Comparison of the Disposition of Hepatically-Generated Morphine-3-glucuronide and Morphine-6-glucuronide in Isolated Perfused Liver from the Guinea Pig

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Purpose. Humans and guinea pigs metabolise morphine extensively, forming the isomers morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in relatively similar ratios. Both metabolites are formed in the liver, and their greater polarity relative to the parent aglycone may limit their permeability across hepatic membranes. This study compared the disposition of hepatically-generated M3G and M6G in perfused livers isolated from guinea pigs.

Methods. Livers were perfused at 30 ml/min in a non-recirculating manner with Krebs bicarbonate buffer containing morphine (6 to 7 μM). Perfusing medium, venous perfusate and bile were collected at regular intervals and concentrations of morphine, M3G and M6G determined by reversed-phase HPLC.

Results. Concentrations of morphine, M3G and M6G in perfusate and the rates of biliary excretion of M3G and M6G were consistent between 20 and 50 min of perfusion. The mean (±s.d.) ratio for the rate of formation of M3G relative to M6G was 3.7 ± 1.5. A mean 33 ± 3% of morphine extracted by the liver was recovered as summed M3G and M6G. Of the M3G and M6G formed during a single passage, 19 ± 11% and 9 ± 9%, respectively, was excreted into bile; the values were significantly different (P = 0.002).

Conclusions. A greater fraction of hepatically-generated M3G excreted into bile compared to that for M6G reflects differences in their relative transport across sinusoidal and canalicular membranes of hepatocytes, possibly via carrier-mediated systems.

KEY WORDS: morphine; morphine-3-glucuronide; morphine-6-glucuronide; liver; membrane transport.

INTRODUCTION

The majority of a dose of morphine administered to humans and animals is metabolized in the liver (1). In humans, the most important metabolites are the isomers morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Between 50% and 60% of an intravenous or oral dose is excreted in urine as M3G, and about 10% as M6G (1). Their formation is believed to be catalyzed by distinct isoforms of UDP-glucuronosyltransferase (UGT) (1).

The two isomers exhibit quite different pharmacological activity: M6G is antinociceptive in animals and analgesic in humans while M3G has no antinociceptive action, but elicits central excitatory activity and opposes the antinociceptive effects of M6G and its parent, morphine (1).

Glucuronides are more polar than the corresponding aglycones. Previous studies have demonstrated a limited permeability for M3G across the hepatic sinusoidal membrane (2), and the possible involvement of carrier-mediated systems for its transport across hepatic membranes (3,4). Differences between M3G and M6G in structure and lipophilicity (5), may result in differential partitioning into the lipophilic environment of cellular membranes and/or binding to carrier-mediated systems. The influence of differences in structure and polarity between glucuronides derived from a common precursor on transport across membranes has not been examined in great detail.

Few studies have compared the disposition of M3G and M6G *in vivo* following their separate administration. After intravenous dosing of rabbits, the total clearance of M6G was approximately 80% greater than that for M3G (6). Also, given that an entire dose of morphine administered by these workers was recovered in the urine as M3G and M6G, it could be assumed that the total clearance for each glucuronide equated to renal clearance. Since both metabolites are poorly bound in plasma (1), differences in renal clearance may reflect dissimilar transport across renal tubular membranes.

The aim of the present work was to compare the hepatic disposition of the isomers M3G and M6G following their formation from morphine in the isolated perfused liver. The guinea pig is a suitable small animal for these studies, since the formation of M6G relative to M3G is comparable to that in humans (1) while the rat, used commonly as a donor in studies with perfused livers, does not form measurable amounts of M6G (1).

MATERIALS AND METHODS

Chemicals

Morphine (morphine HCl.3H_2O) was purchased from McFarlane Smith, Edinburgh, UK, and morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide (dihydrate) from Sigma Chemical Co., St. Louis, MO, USA. Acetonitrile (UV cutoff 190 nm, BDH Laboratory Supplies, Poole UK), trifluoroacetic acid (Sigma Chemical Co.) and Milli-Q water were used for HPLC. All other chemicals for the preparation of perfusing medium were of analytical grade and used as received.

Surgery and Perfusion of Livers

Five male Duncan-Hartley guinea pigs (ranging in weight from 226 g to 257 g) were obtained from the Field Station of the Institute of Medical and Veterinary Science, Gilles Plains, Australia. They were housed in metal cages in a room with a 12 hr cycle of light/dark, and allowed free access to water and food (Mouse Cubes, Rigley Agriproducts, Murray Bridge, Australia). Approval for their use in experiments was obtained from the Animal Ethics Committee of the University. Following fasting for 24 hr, animals were anesthetized by intraperitoneal

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NOTATIONS: M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; SKF 525-A, β-diethylaminoethyl diphenlypropylacetate.

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injection of 30 mg/kg of sodium pentobarbital (Nembutal 60 mg/ml, Boehringer Ingelheim, Artarmon, Australia). After a mid-line abdominal incision and lateral cuts, the intestines were moved to the right to expose the cystic duct, gallbladder and portal vein. The gall bladder was raised with forceps, a small incision made, and medical grade polyethylene tubing (0.58 mm i.d., 0.96 mm o.d., Dural Plastics and Engineering, Dural, Australia) passed through the cystic duct and along the bile duct, 4–5 mm towards the liver. The tubing was held in place with a ligature (Silk 1.5 metric 4/0 USP, Cyanamid Australia, Baulkham Hills, Australia). The bile duct was ligated posterior to its junction with the cystic duct. Two loose ligatures were placed around the portal vein, and another around the posterior vena cava above the entry of the renal vein. A cannula (Insyte® IV catheter, 18 GA, Becton Dickinson, Sandy, UT, USA) was inserted into the portal vein and held in place by tying the two ligatures. Perfusion of the liver was commenced at a flow of 15 ml/min followed immediately by opening of the diaphragm and insertion of a cannula (Insyte® IV catheter, 14 GA, Becton Dickinson) into the superior vena cava through the right atrium. As the liver cleared of blood, the flow of perfusing medium was increased to 30 ml/min and the ligature tightened around the posterior vena cava. The liver *in situ* was transferred to a thermostatically controlled cabinet at 37°C and covered with parafilm to retain moisture.

Livers were perfused, as described previously (7), in a single passage with freshly prepared and filtered (0.2 μm) erythrocyte- and albumin-free Krebs bicarbonate buffer (pH 7.4) supplemented with glucose (16.7 mM) and sodium taurocholate (8.33 μM). The concentration of oxygen in perfusing medium and venous perfusate was measured at regular intervals with an oxygen meter (Model 820, Orion Research Inc., Boston, MA, USA).

After a 15 min period of equilibration with drug-free medium, morphine HCl was added to perfusing medium to generate a nominal concentration between 6 and 7 μM. Samples of venous perfusate (10 ml) were collected into polypropylene centrifuge tubes (Disposable Products, Salisbury, Australia) at 0, 1, 2 and 5 min after the addition of morphine, and every 5 min thereafter up to 50 min. Perfusing medium (10 ml) was taken from the reservoir every 10 min up to 50 min. Bile was collected continuously, over intervals of 10 min, into pre-weighed 1.5 ml polypropylene microcentrifuge tubes (Medos Co., Adelaide, Australia). In preliminary experiments the density of bile was found to be 1.0. Upon completion of the perfusion the liver was excised, blotted dry and weighed.

Viability of the liver was monitored throughout by assessing its gross appearance (uniformly pink to brown) and consumption of oxygen, the flow of bile, and recovery of perfusing medium as venous perfusate. Retrospective viability was assessed also from the consistency in elimination of morphine and recovery of M3G and M6G in venous perfusate and bile.

Analytical Methods

Morphine in perfusing medium, venous perfusate and bile, and M3G and M6G in venous perfusate and bile, were quantified by reversed-phase HPLC. A mobile phase consisting of 4 parts of acetonitrile made up to 100 parts with 0.1% trifluoroacetic acid (adjusted to pH 2.40 with 1 M sodium hydroxide) was pumped (LC-10AS pump, Shimadzu, Kyoto, Japan) at 0.8 ml/min through a 30 cm steel column packed with Econosil C_{18} (10 μm, Alltech, Baulkham Hills, Australia). Venous perfusate, perfusing medium (diluted 1 in 4 with water), or bile (diluted 1 in 100) was injected directly (SIL-10A Auto Injector, Shimadzu) and the chromatographic eluate monitored for fluorescence (λ_{ex} 280 nm, λ_{em} 335 nm) using a Waters Model 470 detector (Millipore Corporation, Milford, MA, USA). Data were collected and processed with a Waters Maxima 820 Workstation. Approximate retentions for M3G, M6G and morphine were 7 min, 11 min and 13 min, respectively. Calibration curves of peak-heights against concentration were linear ($r > 0.99$) for morphine (0.27 μM to 2.7 μM), M3G (0.22 μM to 2.2 μM) and M6G (0.20 μM to 2.0 μM) in venous perfusate, and for morphine (0.27 μM to 4.0 μM), M3G (0.22 μM to 3.3 μM) and M6G (0.20 μM to 3.0 μM) in diluted bile. Analysis of appropriate quality controls spanning the range of concentrations for the calibration standards showed that intra- and inter-day accuracy and reproducibility were within 16%.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated from data collected between 20 min and 50 min after commencing perfusion with morphine.

Hepatic availability of morphine (F_M):

$$F_M = \frac{C_{out}^M}{C_{in}^M} \quad (1)$$

where C_{out}^M and C_{in}^M are the mean concentrations of morphine in venous perfusate and perfusing medium, respectively.

Hepatic clearance of morphine (CL_H):

$$CL_H = Q \times (1 - F_M) \quad (2)$$

where Q is the flow of perfusing medium.

Biliary extraction ratios (E_{bil}^x) for hepatically-generated M3G and M6G:

$$E_{bil}^x = \frac{R_{bil}^x}{R_{bil}^x + R_{out}^x} \quad (3)$$

where R is the rate of recovery of the corresponding glucuronide (x) in bile (bil) or venous perfusate (out).

For calculations of mass-balance:

$$\text{Mass-balance} = \frac{R_{out}^M + R_{tot}^{M3G} + R_{tot}^{M6G}}{Q \times C_{in}^M} \quad (4)$$

where R_{out}^M is the rate of recovery of morphine in venous perfusate, and R_{tot}^{M3G} and R_{tot}^{M6G} are the summed rates of recovery in bile and venous perfusate of M3G and M6G, respectively.

Data are presented as mean ± s.d. Comparisons of means were performed using a paired Student's t-test, and correlations between two or more variables were analyzed using Kendall rank-order correlation. Values of $P < 0.05$ were considered significant.

RESULTS

Functional parameters such as consumption of oxygen and biliary flow, reflecting the viability of each liver, were measured up to 50 min from the addition of morphine to perfusing medium. Mean delivery of oxygen to the five livers was 3.9

± 0.9 μmol/min/g liver. Mean consumption for each liver ranged from 1.1 ± 0.2 μmol/min/g liver to 2.7 ± 0.4 μmol/min/g liver and there were no obvious trends with time; the overall mean was 1.8 ± 0.7 μmol/min/g liver. Mean individual biliary flows ranged from 1.8 ± 0.2 μl/min/g liver to 6.0 ± 0.3 μl/min/g liver, and again there were no obvious trends with time; the overall mean was 3.9 ± 1.9 μl/min/g liver. Recovery of venous perfusate was greater than 95%. The mean weight of the livers was 8.6 ± 1.7 g.

Fig. 1a shows the concentrations of morphine, M3G and M6G in venous perfusate, while Fig. 1b shows the rate of biliary excretion of M3G and M6G, during perfusion of one liver with morphine; the results are typical of the findings from perfusion of the other four livers. No morphine was found in bile. Mean pharmacokinetic parameters determined at steady-state are summarized in Table I.

Of the morphine extracted by the liver, 33 ± 3% was recovered as summed M3G and M6G; 5.0 ± 2.1% of extracted morphine was recovered in bile as summed M3G and M6G. The mean ratio of the summed rates of recovery in venous perfusate and bile of M3G to that of M6G was 3.7 ± 1.5. From 20 min after the addition of morphine to the perfusing medium, there was a significant rank-order correlation between biliary flow and the biliary extraction ratio of M3G (T = 0.95, $P < 0.05$) and M6G (T = 1.0, $P < 0.01$); neither correlation was improved when the additional influence from consumption of oxygen was considered.

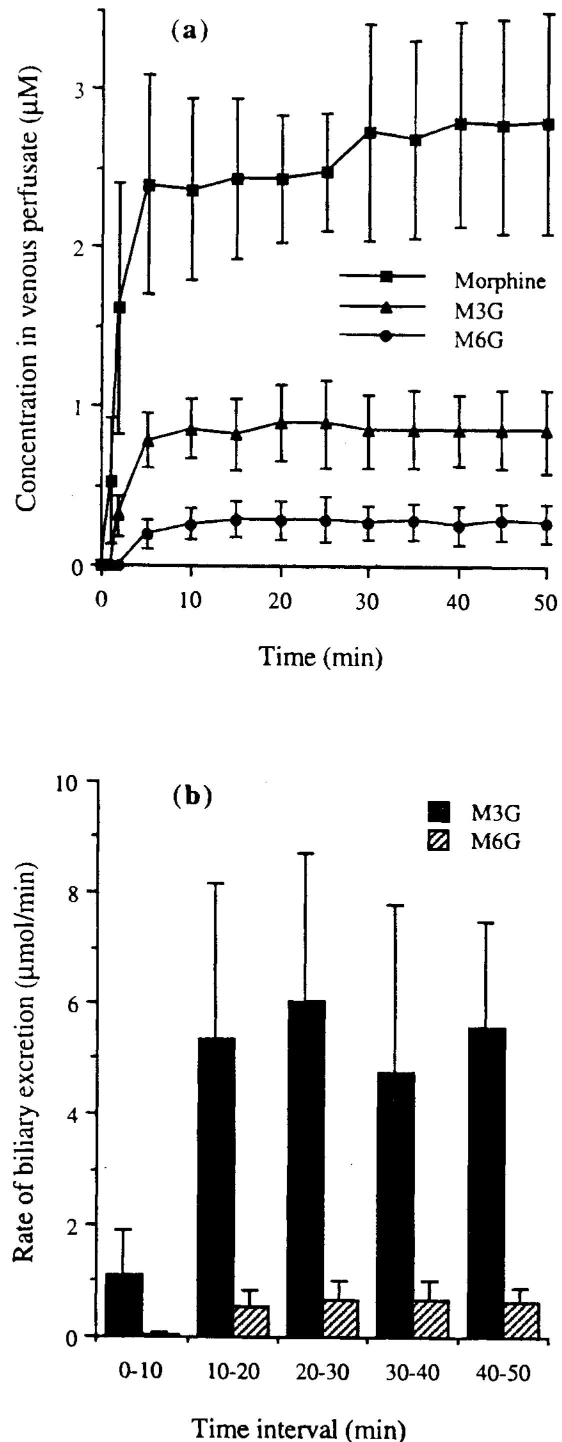

Fig. 1. (a) Mean (±s.d.) concentrations of morphine, M3G and M6G in venous perfusate and, (b) mean (±s.d.) rate of biliary excretion of M3G and M6G, during perfusion of five livers with morphine. Morphine was added to the perfusing medium at time zero.

DISCUSSION

The liver is the main organ for the metabolism of morphine to M3G and M6G (1). These metabolites may be excreted into bile or pass into the hepatic vascular space. The hepatic sinusoidal membrane presents a diffusional barrier to M3G (2) and carrier-mediated systems may be responsible primarily for its transport across hepatic membranes (3,4,8). The principal aim of the present study was to examine the relative disposition of hepatically-generated M3G and M6G in the isolated perfused liver. Most studies investigating the disposition of xenobiotics in perfused livers have utilised rats. However, this species does not form measurable amounts of M6G, but the formation of M6G relative to M3G in the guinea pig is comparable to that occurring in humans (1).

When experiments have been performed with livers from guinea pigs, conditions of perfusion have been adapted from those used for the rat; only a minority have reported physiological parameters. When livers from guinea pigs were perfused previously (9–11) in a single passage with physiologically-based buffers at flows comparable to that used in the present experiments, oxygen was consumed at the rate of 0.9 to 2.2 μmol/min/g liver; values similar to those obtained in the present study. Previous work found that consumption by livers from the guinea pig was 30% to 40% less than that for the rat (9). In contrast, similar consumption of oxygen by both species was noted when data in the present study was compared with unpublished data collected previously in our laboratory from the rat under almost identical conditions of perfusion. Biliary flow in the present study was consistent during the period of an experiment when pharmacokinetic parameters were calculated. There were differences in flow between guinea pigs, but the overall mean (and variability about the mean) were similar to values observed previously (12) during perfusion with a comparable medium. The flow also compares favourably with a value of about 3 μl/min/g liver estimated from measured flows and body-weights of conscious, bile duct-cannulated animals (13). The relativity in biliary flows for the guinea

Table I. Pharmacokinetic Parameters Determined at Steady-State During Perfusion with Morphine of Livers from Guinea Pigs

Pharmacokinetic parameter	Mean (±s.d.)
F_M	0.39 ± 0.06
CL_H (ml/min)	19.5 ± 1.7
E_{bil}^{M3G}	0.19 ± 0.11[a]
E_{bil}^{M6G}	0.09 ± 0.09[a]
Mass-balance	0.58 ± 0.06

[a] Significantly different, P = 0.002.

pig and rat observed by these workers compares well with the relativity in flows from perfused livers of both species in our laboratory.

After 20 min of perfusion with morphine, steady-state concentrations of morphine, M3G and M6G were achieved in venous perfusate (Fig. 1a), and the rate of recovery of M3G and M6G in bile was constant (Fig. 1b). There is no previously reported value for the hepatic availability of morphine in guinea pigs; the mean availability is approximately thrice the value determined in male rats (7) using similar conditions of perfusion, but is comparable to values ranging from 0.11 to 0.55 determined in other studies perfusing livers from rodents (1).

The relative rates of formation of M3G and M6G are comparable to those found previously in the male Duncan-Hartley guinea pig when morphine was incubated with hepatic microsomes, and when their recovery in urine was compared following an intravenous dose of morphine (14). Only 20% of an intravenous dose administered to the guinea pig was collected in urine as summed M3G and M6G and, even with the inclusion of unchanged morphine in urine, approximately 65% of the dose remained unaccounted for (14). In our study, 58% of morphine perfusing the liver was recovered in venous perfusate and bile, leaving 42% unaccounted for. Thus, comparable percentages of the dose in both studies are either retained for a prolonged period within the liver, if a condition of pseudo-steady state rather than true steady state was achieved in the liver during the experiment, or other metabolites were formed and not measured. Morphinone, and a morphinone-glutathione adduct, were identified in bile collected for 4 hr from male Duncan-Hartley guinea pigs administered a single subcutaneous dose of morphine, and together accounted for 10.6% of the dose (15). Interestingly, 1.1% of the dose was excreted in bile as morphine, but no M6G was detected (15). Both of these observations contrast with our findings; excretion of 1.1% of morphine in perfusing medium as unchanged morphine in bile would have been quantifiable even at the highest biliary flow observed.

Dissimilar kinetics of transport across the sinusoidal membrane into venous perfusate and/or across the canalicular membrane into bile probably explains the differential biliary extraction ratios for M3G and M6G (Table I). Previous work in our laboratory (2) found that alterations in the flow of medium perfusing livers isolated from the rat had no significant effect on the biliary extraction ratio for M3G. From this, a limited permeability for M3G across the hepatic sinusoidal membrane was proposed. Furthermore, probenecid, a potential inhibitor of the carrier-mediated transport of organic anions across membranes, reduced the permeability of hepatically-generated M3G across the sinusoidal membrane (8). Additional support for the involvement of a carrier-mediated system of transport comes from the reduced egress of hepatically-generated M3G into bile in the presence of dehydrocholate (3), and of the trapping of M3G within hepatocytes by the organic cation, SKF 525-A (4). No investigations have been performed to date, but limited permeability may exist also for M6G. The dissimilar biliary extraction ratios for M3G and M6G are probably due to their differential affinities for a common carrier-mediated system of transport across each membrane, not unlike their differences in affinity for opioid receptors, or to the existence of independent systems for their transport.

Interestingly, there was a significant correlation between biliary flow and the biliary extraction ratios for M3G and M6G. Higher biliary flow may reflect a greater functional performance of the liver and, therefore, enhanced canalicular transport for M3G and M6G. Alternatively, a reduced biliary excretion at lower flows may arise from a greater residence-time in the biliary tree, increasing their movement from bile via hepatocytes or paracellular paths into the sinusoidal space, or via biliary epithelia (16) into venous perfusate.

In conclusion, livers isolated from guinea pigs and perfused with morphine generated M3G and M6G in proportions similar to those observed when morphine is administered to humans. Differences were observed in the disposition of hepatically-generated M3G and M6G. A greater fraction of M3G excreted into bile compared to that for M6G probably reflects differences in their relative transport across the sinusoidal and canalicular membranes of hepatocytes. Further work is in progress to investigate their mechanisms of transport.

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