The effect of multiple dosage on the kinetics of glucuronidation and sulphation of diflunisal in man

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- 1 The single (250 and 500 mg) and multiple dose (250 and 500 mg twice daily for 15 days) pharmacokinetics of diflunisal were compared in young volunteers.
- 2 The plasma clearance of diflunisal was lowered significantly after multiple dose administration (5.2 ± 1.2 and 4.2 ± 0.7 ml min⁻¹ for the 250 and 500 mg twice daily regimens, respectively) as compared with single dose administration (11.4 ± 3.1 and 9.9 ± 2.0 ml min⁻¹ for the 250 and 500 mg single doses, respectively).
- 3 The partial metabolic clearances of diflunisal by acyl and phenolic glucuronide formation were lowered significantly (> 50%) after multiple dose administration.
- 4 The urinary recovery of diflunisal sulphate increased as a function of dose: 6.1 ± 2.8 and $9.1 \pm 3.5\%$ following the 250 and 500 mg single dose, respectively, and 10.9 ± 3.1 and $15.9 \pm 3.6\%$ following the 250 and 500 mg twice daily regimens. The partial metabolic clearance of diflunisal by sulphate conjugation was unchanged following multiple dose administration.
- 5 The plasma protein binding of diflunisal was concentration-dependent. Analysis of unbound plasma clearances of diflunisal showed that its total plasma clearance following 500 mg twice daily was affected by both saturable glucuronidation and concentration-dependent plasma binding.

Keywords diflunisal multiple dose pharmacokinetics glucuronidation sulphation plasma protein binding

Introduction

Diflunisal [5-(2',4'-difluorophenyl)-salicylic acid] is eliminated in man by formation of an acyl glucuronide (DAG), a phenolic glucuronide (DPG) and a sulphate conjugate (DS) (Loewen *et al.*, 1986; Tocco *et al.*, 1975). In a previous study we showed that the pharmacokinetics of diflunisal were dose-dependent following administration of single doses (100–1000 mg) (Loewen *et al.*, 1988). The gradual decrease in plasma diflunisal clearance following administration of increasing single doses seemed to be due to saturation of the formation of the acyl

glucuronide. The formation clearances of the phenolic glucuronide and sulphate conjugates were relatively constant at the doses studied. The *in vitro* plasma protein binding of diflunisal was high (approximately 99.9%) and relatively constant at plasma concentrations under 80 µg ml⁻¹, but decreased at plasma concentrations above 80 µg ml⁻¹. Since plasma diflunisal concentrations were generally below 80 µg ml⁻¹ in the experiments involving single dose administration, it was concluded that concentration-dependency in plasma binding was unlikely to

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affect significantly the single-dose pharmacokinetics of this drug.

We now compare the pharmacokinetics of diflunisal following 250 mg and 500 mg twice daily administration and after single 250 mg and 500 mg doses. Steady state plasma diflunisal concentrations were in the concentration-dependent plasma binding range. Therefore, the pharmacokinetic parameters of diflunisal were calculated for both total and unbound drug. Furthermore, concentrations of diflunisal conjugates could be measured in plasma at steady state enabling the calculation of the renal clearance of these conjugates.

Methods

Subjects and study protocol

Six male volunteers from 18 to 26 years old and weighing 72.4 ± 4.9 kg (mean $\pm$ s.d.) entered the study. All were nonsmokers with no history of serious illness. Their health was judged to be normal following a physical examination and routine biochemical tests of hepatic and renal function. No medications were permitted for at least 2 weeks immediately before and during each study period. The volunteers were also told to abstain from alcohol 48 h before and throughout each study period. The study was approved by the University Human Ethics Committee, and written informed consent was obtained from each subject prior to the study.

The oral pharmacokinetics of diflunisal were studied in each volunteer on four different occasions in random order: (1) following administration of a single 250 mg diflunisal tablet (Dolobid®, Merck Frosst Canada Inc.), (2) following administration of a single 500 mg dose (two 250 mg tablets), (3) following administration of 250 mg diflunisal twice daily for 15 days, and (4) following administration of 500 mg diflunisal twice daily for 15 days. All study phases were separated by a washout period of at least 2 weeks. One volunteer completed only two of the four study phases and was therefore excluded from the final analysis of the results. Volunteers were instructed to fast the night before the single dose studies and the nights prior to administration of the final diflunisal dose on day 16 of the two multiple dose regimens.

Following administration of the single 250 and 500 mg diflunisal doses, and following administration of the last diflunisal dose on Day 16 of the two multiple dose regimens, blood samples were withdrawn at 0 (pre-dose), 20, 40 min, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, 72,

84, 96 and 108 h. The following additional samples were withdrawn during the multiple dose regimens: 0 and 12 h on Day 1, and pre-morning dose samples on Days 2, 3, 4, 5, 8, 10, 12, 13, and 15. Blood samples were collected in heparinized tubes (Venoject, 143 iu heparin) and centrifuged immediately (800 g for 20 min at 4° C) to separate the plasma. Urine was collected for 108 h in 12 h intervals following administration of the 250 and 500 mg single doses, and during one dosing interval (i.e. 12 h) following administration of the last diflunisal dose of the two multiple dose regimens. Urine (12 h intervals) was always collected over 500 ml of a 0.5 M phosphate buffer solution (pH 4.50). Aliquots of the urine collections were stored at -20° C until analysis.

H.p.l.c. assays of diflunisal and its conjugates

Diflunisal concentrations in plasma were measured by h.p.l.c. using an assay developed by Ray & Day (1983), modified as described previously (Loewen *et al.*, 1988). Urine concentrations of diflunisal and its three conjugates (DAG, DPG, and DS) were measured by the method of Loewen *et al.* (1989). Steady state plasma samples were analyzed for DAG, DPG and DS after protein precipitation with acetonitrile (Dickinson & King, 1989). In the figures, tables and text plasma concentrations of diflunisal conjugates are expressed as diflunisal equivalents.

Plasma protein binding

Blank plasma samples, obtained before the start of each of the four study phases, were pooled for each volunteer. Unlabelled and ^{14}C -labelled diflunisal (specific activity: $52.6 \mu\text{Ci mg}^{-1}$) were added to obtain a series of total diflunisal plasma concentrations between 10 and $300 \mu\text{g ml}^{-1}$. The *in vitro* plasma protein binding of diflunisal was then measured by equilibrium dialysis as described by Loewen *et al.* (1988). ^{14}C -labelled diflunisal was used within 1 week of purification by h.p.l.c.

The *ex vivo* plasma protein binding of diflunisal was measured in all of the plasma samples obtained on Day 16 of the two multiple dose study phases. After adding $3 \mu\text{g}$ of ^{14}C -labelled diflunisal, 1 ml of each steady state plasma sample was dialyzed and the unbound diflunisal fraction was measured as before. All *in vitro* and *ex vivo* plasma protein binding experiments were carried out in duplicate.

Pharmacokinetics analysis

The plasma clearance of diflunisal was calculated according to the following equation:

$$CL/F = \frac{D}{AUC}$$

where D is the oral dose and F is the bioavailability of the oral dosage form. Mass balance studies with ^{14}C -labelled diflunisal showed that 95% of an oral dose is recovered in urine, indicating that absorption from the gastrointestinal tract is virtually complete (Tocco *et al.*, 1975). AUC represents the area under the plasma diflunisal concentration-time curve from 0 to ∞ following single dose administration, and from 0 to 12 h (i.e. during one dosing interval τ) following multiple dose administration. Average steady state plasma drug concentrations were calculated by dividing AUC by τ (12 h). The renal clearance of diflunisal was calculated from:

$$CL_r = \frac{Ae_{DF}}{AUC}$$

where Ae_{DF} is the amount of diflunisal excreted unchanged in urine (from 0 to 108 h after single dose administration, and from 0 to τ following multiple dose administration). The partial metabolic clearance of diflunisal (CL_m) by various routes was calculated according to Houston (1982):

$$CL_m = CL/F \cdot f_m$$

where f_m is the fraction of the dose excreted as a particular metabolite in the urine.

Renal clearances of the diflunisal conjugates at steady state were calculated from:

$$CL_R(m) = \frac{Ae(m)(0,12)}{AUC(m)(0,12)}$$

where $Ae(m)(0,12)$ is the amount of conjugate recovered in urine at steady state during one dosing interval, and $AUC(m)(0,12)$ is the AUC of the conjugate during the same dosing interval.

Predicted and actual accumulation factors for diflunisal were calculated using the following equations (Bjornsson & Shand, 1979):

$$R(\text{predicted}) = \frac{1}{1 - e^{-k\tau}}$$

$$R(\text{actual}) = \frac{C_{ss,\min}}{C_{1,\min}}$$

where k is the rate constant associated with the terminal elimination phase of diflunisal from

plasma estimated from the single dose experiment, $C_{1,\min}$ is the plasma diflunisal concentration at 12 h after administration of the first dose of the multiple dosage experiment, and $C_{ss,\min}$ is the minimum plasma diflunisal concentration at steady state.

The AUC of unbound diflunisal was calculated and used to estimate unbound clearances of diflunisal (i.e. unbound plasma clearance, renal clearance of unbound drug and unbound partial metabolic clearances).

Statistical analysis

All results in the text and tables are expressed as mean $\pm$ standard deviation. Mean pharmacokinetic parameters for the four study phases were compared by ANOVA. Individual differences between pairs of means were assessed by the Student-Newman-Keuls test (Zar, 1984). A P value of 0.05 or less was considered significant.

Results

Trough plasma concentrations of diflunisal, measured at frequent intervals during the 15-day drug administration period, indicated that steady state levels of diflunisal in plasma were reached by Day 10 (Figure 1). Accumulation factors predicted after single dose administration (1.73 ± 0.13 and 1.86 ± 0.11 for the 250 and 500 mg twice daily regimens, respectively) were significantly lower (paired t -test, $P < 0.005$) than the actual values obtained experimentally (3.98 ± 0.92 and 4.91 ± 0.75 for the 250 and 500 mg twice daily experiments).

Relevant single and multiple dose pharmacokinetic parameters (AUC, AUC/DOSE and CL/F) of total (bound + unbound) diflunisal are summarized in Table 1. This table also shows urinary recovery data for unchanged drug and metabolite, and the partial clearances (renal, metabolic) of diflunisal. The pharmacokinetics of diflunisal were non-linear. Thus, the AUC of diflunisal at steady state was more than twice the value obtained after single dose administration of the same dose. As a result, plasma diflunisal clearance at steady state (5.2 ± 1.2 and $4.2 \pm 0.7 \text{ ml min}^{-1}$ following 250 and 500 mg doses, respectively) was significantly lower than that following single administration of the same doses (11.4 ± 3.1 and $9.9 \pm 2.0 \text{ ml min}^{-1}$). Total urinary recovery of diflunisal and its three conjugates (X_u , expressed as % of the administered dose, see Table 1) following mul-

Figure 1 Mean plasma diflunisal concentration-time profiles after single (250 and 500 mg, SD) and multiple dose (250 and 500 mg twice daily, MD) oral administration to five healthy volunteers ($\square$ ----- $\square$: 250 mg SD; $\circ$ — $\circ$: 500 mg SD; $\blacksquare$ ----- $\blacksquare$: 250 mg MD; $\bullet$ — $\bullet$: 500 mg MD).

tiple dose administration was $87.3 \pm 6.8\%$ (250 mg) and $84.6 \pm 7.3\%$ (500 mg). These total recoveries were not significantly different from those found after single 250 or 500 mg doses. The fraction of the dose excreted in urine as unchanged diflunisal was always small (approximately 5%). The urinary recovery of DAG following 250 mg multiple dose administration ($43.5 \pm 2.1\%$) was not significantly different from that following single 250 mg ($47.2 \pm 4.5\%$) or 500 mg doses ($47.7 \pm 2.4\%$). Following 500 mg multiple dose administration, however, f_{DAG} was decreased significantly ($33.9 \pm 2.1\%$). The urinary recovery of DPG constituted approximately 30% of the dose and was relatively constant after single and multiple dose administration. The urinary recovery of DS increased significantly with dose in both the single and multiple dose experiments. The renal clearance of diflunisal and the partial metabolic clearances for formation of DPG and DAG were significantly less at steady state than after single dose administration. However, CL_{DS} (partial metabolic clearance to DS) was dose-independent (Table 1).

In vitro and *ex vivo* estimates of the plasma protein binding of diflunisal were similar, showing that the f_u value of diflunisal is concentration-dependent (Figure 2). Since the 500 mg twice daily steady state diflunisal plasma concentrations were in the range of concentration-dependent plasma binding, pharmacokinetic parameters were also calculated based on unbound plasma diflunisal (Table 2). Taking this into account, a more pronounced reduction (44%) in the steady state clearance of unbound diflunisal following 500 mg ($2.9 \pm 0.8 \text{ ml min}^{-1}$) as compared with 250 mg administration ($5.2 \pm 1.2 \text{ ml min}^{-1}$) was observed. Accordingly, more pronounced differences in the partial clearances of unbound diflunisal were also found between the 250 and 500 mg multiple dose data.

Average steady state plasma concentrations of DS (4.3 and $13.3 \mu\text{g ml}^{-1}$ for the 250 and 500 mg multiple dose administration respectively) were higher than those of DPG (2.8 and $10.0 \mu\text{g ml}^{-1}$). Nevertheless, steady state plasma concentrations of DS and DPG were only a fraction (4 to 8%) of the corresponding plasma diflunisal

Table 1 Mean ($\pm$ s.d.) pharmacokinetic parameters of total (bound + unbound) diflunisal following single dose (SD) and multiple dose (MD) administration of 250 and 500 mg tablets to five healthy volunteers

Parameter	Dosage regimen				ANOVA P
	250 SD	500 SD	250 MD	500 MD	
AUC ($\mu\text{g ml}^{-1}\text{ h}$)	375.4 ± 135.1	875.8 ^a ± 180.8	825.2 ^a ± 216.0	2019.6 ± 366.3	< 0.001
AUC/Dose	1.50 ^a ± 0.54	1.75 ^a ± 0.38	3.30 ^b ± 0.86	4.04 ^b ± 0.73	< 0.001
CL/F (ml min^{-1})	11.4 ^a ± 3.1	9.9 ^a ± 2.0	5.2 ^b ± 1.2	4.2 ^b ± 0.7	< 0.001
X _u (%) *	90.4 ± 9.1	91.6 ± 12.3	87.3 ± 6.8	84.6 ± 7.3	NS
f _e (%) *	5.2 ± 1.4	5.0 ± 1.2	4.2 ± 1.3	3.6 ± 0.9	NS
f _{DAG} (%) *	47.2 ^a ± 4.5	47.7 ^a ± 2.4	43.5 ^a ± 2.1	33.9 ± 2.1	< 0.001
f _{DPG} (%) *	31.8 ± 5.1	29.9 ± 3.3	28.7 ± 3.2	31.2 ± 4.1	NS
f _{DS} (%) *	6.1 ± 2.8	9.1 ^a ± 3.5	10.9 ^a ± 3.1	15.9 ± 3.6	< 0.001
CL _R (ml min^{-1})	0.59 ^a ± 0.24	0.49 ^a ± 0.14	0.22 ^b ± 0.05	0.15 ^b ± 0.02	< 0.001
CL _{DAG} (ml min^{-1})	5.48 ^a ± 1.90	4.73 ^a ± 1.12	2.30 ^b ± 0.56	1.44 ^b ± 0.30	< 0.001
CL _{DPG} (ml min^{-1})	3.51 ^a ± 0.50	2.92 ^a ± 0.50	1.50 ^b ± 0.27	1.31 ^b ± 0.19	< 0.001
CL _{DS} (ml min^{-1})	0.72 ± 0.42	0.91 ± 0.38	0.59 ± 0.24	0.68 ± 0.21	NS

* Percent of administered dose.

NS: not significantly different (ANOVA).

Parameter means with a superscript in common are not significantly different by multiple comparison (Student-Newman-Keuls test).

concentrations. Concentrations of DAG could not be measured in plasma owing to extensive rearrangement of this labile glucuronide to positional isomers (Figure 3). The renal clearance of DS following multiple dose administration of diflunisal was independent of dose (9.8 ± 2.8 and $8.8 \pm 1.3 \text{ ml min}^{-1}$ following the 250 and 500 mg doses, respectively). However, the renal clearance of DPG was significantly ($P < 0.05$, Student's *t*-test) lower following 500 mg compared with 250 mg multiple dose administration ($25.5 \pm 8.4 \text{ ml min}^{-1}$ vs $45.9 \pm 21.3 \text{ ml min}^{-1}$).

Discussion

Previous studies have shown that the pharmacokinetics of diflunisal are concentration-dependent (Davies, 1983; Loewen *et al.*, 1988; Meffin *et al.*, 1983). Following administration of single doses, this concentration-dependency seemed to arise mainly from saturation of the formation of the acyl glucuronide (Loewen *et al.*, 1988). Meffin *et al.* (1983) studied the multiple dose pharmacokinetics of diflunisal in patients with osteoarthritis. They found that the dose-dependent reduction in the oral clearance

Figure 2 Unbound plasma fraction of diflunisal (f_u) as a function of total diflunisal plasma concentration. The dashed line represents the unbound diflunisal plasma fraction determined *in vitro* in blank plasma of the five volunteers. The individual symbols represent the unbound plasma fraction of diflunisal determined *ex vivo* in the steady state plasma samples of the five volunteers following 250 and 500 mg multiple dose administration.

Table 2 Mean ($\pm$ s.d.) pharmacokinetic parameters for unbound diflunisal following single dose (SD) and multiple dose (MD) administration of 250 and 500 mg tablets to five healthy volunteers

Parameter	Dosage regimen				ANOVA P
	250 SD	500 SD	250 MD	500 MD	
AUC ($\mu\text{g ml}^{-1} \text{ h}$)	353.4 ± 118.0	826.0 ^a ± 139.9	865.3 ^a ± 272.7	2970.2 ± 875.4	<0.001
AUC/Dose	1.41 ^a ± 0.47	1.65 ^a ± 0.28	3.46 ± 0.92	5.94 ± 1.76	<0.001
CL/F (ml min^{-1})	12.9 ^a ± 4.4	10.3 ^a ± 1.7	5.2 ± 1.2	2.9 ± 0.8	<0.001
CL _R (ml min^{-1})	0.67 ^a ± 0.29	0.51 ^{a,b} ± 0.14	0.22 ^{b,c} ± 0.07	0.11 ^c ± 0.02	<0.001
CL _{DAG} (ml min^{-1})	6.09 ^a ± 2.21	4.91 ^a ± 0.94	2.23 ± 0.56	1.02 ± 0.24	<0.001
CL _{DPG} (ml min^{-1})	4.10 ^a ± 0.98	3.08 ^a ± 0.49	1.49 ^b ± 0.33	0.94 ^b ± 0.25	<0.001
CL _{DS} (ml min^{-1})	0.79 ± 0.52	0.93 ± 0.40	0.59 ± 0.24	0.49 ± 0.19	NS

NS: not significantly different (ANOVA).

Parameter means with a superscript in common are not significantly different by multiple comparison (Student-Newman-Keuls test).

Figure 3 H.p.l.c. chromatograms of: (a) blank plasma spiked with internal standard (IS), (b) plasma 4 h following a 250 mg oral dose of diflunisal at steady state, and (c) plasma 4 h following a 500 mg dose of diflunisal at steady state (ISO: rearrangement products of DAG).

of diflunisal decreased progressively as the daily dose increased between 250 and 1000 mg, and concluded that the nonlinear kinetics could not be explained entirely by simple saturation of the biotransformation pathways. They realized that a combination of concentration-dependent plasma protein binding and saturable metabolism of diflunisal could explain their findings. However, their *in vitro* binding studies failed to reveal a concentration-dependency in the plasma binding of diflunisal and they concluded that changes in plasma protein binding did not account for the unusual pharmacokinetics of diflunisal.

In the present study, we have shown that both glucuronidation pathways are saturable following multiple dose administration of diflunisal. In addition, we have confirmed our earlier observation that the plasma protein binding of diflunisal becomes concentration-dependent at plasma drug concentrations above $80\text{--}90\text{ }\mu\text{g ml}^{-1}$ (Loewen *et al.*, 1988), which is in contrast to the results obtained by Meffin *et al.* (1983). Since steady state plasma concentrations of diflunisal following daily doses of 1000 mg fall within the concentration-dependent plasma binding range, total plasma clearance at that dosage will be affected in opposite directions by: (1) saturation of glucuronidation (leading to a decrease in plasma clearance), and (2) increased unbound fraction of diflunisal in plasma (leading to an increase in total plasma clearance). As a result, plasma clearances calculated based on unbound rather than total plasma concentrations of diflunisal are better reflections of the intrinsic

capacity of the liver to conjugate the drug (Wilkinson & Shand, 1975). This opposing effect of saturable plasma binding and saturable metabolism has also been shown to occur with salicylic acid in man (Furst *et al.*, 1979), and diflunisal in rats (Lin *et al.*, 1985). The more pronounced reduction in unbound (44%) as compared to total plasma clearance of diflunisal (19%) between the two dosing levels at steady state observed in the present study is compatible with the explanation that decreased plasma binding tends to obscure the effect of saturation of the metabolizing enzymes.

In single-dose studies we found no evidence of saturation of the sulphation of diflunisal (Loewen *et al.*, 1988). The present results show that even after multiple dosing, sulphation of diflunisal is not saturated and thus follows linear kinetics. As a result of the saturation of the two glucuronidation pathways more diflunisal is available for the third parallel pathway (i.e. sulphation) and, consequently, the urinary recovery of DS is increased significantly. This is in marked contrast to observations with many other agents undergoing simultaneous glucuronidation and sulphation, for which sulphation is saturated first as the substrate dose is increased (Mulder, 1981).

Following administration of single doses of diflunisal, plasma concentrations of the drug conjugates are close to the level of analytical sensitivity. However, at steady state DS and DPG reach measurable concentrations in plasma. In the present study, DAG could not be measured in plasma because most of this labile

glucuronide appears in the form of rearrangement products. The rearrangement of diflunisal acyl glucuronide has been well documented (Hansen-Møller *et al.*, 1987; Musson *et al.*, 1985). It is not clear whether the occurrence of these positional isomers of DAG in unbuffered plasma is the result of *in vivo* or *in vitro* rearrangement. Acidifying plasma samples to inhibit rearrangement and hydrolysis of acyl glucuronides has been used successfully to analyze the glucuronides of zomepirac (Langendijk *et al.*, 1984) and tolmetin (Hyneck *et al.*, 1988). In the case of diflunisal the issue is complicated by the presence in plasma of the acid-labile sulphate conjugate (Dickinson & King, 1989; Loewen *et al.*, 1989). From the results of the present study it is clear that plasma concentrations of the diflunisal conjugates (including DAG, unless significant *in vitro* hydrolysis has occurred during sample handling) are low relative to those of diflunisal. The significant reduction in renal clearance of DPG on raising the multiple dose from 250 to 500 mg twice daily indicates saturation of the renal excretion of DPG. The renal clearance of DS did not differ between the two dosage regimens and therefore renal excretion of this conjugate was concentration-independent.

Extensive accumulation of diflunisal conjugates has been shown to occur in patients with impaired kidney function (Eriksson *et al.*, 1989; Verbeeck *et al.*, 1979). However, in these

studies plasma concentrations of unconjugated drug were almost certainly overestimated because of the instability of diflunisal sulphate during extraction of the plasma samples. In both studies diflunisal was extracted with ether following acidification of the plasma samples, a procedure which leads to fast and complete solvolysis of the sulphate conjugate (Dickinson & King, 1989; Loewen *et al.*, 1989). As a result, plasma diflunisal clearances would have been underestimated. The magnitude of the underestimation depends on the extent of accumulation of the sulphate conjugate in the plasma of these patients, which to our knowledge has not been described to date.

Urinary recovery of diflunisal and its three conjugates accounts for 85–90% of an oral dose. The remaining 10–15% may be excreted in the bile and faeces (Verbeeck *et al.*, 1988) or it may be excreted in the urine as the 3-hydroxy metabolite of diflunisal (i.e. hydroxylation in position 3 of the salicylic acid ring), identified recently in human urine by Macdonald *et al.* (1989).

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