

GLUCURONIDATION OF R- AND S-KETOPROFEN, ACETAMINOPHEN, AND DIFLUNISAL BY LIVER MICROSOMES OF ADJUVANT-INDUCED ARTHRITIC RATS

CHRISTINE J. MEUNIER AND ROGER K. VERBEECK

Pharmacokinetics and Drug Metabolism Laboratory, School of Pharmacy, Catholic University of Louvain, Brussels, Belgium

(Received April 15, 1998; accepted July 15, 1998)

This paper is available online at <http://www.dmd.org>

ABSTRACT:

The effect of adjuvant-induced arthritis on hepatic microsomal glucuronidation was studied in the rat. Arthritis was induced by injection of *Mycobacterium butyricum* suspended in liquid paraffin. V_{\max} and the Michaelis-Menten constant values for the in vitro glucuronidation of R- and S-ketoprofen, acetaminophen, and diflunisal by liver microsomes obtained from control and adjuvant-induced arthritic rats were compared. In addition, uridine 5'-diphosphate-glucuronosyltransferase activity toward bilirubin and p-nitrophenol, as well as levels of cytochrome P-450 and β -glucuronidase were determined in these microsomal preparations. Adjuvant-induced arthritis resulted in a significant reduction in hepatic cytochrome P-450 levels and in p-nitrophenol glucuronidation (5.65 ± 0.40 versus $2.58 \pm 0.27 \mu\text{mol}\cdot\text{min}/\text{mg}$ protein in control and arthritic rats, respectively, mean \pm S.E.M.). Glucuronidation of bilirubin and β -glucuronidase activities in liver microsomes and in plasma were

not affected by adjuvant-induced arthritis. V_{\max} (nmol/min/mg protein) for the formation of R-ketoprofen glucuronide, S-ketoprofen glucuronide, diflunisal phenolic glucuronide, and diflunisal acyl glucuronide was significantly decreased in arthritic rats (0.68 ± 0.10 , 0.77 ± 0.12 , 0.044 ± 0.005 , 0.26 ± 0.03 , respectively) compared with control rats (1.45 ± 0.04 , 1.60 ± 0.04 , 0.087 ± 0.008 , 0.46 ± 0.04 , respectively). Glucuronidation of p-nitrophenol, ketoprofen and diflunisal, substrates which seem to be at least partly glucuronidated in the rat by isoenzymes of the UGT2B subfamily, was impaired in adjuvant-induced arthritis. Glucuronidation of bilirubin and acetaminophen, substrates of UGT1- isoenzymes, was not affected by adjuvant-induced arthritis. It seems, therefore, that adjuvant-induced arthritis in the rat leads to impaired glucuronidation of substrates of the UGT2B subfamily.

The pharmacokinetics of a number of drugs is altered in patients with chronic inflammatory diseases, such as rheumatoid arthritis (Harris, 1981). The most likely causes involved are altered distribution and/or abnormal metabolism. Adjuvant-induced arthritis in rats, which resembles the human disease both clinically and histologically, is a widely used animal model to evaluate drug candidates for the treatment of rheumatoid arthritis. This animal model has also been used to investigate the effect of experimentally induced arthritis on drug metabolism. For example, in adjuvant-induced arthritic rats pentobarbital sleeping time was shown to be increased and hepatic activation of cyclophosphamide was reduced (Beck and Whitehouse, 1973; Dipasquale et al., 1974). This abnormal drug metabolism in adjuvant-induced arthritic rats was related to reduced cytochrome P-450 (CYP-450)¹ content in the liver (Cawthorne et al., 1976; Toda et al., 1994). Buchar and Janku (1985) have suggested that adjuvant-induced arthritis alters the phospholipid composition of the endoplasmic reticulum in the liver as a result of decreased synthesis of phosphatidylcholine and phosphatidylethanolamine. Consequently,

this alteration may lead to impairment of microsomal enzymes. Other reports have shown that interleukin-1, a cytokine mediator of inflammation, decreases the activity of hepatic CYP-450 in the rat (Sugita et al., 1990; Poüs et al., 1990).

Although the effect of adjuvant-induced arthritis on drug metabolism has been relatively well documented as far as CYP-450 catalyzed reactions are concerned, little information is available in the literature on the glucuronidation of drugs in experimentally induced arthritis in the rat (Toda et al., 1994). Glucuronidation of endo- and xenobiotics is catalyzed by the uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) system (EC 2.4.1.17), a family of closely related isoenzymes mainly located in the endoplasmic reticulum and exhibiting different, but overlapping, substrate specificities (Clarke and Burchell, 1994). The products of this conjugation reaction, i.e., β -glucuronides, are substrates for the hydrolytic enzyme β -glucuronidase. It has recently become evident that the β -glucuronidase catalyzed hydrolysis of certain glucuronide conjugates is so fast that it can affect the overall glucuronidation of a compound. Such futile glucuronidation-deglucuronidation cycling has been shown to occur in vitro (microsomes, intact cells) as well as in vivo (Brunelle and Verbeeck, 1993; Kauffman, 1994; Brunelle and Verbeeck, 1997). Some reports in the literature indicate that serum and/or β -glucuronidase activities are increased in patients with rheumatoid arthritis (Falkenbach et al., 1993) and in rats with adjuvant-induced arthritis (Reddy and Dhar, 1991). Whether this increase in β -glucuronidase activity in arthritic conditions is related to altered hepatic β -glucuronidase activity is not known.

¹ Abbreviations used are: CYP-450, cytochrome P450; UDP, uridine 5'-diphosphate; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HPLC, high-performance liquid chromatography; AG, acetaminophen glucuronide; R-KG, R-ketoprofen glucuronide; S-KG, S-ketoprofen glucuronide; DPG, diflunisal phenolic glucuronide; DAG, diflunisal acyl glucuronide; UGT-pnp, UGT activity toward p-nitrophenol.

Send reprint requests to: Dr. Roger K. Verbeeck, UCL/FATC 7355, School of Pharmacy, Av. E. Mounier 73, 1200 Brussels, Belgium email: verbeeck@fatc.ucl.ac.be

We therefore decided to investigate the effect of adjuvant-induced

arthritis on the hepatic microsomal glucuronidation of three different substrates. Ketoprofen and acetaminophen were selected because they are glucuronidated by different UGT isoenzymes in the rat (Clarke and Burchell, 1994). In addition, the *in vitro* glucuronidation of diflunisal was investigated by using liver microsomes of control and adjuvant-induced arthritic rats. Diflunisal is an interesting substrate for studying glucuronide conjugation because it forms two different types of glucuronides (i.e., both a phenolic and an acyl glucuronide) and because the *in vitro* formation of its acyl glucuronide is significantly influenced by the microsomal β -glucuronidase activity (Brunelle and Verbeeck, 1993).

Materials and Methods

Chemicals and Reagents. Pure *R*- and *S*-ketoprofen enantiomers were kindly supplied by Dr. M. R. Martinet (Rhône-Poulenc Rorer, Vitry sur Seine, France). UDP-Glucuronic acid (UDPGA), Brij 58, Triton X-100, glucaro-1,4-lactone, acetaminophen, *R*, *S*-ketoprofen, diflunisal, phenolphthalein, phenolphthalein glucuronide, 4-methylumbelliferone, 4-methylumbelliferone glucuronide, bilirubin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Digitonin was obtained from Calbiochem (La Jolla, CA) and tris(hydroxymethyl)-aminomethane (Tris) from Merck AG (Darmstadt, Germany). Glucuronides of the drugs studied (i.e., *R*- and *S*-ketoprofen, acetaminophen and diflunisal) were isolated from human urine and purified by semipreparative high-performance liquid chromatography (HPLC). Acetonitrile (HPLC grade) was purchased from Labsan (Dublin, Ireland). All other chemicals used were of the highest purity available from standard commercial sources.

Induction of Arthritis and Preparation of Liver Microsomes. Male Wistar rats (Janssen Pharmaceutica, Beerse, Belgium), weighing 160 to 180 g, received an injection of *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) suspended in liquid paraffin (0.5 mg/0.1 ml) into the tail base (Awouters et al., 1976). Control animals were injected with an equivalent volume of liquid paraffin. By day 20, the rats injected with *M. butyricum* had developed arthritis. The degree of arthritis was assessed by measuring the circumference of the right and left ankles. The rats were sacrificed by decapitation 20 days after the injection of adjuvant or vehicle. Blood was collected in heparinized tubes (Monovette, Sarstedt, Nümbrecht, Germany) and liver microsomes were prepared according to the method of Amar-Costesec et al. (1974). The protein concentration of the microsomal preparations was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme Assays. The activity of β -glucuronidase in rat liver microsomal suspensions was determined using phenolphthalein glucuronide as substrate. After incubating phenolphthalein glucuronide for 30 min in the microsomal suspension at pH 5.0 (0.1 M sodium acetate buffer), the concentration of released phenolphthalein was determined photometrically (540 nm). The β -glucuronidase activity in the microsomal suspension is expressed as micrograms phenolphthalein liberated per mg protein in 1 h at 37°C. The activity of β -glucuronidase in rat plasma was determined using 4-methylumbelliferone glucuronide as substrate as described by Mead et al. (1955).

Rat liver microsomal UGT activities toward bilirubin and *p*-nitrophenol were determined by the procedure of Mulder et al. (1975) and Heirwegh et al. (1972), respectively. The rat liver microsomal CYP-450 concentration was determined by the method of Omura and Sato (1964).

In Vitro Glucuronidation Kinetics. In preliminary experiments, the effect of different detergents on the activation of rat liver microsomal UGT was studied. Microsomal suspensions were preincubated for 30 min on ice at various detergent/microsomal protein ratios between 0 and 2 mg detergent/mg protein for digitonin, between 0 and 0.4 mg detergent/mg protein for Triton X-100, and between 0 and 0.3 mg detergent/mg protein for Brij 58. Maximal activation of UGT activity toward all substrates studied (ketoprofen, acetaminophen, diflunisal) occurred with Brij 58 at a concentration of 0.15 mg/mg protein. Therefore, in all subsequent experiments pretreatment with Brij 58 (0.15 mg/mg protein) was used to activate the rat liver microsomes.

In preliminary experiments, the linearity of glucuronide formation was checked by varying protein concentration (0–4 mg), time (0–120 min) and UDPGA concentration (0–5 mM). The incubation mixture (total volume 0.5

ml) contained the following: Brij 58 activated rat liver microsomes (1 mg/ml), 0.2 M Tris-HCl buffer (pH 7.4), 3 mM UDPGA, 10 mM MgCl₂, and 4 mM glucaro-1,4-lactone (only for incubations with diflunisal) and as substrate *R*, *S*-ketoprofen (0–1.6 mM), acetaminophen (0–30 mM) or diflunisal (0–1.6 mM). Microsomal suspensions were incubated for 20 min at 37°C in a shaking water bath. The reactions were stopped by adding to 200 μ l of the incubation mixture either 20 μ l of HClO₄ 30% containing 75 μ g/ml 3-acetamidophenol (the internal standard for the acetaminophen glucuronide (AG) assay), or 200 μ l 0.6 M glycine-0.4 M trichloroacetic acid buffer (pH 2.2) containing 10 μ g/ml indoprofen (the internal standard for the assay of the ketoprofen glucuronides) or 80 μ l acetonitrile containing 4% acetic acid and 750 μ g/ml clofibrac acid (internal standard for the assay of diflunisal glucuronides).

Stability of Drug Glucuronides during Incubation with Rat Liver Microsomes. The stabilities of the *R*- and *S*-ketoprofen glucuronides (*R*-KG, *S*-KG) and of AG were studied under the conditions of the microsomal incubations. *R*-KG and *S*-KG (10 μ M) and AG (10 μ M) were incubated in the presence and absence of native rat liver microsomes in incubation medium which did not contain UDPGA. The stability of these glucuronides was tested during a total incubation period of 24 h. At certain time intervals, 100 μ l of the incubation mixture was sampled and either 10 μ l of a 2.5 μ g/ml indoprofen solution in 0.6 M glycine-0.4 M trichloroacetic acid buffer, pH 2.2 (ketoprofen glucuronides assay), or 10 μ l of a 200 μ g/ml 3-acetamidophenol solution in 30% HClO₄ (AG assay) was added. After mixing (vortex) and centrifugation, an aliquot of the supernatant was injected into the HPLC system.

The stability of the two diflunisal glucuronides, diflunisal phenolic glucuronide (DPG) and diflunisal acyl glucuronide (DAG), had already been studied in detail before (Brunelle and Verbeeck, 1993). As a result of the β -glucuronidase catalyzed hydrolysis of DAG during the microsomal incubation, 4 mM glucaro-1,4-lactone was added to the incubation medium (see previous section) to inhibit DAG hydrolysis.

HPLC Assays. To quantify *R*-KG and *S*-KG, the incubation mixtures were vortexed and centrifuged and an aliquot (150 μ l) of the supernatant was passed through a Bond Elut C₁₈ cartridge (Analytical International, Harbor City, CA) which had been previously activated with 3 ml of acetonitrile. After washing with 1 ml of water-trifluoroacetic acid (90:10, v/v), the ketoprofen glucuronides were eluted with acetonitrile (0.75 ml) and the eluate was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was redissolved in mobile phase buffer (400–600 μ l) and a 50- μ l aliquot was injected onto the HPLC system. The HPLC method was based on the method of Chakir et al. (1994) using a Superspher 100 RP-18 (Merck AG) end-capped analytical column (125 \times 4 mm; particle size, 4 μ m). The mobile phase consisted of acetonitrile and 10 mM tetrabutylammonium bromide in 1 mM potassium phosphate buffer (pH 4.3; 30:70, v/v). The flow rate was 1.4 ml/min and the eluent was monitored at 254 nm (Kontron 322 UV detector, Zurich, Switzerland). The column was maintained at a temperature of 35°C. In preliminary experiments, pure *S*- and *R*-ketoprofen enantiomers were incubated in the presence of rat liver microsomes to identify *R*-KG and *S*-KG on the HPLC chromatograms.

AG was quantified in the supernatant of the microsomal incubation mixture at 254 nm (Kontron 322 UV detector) using a C₁₈ column (Hypersil ODS, 250 \times 4.6 mm, 5 μ m particle size; Alltech Associates, Deerfield, IL) according to the method of Miners et al. (1990). The mobile phase was composed of acetonitrile and 0.015 M KH₂PO₄ phosphate buffer (pH 2.7; 1.5:98.5, v/v). The flow rate was 2.25 ml/min.

Diflunisal glucuronides in the supernatant of the microsomal incubation mixtures were quantified according to the method of Dickinson and King (1989). Briefly, the mobile phase, a mixture of methanol and 0.02 M Na₂HPO₄ phosphate buffer (pH 2.7) containing 1% (w/v) Na₂SO₄·10H₂O (55:45, v/v) was delivered to a C18 column (Hypersil ODS, 250 \times 4.6 mm, 5 μ m particle size). The flow rate was 1 ml/min and the eluent was monitored at 254 nm (Kontron 433 UV detector).

Data Analysis. The Michaelis-Menten equation was used to determine apparent V_{\max} and K_m values for the microsomal glucuronidation of *R*- and *S*-ketoprofen, acetaminophen, and diflunisal by using nonlinear regression analysis (Statistica, Statsoft Inc., Tulsa, OK):

$$v = \frac{V_{\max} \cdot S}{K_m + S}$$

TABLE 1

Ankle circumferences (in mm) on day 20 following injection of *Mycobacterium* in control and adjuvant-treated rats

	Control Rats		Adjuvant-treated Rats	
	Right Ankle	Left Ankle	Right Ankle	Left Ankle
1	7.5	7.5	1	12.0
2	7.5	7.5	2	13.5
3	7.5	7.5	3	11.5
4	7.0	7.5	4	12.0
5	7.0	7.0	5	14.0
Mean	7.3	7.4	Mean	12.6
± S.E.M.	0.1	0.1	± S.E.M.	0.5

where v is the initial rate of the glucuronidation reaction, V_{\max} is the maximum rate, K_m is the substrate concentration at which the reaction rate is half of its maximal value, and S is the substrate concentration.

All results are expressed as mean \pm S.E.M. Mean values of ankle circumferences, enzyme activities, or enzyme parameters obtained in control and arthritic rats were compared by unpaired Student's t test. A possible correlation between V_{\max} values for glucuronidation of the drug substrates and UGT activities toward bilirubin and p -nitrophenol was shown by simple linear correlation analysis. A p -value of .05 or less was considered significant.

Results

Induction of Arthritis. Approximately 10 days after injection of *M. butyricum* into the base of the tail, rats showed swelling of the hind limbs and typical skin lesions appeared on the skin and the tail. Table 1 shows the ankle circumferences of rats on day 20 after injection of adjuvant or vehicle. Ankle circumferences were approximately 70% higher ($p < .001$) and body weight was approximately 30% lower ($p < .001$) in adjuvant-treated rats compared with control rats. In addition, on day 20 after injection of *M. butyricum* or vehicle body weights were significantly lower ($p < .001$) in adjuvant-treated (209 \pm 3 g) compared with control rats (296 \pm 3 g).

Effect of Experimental Arthritis on Microsomal Enzyme Activities. Protein concentration, CYP-450 concentration, and UGT activities toward bilirubin and p -nitrophenol were measured in liver microsomes of five control and five arthritic rats. In addition, microsomal and plasma β -glucuronidase activities were determined in the same animals. Microsomal CYP-450 concentration and UGT activity towards p -nitrophenol (UGT-pnp) were significantly reduced in arthritic rats compared with control rats. The other concentrations or activities measured did not show significant differences between the two groups of animals (Table 2).

Hydrolysis of the Glucuronides of R - and S -Ketoprofen and of Acetaminophen by Rat Liver Microsomes. The stability of R -KG, S -KG, and AG was studied in the absence and presence of rat liver microsomes. Incubations were carried out using liver microsomes of three control rats. In the absence of rat liver microsomes, R -KG and S -KG underwent spontaneous hydrolysis with half-lives of 66.3 \pm 2.0 min and 101.2 \pm 1.1 min, respectively. Hydrolysis of R -KG and S -KG was faster when incubated in the presence of microsomes; under such conditions, the half-lives of R -KG and S -KG were 58.5 \pm 0.8 min and 80.3 \pm 3.1 min, respectively (Fig. 1). Addition of 4 mM glucaro-1,4-lactone to the incubation medium containing microsomes resulted in a degradation curve for both R -KG and S -KG which was identical with the control incubation without microsomes (Fig. 1). After a 20-min incubation (i.e., the incubation time selected for the glucuronidation kinetic studies) in the absence of microsomes, 78 \pm 1.5% and 87 \pm 1.1% of R -KG and S -KG, respectively, remained in the medium. These percentages were very similar when the incubation medium contained liver microsomes (75 \pm 0.4% and 84.3 \pm 3.8% for R -KG and S -KG, respectively). Because of this small difference in stability

TABLE 2

Liver microsomal protein concentration, CYP-450 concentration, UGT activities toward bilirubin and p -nitrophenol (UGT-pnp) and β -glucuronidase activity (in microsomes and plasma) of control ($n = 5$) and arthritic rats ($n = 5$)

	Control Rats ($n = 5$)	Arthritic Rats ($n = 5$)
Protein (mg/ml)	6.37 \pm 0.28	6.03 \pm 0.27
CYP-450 (nmol/mg protein)	0.56 \pm 0.03	0.13 \pm 0.02***
UGT-bilirubin (nmol/min/mg protein)	0.080 \pm 0.005	0.089 \pm 0.002
UGT-pnp (μ mol/min/mg protein)	5.65 \pm 0.40	2.58 \pm 0.27***
β -Glucuronidase		
Microsomes (SU/mg protein) ^a	55.1 \pm 1.9	56.6 \pm 2.4
Plasma (units/ml) ^b	1.89 \pm 0.11	1.96 \pm 0.08

*** $p < .005$.

^a SU (Sigma unit): one SU corresponds to 1 μ g of phenolphthalein liberated in 1 h, at 37°C, and per mg protein.

^b One unit corresponds to 1 μ g of 4-methylumbelliferone liberated in 1 h at 37°C.

of the ketoprofen glucuronides during incubations in the presence and absence of rat liver microsomes, there was no need to add the β -glucuronidase inhibitor glucaro-1,4-lactone to the incubation medium during the 20-min incubation to determine the glucuronidation kinetics of R - and S -ketoprofen. AG was much more stable during incubations in the microsomal medium; its half-life was very long (12.4 \pm 1.3 h), even in the presence of rat liver microsomes. Therefore, hydrolysis of AG under the conditions of the in vitro glucuronidation experiments with hepatic microsomes is negligible.

Effect of Experimental Arthritis on the In Vitro Glucuronidation of R - and S -Ketoprofen, Acetaminophen, and Diflunisal. Liver microsomes from control and arthritic rats were incubated in the presence of increasing concentrations of R , S -ketoprofen ($n = 5$), acetaminophen ($n = 5$), and diflunisal ($n = 4$) to determine apparent V_{\max} and K_m values for the formation of the respective glucuronides, i.e., R -KG, S -KG, AG, DPG, and DAG. These experiments were carried out in the presence of glucaro-1,4-lactone (4 mM) for the incubations with diflunisal (Brunelle and Verbeeck, 1993).

Glucuronidation of R - and S -ketoprofen was impaired in arthritic rats (Fig. 2). Apparent V_{\max} values for the formation of R -KG and S -KG were significantly smaller ($p < .005$) in arthritic rats (0.68 \pm 0.10 and 0.77 \pm 0.12 nmol/min/mg protein, respectively) compared with control rats (1.45 \pm 0.04 and 1.60 \pm 0.04 nmol/min/mg protein, respectively). K_m for the glucuronidation of both R -KG and S -KG was not affected by experimentally induced arthritis. Moreover, glucuronidation of R , S -ketoprofen by rat liver microsomes was apparently stereospecific: V_{\max} values for the formation of S -KG in control and arthritic rats (1.60 \pm 0.04 and 0.77 \pm 0.12 nmol/min/mg protein, respectively) were slightly (approximately 10%) but statistically significantly higher ($p < .005$ in control rats, $p < .01$ in arthritic rats) than V_{\max} values for the formation of R -KG (1.45 \pm 0.04 and 0.68 \pm 0.10 nmol/min/mg protein, respectively). No stereospecific differences were observed in K_m . The greater stability of S -KG in the microsomal suspension during the 20-min incubation period may be responsible for this apparent stereoselectivity in the glucuronidation of R , S -ketoprofen.

Adjuvant-induced arthritis had no statistically significant effect on the in vitro glucuronidation of acetaminophen in rat liver microsomes (Table 3; Fig. 2). On the contrary, the in vitro glucuronidation studies with diflunisal showed that adjuvant-induced arthritis significantly decreased the apparent V_{\max} values for the formation of DPG and DAG (Table 3; fig. 2). The apparent K_m values for the formation of DPG and DAG were not significantly different in arthritic rats compared to control animals.

UGT-pnp was significantly correlated with the apparent V_{\max} values for the formation of R -KG, S -KG, DPG, and DAG, but not with

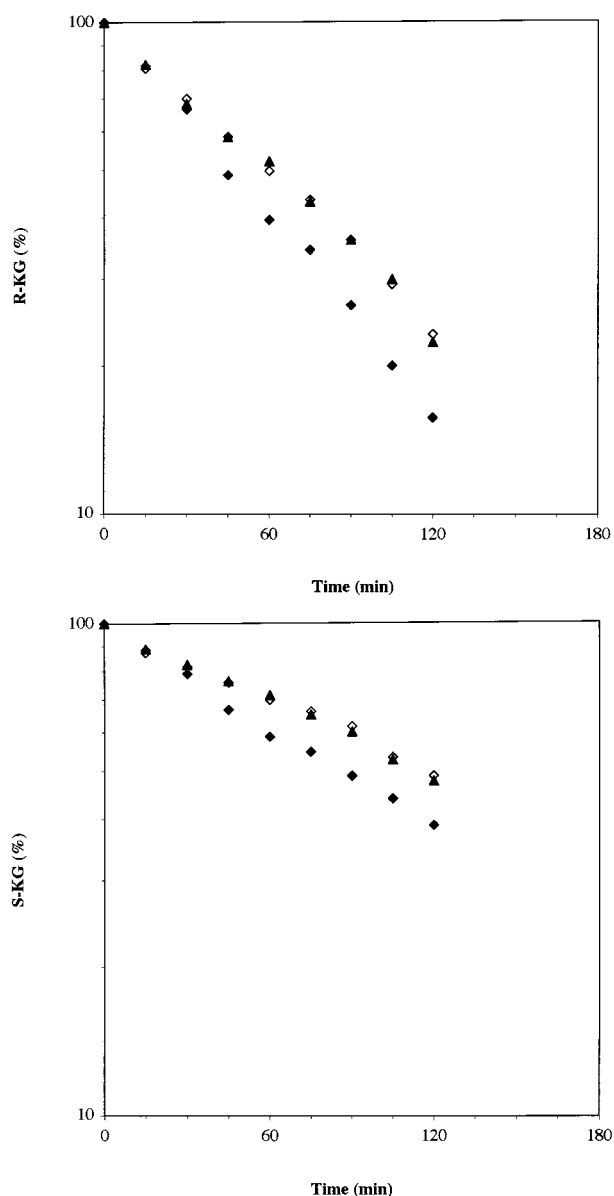


FIG. 1. Hydrolysis of *R*-ketoprofen glucuronide (top) and *S*-ketoprofen glucuronide (bottom) during incubation at 37°C in medium in the absence (\diamond) and presence (\blacklozenge) of rat liver microsomes.

The effect of glucaro-1,4-lactone (4 mM) on the hydrolysis of both glucuronides in the presence of rat liver microsomes was also determined (\blacktriangle). Each point is the average of measurements carried out on rat liver microsomes of three individual rats.

the apparent V_{\max} for the formation of AG. UGT activity toward bilirubin, on the contrary, did not show a significant correlation with the V_{\max} values for the formation of AG, *R*-KG, *S*-KG, DPG, or DAG (Table 4).

Discussion

Although less information is available on rat UGT than on human UGT, two UGT families have been characterized in both species (Burchell et al., 1991; Clarke and Burchell, 1994; Mackenzie et al., 1996; Mackenzie et al., 1997). In the rat, the isozymes of the UGT1 family catalyze the glucuronidation of planar phenols (UGT1A6) and bilirubin (UGT1A1, UGT1A2, and UGT1A4P). Subfamily 2A consists of a unique olfactory UGT present in support cells of the

olfactory epithelium. The various isoenzymes of the UGT2B subfamily (2B1, 2B2, 2B3, 2B6, and 2B12) are responsible for the glucuronidation of steroids, bile acids, and a number of drugs (e.g., profen nonsteroidal anti-inflammatory drugs such as ketoprofen) and other exogenous substances. Of the substrates tested in the present study, bilirubin is glucuronidated by UGT1A1, UGT1A2, and UGT1A4P, acetaminophen is probably also mainly glucuronidated by isoenzymes of the UGT1 family (analogous to the situation in humans where UGT1A6 is the major isoenzyme involved in the glucuronidation of this analgesic), and *p*-nitrophenol is glucuronidated by isoenzymes of the UGT1 family (UGT1A6), UGT2A1, and isoenzymes of the UGT2B subfamily (e.g., UGT2B12) (Antoine et al., 1993; Clarke and Burchell, 1994). Ketoprofen has been shown to be a substrate of the rat UGT2B1 isoenzyme but not of UGT1A1 (Pritchard et al., 1994; King et al., 1996). In humans, diflunisal is glucuronidated by UGT1A9 and to a lesser extent by UGT2B7 (Burchell et al., 1995). The UGT isoenzymes responsible for the glucuronidation of diflunisal in the rat have not been clearly identified, although isoenzymes of the UGT1A subfamily seem to be involved because Gunn rats show a reduced formation of DAG and no longer form DPG (Dickinson et al., 1991).

Adjuvant-induced arthritis in the rat has not the same effect on the various substrates we tested. The glucuronidation of bilirubin and acetaminophen is not affected at all, whereas the glucuronidation of *p*-nitrophenol, *R*- and *S*-ketoprofen, and diflunisal (to form the phenolic and the acyl glucuronide) is significantly impaired in adjuvant-induced arthritis. Because the substrate specificities of the UGT isoenzymes in the rat involved in the glucuronidation of the various substrates tested are not well known, it is difficult to conclude based on our observations which UGT isoenzymes would be affected by adjuvant-induced arthritis in the rat. However, it seems that those substrates which are apparently exclusively glucuronidated by isoenzymes of the UGT1 family (i.e., bilirubin and acetaminophen) are not affected. The substrates *p*-nitrophenol, *R*-ketoprofen, *S*-ketoprofen, and diflunisal, showing reduced glucuronidation in adjuvant-induced arthritis, are substrates for UGT isoenzymes of both family 1 and subfamily 2B, suggesting that only isoenzymes of the UGT2B subfamily are affected by adjuvant-induced arthritis. However, additional studies are needed with more selective substrates to confirm this hypothesis.

Correlation analysis showed that UGT-pnp was significantly correlated to V_{\max} values for the glucuronidation of *R*-ketoprofen, *S*-ketoprofen, and diflunisal (both formation of the phenolic and acyl glucuronides). The r^2 values for these correlations were between 0.53 and 0.66, indicating that UGT-pnp is an important predictor of the V_{\max} values for the formation of these drug glucuronides. This is consistent with observations in the rat that *p*-nitrophenol glucuronidation is catalyzed by several UGT isoenzymes of the UGT1 and UGT2B (sub)families (Antoine et al., 1993; Clarke and Burchell, 1994) and that ketoprofen and diflunisal are also substrates for one or more of the isoenzymes responsible for the glucuronidation of *p*-nitrophenol.

We could not confirm reports that serum and/or tissue β -glucuronidase levels are increased in arthritis (Reddy and Dhar, 1991; Falkenbach et al., 1993). Hepatic microsomal β -glucuronidase activity was also not affected by adjuvant-induced arthritis. This is important to know when studying in vitro microsomal glucuronidation. Certain glucuronides are susceptible to rapid β -glucuronidase catalyzed hydrolysis which may lead to futile glucuronidation-deglucuronidation cycling and significant underestimation of the intrinsic glucuronidation rate of the substrate (Brunelle and Verbeeck, 1993). We showed that *R*-KG and *S*-KG, which are acyl glucuronides, undergo hydrolysis during incubation at 37°C in the presence of rat liver microsomes. This hydrolysis

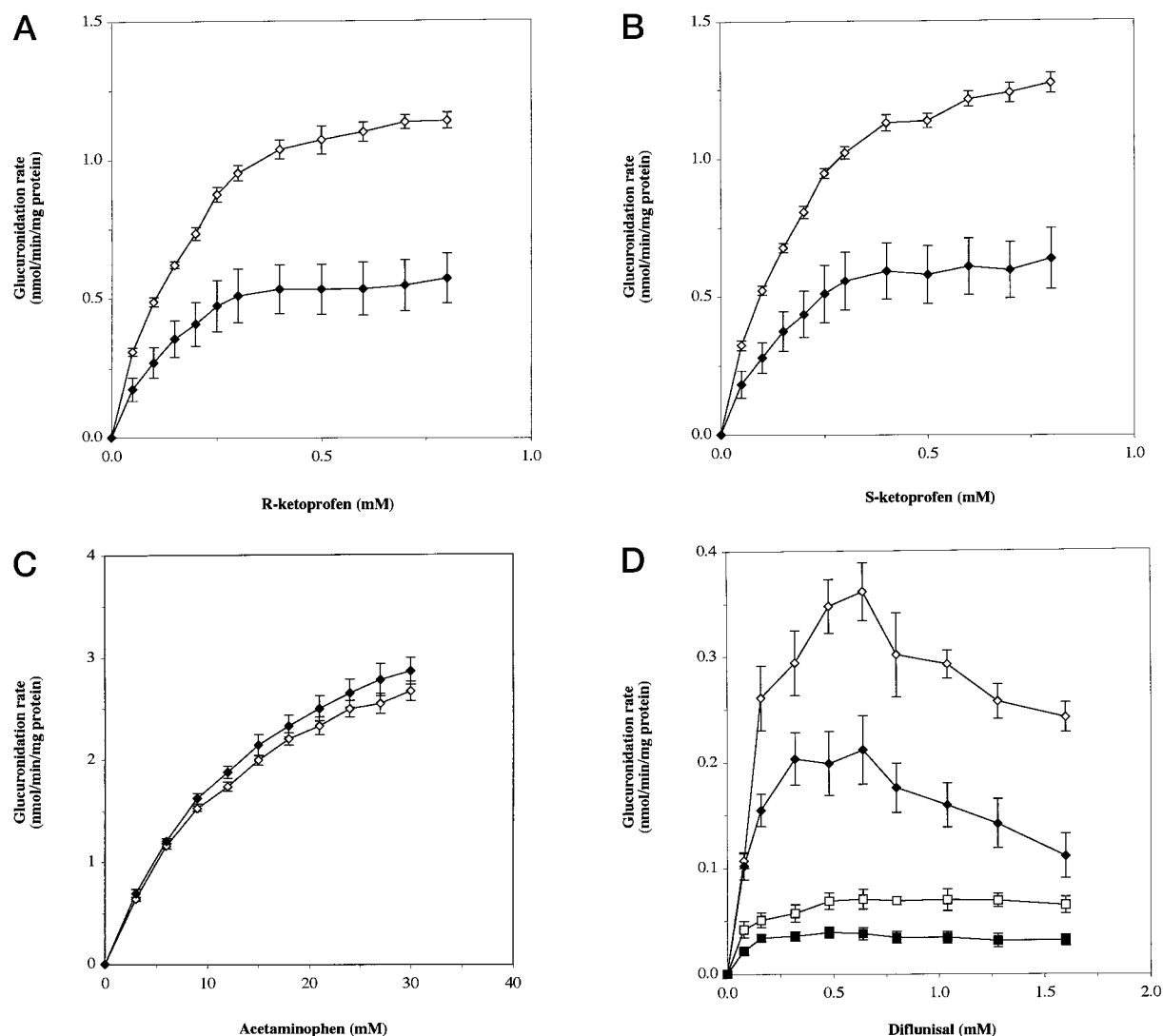


Fig. 2. Michaelis-Menten plots for the glucuronidation of R-ketoprofen (A), S-ketoprofen (B), acetaminophen (C) and diflunisal (D).

Open symbols represent control rats ($n = 5$) and closed symbols arthritic rats ($n = 5$). In case of diflunisal (D) two glucuronides were formed, i.e., diflunisal acyl glucuronide (\diamond, \blacklozenge) and diflunisal phenolic glucuronide (\square, \blacksquare).

TABLE 3

Apparent V_{max} and K_m values for the glucuronidation of R- and S-ketoprofen, acetaminophen, and diflunisal by liver microsomes of control and arthritic rats

Parameter	Substrate	Control Rats ($n = 5$) ^a	Arthritic Rats ($n = 5$) ^a
V_{max} (nmol/min/mg protein)	R-ketoprofen	1.45 ± 0.04	$0.68 \pm 0.10^{***}$
	S-ketoprofen	1.60 ± 0.04	$0.77 \pm 0.12^{***}$
	Acetaminophen	4.0 ± 0.2	4.4 ± 0.3
	Diflunisal: DPG	0.087 ± 0.008	$0.044 \pm 0.005^{***}$
K_m (mM)	Diflunisal: DAG	0.46 ± 0.04	$0.26 \pm 0.03^{**}$
	R-ketoprofen	0.19 ± 0.01	0.16 ± 0.03
	S-ketoprofen	0.19 ± 0.008	0.18 ± 0.03
	Acetaminophen	15.2 ± 1.0	15.7 ± 1.1
	Diflunisal: DPG	0.12 ± 0.03	0.10 ± 0.01
	Diflunisal: DAG	0.17 ± 0.02	0.11 ± 0.02

^a $n = 4$ for the experiments with diflunisal as substrate.

** $p < .01$.

*** $p < .005$.

seems to be mostly spontaneous (chemical instability). AG (a phenolic glucuronide) is much more stable under the same conditions. These results are compatible with the known instability of acyl glucuronides (Faed, 1984). In addition, it seems that acyl glucuronides are better substrates for hepatic microsomal β -glucuronidase than phenolic gluc-

uronides, which was already clearly shown for the phenolic and acyl glucuronides of diflunisal: the half-life of diflunisal acyl glucuronide when incubated in the presence of rat liver microsomes was ± 12 min compared with ± 35 h for the phenolic glucuronide (Brunelle and Verbeeck, 1993). The slightly faster hydrolysis of R-KG compared

TABLE 4

Correlation coefficients, r , between UGT activities toward *p*-nitrophenol and bilirubin and apparent V_{max} values (nmol/min/mg protein) for the formation of AG, R-KF, S-KF, DAG, and DPG

	UGT-bilirubin (nmol/min/mg protein)	UGT-pnp (μ mol/min/mg protein)
V_{max} AG	0.42	0.06
V_{max} R-KG	0.47	0.81**
V_{max} S-KG	0.45	0.81**
V_{max} DAG	0.36	0.73*
V_{max} DPG	0.44	0.76*

* $p < .05$.

** $p < .005$.

with the hydrolysis of the S-KG may explain the observed apparent stereoselectivity in the glucuronidation rate of the ketoprofen enantiomers. This illustrates how careful one should be when carrying out microsomal incubations during which glucuronides are formed that may undergo spontaneous or β -glucuronidase catalyzed hydrolysis. We could not confirm previous reports that rheumatoid arthritis is associated with increased levels of β -glucuronidase in plasma.

Little is known about the mechanisms explaining the impaired metabolism in adjuvant-induced arthritic rats. Interleukin-1, an important cytokine mediator released by monocytes and macrophages in case of inflammatory processes, has been shown to lower CYP-450 levels and activities in rat hepatocytes (Sugita et al., 1990; Poüs et al., 1990). The potential role of interleukin-1, or any other mediator of inflammatory processes, on UGT activity is not known. Our interesting observation that adjuvant-induced arthritis leads to reduced glucuronidation of certain substrates whereas the glucuronidation of other substrates is unaffected requires further investigation into the factors controlling the expression of UGT isoenzyme activities.

Acknowledgments. We thank Martine Petit for her excellent technical assistance.

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