 Phenobarbital N-Glucosylation by human liver microsomes

SHEELA G. PAIBIR, WILLIAM H. SOINE, DIANA F. THOMAS, and ROBERT A. FISHER

Department of Medicinal Chemistry and Department of Surgery, Medical College of Virginia/Virginia Commonwealth University, Richmond, USA.

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SUMMARY

Glucosylation of xenobiotics in mammals has been observed for a limited number of drugs. Generally, these glucoside conjugates are detected as urinary excretion products with limited information on their formation. An in vitro assay is described for measuring the formation of the phenobarbital N-glucoside diastereomers ((5R)-PBG, (5S)-PBG) using human liver microsomes. Human livers (n = 18) were screened for their ability to N-glucosylate PB. Cell viability, period of liver storage, prior drug exposure, serum bilirubin levels, age, sex and ethnicity did not appear to influence the specific activities associated with the formation of the PB N-glucosides. The average rate of formation for both PB N-glucoside was 1.42±1.04 (range 0.11 – 4.64) picomole/min/mg-protein with an (SS)-PBG/(SR)-PBG ratio of 6.75±1.34. The apparent kinetic constants, K_m and V_max, for PB N-glucosylation for eight of the livers ranged from 0.61 – 20.8 mM and 2.41 – 6.29 picomole/min/mg-protein, respectively. The apparent V_max/K_m ratio for PB exhibited a greater than 20 fold variation in the ability of the microsomes to form the PB N-glucosides. It would appear that the formation of these barbiturate N-glucoside conjugates in vitro are consistent with the amount of barbiturate N-glucosides formed and excreted in the urine in prior drug disposition studies.

INTRODUCTION

The importance of microsomal glucosylation of xenobiotics in mammals is not well understood. In humans, microsomal glucosyltransferase(s) has been shown to occur in the metabolism of endogenous compounds, such as bilirubin [1], and in a limited number of xenobiotics, such as pranoprofen [2] or the barbiturates [3]. In humans the barbiturate for which conjugation with glucose appears to be most important for its biodisposition is amobarbital [3]. The urinary excretion of the amobarbital N-glucosides can account for up to 27% of the biodisposition of amobarbital following a single dose [4]. A screening assay had been used for detecting these N-glucoisides in urine to identify the variability in amobarbital metabolism, since an apparent ethnic difference for Orientals versus Caucasians had been observed for their excretion [5,6]. Several subjects have been identified with a limited ability to excrete amobarbital N-glucosides [5], and subjects have been identified that appear to have a genetic deficiency for excretion of the amobarbital N-glucosides [7]. Due to the nature of the previous studies the variability associated with formation, distribution, and further metabolism of the N-glucoside metabolites could not be evaluated. There has been a single report showing that human liver microsomes are capable of forming the amobarbital N-glucoside metabolite [8]. A structurally related barbiturate, phenobarbital, is also partially excreted in urine as an N-glucoside conjugate [3]. When D-glucose is coupled to
either nitrogen of PB, asymmetry is conferred, resulting in the formation of two diastereomers, (5R)-phenobarbital N-glucoside [(5R)-PBG] and (5S)-phenobarbital N-glucoside [(5S)-PBG] (shown in Fig. 1). Based on urinary excretion studies product enantioselectivity has been observed in the excretion of PB by humans with the (5S)-PBG being the major epimer excreted [9,10]. No in vitro study using human liver microsomal enzymes comparable to that with amobarbital has been reported. Previously, a radiochemical HPLC method had been developed to measure the diastereomeric preference for PB N-glucosides formation in mouse liver microsomes [11]. The use of this assay using human liver microsomes may allow the investigation of the variability in the activity of this enzyme while minimizing the potential interference associated with other pathways involved in a drugs biodisposition. This paper describes the evaluation of assay conditions used to measure the formation of both (5R)-PBG and (5S)-PBG in human liver microsomes. Human livers (n = 18) were screened for their ability to N-glucosylate PB in which the influence of age, sex, race and prior drug exposure could be evaluated. Apparent kinetic constants (K_m and V_max) for PB N-glucosylation were determined for eight of the livers. A greater than 20 fold variation was found in the ability of the microsomes to form PB N-glucosides.

**Materials and Methods**

**Chemicals**

ACES (N-[2-acetamido]-2-aminoethanesulfonic acid), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, polyoxyethylene 20 cetyl ether (Brig 58), UDP-Glucose (uridine 5’-diphosphoglucose) ammonium salt, D-gluconic acid lactone, ammonium sulfate, and sodium sulfate (anhydrous) were purchased from Sigma (Milwaukee, WI). Ethyl acetate (HPLC grade), methanol (HPLC grade), monobasic sodium phosphate, dibasic sodium phosphate and ortho-phosphoric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin was purchased from Pierce (Rockford, IL).

Radioactive UDP-[6-3H]-glucose (specific activity of 0.1 mCi/ml or 10 to 15 mCi/nanomole) were purchased from NEN-Dupont (Boston, MA). Sodium phenobarbital, USP, was purchased from Penick Chemical Division (New York). Synthetic standards of the PB N-glucosides and the mephobarbital N-glucosides were synthesized as previously reported [12].

**Hepatic Microsomal Preparation**

**Liver Tissue:** Approximately 10 gm portions of human livers (12 male and 6 female) were obtained from the Division of Liver Transplant Surgery, Medical College of Virginia (Richmond, VA). The sex, age, race, prior drug exposure and bilirubin (total and conjugated) prior to death are listed in Table I. Prior to freezing the liver sample the percent cell viability was determined based on trypan blue uptake [13] and is listed in Table I. The liver samples (except for Subject #18) were flash frozen in liquid nitrogen and stored at -80°C. The liver fragments were stored for the time indicated in Table I prior to initial analysis. At the time of analysis the liver sample was weighed, thawed over a period of 30-45 minutes, and cut into thin slices while still slightly frozen. All the remaining tissue preparation steps were carried out at 0-4 °C. The tissue was minced and homogenized (first with a rotor stator Brinkman Homogenizer, then the Potter-Elvehjem homogenizer) in 0.2M ACES buffer (pH 7.3) to give a 20-25% w/v suspension. The procedure for the isolation, preparation and storage of microsomes was similar to that described for mice [11]. Storage of liver microsomes at -80°C up to a period of one year and freezing and thawing of the microsomal pellets up to four times did not show any change in enzyme activity. All incubations were run in duplicate (unless otherwise indicated) and are reported as the average of the two values.

**Microsomal Incubations**

The microsomal incubations were done in uncovered 16 x 100mm screw-cap test tubes. The labeled solution of UDP-[3H]glucose used in the incubation was prepared by evaporating the required amount of the radioactive solution (available in 7:3 v:v ethanol:water) to dryness under a slow stream of nitrogen, and then reconstituting it with the solution of unlabelled UDP-G at the appropriate concentration. A typical incubation mixture had a total volume of 154 µl and contained 250 µM UDP-D-[6-3H]glucose (6 µCi), 8 mM MgCl_2, 2 mM PB 1.2-1.4 mg/ml human liver microsomal protein buffered to pH 7.3 with 0.2M ACES buffer. The biosynthetic reaction was initiated by the addition of the microsomal protein followed by the solution of UDP-[6-3H]-glucose. The tube was immediately