ELSEVIER

Journal of Chromatography B, 770 (2002) 191–205

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Capillary electrophoresis and capillary electrophoresis–ion trap multiple-stage mass spectrometry for the differentiation and identification of oxycodone and its major metabolites in human urine

Anita B. Wey, Wolfgang Thormann*

Department of Clinical Pharmacology, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland

Abstract

Oxycodone (OCOD) and its metabolites, including oxymorphone (OMOR), noroxycodone (NOCOD) and noroxymorphone (NOMOR), are opioids that carry an OH group at position 14. Using capillary electrophoresis (CE) with a binary phosphate buffer containing 60% ethylene glycol (pH 7.9), the migration order of OCOD and OMOR with respect to their N-demethylated analogs was found to be reversed compared to that observed for codeine, dihydrocodeine, morphine and dihydromorphine, compounds that do not have an OH group at position 14. OCOD and structurally related compounds can also be distinguished from these opioids by their absorbance spectra at low wavelengths and via a characteristic neutral H_2O loss at the MS^2 level. Using the binary phosphate buffer, CE with UV detection is shown to be capable of monitoring OCOD, NOCOD, OMOR (after hydrolysis only) and NOMOR (after hydrolysis and in patient urine only) in alkaline liquid–liquid extracts of urines that were collected after ingestion of 10 mg OCOD hydrochloride and in a patient urine collected at steady state (80 mg OCOD hydrochloride daily). Using an aqueous pH 9 ammonium acetate buffer, these results were confirmed by CE–MS^3. Based on CE–MS, MS^2 and MS^3 data, the absorbance spectra measured across the CE peaks and the relative position within the electropherogram, two peaks monitored in the UV absorbance electropherograms could be assigned to the two keto-reduced metabolites 6oxycodol (6OCOL) and nor6oxycodol, for which no standards were available. Comparison of data obtained with urines pretreated with two different enzyme products (β-glucuronidase and β-glucuronidase/arylsulfatase) suggest that OCOD, NOCOD and 6OCOL are mainly glucuronidated, whereas OMOR mainly forms other conjugates. Furthermore, in a first attempt to directly measure conjugates of the compounds of interest, solid-phase extracts were analyzed by CE–MS^4, which revealed the presence of the acyl glucuronides of 6OCOL and OMOR and an unidentified OMOR conjugate. The quantitation of free OCOD and NOCOD by CE–MS using deuterated internal standards is also discussed briefly.

Keywords: Oxycodone

1. Introduction

Oxycodone (OCOD; for structure, see Fig. 1) is a semisynthetic opioid with structural relations to other opioids, including dihydrocodeine, codeine and morphine. Compared to dihydrocodeine, OCOD carries a

*Corresponding author. Tel.: +41-31-632-3288; fax: +41-31-632-4997.

E-mail address: wolfgang.thormann@ikp.unibe.ch (W. Thormann).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00568-2

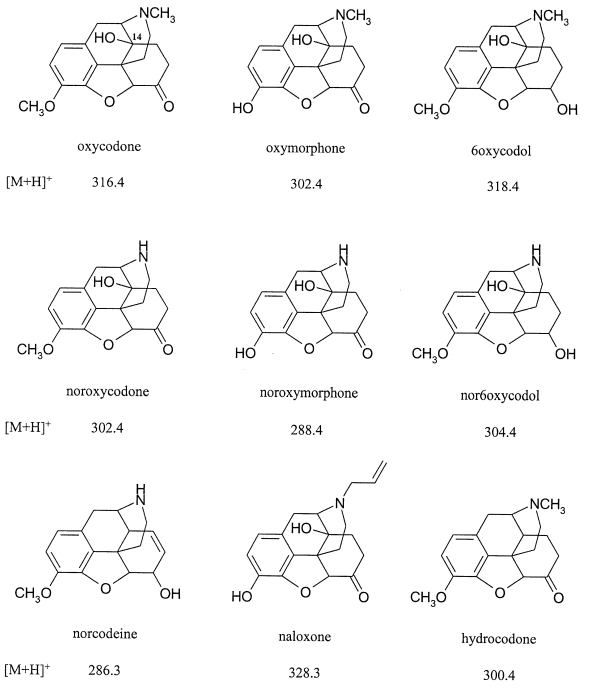

Fig. 1. Chemical structures and $[M+H]^+$ values for OCOD and five metabolites, NCOD (internal standard), naloxone and hydrocodone.

hydroxy group at C14 and a ketone group instead of the hydroxy group at C6. The C14 hydroxy group is the only difference to hydrocodone (Fig. 1). OCOD is a strong opioid analgesic that is used for the management of moderate to severe mainly post-operative or cancer related pain [1–4]. The short half-life of OCOD (4–6 h) requests a dosing every 4 h. For convenience and to improve compliance, OCOD is typically administered in controlled-release formulations that can be taken in a 12 h interval [4,5]. Although OCOD has been reported to be metabolized in animals by N- and O-demethylation, 6-keto reduction and conjugation (for structures, see Fig. 1), the metabolism of OCOD in man is poorly characterized [5–9]. The O-demethylation (formation of oxymorphone (OMOR) from OCOD and noroxymorphone (NOMOR) from noroxycodone (NOCOD)) is catalyzed by the liver enzyme CYP2D6. It is known that this enzyme is expressed as two phenotypes and individuals are therefore referred to as poor and extensive metabolizer phenotypes [10]. In a study performed by Heiskanen et al. [11], no OMOR could be measured in the plasma after selectively blocking CYP2D6 with quinidine in eight out of 10 volunteers. The hypothesis that OMOR plays an active role in the analgesia of OCOD could not be confirmed by Kaiko et al. [5] and Heiskanen et al. [11,12]. Thus, it is still unclear which metabolites play an active role in the analgetic effect.

For the detection and determination of OCOD in plasma or urine, methods based upon high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been employed. Smith et al. [13] and Wright et al. [14] developed HPLC methods with electrochemical detection for the quantitation of OCOD in human plasma, and Kaiko et al. [5] reported the use of gas chromatography–mass spectrometry (GC–MS) for the determination of plasma levels of OCOD, OMOR, NOCOD and NOMOR. Meatherall [15] described a procedure for simultaneous confirmation of codeine, morphine, 6-acetylmorphine, hydrocodone, hydromorphone, OCOD and OMOR in urine specimens by GC–MS, and Nowatzke et al. [16] reported a GC–MS method suitable for the distinction between OCOD, OMOR and six other opiate drugs in urine.

In the past few years, our laboratory has been engaged in the evaluation of capillary electrophoresis (CE) for separation of opioids [17] and analysis of opioids in body fluids [18–27]. Micellar electrokinetic capillary chromatography (MECC) assays with multi-wavelength detection were demonstrated to be suitable for the urinary screening of codeine, morphine and 6-acetylmorphine [18,19] and for the assessment of the metabolism of dihydrocodeine [20,21]. Using capillary zone electrophoresis (CZE), a high-sensitivity approach based upon head-column field-amplified sample stacking (FASS) for the determination of dihydrocodeine in small amounts of plasma [22–24] and urine [23,24] was developed and applied to samples that were collected after intake of pharmaceutical preparations. Finally, an electrokinetic capillary immunoassay was shown to be suitable to recognize the presence of opioids in urine [25] and CE–electrospray ionization multiple-stage ion trap MS was recognized as an attractive confirmatory approach for urinary codeinoids [25] and morphinoids [26]. As a continuation of that work and as the metabolism of OCOD in man has been poorly explored thus far, the CZE behavior of OCOD and its metabolites was studied. Compared to the previ-

ously investigated molecules, namely opioids related to codeine, dihydrocodeine, morphine and heroin, these compounds carry an additional OH group at position 14 of the opioid structure (Fig. 1).

This paper reports for the first time (i) the CZE characterization of OCOD, OMOR, NOCOD and NOMOR in comparison to codeine and dihydrocodeine and their metabolites, (ii) the analysis of OCOD and its metabolites in urinary extracts by CE with UV absorbance detection and by CE–ion trap MS^n, (iii) quantitation of urinary OCOD and NOCOD by CE–MS, and (iv) the characterization of the urinary metabolic pattern of OCOD, including the assignment of the two keto-reduced metabolites 6oxycodol (6OCOL) and nor6oxycodol (N6OCOL) (urinary metabolites that were previously reported to be excreted in rabbits; for structures, see Fig. 1), after single dose administration of 10 mg OCOD hydrochloride and at steady state with a daily dose of 80 mg OCOD hydrochloride.

2. Experimental

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

OCOD, OMOR and norcodeine (NCOD) were purchased as methanolic solutions (1 mg/ml) from Alltech (State College, PA, USA). NOCOD (1 mg/ml), NOMOR (100 μg/ml), and the deuterated standards OCODd3, NOCODd3 and OMORd3 (100 μg/ml each) were purchased as methanolic solutions from Cerilliant (Austin, TX, USA). β-Glucuronidase from *Escherichia coli* (G7646; 25000 units/ml) was obtained from Sigma (St. Louis, MO, USA) and β-glucuronidase/arylsulfatase from *Helix pomatia* (127 060; 100 000/800 000 units/ml) was from Roche (Basel, Switzerland). All other chemicals were of analytical grade. Standard solutions were prepared by diluting appropriate aliquots of the methanolic stock solutions with water. All solutions were stored at −20 °C. Four OCOD-containing urines were analyzed. Two were collected during the 0–8 h (referred to as ua8 and ut8) and one during the 11–14 h (ut14) interval after administration of a 10 mg tablet of OxyContin (Purdue Pharma, Norwalk, CT, USA; 10 mg OCOD hydrochloride controlled release). The fourth sample (ul12) was a morning urine obtained from a person on a steady state level (80 mg of OxyContin daily; morning and evening, 40 mg each) that was collected just before the next dosing. Our own urines were used as blank matrices.

2.2. Sample preparation

The urines were either analyzed after dilution with water or after extraction. Solid-phase extraction (SPE) 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 milliliters 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 2 ml of methylene chloride–isopropanol (80:20, v/v) containing 2% of concentrated ammonia solution, 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 100 μl of water. For CE–MS measurements, the opioids were eluted with 1.5 ml methanol containing 30% of concentrated ammonia solution. For liquid–liquid extraction, the ToxiTube A system (pH 9) from Analytical Systems (Laguna Hills, CA, USA) was employed. Two milliliters of urine were added to this commercial liquid–liquid extraction system, which comprises an organic solvent mixture of CH_2Cl_2 and $C_2H_4Cl_2$. After gently shaking for about 1 min and centrifugation for 5 min at about 1500 *g*, 2 ml of the organic phase were transferred into a glass tube and the solvent was evaporated at 35 °C under a gentle stream of nitrogen. The residue was redissolved in 100 μl of water. For enzymatic hydrolysis, 2 ml of urine, 2 ml of 0.2 *M* sodium acetate buffer (pH 5.4), 200 μl of an aqueous NCOD solution (10 μg/ml; internal standard) and 50 μl of the enzyme product were mixed and incubated at 37 °C for 4 or 24 h. Thereafter, the entire mixture (4.25 ml) was extracted with the ToxiTube A system as described above.

2.3. Instrumentation and running conditions for CZE with UV absorbance detection

For CZE with single-wavelength detection, a P/ACE 5510 capillary electrophoresis system (Beckman Instruments, Fullerton, USA) was used which was equipped with a fused-silica capillary of 50 μm I.D. and 27 cm (20 cm) total (effective) length. Samples were injected hydrodynamically by using a positive pressure of 0.5 p.s.i. or electrokinetically across a water plug by applying 10 kV. The capillary temperature was set at 25 °C, the carousel was at ambient temperature and detection was effected at 210 nm. 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 3 min with running buffer only. If not stated otherwise, the buffer consisted of 75 m*M* Na_2HPO_4 and 25 m*M* NaH_2PO_4 containing 60% (v/v) ethylene glycol (pH 7.9) [17,22]. The voltage applied was 20 kV (current: about 36 μA) and the anode was on the sampling side. For comparison with CE–MS data, a 50 μm I.D. capillary of 87 cm (80 cm) total (effective) length was employed together with a 25 m*M* ammonium acetate buffer that was adjusted to pH 9 with 1 *M* NH_3 [25] and application of 30 kV (current: about 17 μA). For CZE with multi-wavelength detection, a BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) was employed. It was equipped with a 50 μm I.D. fused-silica capillary of 24 cm total length (19.4 cm effective length). Sample was injected by positive pressure (10 p.s.i.*s) and a constant voltage of 20 kV (current: about 40 μA) was applied. The temperatures of the cartridge and the carousel were maintained at 25 °C and detection was effected via fast scanning at 5 nm resolution in the range between 195 and 320 nm. The phosphate buffer mentioned above was used as background electrolyte.

2.4. CE–MS^n 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) run in the positive ion mode (3.5 kV). Sheath gas (N_2) pressure was set at 20 arbitrary units. A mixture of methanol and water (50:50, v/v) containing 1% formic acid at a flow-rate of 5 μl/min was used as sheath liquid. The acidic sheath liquid employed supported the formation of positively charged ions $[M+H]^+$. 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, The Netherlands) equipped with a 50 μm I.D. fused-silica capillary of 80 cm length (Polymicro Technologies, Phoenix, AZ, USA) was interfaced to the LCQ. Sample was either introduced hydrodynamically by applying positive pressure (70 mbar, 18 s) or electrokinetically by applying 10 kV for 90 s. To minimize the effects of siphoning towards the cathode the spray voltage and the sheath gas were set to 0 during both injection modes [27]. 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 during separation was 30 kV (26.5 kV effective voltage). Full-scan mass spectra were acquired in the mass range 100–500 Th. Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS^2, MS^3 and MS^4 were performed using data-dependent scans with an isolation width of 2 Th and a relative collision energy of 35%. For quantification, full-scan MS (*m*/*z* 100–500) was employed. To guarantee a minimum of eight data points across the electrophoretic peak, two microscans and a maximum injection time of 200 ms were chosen.

3. Results and discussion

3.1. Electrophoretic separation of OCOD and its metabolites

The CZE behavior of OCOD and its metabolites was studied using the binary buffer system comprising 0.1 *M* phosphate and 60% ethylene glycol (pH 7.9) that was previously used for other opioids by Zhang and Thormann [17,22]. The data presented in Fig. 2 are those obtained on the BioFocus after

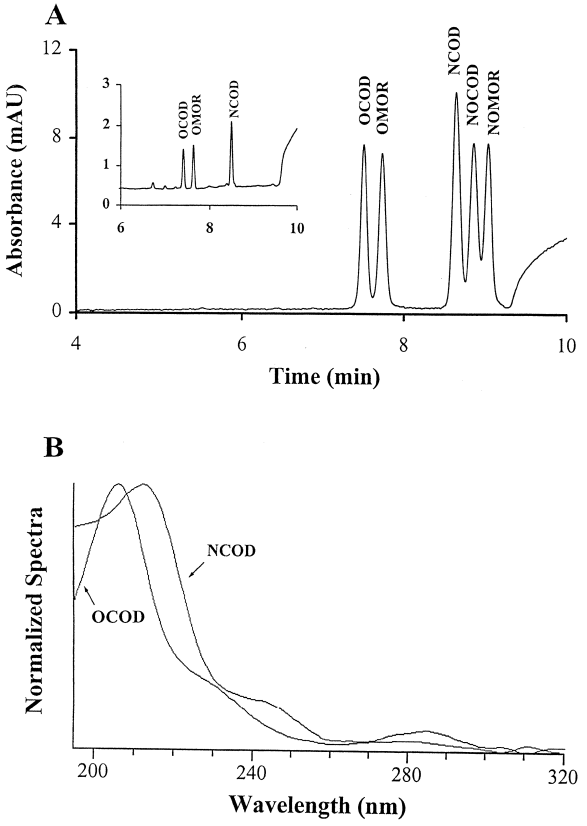

Fig. 2. (A) Single-wavelength (210 nm) electropherogram monitored on the BioFocus with an aqueous standard sample comprising OCOD, OMOR, NCOD, NOCOD and NOMOR (20 μg/ml each) and (B) normalized absorbance spectra of OCOD and NCOD. The insert in panel (A) depicts an electropherogram that was obtained on the P/ACE using head-column FASS injection (10 kV, 20 s) from an aqueous standard solution containing OCOD, OMOR and NCOD (10 ng/ml each).

hydrodynamic injection (10 p.s.i.*s) of an aqueous model mixture comprising OCOD, OMOR, NOCOD, NOMOR and NCOD (20 μg/ml each). Separation of the five compounds is shown to be possible within 9 min (panel (A)). OCOD and OMOR were determined to migrate ahead of NOCOD and NOMOR, respectively, a sequence which is reversed compared to that observed with codeine, dihydrocodeine and morphine with respect to their corresponding N-demethylated analogs. Furthermore, the spectra of OCOD and its metabolites were found to be similar (see below), but distinctly different than those of NCOD (Fig. 2B) and analogs (data not shown). Using the P/ACE and hydrodynamic sample injection, the detection limit with an S/N ratio of 3 was found to be 100 ng/ml. To improve sensitivity, head-column FASS [22–24,27,28] was employed. Before electrokinetic sample injection, the capillary tip of the inlet end was dipped into a vial filled with water for 3 s and a water plug (from a different vial) was injected. To find the proper length of the water plug, a standard solution containing OCOD and OMOR and NCOD (10 ng/ml each) was analyzed. After injecting water for 1, 2 or 3 s leading to plug lengths of 0.19, 0.38 or 0.57 mm, respectively, the sample was introduced onto the capillary by applying 10 kV for 20 s. Best results were obtained with a plug length of 0.19 mm and all further head-column FASS measurements were executed under these conditions. An electropherogram obtained with an aqueous standard solution containing OCOD, OMOR and NCOD (10 ng/ml each) is presented as an insert in Fig. 2A. Using that approach, OCOD and OMOR could be monitored down to a concentration of 1 ng/ml ($S/N = 3$). It is expected that the detection limit could be lowered by increasing the injection time [27]. As it is known that the sample solvent influences the stacking efficiency [28], different solvents were tested. Water appeared to be the most suitable solvent. The use of 50, 100 or 1000 μM HCOOH and the addition of MeOH in concentrations of 50–70% did not lead to any sensitivity improvement (data not shown).

For OCOD, OMOR and NCOD (internal standard) and the ratios OCOD/NCOD and OMOR/NCOD the intra- and inter-day RSD values ($n = 5$) of migration time, peak area and peak height after hydrodynamic injection and head-column FASS of an aqueous standard solution at a concentration of 500 and 50 ng/ml, respectively, were determined and are presented in Table 1. Within a day the detection times for all three compounds are shown to be reproducible (RSD values $<0.7\%$). As expected, RSD values of peak areas and heights were much smaller after hydrodynamic injection ($\leq$ 7%) than after head-column FASS (62–69%). This finding corresponds well with the data presented by Zhang and Thormann [22,28] and it confirms the need of an internal standard for head-column FASS. The ratios of OCOD/NCOD and OMOR/NCOD led to RSD values $<10.5\%$ for head-column FASS and $<3\%$

Table 1
RSD values of intra-day and inter-day measurements of an aqueous standard solution comprising OCOD, OMOR and NCOD ($n=5$)[a]

	Hydrodynamic injection			Head-column FASS injection		
	Detection time	Peak area	Peak height	Detection time	Peak area	Peak height
Intra-day RSD values (%)						
OCOD	0.46	5.76	3.73	0.48	63.29	62.99
OMOR	0.47	7.01	4.15	0.53	68.30	65.96
NCOD	0.62	5.68	3.41	0.64	62.71	63.68
OCOD/NCOD	0.16	3.94	2.90	0.20	4.20	1.00
OMOR/NCOD	0.18	2.14	1.83	0.17	10.44	5.50
Inter-day RSD values (%)						
OCOD	4.91	8.63	5.73	4.99	14.84	19.94
OMOR	5.02	10.36	6.29	5.09	11.24	14.30
NCOD	5.47	8.15	5.97	5.41	15.09	19.91
OCOD/NCOD	0.53	6.12	3.35	0.40	1.56	1.45
OMOR/NCOD	0.44	7.41	3.49	0.30	6.44	3.82

[a] For hydrodynamic and electrokinetic injection, opioid concentrations were 500 and 50 ng/ml, respectively. Injection times were 20 s in both cases.

for hydrodynamic injection, indicating that the use of an internal standard increases the reproducibility for both injection modes. With measurements on different days (inter-day data), detection time RSD values for all three compounds and both injection methods were found to be between 4.9 and 5.5%, whereas the relative values were found to be $<0.5\%$. After hydrodynamic injection, RSD values for peak areas and heights were between 5.7 and 10.4% (for ratios between 3.3 and 7.4%). After head-column FASS, all RSD values for peak areas and heights were determined to be between 11 and 20%. These values are surprisingly low. They were generated with the first head-column FASS runs of each day. Evaluation of the second to the fifth runs revealed RSD values between 50 and 100%. Although the origin of this phenomenon is unknown, it might be the consequence of some contamination of the involved buffer vials as they were not changed between every run.

3.2. Characterization of urinary extracts by CZE with UV detection

The data presented in Fig. 3 were obtained from the analysis of SPE extracts of urine blank and urine ua8 that were injected hydrodynamically at 0.5 p.s.i. for 20 s (Fig. 3A and B) and, after 20-fold dilution with water, electrokinetically using head-column FASS with an injection time of 90 s and otherwise as described above (Fig. 3C and D). Head-column FASS thereby reveals about a 10-fold higher sensitivity. Using both injection modes, OCOD and NOCOD could easily be monitored and their identity was confirmed by spiking the extracts with standards and re-running the samples. A small peak can be seen at the position of OMOR (marked with an asterisk). Identification, however, was not possible. Additionally, N6OCOL was detected (for identification, see below). Furthermore, analysis of a urine blank spiked with OCOD and OMOR (100 ng/ml each) and otherwise identical conditions as described for Fig. 3, revealed small peaks for OCOD and OMOR in both assay formats, peaks that were found to be close to the detection limits ($S/N\sim5$; data not shown). Thus, using SPE from 2 ml urine and hydrodynamic sample injection or head-column FASS, detection limits of about 100 and 5 ng/ml, respectively, can thereby be obtained. Using liquid–liquid extraction with ToxiTube A led to the data presented in Fig. 4. Compared to the case of SPE, cleaner electropherograms are obtained which revealed not only the detection of OCOD, NOCOD and N6OCOL, but also 6OCOL (for identification, see below). Furthermore, the OMOR peak was again very close to the detection limit. To obtain the data presented in Fig. 4D, the ua8 extract was diluted 10-fold with water prior to head-column FASS injection. Comparison with the data of Fig. 3D

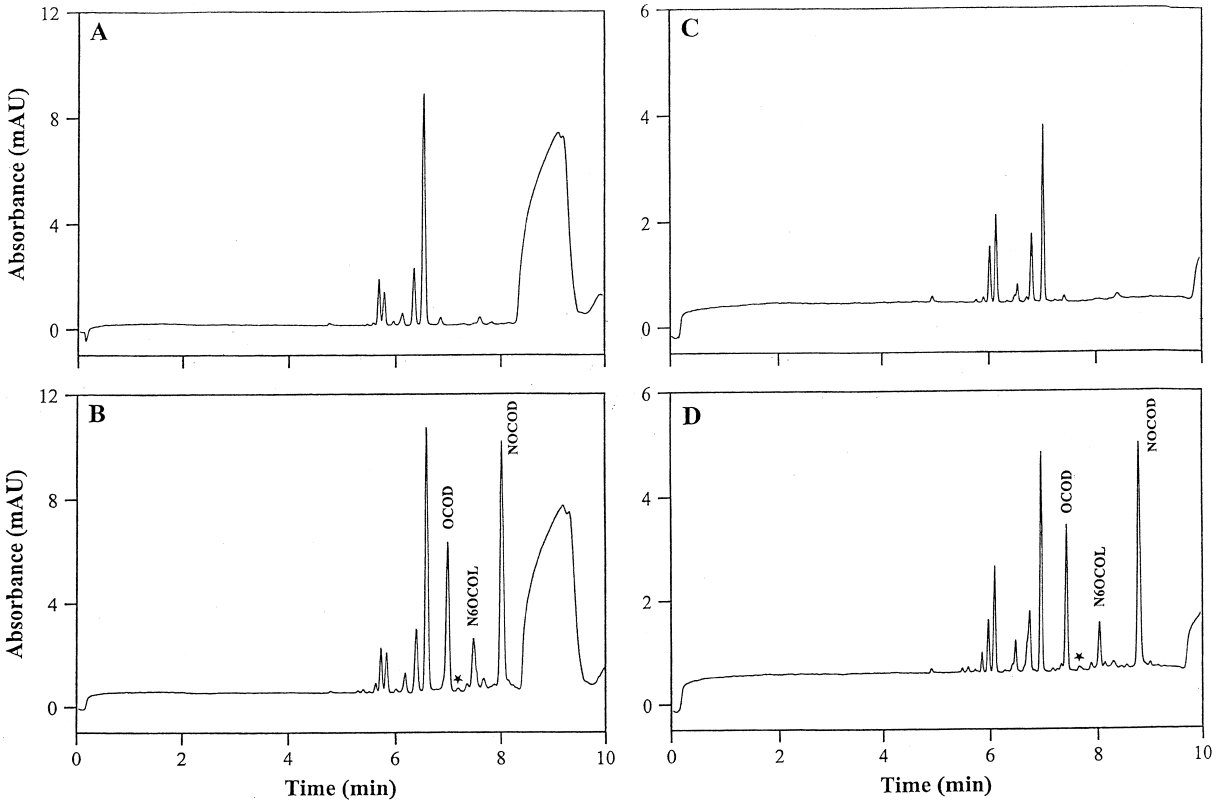

Fig. 3. (A,B) Hydrodynamic (0.5 p.s.i., 20 s) and (C,D) head-column FASS (10 kV, 90 s) injection of SPE extracts of (A,C) blank urine and (B,D) volunteer urine ua8 collected during the 0–8 h interval after ingestion of 10 mg of oxycodone hydrochloride in a controlled-release formulation. For head-column FASS, both extracts were diluted 20-fold with water.

indicates that head-column FASS after liquid–liquid extraction provides higher sensitivity. This was previously noted for dihydrocodeine and related compounds as well [23]. After liquid–liquid extraction, the detection limits for OCOD and OMOR were determined to be about 10 ng/ml using hydrodynamic sample injection. With head-column FASS, sub ng/ml concentrations could be detected (data not shown).

Starting with 2 ml of urine, liquid–liquid extraction and reconstitution in 100 μl of water theoretically leads to a 20-fold concentration of the solutes. This value is diminished by the extraction recovery, which was found to be about 80% for OCOD, 88% for OMOR, 76% for NOCOD, 20% for NOMOR and 77% for NCOD. For this determination, five times 2 ml of urine were spiked with OCOD, OMOR, NOCOD, NOMOR, and NCOD (1 μg/ml each), extracted and reconstituted in 2 ml of water and the peak areas of all compounds were compared with the values of an aqueous 1 μg/ml standard solution. The reproducibility ($n=5$) after liquid–liquid extraction and hydrodynamic injection of 2 ml spiked urine (OCOD, OMOR, NOCOD, NOMOR and NCOD as I.S. at the 1 μg/ml level) was found to be between 2.75 and 4.65% for the OCOD/NCOD, OMOR/NCOD and NOCOD/NCOD peak area ratios and 15.19% for the NOMOR/NCOD peak area ratio.

It is known that OMOR is mostly excreted in a conjugated form [6,9]. Without hydrolysis, it could thus hardly be detected in urines collected after single dose administration of OxyContin (Figs. 3 and 4). To obtain more information on the metabolism of OCOD the urines were hydrolyzed using two different enzyme products (see Section 2.2). The β-glucuronidase/arylsulfatase from *Helix pomatia* has

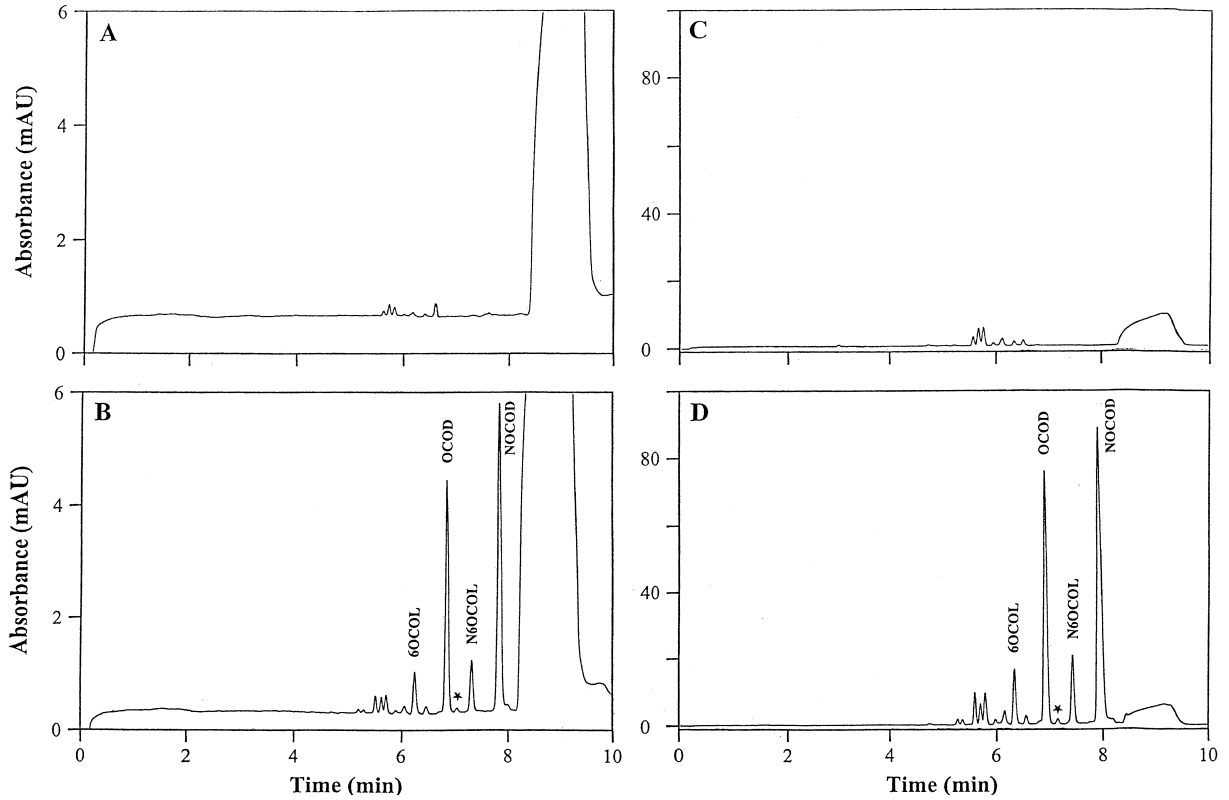

Fig. 4. (A,B) Hydrodynamic (0.5 p.s.i., 20 s) and (C,D) head-column FASS (10 kV, 90 s) injection of liquid–liquid extracts of (A,C) blank urine and (B,D) volunteer urine ua8 collected during the 0–8 h interval after ingestion of 10 mg of oxycodone hydrochloride in a controlled-release formulation. For head-column FASS, extract ua8 was diluted 10-fold with water.

a broader cleaving capability than β-glucuronidase from *Escherichia coli* as it incorporates two enzymes capable of splitting off glucuronic acid (as with β-glucuronidase) and sulfonic acid groups. The ua8 urine and a blank urine were spiked with 1 μg/ml NCOD (internal standard) and incubated for 4 and 24 h with the enzymes before liquid–liquid extraction and analysis of the reconstituted extracts. Peak height ratios measured for urine ua8 are summarized in Table 2 and selected electrophero-

Table 2
Peak height ratios obtained with liquid–liquid extracts of urine ua8 without hydrolysis and after enzymatic hydrolysis with β-glucuronidase and β-glucuronidase/arylsulfatase[a]

Hydrolysis product	Hydrolysis time (h)	OCOD	OMOR	NOCOD	6OCOL	N6OCOL
No hydrolysis	–	0.65	0.02	1.01	0.11	0.19
β-Glucuronidase	4	1.10	0.03	1.48	0.21	0.22
β-Glucuronidase	24	3.20	0.08	2.34	0.58	0.19
β-Glucuronidase/ arylsulfatase	4	1.01	0.21	1.40	0.27	0.24
β-Glucuronidase/ arylsulfatase	24	1.90	0.80	2.49	0.57	0.43

[a] Peak heights divided by the peak height of NCOD (1 μg/ml; I.S.) after hydrodynamic sample injection (conditions as for Fig. 5).

grams are presented in Fig. 5. No major peaks that could interfere with the opioids of interest were detected for the blank urines (data not shown). It was interesting to find that, with β-glucuronidase/arylsulfatase, the OMOR conjugates were readily hydrolyzed and the OMOR peak could easily be detected (Fig. 5B and 5C). Using β-glucuronidase, however, no significant increase in the OMOR amount could be monitored (Fig. 5A, Table 2). For the other compounds, no significant differences between the two enzyme products were noted (Table 2). Thus, these data suggest that OCOD, NOCOD and 6OCOL are mainly glucuronidated, whereas OMOR mainly forms other conjugates. In most cases the incubation time of 24 h led to increased ratios, indicating that 4 h provides incomplete hydrolysis. It was interesting to find that all peaks after β-glucuronidase/arylsulfatase hydrolysis were smaller than in Fig. 4 and 5A. An overload of the liquid–liquid extraction system, previously mentioned by Prost and Thormann [29], is presumed to be the reason for this phenomenon. Furthermore, analysis of urine ul12 revealed higher peaks for all metabolites and β-glucuronidase/arylsulfatase was again noted to be the better product for hydrolysis of the conjugates. However, in contrast to the results obtained with sample ua8, a small increase of the OMOR peak could be seen even after hydrolysis with β-glucuronidase (data not shown). For urines ut8 and ut14, the same metabolite pattern as for urine ua8 (Figs. 3–5) was obtained and the electropherograms are therefore not shown. For the urine collected during the 11–14 h interval after intake of oxycodone (ut14), the OCOD and NOCOD peaks were found to be about half the size of the corresponding peaks in the 0–8 h interval samples (ua8 and ut8).

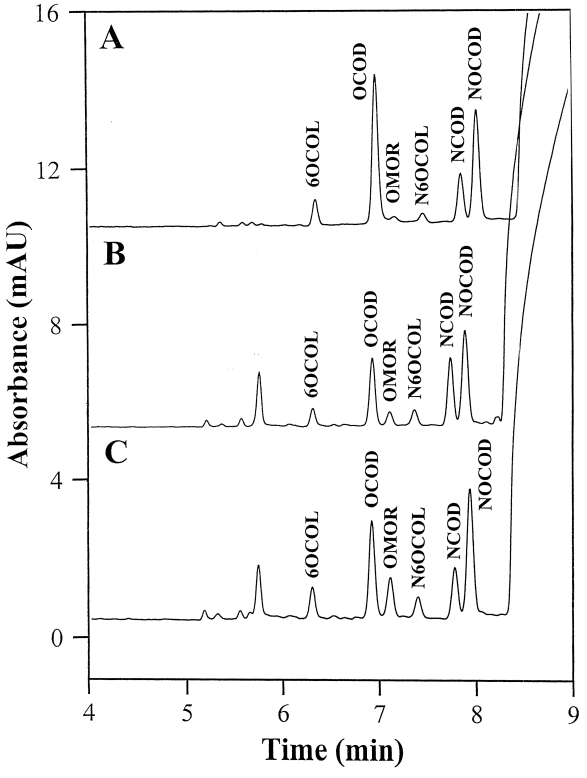

Fig. 5. Electropherograms obtained with liquid–liquid extracts of volunteer urine ua8 fortified with 1 μg/ml NCOD (I.S.) after hydrolysis at 37 °C for (A) 24 h with β-glucuronidase, (B) 4 h with β-glucuronidase/arylsulfatase and (C) 24 h with β-glucuronidase/arylsulfatase. All samples were hydrodynamically injected using 0.5 p.s.i. for 20 s. Electropherograms (A) and (B) are presented with a 10 and 5 mAU *y*-axis offset, respectively.

Finally, extracts were characterized using multi-wavelength detection. The data presented in Fig. 6 were obtained for analysis of a liquid–liquid extract of hydrolyzed (β-glucuronidase/arylsulfatase) urine ul12. Fig. 6A depicts the electropherogram measured at 210 nm. The monitored spectra of OCOD (Fig. 6B), OMOR (Fig. 6C), NOCOD (data not shown) and NOMOR (data not shown) were found to agree well with those obtained via analysis of standards (Fig. 2). Furthermore, the normalized spectra of OCOD and NOCOD were determined to be identical (insert of panel (B)) and the same was found to be true for OMOR and NOMOR (insert of panel (C)). Small differences were noted between the spectra of OCOD and OMOR (peak maximum of OMOR is at slightly lower wavelength; compare data of panels (B) and (C)) and a characteristic difference was observed between the spectra of OCOD and 6OCOL (panel (D)). The peaks assigned to 6OCOL and N6OCOL were found to have identical spectra (insert of panel (D)), indicating that these two substances are indeed related (for structural proof of relation, refer to the MS data below).

3.3. Identification, confirmation and quantitation of urinary OCOD and metabolites with CE–MS^n

In the search for a suitable buffer, an aqueous

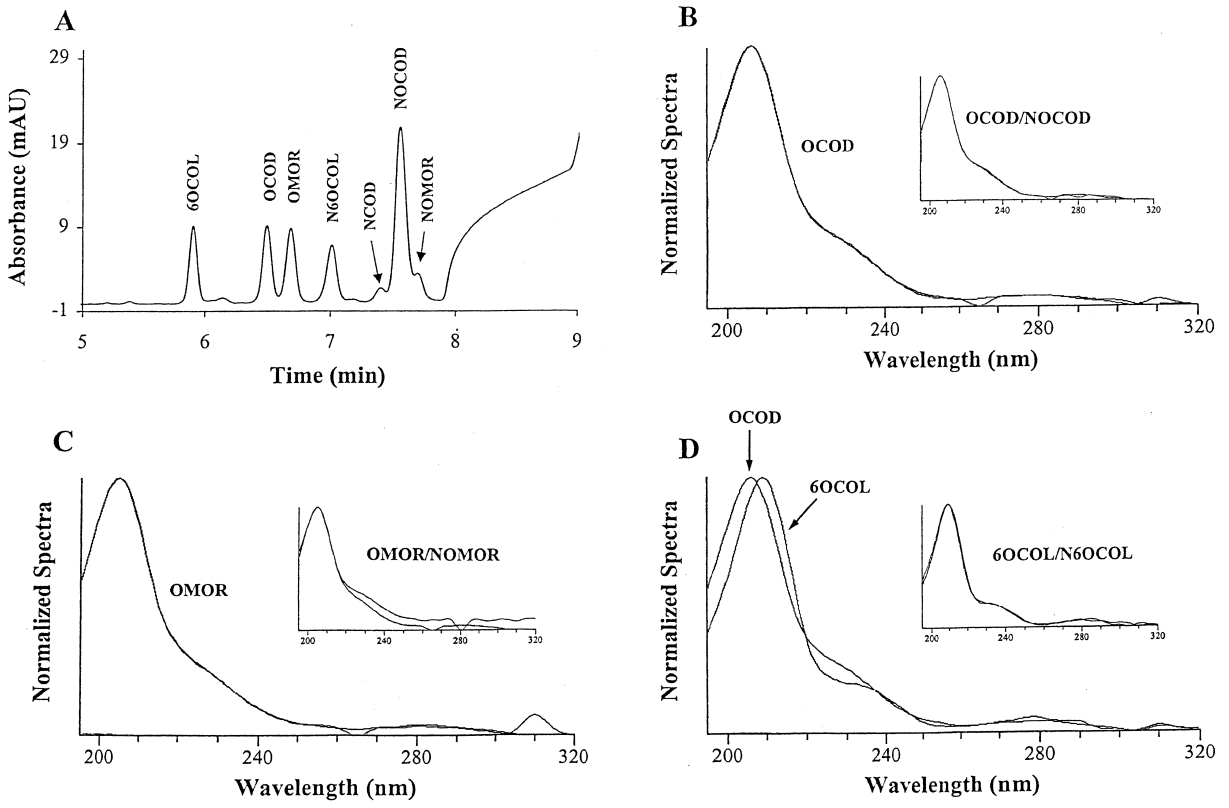

Fig. 6. (A) Electropherogram at 210 nm and (B–D) normalized UV spectra of a hydrodynamically injected hydrolyzed (4 h with β-glucuronidase/arylsulfatase) liquid–liquid extract of urine ul12. The normalized spectra of (B) OCOD and (C) OMOR are shown to agree well with those of the standards, the spectra of OCOD and 6OCOL are shown to be different (D), and the spectra of OCOD and NOCOD, OMOR and NOMOR, and 6OCOL and N6OCOL are depicted to be alike (inserts of panels (B)–(D), respectively).

standard solution containing OCOD and OMOR (10 μg/ml each) was analyzed in the P/ACE using a 50 μm I.D. capillary of 87/80 cm total/effective length and buffers composed of 25 m*M* ammonium acetate solution that were adjusted to pH 7.8, 8.3, 8.8, 9 and 9.3 with 1 *M* NH_3. At pH 7.8 the two solutes were not baseline resolved, and at pH 9.3 there was only one peak. At the three other buffer pH values the resolution was good. The best result in terms of resolution and peak shape was obtained at pH 9, i.e. with the same buffer as previously used for CE–MS of other opioids [25,26]. CE–MS data obtained with an aqueous sample containing seven standards (10 μg/ml each) are presented in the left hand panel of Fig. 7. With the buffer employed, the N-demethylated compounds were found to migrate ahead of their non-N-demethylated counterparts. This is the same order as previously observed for codeinoids and morphinoids [25,26]. Reference mass spectra of OCOD, OCODd3, NOCOD, NOCODd3, OMOR, OMORd3 and NOMOR were analyzed via syringe inlet using aqueous standard solutions of 10 μg/ml and, together with MS^2 and MS^3 spectra, they were stored in a computer library. The MS^3 spectra are presented in the right hand panel of Fig. 7. For 6OCOL and N6OCOL, no standards were available and no MS spectra could be found in the literature. It was interesting to note that all tested compounds lost H_2O at the MS^2 level, i.e. after isolation and fragmentation of the [M+H] ions (for *m*/*z* values, refer to Fig. 1). This neutral loss seems to be a characteristic behavior of opioid structures carrying an OH group at position 14 of the molecule. The same was determined with the structurally related

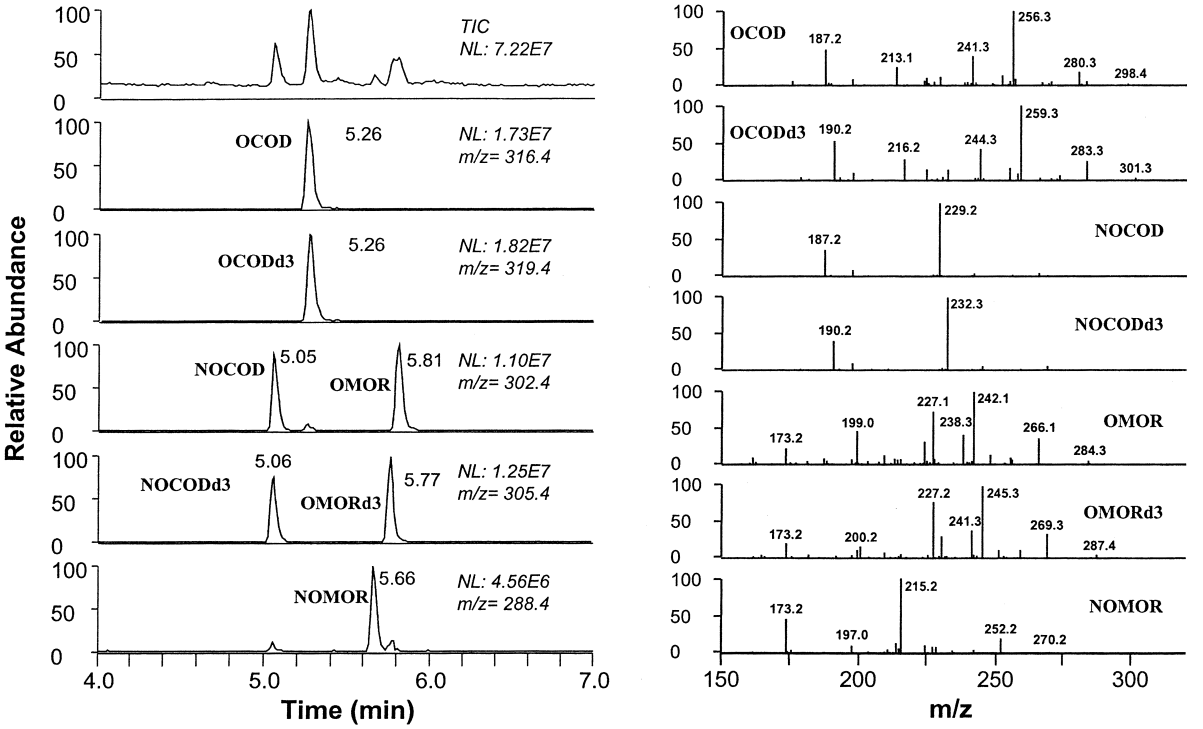

Fig. 7. Mass traces of seven standards (10 μg/ml each in water) and TIC (top graph) obtained after hydrodynamic injection at 70 mbar for 18 s (left panel), and MS^3 spectra of the seven compounds determined after isolation and fragmentation of the $[M+H-18]$ ions (right panel).

opioid antagonist naloxone (data not shown; for chemical structure, refer to Fig. 1). Depending on the spray conditions, the loss of water could be monitored under full-scan MS conditions, resulting in the detection of the $[M+H]^+$ and $[M+H-H_2O]^+$ mass traces (for examples, see Fig. 8B). The detection limits ($S/N=3$) after hydrodynamic injection (70 mbar for 18 s) of urinary liquid–liquid extracts were found to be about 10 ng/ml (spiking level) for OCOD and OCODd3, 50 ng/ml for NOCOD, NOCODd3, OMOR and OMORd3 and about 300 ng/ml for NOMOR. After electrokinetic injection (10 kV for 90 s) of liquid–liquid extracts the values found were 1 ng/ml (spiking level) for OCOD and OCODd3, 10 ng/ml for NOCOD, NOCODd3, OMOR and OMORd3 and about 50 ng/ml for NOMOR. After electrokinetic injection of two-fold diluted urine the detection limits were determined to be 100 ng/ml (spiking level) for OCOD, OCODd3, NOCOD, NOCODd3, OMOR and OMORd3 and about 500 ng/ml for NOMOR. The reproducibility ($n=5$) after liquid–liquid extraction and hydrodynamic injection of 2 ml spiked urine (OCOD, OCODd3, OMOR, OMORd3, NOCOD, NOCODd3 and NOMOR at the 1 μg/ml level) was 6.63% for OCOD/OCODd3, 14.40% for OMOR/OMORd3, 4.46% for NOCOD/NOCODd3 (all signal intensity ratios) and 25.54% for NOMOR (signal intensity). If OCODd3 is taken as I.S. for all compounds, signal intensity ratios are 21.35% for OMOR/OCODd3, 10.94% for NOCOD/OCODd3 and 25.60% for NOMOR/OCODd3. These data suggest that every compound of interest should have its own deuterated I.S.

Mass traces and spectra of a liquid–liquid extract of the enzymatically hydrolyzed (β-glucuronidase/arylsulfatase, 4 h at 37 °C) urine ul12 after electrokinetic injection (10 kV, 90 s) are depicted in Fig. 8. The mass traces for OCOD ($[M+H]^+=316.4$), NOCOD ($[M+H]^+=302.4$), OMOR ($[M+H]^+=$

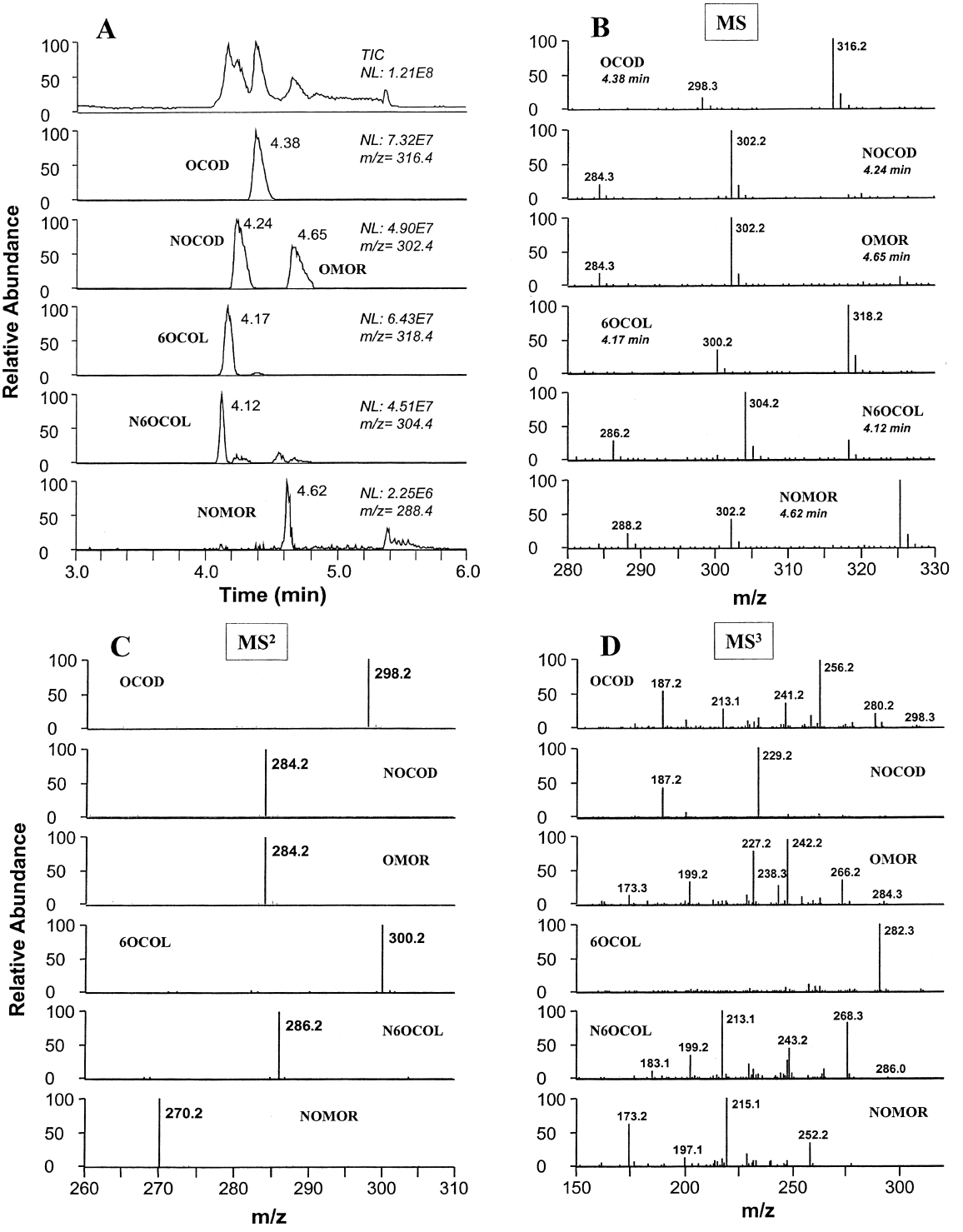

Fig. 8. (A) Mass traces for OCOD and five metabolites and TIC (top graph), (B) MS, (C) MS^2 (isolation and fragmentation of $[M+H]^+$) and (D) MS^3 (isolation and fragmentation of $[M+H-18]^+$) data of an electrokinetically (10 kV, 90 s) injected liquid–liquid extract of ul12 after 4 h enzymatic hydrolysis with β-glucuronidase/arylsulfatase.

302.4) and NOMOR ($[M+H]^+ = 288.4$) could easily be found (Fig. 8A). According to Ishida et al. [7,8], 6OCOL ($[M+H]^+ = 318.4$) and N6OCOL ($[M+H]^+ = 304.4$) should be produced as metabolites and their mass traces could also be detected (Fig. 8A). Furthermore, 6OCOL and N6OCOL are expected to show the same neutral loss in the MS^2 step. Both compounds fragmented in that way, which is a hint for their identity. The MS, MS^2 and MS^3 spectra of all six compounds are presented in Fig. 8B, 8C and 8D, respectively. After analysis of an electrokinetically injected unhydrolyzed liquid–liquid extract of the same urine, all compounds in Fig. 8 could be detected and characterized. Furthermore, with direct electrokinetic injection of the two-fold diluted urine ul12, the presence of OCOD, NOCOD, 6OCOL and N6OCOL could be confirmed from MS^2 and MS^3 spectra. Conjugates, however, could not be detected (for m/z values searched, see below). CE–MS^n of the liquid–liquid extracts of unhydrolyzed urines ua8, ut8 and ut14 provided responses for OCOD, NOCOD, 6OCOL and N6OCOL, but did not reveal the presence of OMOR and NOMOR. For monitoring of OMOR, these three urines had to be hydrolyzed with β-glucuronidase/arylsulfatase. NOMOR could not be detected at all in these samples (data not shown).

As mentioned above, OMOR is mainly excreted in a conjugated form. The data gathered suggest that only a small amount is excreted as glucuronide, whereas urine hydrolysis with β-glucuronidase/arylsulfatase led to a significant increase in the amount of free OMOR (Fig. 5). Therefore, not only mass traces of the glucuronides, but also of sulfates were searched for. The expected mass traces ($[M+H]^+$) for the acyl glucuronides were m/z 492.5 for the glucuronide of OCOD (OCODG), 478.5 for OMORG and NOCODG, 464.5 for NOMORG, 494.5 for 6OCOLG and 480.5 for N6OCOLG. For the sulfates, the anticipated mass traces ($[M+H]^+$) were 396.5 for the sulfate of OCOD (OCODS), 382.5 for OMORS and NOCODS, 368.5 for NOMORS, 398.5 for 6OCOLS and 384.5 for N6OCOLS. After electrokinetic injection (10 kV, 90 s) of two-fold diluted urine ul12, none of the above conjugates could be found. After SPE using methanol with 30% concentrated ammonia solution as eluent, mass traces for the m/z values corresponding to those of 6OCOLG, OMORG and/or NOCODG (same masses) and NOMORG could be detected (bottom, center and top graphs, respectively, of Fig. 9A). The fragmentation of 6OCOLG (MS^4 m/z 494.5→476.2→300.2) led as expected to the MS^3 (m/z 318.4→300.2) spectrum of 6OCOL. The fragmentation of the OMORG/OCODG peak revealed a spectrum difficult to evaluate, as the peak intensity was close to the background. The mass spectrum was found to be related to the MS^3 spectrum of OMOR (NOCOD could thereby be excluded), but the concentration was too low for unambiguous confirmation of its identity. Finally, fragmentation of the m/z 464.5 mass trace was found to represent an interesting case (Fig. 9B). Fragmentation of $[M+H]^+$ led to the characteristic neutral loss of H_2O in the MS^2 step, whereas MS^3 data obtained via isolation and fragmentation of $[M+H-18]^+$ did not provide the expected m/z 270 mass trace (NOMOR–H_2O), but an m/z value of 284.2, which corresponds to OMOR–H_2O (Fig. 8C). Finally, fragmentation of m/z 284.2 led to the MS^3 spectrum of OMOR (compare MS^4 data of Fig. 9B with MS^3 data of Fig. 8D). Thus, these data suggest that the mass trace detected at m/z 464.5 does not represent the glucuronide of NOMOR, but an unidentified conjugate of OMOR. As in the MS^2 step only the $[M+H-H_2O]$ ion is formed it can be concluded that this molecule is not substituted at the OH group at position 14. None of the above mentioned mass traces for sulfates could be found. On analysis of a comparable SPE extract of urine ua8, the same three mass traces could be found and the fragmentation led to the same findings. Again, no sulfates could be detected.

For quantification of free OCOD and NOCOD, blank human urine samples fortified with 0.1, 0.5, 2, 5 and 8 μg/ml of each compound were used as calibrators and urines containing 0.7 and 6 μg/ml of both compounds served as controls. Prior to liquid–liquid extraction (for procedure, refer to Section 2.2), 200 μl of an aqueous I.S. solution containing OCODd3 and NOCODd3 (10 μg/ml each) was added to the urines and extracts were injected hydrodynamically (70 mbar for 18 s). Peak area ratios were employed as the basis for data evaluation. Linear relationships with correlation coefficients (r) for OCOD and NOCOD of 0.9999 and 0.9989, respectively, were obtained, and deviations for the controls were $<4.19\%$ for all values. Urine ul12 was determined to contain 2.96 and 13.6 μg/ml

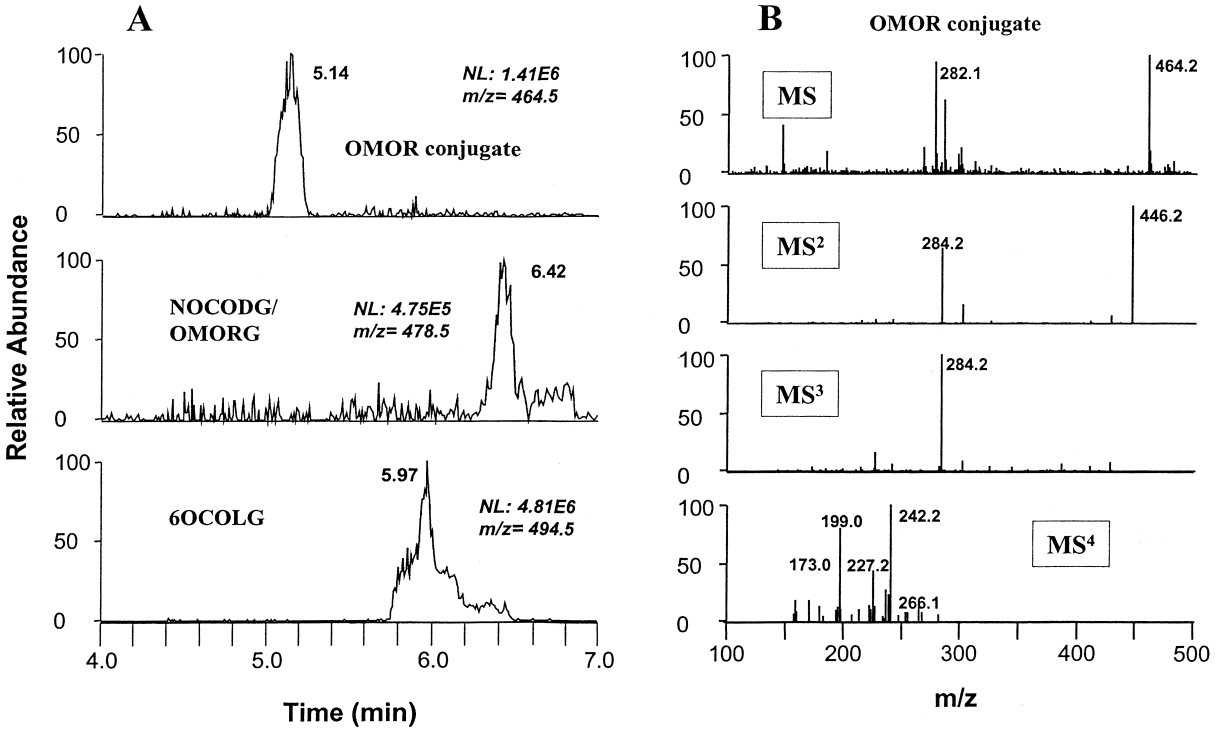

Fig. 9. (A) Mass traces for selected conjugates and (B) MS, MS^2, MS^3 and MS^4 data of an unidentified OMOR conjugate obtained for the analysis of an electrokinetically (10 kV, 90 s) injected SPE extract of unhydrolyzed urine ul12.

of OCOD and NOCOD, respectively. Corresponding values in urines ua8 and ut8 were estimated to be between 0.3 and 0.5 μg/ml.

4. Concluding remarks

The data reported in this paper represent the first account of CE of opioids that carry an OH group at position 14 of the opioid structure. OCOD and its metabolites OMOR, NOCOD and NOMOR are shown to separate well using the binary phosphate buffer at pH 7.9. The migration order of OCOD and OMOR with respect to their N-demethylated analogs was found to be reversed compared to that observed for codeine, dihydrocodeine and morphine. Using an aqueous buffer at pH 9, however, the same order was detected. OCOD and structurally related compounds can also be distinguished from other opioids by their absorbance spectra at low wavelengths and via fragmentation. Using CE–MS, opioids carrying an OH group at position 14 were found to lose H_2O at the MS^2 level, a step that is not observed with codeine, dihydrocodeine, morphine and analogs. Furthermore, compared to the latter compounds, the abundance was determined to be somewhat lower. Using the binary phosphate buffer, CE with UV detection is shown to be capable of monitoring OCOD, NOCOD, OMOR (after hydrolysis only) and NOMOR (after hydrolysis and in patient urine ul12 only) in alkaline liquid–liquid extracts of urines that were collected after ingestion of 10 mg OCOD hydrochloride and in a patient urine collected at steady state (80 mg OCOD hydrochloride daily). Using the ammonium acetate buffer at pH 9, these results were confirmed with $CE–MS^3$. Based on CE–MS, MS^2 and MS^3 data, the absorbance spectra measured across the CE peaks and the relative position within the electropherogram, two peaks monitored in the UV absorbance electropherograms could be assigned to the two keto-reduced metabolites 6OCOL and N6OCOL, for which no standards

were available. Comparison of data obtained with urines pretreated with two different enzyme products (β-glucuronidase and β-glucuronidase/arylsulfatase) suggests that OCOD, NOCOD and 6OCOL are mainly glucuronidated, whereas OMOR mainly forms other conjugates. Furthermore, in a first attempt to directly measure conjugates of the compounds of interest, SPE extracts were analyzed by $CE–MS^4$, which revealed the presence of 6OCOLG, OMORG and an unidentified OMOR conjugate. Quantitation of free OCOD and NOCOD by CE–MS using deuterated internal standards is also discussed briefly. Further work is required to properly elucidate the nature and amount of the major conjugates excreted in urine.

Acknowledgements

A.B. Wey is a recipient of a PhD grant from Mundipharma Medical Company, Basel, Switzerland. The data with multi-wavelength detection were kindly recorded by Francine Prost. This work was partly supported by the Swiss National Science Foundation.

References

[1] E. Kalso, A. Vainio, Clin. Pharmacol. Ther. 47 (1990) 639.
[2] K.P. Leow, M.T. Smith, B. Williams, T. Cramond, Clin. Pharmacol. Ther. 52 (1992) 487.
[3] K.P. Leow, T. Cramond, M.T. Smith, Anesth. Analg. 80 (1995) 296.
[4] N.A. Hagen, N. Babul, Cancer 79 (1997) 1428.
[5] R.F. Kaiko, D.P. Benziger, R.D. Fitzmartin, B.E. Burke, R.F. Reder, P.D. Goldenheim, Clin. Pharmacol. Ther. 59 (1996) 52.
[6] T. Ishida, K. Oguri, H. Yoshimura, Drug Metab. Dispos. 7 (1979) 162.
[7] T. Ishida, K. Oguri, H. Yoshimura, J. Pharm. Dyn. 5 (1982) 134.
[8] T. Ishida, K. Oguri, H. Yoshimura, J. Pharm. Dyn. 5 (1982) 521.
[9] R. Pöyhiä, T. Seppälä, K.T. Olkkola, E. Kalso, Br. J. Clin. Pharmacol. 33 (1992) 617.
[10] S.V. Otton, D. Wu, R.T. Joffe, S.W. Cheung, E.M. Sellers, Clin. Pharmacol. Ther. 53 (1993) 401.
[11] T. Heiskanen, K.T. Olkkola, E. Kalso, Clin. Pharmacol. Ther. 64 (1998) 603.
[12] T.E. Heiskanen, P.M. Ruismäki, T.A. Seppälä, E.A. Kalso, Acta Oncol. 39 (2000) 941.
[13] M.T. Smith, J.A. Watt, G.P. Mapp, T. Cramond, Ther. Drug Monit. 13 (1991) 126.
[14] A.W.E. Wright, J.A. Lawrence, M. Iu, T. Cramond, M.T. Smith, J. Chromatogr. B 712 (1998) 169.
[15] R. Meatherall, J. Anal. Toxicol. 23 (1999) 177.
[16] W. Nowatzke, J. Zeng, A. Saunders, A. Bohrer, J. Koenig, J. Turk, J. Pharm. Biomed. Anal. 20 (1999) 815.
[17] C.-X. Zhang, W. Thormann, J. Chromatogr. A 764 (1997) 157.
[18] P. Wernly, W. Thormann, Anal. Chem. 63 (1991) 2878.
[19] P. Wernly, W. Thormann, Anal. Chem. 64 (1992) 2155.
[20] E. Hufschmid, R. Theurillat, U. Martin, W. Thormann, J. Chromatogr. B 668 (1995) 159.
[21] E. Hufschmid, R. Theurillat, C.H. Wilder-Smith, W. Thormann, J. Chromatogr. B 678 (1996) 43.
[22] C.-X. Zhang, W. Thormann, Anal. Chem. 70 (1998) 540.
[23] A.B. Wey, C.-X. Zhang, W. Thormann, J. Chromatogr. A 853 (1999) 95.
[24] A.B. Wey, W. Thormann, Chromatographia 49 (Suppl. I) (1999) S12.
[25] A.B. Wey, J. Caslavska, W. Thormann, J. Chromatogr. A 895 (2000) 133.
[26] A.B. Wey, W. Thormann, J. Chromatogr. A 916 (2001) 225.
[27] A.B. Wey, W. Thormann, J. Chromatogr. A 924 (2001) 507.
[28] C.-X. Zhang, W. Thormann, Anal. Chem. 68 (1996) 2523.
[29] F. Prost, W. Thormann, Electrophoresis 22 (2001) 3270.