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Chemical reactivity of the naproxen acyl glucuronide and the naproxen coenzyme A thioester towards bionucleophiles

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Abstract

Drugs may be metabolised to reactive electrophilic species that spontaneously react with proteins. The presence of such drug–protein adducts has been associated with drug toxicity. In this study, the reactivity of the major metabolite of naproxen—the 1-β-*O*-glucuronide (Nap-GlcU)—was compared to the corresponding naproxen coenzyme A (Nap-CoA) thioester. The reactivity of the two metabolites was assessed in vitro in a phosphate buffer (pH 7.4; 0.1 M) at 37 °C towards the model bionucleophiles glutathione and human serum albumin (HSA). The reaction between the electrophilic species (Nap-GlcU and Nap-CoA) and glutathione forming the Nap-glutathione conjugate was monitored using LC–MS–MS and LC–UV, respectively. It was shown that Nap-CoA resulted in an approximate 100-fold higher formation of Nap-glutathione conjugate than Nap-GlcU. The presence of Nap-CoA also resulted in acylated HSA with a rate and a yield that was significantly higher than reported for Nap-GlcU. In summary, the data suggest that CoA metabolites may be more reactive species than acyl glucuronides that previously have been associated with severe drug related side effects in vivo.

Keywords: Reactive drug metabolites; Drug protein adduct formation; Naproxen; Acyl glucuronides; Xenobiotic coenzyme A thioesters; LC–MS–MS

1. Introduction

Important non-steroidal anti-inflammatory drugs (NSAIDs) such as naproxen (Nap), tolmetin and ibuprofen are all drugs containing a carboxylic acid group for which glucuronidation is a major metabolic pathway. Glucuronidation of carboxylic acids yields acyl glucuronides and the reactivity of these conjugates has been extensively investigated during the last two decades [1–8]. Literature reveals that some acyl glucuronides react in vivo with proteins resulting in acylated proteins and the presence of these drug–protein adducts has been suggested to cause allergic reactions [9] and hepatotoxicity [10].

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Acidic drugs may also be metabolised to other products that are more electrophilic in nature than glucuronides, e.g. to coenzyme A (CoA) thioesters, which are formed by acyl-CoA synthetases [11]. Acyl-CoA thioesters of xenobiotica have been shown to interfere with several biochemical pathways where the endogenous fatty acid CoA thioesters usually serve as substrates [12–15]. In addition to these pathways, xenobiotic CoA thioesters have also been shown to be intermediates in the in vivo chiral inversion from the *R*- to the *S*-enantiomer of several drugs that belong to the 2-propionic acid derivatives (the profens), a process being catalysed by racemases [16–18]. The pathways that xenobiotic CoA thioesters may enter are summarised in Fig. 1.

The role of xenobiotic acyl-CoA thioesters in acylation processes such as drug–protein adduct formation and acylation of other bionucleophiles is—compared to acyl glucuronides—a relatively unexplored field at the moment. CoA thioesters of endogenous fatty acids are known to acylate proteins and thereby modify the function of proteins. However, whether this process is spontaneous or enzyme mediated still remains to be elucidated [19,20]. Being more electrophilic in nature than acyl glucuronides, xenobiotic CoA thioesters may also contribute to acylation of endogenous proteins. Sallustio et al. [21] have shown that human liver proteins were acylated in the presence of nafenopin-CoA. This recent finding suggests that CoA thioesters may be chemically reactive species, but enzyme mediated acylation could not be entirely ruled out.

In this study, glutathione and human serum albumin (HSA) were chosed as models for potential endogenous nucleophiles for investigation of the spontaneous reaction with the naproxen acyl glucuronide (Nap-GlcU) and the corresponding CoA thioester (Nap-CoA).

2. Experimental

2.1. Materials

S-Nap, ketoprofen, trypsin (TPCK treated), HSA (essentially fatty acid free fraction V) and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*,*N*-carbonyldiimidazol was obtained from Fluka Chemie AG (Buchs, Switzerland), and coenzyme A was supplied by Applichem (Darmstadt, Germany) as the trilithium dihydrate salt. All other chemicals were

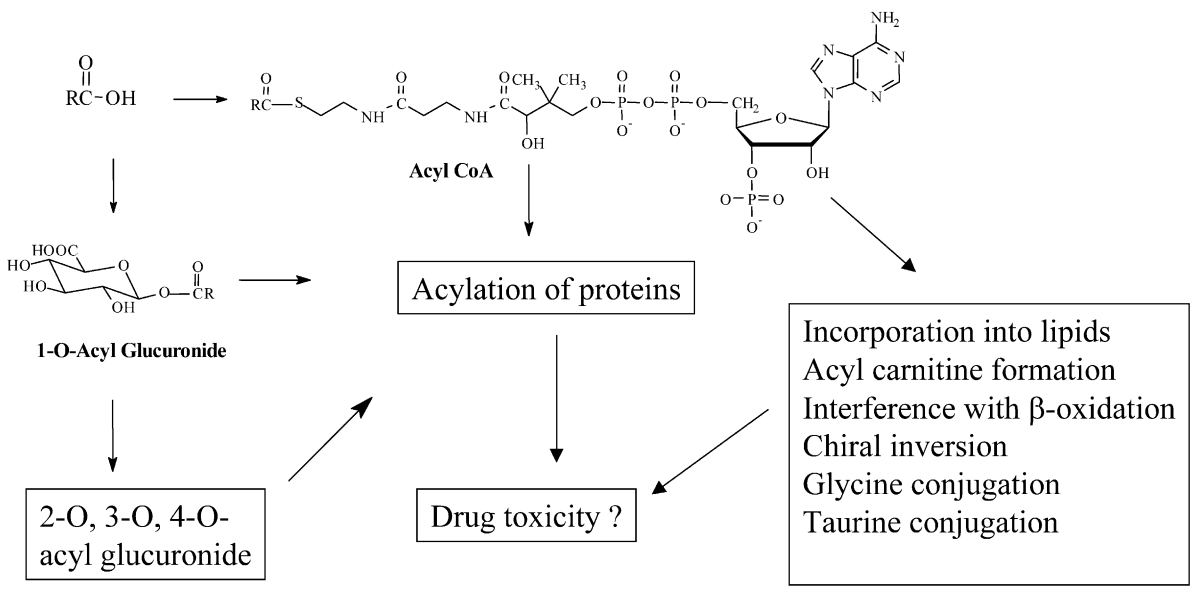

Fig. 1. Potential phase two metabolic routes of xenobiotic carboxylic acids and reactions of acyl glucuronides and acyl-CoA.

of analytical or HPLC grade. The 1-*O*-acyl-glucuronide of Nap was synthesised and provided by Mortensen [22]. HiSEP (500 mg) solid phase extraction (SPE) cartridges were provided by Supelco (Sigma–Aldrich, Stockholm, Sweden) and Ultrafree-CL Centrifugal Filters, 2 ml, were purchased from Millipore (Bedford, MA, USA).

2.2. Methods

2.2.1. Synthesis of Nap-CoA

Synthesis of Nap-CoA was performed using the procedure described by Kawaguchi et al. [23] and the synthesis product was purified by preparative HPLC as described by Tracy and Hall [16]. ESI–MS–MS in positive mode of the purified compound confirmed the structure of Nap-CoA. Characteristic fragments of $[M + H]^+$ (m/z 980.6) were m/z 571.4, 473.4 and 371.3.

2.2.2. Reactivity of Nap-CoA and Nap-GlcU towards glutathione

Incubation conditions: 0.5 mM Nap-GlcU or 0.5 mM Nap-CoA was incubated with 5 mM glutathione at 37 °C. Incubation medium was a phosphate buffer (pH 7.4; 0.1 M) with 5 mM EDTA added. Each incubation experiment with Nap-GlcU and Nap-CoA was performed in triplicate and samples were analysed using LC–MS–MS or LC–UV, respectively.

Half lives of Nap-GlcU and Nap-CoA were determined when incubated in a phosphate buffer (pH 7.4; 0.1 M) at 37 °C.

2.2.3. Reactivity of Nap-CoA towards HSA

The reaction of 0.5 mM Nap-CoA with 0.45 mM HSA was initially performed for 6 h at 37 °C in a phosphate buffer (pH 7.4; 0.1 M). The modified protein was partly purified for Nap using SPE where the reaction mixture was acidified with acetic acid and applied to a HiSEP SPE cartridge. HSA eluted in the application step, whereas Nap was retained by the C-18 sorbent. The fraction containing HSA and the following wash with water (total recovery was 97%, $n = 6$) were combined and subjected to three ultrafiltration cycles after dilution with Tris buffer (pH 8.0; 0.1 M) and the tryptic digest was performed according to Ohmacht et al. [24]. The tryptic digest was analysed using HPLC with UV and fluorescence detection.

To investigate the reaction further, 200 μM Nap-CoA was incubated in a phosphate buffer (pH 7.4; 0.1 M) containing 0.45 mM HSA. At given reaction times, 100 μl of 4% acetic acid in acetonitrile (MeCN) was added to 50 μl of the incubation medium to precipitate the protein ($n = 4$ for each time point). The protein was washed extensively to remove non-specifically bound Nap and Nap-CoA according to the procedure described by Sallustio et al. [21]. To hydrolyse the drug–protein adducts, the pellet was dissolved in 0.2 ml 1 N KOH for 8 h at 80 °C. The samples were cooled to room temperature before adding 20 μl of 85% phosphoric acid and internal standard (5 μg ketoprofen). Extraction of released Nap and internal standard was performed with 2×2 ml of dichloromethane. The organic phase was evaporated and reconstituted in 200 μl of mobile phase and analysed using HPLC. Standards were produced by adding Nap and internal standard to the dried pellet and subsequently the standards underwent the same extraction procedure as the samples which resulted in a linear standard curve ($r^2 > 0.99$) from 10 to 200 μM.

2.2.4. HPLC systems

2.2.4.1. Analysis of Nap-CoA, Nap-glutathione conjugate, Nap-GlcU and Nap. Analytical separations were performed on a Hewlett-Packard 1050 system equipped with a column (120×4.6 mm i.d.) packed with Apex 1 ODS-2, 5 μm (Jones Chromatography, Hengoed, UK). The mobile phase comprised MeCN–ammonium acetate (pH 6.0; 10 mM) (20:80, v/v). The flow rate was 1.0 ml/min and 20 μl was injected onto the column. UV detection was performed at 232 nm, which is the UV max for Nap where the contribution from glutathione, CoA and glucuronic acid was negligible. The retention factor (k) for Nap-CoA was 1.9, for Nap 3.3 and for Nap-glutathione 4.7. The Nap-GlcUs eluted between $k = 0.4$ (4-*O*-isomer) and $k = 6.1$ (2-*O*-isomer). For quantification of Nap and the internal standard in the protein binding experiments, the mobile phase was altered

to MeCN–ammonium acetate (pH 5.0; 10 mM) (30:70, v/v) in order to separate Nap and ketoprofen. k for ketoprofen was 1.9 and for Nap 3.3.

2.2.4.2. LC–MS–MS of glutathione conjugates from incubation studies with Nap-GlcU. The analytical column was a Waters Xterra (C-18, 150 × 4.6 mm i.d) column. The mobile phase consisted of MeCN–water–HCOOH (34:66:1, v/v/v). The flow rate was 0.8 ml/min and UV detection was performed at 232 nm. 30 μl was injected and the flow was split 1/20 to the MS detector where selected reaction monitoring (SRM, m/z 391) was used for quantification. Between each run, the column was washed with MeCN–water–HCOOH (90:10:1, v/v/v) to elute Nap and the column was then re-equilibrated for 10 min. For the LC–MS–MS experiments, signal suppression/enhancement effects were assessed and the recovery ($n = 3$) was found to be 97% at one concentration level (1.72 μM). The repeatability ($n = 5$) was assessed at three concentration levels using purified Nap-glutathione conjugate as standard. The RSD was 11% at 0.172 μM, 2.9% at 1.72 μM and 2.5% at 8.6 μM. The linearity ($n = 2$ at each level) was assessed from 0.34 to 8.6 μM and r^2 was 0.9997. The detection limit ($S/N = 3$) was estimated to 0.02 μM.

2.2.4.3. Analysis of tryptic digest of HSA. For analysis of the tryptic digest of drug–protein adducts, a Hewlett-Packard 1100 system equipped with a Jupiter column (150 × 2.0 mm i.d., Phenomenex) was used. The mobile phases consisted of mobile phase A: MeCN–water–TFA (5:95:0.1, v/v/v) and mobile phase B: MeCN–water–TFA (50:50:0.1, v/v). A linear gradient was run from 100% A to 100% B over 80 min with a flow rate of 0.2 ml/min. The column temperature was 40 °C and UV detection at 214 nm was used for detection of all tryptic peptides. For the selective detection of Nap-containing peptides, fluorescence detection (excitation 331 nm/emission 353 nm) was applied.

2.2.5. Mass spectrometry

MS experiments were performed on a Finnigan MAT LCQ ion trap (San Jose, CA, USA) using ESI in positive mode. MS and MS–MS conditions for verifying the structure of Nap-CoA from the organic synthesis as well as the structure of the Nap-glutathione conjugate were: sheath/auxillary gas (60/0), spray voltage: 4.5 kV, temperature of the heated capillary: 200 °C, capillary voltage: 3 V. The compounds were infused directly into the MS using a flow rate of 3 μl/min.

For the LC–MS–MS determination of Nap-glutathione conjugate, the MS conditions were: sheath/auxillary gas (80/20), spray voltage: 4.5 kV, temperature of the heated capillary: 250 °C, capillary voltage: 3 V. For MS–MS experiments, m/z 520 was fragmented to m/z 391 using a relative collision energy at 25. m/z 391 was used for quantification.

3. Results and discussion

Numerous studies have been performed on acyl glucuronides of NSAIDs and their reactivity towards proteins. Some of the reported techniques were applied in this study using glutathione [25] and HSA [1–7] as model biomolecules. Glutathione is an endogenous tripeptide that reacts with electrophilic substrates—spontaneously or enzyme mediated—which in this study provided a simple system for studying chemical reactivities. As model electrophilic metabolites, the main metabolite of *S*-Nap, the 1-β-*O*-acyl glucuronide, was chosen and compared with the CoA thioester to study similarities/differences in chemical reactivities of such two species.

3.1. Analysis of Nap-glutathione conjugate

The reaction of glutathione with Nap-CoA was monitored using HPLC. For the reaction with Nap-CoA, HPLC with UV detection was found to be applicable for monitoring the transthioesterification process since Nap-CoA was readily separated from the glutathione product. MS and MS–MS of the collected glutathione conjugate is shown in Fig. 2a and b, respectively.

LC–MS–MS was used to detect the small amounts of glutathione conjugate that was formed in the reaction of Nap-GlcU with glu-

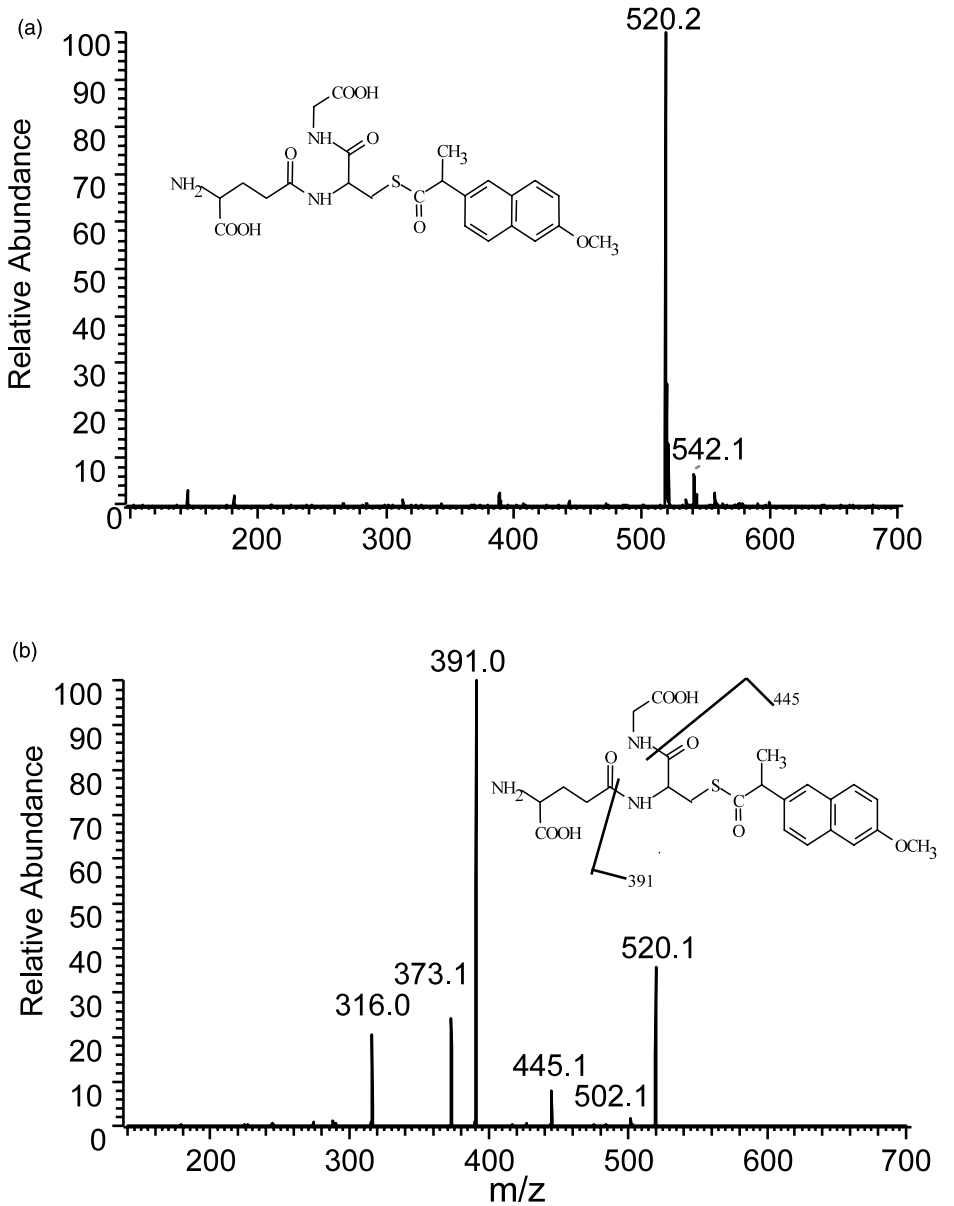

Fig. 2. (a) MS spectrum of the Nap-glutathione conjugate. (b) MS–MS spectrum of the Nap-glutathione conjugate. Experimental details are found in Section 2.

tathione and which in most LC systems co-eluted with acyl glucuronide isomers/anomers. However, when the glutathione conjugate co-eluted with the abundant acyl glucuronide isomers/anomers, the recovery of the Nap-glutathione conjugate was found to be approximately 400% ($n = 3$) due to a signal enhancement effect. Thus, in order to quantify the Nap-glutathione conjugate, the chromatographic conditions were altered as shown in Fig. 3, where the Nap-glutathione conjugate was separated from the acyl glucuronide isomers/anomers. The recovery was found to be 97% in this system.

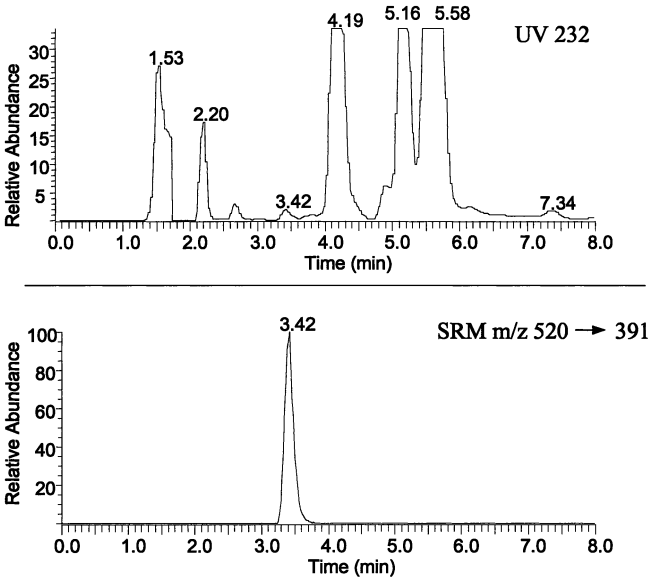

Fig. 3. LC–MS–MS chromatogram of 0.5 mM Nap-GlcU incubated with 5 mM glutathione after 12 h. Top chromatogram shows the UV trace at 232 nm and the buttom chromatogram represents LC–MS–MS, SRM (m/z 520 → 391). Other experimental details are found in Section 2.

Other relevant validation data are listed in Section 2.

3.2. Reactivity of Nap-GlcU and Nap-CoA towards glutathione

The aim of the study was to compare the chemical reactivity of Nap-GlcU and Nap-CoA towards glutathione. Initially, it was investigated if the presence of glutathione changed the overall degradation, acyl migration and hydrolysis rates of the acyl glucuronide, but we could not detect any differences in degradation. Subsequently, the formation of Nap-glutathione conjugate from the reaction of Nap-CoA or Nap-GlcU with glutathione was determined which is shown in Fig. 4. From the figure, it is evident that the reaction of Nap-CoA with glutathione resulted in the highest formation of the glutathione conjugate, the difference being approximately 100-fold in binding yield.

Previously, Shore et al. have reported that the clofibrioyl acyl glucuronide could react with glutathione covalently in vitro and they were furthermore able to detect the formed metabolite in the urine in a very low concentration [25]. In contrast to Shore et al., we did not find any difference in the overall degradation of 1-β-*O*-acyl glucuronide, nor in the acyl migration rates when glutathione was present. However, the binding yield was similar to that found for the clofibrioyl-

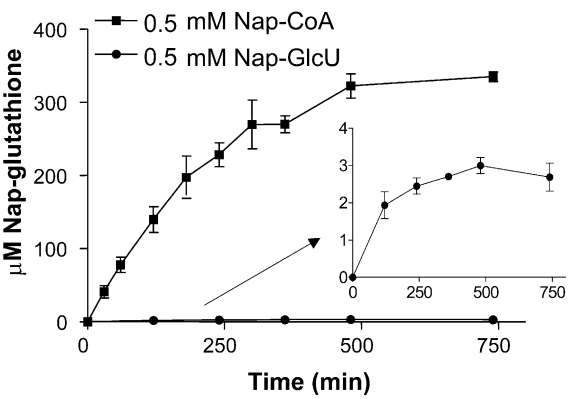

Fig. 4. Formation of Nap-glutathione conjugate from incubation of 0.5 mM Nap-CoA (squares) or 0.5 mM Nap-GlcU (circles) with 5 mM glutathione in a phosphate buffer pH 7.4 at 37 °C. Incubations were performed in triplicate.

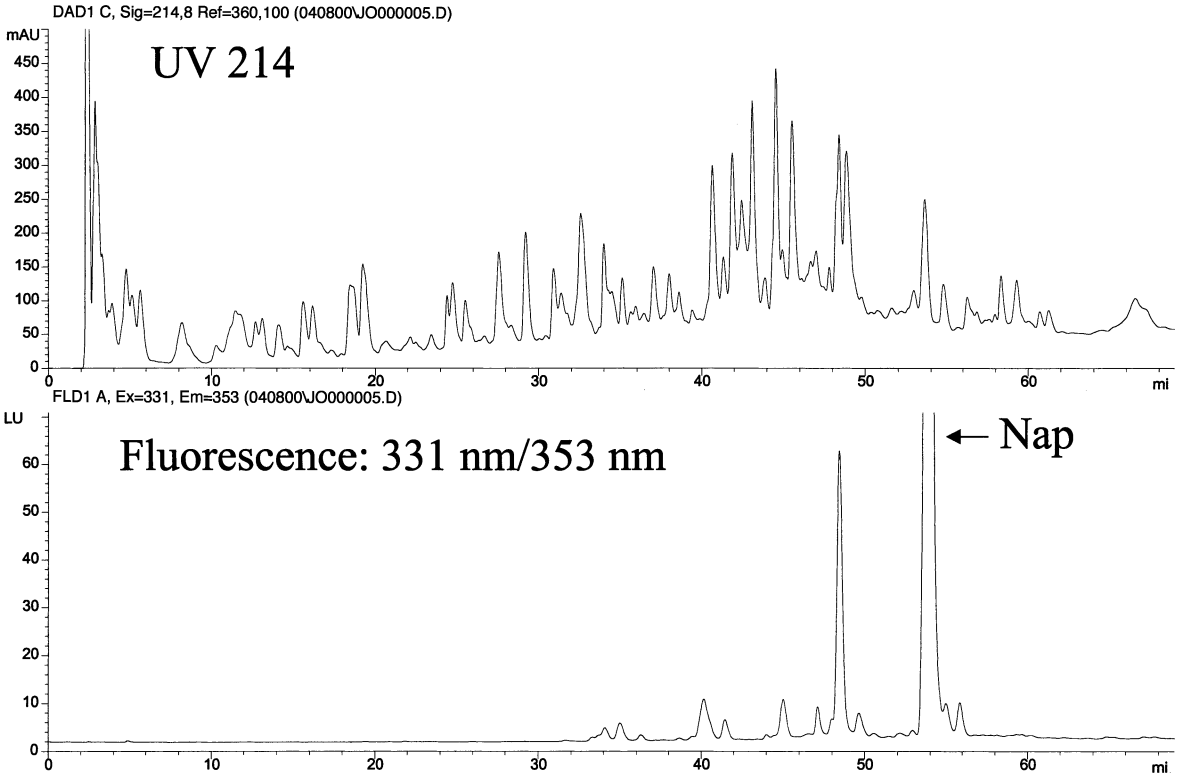

Fig. 5. Chromatogram showing a tryptic digest of HSA that was covalently modified with Nap from 6 h incubation of 1 mM Nap-CoA thioester with 0.45 mM HSA in a 0.1 M phosphate buffer (pH 7.4) at 37 °C. UV detection was performed at 214 nm for detection of all tryptic peptides and for the selective detection of Nap-containing peptides, fluorescence detection (excitation 331 nm/emission 353 nm) was applied. Other experimental details are found in Section 2.

glucuronide which was 0.5% of the initial molar concentration of the acyl glucuronide after 300 min of incubation.

3.3. Covalent modification of a model protein

Nap-CoA was incubated with HSA and a tryptic digest of the drug–protein adduct was performed which provided a preliminary overview of the extent of adduct formation and the number of amino acids involved in the covalent modification of the protein. Fluorescence detection was applied to the study to selectively detect Nap that was covalently bound to the resultant peptides from the tryptic digest. This was confirmed analysing a blank sample where Nap was added to 0.45 mM HSA in incubation buffer instead of Nap-CoA and which resulted in a chromatogram with one peak corresponding to Nap (data not shown).

Several peptides, that were modified with Nap, were observed. It appeared that especially one amino acid was involved in the reaction with Nap-CoA (Fig. 5). Based on the reaction of Nap-CoA with glutathione, the single free cysteine (Cys 34) on HSA may be considered to be involved in the covalent binding which would be in agreement with observations reported in the literature on palmitoyl acyl-CoA which acylates cysteines in posttranslational processes [19]. Additionally, Sallustio et al. have reported that nafenopin-CoA predominantly modified liver proteins via thioester and amide linkages [21]. Further investigations are in progress to identify the drug–protein adducts on the amino acid level. Since numerous studies already have been

performed on acyl glucuronides and their binding sites on HSA, this will allow us to compare the binding pattern of CoA thioesters with acyl glucuronides [5,26–28].

The results from a more detailed study of the reaction of Nap-CoA (200 μM) with HSA (0.45 mM) in a phosphate buffer (pH 7.4; 0.1 M) are shown in Fig. 6. The adduct formation was rapid and the maximum concentration was observed after 30 min where the concentration of Nap–HSA was 130 μM which corresponds to 280 mmol Nap/mol protein. The subsequent decrease in adduct concentration indicated that some of the adducts were labile.

As seen from Fig. 6, Nap-CoA degraded rapidly in the presence of HSA which was in contrast to the half life of Nap-CoA in a 0.1 M phosphate buffer (pH 7.4) where only 2% was hydrolysed after 72 h. The half life of Nap-GlcU was in a similar study determined to 210 min and when adding HSA (0.45 mM), the half life has been reported to decrease fourfold [1], i.e. that the degradation of Nap-GlcU was not enhanced as much as seen in this study for Nap-CoA in the presence of HSA.

In the literature, the maximum adduct formation for the *S*-Nap acyl glucuronide has been reported to be 1 mmol Nap/mol protein, a binding yield that was reported to be constant from 2 to 24 h when incubating 50 μM of *S*-*O*-acyl-Nap glucuronide in the same incubation buffer as used in this study [1]. In another study on Nap-GlcU, it was shown that the binding yield was approximately proportional to the initial concentration of Nap-GlcU [2]. Despite the fact that a fourfold higher concentration of Nap-metabolite (Nap-CoA) was used in this study, it may be concluded that the binding yield was significantly higher than reported for the corresponding Nap-GlcU.

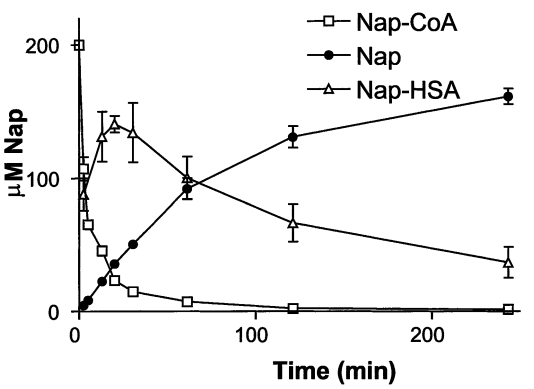

Fig. 6. Profiles of Nap-CoA (squares), Nap–protein adducts (Nap-HSA, triangles) and released Nap (circles) from incubation of 200 μM Nap-CoA with 0.45 mM HSA in 0.1 M phosphate buffer (pH 7.4) at 37 °C ($n = 4$).

4. Conclusions

Our model systems for comparing the chemical reactivity of Nap-GlcU with Nap-CoA show that the CoA thioester was more reactive than the acyl glucuronide in the spontaneous reaction with potential bionucleophiles such as peptides and proteins. The study suggests that even small levels of xenobiotic-CoA thioesters may lead to the same degree of acylation in vivo as a compound that is being extensively glucuronidated. Potential adduct formation may take place in the liver where the acyl-CoA synthetases, that catalyse CoA thioester formation, are located and this may explain some adverse effects in the liver that are associated with the intake of some acidic drugs. However, extensive work on acylation of biomolecules by CoA thioesters of drug substances containing carboxylic acid groups is needed to clarify the possible role of xenobiotic-CoAs as a cause of drug side effects.

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