*Biochemical Pharmacology*, Vol. 33, No. 19, pp. 3081–3087, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00


# EXTRAHEPATIC SULFATION AND GLUCURONIDATION IN THE RAT *IN VIVO*

## DETERMINATION OF THE HEPATIC EXTRACTION RATIO OF HARMOL AND THE EXTRAHEPATIC CONTRIBUTION TO HARMOL CONJUGATION

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(*Received* 7 *March* 1984; *accepted* 28 *May* 1984)

**Abstract**—The phenolic compound, harmol, is metabolized by sulfation and glucuronidation in the rat *in vivo*. In the present study, various harmol infusion rates into the jugular vein were used to delineate first-order conditions whereby total body clearance was maximal and constant; at low infusion rates the steady state harmol concentration in blood varied proportionally with the infusion rate. At infusion rates of 167 nmole/min and below, the steady state clearance of harmol was 60 ml/min or 200 ml/min/kg. Because this value for total body clearance greatly exceeded the value for hepatic blood flow rate (20 ml/min for a 300 g rat), considerable extrahepatic conjugation of harmol was suggested. At higher harmol infusion rates the total clearance decreased.

Since an intraportal infusion of 167 nmole/min to the rat yielded, during steady state, the same arterial harmol blood concentration as a 52 nmole/min jugular infusion, the hepatic extraction ratio of harmol *in vivo* was 0.7. Extrahepatic clearance, therefore, constituted about 77% of total body clearance (after taking the difference between total body clearance and hepatic clearance). Total sulfation clearance was 52 ml/min, and greatly exceed the value for hepatic clearance (14 ml/min). Extrahepatic clearance for sulfation (at least 38 ml/min) therefore accounted for a major proportion of the sulfation activity. Blood platelets did not seem to contribute to sulfation or glucuronidation *in vivo*.

Although the liver is considered to be the major organ for drug biotransformation, in recent years there has been increasing awareness of the contribution of extrahepatic organs to drug metabolism [1, 2]. The importance of extrahepatic sites as contributors to the total metabolism of drugs is often underestimated because the liver has often been presumed to be the sole organ for drug modification, in particular because it has, in general, the highest activities of drug-metabolizing enzymes. Thus, more often than not, only hepatic metabolism is considered during the first-pass effect on oral drug administration, and the contribution of the gastrointestinal mucosa or bacteria is often ignored, despite the fact that these extrahepatic drug-metabolizing activities can account for a sizeable percentage of drug removal during presystemic drug absorption [3, 4].

Little data is available in the literature regarding extrahepatic drug conjugation *in vivo*. Cassidy and Houston [5–7] have recently investigated the fate of phenol administered via various routes to the rat. Although they did not differentiate between the conjugation processes, they concluded that extrahepatic conjugation of phenol was effected by the lung to a high extent at low doses of phenol. Usually, the lung possesses lower drug-metabolizing activities than the liver [1, 2], but the entire cardiac output (four to five times liver blood flow) perfuses the lung. In this respect, clearance by the lung (flow × extraction ratio) may be quite considerable when compared to hepatic clearance [1, 8, 9], even though a higher metabolic activity in the liver prevails. Similarly, Machida *et al.* [10] concluded that 4-nitrophenol was converted to its sulfate and glucuronide conjugates predominantly by extrahepatic mechanisms when the compound was injected intravenously in the rat. They further elaborated that sulfation occurred exclusively in the liver whereas glucuronidation was primarily extrahepatic, basing their findings on *in vitro* activities of the transferases from homogenates of various organs. The activities provided from these *in vitro* studies, however, are often misleading, either because of the nature of the enzymes [11, 12] and the availability of cofactors, or because blood flow rates to the organs need to be considered for evaluation of organ clearances.

In this communication, we investigated the conjugation of harmol, a phenolic substrate, and assessed the contribution of extrahepatic sulfation and glucuronidation, in comparison to hepatic con-

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jugation pathways, to the total metabolism of harmol in the rat *in vivo*. Our results suggest that at low doses, extensive conjugation of harmol occurs extrahepatically.

## MATERIALS AND METHODS

*Rats*. Male Wistar rats (290–310 g) of inbred strain at the Department of Pharmacology, University of Groningen, were used for the studies. The rats had free access to food and water, and were kept at a 12 hr light/dark schedule. For the duration of the experiments, the rats were anesthetized with sodium pentobarbital. The bile duct, urinary bladder and trachea were cannulated as described elsewhere [13]. Artificial respiration was applied through the trachea cannula. The temperature of the rat was maintained between 37.5 and 38.5°. Bolus injections were made via the tail vein whereas drug infusions were given either intravenously (i.v.) into the jugular vein, or intraportally (p.v.) into the portal vein. For the latter, a catheter filled with saline and frozen with liquid nitrogen was inserted directly into the portal vein and kept in position by cyanoacrylate glue [14]. The advantage of this method is a minimal disturbance of liver blood flow by surgical manipulations. Blood sampling was effected through a catheter inserted into the carotid artery. A mannitol infusion via the jugular cannula was given to ensure adequate urinary flow [13].

*Drug assay*. In some experiments, general tritium-labeled harmol (New England Nuclear, Boston, MA) was added to unlabeled harmol (Sigma Chem. Comp., St. Louis, MO) to result in a constant specific radioactivity prior to administration. Harmol and its conjugates were quantified according to the amount of radioactivity present or by a fluorimetric assay. [$^3$H]-Harmol in heparinized whole blood was determined by an extraction procedure with ethyl acetate as previously described [15]. [$^3$H]-Harmol sulfate and [$^3$H]-harmol glucuronide were not measured in blood because of the small volume of blood sampled (0.2 ml). Harmol and its sulfate and glucuronide conjugates in bile and urine were separated by thin-layer chromatography followed by quantitation by fluorimetry (Perkin Elmer Fluorimeter 1000 M) as previously described [16].

*Harmol single intravenous and continuous infusion studies*. The pharmacokinetic parameters of harmol disposition in the rat *in vivo* were initially examined with single intravenous doses of 10 or 100 μmole/kg, given via the jugular vein. Parameter values provided by the curve-fitting procedure, RUGFIT [17], were used for the calculation of the pharmacokinetic parameters, such as total body clearance, CL. The values for total body clearance were in turn used to calculate the loading doses and the infusion rates for the steady state experiments.

Continuous infusion studies with an initial loading dose were conducted to provide estimations of the steady state rates of formation of harmol conjugates; the loading doses and the infusion rates were calculated according to respective volume of distribution and the clearance values obtained for the single dose studies. Moreover, computer simulations (data not shown) indicated that the predicted steady state was reached at around 30 min post infusion and bolus injection, and the time (approx. 40 min) to attain steady state was confirmed in preliminary experiments.

Three groups of rats were used for the infusion studies: Group A rats underwent three infusion periods of 70 min each (bolus doses were 0.75, 2.5 and 5.0 μmole and the corresponding infusion rates were 16.6, 67 and 167 nmole/min, respectively); Group B rats underwent two infusion periods of 70 min each (bolus doses were 3.6 and 4.1 μmole and the corresponding infusion rates were 100 and 135 nmole/min, respectively); Group C rats underwent one infusion period of 70 min, and a bolus dose of 4.8 μmole and infusion rate of 760 nmole/min were used. Blood (0.2 ml) was removed from the carotid artery at the end of each infusion period to determine the steady state harmol concentration in blood. Bile and urine were collected *in toto* in four fractions of 15 min each, and a 10 min fraction for the last interval for each 70 min of infusion. For the determination of the steady state formation rates of the conjugates, the rate of excretion into bile and urine at steady state was summed, and the total rate measured over the last two periods for each infusion period was averaged to provide the steady state rate of formation for each conjugate.

In another set of experiments, harmol (167 nmole/min) was infused either i.v. or p.v. for 3 hr into the rat without a priming dose. Blood was collected at 30 min intervals at 60 min after harmol infusion; urine and bile were collected at 15 min intervals.

*Incubation of harmol with blood platelets*. In order to determine whether cellular components in blood, especially blood platelets [18, 19], metabolize harmol, heparinized whole blood or EDTA-treated blood and plasma obtained thereof were incubated with [$^3$H]-harmol in saline for 60 min at 37°. At the end of the incubation period, ethyl acetate was added to the incubation mixture, and [$^3$H]-harmol and its conjugates were quantified as previously described [15].

## RESULTS

### *Single intravenous doses of harmol*

When 10 or 100 μmole/kg harmol was injected i.v. into the jugular vein, harmol disappeared rapidly from blood (Fig. 1). The fitting-procedure with RUGFIT (obtainable from Dr A. H. J. Scaf, Department of Pharmacology, University of Groningen) [17] revealed that a triexponential equation best described the disposition of harmol (Table 1). The steady state volumes of distribution and clearance for the 10 μmole i.v. dose were found to be 2.15 l/kg and 146 ml/min/kg respectively. At the higher i.v. dose of 100 μmole/kg, clearance was found to be 66 ml/min/kg.

### *Continuous infusion of harmol*

The presence of harmol in bile and urine accounted only for less than 1% of the infused dose. Moreover, the recovery of harmol and its conjugates in bile and urine at steady state was virtually complete (Table 2). The steady state harmol concentration in blood was proportional to the infusion rate of harmol at

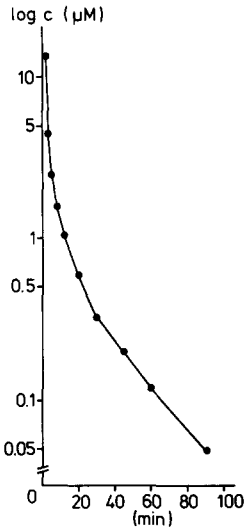


Fig. 1. The blood disappearance curve for harmol in a representative rat after a bolus injection of 10 μmole/kg into the jugular vein. A triexponential decay curve best-fitted the data.

167 nmole/min and below (Fig. 2; Table 2). A linear relationship was also present between harmol infusion rate at 167 nmole/min and below, and harmol sulfation and glucuronidation rates (Fig. 3; Table 2). Analogously, a correspondence between the steady state harmol blood concentration and the

Table 1. Kinetic parameters for harmol elimination in the rat *in vivo* after a single intravenous bolus injection of 10 or 100 μmole/kg

| Dose (μmole/kg) | Parameter | Mean | Range |
|---|---|---|---|
| 10 | $C_1$ (μM) | 21 | (13–34) |
| | $C_2$ (μM) | 2.4 | (1.4–4.3) |
| | $C_3$ (μM) | 0.63 | (0.22–0.90) |
| | $t_{\frac{1}{2}}(\lambda_1)$(min) | 1.1 | (0.8–1.4) |
| | $t_{\frac{1}{2}}(\lambda_2)$(min) | 6.7 | (4.7–9.4) |
| | $t_{\frac{1}{2}}(\lambda_3)$(min) | 26 | (21–36) |
| | $V_c$ (ml/kg) | 587 | (260–960) |
| | $V_{d,ss}$(ml/kg) | 2150 | (1240–2790) |
| | $CL$ (ml/min/kg) | 148 | (100–176) |
| 100 | $C_1$ (μM) | 252 | (244–258) |
| | $C_2$ (μM) | 20.3 | (15–24) |
| | $C_3$ (μM) | 21.8 | (20–22) |
| | $t_{\frac{1}{2}}(\lambda_1)$(min) | 1.1 | (0.9–1.2) |
| | $t_{\frac{1}{2}}(\lambda_2)$(min) | 5.9 | (5.2–6.3) |
| | $t_{\frac{1}{2}}(\lambda_3)$(min) | 31 | (29–34) |
| | $V_c$ (ml/kg) | 340 | (326–357) |
| | $V_{d,ss}$(ml/kg) | 1950 | (1880–2050) |
| | $CL$ (ml/min/kg) | 66 | (57–72) |

The parameters were obtained after a curve-fitting procedure with RUGFIT [17] for each rat individually. The data represent the mean of the individual rat data. $C_1$, $C_2$ and $C_3$ are the coefficients and $\lambda_1$, $\lambda_2$ and $\lambda_3$ are the exponents of the triexponential equation that best-fitted harmol concentrations: $C_1e^{-\lambda_1 t} + C_2e^{-\lambda_2} + C_3e^{-\lambda_3}$; $t_{\frac{1}{2}}$ is the half life; $V_c$ and $V_{d,ss}$ are the volume of the central compartment and the volume of distribution at steady state, respectively; $CL$ is total body clearance.

Table 2. Steady-state sulfation and glucuronidation rates of harmol at various infusion rates in the rat *in vivo*

| Infusion rate (nmole/min) | Harmol blood concentration at steady state (μM) | Steady state clearance of harmol[c] (ml/min) | (ml/min/kg) | Steady state rate of formation: Harmol sulfate (nmole/min) | Harmol glucuronide (nmole/min) | Recovery at steady state (% harmol infused)* |
|---|---|---|---|---|---|---|
| 16.6 (4)† | 0.29 ± 0.02 | 58 | 192 | 12.6 ± 1.3 | 2.17 ± 0.13 | 89 ± 5 |
| 67 (4) | 0.96 ± 0.03 | 70 | 232 | 54 ± 3 | 12.9 ± 1.1 | 100 ± 4 |
| 100 (4) | 1.49 ± 0.04 | 67 | 224 | 69 ± 2 | 18.1 ± 0.2 | 87 ± 1 |
| 135 (4) | 2.60 ± 0.09 | 52 | 173 | 105 ± 2 | 32.6 ± 0.9 | 102 ± 2 |
| 167 (4) | 2.95 ± 0.14 | 57 | 202 | 113 ± 2 | 39.7 ± 2.3 | 91 ± 3 |
| 760 (10) | 28.5 ± 7.0 | 27‡ | 90 | 470 ± 80 | 230 ± 30 | 92 ± 5 |

All results are expressed as the mean ± S.E.M. Group A rats received infusion rates of 16.6, 67 and 167 nmole/min stepwise at 70 min intervals; Group B rats, 100 and 135 nmole/min; Group C rats 760 nmole/min (see Methods).

* Recovery at steady state was calculated as the total excretion rate of harmol conjugates in bile and urine for each infusion period divided by the infusion rate of harmol × 100.

† Number of animals.

‡ Total body clearance for this infusion period was statistically different from harmol infusion rates of 167 nmole/min or below ($P < 0.01$). The mean total body clearance at infusion rates of 167 nmole/min and below was 60.6 ± 1.8 ml/min or 202 ± 5 ml/min/kg.

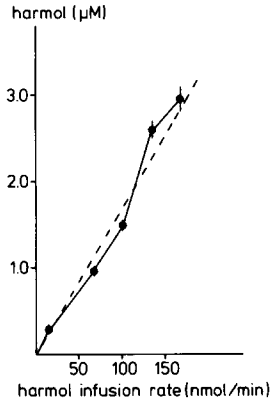


Fig. 2. The relationship between the steady state harmol concentration in arterial blood and intravenous harmol infusion rates. Each data point is the mean ± S.E.M. of four separate experiments. See text for details.

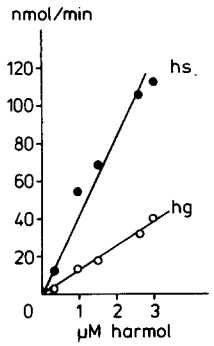


Fig. 4. The relationships between sulfation and glucuronidation rates of harmol and the blood concentration of harmol at steady state. The data of Figs. 2 and 3 were replotted to show the dependence of the formation rates of harmol sulfate and harmol glucuronide on harmol blood concentration.

steady state formation rates for harmol sulfate and harmol glucuronide was found (Fig. 4). Total body clearance for infusion rates of harmol at 167 nmole/min and below was 60 ml/min or 200 ml/min/kg. At higher infusion rates of harmol (760 nmole/min), however, a reduced clearance (27 ml/min or 90 ml/min/kg), as calculated from the steady state blood concentration of harmol (28 μM), was observed. The significant reduction in clearance ($P < 0.01$) at the higher dose or infusion rate is presumably due to the saturation of enzyme systems for harmol conjugation.

Interestingly, the values for total body clearance (60 ml/min or 200 ml/min/kg) greatly exceed the values for hepatic blood flow rate (usually 20 ml/min) *in vivo* [18, 19]. Even when the hepatic extraction ratio of harmol is assumed to be unity and hepatic clearance would equal the value of hepatic blood flow (20 ml/min), extrahepatic clearance which was calculated as the difference between total body clearance (60 ml/min) and hepatic clearance (20 ml/min) was 40 ml/min, which represents 67% of total body clearance.

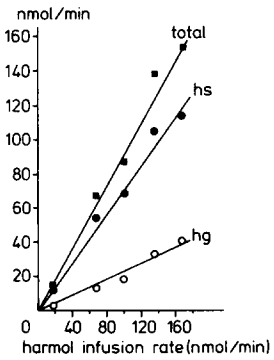


Fig. 3. The steady state rates of formation of harmol sulfate and harmol glucuronide (sum of excretion rates in bile and urine) at various intravenous infusion rates of harmol. These studies were the same as that for Fig. 2. The symbols were: hs, harmol sulfate; hg, harmol glucuronide; total, hs + hg.

The extent of sulfation and glucuronidation (rates/total conjugation rate) changed as the infusion rate increased. The ratio of the steady state formation rates of harmol sulfate/harmol glucuronide (S/G) slowly decreased with increasing infusion rates of harmol (Table 3), and the trend extended to the high infusion rate (760 nmole/min) where the disposition of harmol was non-linear.

*Intravenous versus intraportal infusion of harmol: hepatic extraction ratio of harmol*

To further delineate the role of extrahepatic metabolism of harmol, harmol conjugation rates were examined at a low infusion rate (167 nmole/min) into jugular vein and portal vein. This infusion rate was chosen because linear kinetics of harmol was observed at this infusion rate (Figs 2 and 3). The portal infusion rate can be perceived as an infusion rate which equals the rate of metabolism during the hepatic first-pass effect, plus a resulting lower infusion rate at the jugular vein (the part that 'escapes' first pass uptake by the liver).

Major differences resulting from the route of harmol infusion were the steady state harmol blood concentration and the S/G ratio. The steady state harmol concentration in blood for i.v. and p.v. infusions were respectively 2.85 ± 0.10 μM (N = 10) and 0.86 ± 0.15 μM (N = 11); the corresponding S/G ratios were 3.00 and 1.68 (Table 4 and Fig. 5). Because the steady state harmol arterial blood concentration during intraportal infusion rate (of 167 nmole/min) was 0.86 μM which corresponded to the jugular infusion rate of 52 nmole/min (found from Fig. 2), the rate of first-pass metabolism by the liver was calculated as the difference (167–52) or 115 nmole/min. The hepatic available fraction was 52/167 or 0.31 whereas the hepatic extraction ratio was 115/167 or 0.69. A similar result can be obtained for the hepatic availability by 0.86/2.85 or 0.3; hepatic extraction ratio was 0.70.

*Incubation of* [$^3$H]*-harmol with blood cells*

Because the total body clearance of harmol greatly exceeded hepatic blood flow, blood cells as extra-

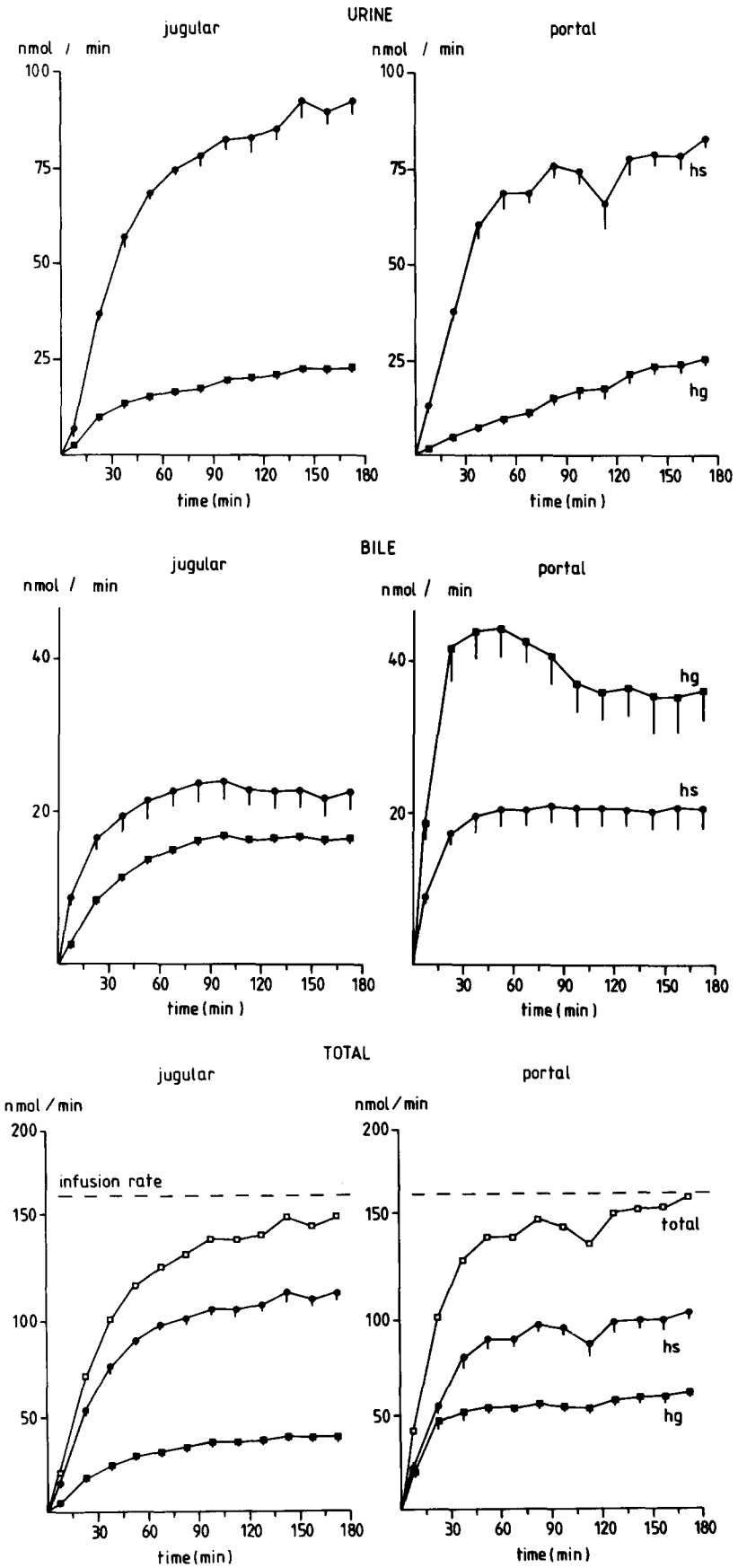


Fig. 5. A comparison of the conjugation patterns of harmol when it is infused either into the jugular vein (i.v.) or the portal vein (p.v.) at 167 nmole/min. The excretion rates of harmol sulfate (●—●) and harmol glucuronide (■—■) in urine (upper panel), bile (middle panel), and the total excretion pattern (bile and urine, lower panel) were shown for five rats in each group. Each data point represented the mean ± S.E.M.

Table 3. Ratio between sulfation and glucuronidation rates of harmol at various infusion rates

| Infusion rate (nmole/min) | Steady state conjugation rate (nmole/min) | Percent of total conjugation | | S/G Ratio |
|---|---|---|---|---|
| | | Sulfation | Glucuronidation (%) | |
| 16.6 | 14.8 | 85 | 15 | 5.7 |
| 67 | 66.9 | 80 | 20 | 4.0 |
| 100 | 87.1 | 79 | 21 | 3.8 |
| 135 | 138 | 76 | 24 | 3.2 |
| 167 | 153 | 74 | 26 | 2.9 |
| 760 | 700 | 67 | 33 | 2.0 |

hepatic site for the metabolism of harmol were investigated by the incubation of [$^3$H]-harmol with whole blood (heparinized or EDTA-treated) for 60 min at 37°. The platelets, in particular, have been reported to contain high activities of the phenol sulfotransferases [20, 21]. But no detectable sulfate or glucuronide conjugate was found at the end of the incubation period.

## DISCUSSION

The present results show that extrahepatic conjugation plays a major role in the metabolism of harmol, primarily because the total clearance of harmol far exceeds hepatic blood flow. Studies with the single intravenous injection of harmol (10 and 100 μmole/kg) and infusion rate (760 nmole/min) revealed that at the higher dose (100 μmole/kg) and infusion rate (760 nmole/min), total body clearance of harmol was less than maximal (60 ml/min for infusion rates at 167 nmole/min or below) (Tables 1 and 2). In fact, the lower clearance value for the 10 μmole/kg single dose (44 ml/min) as compared to the low rate infusion studies may consist of a region of nonlinearity at the early period (the first 10 min) post injection when the blood concentrations of harmol are high. Such a transient period of saturation may result in a smaller time-averaged clearance value. A similar observation was found in other studies with procainamide [22] after rapid intravenous injection and rapid intraportal injection. Consequently, when the clearance value from the single 10 μmole/kg dose (44 ml/min) was used to predict the loading doses and infusion rates, the predicted steady-state harmol concentration in blood was 30% higher than the observed concentration. In this respect, drug infusion at low rates most likely is the better method to examine drug clearances under first-order conditions because the transient saturation at the early period resulting from rapid injections is circumvented.

Total body clearance, as found for infusion rates of harmol at 167 nmole/min and below, was maximal at 60 ml/min. This value exceeds that for hepatic blood flow for the intact rat (approx. 20 ml/min for a 300 g rat). The high total body clearance, therefore, infers extrahepatic conjugation. When the steady state hepatic extraction ratio of harmol was found to be approx. 0.7 from the intraportal infusion of harmol at 167 nmole/min, extrahepatic clearance is calculated to be 46 ml/min (assuming hepatic blood flow of 20 ml/min and, thus, hepatic clearance of 14 ml/min) and represents 77% of total body clearance. The present findings confirmed the conclusions presented by Cassidy and Houston [5–7] and Machida *et al.* [10] that extrahepatic conjugation plays an important role in the total conjugation of phenolic substrates. The steady state hepatic extraction ratio of harmol *in vivo* (0.7) was less than that reported for the perfused rat liver preparation (0.8–0.9) [23, 24]; this discrepancy may be explained by, for instance, the differences in blood flow rate (10 ml/min for the rat liver preparation) and the extent to which harmol will be bound to blood proteins (only 1% (w/v) albumin was used in perfusate for the liver perfusion system).

Taking solely the data on the intravenous and

Table 4. Harmol conjugation rates at intravenous and intraportal infusion rates of 167 nmole/min to the rat *in vivo*

| Route of infusion | Steady state harmol blood concentration (μM) | Steady state rate of formation | | Steady state clearances | | | S/G Ratio |
|---|---|---|---|---|---|---|---|
| | | Harmol sulfate (nmole/min) | Harmol glucuronide (nmole/min) | Total body clearance | Sulfation clearance* (ml/min) | Glucuronidation clearance† (ml/min) | |
| i.v. | 2.85 ± 0.19‡ | 116 | 39 | 58.6 | 51.9 | 6.7 | 3.00 |
| p.v. | 0.86 ± 0.15 | 101 | 60 | — | — | — | 1.68 |

* Sulfation clearance was calculated as (sulfation rate/total conjugation rate) × total body clearance.
† Glucuronidation clearance was calculated as (glucuronidation rate/total conjugation rate) × total body clearance.
‡ i.v. N = 10; p.v. N = 11.

intraportal infusions of harmol (Table 4), total body clearance after intravenous harmol infusion was 59 ml/min. The clearance for the formation of harmol sulfate was 52 ml/min [sulfation rate/total conjugation rate × total body clearance]. Even when hepatic clearance (14 ml/min, or hepatic blood flow × hepatic extraction ratio) is viewed as the clearance solely for formation of harmol sulfate (which is unlikely), extrahepatic clearance for the formation of harmol sulfate is 38 ml/min (52–14 ml/min) and exceeds that for the liver.

At present it is unclear which organ or tissue contributes to the high clearance of harmol. The blood is devoid of conjugative activity on harmol, and other studies in the laboratory with the perfused lung preparation (J. R. Dawson *et al.*, unpublished data) suggest limited activity of lung metabolizing enzymes for harmol conjugation. The extrahepatic sites for harmol conjugation, and sulfation in particular, remain to be elucidated.

The sulfate/glucuronide ratio (Tables 2 and 4) was found to change with infusion rate or dose and route of drug administration, regardless of whether harmol kinetics are linear or non-linear. A similar observation is found in studies with the perfused rat liver preparation [23, 24] in which the S/G ratio decreased with increasing harmol concentrations, despite the fact that the clearance and extraction ratio by the liver remained constant; such changes in S/G ratio have been attributed to an uneven distribution of sulfation and glucuronidation activities in the rat liver [24, 25]. But because extrahepatic conjugation plays a very important role in the total metabolism of harmol in the rat *in vivo,* perturbations in the S/G ratio *in vivo* may be due to factors unexplored. Moreover, harmol glucuronidation rate increased markedly on intraportal infusion compared to intravenous infusion (Table 4). Reasons for this phenomenon have yet to be explored.

*Acknowledgements*—The authors thank Ms Aly Schirring for the preparation of the manuscript and Dr A. H. J. Scaf for calculations with his RUGFIT program. This work was supported by a NATO Travel grant (83.035). J.R.D. was a recipient of a Royal Society European Fellowship (London). K.S.P. was a recipient of a Faculty Development Award (DG 262, 263 and 264) from the Medical Research Council of Canada and a grant from the Canadian Liver Foundation.

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