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LC-^{1}H NMR used for determination of the elution order of S-naproxen glucuronide isomers in two isocratic reversed-phase LC-systems

Rasmus W. Mortensen [a,*], Olivia Corcoran [b], Claus Cornett [a], Ulla G. Sidelmann [c], Jeff Troke [b], John C. Lindon [b], Jeremy K. Nicholson [b], Steen Honoré Hansen [a]

[a] *Department of Analytical and Pharmaceutical Chemistry, Royal Danish School of Pharmacy, 2, Universitetsparken, DK-2100 Copenhagen, Denmark*
[b] *Biological Chemistry, Biomedical Sciences Division, Sir Alexander Fleming Building, Imperial College of Science, Technology and Medicine, Exhibition Road, South Kensington, London SW 7 2AZ, UK*
[c] *Drug Metabolism, Novo Nordisk, DK-2760 Måløv, Denmark*

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Abstract

The reactive metabolite S-naproxen-β-1-*O*-acyl glucuronide was purified from human urine using solid phase extraction (SPE) and preparative HPLC. The structure was confirmed by 600 MHz ^{1}H NMR. Directly coupled 600 MHz HPLC-^{1}H NMR was used to assign the peaks in chromatograms obtained when analysing a sample containing S-naproxen aglycone and the 1-, 2-, 3-, and 4-isomers of S-naproxen-β-1-*O*-acyl glucuronide in two simple isocratic reversed phase HPLC-systems. Using mobile phase I (50 mM formate buffer pH 5.75/acetonitrile 75:25 v/v) the elution order was: 4-*O*-acyl isomers, β-1-*O*-acyl glucuronide, 3-*O*-acyl isomers, 2-*O*-acyl isomers, and S-naproxen aglycone. Using mobile phase II (25 mM potassium phosphate pH 7.40/acetonitrile 80:20 v/v) the elution order was: α/β-4-*O*-acyl isomers, S-naproxen aglycone, β-1-*O*-acyl glucuronide, 3-*O*-acyl isomers, and α/β-2-*O*-acyl isomers. In both systems the elution order for the 2-, 3- and 4-*O*-acyl isomers corresponded with previously published results for 2-, 3-, and 4-fluorobenzoic acid glucuronide isomers determined by reversed phase HPLC-^{1}H NMR [U.G. Sidelmann, S.H. Hansen, C. Gavaghan, A.W. Nicholls, H.A.J. Carless, J.C. Lindon, I.D. Wilson, J.K. Nicholson, J. Chromatogr. B Biomed. Appl. 685 (1996) 113–122]. The α-1-*O*-acyl isomer was found to be present at approximately 3% of the initial S-naproxen-β-1-*O*-acyl glucuronide concentration in the glucuronide isomer mixture after 6 h of incubation at pH 7.40 and 37°C. In both HPLC systems it eluted just before the β-1-*O*-acyl glucuronide well separated from other isomers. Investigators should consider the possible formation of a α-1-*O*-acyl isomer when studying glucuronide reactivity and degradation.

* Corresponding author. Present address: Pharmaceutical Stability Testing, Leo Pharmaceutical Products, 55, Industriparken, DK-2750 Ballerup, Denmark. Tel.: +45-4492-3800; fax: +45-4494-2618.
E-mail address: rasmus.mortensen@leo-pharma.com (R.W. Mortensen).

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1. Introduction

β-1-*O*-acyl glucuronides appear as reactive phase II metabolites of many drugs containing carboxylic acid functionalities, including the widely used non steroidal anti-inflammatory drug (NSAID) S-naproxen ((S)-6-methoxy-α-methyl-naphthalene acetic acid). Upton [1] showed in 1980 that the NSAIDs ketoprofen and S-naproxen are excreted in urine as conjugated metabolites with no renal excretion of the unchanged drug. This was in contrast to earlier observations where 15–50% of the drugs mentioned were reported to be excreted unchanged. At neutral to basic pH values the unstable β-1-*O*-acyl glucuronides will acyl-migrate to the 2-, 3- and 4-positional isomers and eventually hydrolyse to the parent drug (Fig. 1) and this had not been recognised in earlier reports. In order to minimise the degradation Upton performed all analyses within 30 min of urine collection, and thus obtained the correct data for β-1-*O*-acyl glucuronide and the unchanged parent drug excreted. Since this initial report describing β-1-*O*-acyl glucuronide instability many groups has studied the reactivity of β-1-*O*-acyl glucuronides and their acyl-migration products [2–5]. The major concern is the potential of the isomers to react with proteins, which has been studied for a large number of acidic drugs [6–13] including naproxen [14]. Benet [15] showed in 1993 a linear correlation between the in vitro β-1-*O*-acyl glucuronide degradation rate and in vitro protein binding for nine different drug acyl glucuronides, a correlation that has been found also to be valid for a number of other drug acyl glucuronides including the β-1-*O*-acyl glucuronides of R- and S-naproxen [14].

When studying the hydrolysis and acyl-migration of β-1-*O*-acyl glucuronides by high performance liquid chromatography (HPLC) a major problem is the assignment of the chromatographic peaks to the individual isomers.

As the migration products are unstable and interconvert in a dynamic equilibrium no reference compounds are available. Where the isomers have been separated chromatographically from the β-1-*O*-acyl glucuronide, they have typically been termed 'isoglucuronide' [16] or 'rearrangement isomers' [17]. In cases where the 2-, 3- and 4-*O*-acyl isomers have been separated chromatographically the assignment of the chromatographic peaks has usually been based on their

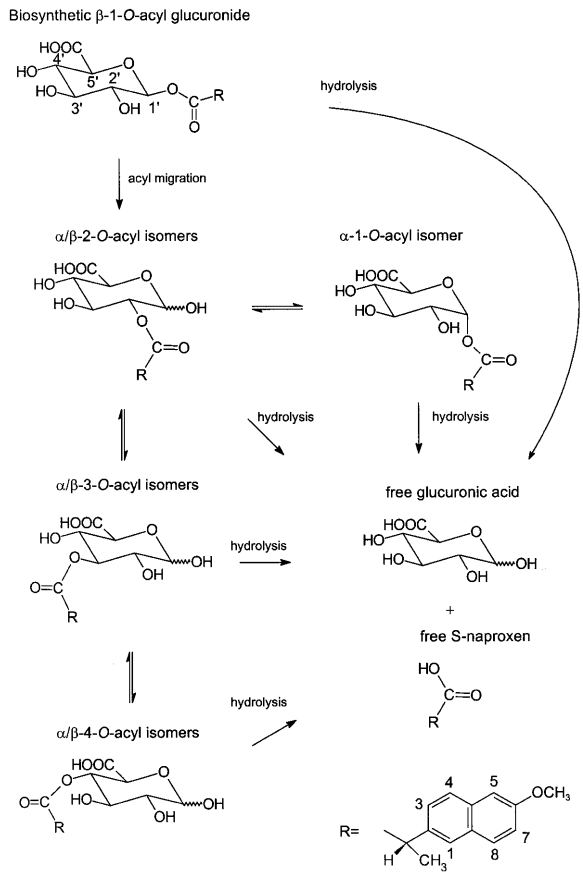

Fig. 1. The degradation rearrangement scheme for S-naproxen-β-1-*O*-acyl glucuronide. The acyl migration and hydrolysis scheme is general for β-1-*O*-acyl glucuronides. The novel α-1-*O*-acyl isomer is included. For clarity the anomerisation reactions between the α/β-2-, α/β-3- and α/β-4-*O*-acyl anomers have been omitted. Proton chemical shifts of S-naproxen-β-1-*O*-acyl glucuronide are given in Section 3.

sequential appearance [18,19]. The acyl-migration scheme depicted in Fig. 1 is general for β-1-*O*-acyl glucuronides and shows the sequential migration of the aglycone from the biosynthetic β-1-*O*-acyl glucuronide via the 2-*O*-acyl isomer to the 3-*O*-acyl isomer and finally the 4-*O*-acyl isomer and eventually hydrolysis of all the acyl-migration isomers to parent drug. The first isomer peak appearing over time in an incubation mixture of β-1-*O*-acyl glucuronide has then been assigned to the 2-*O*-acyl isomer, the next to the 3-*O*-acyl isomer and the last to the 4-*O*-acyl isomer. A more radical approach is to fraction-collect the individual isomers by preparative HPLC and identify them off-line by nuclear magnetic resonance (NMR) spectroscopy [20]. This task is very time-consuming and difficult, as the unstable isomers tend to rearrange during the isolation and purification process. Using HPLC directly coupled to high frequency ^{1}H NMR techniques it has recently been possible to unambiguously identify the individual isomers in a chromatogram of isomerised glucuronide without the tedious fraction-collection and off-line identification of the unstable isomers [21,22]. Another NMR-based approach to study the reactivity of β-1-*O*-acyl glucuronides is to use ^{13}C-labelled compounds [23] and follow the acyl-migration directly by the distinct ^{13}C-NMR signals from the positional isomers.

The degradation of S-naproxen-β-1-*O*-acyl glucuronide has been studied by Vree using HPLC [16]. The major and minor degradation peak in the chromatogram of isomerised S-naproxen-β-1-*O*-acyl glucuronide was named naproxen isoglucuronide and naproxen isoglucuronides, respectively. The major degradation peak (eluting just before the β-1-*O*-acyl glucuronide in the chromatogram) was assigned to the 4-*O*-acyl isomer, as this was regarded to be the end-product of isomerisation. However, no structural evidence for this assignment was reported and all the isomers were not chromatographically separated.

Recently Iwaki [4] studied the degradation kinetics of S-naproxen-β-1-*O*-acyl glucuronide and its 2-*O*-acyl isomer. The 2-*O*-acyl isomer was isolated by preparative HPLC and its degradation kinetics were compared to the β-1-*O*-acyl glucuronide using HPLC. The 3- and 4-*O*-acyl isomers were not structurally assigned to the peaks in the chromatogram, but their sequential appearance over time was used to identify the peaks. A minor 'isomer X' eluting just before the β-1-*O*-acyl glucuronide in their HPLC-system was reported. It was resistant to cleavage by β-glucuronidase but not to base-hydrolysis, which strongly implies an acyl-migration product. No structural information or speculation was given by the authors regarding this minor compound.

In the present paper HPLC-^{1}H NMR has been used to unambiguously assign the elution order of the acyl-migration products in chromatograms of isomerised S-naproxen-β-1-*O*-acyl glucuronide using two different simple isocratic reversed-phase chromatographic systems.

2. Experimental

All chemicals used were of analytical reagent grade or higher from commercial suppliers. Water was purified using a Milli-Q Plus water purification apparatus (Millipore A/S, Glostrup, Denmark). Deuterium oxide for HPLC-NMR was from GOSS Scientific Instruments, Ltd (UK). Acetonitrile for the HPLC-NMR experiments was NMR CHROMASOLV® grade from Riedel de Haën (Sigma-Aldrich UK, Dorset, England).

2.1. Preparation of S-naproxen-β-1-O-acyl glucuronide

S-naproxen-β-1-*O*-acyl glucuronide was isolated from human urine using solid phase extraction (SPE) and preparative HPLC. A total of 402 mg of S-naproxen in the form of two Alleve® tablets (220 mg naproxen sodium each — Bayer Corporation, Morristown, NJ 07960, USA) were given as a single dose to a healthy male volunteer (27 years, 71 kg) and urine for the period 0–30 h was collected (3.8 l). Vree [16] reported 53% of a single dose of S-naproxen excreted as the β-1-*O*-acyl glucuronide with a $T_{\frac{1}{2}}$ for naproxen in plasma of 21 h. Thus it was estimated that urine collection after 30 h would give only minor extra yield of S-naproxen-β-1-*O*-acyl glucuronide.

The urine fractions were acidified with 10 ml/l of 10% formic acid solution immediately after collection and stored at 5°C until purification in order to minimise degradation of S-naproxen-β-1-*O*-acyl glucuronide. The β-1-*O*-acyl glucuronide was stable in the 5°C acidified urine for at least 14 days, as monitored by analytical HPLC.

2.2. SPE-purification

Varian MegaBondElut™ C-18 cartridges with 2 g of chemically bonded C-18 material were used. Eluting buffers: 0.1% formic acid, 20 mM ammonium formate and methanol. The SPE-cartridge was activated with 5 ml methanol, 5 ml 0.1% formic acid and 5 ml 20 mM ammonium formate. A total of 50 ml urine was applied and the cartridge was washed with 10 ml 20 mM ammonium formate. It was then eluted with 10 ml 20 mM ammonium formate/MeOH (60:40, v/v) followed by 15 ml 20 mM ammonium formate/MeOH (15:85, v/v). The 85%-MeOH fraction contained the glucuronide. The MeOH was evaporated under a stream of nitrogen at room temperature and the aqueous residue was lyophilised. The SPE-procedure could be repeated ten times using the same SPE-cartridge with no decrease in yield or purity of isolated S-naproxen-β-1-*O*-acyl glucuronide as determined by analytical HPLC. The pooled lyophilised residues were dissolved in 20 ml 0.1% formic acid prior to injection on the preparative HPLC-system.

2.3. Preparative HPLC

A Waters 6000A pump was used with a flow rate of 10 ml/min. The 250 mm∗16 mm i.d. column was packed in our laboratory with Polygosil 60 C-18, 10 μm particles. A 2 ml injection loop was fitted on a Rheodyne injection valve. An ABI Analytical Spectroflow 757 detector was operated at 272 nm and connected to a HP 3395 integrator. The mobile phase was MeOH/100 mM ammonium formate 50:50 (v/v) adjusted with formic acid after mixing to a pH-meter reading of 5.85, using aqueous pH-standards of pH 4.00 and 7.00 for calibration of the pH-meter. Aliquots of 0.5 ml of the pooled SPE fractions were injected and the HPLC fractions containing the β-1-*O*-acyl glucuronide (t_R 6.50–7.75 min) were collected and pooled. The pooled fractions were then rotary evaporated at room temperature using a Heto MAXI Dry-lyo vacuum centrifuge. A total yield of 210 mg S-naproxen-β-1-*O*-acyl glucuronide (0.52 mmol) gave a total recovery of 30% on a molar basis compared to the amount of drug ingested (1.75 mmol).

HPLC analysis at 272 nm of the biosynthetic S-naproxen-β-1-*O*-acyl glucuronide gave a major peak at t_R 6.17 min which accounted for 94% of the total peak area in the chromatogram, with only minor other peaks, mainly α/β-2-*O*-acyl isomer and solvent front (results not shown). The identity was confirmed by 600 MHz ^{1}H NMR spectroscopy (see Section 3).

2.4. Preparation of glucuronide isomer mixture

A total of 2.8 mg S-naproxen-β-1-*O*-acyl glucuronide was dissolved in 1.0 ml 25 mM potassium phosphate buffer pH 7.40 and incubated at 37°C. Degradation was followed by analytical HPLC and degradation mixtures were stabilised by adding 100 μl cold 10% formic acid. Samples were then stored at 5°C for no more than 72 h prior to analysis by HPLC-NMR. One sample stabilised at $t = 6$ h contained maximum amounts of the putative α/β-2-*O*-acyl isomers, the putative α-1-*O*-acyl isomer and β-1-*O*-acyl glucuronide as determined by HPLC-UV (40, 3 and 20% by chromatographic peak area, respectively). This sample was used for the identification of all isomers using mobile phase I and for the α/β-2-*O*-acyl isomers and the α-1-*O*-acyl isomer using mobile phase II. A second sample stabilised at $t = 24$ h contained maximum amounts of the putative α/β-3-*O*-acyl isomers and α/β-4-*O*-acyl isomers as well as S-naproxen aglycone as determined by HPLC-UV (30, 15 and 25% by chromatographic peak area, respectively) and was used for the identification of the α/β-3-*O*-acyl isomers and α/β-4-*O*-acyl isomers using mobile phase II.

2.5. Analytical HPLC

For analytical HPLC-UV during the S-naproxen-β-1-*O*-acyl glucuronide purification and for the HPLC-NMR method development a HP 1100 series chromatographic system with HP ChemStation software was used (Agilent Technologies Denmark A/S, Birkerød, Denmark). This system was isocratic with a single-wavelength UV/VIS-detector. The column was a Hibar LiCrospher® 100 RP-C18 column with 5 μm particle size 250 mm∗4 mm i.d. (Merck, Darmstadt, Germany). The flow rate was 1 ml/min and the detection wavelength was 272 nm. Mobile phase I consisted of 50 mM ammonium formate buffer adjusted to pH 5.75 with formic acid/acetonitrile 75:25 v/v. Mobile phase II consisted of 25 mM potassium phosphate pH 7.40/acetonitrile 80:20 v/v. 20 μl sample was injected manually using a Rheodyne injection valve.

2.6. Directly coupled 600 MHz HPLC-^{1}H NMR spectroscopy

The LiCrospher® 100 RP-C18 column was used, with the following hardware: A Bruker LC-22 pump (Rheinstetten, Germany), a Bruker photodiode array detector (J & M Analytische Mess- und Regeltechnik GmbH, Aalen, Germany), and a Bruker BPSU-36 flow control unit. The outlet of the detector was connected to the HPLC-NMR flow probe via an inert polyether-ether ketone (PEEK) capillary (3.1 m∗0.25 mm i.d.). HPLC-NMR-MS software (Hystar™ v1.1) controlled the flow dynamics of the system and stored the chromatographic data. 50 μl sample were injected for each chromatogram.

For HPLC-^{1}H NMR the mobile phase buffers were modified compared to the method development system, as they were made up in D_2O. pD of the buffers were adjusted to a pH-meter reading of 5.75 and 7.40, respectively, using normal aqueous calibration standards with pH 4.00, 7.00 and 10.00 to calibrate the pH-meter. The buffer/acetonitrile ratios were unchanged at 75:25 v/v for mobile phase I and 80:20 v/v for mobile phase II.

2.7. NMR spectroscopy

The HPLC-^{1}H NMR spectra were acquired using a Bruker AVANCE600 spectrometer operating at 600.13 MHz ^{1}H frequency equipped with a ^{1}H-^{13}C inverse detection LC flow probe containing a 4 mm i.d., 120 μl cell. NMR spectra of the glucuronide isomers were obtained in stopped-flow mode at 600.13 MHz and probe temperature 25°C with mobile phase I, and 37°C with mobile phase II. Dual solvent suppression of the acetonitrile and residual HDO signals was achieved using a modification of the 1-dimensional NOESYPRESAT presaturation pulse sequence published by Nicholson [24] with relaxation and mixing delays of 2.0 and 0.1 s, respectively. Between 128 and 512 free induction decays (FIDs) were collected into 64 K computer data points with a spectral with of 20 ppm, corresponding to an acquisition time of 2.73 s. Prior to Fourier transformation, an exponential apodization function was applied to the FID, corresponding to a line broadening of 0.5 Hz. Chemical shifts were referenced to the acetonitrile signal at δ 2.0 and thus may differ from signals referenced to HDO at δ 4.7.

3. Results

3.1. NMR data for S-naproxen-β-1-O-acyl glucuronide

600 MHz ^{1}H NMR spectroscopy of the produced S-naproxen-β-1-*O*-acyl glucuronide (in 50 mM ammonium formate in D_2O pD 5.75/MeCN 75:25 v/v) gave the following signals (δ H referenced to acetonitrile at 2.0 ppm, multiplicity, coupling constant, integration, protons numbered in Fig. 1): δ 7.80, d, 8.0 Hz, 2H, H8 + H4; δ 7.76, s, 1H, H1; δ 7.44, d, 8.0 Hz, 1H, H3; δ 7.30, s, 1H, H5; δ 7.17, d, 8.0 Hz, 1H, H7; δ 5.46, d, 8.0, 1H, H1′; δ 4.03, q, 7.5 Hz, 1H, -CH-; δ 3.89, s, 3H, -OCH_3; δ 3.66, d, 9.5 Hz, 1H, H5′; δ 3.47, t, 9.5 Hz, 1H, H3′; δ 3.39, 2*t, 8.8/9.5 Hz, 2H, H2′ + H4′; δ 1.54, d, 7.0 Hz, 3H, -CH_3.

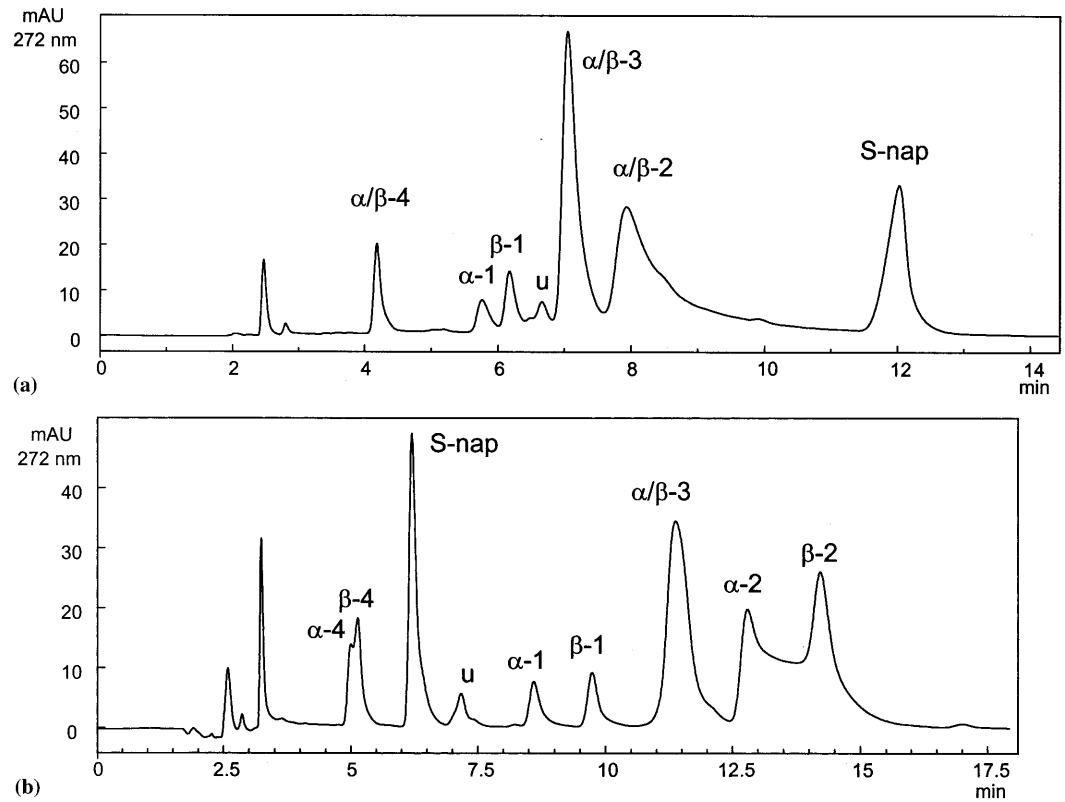

Fig. 2. (a) Chromatogram of a 6-h glucuronide isomer degradation mixture using mobile phase I: 50 mM ammonium formate buffer pH 5.75/acetonitrile 75:25 (v/v), UV-detection at 272 nm. Peaks assigned by stopped-flow NMR: α/β-4-*O*-acyl isomers (t_R 4.18 min), α-1-*O*-acyl isomer (t_R 5.76 min), β-1-*O*-acyl glucuronide (t_R 6.17 min), α/β-3-*O*-acyl isomers (t_R 7.05 min), α/β-2-*O*-acyl isomers (t_R 7.93 min) and S-naproxen aglycone (t_R 12.03 min). u: unknown impurity from starting biological material. (b) Chromatogram of a 6-h glucuronide isomer degradation mixture using mobile phase II: 25 mM phosphate buffer pH 7.40/acetonitrile 80:20 (v/v), UV-detection at 272 nm. Peaks assigned by stopped-flow NMR: α-4-*O*-acyl isomer (t_R 4.99 min), β-4-*O*-acyl isomer (t_R 5.13 min), S-naproxen aglycone (t_R 6.20 min), α-1-*O*-acyl-isomer (t_R 8.59 min), β-1-*O*-acyl glucuronide (t_R 9.73 min), α/β-3-*O*-acyl isomers (t_R 11.38 min), α-2-*O*-acyl isomer (t_R 12.79 min) and β-2-*O*-acyl isomer (t_R 14.21 min). u: unknown impurity from starting biological material.

3.2. HPLC analysis of glucuronide isomer mixture

UV-chromatograms of the 6-h degradation mixtures with assigned peaks are shown in Fig. 2a (mobile phase I) and 2b (mobile phase II). The elution order for the glucuronide isomers was unchanged when substituting H_2O with D_2O in the HPLC mobile phase buffers, based on retention times and relative peak areas using UV-detection at 272 nm. Thus, the HPLC-^{1}H NMR-assignment of the chromatographic peaks can be transferred directly to the non-deuterated mobile phases used for HPLC-UV.

Mobile phase I was based on ammonium formate buffer at pD 5.75 to be used for preliminary identification as well as being HPLC-MS compatible. pD 5.75 was chosen as the glucuronide isomers are stable at this pD, and will not interconvert once isolated in the NMR-probe. This system could separate the α/β-4-*O*-acyl isomers (t_R 4.18 min), the putative α-1-*O*-acyl isomer (t_R 5.76 min), the β-1-*O*-acyl glucuronide (t_R 6.17 min), the α/β-3-*O*-acyl isomers (t_R 7.05 min) and the α/β-2-*O*-acyl isomers (t_R 7.93 min) from S-naproxen aglycone (t_R 12.03 min). Mobile phase II was based on 25 mM phosphate buffer at pD

7.40. This system was developed in order to monitor the degradation of isolated glucuronide isomers when stopped in the flow-probe (dynamic stopped-flow HPLC-NMR) [25]. At pD 7.40 the same elution order was found for the glucuronide isomers, but S-naproxen aglycone eluted faster due to higher ionisation of the carboxylic acid group at the higher pD. Also it was possible to separate the α/β-2-*O*-acyl anomers, which was not possible at pD 5.75. The order of elution was as follows: α-4-*O*-acyl isomer (t_R 4.99 min), β-4-*O*-acyl isomer (t_R 5.13 min), S-naproxen (t_R 6.20 min), α-1-*O*-acyl isomer (t_R 8.59 min), β-1-*O*-acyl glucuronide (t_R 9.73 min), α/β-3-*O*-acyl isomers (t_R 11.38 min), α-2-*O*-acyl isomer (t_R 12.79 min), and β-2-*O*-acyl isomer (t_R 14.21 min). The high anomerisation rate between the α-2- and β-2-*O*-acyl anomers is responsible for the incomplete chromatographic separation of the α/β-2-*O*-acyl anomers, as they interconvert on-column during the chromatographic run.

When using mobile phase I (pD 5.75) for HPLC-NMR a single sample injection could be used for 1D-spectral assignment of all isomers, as no acyl-migration on-column of the late eluting isomers was observed. Thus, no signals from the α/β-3- and α/β-4-*O*-acyl isomers were detectable in the spectrum of the late eluting α/β-2-*O*-acyl isomers although the flow had been stopped several times to acquire the spectra for the α/β-4-, α-1-, β-1- and α/β-3-*O*-acyl glucuronide isomers.

When using mobile phase II (pD 7.40), new injections had to be made for every peak studied by stopped-flow NMR, as the late eluting isomers would acyl-migrate on-column during the acquisition of the spectrum of the earlier eluting peaks.

The novel α-1-*O*-acyl isomer (Corcoran 2000, in preparation) was found to be very unstable at pD 7.40 and more than 50% was isomerised to the α/β-2-*O*-acyl isomers during the 256 scans (24 min acquisition time) on the α-1-*O*-acyl glucuronide peak (t_R 8.59 min) in mobile phase II.

3.3. Identification of the glucuronide isomers

The identification of the isomers was based on the glucuronide ring protons, as they show a characteristic pattern [22]: (i) all α-anomers show H1′-signals approximately 0.5 ppm downfield compared to the corresponding β-anomer. Also, the equatorial α-anomer H1′ coupling to the axial H2′ is 2–3 Hz compared to the axial-axial coupling of 8–9 Hz of the β-anomer H1′-H2′. (ii) The signal of the H1′-proton moves upfield as the aglycone migrates down the glucuronide ring and the de-shielding effect diminishes. (iii) The signal of the proton attached to the carbon with the aglycone moves downfield due to the proximity of the de-shielding carbonyl group.

The glucuronide-ring ^{1}H NMR signals of the separated isomers in mobile phase I are assigned according to Ref. [22] in Table 1. Only minor differences in the chemical shift of the glucuronide-ring ^{1}H NMR signals between mobile phases I and II were observed (data not shown). The signals of the novel α-1-*O*-acyl isomer are assigned in Corcoran 2000 (in preparation).

As the α/β-anomers of the 2-, 3- and 4-*O*-acyl isomers were not separated using mobile phase I the recorded spectra of these isomers consist of mixtures of the α/β-anomers. Using mobile phase II the α/β-anomers of the 2-*O*-acyl isomer were separated, however, the anomerisation rate was so fast that the 256-scan spectrum taken over 24 min (stopped on the putative α-2-*O*-acyl anomer peak at t_R 12.79 min) showed a mixture of α-2- and β-2-*O*-acyl anomer. Thus, the assignment of the anomers of the 4-*O*-acyl isomer and the 2-*O*-acyl isomer as α or β in Fig. 2b is based on Sidelmann (1996) [26] and not on experimental data.

4. Discussion

Two simple isocratic reversed-phase HPLC-systems were used to separate the rearrangement isomers of S-naproxen-β-1-*O*-acyl glucuronide. For HPLC-NMR experiments isocratic systems are preferable, as the chemical shift of compounds eluting at different times do not have to be corrected for the change in solvent composition during the HPLC-run.

The elution order of the glucuronide isomers was found to be identical using both mobile phases and corresponded with the previously pub-

lished elution order for 2-, 3- and 4-fluorobenzoic acid glucuronide isomers by HPLC-^{1}H NMR [26]: α/β-4-, α/β-3-, α/β-2-*O*-acyl isomers, with the β-1-*O*-acyl glucuronide and aglycone elution order depending on the aglycone. The minor α-1-*O*-acyl isomer eluted before the corresponding β-1-*O*-acyl glucuronide in both mobile phase I and II.

The α-1-*O*-acyl isomer was not detectable in the purified biosynthetic β-1-*O*-acyl glucuronide at time zero prior to incubation, so it is not a hitherto unknown biosynthetic metabolite. Consequently, it must form spontaneously from the β-1-*O*-acyl glucuronide in solution along with the α/β-2-, α/β-3- and α/β-4-*O*-acyl isomers. Fig. 1 depicts the overall acyl migration scheme of S-naproxen-β-1-*O*-acyl glucuronide including the α-1-*O*-acyl isomer which is formed from the α-2-*O*-acyl isomer as shown in Corcoran 2000 (in preparation).

HPLC-NMR is a very powerful technique when working with unstable unknown compounds. With traditional preparative HPLC combined with off-line NMR there is a high risk that the purified fractions may degrade during the work-up. Also the resolution of preparative HPLC is usually poorer than analytical HPLC, making it impossible to separate the compounds of interest. A high-resolution analytical HPLC-UV-method can be transferred directly to the HPLC-NMR set-up with no loss of chromatographic resolution. All the time-consuming work on fraction collection and sample concentration can then be omitted.

HPLC-MS carries a number of the same advantages, however, an NMR-spectrum gives more structural information than an MS-spectrum, and in the present case with isomeric compounds having the same molecular weight no assignment could be made with MS.

The acyl-migration from the β-1-*O*-acyl glucuronide to the α/β-2-*O*-acyl isomers has been believed to be unidirectional [5], i.e. no α- or β-1-*O*-acyl isomer is re-formed from the α/β-2-*O*-acyl isomers. In this paper the chromatographic separation of the α-1-*O*-acyl isomer at a concentration of 3% of the total glucuronide-isomer material at $t = 6$ h is presented in two different HPLC-systems at pH 5.75 and 7.40. The minor 'isomer X' reported by Iwaki was possibly the α-1-*O*-acyl isomer reported here. It eluted just before the biosynthetic β-1-*O*-acyl glucuronide in the chromatograms presented (mobile phase pH 6.0), similarly to the results presented here with mobile phases at pH 5.75 and 7.40.

Table 1
^{1}H NMR chemical shifts and multiplicity of the glucuronide ring protons of the positional isomers of S-naproxen-β-1-*O*-acyl glucuronide obtained by stopped-flow 600 MHz HPLC-^{1}H NMR in deuterated ammonium formate buffer pD 5.75/MeCN 75:25 v/v[a]

Isomer	t_R (min)	1′	2′	3′	4′	5′
α-4	4.18	5.15 d	3.57 (2) m	3.78 t	4.86 (2) t	(1)
β-4	4.18	(1)	3.57 (2) m	3.74 d	4.86 (2) t	4.09 d
β-1	6.17	5.46 d	3.39 (3) t	3.47 t	3.39 (3) t	3.66 d
β-3	7.05	4.57 d	3.58 (2) d	4.93 t	3.20 t	3.67 d
α-3	7.05	5.10 d	3.58 (2) d	5.11 t	3.50 dd	4.02 d
α-2	7.93	5.29 d	4.66 (2) m	3.80 t	3.47 (2) t	3.99 d
β-2	7.93	4.62 dd	4.66 (2) m	3.51 t	3.47 (2) t	3.60 d

[a] 1, obscured by HDO signal at δ 4.45 ppm; 2, signals between α- and β-anomers not resolved; and 3, 2H′ and 4H′ signals at δ 3.39 ppm not resolved.

This work shows the value of the direct coupling of HPLC to NMR spectroscopy when dealing with reactive metabolites and illustrates the general characteristic pattern of glucuronide isomer elution order in reversed phase HPLC, irrespective of the aglycone drug structure.

Investigators of acyl glucuronide reactivity should consider the possible formation of a α-1-*O*-acyl isomer when handling their samples and analysing their NMR- or HPLC-data.

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