J. Pharm. Pharmacol. 1999, 51: 1289–1297
Received February 11, 1999
Accepted June 1, 1999

Application of a Loading Wash-out Method for Investigating the Hepatocellular Efflux of a Hepatically-generated Metabolite, Morphine-3-glucuronide

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Abstract

Previous studies using the rat isolated perfused liver demonstrated that the hepatic disposition of morphine-3-glucuronide is membrane permeability-rate limited, and that the movement of the metabolite across hepatic sinusoidal and canalicular membranes is partly via carrier-mediated transport systems. As a consequence of the membrane permeability-limitation, the biliary excretion of hepatically-generated morphine-3-glucuronide is much more efficient than that of morphine-3-glucuronide reaching the liver via the vasculature. We have quantitated the cellular efflux kinetics (cell-to-blood and cell-to-bile) of morphine-3-glucuronide in the rat isolated perfused liver using a loading wash-out design. In the 'loading' phase, morphine was infused into the liver (2·7 μM) and the biliary excretion and sinusoidal efflux of morphine-3-glucuronide was assessed under steady-state conditions. Subsequently, the infusion was stopped and the concentration vs time profile of morphine-3-glucuronide in outflow perfusate (the wash-out phase) was determined. A physiologically-based pharmacokinetic model was used to determine the rate-constants for the movement of hepatically-generated morphine-3-glucuronide into the sinusoidal and canalicular spaces of the liver, and the associated membrane permeability terms.

The mean ($\pm$ s.d.) rate constants for the biliary excretion and sinusoidal efflux of morphine-3-glucuronide were determined to be $0{\cdot}160 \pm 0{\cdot}043$ and $0{\cdot}169 \pm 0{\cdot}068\ \text{min}^{-1}$, respectively, and the corresponding membrane permeability parameters were 1·12 and 1·18 mL min^{-1}, respectively. The sinusoidal membrane permeability term was significantly less than hepatic blood flow in the rat. The volume of distribution of hepatically-generated morphine-3-glucuronide ($207{\cdot}5 \pm 74{\cdot}8$ mL) was found to be approximately 50-times the intracellular space of the rat liver, suggesting that hepatically-generated morphine-3-glucuronide accumulates within hepatocytes. The results indicate that hepatically-generated morphine-3-glucuronide undergoes intracellular accumulation, probably as a consequence of poor membrane permeability.

Metabolites of drugs are invariably more polar than their precursors and rather than being able to freely diffuse across plasma membranes, their movement into and out of cells is more likely to be limited by cell membrane permeability. Consequently, the fate of a metabolite generated from a precursor within the liver (generated metabolite) may differ from that which reaches the organ via the hepatic vasculature (pre-formed metabolite), and the hepatic elimination of pre-formed metabolite might be less efficient than that of generated metabolite (Miyauchi et al 1987; Pang et al 1992; Evans 1996). Metabolites where such differences have been demonstrated include enalaprilat (de Lannoy & Pang 1986, 1987; Schwab et al 1990), paracetamol glucuronide (Brouwer & Jones 1990), morphine-3-glucuronide (Evans & Shanahan 1993), and conjugates of the experimental compound E3040 (Takenaka et al 1997).

Morphine-3-glucuronide reaching the liver via the bloodstream (including that returning to the liver via recirculation) experiences membrane-limited hepatic uptake (Evans & Shanahan 1993;

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O'Brien et al 1996) and relies partly on carrier-mediated transport systems for movement into hepatocytes (Imamura & Fujimoto 1980a, b; Brock & Vore 1982). However, there is no such reliance in the case of morphine-3-glucuronide generated inside hepatocytes from its precursor, morphine. As a consequence, whereas about 50% of hepatically-generated morphine-3-glucuronide is excreted in bile during a single passage of morphine through the rat liver, the extraction ratio of pre-formed morphine-3-glucuronide is less than 1% (Evans & Shanahan 1993).

For a hepatically-generated metabolite, the kinetics of efflux into blood and bile will dictate the intracellular concentrations of the metabolite, and will also influence its ultimate fate, i.e. the relative extents of further metabolism, biliary excretion and movement into the bloodstream. Currently, very little information is available on the cellular efflux of hepatically-formed metabolites and methods of quantifying this efflux from a kinetic perspective in the intact liver. In this study, the cellular efflux kinetics (cell-to-blood and cell-to-bile) of morphine-3-glucuronide were quantified in the rat isolated perfused liver using a loading wash-out design. In the 'loading' phase, morphine was infused into the liver and the biliary excretion and sinusoidal efflux of morphine-3-glucuronide were assessed under steady-state conditions. Subsequently, the infusion was stopped and the concentration vs time profile for morphine-3-glucuronide in the outflow perfusate (the wash-out phase) was determined. Kinetic analysis was used to determine the rate-constants for the movement of hepatically-generated morphine-3-glucuronide from hepatocytes into the sinusoidal and canalicular domains of the liver.

Materials and Methods

Chemicals

Morphine hydrochloride was purchased from McFarlane Smith, (Edinburgh, UK) and morphine-3β-D-glucuronide and sodium taurocholate from Sigma Chemical Company (St Louis, MO). Water was purified using a Milli-R/Q purifier system (Millipore Australia Pty. Ltd, Victoria, Australia). Acetonitrile (UV cut-off 190 nm) and methanol were HPLC grade (Waters Associates, Lane Cove, NSW, Australia). Other compounds were of analytical grade, purchased from Sigma Chemical Company, and used as received.

Liver perfusions and experimental design

Experiments were conducted using isolated perfused livers from four female Sprague–Dawley rats (260–280 g) obtained from Gilles Plains Animal Resource Centre, South Australia. The surgical procedure and perfusion conditions have been described previously (Evans & Shanahan 1995). Briefly, the liver remained in-situ while an erythrocyte- and albumin-free perfusion medium was delivered, via the portal vein, at a constant flow rate of 30 mL min^{-1}. This flow rate provided an oxygen delivery rate of approximately 2 μmol min^{-1} (g liver tissue)$^{-1}$. Outflow perfusate and bile samples were collected via cannulas placed into the vena cava and bile duct, respectively. Inflow perfusate samples (1 mL) were also collected from a pre-portal sampling site distal to the infusion port. Bile was collected into pre-weighed tubes and bile flow rate was determined by gravimetric assessment of the volume of bile collected per unit time (assuming 1 g mL^{-1}).

After cannulation, each liver was perfused with drug-free medium at a flow rate of 30 mL min^{-1}, for an equilibration period of 15 min. Subsequently, a solution of morphine hydrochloride was infused into the inflow perfusate (immediately proximal to the portal vein cannula) via a calibrated syringe pump to produce a final nominal inflow concentration of 2·7 μM (1000 ng mL^{-1}). Inflow and outflow perfusate samples (1 mL) were collected before the infusion (blank) and 17, 18, 19 and 20 min after starting the infusion (steady-state samples). It should be noted that earlier experiments indicated that steady-state was achieved within this time. The morphine infusion was ceased at 20 min, and venous perfusate samples (1 mL) were collected at 10-s intervals for 2 min and then at 2·5, 3, 4, 5, 7·5, 10, 15 and 19 min after stopping the infusion (wash-out samples). Bile samples were collected at 5-min intervals during (0–20 min) and after (20–40 min) the infusion. In each perfused liver, the infusion-wash-out experiment was performed in duplicate i.e. two periods. Period 2 commenced 20 min after stopping the morphine infusion in period 1.

During an experiment, a perfused liver was not considered acceptable unless the viability measurements were within the following acceptance limits: oxygen consumption > 10 μmol min^{-1}; bile flow > 5 μL min^{-1}; and recovery of perfusate from the venous cannula $> 98\%$ of inflow rate. In addition, observations of gross liver appearance required that the organ be uniformly perfused with an absence of necrotic patches.

Analytical methods

The concentrations of morphine and morphine-3-glucuronide in perfusate and bile were determined

using an HPLC method involving direct injection of perfusate and diluted bile samples into an HPLC system. The calibration ranges for morphine and morphine-3-glucuronide in perfusate were 50–1000 ng mL^{-1}, whereas the calibration range for diluted bile was 1–10 μg mL^{-1}, with dilution of bile in water being performed where necessary. The chromatographic conditions were based upon those described by Venn & Michalkiewicz (1990), and involved the use of a Model M45 pump (Waters Associates), a Wisp 712 Autosampler (Waters Associates), an Alltech EconosilC8 10 μm column (250 mm × 4·6 mm) (Alltech Associates, Homebush, NSW, Australia) heated to 60°C and a Model 821-FP Jasco Intelligent Spectrofluorometric Detector (excitation wavelength 280 nm, emission wavelength 335 nm). The signal was recorded using a CR-3A Chromatopak integrator (Shimadzu, Tokyo, Japan). The mobile phase consisted of 0·1% trifluoroacetic acid and 3% acetonitrile in water and was recirculated through the HPLC system at 1 mL min^{-1}. Under these conditions, the retention times of morphine and morphine-3-glucuronide were typically 9·5 and 6·5 min, respectively. Analysis of low (100 ng mL^{-1}), medium (250 ng mL^{-1}) and high (1000 ng mL^{-1}) quality control samples for each analyte in perfusate indicated inter- and intra-day accuracy and precision values of less than 8%. The accuracy and precision values for morphine-3-glucuronide in bile were less than 4%.

Data analysis

Model-independent analysis. The average concentrations of morphine in inflow and outflow perfusate at steady-state, $C^{M}_{IN}(ss)$ and $C^{M}_{OUT}(ss)$, respectively, were calculated as the arithmetic mean values of results from samples collected during the steady-state period (17–20 min). The fraction escaping hepatic extraction (availability, F^{M}) was taken to be the ratio of $C^{M}_{OUT}(ss)$ to $C^{M}_{IN}(ss)$; the hepatic extraction ratio of morphine (E^{M}) was calculated as $(1-F^{M})$ and the hepatic clearance of morphine (CL^{M}) was taken to be the product of E^{M} and perfusate flow rate, Q. The rate of morphine-3-glucuronide formation was assumed to represent the sum of the rates of recovery of the compound, at steady-state, in perfusate ($R^{M3G}_{perf}(ss)$) and bile ($R^{M3G}_{bile}(ss)$). The partial clearance of morphine to morphine-3-glucuronide was calculated by the following equation:

$$CL^{M3G}_{f} = \frac{R^{M3G}_{perf}(ss) + R^{M3G}_{bile}(ss)}{C^{M}_{IN}} \qquad (1)$$

and the biliary extraction ratio of morphine-3-glucuronide (E^{M3G}_{B}) was calculated by equation 2:

$$E^{M3G}_{B} = \frac{R^{M3G}_{bile}(ss)}{R^{M3G}_{bile}(ss) + R^{M3G}_{perf}(ss)} \qquad (2)$$

The fraction of eliminated morphine which was converted to morphine-3-glucuronide (f^{M3G}_{M}) was taken to be the ratio of the rate of recovery of morphine-3-glucuronide $[R^{M3G}_{bile}(ss) + R^{M3G}_{perf}(ss)]$ to the rate of elimination of morphine. Mass-balance was calculated at steady-state as the total rate of recovery of morphine, and morphine-3-glucuronide in outflow perfusate and bile relative to the rate of morphine input.

Model-dependent analysis. In deriving a model for describing the wash-out profiles for morphine assumptions were made. Firstly, it was assumed that the hepatic uptake of morphine was flow-limited and was not a rate-limiting factor in terms of its disposition kinetics in the liver — this assumption was not made for morphine-3-glucuronide. Secondly, it was assumed that morphine and morphine-3-glucuronide were completely unbound in perfusate, a realistic assumption given that albumin-free perfusion medium was utilized. Thirdly, it was assumed that hepatically-generated morphine-3-glucuronide was either excreted into bile or appeared in outflow perfusate (in other words, it was assumed that morphine-3-glucuronide did not undergo further metabolism or irreversible sequestration within hepatocytes). Preliminary experiments involving the perfusion of livers with pre-formed morphine-3-glucuronide supported this third assumption. The average concentrations of morphine and morphine-3-glucuronide within the vascular space of the liver were assumed to equal the respective concentrations in outflow perfusate. Finally, it was assumed that once morphine-3-glucuronide exited an hepatocyte it did not enter downstream hepatocytes — this assumption was supported by the fact that the hepatic extraction ratio of pre-formed morphine-3-glucuronide was less than 0·01 (Evans & Shanahan 1993). Based upon these assumptions, a two-compartment venous equilibration model was derived (Figure 1). This model had been used previously to describe the effects of altered perfusate flow rate on the hepatic disposition of morphine and morphine-3-glucuronide (O'Brien et al 1996). Use of this model allowed the following equations to be derived for morphine and morphine-3-glucuronide (refer to Appendix):

For morphine (M)

$$CL^{M}_{int} = Q \cdot \left[\frac{E^{M}}{1 - E^{M}}\right] \qquad (3)$$

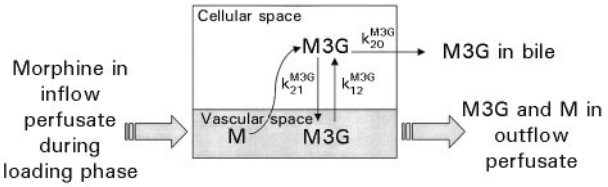

Figure 1. Diagrammatic representation of the physiological model used to describe the hepatic disposition of morphine and hepatically-generated morphine-3-glucuronide. During the loading phase of the experiment, morphine was delivered to the liver, via perfusate, at a constant concentration. Morphine was assumed to exhibit flow-limited distribution within the liver, and morphine-3-glucuronide formed within the cellular space was assumed to undergo excretion into bile and movement into the vascular space of the liver.

where CL_{int}^{M} is the intrinsic clearance of morphine.

For morphine-3-glucuronide

$$\lambda_Z^{M3G} = k_{20}^{M3G} + k_{21}^{M3G} \quad (4)$$

$$\frac{R_{bile}^{M3G}(ss)}{R_{perf}^{M3G}(ss)} = \frac{k_{20}^{M3G}}{k_{21}^{M3G}} \quad (5)$$

$$V_{ss}^{M3G} = \frac{Q}{C_{OUT}^{M3G}(ss)} \cdot AUC_{20-\infty}^{M3G}\left(1 + \frac{k_{20}^{M3G}}{k_{21}^{M3G}}\right) \quad (6)$$

where λ_Z^{M3G} is the terminal rate constant for morphine-3-glucuronide calculated from the slope of the ln-transformed plasma concentration vs time profile during the wash-out phase; k_{20}^{M3G} and k_{21}^{M3G} are the first-order rate-constants for the movement of morphine-3-glucuronide from hepatocytes into bile and perfusate, respectively; V_{SS}^{M3G} is the volume of distribution (with respect to outflow perfusate) of morphine-3-glucuronide at steady-state; and $AUC_{20-\infty}^{M3G}$ is the area under the morphine-3-glucuronide concentration (in outflow perfusate) vs time curve from the end of the infusion (i.e. 20 min after the start of the infusion) until infinite time, determined using the linear trapezoidal method with extrapolation. Using equations 3–6 it was possible to determine the following model-dependent parameters:

$$CL_{int}^{M}, k_{21}^{M3G}, k_{20}^{M3G} \text{ and } V_{SS}^{M3G}.$$

All data are presented as mean ± s.d. Paired Student's *t*-tests were used to test for differences in the hepatic disposition of morphine and morphine-3-glucuronide during period 1 and period 2 of each perfusion; $P < 0{\cdot}05$ was taken to represent statistical significance.

Results

Bile production and oxygen consumption remained constant over the course of each perfusion experiment and there were no significant differences for these viability parameters (Table 1) between period 1 and period 2 ($P > 0{\cdot}05$). The recovery of inflow perfusate was greater than 98% in each perfusion, indicating that perfusate leakage within the liver was negligible.

In keeping with previous studies (Evans & Shanahan 1995), the concentrations of morphine and morphine-3-glucuronide in outflow perfusate, and morphine-3-glucuronide concentration in bile, were constant 15 min after starting the infusion of morphine, signifying that steady-state had been attained. Morphine could not be detected in bile and normorphine was not detected in outflow perfusate or bile. Figure 2 is a representative plot of the concentration of morphine and morphine-3-glucuronide in outflow perfusate vs time after stopping the infusion of morphine. Typically, morphine concentrations were quantifiable for less than 30 s, whereas the profile for morphine-3-glucuronide could be followed for 5–10 min, with a half-life of 2–4 min.

The model-independent pharmacokinetic parameters for morphine and morphine-3-glucuronide are summarized in Table 1. In period 1, the mean availability of morphine at steady-state was 0·31 and the mean hepatic clearance of morphine was relatively high (20·9 mL min^{-1}). Replication of the "loading wash-out" study for each liver demonstrated no significant alteration in morphine availability or clearance between period 1 and 2 (Table 1).

Mass balance calculations indicated that approximately 90% of morphine in inflowing perfusate was recovered as morphine-3-glucuronide and morphine in outflow perfusate and morphine-3-glucuronide in bile. During period 1, the mean fraction of eliminated morphine converted to morphine-3-glucuronide was 0·85 (Table 1) indicating

Table 1. Model-independent steady-state pharmacokinetic parameters describing the hepatic disposition of morphine and hepatically-generated morphine-3-glucuronide, and functional parameters of the isolated perfused rat liver during a constant infusion of morphine.

Parameter	Period 1	Period 2
F^M	0·31 ± 0·09	0·36 ± 0·14
CL^M (mL min^{-1})	20·9 ± 2·6	19·1 ± 4·2
CL_f^{M3G} (mL min^{-1})	17·7 ± 2·5	17·4 ± 1·5
f_M^{M3G}	0·85 ± 0·06	0·94 ± 0·15
E_B^{M3G}	0·50 ± 0·04	0·33 ± 0·06*
Mass balance (%)	90 ± 4	94 ± 9
Bile flow rate (μL min^{-1})	9·59 ± 1·56	8·07 ± 2·64
O_2 consumption (μmol min^{-1} g^{-1})	1·86 ± 0·34	2·01 ± 0·16

Values are mean ± s.d., n = 4. *$P < 0{\cdot}05$ compared with the corresponding value in period 1.

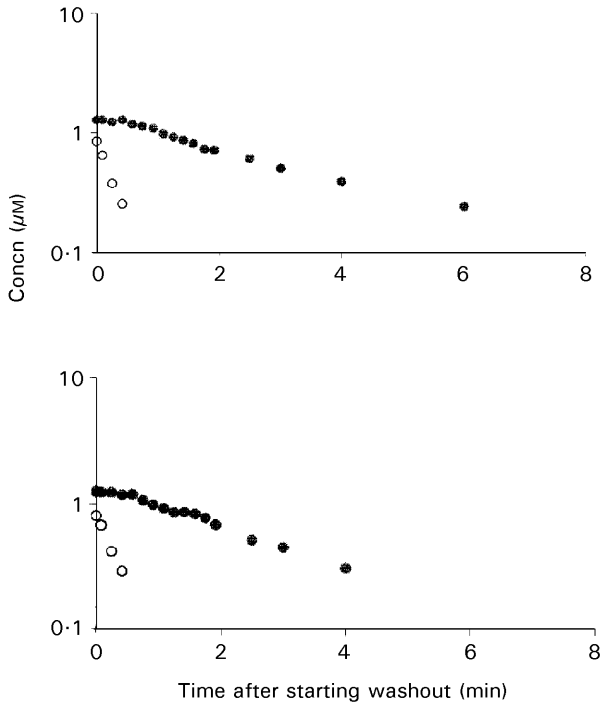

Figure 2. Representative plot of the outflow perfusate concentration of morphine (○) and morphine-3-glucuronide (●) vs time after stopping a constant infusion of morphine into the rat isolated perfused liver. The upper and lower panels represent data from period 1 and period 2, respectively.

that morphine-3-glucuronide was the major metabolite of morphine. A mean biliary extraction ratio of 0·50 for morphine-3-glucuronide in period 1 (Table 1) indicated that half of the hepatically-generated metabolite was excreted into bile, with the remainder exiting the liver via perfusate. While there was no significant difference between period 1 and 2 in the partial clearance of morphine to morphine-3-glucuronide or the fraction of eliminated morphine recovered as morphine-3-glucuronide, the mean biliary extraction ratio of hepatically-generated morphine-3-glucuronide was significantly reduced (from 0·50 to 0·33) in period 2 ($P < 0·05$; Table 1).

Table 2 summarizes the pharmacokinetic parameters calculated using the physiologically-based model (see Figure 1 and Appendix). The intrinsic clearance of morphine was high (74·7 mL min^{-1} in period 1), relative to perfusate flow rate (30 mL min^{-1}), and did not differ significantly between periods 1 and 2. Similarly, there was no difference between period 1 and 2 in the volume of distribution of morphine-3-glucuronide or the rate constant for the movement of morphine-3-glucuronide across the sinusoidal membrane (k_{21}^{M3G}). However, there was a 44% reduction in the rate constant for the movement of morphine-3-glucuronide across the canalicular membrane (k_{20}^{M3G}) in period 2 ($P < 0·05$).

Discussion

The rat isolated perfused liver has been used in a number of previous studies to investigate the hepatic handling of morphine and morphine-3-glucuronide (Evans & Shanhan 1995; O'Brien et al 1996). Experiments in our laboratory have demonstrated that morphine-3-glucuronide exhibits barrier-limited disposition, leading to substantial differences in the fate of pre-formed and hepatically-generated metabolite (Evans & Shanhan 1993). These findings are in keeping with other in-vitro experiments, which suggest that the hepatic uptake of morphine-3-glucuronide involves carrier-mediated transport (Imamura & Fujimoto 1980a, b; Brock & Vore 1982).

Previous experiments in the rat isolated perfused liver applied a steady-state approach to study the hepatic disposition of morphine and morphine-3-glucuronide (Evans & Shanahan 1995; O'Brien et al 1996). In this study, morphine was infused into the liver until steady-state conditions were achieved (loading phase) and after stopping the infusion, the venous outflow profiles for morphine and morphine-3-glucuronide were determined (wash-out phase). Kinetic analysis of the wash-out data, using a two-compartment venous-equilibrium (well-stirred) model, was used to determine the rate constants for the movement of hepatically-generated morphine-3-glucuronide out of hepatocytes into perfusate and bile. The study was designed to assess the applicability of the loading-wash-out method for investigating the membrane transport kinetics of a hepatically-generated metabolite.

Previous experiments demonstrated that the disposition of morphine and morphine-3-glucuronide in the in-situ rat isolated perfused liver, under

Table 2. Pharmacokinetic parameters describing the hepatic disposition of morphine and hepatically-generated morphine-3-glucuronide derived from the wash-out outflow perfusate profiles for morphine and morphine-3-glucuronide using a physiological model.

Parameter	Period 1	Period 2
CL_{int}^{M} (mL min^{-1})	74·7 ± 29·7	60·8 ± 30·0
V_{SS}^{M3G} (mL)	207·5 ± 74·8	151·2 ± 60·7
k_{20}^{M3G} (min^{-1})	0·160 ± 0·043	0·093 ± 0·025*
k_{21}^{M3G} (min^{-1})	0·169 ± 0·068	0·190 ± 0·047

Values are mean ± s.d., n = 4. *$P < 0·05$ compared with the corresponding value in period 1.

identical conditions to those used in the current experiments, remained constant for up to 1 h (Evans & Shanahan 1995) allowing studies to be conducted examining the effects of perfusate flow rate (O'Brien et al 1996) and perfusate protein content (O'Brien et al 1997) on the hepatic extraction of morphine. In the current experiments, perfusions lasted for approximately 95 min to allow replication of the loading-wash-out experimental design, and liver viability remained constant over this period (Table 1). In addition, the hepatic clearance of morphine remained constant, confirming that the metabolic activity of the liver was not impaired. The only steady-state pharmacokinetic parameter to change significantly from period 1 to period 2 was the mean biliary extraction ratio of morphine-3-glucuronide, which decreased significantly ($P < 0{\cdot}05$) from 0·50 to 0·33 (Table 1). Therefore, as the experiment progressed, there was a relative increase in the sinusoidal efflux of morphine-3-glucuronide compared with biliary excretion. In theory, such a change could be due to a reduction in the biliary excretion rate constant (k_{20}^{M3G}), an increase in the sinusoidal efflux rate constant (k_{21}^{M3G}), or a combination of both. The kinetic analysis using the morphine-3-glucuronide wash-out profiles was vital for determining the causative mechanism.

A number of physiological-based models have been used for describing the disposition of drugs and metabolites in isolated organs. These models vary in complexity from single compartment venous-equilibrium and parallel-tube models, through to more complex dispersion and distributed models. Among these models, the primary difference is the assumptions that are made regarding the magnitude of axial dispersion of drug as it travels through the liver (Rowland & Evans 1991). The most appropriate model depends on the substrate being studied and the experimental design being utilized. For example, with impulse–response experiments, a distributed or dispersion model is most appropriate, whereas with infusion wash-out designs, simpler models normally suffice. In this study, the wash-out curve for morphine-3-glucuronide displayed monoexponential behaviour (Figure 2), meaning that the liver was, in essence, operating as a single well-mixed compartment. It could be argued that this observation for morphine-3-glucuronide is in conflict with the concept that the compound exhibits membrane-limited disposition in the liver (since a membrane barrier implies at least two distinct compartments). However, the hepatic uptake morphine-3-glucuronide that exits from hepatocytes is negligible and the hepatic extraction ratio of pre-formed morphine-3-glucuronide is less than 0·01 (Evans & Shanahan 1993). Therefore, when considering the kinetics of generated-morphine-3-glucuronide, the flux of morphine-3-glucuronide from the sinusoids to the cells is negligible relative to the flux in the opposite direction (cells to sinusoids). Under these conditions, the rate constant for sinusoidal uptake (k_{12}^{M3G}) can be assumed to approach zero, and the two-compartment model for morphine-3-glucuronide (Figure 1) behaves as a single compartment system.

From the kinetic analysis of the morphine-3-glucuronide data, the mean volume of distribution of morphine-3-glucuronide during period 1 was found to be $207{\cdot}5 \pm 74{\cdot}8$ mL, which greatly exceeds the aqueous volume of the liver (approx. 7 mL). This indicates that under steady-state conditions, morphine-3-glucuronide accumulates within hepatocytes to concentrations much higher than those in hepatic outflow. This observation is consistent with the concept that morphine-3-glucuronide, a highly polar molecule, does not permeate freely across hepatocyte membranes. This also explains why the biliary extraction ratio of morphine-3-glucuronide formed in-situ during morphine administration greatly exceeds that for pre-formed morphine-3-glucuronide infused into the liver via the portal route (Evans & Shanahan 1993). The low membrane permeability of morphine-3-glucuronide is consistent with findings for other glucuronide conjugates including paracetamol–glucuronide (Brouwer & Jones 1990), oxazepam–glucuronide (St-Pierre et al 1990) and the conjugate of E3040 (Takenaka et al 1997).

Using the physiological model, the rate constants for biliary excretion (k_{20}^{M3G}) and sinusoidal efflux (k_{21}^{M3G}) of morphine-3-glucuronide were quantified. The significance of being able to quantify these rate constants for the membrane transport of hepatically-generated polar metabolites such as morphine-3-glucuronide is that intracellular interactions at the level of membrane transport can be evaluated (Evans 1996). The mean rate constant for the sinusoidal efflux of morphine-3-glucuronide was not significantly different between periods 1 and 2, suggesting that the movement of morphine-3-glucuronide from hepatocytes into the sinusoids remained constant with time. However, k_{20}^{M3G} was significantly reduced in period 2 ($P < 0{\cdot}05$) indicating a reduction in the ability of the liver to remove morphine-3-glucuronide from cells into bile. Thus, despite liver viability tests indicating acceptable liver function and performance throughout the course of the perfusions, the biliary excretion of morphine-3-glucuronide did not remain constant for 90 min. Although the cause of the reduction in k_{20}^{M3G} is unknown, possible

mechanisms include intracellular accumulation of endogenous competitors for the canalicular membrane transport of morphine-3-glucuronide or non-specific deterioration of the canalicular membrane transport systems. One could also postulate that there might have been a weakening of the tight junctions between hepatocytes, thereby allowing morphine-3-glucuronide within the bile canaliculus to leak out into the sinusoids.

The rate constants for morphine-3-glucuronide can be expressed in physiological terms as:

$$k_{21}^{M3G} = \frac{fu_{cell} \cdot P_{sin}}{V_{cell}} \quad (7)$$

$$k_{20}^{M3G} = \frac{fu_{cell} \cdot P_{can}}{V_{cell}} \quad (8)$$

where fu_{cell} is the fraction unbound of morphine-3-glucuronide within hepatocytes, V_{cell} is the physical volume of the cell which is occupied by morphine-3-glucuronide, and P_{sin} and P_{can} are the intrinsic permeability clearance terms for the movement of morphine-3-glucuronide across the sinusoidal and canalicular membranes, respectively. Assuming that hepatically-generated morphine-3-glucuronide occupies liver cell water, which is about 0·55 mL (g liver weight)$^{-1}$ (Pang et al 1990) a value of 7 mL (for a 12·5-g liver) can be assigned for V_{cell}. Given that morphine-3-glucuronide does not bind to plasma proteins (Milne et al 1996) and does not accumulate within hepatocytes during perfusions with the pre-formed metabolite, a value of unity can be assumed for fu_{cell}. The resultant values for P_{sin} and P_{can} are 1·18 and 1·12 mL min^{-1}, respectively. Although these permeability terms incorporate the contribution of passive and carrier-mediated movement, it is notable that the value for P_{sin} is substantially less than perfusate flow rate (30 mL min^{-1}) or hepatic blood flow in the rat (10 mL min^{-1}), which is further confirmation that the hepatic disposition of this metabolite is membrane-limited rather than flow-limited.

In conclusion, this study has demonstrated that loading wash-out experiments in the rat isolated perfused liver can be used to derive information about kinetic events involved in the hepatic disposition of hepatically-generated metabolites such as morphine-3-glucuronide. The rate constants for the biliary excretion and sinusoidal efflux of morphine-3-glucuronide were found to be 0·160 and 0·169 min^{-1}, and the corresponding membrane permeability parameters were 1·12 and 1·18 mL min^{-1}, respectively. The mean volume of distribution of morphine-3-glucuronide was found to be approximately 50-times greater than the intracellular space, which is in keeping with the concept that morphine-3-glucuronide experiences difficulty in permeating hepatocyte membranes and therefore accumulates within hepatocytes.

Acknowledgement

This study was supported by the National Health and Medical Research Council of Australia, grant number 940330.

Appendix

The disposition of morphine and hepatically-generated morphine-3-glucuronide in the single-pass rat isolated perfused liver is described by a 2-compartment model in which morphine displays flow-limited disposition and morphine-3-glucuronide exhibits permeability-limited disposition (Figure 1). It is assumed that morphine-3-glucuronide is formed in the peripheral compartment and undergoes either biliary excretion or efflux into the vascular space. In addition, it is assumed that there is no protein binding of morphine and morphine-3-glucuronide in the vascular space and the equations derived below therefore apply only to the condition in which the fraction unbound within the vascular space is unity (i.e. $f_u = 1$). Further assumptions and definitions of symbols are given in the text. According to this model, the following differential equations can be derived;

$$\frac{dM}{dt} = Q \cdot C_{IN}^{M} - (Q + CL_{int}^{M}) \cdot C_{OUT}^{M} \quad (A1)$$

$$\frac{dM3G_1}{dt} = k_{21}^{M3G} \cdot M3G_2 - k_{12}^{M3G} \cdot M3G_1 - Q \cdot C_{OUT}^{M3G} \quad (A2)$$

$$\frac{dM3G_2}{dt} = f_M^{M3G} \cdot CL_{int}^{M} \cdot C_{OUT}^{M} + k_{12}^{M3G} \cdot M3G_1 - (k_{21}^{M3G} + k_{20}^{M3G}) \cdot M3G_2 \quad (A3)$$

Assuming that the re-uptake of morphine-3-glucuronide from the sinusoid into the cell is negligible (i.e. $k_{12}^{M3G} = 0$), which is justified on experimental grounds (see text), equations A2 and A3 simplify to equations A4 and A5, respectively:

$$\frac{dM3G_1}{dt} = k_{21}^{M3G} \cdot M3G_2 - Q \cdot C_{OUT}^{M3G} \quad (A4)$$

$$\frac{dM3G_2}{dt} = f_M^{M3G} \cdot CL_{int}^{M} \cdot C_{OUT}^{M} - (k_{21}^{M3G} + k_{20}^{M3G}) \cdot M3G_2 \quad (A5)$$

Under steady-state conditions, during a constant input of morphine, the following equations can be derived:

$$\text{Intrinsic clearance of morphine} = Q \cdot \left[\frac{E^M}{1 - E^M}\right] \quad \text{(A6)}$$

$$\frac{R_{bile}^{M3G}(ss)}{R_{perf}^{M3G}(ss)} = \frac{k_{20}^{M3G}}{k_{21}^{M3G}} \quad \text{(A7)}$$

where E^M is the steady-state extraction ratio of morphine, and $R_{bile}^{M3G}(ss)$ and $R_{perf}^{M3G}(ss)$ are the rates of recovery of morphine-3-glucuronide in bile and outflow perfusate, respectively, at steady-state. It should be noted that equation A6 is reliant on the unbound fraction of morphine, in perfusate, being equal to 1.

Recognizing that $M3G_1 = V_1 \cdot C_{OUT}^{M3G}$ and assuming that the formation of morphine-3-glucuronide ceases once the infusion of morphine is stopped, then the equation describing the outflow concentration of morphine-3-glucuronide, as a function of time after stopping the input of morphine, is:

$$C_{OUT}^{M3G}(t) = A \cdot \exp^{-\left(\frac{Q}{V_1}\right)t} + B \cdot \exp^{-\left(k_{21}^{M3G} + k_{20}^{M3G}\right)\cdot t} \quad \text{(A8)}$$

where t is the time after stopping the infusion. Hence, the model predicts a biexponential decline in the outflow concentrations of morphine-3-glucuronide. However, the rate constant for the first process is so large ($30\,min^{-1}$, estimated as a perfusate flow rate of $30\,mL\,min^{-1}$ divided by the likely value of V_1, about 1 mL), and the amount of morphine-3-glucuronide in compartment 1 relative to compartment 2 is so small, that the first exponent would not be detected, and a monoexponential outflow profile would be observed in practice. Therefore, from the terminal rate constant for morphine-3-glucuronide (equation A8) and the rates of appearance of morphine-3-glucuronide in bile and perfusate at steady-state (equation A7), the two rate constants, k_{21}^{M3G} and k_{20}^{M3G} can be derived.

The steady-state volume of distribution of morphine-3-glucuronide, relative to outflow perfusate, is given by the amount of morphine-3-glucuronide in the liver at steady-state divided by the concentration of morphine-3-glucuronide in outflow perfusate at steady-state. If morphine-3-glucuronide formation ceases when the morphine infusion is stopped, and morphine-3-glucuronide is not metabolized further, then the amount of morphine-3-glucuronide in the liver at steady-state (A_{SS}^{M3G}) is equal to the total amount which leaves the liver, via perfusate and bile, from the end of the infusion until infinite time:

$$A_{SS}^{M3G} = (\text{M3G in perfusate})_{20-\infty} + (\text{M3G in bile})_{20-\infty} \quad \text{(A9)}$$

which can also be expressed as:

$$A_{SS}^{M3G} = (\text{M3G in perfusate})_{20-\infty} \cdot \left[1 + \frac{(\text{M3G in bile})_{20-\infty}}{(\text{M3G in perfusate})_{20-\infty}}\right] \quad \text{(A10)}$$

It is assumed that the relative amount of morphine-3-glucuronide recovered in bile vs perfusate, after stopping the infusion, is equal to the relative rates of recovery at steady-state, i.e.

$$\frac{(\text{M3G in bile})_{20-\infty}}{(\text{MeG in perfusate})_{20-\infty}} = \frac{R_{bile}^{M3G}(ss)}{R_{perf}^{M3G}(ss)} = \frac{k_{20}^{M3G}}{k_{21}^{M3G}} \quad \text{(A11)}$$

then,

$$A_{SS}^{M3G} = Q \cdot AUC_{20-\infty}^{M3G}\left(1 + \frac{k_{20}^{M3G}}{k_{21}^{M3G}}\right) \quad \text{(A12)}$$

where $AUC_{20-\infty}^{M3G}$ is the area under the outflow perfusate vs time curve for morphine-3-glucuronide from the time of stopping the infusion ($t = 20\,min$) until infinite time. Therefore, the steady-state volume of distribution of morphine-3-glucuronide is given by

$$V_{SS}^{M3G} = \frac{Q}{C_{OUT}^{M3G}(ss)} \cdot AUC_{20-\infty}^{M3G}\left(1 + \frac{k_{20}^{M3G}}{k_{21}^{M3G}}\right) \quad \text{(A13)}$$

where $C_{OUT}^{M3G}(ss)$ is the steady-state concentration of morphine-3-glucuronide in the outflow perfusate.

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