0090-9556/03/3106-762–767$7.00
DRUG METABOLISM AND DISPOSITION
Vol. 31, No. 6
1031/1067920
DMD 31:762–767, 2003
Printed in U.S.A.

THE EFFECT OF INCUBATION CONDITIONS ON THE ENZYME KINETICS OF UDP-GLUCURONOSYLTRANSFERASES

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(Received December 9, 2002; accepted March 11, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Traditionally, the Michaelis-Menten equation has been used to determine kinetic parameters for in vitro glucuronidation assays. Recently, estradiol-3-glucuronide formation was shown to exhibit non-Michaelis-Menten kinetics consistent with autoactivation. A concern with the observation of nontraditional kinetics is that they may result as an artifact of the incubation conditions. To examine this concern, the formation of estradiol-3-glucuronide was investigated using human liver microsomes prepared by two different methods, a range of assay conditions, and activation by alamethecin, sonication, or Brij 58 (polyoxyethylene monocetyl ether). Interestingly, holding the other assay components constant, estradiol-3-glucuronide formation was up to 2.5-fold greater using microsomes prepared in phosphate buffer compared with those prepared in sucrose. Incubations activated by alamethecin consistently exhibited the highest rates of estradiol glucuronidation versus the other activators. Furthermore, estradiol-3-glucuronidation exhibited autoactivation kinetics in all of the conditions investigated (n = 1.2–1.7). Nontraditional kinetics were also observed when other UGT1A1 substrates such as ethinylestradiol, buprenorphine, and anthraflavic acid were studied with both human liver microsomes and recombinant UGT1A1. Naphthol, propofol, morphine, and androstanediol were used as probe UGT substrates selective for UGT1A6, UGT1A9, UGT2B7, and UGT2B15, respectively. Of these substrates, only androstanediol exhibited nontraditional kinetics using human liver microsomes. In conclusion, the Hill and/or Michaelis-Menten equations should be used to fit kinetic data to obtain an accurate assessment of in vitro glucuronidation.

Glucuronidation, catalyzed by the uridine diphosphate glucuronosyltransferases (UGTs[1]), is a major detoxification mechanism for both endo- and xenobiotics (Dutton, 1980). The large number of chemically diverse drug substrates eliminated as glucuronides in either urine or bile necessitates a thorough understanding of the process of glucuronidation for efficient drug development and to better understand the role of glucuronidation in the metabolic clearance of compounds (Clarke and Burchell, 1994; Bertz and Granneman, 1997). To date, several in vitro approaches, incorporating different assay conditions, have been utilized to investigate the glucuronidation of new chemical entities.

Advances in molecular biology have allowed the cloning and expression of 15 human UGTs in mammalian cell lines (King et al., 2000). The use of human UGT recombinant cell lines has enabled the examination of substrate specificity/selectivity of many of the UGT isoforms and potentially enables the routine identification of the UGTs responsible for the glucuronidation of new chemical entities (Ethell et al., 2001; Kemp et al., 2002). However, studies bridging in vitro enzyme kinetic results to in vivo clearance for highly glucuronidated drugs (Soars et al., 2002a) have not been as successful as those with oxidative metabolism (Riley, 2001). A potential drawback of these past bridging studies may have been that the assay conditions were not completely optimized for determination of enzyme kinetic parameters. For example, the membrane orientation of UGTs has had a confounding effect on glucuronidation studies for which several solutions have been proposed.

UGTs are primarily located in the endoplasmic reticulum of both hepatic and extrahepatic tissues (Dutton, 1980). By contrast to the cytochromes P450, the active site of UGTs resides in the lumen of the endoplasmic reticulum. Therefore, in vitro UGT activity is latent, and maximal activity is not obtained until the integrity of the membrane is disrupted (Dutton, 1980). Investigators have removed latency in microsomal UGT assays using many different techniques ranging from the traditional use of detergent (Shepherd et al., 1989; Coughtrie et al., 1991) to sonication (Vanstapel and Blanckaert, 1988; Soars et al., 2001) and, more recently, the pore-forming agent alamethecin (Fulceri et al., 1994; Fisher et al., 2000a). However, the relative effects of the various membrane-disrupting techniques on the enzyme kinetics of UGTs has not been thoroughly examined.

To date, the majority of studies examining the enzyme kinetics of glucuronidation, performed using HLM, have fit the enzyme velocity data to the Michaelis-Menten equation (Pacifici and Back, 1988; Soars et al., 2001). However, recent reports have described non-Michaelis-Menten kinetics for the glucuronidation of a number of drugs. The formation of the quaternary ammonium glucuronides of imipramine, amitriptyline, and diphenhydramine by HLM have been shown to exhibit biphasic kinetics (Breyer-Pfaff et al., 1997; Nakajima et al., 2002), whereas autoactivation kinetics have been described

[1] Abbreviations used are: UGT, uridine diphosphate glucuronosyltransferase; UDPGA, uridine diphosphate glucuronic acid; HLM, human liver microsomes; CL_{int}, intrinsic clearance; LC/MS, liquid chromatography/mass spectrometry.

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for estradiol-3-glucuronidation (Fisher et al., 2000a) and acetaminophen glucuronidation (Fisher et al., 2000a; Court et al., 2001). However, when determining the appropriate fit of the data to various enzyme kinetic models, there are a number of potential artifacts that may influence the fit including a lack of analytical sensitivity, nonspecific binding of the substrate to the incubation matrix, and the involvement of multiple enzymes in the reaction (Hutzler and Tracy, 2002). It is therefore imperative that detailed kinetic studies are performed to confirm that the non-Michaelis-Menten kinetics observed to date with UGTs actually reflect the intrinsic properties of the enzyme.

The aims of this report were severalfold: to investigate the effects of incubation conditions on estradiol glucuronidation and to determine whether the non-Michaelis-Menten kinetics observed previously with estradiol-3-glucuronidation (Fisher et al., 2000a) were an artifact of the incubation conditions used; to ascertain whether the kinetics of estradiol-3-glucuronidation would also be observed in incubations containing recombinantly expressed UGT1A1; to determine whether non-Michaelis-Menten kinetics would be associated with other UGT1A1 substrates; and, finally, to determine whether reactions catalyzed by other UGT isoforms also exhibited this phenomenon.

Materials and Methods

Materials. Ethinylestradiol, estradiol, estradiol-3-glucuronide, estradiol-17-glucuronide, naphthol, naphthyl-glucuronide, androstanediol, androstanediol-17-glucuronide, morphine-3-glucuronide, morphine-6-glucuronide, buprenorphine, saccharic acid 1,4-lactone, and UDPGA were purchased from Sigma-Aldrich (St. Louis, MO). Propofol, anthraflavic acid, and Brij 58 (polyoxyethylene monocetyl ether) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Morphine was purchased from Sigma/RBI (Natick, MA). All aglycone substrates for glucuronidation were of the highest grade available. Propofol-glucuronide, ethinylestradiol-3-glucuronide, buprenorphine-3-glucuronide, and anthraflavic acid-4-glucuronide were produced and fully characterized as described previously (Soars et al., 2002b).

Human liver samples from six separate donors were obtained from the liver transplant unit at the Medical College of Wisconsin (Milwaukee, MI) or Indiana School of Medicine (Indianapolis, IN) under protocols approved by the appropriate committee for the conduct of human research. Two separate batches of hepatic microsomes were prepared by differential centrifugation using either phosphate-based buffers (van der Hoeven and Coon, 1974) or 0.25 M sucrose in 5 mM HEPES (Coughtrie et al., 1987). Two separate mixtures (prepared using phosphate or sucrose buffers) were produced using equal amounts of protein from each of the six donors.

Recombinant UGT1A9, UGT2B7, and UGT2B15 were purchased from BD Gentest (Woburn, MA). Recombinant UGT1A1 and UGT1A6 expressed in V79 cells were kindly provided by Professor Brian Burchell (Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee, UK). Pellets containing cells harvested from two 75-cm^2 tissue culture flasks were thawed before assaying and resuspended in 200 μl of phosphate-buffered saline.

Methods. *UGT assay A.* UGT assays were performed as described previously (Fisher et al., 2000a). Each incubation contained 100 mM potassium phosphate buffer (pH 7.1), 1 mM $MgCl_2$, 5 mM saccharic acid 1,4-lactone, typically 5 to 1000 μM substrate, and 25 to 300 μg of either native or activated HLM/UGT cell line (see below). After a 3-min preincubation at 37°C, the reaction was initiated with the addition of UDPGA (5 mM final concentration) to make a total incubation volume of 200 μl. After incubation at 37°C for 10 to 60 min (initial rate conditions were used for each assay), the assays were quenched with the addition of 50 μl of ice-cold formic acid (25%, v/v, in 100 mM potassium phosphate buffer). Morphine reactions were quenched with 100 μl of ice-cold methanol. Naphthyl-glucuronide (2 nmol) was added as an internal standard (estradiol-3-glucuronide was added for reactions containing naphthol), and reactions were kept on ice for 30 min before centrifugation. The resultant supernatants were then analyzed for glucuronide formation by liquid chromatography/mass spectrometry (LC/MS; see below). The buffer used in this assay method (100 mM potassium phosphate, pH 7.1, 1 mM $MgCl_2$, 5 mM saccharic acid 1,4-lactone) will subsequently be called buffer A.

UGT assay B. UGT assays were adapted from the method of Soars et al. (2001). Each assay contained 100 mM Tris/malate buffer (pH 7.4), 10 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone, typically 5 to 1000 μM substrate, and 25 to 300 μg of either native or activated HLM/UGT cell line (see below). After a 3-min preincubation at 37°C, the reaction was initiated with the addition of UDPGA (5 mM final concentration) to make a total incubation volume of 200 μl. After incubation at 37°C for 10 to 60 min (initial rate conditions were used for each assay), the assays were quenched with the addition of 100 μl of ice-cold methanol. Naphthyl-glucuronide (2 nmol) was added as an internal standard, and reactions were kept on ice for 30 min before centrifugation. The resultant supernatants were then analyzed for glucuronide formation by LC/MS (see below). The buffer used in this assay method (100 mM Tris/malate, pH 7.4, 10 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone) will subsequently be called buffer B.

Optimization of activation. Estradiol glucuronidation was used to investigate three separate methods of activation: sonication, alamethecin, and treatment with Brij 58. Optimization of activation was performed for each separate enzyme source (HLM/cell line) and assay method.

Activation by sonication was performed as described previously (Soars et al., 2001). HLM/cell lines were sonicated using a sonic probe (setting 10, 60 Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA) for up to five, 5-s bursts with 1 min on ice between bursts. The resultant preparations were used to determine the optimal level of sonication required for maximal estradiol glucuronidation.

Activation by alamethecin treatment was performed as described previously (Fisher et al., 2000a). Alamethecin concentrations of 0, 25, 50, 100, and 200 μg/mg protein were incubated with HLM/cell lines for 15 min on ice. The activated preparations were then used to determine the optimal concentration of alamethecin required for maximal estradiol glucuronidation.

Activation by detergent treatment was performed as described previously (Dutton, 1980). Detergent (Brij 58) was incubated for 30 min on ice at detergent/protein ratios (w/w) of 0, 0.1, 0.15, 0.2, 0.25, and 0.3. The activated preparations were used to determine the optimal concentration of Brij 58 required for maximal estradiol glucuronidation.

LC/MS analyses of glucuronide formation. Analyses of glucuronide formation were conducted on a Waters Micromass Z_Q single quadrupole mass spectrometer with a Waters Alliance 2690 HPLC system for separation. Electrospray ionization was used for all mass spectrometry methods with a cone voltage of −30 V and a capillary voltage of 2.7 kV. Analyses were performed in negative ion mode monitoring: estradiol-3- and 17-glucuronides (*m*/*z* 447.1), naphthyl-glucuronide (*m*/*z* 319), propofol-glucuronide (*m*/*z* 353.3), morphine-3- and 6-glucuronides (*m*/*z* 460.3), androstanediol-17-glucuronide (*m*/*z* 466.6), buprenorphine-glucuronide (*m*/*z* 642.6), ethinylestradiol-3-glucuronide (*m*/*z* 471), and anthraflavic acid-4-glucuronide (*m*/*z* 415).

Chromatographic separation for morphine glucuronides was obtained using a 5-μm, 150 × 3 mm Prodigy ODS (3) HPLC column (Phenomenex, Torrance, CA) and a SecurityGuard column. All other glucuronides were resolved using a 3-μm, 100 × 2 mm Prodigy ODS (3) HPLC column (Phenomenex) and a SecurityGuard column. The mobile phase (solvent A) consisted of 10 mM ammonium acetate; the organic phase (solvent B) consisted of 10% (v/v) 10 mM ammonium acetate and 90% (v/v) acetonitrile. Several HPLC gradients were used in this study. Estradiol glucuronides were resolved using the following gradient (t = 0 min, % B = 15; t = 8 min, % B = 31; t = 8.1 min, % B = 100; t = 9.1 min, % B = 100; t = 9.2 min; % B = 15; total run time = 15 min). Glucuronidation kinetics of naphthol, propofol, androstanediol, buprenorphine, and ethinylestradiol were analyzed using the following gradient (t = 0 min. % B = 15; t = 3 min, % B = 100; t = 4 min, % B = 100; t = 4.1 min, % B = 15; total run time = 11 min). Morphine glucuronides were resolved using the following gradient (t = 0 min, % B = 10; t = 11 min, % B = 100; t = 12 min, % B = 100; t = 12.1 min, % B = 10; total run time = 18 min). Anthraflavic acid glucuronidation kinetics were analyzed by the following gradient (t = 0 min, % B = 15; t = 1.5 min, % B = 100; t = 2.5 min, % B = 100; t = 2.6 min, % B = 15; total run time = 6 min). The flow rate for all HPLC methods was 0.25 ml/min. Metabolite formation was quantitated by comparing peak area ratios (metabolite/internal standard) in incubations to ratios obtained from a standard curve containing known

TABLE 1

Effect of assay conditions on estradiol glucuronidation

Two separate microsomal preparations (phosphate or sucrose), each composed of a mixture of six human livers, were incubated with 100 μM estradiol using varying assay buffers (A, 100 mM potassium phosphate, pH 7.1, 1 mM $MgCl_2$, 5 mM saccharic acid 1,4-lactone; B, 100 mM Tris/malate, pH 7.4, 10 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone) and activation conditions (see *Materials and Methods*). Each value is the mean of duplicate incubations. The fold activation was calculated as estradiol glucuronidaton at the maximal level of activation divided by estradiol glucuronidation obtained with untreated microsomes. Maximal activation was obtained with 50 μg/mg alamethecin and 5 s of sonication, done four times, in all cases. However, the concentration of Brij 58 required varied with different assay conditions.

Microsomal Preparation	Assay Buffer	Activation	Estradiol-3-glucuronidation		Estradiol-17-glucuronidation	
			Fold Activation	Activity	Fold Activation	Activity
				pmol/min/mg		*pmol/min/mg*
Phosphate	A	Alamethecin	3	870	3	150
Phosphate	B	Alamethecin	2.5	1230	2.2	240
Sucrose	A	Alamethecin	3	540	2.2	150
Sucrose	B	Alamethecin	2.8	740	2	180
Phosphate	A	Sonication	2.5	480	2.8	60
Phosphate	B	Sonication	1.8	710	2.2	120
Sucrose	A	Sonication	1.4	230	1.5	40
Sucrose	B	Sonication	1.8	280	2	70
Phosphate	A	Brij 58	2.6	770	3.8	120
Phosphate	B	Brij 58	1.6	780	2.5	160
Sucrose	A	Brij 58	2.3	390	3	80
Sucrose	B	Brij 58	2.5	370	2.9	80

amounts of glucuronide. Standard curve correlation coefficients (r^2) were $\geq$0.99.

Kinetic determinations. Preliminary experiments (data not shown) were performed to ensure that all kinetic determinations were carried out under initial rate conditions with respect to time and protein. The following ranges of substrate concentrations were used to determine kinetic data for each substrate: 100 to 1 μM estradiol, 150 to 0.5 μM buprenorphine, 75 to 0.25 μM ethinylestradiol, 100 to 0.25 μM anthraflavic acid, 400 to 1 μM naphthol, 600 to 0.5 μM propofol, 4000 to 10 μM morphine, and 150 to 1 μM androstanediol. Each set of data was fit to both the Michaelis-Menten and Hill equations using nonlinear regression analysis (WinNonlin; Pharsight, Mountain View, CA). The quality of fit to a particular model was determined by evaluation of three criteria that are listed in decreasing order of importance: 1) the randomness of the residuals; 2) the size of the sum of the squares of the residuals; and 3) the standard error of the parameter estimates (Ring et al., 1996; Mayhew et al., 2000; Court et al., 2001).

Results

Effect of Assay Conditions on Estradiol Glucuronidation. Incubation conditions for UGT assays vary significantly among different laboratories. Several groups use hepatic microsomes prepared in phosphate buffers (Fisher et al., 2000a; Court et al., 2001), whereas others prepare microsomes in sucrose buffers (Bock et al., 1984; Soars et al., 2001). The composition of assay buffers varies between groups, with both phosphate- (Fisher et al., 2000a; Court et al., 2001) and Tris-based buffers being used frequently (Bock et al., 1984; Soars et al., 2001). The activation of UGTs also differs among laboratories, with sonication (Soars et al., 2001), alamethecin (Fisher et al., 2000a), and detergent treatments (Shepherd et al., 1989; Coughtrie et al., 1991) used most frequently.

The effects of the preparation of microsomes and assay conditions on UGT activity were investigated using estradiol-3- and 17-glucuronidation as endpoints (Table 1). Interestingly, estradiol-3-glucuronidation was greater (up to 2.5-fold) when microsomes prepared in phosphate were used compared with those prepared in sucrose, with all other assay components held constant. This difference in activity was also apparent (albeit to a lesser extent) for estradiol-17-glucuronidation. Differences in estradiol activities due to assay buffer conditions (buffer A versus buffer B) were also apparent (Table 1). However no simple trend adequately described these effects. Activation by sonication, alamethecin, and treatment with Brij 58 was optimized for each HLM/cell type and assay condition (see *Materials and Methods* for details). In each case, maximal estradiol glucuronidation (approximately a 2.5-fold activation) was obtained with 50 μg alamethecin/mg protein. A similar trend was observed when HLM were activated with sonication. Maximal estradiol glucuronidation (on average a 2-fold activation) was always attained after 5 s of sonication performed four times. Conversely, for each specific microsomal preparation and assay condition, a different concentration of Brij 58 was required for optimal activation.

The effects of different incubation conditions on the kinetics of estradiol glucuronidation are summarized in Table 2 for estradiol-3-glucuronidation and Table 3 for estradiol-17-glucuronidation. Estradiol-3-glucuronidation exhibited non-Michaelis-Menten kinetics consistent with autoactivation in all of the conditions investigated (Table 2). Kinetic analyses performed with microsomes prepared in phosphate buffer yielded V_{max} values for estradiol-3-glucuronidation that were 2- to 3-fold greater than those prepared in sucrose (consistent with previous activity data, Table 1). However, both the S_{50} values and the degree of sigmoidicity (n) demonstrated little variability throughout the conditions used in this study. By contrast, estradiol-17-glucuronidation best fit Michaelis-Menten kinetics under all assay conditions (Table 3). The V_{max} values determined after treatment with alamethecin or Brij 58 were comparable, with the V_{max} following activation by sonication, being slightly lower. The K_m values determined for estradiol-17-glucuronidation were relatively consistent for all assay conditions investigated.

Glucuronidation Kinetics Determined Using HLM and Recombinant UGTs. Due to the lack of variability of S_{50}/K_m values obtained using different assay conditions, all subsequent kinetic determinations used HLM prepared in phosphate buffer (or cell lines expressing recombinant UGTs), assay method A, and activation by alamethecin treatment to be consistent with previous work (Fisher et al., 2000a,b). The kinetic parameters for eight compounds were determined (Table 4) using both HLM and the recombinant UGT primarily involved in the hepatic glucuronidation of each particular substrate. Kinetic data obtained using both HLM and UGT1A1 for buprenorphine, ethinylestradiol, anthraflavic acid, and estradiol-3-glucuronidation best fit the Hill model yielding an n_H value between 1.2 and 1.4. Interestingly, S_{50} values determined using HLM and UGT1A1 were similar for all four biotransformations studied; however, the V_{max} values were severalfold greater in HLM than in UGT1A1, with the exception of

TABLE 2

Effect of assay conditions on estradiol-3-glucuronidation by human liver microsomes

Kinetic parameters were determined using HLM under varying assay conditions as described under *Materials and Methods*. Results are from two separate experiments (each kinetic determination was performed using nine substrate concentrations in duplicate). Assay buffer A contained 100 mM potassium phosphate, pH 7.1, 1 mM $MgCl_2$, 5 mM saccharic acid 1,4-lactone. Assay Buffer B contained 100 mM Tris/malate, pH 7.4, 10 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone.

Microsomal Preparation	Assay Buffer	Activating Agent								
		Alamethecin			Sonication			Brij 58		
		V_{max}	S_{50}	n	V_{max}	S_{50}	n	V_{max}	S_{50}	n
		pmol/min/mg	*μM*		*pmol/min/mg*	*μM*		*pmol/min/mg*	*μM*	
Phosphate	A	1670, 810	50, 30	1.3, 1.3	1080, 1070	40, 39	1.4, 1.4	1170, 710	44, 49	1.5, 1.4
Phosphate	B	2190, 1150	50, 38	1.3, 1.3	730, 730	50, 50	1.2, 1.2	840, 870	38, 83	1.7, 1.3
Sucrose	A	580, 480	35, 30	1.3, 1.4	330, 300	33, 30	1.2, 1.4	480, 470	37, 50	1.5, 1.3
Sucrose	B	820, 690	30, 43	1.3, 1.3	360, 360	53, 61	1.2, 1.2	420, 390	50, 48	1.6, 1.5

TABLE 3

Effect of assay conditions on estradiol-17-glucuronidation by human liver microsomes

Kinetic parameters were determined using HLM under varying assay conditions as described under *Materials and Methods*. Results are from two separate experiments (each kinetic determination was performed using nine substrate concentrations in duplicate). Assay buffer A contained 100 mM potassium phosphate, pH 7.1, 1 mM $MgCl_2$, 5 mM saccharic acid 1,4-lactone. Assay buffer B contained 100 mM Tris/malate, pH 7.4, 10 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone.

Microsomal Preparation	Assay Buffer	Activating Agent								
		Alamethecin			Sonication			Brij 58		
		V_{max}	K_m	CL_{int}	V_{max}	K_m	CL_{int}	V_{max}	K_m	CL_{int}
		pmol/min/mg	*μM*	*μl/min/mg*	*pmol/min/mg*	*μM*	*μl/min/mg*	*pmol/min/mg*	*μM*	*μl/min/mg*
Phosphate	A	110, 110	11, 8	10, 13	100, 70	12, 8	8.2, 9.1	120, 120	12, 9	10, 13
Phosphate	B	230, 200	11, 11	21, 18	100, 100	14, 11	6.9, 8.9	190, 200	13, 15	15, 13
Sucrose	A	80, 120	13, 15	6.4, 7.9	60, 60	9, 13	6.2, 4.5	90, 110	13, 13	7.2, 8.4
Sucrose	B	190, 180	16, 18	12, 9.8	80, 70	19, 16	4.0, 4.3	120, 150	26, 18	4.5, 8.4

TABLE 4

Comparison of kinetic parameters determined using human liver microsomes and recombinant enzymes for several substrates

The human liver microsomes used in these experiments were prepared in phosphate buffer. Kinetic parameters were determined using assay method A and activation by incubation for 15 min on ice with alamethecin (50 μg/mg protein). Each number represents an individual experiment or the mean ± standard deviation of three experiments (each kinetic determination was performed using nine substrate concentrations in duplicate).

Compound	Enzymes	Human Liver Microsomes			Recombinant Enzymes		
		V_{max}	S_{50}, K_m	n	V_{max}	S_{50}, K_m	n
		pmol/min/mg	*μM*		*pmol/min/mg*	*μM*	
Estradiol-3-glucuronide	UGT1A1	1,670, 810	50, 30	1.3, 1.3	310, 300	20, 22	1.4, 1.2
Buprenorphine	UGT1A1	4,900 ± 1,400	57 ± 14	1.2 ± 0.01	2,300, 2,700	45, 68	1.2, 1.2
Ethinylestradiol	UGT1A1	1,500, 1,200	21, 13	1.3, 1.2	700, 600	11, 9.7	1.2, 1.3
Anthraflavic Acid	UGT1A1	170, 340	4, 5	1.3, 1.3	830, 810	12, 9.8	1.2, 1.2
Naphthol	UGT1A6	17,000, 18,000	38, 45	1.0, 1.0	33,000, 27,000	78, 67	1.0, 1.0
Propofol	UGT1A9	4,100, 3,500	190, 280	1.0, 1.0	3,200, 2,400	28, 45	1.0, 1.0
Morphine-3-glucuronide	UGT2B7	2,500, 2,700	1,900, 3,100	1.0, 1.0	440, 410	290, 200	1.1, 1.2
Morphine-6-glucuronide	UGT2B7	160, 140	690, 960	1.0, 1.0	81, 74	240, 240	1.2, 1.0
Androstanediol	UGT2B15	6,700, 5,800	25, 21	1.2, 1.2	980, 980	16, 13	1.1, 1.0

anthraflavic acid, where this trend was reversed. Conversely, naphthol and propofol glucuronidation data generated using both HLM and recombinant cell lines expressing UGT1A6 and UGT1A9, respectively, were found to best fit traditional Michaelis-Menten kinetics. The K_m values determined for naphthol using HLM were similar to those obtained using expressed UGT1A6, whereas the K_m values generated for propofol using expressed UGT1A9 were almost 10 times lower than those observed using HLM. The formation of morphine-3- and morphine-6-glucuronides by HLM best fit the Michaelis-Menten equation. However, morphine glucuronidation catalyzed by expressed UGT2B7 exhibited a slight tendency toward sigmoidicity in three of the four determinations (Table 4). Interestingly, the S_{50}/K_m values obtained using expressed UGT2B7 for the formation of both morphine-3- and 6-glucuronides were similar, whereas the K_m values generated for morphine-3-glucuronidation using HLM were about 3-fold greater than that obtained for morphine-6-glucuronidation. Androstanediol glucuronidation catalyzed by HLM and UGT2B15 best fit different models (Hill for HLM and Hill and Michaelis-Menten for UGT2B15). However the S_{50}/K_m values, produced for androstanediol glucuronidation, using both HLM and expressed UGT2B15, were similar (Table 4).

Discussion

Bock et al. (1983) proposed a set of assay conditions for conducting glucuronidation assays. However, as outlined under *Results*, a variety of different assay conditions have subsequently been utilized by investigators around the world. In the current study the effects of these assay conditions have been assessed using estradiol glucuronidation (Table 1). The consistently high levels of activation obtained using alamethecin treatment (Table 1) and its ease of use (50 μg/mg protein

always gave maximal activation in these studies) indicate that this method of activation appears to be the most appropriate for future UGT studies. It should be noted that different concentrations of alamethecin may be required under different experimental conditions for optimal activation, particularly when glucuronidation in different species is being considered (Vashishtha et al., 2002). Although differences in estradiol glucuronidation were noted using both buffer A and buffer B, neither buffer could be judged to be superior because this particular investigation used varying pH and magnesium levels in the different buffers, which may have resulted in different rates of estradiol glucuronidation. Interestingly, incubations performed with microsomes prepared in phosphate buffer gave 2- to 3-fold greater estradiol-3-glucuronidation than those prepared in sucrose (Table 1). This agrees with the work of Graham and Wood (1973), who observed a similar trend in rat and guinea pig liver microsomes. Perhaps phosphate activates UGTs by increasing the permeability of the microsomal membrane to substrates and UDPGA. However, incubations performed using microsomes (prepared in either sucrose or phosphate) that have been maximally activated (see *Materials and Methods*) should yield similar activities but did not. These results suggest that phosphate may have an additional effect on the conformation of the enzyme, thus increasing catalytic activity. Although the variation in estradiol glucuronidation observed due to different assay conditions is interesting, the focus of this study was to assess the impact of these in vitro conditions on kinetic determinations.

Fisher et al. (2000a) first described autoactivation kinetics associated with glucuronidation when investigating estradiol-3-glucuronidation with HLM. However, there are many potential artifactual causes of non-Michaelis-Menten kinetics (Hutzler and Tracy, 2002). To determine whether atypical kinetics are an artifact of the in vitro system utilized, a variety of conditions were used to examine glucuronidation. Estradiol-3-glucuronidation by HLM exhibited autoactivation kinetics throughout the range of in vitro incubations examined in this study (Table 2). Therefore, the non-Michaelis-Menten kinetics observed previously were not an artifact of the incubation conditions used. Another potential cause of the atypical kinetics observed with estradiol-3-glucuronide formation could be saturable protein binding at low substrate concentrations. However, this does not appear to be the case for estradiol since estradiol-17-glucuronide formation (determined from the same incubations as estradiol-3-glucuronidation) displayed Michaelis-Menten kinetics. The use of multienzyme systems like HLM might also have an impact on the type of enzyme kinetics observed. Therefore, it was important to investigate glucuronidation in simpler in vitro systems.

Glucuronidation of estradiol at the 3-position has been shown previously to be primarily catalyzed in humans by UGT1A1 (Senafi et al., 1994) and has subsequently been used as a selective substrate for this isoform (Fisher et al., 2000a,b). Estradiol kinetics determined using recombinant UGT1A1 exhibited non-Michaelis-Menten kinetics consistent with autoactivation. Thus, the atypical kinetics observed previously using HLM (Table 2; Fisher et al., 2000a,b) were not due to the complex in vitro matrix used.

To further investigate the potential association of autoactivation kinetics with UGT1A1, the kinetics of three chemically diverse UGT1A1 substrates, buprenorphine, ethinylestradiol, and anthraflavic acid were determined using both HLM and recombinant UGT1A1. Although none of these substrates are exclusively glucuronidated by UGT1A1 in HLM, a significant proportion of their hepatic glucuronidation has been shown to be catalyzed by this isoform (Ebner et al., 1993; King et al., 1996). Interestingly, the glucuronidation of buprenorphine, ethinylestradiol, and anthraflavic acid catalyzed by HLM and recombinant UGT1A1 was consistent with autoactivation in each case (Table 4). The S_{50} values determined for buprenorphine and anthraflavic acid in these experiments agreed well with those obtained previously by King et al. (1996), using recombinant UGT1A1 (69 and 9 μM, respectively). By contrast, the S_{50} for ethinylestradiol obtained here (10 μM) was significantly lower than the K_m (130 μM) observed previously by Ebner et al. (1993). The use of LC/MS technology in the present study appears to have afforded greater sensitivity at low ethinylestradiol glucuronide concentrations and hence allowed a more complete kinetic profile to be determined. The S_{50} values determined for buprenorphine, ethinylestradiol, and anthraflavic acid using HLM and recombinant UGT1A1 were similar, further suggesting that UGT1A1 is the predominant isoform involved in their hepatic metabolism. Senafi et al. (1994) demonstrated that UGT1A1 has the capacity to glucuronidate compounds covering many different chemical classes. Although the present study only investigated the glucuronidation of four different UGT1A1 reactions, the kinetics of two steroids, an opioid, and an anthraquinone have all exhibited autoactivation kinetics. In addition, Bruni and Chang (1999) showed that the glucuronidation kinetics of bilirubin (an endobiotic known to be specifically metabolized by UGT1A1) were consistent with autoactivation. These results indicate that for all UGT1A1 substrates, sensitive bioanalytical assays capable of detecting the formation of glucuronide at low substrate concentrations are necessary so that kinetic analyses may be performed that more fully define the kinetic relationship.

The mechanism of autoactivation kinetics associated with UGT1A1 is currently unknown. However, one potential explanation is that UGT1A1 acts in a multimeric form, where the binding of one substrate molecule to the active site of UGT1A1 aids the binding of a second substrate to a second UGT1A1 active site. Ghosh et al. (2001) have used cross-linking studies in conjunction with two-hybrid analysis in both yeast and mammalian systems to investigate directly any potential interactions between UGT1A1 molecules. They concluded that UGT1A1 was capable of homodimerization and that this intermolecular association might be functionally significant.

Enzymes involved in the regulation of physiological processes often demonstrate substrate activation. In these situations, the catalytic activation of the regulatory enzyme increases immediately upon an increase in substrate concentration, thus rapidly lowering the levels of the physiologic regulator (substrate) to desired levels (Ekins et al., 1998). Therefore, in the current situation with UGT1A1, increased levels of bilirubin would activate UGT1A1 resulting in a more rapid detoxification of bilrubin via glucuronidation. This premise is consistent with the recent views on enhanced detoxification via autoactivation of cytochromes P450 proposed by Atkins et al. (2002).

To further examine potential non-Michaelis-Menten kinetics associated with UGTs, the glucuronidation kinetics of naphthol, propofol, morphine, and androstanediol were determined using HLM and recombinant cell lines expressing UGT1A6 (1-naphthol), UGT1A9 (propofol), UGT2B7 (morphine), and UGT2B15 (androstanediol). These substrates were chosen because they have been recommended previously as relatively selective probes for the relevant human UGT isoforms (Burchell et al., 2001). Naphthol and propofol kinetics exhibited Michaelis-Menten kinetics in both HLM and expressed UGT1A6 and UGT1A9, respectively. Interestingly, earlier studies that used acetaminophen as a probe substrate for UGT1A6 exhibited non-Michaelis-Menten kinetics (Fisher et al., 2000a). However, subsequent experiments by Court et al. (2001) determined that acetaminophen glucuronidation was primarily catalyzed in the liver by UGT1A1, UGT1A6, and UGT1A9 and therefore was not a selective probe for UGT1A6 in HLM. Furthermore, the Court et al. (2001) study also showed that UGT1A1-mediated glucuronidation of acetaminophen displayed autoactivation kinetics ($n = 1.6$), whereas glu-

curonidation catalyzed by UGT1A6 was consistent with Michaelis-Menten kinetics. The formation of morphine-3- and morphine-6-glucuronides by HLM exhibited Michaelis-Menten kinetics in the current study, which agrees with previous studies (Fisher et al., 2000a; Soars et al., 2001). However, morphine glucuronidation, catalyzed by UGT2B7, displayed a slight tendency toward sigmoidicity in three of the four determinations, although the S_{50} values determined in this study were similar to the K_m values obtained previously (Coffman et al., 1998). Androstanediol glucuronidation displayed autoactivation kinetics with HLM. However, androstanediol glucuronidation exhibited only a slight sigmoid nature in one determination with expressed UGT2B15 and Michaelis-Menten in another determination. The inconsistency in models used to fit the UGT2B15 data appears to be due to the small degree of sigmoidicity observed. However, both the S_{50} and K_m values determined for androstanediol in this study agree with those obtained previously (Green et al., 1994).

In conclusion, the glucuronidation of estradiol was investigated using a range of in vitro assay conditions. Although the rate of glucuronidation varied with different methods of microsome preparation, assay buffers, and activation methods, estradiol-3-glucuronidation routinely exhibited autoactivation kinetics using HLM and recombinant UGT1A1. Therefore, the non-Michaelis-Menten kinetics observed previously were not an artifact of the incubation conditions utilized. Autoactivation kinetics were also observed with several other UGT1A1 substrates. However, with the exception of androstanediol, the glucuronidation of all other substrates investigated using HLM displayed Michaelis-Menten kinetics. Therefore, both the Hill and Michaelis-Menten equations should be used to fit kinetic data to obtain an accurate assessment of in vitro glucuronidation. The determination of the best-fit enzyme kinetic parameters for the formation of glucuronides will theoretically improve the predictability of in vivo clearance from in vitro enzyme kinetic determinations (Houston and Kenworthy, 2000).

Acknowledgments. We thank Professor Brian Burchell for providing the recombinant UGT1A1 and UGT1A6 used in this study.

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