Initial Studies on the *N*-Glucosylation of Phenobarbital by Mouse Liver Microsomes Using a Radiochemical High-Performance Liquid Chromatographic (HPLC) Method

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A method is described for the assay of phenobarbital *N*-glucosylation using UDP-D-[6-^{3}H]glucose. The radioactive phenobarbital *N*-glucoside conjugates [(5*R*)-PBG, (5*S*)-PBG] formed during the incubations were resolved from each other and from uncharacterized radioactive products by semipreparative HPLC. The product ratio of the *N*-glucosides of (5*R*)-PBG/(5*S*)-PBG was 2.9 for the crude liver homogenate and 3.0 ± 0.5 for the microsomes. Magnesium was necessary for optimal activity. The K_m values for formation of (5*R*)-PBG, (5*S*)-PBG, and (5*R* + 5*S*)-PBG were 1.55 ± 0.35, 1.27 ± 0.14, and 1.47 ± 0.21 m*M*, respectively. The V_{max} values for formation of (5*R*)-PBG, (5*S*)-PBG, and (5*R* + 5*S*)-PBG were 1.34 ± 0.05 × 10^{-6}, 0.43 ± 0.01 × 10^{-6}, and 1.77 ± 0.04 × 10^{-6} μmol/min/mg microsomal protein, respectively. It was observed that at concentrations greater than 5 m*M* sodium phenobarbital, inhibition of formation of phenobarbital *N*-glucosides occurred. The product ratio of (5*R*)-PBG/(5*S*)-PBG is comparable to that observed in the urinary excretion studies with the mouse and opposite to that observed in urinary excretion studies in humans.

KEY WORDS: product enantioselectivity; phenobarbital; 1-(β-D-glucopyranosyl)phenobarbital; mouse microsome; radiochemical; high-performance liquid chromatography reverse-phase (HPLC).

INTRODUCTION

It has been shown that *N*-glucosylation of phenobarbital occurs during the metabolism of phenobarbital by humans (1–3) and mice (4). This is based on the identification of phenobarbital *N*-glucosides in the urine of both species following dosing with phenobarbital. When D-glucose is coupled to either nitrogen of phenobarbital, asymmetry is conferred on C_5 of the barbiturate ring, resulting in the formation of two epimers, (5*R*)-phenobarbital *N*-glucoside [(5*R*)-PBG] and (5*S*)-phenobarbital *N*-glucoside [(5*S*)-PBG]. Product enantioselectivity has been observed in the excretion of phenobarbital *N*-glucosides by both humans (5,6) and mice (4), suggesting that mice may serve as a suitable animal model for study of the barbiturate *N*-glucosylation pathway. The major epimer excreted in the urine by humans is (5*S*)-PBG, whereas the (5*R*)-PBG is the primary epimer excreted by mice. Since little is known about the glucosylation pathway in general and nothing is known about the formation of the phenobarbital *N*-glucosides, it was desirable to develop an assay suitable for studying the formation of the phenobarbital *N*-glucosides *in vitro*. This would minimize the problems due to absorption, metabolism, distribution, and excretion of the phenobarbital *N*-glucosides.

This paper describes a radiochemical HPLC assay that is capable of monitoring the formation of both 5(*S*)- and 5(*R*)-PBG using a mouse liver homogenate or microsomal fraction. Investigations are described on assay conditions that were important in measuring the formation of the phenobarbital *N*-glucosides.

MATERIALS AND METHODS

Chemicals

Uridine diphospho-D-[6-^{3}H]glucose, ammonium salt (4.2 Ci/mmol), was purchased from Amersham Corp., Arlington Heights, IL. Sodium phenobarbital, U.S.P., was purchased from Penick Chemical Division, New York. Uridine diphosphoglucose (UDP-glucose), ammonium salt and ammonium sulfate, was purchased from Sigma Chemical Co., St. Louis, MO. The 5(*S*)-5-ethyl-1-(1-β-D-glucopyranosyl)-5-phenyl-2,4,6(1H,3H,5H)pyrimidinetrione [(5*S*)-PBG] and (5*R*)-5-ethyl-1-(1-β-D-glucopyranosyl)-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione [(5*R*)-PBG] were prepared as previously reported (7).

Enzyme Preparation

Freshly excised livers of adult male ICR mice (body wt, 25–35 g) were used for the preparations. All manipulations were carried out at 0–4°C. The mice were euthanized with CO_2. The combined livers of three mice were rinsed in ice-cold 0.85% NaCl, blotted with paper towels, and homogenized in 0.25 *M* sucrose or in 0.2 *M* Tris–HCl buffer to give 25–40% (w/v) homogenates. Homogenization was carried out by four passes in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle at low speed. In the cell fractionation experiment the cell fractions were prepared as described by Gessner *et al.* (8). When only the microsomal preparation was needed, the fraction was obtained by centrifuging the homogenate at 12,100g_{av} for 20 min, discarding the pellet, and centrifuging the supernatant at 105,000g_{av} for 90 min. The microsomal fraction was transferred to the homogenizer described above and resuspended in 2–3 ml of 0.2 *M* Tris–HCl buffer, pH 7.3. Cell fractions were used immediately for the phenobarbital *N*-glucosylation studies. Protein concentrations were determined as specified using the commercially available Coomassie Protein Assay (Pierce Chemical Co., Rockford, IL).

Assays of Phenobarbital *N*-Glucosylation

All assays were performed on freshly prepared homogenates and subcellular fractions. Biosynthesized (5*R*)-PBG and (5*S*)-PBG were detected and quantified by measuring incorporation of ^{3}H from UDP-D-[6-^{3}H]glucose. The incubations were done in duplicate and conducted in uncovered 16 × 100-mm screw-cap test tubes in a total volume of 154 μl.

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A typical incubation mixture contained 2 μCi UDP-D-[6-^{3}H]glucose, 250 μ*M* UDP-glucose, 8 m*M* $MgCl_2$, 5 m*M* sodium phenobarbital, and 2.4–6.5 mg microsomal protein buffered to pH 7.3 with Tris–HCl buffer. The incubations were conducted at 37°C in a metabolic shaker for 0–60 min as indicated. Reaction mixtures were stopped by the addition of 100 μl 21% H_3PO_4. This was followed by the addition of 25 μl of a mixture of the unlabeled phenobarbital *N*-glucosides [0.2 m*M* (5*S*)-PBG, 0.6 m*M* (5*R*)-PBG] dissolved in 21% H_3PO_4 solution. The incubation mixtures were heated to 95°C for 1 hr. Approximately 100 mg $(NH_4)_2SO_4$ was added to the incubation mixtures, followed by 3 ml ethyl acetate. The mixtures were extracted on a rotary extractor for 30 min, then centrifuged at 725*g* for 5 min. The ethyl acetate layers were dried over Na_2SO_4 (anhyd), then transferred, and the duplicate samples were pooled. The ethyl acetate was evaporated under a stream of nitrogen or allowed to evaporate overnight. The residues were resuspended first in 50 μl methanol, followed by the addition of 150 μl 20% acetonitrile/water (v/v) immediately prior to analysis.

HPLC Determination of Phenobarbital *N*-Glucosylation

A semipreparative HPLC system was used to separate the phenobarbital *N*-glucosides from other uncharacterized radioactive products. The system consisted of a Rheodyne Model 7125 syringe loading injector (100 μl loop), a Pellicular C_{18} guard column (20 mm × 2-mm i.d.; particle size, 37–53 μm; Whatman), and an Econosil C_{18} reverse-phase column (250 mm × 10 mm; particle size, 10 μm; Alltech Assoc.). The mobile phase was 15% acetonitrile in 0.025 *M* sodium phosphate buffer (v/v), pH 6.5, at a flow rate of 4 ml/min. The effluent was monitored at 220 nm using a Gilson Holochrome detector. The retention volumes of (5*S*)-PBG and (5*R*)-PBG were 80–88 and 88–104 ml, respectively. Fractions were collected every minute and dissolved in 15 ml Econo-Safe scintillation cocktail (Research Products International, Mount Prospect, IL). The radioactivity in the fractions was determined using a Beckman LS-1801 liquid scintillation counter.

RESULTS

Phenobarbital *N*-glucosylation activity was present in the mouse liver homogenate. The specific activity in the whole liver homogenate was 0.098 picomol/min/mg protein. The ratio of (5*R*)-PBG/(5*S*)-PBG was equal to 2.9. Upon cell fractionation, no detectable product formation was associated with the nuclear (775*g*), mitochondrial (5090*g*), lysosomal (12,100*g*), and soluble (105,000*g*) fractions. The specific activity in the microsomal fraction (105,000*g*) was 0.25 picomol/min/mg protein with the ratio of (5*R*)-PBG/(5*S*)-PBG equal to 3.3. Since activity resided exclusively in the microsomal fraction and the product ratio of (5*R*)-PBG/(5*S*)-PBG for both the crude liver homogenate and the microsomes was comparable, the remainder of the studies were done with the microsomal fraction.

Radiolabeled product cochromatographed with unlabeled standards, indicating that the radioactive products were the phenobarbital *N*-glucosides (Fig. 1). The radioactivity associated with the diastereomers ranged from 650 to 2750 dpm in the 30-min assay (background of ≈40 dpm) and

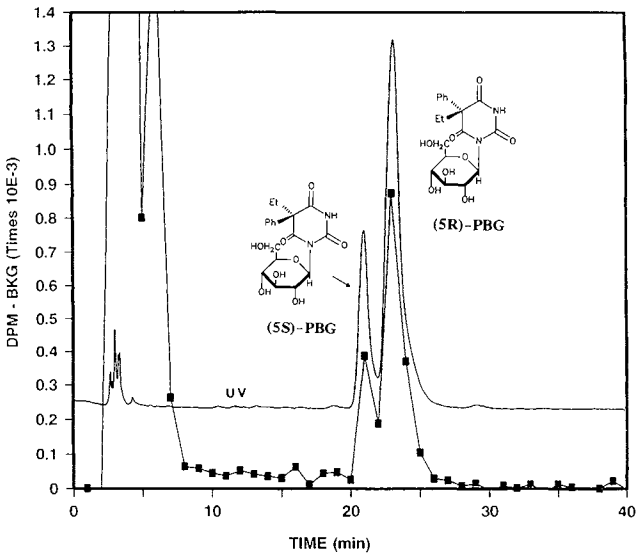

Fig. 1. Overlapping radiochromatogram and UV chromatogram of the diastereomers of 1-(β-D-glucopyranosyl)phenobarbital. Chromatographic conditions are described in the text. UV full-scale deflection is 0.5 AU at 220 nm. (■) The radiochromatographic tracing.

accounted for approximately 0.05% of the radiolabel used in the assay. Radioactivity did not cochromatograph with standards when phenobarbital was omitted from the incubation mixture or with boiled microsomal protein.

Initial studies with 10 m*M* UDP-glucose (0.2 μCi/mmol UDP-D-[6-^{3}H]glucose) produced radioactivity only two to three times above background that cochromatographed with the phenobarbital *N*-glucoside standards. The concentration of UDP-glucose was arbitrarily decreased to 250 μ*M* UDP-glucose (8 μCi/mmol UDP-D-[6-^{3}H]glucose) to improve the ratio of labeled to unlabeled UDP-glucose. After this modification, radioactivity that coeluted with standards increased to approximately 2000 dpm for each assay. No further studies were done to optimize the concentration of the UDP-glucose cofactor.

Studies to optimize assay conditions further included variation of the incubation time, concentration of protein, cofactors, and substrate as well as workup conditions. For each study phenobarbital *N*-glucosylation rates were obtained under standard reaction conditions: 10 m*M* sodium phenobarbital, 250 μ*M* UDP-D-[6-^{3}H]glucose (8 μCi/mmol), 8 m*M* $MgCl_2$, and 2–4 mg microsomal protein were incubated for 30 min. Under these conditions the formation of the phenobarbital *N*-glucosides averaged 0.83 ± 0.09 picomol/min/mg microsomal protein (n = 7). When it was observed that above 5 m*M* sodium phenobarbital the formation of phenobarbital *N*-glucosides was decreased, most of the studies were repeated at 5 m*M* sodium phenobarbital. At 5 m*M* sodium phenobarbital the rate of formation of both phenobarbital *N*-glucosides increased to 1.02 ± 0.09 picomol/min/mg microsomal protein (n = 6).

The extraction protocol and HPLC analysis were adapted from the synthetic method (7) used for isolation of the phenobarbital *N*-glucosides and the analytical method (4–6) used for quantification of the phenobarbital *N*-glucosides. The acid hydrolysis was included in the workup be-

cause it increased the recovery of the radioactivity that coeluted with standards by 35% versus a sample that was not treated. The extraction efficiency for both (5*R*)- and (5*S*)-PBG from the microsomal incubation mixture was 84 ± 5% (n = 5).

The formation of phenobarbital-*N*-[6-^{3}H]glucosides was linear for the first 30 min, with very few *N*-glucoside metabolites formed from 30 to 60 min at a concentration of 5 m*M* sodium phenobarbital. All subsequent incubations were carried out for 30 min. The rate of formation of phenobarbital-*N*-[6-^{3}H]glucosides increased linearly up to 4.6 mg microsomal protein/ml at a concentration of 10 m*M* sodium phenobarbital. At higher protein concentrations the formation of the *N*-glucoside metabolites no longer increased. All subsequent incubations were carried out at protein concentrations less than 5.0 mg microsomal protein/ml. Magnesium was found to be necessary for optimal activity, as reduced glucoside formation (47 ± 9%, n = 3) was observed when $MgCl_2$ was omitted and EDTA was added to the incubation mixture. Except for the prior experiment, 8 m*M* $MgCl_2$ was present in all of the assays.

Various concentrations of sodium phenobarbital were incubated with a constant concentration of UDP-glucose to determine the optimal concentration of sodium phenobarbital for studying the *N*-glucosylation of phenobarbital (Fig. 2). The conversion rate increased with increasing concentration of sodium phenobarbital up to 5 m*M*. The rate of formation of phenobarbital *N*-glucosides decreased at concentrations greater than 5 m*M* sodium phenobarbital. Since inhibition was observed above 5.0 m*M* sodium phenobarbital, the K_m and V_{max} were calculated from the results obtained at concentrations of 0.01–5.0 m*M* sodium phenobarbital. Kinetic data were fitted to the Michaelis–Menten equation using the nonlinear regression program described by Duggleby (9). The K_m values for formation of (5*R*)-PBG, (5*S*)-PBG and (5*R*+5*S*)-PBG were 1.55 ± 0.35, 1.27 ± 0.14, and 1.47 ± 0.21 m*M*, respectively. The V_{max} values for formation of (5*R*)-PBG, (5*S*)-PBG, and (5*R*+5*S*)-PBG were 1.34 ± 0.05 × 10^{-6}, 0.43 ± 0.01 × 10^{-6}, and 1.77 ± 0.04 × 10^{-6} μmol/min/mg microsomal protein, respectively. In the studies described above, the average product ratio of the specific activity for formation of (5*R*)-PBG/(5*S*)-PBG by the microsomal fraction was 3.0 ± 0.5 (n = 42).

DISCUSSION

The assay that was developed provides a specific and sensitive method for measuring the formation of both phenobarbital *N*-glucoside epimers. Prior *in vitro* studies of amobarbital *N*-glucosylation have been done with radiolabeled amobarbital or glucose (10–12). It was not feasible to develop the assay using radiolabeled phenobarbital, since it is no longer readily available commercially. The alternative approach was to have the label incorporated into the glycoside portion of the phenobarbital *N*-glucoside metabolites by using commercially available UDP-[6-^{3}H]glucose that was of a high specific activity. A similar concept was used in the development of a sensitive and specific assay for glucuronide conjugates of numerous xenobiotics using uridine diphosphate [^{14}C-U]glucuronic acid (13) or for amobarbital using UDP-[6-^{3}H]glucose (11). However, having the radiolabel present in the glucose portion of the metabolite could potentially cause problems due to nonspecific glucosylation of microsomal components. To minimize possible interfer-

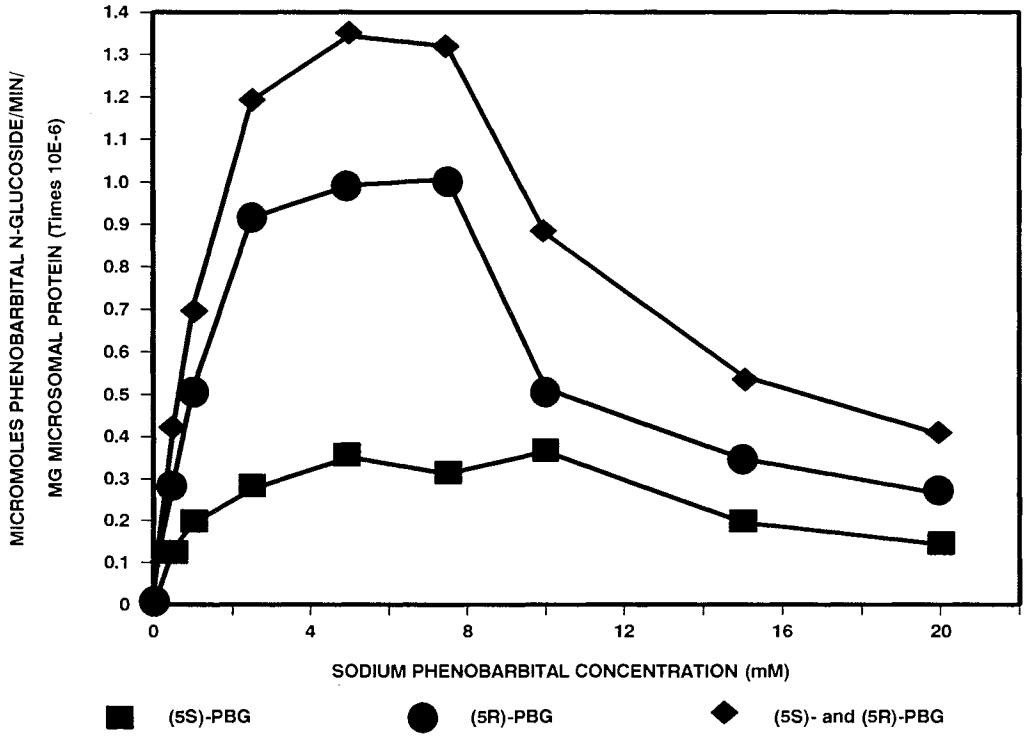

Fig. 2. Effect of sodium phenobarbital concentration on rate of formation of phenobarbital-*N*-[6-^{3}H]glucosides.

ences by nonspecific glucosylation, an acid hydrolysis step was incorporated into the assay. Since the phenobarbital *N*-glucosides are stable under strongly acidic conditions (1,5,7), the acid hydrolysis step should hydrolyze any *O*-glucosides that formed during the incubation, in addition to disrupting the microsomal matrix further. Evidence that the assay described is measuring formation of phenobarbital-*N*-[6-^{3}H]-glucosides is as follows: (i) phenobarbital *N*-glucosides have been shown previously to be excreted in mouse urine following administration of sodium phenobarbital, at a ratio comparable to that observed in this study; (ii) the radioactivity measured in the assay cochromatographs with synthetic standards of phenobarbital *N*-glucosides; (iii) radioactivity did not cochromatograph with standards when sodium phenobarbital was omitted from the incubation or with boiled protein; and (iv) the formation of phenobarbital-*N*-[6-^{3}H]glucosides was proportional to increasing protein concentration and increasing time. Therefore, this method is measuring the formation of the individual epimers of phenobarbital *N*-glucosides *in vitro*.

The enzyme that is responsible for the formation of the phenobarbital *N*-glucosides is primarily a microsomal protein. Formation of β-linked glucosides of xenobiotic compounds by a liver or microsomal UDP-glucosyltransferase has been shown for 4-nitrophenol (8) and pranoprofen (14) by mice, isoflavones and phenols by rabbits (15), and amobarbital by humans (10) and cats (11,12). Amobarbital *N*-glucosylation by cat liver microsomes exhibited a product enantioselectivity of 2:1 (5R:5S), comparable to what was observed for phenobarbital *N*-glucosylation by the mouse in this study. Also, stereoselective acyl glucosidation by mouse kidney microsomes has been shown for *S*(+)-pranoprofen over *R*(−)-pranoprofen (14,16). It appears that there may be a stereochemical component to the formation of xenobiotic glucoside conjugates.

In the study to determine K_m and V_{max} it was observed that at high concentrations of sodium phenobarbital (above 5.0 m*M* sodium phenobarbital) the rate of formation of the phenobarbital *N*-glucosides decreased. This was unexpected, however, substrate inhibition is not unusual for multireactant enzymes (17). Given the structural resemblance between the barbiturate ring system and the uridine moiety of UDP-glucose, it is possible that substrate inhibition arises from formation of an enzyme–substrate complex in which the barbiturate is bound nonproductively to a portion of the UDP-glucose site. Although no definitive conclusions can be drawn at this time, this study suggests that at toxic levels of phenobarbital (2), the *N*-glucosylation of phenobarbital [a major metabolic route for the detoxification of phenobarbital (2,6)], could be inhibited.

These studies are the first to show that the product enantioselectivity observed for urinary excretion of the phenobarbital *N*-glucoside metabolites also occurs for the formation of metabolites in the liver. The product ratio of (5*R*)-PBG/(5*S*)-PBG of 3.0 is comparable to that observed in the urinary excretion studies in the mouse, which was 10.2 [91% (5*R*)-PBG, 9% (5*S*)-PBG (4)]. Since deconjugation of the phenobarbital *N*-glucosides to release phenobarbital has been observed in mice, it is possible that stereoselective metabolism, renal absorption, or excretion may also be occurring with the phenobarbital *N*-glucosides.

In conclusion, these studies have shown that mouse liver microsomes are capable of catalyzing the *N*-glucosylation of phenobarbital. The product stereoselectivity observed for the formation of phenobarbital *N*-glucosides is opposite that observed in humans, where (5*S*)-PBG is the major diastereomer formed and/or excreted.

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