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Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection

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Abstract

Chromatographic analyses play an important role in the identification and determination of phase I and phase II drug metabolites. While the chemical standards of phase I metabolites are usually available from commercial sources or by various synthetic, degradation or isolation methods, the phase II drug metabolites have usually more complicated structures, their standards are in general inaccessible and their identification and determination require a comprehensive analytical approach involving the use of xenobiochemical methods and the employment of hyphenated analytical techniques. In this work, various high-performance liquid chromatography (HPLC) methods were employed in the evaluation of xenobiochemical experiments leading to the identification and determination of phase II nabumetone metabolites. Optimal conditions for the quantitative enzymatic deconjugation of phase II metabolites were found for the samples of minipig bile, small intestine contents and urine. Comparative HPLC analyses of the samples of above-mentioned biomatrices and of the same biomatrices after their enzymatic treatment using β-glucuronidase and arylsulfatase afforded the qualitative and quantitative information about phase II nabumetone metabolites. Hereby, three principal phase II nabumetone metabolites (ether glucuronides) were discovered in minipig's body fluids and their structures were confirmed using liquid chromatography (LC)–electrospray ionization mass spectrometric (MS) analyses.

Keywords: Nabumetone; Nonsteroidal anti-inflammatory drugs

1. Introduction

Nabumetone (4-(6-methoxy-2-naphthyl)-2-butanone) is a nonacidic, nonsteroidal, anti-inflammatory prodrug, used for the treatment of rheumatic and inflammatory conditions [1–7]. An actual overview of the knowledge of nabumetone pharmacokinetics and biotransformation, as well as of the ways of nabumetone metabolites determination in various biomatrices has been presented in our previous paper [8].

In the above-mentioned communication, a comparison of nabumetone pharmacokinetics and biotransformation in humans and minipigs has been done. The syntheses of five phase I nabumetone metabolites including their nuclear magnetic resonance (NMR) and mass spectrometric (MS) analyses have also been reported. These standards were employed in the development and validation of a bioanalytical method involving liquid–liquid extraction (or alternatively solid-phase extraction) and high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence and MS detection.

The goal of this subsequent study was to develop methods of the identification and determination of phase II nabumetone metabolites, the conjugates of phase I nabumetone metabolites with various endogenous compounds.

2. Experimental

2.1. Chemicals, preparations and materials

Nabumetone ($C_{15}H_{16}O_2$, molecular mass = 228.3 g/mol, Fig. 1, compound 1) was obtained from PRO.MED.CS,

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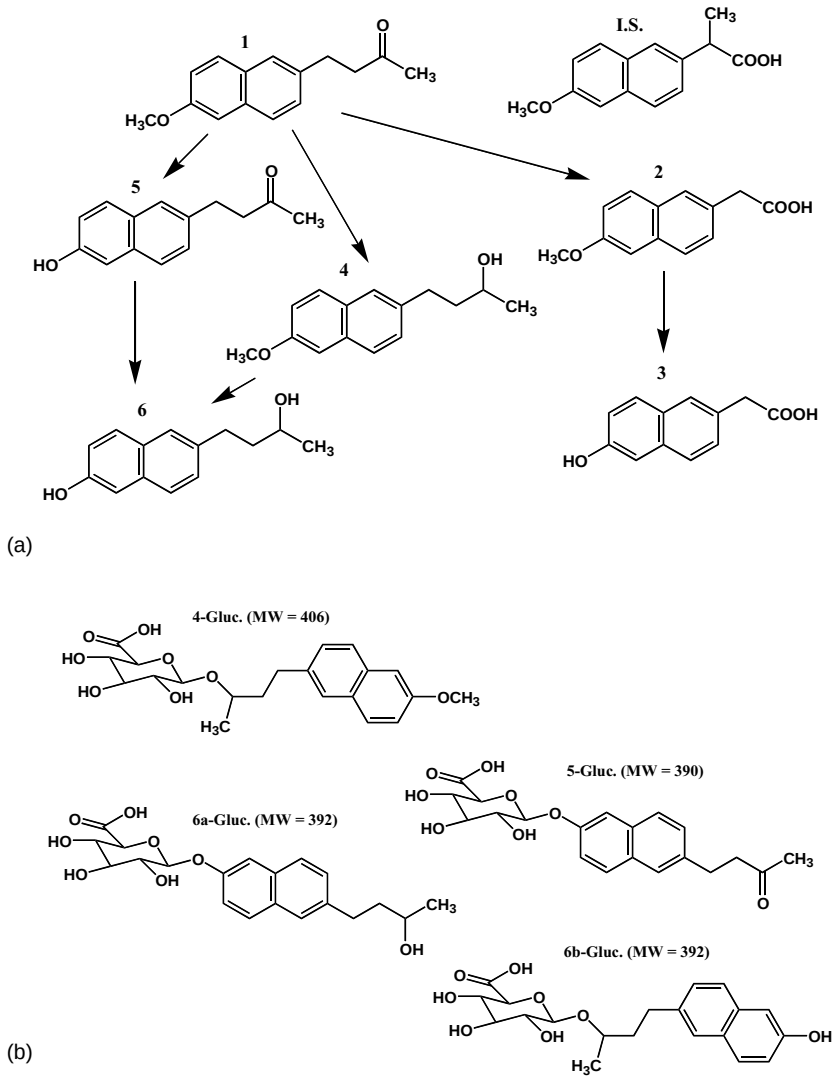

Fig. 1. (a) Chemical structures of nabumetone (1), naproxen (I.S.) and phase I nabumetone metabolites (2 = 6-MNA; 3 = 6-HNA; 4 = 6-MeOnphBu–OH; 5 = 6-HOnphBu=O; 6 = 6-HOnphBu–OH). (b) Chemical structures of the principal phase II nabumetone metabolites found in body fluids.

Prague, Czech Republic; naproxen, (+)-6-methoxy-α-methyl-2-naphthaleneacetic acid ($C_{14}H_{14}O_3$, molecular mass = 230.3 g/mol, I.S. in Fig. 1, Sigma–Aldrich, Prague, Czech Republic) was used as the internal standard for HPLC determination. 6-Methoxy-2-naphthylacetic acid (6-MNA, $C_{13}H_{12}O_3$, 216.24 g/mol, Fig. 1, compound 2), 6-hydroxy-2-naphthylacetic acid (6-HNA, $C_{12}H_{10}O_3$, 202.21 g/mol, Fig. 1, compound 3), 4-(6-methoxy-2-naphthyl)-2-butanol (6-MeOnphBu–OH, $C_{15}H_{18}O_2$, 230.31 g/mol, Fig. 1, compound 4), 4-(6-hydroxy-2-naphthtyl)-2-butanone (6-HOnphBu=O, $C_{14}H_{14}O_2$, 214.26 g/mol, Fig. 1, compound 5), 4-(6-hydroxy-2-naphthyl)-2-butanol (6-HOnphBu–OH, $C_{14}H_{16}O_2$, 216.28 g/mol, Fig. 1, compound 6) were synthesized in the laboratories of our Institute, characterized by NMR and LC–MS and used as the standards of phase I nabumetone metabolites. Acetonitrile, methanol (both HPLC grade, Merck, Darmstadt, Germany), diethyl ether (analytical grade, Merck), hydrochloric acid (35% water solution, analytical grade), sodium hydrogenphosphate dodecahydrate, potassium dihydrogenphosphate and sodium hydroxide (all analytical grade, Lachema Brno, Czech Republic), acetic acid (99%, analytical grade, HiChem, Prague, Czech Republic), ultra-high-quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga, Bucks., UK) were used for the liquid–liquid extraction of body fluids and chromatography of nabumetone derivatives.

β-Glucuronidase (EC 3.2.1.31, type HP-2, 137,800 units/ml) and sulfatase (EC 3.1.6.1, type H-2, 4100 units/ml), both fluid preparations were made from *Helix pomatia* by Sigma, USA.

A phosphate buffer (pH 7.4) was prepared by mixing 800 ml of 0.067 M sodium hydrogenphosphate dodecahydrate (23.976 g in 1000 ml of UHQ water) and 200 ml of 0.067 M potassium dihydrogenphosphate (2.2695 g in 250 ml of UHQ water).

An acetate buffer (pH 5) was made from 59 ml of 0.2 M acetic acid and 141 ml of 0.2 M sodium acetate. An oral nabumetone formulation (coated tablets containing 500 mg of the parent compound) was tested in this study.

2.2. Minipigs and biological material

A white castrated male minipig was used in the experiments approved by the regional ethics committee. The animal received a dose of 1 g of nabumetone orally (two 500 mg tablets). Five hours after the administration (corresponding approximately to t_{max} determined in the preceding pharmacokinetic experiments [8]), the animal was sacrificed and fluid samples were taken immediately: urine from urinary bladder, bile from gall bladder, small intestine content. Body fluids from a drug-free minipig were taken for a comparison. All these biomatrices were stored at −70 °C until the analysis.

2.3. Sample preparation

2.3.1. Liquid–liquid extraction of body fluids

The frozen samples of bile, small intestine content or urine were thawed out and centrifuged (2000 × *g* for 10 min at 20 °C). Each individual sample was divided into three portions of the same volumes. Each portion (0.5 ml) was spiked with 150 μl 10^{-4} M naproxen (I.S.) and 0.5 ml of phosphate buffer (pH 7.4) was added. After centrifugation (3400 rpm for 20 min), 1 ml of the supernatant was transferred into a clean tube, 400 μl of acetate buffer (pH 5) was added and the incubation with β-glucuronidase (in the first portion) and sulfatase (in second portion) was performed. According to our previous experiments, 32 μl of the respective enzymatic preparation in case of intestinal content and 8 μl in case of bile or urine were needed for quantitative accomplishment of the enzymatic deconjugation during 18 h at 37 °C. To the third portion, only adequate volume of acetate buffer was added for comparison (no deconjugation ran through in this portion). The enzymatic reactions were terminated by the addition of 36% aqueous HCl (25 μl). Each of the portions was vigorously shaken for a short time. After centrifugation (2000 × *g* for 20 min), the supernatant was separated by decantation from the sediment. Diethyl ether (3 ml) was added and the content of the tubes was vortex-mixed for 1 min. After centrifugation (2000 × *g* for 12 min), the tubes were stored in a deep freezer until the water layer froze to ice. The upper diethyl ether layer containing the analytes (nabumetone, its phase I metabolites and naproxen) was decanted into another clean tube and the solvent was evaporated (water bath 40 °C, stream of nitrogen). The dry extract in the glass tube was reconstituted in 600 μl of the mobile phase and transferred into the vial of the autosampler. One hundred microliters of the sample were injected into the chromatographic column.

2.3.2. Sample preparation of body fluids before their direct injection

For the HPLC–photodiode array detection (DAD)–MS identification of phase II nabumetone metabolites (conjugates) in body fluids, direct injection of treated and diluted samples into chromatographic column was employed.

Two hundred and fifty microliters of urine (or another body fluid) in a tube was mixed with 100 μl of acetate buffer (pH 5); no internal standard was used in this case. Eight microliters of β-glucuronidase (or sulfatase, or only acetate buffer pH 5) was added. The content of the tube was mixed and incubated at 37 °C for 18 h. The enzymatic reaction was then terminated by 5 μl of concentrated HCl, 240 μl of mobile phase was added. After centrifugation (10,000 rpm for 5 min) and filtration, the direct injection of diluted bile samples into the chromatographic column followed. Urine samples had to be diluted with mobile phase before injection approximately 60 times.

2.4. HPLC–DAD

Routine chromatographic analyses were performed using a Thermo Electron (formerly Thermo Finnigan) chromatograph (San Jose, CA, USA). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100 μl sample loop, UV6000 LP photodiode array detector with Light Pipe Technology, SN4000 system controller and a data station (Intel Pentium 4 CPU 1.6 GHz, RAM 256 MB, HDD 40 GB) with the ChromQuest 4 analytical software (Thermo Electron) working under the Windows 2000 operating system (Microsoft Corporation).

A LiChroCART 125 mm × 4 mm column packed with LiChrospher 100 RP-C_{18}, 5 μm, precolumn LiChroCART 4 mm × 4 mm with the same stationary phase (Merck) and two various mobile phases were used for the HPLC analyses.

Mobile phase A (acetonitrile–UHQ water–acetic acid, 45:55:1, v/v/v) was applied to the separation and determination of nabumetone and its phase I metabolites in body fluid samples. In this mobile phase, the parent prodrug, its five metabolites described in Section 2.1 and naproxen (the internal standard) were separated over 12 min.

Mobile phase B (acetonitrile–UHQ water–acetic acid, 10:90:1, v/v/v) was employed for the separation of phase II metabolites (the conjugates of nabumetone phase I metabolites) in treated and diluted body fluids directly injected into the chromatographic column. The above-mentioned mobile phase composition was used during the analytical period (0–65 min), when phase II metabolites were gradually eluted from the chromatographic column. A washout period followed (65–85 min; 80% acetonitrile in UHQ water), so that phase I nabumetone metabolites remaining in the chromatographic column were quickly removed. After equilibration (time interval 85–100 min; mobile phase B) preceding the initial chromatographic conditions, the next sample could be analyzed.

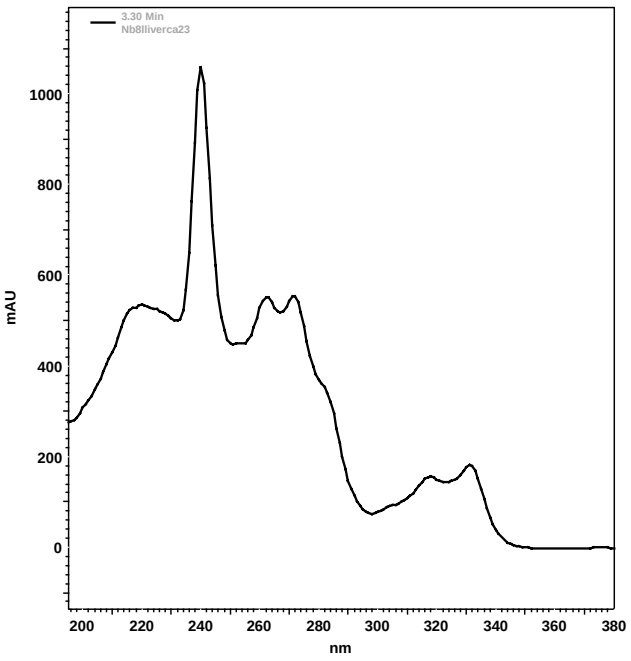

Fig. 2. UV spectrum of nabumetone derivatives obtained on a UV6000LP photodiode array detector during the HPLC analysis. UV maxima: 220, 240, 265, 272, 318 and 333 nm.

The flow rate of both mobile phases was 1 ml min. UV detection was performed at 265 nm (see Fig. 2). For the collection of the UV spectra, a photodiode array mode (in the range 195–380 nm with a 1 nm distance) was used.

2.5. HPLC–MS analyses

HPLC–MS analyses were performed on an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) and a liquid chromatograph consisting of a model 616 pump, a model 717+ autosampler, a four-channel solvent delivery system (low-pressure gradient system), a thermostated column compartment, a model 996 photodiode array detector and a Millenium chromatography manager (all from Waters, Milford, MA, USA). Mass spectra were recorded in the range *m*/*z* 50–1200, the mass spectrometer was tuned to give a maximum response for *m*/*z* 230. The phase I metabolites were separated in the mobile phase A and identified by HPLC–MS with positive-ion and negative-ion atmospheric pressure chemical ionization (APCI). The pressure of the nebulizing gas was 70 psi and the flow rate of the drying gas was 4 l/min (1 psi = 6894.76 Pa). The temperatures of the drying gas and APCI heater were 350 and 450 °C, respectively. The phase II metabolites were separated in the mobile phase B and identified by HPLC–MS with positive-ion and negative-ion electrospray ionization (ESI). The pressure of the nebulizing gas was 70 psi and the flow rate of the drying gas was 12 l/min and the ion source temperature was 300 °C. The samples were dissolved in the mobile phase and 100 μl of the solution was injected into the HPLC–MS system.

3. Results and discussion

3.1. Sample preparation procedures

All phase II nabumetone metabolites (for example, the expected conjugates of phase I nabumetone metabolites with glucuronic or sulphuric acid) were extremely polar and they have not practically been transferred from biomatrices into diethyl ether during the liquid–liquid extraction procedure. Thus, two approaches were applied in the sample preparation, when phase II metabolites were analyzed.

The experiments with β-glucuronidase (EC 3.2.1.31) and sulfatase (EC 3.1.6.1) gave us the initial information about the presence of phase II nabumetone metabolites in individual body fluids. Both these enzymes converted the phase II nabumetone metabolites (conjugates of xenobiotics with glucuronic or sulfuric acid) back to the phase I nabumetone metabolites. The incubation mixtures were then processed using a liquid–liquid extraction as described in Section 2.3.1. Differences in the amounts of individual phase I nabumetone metabolites, which were found in a β-glucuronidase(sulfatase)-treated sample and in a sample incubated without enzyme, correspond to the amounts of phase II metabolites (conjugates with glucuronic or sulfuric acid).

The final information about the structures of individual phase II metabolites was obtained from HPLC–MS analyses of the treated, diluted and filtered samples of body fluids after their direct injection into the chromatographic column.

3.2. HPLC–DAD

Reversed-phase HPLC method employing the mobile phase A has been validated in our previous communication [8]. This method was used for the determination of nabumetone and its five phase I metabolites but also for the indirect determination of phase II nabumetone metabolites after their conversion to phase I metabolites, as it is described in Sections 2.3.1, 3.1 and 3.3 and demonstrated in Figs. 3 and 4b. The individual analyses lasted 12 min, when the extracts from bile and urine were measured. In the case of intestinal content extracts, the presence of several peaks of ballast compounds was observed (in the t_R interval between 20 and 38 min). Because these peaks could interfere in the analyses of the next samples in the queue, the length of these analyses was extended to 45 min.

Direct analyses of polar phase II nabumetone metabolites were more complicated, because no standards of these compounds were available. It was only expected that these conjugates would be eluted from the chromatographic column before or close to the most polar phase I metabolite, 6-hydroxy-2-naphthylacetic acid, having $t_R \cong 1.6$ min in the mobile phase A. The time interval of 1.6 min was too short for the satisfactory resolution of an unknown number of phase II metabolites. Hence, a new mobile phase

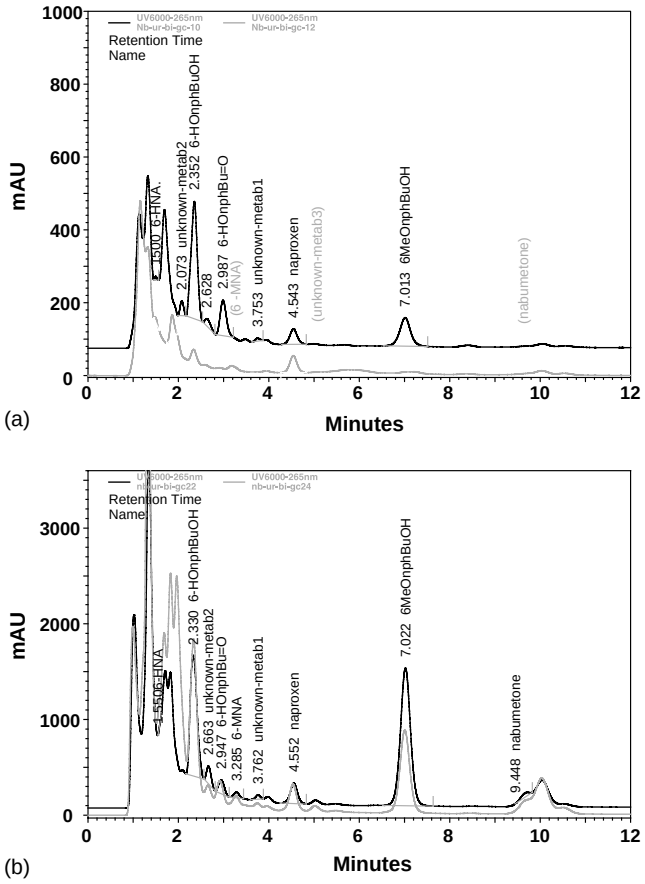

Fig. 3. Typical chromatograms of a diluted minipig bile withdrawn 5 h after the administration of 1 g nabumetone without any enzymatic treatment (a, lower gray chromatogram) and after the β-glucuronidase incubation of the sample (a, upper black chromatogram). Typical chromatograms of the extracts from minipig intestinal contents without any enzymatic treatment (b, lower gray chromatogram) and after the β-glucuronidase incubation of the sample (b, upper black chromatogram).

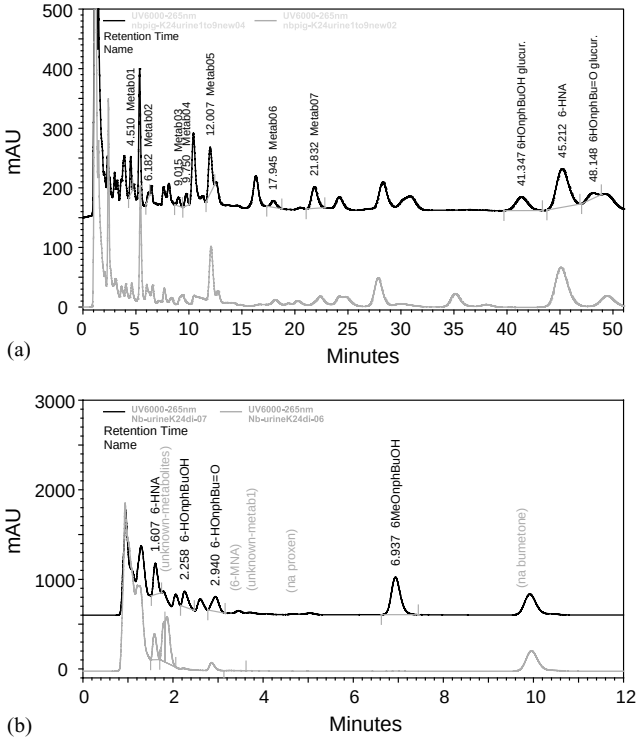

Fig. 4. Typical chromatograms of diluted minipig urine in the mobile phase B (after direct injection of the sample) without any enzymatic treatment (a, upper black chromatogram) and after the β-glucuronidase incubation of the sample (a, lower gray chromatogram). Typical chromatograms of diluted minipig urine in the mobile phase A (after direct injection of the sample) without any enzymatic treatment (b, lower gray chromatogram) and after the β-glucuronidase-incubation of the sample (b, upper black chromatogram).

for the separation of phase II nabumetone metabolites was searched for.

The composition of a new mobile phase designed for the sufficient separation of the expected phase II nabumetone metabolites was selected according to the retention time of 6-hydroxy-2-naphthylacetic acid. In the mobile phase B (containing only 10% of acetonitrile, see Section 2.4), the retention time of 6-HNA was approximately 45 min. A 65 min chromatographic analysis of a sample in the mobile phase B seemed to be long enough for the resolution of all phase II nabumetone metabolites and ballast compounds having retention times shorter or close to 6-hydroxy-2-naphthylacetic acid. Then, a 20 min washout period followed by a 15 min equilibration to the starting analytical conditions was applied, as it was described in Section 2.4.

The routine chromatographic analyses were performed using a UV6000LP system DAD (in the range 195–380 nm with a 1 nm distance). In our previous paper [8], a SpectraFOCUS high-speed scanning UV-Vis detector having only 0.6 cm optical path length of the flow cell was employed for HPLC analyses. In the new UV6000LP system, the optical path length of the flow cell was 5 cm, thus almost a 10-fold increase of the response was achieved. In addition, a light pipe (LP) technology (a total reflexion of the beam inside of the flow cell) minimized the loss of the light stream in the UV6000LP system.

Fig. 2 shows a characteristic UV spectrum of nabumetone and all its phase I and phase II metabolites and naproxen (I.S.). All these compounds possessing the same 2-methoxynaphthalene chromophore have an identical UV spectrum. Minor differences between the UV spectrum published formerly [8] and the spectrum presented here (in Fig. 2) are probably a result of two different ways of spectra acquisition (a high-speed scanning technology in the SpectraFOCUS system versus a photodiode array technology in the UV6000LP system).

The presence of nabumetone metabolites in an acquired spectrochromatogram (displayed in the form of a contour or three-dimensional scan) was indicated by the finding of the above-mentioned characteristic spectrum at a certain retention time. The HPLC–DAD results were correlated with the results from an HPLC–MS analyses (see Section 3.4).

3.3. Preliminary identification of phase II nabumetone metabolites in body fluids using HPLC–DAD

According to Haddock et al. [2], the glucuronides and sulfates of phase I nabumetone metabolites were reported as the principal conjugates found in biomatrices of various species. Standards of the phase II nabumetone metabolites were not available. Thus, a combination of various xenobiochemical and bioanalytical methods had to be applied for the purpose of identification and determination of conjugates in our study. For these purposes, the influence of sample incubation with β-glucuronidase and sulfatase on the amount of phase I nabumetone metabolites found in biomatrices was studied.

The quantitative enzymatic hydrolysis of conjugates presented in a biomatrix led to the formation of phase I metabolites, which were well determinable in the mobile phase A (acetonitrile–UHQ water–acetic acid, 45:55:1, v/v/v).

Chromatograms from the HPLC analyses of minipig bile and intestinal contents are presented in Fig. 3a and b. In Fig. 3a, the lower gray chromatogram corresponds to the analysis of a diluted bile sample, which was not enzymatically treated. Only the peak of naproxen (I.S.) and virtually no phase I metabolites were observed in this chromatogram. In case the same bile sample was incubated with β-glucuronidase, new peaks of 6-HOnphBu–OH, 6-HOnphBu=O, 6-MeOnphBu–OH and some unknown minor metabolites appeared, as shown in upper black chromatogram in Fig. 3a. The comparison of these two chromatograms led to the conclusion that the glucuronides of 6-HOnphBu–OH, 6-HOnphBu=O and 6-MeOnphBu–OH prevail among the nabumetone metabolites in the minipig bile.

Analogously, in Fig. 3b, the lower gray chromatogram of an extract from enzymatically untreated minipig intestinal contents was compared with the upper black chromatogram of an extract from β-glucuronidase-treated intestinal contents. In both analyses, comparable amounts of phase I nabumetone metabolites 6-HOnphBu–OH and 6-HOnphBu=O were detected. A higher amount of 6-MeOnphBu–OH present after the incubation with β-glucuronidase (compare the peaks at 7.022 min in both chromatograms in Fig. 3b) gave us indirect evidence that both 6-MeOnphBu–OH (compound 4 in Fig. 1a) and its glucuronide (compound 4-Gluc. in Fig. 1b) were present in the intestinal contents.

To obtain a direct proof of the phase II metabolites, the mobile phase B (acetonitrile–UHQ water–acetic acid, 10:90:1, v/v/v), which enables a better resolution of the polar conjugates, was used for the chromatography of diluted urine samples injected directly into the column (see Fig. 4a). In this mobile phase, the conjugates having mostly a shorter retention time than 6-HNA (t_R = 45.2 min) were searched for in the spectrochromatogram on the basis of the typical UV spectrum of nabumetone derivatives (see Fig. 2). At least nine peaks with the same UV spectrum as nabumetone were found in the chromatogram (upper chromatogram in Fig. 4a), but only two of these compounds (t_R = 41.3 min and 48.1 min) were found in higher concentrations. After the incubation of urine with β-glucuronidase, just these two compounds disappeared, as shown in the lower chromatogram in Fig. 4a. This experiment confirmed that the above-mentioned peaks belong to the glucuronides of some phase I nabumetone metabolites. Following the HPLC–MS (ESI) analysis, the peak with t_R = 41.3 min was identified from the molecular ion as 6-HOnphBu–OH glucuronide (compound 6-Gluc., M_r = 392, see Fig. 1b) and the peak with t_R = 48.1 min was identified as 6-HOnphBu=O glucuronide (compound 5-Gluc., M_r = 390, see Fig. 1b).

Fig. 4b shows the same urine samples mentioned in the previous paragraph, which were analyzed in the mobile phase A. The lower gray chromatogram is a result of the analysis of a directly injected, enzymatically untreated minipig urine. Only 6-HNA (t_R = 1.6 min) and a peak group of unknown metabolites with t_R = 1.7–2 min were identified on the basis of the typical UV spectra. The upper black chromatogram corresponds to the analysis of a directly injected, β-glucuronidase-treated minipig urine. The peak group of unknown metabolites with t_R = 1.7–2 min disappeared and a new peak of 6-MeOnphBu–OH with t_R = 6.94 min was found in this chromatogram. In accord with these results, the HPLC–MS (ESI) analysis identified a molecular ion of M_r = 406 corresponding to 6-MeOnphBu–OH glucuronide in the peak group with t_R = 1.7–2 min (see compound 4-Gluc. in Fig. 1b).

3.4. Mass spectra of the nabumetone metabolites

Both mobile phases A and B mentioned in Sections 2.4 and 2.5 were used in HPLC–MS methods for the identification of nabumetone metabolites. The first method with the mobile phase A was applied for the separation of phase I metabolites followed by APCI–MS identification on the basis of our previous work [8]. The sensitivity of APCI for phase I metabolites is much better than with ESI, but still only comparable with the UV detection due to the low ionization efficiency of parent drug and phase I metabolites. The second method with a lower content of acetonitrile in the mobile phase (the mobile phase B) yielded a better separation of more polar phase II metabolites, coupled with ESI-MS in both positive-ion and negative-ion mode for their identification.

The mass spectrometric identification procedure is composed of two basic steps. Firstly, the molecular masses of individual peaks are determined using both positive-ion and negative-ion first-order ESI mass spectra. Typical ions observed in the positive-ion ESI mode are the protonated molecules $[M + H]^+$ and also adducts ions, such as $[M + NH_4]^+$, $[M + Na]^+$, $[M + K]^+$, etc. (see Table 1). Complementary information from the negative-ion ESI mass spectra, i.e. the $[M - H]^-$ ions as the base peaks and also the molecular adducts such as $[2M - H]^-$, $[2M - 2H + Na]^-$,

Table 1
Ions observed in MS and MS–MS spectra of individual peaks in the positive-ion and negative-ion ESI mode with their tentative identification

t_R (min)	M_r	Positive-ion ESI-MS[a]	Positive-ion ESI-MS–MS[b]	Negative-ion ESI-MS[a]	Negative-ion ESI-MS–MS[c]
4.51	408	**426** $[\boldsymbol{M} + \mathbf{NH_4}]^+$, 431 $[M + Na]^+$, 447 $[M + K]^+$	409 $[M + H]^+$, 391 $[M + H - H_2O]^+$, 381, 337, 281, 233 $[M + H - 176]^+$, 171	**407** $[\boldsymbol{M} - \mathbf{H}]^-$, 815 $[2M - H]^-$	389 $[M - H - H_2O]^-$, 231 $[M - H - 176]^-$, 175, 113
5.28	179	**180** $[\boldsymbol{M} + \mathbf{H}]^+$, 202 $[M + Na]^+$, 381 $[2M + Na]^+$, 397 $[2M + K]^+$, 560 $[3M + Na]^+$, 576 $[3M + K]^+$	162 $[M + H - H_2O]^+$, 134 $[M + H - HCOOH]^+$, 105 $[M + H -$ glycine$]^+$	**178** $[\boldsymbol{M} - \mathbf{H}]^-$	134 $[M - H - CO_2]^-$
5.72	406	424 $[M + NH_4]^+$, 429 $[M + Na]^+$, 467 $[M + Na + K - H]^+$	–	**405** $[\boldsymbol{M} - \mathbf{H}]^-$	387 $[M - H - H_2O]^-$, 347 $[M - H - 58]^-$, 229 $[M - H - 176]^-$, 175, 113
6.18	426	**427** $[\boldsymbol{M} + \mathbf{H}]^+$, 409 $[M + H - H_2O]^+$	409 $[M + H - H_2O]^+$, 391 $[M + H - 2H_2O]^+$, 345, 233 $[M + H - 176 - H_2O]^+$, 174, 146	**425** $[\boldsymbol{M} - \mathbf{H}]^-$	354, 249 $[M - H - 176]^-$, 193, 175
7.43	193	**194** $[\boldsymbol{M} + \mathbf{H}]^+$, 216 $[M + Na]^+$, 409 $[2M + Na]^+$, 425 $[2M + K]^+$, 602 $[3M + Na]^+$, 618 $[3M + K]^+$	176 $[M + H - H_2O]^+$, 148 $[M + H - HCOOH]^+$, 91 $[C_7H_7]^+$, 77 $[C_6H_5]^+$	**192** $[\boldsymbol{M} - \mathbf{H}]^-$, 385 $[2M - H]^-$, 600 $[3M - 2H + Na]^-$, 616 $[3M - 2H + K]^-$	74 $[NH_2CH_2COO]^-$
9.02	408	**409** $[\boldsymbol{M} + \mathbf{H}]^+$, 426 $[M + NH_4]^+$, 431 $[M + Na]^+$, 447 $[M + K]^+$	375, 260, 221, 176	**407** $[\boldsymbol{M} - \mathbf{H}]^-$	231 $[M - H - 176]^-$, 175, 113
10.51	284	302 $[M + NH_4]^+$, 307 $[M + Na]^+$, 323 $[M + K]^+$, 591 $[2M + Na]^+$, 607 $[2M + K]^+$	–	**283** $[\boldsymbol{M} - \mathbf{H}]^-$, 567 $[2M - H]^-$	265 $[M - H - H_2O]^-$, 175, 113, 95, 85
12.01	232	**233** $[\boldsymbol{M} + \mathbf{H}]^+$, 255 $[M + Na]^+$, 215 $[M + H - H_2O]^+$, 197 $[M + H - 2H_2O]^+$	215 $[M + H - H_2O]^+$, 192, 158, 130, 76	**231** $[\boldsymbol{M} - \mathbf{H}]^-$, 485 $[2M - 2H + Na]^-$	171 $[M - H - 60]^-$, 156, 74
16.24	390	**391** $[\boldsymbol{M} + \mathbf{H}]^+$	349, 331, 255, 149	**389** $[\boldsymbol{M} - \mathbf{H}]^-$	329 $[M - H - 60]^-$, 213 $[M - H - 176]^-$, 175, 113
17.95	406	**424** $[\boldsymbol{M} + \mathbf{NH_4}]^+$, 429 $[M + Na]^+$, 489 $[M + 2Na + K - 2H]^+$, 505 $[M + Na + 2K - 2H]^+$	366, 247, 213, 187	**405** $[\boldsymbol{M} - \mathbf{H}]^-$	323, 193, 175, 157, 149, 131, 113
21.83	364	**382** $[\boldsymbol{M} + \mathbf{NH_4}]^+$, 387 $[M + Na]^+$, 403 $[M + K]^+$, 425 $[M + Na + K - H]^+$, 447 $[M + 2Na + K - 2H]^+$, 463 $[M + Na + 2K - 2H]^+$	365 $[M + H]^+$, 347 $[M + H - H_2O]^+$, 329 $[M + H - 2H_2O]^+$, 311 $[M + H - 3H_2O]^+$, 293 $[M + H - 4H_2O]^+$, 189 $[M + H - 176]^+$, 171 $[M + H - 176 - H_2O]^+$,	**363** $[\boldsymbol{M} - \mathbf{H}]^-$, 407 $[M - 3H + 2Na]^-$, 445 $[M - 4H + 2Na + K]^-$, 727 $[2M - H]^-$, 749 $[2M - 2H + Na]^-$	345 $[M - H - H_2O]^-$, 302, 220, 193, 175, 131, 113
28.10	436	**437** $[\boldsymbol{M} + \mathbf{H}]^+$	419 $[M + H - H_2O]^+$, 393 $[M + H - CO_2]^+$, 261 $[M + H - 176]^+$, 217 $[M + H - 176 - CO_2]^+$, 201, 185	**435** $[\boldsymbol{M} - \mathbf{H}]^-$	393, 259 $[M - H - 176]^-$, 215 $[M - H - 176 - CO_2]^-$, 175, 113
41.35	392	**410** $[\boldsymbol{M} + \mathbf{NH_4}]^+$, 415 $[M + Na]^+$, 431 $[M + K]^+$, 453 $[M + Na + K - H]^+$	393 $[M + H]^+$, 375 $[M + H - H_2O]^+$, 357 $[M + H - 2H_2O]^+$, 339 $[M + H - 3H_2O]^+$, 217 $[M + H - 176]^+$, 199 $[M + H - 176 - H_2O]^+$, 157 $[M + H - 176 - 60]^+$	**391** $[\boldsymbol{M} - \mathbf{H}]^-$	373 $[M - H - H_2O]^-$, 330, 215 $[M - H - 176]^-$, 175, 113
45.21	202	**203** $[\boldsymbol{M} + \mathbf{H}]^+$, 157 $[M + H - HCOOH]^+$	157 $[M + H - HCOOH]^+$	**201** $[\boldsymbol{M} - \mathbf{H}]^-$, 157 $[M - H - CO_2]^-$	173 $[M - H - CO]^-$, 157 $[M - H - CO_2]^-$
48.15	390	**408** $[\boldsymbol{M} + \mathbf{NH_4}]^+$, 413 $[M + Na]^+$, 429 $[M + K]^+$, 451 $[M + Na + K - H]^+$	373, 349, 255, 232, 215 $[M + H - 176]^+$, 157 $[M + H - 176 - 58]^+$	**389** $[\boldsymbol{M} - \mathbf{H}]^-$	371 $[M - H - H_2O]^-$, 329, 213 $[M - H - 176]^-$, 175, 113

[a] Precursor ions for MS–MS measurements are in bold.
[b] Neutral losses are as follows: m/z 58 is CH_3COCH_3, m/z 60 is $CH_3CH(OH)CH_3$, and m/z 176 is a neutral glucuronide moiety.
[c] The fragment ion at m/z 175 is a negatively charged glucuronide moiety $[CH_3COCH(OH)CH(OH)COCOO]^-$, and m/z 113 ion corresponds to glucuronide minus glycol.

Table 2
Concentrations (nmol/ml) of nabumetone and its phase I and phase II metabolites (compare Fig. 1) in minipig body fluids

Fluid	6-HNA	6-HOnphBu–OH (6-Gluc.)	6-HOnphBu=O (5-Gluc.)	6-MNA	6-MeOnphBu–OH (4-Gluc.)	Nabumetone
Intestinal contents	0.3	124 (41.4)	17	7.2	129 (153)	2.1
Urine	1954	– (448)	–	–	– (1378)	–
Bile	5.9	– (375)	– (112)	–	– (162)	–

etc., enables us an unambiguous assignment of molecular masses. The fragment ions were not observed in the first-order mass spectra, which simplified the molecular mass determination.

The positive-ion MS–MS spectra of $[M + H]^+$ or $[M + NH_4]^+$ ions yielded abundant fragment ions, which supports the initial structural proposals based on the M_r determination (Table 1). The consecutive neutral losses of water bring little information about the structure, but, for example, the neutral loss of the glucuronide moiety (*m*/*z* 176) is an excellent tool for the positive identification of glucuronide presence in the molecular structure. Other characteristic neutral losses for the identification of particular structural features are CO_2 ($\Delta m/z$ 44, carboxylic acid), CH_3COCH_3 ($\Delta m/z$ 58) and $CH_3CH(OH)CH_3$ ($\Delta m/z$ 60). The negative-ion MS–MS spectra yielded again the characteristic features for the confirmation of the presence or absence of glucuronides, such as the neutral losses of $\Delta m/z$ 176 and the fragment ions at *m*/*z* 175 (negatively charged glucuronide residue) and *m*/*z* 113 (i.e. *m*/*z* 175 minus the glycol molecule). In summary, from both positive-ion and negative-ion MS–MS spectra, we can conclude that glucuronides correspond to eleven peaks in the chromatogram with the retention times 4.51 min (M_r = 408), 5.72 (406), 6.18 (426), 9.02 (408), 10.51 (284), 16.24 (390), 17.95 (406), 21.83 (364), 28.10 (436), 41.35 (392), and 48.15 (390). The structures of some glucuronides have been elucidated in this work (41.35 min for 6-Gluc. and 48.15 min for 5-Gluc. in Fig. 1b), but others require further study. In accordance with the results obtained with the mobile phase A for phase I metabolites, the most abundant phase II metabolites are glucuronides derived from 6-HOnphBu–OH, 6-HOnphBu=O and 6-MeOnphBu–OH. In case of compounds with M_r = 406 (17.95 min) and M_r = 364 (21.83 min), the additional glucuronide fragment ions at *m*/*z* 193 and 131 were observed in the negative-ion ESI-MS–MS, which may reflect different ways of glucuronide bonding in comparison to other glucuronides found.

The presence of any sulfate conjugates was not confirmed in our HPLC–MS experiments, although the incubation of some biomatrices with sulfatase gave positive results as well as in the experiments with β-glucuronidase. We assume that the enzyme specificity of β-glucuronidase and sulfatase is lower and these enzymes isolated from the same source (snail *H. pomatia*) are probably capable of hydrolyzing both glucuronides and sulfates each other.

4. Conclusions

Chemical structures of three principal ether glucuronides of phase I nabumetone metabolites (see Fig. 1b) were elucidated using a combination of both xenobiochemical (treatment of the biomatrices with β-glucuronidase) and analytical (HPLC–DAD, HPLC–MS) approaches. The expected acylglucuronides (glucuronides of 6-MNA or 6-HNA) and sulfate conjugates were not found in the samples of minipig's body fluids (Table 2). A number of further polar compounds, as yet unidentified minor nabumetone metabolites were detected in body fluids according to their characteristic UV spectra. The determination of three identified phase II nabumetone metabolites was performed by comparative HPLC–DAD analyses of the body fluids samples and of the same samples after their quantitative enzymatic treatment by β-glucuronidase.

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