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Identification of new oxycodone metabolites in human urine by capillary electrophoresis–multiple-stage ion-trap mass spectrometry

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Abstract

Capillary electrophoresis–electrospray ionization multiple-stage ion-trap mass spectrometry (CE–MS^n) and computer simulation of fragmentation are demonstrated to be effective tools to detect and identify phase I and phase II metabolites of oxycodone (OCOD) in human urine. OCOD is a strong analgesic used for the management of moderate to severe mainly postoperative or cancer-related pain whose metabolism in man is largely unknown. Using an aqueous pH 9 ammonium acetate buffer and CE–MS^n ($n \leq 5$), OCOD and its phase I metabolites produced by *O*-demethylation, *N*-demethylation, 6-ketoreduction and *N*-oxidation (such as oxymorphone, noroxycodone, noroxymorphone, 6-oxycodol, nor-6-oxycodol, oxycodone-*N*-oxide and 6-oxycodol-*N*-oxide) and phase II conjugates with glucuronic acid of several of these compounds could be detected in alkaline solid-phase extracts of a patient urine that was collected during a pharmacotherapy episode with daily ingestion of 240–320 mg of OCOD chloride. The data for three known OCOD metabolites for which the standards had to be synthesized in-house, 6-oxycodol, nor-6-oxycodol and oxycodone-*N*-oxide, were employed to identify two new metabolites, the *N*-oxidized derivative of 6-oxycodol and an *O*-glucuronide of this compound. CE–MS^n and computer simulation of fragmentation also led to the identification of the *N*-glucuronide of noroxymorphone, another novel OCOD metabolite for which no standard compound or mass spectra library data were available.

Keywords: Oxycodone; 6-Oxycodol; Oxycodone-*N*-oxide; 6-Oxycodol-*N*-oxide; *N*-Glucuronide; *O*-Glucuronide; Simulation of fragmentation

1. Introduction

Oxycodone (4,5-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one, or 14-hydroxy-7,8-dihydrocodeinone, OCOD; for the chemical structure refer to Fig. 1) is a semisynthetic opioid, structurally related to other compounds of the same class, including codeine and morphine. The main differences between morphine and OCOD are the saturation of the C7–C8 bond and the presence of a hydroxyl group in position 14. The absence of the double bond between C7 and C8 allows ring C to assume preferentially a chair conformation [1], while the C14-hydroxyl is in axial position due to the *cis*-fusion between rings C and B (Fig. 1). OCOD is a strong analgesic opioid used for the treatment of moderate to severe postoperative or cancer-related pain and is typically orally administered as a controlled release formulation [2–6]. OCOD is also a drug of abuse and reported to be found, often together with other licit or illicit drugs, in many intoxication reports [7,8].

It is known that OCOD is *N*-demethylated to noroxycodone (NOCOD, Fig. 1) [8–16], a biotransformation that is most likely catalyzed by the liver enzyme cytochrome P450 3A4 [14]. OCOD and NOCOD can be *O*-demethylated in presence of the cytochrome P450 2D6 enzyme [14,18,19] thereby producing oxymorphone (OMOR, Fig. 1) [8,9,11–17] and noroxymorphone (NOMOR, Fig. 1), respectively [15]. The 6-keto reduction of OCOD and NOCOD leads to 6-oxycodol (6OCOL, Fig. 1) and nor-6-oxycodol (N6OCOL, Fig. 1), respectively [8,9,11,15], metabolites that may be formed as two diastereoisomers. The enzymes involved in the 6-keto reduction are not yet known. Furthermore, flavin containing monoxygenase is assumed to catalyze the formation of the *N*-oxidized derivative of OCOD, oxycodone-*N*-oxide (OCODNO, Fig. 1), and of its phase I metabolites [8,9,11], compounds that may exist as

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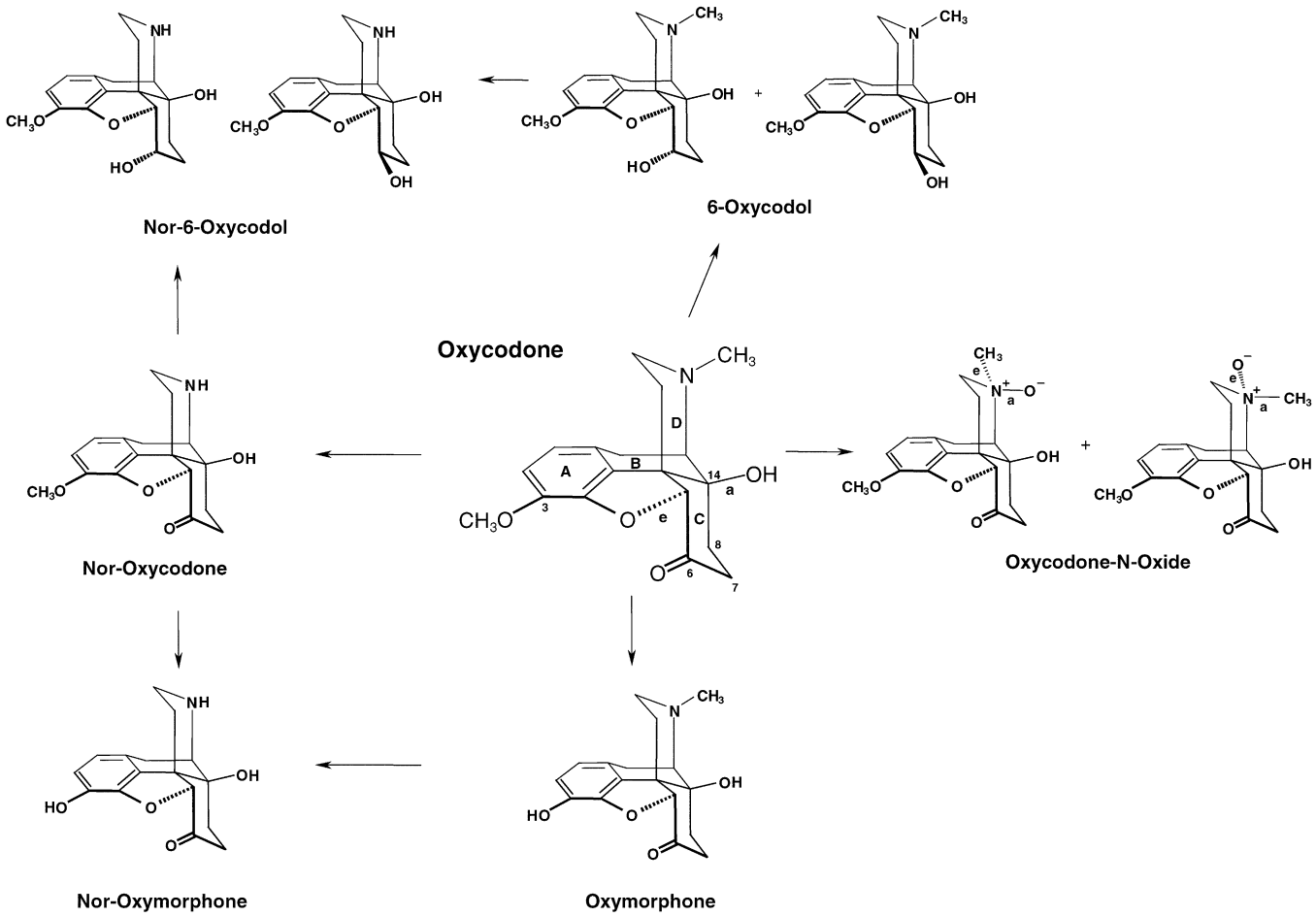

Fig. 1. Chemical structures of oxycodone and selected phase I metabolites. Except for oxycodone-*N*-oxide, the *N*-oxide metabolites are not shown. Letters a and e refer to axial and equatorial, respectively.

two diastereoisomers (Fig. 1). Among the phase II reactions, it is known that conjugation with glucuronic acid occurs with several OCOD metabolites [11,15]. The information regarding the metabolism of OCOD was gained from animal studies [9–11,20], via analysis of human body fluids, including urine [8,12,15,17], and in vitro incubations of OCOD with human liver microsomes [14,16].

For the analysis of OCOD and some of its metabolites in various human biological matrices, methods based upon gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been developed. Modern GC-based assays described in the literature feature mass spectrometry (MS) for solute detection [8,13,17,21,22], HPLC methods are based upon electrochemical [23,24] and MS [25] detection and the CE assays reported employ multiwavelength UV absorption and MS detection [15]. Furthermore, HPLC with a UV absorbance detector was used for the study of the OCOD metabolism in vitro via incubations with human liver microsomes [16].

There are several reasons why one would have or want to assess the fate of a drug in man, including the elucidation of the drug's metabolism, activity, toxicity and bioavailability. Furthermore, metabolite monitoring is a topic of high interest in forensic science. During the past few years, our laboratory investigated the use of CE for the assessment of the metabolism of opioids via analysis of these compounds and their metabolites in human urine [15,26–31]. The work with OCOD led to the monitoring of OCOD, NOCOD, OMOR, NOMOR, 6OCOL, N6OCOL and glucuronides of some of these compounds in extracts of urines that were collected after intake of 10 mg OCOD chloride and from a patient at steady state (daily intake of 80 mg OCOD chloride) [15]. CE assays with UV absorption and MS detection were thereby shown to represent attractive approaches for the detection of these compounds. As the metabolism of OCOD in man has not yet been completely elucidated, our efforts were continued using CE–MS^n and computer simulation of fragmentation. The data for three known OCOD metabolites for which the standards had to be synthesized in-house, 6OCOL, N6OCOL and OCODNO, were gathered and compared to those monitored previously. Then, the two technologies were successfully applied to the detection and identification of unknown phase I and phase II OCOD metabolites, namely the *N*-oxidized derivative of 6OCOL (6OCOLNO), an *O*-glucuronide of

6OCOLNO and the *N*-glucuronide of NOMOR, in urine of a patient with a daily ingestion of 240–320 mg OCOD chloride.

2. Experimental

2.1. Chemicals and samples

OCOD and NOCOD were purchased as methanolic solutions (1.0 mg/mL) from Cambridge Isotope Labs. (Andover, MA, USA). All other OCOD metabolite standards were the same as employed previously [15]. $NaBH_4$ was a kind gift of the Fachhochschule Burgdorf, Switzerland. 3-Chloroperbenzoic acid and tetrahydrofuran were from Fluka (Buchs, Switzerland), and ammonium acetate, ammonium chloride, sodium acetate, KOH, ammonia, acetic acid, formic acid, isopropanol and chloroform were from Merck (Darmstadt, Germany). Methanol (HPLC supra gradient grade) and dichloromethane (HPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Aluminum oxide for chromatography (No. 015.0811 of Camag, Muttenz, Switzerland) was a kind gift of the Department of Chemistry (Bern, Switzerland). β-Glucuronidase/arylsulfatase (aqueous solution, stabilized with thiomerosal, Catalog No. 127060) was purchased from Roche (Mannheim, Germany) and bidistilled water was obtained from a Fitstream Cyclon apparatus (Loughborough, UK). The urine stemmed from a patient under polydrug pharmacotherapy with OxyContin (urine KR062801, daily intake of 240–320 mg of oxycodone chloride, Mundipharma Medical Company, Basel, Switzerland), morphine, dipyrone (metamizol) and citalopram and was stored at −20 °C.

2.2. Synthesis of 6OCOL and N6OCOL

6OCOL and N6OCOL were synthesized according to a protocol employed by Hahn and Fishman [32] for the reduction of naloxone. Briefly, 0.5 mL of the methanolic OCOD or NOCOD solution (1.0 mg/mL each) was diluted with 1.5 mL methanol in a disposable 5 mL glass tube. Six hundred micrograms (or 560 μg in case of NOCOD) of $NaBH_4$ was added and the solution was left at room temperature for 5 h and periodically stirred. Thereafter, 0.5 mL of a saturated solution of NH_4Cl was added and a precipitation of white crystals was observed. As both phases were found to contain the product, 0.5 mL water was added for complete dissolution of the precipitate. Prior to analysis by CE–MS, the sample was purified via solid-phase extraction (SPE) as described below for urine preparation.

2.3. Synthesis of OCODNO

The synthesis of OCODNO was performed according to a protocol employed by Cymerman Craig and Purushotaman for the synthesis of several tertiary amine *N*-oxides [33]. The proposed procedure was followed except for the elimination of the unreacted parent compound. 0.200 mL of methanolic OCOD solution (1.0 mg/mL) was diluted with 1.5 mL tetrahydrofuran in a 5 mL glass tube. One hundred and twenty micrograms of 3-chloroperbenzoic acid was added and the solution was left at room temperature for 5 h and periodically stirred. Thereafter, the solution was purified using a hand packed column of aluminum oxide (ca. 200 mg) conditioned with 2.0 mL of methanol–chloroform (1:3, v/v). The product was eluted with 1.5 mL methanol–chloroform (1:3, v/v) and evaporated to dryness at 35 °C under a gentle stream of air. The residue was reconstituted with 100 μL of water and stored at −20 °C.

2.4. Urine preparation

The patient urine was analyzed either after SPE or after enzymatic hydrolysis followed by SPE. Enzymatic hydrolysis was performed by mixing 2 mL of urine with 2 mL of a pH 5.4 sodium acetate buffer (prepared by dissolving sodium acetate in water, such that the obtained concentration was 0.2 M, and subsequently adjusting the pH with acetic acid) and 50 μL β-glucuronidase/arylsulfatase. Incubation occurred at 37 °C for 24 h. SPE was carried out according to a modification of a protocol established by Wey and Thormann [15] using disposable, mixed mode polymer cartridges (Bond Elut Certify, No. 1211–3050, Varian, Harbor City, CA, USA) together with the Vac-Elut setup (Varian). Briefly, the pH of the sample (either 2 ml of non-hydrolyzed urine or the entire hydrolysis mixture) was adjusted to 7 with 1 M KOH. The cartridge was conditioned with 2 mL methanol and 2 mL water, and the sample was slowly drawn through the cartridge. It was then sequentially rinsed with 2 mL water, 1 mL acetate buffer (0.1 M at pH 4.0, prepared according to the Varian Instruction Manual for Bond Elut Certify) and 2 mL methanol. The bed was dried under light vacuum after the last rinsing step. Analytes were first eluted with 2.0 mL of a mixture of dichloromethane–isopropanol–concentrated ammonia solution (78.4:19.6:2.0, v/v/v) by applying a light vacuum and letting the bed to dry. Then, elution was continued using 1.5 mL of a mixture of methanol–concentrated ammonia solution (70:30, v/v). Both eluates were combined (improved extraction efficiency for phase I metabolites) and evaporated to dryness at 35 °C under a gentle stream of air. The sample was reconstituted with 100 μL of water and stored at −20 °C.

2.5. 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 ionization interface (Finnigan) run in the positive mode (4.0 kV). Sheath gas (N_2) pressure was set at 40 arbitrary units. The sheath liquid consisted of a mixture of water–methanol (1:1, v/v) containing 1% of formic acid in order to support the formation of positively charged ions $[M + H]^+$. The sheath liquid was infused into

the interface at a flow rate of 5.0 μL/min employing a 250 μL Unimetrics syringe. The temperature of the heated capillary was kept at 200 °C. The instrument was computer controlled using the Xcalibur 1.2 software (Finnigan). A Prince Instrument (Lauerlabs, Emmen, The Netherlands) equipped with 50 μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 80 cm length was connected to the electrospray ionization interface of the LCQ. The sample was either introduced hydrodynamically by applying a positive pressure (70 mbar, 18 s) or electrokinetically by applying a positive voltage of 10 kV for 30 s.

The background electrolyte (BGE) was prepared daily as follows. Ammonium acetate powder was weighted and dissolved in water in order to obtain a concentration of 20 mM and the pH was adjusted to 9 with 1 M NH_3. The applied voltage during separation was 30 kV. Full scan mass spectra were collected in the mass range 100–600 Th. Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS^2, MS^3, MS^4 and MS^5 experiments were performed targeting selectively the *m*/*z* ratios of the compounds of interest with an isolation width of 2 Th, and applying different collision energies.

2.6. Software

Molecular masses were calculated with the software ISIS/Draw 2.1.3d (MDL Information Systems, San Leandro, CA, USA) and fragmentation was simulated using the HighChem Mass Frontier version 1.0 software package (HighChem, Bratislava, Slovak Republic).

3. Results and discussion

3.1. Characterization of synthesized oxycodone metabolites

The identification of the synthesized substances was carried out with CE–MS^n such that the compound of interest could be separated from the non-reacted parent compound and other products prior to the gathering of the mass spectra. All three compounds were analyzed separately and each full scan spectrum revealed the presence of the expected OCOD metabolite, the parent compound and some undesired products. In each case, the signal corresponding to the expected oxycodone derivative was found to be predominant and strong enough to allow the collection of MS^2, MS^3 and MS^4 spectra (Figs. 2–4). For 6OCOL and N6OCOL, electrokinetic sample injection was required in order to reach a high enough sample concentration that permitted the collection of the MS^4 spectra. OCODNO peak magnitudes were not much affected by the injection mode, confirming that this metabolite is neutral at the pH employed.

The MS^2 data of 6OCOL showed the expected precursor–product ion transition between *m*/*z* 318 and *m*/*z* 300, corresponding to a loss of 18 mass units (water) which

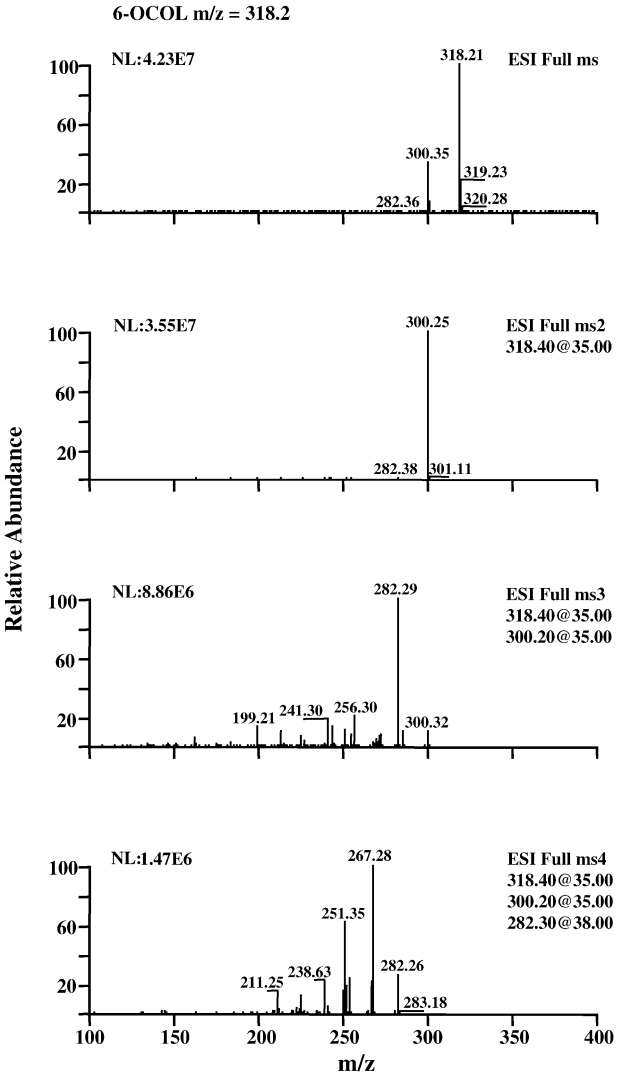

Fig. 2. MS to MS^4 spectra (from top to bottom, respectively) for synthesized 6OCOL. The right labels indicate the power of each mass spectrum, the *m*/*z* ratio of the targeted ion and the corresponding collision energy. The collision energies are expressed in arbitrary units in a percentile scale.

was previously noted to be typical for this class of compounds bearing a hydroxyl group in position 14 [15]. MS^3 led to the fragmentation 300 → 282 (second water loss, in agreement with the data of [15]), while the MS^4 data, obtained via fragmentation of the *m*/*z* = 282 ion, showed a more complex pattern that is dominated by ions with *m*/*z* = 267, 251 and 239 (Fig. 2). The *m*/*z* = 282 ion shows a stability that is higher than those of the other targeted fragments (higher collision energy is required for fragmentation). The expected water loss is also seen in the MS^2 data of N6OCOL (304 → 286), whereas MS^3 obtained by fragmentation of the *m*/*z* = 286 ion resulted in a more complex pattern than that obtained for 6OCOL. Although the spectrum is dominated by the *m*/*z* = 268 ion, corresponding to the second water loss, there are also peaks at *m*/*z* = 243 and 213, whose intensities are not negligible. The MS^4 data produced from the *m*/*z* = 268 ion

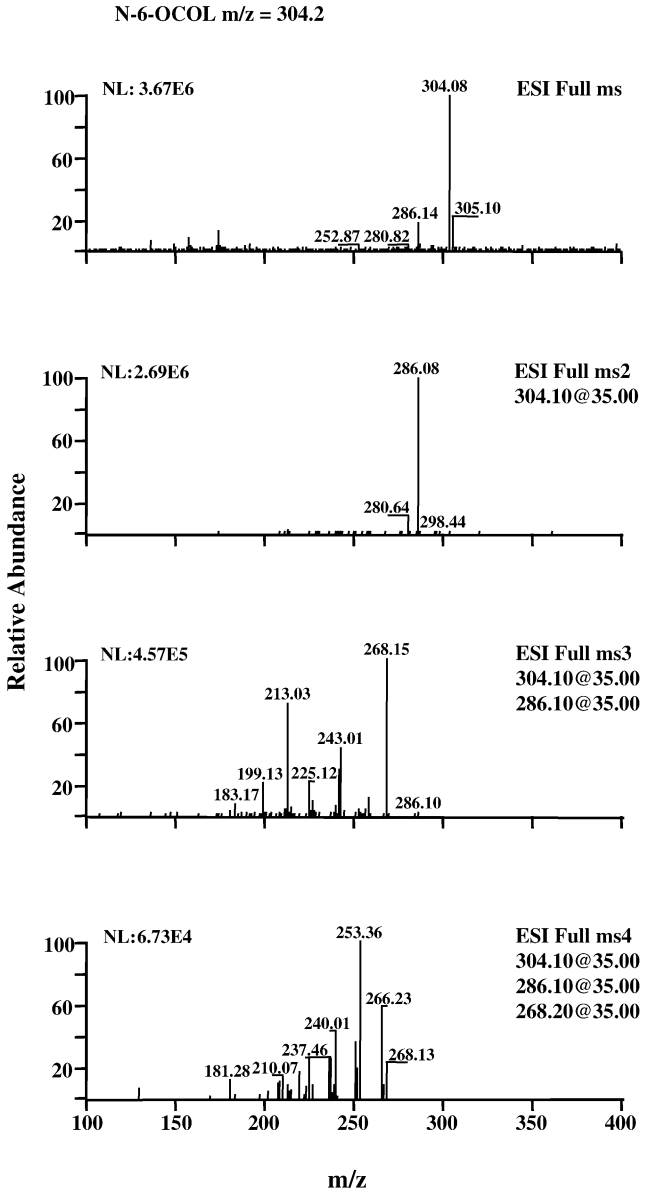

Fig. 3. MS to MS^4 spectra (from top to bottom, respectively) for synthesized N6OCOL. The right labels indicate the power of each mass spectrum, the *m/z* ratio of the targeted ion and the corresponding collision energy. The collision energies are expressed in arbitrary units in a percentile scale.

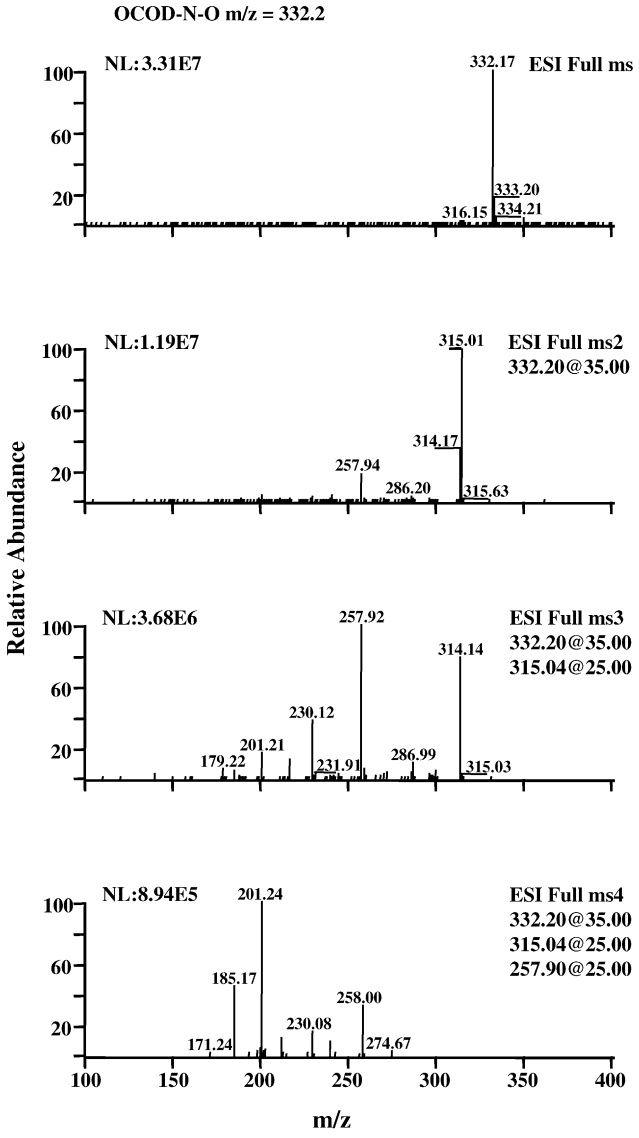

Fig. 4. MS to MS^4 spectra (from top to bottom, respectively) for synthesized OCODNO. The right labels indicate the power of each mass spectrum, the *m/z* ratio of the targeted ion and the corresponding collision energy. The collision energies are expressed in arbitrary units in a percentile scale.

led to a complex mass spectrum in which the *m/z* = 253 ion has the highest abundance (Fig. 3). Thus, it appeared that the double water loss, resulting in a predominant peak at $[M + H - 36]^+$ at the MS^3 level, followed by a transition $[M + H - 36]^+ \rightarrow [M + H - 51]^+$, is a common pattern for both C6-reduced oxycodone metabolites. On the other hand, the double water loss was also observed for other metabolites and for OCOD itself. The $[M + H - 36]^+$ peak, however, was not the most abundant ion of these MS^3 spectra [15]. The analysis of OCODNO revealed a loss of 17 mass units on the MS^2 level (*m/z* = 315 Th). The expected reduction of 18 Th is present as well (*m/z* = 314), but with secondary abundance. Complex spectra were obtained after MS^3 (fragmentation of the most abundant ion) and MS^4 (Fig. 4). All spectra were stored in a library and used as reference for the recognition of these compounds in the urinary extracts.

The fragmentation patterns for 6OCOL and N6OCOL were simulated and the results could be compared to the collected data. It was not possible to simulate the fragmentation of OCODNO because the software does not handle structures with charge separation. For 6OCOL, simulated MS, MS^2 and MS^3 spectra were found to be in complete agreement with the monitored spectra shown in Fig. 2. Simulation, however, did not reveal the *m/z* = 267 ion at the MS^4 level. This 282 → 267 precursor–product ion transition corresponds to a loss of 15 mass units, possibly the elimination of a methyl group as a radical. A very similar situation was found for N6OCOL for which the 268 → 253 transition observed at the MS^4 level

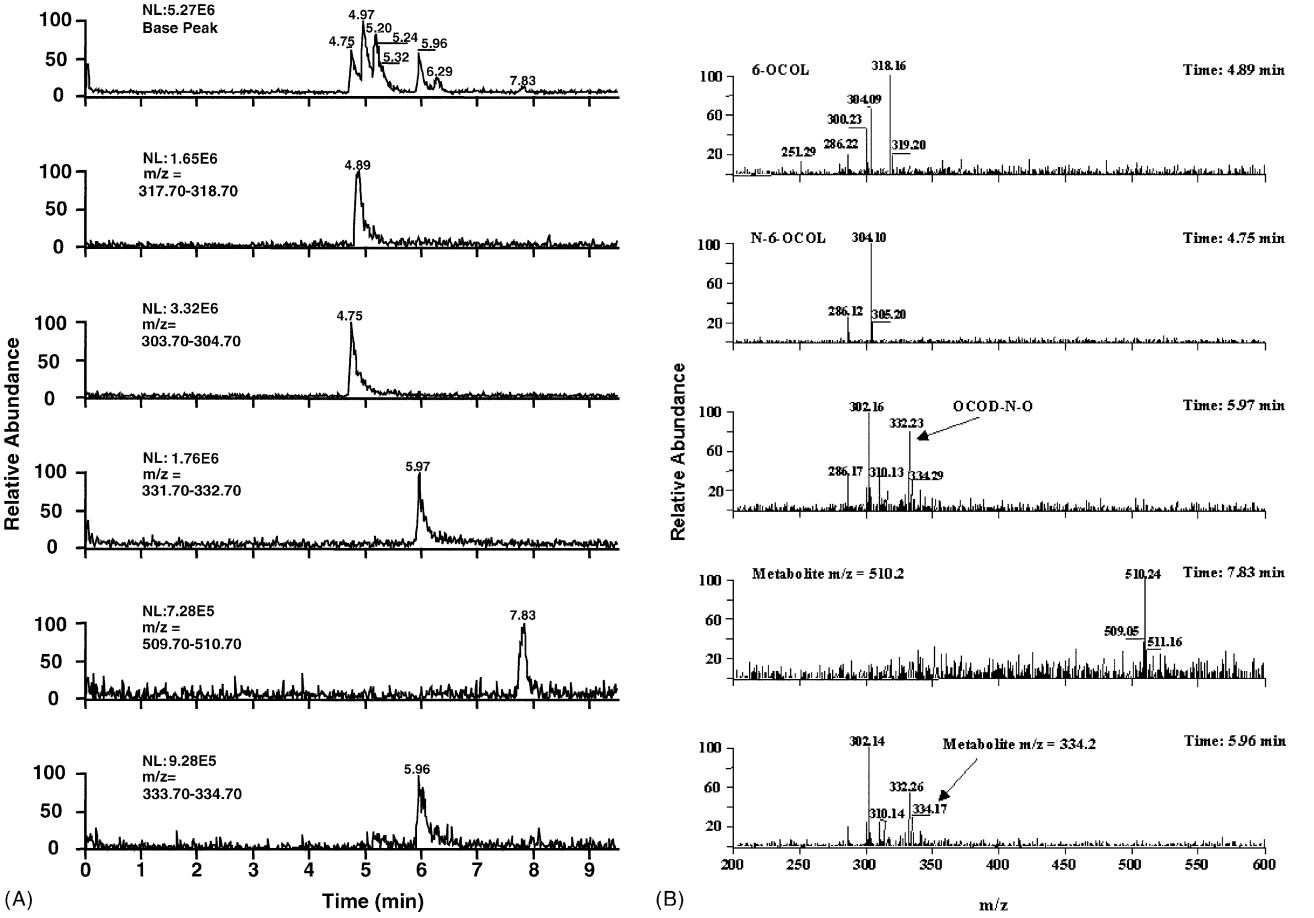

Fig. 5. Electropherograms for selected masses of targeted OCOD metabolites (panel A) and corresponding mass spectra at the indicated time points (panel B) obtained for analysis of an extract of the non-hydrolyzed patient urine. The top electropherogram in panel A depicts the base peak, which is a normalized plot of the intensity of the largest peak vs. time.

could not be simulated. Thus, the loss of 15 units at the MS^4 level could be a peculiar reaction for structures related to the 6-reduced OCOD metabolites. Although the simulated MS, MS^2 and MS^3 spectra of 6OCOL and N6OCOL were found to be in agreement with the experimentally observed spectra, the exact structure of the fragments cannot be predicted. Multiple fragments with equal *m/z* values are predicted in most cases.

3.2. Identification of 6OCOL, N6OCOL and OCODNO in urine

CE–MS analysis of the urine after SPE extraction showed mass traces at *m/z* = 318, 304, 332 corresponding to the masses of 6OCOL, N6OCOL and OCODNO, respectively (Fig. 5A), mass traces at *m/z* values of 510 and 334 (Fig. 5A), as well as mass traces for OCOD, NOCOD, OMOR, NOMOR and various glucuronic acid conjugates that were previously discussed by Wey and Thormann ([15], data not shown). Corresponding mass spectra at the indicated time points are presented in Fig. 5B. OCOD and several of these metabolites were also monitored in extracts of rabbit urine [9] and human urine [8] using chromatographic approaches.

The identification of the masses found in urine was carried out by matching the monitored MS^n spectra ($n \leq 4$) with those of the standards stored in the library. The Xcalibur software supports two different modalities for that purpose, called 'Identity' and 'Similarity', the former being used for known substances, the latter for unknown compounds. Because of the presence of possible diastereoisomers that might affect the fragmentation patterns, especially at the MS^4 level, the 'Similarity' option was employed in our work.

The result of a 'Similarity' matching search consists of a ranking list where the spectra stored in the library are shown in order of decreasing degree of similarity to the unknown spectrum, according to two different software regulated indexes, called SI and RSI. The SI index, expressed in the 0–999 range, is a direct matching factor which considers all signals whereas the RSI index, also expressed in the 0–999 range, is a reverse matching factor which ignores any peak in the unknown that is not present in the library spectrum [34]. The RSI index is typically higher then the SI. For the compounds of in-

Table 1
Similarity between mass spectrometric data of urinary OCOD metabolites and their standard compounds

Urinary compound	Mass power	Matching parameters		Library data
		SI	RSI	
6OCOL	MS	340	818	6OCOL standard MS
	MS^2	930	989	6OCOL standard MS^2
	MS^3	941	976	6OCOL standard MS^3
	MS^4	828	910	6OCOL standard MS^4
N6OCOL	MS	502	734	N6OCOL standard MS
	MS^2	967	982	N6OCOL standard MS^2
	MS^3	930	950	N6OCOL standard MS^3
	MS^4	721	850	N6OCOL standard MS^4
OCODNO	MS	401	648	OCODNO standard MS
	MS^2	888	955	OCODNO standard MS^2
	MS^3	916	963	OCODNO standard MS^3
	MS^4	871	919	OCODNO standard MS^4

terest (6OCOL, N6OCOL and OCODNO), the mass spectra were found to match well (Table 1). SI and RSI results greater than 900 are considered excellent matches, the range 800–900 is considered to be good, 700–800 is fair, while SI and RSI values below 600 indicate a very poor match [34]. In each group, it is apparent that the degree of similarity increases from the full scan mass spectrum to MS^2 and MS^3. For example, the full scan spectrum at 5.97 min (Fig. 5B) comprises not only an ion with the $[M + H]^+$ value of OCODNO (m/z = 332) but also those of other OCOD metabolites, including that of OMOR (m/z = 302). Fragment isolation with a small window eliminates matrix interferences. SI values are thereby becoming much higher and SI and RSI values very similar. The worsening of the results for MS^4 can be attributed to the lower intensity of the signals and the possible presence of diastereoisomers ([9], Fig. 1) whose fragmentation might only differ at elevated MS levels (MS^n with $n \geq 4$) and whose presence could not be assessed in the synthesized compounds. The decreasing SI index indicates that the actual spectrum is more complex than that in the computer library.

3.3. Identification of two new metabolites of OCOD

Table 2 provides a list of known and possible phase I and phase II metabolites of OCOD. The first eight compounds are reported as being found in humans and/or animals [8,9,11,15] and could be identified in our urine sample. The existence of the last four compounds, namely nor-6-oxycodol-*N*-oxide (N6OCOLNO), nor-oxycodone-*N*-oxide (NOCODNO), oxymorphone-*N*-oxide (OMORNO) and nor-oxymorphone-*N*-oxide (NOMORNO), has never been reported and these substances could not be detected with our CE–MS-based assay. Among the possible phase II conjugates, only glucuronides have been reported in the literature [9,11,15]. The same applies for our efforts in trying to find these compounds in our samples. The CE–MS data obtained with the urine extract revealed two interesting peaks with m/z values of 510 and 334 (Fig. 5A and B). It was interesting

Table 2
List of protonated molecular ions of OCOD and possible phase I and II metabolites[a]

Parent compound and phase I metabolites		Phase II metabolites					
Compound	$[M + H]^+$	Glucuronide metabolite $[M + H]^+$	Acetyl metabolite $[M + H]^+$	Glycine metabolite $[M + H]^+$	Sulfate metabolite $[M + H]^+$	*N*-Methyl metabolite $[M + H]^+$	Glutathione metabolite $[M + H]^+$
OCOD	316.38	492.50	358.41	373.43	396.44	331.42	622.70
6OCOL	318.40	494.50	360.43	375.45	398.46	333.44	624.72
N6OCOL	304.37	480.49	346.40	361.42	384.43	[b]	610.69
NOCOD	302.36	478.48	344.39	359.41	382.42	[b]	608.68
OMOR	302.36	478.48	344.39	359.41	382.42	317.40	608.68
NOMOR	288.33	464.45	330.36	345.38	368.39	[b]	594.65
OCODNO	332.38	508.50	374.41	389.43	412.44	[c]	638.70
6OCOLNO	334.40	510.52	376.43	391.45	414.46	[c]	640.72
N6OCOLNO	320.37	496.49	362.40	377.42	400.43	[c]	626.69
NOCODNO	318.35	494.47	360.38	375.4	398.41	[c]	624.67
OMORNO	317.34	493.47	359.37	374.39	397.4	[c]	623.66
NOMORNO	303.32	479.44	345.35	360.37	383.38	[c]	609.64

[a] All masses were calculated with the ISIS/Draw software.
[b] *N*-Methylation is meaningless as produced compounds are part of this list.
[c] *N*-Methylation not possible.

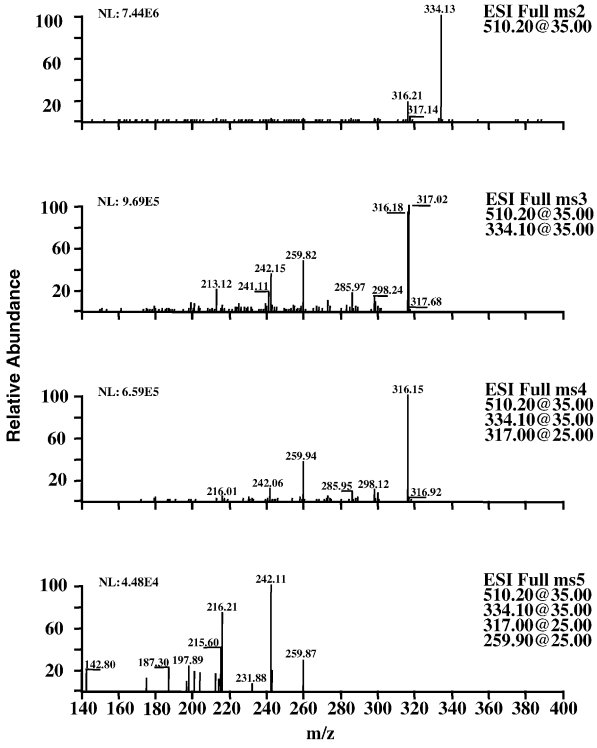

Fig. 6. MS^2 to MS^5 spectra (from top to bottom, respectively) of the *m/z* 510 ion monitored with the extract of the non-hydrolyzed patient urine. Right labels as for Fig. 2.

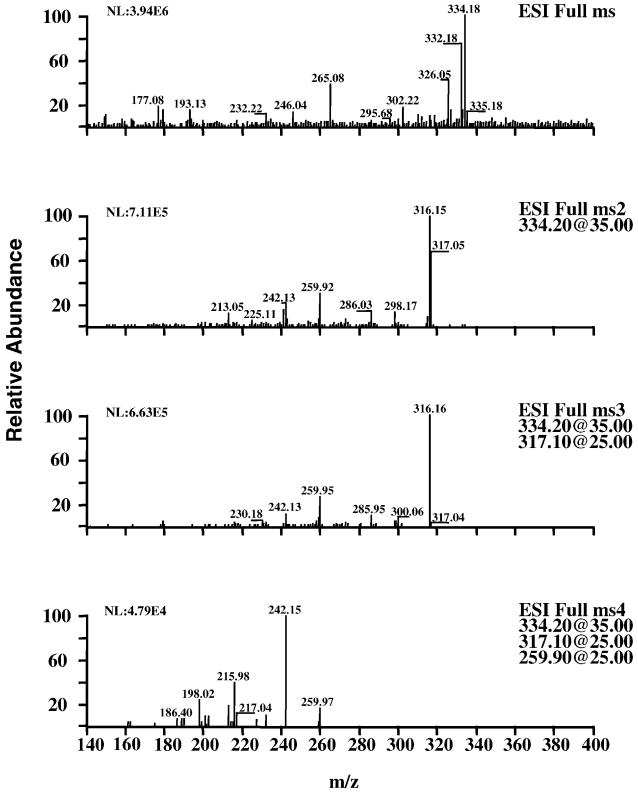

Fig. 7. MS to MS^4 spectra (from top to bottom, respectively) of the *m/z* = 334 ion detected with the extract of the hydrolyzed urine. Right labels as for Fig. 2.

to note that the combined elution principle (cf. Section 2.4) was required for extraction of the molecule with a mass of 510. Only traces of this compound were recovered using only methanol–concentrated ammonia (70:30, v/v) as eluent.

The difference between the two masses is 176 mass units. This leads to the assumption of a structural relationship with the *m/z* 510 ion being the *m/z* = 334 ion plus glucuronidation. In order to confirm or deny this assumption, aliquots of the urine were incubated for 24 h with β-glucuronidase/arylsulfatase at 37 °C, then extracted as described in Section 2.4 and analyzed. The obtained electropherogram showed a decreased signal for *m/z* = 510 peak and an increased response for *m/z* = 334, thus indicating the presence of a β-glycosidic bond in the molecule (data not shown). Furthermore, MS^2 data obtained with the extract of the non-hydrolyzed urine revealed the 510 → 334 transition (loss of 176 mass units, Fig. 6), a transition that corresponds to the excision of a glucuronic acid moiety as was previously described for *O*-glucuronides of various phase I OCOD metabolites [15]. MS^3 revealed the formation of both a *m/z* = 317 and a *m/z* = 316 ion, in analogy of what was observed with OCODNO, thus suggesting the presence of the *N*-oxide group (loss of 17 mass units) and a hydroxyl group in position 14 (loss of 18 mass units). MS^4 of the *m/z* 317 ion provided a mass spectrum with the *m/z* = 260 and 316 ions (Fig. 6), the difference of 56 mass units between the two fragments being the same as was previously observed for the two most abundant ions in the MS^3 data of OCODNO (Fig. 4). Furthermore, fragmentation of the *m/z* = 316 ion at the MS^3 level showed the loss of 18 mass units with the production of a *m/z* = 298 fragment, suggesting thus a similarity to the C6-reduced compounds (data not shown). A 298 → 283 transition was subsequently observed, as previously noted for various other oxycodone metabolites (data not shown). Fragmentation of the *m/z* = 260 peak led to a complex pattern whose intensity was too weak for a reliable evaluation (MS^5 data of Fig. 6).

The mass to charge ratio of 334 corresponds to the one expected for the 6-reduced, *N*-oxidized metabolite of OCOD, i.e. to 6-oxycodol-*N*-oxide (6OCOLNO). This compound is theoretically present in four different diastereoisomeric forms. The presence of one of these four possible metabolites in rabbit urine was previously discussed by Ishida et al. [9]. Commencing with the *m/z* 334 ion obtained with the extract of the hydrolyzed urine, fragmentation was found to produce MS^n mass spectra (Fig. 7) that compare well to the MS^{n+1} data of Fig. 6 (Table 3). Without removal of the additional ions seen in the MS data of Fig. 5B, the SI value was noted to be rather low and the SI and RSI indices are shown to be substantially different (SI = 137, RSI = 714). Good agreement between the spectra is obtained at higher MS levels.

On the basis of all the findings, it is possible to postulate the urinary presence of at least one of the diastereoisomers of 6OCOLNO and an *O*-glucuronide of 6OCOLNO. As the

Table 3
Report of the similarity between MS spectra of the *m/z* = 334 ion with those of the *m/z* = 510 ion as reference

Metabolite ion *m/z* = 510	Metabolite ion *m/z* = 334	Matching parameters	
		SI	RSI
MS^2	MS	137	714
MS^3	MS^2	843	922
MS^4	MS^3	816	907
MS^5	MS^4	548	744

initial water loss was not observed in the MS^2 data of the *m/z* 510 ion (Fig. 6, data not shown up to *m/z* of 510), conjugation of that compound is most likely occurring at the C14 hydroxyl group.

3.4. Identification of NOMOR-N-glucuronide

In the work of Wey and Thormann [15], a urinary metabolite of OCOD with an *m/z* ratio of 464 Th was observed but could not be unambiguously assigned to a molecular structure (for CE–MS^n data refer to [15]). It was interesting to find that this metabolite was detected only after SPE extraction using methanol/concentrated ammonia solution (7:3) as the elutant. Although the *m/z* value indicated that this compound was NOMOR glucuronide (Table 2), their conclusions, based on the fragmentation behavior compared to other glucuronides, led them to assign the metabolite to an OMOR derivative, without further specification of the conjugated chemical group. The possibility of elucidating this structure via fragmentation simulation and comparison with actual data was thus explored.

Considerations based on the *m/z* ratio, the susceptibility to hydrolysis with β-glucuronidase/arylsulfatase and the peculiar fragmentation pattern, led to formulate the hypothesis of a *N*-glucuronide derivative of NOMOR, featuring a β-glucosidic bond between the C1′ of the glucuronic acid moiety and the *N* of NOMOR (Fig. 8A). *N*-Glucuronide conjugates of drugs are not so well known as the *O*-glucuronides, although a large number of biologically active amines is reported to be metabolized in such a form in a variety of animal species, including man [35,36]. The enzyme responsible for this phase II reaction is the UDP-glucuronosyl transferase, existing in different isoforms [37–39]. The *N*-glucuronides

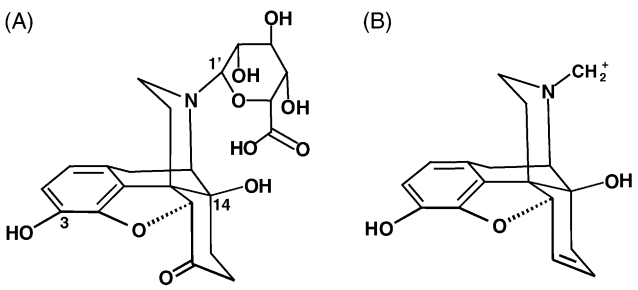

Fig. 8. Suggested chemical structures for (A) NOMOR-*N*-glucuronide and (B) the *m/z* = 284 fragment.

can be roughly distinguished in two groups, according to the degree of substitution in the amine residue. Quaternary *N*-glucuronides exhibit a permanent positive charge and their chemical properties are different compared to those of non-quaternary *N*-glucuronides [40].

The simulation of the MS spectrum of this molecule leads to the *m/z* = 464 ion and to fragments with *m/z* values of 282 and 284, ions that were also detected experimentally (data of top graph in Fig. 9B of [15]). MS^2 simulation predicts the H_2O loss (*m/z* = 446) and the elimination of the residual portion of the former glucuronic acid group leading, after rearrangements, to the *m/z* = 284 ion. Very interestingly, although 40 possibilities for the H_2O elimination process were predicted, the further examination of each of them revealed that only in seven cases it was possible to proceed to the MS^3 spectrum with results agreeing with the collected data. In all these seven cases, the water loss takes place in the glucuronic acid residue of the conjugate, involving different hydroxyl groups and various rearrangements. Simulated fragmentation leads to the same *m/z* = 284 ion in any case, which is identical to the one simulated for both MS and MS^2 (Fig. 8B). The same ion was monitored experimentally (Fig. 9B of [15]). The simulation of the fragmentation of the *m/z* = 284 peak leads to a spectrum matching the MS^4 data presented by Wey and Thormann [15]. The only signal which was not reproduced is that of the *m/z* = 199 ion.

Fragmentation of NOMOR β-*O*-glucuronides in which the glucuronic acid is linked to the C3 or C14 was also simulated. The experimentally acquired MS^2 and MS^3 spectra were thereby predicted. However, the simulated MS^4 data did not match the monitored spectrum depicted in [15]. Thus, considering experimental and simulated data, it appears reasonable to suggest that OCOD might undergo a bio-transformation leading to the formation of a NOMOR-*N*-glucuronide.

4. Conclusions

In this work, CE–MS^n (up to *n* = 5) and computer simulation of fragmentation are shown to be complementary approaches for the characterization and identification of phase I and phase II metabolites of OCOD for which standards are not available. 6OCOL, N6OCOL and OCODNO were synthesized in house and unambiguously identified in an extract of a urine that stemmed from a patient under pharmacotherapy with OCOD (administration of 240–320 mg of OCOD chloride daily). The two C6-reduced OCOD metabolites with $[M + H]^+$ ions of *m/z* = 318 and 304, respectively, show a characteristic fragmentation pattern, comprising a double water loss and a further decrease of 15 mass units on the MS^2, MS^3 and MS^4 level, respectively. Mass spectra for these compounds were predicted up to MS^3 (partial prediction of MS^4) by computer simulation using the HighChem Mass Frontier version 1.0 software package. OCODNO (*m/z* = 332) was found to behave differently with the MS^2 spectrum being character-

ized with the loss of 17 mass units (m/z = 315, most abundant ion) and the expected neutral loss of 18 mass units (m/z = 314). Based upon these MS^n data and experimental CE–MS^n data obtained with the non-hydrolyzed and enzymatically (β-glucuronidase/arylsulfatase) hydrolyzed patient urine, the presence of two new OCOD metabolites, 6OCOLNO (m/z = 334) and an *O*-glucuronide of 6OCOLNO (m/z = 510), could be identified. Furthermore, CE–MS^n and computer simulation led to the identification of NOMOR-*N*-glucuronide, a previously detected OCOD metabolite that could not be identified solely based upon the CE–MS^n data.

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