Studies on the renal excretion of the acyl glucuronide, phenolic glucuronide and sulphate conjugates of diflunisal

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- 1 In five healthy male volunteers given multiple doses of diflunisal (DF), renal clearances (CL_R) of the acyl glucuronide (DAG), phenolic glucuronide (DPG) and sulphate (DS) conjugates were about 42, 25 and 13 ml min⁻¹, respectively.
- 2 These relatively low CL_R values are probably due largely to the very high plasma protein binding of the conjugates, found *in vitro* to be 99.0%, 97.8% and 99.45%, respectively.
- 3 Thus glomerular filtration plays the minor and active tubular secretion the major role in renal excretion of the three conjugates.
- 4 This conclusion was supported by the effect of probenecid co-administration, which decreased CL_R of DAG and DPG by about 70%. CL_R for DS could not be calculated when probenecid was co-administered (because of interference by probenecid metabolites in the analysis of DS in urine).
- 5 Water-induced diuresis had no effect on CL_R of the DF conjugates, consistent with tubular reabsorption being negligible.

Keywords diflunisal renal clearance, glucuronide sulphate protein binding diuresis probenecid

Introduction

The anti-inflammatory agent diflunisal (DF), a difluorophenyl derivative of salicylic acid, is eliminated from the body primarily by biotransformation to its acyl glucuronide (DAG), phenolic glucuronide (DPG) and sulphate (DS) conjugates (Loewen *et al.*, 1988; Verbeeck *et al.*, 1990). In earlier work in renal failure patients given a single dose of DF (Dickinson *et al.*, 1991b), we showed that DPG and DS (which are chemically stable at physiological pH) had the capacity, unlike DAG (which is unstable at physiological pH), to accumulate in plasma as a result of the renal impairment. That study, however, provided no information about plasma protein binding and renal clearances (CL_R) of the conjugates themselves. Indeed, such data on drug conjugates are relatively rare. The present study, in healthy volunteers given multiple doses of DF, was carried out to examine the mechanisms involved in the renal excretion of the three DF conjugates.

Methods

Materials

DF pure substance was a gift from Merck, Sharp & Dohme, Sydney, NSW. Also gifts were clofibrilic acid (ICI Pharmaceuticals, Macclesfield, England), naproxen (Syntex, Sydney, NSW) and ketoprofen (May & Baker, Melbourne, VIC), which were used as internal standards for h.p.l.c. analyses. Authentic samples of DAG, DPG and DS were obtained as described previously (Dickinson *et al.*, 1991a; Watt *et al.*, 1991). β -Glucuronidase (from *Helix pomatia*, type H-2) was purchased from Sigma, St Louis, MO. Solvents were h.p.l.c. grade and obtained from Mallinckrodt, Melbourne, VIC. Reagents were of analytical grade purity.

Diuresis study

Four healthy, informed and consenting adult male volunteers (28–45 years, 64–74 kg, creatinine clear-

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ances 82–119 ml min⁻¹) participated in the study, which was approved by the Medical Research Ethics Committee of The University of Queensland. Subjects took oral doses of DF (Dolobid® 250 mg tablets, Merck, Sharp & Dohme) at *circa* 07.00 h and 19.00 h according to the following schedule: day 1, 250 mg p.m.; day 2, 250 mg a.m., 500 mg p.m.; day 3, 500 mg a.m. and p.m.; day 4, 1000 mg a.m. Blood samples (10 ml) were drawn hourly from 3 to 11 h (inclusive) after the last dose, immediately centrifuged, and the plasma snap-frozen (dry ice). Fluid intake was normal until 5 h after the dose, when subjects drank copious quantities of water for the next 4 h to induce diuresis. All urine produced during the intervals 3–5, 5–7, 7–9 and 9–11 h after the dose was collected, the pH adjusted to *circa* 4.5, and a sample (10 ml) frozen until analysis.

Probenecid interaction study

On a later (*circa* 3 months) occasion, the same four and one additional (44 years, 89 kg, creatinine clearance 129 ml min⁻¹) male subjects participated in the following study, also approved by the ethics committee noted above. DF (Dolobid® 250 mg tablets) were taken at *circa* 07.00 h and 19.00 h according to the following schedule: day 1, 250 mg p.m.; day 2, 250 mg a.m. and p.m.; day 3, 250 mg a.m., 500 mg p.m.; days 4–6, 500 mg a.m. and p.m.; day 7, 500 mg a.m. Probenecid (Benemid® 500 mg tablets, Charles Frosst) doses of 500 mg were taken at *circa* 19.00 h on day 5; 01.00 h, 07.00 h, 13.00 h and 19.00 h on day 6, and 01.00 h and 07.00 h on day 7. Blood samples were collected each 1.5 h from 2 to 8 h (inclusive) after the 07.00 h doses on days 5 and 7. All urine produced during the intervals 2–5 and 5–8 h after these doses was collected. Blood and urine samples were processed for storage as described in the diuresis study.

Analysis of plasma and urine samples

Samples of plasma (50 µl) and urine (200 µl) were analysed for DF, DAG, DPG and DS using the non-extractive h.p.l.c. method previously described (Dickinson & King, 1989). Isomers of DAG formed by intramolecular acyl migration were measured using the same molar extinction at 226 nm as DAG, an approach which has been validated (King & Dickinson, 1991). Clofibric acid was used as the internal standard (as described), except for samples from the probenecid interaction study, where chromatographic interference by probenecid necessitated use of an alternative internal standard, naproxen. Interference by probenecid metabolites precluded reliable measurement of DS in urine. Analysis of samples from appropriate control experiments (administration of probenecid alone to the volunteers on a later occasion) verified that interference by probenecid metabolites in the analysis of DS in plasma samples was minimal.

Plasma protein binding studies

Solutions (30 µl) of purified DAG, DPG and DS each at *circa* 360 µg DF equivalents ml⁻¹ 0.01 M phosphate buffer pH 5.0 were added to 0.5 ml samples of fresh

human plasma, to yield final concentrations of *circa* 20 µg DF equivalents ml⁻¹. DPG was also studied at final concentrations of *circa* 5 and 10 µg DF equivalents ml⁻¹. Aliquots (25 µl) were taken for direct analysis of the conjugates as previously described (Dickinson & King, 1989).

The plasma samples (0.5 ml) containing DAG were immediately centrifuged at 25° C for 15 min at 1000 g in an Amicon MPS-1 apparatus equipped with a YMT membrane (Amicon, Danvers, MA). Ultrafiltrates were tested for protein leakage (Bradford, 1976). To a 300 µl aliquot of ultrafiltrate was added 100 µl 2 M NaOH and the solution was heated at 90° C for 30 min (to achieve alkaline hydrolysis of DAG and its isomers). After cooling and acidification with 600 µl of 2 M HCl, 50 µl internal standard solution (100 µg ml⁻¹, ketoprofen in methanol) was added and the solution equilibrated with 5 ml ether:hexane (1:1 by volume). After centrifugation, the organic layer was removed and evaporated to dryness at room temperature under a stream of air. The residue was reconstituted in 150 µl h.p.l.c. mobile phase, and a 100 µl aliquot taken for analysis of DF using the chromatographic conditions previously described (Dickinson & King, 1989).

Plasma samples (0.5 ml) containing DPG or DS were dialysed against an equal volume of buffer solution (0.01 M phosphate, 0.15 M NaCl, pH 7.4) at 25° C for 18 h. Dialysates were tested for protein leakage. Control experiments confirmed the stability of DPG and DS, and the attainment of equilibrium, under these conditions. For analysis of dialysates (300 µl aliquots) containing DS, water (200 µl), 2 M HCl (500 µl), ketoprofen internal standard solution (50 µl) and ether:hexane (1:1 by volume, 5 ml) were added, and the mixture shaken for 30 min (to achieve acidic solvolysis of DS and extraction of liberated DF). The organic layer was then removed and analysed as described above. For analysis of dialysates (300 µl aliquots) containing DPG, acetate buffer (0.25 M, pH 5.0, 500 µl) containing β-glucuronidase (6300 units) was added, followed by overnight incubation at 37° C (to achieve β-glucuronidase hydrolysis of DPG). After acidification (10 M HCl, 200 µl) and addition of internal standard, the mixture was extracted with ether:hexane for analysis of DF as described above.

Data analysis

Values for CL_R over the 2 h intervals of the diuresis study or 3 h intervals of the probenecid interaction study were calculated as the quotients of urinary recoveries and average plasma concentrations of the specified analytes over these periods.

Statistical analyses were carried out using the two-tailed paired *t*-test and confidence interval analysis.

Results

Diuresis, induced by drinking copious quantities of water, had no effect on the renal clearance (CL_R) of DF conjugates (Table 1). Inter-subject variation in CL_R of the individual conjugates was greatest for DPG (a

Table 1 Renal clearance of diflunisal conjugates in healthy subjects

<i>Time interval after dose (h)</i>	<i>Urine flow (ml min⁻¹)</i>	<i>DAG†</i>	<i>Renal clearance (ml min⁻¹)</i>		
			<i>DPG</i>	<i>DS</i>	<i>DF</i>
<i>Diuresis study</i>					
3–5	1.3 ± 0.7	44.7 ± 6.9	23.4 ± 6.2	12.8 ± 2.7	0.03 ± 0.01
5–7	4.8 ± 2.3	39.6 ± 9.7	26.9 ± 16.1	11.5 ± 4.5	0.03 ± 0.01
7–9	8.6 ± 1.1	44.1 ± 16.5	21.7 ± 8.6	12.6 ± 3.4	0.04 ± 0.01
9–11	6.5 ± 1.8	43.3 ± 21.3	21.5 ± 10.2	12.6 ± 5.1	0.04 ± 0.01
<i>Interaction study—no probenecid</i>					
2–5		41.1 ± 18.1	27.7 ± 9.3	13.5 ± 3.7	0.08 ± 0.05
5–8		39.1 ± 14.6	27.8 ± 7.2	14.8 ± 3.9	0.11 ± 0.07
<i>Interaction study—with probenecid</i>					
2–5		12.1 ± 5.6	10.4 ± 2.8	—*	0.04 ± 0.02
5–8		10.7 ± 3.4	8.7 ± 2.4	—	0.05 ± 0.01

Results are means ± s.d., $n = 4$ for the diuresis study and $n = 5$ for the probenecid interaction study.

†Measured as the sum of DAG and its isomers formed by intramolecular acyl migration.

*Measurement not reliable because of chromatographic interference by probenecid metabolites in analysis of DS in urine samples.

chemically-stable conjugate): across the four 2 h intervals of the diuresis study, CL_R ranged from $13.7 \pm \text{s.d. } 1.7 \text{ ml min}^{-1}$ for subject 3 to $37.0 \pm \text{s.d. } 9.0 \text{ ml min}^{-1}$ for subject 2. Intra-subject variation in CL_R was greatest for DAG (a chemically-labile conjugate): values calculated for subjects 1, 2, 3 and 4 were 47.4 ± 8.8 , 78.6 ± 20.4 , 48.9 ± 8.3 and $48.2 \pm 7.0 \text{ ml min}^{-1}$ (means ± s.d.), respectively. Precautions (cooling, pH adjustment) to minimise *ex vivo* degradation of DAG were taken as soon as practicable after collection of plasma and urine samples. Nonetheless, isomers of DAG (formed by intramolecular acyl migration) were present in low concentrations, particularly in plasma

samples. Concentrations of DAG were therefore calculated throughout using the sum of DAG and its isomers. Over the total 8 h collection period, the proportions of the measured DF-related species recovered in urine were $37 \pm 5\%$ DAG, $36 \pm 5\%$ DPG, $26 \pm 9\%$ DS and $1.0 \pm 0.3\%$ DF (means ± s.d.).

Plasma concentrations of DF and its conjugates were higher after probenecid coadministration (Figure 1), though it should be noted that the attainment of steady state prior to probenecid co-administration was not confirmed. Assessed in terms of the average plasma concentrations over the 6 h study periods, the increases [means ± s.d. (95% confidence intervals)] were $45 \pm$

Figure 1 Plasma concentrations of diflunisal (●) and its acyl glucuronide (△), phenolic glucuronide (□) and sulphate (○) conjugates in healthy volunteers taking diflunisal before (panel A) and after (panel B) co-administration of probenecid. Time zero is the time of the 500 mg dose of diflunisal taken at 07.00 h on day 5 (panel A) and of the 500 mg doses of diflunisal and probenecid taken at 07.00 h on day 7 (panel B). The acyl glucuronide concentrations refer to the sum of glucuronide itself plus its isomers formed by intramolecular acyl migration.

13% (28–61%) for DF, $28 \pm 20\%$ (3–53%) for DAG, $80 \pm 17\%$ (59–102%) for DPG and $150 \pm 45\%$ (95–205%) for DS ($P < 0.01$ except for DAG, $P = 0.035$). There were corresponding decreases [means $\pm$ s.d. (95% confidence intervals)] in CL_R of DAG and DPG [by $70 \pm 5\%$ (64–76%) and $65 \pm 5\%$ (58–71%), respectively, $P < 0.01$]. Probenecid-induced change in CL_R of DS could not be calculated because of chromatographic interference by probenecid metabolites in the analysis of DS in urine.

The plasma protein binding of DF conjugates was examined initially by ultrafiltration of plasma samples from the diuresis study. However, levels of the conjugates in the ultrafiltrates were below the limit of quantification of the direct, non-extractive method which analyses the intact conjugates (Dickinson & King, 1989). This indicated very high binding ($> 97\%$). In order to quantify precisely the free fraction of each conjugate, it was therefore necessary to use a more sensitive extractive analytical method employing prior hydrolysis of the conjugate to DF. This, therefore, required plasma samples containing a single conjugate (and no DF), and consequently precluded use of samples from the *in vivo* studies. Fresh human plasma samples spiked with DAG were subjected to ultrafiltration (because of the relative instability of DAG under physiological conditions), and those spiked with DPG or DS were subjected to equilibrium dialysis. At $20 \mu\text{g}$ DF equivalents ml^{-1} , the percentage unbound fractions were: DAG, $1.00 \pm 0.05\%$; DPG $2.20 \pm 0.15\%$; DS $0.55 \pm 0.05\%$ (means $\pm$ s.d., $n = 3$). The DPG unbound fraction was unchanged at lower total concentrations of 10 and $5 \mu\text{g}$ DF equivalents ml^{-1} .

Discussion

Relatively little has been published on plasma protein binding and CL_R of drug conjugates, due in part to the limited availability of appropriate analytical methods for the conjugates themselves. In the case of acyl glucuronides, the situation is further complicated by their potential reactivity i.e. an ability to undergo hydrolysis, rearrangement (isomerisation via acyl migration) and covalent binding reactions to proteins under physiological conditions (Spahn-Langguth & Benet, 1992). Although such reactions are quenched *ex vivo* by mild acidification of biological samples, this reactivity can make accurate measurements of pharmacokinetic parameters for acyl glucuronides considerably more difficult. DF is a useful model drug for the study of conjugate disposition. It forms an acyl glucuronide conjugate (DAG), which is relatively reactive (Dickinson & King, 1989 & 1991; McKinnon & Dickinson, 1989). As well, it forms a phenolic glucuronide (DPG) and a sulphate conjugate (DS), which are systemically stable, at least in the rat (Dickinson *et al.*, 1991a; Watt *et al.*, 1991).

DF itself is very highly protein bound, to the extent of about 99.9% in human plasma at concentrations up to about $100 \mu\text{g}$ ml^{-1} (Verbeeck *et al.*, 1990). In the present study, high plasma protein binding was also

demonstrated by all three DF conjugates—99.0% for DAG, 97.8% for DPG and 99.45% for DS, at concentrations of *circa* $20 \mu\text{g}$ DF equivalents ml^{-1} . To our knowledge such levels of binding are higher than hitherto reported for drug conjugates, with the exception of the diastereomeric carprofen acyl glucuronides (Iwakawa *et al.*, 1990), where binding to human serum albumin was calculated to exceed 99%. That DS was the most highly bound of the DF conjugates was perhaps not surprising, since sulphate conjugation has been reported to enhance reversible binding of certain model phenols (Mizuma *et al.*, 1990). Binding of DPG was concentration-independent over the range $5\text{--}20 \mu\text{g}$ DF equivalents ml^{-1} . Analytical sensitivity precluded similar assessment for DAG and DS. The binding studies were carried out, because of analytical necessity, using plasma samples spiked with a single conjugate. Consequently, the effect on the binding of a particular conjugate caused by the presence of both DF itself and the other conjugates (i.e. the situation found in plasma *in vivo*) could not be established.

CL_R values for the DF conjugates (Table 1) were relatively low in comparison to most reported values for drug conjugates e.g. CL_R values of 540, 406 and 955 ml min^{-1} have been reported for the acyl glucuronides of ketoprofen (Upton *et al.*, 1982), zomepirac (Smith *et al.*, 1985) and tolmetin (Hyneck *et al.*, 1988), respectively. The exception again is carprofen, where CL_R values of 26 and 36 ml min^{-1} were reported for the diastereomeric (R)- and (S)-carprofen glucuronides, respectively (Spahn *et al.*, 1989). For carprofen glucuronides and DF conjugates, the low CL_R values are probably attributable to the very high protein binding. Based on the unbound fractions obtained *in vitro*, glomerular filtration should contribute only 1.2, 2.6 and 0.7 ml min^{-1} to the total CL_R of DAG, DPG and DS, respectively (assuming glomerular filtration rate is 120 ml min^{-1}). Thus active tubular secretion must be responsible for the majority of the renal excretion of each conjugate. This contention is supported by the probenecid interaction study (Table 1), where competition for the tubular organic anion pump (Cunningham *et al.*, 1981) resulted in decreases in CL_R of DAG and DPG. For both glucuronides, the decrease was about 70%, and so there was no apparent gross differentiation between the glucuronides by the active tubular secretion mechanism. That diuresis failed to alter CL_R of any of the conjugates (Table 1) is consistent with tubular reabsorption being negligible in each case.

Values for CL_R of DF itself (Table 1), although somewhat smaller than those reported earlier in healthy volunteers (Balali-Mood & Prescott, 1989; Verbeeck *et al.*, 1990) are probably still overestimates because of probable small contributions to urinary DF concentrations from hydrolysis of DAG during urine retention in the bladder and perhaps of DS during acidic workup for analysis (Dickinson *et al.*, 1991b). Even a relatively trivial amount of conjugate hydrolysis would cause a substantial increase in the apparent amount of DF excreted in urine.

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