Biosynthesis of Dobutamine Monoglucuronides and Glucuronidation of Dobutamine by Recombinant Human UDP-Glucuronosyltransferases

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ABSTRACT:

Selected aspects of dobutamine glucuronidation were studied in detail. There are potentially four sites at which dobutamine can be conjugated to glucuronic acid. Three of the four dobutamine monoglucuronides that can be formed were enzymatically synthesized using pig liver microsomes, isolated, and characterized by tandem mass spectrometry, and 1H and 13C NMR spectroscopy. Analysis of dobutamine glucuronidation by liver microsomes from various sources revealed large variability in the ratios of the three regioisomers. Interestingly, catecholic dobutamine meta-O-glucuronide, by far the major product synthesized with human liver microsomes, was only a minor product for rat liver microsomes. Rabbit liver microsomes yielded dигlucuronides, in addition to monoglucuronides. Activities of individual recombinant human UDP-glucuronosyltransferases (UGTs) were investigated, and the results suggested that dobutamine glucuronidation in the human liver is mainly carried out by UGTs 2B7 and 1A8. Among the extrahepatic UGTs, the formation of monoglucuronides, mainly catecholic meta-O-glucuronide, by UGTs 1A7 and 1A8 was similar to that by 1A9, whereas UGT1A10 also efficiently catalyzed the formation of catecholic dobutamine para-O-glucuronide.

Enzyme-catalyzed drug metabolism occurs in phase I and phase II reactions. Phase I reactions add or expose a polar functional group, and phase II reactions, known as conjugation reactions, increase the hydrophilic nature of the molecule. Typically, the reaction order is from phase I to phase II, but a compound may be excreted directly after phase I reaction, or the phase II reaction may occur without the phase I reaction if the substrate contains an appropriate functional group. The products of these metabolic reactions are often inactive, hydrophilic compounds that are easily excreted via urine or bile. Sometimes, however, the metabolites are biologically active or even toxic.

Glucuronidation is an important conjugation reaction catalyzed by the UDP-glucuronosyltransferases (UGTs). The human UGTs are classified into UGT1 and UGT2 families according to their amino acid sequence and gene organization (Mackenzie et al., 1997; Tukey and Strassburg, 2000). The UGT2 family is divided further into two subfamilies, UGT2A and UGT2B. The UGTs are mainly expressed in the liver but also in other organs, such as intestine and kidney (Tukey and Strassburg, 2000). The aglycone substrates of the UGTs must have a functional group to which the sugar can be linked. The O-glucuronides are formed when the substrate contains an aryl, alkyl, enolic, or acyl hydroxy group, whereas N-glucuronides are formed with amines and sulfonamides. S-Glucuronides and C-glucuronides are also known, but usually as minor conjugates. The UGTs catalyze the glucuronidation of a large number of endobiotics and xenobiotics, and there is considerable overlap in the substrate specificity of some UGTs (Burchell et al., 1995; Ritter, 2000).

Glucuronidation of certain substrates may be dissimilar between humans and laboratory animals; for example, the glucuronidation of angiotensin II receptor antagonists (Huskey et al., 1993), 1-substituted imidazoles (Kaivosaari et al., 2002; Vashishtha et al., 2002), and statins (Prueksaritanont et al., 2002). Because this topic has not been systematically studied, despite the widespread use of laboratory animals in toxicity tests, a general summary of interspecies glucuronidation is not available. Still, glucuronidation of amines, especially tertiary amines, has been shown to exhibit species differences (Chiu and Huskey, 1998). Earlier, metabolism of tertiary amines to form quaternary N-glucuronides was thought to be the exclusive ability of humans and higher primates, but recently it has been found that rabbits (Li et al., 2001) and rats (Singh et al., 2001; Vashishtha et al., 2002) also glucuronidate tertiary amines.

In this work we studied the glucuronidation of dobutamine, a synthetic sympathomimetic agent derived from the endogenous catecholamine dopamine. Dobutamine strongly activates adrenergic β1-receptors and moderately activates α1- and β2-receptors, and it has more inotropic than chronotropic action on the heart. Dobutamine glucuronides have been detected in human (Yan et al., 2002) and dog (Murphy et al., 1976) urine. However, the site of glucuronidation was not then discovered because the glucuronides were measured indirectly, after enzymatic hydrolysis. The lack of commercially available

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; glu, glucuronic acid; MS/MS, tandem mass spectrometry; UDPGA, uridine-5’-diphosphoglucuronic acid; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; LC, liquid chromatography; MS, mass spectrometry; DQFCOSY, double quantum filtered correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation.

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reference materials is a general problem that limits metabolism research as well as pharmacological and toxicological studies of the metabolites. Enzyme-assisted synthesis has proven to be an easy and rapid method to produce metabolites, for example glucuronides, at milligram scale.

Dobutamine is an interesting molecule for investigation of the site of glucuronidation since the formation of three O-glucuronides and one N-glucuronide is theoretically possible (Fig. 1). One aim of this study was to synthesize the dobutamine glucuronide regioisomers enzymatically and to characterize their structures by tandem mass spectrometry (MS/MS) and NMR spectroscopy. In addition, the site-specific glucuronidation of dobutamine was compared among human, rabbit, rat, and pig liver microsomes as well as between individual recombinant human UGTs, using the enzymatically synthesized glucuronides as reference standards.

**Materials and Methods**

**Reagents.** Dobutamine hydrochloride was purchased from Eli Lilly & Co. (Indianapolis, IN), and saccharic acid 1,4-lactone and uridine-5'-diphosphoglucuronic acid (UDPGA, trisodium salt) were obtained from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade solvents and analytical-grade reagents were used in all experiments. Pooled human liver microsomes were purchased from BD Gentest (Woburn, MA), and male New Zealand White rabbit liver microsomes from In Vitro Technologies (Baltimore, MD). Rat liver microsomes were prepared from Aroclor 1254-induced male Sprague-Dawley rats and pig liver microsomes from untreated pig, as previously described (Luukkanen et al., 1997), in our laboratory. The human UGTs 1A1, 1A3, 1A4, 1A6–1A10, 2B4, 2B7, and 2B15). The substrate in dimethyl sulfoxide (DMSO) solution was added so that the final concentration of DMSO was 2% of the reaction volume. Incubation mixtures contained 5 mM saccharic acid 1,4-lactone, 5 mM MgCl₂, 50 mM phosphate buffer (pH 7.4), 625 μg of protein, and 5 mM UDPGA. Enzyme reactions (250 μl) were initiated by the addition of UDPGA, and mixtures were incubated at 37°C for 240 min. Samples, except reference samples, were prepared in duplicates. Reference samples contained 1000 μM dobutamine and the tested microsomes or enzyme, but no UDPGA was added. The reactions were terminated by the addition of 25 μl of ice-cold 4 M perchloric acid. The test tubes were kept on ice at least 10 min and then centrifuged (14,000 rpm, 5 min). Supernatants were analyzed by HPLC.

**Chromatography.** The same instrument and method were used in fractionating, in purity analysis of synthesis products, and in activity assays. The HPLC apparatus was an Agilent 1100 Series HPLC equipped with an autosampler and the Agilent 1100 Series Fluorescence and UV diode array detectors (Agilent Technologies, Waldbronn, Germany). An HP Hypersil BDS C18 column (5 μm 250 × 4.0) (Agilent Technologies, Palo Alto, CA) was used for the chromatographic separation, and the oven temperature was 40°C. The eluent was 20 mM ammonium acetate (pH 4.5)-methanol 82:18, used at a flow rate of 1 ml/min. In fractionation, the separated glucuronides were collected with the Agilent 1100 Series fraction collector. Automated collection of

![Fig. 1. Dobutamine and its theoretical monoglucuronide metabolites: phenolic dobutamine O-glucuronide (A), dobutamine N-glucuronide (B), and two catecholic dobutamine O-glucuronides (C and D).](image-url)
fractions was carried out overnight (10 h and 25 min) by injecting 50 μl of purified synthesis mixture 25 times. The purity tests were carried out with the diode array detector, and in activity tests, the glucuronides were measured with the fluorescence detector (excitation and emission wavelengths 285 nm and 313 nm). The injection volume in these analyses was 40 μl, and in activity tests, a signal to noise ratio of 3 was set as the detection limit.

**Structure Characterization.** The mass spectrometric analyses were carried out on a PerkinElmerSciex API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex, Boston, MA) equipped with an ionspray source. Compressed air (Atlas Copco CD-2 system, Wilrijk, Belgium) was used as a nebulizing gas with a flow rate of 1.23 l/min. Nitrogen (Whatman 75-72 nitrogen generator; Whatman, Clifton, NJ) was used as both collision and curtain gas (1.25 l/min). In positive ion mode, the ionspray needle voltage was 5000 V and the orifice (declustering) voltage 30 V; in negative ion mode, the corresponding values were −4200 V and −30 V. The mass spectra were measured with a liquid chromatograph-mass spectrometer operating in positive ion mode and using the LC method described above. The flow was split 1:16 before the MS analyses. The MS/MS spectra were measured by direct infusion of sample solution [20 mM ammonium acetate (pH 4.5)-methanol 1:1, in positive ion mode and 10 mM ammonium acetate-methanol 1:1 in negative ion mode] using a Harvard Apparatus microsyringe pump (Harvard Apparatus Inc., Holliston, MA) with a flow rate of 10 μl/min. The scan range in the MS measurements varied between m/z 50 and 700, with a scan speed of 1 s/scan. In MS/MS experiments, the protonated or deprotonated molecule was selected as precursor ion, and the collision offset voltage was 40 eV in positive ion mode and 35 eV in negative ion mode. Analyst 1.3.1 software (PerkinElmer Sciex) was used for instrument control and data processing.

Double quantum filtered correlated spectroscopy (DQFCOSY), nuclear Overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra were recorded for fractions I, II, and III (see Results). The NMR experiments for fractions I and II, as well as NOESY and DQFCOSY spectra for fraction III, were performed on a Varian Unity 500 MHz spectrometer (Varian, Inc., Palo Alto, CA). The heteronuclear experiments were measured using a gHX nano-NMR probe. For the HSQC and HMBC spectra (16 and 128 scans per t1 value, respectively), matrices of 4096 × 256 points were recorded and zero-filled to 4096 × 512 points. The average 1H-13C coupling constant was estimated to be 140 Hz, and Δ2 was 60 ms. For the DQFCOSY and NOESY spectra (8 and 16 scans per t1 value, respectively), matrices of 4k × 256 points were recorded and zero-filled to 4k × 512 points. In recording the NOESY spectra, a mixing time of 200 ms was used. The rest of the NMR experiments of fraction III were carried out with a Varian Unity 300 MHz spectrometer. The HSQC and HMBC spectra were recorded similarly to those for fractions I and II. One-dimensional 1H spectra were also acquired for each fraction at 23°C and at 40°C to resolve overlapping signals for the two diastereomers and to determine coupling constants. One-dimensional 13C spectra were measured for fractions I and III. For comparisons, one-dimensional DQFCOSY, HSQC, and HMBC spectra were also recorded for dobutamine. Two-dimensional spectra for fractions I, II, III, and dobutamine were measured at 23°C, except DQFCOSY, measured at 40°C. DMSO-d6 was used as solvent in all experiments, and the 1H and 13C chemical shifts were referenced to residual DMSO, 2.50 ppm and 39.5 ppm, respectively.

**Results**

**Synthesis of Dobutamine Monoglucuronides.** Human, rabbit, rat, and pig liver microsomes, and 11 recombinant human UGTs were tested as sources for the enzymatic synthesis of dobutamine glucuronides. Pig liver microsomes produced three regioisomers of the dobutamine glucuronides with good and equal efficiency and were chosen as the biocatalyst. The HPLC chromatogram of the purified synthetic mixture showed dobutamine monoglucuronides as three main peaks at retention times of 4.9, 7.7, and 12.1 min and one minor peak at a retention time of 7.1 min (Fig. 2). The main peaks were collected (fractions I, II, and III, respectively) by LC. The minor peak at retention time 7.1 min was not collected as a separate fraction because its concentration was very low, and it was poorly separated from the large peak at retention time 7.7 min (Fig. 2). After purification and freeze-drying, fractions I, II, and III contained 1.7 mg, 3.5 mg, and 6.2 mg of dobutamine monoglucuronides, respectively.

**Mass Spectra.** The electrospray ionization-MS spectra of the four compounds detected by LC-MS, as well as the direct infusions of glucuronide fractions I, II, and III, showed abundant ion at m/z 478 in positive ion mode, corresponding to the protonated molecule of dobutamine monoglucuronide. The direct infusion of glucuronide fractions I, II, and III in negative ion mode showed ion m/z 476 that corresponds to deprotonated dobutamine monoglucuronide. These two ions were selected as the precursor ions for the MS/MS analyses. All three glucuronides showed similar product ions, but with slightly different relative abundances. The main product ions of the protonated molecules were m/z 302, 166, 137, and 107, whereas those of the deprotonated molecules were m/z 300, 175, and 113 (Table 1).

**NMR Spectra.** Glucuronidation of dobutamine produced diastereomers, since the dobutamine that was used as the starting material was a racemate. Although enzymes are often stereospecific, pig liver microsomes, which were used as a biocatalyst, produced both diastereomers. This was observed in the NMR spectra of glucuronide fractions I, II, and III as two sets of signals produced by a mixture of two diastereomers. The signals of the two diastereomers were extensively overlapping, resulting in more complex one-dimensional spectra than expected. Only a few nuclei gave two distinguishable signals corresponding to the two forms (Table 2), either at 23°C or at 40°C. The signals shifted relative to each other when the temperature was changed, ruling out the possibility that the shape of the signals resulted from scalar coupling and, rather, indicated the presence of two compounds. The 1H signals of the dobutamine glucuronides were assigned from their DQFCOSY spectra, and the corresponding 13C
resonances were located in the HSQC spectra. HMBC spectra were used to assign the chemical shifts of quaternary carbons. G1 protons and carbons were identified by the characteristic chemical shifts near 5 ppm and 100 ppm, and the nuclei G2 to G6 were assigned on the basis of the DQFCOSY, HSQC, and HMBC spectra. The NMR spectra indicate that fractions I, II, and III (detected at retention times of 4.9, 7.7, and 12.1 min) were phenolic O-glucuronide (compound A), catecholic para-O-glucuronide (compound C), and catecholic meta-O-glucuronide (compound D) of dobutamine (Fig. 1), respectively. The reasons for these assignments are detailed under Discussion.

**Purity and Yields.** The purity of the products synthesized with pig
Liver microsomes was assessed by HPLC with diode array and fluorescence detection, and by direct infusion MS. The HPLC studies indicated that the three main products synthesized, phenolic dobutamine O-glucuronide (A), catecholic dobutamine para-O-glucuronide (C), and catecholic dobutamine meta-O-glucuronide (D), contained 1.2%, 2.2%, and 0.12% of other monoglucuronides, respectively. Neither residual dobutamine nor by-products were detected. The yields of compounds A, C, and D were 4.6% (1.7 mg), 9% (3.5 mg), and 17% (6.2 mg), respectively.

**Activity Assays.** The glucuronidation of dobutamine was studied with human, rabbit, rat, and pig liver microsomes and with 11 human recombinant UGTs. As shown in Fig. 3, all the animal liver microsomes produced the three glucuronides, A, C, and D, but in significantly different relative amounts. In particular, the human liver microsomes differed markedly from the other tested microsomes in their glucuronide profile. The main product in the reactions catalyzed by human liver microsomes was D, and the relative amounts of the other glucuronides were much lower than those for rabbit, rat, or pig liver microsomes. Glucuronide C was the major product of the rat and rabbit liver microsomes, but the rat liver microsomes produced significantly more A and less D than the rabbit liver microsomes. In the reactions catalyzed by pig liver microsomes, the three monoglucuronides, A, C, and D, were produced in similar amount (Fig. 3).

In addition to compounds A, C, and D, rabbit liver microsomes produced peaks at retention times of 2.9 and 4.2 min, which were identified as dobutamine diglucuronides by positive ion LC-MS (MH$^+$ = m/z 654). Relative to the peak area of compound C, the amounts of diglucuronides were 61% and 11% when the dobutamine concentration was 250 μM. Traces of diglucuronides were also detected in reactions catalyzed by rat liver microsomes.

The formation of dobutamine glucuronides A, C, and D by 11 recombinant human UGTs was also tested. The relative expression level of the UGTs was determined and taken into account, and the normalized efficiencies of these enzymes are presented in Fig. 4. All the human UGTs, with the exception of UGT1A4, catalyzed the formation of dobutamine monoglucuronides A, C, and D (Fig. 4). In the reaction catalyzed by UGT1A4, only glucuronide A was detected. UGT1A6 produced the glucuronide C only at a substrate concentration of 1000 μM, and with UGT2B15, the glucuronide D was detected only when the substrate concentration was 250 μM or 1000 μM. Trace amounts of diglucuronides were detected in the UGT1A1 reaction. The catalytic activity of the UGTs varied widely. The most active ones were UGTs 1A7, 1A10, and 2B7. The UGTs 1A8, 1A9, and 2B4 showed considerable activity, whereas the activity of 1A1 and 1A3 was low. UGTs 1A4, 1A6, and 2B15 exhibited only poor activity.

UGTs 2B7 and 1A7, and, to a lesser extent, also 1A9 and 2B4, were highly selective, producing glucuronide D almost exclusively (Fig. 4). The UGTs 1A1, 1A3, and 1A10 produced all three glucuronides, C being the major product with 1A1 and 1A10, but there was a major difference in relative efficiency of UGTs 1A1 and 1A10. Glucuronide A was the main product with UGTs 1A4 and 2B15.

**Discussion**

Human, rabbit, rat, and pig liver microsomes were tested as biocatalyst for the synthesis of dobutamine glucuronides. The results in Fig. 3 show that human microsomes catalyze catecholic glucuronide D more regioselectively than did animal microsomes and that the correlation between animal and human liver microsomes is poor. Since pig liver microsomes produced the three glucuronides A, C, and D with relatively high activity, they were selected as biocatalyst for the enzymatic synthesis. The use of pig liver microsomes was also favored by the availability.

From 77.9 μmol of dobutamine, the optimized synthesis produced 3.6 μmol (1.7 mg, 5%), 7.3 μmol (3.5 mg, 9%), and 13.0 μmol (6.2 mg, 17%) of glucuronides A, C, and D, respectively. The purities of the glucuronides were determined by HPLC with UV diode array detection and by MS. The results showed that the purities of all the glucuronides were at least 97.8% and high enough to be used as standard reference compounds. Hence, the enzyme-assisted synthesis method developed here offers a convenient and easy way to synthesize dobutamine glucuronides in milligram amounts.

The fractionated and purified glucuronides were characterized by MS/MS and NMR. The synthesis products A, C, and D were characterized as dobutamine monoglucuronides, since their positive and negative ion electrospray mass spectra showed abundant protonated [M + H]$^+$ and deprotonated [M – H]$^-$ ions with the corresponding masses. The MS/MS spectra of the protonated molecule of all the glucuronides showed abundant product ion at m/z 302 formed by the neutral loss of glucuronide moiety (glu; 176 Da). After this, the N–C(11) bond was dissociated, and ions [M + H – glu – OH$_2$PhCH$_2$Cl]$^+$ (m/z 166) and [M + H – glu – NH$_3$CH$_2$(CH$_3$)$_2$CH$_2$PhOH]$^+$ (m/z 137) were...
produced. The product ion at m/z 107, which was formed by further fragmentation of m/z 166, was CH$_2$PhOH$^+$. The MS/MS spectra of the deprotonated molecules of all the glucuronides showed a main product ion at m/z 300 due to loss of the glucuronide moiety. The ion m/z 175 was a deprotonated glucuronic acid, which was further fragmented by the loss of H$_2$O and CO$_2$ to give the ion at m/z 113. The MS/MS spectra confirmed that compounds A, C, and D were dobutamine monoglucuronides. However, similar to early work (Keski-Hynnila et al., 2002), the MS/MS spectra did not show any diagnostic product ion for the determination of the glucuronidation site.

The glucuronidation site was determined from the NMR spectra of glucuronides A, C, and D. The comparison of $^1$H chemical shifts of dobutamine with that of compound A revealed that the largest change in chemical shift upon glucuronidation occurred for H2/H6 (0.23 ppm downfield) (Table 2). In its NOESY spectrum, the only observed interglycosidic correlation was between GH1 (4.87 ppm) and H2/H6 (6.93 ppm), and the only interglycosidic correlation in the HMBC spectrum was between GH1 (4.87 ppm) and C1 (155.7 ppm). These results confirmed that compound A was the phenolic O-glucuronide. In the case of compound D, the most significant change in chemical shift in comparison to dobutamine occurred for H14 (0.35 ppm downfield). The correlation between GH1 (4.74 ppm) and C15 or C16 (145.1 ppm) in the HMBC spectrum showed that its glucuronidation site was one of the catecholic hydroxyls. The carbon was most probably C15, since correlations between this carbon and H14 and H17, but not H18, were observed in the HMBC spectrum. NOE correlations between each proton in the sugar moiety and H14 were observed. The NOE between GH1 and H14 was almost as strong as the one between GH1 and GH5, indicating that the average distance between these pairs of protons was similar. The other interglycosidic NOEs were significantly weaker. In addition, no interglycosidic NOEs involving H17 or H18 were observed. Taken together, the catecholic hydroxyl of C15 was glucuronidated in compound D. In the $^1$H spectrum of compound C, the resonance of H17 shifted 0.35 ppm downfield upon glucuronidation. Interestingly, no interglycosidic cross-peaks could be detected in the HMBC spectrum of compound C, probably due to small $^3$J$_{CH}$. However, the NOESY spectrum demonstrated that compound C was the other catecholic O-glucuronide with the hydroxyl of C16 glucuronidated, since the only interglycosidic correlations observed were between the sugar protons and H17. Here also, the NOE between GH1 and H17 was almost as strong as the one between GH1 and GH5. It was also noted that the largest changes in

![FIG. 4. The actual (A) and normalized (B) formation of dobutamine monoglucuronides A (white bar), C (gray bar), and D (black bar) by 11 recombinant human UGTs. The relative expression levels of the different recombinant UGTs are presented in parentheses (B) (see Materials and Methods). The expression level of UGT1A4 was the lowest in this set, and it was assigned the relative value 1.00. Activity results are averages of duplicate samples, where the dobutamine and protein concentrations were 1000 μM and 2.5 mg/ml, respectively, and the incubation time was 240 min.](image-url)
3C chemical shifts for each glucuronide were observed for aromatic carbons para to the site of glucuronidation. The carbon ortho to the site sited less, whereas carbons bound to the glucuronidated hydroxyl were not affected.

The results of the glucuronidation with the recombinant human isoenzymes (Fig. 4) were in good agreement with the results for human liver microsomes (Fig. 3), since D was overwhelmingly the major product in the reactions catalyzed by the isoforms expressed in the liver, particularly 2B7 and 1A9. The relative activities of the isoenzymes suggest that, when administered orally to humans, dobutamine would be efficiently glucuronidated by UGTs 1A7, 1A8, and 1A10 in the gastrointestinal tract. Dobutamine is usually administered as an infusion, however, and the isoenzymes responsible for its glucuronidation would then be UGTs 1A9 and 2B7 and, to a lesser extent, UGTs 1A1, 1A3, and 2B4 (Fig. 4).

UGTs 1A7, 1A8, 1A9, and 1A10 exhibit 83% identity at the level of amino acid sequences (Tukey and Strassburg, 2000), and this was reflected in their ability to form catecholic dobutamine O-glucuronide D. Nevertheless, it is interesting to note that, despite the high sequence similarity of these four isoforms, only UGT1A10 was highly active in glucuronidating both catecholic hydroxyls of dobutamine (Fig. 4).

Glucuronidation of dobutamine is species-specific (Fig. 3). Comparable species differences have also been found along with regioselectivity in denopamine glucuronidation (Kaji and Kume, 2005). Although the present investigation has dealt only with in vitro glucuronidation, the question clearly arises whether the use of laboratory preparable species differences have also been found along with regioselectivity in denopamine glucuronidation (Kaji and Kume, 2005). Although the present investigation has dealt only with in vitro glucuronidation, the question clearly arises whether the use of laboratory preparations and expression and disease. Annu Rev Pharmacol Toxicol 40:581–616.


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