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Isolation and characterization of a new human urinary metabolite of diclofenac applying LC–NMR–MS and high-resolution mass analyses

Dele Stülten, Marc Lamshöft, Sebastian Zühlke, Michael Spiteller *

Institute of Environmental Research (INFU), University of Dortmund, Otto-Hahn-Straße 6, D 44221 Dortmund, Germany

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Abstract

The nonsteroidal anti-inflammatory drug diclofenac is widely used. Diclofenac is extensively metabolized to several hydroxylated derivatives and their conjugates. The lactam-dehydrate of 4′-hydroxy diclofenac (4′-OHDD) has now been detected as a new urinary metabolite of diclofenac. Isolation was successfully performed using preparative HPLC in three different steps using water, methanol, and acetonitrile, respectively. The structural characterization of 4′-OHDD was achieved by LC–NMR–MS. In addition, specific mass fragmentation pattern could be obtained using LC–high-resolution MS with both positive and negative electrospray ionization.

Keywords: Diclofenac; Biotransformation; Preparative LC; LC–NMR; HRMS; Accurate mass

1. Introduction

After application to humans, the active pharmaceutical ingredients and their metabolites are excreted and as a result reach the municipal sewage treatment works. Externally applied medications are directly washed into discharges. But not all of them are removed by the sewage treatment. Diclofenac (DF) is a COX inhibitor with anti-inflammatory and analgesic effects, and is a widely used over-the-counter drug. In surface waters DF has thus been reported in concentrations up to 15 μg/l [1–5]. Considering the common use and the ubiquity of DF in the environment, the biological fate after intake is of great interest.

The metabolism of DF depends on the type of application. After oral administration, the drug is metabolized to the following substances (Table 1) and their conjugates (mainly glucuronides and sulfates): principally formed 4′-hydroxy DF (4′-OHD, 16.0% of urine excretion) but also 3′-hydroxy DF (3′-OHD, 2.0%), 5-hydroxy DF (5-OHD, 6.1%), and 3′-hydroxy-4′-methoxy DF (3′-OH-4′-OMeD, <0.01%). About 6.2% remain unchanged as DF [6]. But in subjects with reduced renal function, urinary excretion of 3′-OHD, 5-OHD, 3′-OH-4′-OMeD, and DF accounted for less than 2% of the dose [6]. In addition, Schneider and Degen [7] analyzed the urinary amount of 4′,5-dihydroxydiclofenac (4′,5-$(OH)_2$D) and conjugates after oral administration and found it was 9.4% of the dose. On administering DF i.m., they detected 6.5% DF, 18.1% 4′-OHD, 8.2% 5-OHD, 1.4% 3′-OHD, and 15.4% 4′,5-$(OH)_2$D in urine samples (including conjugates). The biliary excretion of DF and its metabolites in humans account for a maximum of 42% of the dose, mainly 10–20% of 4′-OHD and 5–10% of 5-OHD (including conjugates), DF accounting for less than 5% of the dose [8]. After application on the skin, only 6% (compared to renal excretion after oral intake) is resorbed, the rest is washed away [9].

In earlier studies, the formation of dehydrated hydroxyl metabolites of DF (lactams) was observed, but only after vigorous alkaline or acidic treatment and after extractive alkylation [10,7]. The lactam of DF has been recently detected as one of the main degradation products after solar radiation [11].

This paper describes the separation of a new minor urinary lactam metabolite of DF, 4′-OHD dehydrate (4′-OHDD), as well as 5-OHD and 4′-OHD, to obtain pure substances as standards for future quantifications. 4′-OHDD was characterized by means of LC–NMR–MS as well as LC–high-resolution MS^n studies.

* Corresponding author. Tel.: +49 231 7554081; fax: +49 231 7554084.
E-mail address: m.spiteller@infu.uni-dortmund.de (M. Spiteller).

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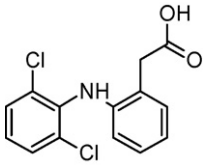
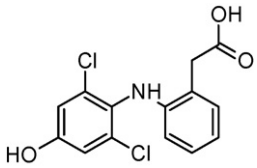
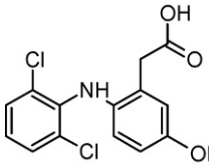
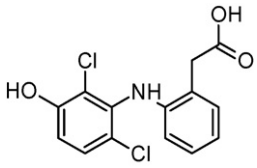
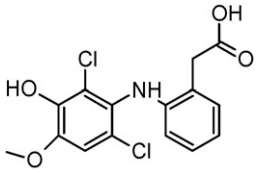
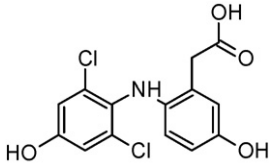
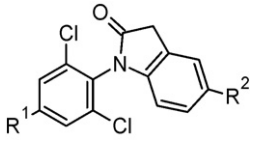
Table 1
Derivatives of diclofenac (conjugates not shown)

Compound	Structure	Origin	Literature
Diclofenac (DF)		Analgesic, anti-inflammatory drug	[6,7,10]
4′-Hydroxy-diclofenac (4′-OHD)		Metabolite of DF	[6,7,10]
5-Hydroxy-diclofenac (5-OHD)		Metabolite of DF	[6,7,10]
3′-Hydroxy-diclofenac (3′-OHD)		Metabolite of DF	[6,7,10]
3′-Hydroxy-4′-methoxy-diclofenac (3′-OH-4′-OMeD)		Metabolite of DF	[6]
4′,5-Dihydroxy-diclofenac (4′,5-$(OH)_2$D)		Metabolite of DF	[7,10]
Lactam-dehydrates of diclofenac-metabolites[a]		Derivatives of hydroxyl metabolites after alkaline or acidic treatment and after extractive alkylation	[7,10]

[a] Lactams: R^1 = OH, R^2 = H: 4′-hydroxy-diclofenac-dehydrate (4′-OHDD); R^1 = H, R^2 = OH: 5-hydroxy-diclofenac-dehydrate; R^1 = OH, R^2 = H: 4′,5-dihydroxy-diclofenac-dehydrate.

2. Experimental

2.1. Materials

2.1.1. Chemicals and standards

Acetonitrile and methanol were purchased from Baker (Deventer, Netherlands). Formic acid and ammonium acetate were of analytical grade, d_4-methanol was of Uvasol-Grade (99.8%) and all supplied by Merck (Darmstadt, Germany).

DMSO (99.5%) was obtained from Fluka (Buchs, Switzerland).

2.2. Urine samples

The urine of three patients who had taken 50–150 mg DF-Na daily was collected over 24 h by the municipal hospital in Dortmund. To avoid possible degradation, the samples have been collected and stored in amber-colored glass bottles at 4 °C.

Extraction and concentration (by solid-phase extraction, SPE) were done closely to the collection.

2.3. Proceeding

After extraction and concentration of the urine by SPE, the extracts were screened by LC–MS for the diclofenac metabolites. These metabolites were separated using preparative HPLC in three steps. After the first preparative separation step, the raw fraction containing 5-OHD, 4′-OHD, and 4′-OHDD was subjected to an LC–NMR–MS experiment, leading to the identification of the three compounds. Pure 4′-OHDD was obtained after the third preparative separation step. It was further characterized by LC–high-resolution MS^n measurements.

2.4. Extraction and concentration of urine by SPE

The urine samples (12 l) were centrifuged at 15,000 × *g*/10,000 rpm for 15 min (Beckman Coulter, Krefeld, Germany) and the supernatant was extracted in 200 ml aliquots on BakerBond PolarPlus columns (C18, 2000 mg, Baker, Griesheim, Germany) that had been preconditioned each with 3× 4 ml methanol and 3× 4 ml Millipore-water (Millipore, Schwalbach, Germany). After percolation of the urine, the cartridges were washed with 2× 4 ml Millipore-water and dried under vacuum for 30 min. Elution has been carried out using 3× 2 ml methanol.

The pooled extracts were concentrated to 10 ml under a gentle flow of nitrogen and stored at 4 °C. Before analysis or separation, samples were centrifuged (10 min at 15,000 × *g*) and only the supernatant was analyzed.

2.5. Purification of urine by preparative LC

The metabolites detected were isolated from the SPE eluate in three steps. The first consisted of a gradient separation followed by two isocratic separations with different solvent systems.

Gradient separation (step 1) was performed using a Gilson (Middleton, USA) preparative HPLC system with a model 322 pump, a model 152 UV/vis detector (λ = 254 nm), a model 204 fraction collector (collection: 1 fraction every 2 min) and the Gilson-Unipoint software. Compounds were separated at a flow rate of 14 ml/min on a Waters Sunfire-C18 column, 5 μm, 190 mm × 15 mm using a Millipore-water (0.1% formic acid; solvent A)–distilled methanol (0.1% formic acid; solvent B) gradient: 100% A isocratic for 3 min, linear gradient to 40% B within 12 min, and to 100% B within another 10 min. After 100% B for 5 min, the system was returned to its initial conditions (100% A) within 3 min and held for 4 min.

Further isocratic separation (steps 2 and 3) has been realized on a semi-preparative HPLC system from Dionex Softron (Germering, Germany) with a Gynkotech pump, an UV340S DAD-detector (collection at λ = 254 nm), the Chromeleon 6.00 (Build 435) software and equipped with a Foxy Jr. fraction collector (ISCO, Lincoln, USA). Compounds were separated on an Alltima column, 5 μm, 250 mm × 10 mm (Alltech Associates, Deerfield, USA) at a flow rate of 4 ml/min. A first isocratic separation (step 2) was carried out with Millipore-water (0.1% formic acid)–distilled methanol (0.1% formic acid) (45:55, v/v).

The second isocratic separation (step 3) was performed with Millipore-water (0.1% formic acid)–acetonitrile (0.1% formic acid) (65:35, v/v).

The solvents were removed from the fractions using a rotary evaporator. The relevant fraction residues were redissolved in the suitable solvent mixture before the next separation step.

The amounts of 4′-OHD and 4′-OHDD obtained after isolation were 1.5 mg and 1.9 mg, respectively. The amounts were calculated after quantitative ^{1}H NMR analysis using DMSO as internal standard. 5-OHD was not isolated in sufficient amount for these measurements.

2.6. LC/MS analysis of urine and fractions

The analytical LC–MS measurements of the urine extracts and the fractions from the preparative LC-system were done on a system consisting of a Surveyor HPLC (Thermo Finnigan, USA) coupled to a TSQ 7000 spectrometer (Thermo Finnigan, USA).

The separations for analytical spectra were performed using a Nucleodur Sphinx RP column, 5 μm, 250 mm × 2 mm (Macherey & Nagel, Düren, Germany). The HPLC system was equipped with a pump, an autosampler, and a PDA detector (254 nm) and operated at a flow rate of 0.2 ml/min. The mobile phase consisted of double distilled water (0.1% formic acid; solvent A)–acetonitrile (0.1% formic acid; solvent B). Analyses were carried out by the following gradient program: linear gradient from 20 to 45% B for 5 min; isocratic at 45% B for 25 min, followed by a linear gradient from 45 to 55% B within 2 min, another linear gradient from 55 to 65% B within 14 min, and from 65 to 100% B within 1 min. After 100% acetonitrile for 3 min, the system was held at the initial conditions (20% B) 10 min.

The mass spectrometer was operated in negative ESI-FS mode (mass range: *m/z* 100–800). Nitrogen was used as sheath gas (450 kPa), and argon served as collision gas (0.27 Pa). 5-OHD, 4′-OHD and 4′-OHDD were detected in FS spectra by *m/z* 309.8, 309.8 and 291.8, respectively.

2.7. LC–NMR–MS analysis of (partially) purified metabolites

The LC–NMR–MS analysis of the relevant fraction (containing 5-OHD, 4′-OHD, and 4′-OHDD) was performed after the first preparative separation (step 1). The sample was dissolved in 300 μl acetonitrile–water (70:30, v/v).

The system consisted of an HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an Esquire 3000 plus mass spectrometer (Bruker Daltonics, Bremen, Germany) between the HPLC instrument and its UV detector with a split of 1:20. A Prospekt2 SPE unit (Spark, AJ Emmen, Holland) was connected to the HPLC system. Elution with deuterated solvent was used for the transfer

to an AV600 NMR spectrometer (Bruker, Karlsruhe, Germany).

The HPLC system was equipped with a G1367A autosampler and a G1314A VWD Detector (254 nm); the injection volume was 10 μl. The column oven temperature was set at 40 °C. Separation was carried out on a Nucleodur C18 Gravity column, 5 μm, 250 mm × 2 mm (Macherey & Nagel, Düren, Germany) using water (0.1% formic acid; solvent A)–acetonitrile (0.1% formic acid; solvent B) at a flow rate of 0.2 ml/min with linear gradient from 20 to 40% B in 1 min, followed by linear gradient to 60% B within 33 min and another to 95% B within 1 min. B was held at 95% for 4 min followed by a conditioning of 16 min at 20% B.

The mass spectrometer was used in positive electrospray ionization mode to produce FS mass spectra with a scan range of 50–1000 (*m/z*). The maximum acquisition time was 200,000 μs, the capillary voltage was at 94 V, the HV capillary was at 4500 V, and the drying temperature was at 250 °C.

The compounds were separately trapped in the SPE unit on a polymer phase (Hysphere Resin GP, 10–12 μm, 10 mm × 1 mm, Spark) triggered by their quasi-molecular ion detected by the mass spectrometer. After trapping the corresponding HPLC peaks, the SPE cartridges were dried (30 min) by a stream of nitrogen and eluted each with 0.3 ml d_4-methanol.

The NMR spectra recorded included ^{1}H, ^{1}H-^{1}H-COSY and ^{1}H-^{13}C (HMQC and HMBC) NMR analyses. All were acquired using standard procedures on a 3-mm probe head at a temperature of 298 K using TopSpin 1.3 software (Bruker). ^{1}H NMR spectroscopy was performed with double solvent suppression (methanol, $\delta = 3.328$ ppm and 4.940 ppm).

2.8. High-resolution mass analysis of purified 4′-OHDD

The relevant fraction from the second isocratic separation (step 3) was injected to a Dionex μ-HPLC system Ultimate 3000 (Dionex, Idstein, Germany) coupled to a LTQ-Orbitrap spectrometer (Thermo Scientific, USA) for the analysis of 4′-OHDD.

The μ-HPLC system consisted of pump, UV detector ($\lambda = 254$ nm), flow manager, and an autosampler (injection volume 1 μl). The separation was performed using a Phenomenex Gemini C18 column, 3 μm, 0.3 mm × 150 mm (Torrance, CA, USA), with a double distilled water (0.1% HCOOH; solvent A)–acetonitrile (0.1% HCOOH; solvent B) gradient (flow rate 4 μl min^{-1}): linear gradient from 5% B to 45% B for 1 min, followed by a linear gradient to 70% B within 12 min and to 100% B within 2 min, after 100% B isocratic for 5 min, the system returned to its initial condition (5% B), and was equilibrated for 9 min.

The mass spectrometer was operated in positive and negative ESI–FT mode (mass range: *m/z* 100–1000, with nominal mass resolving power of 60,000 at *m/z* 400 and a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements in positive mode within 2 ppm deviation using an internal lock mass, bis(2-ethylhexyl)phthalate, *m/z* = 391.284286. Nitrogen was used as sheath gas (5 A.U.), and helium served as the collision gas.

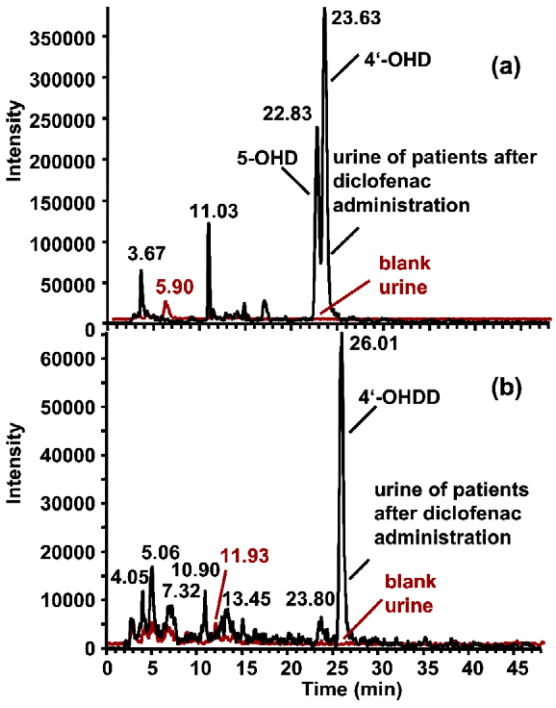


Fig. 1. SIM chromatograms of diclofenac metabolites in extracts of patient urine compared to blank urine: (a) mass trace of *m/z* 309.8 and (b) mass trace of *m/z* 291.8.

3. Results and discussion

The analytical LC–MS FS run of urine extracts of diclofenac-treated patients revealed the metabolites 5-OHD, 4′-OHD, and 4′-OHDD at retention times of 22.8 min, 23.6 min, and 26.1 min, respectively (Fig. 1). The UV spectrum of the latter exhibited two maxima at 240 nm and 280 nm.

To obtain pure substances from preparative HPLC, separations were carried out with different organic solvents (methanol in steps 1 and 2, acetonitrile in step 3). When using methanol as organic solvent in isocratic separation, the hydroxy diclofenac isomer 5-OHD was isolated (from 26 min to 30 min), but 4′-OHD and 4′-OHDD were still eluted within the same fractions (from 31 min to 34 min). Isocratic separation of 4′-OHD and 4′-OHDD (from 38 min to 40 min and from 43 min to 45 min, respectively) was achieved by using water–acetonitrile as mobile phase (step 3).

In LC–NMR–MS studies, all metabolites could be identified by ^{1}H, ^{1}H-^{1}H and ^{1}H-^{13}C NMR (COSY, HMQC, and HMBC mode). The structure of 4′-OHDD (Fig. 2) was elucidated by two-dimensional NMR spectroscopy. ^{1}H-^{1}H-COSY analysis identified the signals of the two isolated aromatic systems. Further detail of the structure could be observed in HMQC. The protons of the aromatic ring close to the lactam ring resonated at 6.42 ppm (H-13; coupling to C-13 at 110.62 ppm), 7.08 ppm (H-11; C-11 at 124.48 ppm), 7.19 ppm (H-12; C-12 at 129.68 ppm), and 7.33 ppm (H-10; C-10 at 126.21 ppm), respectively. The

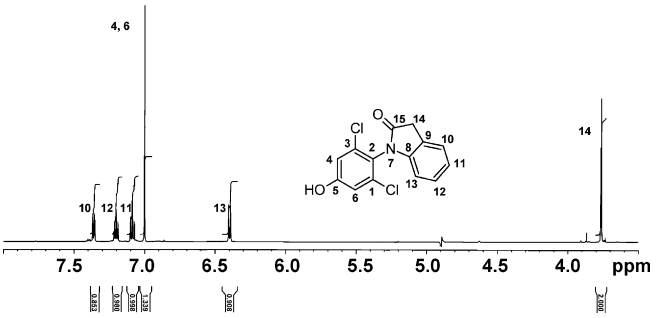


Fig. 2. ^{1}H NMR spectrum (600 MHz) of 4′-OHDD in methanol-d_4.

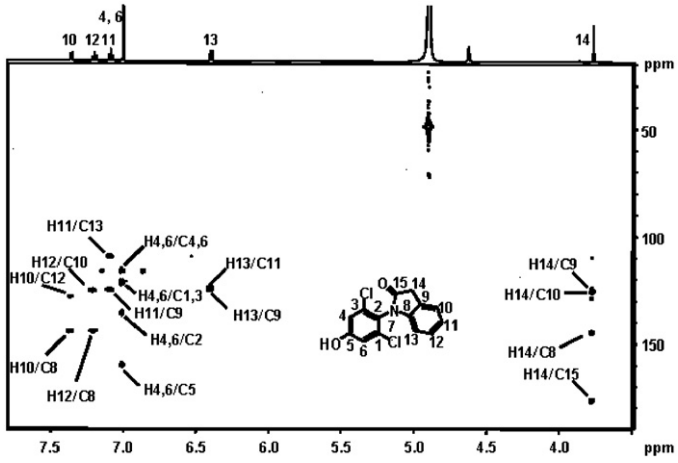


Fig. 3. ^{1}H-^{13}C-NMR spectrum (HMBC; 600 MHz) of 4′-OHDD in methanol-d_4.

HMBC spectrum (Fig. 3) exhibits the symmetric protons of the hydroxyl aromatic ring (H-4,6; C-4,6 at 117.55 ppm) as a singlet at 6.99 ppm coupling to the carbon of the oxidized ring system (C-5) at 160.85 ppm. The protons of the CH_2 group at 3.72 ppm (C-14 at 35.34 ppm) couple to the carbonyl carbon at 176.44 ppm (C-15) of the characteristic lactam ring system. The carbon of the carboxyl acid function of diclofenac shows a ^{13}C-shift of 181 ppm. By identification of the coupling of protons H-4,6, and H-14 in HMBC, the chemical shift of C-1,3 (121.02 ppm), C-2 (136.60 ppm), C-5 (160.85 ppm), C-9 (124.48 ppm), and C-8 (145.26 ppm), respectively, could be determined, leading to the elaboration of the complete structure of the new metabolite.

After isolation of 4′-OHDD (third preparative LC run), high-resolution MS^n experiments in both positive and negative mode revealed a specific fragmentation pattern which is reported in Figs. 4 and 5, respectively. One example for MS^n fragmentation is shown in Fig. 6. In positive ESI, 4-OHDD gave a molecular ion peak of *m/z* 294.00789 $[M+H]^+$, confirming the molecular composition of $C_{14}H_{10}Cl_2NO_2$ [M]. The main fragment (MS^2) was found at *m/z* 231.04490 (loss of CO and Cl). Other fragments showed masses of *m/z* 259.03949 (–Cl), 224.07076 (–Cl_2), and 230.03679 (–CO and HCl), respectively. A further MS^3 scan on *m/z* 259.03949 leads to fragments at *m/z* 224.07071 (–Cl), 230.03684 (–HCO), and 231.04471 (–CO), respectively.

To give an idea of the amounts of unconjugated metabolites, the UV chromatograms of two urine extracts at λ = 240 nm were recorded. The area integrals of the metabolites and DF were

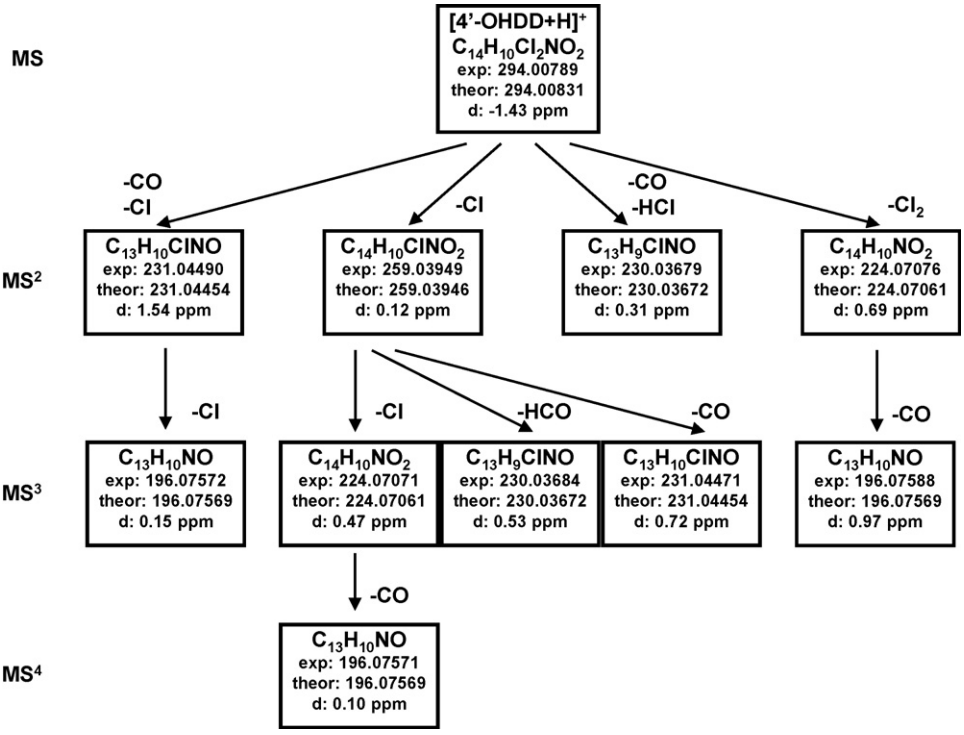


Fig. 4. Fragmentation scheme of 4′-OHDD in positive LC–high-resolution MS^n studies (exp: experimental mass; theor: theoretical mass; d: difference).

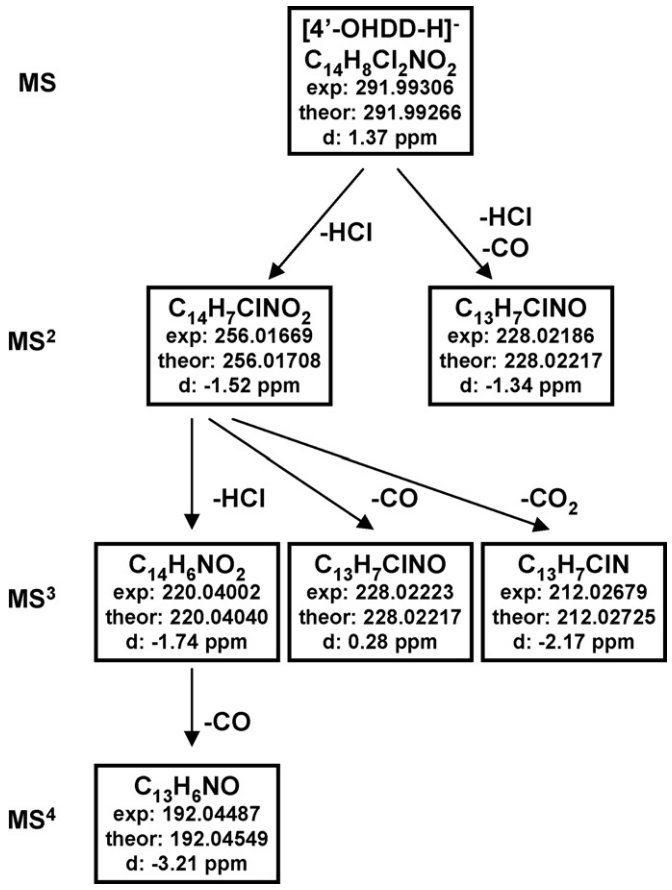


Fig. 5. Fragmentation scheme of 4′-OHDD in negative LC–high-resolution MS^n studies (exp: experimental mass; theor: theoretical mass; d: difference).

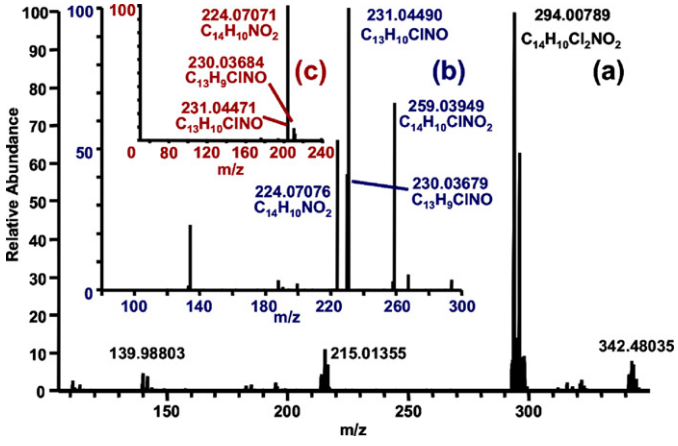


Fig. 6. Positive ESI high-resolution mass spectrum of 4′-OHDD: (a) MS, (b) MS^2 of $m/z = 294.00789$ (25 eV) and (c) MS^3 of fragment ion $m/z = 259.03949$ (25 eV).

referred to the value of the 4′-OHD integral (100%). Hence 5-OHD, 4′-OHDD, and diclofenac excretions corresponded to about 47 ± 5, 5 ± 0.7, and $34 \pm 24\%$ of the 4′-OHD amount, respectively.

In a single measurement of sewage treatment plant outlet samples with the analytical method (2.6) 5-OHD, 4′-OHD, 4′-OHDD could be detected, revealing the importance of metabolite monitoring in environmental systems.

4. Conclusion

This study reports the occurrence of the diclofenac metabolite 4′-OHDD in human urine. 4′-OHDD as well as the hydroxy metabolites of diclofenac have been isolated. The proposed structure of 4′-OHDD was confirmed by LC–NMR–MS and LC–high-resolution MS^n measurements. 4′-OHDD, 4′-OHD, and 5-OHD were detected in wastewater treatment plant outlets.

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