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Determination of drug glucuronidation and UDP-glucuronosyltransferase selectivity using a 96-well non-HPLC radiometric assay

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Running Title: Radiometric UGT assay
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Discussion: 996 words
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Abbreviations used are: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HLM, human liver microsomes; RLM, rat liver microsomes; DLM, dog liver microsomes; SPE, solid phase extraction; MeOH, methanol; 1NP, 1-naphthol; 4MU, 4-methylumbelliferone; HFC, 7-hydroxy-4-trifluoromethylcoumarin; HPLC, high performance liquid chromatography; MS, mass spectrometry.

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ABSTRACT

A rapid and sensitive radiometric assay for UDP-glucuronosyltransferase (UGT) is described. UGT substrates are incubated in 96-well plates with microsomes in the presence of [^{14}C]UDPGA, and [^{14}C]-labelled glucuronidation products are separated from the unreacted nucleotide sugar by solid phase extraction using 96-well extraction plates. The assay was validated with 15 structurally diverse UGT substrates containing acidic, phenolic and hydroxyl reacting groups. Glucuronidation velocities for these compounds were determined using human, rat, and dog liver microsomes, and reaction kinetics was studied with 1-naphthol and 4-methylumbelliferone. Results obtained with the new assay confirmed the previously reported rank order of glucuronidation velocity of several typical UGT substrates and the finding that glucuronidation of most of these compounds is significantly faster in dog than in human liver microsomes. UGT specificity of 5 compounds was determined using recombinant human UGTs. The major UGT isoforms identified were UGT1A6, UGT1A7, and UGT1A9 for 4-methylumbelliferone, UGT1A6 and UGT1A8 for 1-naphthol, UGT2B7 for naloxone, UGT1A3 and UGT2B7 for ketoprofen, and UGT1A4 for trifluoperazine. Identical results were obtained with a conventional HPLC method coupled to mass spectrometric detection. The new assay should prove to be valuable for rapidly benchmarking recombinant UGTs and microsomal preparations from different species and tissues, for identifying high turnover compounds during drug discovery, and for reaction phenotyping studies.

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INTRODUCTION

UDP-glucuronosyltransferases (UGT) play an important role in the metabolism and detoxification of a variety of endobiotics and xenobiotics (Burchell et al., 1995; King et al., 2000; Tukey and Strassburg, 2000; Bock, 2003; Wells et al., 2004). Over 46 UGT enzymes belonging to 2 gene families, *UGT1* and *UGT2* have been identified. These enzymes are expressed in the liver and some extrahepatic tissues, where they are localized to the luminal side of the endoplasmic reticulum and the nuclear envelope. UGTs catalyze the transfer of a glucuronic acid moiety to a variety of acceptor groups such as phenols, alcohols, carboxylic acids, amines, carbamic acids, hydroylamines, hydroxylamides, carboxamides, sulfonamides, thiols, dithiocarboxylic acids and nucleophilic carbon of 1,3-dicarbonyl compounds. Typical endogenous UGT substrates include bilirubin, bile acids and steroid hormones, while xenobiotic substrates include phenols and coumarins as well as many therapeutic drugs of different structure. Most UGTs exhibit partially distinct, but frequently overlapping substrate specificities (Burchell et al., 1995; King et al., 2000; Tukey and Strassburg, 2000; Bock, 2003; Wells et al., 2004).

Because of the great number of different enzyme isoforms, their wide tissue distribution, and the multiplicity of substrates, the availability of robust, simple, and sensitive assay methods is crucial for studies of the biochemistry, function and regulation of this enzyme family. As a consequence of the major role of glucuronidation in drug elimination, rapid UGT assays are also needed during the drug discovery process to identify metabolic liabilities, to define the UGT specificity and reaction kinetics, and to predict human pharmacokinetics of new chemical entities (Lin and Wong, 2002; Miners et al., 2004). All currently used techniques for the determination of UGT activity involve separation of glucuronide conjugation products from the parent aglycone, followed by product quantification using ultraviolet, mass spectrometric or radiochemical detection. Two important advantages of radiometric methods based on the use of [^{14}C]UDPGA is that they are

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sensitive and do not require synthetic glucuronide standards for product quantification. However, most of the current methods for product separation, such as TLC (Bansal and Gessner, 1980), HPLC (Ethell et al., 1998), or organic solvent extraction (Matern et al., 1994), are relatively laborious and not ideally suited for processing large number of samples in a short time. A radiometric SPE method using C18 cartridges for determination of acyl glucuronide formation has been described (Pritchard et al., 1993). This method is much more rapid than alternative separation methods, but it has not been investigated whether it could be applied to determine glucuronidation drugs other than carboxylic acids. In the present report, we describe a 96-well radiometric assay for the determination of UGT activity toward compounds containing carboxyl, phenol and hydroxyl moieties, based on the conjugation of test compounds with [^{14}C]UDPGA and separation of reaction products on 96-well SPE plates. The assay was validated with 15 structurally diverse substrates, used to compare reaction velocities in microsomes from 3 different species, and to determine the reaction kinetics and UGT specificity of typical substrates.

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MATERIALS AND METHODS

Reagents. [^{14}C]UDPGA (418.3 mCi/mmol) was purchased from PerkinElmer Life Sciences (Monza, Italy). Other chemicals were purchased from Sigma-Aldrich (Milan, Italy) and were of the highest purity available. HLM (pooled from 22 donors) and RLM (pooled from 60 male Sprague Dawley rats) were obtained from BD Gentest (Franklin Lakes, NJ), and DLM (pooled from 9 male Beagle dogs) from Xenotech (Lenexa, KA). All microsomal stocks contained ~ 20 mg protein/ml. Recombinant human UGTs expressed in baculovirus-infected cells (Supersomes) were purchased from BD Gentest, with the exception of UGT1A7, which was obtained from PanVera (Madison, WI).

Assay Procedure. Assays were carried out in polystyrene 96 well plates (Costar 96-well round bottom plate, Corning Life Sciences, Acton, MA) containing 100 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 10 or 50 µg of microsomal protein, 2 mM UDPGA, 50-100 nCi of [^{14}C]UDPGA, 5 mM D-saccharic acid 1,4 lactone, 1 mM adenosine-5'-monophosphate, 1 mM ethylene glycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid and 50 µg/mg of protein of alamethicin in a final volume of 100 µl. Adenosine-5'-monophosphate and ethylene glycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid were added to inhibit nucleotide pyrophosphatases (Torp-Pedersen et al., 1979; Faltynek et al., 1981; Matern et al., 1994). All substrates were dissolved in DMSO resulting in a final solvent concentration of 1%, v/v. Blank controls for each batch of microsomes were incubated in the presence of vehicle only. Plates were incubated at 37^{0}C for up to 40 min and reactions were terminated by the addition of 100 µl of 0.2 N HCl. For zero time controls, HCl was added to the reaction mixture prior to the addition of UDPGA. Proteins were removed by centrifugation at 1750 x g for 30 minutes using a microplate rotor.

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Separation of glucuronide products. Oasis™ HLB 30mg solid phase 96 well extraction plates (Waters, Milford, MA) were used for the SPE process on a 96 well vacuum manifold. Prior to loading the assay supernatant, plates were washed with MeOH (2 x 1.5 ml washes) and water (2 x 1.5 ml washes) and the solvent was drawn through the column by applying vacuum. Deproteinized assay mixtures (180 µl) were loaded onto the plate and vacuum was applied again. The plates were then washed twice with 0.5 ml of water followed by 3 further washes with 1 ml of water to remove UDPGA and [^{14}C]UDPGA. The waste tray in the vacuum manifold was replaced with a 2 ml, deep format 96-well plate and the radioactive glucuronide was eluted with 2 x 0.5 ml of MeOH. The MeOH eluate was evaporated to dryness under nitrogen (Micro DS96 sample concentrator, Porvair Sciences Ltd, Shepperton, UK), resuspended in 120 µl of acetonitrile/water (50:50, v/v) and 100 µl of this solution was transferred to 24 well scintillation plates (Packard- PerkinElmer Life Sciences). Scintillation fluid (1.5 ml of Microscint 20, Packard) was added to each well before sealing, mixing and counting for 2 minutes per well using a microplate scintillation counter (Topcount NXT1, Packard Biosciences).

Validation of the SPE procedure. To assess whether glucuronide conjugates were selectively retained on the SPE plate and eluted only in the MeOH elution step, a second extraction was carried out. Briefly, MeOH eluates collected from the first extraction (which contained the glucuronide product) were dried under nitrogen and reconstituted in a 220 µL mixture of 0.2 N HCl and 100 mM Tris-HCl, pH 7.4 containing 2 mM cold UDPGA (in a 1:1 ratio). Thirty µl of this mixture were counted to determine the total radioactivity, while 180 µl were loaded onto a second extraction plate, which was washed and eluted as described for the standard procedure. Product recovery was calculated from the ratio of counts recovered in the MeOH eluate of the second extraction relative to the total input radioactivity.

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Determination of substrate glucuronidation by recombinant UGTs. Compounds were incubated with recombinant UGT preparations containing 10 or 50 µg of microsomal protein for 30 min, except for trifluoperazine, which was incubated for 120 min. Following addition of stopping solution and centrifugation, 150 µl aliquots of deproteinized supernatants were subjected to SPE and processed as described above. Aliquots of 30 µl aliquots were diluted with an equal volume of acetonitrile and analyzed by HPLC-MS/MS using an Agilent HP1100 liquid chromatograph equipped with a CTC Analytics PAL Autosampler. Chromatography was performed on an XTERRA MS C_{18} column (4.6 mm x 5 cm; 5µm; Waters) at a flow rate of 2 mL/min, using a linear gradient from water/ 0.1% formic acid (A) to 20% A, 80% acetonitrile/ 0.1% formic acid. The eluate was diverted to a Sciex API-3000 triple quadrupole mass with a Turbo Ionspray ionization source operated in the positive ion mode (glucuronides of ketoprofen, naloxone, trifluoperazine) or negative ion mode (glucuronides of 1NP and 4MU).

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RESULTS

Separation of glucuronide conjugates from unreacted UDPGA. To generate glucuronide conjugates of a series of typical UGT substrates, compounds were incubated for 2 h at 37° with alamethicin-treated DLM in the presence of a trace amount (1.5-2 x 10^5 dpm) of [^{14}C]UDPGA and 2 mM of unlabelled UDPGA. Reaction mixtures were deproteinized and loaded on 96-well SPE plates containing Oasis^{TM} polymeric sorbent. Radioactivity was eluted from the plates by stepwise washes with 1 ml fractions of water followed by MeOH. Over 99.9 % of [^{14}C]UDPGA eluted in the combined void volume and aqueous fractions, with less than 0.15% of the radioactivity eluting in the organic solvent fractions. Similarly, over 99% and < 1% of the radioactivity in control microsomal reaction mixtures incubated in the absence of aglycone eluted in the aqueous and organic fractions, respectively (Table 1 and Figure 1A). In contrast, when incubation mixtures contained the UGT substrate 1NP, radioactivity eluting in the aqueous fraction was significantly reduced, indicating that part of the [^{14}C]UDPGA had been consumed in the reaction. Significant amounts of radioactivity eluted in the MeOH fraction (Table 1 and Fig. 1B). Since radioactivity was detected in the organic fraction only when both 1NP and microsomes were present in the incubation, this radioactivity must correspond to the reaction product of 1NP with [^{14}C]UDPGA, namely 1NP-O-glucuronide. Our results indicate that the glucuronide conjugate of 1NP, by virtue of being more hydrophobic than UDPGA, can be separated from the labelled substrate by retention on Oasis^{TM} SPE resin, and subsequently eluted and recovered by washing the resin with organic solvent.

To assess whether this is true also for other UGT substrates, several structurally diverse compounds were incubated with [^{14}C]UDPGA and DLM and reaction mixtures were fractionated on SPE plates as described above. The compounds tested included drugs containing carboxylic acid moieties that are conjugated with UDPGA to form acyl glucuronides, such as naproxen, ketoprofen,

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furosemide, gemfibrozil and valproic acid, and endogenous compounds, chemicals or drugs containing hydroxyl or phenolic groups, such as the coumarin 4MU, the phenols 1NP and octyl gallate, the steroids 5α-androstane-3α,17α-diol, 5α-androstane-3α,17β-diol, β-estradiol, and 17α-ethynylestradiol, the bile acid hyodeoxycholic acid, and the drugs naloxone and propofol. As shown in Table 1, all of these compounds formed reaction products that eluted in the MeOH fraction.

One possible concern with the SPE extraction method is that not all of the glucuronide conjugates formed are retained on the SPE resin and/or that glucuronide conjugates are not quantitatively recovered in the MeOH eluate. To address this issue, the MeOH fractions containing glucuronide conjugates of the drugs described above were collected, evaporated to dryness, reconstituted in water and loaded on a second SPE plate. If glucuronide conjugates are not completely adsorbed to the resin, part of the radioactivity would be expected to elute in the water fraction of the second extraction, with a corresponding decrease of radioactivity in the MeOH fraction. The amount of radioactivity recovered in the MeOH fraction also provides an estimate for eventual losses due to compound remaining associated with the resin. For all tested compounds, radioactivity in the water fraction of the second extraction was not significantly higher than background (data not shown), indicating that all of the glucuronide conjugates quantitatively bound to the extraction resin. As shown in Table 1, [^{14}C]-labelled glucuronide conjugates were recovered in good yield in the MeOH fraction, with recoveries of 70-100% for all compounds.

Assay sensitivity. Assay sensitivity is limited by the specific radioactivity of the [^{14}C]UDPGA substrate and the signal to noise ratio. The specific radioactivity of [^{14}C]UDPGA can be increased either by increasing the amount of tracer radioactivity (which obviously has an impact on the cost of the assay), or by decreasing the concentration of unlabelled substrate. The concentration of UDPGA must remain sufficiently high to provide conjugating equivalents to aglycones, which are typically assayed at concentrations in the 0.1-1 mM range. Moreover,

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UDPGA is unstable in microsomal systems (Puhakainen and Hanninen, 1976), and preliminary experiments indicated that reactions were not linear for more than 10 minutes when total UDPGA concentrations < 0.5 mM were used. Most published UGT assays contain UDPGA at concentrations of 1-10 mM, and we used 2 mM in the present study. Under these conditions, with 2 x 10^5 dpm of [^{14}C]UDPGA, the specific radioactivity of the nucleotide sugar is 1000 dpm/nmol. For low turnover substrates, the UDPGA concentration can be decreased to 0.5 mM, resulting in a specific radioactivity of 4000 dpm/nmol.

Background radioactivity (noise) in the present assay had 2 distinct origins. First, as shown for the zero-time control in Table 1, a small amount (0.1% of total radioactivity) of [^{14}C]UDPGA eluted in the product (MeOH) fraction from the SPE plates. Second, there was a significant time- and microsome - dependent increase of counts eluting in the MeOH fraction in the absence of added aglycone (see blank controls in Table 1). The origin of this background activity, which was highest in DLM, is not known. One possibility is that it represents glucuronidation of a contaminant present in the assay reagents or in microsomes. Blank values were 0.27 ± 0.03, 0.66 ± 0.13, and 0.34 ± 0.04 percent of total radioactivity (average ± SEM, n = 9-14) after 40 min incubation with 0.5 mg/ml of rat, dog and human liver microsomes, respectively. The limit of detection of the assay (product counts 2-fold above blank values) was 0.3 nmol/min/mg microsomal protein for RLM and HLM, and about 2-fold higher for DLM.

Reaction kinetics. The formation rates of glucuronide conjugates for a series of typical UGT substrates were determined in rat, human, and dog liver microsomes at 2 different protein concentrations, 0.1 and 0.5 mg/ml. Results for 1NP and 4MU, are depicted in Fig. 2. At a microsome concentration of 0.1 mg/ml, 1NP glucuronidation was linear with incubation time up to 40 min in all species. Deviation from linearity was observed in RLM and DLM at the higher microsome concentration, likely due to substrate depletion (Figure 2A). Similar results were

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obtained for 4MU (Fig. 2B). The concentration dependence of 1NP and 4MU glucuronidation is shown in Fig. 3A and 3B, respectively. 1NP glucuronidation did not follow Michaelis Menten kinetics in microsomes from any of the species tested. Bell-shaped curves consistent with substrate inhibition were observed. To exclude that the decreased velocity at high (> 1 mM) substrate concentrations was due to depletion of the co-substrate UDPGA or to overloading of the SPE resin, incubations were also performed at a 5-fold higher UDPGA concentration (10 mM). Even though reaction velocities slightly increased at 10 mM UDPGA, similar bell shaped curves were observed (Fig 3A). Substrate inhibition kinetics has been described for several human UGT substrates (Court et al., 2002; Watanabe et al., 2002), including 1NP (Uchaipichat et al., 2004). The number of data points at high substrate concentration was insufficient for reliable curve fitting to a substrate inhibition model and kinetic parameters could not be calculated. The glucuronidation of 4MU followed Michaelis Menten kinetics in RLM and DLM, with K_m and V_{max} values of 1.1 mM and 160 nmol/min/mg, and 0.3 mM and 220 nmol/min/mg, respectively (Fig. 3B). In HLM, velocity increased linearly with substrate concentration, and no saturation was observed at concentrations up to 2 mM.

For all other compounds tested, glucuronide formation was linear for up to 40 min at one or both of the microsome concentrations used. Reaction rates are summarized in Table 2. Kinetic constants for glucuronidation in RLM, DLM and HLM of several of these substrates, namely furosemide, gemfibrozil, hyodeoxycholic acid, naloxone, 1NP, naproxen, valproic acid, ethynylestradiol, and propofol, have been published (Soars et al., 2001a; Soars et al., 2001b). Reaction velocities observed in the present study were on average 5-fold higher than those extrapolated from the V_{max} and K_m values determined by these authors using a radiometric HPLC method (data not shown). This could be due to a number of reasons, such as potential formation of multiple metabolites with some substrates, use of different microsome preparations, and different incubation conditions (assay buffer, use of alamethicin vs. sonication for activation of microsomes),

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which can affect glucuronidation velocity (Soars et al., 2003). When enzymatic activity was expressed relative to that of 1NP, a good agreement was observed between the results obtained with the present method and those calculated from the data of Soars and coworkers (Soars et al., 2001a; Soars et al., 2001b), as summarized in Table 3 for relative activities in HLM. A greater than 2-fold difference with the published data was observed only for hyodeoxycholic acid. The reason for this discrepancy is not known; possible explanations include different incubation conditions and enzyme source, as pointed out above. Our results also confirm the previous finding that glucuronidation velocities for most of these drugs are significantly higher in dog than in human liver microsomes (Soars et al., 2001b).

Reaction phenotyping. We next assessed whether the assay can be used for reaction phenotyping, i.e. the determination of which UGT isoforms metabolize a particular substrate. The activity of commercial recombinant human UGT preparations was determined using the non-selective substrate HFC (at 100 µM), whose glucuronidation is catalyzed by all isoforms tested, with the exception of UGT1A4. As shown in Table 4, HFC glucuronidation activities were between 0.6 and 10 nmol/min/mg microsomal protein. The activity of UGT1A4, determined using 200 µM of the specific substrate trifluoperazine was 0.7 nmol/min/mg. These results are in agreement with published data (Ghosal et al., 2004).

Glucuronidation of 5 different substrates by recombinant human UGTs was determined and results were compared to those of a conventional separation method using HPLC coupled to triple quadrupole mass spectrometric analysis for the detection of glucuronide conjugates. Glucuronidation activities determined by radiometric assay are reported in Table 4. Since synthetic glucuronide standards were not available, results were expressed as the amount of glucuronide conjugate (dpm or peak area) formed with each UGT isoform relative to that obtained in incubations with HLM. As shown in Fig 4, relative activities determined with the radiometric assay

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were similar to those of the LC-MS/MS method. In particular, the 2 methods identified the same major metabolizing enzymes for the different substrates tested, namely: UGT1A6, UGT1A7, and UGT1A9 for 4MU, UGT1A6 and UGT1A8 for 1NP, UGT2B7 for naloxone, UGT1A3 and UGT2B7 for ketoprofen, and UGT1A4 for trifluoperazine.

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DISCUSSION

It has previously been reported that acyl glucuronide conjugates of a series of carboxylic acid drugs can be separated from [^{14}C]UDPGA using C18 SPE cartridges (Pritchard et al., 1993). Using 15 structurally different UGT substrates, we demonstrated that Oasis™ SPE resin can be used to isolate not only acyl glucuronide conjugates but also ether glucuronates. The use of a 96-well extraction plate format represents a significant advantage in terms of ease, speed and throughput and allowed to miniaturize the assay, which is carried out in a reaction volume of 100 µl containing 10-50 µg of microsomal protein, vs. 200 µl and 200 µg respectively, for the C18 cartridge extraction method. There was good agreement between relative activities of typical UGT substrates obtained with the present assay and those determined using conventional HPLC-based methods. Moreover, the new method was used to confirm that glucuronidation velocities for many drugs are significantly higher in dog than in human liver microsomes (Soars et al., 2001b). These results provide evidence for the reliability of the assay and its usefulness to determine UGT activity with a variety of substrates in microsomes from different species.

The limit of detection of the new assay (0.3 nmol/min/mg in HLM) is identical to that of a previously described SPE method for the determination of acyl glucuronide formation (Pritchard et al., 1993). If reactions are carried out at substrate concentrations of 100 µM, and assuming that first order kinetics apply, this corresponds to a detection limit for intrinsic clearance of 3 µl/min/mg, similar to that of commonly used substrate depletion methods. The reported sensitivity of an HPLC-based radiometric glucuronidation assay (Ethell et al., 1998) is significantly higher (8 pmol/min/mg of HLM), but this value was obtained at a 6-fold higher microsome concentration than that used in the present work (3 mg/ml vs. 0.5 mg/ml), and represents product counts of about 100 dpm, which may not be easily quantifiable with many commonly used online radiochemical detectors.

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Normalizing for the different microsome concentrations, the present assay appears to be about 6-fold less sensitive than the HPLC-based method. Despite its somewhat decreased sensitivity, which limits its use with very low turnover substrates, the radiometric SPE method offers the advantage of greatly increased speed and throughput, since the entire procedure, namely incubation and product separation is carried out in 96-well plates, and does not require specialized equipment, such as HPLC and online radiochemical, mass spectrometric or fluorimetric detectors. It should be noted that assay sensitivity can be increased at least 4-fold by decreasing the concentration of unlabelled UDPGA to 0.5 mM and/or using higher amounts of radiolabelled substrate or microsomes.

The results of reaction phenotyping studies with the present method are in general agreement with previous studies, in particular for the known UGT specificity of ketoprofen (Sakaguchi et al., 2004), naloxone (Coffman et al., 1998), and trifluoperazine (Ghosal et al., 2004), although some differences were observed with the reported ranking of the activities of different UGTs toward 4MU and 1NP (Uchaipichat et al., 2004). Thus, in our hands the activity of UGT2B7 toward 4MU and 1NP and that of UGT1A6 toward 1NP were significantly (> 20-fold) higher than reported by Uchaipichat et al. (2004), while that of UGT1A10-mediated 4MU glucuronidation was significantly (14-fold) lower. The authors of this study had noted a similar difference between their data and the high UGT1A6 activity toward 1NP observed by Soars et al. (2003). Consistent with the present data, relatively high activity of UGT1A6 and UGT2B7 with 1NP (Gschaidmeier et al., 1995; Kurkela et al., 2003) and low activity of UGT1A10 with 4MU (Strassburg et al., 1998; Cheng et al., 1999) has been reported. It should be noted that Uchaipichat et al. (2004) used cell lysates from a mammalian UGT-expressing cell line while baculovirus-expressed enzymes were used in the present study. The amount of UGT protein in different recombinant preparations is not known, which precludes normalization of enzymatic activities that are typically expressed relative to the total microsomal or cellular protein content. Sequence variability (Uchaipichat et al., 2004), different membrane composition or environment (Nakajima et al., 2002), or different methods for

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microsome permeabilization (Soars et al., 2003) may be additional factors contributing to interlaboratory differences in enzyme activity. For commercial UGT preparations, enzymatic activities reported by different suppliers are frequently obtained using different expression systems, substrates, incubation conditions and analytical methods. On the basis of these considerations, it appears advisable to determine the activity of recombinant UGT preparations with several well characterized reference compounds and a standard assay method before using them for reaction phenotyping studies.

In conclusion, we developed a 96-well non HPLC assay method that allows rapid determination of UGT activities with a variety of substrates. At the present time, we cannot claim that the method can be universally applied to all UGT substrates, since a limited number of compounds were tested. It cannot be excluded that some very hydrophilic glucuronide conjugates would not be retained by the SPE resin, although this was not observed with the compounds analyzed so far. More extensive studies with a large number of UGT substrates will be carried out to clarify this point. A significant difference with HPLC methods is that if a substrate contains several glucuronidation sites (such as β-estradiol and androstanediol, which form 3α- and 17β-glucuronides), the different conjugation products are not resolved and only total glucuronidation is measured. Despite these potential limitations, the new assay has several important applications. First, it can be used for benchmarking, i.e. for easily and rapidly determining the quality of microsome preparations from several species or organs and of recombinant UGT batches using reference compounds. Second, in a drug discovery setting, the assay should prove valuable to screen chemical series with known glucuronidation properties in order to identify and flag high turnover compounds. Finally, the method offers a convenient and simple alternative to existing technologies for carrying out reaction phenotyping studies with new chemical entities, comparison of UGT activities with those of reference compounds, and determination of enzyme kinetics.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Separation of the [^{14}C]-labelled glucuronide conjugate of 1NP from [^{14}C]UDPGA by SPE. DLM were incubated for 120 min with ~0.1 µCi [^{14}C]UDPGA/2 mM UDPGA in the absence (empty bars) or presence (solid bars) of 1 mM 1NP. Incubation mixtures were deproteinized and loaded on Oasis™ SPE plates. Plates were eluted with 2 x 0.5 water (pooled eluates denominated "water 1"), followed by 4 additional washes with 1 ml of water (eluate fractions "water 2-5"), 2 x 0.5 ml of MeOH (pooled eluate fraction "MeOH 1"), and 2 x 1 ml of MeOH (fractions "MeOH 2-3). Radioactivity in the eluates was expressed as a percentage of the total radioactivity in each incubation mixture and represents the mean ± SD, n = 3.

Figure 2. Effect of incubation time and microsome concentration on [^{14}C]glucuronide formation from 1NP and 4MU. A, incubations with 1 mM 1NP. B, incubations with 0.5 mM 4MU. Compounds were incubated for the indicated times with RLM (upper panels), DLM (middle panels) or HLM (lower panels). Microsome concentrations were 0.1 mg/ml (filled symbols) and 0.5 mg/ml (empty symbols). Each point is the mean ± half-range from duplicate determinations.

Figure 3. Concentration dependence of 1NP- and 4MU-[^{14}C]glucuronide formation rates. A, Reaction with 1NP. B, Reaction with 4MU. Compounds were incubated for 30 min with RLM (upper panels), DLM (middle panels) or HLM (lower panels). Incubations contained a total UDPGA concentration of 2 mM (empty symbols) or 10 mM (filled symbols). Each point is the mean ± half-range from duplicate determinations.

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Figure 4. Glucuronidation by individual UGTs. Recombinant UGTs and HLM were incubated with 1 mM 1NP (A), 0.5 mM 4MU (B), 1 mM ketoprofen (C), 0.2 mM trifluoperazine (D) and 1 mM naloxone (E). Aliquots of the reaction mixtures were analyzed by LC-MS/MS (empty bars) or SPE and scintillation counting (solid bars). For each UGT, glucuronide peak areas from MS analysis and radioactive product counts from SPE were expressed as a percentage of the corresponding signals from incubations with HLM. Results are mean values ± half-range from duplicate determinations.

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TABLE 1

Validation of SPE method

Compound	Concentration	Radioactivity eluted in first extraction[a]			Recovery of glucuronide in second extraction[b]
		Water fraction	MeOH fraction	Total	
	mM	*% of total [^{14}C]UDPGA in incubation*			*%*
4MU	0.5	19.5 ± 7.3	70.3 ± 5.8	89.8	69 ± 3
HFC	1	98.7 ± 2.4	8.5 ± 2.4	107.2	86 ± 25
Octyl Gallate	0.5	74.5 ± 1.8	8.3 ± 0.5	82.8	73 ± 10
1NP	1	45.4 ± 22.9	64.6 ± 16.0	110.0	95 ± 6
5α-Androstane-3α,17α-diol	0.25	99.9 ± 15.8	4.9 ± 0.4	104.8	89 ± 6
5α-Androstane-3α,17β-diol	0.25	80.4 ± 2.1	6.6 ± 0.3	87.0	94 ± 4
Hyodeoxycholic acid	1	75.0 ± 10.0	9.2 ± 0.1	84.2	86 ± 16
β-estradiol	1	101.7 ± 3.1	5.8 ± 0.4	107.6	87 ± 7
Ketoprofen	1	84.0 ± 10.9	21.7 ± 4.7	105.6	82 ± 13
Naproxen	1	82.0 ± 3.3	14.1 ± 0.4	96.0	89 ± 12
Naloxone	1	97.4 ± 2.5	12.8 ± 0.5	110.3	78 ± 14
Furosemide	1	82.5 ± 6.3	10.2 ± 1.4	92.8	76 ± 8
Gemfibrozil	1	45.6 ± 2.3	41.7 ± 1.7	87.0	84 ± 1
Valproic acid	1	62.0 ± 4.1	29.7 ± 1.1	91.7	69 ± 1
17α-Ethynylestradiol	0.25	ND	4.6 ± 0.2	ND	115 ± 25
None (blank)		98.8 ± 1.8	0.72 ± 0.07	99.4	
None (Time zero)		100.1 ± 1.8	0.11 ± 0.02	100.2	

ND, not determined. Results are mean values ± SD, n = 3, except for the blank and time zero controls (SEM, n = 15)

[a] Compounds were incubated for 120 min with 0.5 mg/ml of DLM. Deproteinized reaction mixtures were loaded on SPE plates which were eluted with 4 x 1 ml of water and 2 x 0.5 ml of MeOH. Results represent the percentage of radioactive counts eluting in the water and methanol fractions relative to the total [^{14}C]UDPGA present in incubations.

[b] MeOH fractions from the first incubation were dried, reconstituted in water and loaded on a second extraction plate, which was eluted with water followed by MeOH. Results represent the % of radioactivity recovered in the MeOH eluate of the second plate relative to the total input.

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TABLE 2

Glucuronidation of compounds in human, dog and rat liver microsomes

Compound	Concentration	Reaction velocity[a]		
		HLM	DLM	RLM
	mM		*nmol/min/mg*	
4MU	0.5	37 ± 7	120 ± 11	34 ± 9
Octyl Gallate	0.5	6.7 ± 2.3	3.9 ± 1.3	9.4 ± 0.15
1NP	1	27 ± 4	121 ± 15	38.1 ± 2.2
5α-Androstane-3α,17α-diol	0.25	1.4 ± 0.04	1.4 ± 0.2	1.0 ± 0.04
5α-Androstane-3α,17β-diol	0.25	5.8 ± 0.6	6.8 ± 2.2	7.4 ± 0.8
Hyodeoxycholic acid	1	3.9 ± 0.2	3.8 ± 0.4	<0.3
β-estradiol	1	1.3 ± 0.03	21.1 ± 1.7	4.1 ± 0.2
Naproxen	1	0.8 ± 0.14	4.6 ± 0.3	4.10 ± 1.0
Naloxone	1	1.4 ± 0.2	5.6 ± 0.5	5.0 ± 0.3
Furosemide	1	0.49 ± 0.04	1.3 ± 0.1	<0.3
Gemfibrozil	1	2.4 ± 1.0	13.8 ± 2.2	3.9 ± 0.3
Valproic acid	1	0.6 ± 0.2	5.0 ± 1.4	11 ± 5
17α-Ethynylestradiol	0.25	0.6 ± 0.1	<0.6	0.6 ± 0.1
Propofol	0.5	1.3 ± 0.3	<0.6	1.4 ± 0.4

[a] Incubations were performed in duplicate at 2 different microsome concentrations (0.1 and 0.5 mg/ml) and stopped after 10, 20, and 40 min. Reactions were linear with time at one or both microsome concentrations. Reaction velocities were determined by linear regression analysis from the slope of the product formation time course. Results are mean values ± standard error of the fit, or mean values ± half-range of the velocities obtained at 2 microsome concentrations.

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TABLE 3

Comparison of relative reaction rates with literature data

Compound	Relative Activity[a] in HLM			Activity ratio[b] DLM/HLM	
	HPLC method[c]	SPE method[d]	Ratio SPE/HPLC	HPLC method[c]	SPE method[d]
Furosemide	24	35	1.5	6.4	2.7
17α-Ethynylestradiol	28	43	1.5	7.4	nd
Naproxen	52	57	1.1	8.9	5.8
Propofol	58	93	1.6	nd	nd
Valproic acid	62	43	0.7	6.5	8.3
Naloxone	100	100	1.0	15.4	3.9
Gembfibrozil	182	136	0.7	10.0	5.8
Hyodeoxycholic acid	1422	207	0.15	0.3	1.0
Naphtol	2015	1929	1.0	10.6	4.5

[a] Reaction velocities are expressed relative to that of naloxone (relative activity = 100).
[b] ratio of reaction velocity in dog vs. human liver microsomes
[c] Radiometric HPLC method. Velocities at the drug concentration used were calculated from the V_{max} and K_m values given in Soars et al. (2001b).
[d] Radiometric SPE method (present work). Velocities are given in Table 2.
The concentration of compounds in microsomal incubations was 1 mM, except for propofol (0.5 mM) and 17α-ethynylestradiol (0.25 mM).
nd, not determined

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Figure 1

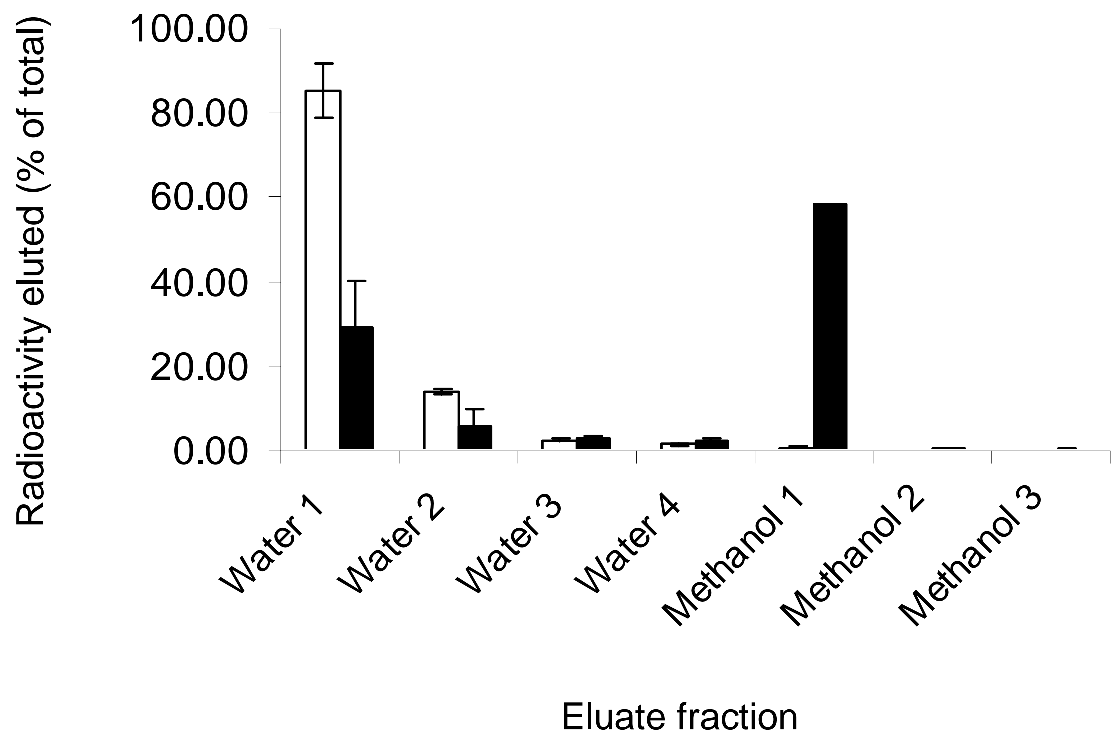

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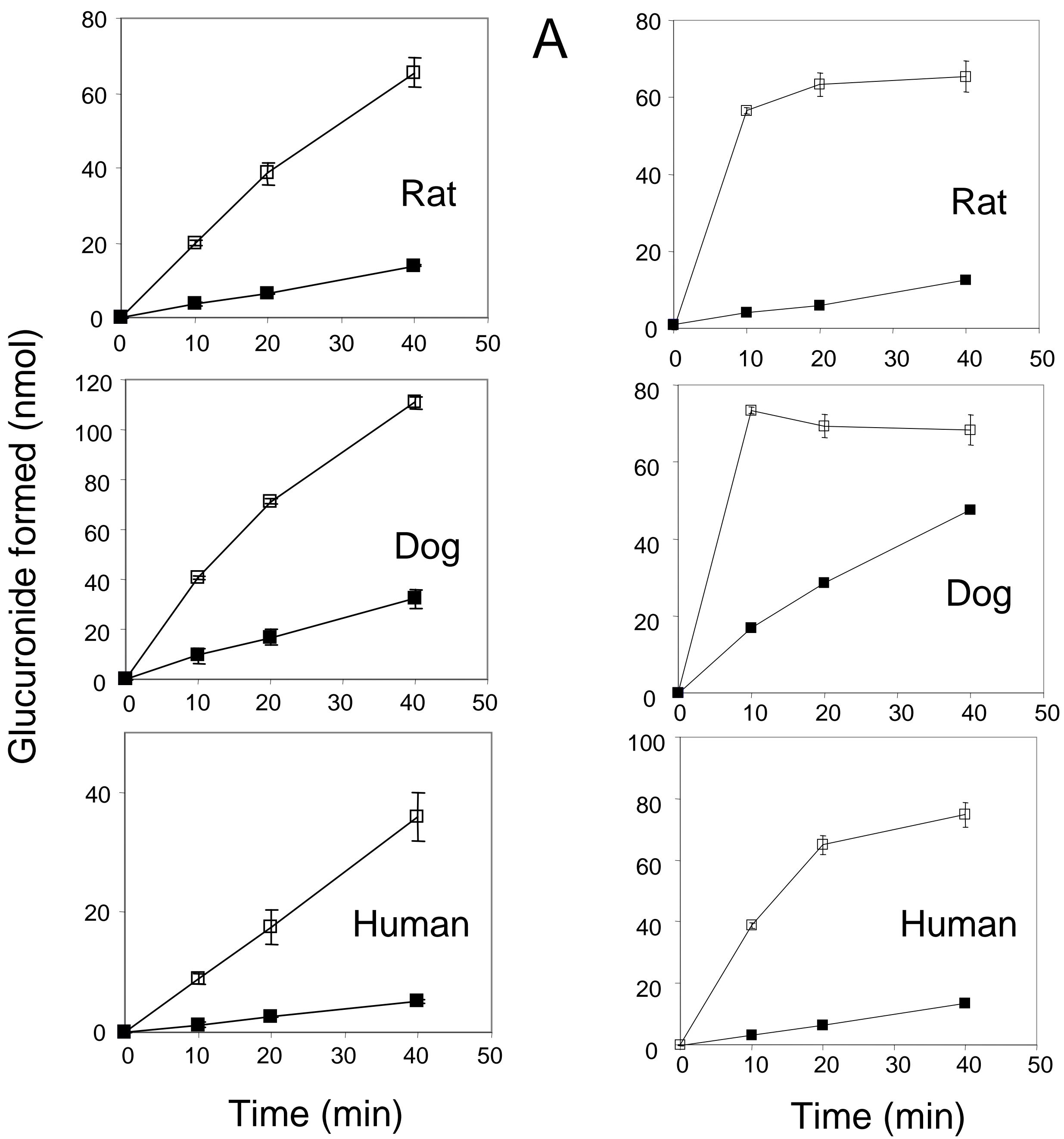
Figure 2



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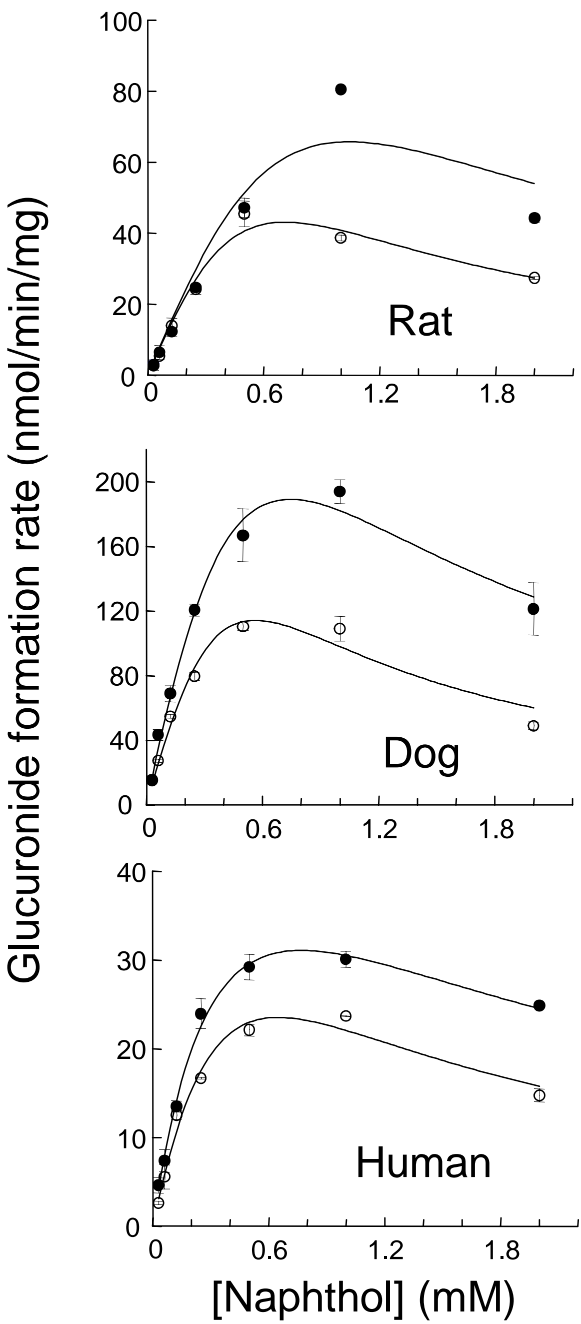

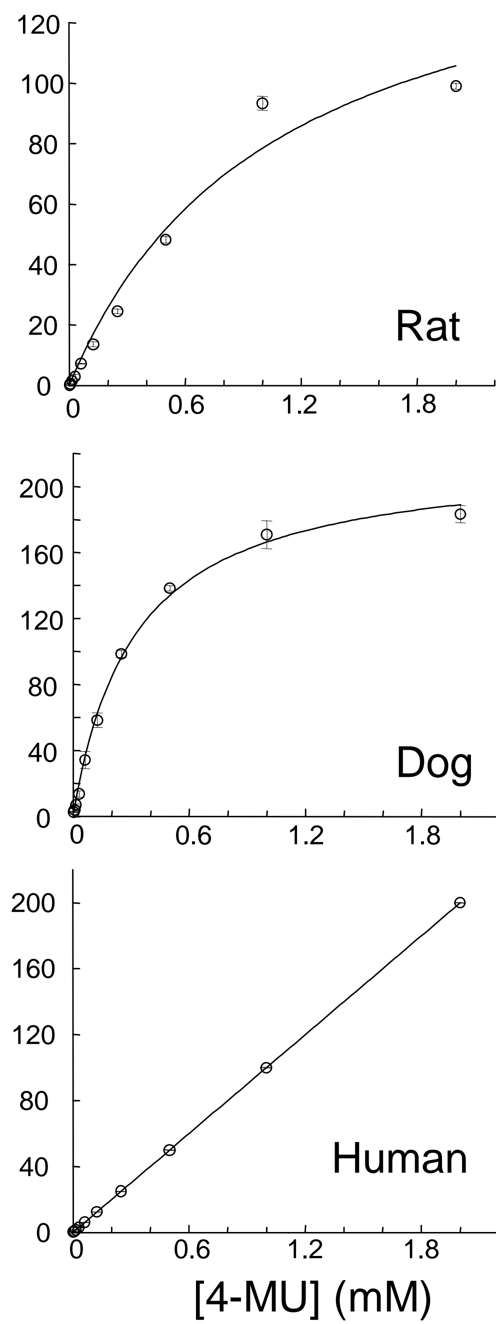

Figure 3

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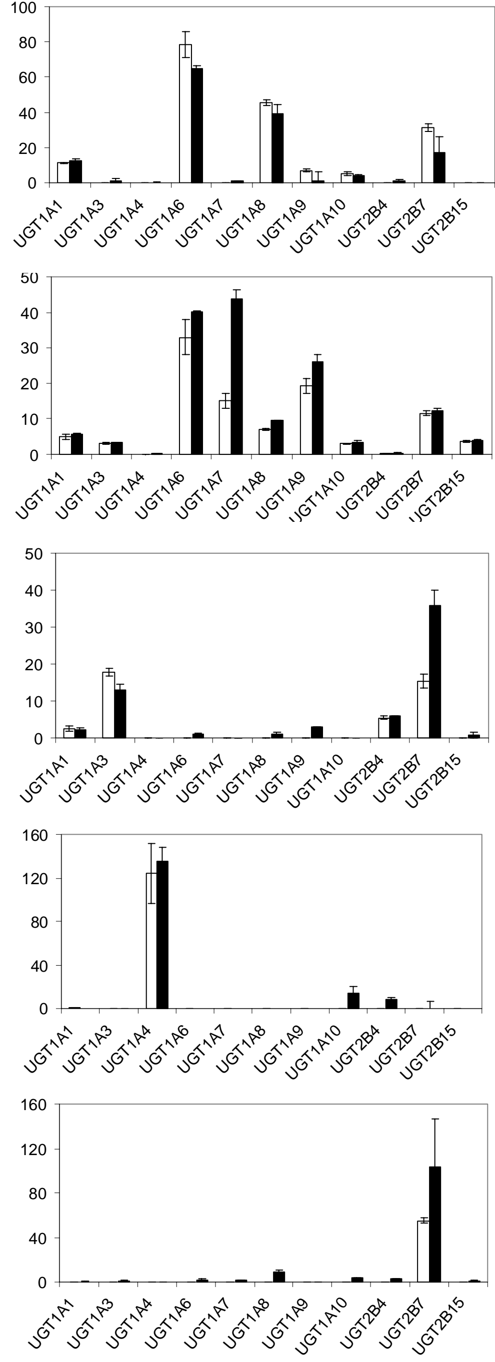
Figure 4

Glucuronide formation (% of HLM signal)



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