (Department of Clinical Research, University of Berne, Berne, Switzerland). Methadone (MET) was purchased from the Universi-

ty Hospital Pharmacy (Berne, Switzerland). All other chemicals were of analytical grade.

Four human urine samples were analyzed. Urine u94 was collected during the 0–8 h time interval after administration of 75 drops of Paracodin (Knoll, Liestal, Switzerland, containing 25 mg DHC), urine u91 was collected after administration of one tablet of Pretuval C (Roche, Reinach, Switzerland, containing 30 mg pseudoephedrine, 20 mg dextrometorphan, 300 mg paracetamol and 250 mg ascorbic acid) and 30 drops of Resyl Plus (Novartis Consumer Health, Nyon, Switzerland, containing about 7 mg COD) and urine mb94 originated from a controlled study [12,31] where healthy volunteers, who gave their consent, ingested 60 mg of DHC in form of a slow release tablet (DHC Continus tablet containing 90 mg DHC hydrogentartrate, Napp Labs., Cambridge, UK) and collected the urine between 0 and 12 h after drug administration. An external quality control urine, referred to as sample c106, was purchased from Cardiff Bioanalytical Services (UKNEQAS for drugs of abuse, Cardiff, UK). This sample is reported to be prepared from urine collected over 4 month from two polydrug abusers consuming heroin cut with phenobarbitone, methadone and diazepam. Additionally cannabinoid metabolites, amphetamine, buprenorphine and phencyclidin were added prior to sample distribution. Our own urine was employed as blank and fortified urines were prepared by adding appropriate aliquots of stock solutions to this urine blank. All urines were stored at −20°C. Stock solutions of free drugs (1 mg/ml) were prepared with methanol–water (50:50, v/v) containing 1% of formic acid. Conjugates were dissolved in water (20 μg/ml). Standard solutions were prepared by diluting appropriate aliquots of the stock solutions with the same solvent or with sample solvent that was composed of 20 m*M* ammonium acetate and 20 m*M* acetic acid (pH 4.6). All solutions were stored in glass vials at −20°C.

2.2. Sample preparation

Urine pretreatment included dilution, solid-phase extraction or liquid–liquid extraction. Dilution was effected by mixing the urine 1:1 (v/v) with water. Solid-phase extraction was performed in a similar way as described previously [11–13] using disposable, mixed-mode polymer cartridges (Bond Elut Certify, No. 1211-3050, Varian, Harbor City, CA, USA) together with the Vac-Elut setup from Analytichem International (Harbor City, CA). Briefly, the cartridges were conditioned with 2 ml of methanol and 2 ml of water using vacuum aspiration without drying the sorbent bed. A measure of 2 ml of urine (adjusted to pH 7 with 1 *M* KOH solution) was loaded onto and slowly drawn through the cartridges. Prior to elution of the adsorbed opioids with 1.5 ml of methanolic solution containing 30% of ammonia, the cartridges were sequentially washed with 2 ml of water, 1 ml of 0.1 *M* acetate buffer (pH 4) and 2 ml of methanol applying vacuum aspiration. The eluate was collected in a glass tube and evaporated to dryness at 35°C under a gentle stream of nitrogen. The residue was redissolved in 200 μl of sample solvent. For liquid–liquid extraction, the commercially available Toxi-Tube A system comprising about 2 ml of an organic solvent mixture of CH_2Cl_2 and $C_2H_4Cl_2$, (pH 9) from Analytical Systems (Laguna Hills, CA, USA) was employed. After adding of 2 ml of urine, gently shaking for about 1 min and centrifugation for 5 min at about 750 *g*, 2 ml of the organic phase were transferred into a glass tube, two drops of 2 *M* acetic acid in ethyl acetate were added, and the solvent was evaporated in a water bath at 35°C under a gentle stream of nitrogen. The residue was redissolved in 200 μl of sample solvent.

2.3. CE analysis with UV detection

CE was performed on a P/ACE 5510 capillary electrophoresis system (Beckman, Fullerton, CA, USA) equipped with a 87 cm (80 cm effective length)×50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). A new capillary was first flushed with 1 *M* NaOH for about 30 min and capillary conditioning between runs was effected by flushing with running buffer for 3 min (application of 20 p.s.i. pressure at the inlet end; i.e. 1 p.s.i.=6894.76 Pa). If not stated otherwise, the running buffer was composed of 25 m*M* ammonium acetate adjusted to pH 9 with 1 *M* ammonia solution. Sample was introduced hydrodynamically via application of positive pressure (0.5 p.s.i.) for 20 s. The run voltage was 30 kV (anode on injection end) and

the current was about 17 μA. Solute detection was effected by UV absorbance at 214 nm. All operations were computer controlled using the Beckman P/ACE station software (version 1.0).

2.4. CE–MS analysis

MS was performed on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an ESI (Finnigan) source that was run in the positive ion mode (3.5 kV). Sheath gas (N_2) pressure was set at 20 arbitrary units and a mixture of methanol–water (60:40, v/v) containing 1% of acetic acid at a flow-rate of 3 μl/min was used as sheath liquid. The temperature of the heated capillary was at 200°C. The instrument was computer controlled using the XCalibur 1.0 software (Finnigan). A Prince Instrument (Lauerlabs, Emmen, Netherlands) equipped with a 80 cm×50 μm I.D. fused-silica capillary (Polymicro Technologies) was interfaced. Sample was introduced hydrodynamically by applying a positive pressure of 70 mbar for 18 s. Separation was effected with the running buffer employed for CE–UV measurements mentioned above and by applying a voltage of 30 kV (26.5 kV effective voltage for separation). Full scan mass spectra were acquired in the mass range of 100–500 or 200–500 Th. Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS–MS was performed using data dependent scans with an isolation width of 2 and a relative collision energy of 35%. In these experiments the instrument automatically switches to MS–MS as soon as a defined mass peak exceeds a predefined threshold. Triple MS (MS^3) experiments were performed with an isolation width of 2 and a relative collision energy of 35%.

3. Results and discussion

3.1. CE with UV detection

A standard sample comprising eight opioids (Fig. 1, 10 μg/ml each) dissolved in sample solvent was employed for the search of suitable CE separation and extraction conditions. It was necessary to find a volatile buffer to ensure its ability to be used for CE–MS measurements. Therefore the phosphate buffer previously recommended by Zhang and Thormann [5,32] could not be employed. Several aqueous and non-aqueous media comprising acetonitrile, methanol or a mixture of both of these solvents containing 20–100 m*M* ammonium acetate and pH values between 3 and 9 [22,26–28] were tested and found to be either unsuitable to resolve the eight test compounds of interest or provided unexpected operational problems during the use with CE–MS. The latter effect was particularly observed with acidic buffers comprising large amounts of acetonitrile or equivolume mixtures of acetonitrile and methanol. An aqueous buffer consisting of 25 m*M* ammonium acetate adjusted to pH 9 with 1 *M* NH_3 solution was found to provide good resolution for the eight opioids (Fig. 2) and permitted trouble-free operation

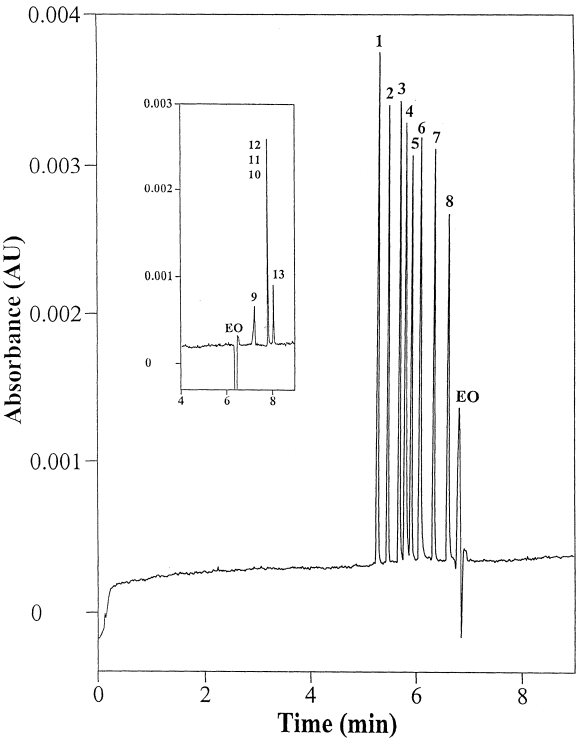

Fig. 2. CE–UV electropherogram of a standard mixture of eight opioids (10 μg/ml each) dissolved in sample solvent and a standard mixture of conjugates of selected opioids (inset). EO marks the fluid element transported by electroosmosis. Key: 1, NDHC; 2, NCOD; 3, NDHM; 4, DHC; 5, NMOR; 6, DHM; 7, COD; 8, MOR; 9, DHC-6-G, 10, MOR-3-G; 11, COD-6-G; 12, naloxon-6-glucuronide; 13, MOR-6-G.

of CE–MS (see below). At that pH, all eight opioids are cations [32]. It is important to note, however, that the pH has to be properly adjusted.

The data depicted in Fig. 3 represent selected electropherograms that were obtained after injection of two-fold diluted urine samples (panels A–C) and a urine extract prepared by liquid–liquid extraction (panel D). Urine samples had to be diluted two-fold with water, otherwise electropherograms were found not to provide sharp sample peaks. Analysis of urine blank (panel A) is shown to provide a strong peak comprising neutral components (electroosmotic flow (EOF) marker) and otherwise a relatively clean electropherogram such that ppm levels of opioids can be analyzed without extraction (panel B). This is further illustrated with the data obtained after injection of two-fold diluted urine mb94. In this urine, DHC (peak 4; urine concentration: 9.77 μg/ml) and

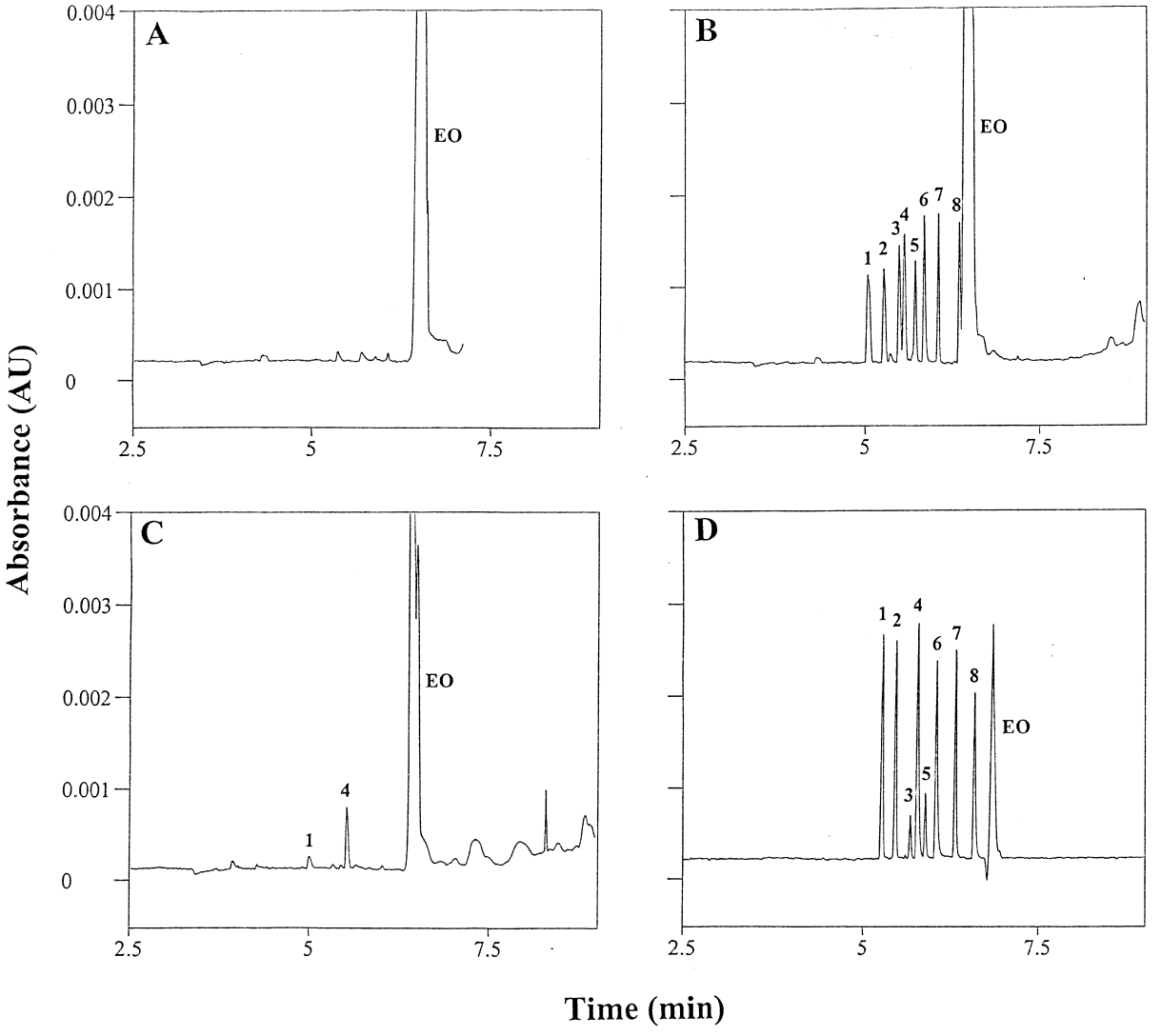

Fig. 3. CE–UV electropherograms obtained with (A) two-fold diluted blank urine, (B) two-fold diluted fortified urine containing eight opioids (10 μg/ml each), (C) two-fold diluted urine mb94 and (D) the extract of the fortified urine that was prepared by liquid–liquid extraction and reconstituted in the same volume. Experimental conditions and key as for Fig. 2.

NDHC (peak 1; 3.94 μg/ml) could be easily recognized which is in agreement with micellar electrokinetic capillary chromatography (MECC) data reported previously [11,12]. The presence of DHC-6-G (no sharp peak at expected location; about 30 μg/ml), however, could not be confirmed, this being different to the MECC approach [12]. More work is required to elucidate the behavior of glucuronides in presence of the urine matrix. After sample clean-up, a nicer electropherogram with a much reduced peak for the neutrals (EOF marker) is obtained. This is illustrated with the data presented in panel D of Fig. 3, data that were obtained with a liquid–liquid extract of 2 ml urine spiked with eight opioids (10 μg/ml each) and reconstituted in 2 ml sample solvent.

CE with UV detection was also employed for the determination of extraction recoveries (Table 1). For that purpose, 12 ml of urine were spiked at a concentration of 10 μg/ml for each of the 8 opioids. A measure of 3×2 ml were applied to solid-phase extraction and 3×2 ml to liquid–liquid extraction following the procedures described in Section 2.2. After evaporation, the residues were reconstituted in 2 ml of sample solvent. Each sample was analyzed by CE using hydrodynamic injection (20 s, 0.5 p.s.i.). Recoveries were determined via comparison of the mean ($n=3$) of the peak areas with those obtained with a sample comprising the eight opioids dissolved in sample solvent at 10 μg/ml (for an example refer to Fig. 2). The extraction recoveries found after solid-phase extraction were >92% for all eight opioids tested. Using liquid–liquid extraction, the recoveries for NDHM and NMOR were ≤25% and the recoveries for the other opioids were found to be between 76 and 86% (Table 1, Fig. 3D).

Table 1
Extraction recoveries of opioids

Compound	Recovery (%)	
	Liquid–liquid extraction	Solid-phase extraction
NDHC	76	93
NCOD	81	94
NDHM	14	83
DHC	86	95
NMOR	25	92
DHM	76	95
COD	86	96
MOR	81	94

The behavior of selected glucuronides was also investigated. At pH 9, all glucuronides are negatively charged [32] and are thus detected after the EOF marker (inset in Fig. 2). MOR-3-G, COD-6-G and naloxon-6-glucuronide could not be separated in this buffer. However, they were found to be well separated from DHC-6-G and MOR-6-G. Urinary glucuronides cannot be extracted by liquid–liquid extraction at pH 9 (as was used in this work). However, the solid-phase extraction procedure employed was found to extract glucuronides of opioids with a recovery of >80% [11].

3.2. CE with MS detection

For analysis of opioids in urine, CE–MS was performed with the LCQ ion trap MS that is capable of measuring up to MS^9. A volatile background electrolyte composed of 25 m*M* ammonium acetate adjusted to pH 9 with 1 *M* NH_3 was employed. The use of an acidic sheath liquid supported the formation of positively charged molecule ions $[M+H]^+$. The data presented in Fig. 4 depict the mass traces (left panel) and MS spectra (right panel) of a CE–MS run performed with a sample comprising eight opioids (20 μg/ml each) that were dissolved in water. The total ion current (TIC) electropherogram representing the sum of the currents of all masses is given as top graph in the left hand panel. These data nicely show the CE separability of the eight compounds in the pH 9 buffer (compare with Fig. 2) and reveal that selected opioids cannot be distinguished by MS spectra. Both NCOD and MOR are shown to produce an $[M+H]^+$ ion with m/z of 286.3. A similar relationship exists between NDHC and DHM ($m/z=288.4$). In this configuration, these compounds can thus only be identified by their migration times. Using MS–MS, however, different mass spectra are obtained for all eight compounds. This is illustrated with the data presented in Fig. 5. These data were obtained for the CE–MS analysis of a solid-phase extract prepared from a urine blank that was fortified with all eight opioids at 10 μg/ml each. MS spectra

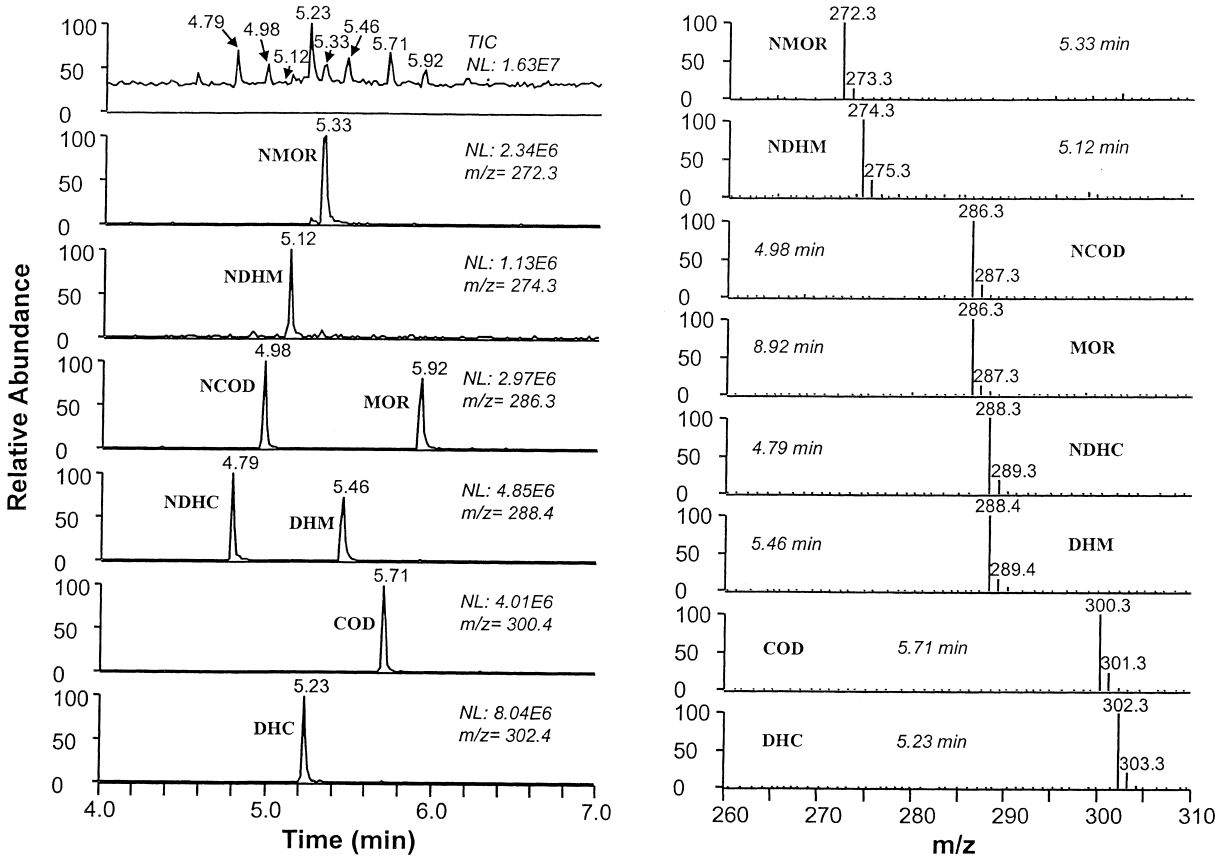

Fig. 4. CE–MS mass traces and TIC (left panel) and MS mass spectra (right panel) obtained with an aqueous mixture of eight opioids (20 μg/ml each).

were found to be identical to those shown in Fig. 4 and are thus not shown.

The easiest and fastest way to measure urine samples would be by direct injection of a small aliquot onto the CE column, i.e. without any preparation step. Experiments performed with spiked urines and UV detection showed that urines have to be diluted 1:1 with water prior to injection in order to provide good, narrow peak shapes (Fig. 3). Therefore all samples used were prepared in that way. As expected, no opioids were detected in the blank urine. In the fortified urine containing the eight opioids (10 μg/ml each), after CE–MS of two-fold diluted urine, all eight compounds could be found according to their m/z values and their presence was further confirmed by MS–MS spectra (data not shown). However, the concentration of 5 μg/ml was found to be close to the detection limit of NMOR and NDHM ($S/N \approx 3$) and for the other compounds the S/N ratios were between 4 and 10. Thus, the detection limits for these compounds are expected to be between 2 and 5 μg/ml. These limits are similar to those observed with UV detection (Fig. 3). For assessing lower concentrations, urines have to be extracted. With the sample preparation procedures employed (cf. Section 2.2), detection limits were determined to be between 100 and 200 ng/ml.

The reproducibility of the CE–MS system was assessed via analysis of a sample comprising the eight opioids (10 μg/ml each) in sample solvent.

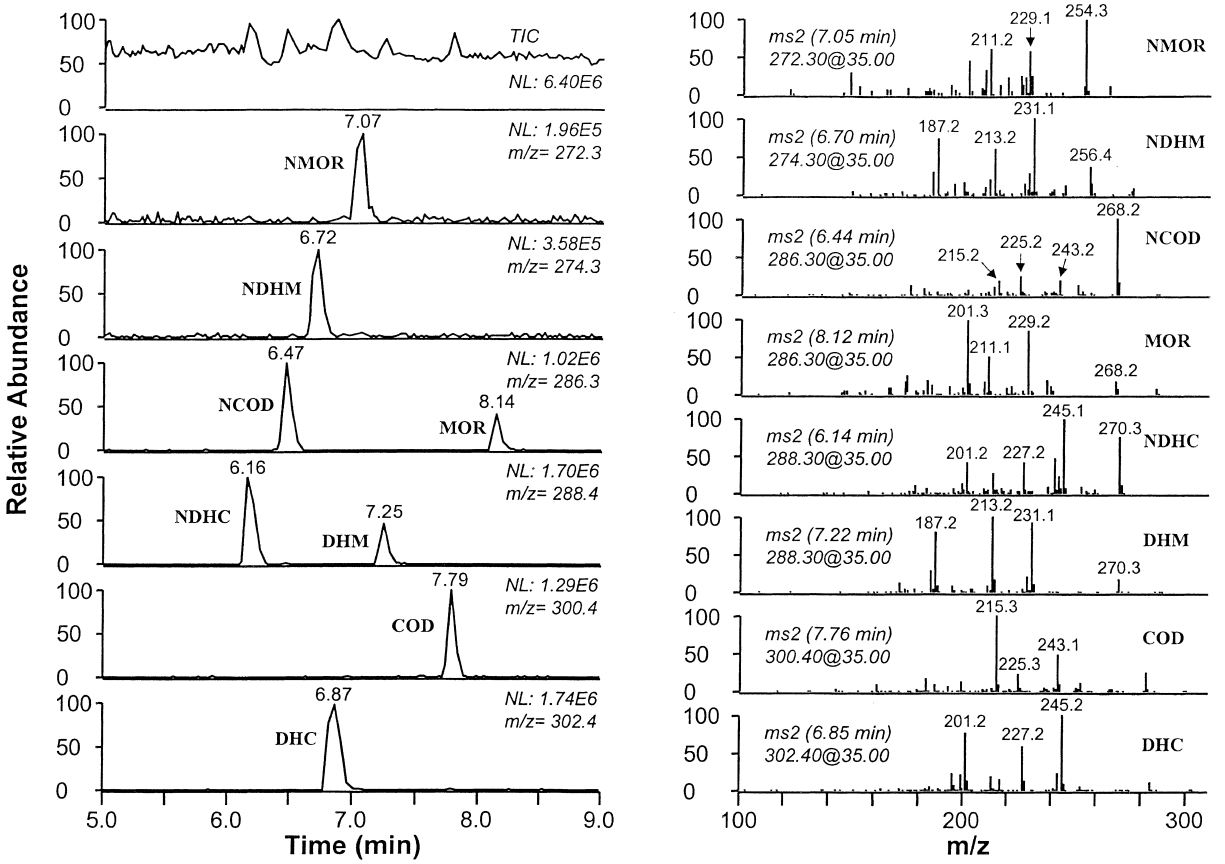

Fig. 5. CE–MS mass traces and TIC (left panel) and MS–MS mass spectra (right panel) obtained with an extract of fortified blank urine (eight opioids, 10 μg/ml each) that was prepared by solid-phase extraction according to the procedure described in Experimental.

The intra-day RSD values ($n=5$) found for run times were typically <1.0% for all eight opioids, whereas the RSD values for the peak intensities were typically between 20 and 40% (Table 2). Inter-day RSD values ($n=3$) for run times were determined to be between 5 and 7% (Table 2) and for the intensities

Table 2
Typical intra-day and inter-day CE–MS imprecision data

Compound	Intra-day RSD (%), $n=5$		Inter-day RDS (%), $n=3$	
	Detection time	Peak intensity	Detection time	Peak intensity
NDHC	0.52	38.66	5.65	32.71
NCOD	0.66	32.73	5.52	34.16
NDHM	0.85	35.70	5.61	13.25
DHC	0.65	30.30	5.68	40.78
NMOR	0.66	33.31	6.03	24.87
DHM	0.67	22.77	6.05	22.58
COD	0.62	25.54	5.73	31.19
MOR	0.59	36.78	6.17	44.51

between 20 and 40%. It was interesting to find that intra-day and inter-day RSD values of peak intensities did not differ.

3.3. Confirmation testing of urinary opioids by $CE–MS^n$ ($n>1$)

For the determination and identification of opioids in urine, CE–MS analysis of diluted and extracted samples were performed with the LCQ ion trap MS that is capable of measuring up to MS^9. Typically, the identity of a substance can be confirmed via gathering and comparing MS–MS or MS^3 spectra with those of standards. Aqueous standard solutions (10 μg/ml) of DHC, NDHC, DHM, NDHM, MOR, COD, NMOR, NCOD, 6-MAM, DHC-6-G, COD-6-G, MOR-6-G and MOR-3-G were directly analyzed (via syringe inlet) and their MS, MS–MS and MS^3 spectra were stored in a computer library. This library is capable of comparing a selected spectrum with all stored spectra and the probability (%) of a match is automatically calculated by the computer. A total of six urines were analyzed by $CE–MS^n$ (Table 3). Blank urine and fortified blank urine were expected to contain no opioids and all spiked compounds, respectively. The volunteer urine u91 should contain COD and metabolites, whereas u94 and mb94 should comprise DHC and metabolites. Major urinary metabolites in the latter three samples are the glucuronides (COD-6-G and DHC-6-G) and the desmethylated products NCOD and NDHC [11,12]. Urine c106 was reported to contain MOR (content determined by chromatography: 5.64 μg/ml), DHC (0.19 μg/ml), COD (0.20 μg/ml), 6-MAM (0.38 μg/ml) and their conjugates, as well as MET (2.29 μg/ml), EDDP (1.86 μg/ml), amphetamine (A, 2.38 μg/ml) and other drugs [33].

Using $CE–MS^n$ with injection of two-fold diluted urine, no opioids were found in the urine blank and, in the fortified blank urine, all eight substances were observed (Table 3). However, in the ''real world'' samples very few opioids could be detected (Table 3). No opioid mass traces could be observed for samples u91 and u94, whereas DHC (m/z 302.4) and EDDP (m/z 278.4) could be found in urines mb94 and c106, respectively. The presence of these two compounds could be confirmed via their MS–MS spectra (data not shown, spectra are identical to those given in Refs. [30] and [24,25], respectively). As the urinary concentrations of the compounds were typically below 5 μg/ml, samples had to be extracted. All six urine samples were applied to liquid–liquid

Table 3
Confirmed presence of opioids in urines using CE–MS–MS or $CE–MS^3$

Urine sample	Compounds detected by CE–MS–MS or $CE–MS^3$		
	Two-fold diluted urine	Liquid–liquid extract	Solid-phase extract
Blank urine	No opioid	No opioid	No opioid
Blank urine fortified with eight opioids (10 μg/ml each)	All eight opioids	All eight opioids	All eight opioids
u91	No opioids	COD	COD, COD-6-G
u94	No opioids	DHC, NDHC	DHC, DHC-6-G
mb94	DHC	DHC, NDHC	DHC, NDHC, DHC-6-G
c106[a]	No opioids, EDDP	MOR, COD, DHC, 6-MAM, MET, EDDP	MOR, MOR-3-G, COD, COD-6-G, MET, EDDP

[a] Amphetamine was detected after liquid–liquid extraction. In the case of solid-phase extraction, the eluent was not acidified prior to evaporation and A was thus not monitored.

extraction using the commercial ToxiTube A system (Table 3), a simple method which requires about 30 min (evaporation included). Starting with 2 ml of urine and reconstitution in 200 μl sample solvent theoretically leads to a ten-fold concentration of the solutes. This of course is diminished by the extraction recoveries (Table 1). For blank urine, no opioids could be found, whereas in the case of the urine containing the eight opioids, all eight compounds could easily be recognized. COD in urine u91 could be detected and confirmed and DHC together with its metabolite NDHC were measured in urines u94 and mb94. The control urine c106 revealed four of the anticipated free opioids, namely MOR, COD, DHC and 6-MAM (Fig. 6, Table 3). MS and MS–MS spectra were found to match those stored in the library with a probability of >95%. Metabolites of MOR, COD and DHC were not detected. However, in analogy to previous work form

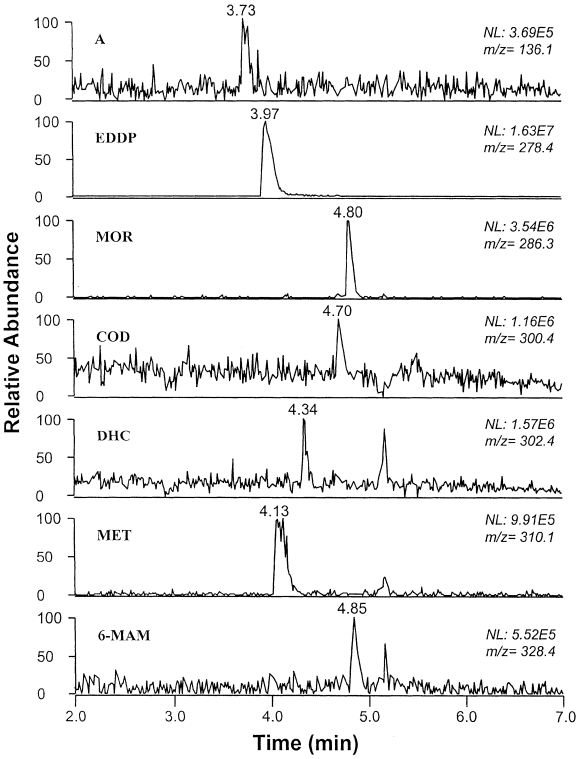

Fig. 6. Selected CE–MS mass traces obtained for the liquid–liquid extract of urine c106. Experimental conditions as described in Experimental.

our laboratory [23–25], the presence of A, MET and EDDP could also be confirmed. Other non-opioid compounds were not investigated. This example demonstrates that drug levels around 200 ng/ml (DHC, COD and 6-MAM) can be properly recognized. Mass spectra for 6-MAM are presented in Fig. 7B and compared to those stored in the library (Fig. 7A). It is worth mentioning that 6-MAM is a specific metabolite of heroin and thus represents a marker for heroin consumption. After further degradation to MOR it is impossible to make the relation back to heroin as MOR can be derived from various sources. The strong mass appearing in the MS spectrum of 6-MAM that was registered for the extract of c106 (top graph of Fig. 7B) was found to have an m/z value of 286.3. This is identical to the m/z value of the $[M+H]^+$ ion of MOR, indicating that 6-MAM and MOR are not completely separated by CE (Fig. 6).

Using solid-phase extraction with Bond Elut Certify cartridges, the presence of free and conjugated opioids could be confirmed (Table 3). This method is much more time consuming than the procedure based upon liquid–liquid extraction because the eluate was composed of methanol containing 30% ammonia which itself contains around 70% of water. Therefore the evaporation step is longer than after liquid–liquid extraction and the entire sample preparation process lasts at least 1 h. As above, the blank urine did not show any substances of interest and all eight opioids could be detected in the extract of the fortified blank urine (Fig. 5). As was discussed previously [30], COD and its glucuronide COD-6-G could be detected in u91, as well as DHC and DHC-6-G were monitored in the extract of urine u94. Similarly, the extract of urine mb94 revealed the presence of DHC, NDHC and DHC-6-G (Table 3, data not shown). In the extract of urine c106 several free opioids, COD-6-G and MOR-3-G were recognized (Table 3). CE–MS data for MOR and the MOR conjugate are presented in Fig. 8. The mass trace for $[M+H]^+ =$ 462.5 (MOR-3-G and MOR-6-G) revealed only one peak and this despite that the two MOR glucuronides can be separated with the CE system used (see inset of Fig. 2). It is known that urines contain much larger amounts of MOR-3-G [9,14], this suggesting that the peak detected after 5.2 min of current flow (Fig. 8) is more likely to represent MOR-3-G and

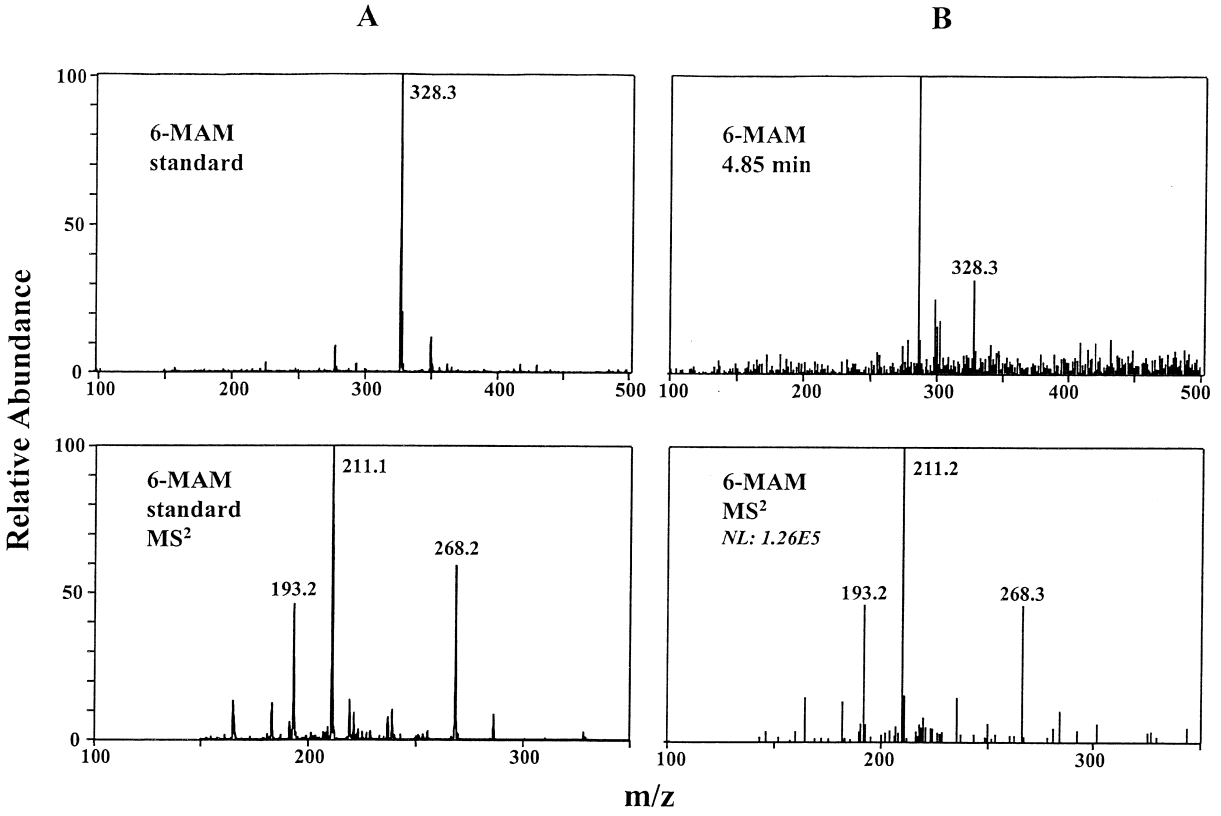

Fig. 7. MS spectra (top graphs) and MS–MS spectra (bottom graphs) of 6-MAM obtained (A) by MS analysis of a standard (spectra stored in the library) and (B) by CE–MS of the extract of urine c106 that was prepared by liquid–liquid extraction (for mass trace refer to Fig. 6).

MOR-6-G is assumed to remain undetected. Furthermore, the peak produced by the $[M+H]^+$ ion was found to comigrate with that of COD-6-G, i.e. the ion with m/z=476.5 (last peak in right panel of centre graph of Fig. 8). As MOR-3-G and COD-6-G were found to be inseparable in this configuration (inset of Fig. 2), this suggests that the detected MOR glucuronide is indeed MOR-3-G (Fig. 8). The fragmentation principle of the two MOR glucuronides was found to be comparable to that of COD-6-G and DHC-6-G [30]. For both $[M+H]^+$ ions with m/z of 462.5, MS–MS lead to the typical mass spectrum of MOR with an m/z value of 286.3 (Fig. 4) and MS^3 data provided the structural proof via showing the MS–MS spectrum of MOR (Fig. 5). It is worth mentioning, that the same degradation principle was previously observed for the glucuronides of MOR using LC–MS with electrospray ionization [34]. Thus, according to the fragmentation pattern, MOR-3-G and MOR-6-G cannot be distinguished. For direct, unambiguous assignment, the urinary extract would have to be spiked with the two glucuronides prior to reanalysis of the fortified extract.

3.4. Direct MS analysis of urinary opioids

The use of the LCQ ESI ion trap MS system without the CE capillary for analysis of urinary opioids was also studied. In that case, samples were introduced via the syringe of the infusion pump otherwise used to apply the sheath liquid and solute discrimination is provided by MS only. Direct urine injection is not possible as the high salt content would tremendously pollute the source and the heated capillary. Introduction of extracts, however, was found to lead to interesting data. As this approach requires more sample than CE–MS, 5 ml of urine was pretreated using solid-phase extraction

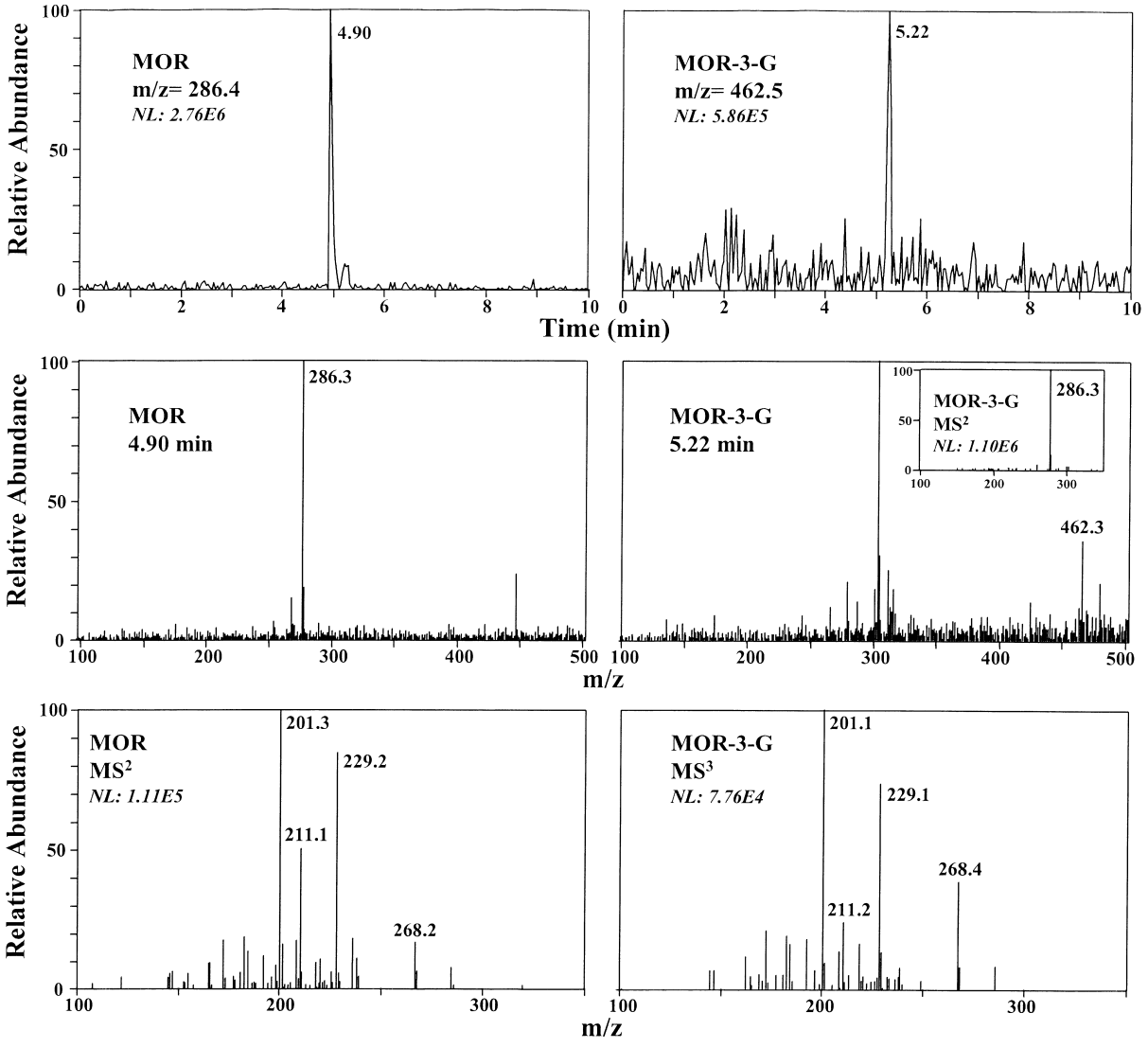

Fig. 8. Selected CE–MS data for MOR (left panel) and MOR-3-G (right panel) obtained for the solid-phase extract of urine c106. The top graphs represent mass traces for MOR (m/z 286.4) and MOR-3-G (m/z 462.5), respectively. The mass spectra depicted as center graphs are MS data for MOR and MOR-3-G (inset: MS–MS data) whereas those presented in the bottom panels are the MS–MS data of MOR and the MS^3 data of MOR-3-G, respectively.

with Bond Elut Certify cartridges and the dried residue was reconstituted in 1 ml of a 1:1 mixture of methanol and water containing 1% formic acid (about five-fold concentration). Detection limits for urinary free opioids were determined to be lower than 50 ng/ml. At that drug level, the presence of DHC, NDHC, DHM and NDHM could still be confirmed via MS–MS and MS^3 spectra. Application to the analysis of urine u91 revealed the unambiguous presence of COD, COD-6-G and NCOD in that sample. For all three compounds, the match of MS–MS and MS^3 spectra was between 96.40 and 98.98%. Note that NCOD was not detected by CE–MS and extract preparation commencing with 2 ml of urine and featuring about ten-fold concentration (Table 3). Thus, compared to the CE–MS method

described above, injection of urinary extracts via continuous infusion using the motor-driven syringe was found to provide higher sensitivity. This approach, however, cannot be employed for unambiguously recognizing compounds with equal fragmentation behavior, including MOR-3-G and MOR-6-G. Furthermore, a larger amount of extract is required and the MS system becomes more likely polluted in a short time.

4. Conclusions

CE–MS is shown to comprise an attractive method for analysis and confirmation testing of MOR and related compounds in urine. For rapidly gathering CE–MS data of a ''real world'' urine sample there is the possibility to directly inject plain or diluted urine onto the CE–MS system. If the concentration of opioids is sufficiently high (a few μg/ml or higher) they can be monitored and identified by MS, MS–MS or MS^3. For detection of free opioids below about 2–5 μg/ml, solute extraction and concentration is required. Liquid–liquid extraction at alkaline pH is shown to be suitable for analysis of free opioids only whereas solid-phase extraction using a mixed-mode polymer phase is demonstrated to permit analysis of both free and conjugated opioids. Commencing with 2 ml of urine and reconstitution in 0.2 ml sample solvent, detection limits for free opioids were found to be on the 100 – 200 ng/ml drug level. This sensitivity is comparable to that obtained by UV absorption detection. Lower (ppb or sub-ppb) concentrations would be accessible by having a larger ratio between initial urine volume and the volume after redissolution of the dried residue or by applying electrokinetic sample injection (work in progress). The same was found to be true after directly infusing the urinary extract into the ESI source. This approach, however, does not permit discrimination of solutes that produce equal fragments using MS^n ($n>1$). Hyphenation of CE to the LCQ system appears to be somewhat tricky. The position of the capillary tip was found to have a high influence on the quality of the spray and therefore on the sensitivity which can easily vary by a factor of more than ten. The intra-day reproducibility assessed on the basis of five consecutive injections of a standard mixture provided RSD values <2% for run times but up to 40% for the peak intensities. The latter variability can be a problem if the concentration of the compound of interest is close to the detection limit. Inter-day variations of detection times and peak intensities were found to be somewhat larger (<7%) and of the same magnitude, respectively. In the work described here, qualitative data were generated only. However, with inclusion of an internal standard, quantitative data could also be obtained. Using CE–ion trap MS with the LCQ system, Bach and Henion [22] reported quantitation of urinary methylphenidate on the ppb level.

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