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Determination of UDP-glucuronosyltransferase UGT1A6 activity in human and rat liver microsomes by HPLC with UV detection

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Abstract

A simple and sensitive method for the determination of UDP-glucuronosyltransferase UGT1A6 activity using 4-methylumbelliferone (4-MU) and 4-nitrophenol (4-NP) as substrates in human and rat liver microsomes by high-performance liquid chromatography (HPLC) with uv detection is reported. The method was validated for the determination of 4-methylumbelliferyl β-D-glucuronide (4-MUG) and 4-nitrophenyl β-D-glucuronide (4-NPG) with respect to specificity, linearity, detection limit, recovery, stability, precision and accuracy. There was no interference from matrix and non-enzymatic reactions. Calibration curves for 4-MUG and 4-NPG are linear from 0.5 to 500 μM. Average recoveries ranged from 98 to 100% in spiked liver microsomes samples. 4-MUG and 4-NPG were stable at 4°C for at least 72 h in spiked liver microsomes samples. The method was found to be more sensitive than previous methods using a spectrophotometer, a spectrofluorometer and HPLC. The detection limit for 4-MUG and 4-NPG (signal-to-noise ratio of 3) was 14 and 23 nM, respectively. The intra- and inter-day precision (relative S.D. (RSD)) and accuracy (relative mean error (RME)) was <5 and 9%, respectively. The intra- and inter-day reproducibility (RSD) of UGT1A6 enzyme assay in liver microsomes was <6%. With this improved sensitivity, the kinetics of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes could be determined more precisely. In addition, the method could determine the non-inducible, and 3-methylcholanthrene- and phenobarbital-inducible activities of UGT1A6 in rat liver microsomes under the same assay conditions. Therefore, this method is applicable to in vivo and in vitro studies on the interaction of xenobiotic chemicals with UGT1A6 isoform in mammals using small amounts of biological samples. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: UDP-Glucuronosyltransferase; 4-Methylumbelliferone; 4-Methylumbelliferyl β-D-glucuronide; 4-Nitrophenol; 4-Nitrophenyl β-D-glucuronide; Liver microsomes

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1. Introduction

In mammals, glucuronidation is a major conjugation reaction providing for metabolic elimination of exogenous compounds (e.g. drugs, environmental chemicals and dietary constituents) and endogenous compounds (e.g. bile acid, bilirubin and steroids). This reaction is catalyzed by a family of enzymes known as UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) [1–4]. UGTs are mainly located in the endoplasmic reticulum of liver and the activities are only detected after perturbation with detergent, phospholipase or sonication [1]. To date, ≈ 40 UGT isoforms have been identified from various mammalian species by protein purification or cDNA cloning and classified into two families of protein, termed UGT1 and UGT2 on the basis of amino acid sequence similarities and gene structure [3,5]. In humans and rats, UGT1 family isoforms are encoded by one gene which has multiple unique exons located upstream of four common exons. By contrast, UGT2 family isoforms are encoded by a separate gene comprised of six exons [3,6–8].

The reports of Ritter et al. and Emi et al. on the *UGT1A* gene in humans and rats showed that exon 1 consists of bilirubin-like and phenol-like exons. The main expressed isoforms in liver are UGT1A1 and UGT1A6, respectively [6,7]. In rats, the expression level of UGT1A1 mRNA is increased by dexamethasone and clofibrate, but not by 3-methylcholanthrene and phenobarbital, whereas the expression level of UGT1A6 mRNA is increased by 3-methylcholanthrene [7]. The UGT1A6 enzyme is also co-induced with cytochrome P450 1A isoforms (CYP1A1 and CYP1A2) via arylhydrocarbon receptor protein [7,9,10]. In addition to the liver, UGT1A6 is also expressed in extrahepatic tissues such as kidney, epididymis, testis, lung, small intestine and stomach in humans and rats; however, the enzyme activities are lower than that in liver or undetectable [7,11–13]. Therefore, the determination of UGT1A6 activity in liver is an integral part of toxicological research for xenobiotic chemicals.

Planar phenol derivatives, such as 4-methylumbelliferone (4-MU) and 4-nitrophenol (4-NP), have been reported to be mainly conjugated by UGT1A6 isoform in mammals, although the contribution ratio differed extensively among the animal species [3,4]. Most laboratories use an assay of UGT activities toward 4-MU and 4-NP that is adapted from a method based on the disappearance of substrate [14,15]. However, the background originating from incubation factors, such as enzyme source and UDP-glucuronic acid (UDPGA) as donor, interferes with the determination of UGT activities. On the other hand, Lilienblum et al. have reported a spectrofluorimetric method to determine glucuronide metabolite for assay of UGT activity toward 4-MU [16], but it requires an extraction step to remove unconjugated substrate. Since these methods also suffer from low sensitivity, they do not allow for the determination of low activity samples. More recently, methods to determine 4-methylumbelliferyl β-D-glucuronide (4-MUG) and 4-nitrophenyl β-D-glucuronide (4-NPG) in rat liver perfusates or cells (in situ system) using high-performance liquid chromatography (HPLC) have been developed, however, the sensitivity of these methods is still low [17–19]. Thus, a highly sensitive HPLC method for the assay of UGT activities toward 4-MU and 4-NP in mammalian tissues (in vitro system) has not been developed.

The present paper describes a simple and sensitive method for the determination of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes by HPLC with uv detection. This method was validated with respect to specificity, linearity, detection limit, recovery, stability, precision and accuracy.

2. Experimental

2.1. Chemicals and materials

4-MU sodium salt (99.0% pure) was obtained from Sigma (St. Louis, MO). 4-NP (98.5% pure), 4-MUG (98.8% pure), 4-NPG (99.2%), UDPGA (trisodium salt) and Brij 58 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Pooled liver microsomes from ten human donors (16–73-years-old) were purchased from Gentest (Woburn, MA). Pooled liver microsomes from

eight male Sprague–Dawley rats (8-weeks-old) were prepared, as previously described [20]. Administration and liver microsomal preparations for the induction study using rats were performed as previously reported [20,21]. The microsomal protein content was determined according to Lowry et al. [22] using bovine serum albumin as standard. The microsomal samples were stored at −80°C until use. All other materials were of the highest quality commercially available.

2.2. Standards

Stock solutions at a concentration of 50 mM in methanol were prepared separately for 4-MUG and 4-NPG. Stock solutions (0.025–25 mM) for calibration curves and quality control samples were prepared by serial dilutions of each 50 mM stock solution with methanol. These solutions were stored at −20°C protected from light and were stable for at least 4 weeks. Working solutions were freshly prepared each day and were obtained by dilution from the stock solutions with 50 mM Tris–HCl buffer (pH 7.4)–15% (w/v) perchloric acid (90:10, v/v) or the solution containing liver microsomal protein (10 μg).

2.3. Incubations

UGT activities toward 4-MU and 4-NP were determined by quantification of the 4-MUG and 4-NPG production from glucuronidation by liver microsomes, respectively. Incubations were performed in 5-ml glass test tubes. The standard incubation mixture contained 4-MU (10–3000 μM) or 4-NP (10–3000 μM) each spiked separately as substrate, liver microsomal proteins from humans and rats (0–200 μg), 10 mM $MgCl_2$, Brij 58 (1 mg/mg microsomal protein) and 3 mM UDPGA in a final volume of 400 μl of 50 mM Tris–HCl buffer (pH 7.4). After preincubation at 37°C for 1 min, the reaction was started by the addition of UDPGA. The mixture was incubated at 37°C for 0–120 min and the reaction terminated with 50 μl of ice-cold 15% (w/v) perchloric acid and 50 μl of 1% (w/v) bovine serum albumin with vortexing. Deproteined samples were placed on ice for 30 min. The contents of the tubes were transferred to 1.5-ml polypropylene test tubes and centrifuged at 12 000 × g for 10 min at 4°C. The supernatant was filtered with a PTFE membrane filter of 0.45 μm pore size (Millipore, Bedford, MA) and analyzed by HPLC within 8 h. Blank samples contained all components except the UDPGA which was added after termination of the reaction.

2.4. Apparatus and HPLC conditions

HPLC analysis was performed using a Shimadzu SCL-10A system controller (Kyoto, Japan) consisting of two LC-10AD pumps, an SIL-10A auto injector with sample cooler, an SPD-10AV UV-Vis detector, a CTO-10A column oven, a DGU-3A degasser and a C-R4A chromatopac integrator. The samples were cooled at 4°C and 20-μl aliquots were injected into an Inertsil ODS-80A column (5 μm, 150 × 4.6 mm i.d., GL Sciences, Tokyo, Japan). The column was kept at 40°C. The product was eluted isocratically with water containing 25 mM triethylamine–acetonitrile (80:20, v/v) at a flow rate of 1.0 ml/min. The pH of the aqueous portion was adjusted to 2.1 with 60% perchloric acid. The UV wavelengths were fixed at 318 and 305 nm for 4-MUG and 4-NPG, respectively. The calibration curves for 4-MUG and 4-NPG were prepared from stock standard solutions with concentrations of 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100, 200 and 500 μM, as described above.

2.5. Kinetic analysis

Incubation conditions were chosen such that product formation was linear with respect to both microsomal protein amount and incubation time for the determination of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes. The microsomal protein amounts and incubation time for the determination of both UGT activities were 10 μg and 15 min, respectively. Substrate concentrations for the determination of both UGT activities were 10–3000 μM. The Michaelis–Menten parameters,

such as K_m and V_{max}, were estimated by analyzing Eadie–Hofstee plots using the software Enzyme-Kinetics v.1.4 (Trinity Software, Campton, NH).

2.6. Induction study

UGT activities toward 4-MU and 4-NP in control, 3-methycholanthrene- and phenobarbital-pretreated liver microsomes from rats were measured separately, as described above. The reactions were performed with a substrate concentration of 500 μM for 4-MU or 1000 μM for 4-NP and liver microsomal protein amounts of 10 μg at 37°C for 15 min.

3. Results and discussion

3.1. Specificity

Liver microsomes from humans and rats were incubated with 4-MU or 4-NP in the presence of UDPGA and the amount of 4-MUG or 4-NPG formed was determined by HPLC (Figs. 1 and 2). 4-MUG and 4-NPG were detected just after the peak of UDPGA with retention times of 3.0 and 3.5 min, respectively. Unconjugated 4-MU and 4-NP were eluted in 10.4 and 14.1 min, respectively. The specificity of the method was assessed to evaluate the influence of the matrix from liver microsomes and non-enzymatic reactions. The in-

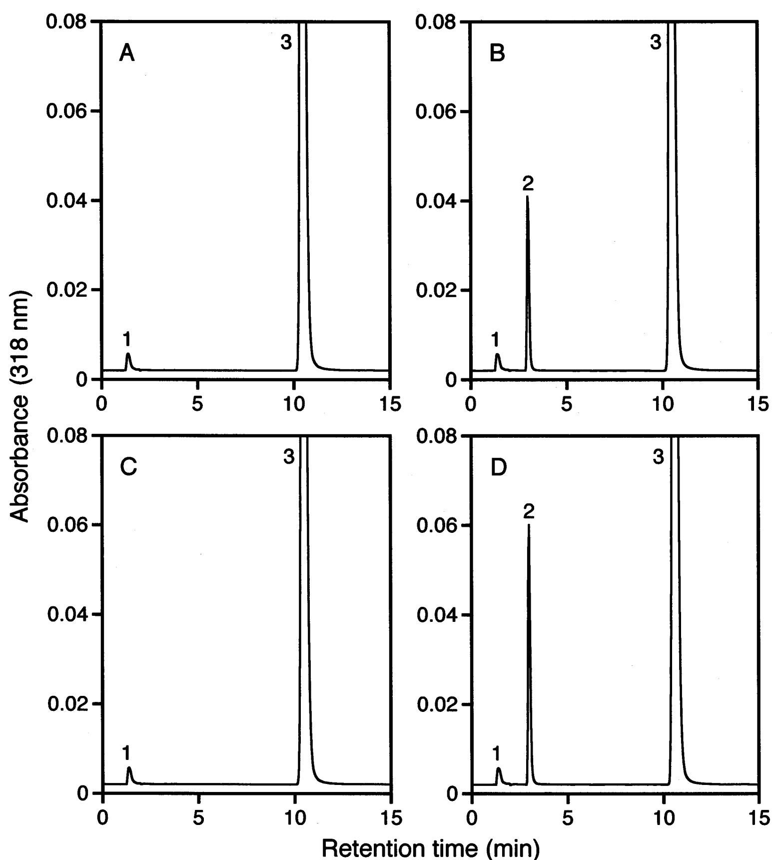

Fig. 1. HPLC analysis of UGT activity toward 4-MU in human and rat liver microsomes. Reactions were performed in the presence of 4-MU (500 μM) and liver microsomal proteins (10 μg) in a total volume of 400 μl for 15 min at 37°C. The method of sample preparation and the HPLC conditions are described in Section 2. Panels are: (A) blank sample from human liver microsomes; (B) incubation sample from human liver microsomes; (C) blank sample from rat liver microsomes; and (D) incubation sample from rat liver microsomes. Peaks are: (1) UDPGA; (2), 4-MUG; and (3) 4-MU.

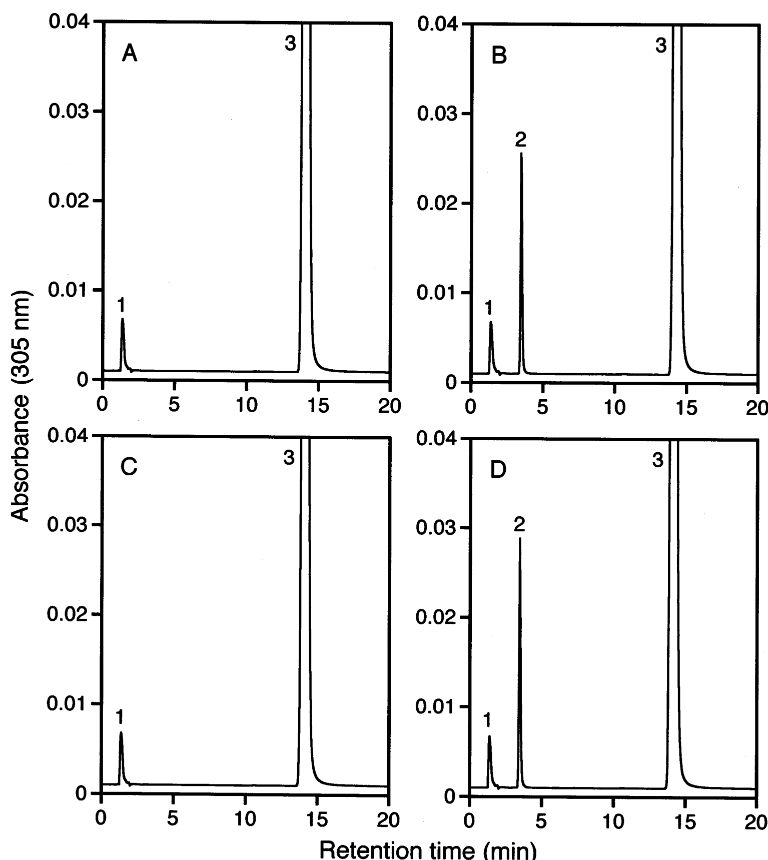

Fig. 2. HPLC analysis of UGT activity toward 4-NP in human and rat liver microsomes. Reactions were performed in the presence of 4-NP (1000 μM) and liver microsomal proteins (10 μg) in a total volume of 400 μl for 15 min at 37°C. The method of sample preparation and the HPLC conditions are described in Section 2. Panels are: (A) blank sample from human liver microsomes; (B) incubation sample from human liver microsomes; (C) blank sample from rat liver microsomes; and (D) incubation sample from rat liver microsomes. Peaks are: (1) UDPGA; (2) 4-NPG; and (3) 4-NP.

terfering peaks for the determination of 4-MUG and 4-NPG were not detected in blank samples from human and rat liver microsomes (Figs. 1 and 2).

3.2. Calibration curves and detection limit

Calibration curves ($y = ax + b$) were prepared from solution to which 4-MUG and 4-NPG had been added in the range of 0.5–500 μM. A high correlation was found between the amount of 4-MUG and 4-NPG (x: μM), and the peak area (y: peak area/1000). The means of their calibration curves yielded the following equations ($n = 5$): $y = 4.954x + 0.733$ ($r^2 = 0.999$) for 4-MUG and $y = 3.216x + 0.316$ ($r^2 = 1.000$) for 4-NPG. The detection limits (signal-to-noise ratio of 3) were assessed using the 0.05 μM standard solutions, the detection limits of 0.014 μM for 4-MUG and 0.023 μM for 4-NPG were back-calculated, respectively. These values were lower than previous results obtained using the spectrofluorimetric or HPLC method (0.1–0.2 μM for 4-MUG and 1.0–1.5 μM for 4-NPG, respectively) [16–19].

3.3. Recovery

Recoveries from human and rat liver microsomes spiked with 4-MUG and 4-NPG at concen-

trations of 0.5, 20 and 500 μM were studied ($n = 5$). The recoveries of 4-MUG and 4-NPG were 98.5–99.8 and 97.9–99.7%, respectively. The recoveries for both 4-MUG and 4-NPG are not only high, but also similar at all concentrations studied. When the enzyme reactions were stopped with 50 μl of 20% (w/v) zinc sulfate in a preliminary study, the recoveries of 4-MUG and 4-NPG from liver microsomes were 72–79 and 81–84%, respectively. Although trichloroacetic acid is used extensively to stop enzyme reactions for UGT assay [14,15,23], 50 μl of 20% (w/v) trichloroacetic acid interfered with the peaks of 4-MUG and 4-NPG under these HPLC conditions.

3.4. Stability

To check the stability of the biological matrix, liver microsomes from humans and rats were spiked with 4-MUG and 4-NPG separately at three concentrations (0.5, 20 and 500 μM). The stability was assessed after storage in a refrigerator (4°C) and at room temperature (25°C) for various intervals (8, 24 and 72 h), by comparing peak areas from initial and subsequent determinations. 4-MUG and 4-NPG were stable for 72 h at 4°C in both human and rat liver microsomes (97.5–99.9%). The percentage recoveries were similar at all concentrations. 4-MUG and 4-NPG were also stable for 24 h at 25°C in both liver microsomes (97.0–99.7%). After storage for 72 h at 25°C, 4-NPG was slightly degraded 5.4–7.0% in liver microsomes, although 4-MUG was stable.

3.5. Precision and accuracy

The quality control samples were prepared by spiking liver microsomes with 4-MUG and 4-NPG at three different concentrations (0.5, 20 and 500 μM), and were analyzed with the calibration standards (0.5–500 μM). Intra-day precision and accuracy were determined by analyzing five times in triplicate on the same day. Inter-day precision and accuracy were determined by analyzing once in triplicate daily for 5 days. Precision was expressed as relative S.D. (RSD). Accuracy was calculated as relative mean error (RME) of back-calculated concentrations from nominal concentrations. The results are shown in Table 1. Intra- and inter-day precision was $< 5\%$ for all concentrations of 4-MUG and 4-NPG in both human and rat liver microsomes. Accuracies ranged from -6.4 to 7.9% for 4-MUG and -8.2 to 6.1% for 4-NPG.

3.6. Linearity of glucuronide formation

To determine the optimal reaction conditions for the assay of UGT activities toward 4-MU and 4-NP in liver microsomes, the dependence of the enzyme activities on incubation time and microsomal protein amount was studied. For these studies, substrate concentrations of 500 μM for 4-MU and 1000 μM for 4-NP were used. Fig. 3 shows time-dependent formation of 4-MUG and 4-NPG by human and rat liver microsomes (10 μg of microsomal protein). The 4-MUG formation was linear for at least 20 min in both liver microsomes ($r^2 > 0.999$). On the other hand, the 4-NPG formation was linear for at least 40 min ($r^2 > 0.995$) in both liver microsomes. Similarly, in both assays of UGT activities toward 4-MU and 4-NP in liver microsomes, the formations of 4-MUG and 4-NPG were found to be linear up to 100 and 200 μg microsomal protein, respectively, in both liver microsomes ($r^2 > 0.995$) (Fig. 4). The sensitivities of detection are 7.1 pmol of 4-MUG and 12 pmol of 4-NPG/incubation and allow UGT activities toward 4-MU and 4-NP as low as 3.6 and 1.5 pmol/min per miligram protein to be determined, respectively.

3.7. Reproducibility

The reproducibility of the assay was determined by analysis of UGT activities toward 4-MU and 4-NP of microsomal proteins from human and rat liver samples. Three samples were analyzed five times for the intra-day reproducibility and once in triplicate daily for 5 days for the inter-day reproducibility. The results are summarized in Table 2. Intra- and inter-day reproducibilities (expressed as RSD) were $< 6\%$ for both enzyme activities.

Table 1
Intra- and inter-day precision and accuracy of the determination for 4-MUG and 4-NPG in human and rat liver microsomes[a]

Compound	Nominal concentration (μM)	Human (n = 5)			Rat (n = 5)		
		Mean calculated concentration (μM)	RSD (%)	RME (%)	Mean calculated concentration (μM)	RSD (%)	RME (%)
Intra-day							
4-MUG	0.50	0.474	3.8	−5.2	0.486	2.7	−2.8
	20.0	21.45	0.6	7.3	21.23	1.5	6.2
	500	504.5	1.1	0.9	492.1	1.7	−1.6
4-NPG	0.50	0.459	4.6	−8.2	0.477	3.4	−4.6
	20.0	21.21	0.8	6.1	20.78	2.1	3.9
	500	495.3	1.3	−0.9	497.1	0.5	−0.6
Inter-day							
4-MUG	0.50	0.468	3.4	−6.4	0.476	4.2	−4.8
	20.0	21.58	1.5	7.9	21.46	1.8	7.3
	500	494.9	2.7	−1.0	499.3	2.0	−0.1
4-NPG	0.50	0.463	1.3	−7.4	0.469	3.0	−6.2
	20.0	21.18	0.6	5.9	21.05	2.0	5.3
	500	498.7	0.8	−0.3	501.1	1.1	0.2

[a] 4-MUG or 4-NPG was added to 50 mM Tris–HCl buffer (pH 7.4) containing liver microsomal protein (10 μg) in a total volume of 400 μl. The method of sample preparation and the HPLC conditions are described in Section 2.

3.8. Kinetic analysis

To compare the enzymology of UGT toward 4-MU and 4-NP in liver microsomes from human and rat, Eadie–Hofstee plots were constructed. The curves and Michaelis–Menten parameters for the enzymes are shown in Fig. 5 and Table 3, respectively. The curves were all linear ($r^2 =$ 0.984–0.996). Both of the UGT activities toward 4-MU and 4-NP in human and rat liver micosomes indicated a monophasic kinetic pattern. The V_{max} for UGT activity toward 4-MU in rat liver microsomes was slightly higher than that of human liver microsomes, although the K_m was similar. No notable difference was found in K_m and V_{max} for UGT activity toward 4-NP between human and rat liver microsomes. Thus, the kinetics patterns for UGT activities toward 4-MU and

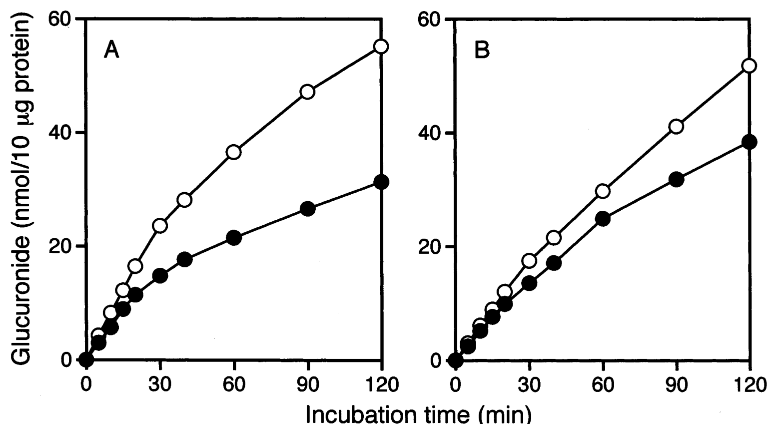

Fig. 3. Dependence on incubation time of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes. Reactions were performed in the presence of 4-MU (500 μM) or 4-NP (1000 μM) and liver microsomal proteins (10 μg) in a total volume of 400 μl for 0–120 min at 37°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Section 2. Panels are: (A) UGT activity toward 4-MU; and (B) UGT activity toward 4-NP. Symbols are: (●) human; and (O) rat. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

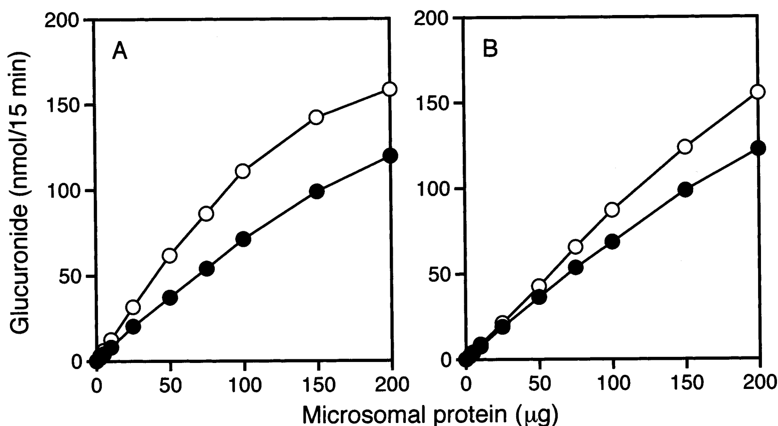

Fig. 4. Dependence on protein amount of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes. Reactions were performed in the presence of 4-MU (500 μM) or 4-NP (1000 μM) and liver microsomal proteins (0–200 μg) in a total volume of 400 μl for 15 min at 37°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Section 2. Panels are: (A) UGT activity toward 4-MU; and (B) UGT activity toward 4-NP. Symbols are: (●) human; and (O) rat. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

Table 2
Intra- and inter-day reproducibility of the assay for UGT activities toward 4-MU and 4-NP in human and rat liver microsomes[a]

Substrate	Human ($n=5$)		Rat ($n=5$)	
	Activity (mean ± S.D.) (nmol/min per miligram protein)	RSD (%)	Activity (mean ± S.D.) (nmol/min per miligram protein)	RSD (%)
Intra-day				
4-MU	54.4 ± 1.0	1.8	83.4 ± 2.2	2.6
4-NP	50.9 ± 2.7	5.3	56.4 ± 2.1	3.7
Inter-day				
4-MU	55.5 ± 2.5	4.5	82.8 ± 3.3	4.0
4-NP	51.4 ± 1.7	3.3	57.3 ± 3.4	5.9

[a] Reactions were performed in the presence of 4-MU (500 μM) or 4-NP (1000 μM) and liver microsomal protein (10 μg) in a total volume of 400 μl for 15 min at 37°C. The method of sample preparation and the HPLC conditions are described in Section 2.

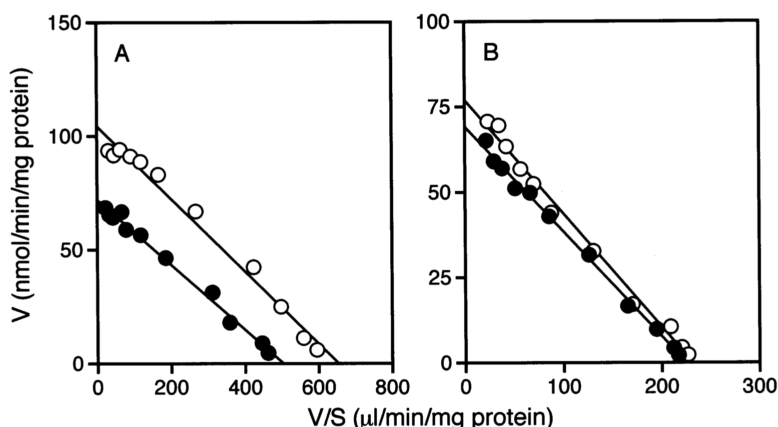

Fig. 5. Eadie–Hofstee plots of UGT activities toward 4-MU and 4-NP in human and rat liver microsomes. Reactions were performed in the presence of 4-MU (10–3000 μM) or 4-NP (10–3000 μM) and liver microsomal proteins (10 μg) in a total volume of 400 μl for 15 min at 37°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Section 2. Panels are: (A) UGT activity toward 4-MU; and (B) UGT activity toward 4-NP. Symbols are: (●) human; and (O) rat. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

Table 3
Michaelis–Menten parameters for UGT activities toward 4-MU and 4-NP in human and rat liver microsomes[a]

Substrate	Human		Rat	
	K_m (μM)	V_{max} (nmol/min per miligram protein)	K_m (μM)	V_{max} (nmol/min per miligram protein)
4-MU	143	72.1	160	105
4-NP	308	69.2	333	77.0

[a] Reactions were performed in the presence of 4-MU (10–3000 μM) or 4-NP (10–3000 μM) and liver microsomal protein (10 μg) in a total volume of 400 μl for 15 min at 37°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Section 2. Each value represents the mean of two separate experiments and was obtained from Eadie–Hofstee plots. Similar results were obtained in both experiments.

4-NP in liver microsomes are generally similar between humans and rats.

3.9. Induction study

Table 4 shows the UGT activities toward 4-MU and 4-NP in control, 3-methylcholanthrene-and phenobarbital-pretreated liver microsomes from rats. UGT activity for 4-MU in rat liver microsomes was significantly induced 4.5- and 2.6-fold by 3-methylcholanthrene and phenobarbital, respectively. 3-Methylcholanthrene and phenobarbital also significantly induced UGT activitiy toward 4-NP in rat liver microsomes (4.1- and 2.4-fold, respectively). These induction profiles were similar to the results in previous reports [23,24].

4. Concluding discussion

4-MU and 4-NP have been reported to be good substrates for UGT1A6 in mammals [3,4]. Furthermore, as UGT1A6 isoform in mammalian tissues is induced by some xenobiotic chemicals, as well as CYP1A1/2 isoforms, UGT activities toward 4-MU and 4-NP have been often used as toxicological markers for environmental chemicals [25,26]. However, the activity range of UGT1A6 in mammal is very broad because of its extensive tissue distribution [1,7,11–13] and high sensitivity is needed to determine UGT1A6 activity from small amounts of biological samples. Furthermore, no method for the determination of low and high UGT1A6 activities, such as kinetic and induction studies under the same assay conditions has been developed, because the determination is practically based on the disappearance of substrate [14,15]. With the method described here, the kinetics and inducibility of UGT activities toward 4-MU and 4-NP in human and rat liver were precisely determined using small amounts of microsomal proteins without extraction. Therefore, this method for the determination of UGT activities for 4-MU and 4-NP is sensitive and efficient and should be useful for in vivo and in vitro studies on the interaction of xenobiotic chemicals with UGT1A6 isoform in mammals using small amounts of biological sample.

Table 4
Induction of UGT activities toward 4-MU and 4-NP by 3-methylcholanthrene and phenobarbital in rat liver microsomes[a]

Substrate	UGT activity (nmol/min per miligram protein)		
	Control	3-Methyl-cholanthrene	Phenobarbital
4-MU	84.0 ± 7.4	375 ± 42**	217 ± 21**
4-NP	56.1 ± 3.9	229 ± 41**	137 ± 15*

[a] Reactions were performed in the presence of 4-MU (500 μM) or 4-NP (1000 μM) and liver microsomal protein (10 μg) in a total volume of 400 μl for 15 min at 37°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Section 2. Each value represents the mean ± S.D. of three individual animals.

* $P < 0.05$, statistical significance of differences was calculated using analysis of variance with Dunnett's post-hoc test.

** $P < 0.01$, statistical significance of differences was calculated using analysis of variance with Dunnett's post-hoc test.

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References

[1] G.J. Dutton, Glucuronidation of Drugs and Other Compounds, CRC Press, Boca Raton, 1980.
[2] B. Burchell, M.W. Coughtrie, Pharmacol. Ther. 43 (1989) 261–289.
[3] P.I. Mackenzie, I.S. Owens, B. Burchell, K.W. Bock, A. Bairoch, A. Belanger, S. Fournel-Gigleux, M. Green, D.W. Hum, T. Iyanagi, D. Lancet, P. Louisot, J. Magdalou, J.R. Chowdhury, J.K. Ritter, H. Schachter, T.R. Tephly, K.F. Tipton, D.W. Nebert, Pharmacogenetics 7 (1997) 255–269.
[4] S.N. de Wildt, G.L. Kearns, J.S. Leeder, J.N. van den Anker, Clin. Pharmacokinet. 36 (1999) 439–452.
[5] B. Burchell, C.H. Brierley, G. Monaghan, D.J. Clarke, Adv. Pharmacol. 42 (1998) 335–338.

[6] J.K. Ritter, F. Chen, Y.Y. Sheen, H.M. Tran, S. Kimura, M.T. Yeatman, I.S. Owens, J. Biol. Chem. 267 (1992) 3257–3261.
[7] Y. Emi, S. Ikushiro, T. Iyanagi, J. Biochem. (Tokyo) 117 (1995) 392–399.
[8] G. Monaghan, D.J. Clarke, S. Povey, C.G. See, M. Boxer, B. Burchell, Genomics 23 (1994) 496–499.
[9] K. Sogawa, O. Gotoh, K. Kawajiri, T. Harada, Y. Fujii-Kuriyama, J. Biol. Chem. 260 (1985) 5026–5032.
[10] I.S. Owens, J.K. Ritter, Prog. Nucleic Acid Res. Mol. Biol. 51 (1995) 305–338.
[11] P.A. Münzel, M. Brück, K.W. Bock, Biochem. Pharmacol. 47 (1994) 1445–1448.
[12] C.P. Strassburg, M.P. Manns, R.H. Tukey, J. Biol. Chem. 273 (1998) 8719–8726.
[13] P.A. Münzel, G. Bookjans, G. Mehner, T. Lehmköster, K.W. Bock, Arch. Biochem. Biophys. 335 (1996) 205–210.
[14] G.W. Lucier, O.S. McDaniel, H.B. Matthews, Arch. Biochem. Biophys. 145 (1971) 520–530.
[15] A. Winsnes, Biochim. Biophys. Acta 191 (1969) 279–291.
[16] W. Lilienblum, A.K. Walli, K.W. Bock, Biochem. Pharmacol. 31 (1982) 907–913.
[17] C.I. Zimmerman, S. Ratna, E. Leboeuf, K.S. Pang, J. Chromatogr. 563 (1991) 83–94.
[18] M.J. Thompson, L.N. Ballinger, S.E. Cross, M.S. Roberts, J. Chromatogr. B. 677 (1996) 117–122.
[19] K. Jemnitz, Z. Veres, K. Monostory, L. Vereczkey, Drug Metab. Dispos. 28 (2000) 34–37.
[20] N. Hanioka, H. Jinno, T. Nishimura, M. Ando, Arch. Toxicol. 72 (1998) 387–394.
[21] N. Hanioka, H. Jinno, T. Tanaka-Kagawa, T. Nishimura, M. Ando, Toxicol. Appl. Pharmacol. 156 (1999) 195–205.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[23] Y. Ishii, K. Tsuruda, M. Tanaka, K. Oguri, Arch. Biochem. Biophys. 315 (1994) 345–351.
[24] N. Hanioka, Y. Hoshikawa, T. Mitsui, K. Oguri, H. Yoshimura, J. Pharmacobio.-Dyn. 13 (1990) 712–717.
[25] T.M. Buetler, E.P. Gallagher, C. Wang, D.L. Stahl, J.D. Hayes, D.L. Eaton, Toxicol. Appl. Pharmacol. 135 (1995) 45–57.
[26] P.A. Münzel, S. Schmohl, H. Heel, K. Kalberer, B.S. Bock-Hennig, K.W. Bock, Drug Metab. Dispos. 27 (1999) 569–573.