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Rapid high-performance liquid chromatographic assay for the simultaneous determination of probenecid and its glucuronide in urine. Irreversible binding of probenecid to serum albumin

JENS HANSEN-MØLLER* and ULLA SCHMIT

Department of Organic Chemistry, Royal Danish School of Pharmacy, 2-Universitetsparken, DK-2100 Copenhagen, Denmark

Abstract: A reversed-phase high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of probenecid and its glucuronide in urine has been developed. The genuine glucuronide conjugate was isolated from urine by the use of solid-phase extraction on Amberlite XAD-2 and finally purified by the use of preparative HPLC on a Sepharon Hema 1000 RP-18 column. The purity of the product obtained was 88.9%. The isolated glucuronide was used as a standard sample. Of a p.o. dose of 500 mg to two volunteers, 26 and 29% were excreted as the ester glucuronide, while 1.0 and 2.7% were excreted unmetabolized.

The stability of the ester glucuronide was investigated in aqueous buffers, buffered urine and human serum albumin solutions. The glucuronide was unstable in neutral and mildly alkaline solutions, and special precautions have to be taken during sampling and sample treatment in order to preserve the genuine glucuronide.

The presence of human serum albumin in the solution stabilized the glucuronide against isomerization/rearrangements but catalysed the hydrolysis of the glucuronide. When incubating human serum albumin with the ester glucuronide, probenecid was shown to be covalently bound to the protein probably via a transacylation reaction.

Keywords: *Reversed-phase liquid chromatography; quantitative determination; acyl glucuronide; isolation; probenecid; irreversible binding.*

Introduction

Probenecid (PR) (4-[(dipropylamino)sulphonyl]benzoic acid) is an uricosuric compound. It is metabolized in the liver to form an acyl glucuronide (PAG) (Fig. 1) as well as several phase I metabolites, which are excreted in the urine together with the parent compound. Of the dose, 25–47% is excreted as an ester glucuronide. Only a small amount is excreted as the parent drug.

Different methods for quantitative determination of PR have been reported. Among these are spectrophotometric, RIA, gas chromatographic as well as high-performance liquid chromatographic (HPLC) methods. The older spectrophotometric methods [1, 2] lack the required specificity and sensitivity. Whereas a published RIA method [3] has the required specificity and sensitivity. Several gas chromatographic methods have been published [4–7], the disadvantages of the gas chromatographic methods are, that tedious derivatization of the sample is necessary. Several reversed-phase HPLC methods for the determination of PR in biological fluids have been published [8–12]. However, the direct determination of PR and PAG is reported in none of the above mentioned methods.

Eggers and Dourst [13] have reported a method for liquid–liquid extraction and purification of PAG by the use of preparative HPLC. They reported the glucuronide to be unstable in 50 mM ammonia, but no quantitative determination of PR or PAG in urine was reported.

In the last few decades an increasing interest in drugs excreted as acyl glucuronides has emerged, because they are potentially reactive metabolites. Their reactivity stems from the fact, that the ester group is susceptible to nucleophilic substitution reactions [14, 15]. The reactivity may result in (1) intramolecular rearrangements where the acyl group migrate from the original 1-β position to the 2-, 3- and 4-position of the glucuronic acid via an 1,2-ortho ester [16, 17]; (2) hydrolysis of the glucuronide or the rearrangement products

*Author to whom correspondence should be addressed. Present address: Danish Meat Research 2-Maglegårosvez, DK-4000 Roskilde, Denmark.

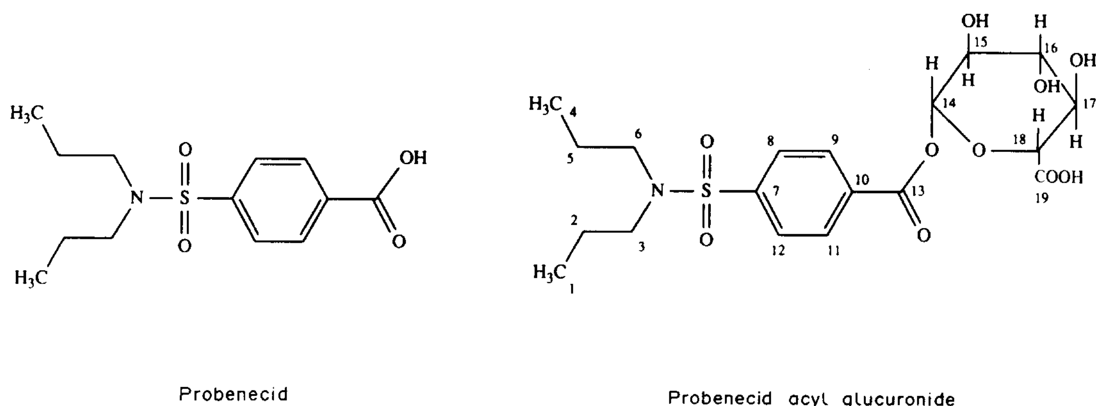

Figure 1
PR is metabolized in the liver to form an ester glucuronide and several phase I metabolites. 25–47% of the parent compound is excreted as an ester glucuronide. Only a small amount is excreted as the parent compound.

[14]; or (3) transacylation of nucleophilic compounds, e.g. plasma proteins like serum albumin [18–21].

Recently, PR has been shown to be covalently bound to plasma proteins *in vivo* after dosage of probenecid and diflunisal. The covalent adduct was postulated to be the result of a transacylation of the proteins by the PAG. The adduct had a half-life of about 13 days [22].

In the present work, we have developed a reversed-phase HPLC assay for the simultaneous determination of PR and PAG in urine. Furthermore, a method for the isolation and purification of PAG from human urine is described. The stability of PAG has been investigated in aqueous buffers, buffered urine and albumin solutions. The glucuronide has been shown to react covalently with albumin *in vitro*, which has not been reported earlier.

Experimental

Chemicals

Probenecid and β-glucuronidase from *Helix pomatia*, containing 100,000 Fishman units mg^{-1} and human serum albumin fraction V (HSA), were obtained from Sigma (St. Louis, MO, USA). Piratos®, a soft liquorice pastille containing NH_4Cl, was obtained from Haribo (Faxe, Denmark). Amberlite XAD-2 (20–50 mesh) and XAD-4 (20–50 mesh) from Serva (Heidelberg, FRG) were purified by the method described by Dieterle *et al.* [23]. Tetrabutylammonium bromide was obtained from Fluka (Buchs, Switzerland). Methanol and acetonitrile were of HPLC grade, and all other chemicals were of analytical grade.

Thin-layer chromatography

Thin-layer chromatography was performed on silica gel 60 F-254 sheets (5 × 7.5 cm) from E. Merck (Darmstadt, FRG) with a mobile phase consisting of toluene–butanol–water–methanol (4:8:4:5, v/v/v/v). When viewed under UV light (254 nm) both of the compounds appeared as blue spots on a yellow background. The glucuronide appeared as a red–blue spot on a pink background when the plates were sprayed with a 1:1 (v/v) mixture of 0.2% naphthoresorcinol in ethanol and 4 N sulphuric acid. After the plates were heated at 100°C for 30 min and subsequently spraying with 1 N sodium hydroxide the glucuronide turned blue–violet. The R_f values obtained were 0.41 for the ester glucuronide and 0.52 for probenecid.

High-performance liquid chromatography

A HPLC system consisting of a Hitatchi 655A-12 HPLC-pump, a Hitatchi 655A UV detector operated at 254 nm (Tokyo, Japan), and a Kontron MSI 660 autosampler (Zurich, Switzerland) were used. Chromatograms were recorded on a Hitatchi D-2000 integrator.

In all assays 20 μl of the sample were injected. The analytical column (120 × 4.6 mm i.d.) was packed with Spherisorb Octyl 5 μm (Phase Sep, Clwyd, UK) and operated at 37°C. The composition of the mobile phase was methanol–water–acetic acid (50:50:1, v/v/v) with 40 mM tetrabutylammonium bromide added. The flow rate was 1.2 ml min^{-1}. Standard curves (based on peak-height) were obtained in the range of 0.1–400 and 0.1–100 mg l^{-1} for PAG and PR, respectively.

The preparative HPLC system. This consisted of a column (250 × 16 mm i.d.) packed with Polygosil ODS 10 μm (Machery–Nagel, Düren, FRG). The mobile phase contained methanol–water–acetic acid (50:50:1, v/v/v). The flow rate was 10 ml min^{-1}.

The semi-preparative HPLC system. This consisted of a Sepharon Hema 1000 C_{18} column (10 μm; 80 × 8 mm i.d.) (Tesseck, Århus, Denmark). The mobile phase contained methanol–water–acetic acid (44:55:1, v/v/v). The flow rate was 1.5 ml min^{-1}.

Isolation of the glucuronide

Urine from two volunteers was collected for 12 h after ingestion of 1 g of probenecid each. The urines were combined, total volume 1800 ml. During the collection of urine about 25 g of Piratos® was ingested in order to lower the pH of the urine. The pH of the urine was adjusted to 4.0 with acetic acid. After filtration the urine was passed through a solid-phase extraction column (1000 × 25 mm i.d.) packed with 400 ml of Amberlite XAD-2 by pumping the urine through the column at a flow rate of 5 ml min^{-1}. The glucuronide was eluted with a linear gradient ranging from 100% (1% acetic acid in water) to 100% methanol for 12 h by the use of an Ultrograd 1300 gradient mixing kit (LKB, Bromma, Sweden). The fractions containing the glucuronide (investigated by HPLC) were rotoevaporated at 40°C max., until they could be frozen and lyophilized.

In another study urine from two volunteers was collected for 12 h after ingestion of 1 g of probenecid each. The urines were combined, total volume 2000 ml. During the collection period *ca* 25 g of Piratos® was ingested. The urine was treated as described earlier, except that a column (1000 × 25 mm i.d.) packed with 400 ml of Amberlite XAD-4 was used.

The glucuronide was purified by the use of the preparative HPLC system. The collected fractions were pooled, rotoevaporated at max. 40°C and lyophilized. The glucuronide was recrystallized from ethanol–water. PAG was further purified by the use of the semi-preparative HPLC system. The fractions were rotoevaporated and the compound recrystallized as above. The melting point was 82–87°C. The total yield was 210 mg.

Identification of the glucuronide

The identity of the glucuronide was confirmed by the use of ^{13}C and ^{1}H NMR spectroscopy. ^{1}H and ^{13}C spectra were obtained on a Brucker (Rheinstetten, FRG) AM 250 operated 250 and 62.9 MHz, respectively. A sample of 8 mg of the glucuronide was dissolved in 400 μl of [$^{2}H_6$] acetone containing 2 μl tetramethylsilane as internal standard.

The signals in the ^{13}C spectrum and the proton signals are in good agreement with published data [13].

Purity of the glucuronide

Several approaches were made to establish the purity of the glucuronide.

β-Glucuronidase. 1000 μl of a freshly prepared solution of the glucuronide, containing approximately 50 μg ml^{-1} PAG were mixed with 1000 μl 0.5 M of acetic acid buffer, pH 5.0, containing 20 μl ml^{-1} of β-glucuronidase (100,000 units ml^{-1}). The mixture was incubated at 37°C for 1 h and the amount of liberated PR assayed in the analytical HPLC system.

Sodium hydroxide. 700 μl of a solution containing 200 mg ml^{-1} of PAG were mixed with 200 μl of 2 N sodium hydroxide. After 2 h incubation at 37°C 100 μl of 4 N acetic acid was added, and the amount of liberated PR assayed by the use of the analytical HPLC system. An aqueous solution of PR was treated in the same way for 18 h. No decrease in the PR concentration could be detected.

Stability of the glucuronide

The stability of the glucuronide in different solutions was investigated at different pH values. In one series of experiments, 1.00 ml of an aqueous solution containing 400 mg l^{-1} of PAG, was mixed with 1.00 ml of a 0.2 M phosphoric acid buffer with a pH value of 4.0, 5.0, 6.0 or 7.4, and 2.00 ml of water or methanol and incubated at ambient temperature; 20 μl of the solution was assayed at appropriate intervals. In a second series, 1.00 ml of the PAG solution was mixed with 1.00 ml of the mentioned buffers, 2.00 ml of water or blank urine and 4.0 ml of methanol and incubated at ambient temperature; 20 μl of the solution was assayed at appropriate intervals. In a third series, 2.00 ml of a solution containing 0.43 mg ml^{-1} of the PAG was mixed with 2.00 ml of a 80.0 mg ml^{-1} HSA solution (the HSA was dissolved in 0.2 M

potassium phosphate buffer pH 7.4 and the pH adjusted to 7.4) and incubated at 37°C; 400 μl of the solution was withdrawn at appropriate intervals and the albumin precipitated by the addition of 400 μl of acetonitrile containing 2% of acetic acid. After centrifugation for 4 min at 18,000 *g*, 20 μl of the supernatant was analysed.

Covalent binding of PR to albumin

The protein pellet from the above experiment, was resuspended in 500 μl of acetonitrile containing 2% acetic acid and 10% water, and shaken for 20 min. After centrifugation for 4 min at 18,000 *g* the supernatant was discharged. The washing procedure was repeated three times. The pellet was then digested by the addition of 400 μl 0.1 N sodium hydroxide. After 4 h at ambient temperature, 150 μl of acetonitrile containing 4% acetic acid was added and 20 μl of the solution assayed.

Assay validation

Validation and recovery studies were conducted by adding known amounts of PR and PAG to drug-free urine at three different concentrations. Within-day reproducibility was determined by assaying six different samples at three concentrations. In between day reproducibility was determined by assaying four different samples on 5 successive days.

Sampling and sample treatment

Immediately after collection, the urine was acidified with 300 mg of citric acid per 100 ml of urine (resulting in a pH of about 4) and frozen at −18°C. Before analysis the samples were diluted with one part of methanol, and centrifuged at 18,000 *g* for 4 min.

Application

Two healthy male volunteers aged 27 and 32, with a body weight of 72 and 67 kg, respectively, did not ingest any other drugs for 1 week prior to the ingestion of PR and during the period of urine collection. A dose of 500 mg PR, suspended in 200 ml water, was given p.o. to each volunteer. During the period of urine collection *ad libitum* consumption of Piratos®, was allowed (*ca* 25 g was ingested during the 48 h period). Urine was collected at separate intervals during 48 h.

Results and Discussion

The chromatographic system

In order to improve the selectivity of the separation of PAG and PR from compounds in blank urine, different pH values of the mobile phase as well as different ion-pairing agents were investigated. A mobile phase containing *ca* 1% acetic acid and 40 mM tetrabutylammonium bromide turned out to give the best selectivity of the system as well as a rapid assay (Fig. 2). Because of the low pH in the mobile phase the system cannot be determined as an ion-pairing system with respect to PR or PAG, but the ion-pairing effect was useful in order to improve the selectivity between PR, PAG and interfering peaks in blank urine.

Isolation of the glucuronide

Both Amberlite XAD-2 and Amberlite XAD-4 were investigated as adsorbents for the solid-phase extraction of the glucuronide. From the XAD-2 column the peak concentration eluted at *ca* 77% methanol, whilst the peak concentration eluted at 93% methanol when using the XAD-4 column. This is in good agreement with the XAD-4 resin, having a larger surface area and a greater affinity for smaller molecules compared with XAD-2 [25]. With the model compound *p*-nitrophenyl glucuronide, XAD-4 also had the strongest affinity [26].

At the peak concentrations the % area of the glucuronide of the total area of the HPLC UV tracks were 56.2 and 49.6% for XAD-2 and XAD-4, respectively. Comparing this with the % area of PAG in the urines prior to passage of the solid-phase extraction columns of 12.3 and 14.5% for the XAD-2 and XAD-4 resins the XAD-2 resin was preferred to the XAD-4 resin because of the superior purity of the extract.

When purifying the glucuronide by the use of preparative HPLC on the Polygosil column the purity was 81.4 or 81.9%, when assaying the liberated probenecid, after cleavage by the use of β-glucuronidase and sodium hydroxide, respectively. Further purification, using the polymer-based Hema column resulted in a purity of 88.9%.

It is known [27, 28] that it may be difficult to cleave glucuronides quantitatively. However, in our investigations we did not find any trace of the original glucuronide in any of the chromatograms obtained after cleavage with

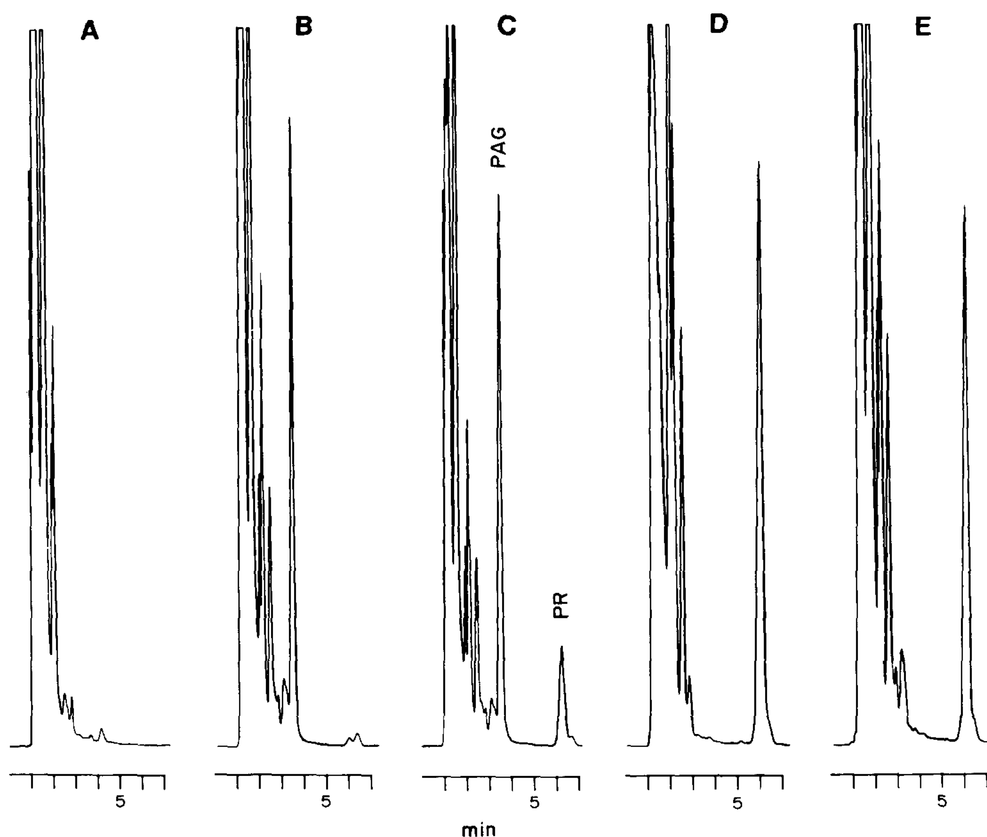

Figure 2
Selectivity of the chromatographic assay: A, blank urine; B, urine collected 1–2 h after the ingestion of 500 mg of PR; C, same as B, added 51.0 and 64.0 mg l^{-1} of PAG and PR, respectively; D, urine from B treated with β-glucuronidase from *Helix pomatia* (1300 units ml sample) at pH 5.0 for 30 min at 37°C; E, urine from B treated with 0.4 N sodium hydroxide for 30 min.

base or β-glucuronidase. Degradation of probenecid, treated in the same way was found to be negligible.

Stability of the glucuronide

In the literature several examples of drugs partly excreted as unstable ester glucuronides can be found, among these drugs are zomepirac [29], clofibrate [30], probenecid [13], valproate [31], fenclofenac [32], isoxepac [33] wy-18,251 (3-(*p*-clororophenyl)thiazolo-[3,2-a]benzimidazole-2-acetic acid) [34], furosemide [35], oxaprozin [19] and tolmetin [36]. Bilirubin [17] is an example of an endogenous compound excreted as an ester glucuronide. At mild alkaline conditions the aglycon starts to migrate from the first OH-group of the glucuronic acid moiety through an 1,2-*ortho* ester to the second OH-group and then to the third and fourth OH-group [17, 37], thereby giving the possibility of several isomers. In a study with diflunisal eight isomers of the ester glucuronide were isolated, and identified by the use of COSY–NMR, as α- and β-forms of positional isomers in which the aglycon is forming an ester with one of the four hydroxyl groups of glucupyranuronic acid, as well as the reversibility of the migration back to the original β-glucuronide was demonstrated [16].

The stability of PAG in buffered urine diluted with methanol or water is shown in Fig. 3. When methanol was added the stability was decreased. The stability of the glucuronide in buffers and buffers containing methanol is equal to the stability in diluted urine (data not shown). From Fig. 3 it is seen that the glucuronide is stable for at least 35 h at pH 4.0, which is sufficient, when an autosampler is used.

Investigations with the acyl glucuronide of zomepirac have shown that the degradation of the glucuronide is accelerated in blood and plasma [38]. Ruelius *et al.* [19] also found that the hydrolysis as well as the rearrangement reactions of oxaprozin glucuronide were accelerated by human serum albumin solutions and

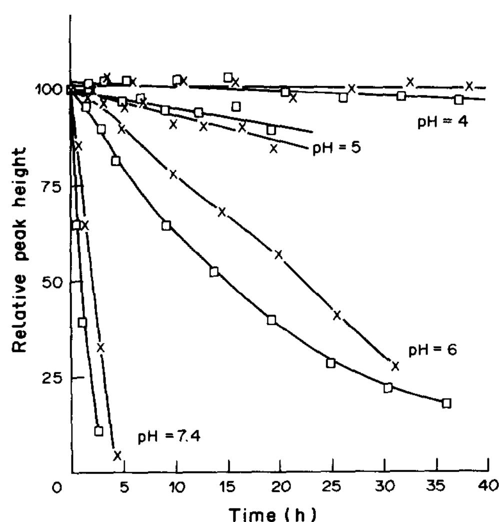

Figure 3
Stability of PAG as a function of time at ambient temperature in buffered blank urine with different pH values. The urine was diluted with methanol (□) or water (×) (1:1, v/v). The incubation media was 0.07 M with respect to phosphate, whilst the initial concentration of PAG was 133 mg l^{-1}.

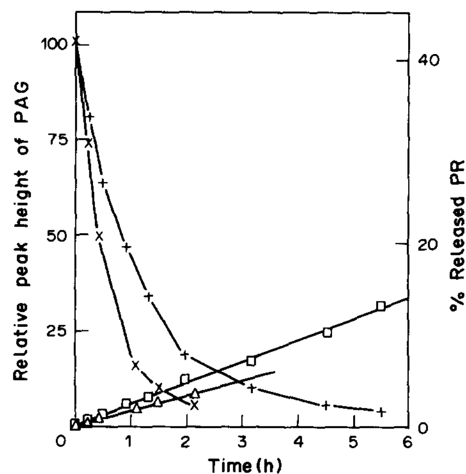

Figure 4
Stability of PAG in buffer (×) and buffer containing 40.0 mg ml^{-1} of human serum albumin (+) at a pH of 7.4 at 37°C. The buffer was 0.1 M with respect to potassium phosphate. The initial concentration of glucuronide was 0.28 mg ml^{-1}. Amount of PAG hydrolysed to PR in buffer (△) and albumin solution (□) as a function of time.

plasma. They explain these findings as the binding site of the glucuronide (the benzodiazepin binding site on the albumin molecule) is also the site of the degradation/rearrangement of the glucuronide [19].

In a study of the interaction between the acyl glucuronide of diflunisal and human serum albumin Watt and Dickinson [39] found, that the degradation of the glucuronide was decelerated rather than accelerated when the glucuronide was incubated in pure albumin solutions, whereas the degradation in human plasma was equal to the degradation in buffer solutions.

In our investigations we find that the rearrangement of PAG is reduced in the presence of HSA, whilst the hydrolysis of PAG or the rearrangement products of PAG are catalysed by HSA (Fig. 4). In the buffer solution and the HSA solutions the half-lives of PAG were 24 and 40 min, while 3.2 and 7.7% of the PAG were hydrolysed, respectively. Thus when HSA is present it is capable of stabilizing the PAG against rearrangement reactions while it concomitantly catalyses the hydrolysis of PAG to free PR. A possible explanation for this may be that the site of protein binding, because of steric hindrance, cannot catalyse the rearrangement reactions but only the hydrolysis. Further investigations are needed to clarify these findings.

Covalent binding of probenecid to albumin

In Fig. 5 the amount of PR liberated upon basic digestion of HSA after incubation with PAG is shown. When PR was incubated with HSA under the same conditions, only traces of PR were released from the protein pellet upon basic digestion. From Fig. 5 it is seen that the amount of PR bound to HSA is increasing

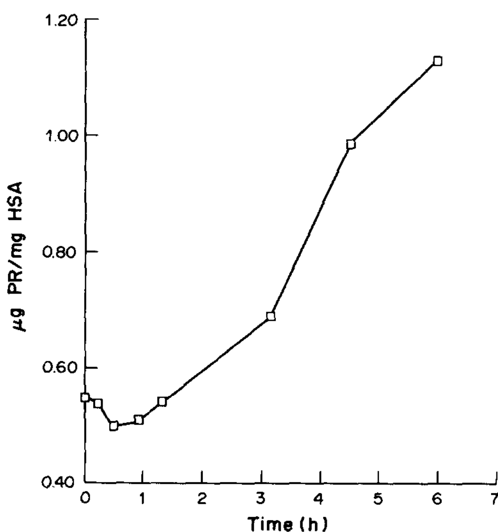

Figure 5
Covalent binding of PR to human serum albumin (HSA) as a function of time at 37°C and pH 7.4 (0.1 M potassium phosphate) (□). The initial concentration of the glucuronide was 0.28 mg ml^{-1}, whilst the concentration of the serum albumin was 40.0 mg ml^{-1}.

approximately linearly as a function of time, after a short lag time.

The mechanism for the covalent binding of aglycon from acyl glucuronides is mainly unknown, although transacylation to —OH [19], —SH [18] or —NH_2 [40] groups on proteins have been postulated. An alternative mechanism, in which an isomer of zomepirac acyl glucuronide is forming an imine (Schiff's base) between the aglycone and a lysine residue on the albumin molecule, has been proposed by Smith *et al.* [20]. The site of the covalent binding on the albumin molecule is largely unknown, although Ruelius *et al.* [19] have shown, that the site of covalent binding of oxaprozin is the —OH group of the tyrosine-411 located at binding site II (as classified by Sudlow *et al.* [41]) on the albumin molecule. Further investigations are in progress to clarify the mechanism of the covalent binding of PR to HSA and the binding site on the HSA molecule.

Assay validation

Standard curves (based on peak-height) were investigated in the range of 0.1–400 and 0.1–100 mg l^{-1} for PAG and PR, respectively. The standard curves had a coefficient of correlation above 0.999. The limit of detection was found to be 0.1 mg l^{-1} for both compounds at a signal-to-noise ratio of 2.

Validation and recovery studies were performed by adding known amounts of probenecid and its glucuronide to blank urine at three different concentrations (Table 1).

Application

The urine excretion data are shown in Fig. 6. From the terminal part of the excretion curves, half-lives of 5.0 and 4.6 h and 4.2 and 5.8 h could be found for PAG and PR, respectively. Of the administered dose of 500 mg, 26 and 29% of the dose was excreted as the ester glucuronide, while 1.0 and 2.7% is excreted unmetabolized. In a study by Melethil and Conway [5], they found the half-life of PAG to be 4.72 or 4.94 h. They found the PAG to account for 41.8–55.6%, whilst PR accounted for 4.8–16.5% at a dose of 500 mg.

The coadministration of NH_4Cl is done in order to lower the pH value of the urine at the time of production to preserve the intact glucuronide. When comparing Figs 2D and 2E, it is seen, that the glucuronide is intact, as the amounts of PR liberated after cleavage by the use of 0.4 N sodium hydroxide and β-glucuronidase are practically identical. Thus the

Table 1
Validation studies were performed by adding known amounts of PR and PAG to blank urine at three different concentrations. For the validation three different blank urine samples were used

Conc. (mg l^{-1})	Urine	Assay conc. mean ± SD (n = 6, mg l^{-1})	Recovery (%)	Relative standard deviation Within-day (n = 6)	Between-day (n = 5)
		PAG			
17.64	A	16.94 ± 0.30	96.0	1.8	1.0
	B	16.64 ± 0.36	94.6	2.1	4.1
	C	17.92 ± 0.20	101.7	1.1	5.7
88.20	A	88.26 ± 0.49	100.1	0.6	0.8
	B	85.50 ± 0.72	96.9	0.4	0.6
	C	90.28 ± 1.56	102.4	1.7	1.1
229.88	A	234.24 ± 0.32	101.9	0.1	0.9
	B	230.76 ± 2.06	100.4	0.9	1.4
	C	229.26 ± 2.48	99.7	1.6	1.6
		PR			
3.90	A	3.45 ± 0.07	88.5	2.3	3.3
	B	3.30 ± 0.16	84.6	4.9	5.6
	C	3.36 ± 0.16	86.2	4.9	5.6
19.64	A	18.33 ± 0.16	93.3	0.9	1.2
	B	18.55 ± 0.17	94.5	0.9	1.9
	C	18.18 ± 0.22	92.6	1.2	0.5
48.87	A	48.16 ± 0.28	98.6	0.5	2.1
	B	47.49 ± 0.38	92.2	0.8	1.0
	C	46.44 ± 0.15	95.0	0.3	6.8

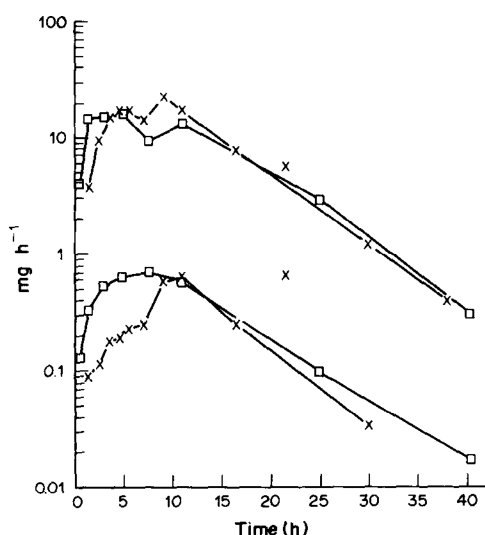

Figure 6
Semilogarithmic plot of urinary excretion rate of PR (lower curves) and PAG (upper curves). 500 mg of PR were given p.o. to two healthy male volunteers aged 27 (□) and 32 (×) with a body weight of 72 and 67 kg, respectively.

glucuronide has not rearranged into isomers resistant to cleavage by the use of β-glucuronidase. The low recovery of PR as PAG, in the present study compared with the findings of Melethil and Conway [5] can, therefore, not be explained by rearrangement of the glucuronide. The urinary excretion of PR is pH dependent, being lower at low pH values [42]. This may be the explanation of the low recoveries of PAG and PR in our investigations, because of the coadministration of NH_4Cl.

Conclusion

The inherent instability of acyl glucuronides is a challenge to the analytical chemist. In the present study we have shown, that it is possible to develop a quantitative analytical assay for the direct determination of PR and its unstable acyl glucuronide; and we have shown that it is possible to preserve the intact glucuronide in the urine, if the urine is acidified at the time of production by coadministration of NH_4Cl, and that careful sample treatment is needed.

The biological significance of the covalent binding of aglycons from acyl glucuronides is at present unknown. Ruelius *et al.* [19] concluded that the covalent binding *in vivo* of oxaprozin has no toxicological significance, because the acyl glucuronide of oxaprozin has not been detected in human plasma. The acyl glucuronide of diflunisal has, however, been detected and determined in human serum [43] and covalent adducts of diflunisal to plasma proteins have been detected *in vivo* [22]; but only speculations were made on the findings [22] and the three cases of diflunisal hypersensitivity recently reported [44].

The non-steroidal anti-inflammatory drug zomepirac has been withdrawn from the market because of unexplained anaphylactic reactions; irreversible binding of zomepirac via its acyl glucuronide was proposed as an explanation [20].

Immune mediated haemolytic anaemia has been reported for PR [45] as well as covalent binding of PR to plasma proteins *in vivo* [22], although PAG has not to our knowledge been reported in human plasma.

More investigations are obviously needed in order to elucidate the site and mechanism of the covalent binding of acidic drugs via their acyl glucuronides, and its biological significance.

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