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Analysis of codeine, dihydrocodeine and their glucuronides in human urine by electrokinetic capillary immunoassays and capillary electrophoresis–ion trap mass spectrometry

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Abstract

Screening for and confirmation of illicit, abused and banned drugs in human urine is a timely topic in which capillary separation techniques play a key role. Capillary electrophoresis (CE) represents the newest technology employed in this field of analysis. Two rapid competitive binding, electrokinetic capillary-based immunoassays are shown to be capable of recognizing the presence, but not the identity, of urinary opioids, namely codeine (COD), codeine-6-glucuronide, dihydrocodeine (DHC), dihydrocodeine-6-glucuronide, morphine (MOR), morphine-3-glucuronide and ethylmorphine (EMOR). In these approaches, aliquots of urine and immunoreagents of a commercial, broadly cross-reacting fluorescence polarization immunoassay for opiates were combined and analyzed by capillary zone electrophoresis or micellar electrokinetic capillary chromatography with laser induced fluorescence detection. With the fluorescent tracer solution employed, the former method is shown to provide simple electropherograms which are characterized by an opioid concentration dependent magnitude of the free tracer peak. In presence of dodecyl sulfate micelles, however, two tracer peaks with equal opioid concentration sensitivity are monitored. These data suggest the presence of two fluorescent tracers which react competitively with the urinary opioids for the binding sites of the antibody. Assay sensitivities for COD and MOR are comparable (10 ng/ml), whereas those for DHC and EMOR are about four-fold lower. Furthermore, glucuronides are shown to react like the corresponding free opioids. Analysis of urines that were collected after administration of 7 mg COD and 25 mg DHC tested positively in both assay formats. The presence of the free and conjugated codeinoids in these urines and their identification was accomplished by capillary electrophoresis–ion trap mass spectrometry (CE–MS). This confirmatory assay is based upon solid-phase extraction using a mixed-mode polymer cartridge followed by CE hyphenated to the LCQ mass spectrometer with electrospray ionization in the positive ion mode. With this technology, MS^2 is employed for proper identification of COD (m/z 300.4) and DHC (m/z 302.4) whereas MS^3 provides unambiguous identification of the glucuronides of COD (m/z 476.5) and DHC (m/z 478.5) via their fragmentation to COD and DHC, respectively. MS^n ($n \geq 2$) is shown to be capable of properly identifying the urinary codeinoids on the 100–200 ng/ml concentration level.

Keywords: Immunoassay; Codeine; Dihydrocodeine; Glucuronides; Opioids

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1. Introduction

Opioids, including codeine (COD), dihydrocodeine (DHC), morphine (MOR) and heroin, are being used therapeutically and/or consumed illicitly for many years. Interest in elucidating the pharmacokinetic, pharmacogenetic and pharmacodynamic properties of these opioids and their metabolites, as well as assessment of compliance, control of drug abuse and assessment of intoxications, have prompted analysis of these substances in body fluids, tissue extracts, post mortem specimens and seizure samples. For the monitoring of opioids in urine, inumerable analytical techniques based on chromatographic and immunological principles have been developed and are widely used. Due to appealing advantages of employing capillary electrophoresis (CE) instead of a chromatographic approach, quite a number of papers discussing the CE analysis of urinary morphine-like compounds have been published in the past years [1–8].

The combination of immunochemistry and CE has recently attracted considerable attention and competitive binding, CE-based assays using labelled drugs as fluorescent tracers emerged. Chen and co-workers reported principles for immunological determination of MOR and other drugs of abuse in urine by capillary zone electrophoresis (CZE) [9–11] and Choi et al. discussed the use of various antisera for monitoring of methamphetamine [12]. Our laboratory evaluated micellar electrokinetic capillary chromatography (MECC) and CZE based immunoassays for urinary methadone [13], amphetamines [14] and benzoylecgonine [15] using reagents from commercial fluorescence polarization immunoassays (FPIA). Although the structural specificity in antibody–antigen reactions of MOR, normorphine, hydromorphone and morphine-3-glucuronide has been discussed [9] and the feasibility of CZE monitoring of urinary MOR in multianalyte CE-based immunoassays has been reported [10,11,16], no detailed study dealing with urinary screening for opioids (particularly codeinoids) by MECC and CZE based immunoassays (MECCIA and CZEIA, respectively) could be found in the literature. Furthermore, unexplained multi-peak responses associated with MOR in a multianalyte MECCIA [17] prompted us to further investigate this immunochemical system.

In other efforts and in analogy to the widespread use of gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–MS (LC–MS), hyphenation of CE with MS (CE–MS) has been shown to be an attractive approach for identification of urinary drugs, including *N*-1-hydroxyethylflurazepam (major metabolite of flurazepam) [18], haloperidol [19], paracetamol and metabolites [20,21], nonopioid analgesics and metabolites [22], methylphenidate [23], methadone [13,24,17] and amphetamines [17,24]. With the exception of a brief link to MOR [24], no paper dealing with the CE–MS of urinary opioids could be found. However, CE–MS was employed for the characterization of various alkaloid classes [25] and the determination of opioids in crude extracts from opium [26,27].

This paper reports (i) an investigation of MECCIA and CZEIA of various urinary codeinoids, namely COD, codeine-6-glucuronide (COD-6-G), DHC and dihydrocodeine-6-glucuronide (DHC-6-G) in comparison to selected morphinoids, namely MOR, morphine-3-glucuronide (MOR-3-G) and ethylmorphine (EMOR) and (ii) discusses the first approach of monitoring urinary codeinoids and their glucuronides using CE–ion trap mass spectrometry. Data obtained with urine blank, urine blank fortified with opioids and urines collected after selfadministration of COD and DHC containing pharmaceutical preparations are compared.

2. Experimental

2.1. Chemicals, reagents for immunoassays, urine samples, blank matrices and standard solutions

All chemicals were of analytical grade. COD, COD-6-G, MOR and MOR-3-G were a gift of Professor R. Brenneisen (University of Berne, Berne, Switzerland) and DHC, nordihydrocodeine (NDHC) and DHC-6-G were kindly provided by Mundipharma (Basel, Switzerland). Norcodeine (NCOD) and EMOR were purchased as methanolic solutions (1 mg/ml) from Alltech (State College, PA, USA). Na_2HPO_4 and $Na_2B_4O_7$ were from Merck (Darmstadt, Germany) and sodium dodecylsulfate (SDS) from BDH (Poole, UK).

The TDx/TDxFLx opiates reagent pack (No.

9673-60) and the human urine calibrators (No. 9673-06) were from Abbott (Baar, Switzerland). The reagent pack comprises an antibody containing solution S (<1% sheep opiates antiserum) and opiates fluorescein tracer solution T (<0.01% tracer concentration). All TDxFLx products contain sodium azide as preservative. FPIA analyses on the TDxFLx (Abbott) were executed with the reagent kit provided by Abbott (see above) and following the manufacturer's instructions. This assay uses six urine calibrators containing 0 to 1000 ng/ml MOR.

Two urines were analyzed, one collected about 5 h after selfadministration of 1 tablet of Pretuval (Roche, Reinach, Switzerland, containing 30 mg pseudoephedrine, 20 mg dextromethorphan, 300 mg paracetamol and 250 mg ascorbic acid) and 30 drops of Resyl Plus (Novartis Consumer Health, Berne, Switzerland, comprising about 7 mg COD) and one collected during the 0–8 h interval after administration of 75 drops of Paracodin (Knoll, Liestal, Switzerland, containing 25 mg DHC). These samples are referred to as urines u91 and u94, respectively, throughout this text. Our own urines were used as blank matrices.

For the capillary based immunoassays, stock solutions of 1 μg/ml were prepared in blank urine and diluted with urine to appropriate concentrations before use. The commercially available human urine calibrators from Abbott containing MOR were also used. For CE–MS stock solutions of 1 mg/ml were prepared in methanol–water (50:50, v/v) containing 1% of formic acid. Standard solutions were prepared by diluting appropriate aliquots of the stock solutions with the same solvent and blank urine was fortified via addition of aliquots of these standard solutions. All solutions were stored at −20°C.

2.2. Sample preparation

For the immunoassays, aliquots of urine, solution T and solution S (25 μl each) were given into a 0.5 ml plastic vial, vortex-mixed for 10 s and transferred into the sample vial of the P/ACE instrument. Injection occurred after a 15 to 16 min incubation at room temperature. For CE–MS, solid-phase extraction was effected using disposable, mixed-mode polymer cartridges (Bond Elut Certify, No. 1211-3050, Varian, Harbor City, CA, USA) together with the Vac-Elut setup (Varian). The cartridges were conditioned with 2 ml of methanol and 2 ml of water using vacuum aspiration without drying the sorbent bed. Two ml of urine (adjusted to pH 7 with 1 *M* KOH solution) were 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 rinsed with 2 ml of water, 1 ml of 0.1 *M* acetate buffer (pH 4) and 2 ml of methanol by applying vacuum aspiration. The eluates were collected in glass tubes and evaporated to dryness at 35°C under a gentle stream of nitrogen. The residues were redissolved in 200 μl of sample solvent composed of 20 m*M* acetic acid and 20 m*M* ammonium acetate (pH 4.6).

2.3. Instrumentation and running conditions for CE-based immunoassays

CE-based immunoassays were performed on a P/ACE 5510 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a fused-silica capillary of 47 cm (40 cm effective) length 75 μm I.D. If not stated otherwise, applied voltages for CZEIA and MECCIA were 11 kV (current: about 83 μA) and 14 kV (current: about 83 μA), respectively, and the anode was on the sampling side. Samples were hydrodynamically injected by applying a positive pressure of 0.5 p.s.i. for 4 s (1 p.s.i.=6894.76 Pa). The capillary temperature was set at 30°C, the carousel was at room temperature. Detection was effected by a laser-induced fluorescence (LIF) detector assembly (Beckman) powered by an air-cooled argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) at 488 nm and equipped with a 520 nm emission filter. Data were evaluated using the P/ACE station software (version 1.0). The capillary was conditioned by sequentially rinsing (positive pressure, 20 p.s.i.) with 0.1 *M* NaOH, water and running buffer (5 min each). Between runs the capillary was flushed for 5 min with running buffer only. For CZEIA the running buffer was composed of 50 m*M* $Na_2B_4O_7$ (pH 9.4) and for MECCIA of 6 m*M* $Na_2B_4O_7$, 10 m*M* Na_2HPO_4 and 75 m*M* SDS (pH 9.4).

2.4. CE–MS instrumentation and running conditions

Mass spectrometry was performed on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray interface (Finnigan) 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-acetic acid (60:39:1, v/v/v) at a flow-rate of 3 μl/min was used as sheath liquid. The temperature of the heated capillary was kept at 200°C. The instrument was computer controlled using the XCalibur 1.0 software (Finnigan). A Prince Instrument (Lauerlabs, Emmen, NL) equipped with a 80 cm×50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was interfaced to the LCQ. Sample was introduced hydrodynamically by applying a positive pressure of 70 mbar for 12 s. The background electrolyte (BGE) was composed of 25 m*M* ammonium acetate adjusted to pH 9 with 1 *M* NH_3 and the applied voltage was 30 kV. Full scan mass spectra were acquired in the mass range of 100–500 or 200–500 Th (1 Th=1 *m*/*z*). Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS^2 was performed using data dependent scans with an isolation width of 2 Th and a relative collision energy of 35%. In these experiments the instrument automatically switches to MS^2 as soon as a defined mass peak exceeds a predefined threshold.

3. Results and discussion

3.1. CE-based immunoassays

As a continuation of our work characterizing competitive binding, electrokinetic capillary-based immunoassays for various drugs in human urine [13–17] using reagents which were commercialized for FPIA, analysis of urinary opioids by MECCIA and CZEIA was investigated. These approaches are based upon a 15 min incubation of equal amounts of urine with the reagents T and S (25 μl each) prior to injection of a small aliquot of the mixture onto the capillary and analysis of the fluorescein labeled tracer via on column LIF detection. Samples were typically injected during 1 s (at 0.5 p.s.i.) and electrophoretic runs were performed at 20°C. These conditions were also applied to the opioid system. Data obtained under MECC conditions are presented in panel A of Fig. 1. The opioid tracer was determined to produce more than one peak (top graph), indicating that the tracer may consist of a mixture of fluorescing antigens. This was verified in the MECCIA format which is exemplified with data obtained with urine blank (center graph) and urine blank fortified with 50 ng/ml MOR (bottom graph). Comparison of the two MECCIA electropherograms reveals that two of the peaks are becoming significantly higher in presence of urinary MOR, indicating that the competitive reaction is indeed taking place. Blank urine analyzed without immunoreagents did provide a few small peaks only or no peaks at all (data not shown). In the MECCIA electropherograms, allocation of the antibody-tracer complex was not possible. Furthermore, evaluation of the peak pattern was not trivial. Thus, conditions with increased injection time intervals and running temperature were evaluated. The data depicted in panel B of Fig. 1 were obtained with an injection time interval of 4 s. MECCIA data of blank urine at 20°C (as in Fig. 1A) revealed a complex pattern as well (bottom graph). This response, however, could be somewhat simplified via increase of the temperature to 30°C (center graph).

An MECCIA pattern with multiple peaks could contain more than one immunological reaction scheme, i.e. it could contain more than one antibody and separate tracers. This was previously documented for amphetamine and methamphetamine [14] and via mixing of FPIA reagents of different tests [16,17]. Thus, the MECCIA configuration with a 4 s injection time and 30°C was further evaluated with different opioids. Electropherograms presented in Fig. 2A represent those obtained with blank urine, the 100 ng/ml urine calibrator that was diluted two-fold with urine blank and two undiluted human urine calibrators that are reported to contain MOR in the concentrations of 350 and 1000 ng/ml. Corresponding data for blank urine that was fortified with DHC, EMOR and COD are depicted in panels B, C and D, respectively, of Fig. 2. In all cases, the two peaks labeled FT were found to become increased as the drug level was increased. Thus, it appears that

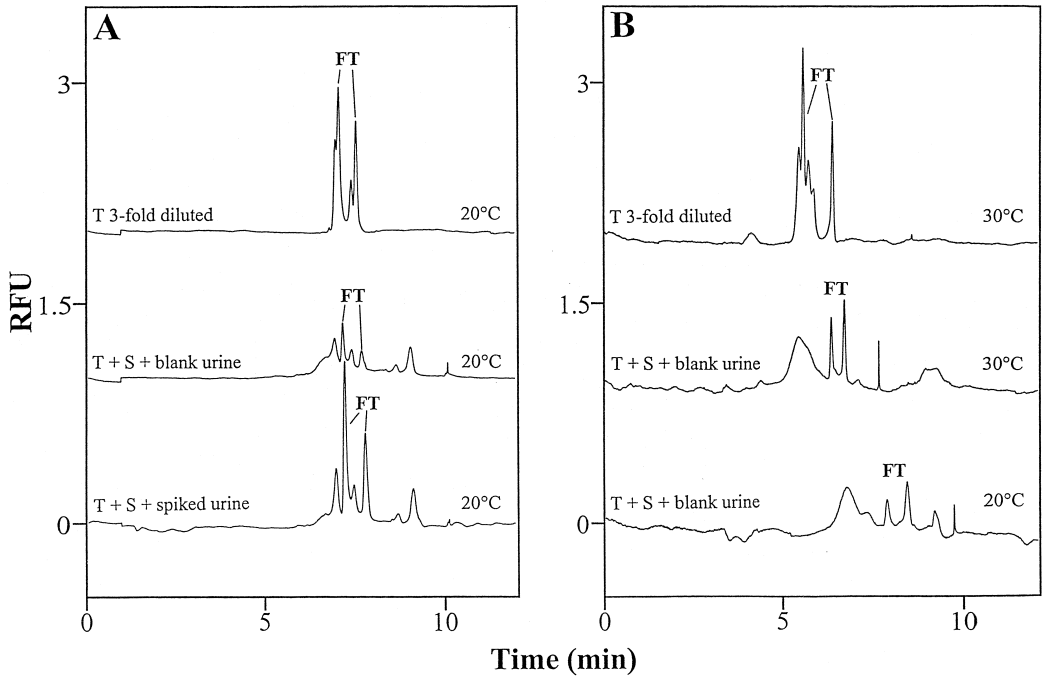

Fig. 1. MECC electropherograms obtained with (A) 1 s injection and (B) 4 s injection and at the temperatures indicated. The graphs in panel A depict MECC data obtained with 3-fold diluted tracer solution, MECCIA data of blank urine and MECCIA data of a blank urine spiked with 50 ng/ml MOR (from top to bottom, respectively). Electropherograms of panel B are those of 3-fold diluted tracer solution (top graph) and MECCIA of urine blank. All electropherograms were generated via application of a constant 15 kV. For experiments at 20°C and 1 s injection, currents were about 70 μA. With 4 s injection and run temperatures of 20 and 30°C, currents were 74 and 92 μA, respectively.

the system does not contain multiple reaction schemes that show differences in selectivity towards the tested compounds and tracers. However, it comprises two tracer molecules that appear to take part equally in the competitive reaction. Furthermore, the data presented in Fig. 2 indicate a drug dependent cross reactivity. During incubation a competitive reaction takes place between the tracers (solution T) and the antigen present in the sample for the limited amount of antibody (solution S) binding sites. Therefore if the free tracer peaks increase it indicates that a substance in the sample is reacting with the antibody and displacing the tracers bond to fluorescein. COD and MOR are shown to react more strongly than DHC and EMOR. This finding is in agreement with the cross reactivities listed in the TDx/TDxFLx pamphlet [28] and those reported by Cone et al. [29,30].

Employing the same reactants, CZE conditions were also evaluated. In the absence of the micelles, the tracer solution was found to provide one peak only (top graph in Fig. 3). The same was found to be true under CZEIA conditions. Selected electropherograms showing a single free tracer peak FT are presented in Fig. 3. These data were obtained with a 1 s sample injection and 20°C as running temperature. Not surprisingly, with increase of the MOR concentration, the FT response became larger, whereas the magnitude of the antibody-tracer complex (marked as C in Fig. 3) became smaller. At 1000 ng/ml MOR, the magnitude of the FT peak was determined to be about the same as was obtained for the 3-fold diluted tracer solution. The detection limit for MOR was estimated to be about 10 ng/ml, a value that compares favorably with the 25 ng/ml sensitivity reported for FPIA [28]. Similar data were obtained with a 4 s injection and operation at 30°C (panel A of Fig. 4). As was the case in MECCIA, responses of various opioids were also investigated by CZEIA and patterns observed in presence of

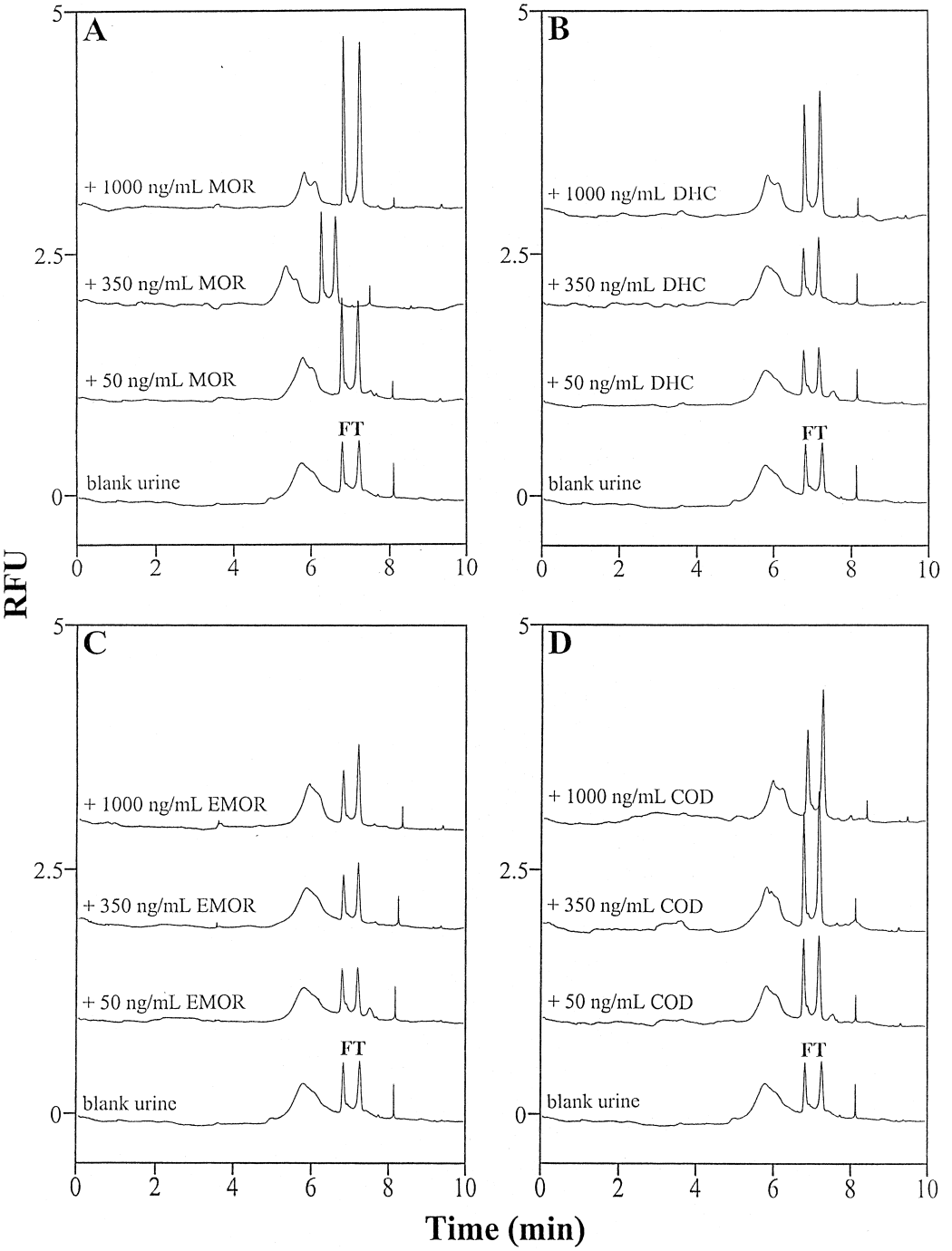

Fig. 2. MECCIA electropherograms obtained with blank urine (bottom graphs in each panel) and urines containing 50, 350 and 1000 ng/ml of (A) MOR, (B) DHC, (C) EMOR and (D) COD. Each sample consists of 25 μl urine, 25 μl solution T and 25 μl solution S. The electropherograms are depicted with a *y*-axis shift of 1 RFU. Key: RFU, relative fluorescence unit; FT, free tracer. For other experimental conditions see Section 2.3.

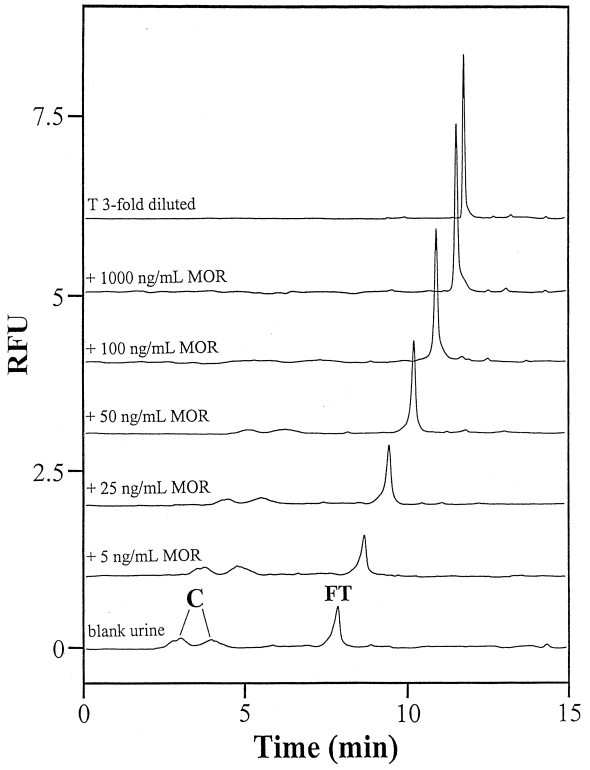

Fig. 3. CZEIA data obtained with 1 s injection and a temperature of 20°C of urine blank (bottom graph) and urines containing 5–1000 ng/ml MOR. Urines analyzed comprised calibrator F (1000 ng/ml MOR) and calibrator F diluted with urine blank to reach the indicated MOR concentrations. The data presented as top electropherogram represent CZE data of 3-fold diluted tracer solution. All electropherograms were generated via application of a constant 13 kV (currents: about 87 μA). C refers to the antibody-tracer complex. For presentation purposes, data are depicted with *x*-axis and *y*-axis shifts of 0.5 min and 1 RFU, respectively (from bottom to top).

DHC, EMOR and COD are depicted in panels B, C and D, respectively, of Fig. 4. Contrary to the MECCIA measurements, only one major FT peak was monitored in all cases, indicating that the two tracer molecules have equal electrophoretic mobilities and thus comigrate in the alkaline buffer system employed here. It is interesting to note that in CZEIA the antibody–tracer complex (marked as C in Fig. 4) could be clearly identified at drug concentrations which lead to small and intermediate FT peaks. This, of course, is dependent on the cross reactivity of the urinary drug present. For a 4 s injection, a running temperature of 30°C and a drug concentration of 350 ng/ml, the antibody–tracer complex was detected in the case of EMOR and DHC, but was not observed in presence of MOR and COD (Fig. 4). This indicates that the latter two compounds have a higher affinity to the antibody. As was the case in MECCIA, cross reactivities were found to increase in the order of EMOR, DHC, COD and MOR. Reactivities of the antibody with glucuronides of opioids was also investigated. The data presented in Fig. 5 represent those obtained for CZEIA analysis of urine blank and urines that were fortified with DHC-6-G, DHC, COD-6-G, COD, MOR-3-G and MOR (350 ng/ml each). The peak heights of FT in the presence of the glucuronides was found to be smaller than those registered with the unconjugated opioids. Consideration of molar concentrations, however, reveals that glucuronides react like the corresponding free opioids. Having a 4 s injection and 30°C, detection limits for MOR, COD and DHC were determined to be about 10, 10 and 40 ng/ml, respectively (data not shown).

MECCIA and CZEIA data of the two urines that were collected after drug administration are presented in Fig. 6. Sample u91 stems from a volunteer who took cold medication (Pretuval and Resyl Plus, the latter containing COD) and specimen u94 is the 0–8 h urine that was collected after intake of 25 mg DHC. FPIA screening in our departmental drug assay laboratory gave positive results for both urines. Without dilution, u91 and u94 were found to contain opiates that provided FPIA responses of 847 ng/ml and $>$1000 ng/ml, respectively. FPIA data of 10-fold diluted urines were 195 and 339 ng/ml, respectively. The electropherograms obtained by MECC are presented in panel A of Fig. 6. The first two graphs from bottom represent MECC data that were measured with three-fold diluted urines (without any immunoreagents), the center graph depicts MECCIA data of a blank urine and the upper two graphs comprise the MECCIA responses of the two urines. The opioid containing urines are shown to test positive (increased FT peaks compared to those seen in the electropherogram of the blank urine). CZE data are depicted in panel B of Fig. 6. The first two graphs from the bottom represent data obtained after injection of the three-fold diluted urines, the center graph represents the CZEIA pattern of a blank urine and the upper two graphs the CZEIA patterns of the

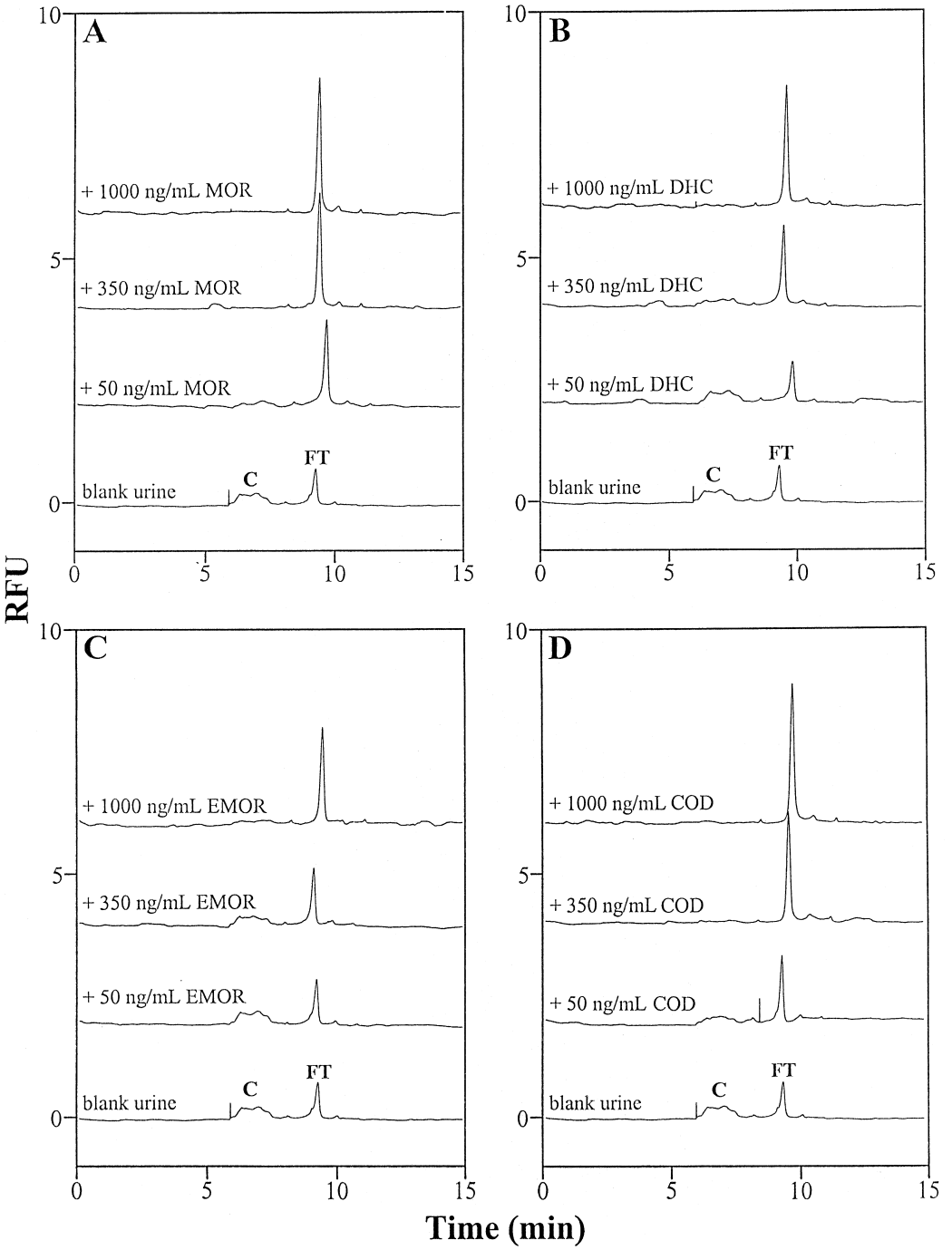

Fig. 4. CZEIA data for blank urine (bottom graphs) and urines containing 50, 350 and 1000 ng/ml of (A) MOR, (B) DHC, (C) EMOR and (D) COD depicted with a *y*-axis offset of 2 RFU units. C, antibody-tracer complex. Other conditions as in Section 2.3.

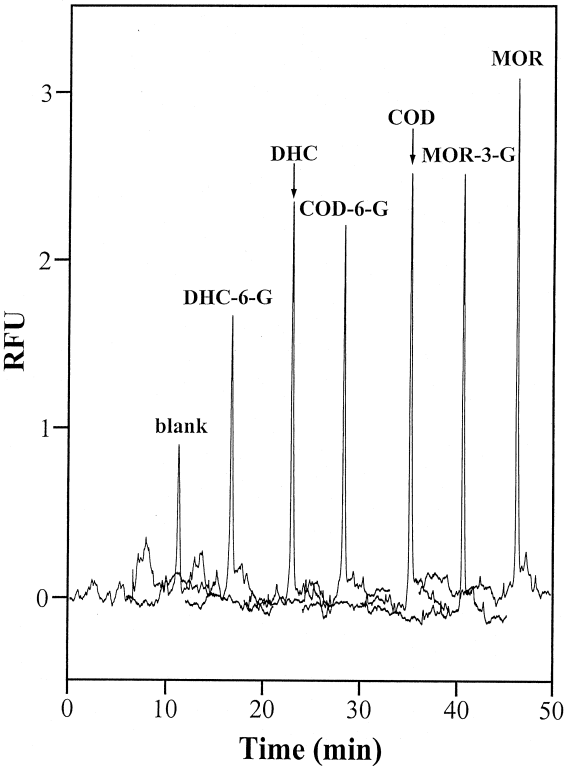

Fig. 5. CZEIA data for blank urine and blank urines fortified with DHC-6-G, DHC, COD-6-G, COD, MOR-3-G and MOR (350 ng/ml each), respectively. For the sake of FT peak comparison, data are presented on the same *y*-axis scale with successive graphs being displayed with a *x*-axis offset of 6 min. Other conditions as in Section 2.3.

two urines. Both urines show an increased FT peak in comparison to the FT peak of blank urine. Thus, it can again be concluded that the two urines contain molecules which react with the employed antibody against opioids. Due to the lack of specificity, however, identification of the compounds involved is not possible. Furthermore, it was interesting to find that the fluorescing compound seen in the CZE electropherograms of the urines (bottom graphs) were detected just ahead of FT. For the case of MECC, this compound is shown to coelute with the first tracer peak which is thus becoming somewhat taller than the second peak. Overall, the CZEIA format provides a simpler pattern and is thus preferred compared to the MECC conditions. Repeatabilities of both assays are as described before [13–16], namely better than 10%.

3.2. Identification and confirmation with CE–MS

For the determination of single urinary codenoids and their identification, urine extracts were analyzed by CE–MS using the LCQ ion trap MS. This instrument features the possibility of measuring up to MS^9. In most cases, the identity of a substance can already be confirmed via MS^2 or MS^3 spectra. The possibility that another ion is being fragmented in the same way beyond this point is rare. Thus, urinary extracts were analyzed by MS^2 or MS^3 only. A volatile BGE composed of 25 m*M* ammonium acetate that was adjusted to pH 9 with 1 *M* NH_3 solution was employed for solute separation. The sheath liquid was acidic and thus guaranteed the formation of positively charged molecule ions $[M+H]^+$. The volunteer urines u91 and u94 were expected to contain COD and metabolites and DHC and metabolites, respectively. Major urinary metabolites are the glucuronides (COD-6-G and DHC-6-G, respectively) and the desmethyled products NCOD and NDHC, respectively [6,7]. To get reference spectra aqueous standard solutions (10 μg/ml) of DHC, DHC-6-G, NDHC, COD, COD-6-G and NCOD were analyzed and their MS, MS^2 and MS^3 (glucuronides only) spectra were stored in a computer library. Not surprisingly, molecule ions $[M+H]^+$ and mass spectra for COD (m/z 300.4) and COD-6-G (m/z 476.5) were found to be identical to those reported for LC–MS with electrospray ionization [31], whereas the molecule ion of DHC (m/z 302.4) was determined to be the same as that previously identified with thermospray MS [32]. No MS^2 and MS^3 spectra of the codeinoids could be found in the literature. Fragmentation of COD-6-G and DHC-6-G to COD and DHC, respectively, was found to follow the same degradation principle as was previously observed for the glucuronides of MOR using LC–MS with electrospray ionization [33].

Urine samples were extracted via solid-phase extraction according to the procedure described in Section 2.2. Starting with 2 ml of urine and reconstitution in 200 μl sample solvent theoretically leads to a 10 fold concentration of the solutes. This is diminished by the extraction recoveries found to be 95%, 93%, 96% and 94% for DHC, NDHC, COD and NCOD, respectively. Based upon previous in-

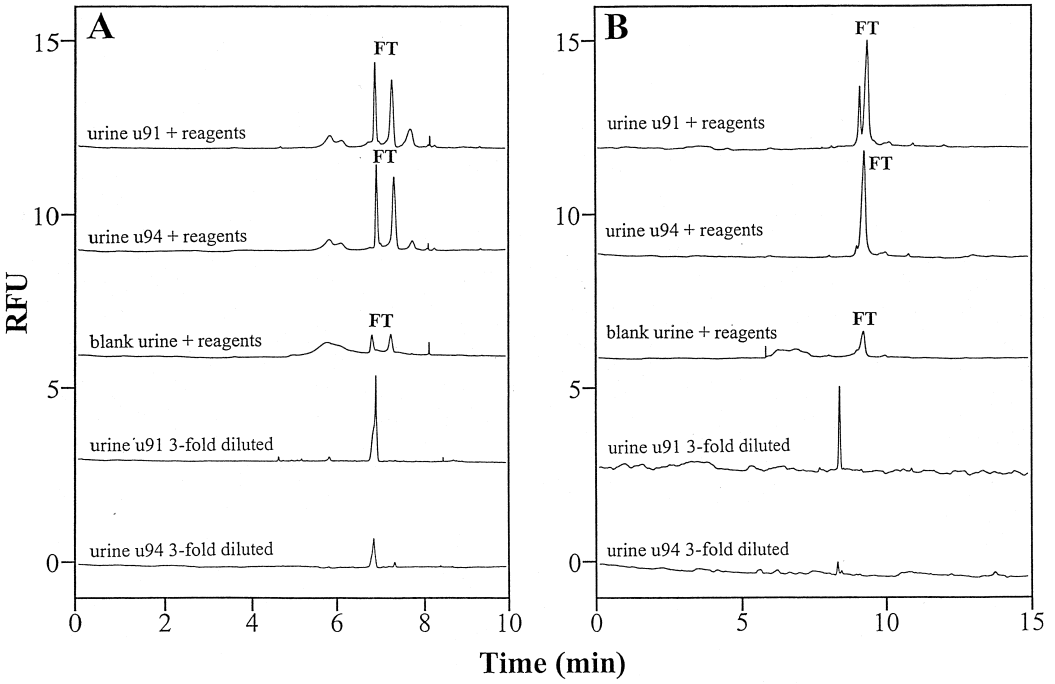

Fig. 6. (A) MECC and (B) CZE data of 3-fold diluted urines u94 (bottom graphs) and u91 (2nd graphs from the bottom) and (A) MECCIA and (B) CZEIA data of the same urines (4th and top graphs). The center graphs depict the electropherograms of blank urine with immunoreagents. The *y*-axis offset is 3 RFU units. Other conditions as for Figs. 2 and 4, respectively.

vestigations, recoveries of the glucuronides are expected to be >80% [6,7]. As examples, mass traces, mass spectra and MS^2 data for DHC (top graphs) and COD (bottom graphs) determined by CE–MS of an extract of blank urine fortified with DHC and COD (10 μg/ml each) are presented in Fig. 7. Under the conditions used DHC ($[M+H]^+$: *m/z* 302.4) was found to migrate ahead of COD ($[M+H]^+$: *m/z* 300.4) and fragmentation of the two compounds was determined to be different (compare MS^2 data). The monitored MS^2 spectra (Fig. 7) were found to compare well with those of the library (see above). The detection limit for 2 ml of spiked urine with an *S/N* ratio of 3 (and MS^2 could still be performed) was found to be at a concentration of 100 ng/ml for DHC, NDHC, and COD and at 200 ng/ml for NCOD (data not shown). Detection limits for the glucuronides were not determined. Furthermore, based on a siphoning effect (cathodic co-flow within separation capillary) which is difficult to control [17,24], detection time intervals were noted to vary significantly when compared with those observed in different assemblies (see e.g. data of Figs. 7 and 8). However, working with the same capillary mounting, intra-day RSD values ($n=5$) of detection times of COD and DHC were determined to be 0.52 and 0.33%, respectively (compare data of Figs. 8 and 9).

The data presented in Fig. 8 represent those obtained for the analysis of the extract of urine u91. This sample was found to comprise COD and COD-6-G. NCOD and MOR could not be detected with the setup employed. The presence of COD was confirmed via comparison of MS^2 data with those shown in Fig. 7 and those of a standard in the computer library (hit: 98%). For COD-6-G ($[M+H]^+$: *m/z* 476.5), MS^2 lead to the typical mass spectrum of COD with an *m/z* value of 300.3 (inset of center panel) and MS^3 data provided the structural proof (bottom graph; spectra match: 98.8%). Thus, the mass trace for *m/z* 476.5 monitored after 5.11

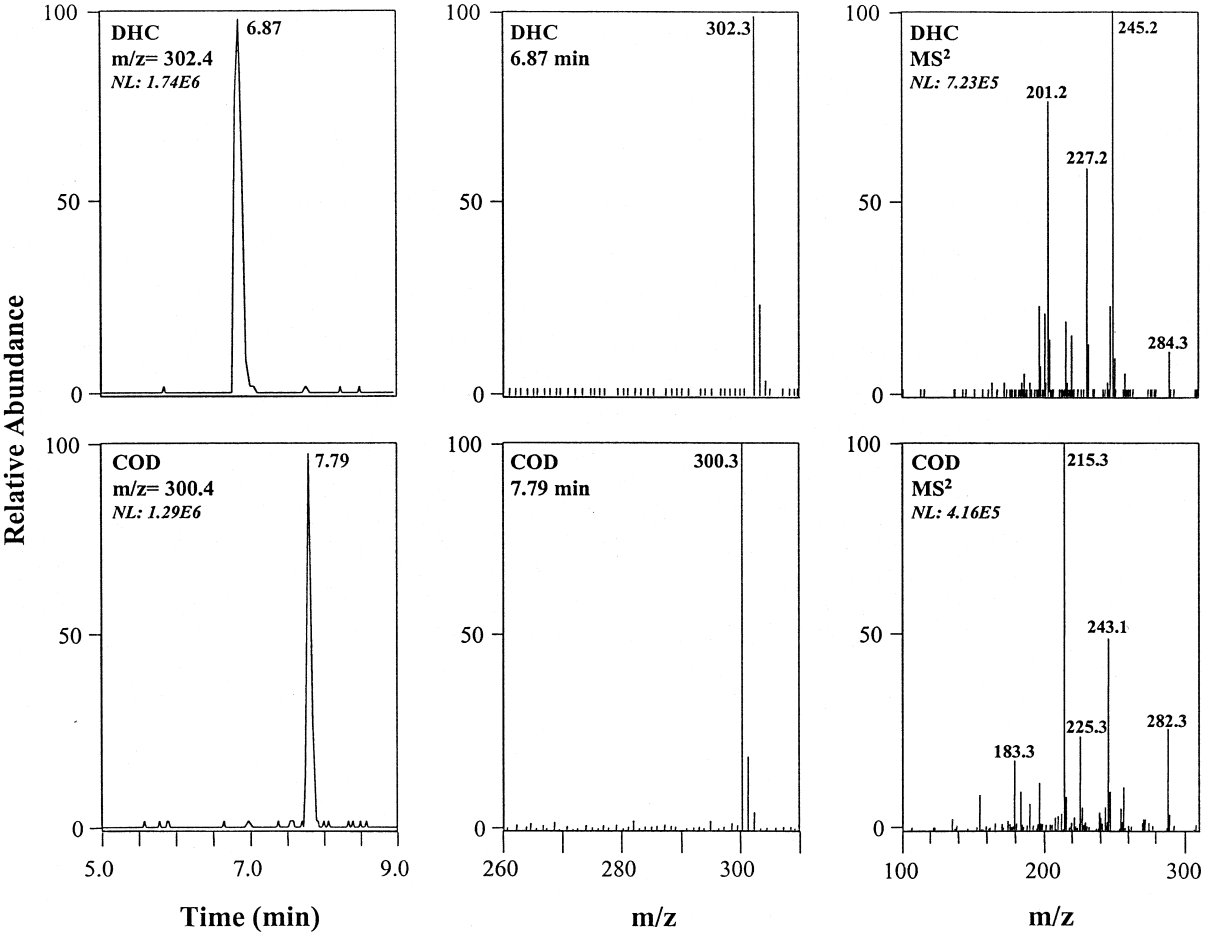

Fig. 7. Mass traces, mass spectra and MS^2 data for DHC (top graphs) and COD (bottom graphs) determined by CE-MS of an extract of blank urine fortified with DHC and COD (10 μg/ml each).

min was found to contain COD-6-G. Based upon the MS data (center panel), other compounds are likely to comigrate with COD-6-G. No further work, however, was geared towards identification of the origin of the other masses in that spectrum.

CE–MS data obtained with the extract of urine u94 are depicted in Fig. 9. As was the case with sample u91, the presence of DHC (*m/z* 302.4) could be confirmed via comparison of MS^2 data with those registered previously (see Fig. 7). Furthermore, the mass trace for *m/z* 478.5 was found to contain a pronounced peak at 5.02 min (top graph in right hand panel). MS data for that time point revealed *m/z* values of 487.3 and 302.3 (center graph), values that correspond to those of DHC-6-G and DHC, respectively. Isolation of *m/z* 478.3 and fragmentation lead to the MS^2 data presented as inset in the center panel. Furthermore, isolation and fragmentation of the obtained *m/z* 302.3 ion lead to the MS^3 data presented in the bottom graph of the right hand panel. These patterns are identical to those obtained with MS and MS^2 of DHC, respectively. Spectral matches of the MS^2 and MS^3 data presented in the bottom panels with those stored in the computer library were 98 and 99%, respectively. Thus, the presence of DHC and DHC-6-G in that urine extract

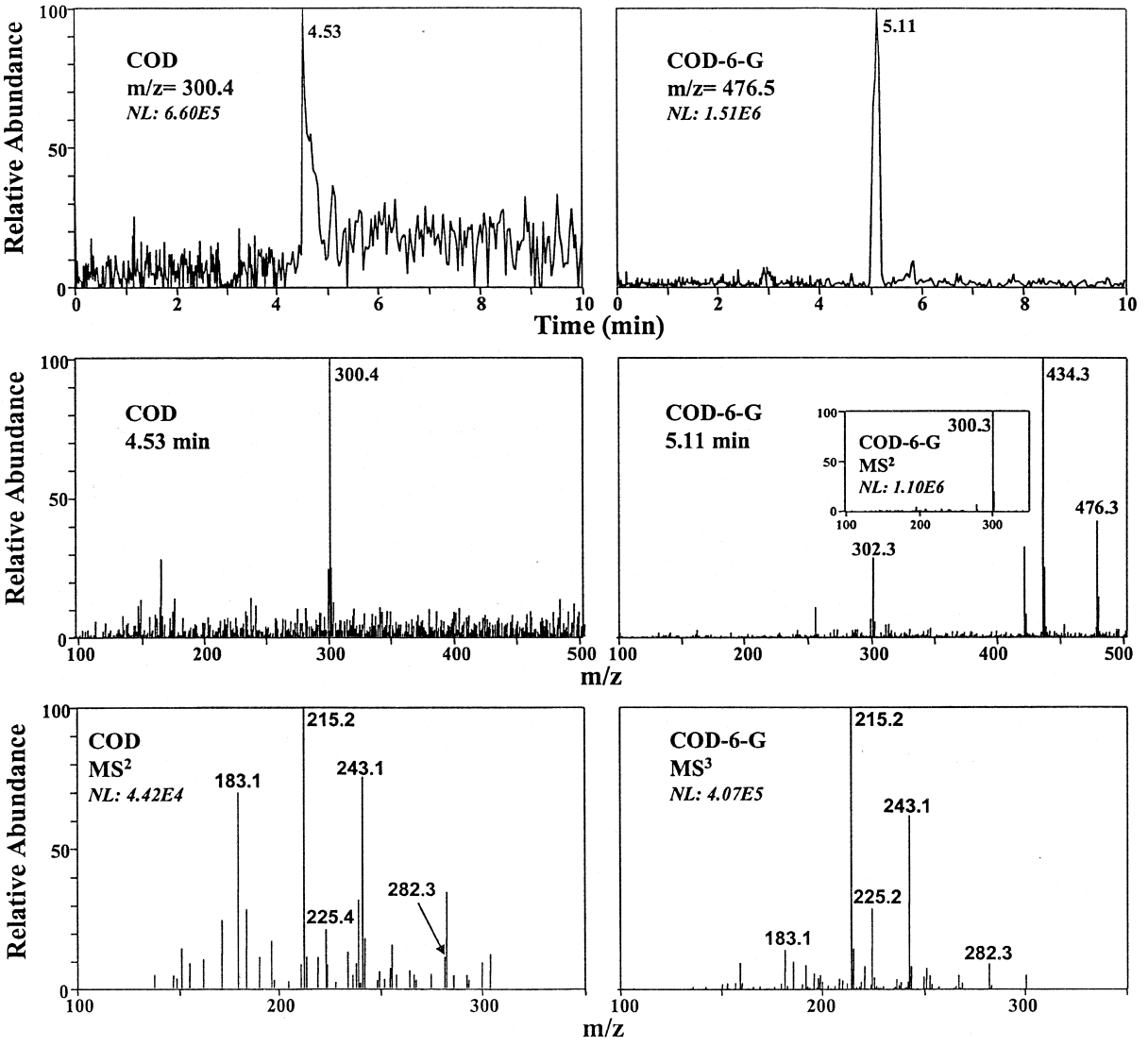

Fig. 8. CE–MS data obtained for the extract of urine u91. The top graphs represent mass traces for COD and COD-6-G. The mass spectra depicted as center graphs are MS^1 data for COD and COD-6-G (inset: MS^2 data) whereas those presented in the bottom panels are the MS^2 data of COD and the MS^3 data of COD-6-G.

is thereby confirmed. NDHC and dihydromorphine were not detected.

4. Concluding remarks

Using the commercial, broadly cross-reacting FPIA reagents for CE analysis of urinary opiates, data presented in this paper indicate that the presence of urinary codeinoids can be recognized by both MECCIA and CZEIA. Codeinoids, however, cannot be identified or distinguished from morphinoids. The same is true for free and conjugated codeinoids. This is equal to the findings reported for FPIA [28–30]. Distinction from morphinoids would require codeinoid-specific antibodies as were described for MOR and its glucuronides [9,34,35]. CZEIA is shown to provide simple electropherograms which

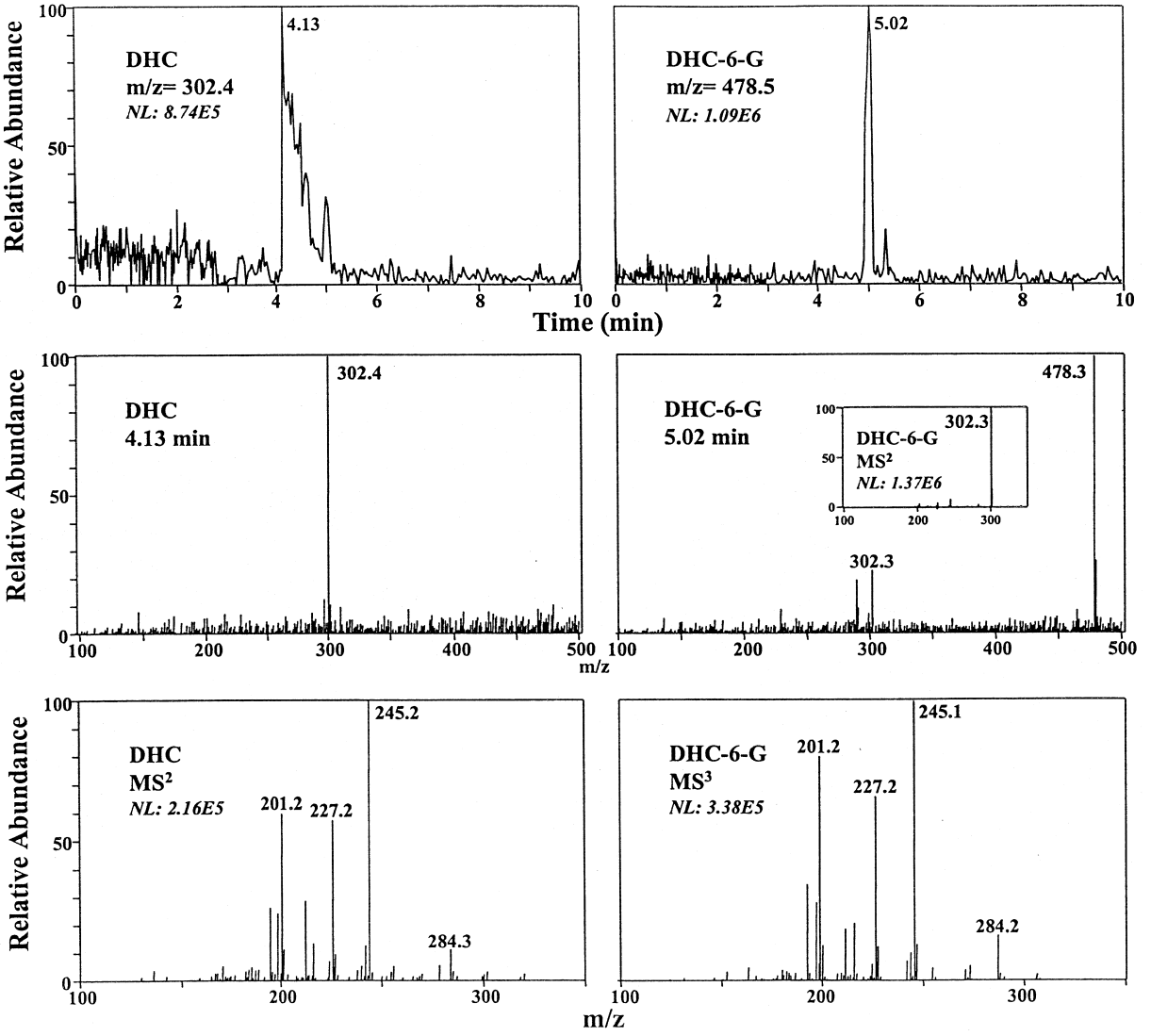

Fig. 9. CE–MS data obtained for the extract of urine u94. The top graphs represent mass traces for DHC and DHC-6-G. The mass spectra depicted as center graphs are MS^1 data for DHC and DHC-6-G (inset: MS^2 data) whereas those presented in the bottom panels are the MS^2 data of DHC and the MS^3 data of DHC-6-G.

are characterized by an opioid concentration dependent magnitude of the free tracer peak. In presence of dodecyl sulfate micelles, however, two tracer peaks with equal opioid concentration sensitivity are monitored. The MECCIA data suggest the presence of two fluorescent tracers which react competitively with the urinary opioids for the binding sites of the antibody. Assay sensitivity for COD is comparable to that of MOR (10 ng/ml) and the detection limits for DHC and EMOR are about 40 ng/ml. Analysis of urines that were collected after administration of 7 mg COD and 25 mg DHC tested positively in both assay formats. The presence of the codeinoids in these urines and their identification was accomplished by CE–MS using an instrument with electrospray ionization and an ion trap MS. Analysis of solid-phase urinary extracts by MS^2 provided the proof for the presence of COD and DHC, whereas

the unambiguous identification of their glucuronides was obtained via MS^3 data. Commencing with 2 ml urine and reconstitution in 200 μl of sample solvent, COD, DHC and their glucuronides could be detected on the 100–200 ng/ml concentration level, a sensitivity that is sufficient for toxicological confirmation case work. Access to lower concentrations (ppb and sub-ppb) would be obtained via (i) use of larger urine volumes followed by reconstitution of the dried extract in sample solvent volumes that are smaller than 1/10 of the original urine volume, (ii) injection of larger sample volumes or (iii) using electrokinetic injection from an extract prepared in a low conductivity sample solvent [8]. 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, Bach and Henion [23] reported quantitation of urinary methylphenidate on the ppb level. Furthermore, Caslavska et al. [16] described quantitation in the immunoassay format, an approach that can only be quantitative for solutes that are monitored with high specificity and selectivity.

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