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Short communication

NMR spectroscopic studies of the transacylation reactivity of ibuprofen 1-β-*O*-acyl glucuronide

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Abstract

The products arising from the intra-molecular acyl migration reactions of drug ester glucuronides can be reactive towards cellular proteins and have been proposed to cause toxic side effects. The relative reactivity of a range of drug and model glucuronides have previously been determined by measuring the rate of disappearance of a peak characteristic of the 1-β-*O*-acyl glucuronide using ^{1}H NMR spectroscopy. Here the degradation rate of ibuprofen 1-β-*O*-acyl glucuronide has been investigated using NMR spectroscopy for the first time using material isolated from human urine with solid-phase extraction chromatography (SPEC). The degradation rate was measured by following the disappearance of the ^{1}H NMR signal from the 1-β-anomeric proton of the glucuronic acid moiety as the reaction progressed in pH 7.4 buffer inside an NMR tube. The measured degradation rate represents a pseudo-first order rate constant, a combination of the transacylation rate (1-β-isomer to 2-β-isomer) and the hydrolysis rate, and is presented as a half-life of 3.5 h. This value is compared to those from drug glucuronides where adverse effects have been observed in patients after administration of the drug.

Keywords: Ester glucuronide; Transacylation; SPEC; NMR

1. Introduction

There is a continuing interest in the transacylation reactivity of drugs, which form ester glucuronides that might be associated with toxicity. The development of quantitative structure-reactivity relationships (QSRRs) for this group of compounds could be of potential benefit when designing new drugs as with an appropriate model it might be possible to eliminate the potential for reactivity from new compounds. As part of research into the QSRRs of these metabolites, the degradation rates of a series of model glucuronides (based on substituted benzoic acids) have been determined and the results used to build several promising QSRRs based on both experimental and calculated properties, e.g. ^{13}C chemical shifts, partial atomic charges on the ester carbonyl carbons and the Hammett substituent parameter, σ_p [1,2].

Drugs with a carboxylic acid functional group, such as the non-steroidal anti-inflammatory drug (NSAID) ibuprofen, can readily form 1-β-*O*-acyl glucuronides in vivo, and this represents a major route for the metabolism and thence elimination of such compounds [3–5]. Depending on the nature of the aglycone, 1-β-*O*-acyl glucuronides can be unstable in aqueous solution under acidic, neutral and alkaline pH conditions and exhibit a range of reactivities [4]. Under physiological conditions, at pH 7.4, the carbonyl carbon of the acyl ester is susceptible to nucleophilic attack. The 1-β-*O*-acyl glucuronide can hydrolyse, liberating the aglycone, but importantly the molecule can undergo an intramolecular rearrangement, whereby the hydroxyl group at C2 of the glucuronic acid moiety can attack the acyl carbonyl carbon to form a cyclic *ortho*-ester intermediate. This can then ring open to form the 2-*O*-acyl ester. The acyl migration reaction can then proceed in a similar fashion to produce 3-*O*- and 4-*O*- position esters. The glucuronide ring of 2-, 3- and 4-*O*-acyl positional isomers can open and recyclise via the aldehyde form, enabling mutarotation to occur yielding the α- and β-anomers of the iso-

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mers. Experiments have also shown the formation of 1-α-*O*-acyl glucuronides in vitro and these are presumably formed from a α-2-*O*-acyl glucuronide (themselves formed after transacylation from the 1-β-*O*-acyl anomers followed by mutarotation of the β-2-*O*-acyl isomers) [6]. The analogous reaction of formation of 1-β-*O*-acyl from the β-2-*O*-acyl isomers (again via the cyclic *ortho*-ester intermediate with 1,2-*trans*-(eq,eq) fused rings) cannot be ruled out in light of this new evidence, and was first proposed on the basis of HPLC/UV analysis of diflunisal 1-β-*O*-acyl glucuronides and enzyme hydrolysis experiments [7]. The full reaction scheme has been shown several times previously [1,2].

The resulting transacylation equilibrium mixture contains six positional isomers and anomers, plus residual amounts of the 1-β-*O*-acyl/1-α-*O*-acyl isomers formed by back-reaction. In addition, all of these isomers are, irreversibly, undergoing hydrolysis. The resulting glucuronides (particularly the isomers in the 3- and 4-position) may act as haptens and stimulate immune responses/allergic reactions in vivo as they can react to form covalently bound adducts with cellular macromolecules, via imine formation/Amadori rearrangement or nucleophilic displacement [3–5]. Circumstantial evidence suggests that drugs that undergo these rapid acyl migrations appear to be related to the incidence of hypersensitivity in clinical use, and these reaction products have also been linked to hepatotoxicity [5,8]. Measurable covalent binding to human serum albumin in vitro and to mouse hepatic proteins by the 1-β-*O*-acyl glucuronides of a number of drugs has been shown [8–11].

For the simplest situation in this reaction scheme, the chemical degradation of the 1-β-*O*-acyl glucuronide, there are two parallel reactions, namely acyl migration which is a unimolecular first order process, and hydrolysis which is a second order reaction but because $[H_2O] \gg$ [1-β-*O*-acyl glucuronide] this is also essentially a first order process. The rate at which the 1-β-*O*-acyl component is depleted – the degradation rate (k_d) – is therefore the sum of these two reaction rates. This rate can be easily measured by following the disappearance of the ^{1}H NMR resonance arising from the anomeric proton of the 1-β-*O*-acyl isomer as the reaction progresses in an NMR tube at pH 7.4.

HPLC could also be used to monitor the reaction using UV detection but that this does not provide the molecular identification that the somewhat slower NMR method can yield. In addition, for drug glucuronides of widely varying log *P* values, different HPLC conditions have to be used and this can make comparisons between compounds difficult and thus can minimise the usefulness of the results for structure-reactivity relationship development.

The metabolism of ibuprofen has been studied extensively and the major metabolic products have been identified as the oxidation products hydroxyl-ibuprofen and carboxyl-ibuprofen and the acyl glucuronides of these as well as of ibuprofen itself [12,13]. The drug is administered as a racemate but there is considerable epimerisation in vivo and the ratio of *S*-ibuprofen to *R*-ibuprofen glucuronide in plasma is about 7.1 [14]. It has been shown in vitro that ibuprofen glucuronide is labile and can form covalent adducts with proteins, and in the case of elderly patients receiving long term administration of ibuprofen, covalent binding to plasma proteins has been shown in vivo [15].

Here we have measured the degradation rate of ibuprofen glucuronide in vitro and have compared the value obtained with those from other NSAID glucuronides.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich Ltd. (Dorset, UK) and used as received. C18 mega Bond-Elut™ SPEC columns (12 ml reservoir volume/2 g sorbent mass) were obtained from Varian Ltd., (Walton-on-Thames, UK).

A sample of urine was collected 7 h following administration of a single oral dose of 200 mg of ibuprofen to a healthy male subject. The sample (∼300 ml) was then acidified to pH 5.6 using 0.1 M hydrochloric acid and stored at −40 °C until required. A C18 Bond-Elut® column (Varian) was activated prior to sample application by first washing with one column volume (∼12 ml) of methanol followed by two column volumes (∼25 ml) of 0.1 M hydrochloric acid. A 12 ml aliquot of the sample was then applied to the column, which was washed with 2 ml × 12 ml of 0.1 M hydrochloric acid. The non-retained urine and acid washings were discarded. The retained material was then eluted using a step-wise gradient with 12 ml of each of the following mixtures (v:v) of 0.1 M hydrochloric acid and methanol: 80:20, 60:40, 40:60, 20:80 and 0:100. Each fraction was collected separately and the solvent removed under a stream of nitrogen. Once dry, the residues were redissolved in 0.5 ml of deuterated methanol and analysed by ^{1}H NMR spectroscopy at 600 MHz. The only samples which contained glucuronides, as shown by characteristic resonances for the anomeric protons, were the last two fractions, 20:80 (HCl:MeOH v/v) and 0:100 (HCl:MeOH v/v).

2.2. Degradation rate measurements

NMR measurements were carried out using a Bruker Avance 600 spectrometer operating at 600.13 MHz for proton detection and equipped with an inverse triple resonance (TXI) 5 mm probe. In order to obtain results quickly on glucuronide samples, a blank sample was measured first. This contained pH 7.4 buffer (0.5 ml) plus D_2O/TSP (0.05 ml 0.1 mg/ml solution) with a total volume equal to that of the test sample (0.55 ml). TSP (3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionic acid, sodium salt is a standard material for NMR chemical shift referencing and for quantitation. Carrying out this procedure ensured that the initial field homogeneity settings (shims) were optimised on a sample as similar as possible to the glucuronide sample being analysed subsequently, thereby allowing faster measurement of the first spectrum in the series.

A 5 ml of eluent from the C18 SPEC extraction (80% methanol fraction), pH adjusted to a value of ∼6.0, was used to prepare the sample, which was freeze-dried overnight. A buffer solution (0.5 ml of pH 7.4 buffer–100 mM phosphate in H_2O) was quickly added to the prepared residue and the time noted

using the spectrometer software (this time was used as the starting point for the rate measurement and compared with starting times of subsequent NMR acquisitions). The buffered solution (0.5 ml) was transferred to a 5 mm NMR tube containing 0.05 ml D_2O/TSP (0.05 ml 0.1 mg/ml solution).

The prepared NMR sample was quickly placed in the spectrometer magnet and allowed to reach thermal equilibrium at 310 K for 10 min. The sample was then locked and shimmed and a test spectrum acquired using a standard presaturation solvent suppression pulse sequence (NOESYPR1D) into 64k data points over a spectral width of 10 ppm. Once the shimming and solvent suppression were satisfactory (usually taking between 15 and 20 min after addition of buffer), automated acquisition of a series of 1D spectra was carried out using standard Bruker software. This program acquired 16 scans every 3 min for the first 60 experiments after which time a spectrum was acquired every 15 min until the initial concentration of the glucuronide, as measured by the area of the peak from the 1-β-anomeric proton, had approximately halved. During acquisition and measurement, two axial shims (z_1 and z_2) were monitored and automatically adjusted by the Bruker software. This was necessary in order to maintain magnet homogeneity during overnight experiments.

Following acquisition, the first experiment was phase-corrected, zero-filled once to 64k data points and treated with an exponential window function equivalent to a line broadening of 0.1 Hz. The same phase parameters (and data treatment) were then used on all subsequent experiments for the rate measurement. Analysis of the resulting spectra and measurement of the rate of decrease in intensity of the 1-β-anomeric proton doublet peak area allowed the degradation rate constant (k_d) and half-life ($t_{1/2}$) to be calculated from a plot of $\log_e$[H1 1-β-*O*-acyl integral value] versus NMR data set acquisition start time (hours) using Microsoft Excel, where k_d was given by the slope of the line and $t_{1/2} = \log_e(2)/k_d$. It was found to be sufficient to analyse every tenth spectrum, up to and including the last spectrum acquired, in order to describe the degradation curve adequately. The TSP chemical shift was set to 0 ppm and the integral set to a value of one unit at the beginning of each analysis. This, in combination with keeping both the NMR acquisition and processing parameters identical for each analysis, allowed an easy comparison of the results from each experiment within this dataset. Extraction of the acquisition start-time from the appropriate Bruker data file was achieved for each experiment by use of a Unix c-shell script.

3. Results

The 1-β-*O*-acyl glucuronide of ibuprofen was isolated using solid-phase extraction chromatography (SPEC) and characterised by NMR spectroscopic analysis [1]. After administration of racemic ibuprofen it has been reported that the drug excreted in human urine is largely in the (*S*)-form, due to chiral inversion in vivo. If both *R*- and *S*-ibuprofen glucuronides had been present in the sample (as a diastereoisomeric mixture) [14] a pair of doublets would have been observed for the anomeric proton resonances, since these are clearly resolvable although there is some overlap of the J-coupled multiplets as shown by previous work on the chemically-related flurbiprofen [16] and ketoprofen [17]. However, from the measurements made here, the resonance observed for the anomeric proton showed only a single doublet. It is therefore likely to be the case that the proportion of the *R*-ibuprofen glucuronide in this sample was very low. The other NMR signals that account for around 20% of the spectral intensity do not arise from *R*-ibuprofen glucuronide and, based on their NMR chemical shifts, almost certainly arise from other known minor metabolites of ibuprofen, namely the hydroxy- and carboxy-metabolites formed from the *sec*-butyl moiety, and their respective glucuronides and this also accounts for some of the extra methyl doublet peaks seen. Fig. 1 shows the ^{1}H NMR spectrum for the 80% methanol fraction with the

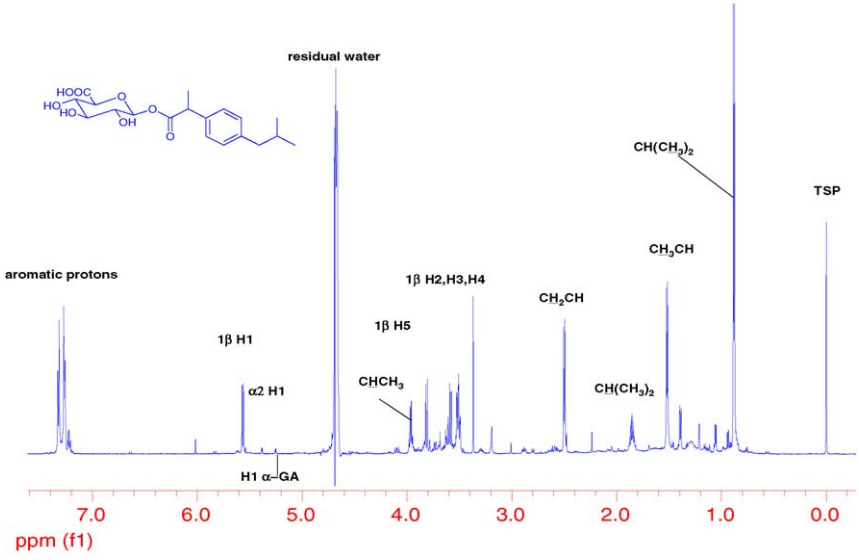

Fig. 1. The 600 MHz ^{1}H NMR spectrum of ibuprofen 1-β-*O*-glucuronide extracted from human urine, with peaks assigned as shown. GA: glucuronic acid, TSP: 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionic acid sodium salt.

relevant peaks labelled for the sugar ring. These were confirmed by inspection of the results from a 2D TOCSY spectrum and allowed assignment of the signals that make an unbroken chain of coupling to the 1-β-proton signal from the ^{1}H-^{1}H TOCSY NMR experiment.

As can be seen from Fig. 1, the major component (>80%) is the expected β-1-*O*-acyl glucuronide of ibuprofen with distinctive NMR resonances at δ 0.88 ($2CH_3$), δ 1.52 (CH_3), δ 1.85 (CH of *sec*-butyl group), δ 2.50 (CH_2), δ 3.96 (CH of $CHCH_3$ moiety), δ 5.57 (H1 of glucuronide), δ 3.81 (H5 of glucuronide), δ 3.5–3.60 (H2, H3, H4 of glucuronide), δ 7.27–7.32 (aromatic protons). In addition, a second ibuprofen-related substance is present at about 15% of that of the main glucuronide as estimated from the relative areas of the CH_3 doublet resonances at δ 1.52 and 1.39 and the partially-resolved second set of aromatic proton peaks. This was probably ibuprofen itself, present as a result of hydrolysis of the glucuronide, since peaks from free glucuronic acid were also present. Other small peaks were seen in the spectrum shown in Fig. 1, and the small doublets observed between δ 5.0 and 6.0 arise from other glucuronides, either those of other minor metabolites present in this fraction or the result of some early transacylation reactions as mentioned below.

Fig. 2 shows partial ^{1}H NMR spectra for the 80% methanol SPEC fraction at pH 7.4 at time zero (a) and after ~6 h at 310 K (b). The spectra are shown at the same vertical scale relative to the TSP used as an internal chemical shift reference and quantitation standard (not shown) and clearly illustrate the transacylation reaction in progress. The decrease in the amount of the 1-β-anomeric peak and corresponding increase in the levels of the other isomers is easily seen. The H1 anomeric proton signal for α-glucuronic acid (at δ 5.25) also increased as a result of hydrolysis. Low concentrations of the α-2-isomer of ibuprofen glucuronide were present in the time-zero spectrum as seen by the doublet signal at δ 5.38.

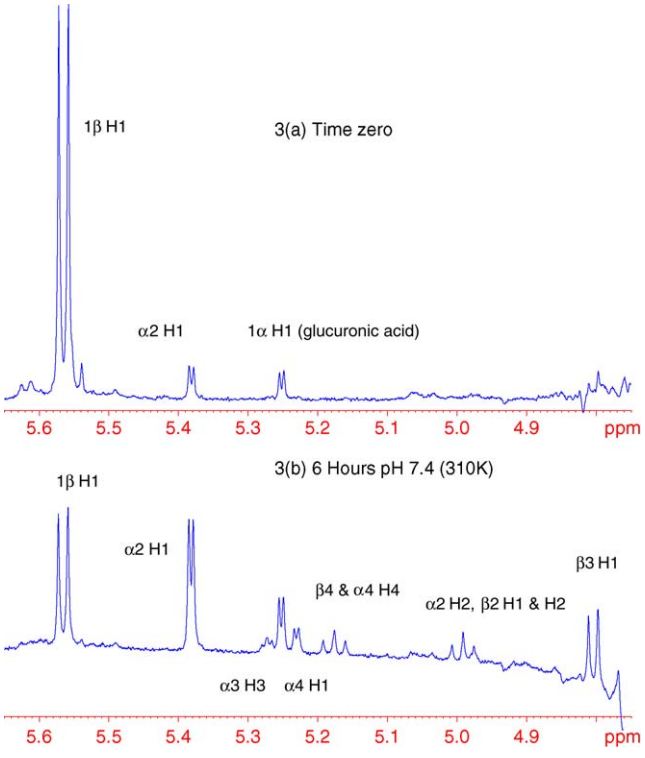

Fig. 2. Partial 600 MHz ^{1}H NMR spectra of the transacylating reaction mixture from ibuprofen 1-β-*O*-glucuronide.

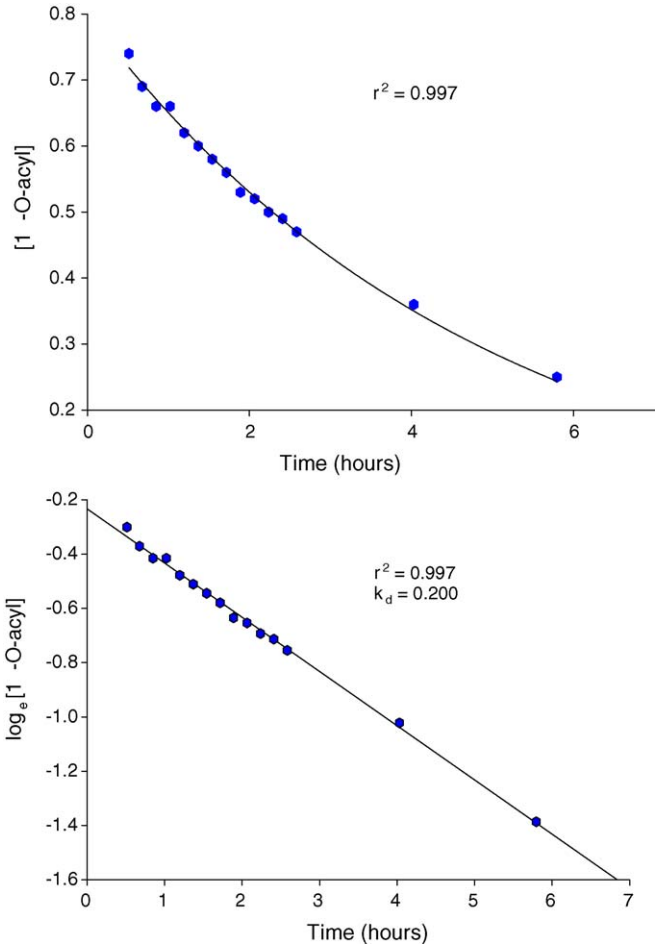

Fig. 3. Degradation rate measurements at 310 K for ibuprofen 1-β-*O*-acyl glucuronide.

Fig. 3 shows the results of plotting the 1-α-anomeric proton signal integral values from every fifth experiment of 70 acquisitions carried out over a period of 6 h and a half-life of 3.5 ± 0.1 h (95% confidence limit) was calculated from the derived ibuprofen 1-β-*O*-glucuronide degradation rate.

4. Discussion

The isolation of glucuronides using SPEC extraction techniques proved to be relatively easy for ibuprofen and, once a suitable solvent elution system had been developed, provided a rapid route to providing the target compound in quantities and purity suitable for analysis.

The synthesis, kinetic studies and QSRR results for a series of benzoic acid glucuronides compounds have also been described recently [1,2] and it would be interesting to extend this approach to arylpropionic acid glucuronides once a suitable database of reactivity indices has been obtained.

A previous study of the degradation of ibuprofen glucuronide using solid-phase extraction followed by HPLC under carefully controlled conditions of pH 7.4 and 37 °C in buffer found a half-life of 3.3 h and the NMR-based determination carried out here is in good agreement [18]. The previous study also investigated the degradation of ibuprofen glucuronide in human serum albumin solution and in blood plasma and found corresponding half-lives of 4.5 and 1.8 h, respectively.

Nevertheless, even in the absence of a large database of drug glucuronide half-life data, the half-life of 3.5 h determined here for ibuprofen glucuronide can also be compared with the value of 0.44 h for tolmetin [17] a drug withdrawn from the market because of adverse side effects. The half-life for the degradation of the diastereopmeric ester glucuronides of ketoprofen, an arylpropionic acid NSAID currently on the market, have also been derived recently at 1.45 h for the *S*-isomer and 0.67 h for the *R*-isomer [17]. It is interesting that the half-life for the glucuronide of ibufenac, the arylacetic acid analogue of ibuprofen has a value of 1.1 h [17]. Ibufenac was withdrawn from the market because of hepatotoxicity in a few patients on chronic use of the drug [19]. It can be surmised that if acyl glucuronides of drugs are to show any degree of toxicity caused by protein binding of the transacylated isomers as hypothesised [1–5], then the transacylation rate must be sufficiently fast (or the isomer half-life sufficiently long) to allow such binding before the drug glucuronide isomer is eliminated from the body. Hence there is probably a balance of the kinetics of glucuronide transacylation, protein binding and elimination that is required for toxicity to be demonstrated. Further studies are in progress to increase the number of arylpropionic acid glucuronides for which the transacylation rate can been determined to enable QSRRs to be constructed and to examine the relationship, if any, between the observed reactivity and toxicity.

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