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Analytical power of LLE–HPLC–PDA–MS/MS in drug metabolism studies: Identification of new nabumetone metabolites

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ABSTRACT

Nabumetone is a non-acidic, nonsteroidal anti-inflammatory prodrug. Following oral administration, the prodrug is converted in the liver to 6-methoxy-2-naphthylacetic acid (6-MNA), which was found to be the principal metabolite responsible for the NSAID effect. The pathway of nabumetone transformation to 6-MNA has not been clarified, with no intermediates between nabumetone and 6-MNA having been identified to date.

In this study, a new, as yet unreported *phase I* metabolite was discovered within the evaluation of nabumetone metabolism by human and rat liver microsomal fractions. Extracts from the biomatrices were subjected to chiral LLE–HPLC–PDA and achiral LLE–UHPLC–MS/MS analyses to elucidate the chemical structure of this metabolite. UHPLC–MS/MS experiments detected the presence of a structure corresponding to elemental composition $C_{15}H_{16}O_3$, which was tentatively assigned as a hydroxylated nabumetone. Identical nabumetone and HO-nabumetone UV spectra obtained from the PDA detector ruled out the presence of the hydroxy group in the aromatic moiety of nabumetone. Hence, the most likely structure of the new metabolite was 4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one (3-hydroxy nabumetone). To confirm this structure, the standard of this nabumetone metabolite was synthesized, its spectral (UV, CD, NMR, MS/MS) and retention properties on chiral and achiral chromatographic columns were evaluated and compared with those of the authentic nabumetone metabolite.

To elucidate the subsequent biotransformation of 3-hydroxy nabumetone, the compound was used as a substrate in incubation with human and rat liver microsomal fraction. A number of 3-hydroxy nabumetone metabolites (products of conjugation with glucuronic acid, *O*-desmethylation, carbonyl reduction and their combination) were discovered in the extracts from the incubated microsomes using LLE–HPLC–PDA–MS/MS experiments. On the other hand, when 3-hydroxy nabumetone was incubated with isolated rat hepatocytes, 6-MNA was detected as the principal metabolite of 3-hydroxy nabumetone. Hence, 3-hydroxy nabumetone could be the missing link in nabumetone biotransformation to 6-MNA (*i.e.* nabumetone → 3-hydroxy nabumetone → 6-MNA).

1. Introduction

The nonsteroidal anti-inflammatory prodrug nabumetone possesses only a weak cyclo-oxygenase 2 (COX-2) inhibitory activity [1]. The originality of the neutral nabumetone structure arises from a relatively low incidence of gastric irritancy, bleeding, ulcerations and even gastrointestinal perforations, which were often observed following a long-term use of various NSAID active arylalkane acids. Lacking the mentioned adverse effects, neutral nabumetone is well absorbed from the GIT, and transported in the liver, where it undergoes an extensive first-pass biotransformation. Oxidative cleavage of nabumetone side-chain leads to the main

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pharmacodynamically active metabolite, a very strong COX-2 inhibitor, 6-methoxy-2-naphthylacetic acid (6-MNA). Similar to other arylalkane acids, 6-MNA displays anti-inflammatory and analgesic effects. *O*-Desmethylation and carbonyl reduction have also been reported as additional metabolic conversions of nabumetone [2,3]. Hydrophilic *phase II* nabumetone metabolites, namely acyl glucuronide from 6-MNA, ether glucuronides from *O*-desmethyl and *phase I* nabumetone metabolites with reduced carbonyl were found in the urine, bile and duodenal mucosa of minipigs as well as in human urine [4].

An overview of analytical approaches to the determination of nabumetone in pharmaceutical applications and nabumetone biotransformation products in bioanalytical studies has been provided in our previous communication [3]. Recent analytical methods for nabumetone determination based on voltammetric techniques [5], heavy atom-induced phosphorescence [6], spectrophotometric methods [7], TLC or HP TLC densitometry [8,9] or HPLC [10–13] have also been reported in the last decade. Characterization of the enzymes involved in the biotransformation of nabumetone to 6-MNA [14] and in *O*-desmethylation of 6-MNA to 6-HNA [15] has already been performed.

It is very surprising that no biotransformation intermediate(s) between nabumetone and 6-MNA have been discovered since 1984, when Haddock et al. published an overview of nabumetone metabolites for the first time [2]. The loss of two carbon atoms indicates an initial conversion of nabumetone to oxidative product(s), the identification of which could help to elucidate the detailed course of nabumetone conversion to 6-MNA.

Our attempt to find the missing link(s) in nabumetone → 6-MNA conversion commenced by *in vitro* evaluation of nabumetone biotransformation using human and rat liver subcellular fractions (microsomes and cytosol), and rat isolated hepatocytes. This xenobiochemical approach combined with LLE–HPLC–PDA–MS/MS enabled the detection and identification of new nabumetone metabolite(s).

2. Experimental

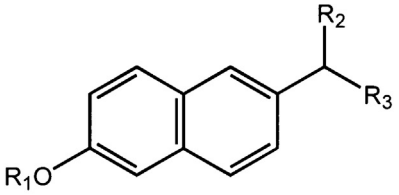
2.1. Chemicals and solutions

Chemical structures of nabumetone, its *phase I* metabolites and I.S. are shown in Fig. 1: 6-hydroxy-2-naphthylacetic acid ($C_{12}H_{10}O_3$, 202.21 g/mol, **6-HNA**, compound **1**); racemic 4-(6-hydroxy-2-naphthyl)-butan-2-ol ($C_{14}H_{16}O_2$, 216.28 g/mol, **6-HO-nb—OH**, enantiomers of compound **2**); 4-(6-hydroxy-2-naphthyl)-butan-2-one ($C_{14}H_{14}O_2$, 214.26 g/mol, **6-HO-nb═O**, compound **3**); 6-methoxy-2-naphthylacetic acid ($C_{13}H_{12}O_3$, 216.24 g/mol, **6-MNA**, compound **4**); 4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one ($C_{15}H_{16}O_3$, 244.29 g/mol, **3-HO-nabumetone,** compound **5**); (*S*)-(+)-2-(6-methoxy-2-naphthyl)propionic acid ($C_{14}H_{14}O_3$, 230.26 g/mol, **naproxen**, I.S. for achiral chromatography, compound **6**); racemic 4-(6-methoxy-2-naphthyl)-butan-2-ol ($C_{15}H_{18}O_2$, 230.31 g/mol, **6-MeO-nb—OH**, enantiomers of compound **7**); 4-(6-methoxy-2-naphthyl)-butan-2-one ($C_{15}H_{16}O_2$, FW = 228.3 g/mol, **nabumetone**, compound **8**). The parent prodrug nabumetone was obtained from PRO.MED.CS Prague, Czech Republic. Synthesis of nabumetone *phase I* metabolites (compounds **1**–**4**, compound **7**) has been described in our previous paper [3]. Methyl ester of naproxen (an internal standard (I.S.) for chiral chromatographic analyses) was prepared by the esterification of compound **6**. Synthesis of 4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one (**3-HO-nabumetone**) is described herein (Section 2.2).

Stock solutions (10^{-3} M) of nabumetone derivatives were prepared as follows: **6-HNA, 6-MNA** and **naproxen** in 10^{-3}M aqueous NaOH–methanol (1:1, v/v); **6-HO-nb—OH** in methanol; **6-HO-nb═O**, 6-**MeO-nb—OH**, **naproxen**, **methyl naproxen** and **nabumetone** in acetonitrile. Lower concentrations (10^{-4}, 10^{-5}, 10^{-6} M) were further prepared by diluting the stock solutions with UHQ water.

Acetonitrile, methanol (both HPLC grade, Merck, Darmstadt, Germany), diethyl ether (analytical grade, Merck), hydrochloric acid (35% water solution of HCl, analytical grade), sodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate and sodium hydroxide (all analytical grade, Lachema Brno, Czech Republic), orthophosphoric acid (85%, analytical grade, HiChem s r.o., Prague, Czech Republic), perchloric acid 70%, sodium perchlorate monohydrate (puriss. p.a. >98%, Fluka, Sigma–Aldrich, Prague, Czech Republic), ultra-high-quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga Ltd, Bucks, UK) were used for the extraction of the biomatrices and both achiral and chiral chromatography with UV-PDA detection of nabumetone derivatives.

Acetonitrile (HPLC gradient grade), acetic acid and sodium formate (all from Sigma–Aldrich St. Louis, MO, USA), de-ionized water (prepared by Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) were used in UHPLC–MS/MS.



Compound No.	Code	R_1	R_2	R_3
1	6-HNA	H-	-H	-COOH
2	6-HO-nb-OH	H-	-H	$-CH_2-CH(OH)-CH_3$
3	6-HO-nb=O	H-	-H	$-CH_2-C(=O)-CH_3$
4	6-MNA	CH_3-	-H	-COOH
5	3-HO-nabumetone	CH_3-	-H	$-CH(OH)-C(=O)-CH_3$
6	naproxen	CH_3-	$-CH_3$	-COOH
7	6-MeO-nb-OH	CH_3-	-H	$-CH_2-CH(OH)-CH_3$
8	nabumetone	CH_3-	-H	$-CH_2-C(=O)-CH_3$

Fig. 1. Chemical structures of synthetic standards of nabumetone derivatives.

Tetrahydrofuran (THF, reagent plus ≥99.0%, ALDRICH, distilled from benzophenone ketyl); pyridinium tribromide, technical grade 90%, ALDRICH; ethyl acetate p.a., PENTA Prague; sodium thiosulfate ($Na_2S_2O_3$, saturated aqueous solution, Alfa Aesar, UK); brine (10% solution of NaCl in water); anhydrous sodium sulfate (Na_2SO_4, p.a., PENTA, Prague); silica gel 60 (0.040–0.063 mm, MERCK); petrolether (p.a., PENTA, Prague); $CDCl_3$ for NMR (99.8% atom D, stabilized with silver foil, ACROS ORGANICS); silver perchlorate monohydrate (99%, ALDRICH) were employed in the synthesis of 3-hydroxy nabumetone (see Section 2.2).

0.1 M Phosphate buffer, pH = 7.4 (made from 0.1 M $NaH_2PO_4{\cdot}2H_2O$/0.1 M $Na_2HPO_4{\cdot}12H_2O$ and H_3PO_4) was used in the preparation of the subcellular fraction (see Section 2.3).

0.1 M Phosphate buffers, pH = 3 and pH = 10 (made from 0.1 M $NaH_2PO_4{\cdot}2H_2O$ or 0.1 M $Na_2HPO_4{\cdot}12H_2O$ and H_3PO_4 or NaOH) were used for biomatrix extraction and reextraction (see Section 2.6).

1 M aqueous $NaClO_4$, pH = 6.74 and 1 M aqueous $NaClO_4/HClO_4$, pH = 3 were used as polar components of the mobile phases in chiral chromatography.

β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH, Merck, Prague, Czech Republic), dimethyl sulfoxide (DMSO), diethyl ether, bicinchoninic acid, HAM F12 medium, Williams' E medium and foetal calf serum (Sigma–Aldrich, Prague, Czech Republic) were used in the xenobiochemical experiments.

2.2. Synthesis of 4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one, NMR, IR and MS analyses

^1H and ^{13}C NMR spectra were recorded on a Varian Vx BB 300 spectrometer. Chemical shifts were recorded as δ values in parts per million (ppm), and were indirectly referenced to tetramethylsilane (TMS) *via* the solvent signal (3.30 ppm for ^1H and 49.0 ppm for ^{13}C). Mass spectra were recorded on an LCMS Agilent 500 instrument. Infrared spectra were recorded on a NICOLET 6700 FT-IR/ATR-Ge spectrometer and are reported in wavenumbers (cm^{-1})

2.2.1. 3-Bromo-4-(6-methoxy-2-naphthyl)butan-2-one

A solution of 4-(6-methoxy-2-naphthyl)butan-2-one (228 mg, 1.0 mmol) in THF (5 ml) was added to the solution of pyridinium tribromide (320 mg, 1.0 mmol) in THF (5 ml) dropwise at 0 °C. The reaction was maintained at 0 °C for another 2 h and then allowed to proceed to completion at room temperature for an additional hour. The mixture was subsequently diluted with ethyl acetate and worked up with brine (2× 10 ml), and a solution of $Na_2S_2O_3$ (2× 10 ml). The organic phase was dried over anhydrous Na_2SO_4, and concentrated under vacuum. The crude product was purified on silica gel using a mixture of petrolether and ethyl acetate (9:1) as a mobile phase to yield the title compound as a yellowish oil (240 mg, 78% yield): **^{1}H NMR** (300 MHz, $CDCl_3$) δ 2.32 (s, 3H, CH_3), 3.29 (dd, J = 7.4, 14.4 Hz, 1H, CH_2), 3.58 (dd, J = 7.4, 14.4 Hz, 1H, CH_2), 3.91 (s, 3H, OCH_3), 4.55 (t, J = 7.4 Hz, 1H, CH), 7.18–7.09 (m, 2H, Ar), 7.28 (dd, J = 1.9, 8.5 Hz, 1H, Ar), 7.61–7.57 (m, 1H, Ar), 7.73–7.65 (m, 2H, Ar); **^{13}C NMR** (75 MHz, $CDCl_3$) δ 27.0, 39.4, 53.1, 55.2, 105.5, 119.0, 127.1, 127.6, 127.8, 128.8, 129.1, 132.1, 133.1, 157.6, 201.3; **IR** (ATR) ν 3007, 2962, 2907, 2838, 1709, 1605, 1506, 1486, 1359, 1263, 1221, 1163, 1030, 870, 851, 816, 757 cm^{-1}; **MS** (APCI) m/z (%) 306.6 ($[M+H]^+$, 100), 290.6 (85), 288.5 (68), 242.1 (16), 228.2 (23), 198.1 (35).

2.2.2. 4-(6-Methoxy-2-naphthyl)-3-hydroxybutan-2-one

A solution of 3-bromo-4-(6-methoxy-2-naphthyl)butan-2-one (120 mg, 0.39 mmol) in THF (5 ml) was added into a round bottom flask loaded with silver perchlorate monohydrate (106 mg, 0.47 mmol). Water (1 ml) was subsequently added, and the resultant mixture stirred at 50 °C for 72 h. The mixture was diluted with ethyl acetate and washed with brine (3× 10 ml). The organic phase was dried over anhydrous Na_2SO_4, and concentrated under vacuum. The crude product was purified on silica gel with a mixture of petrolether and ethyl acetate (8:2) as a mobile phase to yield the title compound as a yellowish amorphous solid (34 mg, 35% yield): **^{1}H NMR** (300 MHz, $CDCl_3$) δ 2.21 (s, 3H, CH_3), 3.00 (dd, J = 7.1, 14.1 Hz, 1H, CH_2), 3.26 (dd, J = 4.5, 14.1 Hz, 1H, CH_2), 3.90 (s, 3H, OCH_3), 4.48 (dd, J = 4.5, 7.1 Hz, 1H, OCH), 7.18–7.08 (m, 2H, Ar), 7.37–7.31 (m, 1H, Ar), 7.76–7.58 (m, 3H); **^{13}C NMR** (75 MHz, $CDCl_3$) δ 25.9, 39.9, 55.2, 77.7, 105.6, 118.9, 127.0, 127.7, 127.8, 127.9, 129.0, 131.5, 133.5, 157.5, 209.2; **IR** ν 3383, 3010, 2940, 1694, 1606, 1488, 1393, 1358, 1268, 1231, 1173, 1085, 1030, 854, 818, 688, 661 cm^{-1}; **MS** (APCI) m/z (%) 244.9 ($[M+H]^+$, 100), 229.8 (34), 228.8 (48), 226.9 (73), 198.2 (25).

2.3. Preparation of liver microsomal and cytosolic fractions

Male laboratory rats (*Rattus norvegicus*, Wistar, 16–18 weeks old) were obtained from BioTest (Konárovice, Czech Republic). They were kept on a standard rat diet with free access to tap water, in animal quarters under a 12-h light–dark cycle. The rats were treated in accordance with the *Guide for the care and use of laboratory animals* (Protection of Animals from Cruelty Act. No. 246/92, Czech Republic).

Human liver samples were obtained from the livers excluded from transplantation for medical reasons (Cadaver Donor Programme of the Transplant Centre of the Faculty of Medicine, Charles University, Hradec Králové, Czech Republic), and were used in compliance with the National Ethic Laws.

Frozen liver (both rat and human) was thawed at ambient temperature within 15 min, and processed individually to furnish microsomal and cytosolic fractions. The liver was homogenized using Potter–Elvehjem homogenizer with 0.1 M sodium phosphate buffer, pH 7.4, at the w/v ratio of 1:6. The subcellular fractions were isolated by differential centrifugation of the tissue homogenate in the same buffer. The first centrifugation (5000 × *g*, 20 min) removed un-homogenized pieces and the nuclei. The supernatant was further centrifuged (20,000 × *g*, 60 min), the pellet corresponding to the mitochondrial fraction was discarded, and the second supernatant ultracentrifuged at 105,000 × *g* for 60 min; the resultant pellet and the supernatant represented the microsomes and the cytosol, respectively. A re-washing step (followed by the second ultracentrifugation) was included at the end of the microsome preparation procedure. The pellets of the microsomes were resuspended in a phosphate buffer containing 20% glycerol (v/v) and both the microsomes and the cytosol were stored at −80 °C. Protein concentrations were assayed using the bicinchoninic acid method (see [16]).

2.4. Incubation of 3-hydroxy nabumetone and nabumetone with rat and human liver cytosolic and microsomal fractions (in vitro biotransformation of nabumetone and 3-hydroxy nabumetone)

In vitro biotransformation of the substrates was performed in 1.5 ml Eppendorf tubes. Three microlitres of 10 mM substrate (3-hydroxy nabumetone or nabumetone for comparison) in DMSO were mixed with 97 μl of 0.1 M phosphate buffer of pH = 7.4. One hundred microlitres of 3 mM NADPH in redistilled water and 100 μl of rat (or human) microsomes (or cytosol) were added. All the above described experiments were made in triplicate. For comparison, biological blank samples (3 μl of DMSO, 97 μl of 0.1 M phosphate buffer of pH = 7.4, 100 μl of aqueous 3 mM NADPH and 100 μl of a rat or human subcellular fraction) and chemical blank samples (3 μl of a substrate in DMSO, 197 μl of 0.1 M phosphate buffer of pH = 7.4,

100 μl of aqueous 3 mM NADPH and no subcellular fraction) were prepared in duplicate.

The samples in the closed Eppendorf tubes were shaken over a 60 min incubation at 37 °C. The incubation was terminated by cooling down to 0 °C and by the addition of 15 μl of 35% hydrochloric acid.

2.5. Preparation of isolated rat hepatocytes and their incubation with 3-hydroxy nabumetone and nabumetone

The hepatocytes were obtained from the liver of two male rats by the two-step collagenase method [17], and the isolated hepatocytes were mixed. Three million of viable (83%) cells in 3 ml of the culture medium ISOM (1:1 mixture of Ham F12 and Williams' E) were placed into 60-mm plastic dishes pre-coated with collagen. To facilitate cell attachment within a 4 h period, foetal calf serum was added in the culture medium (5%). Following the attachment of the hepatocytes, the ISOM medium was replaced with fresh serum-free ISOM medium containing 10 μM nabumetone or 3-hydroxy nabumetone, predissolved in DMSO. The concentration of DMSO in the medium was 0.1% (v/v). The hepatocytes were incubated with the drugs for 24 h at 37 °C in a humid atmosphere of air and 5% CO_2. At the end of the incubation, the medium containing the hepatocytes was collected and stored at −80 °C prior to extraction.

2.6. Sample handling and extraction of the incubated subcellular fractions and isolated hepatocytes

Internal standard (6 μl of 10^{-3} M solution) and diethyl ether (900 μl) were added to an incubated subcellular fraction (300 μl of the microsomes or cytosol, see Section 2.4) and the content of the Eppendorf tubes was shaken intensely for 12 min. The samples were then centrifuged (3000 × *g* for 5 min), and stored at −20 °C for 60 min until the aqueous layer froze to ice. The organic layer was decanted into another clean Eppendorf tube, and the solvent was evaporated (water bath 45 °C, stream of nitrogen). The dry extract in the tube was reconstituted in 600 μl of the mobile phase and transferred into the vial of the autosampler. One hundred microlitres of the sample were injected into the chromatographic column.

For the extracting separation of co-eluted 6-MNA and (−)-3-hydroxy nabumetone enantiomer (see Section 3.2), the following reextraction approach was used: a mixture of the incubated rat hepatocytes or microsomes (1 ml) and 0.1 M phosphate buffer (pH = 3, 1 ml) was spiked by the internal standard (6 μl of 10^{-3} M solution). Diethyl ether (3 ml) was added and the analytes were extracted from the diluted biomatrix (vigorous shaking for 12 min). The samples were centrifuged (3000 × *g* for 5 min), and stored at −20 °C for 60 min until the aqueous layer froze to ice. The organic layer containing the analytes was decanted into another clean tube, and the solvent was evaporated (water bath 45 °C, stream of nitrogen). One millilitre of 0.1 M phosphate buffer (pH = 10) and 3 ml of diethyl ether were added to the dry extract in the tube (containing both acidic and neutral nabumetone derivatives), the content of the tube was again vigorously shaken and then centrifuged, followed by the separation of organic and aqueous layers. The organic layer contained only neutral analytes (including 3-hydroxy nabumetone), whereas 6-MNA was reextracted in the form of the sodium salt into the aqueous layer with sodium phosphate buffer of pH = 10. After the adjustment of pH of the aqueous layer to 3 (addition of 150 μl of 1 M HCl), diethyl ether (3 ml) and the internal standard (6 μl of 10^{-3} M) were added, and 6-MNA was transferred into organic layer as a free acid. Ethereal layers containing both neutral (3-hydroxy nabumetone *etc.*) and acidic metabolites (6-MNA *etc.*) were evaporated to dryness. The dry extracts in the tubes were reconstituted in 1 ml of the mobile phase and transferred into the vial of the autosampler. One hundred microlitres of the sample was injected into the chromatographic column.

2.7. Chiral and achiral HPLC with ultraviolet photodiode-array detector

Chromatographic analyses were performed using a Thermo Electron chromatograph (San Jose, CA, USA), composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100-μl sample loop, UV6000 LP photodiode array detector (UV-PDA) with Light Pipe Technology, SN4000 system controller and a PC data station (Pentium Dual-Core CPU E5300 @ 260 GHz, 3.49 GB of RAM, HDD 500 GB) equipped with the ChromQuest-4.0 software (Thermo Electron, San Jose, CA, USA) for data evaluation.

A Daicel 250 mm × 4.6 mm chromatographic column packed with Chiralcel OD-R (cellulose tris(3,5-dimethylphenyl carbamate) coated on 10 μm silica gel spheric particles, Daicel, Japan) was used for the chiral chromatography of 6-HNA, two enantiomers of 6-HO-nb—OH, 6-HO-nb=O, 6-MNA, two enantiomers of 3-hydroxy nabumetone, two enantiomers of 6-MeO-nb—OH, methyl naproxen and nabumetone (Fig. 3). The following mobile phases for the chiral separations were tested during method development:

mobile phase A: methanol – 1 M aqueous $NaClO_4$, pH = 3 (75:25, v/v),
mobile phase B: methanol – 1 M aqueous $NaClO_4$, pH = 6.85 (75:25, v/v), flow rate was 0.5 ml/min (5–6 MPa). Chromatographic analyses using the mobile phase A and B lasted for less then 60 min.

Chromatographic conditions for achiral HPLC method were described in our previous paper [3]. The analysis of nabumetone, its six metabolites and naproxen (IS) lasted for 12 min (Fig. 4).

UV detection was performed in single wavelength mode (265 nm). For the collection of the UV spectra, a photodiode array scan mode (in the range 195–385 nm with a 1 nm distance) was employed.

2.8. HPLC with polarimetric and CD-detectors, circular dichroism spectra of 3-hydroxy nabumetone

Circular Dichroism (CD, a Jasco J-710 spectropolarimeter) with a flow cell for the LC-coupling (LCCD-311) connected to a Jasco HPLC System composed of a DG-980-50 degasser and a PU-980 intelligent HPLC-pump, was employed to collect the CD-spectra of 3-hydroxy nabumetone.

The CD-spectra were recorded at room temperature using quartz cells with path length of 5 mm, under the following conditions: scan rate (speed) 20 nm/min, bandwidth 1 nm, response 1 s, step resolution 0.5 nm, accumulation 3 and wavelength range 180–400 nm. The spectra are average computed over three instrumental scans and the intensities are presented as ellipticity values (mdeg).

The conditions of the chiral chromatography are described in Section 2.7.

2.9. Achiral UHPLC coupled with MS/MS detector

Individual samples were quantitatively dissolved in a mixture of acetonitrile – water (45:55, v/v). UHPLC–MS/MS chromatograms of the samples were measured in both positive and negative polarity modes using electrospray ionization (ESI) and hybrid quadrupole-time-of-flight (QqTOF) mass analyzer (micrOTOF-Q, Bruker Daltonics, Germany). UHPLC was performed on an Agilent 1290 infinity liquid chromatograph (Agilent Technology, Waldbronn, Germany) using Kinetex C18 column 150 × 2.1 mm, 1.7 μm

(Phenomenex, Torrance, CA, USA), temperature 25 °C, flow rate 0.3 ml/min and the injection volume 1 μl. The separation was performed in the isocratic mode. The mobile phase was composed of acetonitrile (45%) and water (55%), both with the addition of 0.1% acetic acid. The QqTOF mass spectrometer was used with the following setting of the tuning parameters: capillary voltage 4.5 kV, drying temperature 220 °C, the flow rate and pressure of nitrogen were 8 l/min and 1.3 bar, respectively. The external calibration was performed with sodium formate clusters before individual measurements. ESI mass spectra were recorded in the range of m/z 50 – 800 both in positive – and negative-ion modes. The isolation width of $\Delta m/z$ 4 and the collision energy of 25 eV using argon as the collision gas were used in the MS/MS experiments.

3. Results and discussion

3.1. Relevance of in vitro drug metabolism experiments in the identification of new nabumetone metabolites

Oxidative conversion of nabumetone to 6-methoxy-2-naphthylacetic acid (6-MNA) is accompanied by the loss of two carbon atoms. Therefore, the existence of further metabolite(s) is certain. Probably because of a fast biotransformation to 6-MNA, the intermediate(s) have not been detected either in the blood plasma or urine so far.

To detect the above-mentioned intermediate(s), HPLC analyses of the extracts from the subcellular fractions incubated with nabumetone were necessary.

Subcellular fractions are available by the sequential fractional centrifugation of the homogenate from human, rat and other liver tissues. In the last step at 105,000 × g, the pellets of the microsomal fraction containing mainly oxidative cytochrome P450 (CYP) enzymes were obtained, whereas the supernatant (cytosol) contained predominantly reductive enzymes.

The preparation of the microsomal fraction enables preferential participation of the oxidative enzymes located on the smooth endoplasmatic reticulum in the liver cells. Following the fractional centrifugation of the liver homogenate, these oxidases are embedded in the tattered vesicles of the smooth endoplasmatic reticulum (microsomal fraction).

Due to some undisputable advantages of the *in vitro* biotransformation experiments (see Sections 2.3–2.5), their execution prior to *in vivo* experiments is often useful. A lower consumption of the substrate, a better modulation of the experimental conditions (temperature, anaerobic/aerobic conditions, selective additions of suitable coenzymes, substrates, inhibitors) are typical attributes of the *in vitro* biotransformation experiments. In addition, only specific components of the cells exhibiting a biotransformating activity can be selected for the *in vitro* experiments (isolated enzymes, microsomal or cytosolic fractions, mitochondrial fractions). Alternatively, the whole isolated hepatocytes can be used. These *in vitro* approaches increase the chance to capture an unstable metabolite or intermediate.

In the analytical part of the study, HPLC analyses of the extracts from the *in vitro* biomatrix provide more simple chromatograms with fewer interfering peaks.

3.2. Identification of new nabumetone metabolite via chiral and achiral HPLC–PDA–(MS/MS)

When nabumetone was incubated with the microsomal fraction of rat and human liver tissues, the chromatographic analyses of the extracts from microsomes revealed the presence of a new, as yet undescribed, nabumetone metabolite with the retention time t_R = 27.5 min (see the peak denoted as "unknown 1, 2" in Fig. 2).

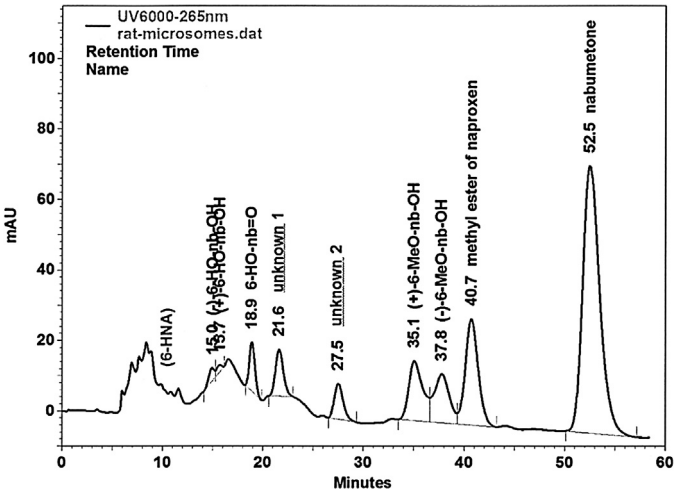


Fig. 2. Chiral chromatogram of the extract from rat liver microsomes incubated with nabumetone. The peaks t_R = 21.6–22 min and t_R = 27.5–28.6 min denoted as "unknown 1" and "unknown 2" were identified as the enantiomers of 3-hydroxy nabumetone (compare chromatograms in Fig. 3 and CD-spectra in Fig. 5).

As this nabumetone metabolite was found in the extracts from the microsomal fraction but not in the extracts from cytosolic fractions, a product of nabumetone oxidation, probably hydroxy nabumetone (HO-nabumetone) was anticipated.

The newly detected metabolite had retention time different from those of nabumetone and all of its *phase I* metabolites.

Identical UV spectra of new metabolite and all nabumetone derivatives ruled out the presence of a phenolic hydroxy group in the aromatic moiety of nabumetone. An auxochromic hydroxy group attached to the naphthalene ring would alter both the wavelength maxima and intensity of absorption of the naphthalene moiety. However, no bathochromic shift was observed in the UV spectrum of new metabolite, hence, the oxidative biotransformation took place on the aliphatic chain of the drug.

Nabumetone oxidation leading to newly found metabolite must have been performed on the aliphatic chain of the molecule, with C1, C3 and C4 being available. The detection of two separable HO-nabumetone enantiomers (see Section 3.4) in the chiral chromatograms (see Fig. 3) excluded hydroxylation at C1 (1-HO-nabumetone is achiral). Because HO-nabumetone was found to

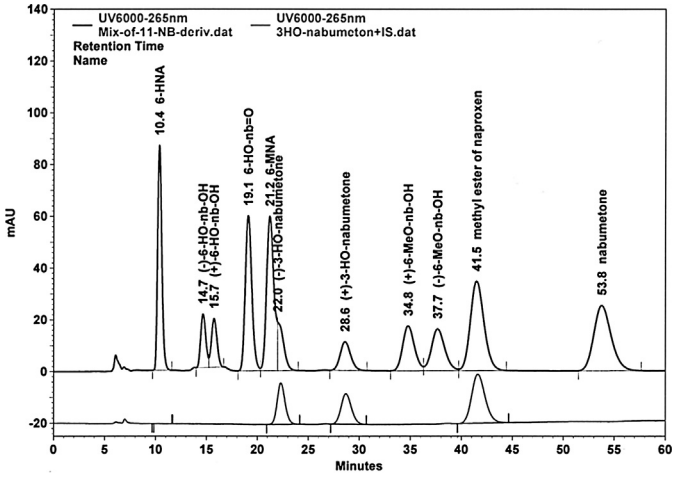


Fig. 3. Chiral separation of the standard mixture of nabumetone and its derivatives (see upper black chromatogram and Fig. 1) compared with the separation of racemic 3-hydroxy nabumetone (enantiomers with t_R = 22 min and t_R = 28.6 min) spiked with internal standard with t_R = 41.5 min (see lower grey chromatogram).

Table 1
List of the main peaks for 3-OH nabumetone biotransformation samples (HM = human microsomes; RM = rat microsomes; RH = rat hepatocytes) detected by UHPLC/MS/MS with their retention times, observed ions using ESI in both polarity modes, molecular weights, elemental composition and description of present metabolites (—CH_2 = demethylation; +H_2 = carbonyl reduction; +$C_6H_8O_6$ = glucuronidation).

t_R (min)	Important observed ions		MW	Elemental composition	Description of metabolite formation	Biotransformation sample
	ESI^+ MS	ESI^- MS				
1.5	*m/z* 255 $[M+Na]^+$ *m/z* 233 $[M+H]^+$ *m/z* 157	*m/z* 231 $[M-H]^-$	232	$C_{14}H_{16}O_3$	3-OH-nabumetone + H_2 − CH_2	HM, RM
1.6	*m/z* 459 $[M+K]^+$ *m/z* 443 $[M+Na]^+$ *m/z* 438 $[M+NH_4]^+$ *m/z* 245	*m/z* 419 $[M-H]^-$	420	$C_{21}H_{24}O_9$	3-OH-nabumetone + $C_6H_8O_6$	RH
1.9	*m/z* 253 $[M+Na]^+$ *m/z* 231 $[M+H]^+$ *m/z* 213 $[M+H-H_2O]^+$ *m/z* 157	*m/z* 229 $[M-H]^-$	230	$C_{14}H_{14}O_3$	3-OH-nabumetone − CH_2	HM, RM
2.4	*m/z* 269 $[M+Na]^+$ *m/z* 247 $[M+H]^+$ *m/z* 229 $[M+H-H_2O]^+$ *m/z* 211 $[M+H-2H_2O]^+$ *m/z* 171	–	246	$C_{15}H_{18}O_3$	3-OH-nabumetone + H_2	HM, RM, RH
2.6	*m/z* 269 $[M+Na]^+$ *m/z* 247 $[M+H]^+$ *m/z* 229 $[M+H-H_2O]^+$ *m/z* 211 $[M+H-2H_2O]^+$ *m/z* 171	–	246	$C_{15}H_{18}O_3$	3-OH-nabumetone + H_2	HM, RM, RH
3.3	*m/z* 239 $[M+Na]^+$ *m/z* 217 $[M+H]^+$ *m/z* 171	*m/z* 215 $[M-H]^-$ *m/z* 171	216	$C_{13}H_{12}O_3$	6-MNA	RH
3.6	*m/z* 267 $[M+Na]^+$ *m/z* 245 $[M+H]^+$ *m/z* 227 $[M+H-H_2O]^+$ *m/z* 171	–	244	$C_{15}H_{16}O_3$	3-OH-nabumetone	HM, RM, RH

undergo metabolic conversion to 6-methoxy-2-naphthylacetic acid (6-MNA), the structure of which preserves the C4 methylene group intact, C3 was almost certainly the hydroxylation site.

The UHPLC/ESI-MS/MS analyses of the extracts from nabumetone incubation with the microsomal fraction revealed the presence of a structure corresponding to elemental composition **$C_{15}H_{16}O_3$** (see Table 1). Characteristic fragments (see Section 3.5) confirmed the oxidation of the aliphatic chain. Furthermore, the ultimate formation of 6-MNA from nabumetone requires that the C2—C3 bond be cleaved. Thus, the structure of newly found metabolite was confirmed as **4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one** (*i.e.* **3-HO-nabumetone**).

A synthetic standard of racemic 3-hydroxy nabumetone was prepared (see Section 2.2. for experimentals, and Section 3.3 for discussion) to corroborate the proposed structure and to enable further xenobiochemical and pharmacokinetic studies. 3-Hydroxy nabumetone is a chiral compound; the chromatographic behaviour of its enantiomers is apparent from Fig. 3.

Chiral separation of all nabumetone derivatives (see Fig. 1) under the experimental conditions mentioned in Section 2.7 (Chiralcel OD-R column, **mobile phase A**) is demonstrated in Fig. 3 (upper chromatogram). The identification of both 3-HO-nabumetone enantiomers is described in Section 3.4. As apparent from the chromatograms in Fig. 3, the above mentioned "unknown 2" nabumetone metabolite (t_R = 27.5 min in Fig. 2) was identified as (+)-3-hydroxy nabumetone. The second enantiomer, (−)-3-hydroxy nabumetone (t_R = 22 min, denoted as "unknown 1" in Fig. 2) was partly co-eluted with 6-MNA. Fortunately, these two metabolites possess different acidobasic properties, which enables their facile separation. While (−)-3-hydroxy nabumetone is neutral, 6-MNA is acidic and therefore well ionizable in basic media. Two approaches were used to separate these two co-eluting nabumetone metabolites.

Approach 1: When chiral **mobile phase B** [methanol – 1 M aqueous $NaClO_4$, pH = 6.85 (75:25, v/v)] was used instead of the routinely employed acidic **mobile phase A** (see Section 2.7), 6-MNA was ionized, and its polar form had a shorter retention time than that of (−)-3-hydroxy nabumetone, the retention characteristics of which in both mobile phases (A and B) remained practically the same.

Approach 2: pH-dependent extraction and reextraction of the biomatrix (see the last paragraph of Section 2.6).

Achiral separation of six *phase I* metabolites, the parent nabumetone and naproxen within 12 min is displayed in Fig. 4. The newly found metabolite, 3-hydroxy nabumetone (t_R = 3.9 min), was

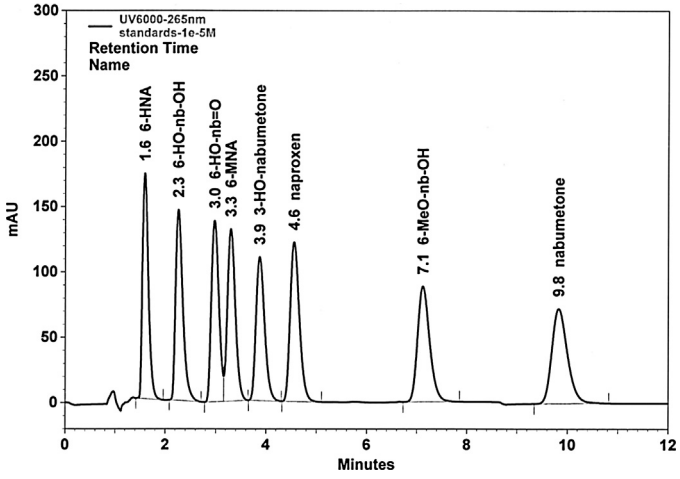


Fig. 4. Achiral chromatogram of standard mixture 3-HO nabumetone (t_R = 3.9 min) and the other nabumetone metabolites under conditions mentioned in [3]. Naproxen was used as an internal standard.

well separated from the two nearest biotransformation products (6-MNA and naproxen). Further metabolism of 3-hydroxy nabumetone (*via* carbonyl reduction and *O*-desmethylation) was also anticipated. As evident from the chromatogram in Fig. 4, the peak capacity in the time interval 1.4–3.9 min is limited, and (as yet uncovered) potential metabolites of 3-hydroxy nabumetone together with those in the chromatogram (Fig. 4) could have been insufficiently separated under the chromatographic conditions described in Section 2.7 and in our previous paper [3]. For this reason, UHPLC–MS/MS was employed in the search for further metabolites derived from 3-hydroxy nabumetone (see Sections 2.9 and 3.5).

3.3. Synthesis of 3-hydroxy-nabumetone standard

Standard procedures were employed to prepare 3-hydroxy nabumetone (see Section 2.2). Thus, α-bromination of nabumetone proceeded *via* the thermodynamically more stable enolate thereby furnishing 3-bromo-4-(6-methoxy-2-naphthyl)butan-2-one, which was converted into the title compound *via* silver-assisted hydrolysis in an aqueous medium.

Assignments of signals were included in the description of the NMR spectra (see Sections 2.2.1 and 2.2.2). Compared to the parent nabumetone, the signal of a —C**H$_2$**— group adjacent to carbonyl (C3) disappeared from the ^{1}H NMR spectrum of 3-bromo-4-(6-methoxy-2-naphthyl)butan-2-one obtained after the treatment of nabumetone with pyridinium tribromide. A new triplet of a methine proton at 4.55 ppm in the spectrum of the compound clearly indicated bromination at C3. Following the treatment of the bromo ketone with H_2O in the presence of Ag^+ ions, the replacement of bromine by an OH group was evident from the change of both chemical shift and multiplicity of this —C**H**— proton, which moved to 4.48 ppm and changed into doublet of doublets. ^{13}C NMR, IR and MS spectra were fully in agreement with this assignment.

3.4. Identification of (+)- and (−)-enantiomers of 3-hydroxy-nabumetone using chiral HPLC with CD and polarimetric detectors

Positive experiences with polarimetric identification of the enantiomers of a drug *phase I* metabolite were recorded in our previous papers [18–20].

However, circular dichroism spectroscopy (CD) is the technique of choice for the determination of stereochemistry of chiral drugs, and may well be coupled with chiral columns to verify the elution order and to measure the spectra of the compounds. Selectivity and short response time also make CD an excellent detector for HPLC [21].

Fig. 5 shows the CD-spectra of the isolated enantiomers of 3-hydroxy nabumetone. To interpret the CD-spectra, the range between 325 and 235 nm was selected, because of being the lowest wavelength which would show little interference with the solvents used throughout this study (UV cut-off for water: 190 nm, for methanol: 220 nm) and with $NaClO_4$.

Since positive chirality gives the first CD Cotton effect positive and the second one negative, while negative chirality gives rise to a reversed order of the Cotton effects [22], the CD-spectra in Fig. 5 indicate that the elution order of the enantiomers is (−,+).

As shown in Fig. 5, the CD spectra of the two eluted peaks were mirror images of each other, thus confirming their enantiomeric nature.

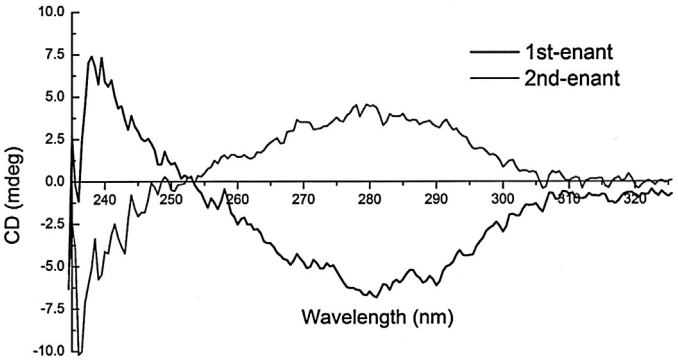


Fig. 5. CD-spectra of (−)-3-hydroxy nabumetone (1st-enantiomer, t_R = 22 min) and (+)-3-hydroxy nabumetone (2nd-enantiomer, t_R = 27.5–28.6 min).

3.5. UHPLC–MS/MS identification of 3-hydroxy nabumetone metabolites

The analytical characterization of 3-hydroxy nabumetone and its metabolites in the samples (see Section 2.9) was performed using UHPLC/ESI-MS/MS. The co-elution of both 3-hydroxy nabumetone enatiomers was observed in the achiral chromatographic system. However, the subsequent carbonyl reduction resulted in the formation of two diastereoisomers, which were separated on a C18 column (see Table 1). Emphasis was given on the accurate calibration of the mass scale to achieve a high mass accuracy (typically better than 1 mDa) and reproducible results, allowing the determination of the exact *m*/*z* values of the observed ions as well as providing information about the elemental composition of the metabolites. The combination of different UHPLC/MS/MS scans, such as the reconstructed ion current chromatograms, constant neutral loss chromatograms or exact mass filtration scans, were used for the unambiguous detection and identification of individual metabolites. To eliminate falsely positive results, chromatograms from *in vitro* biotransformation were compared with those from the chemical and biological blank samples. Altogether, five *phase I* metabolites arising from oxidation and reduction reactions were identified using UHPLC/MS/MS in both ESI polarity modes. The demethylation, carbonyl reduction and their combinations were among the metabolic pathways observed in the rat and human microsomes. On the other hand, the formation of 6-MNA as well 3-hydroxy nabumetone glucuronide was the major metabolic process in the rat hepatocytes (see Table 1). Protonated molecules together with sodium adducts were the most significant ions observed in the positive-ion full scan mass spectra for all metabolites, while a signal in the negative-ion polarity was provided only by two demethylated metabolites eluted at 1.5 and 1.9 min, glucuronide and 6-MNA, as evident from Table 1. The demethylated metabolites could also be identified on the basis of the high mass accuracy measurement in the full scan mass spectra, because the demethylation can occur only for aromatic methoxy groups. Tandem mass spectra for 3-hydroxy nabumetone and two selected metabolites are shown in Fig. 6. In general, characteristic fragment ions were formed by the cleavage of the side chain between the C3 and C4 carbon atom attached to the aromatic ring, yielding the ion at *m*/*z* 171 (*m*/*z* 157 for the *O*-demethylated metabolites), as published in our previous work [3].

3.6. Concluding comments on the newly found nabumetone biotransformation pathways

A survey of nabumetone *phase I* biotransformation involving the newly found metabolic structures is shown in Fig. 7. The new metabolites were discovered using achiral and chiral

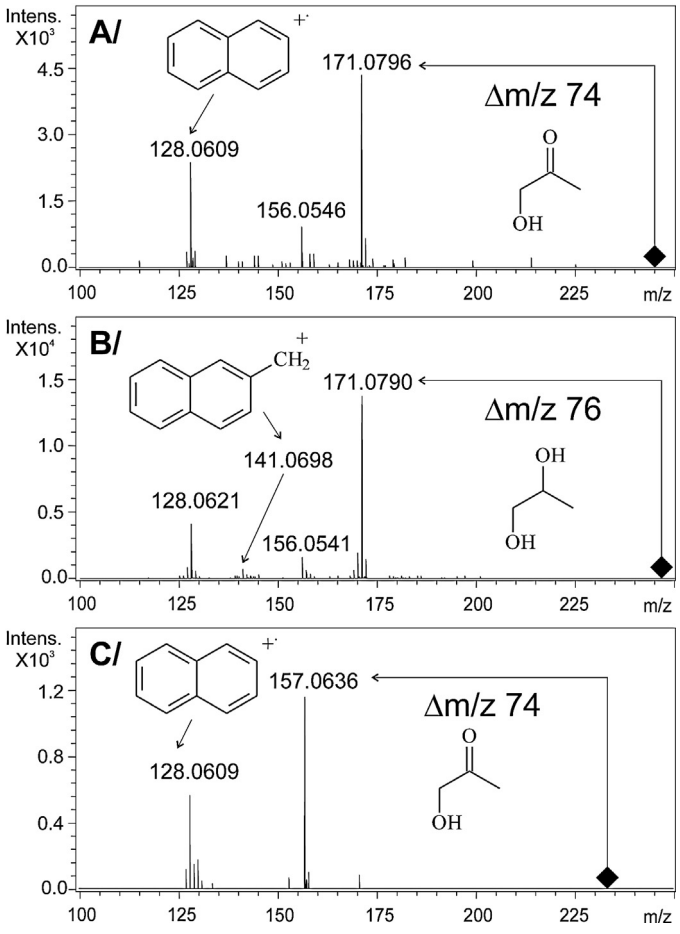


Fig. 6. Positive-ion ESI-MS/MS spectra of protonated (A) 3-HO-nabumetone, (B) reduced 3-HO-nabumetone and (C) *O*-demethylated 3-HO-nabumetone.

HPLC–PDA, HPLC–CD and UHPLC–MS/MS methods. Liquid–liquid extraction and liquid–liquid reextraction were employed in biomatrix handling. The *in vitro* biotransformation experiments (*i.e.* the incubation of nabumetone or 3-hydroxy nabumetone with the liver microsomal fraction and the isolated hepatocytes) were crucial for the detection of new, as yet undescribed, nabumetone metabolites (3-hydroxy nabumetone, its *O*-desmethyl derivative and those with reduced carbonyl). These experiments uncovered an important relationship between 3-hydroxy nabumetone and 6-MNA.

Following the incubation of nabumetone with the liver microsomal fraction, 3-hydroxy nabumetone, its *O*-desmethyl and carbonyl reduced metabolites, but no 6-MNA were detected in this biomatrix.

On the other hand, when nabumetone was incubated with the isolated hepatocytes, 6-MNA was formed as the principal metabolite. This observation supports our hypothesis that 3-hydroxy nabumetone is an unstable metabolic intermediate which undergoes fast conversion to 6-MNA in the liver cells.

To verify this hypothesis, 3-hydroxy nabumetone was incubated with freshly isolated hepatocytes. In the extracts from the hepatocytes, 6-MNA was detected as the principal metabolite, together with the *O*-glucuronide of 3-hydroxy nabumetone and low concentrations of the reduced forms of 3-hydroxy nabumetone (see Table 1). Hence, 3-hydroxy nabumetone must be an intermediate between nabumetone and 6-MNA.

The above-mentioned experiments have proven that the microsomes contain the cytochromes responsible for the oxidation of nabumetone to 3-hydroxy nabumetone. However, further oxidative cleavage of 3-hydroxy nabumetone to 6-MNA was observed only in the isolated hepatocytes and not in the microsomal fraction. Therefore, this oxidative process is likely to take place in other parts of the liver cells than the smooth endoplasmic reticulum (for example in mitochondria). The exact chemism of these two processes as well as their enzymology will be subjected to a more detailed investigation in order to find the reticular CYP-450(s) responsible for the

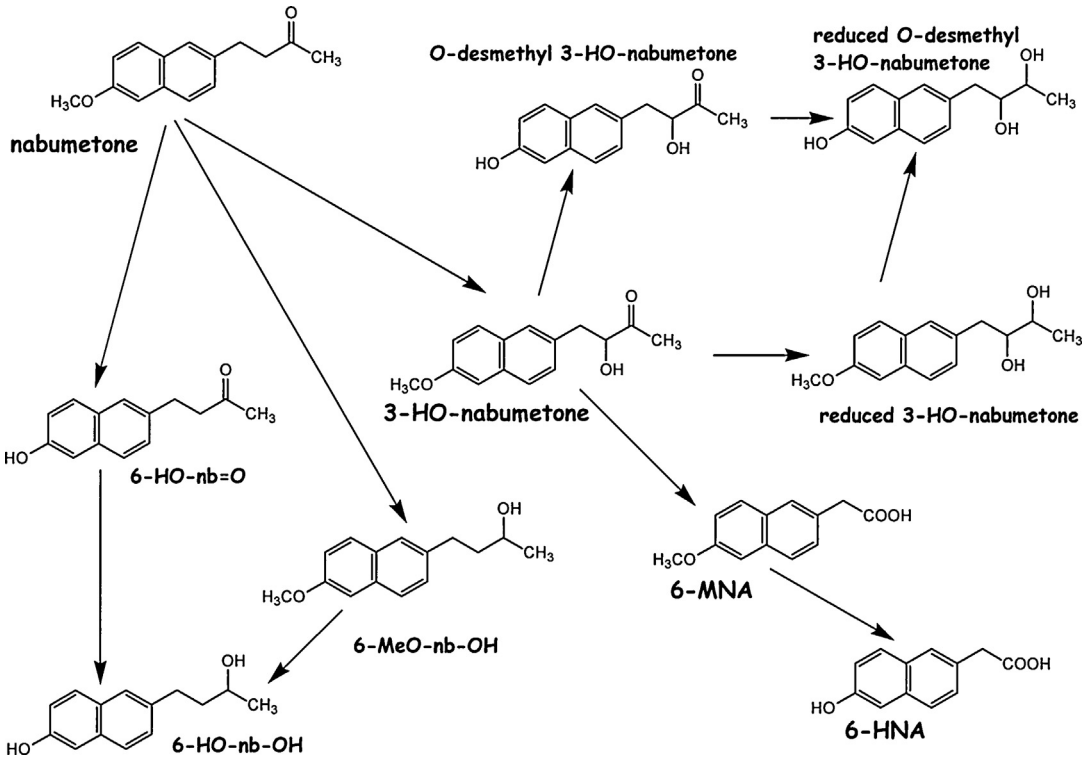


Fig. 7. A survey of nabumetone *phase I* biotransformation including the newly found metabolic structures.

oxidation of nabumetone to 3-hydroxy nabumetone, and to characterize the cellular location of the enzyme(s) converting the latter to 6-MNA.

The structures of 3-hydroxy nabumetone (*4-(6-methoxy-2-naphthyl)-3-hydroxybutan-2-one*) and reduced 3-hydroxy nabumetone (*4-(6-methoxy-2-naphthyl)-butan-2,3-diol*) were registered in a US patent [23] as the compounds exhibited a weak anti-inflammatory activity. However, the authors almost certainly lacked the information that these compounds are formed *via* metabolism of nabumetone, and that their antiinflammatory activity is a result of their biotransformation to 6-MNA. The reduced 3-hydroxy nabumetone was found to be enzymaticaly reoxidizable to 3-hydroxy nabumetone and further on to 6-MNA.

4. Conclusion

A combination of purposefully chosen xenobiochemical experiments with nabumetone and its derivatives *in vitro*, and the use of the hyphenated chromatographic techniques (LLE–HPLC–PDA, HPLC–CD, LLE–UHPLC–MS/MS) applied to the analyses of the extracts from biomatrices enabled the disclosure of new nabumetone metabolites (3-hydroxy nabumetone, its derivative with reduced carbonyl, its *O*-desmethylated derivative and *O*-glucuronide) which are reported for the first time. The standard of racemic 3-hydroxy nabumetone was synthesized for more detailed analytical and ADME studies. The chromatographic behaviour in chiral and achiral HPLC methods, ultraviolet (PDA), circular dichroism (CD) and ESI-MS/MS spectra of the synthetic standards and authentic metabolites extracted from the biomatrices were compared, and the identities of new nabumetone *phase I* and *phase II* metabolites confirmed.

The above described bioanalytical results have important xenobiochemical implications. 3-Hydroxy nabumetone was found in the extracts from the rat and human liver microsomal fraction. Hence, CYP-450s located on the smooth endoplasmic reticulum (ER) of the liver cells are probably the enzymes responsible for the oxidation of nabumetone to 3-hydroxy nabumetone in both species tested. No 6-MNA was detected in the extracts from the liver microsomes, but high concentrations of this compound were found in the rat isolated hepatocytes following their incubation with both nabumetone or racemic 3-hydroxy nabumetone. The subsequent fast conversion of 3-hydroxy nabumetone to the anti-inflammatory active 6-MNA therefore results from the enzymatic activity located in liver cell organelles other than the smooth ER. 3-Hydroxy nabumetone was found to be an intermediate in the conversion of nabumetone to 6-MNA. The racemic 3-hydroxy nabumetone or one of its enantiomers could be considered as an alternative non-steroidal antiinflammatory prodrug instead of the lipophilic nabumetone.

Acknowledgements

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References

[1] T. Hedner, O. Samulesson, P. Währborg, H. Wadenvik, K.-A. Ung, A. Ekbom, Nabumetone – therapeutic use and safety profile in the management of osteoarthritis and rheumatoid arthritis, Drugs 64 (2004) 2315–2343.
[2] R.E. Haddock, D.J. Jeffery, J.A. Lloyd, A.R. Thawley, Metabolism of nabumetone (BRL-14777) by various species including man, Xenobiotica 14 (1984) 327–337.
[3] M. Nobilis, J. Kopecký, J. Květina, Z. Svoboda, M. Pour, J. Kuneš, M. Holčapek, L. Kolářová, Comparative biotransformation and disposition studies of nabumetone in humans and minipigs using high-performance liquid chromatography with ultraviolet, fluorescence and mass spectrometric detection, J. Pharm. Biomed. Anal. 32 (2003) 641–656.
[4] M. Nobilis, M. Holčapek, L. Kolářová, J. Kopecký, M. Kuneš, Z. Svoboda, J. Květina, Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection, J. Chromatogr. A 1031 (2004) 229–236.
[5] Y. Altun, B. Dogan, S.A. Ozkan, B. Uslu, Development and validation of voltammetric techniques for nabumetone in pharmaceutical dosage form, human serum and urine, Acta Chim. Slov. 54 (2007) 287–294.
[6] J.A.M. Pulgarin, A.A. Molina, I.S.F. Robles, Simple and rapid determination of the active metabolite of nabumetone in biological fluids by heavy atom-induced room temperature phosphorescence, Anal. Chim. Acta 554 (2005) 37–42.
[7] A.R. Rote, S.R. Bhalerao, Development and statistical validation of spectrophotometric methods for the estimation of nabumetone in tablet dosage form, E-J. Chem. 7 (2010) 1463–1467.
[8] M. Starek, S. Laskawski, M. Dabrowska, Identification and quantitative determination of nabumetone in pharmaceutical preparations by TLC-densitometry, JPC-J. Planar Chromatogr.-Mod. TLC 24 (2011) 513–519.
[9] S.V. Gandhi, S.S. Ranher, P.B. Deshpande, D.K. Shah, Simultaneous HPTLC determination of nabumetone and paracetamol in combined tablet dosage form, J. Braz. Chem. Soc. 22 (2011) 1068–1072.
[10] P.K. Sahu, M.M. Annapurna, Analysis of nabumetone in bulk and tablet formulation by a new and validated reverse phase high performance liquid chromatography, E-J. Chem. 6 (2009) S59–S64.
[11] B.N. Patel, N. Sharma, M. Sanyal, A. Prasad, P.S. Shrivastav, High-throughput LC–MS/MS assay for 6-methoxy-2-naphthylacetic acid, an active metabolite of nabumetone in human plasma and its application to bioequivalence study, Biomed. Chromatogr. 22 (2008) 1213–1224.
[12] A. Zerzaňová, P. Císař, J. Klimeš, Comparison of different stationary phases for bioanalytical studies of biologically active compounds, J. Sep. Sci. 29 (2006) 2126–2135.
[13] J.F. Sheen, G.R. Her, Application of pentafluorophenyl hydrazine derivatives to the analysis of nabumetone and testosterone in human plasma by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spektrometry, Anal. Bioanal. Chem. 380 (2004) 891–897.
[14] M. Turpeinen, U. Hofmann, K. Klein, T. Muerdter, M. Schwab, U.M. Zanger, A predominate role of CYP1A2 for the metabolism of nabumetone to the active metabolite, 6-methoxy-2-naphthylacetic acid, in human liver microsomes, Drug Metab. Dispos. 37 (2009) 1017–1024.
[15] K. Matsumoto, E. Nemoto, T. Hasegawa, M. Akimoto, K. Sugibayashi, In vitro characterization of the cytochrome P450 isoforms involved in the metabolism of 6-methoxy-2-napthylacetic acid, an active metabolite of the prodrug nabumetone, Biol. Pharm. Bull. 34 (2011) 734–739.
[16] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[17] M.N. Berry, A.M. Edwards, G.J. Barritt, Isolated hepatocytes preparation, properties and applications, in: R.H. Burdow, P.H. van Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science, Amsterdam, 1991, pp. 15–35.
[18] M. Nobilis, T. Jira, M. Lísa, M. Holčapek, B. Szotáková, J. Lamka, L. Skálová, Achiral and chiral high-performance liquid chromatographic determination of flubendazole and its metabolites in biomatrices using UV photodiode-array and mass spectrometric detection, J. Chromatogr. A 1149 (2007) 112–120.
[19] M. Nobilis, Z. Vybíralová, V. Křížová, V. Kubíček, M. Soukupová, J. Lamka, B. Szotáková, L. Skálová, Sensitive chiral high-performance liquid chromatographic determination of anthelmintic flubendazole and its phase I metabolites in blood plasma using UV photodiode-array and fluorescence detection application to pharmacokinetic studies in sheep, J. Chromatogr. B 876 (2008) 89–96.
[20] R. Kučera, M. Nobilis, L. Skálová, B. Szotáková, P. Císař, T. Jira, J. Klimeš, V. Wsól, Use of chiral liquid chromatography for the evaluation of stereospecificity in the carbonyl reduction of potential benzo[*c*]fluorene antineoplastics benfluron and dimefluron in various species, J. Pharm. Biomed. Anal. 37 (2005) 1049–1057.
[21] C. Bertucci, M. Pistolozzi, A.D. Simone, Circular dichroism in drug discovery and development: an abridged review, Anal. Bioanal. Chem. 398 (2010) 155–166.
[22] N. Harada, K. Nakanishi, Circular Dichroic Spectroscopy Exciton Coupling in Organic Stereochemistry, University Science Books, Mill Valley, CA, 1983.
[23] A.C. Goudie, Naphthalene derivatives, a process for their preparation and their use in anti-inflammatory pharmaceutical compositions, United States Patent 4382959 (10 May 1983).