Biochemical Pharmacology, Vol. 34, No. 8, pp. 1325–1329, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

GLUCURONIDATION AND SULFATION IN THE RAT *IN VIVO*

THE ROLE OF THE LIVER AND THE INTESTINE IN THE *IN VIVO* CLEARANCE OF 4-METHYLUMBELLIFERONE

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(*Received* 11 *September* 1984; *accepted* 30 *October* 1984)

Abstract—The role of the liver in the conjugation of 4-methylumbelliferone (4MU), mainly glucuronidation, was investigated in the rat *in vivo*. The liver extracted 4MU almost completely (97%) during steady-state infusion, as measured by the difference between 4MU concentration in portal and hepatic venous blood. Previously, it was shown that the intestinal region extracts 40% of the 4MU of the incoming arterial blood. The liver and the gastrointestinal region are so efficient that their conjugation activity can account for total body clearance of 4MU (50–60 ml/min per kg). These results and other evidence on extrahepatic conjugation of phenolic substrates suggest that glucuronidation may be limited to the liver, (the kidney) and the gastrointestinal region, while sulfation may occur more widespread throughout the body. Protein binding studies showed the sulfate conjugate to be even more protein-bound than unconjugated 4MU, while 4MU glucuronide was much less bound to rat plasma proteins.

The role of the liver and extrahepatic tissues in the conjugation of various phenolic compounds after their intravenous administration has been studied recently by several investigators. It was shown that phenol [1–3], 4-nitrophenol [4], and harmol [5] are conjugated not only by the liver but also by extrahepatic organs; this accounts for up to 75% of the total conjugation of harmol at low doses. Although the separate contribution of sulfation and glucuronidation in the total conjugation has not been determined for phenol [1–3], evidence from other work [6] suggests that at the low doses of phenol as employed, sulfation is more predominant than is glucuronidation. The same applies to 4-nitrophenol [4]. For harmol, sulfation is by far the most important conjugation at low doses [5].

By contrast, Gerkens *et al.* [7] have reported that glucuronidation of lorazepam represents almost exclusively the total conjugation of this drug in the dog, with the reaction occurring primarily in liver and to a minor extent, in the splanchnic region. This finding on gastrointestinal metabolism in the dog agrees well with our recent observation on 4-methylumbelliferone (4MU), a substrate which is primarily glucuronidated as well [8, 9] and which is subject to pre-hepatic conjugation in the gastrointestinal tissues in the rat: 40% of the incoming 4MU in the arterial blood to the intestine/spleen/stomach is conjugated before the drug reaches the liver [10]. It should also be noted that these extrahepatic conjugation reactions observed for lorazepam and 4 MU occur after intravenous drug administration.

Because of the varying degrees of extrahepatic conjugation and the different extents of sulfation and glucuronidation exhibited among these various phenolic substrates, we have further studied the conjugation of 4MU by the liver to discern the relative roles of the liver and the intestine in the overall conjugation of the drug.

4MU (Hymecromone) has a pronounced choleretic effect at low doses [8] and is still used in several European countries for that purpose [11]. The extent of choleresis has been found to be related to the biliary excretion of the glucuronide conjugate: a linear correlation exists between the amount of glucuronide in bile and choleresis, the latter being measured by the extra volume of bile produced in the rat [8]. Glucuronidation is the preferred conjugation reaction in rat and man [8, 9, 12, 13]. The minor metabolite, the sulfate conjugate of 4MU, however, was excreted exclusively into urine [9].

In the course of our studies on the kinetics of glucuronidation and sulfation of various phenolic substrates in the rat *in vivo* and in perfused organ system [14, 15], we have investigated the extent of extrahepatic conjugation of 4MU. The results from the present study, together with previous observations in the laboratory on the contribution of the splanchnic tissues in the rat, suggest that the site for extrahepatic conjugation of 4MU is only in the prehepatic region, namely the gastrointestinal tissues.

MATERIALS AND METHODS

Chemicals. 4MU (sodium salt), 4MU-glucuronide and 4MU-sulfate were obtained from Sigma Chemical Corp. (St. Louis, MO). All other reagents used were of analytical quality grade.

Rats. Male Wistar rats of the inbred colony of the Dept. of Pharmacology were used. They had free

access to food and water; rats of 290–300 g body wt were used. They were anesthesized with pentobarbital sodium (60 mg/kg intraperitoneally) prior to cannulation of the artery, vein, bladder and bile duct. All surgical procedures have been described extensively elsewhere [16]. Briefly, the animals were respirated artificially through a cannula in the trachea, and cannulae in the bile duct and urine bladder were inserted to collect bile and urine, respectively. Urine flow was increased by an infusion of mannitol. Drug infusions were given into the external jugular vein, and blood was drawn from the carotid artery for systemic sampling. Sampling of blood from the portal vein and the hepatic vein was done as previously described [17] *via* catheters inserted into the portal vein and hepatic vein, while blood flow through the liver is not interrupted. It is important to ensure that the tip of the catheter into a branch of the hepatic vein is positioned well towards the liver such that any dilution effect by blood from the vena cava is avoided. The body temperature was kept between 37.5 and 38.5° by a heating pad.

Infusion studies. Solutions for infusion were prepared by dissolving 4MU (sodium salt) in 0.9% (w/v) sodium chloride in water, containing 1% (w/v) bovine serum albumin to compensate for the loss of protein during blood sampling. The pH of the final solution was kept about 9–9.5, to avoid precipitation of 4MU. An infusion volume of 3.8 ml/hr per 300 g rat was employed during the experiments. For these experiments, different infusion rates were used. Each infusion period was accompanied with a priming dose. For the infusion rates of 1.25, 2.5, 5.0, 6.2, 8.7 and 12.4 μmoles/min per kg were given priming doses of 9, 18, 36, 45, 63 and 90 μmoles/kg, respectively. These priming doses for infusion to arrive at the various steady-state concentrations were based on clearance values obtained from a computer-assisted curve fitting procedure (RUGFIT) on blood data from the single i.v. dose experiments (190 and 570 μmoles/kg). Under these conditions, steady state was estimated to be attained within 40 min for 4MU. Each infusion period, however, lasted 60 min, and arterial blood samples were collected at 40, 50 and 60 min. The experimental data indeed confirmed that steady state was reached ≤40 min, and the mean of the concentrations at the three sampling time points was taken as the steady-state blood concentration.

Assay. 4MU was determined fluorimetrically by a Perkin Elmer Fluorimeter 1000M, with an excitation wavelength at 374 nm and an emission filter at 430–3000 nm. The extraction procedure of 4MU from blood was as follows. A 50 μl blood sample was added to 1.0 ml of 75 mM sodium acetate buffer (pH 5.0). Then 4 ml of ethyl acetate was added, followed by vigorous mixing for 30 sec. A 1.0 ml sample of the ethyl acetate layer (which contained 4MU) was dried under air, and the residue was reconstituted in 3 ml of 0.4 M glycine–NaOH buffer (pH 10.4) before detection by fluorescence.

For the determination of 4MU glucuronide and 4MU sulfate in bile or urine, a 10 μl sample was applied to Silica Gel F254 (Merck, Darmstadt, F.R.G.) thin layer plates for chromatography. The plates were developed in 1-butanol/ethanol/water (80:10:20; v/v/v). After development, the spots were located by u.v. light: 4MU glucuronide had an R_f value of 0.20; 4MU sulfate, 0.70; 4MU, 0.94. The spots were scraped and the conjugates were eluted in 2 ml 75 mM sodium acetate buffer, pH 5.0 overnight at 4°. After centrifugation, a 0.5 ml sample was incubated with 5 μl β-glucuronidase/arylsulfatase (Boehringer Co., Mannheim, F.R.G.) for 1 hr at 37°, which hydrolysed the conjugates completely. The incubation was terminated by the addition of 2.0 ml 0.4 M glycine–NaOH buffer, pH 10.4, and the fluorescence of each sample was measured. For plasma samples the same procedure was used, after the initial precipitation of the plasma proteins by addition of an equal volume of methanol on ice. A 50 μl sample was then applied to the plate, after centrifugation. Varying known amounts of authentic 4MU, 4MU glucuronide or 4MU sulfate in bile, urine or plasma blood were subjected to the same procedure for quantitation of 4MU, 4MU glucuronide and 4MU sulfate in bile, urine and plasma.

Protein binding. Protein binding of 4MU and its conjugates to rat plasma proteins was determined by the method of Toribari *et al.* [18]. A 100 μl of the test compound in saline (pH 7.4) was added to 900 μl rat plasma. Ultrafiltration through dialysis tubing by centrifugation was performed at 37° as previous described [19].

Curve fitting. Curve-fitting of the blood data and subsequent calculation of the pharmacokinetic parameters were performed with the RUGFIT program [20], which can be obtained from Dr. A. H. J. Scaf, Dept, of Pharmacology, State University of Groningen, The Netherlands. The data were fitted with the equation $C_t = A_1e^{-\lambda_1 t} + A_2e^{-\lambda_2 t}$. The steady-state volume of distribution was calculated as described by Gibaldi and Perrier [21].

RESULTS

Single intravenous dosing

The disposition of 4MU at two single i.v. doses (190 and 570 μmoles/kg) was followed in the arterial blood in the rat. At the low dose, a very rapid initial phase with a half-life ($t_{\frac{1}{2}}\lambda_1$) of 1 min was followed by a slower phase ($t_{\frac{1}{2}}\lambda_2$) of about 10 min. At the high dose, however, these $t_{\frac{1}{2}}$'s seemed somewhat prolonged, as shown by the derived parameters obtained after fitting the data with RUGFIT, although this was not statistically significant (Table 1, Fig. 1). The clearance at the low dose was sig-

Table 1. Pharmacokinetic parameters of 4MU elimination in the rat

Parameter	Dose 190 μmoles/kg	Dose 570 μmoles/kg
$t_{\frac{1}{2}}$ (λ_1) (min)	1.0 ± 0.04	3.7 ± 0.8*
$t_{\frac{1}{2}}$ (λ_2) (min)	9.3 ± 1.4	12.2 ± 5.0 n.s.
V_{dss} (ml/kg)	420 ± 35	420 ± 40 n.s.
CL (ml/min)kg)	48 ± 2	32 ± 2*
No. of rats	3	4

* Significantly different at $P < 0.05$.

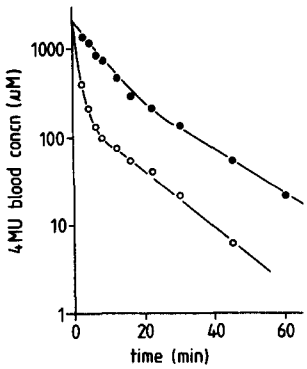

Fig. 1. Blood disappearance curve for 4MU. 4MU was administered at 2 doses, 190 (○—○) and 570 (●—●) μmole/kg i.v. The concentration of unconjugated 4MU in blood is given over a 60 min period.

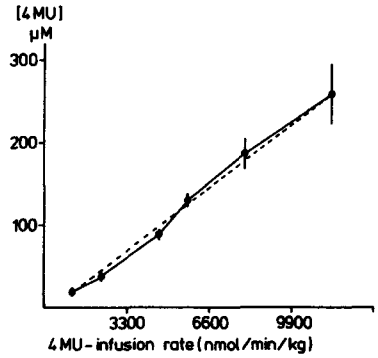

Fig. 2. Correlation between infusion rate of 4MU and steady-state blood levels of 4MU in the rat *in vivo*. The steady-state blood concentration of 4MU is given at 6 different infusion rates (see Methods). The S.E.M. is indicated by the vertical bar. The dashed line was calculated by the least-squares method.

nificantly greater than that at the high dose (48 vs 32 ml/min per kg; $P < 0.05$). The steady-state volume of distribution, however, was unchanged (Table 1).

Only a trace amount of unconjugated 4MU was excreted in urine. The excretion data, both in bile and urine, showed that both 4MU glucuronide and 4MU sulfate decayed with almost the same $t_{\frac{1}{2}}$ in bile (approx. 10 min) whereas the excretion in urine was much prolonged. The sulfate conjugate was almost exclusively excreted in urine, while at these doses about equal amounts of the glucuronide were excreted in bile and urine, respectively. At the doses used, approx. 80–90% of the dose was excreted in urine and bile as the glucuronide conjugate, while 10–20% was the sulfate conjugate [9].

Infusion

In order to determine the total body clearance under steady-state conditions, we administered infusions of 4MU until steady state (constancy in arterial blood concentration of 4MU) was achieved. Computer simulations with the RUGFIT program revealed that steady state was expected to be established within 40 min after start of the infusion. This was confirmed experimentally. The clearance values (50–60 ml/min per kg) were comparable to those found in the single dose experiments (48 ml/min per kg at the lower dose; Table 2). Moreover, over the range of infusion rates there was a linear correlation between infusion rate and steady-state arterial blood concentration of 4MU, indicating that over this range no saturation of the overall conjugating process occurred (Fig. 2).

Protein binding of 4MU and its conjugates

In order to characterize the extent of binding to plasma proteins, we also determined the extent of binding of 4MU, and its conjugates to plasma proteins. 4MU and its sulfate conjugate were highly protein bound: over 90% in the concentration range used in the infusion experiments. The 4MU glucuronide conjugate, however, was less bound (Table 3).

Extraction of 4MU by the liver in vivo

The extraction of 4MU by the rat liver *in vivo* was measured during an infusion of 6.2 μmoles 4MU/min per kg into the jugular vein. At 60 min after start of the infusion, when steady state had been reached, simultaneous sampling from the portal vein and hepatic vein was effected. Hepatic blood flow, however, was left intact, as described recently for this catheterization technique [17]. The concentration of 4MU in the hepatic vein blood had decreased by 97% during the passage across the liver (Table 4)

Table 2. Steady state blood levels and clearance of 4MU during infusion of 4MU in anesthesized rats

Infusion rate (μmoles/min/kg)	4MU arterial blood level (μM)	4MU clearance at steady state (ml/min per kg)
1.25 (6)*	19 ± 1.5†	64 ± 7
2.5 (9)	37 ± 3.3	67 ± 7
5.0 (7)	88 ± 6.4	57 ± 7
6.2 (7)	130 ± 8.8	46 ± 7
8.7 (5)	186 ± 19	46 ± 7
12.4 (5)	259 ± 36	50 ± 7

The total body mean clearance is 55 ml/min per kg among doses studied.
* Number of preparations.
† Means ± S.E.M.

Table 3. Protein binding of 4MU and its conjugates to rat plasma proteins

Compound	Percentage bound at a concn of 0.2 mM	2.0 mM
4MU	90	62
4MU glucuronide	32	9
4MU sulfate	97	58

Protein binding was determined by centrifugation at 37° in undiluted rat plasma in dialysis tubing [18, 19].

and only 3 μM 4MU was found in the hepatic blood. The concomitant 4MU concentration in the vena cava blood (taken above the left kidney) was of the order of 140 μM.

DISCUSSION

The present results on intravenous administration of 4MU to the rat are similar to the findings of Gerkens *et al.* [7] on lorazepam glucuronidation in the dog: the conjugation of 4MU, also mainly glucuronidation, occurs predominantly in the liver and the gastrointestinal organs draining into the portal vein. The evidence for this is the following. The present work shows that the total body clearance of 4MU is about 50–60 ml/min per kg. The high efficiency of extraction (97%) by the liver *in vivo* (Table 4) implies that very little 4MU escaped the liver unconjugated. Therefore, total body clearance can be accounted for by conjugation solely in the liver (assuming a hepatic blood flow of 60–80 ml/min per kg).

Previously, however, we have reported that the gastrointestinal region extracted 40% of the incoming 4MU from the arterial blood at steady state, converting it into the conjugates, as determined from arterial-portal differences [10]. If we assume a portal blood flow of 45–60 ml/min per kg (75% of total liver blood flow), the clearance contribution by the gastrointestinal region is 18–24 ml/min per kg. Obviously, the total clearances by the liver and the intestinal region should not be summed, because the intestinal region is located anterior to the liver, and these two organs are arranged in series.

Table 4. Hepatic extraction ratio in the rat *in vivo*

	Concentration of 4MU (μM)
Portal vein blood (C_{Pv})	86 ± 4
Hepatic vein blood (C_{Hv})	3 ± 1
Hepatic extraction ration (E)*	0.97 ± 0.02

During infusion of 6.2 μmol/min per kg in the jugular vein blood samples were withdrawn at the same time from the portal vein and hepatic vein after steady-state conditions were attained, while the liver flow was not interrupted (N = 4; mean ± S.E.M.)

* $E = \frac{C_{Pv} - C_{Hv}}{C_{Pv}}$.

Rather, an effective clearance, which at best equals total blood flow to these regions, represents the clearance by these organs. The present results indicate that a total body clearance of a drug which approximates liver blood flow should not be taken unequivocally as hepatic clearance when the drug is excreted minimally into urine [22]. As exemplified by 4MU, the intestinal contribution is masked by its high hepatic clearance.

The total body clearance of another phenolic drug, harmol, in the rat was much higher: 200 ml/min per kg [5]; approximately 75% of total conjugation occurs extrahepatically. This extrahepatic conjugation of harmol, mostly sulfation, takes place in tissues other than the intestine, as deduced from the reasoning outlined previously that the effective clearance by the intestine and liver cannot exceed total liver blood flow. This predominance of extrahepatic sulfation of harmol in tissues other than the intestinal region, and the occurrence of extrahepatic glucuronidation of 4MU and lorazepam (almost) exclusively in intestinal tissue suggests that glucuronidation may be limited to the hepatic and intestinal regions, while sulfation may occur more widespread throughout the body. This speculation, however, requires further systematic investigation inasmuch as no data are available on the extents of intestinal and extrahepatic conjugation at other sites on other phenolic substances such as phenol. Recently, Tremaine *et al.* [23] showed that 1-naphthol and 4-nitrophenol are both sulfated and glucuronidated by the kidney in the rat in *in vivo*; this indicates that the kidney certainly has the potential to contribute to total glucuronide conjugation *in vivo*.

Another difference between harmol and 4MU is the volume of distribution at steady state: 420 ml/kg for 4MU and 2000 ml/kg for harmol [5]. A difference in protein binding may, in part, be responsible for this difference. At the lower concentrations 4MU is 90% bound, while harmol at comparable concentrations was 40–50% bound [24]. It is impossible to predict, whether these differences would affect the extent of extrahepatic conjugation.

Our present data also show that the preferred routes of excretion for the sulfate and glucuronide conjugates of 4MU are different, as observed for other substrates. The glucuronide is excreted both in the bile and urine; when the biliary pathway is saturated (or blocked by cholestasis) urinary excretion will increase. 4MU sulfate is only sparingly excreted in bile but appears predominantly in urine. Differences in the degree of binding to plasma pro-

teins of the 4MU conjugates are found (Table 3), but these would have the opposite effect on filtration of the conjugates by the kidney. The preference of 4MU sulfate for urinary excretion, therefore, may be due to its low propensity for biliary excretion as a result of its molecular weight of 225, which is borderline for biliary excretion of anions in the rat [25].

In summary, we found that total clearance of 4MU, mainly glucuronidation, has to be attributed to clearance by both the intestine and the liver, despite that total body clearance approximates total liver blood flow. The observation that extrahepatic glucuronidation of 4MU occurs mainly in intestinal region matched well with the extrahepatic glucuronidation of lorazepam exclusively in the intestinal region of the dog. By contrast, extrahepatic sulfation clearance of harmol greatly exceeds liver blood flow and can be taken as contribution from tissues other than the intestine and liver. This trend on the preferential localization of glucuronidation activity in intestine and liver, and sulfation activity more ubiquitous in tissues other than both the intestine and liver, remains to be further established.

Acknowledgements—This work was supported by a NATO grant (83.035) (KSP and GJM) and a grant from the Canadian Liver Foundation (KSP). KSP is a recipient of a Faculty Department Award (DG 262, 263 and 264) from the Medical Research Council of Canada.

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