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KINETIC AND INHIBITOR STUDIES OF 4-METHYLUMBELLIFERONE AND 1-NAPHTHOL GLUCURONIDATION IN HUMAN LIVER MICROSOMES

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Abstract—The glucuronidation kinetics of 4-methylumbelliferone (4MU) and 1-naphthol (1NP) have been investigated in human liver microsomes to determine the validity of using these compounds as probes for specific UDP-glucuronosyltransferase (GT) activities in human liver. 4MU glucuronidation followed Michaelis–Menten kinetics, whereas 1NP glucuronidation kinetics were biphasic. Cross inhibition studies were performed with 4MU and 1NP to determine the relationship between 4MU glucuronidation and the two phases of 1NP glucuronidation. 4MU glucuronidation was competitively inhibited by 1NP but 4MU inhibited only the high affinity component of 1NP glucuronidation. There was good agreement between the apparent K_m values for 4MU and the high affinity component of 1NP glucuronidation and their respective apparent K_i values determined in the cross inhibition studies. These data suggest that the same form(s) of human liver GT is involved in 4MU glucuronidation and the high affinity component of 1NP glucuronidation.

A number of compounds known to be specific substrates for purified rat liver GTs were screened for inhibitory effects on 4MU glucuronidation in human liver microsomes. 4-Nitrophenol, 2-aminophenol and androsterone inhibited 4MU glucuronidation whereas bilirubin, chloramphenicol, digitoxigenin monodigitoxoside, morphine, oestrone and testosterone had no effect. 4-Nitrophenol and 2-aminophenol were competitive inhibitors of 4MU glucuronidation but the inhibition of 4MU glucuronidation by androsterone followed atypical kinetics. Overall, the substrate specificity of the human liver 4MU/high affinity 1NP-GT activity appears to be broadly similar to that of the 3-methylcholanthrene inducible rat hepatic microsomal GT.

UDP-Glucuronosyltransferase (GT; EC 2.4.1.17) is responsible for the metabolism of a variety of drugs, drug metabolites, environmental chemicals and endogenous compounds, such as bilirubin, bile salts and steroid hormones. Accumulating evidence suggests that glucuronidation reactions in rat liver are catalysed by at least eight independently regulated enzyme forms. This evidence is largely based on the functional heterogeneity of enzyme activities observed in studies of their perinatal development [1], the differential induction of GT activities by microsomal enzyme inducers [2–4], the physical separation of enzyme forms [5–12] and, more recently, by the isolation of cloned cDNAs [13–16]. Heterogeneity of GT has also been established in mouse [17] and rabbit [18] liver.

Despite the advances made in characterising multiple forms of GT in various animal species, comparatively little is known about the multiplicity of this enzyme and the function of individual isozymes in humans. A cDNA clone for a human liver GT has recently been isolated [19] but the substrate specificity of the enzyme encoded by this cDNA has not been determined. While it has been possible to differentiate multiple forms of cytochrome P-450 in human oxidative metabolism by the use of inducers and inhibitors *in vivo*, no selective inducers and inhibitors of glucuronidation reactions in man appear to have been identified to date [20, 21]. Sex-related differences in the *in vivo* glucuronidation of certain drugs have been reported [22, 23] but it is not known whether this reflects the involvement of differentially regulated forms of GT. A limited number of studies have been performed with human liver tissue to determine whether substrates specific for rat liver isozymes may be used to delineate different GT activities in humans. In particular, Bock *et al.* [24] demonstrated that the *in vitro* GT activities towards 1-naphthol (1NP) and 4-methylumbelliferone (4MU), both substrates for the 3-methylcholanthrene-inducible form(s) of GT in rat liver, were highly correlated, whereas activities towards 4MU and substrates for certain other rat liver GT isozymes (e.g. morphine) were not significantly correlated. While this observation suggests that 1NP and 4MU may be useful probes for a particular human liver GT activity, interpretation of the *in vitro* 1NP glucuronidation data is difficult since this reaction exhibits biphasic kinetics in human liver microsomes and homogenates [24–26]. To determine the validity of using 4MU and 1NP as probes for specific GT activities in human liver we have investigated the relationship between 4MU glucuronidation and the two phases of 1NP glucuronidation in human liver microsomes. Moreover, the extent of inhibition of 4MU metabolism by a range of com-

pounds known to be specific substrates for the various rat liver GT isozymes characterised to date has been determined.

MATERIALS AND METHODS

4MU, 4MU-β-D-glucuronide, 1NP, 1NP-β-D-glucuronide, androsterone, bilirubin, chloramphenicol, oestrone, polyoxyethylene 20-cetyl ether (Brij 58), testosterone and UDP-glucuronic acid (sodium salt) were purchased from the Sigma Chemical Co. (St Louis, MO). 4-Nitrophenol was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and morphine hydrochloride from Glaxo Australia (Melbourne, Australia). Digitoxigenin monodigitoxoside was purchased from Atomergic Chemicals (Plainview, NY) and 2-aminophenol from Ajax Chemicals (Sydney, Australia). All other reagents and solvents were of analytical reagent grade.

Human liver samples were obtained from renal transplant donors. The approval of the Flinders Medical Centre Ethical Review Committee was obtained to use such material for drug metabolism studies. Relevant details of the donors of livers F5–F9 have been published elsewhere [27]. The donor of liver F10 was a 62-year-old non-smoking female who died as the result of a subarachnoid haemorrhage. Liver samples were stored at −80° until used. Microsomes were prepared by differential centrifugation as previously described [27]. It has been demonstrated [24] that GT activities and their activation characteristics are preserved during prolonged storage at −80° and it has also been found in this laboratory that GT activities towards 4MU and 1NP are stable in microsomes from human liver samples stored at −80° for at least two years.

1NP glucuronidation activity in human liver microsomes was measured using modifications of the methods of Bock *et al.* [25] and Lilienblum *et al.* [3]. Microsomal incubations typically contained UDP-glucuronic acid (UDPGA: 5 mM), $MgCl_2$ (5 mM), microsomal protein (5 μg), Tris–HCl (0.1 M, pH 7.4) and 1NP (1.5–600 μM) in a total volume of 1 ml. 1NP was dissolved in 5% dimethylsulphoxide (DMSO) and the final concentration of DMSO in the incubation mixture was 0.5% v/v. Incubations were performed for 15 min at 37°. Reaction rates were shown to be linear for incubation times to at least 30 min and for microsomal protein concentrations up to 0.5 mg/ml. The reaction was stopped by the addition of 0.6 M glycine–0.4 M trichloroacetic acid (0.35 ml) and cooling on ice. The incubation mixture was then extracted with 5 ml of chloroform and centrifuged (3000 rpm for 5 min). A 0.4 ml aliquot of the aqueous phase was mixed with 1.6 ml of 0.35 M Na_2HPO_4 and the fluorescence of this solution was measured at 330 nm using an excitation wavelength of 290 nm. Fluorescence was determined using a Perkin-Elmer model 3000 fluorescence spectrometer calibrated with quinine sulphate. A standard curve was prepared using 1NP glucuronide concentrations in the range 0.1–5 μM. The limit of sensitivity of the assay was 0.1 μM (i.e. 0.1 nmol per incubation). A final concentration of 0.00015% w/v Brij 58 in the incubation mixture was shown to result in maximum activation of 1NP glucuronidation activity at a protein concentration of 5 μg/ml. Using native and activated microsomes prepared from a single liver, the within-day coefficient of variation for the assay was determined by measuring 1NP glucuronidation in twelve separate incubations; the coefficients of variation were 3.2% and 1.9% for native and activated microsomes, respectively. In experiments performed to determine the apparent K_m and V_{max} for 1NP glucuronidation the UDPGA concentration was kept constant (5 mM) and activity was measured for eighteen 1NP concentrations over the range 1.5–600 μM. It should be noted that the concentration of UDPGA used (5 mM) in the 1NP and 4MU (see below) kinetic studies was 30- to 140-fold greater than the apparent K_ms for UDPGA (Results section). Similarly, to determine the apparent K_m for UDPGA the concentration of 1NP was kept constant (800 μM) and activity was measured for 16 UDPGA concentrations over the range 10–2000 μM. In experiments investigating the effect of 4MU on 1NP glucuronidation, 1NP kinetic studies (1.5–600 μM) were performed in the absence and presence of three different concentrations (100, 250 and 1000 μM) of 4MU.

The procedure followed to measure 4MU glucuronidation in human liver microsomes was similar to that described above for 1NP, except that incubations contained 4MU (5 μM–800 μM) and 10 μg of microsomal protein. The fluorescence of 4MU glucuronide was determined at 365 nm using an excitation wavelength of 315 nm. Reaction rates were linear for incubation times to at least 30 min and for microsomal protein concentrations to 0.5 mg/ml. Calibration was performed using 4MU glucuronide concentrations in the range 0.2–5 μM. The limit of sensitivity of the assay was 0.2 μM (i.e. 0.2 nmol per incubation). Maximum activation of 4MU glucuronidation occurred in the presence of 0.0003% w/v Brij 58 (for a protein concentration of 10 μg/ml). The within-day coefficients of variation (from 12 replicate measurements) for 4MU glucuronidation in native and activated microsomes were 2.8% and 1.6%, respectively. Experiments to determine the kinetic parameters for 4MU and UDPGA (with 4MU as the fixed substrate) were carried out as described above for 1NP, except that the substrate concentration range in the 4MU kinetic study was 5–800 μM. To characterise the effect of 1NP on 4MU glucuronidation, 4MU kinetic studies were performed in the absence and presence of three concentrations (0.25, 1 and 5 μM) of 1NP. In addition, the extent of inhibition of 4MU glucuronidation by a range of compounds (viz. androsterone, bilirubin, chloramphenicol, digitoxigenin monodigitoxoside, morphine, 4-nitrophenol and oestrone) which are known to be substrates for certain purified forms of rat liver GT was screened in native and activated microsomes from three livers (F5, F6, F7). The concentrations of 4MU and the possible inhibitors used in the screening study are indicated in the text (Table 3). Where inhibition was observed, full kinetic studies were performed to determine apparent K_i values (Fig. 3).

Microsomal protein concentration was measured by the method of Lowry *et al.* [28] using crystalline bovine serum albumin as standard.

All results are expressed as mean ± SD. Apparent K_m, V_{max} and K_i values for 1NP and 4MU were calculated using data from the cross-inhibition studies with these substrates. Apparent K_m and V_{max} values were obtained initially by graphical analysis of Eadie–Hofstee plots. These values were then used as first estimates in an iterative programme based on non-linear least squares regression analysis to calculate values of apparent K_m, V_{max} and apparent K_i to fit the equation:

$$V = \frac{V_{max1} \times S}{S + K_{m1}(1 + I/K_{i1})} + \frac{V_{max2} \times S}{S + K_{m2}(1 + I/K_{i2})} \quad (1)$$

Non-linear regression was carried out on a Prime 9955 computer using the Harwell Subroutine Library procedure VA05A to minimise the residual sum of squares. The method of Dixon [29] was used to determine the apparent K_i values for the inhibition of 4MU glucuronidation by 4-nitrophenol and 2-aminophenol. Differences in kinetic parameters between native and activated microsomes were assessed using Student's paired t-test.

RESULTS

1NP exhibited biphasic glucuronidation kinetics (Fig. 1A) in both native and activated microsomes of all six human livers studied. In contrast, linear Eadie–Hofstee plots were obtained for 4MU glucuronidation (Fig. 2) in native and activated microsomes from these livers. To determine the relationship between 4MU glucuronidation and the two phases of 1NP glucuronidation, cross inhibition studies with these substrates were performed using native and activated microsomes from 4 livers (F5–F8). 4MU selectively inhibited the high affinity component of 1NP glucuronidation in a concentration-dependent manner (Fig. 1), but had no effect on the low affinity component of 1NP glucuronidation (see below). 1NP inhibited 4MU glucuronidation in a competitive manner (Fig. 2).

Data from the cross inhibition studies performed with microsomes from livers F5–F8 and from the kinetic studies performed with microsomes from livers F9 and F10 were fitted to equation (1) to obtain estimates of the Michaelis–Menten parameters for 1NP and 4MU glucuronidation. In addition, apparent K_i values for the inhibition of 1NP glucuronidation by 4MU and for the inhibition of 4MU glucuronidation by 1NP were obtained for livers F5–F8. Based on the residual sum of squares, data for 1NP glucuronidation were best fitted to the Michaelis–Menten expression for a two enzyme system (equation 1). Moreover, residuals from fitting the 1NP data to the two enzyme system were evenly distributed around zero across the whole concentration range whereas residuals from fitting the data to the Michaelis–Menten expression for a single enzyme exhibited a bell-shaped distribution. In contrast, based on both the residual sum of squares and distribution of residuals about zero the data for 4MU were best fitted to the Michaelis–Menten expression for a single enzyme. The closeness of the computer-

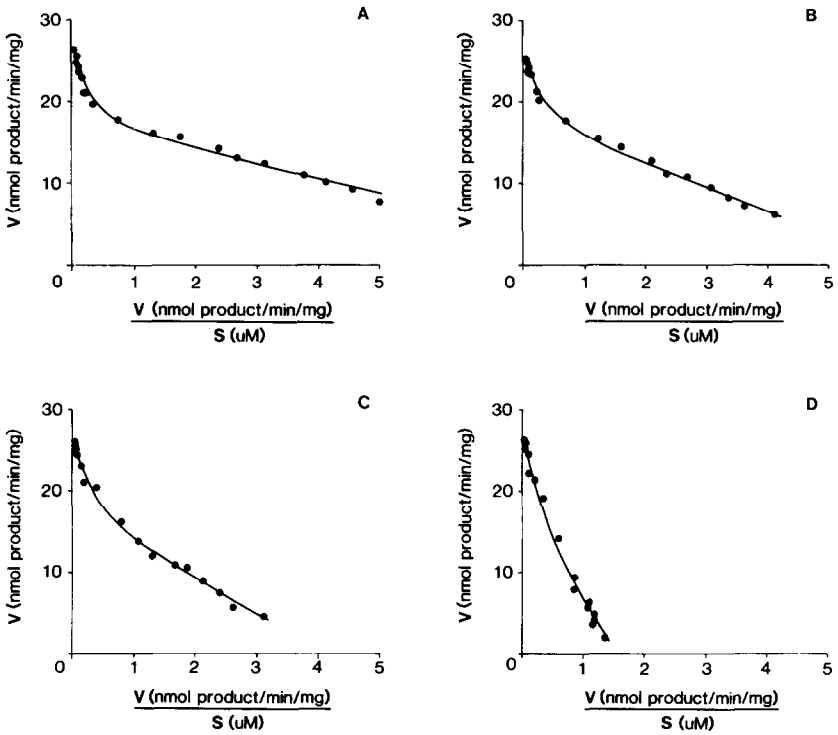

Fig. 1. Representative Eadie–Hofstee plots for 1-naphthol glucuronidation by human liver microsomes (F6, activated) in the absence (panel A) and presence of 100 μM (panel B), 250 μM (panel C) and 1000 μM (panel D) 4-methylumbelliferone. Points are experimentally determined values while solid lines are the computer-generated curves of best-fit.

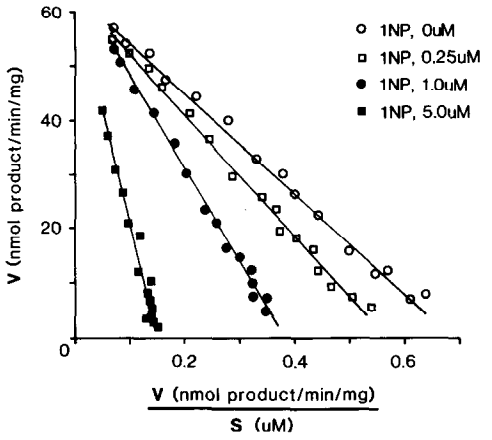

Fig. 2. Representative Eadie–Hofstee plots for 4-methylumbelliferone glucuronidation by human liver microsomes (F6, activated) in the absence and presence of 0.25 μM, 1.0 μM and 5.0 μM 1-naphthol. Points are experimentally determined values while solid lines are the computer-generated curves of best fit.

generated best fit curves to the experimental data for 1NP and 4MU is shown for a representative liver (F6, activated) in Figs 1 and 2. Values for the computer derived Michaelis–Menten parameters for 1NP and 4MU glucuronidation are summarised in Table 1. For native microsomes the apparent K_m for the high affinity component of 1NP glucuronidation was 0.7 ± 0.3 μM while that for the low affinity component was 126.9 ± 21.5 μM. Detergent activation increased ($P < 0.05$) the apparent K_m of the high affinity component to 1.8 ± 0.1 μM but decreased ($P < 0.05$) the apparent K_m for the low affinity component to 87.2 ± 19.0 μM. The apparent K_m for 4MU glucuronidation was increased ($P < 0.05$) from 36.0 ± 9.6 μM in native microsomes to 91.8 ± 7.5 μM in activated microsomes. Activation increased the V_{max} values for both 1NP and 4MU glucuronidation by 3- to 4-fold.

Apparent K_i values obtained in the cross inhibition studies with 1NP and 4MU are summarised in Table 2. The apparent K_i values for inhibition of the high affinity component of 1NP glucuronidation by 4MU were 46.3 ± 14.3 μM and 159.2 ± 16.0 μM in native and activated microsomes, respectively. The computer estimated apparent K_i value for 4MU inhibition of the low affinity component of 1NP glucuronidation was >1M for all four livers, indicating that there is no significant interaction of 4MU with this component of 1NP glucuronidation. The apparent K_i values for the inhibition of 4MU glucuronidation by 1NP were 0.5 ± 0.2 μM and 1.4 ± 0.2 μM for native and activated microsomes, respectively.

Table 2. Computer derived apparent K_i values for cross inhibition of 1-naphthol and 4-methylumbelliferone glucuronidation in native and activated human liver microsomes

Substrate, inhibitor/ microsomal treatment	K_i (μM)
Inhibition of 4MU by 1NP	
native	0.5 ± 0.2
activated	1.4 ± 0.2
Inhibition of 1NP (high affinity component) by 4MU	
native	46.3 ± 14.3
activated	159.2 ± 16.0

Values are mean ± SD in microsomes from four livers (F5–F8).

Apparent K_m values for UDPGA were determined in microsomes from three livers (F5–F7). Using a fixed concentration of 1NP (800 μM), biphasic Eadie–Hofstee plots were observed. For native microsomes the estimated apparent K_m (UDPGA) values were 40.3 ± 8.7 μM and 143.5 ± 16.8 μM, while for activated microsomes the two apparent K_m (UDPGA) values were 65.5 ± 9.0 μM and 156.9 ± 16.3 μM. Using a fixed concentration of 4MU (800 μM), linear kinetics were observed for UDPGA. Apparent K_m (UDPGA) values were 36.0 ± 3.7 μM and 59.6 ± 6.1 μM for native and activated microsomes, respectively.

The effects of added 2-aminophenol, androsterone, biliribin, chloramphenicol, digitoxigenin monodigitoxoside, morphine, 4-nitrophenol, oes-

Table 1. Computer derived Michaelis–Menten parameters for 1-naphthol and 4-methylumbelliferone glucuronidation by native and activated human liver microsomes

Substrate/ microsomal treatment	K_{m1} (μM)	V_{m1} (nmol/min/mg)	K_{m2} (μM)	V_{m2} (nmol/min/mg)
1NP:				
native	0.7 ± 0.3	3.3 ± 1.8	126.9 ± 21.5	3.4 ± 1.6
activated	1.8 ± 0.1	12.8 ± 4.5	87.2 ± 19.0	10.2 ± 4.9
4MU:				
native	36.0 ± 9.5	14.9 ± 9.1	—	—
activated	91.8 ± 7.5	59.9 ± 24.4	—	—

Values are mean ± SD for microsomes from 6 livers (F5–F10).

K_{m1}, V_{m1}, K_{m2} and V_{m2} are apparent K_m and V_{max} values determined from fitting experimental data to equation 1 (Materials and Methods).

Table 3. Effects of various compounds on 4-methylumbelliferone glucuronidation in native and activated human liver microsomes

Compound	Percent control activity	
	Native	Activated
4-Nitrophenol	50 ± 6	60 ± 5
2-Aminophenol	55 ± 4	63 ± 3
Chloramphenicol	96 ± 5	101 ± 4
Morphine	97 ± 4	98 ± 5
Bilirubin	162 ± 11	101 ± 5
Digitoxigenin monodigitoxoside	92 ± 6	93 ± 5
Oestrone	98 ± 4	94 ± 4
Androsterone	73 ± 6	79 ± 5
Testosterone	92 ± 2	96 ± 4

Values mean ± SD from 3 livers (F5–F7). The concentration of 4MU was 50 μM and that of inhibitor was 200 μM, except for experiments with oestrone, androsterone and testosterone where the concentration of 4MU was 10 μM and that of inhibitor was 40 μM.

trone and testosterone on 4MU glucuronidation in native and activated microsomes from three livers (F5–F7) are summarised in Table 3. Only three compounds, 4-nitrophenol, 2-aminophenol and androsterone, caused >10% inhibition of 4MU glucuronidation and this effect was apparent in both native and activated microsomes. Kinetic studies performed in native and activated microsomes from livers F5–F7 demonstrated that inhibition of 4MU glucuronidation by 4-nitrophenol and 2-aminophenol was competitive (Fig. 3). Mean apparent K_i values in native and activated microsomes

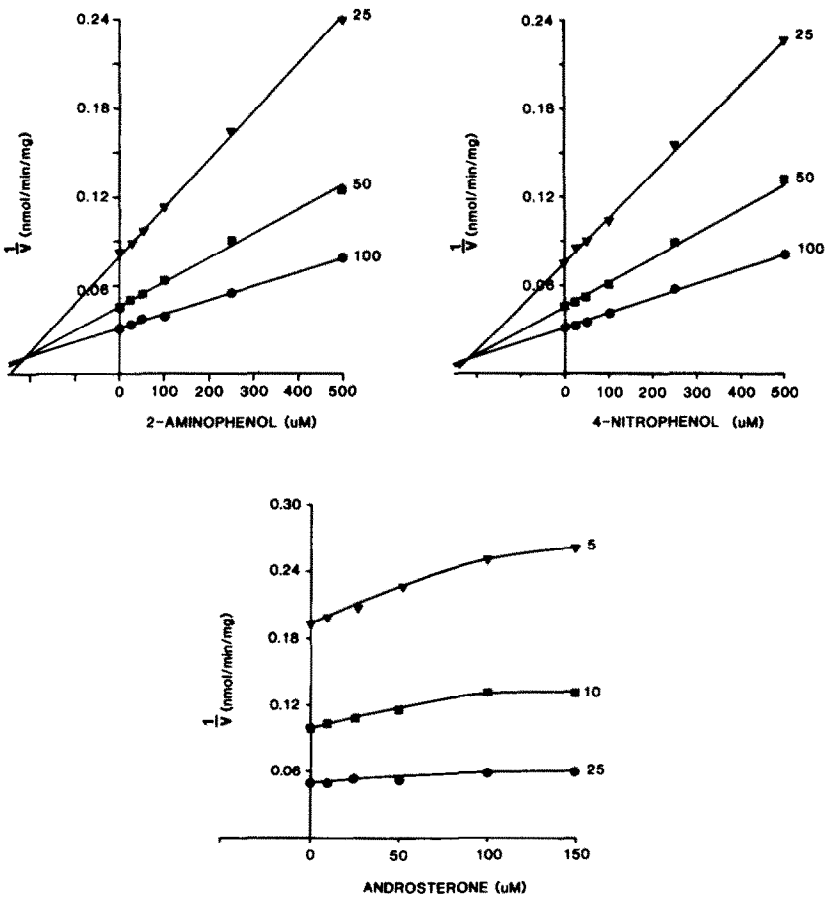

Fig. 3. Representative Dixon plots for the inhibition of 4-methylumbelliferone glucuronidation in human liver microsomes (F6, activated) by 4-nitrophenol, 2-aminophenol and androsterone.

respectively, were; 82 ± 7 μM and 183 ± 31 μM for 4-nitrophenol and 95 ± 10 μM and 191 ± 16 μM for 2-aminophenol. The inhibition of 4MU glucuronidation by androsterone followed atypical kinetics (Fig. 3).

DISCUSSION

In both native and detergent activated microsomes 4MU glucuronidation followed Michaelis–Menten kinetics whereas 1NP glucuronidation kinetics were biphasic. 4MU glucuronidation was competitively inhibited by 1NP while 4MU inhibited only the high affinity component of 1NP glucuronidation. Moreover, there was good agreement between estimates for the apparent K_ms for 4MU and the high affinity component of 1NP glucuronidation and their respective apparent K_i values determined in the cross-inhibition studies, strongly suggesting that the 4MU-GT activity and the high affinity 1NP-GT activity are the same enzyme forms. The kinetic studies with UDPGA support the above conclusions. Linear UDPGA kinetics were observed using 4MU as the fixed substrate while UDPGA kinetics were biphasic when 1NP was the fixed substrate. The apparent K_m for UDPGA determined in the presence of 4MU was similar to the high affinity apparent K_m for UDPGA determined in the presence of 1NP. The 4MU/high affinity 1NP-GT activity and low affinity 1NP-GT activity may be further distinguished by the effect of detergent. Apparent K_m values for 4MU and the high affinity component of 1NP glucuronidation were both increased 2.5-fold on activation whereas activation decreased the apparent K_m for the low affinity component of 1NP glucuronidation by 31%.

Although 4MU kinetics and the relationship between 4MU glucuronidation and the two phases of 1NP glucuronidation in human liver microsomes have not previously been defined, biphasic kinetics for 1NP have previously been reported [25, 26]. Previous estimates [25, 26] of the apparent K_m values for the high and low affinity components of 1NP glucuronidation in activated microsomes have been 30–50 μM and 80–470 μM, respectively. The apparent K_m for the low affinity component of 1NP glucuronidation determined in this study (87.2 ± 19.0 μM) is in agreement with that reported by Bock *et al.* [25] (i.e. 80 μM) but the apparent K_m for the high affinity 1NP transferase determined here is an order of magnitude lower than previous estimates (1.8 ± 0.1 μM vs 30–50 μM). The reason for this discrepancy is not immediately clear, although it should be noted that previous estimates of the apparent K_m values for 1NP were based on relatively few data points and the lowest substrate concentrations used were 20 μM and 32 μM.

After submission of this paper Irshaid and Tephly [30] reported the purification of two GT isozymes from human liver microsomes. Both enzymes had similar K_m values for 4MU (viz. 170 μM and 230 μM); these are approximately 5-fold and 2-fold higher than the K_ms determined here for 4MU in native and activated microsomes, respectively. The apparent K_m values of the purified GTs for 4MU are so similar that the two isozymes would not have been differentiated by us in the kinetic studies. It is likely, but not certain, that the 4MU/high affinity 1NP-GT activity characterised in the present study represents a composite of the two isozymes purified by Irshaid and Tephly [30]. The low affinity 1NP-GT activity observed in the present work indicates the existence of at least one additional form of human liver GT which glucuronidates 1NP.

A number of compounds have been shown to be specific substrates for purified rat liver GT isozymes. These include androsterone [8, 11], bilirubin [6, 10], digitoxigenin monodigitoxoside [9], morphine and chloramphenicol [5, 12], oestrone [7, 11] and testosterone [8, 11]. 4MU, 1NP and 4-nitrophenol are all substrates for a 3-methylcholanthrene inducible form of rat liver GT [5, 8, 11], although 1NP and 4-nitrophenol are also metabolised by the 17-hydroxysteroid transferase [8, 11] and perhaps other rat liver GT isozymes [10]. In the present study bilirubin, chloramphenicol, digitoxigenin monodigitoxoside, morphine, oestrone and testosterone were found not to inhibit 4MU glucuronidation, indicating that these substrates are metabolised by forms of human liver GT other than those involved in 4MU glucuronidation. Interestingly, bilirubin enhanced 4MU glucuronidation in native microsomes but was without effect in activated microsomes. A similar effect of bilirubin on morphine glucuronidation in rat liver microsomes has previously been reported [31]. 4MU glucuronidation was competitively inhibited by 1NP and 4-nitrophenol. 2-Aminophenol, another widely used probe for GT activity, also competitively inhibited 4MU glucuronidation. If the 4MU-GT activity measured in the present study represents a composite of the two isozymes purified by Irshaid and Tephly [30], then linear Dixon plots for the inhibition of 4MU glucuronidation by 1NP, 4-nitrophenol and 2-aminophenol suggests similar affinity of each individual compound for the two isozymes. Indeed, Irshaid and Tephly [30] found that the apparent K_m values of 4-nitrophenol for the two isozymes differed only by 20%. In contrast to the xenobiotic inhibitors, androsterone inhibited microsomal 4MU glucuronidation with atypical kinetics. Androsterone was not a substrate for two purified 4MU glucuronidating isozymes from human liver [30]. The atypical inhibition kinetics observed here may indicate an allosteric mechanism for the inhibition of 4MU metabolism by androsterone, although other explanations cannot be excluded.

The substrate specificity of the human liver microsomal 4MU-GT activity seems to be broadly comparable to that of the 3-methylcholanthrene inducible rat hepatic GT [5, 8, 11]. Both the human and rat enzyme(s) appear to conjugate 4MU, 1NP and 4-nitrophenol, but not bilirubin, chloramphenicol, digitoxigenin monodigitoxoside, morphine, oestrone and testosterone and, probably, androsterone. Irshaid and Tephly [30] noted that the purified human liver GT isozymes which metabolised 4MU differed from the 3-methylcholanthrene inducible rat liver GT in terms of pI values and the ability to glucuronidate estriol and 4-aminobiphenyl. However, identification of orthologous forms of GT in rat and human liver requires identification of amino acid sequences and further comparison of substrate specificities.

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