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IN VITRO EVIDENCE FOR THE INVOLVEMENT OF AT LEAST TWO FORMS OF HUMAN LIVER UDP-GLUCURONOSYLTRANSFERASE IN MORPHINE 3-GLUCURONIDATION

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Abstract—Morphine 3-glucuronidation kinetics and the inhibitory effects of a number of xenobiotics on morphine glucuronidation in human liver microsomes have been investigated. In both native and detergent-activated microsomes morphine glucuronidation exhibited biphasic kinetics, with a high affinity, low capacity component and a low affinity, high capacity component. These data suggest the involvement of at least two forms of human liver UDP-glucuronosyltransferase (UDPGT) in morphine glucuronidation. The high affinity morphine-UDPGT activity is likely to be of most importance in morphine glucuronidation *in vivo*. Chloramphenicol, 4-hydroxybiphenyl, 4-methylumbelliferone, 1-naphthol and 4-nitrophenol were all shown to inhibit the low affinity morphine-UDPGT activity, but only chloramphenicol and 1-naphthol were competitive inhibitors. Each of these xenobiotics were shown to be a non-inhibitor of the high affinity morphine-UDPGT activity, or at least to have considerably lower affinity for this enzyme form(s) than morphine itself. Overall the results provide further evidence for the heterogeneity of human liver UDPGT and, in conjunction with other recent studies (Miners JO *et al.*, Kinetic and inhibitor studies of 4-methylumbelliferone and 1-naphthol glucuronidation in human liver microsomes, *Biochem Pharmacol* **37**: 665–671, 1988) of the kinetics of human liver glucuronidation reactions, indicate that xenobiotic substrates such as morphine, 4-methylumbelliferone and 1-naphthol may be used to differentiate UDPGT isozyme activities in human liver microsomes.

Both in terms of the variety of functional groups metabolised and its distribution throughout living organisms, UDP-glucuronosyltransferase (UDPGT) is the most versatile of the conjugating enzymes. UDPGT is responsible for the metabolism of a variety of drugs, drug metabolites, environmental chemicals and endogenous compounds, such as steroid hormones, bile acids and bilirubin. In recent years it has become apparent that the versatility of UDPGT is due to the existence of multiple forms of the enzyme which have different but overlapping substrate specificities. Evidence from differential induction studies [1–3], the physical separation of enzyme forms [4–10] and from the isolation of cloned cDNAs to individual forms [11–13] suggests the existence of at least eleven rat liver UDPGT isozymes. Morphine has been widely used as a model substrate for *in vitro* UDPGT activity and one of the recently purified rat liver UDPGT isozymes has been shown to glucuronidate morphine, but not substrates such as androsterone, bilirubin, oestrone, 4-nitrophenol and testosterone [8]. Recent studies [14, 15] in this laboratory have investigated whether substrates for individual rat liver UDPGT isozymes may be used to differentiate multiple forms of the enzyme in human liver. Here we describe the results of human liver microsomal kinetic and inhibitor studies with morphine.

MATERIALS AND METHODS

Chemicals and reagents. Chloramphenicol, 4-hydroxybiphenyl, 4-methylumbelliferone, morphine-3-β-D-glucuronide, 1-naphthol, phenolphthalein-β-D-glucuronide, polyoxyethylene 20-cetyl ether (Brij 58) and UDP-glucuronic acid (sodium salt) were purchased from the Sigma Chemical Co. (St. Louis, MO). Morphine HCl was obtained from Glaxo Australia (Melbourne, Australia) and 4-nitrophenol was purchased from the Aldrich Chemical Co. (Milwaukee, WI). All other reagents and solvents were of analytical reagent grade.

N-methyl-^{14}C-morphine hydrochloride (2.07 GBq/mmol) was purchased from Amersham Australia (Sydney, Australia). The ^{14}C-morphine was only 76% radiochemically pure and was therefore purified by high performance liquid chromatography (HPLC). Aliquots (200 μl) of an aqueous solution of the labelled material were injected on to a C-18 reversed-phase column (μ-Bondapak; 30 cm × 3.9 mm, i.d.; Waters Associates). The mobile phase was phosphate buffer (10 mM, pH 2.1)–acetonitrile (98.5:1.5) at a flow rate of 2.0 ml/min. Under these conditions the retention times of morphine and morphine glucuronide were 9.3 min and 6.4 min, respectively. The eluent under the morphine peak was collected, pooled, alkalinised (pH

9.0) and extracted five times with redistilled chloroform (aqueous-chloroform volume ratio 1:15). The organic phase was evaporated to dryness and the purified ^{14}C-morphine reconstituted in water to give a final concentration of 0.63 mM. Subsequent radiometric HPLC analysis showed the ^{14}C-morphine to be greater than 99.7% pure. This purified ^{14}C-morphine was used in experiments determining the extent of inhibition of morphine glucuronidation by various xenobiotics (see below).

Liver samples. Human liver samples were obtained from renal transplant donors and the approval of the Flinders Medical Centre Ethical Review Committee was obtained to use donor liver tissue for drug metabolism studies. Details of the donors of the livers and of the method of preparation of microsomes have been published previously [14, 16]. Liver samples were stored at −80° until used; experience in this laboratory has shown that UDPGT activity towards morphine is stable in liver samples stored at −80° for at least 2.5 years.

Measurement of morphine glucuronidation by human liver microsomes. The 3-glucuronidation of morphine by human liver microsomes was measured using a modification of the assay described by Rane *et al.* [17] for the determination of morphine glucuronidation by hepatic microsomes from the rhesus monkey. Microsomal incubations typically contained UDP-glucuronic acid (UDPGA; 30 mM), $MgCl_2$ (5 mM), Tris–HCl (0.1 M, pH 7.4), microsomal protein (125 μg of native microsomes or 62.5 μg of detergent-activated microsomes; see below), and morphine (2.5–5000 μM) in a final volume of 0.25 ml. The reaction was started by the addition of UDPGA and the samples were then incubated for 20 min at 37°; reaction rates were shown to be linear for incubation times to at least 30 min and for microsomal protein content up to 0.5 mg. A final concentration of 0.004% w/v Brij 58 in the incubation mixture was shown to result in maximum activation of morphine glucuronidation at a protein concentration of 0.25 mg/ml. The incubation was stopped by the addition of 0.1 ml of ammonium sulphate (1.0 M, pH 9.3) and cooling on ice. Phenolphthalein glucuronide (1 μg), the HPLC assay internal standard, was added to each incubation tube. The contents of each incubation tube were loaded onto a C-18 Sep-pak minicolumn (Waters Associates) which was eluted sequentially within 5 mM ammonium sulphate (20 ml) and methanol (4 ml). The methanol fraction was collected and evaporated to dryness under a stream of N_2. The residue was reconstituted in 0.2 ml of water and injected into the chromatograph. The chromatograph used was fitted with a μ-Bondapak 10 micron C-18 column (30 cm × 3.9 mm, i.d.; Waters Associates) and operated at ambient temperature. Morphine glucuronide and the internal standard (phenolphthalein glucuronide) were monitored by UV absorbance at 210 nm. The mobile phase was 17.5:82.5 acetonitrile–phosphate buffer (10 mM, pH 2.1) containing sodium dodecyl sulphate (SDS; 0.6 mM) at a flow rate of 2.0 ml/min. Under these conditions the retention times of morphine glucuronide, internal standard and morphine were 5.5 min, 10.2 min and 12.3 min, respectively. Unknown concentrations of morphine glucuronide were determined by comparison of peak height ratios with those of a calibration curve prepared using morphine glucuronide concentrations in the range 0.5–20.0 μM. Using this procedure the mean recovery of morphine glucuronide was 85.6 ± 2.1% (for six samples over the standard curve concentration range) while that of the internal standard was 89.3 ± 0.9%. The within-day coefficient of variation of the assay, determined by measuring morphine glucuronide formation (at a substrate concentration of 0.5 mM) in twelve separate incubations of the same batch of hepatic microsomes, was 7.1% and 4.9% for native and activated microsomes, respectively.

Other assays. Microsomal protein concentration was measured by the procedure of Lowry *et al.* [18] using crystalline bovine serum albumin as standard.

Kinetic and inhibitor studies. In experiments performed to determine the apparent K_m and V_{max} values for morphine glucuronidation the UDPGA concentration was kept constant (30 mM) and activity was measured for 14 morphine concentrations over the range 2.5–5000 μM. Kinetic experiments were performed with both native and detergent activated microsomes from four livers (F5, F6, F8 and F9). The concentration of UDPGA used here (i.e. 30 mM) was approximately 9–160-fold greater than the apparent K_m values for UDPGA (Results section). To determine the apparent K_m values for UDPGA, the concentration of morphine was kept constant (7.5 mM) and activity was measured for 14 UDPGA concentrations over the range 0.1–5.0 mM. UDPGA kinetic experiments were performed using both native and activated microsomes from three livers (F6, F8 and F9).

Possible inhibitory effects of chloramphenicol, 4-hydroxybiphenyl, 4-methylumbelliferone (4MU), 1-naphthol (1NP) and 4-nitrophenol on morphine glucuronidation were assessed using activated microsomes from three livers (F5, F6, F8). These inhibitor studies were performed at two morphine concentrations, 1.0 μM and 250 μM; concentrations of the putative inhibitors are indicated in the text (Table 2). Due to limitations of assay sensitivity, it was necessary to utilise ^{14}C-*N*-methylmorphine (3.18×10^4 dpm/incubation) in the low substrate concentration experiments. In the experiments using labelled substrate, HPLC column eluent under the morphine glucuronide peak was collected and counted on a liquid scintillation counter (Beckman LS3801) to determine the extent of morphine glucuronidation. In addition, in the high substrate concentration (250 μM) studies chloramphenicol, 4MU, 1NP and/or their respective glucuronides were found to interfere with the morphine glucuronide peak or that of the internal standard and this necessitated minor changes in mobile phase composition. For experiments performed in the presence of chloramphenicol and 4MU the proportion of acetonitrile in the mobile phase was reduced to 15% and 12.5%, respectively, while for experiments performed in the presence of 1NP the concentration of SDS in the mobile phase was increased to 1.1 mM. Where inhibition was observed in the high substrate concentration (250 μM) experiments full kinetic studies were performed (using microsomes from liver F8) to

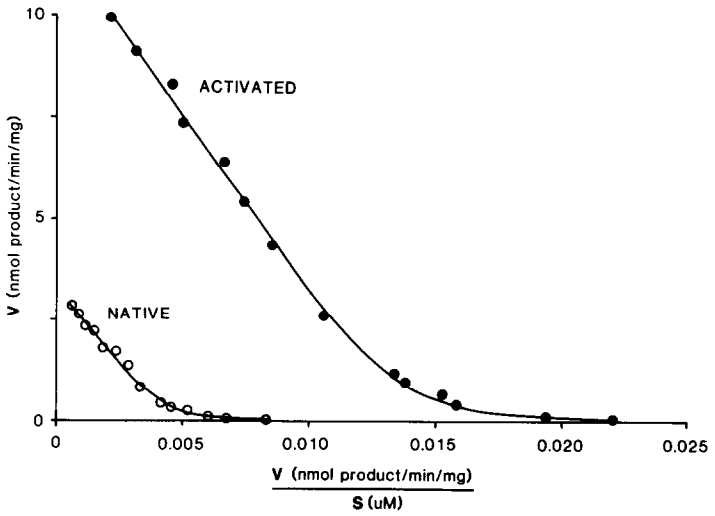

Fig. 1. Representative Eadie–Hofstee plots for morphine 3-glucuronidation by native and detergent activated human liver (F8) microsomes. Points are experimentally determined values while solid lines are the computer-generated curves of best-fit.

determine the type of inhibition and, where appropriate, apparent K_i values.

Analysis of results. All results are presented as mean ± SD. Values of the Michaelis–Menten parameters apparent K_m and V_{max} were initially obtained from graphical analysis of Eadie–Hofstee plots. These values were then used as initial estimates in an iterative programme [14] based on non-linear least squares regression analysis. Where appropriate, data were fitted to the expression for competitive inhibition [19] to obtain estimates of apparent K_i values. Differences in kinetic parameters between native and activated microsomes were assessed using Student's paired *t*-test.

RESULTS

Morphine-3-glucuronide formation showed biphasic kinetics in native and detergent activated microsomes of all 4 human livers studied (Fig. 1). Based on residual sums of squares, data for morphine glucuronidation were best fitted to the Michaelis–Menten expression for a two enzyme system. In addition, only the residuals from data fitted to the expression for a two enzyme system were evenly distributed around zero across the entire substrate concentration range. Computer derived Michaelis–Menten parameters for morphine glucuronidation in native and activated microsomes from each liver

Table 1. Computer derived Michaelis–Menten parameters for morphine glucuronidation by native and detergent-activated human liver microsomes

Liver No./ microsomal treatment	K_{m1} (μM)	V_{m1} (nmol/min/mg)	K_{m2} (μM)	V_{m2} (nmol/min/mg)
F5:				
native	2.4	0.11	922	5.7
activated	4.1	0.30	1589	12.1
F6:				
native	1.9	0.10	886	3.0
activated	3.8	0.28	1260	8.5
F8:				
native	2.3	0.08	688	3.2
activated	6.2	0.29	991	11.8
F9:				
native	3.3	0.08	695	2.6
activated	7.1	0.33	970	11.0

K_{m1}, V_{m1}, K_{m2} and V_{m2} refer to apparent K_m and V_{max} values calculated by fitting experimental data to the Michaelis–Menten expression for a two enzyme system (i.e. enzymes 1 and 2; for details see Materials and Methods).

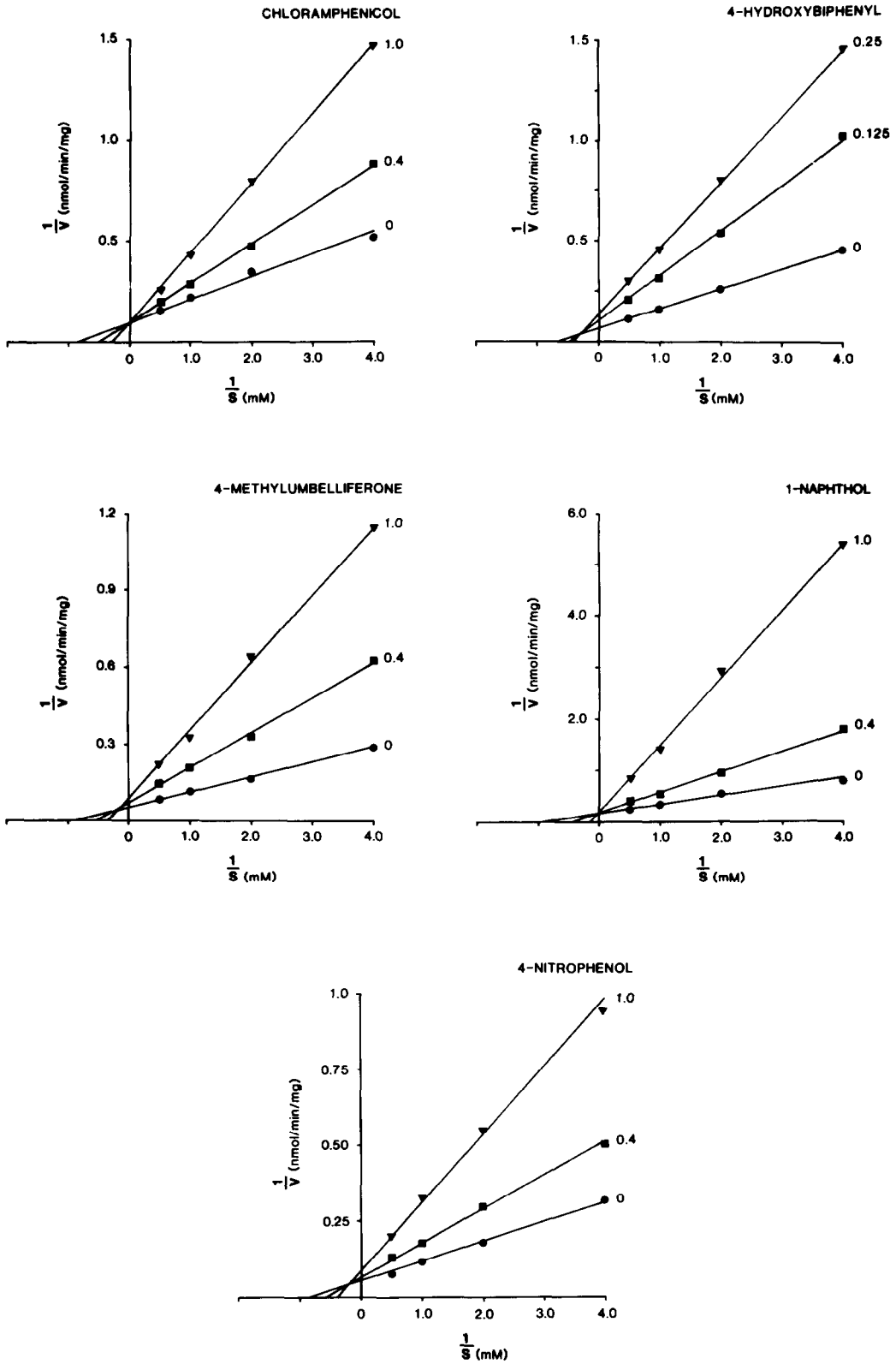

Fig. 2. Lineweaver–Burke plots for inhibition of the low affinity morphine-UDPGT activity in detergent activated human liver (F8) microsomes by chloramphenicol, 4-hydroxybiphenyl, 4-methylumbelliferone, 1-naphthol and 4-nitrophenol. Reciprocal substrate (morphine) concentrations are shown on the abscissa and inhibitor concentrations on the right hand side of each plot.

Table 2. Inhibitory effects of various xenobiotics on the high- and low-affinity components of morphine glucuronidation in detergent activated human liver microsomes

Xenobiotic	Percent control activity	
	High affinity component	Low affinity component
Chloramphenicol	90.2 ± 6.7	50.4 ± 11.5
4-Hydroxybiphenyl	87.2 ± 1.6	7.5 ± 1.4
4-Methylumbelliferone	97.1 ± 2.8	37.1 ± 4.0
1-Naphthol	92.9 ± 4.3	18.9 ± 3.8
4-Nitrophenol	91.3 ± 2.6	40.3 ± 3.3

Values are mean ± SD from 3 livers (F5, F6, F8). High affinity component; morphine concentration 1 μM, inhibitor concentration 100 μM. Low affinity component; morphine concentration 250 μM, inhibitor concentration 500 μM.

studied are shown in Table 1. The mean apparent K_m for the high affinity component (i.e. K_{m1}) of morphine glucuronidation in native microsomes was 2.5 ± 0.6 μM and this increased ($P < 0.05$) to 5.3 ± 1.6 μM on detergent activation. Similarly, the mean apparent K_m for the low affinity component (i.e. K_{m2}) of morphine glucuronidation was increased ($P < 0.05$) from 798 ± 123 μM in native microsomes to 1203 ± 289 μM in detergent activated microsomes. Detergent activation increased ($P < 0.05$) the mean V_{max} for both components of morphine glucuronidation; from 0.09 ± 0.02 nmol/min/mg to 0.30 ± 0.02 nmol/min/mg and from 3.63 ± 1.42 nmol/min/mg to 10.86 ± 1.64 nmol/min/mg for the high- and low-affinity components, respectively.

Apparent K_m values for UDPGA were determined in microsomes from 3 livers (F6, F8, F9). Using a fixed concentration of morphine (7.5 mM), biphasic Eadie–Hofstee plots were observed in microsomes from all three livers. The estimated mean apparent K_m values for UDPGA in native microsomes were 346 ± 76 μM and 1470 ± 670 μM. Detergent activation decreased the apparent K_m of the high affinity UDPGA component to 189 ± 68 μM but increased that of the low affinity UDPGA component to 3280 ± 1200 μM.

A number of xenobiotics were screened for inhibitory effects on morphine glucuronidation in activated microsomes from 3 livers (F5, F6, F8). The effects of the xenobiotics were determined at two morphine concentrations, 1 μM and 250 μM, and results are summarised in Table 2. At a morphine concentration of 1 μM, where 85% of morphine glucuronidation is carried out by the high affinity activity, chloramphenicol, 4-hydroxybiphenyl, 4MU, 1NP and 4-nitrophenol (at a concentration of 100 μM) all caused <13% inhibition of morphine glucuronidation. By contrast, at a morphine concentration of 250 μM, where over 85% of morphine glucuronidation is carried out by the low affinity activity, these xenobiotics (at a concentration of 500 μM) caused 50%–93% inhibition of morphine glucuronidation. Kinetic studies performed in activated microsomes from liver F8 indicated that only chloramphenicol and 1NP were competitive inhibitors of the low affinity component of morphine glucuronidation (Fig. 2). Apparent K_i values for chloramphenicol and 1NP were 980 μM and 93 μM, respectively. 4-Hydroxybiphenyl, 4MU and 4-nitrophenol were mixed-type inhibitors of the low affinity component of morphine glucuronidation.

DISCUSSION

Morphine is largely metabolised in humans and other mammalian species by glucuronidation in the 3-position [20, 21]. This study has demonstrated the involvement of at least two forms of human liver UDPGT in morphine 3-glucuronidation. In both native and activated microsomes morphine glucuronidation comprised two components, a high affinity, low capacity activity and a low affinity, high capacity activity. Biphasic kinetics were also observed for UDPGA using morphine as the fixed substrate. The contribution of each of the morphine-UDPGT activities towards total morphine 3-glucuronidation as a function of morphine concentration was calculated from the Michaelis–Menten equation for a two enzyme system using mean kinetic data from the studies with native microsomes and the simulated plots are shown in Fig. 3. It is apparent from Fig. 3 that the low affinity morphine-UDPGT activity contributes <25% towards total morphine 3-glucuronidation for concentrations < 5 μM. Even in cancer patients who receive high doses of morphine, plasma concentrations rarely exceed 1 μmol/l [22] and the low affinity morphine-UDPGT is therefore unlikely to be involved to any major extent in the *in vivo* glucuronidation of morphine in man.

There was relatively little variability in the apparent K_m and V_{max} values for morphine glucuronidation in the four livers studied. In both native and activated microsomes the apparent K_m values for each component of morphine glucuronidation varied less than 1.8-fold and V_{max} values varied less than 2.2-fold. The apparent K_m (viz. 7.4 mM) of a recently purified [8] morphine-UDPGT from rat liver is similar in magnitude to the apparent K_m for the low affinity component of morphine glucuronidation in activated human liver microsomes but three orders of magnitude greater than the apparent K_m of the high affinity component of morphine glucuronidation in human liver. Detergent activation decreased the

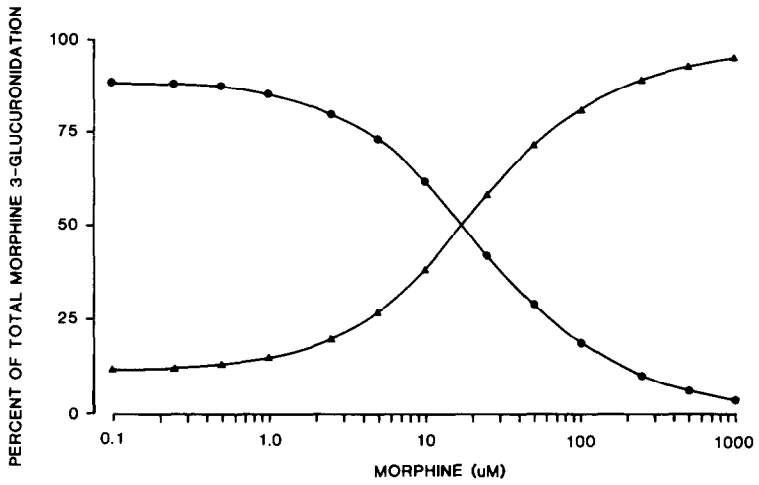

Fig. 3. Plots of the calculated contribution of each of the morphine-UDPGT activities towards total morphine 3-glucuronidation in native human liver microsomes as a function of morphine concentration. Circles and triangles show contribution of the high- and low-affinity morphine-UDPGT activities, respectively.

affinity of both the high-and low-affinity morphine-UDPGT activities of human liver. This effect has also been observed [8] with rat liver microsomal morphine-UDPGT activity. Morphine glucuronidation has previously been reported [23] to follow Michaelis–Menten kinetics in human liver microsomes. However, the lowest substrate concentration used in the earlier study was 750 μM (cf. 2.5 μM in the present study) and relatively insensitive assay conditions were employed; under these conditions the high affinity UDPGT-activity would not be differentiated. The apparent K_m (820–1690 μM) and V_{max} (3.10–7.36 nmol/min/mg) values reported earlier [23] are, however, in good agreement with those determined here for the low affinity morphine-UDPGT activity in activated human liver microsomes.

Recent studies [14, 15] in this laboratory have demonstrated that 4MU glucuronidation follows Michaelis–Menten kinetics in human liver microsomes whereas 1NP glucuronidation kinetics are biphasic. Cross-inhibition studies [14, 15] demonstrated that the 4MU- and high affinity 1NP- activities represented the same UDPGT isozyme, or perhaps a composite of two isozymes with similar kinetic properties [24], and that this isozyme(s) was clearly different to the form(s) associated with the low affinity 1NP-UDPGT activity. 4-Nitrophenol, but not morphine, was apparently a substrate for the 4MU/high affinity 1NP-UDPGT activity. To confirm that the morphine-UDPGT activity described here and the 4MU/high affinity 1NP-UDPGT activity characterised previously represent different enzyme forms 4MU, 1NP and 4-nitrophenol were screened for inhibitory effects on morphine glucuronidation. In addition, since morphine, chloramphenicol and 4-hydroxybiphenyl glucuronidation are all induced by phenobarbitone in the rat [5], the latter two compounds were also screened for possible inhibitory effects on morphine glucuronidation. Chloramphenicol, 4-hydroxybiphenyl, 4MU, 1NP and 4-nitrophenol were all found to inhibit the low affinity morphine-UDPGT activity, but only chloramphenicol and 1NP were competitive inhibitors. Since 4MU (and 4-nitrophenol) was not a competitive inhibitor, as would be expected of an alternative substrate, these data would appear to confirm that the low affinity morphine-UDPGT activity and the previously characterised 4MU/high affinity 1NP-UDPGT activity represent different forms of human liver UDPGT. Similarly, the lack of competitive inhibition by 4-hydroxybiphenyl also suggests that this compound is not a substrate for the low affinity morphine-UDPGT activity. Puig and Tephly reported [8] that 4-hydroxybiphenyl was not a substrate for a purified rat liver morphine-UDPGT. However, since 1NP was a competitive inhibitor of the low affinity morphine UDPGT-activity, it is possible that this component of morphine glucuronidation and the low affinity 1NP-UDPGT activity characterised previously [14, 15] represent the same enzyme form(s). This possibility is supported by the observation that the apparent K_i value for 1NP determined in the present study (i.e. 93 μM) is similar to the apparent K_m for the low affinity component of 1NP glucuronidation (viz. 87.2 ± 19.0 μM) in activated human liver microsomes [14, 15]. Chloramphenicol, which was a relatively weak competitive inhibitor (apparent K_i 980 μM), may also be a substrate for the low affinity morphine UDPGT-activity.

In contrast to the effects of chloramphenicol, 4-hydroxybiphenyl, 4MU, 1NP and 4-nitrophenol on the low affinity morphine-UDPGT activity, each of these compounds (at a concentration of 100 μM) caused <13% inhibition of morphine glucuronidation at a substrate concentration of 1 μM. Under these conditions 85% of morphine glucuronidation is carried out by the high affinity morphine-UDPGT activity and putative inhibitors with apparent K_i values $\leq$475 μM would produce $\geq$15% inhibition [25]. The modest inhibitory effect observed with the

various xenobiotics at the low morphine concentration is most likely due to inhibition of residual low affinity morphine-UDPGT activity (which accounts for 15% of morphine glucuronidation at a substrate concentration of 1 μM). Thus, it would appear that chloramphenicol, 4-hydroxybiphenyl, 4MU, 1NP and 4-nitrophenol do not inhibit the high affinity morphine-UDPGT activity, or at least have considerably lower affinities (i.e. >475 μM) for this enzyme form(s) than morphine itself. While it is not possible to assert unequivocally that the high affinity morphine-UDPGT activity is distinct from the various enzyme forms that conjugate chloramphenicol, 4-hydroxybiphenyl, 4MU, 1NP and 4-nitrophenol, when taken with the previous observation [14, 15] that morphine does not inhibit 4MU glucuronidation, these data strongly suggest that the high affinity morphine-UDPGT activity and the 4MU/high affinity 1NP-UDPGT activity characterised earlier represent different isozymes of human liver UDPGT.

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