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GLUCURONIDATION OF DIFLUNISAL BY RAT LIVER MICROSOMES

EFFECT OF MICROSOMAL β-GLUCURONIDASE ACTIVITY

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Abstract—The *in vitro* formation rates of the phenolic (DPG) and acyl (DAG) glucuronides of diflunisal were investigated using rat liver microsomes. Preliminary studies showed that DAG hydrolysed rapidly (T_i = 12 min) when incubated in the presence of rat liver microsomes at pH 7.4 and 37°. DPG was much more stable under the same conditions (T_i = 35 hr). Hydrolysis of DAG and DPG by rat liver microsomes was inhibited by 4 mM saccharolactone, a β-glucuronidase inhibitor. The apparent K_m and V_{max} values for the formation of DAG in the absence and presence of 4 mM D-saccharic acid-1,4-lactone (saccharolactone) were the following: K_m = 0.05 ± 0.02 vs 0.08 ± 0.02 mM and V_{max} = 0.20 ± 0.06 vs 0.43 ± 0.07 nmol/min/mg protein (0 and 4 mM saccharolactone, respectively). The significant increase in apparent V_{max} for DAG formation in the presence of saccharolactone can be explained by the inhibition of β-glucuronidase-catalysed hydrolysis of DAG. Apparent K_m and V_{max} values for the formation rate of DPG were not affected by addition of saccharolactone to the incubation medium. These results indicate that β-glucuronidase-catalysed hydrolysis of certain glucuronides formed during microsomal incubations may significantly affect the apparent glucuronidation rate due to the presence of a glucuronidation–deglucuronidation cycle.

For many xenobiotics as well as endogenous compounds glucuronidation is an important metabolic pathway [1]. Conjugation with glucuronic acid is mediated by UDP-glucuronosyltransferase (UGT,† EC 2.4.1.17), a family of isoenzymes with different but overlapping substrate specificities. UGTs are predominantly located in the endoplasmic reticulum of liver cells, although appreciable UGT activity has also been shown in a variety of other organs including the kidney, gut wall and lung [2]. Hydrolysis of glucuronides may be catalysed by β-glucuronidase, an enzyme widely distributed in mammalian tissues with a particularly high activity in the liver [3]. The physiologic function of β-glucuronidase is not clear although it has been suggested that it may play a role in the release of active steroid hormone from the steroid hormone glucuronide pool.

Diflunisal, a difluorophenyl derivative of salicylic acid, undergoes glucuronide conjugation in man and rat to form the phenolic glucuronide (DPG) and the acyl glucuronide (DAG) [4, 5]. In recent years acyl glucuronides, including DAG, have attracted a lot of attention because they can be considered as potentially reactive intermediates capable of undergoing hydrolysis, intramolecular rearrangement via acyl migration, and covalent binding to plasma proteins and tissue macromolecules [6–8]. *In vivo*, acyl glucuronides may not only hydrolyse spontaneously (chemically) but may also be cleaved enzymatically by esterases [9, 10] and by β-glucuronidase [11, 12].

Spontaneous (chemical) or enzymatic hydrolysis of drug glucuronides during *in vitro* experiments may lead to a glucuronidation–deglucuronidation futile cycle which would complicate the interpretation of the results. Since both UGT and β-glucuronidase activities are present in liver microsomes [13], we investigated the stability of the phenolic and acyl glucuronides of diflunisal during standard microsomal incubation procedures. The advantage of using diflunisal as substrate for these *in vitro* microsomal experiments is that two types of glucuronides of the same aglycon can be simultaneously investigated to test their susceptibility to chemical and enzymatic hydrolysis under standard incubation conditions.

MATERIALS AND METHODS

Chemicals and reagents. UDPGA (UDP-glucuronic acid), D-saccharic acid-1,4-lactone (saccharolactone), Brij 58, Triton X-100, diflunisal, phenolphthalein, phenolphthalein glucuronide, and bovine serum albumin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Digitonin was obtained from Calbiochem (La Jolla, CA, U.S.A.) and Tris from Merck AG (Darmstadt, Germany). Diflunisal glucuronides were isolated from human urine and purified as described before [14]. Methanol and acetonitrile (HPLC grade) were purchased from Labscan (Dublin, Ireland). All other

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† Abbreviations: UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; DAG, diflunisal acyl glucuronide; DPG, diflunisal phenolic glucuronide.

chemicals were of the highest purity available from standard commercial sources.

Animals and preparation of liver microsomes. Male Wistar rats (Janssen Pharmaceutica, Beerse, Belgium), weighing 250–300 g, were used to prepare liver microsomes. The rats were killed by decapitation and the livers homogenized with 3 vol. of 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole. Microsomes were prepared according to the method of Amar-Costesec *et al.* [15]. The final microsomal pellet was homogenized in 0.25 M sucrose-imidazole buffer and stored at −30°. The protein concentration of the microsomal preparations was determined by the method of Lowry *et al.* [16] using bovine serum albumin as standard.

Incubation conditions for UGT activity. The incubation mixture (total volume of 0.5 mL) contained: activated microsomes (1 mg protein), 0.2 M Tris–HCl buffer pH 7.4, 2 mM UDPGA, 2 mM $MgCl_2$, 0.037 mM diflunisal and saccharolactone (0 or 4 mM). Microsomal suspensions were incubated for 15 min at 37° in a shaking water bath. In preliminary experiments reaction rates were shown to be linear for incubation times up to 45 min and for microsomal protein content to at least 3 mg/mL. The reaction was stopped by adding 200 μL acetonitrile containing 4% acetic acid and the internal standard (naproxen or clofibric acid). The mixture was vortexed and centrifuged and 20 μL of the supernatant were injected onto the HPLC column.

Effect of detergents on UGT activity. To study the effect of different detergents on the activation of UGT, microsomal suspensions were preincubated for 30 min on ice at various detergent/protein ratios between 0 and 0.2 mg/mg protein for Triton X-100, 0 and 0.25 mg/mg protein for Brij 58, and 0 and 1.5 mg/mg protein for digitonin. The activated microsomes were then incubated under the conditions described in the previous section. Maximal activation occurred at a digitonin concentration of 0.5 mg/mg protein. Therefore, in all subsequent experiments pretreatment with digitonin (0.5 mg/mg protein) was used to activate the rat liver microsomes.

Effect of pH on UGT activity. Activated microsomes were incubated as described above in the absence of saccharolactone at a diflunisal concentration of 0.3 mM. A 0.05 M sodium–potassium phosphate buffer was used instead of Tris buffer and the pH varied between 5.5 and 7.5.

DPG and DAG stability during incubation with rat liver microsomes. The stability of DPG and DAG was studied under the conditions of the microsomal incubations. DAG (9.7 μM) and DPG (9.9 μM) were incubated in the presence and absence of liver microsomes for between 0 and 24 hr in incubation medium which did not contain UDPGA. The effect of the β-glucuronidase inhibitor saccharolactone (4 and 20 mM) on the stability of both diflunisal glucuronides under these above conditions was tested.

DPG and DAG hydrolysis by rat liver microsomes: enzyme kinetics. The following incubations were carried out: native microsomes (1 mg protein/mL), 0.2 M Tris buffer (pH 7.4), $MgCl_2$ (2 mM), and DPG (0–2.35 mM) or DAG (0–0.5 mM). The mixture was incubated for 60 min for DPG and 10 min for DAG at 37°. In preliminary experiments reaction rates were shown to be linear for incubation times to at least 8 hr for DPG and up to 10 min for DAG, and for microsomal protein contents up to 3 mg/mL for DPG and 1.5 mg/mL for DAG. The reaction was stopped and the samples prepared for HPLC analysis as described above.

Effect of saccharolactone on the glucuronidation of diflunisal by rat liver microsomes. Activated microsomes were incubated under the same conditions as described above in the presence of saccharolactone concentrations ranging from 0 to 16 mM and at a diflunisal concentration of 0.3 mM. Since the pH of the incubation medium (0.2 M Tris–HCl pH 7.4) was not sufficiently buffered at the high saccharolactone concentrations tested, a series of incubations was also carried out in 0.3 M Tris–HCl buffer pH 7.4.

Diflunisal glucuronidation by rat liver microsomes: enzyme kinetics. Incubations were carried out using digitonin-activated microsomes at diflunisal concentrations between 0 and 1.6 mM and in the absence and presence (4 mM) of saccharolactone.

HPLC. In the absence of UDPGA, the diflunisal glucuronides were measured based on the direct method of Hansen-Moller *et al.* [17]. In case DPG and DAG had to be quantified in microsomal incubation mixtures containing UDPGA an alternative direct method was used [18].

β-Glucuronidase assay. 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 nmol phenolphthalein liberated per mg protein in 1 min at 37° at the optimal pH.

Analysis of results. Unless otherwise stated results are the means ± SD of incubations performed on individual microsomal suspensions of six rats. V_{max} and K_m values for hydrolysis of DAG by rat liver microsomal β-glucuronidase were estimated using an iterative program based on non-linear least squares regression analysis (GraphPad Inplot, San Diego, CA, U.S.A.). Since initial formation rates of DAG and DPG versus diflunisal concentrations showed clear inhibition at high substrate concentration, apparent V_{max} and K_m values were estimated using Hanes plots which were linear at low substrate concentrations [19]. Linear regression analysis was used to demonstrate a correlation between the rates of hydrolysis of DPG and DAG and the β-glucuronidase of the microsomal suspension. A P-value of 0.05 or less was considered significant.

RESULTS

Effect of pH on UGT activity

Experiments with liver microsomes of several rats (N = 6) showed maximal UGT activities for the formation of DPG and DAG at pH values between 6.0 and 6.5. All subsequent incubations, however,

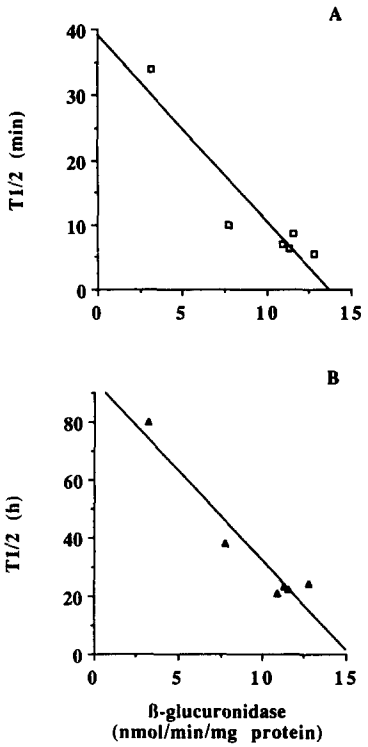

Fig. 1. Correlation between hydrolysis half-life of DAG (A) and DPG (B) and the microsomal β-glucuronidase activity of six rats. DAG: $r = 0.93$, $P < 0.01$; DPG: $r = 0.96$, $P < 0.005$.

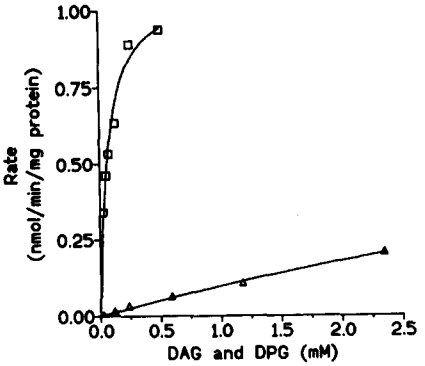

Fig. 2. Initial rates of DAG (□) and DPG (△) hydrolysis by rat liver microsomes (N = 3) as a function of substrate concentration (i.e. DAG or DPG).

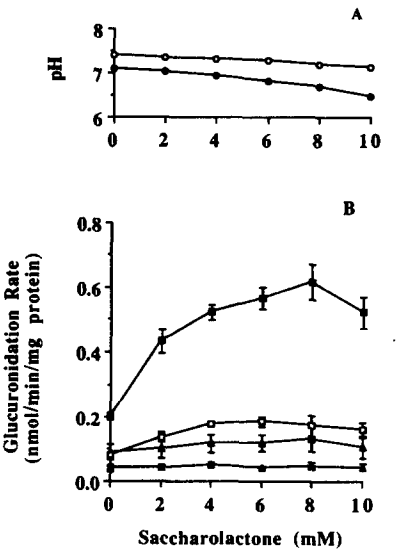

Fig. 3. Effect of saccharolactone on the pH of the microsomal incubation medium (A), and on the formation rates of DAG (■, □) and DPG (▲, △) at a diflunisal concentration of 0.3 mM (B). Two different Tris buffer concentrations were used: 0.2 (closed symbols) and 0.3 (open symbols) M.

were carried out at physiological pH. Since addition of saccharolactone lowered the pH of the incubation medium, Tris buffers of different strengths (0.1–0.3 mM) were used and the pH was always carefully monitored to interpret correctly any changes in UGT activity observed.

Hydrolysis of DAG and DPG by rat liver microsomes

The rate of hydrolysis of DAG and DPG was determined following incubation of the diflunisal glucuronides under standard conditions using rat liver microsomes from six individual rats. The half-life of DAG during incubation in the presence of rat liver microsomes ranged from 5 to 34 min (12 ± 10 min). Hydrolysis of DPG by rat liver microsomes was much slower (35 ± 21 hr). The hydrolysis of both glucuronides by rat liver microsomes was inhibited by adding 4 or 20 mM of saccharolactone. The rates of hydrolysis ($T_{\frac{1}{2}}$) of DAG and DPG by rat liver microsomes were highly correlated ($r = 0.93$, $P < 0.01$ and $r = 0.96$, $P < 0.005$, respectively) to the β-glucuronidase activity of the individual microsomal preparations (Fig. 1).

The rate of hydrolysis of DAG by rat liver microsomes showed simple Michaelis–Menten kinetics (Fig. 2). K_m and V_{max} values for DAG hydrolysis, determined in hepatic microsomes from three rats, were 81.3 ± 9.5 μM and 1.3 ± 0.2 nmol/min/mg protein, respectively. Due to solubility limitations, the highest DAG concentration tested was 0.5 mM. For DPG, concentrations studied were limited to 2.5 mM. DPG at concentrations above 2.5 mM significantly lowered the pH of the incubation medium thus preventing the estimation of K_m and V_{max} values. The available results, however, show a significant difference in affinity of the microsomal β-glucuronidase for the two diflunisal glucuronides.

Effect of saccharolactone on the glucuronidation of diflunisal by rat liver microsomes

The effect of saccharolactone addition to the incubation medium during experiments to investigate the glucuronidation of diflunisal by rat liver microsomes is shown in Fig. 3. Since the increasing

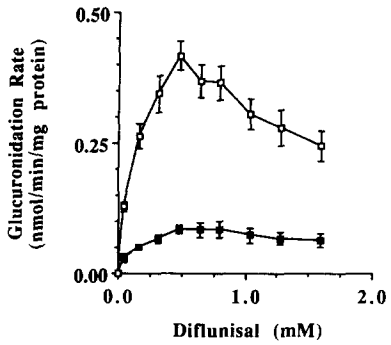

Fig. 4. Initial formation rates of DAG (□) and DPG (■) as a function of diflunisal concentration in the presence of 4 mM saccharolactone.

Table 1. Apparent Michaelis–Menten parameters for glucuronidation of diflunisal to DAG and DPG by rat liver microsomes: effect of saccharolactone

Parameter	Saccharolactone 0 mM	Saccharolactone 4 mM
DAG formation		
K_m (mM)	0.05 ± 0.02	0.08 ± 0.02*
V_{max} (nmol/min/mg)	0.20 ± 0.06	0.43 ± 0.07†
DPG formation		
K_m (mM)	0.12 ± 0.06	0.15 ± 0.10
V_{max} (nmol/min/mg)	0.09 ± 0.03	0.10 ± 0.04

* Significantly different from control (0 mM saccharolactone) using paired t-test: *$P < 0.05$, †$P < 0.005$.

saccharolactone concentrations resulted in a small but gradual pH reduction of the incubation medium, this series of experiments was carried out using two different concentrations of Tris buffer: 0.2 and 0.3 M. The formation rate of DAG increased when saccharolactone was added to the incubation medium (0.2 mM Tris) up to a concentration of 8 mM. This "activating" effect of saccharolactone was also observed, but to a small extent, for the formation of DPG. At saccharolactone concentrations above 8 mM, the DAG formation rate dropped substantially. A further reduction in the glucuronidation rate of diflunisal at saccharolactone concentrations above 12 mM coincided with a significant drop in pH to values under the pH for maximal UGT activity (data not shown). When using a stronger buffer (0.3 M Tris) in the incubation medium a smaller decrease in pH and no reduction in DAG and DPG formation rates were observed at the higher saccharolactone concentrations. These results indicate that the initial increase in formation rate of DAG between 0 and 4 mM saccharolactone is due to the inhibitory effect of β-glucuronidase with no interference of the pH effect of saccharolactone. A saccharolactone concentration of 4 mM was therefore considered sufficient to inhibit most of the β-glucuronidase during the microsomal incubations.

Glucuronidation of diflunisal by rat liver microsomes: enzyme kinetics

Rat liver microsomes were incubated in the presence of increasing concentrations of diflunisal to determine apparent K_m and V_{max} values for the formation of DAG and DPG. These experiments were carried out in the absence and presence (4 mM) of saccharolactone using an incubation medium buffered at pH 7.4 with a 0.2 mM Tris buffer. All plots of the initial formation rates versus diflunisal concentration showed inhibition at high substrate concentrations (Fig. 4). Saccharolactone had no significant effect on apparent V_{max} and K_m values for the formation of DPG, but significantly altered the kinetic parameters for glucuronidation of diflunisal to DAG (Table 1). The formation rate of DAG was least affected in the microsomal preparation which had a much lower β-glucuronidase activity (3.2 nmol/min/mg protein) as compared to the five other microsomal preparations (10.9 ± 1.9 nmol/min/mg protein).

DISCUSSION

A number of factors such as UGT latency, ionic composition and pH of the incubation medium, and substrate and cosubstrate (UDPGA) concentration are known to influence the *in vitro* glucuronidation rate. Another factor which must be taken into account is the possible hydrolysis of the formed glucuronide which could lead to a glucuronidation–deglucuronidation cycle. Enzymes such as β-glucuronidase and esterases are present in microsomal preparations and may hydrolyse certain glucuronides. Although previous reports have clearly shown that UGT and β-glucuronidase are active in rat liver microsomes at physiological pH [13], this problem has not been adequately addressed when estimating *in vitro* glucuronidation rates as pointed out recently by Benet and co-workers [6, 20–22]. Although several investigators have added saccharolactone to the incubation medium when estimating the *in vitro* glucuronidation rate of certain drugs [20, 21, 23, 24], the concentrations of this β-glucuronidase inhibitor used varied considerably from one study to another. Other investigators have buffered the incubation medium at pH values below 7 in an attempt to stabilize acyl glucuronides [25, 26]. Since most mammalian β-glucuronidases exhibit a maximal activity around a pH of 4.5–5.0, reducing the pH of the incubation medium to subneutral values may lead to higher β-glucuronidase-catalysed hydrolysis of the acyl glucuronide thus affecting the apparent glucuronidation rate. We therefore decided to perform all our microsomal incubations at physiological pH.

Addition of saccharolactone to the incubation medium, especially at higher concentrations, may significantly reduce the pH of the medium and thus influence the formation rate of glucuronide conjugates. Indeed, such a shift in pH will not only affect the chemical stability of certain glucuronides (acyl glucuronides), but also the microsomal β-glucuronidase and UGT activities. In case of diflunisal, formation rates of DAG and DPG are

optimal at a pH of approximately 6–6.5. At pH values under 6, UGT activity towards diflunisal decreases very rapidly. To minimize this pH-lowering effect of saccharolactone, the concentration of the buffer in the incubation medium has to be sufficiently high, and the saccharolactone concentration used to inhibit the β-glucuronidase activity should be kept to a minimum. Based on our findings a saccharolactone concentration of 4 mM was considered sufficient to inhibit most β-glucuronidase activity in the microsomal suspension thus preventing β-glucuronidase-catalysed hydrolysis of DAG during the microsomal incubation.

The results of our *in vitro* studies on the glucuronidation of diflunisal by rat liver microsomes show that the acyl glucuronide of diflunisal, unlike the phenolic glucuronide, undergoes rapid hydrolysis at pH 7.4. Addition of saccharolactone to the incubation medium almost completely prevents this microsomal hydrolysis of DAG. Esterases, therefore, are not or only marginally involved in DAG hydrolysis by rat liver microsomes. The rate of hydrolysis of DAG shows substantial interindividual variability and is highly correlated with the β-glucuronidase activity of the microsomal preparation. Hydrolysis of DAG by rat liver microsomes is so fast (average $T_{\frac{1}{2}} = 12$ min) that even during the short incubation time of 15 min on average more than 50% of DAG is hydrolysed. Consequently, during microsomal incubations to determine the initial rates of DAG and DPG formation, the hydrolysis of DAG has to be prevented. If DAG hydrolysis is not inhibited by saccharolactone estimation of the kinetic parameters describing the rate of formation of DAG is significantly influenced by hydrolysis (Table 1). The effect of saccharolactone on the apparent K_m for DAG formation, although statistically significant, is rather small. This makes sense since saccharolactone should not interact with UGT. The difference in apparent V_{max} for DAG formation in the absence and presence of saccharolactone, however, is much more pronounced. This can be explained by the inhibition of DAG hydrolysis in the presence of saccharolactone. Studies using more physiological conditions such as isolated rat liver perfusion or *in vivo* experiments indicate that β-glucuronidase could also affect the *in vivo* glucuronidation rate of drugs and/or endogenous substances [11, 27].

Recent work by Benet and co-workers [20, 21] has shown that benoxaprofen and fenoprofen glucuronide, two acyl glucuronides, rapidly hydrolyse in the presence of sheep liver microsomes, with half-lives ranging from approximately 15 to 60 min depending on the pH of the medium (7.4, 7.0 and 6.6). Based on these results and on the findings of our own studies it would be tempting to hypothesize that acyl glucuronides might be better substrates for β-glucuronidase-catalysed hydrolysis than phenolic glucuronides. Tomasic and Keglevic [12], however, investigated the hydrolysis of a number of ester and ether glucuronides by bovine liver and bacterial β-glucuronidases and came to the conclusion that neither the type of sugar–aglycon linkage nor the aglycon structure could explain the differences they observed in V_{max} and K_m values. More studies are obviously needed to resolve this issue.

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