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Barbital *N*-glucoside is not detected as a urinary excretion product of barbital in humans

WILLIAM H. SOINE,* PHYLLIS J. SOINE and TERRY M. ENGLAND

Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298-0540, USA

Abstract: A study was undertaken to determine if humans excreted barbital *N*-glucoside as a urinary metabolite following oral administration of barbital. A liquid chromatography method using gradient elution was developed for detecting and quantifying barbital *N*-glucoside and barbital in urine. Following a single oral dose of barbital to male caucasian and oriental subjects that had previously been shown to excrete amobarbital and phenobarbital *N*-glucosides, no barbital *N*-glucoside conjugate was observed in the urine. This result indicates that *N*-glucosylation of barbiturates is not a general pathway for the biodisposition of barbiturates in man.

Keywords: *Urine; barbital; 1-(β-D-glucopyranosyl)barbital; reverse-phase chromatography.*

Introduction

It has been proposed that *N*-glucosylation of barbiturates is a general route for the biodisposition of barbiturates in man [1, 2]. This was based on the unambiguous characterization of amobarbital [3, 4] and phenobarbital [5, 6] *N*-glucosides as major urinary excretion products by human subjects following oral administration of amobarbital and phenobarbital, respectively (Fig. 1). In man it was anticipated that barbital, in which the sterically smaller ethyl group replaces the larger alkyl or aryl substituent at the 5 position of the barbiturate ring, would also undergo *N*-glucosylation and be excreted in the urine.

There have been no recent studies on the metabolic fate of barbital, the majority of the studies being done prior to 1955. The results from the early studies are questionable because of the poor specificity of the qualitative and quantitative methods used (gravimetric, colorimetric and ultraviolet spectrophotometric) [7]. In man it was believed that barbital underwent no degradation in the body and its elimination was dependent entirely on renal excretion [7]. The renal excretion of barbital extended over many days and accounted for 75–90% of the barbital dose administered [8]. In rats, barbital is also excreted primarily unchanged in the urine, although a small portion of the dose (approximately 5%) was found to undergo oxidative metabolism to 5-ethylbarbituric acid, 5-β-hydroxyethyl-5-ethyl barbituric acid, and a conjugate of the hydroxylated metabolite [9, 10]. Whether other species produce these

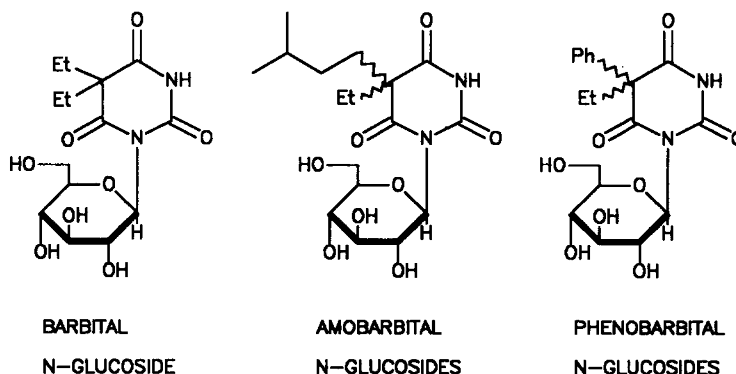

Figure 1
Structures of barbiturate *N*-glucosides

*Author to whom correspondence should be addressed.

metabolites, including man, is unknown. Based on these early studies of barbital biodisposition in humans, a significant portion of the dose remains to be identified.

Since amobarbital and phenobarbital undergo both N-glucosylation and oxidation of a substituent at the 5 position, the prior literature was consistent with possible formation of barbital N-glucoside by man followed by its excretion in the urine. This study was done to determine if N-glucosylation was a general metabolic pathway for the metabolism of a more hydrophilic barbiturate; barbital. This paper describes an assay utilizing HPLC that is capable of detecting barbital N-glucoside at levels of 4 μM in urine, concentrations at which amobarbital and phenobarbital N-glucosides were detected. After human subjects received an oral dose of barbital, barbital N-glucoside was not detected as a urinary excretion product. It would appear that N-glucosylation may not be a significant pathway for the metabolism and urinary excretion of all barbiturates. Barbiturates that undergo N-glucosylation contain some structural parameter(s) or physicochemical property, that is yet undefined, that is important in determining if a barbiturate will form and be excreted in the urine as a glucoside conjugate.

Experimental

Materials

Barbital N-glucoside, 5,5-diethyl-1-β-D-glucopyranosyl-2,4,6-(1H,3H,5H)pyrimidinetrione was synthesized as previously described [11]. Barbital, U.S.P. was purchased from Mallinckrodt, Inc. (St Louis, MO, USA). The 2-ethyl-2-phenylmalonamide monohydrate (EPM), 99%, was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Acetonitrile (MeCN), ethyl acetate, methanol and monobasic and dibasic sodium phosphate were HPLC grade.

Chromatographic conditions

The LC equipment used has been previously described [12]. The analysis was done with an Econosphere C_{18} column (5 μm, 250 × 4.6 mm i.d.; Alltech Associates, Deerfield, IL, USA). The analysis required gradient elution. The eluents were: Solvent A, MeCN–sodium phosphate (pH 6.5, 25 mM), (5:95, v/v); Solvent B, MeCN–sodium phosphate (pH 6.5, 25 mM), (25:75, v/v). The gradient used was 15 min of 100% A, linear programming over 19 min to 20% B, linear programming over 2 min to 100% B, 12 min of 100% B, reequilibration over 2 min to 100% A. The injection volume was 20 μl, the flow rate was 1.4 ml min^{-1}, and the eluate was monitored at 198 nm (the λ_{max} for barbital N-glucoside). The analysis was carried out at 25°C. The mobile phase was degassed by purging with helium.

Sample standards

Individual stock solutions of barbital (4.2 mM), barbital N-glucoside (4.1 mM) and EPM (330 μM) were prepared in methanol and stored at −20°C. When stored under these conditions, no decomposition of the stock solution was detectable during the study. Standard solutions of barbital N-glucoside, barbital and EPM were prepared by dilution of known amounts of stock solution with methanol, transferring known amounts of this solution to a screw cap test tube, evaporating the methanol under a stream of nitrogen and dissolving the residue in acidified reconstituting solution [H_3PO_4 (1 M)–MeCN–water (1:5:94, v/v/v)] or blank urine acidified with citric acid (urine collected over citric acid).

Sample preparation

To prepare the samples for analysis, 33 nmoles (100 μl) of EPM in methanol were transferred a screw-cap test tube (100 × 13 mm), evaporated to dryness and reconstituted with 200 or 400 μl of urine. To the urine sample were added 1.0 ml of a saturated solution of ammonium sulphate and 3.0 ml of ethyl acetate. The solution was extracted using a rotary mixer for 30 min. After centrifugation, the ethyl acetate layer was transferred and dried with anhydrous sodium sulphate, after which 2.0 ml was transferred to a test tube and evaporated to dryness in a vortex evaporator at 30 mm Hg and room temperature. The residue was reconstituted with 200 μl of an acidified reconstituting solution. To ensure dissolution of the analytes the sample was left for 4 h at room temperature or overnight in the refrigerator before transfer to the LC vial for analysis. The samples were stored frozen until just prior to analysis.

Collection of urine samples

Eight adult male volunteers were enrolled in the study after informed consent, medical history and laboratory screening tests (free and

conjugated bilirubin, γ-glutamyltransferase) were performed. Five subjects were caucasian and three subjects were oriental, with ages ranging from 24 to 39 years of age (mean, 26.5 years). All of these subjects had been volunteers in studies in which the *N*-glucosides of phenobarbital [12] and amobarbital [13] had been identified and quantified. These subjects had received no barbiturate or imide drug for a minimum of 30 days prior to this study.

The subjects were instructed not to take any medication for 3 days before taking the drug and during the study. Alcohol consumption was not allowed 24 h before and after taking the drug and was restricted during the remainder of the urine collection period. All subjects received a 300 mg oral dose of barbital, U.S.P. (1.63 mmole, capsule) just prior to retiring for the night. A urine sample was obtained just prior to taking the drug and was used as the blank. The total urine was collected as individual samples at natural periods for 30 h, followed by collection of morning urine (first urine sample upon awakening) for 5 additional days. The subjects collected the urine in the presence of citric acid (final pH of the urine ranged from 2.3 to 2.6). The urine samples were refrigerated immediately upon collection, frozen within 48 h, and kept at −20°C until analysed.

Results

Analytical methodology and assay validation

A chromatogram of EPM, barbital *N*-glucoside and barbital dissolved in the acidified reconstituting solution is shown in Fig. 2a. A chromatogram of an extract of blank urine containing EPM is shown in Fig. 2b. A chromatogram of EPM, barbital *N*-glucoside and barbital extracted from blank urine is shown in Fig. 2c. A chromatogram of a urine

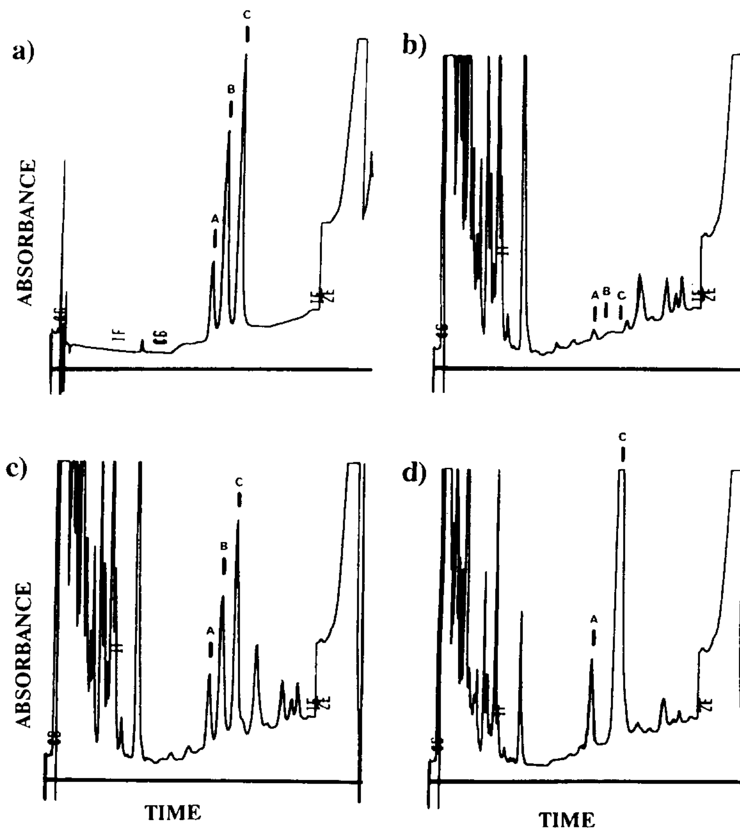

Figure 2
Chromatograms of (a) the standards of 2-ethyl-2-phenylmalonamide (A, 3.3 μM, 25.7 min), barbital *N*-glucoside (B, 41.2 μM, 26.8 min), and barbital (C, 41.5 μM, 28.2 min) dissolved in acidified reconstituting solution; (b) a 400-μl blank urine extract that was acidified with citric acid (2-ethyl-2-phenylmalonamide was added as a standard prior to extraction, 25.8 min); (c) the 41.2 μM standard of barbital *N*-glucoside (26.8 min) and the 41.5 μM standard of barbital (28.2 min) extracted from 200 μl of urine; (d) urine obtained 12 h after an oriental subject had taken a 300 mg oral dose of barbital (A, 25.8 min; B, not detected; C, >83 μM, 28.2 min). Chromatographic conditions are described in text. Chart speed was 0.1 mm min^{-1} for 20 min, then increased to 0.2 mm min^{-1} for the remainder of the analysis.

extract from an oriental subject following oral administration of barbital is shown in Fig. 2d.

EPM was initially used as an internal standard to quantify barbital and barbital *N*-glucoside. The recovery of EPM was found to be highly variable and it was necessary to estimate the amount of barbital *N*-glucoside using the area normalization method. The ordinate was the peak area obtained for the analytes following a 20-μl injection. The abscissa was metabolite concentration (μM). Analysis of the standard (4, 10, 20, 40 and 80 μm) extracted from blank urine samples gave variable slopes for barbital *N*-glucoside (m = 1.15 ± 0.06 × 10^5 to 1.46 ± 0.03 area units per μM) and barbital (m = 1.50 ± 0.03 to 1.80 ± 0.14 × 10^5 area units per μM) depending on the individuals urine being analysed. The ordinate intercept ranged from −2.83 ± 1.36 × 10^5 to 7.41 ± 5.15 × 10^5 area units for barbital *N*-glucoside and −4.90 ± 5.61 × 10^5 to 7.52 ± 2.79 × 10^5 area units for barbital. For each individual analysed, the standard response was linear with a $r^2 \geq 0.983$ for barbital *N*-glucoside and $r^2 \geq 0.963$ for barbital. In a single run of 40 analyses the retention time for barbital *N*-glucoside was 28.1 ± 0.1 min and barbital was 29.2 ± 0.1 min. A small peak was occasionally observed in certain individuals that eluted at 0.3–0.5 min prior to the elution of barbital *N*-glucoside. This could be observed as a shoulder on the leading edge of the barbital glucoside and required careful monitoring of the integration parameters. Because of potential interference from this absorbance, standards of barbital *N*-glucoside and barbital were run every sixth sample to monitor any shift in retention time for the standards. A small peak was also observed in most individuals that co-eluted with barbital. The interfering substance corresponded to 2 μM or less barbital in all urines tested. In urine the lower limit of detection for barbital *N*-glucoside and barbital was 4 and 2 μM, respectively.

Recovery of barbital *N*-glucoside and barbital from urine was determined by comparison of quadruplicate analysis of standards prepared in blank urine at 4, 20 and 40 μM with equivalent quantities of compounds dissolved in the acidified reconstituting solution. By comparison of peak areas the recovery from urine was 108 ± 8% barbital *N*-glucoside and 89 ± 9% barbital.

The within-run precision was evaluated by analysing urine samples containing either 4.1 or 20.2 μM of barbital *N*-glucoside (n = 10 samples at each concentration). The samples were analysed for 4.3 ± 0.6 and 20.2 ± 2.2 μM barbital *N*-glucoside. For barbital standards at 4.2 or 20.8 μM barbital the samples were analysed for 5.1 ± 1.9 and 19.8 ± 1.9 μM barbital.

Discussion

The development of an LC method for detecting barbital *N*-glucoside in urine was considerably more difficult than that encountered for amobarbital and phenobarbital *N*-glucosides. Considerable difficulty was encountered in finding a chromatographic solvent system in which no significant interfering absorbances were observed when monitoring the blank urine extract at 198 nm (optimal for detecting barbital *N*-glucoside). Due to the hydrophilic nature of barbital and barbital *N*-glucoside, isocratic conditions were unsuccessful. Even using solvent programming, only partial success was achieved in minimizing the interferences due to other UV absorbing substances present in the urine extract. No effort was made to modify the analytical procedure for barbital when it was observed that it was present in concentration greater than 83 μM barbital, since barbital *N*-glucoside was of primary interest. In prior studies in which amobarbital and phenobarbital *N*-glucosides were quantified, the oral dose was 90 and 100 mg of the respective barbituate. At those doses the subjects had excreted *N*-glucosides of amobarbital and phenobarbital at concentrations greater than 4 μM. In this study the dose of barbital was 300 mg, therefore, the assay should have been adequate for detecting the barbital *N*-glucoside if barbital was being metabolically transformed and excreted in a manner comparable to amobarbital and phenobarbital. In all subjects studied, no evidence for the urinary excretion of barbital *N*-glucoside was observed using this assay. Although very little is known concerning the glucosylation pathway in man, it has been shown that here is a genetic deficiency [14, 15] as well as an ethnic difference (caucasians versus orientals) [16] for the *N*-glucosylation of amobarbital. In the present study, this problem was addressed since both caucasians and orientals subjects were studied that had been previously documented as being capable of form-

ing both amobarbital and phenobarbital *N*-glucosides. Therefore, the failure to detect barbital *N*-glucoside in the urine should not be attributed to a genetic or ethnic element. Prior studies have indicated that minimal excretion of barbital and its metabolites occurs via fecal excretion [7]. In addition, excretion of amobarbital [17] and phenobarbital [18] *N*-glucosides has only been detected via the renal pathway and it is unlikely that biliary and fecal excretion would become dominant for a more hydrophilic barbiturate *N*-glucoside of lower molecular weight [19]. In this study barbital *N*-glucoside was not detected in the urine following oral administration of barbital. Apparently, in man the disposition of barbital does not parallel the metabolism previously observed for amobarbital and phenobarbital.

Two hypothesis can be proposed to rationalize why barbital *N*-glucoside is not detected in the urine. First, it could be due to failure to form the *N*-glucoside or secondly, if the barbital *N*-glucoside is formed it is a substrate for additional biotransformation pathway(s). Consistent with the first hypothesis is that primarily lipophilic endogenous and exogenous substances form glucoside conjugates; e.g. steroids [20], bilirubin [21], cannabidiol [22], pranoprofen [23] and sulphonamides [24]. An exception to this trend is the observation that the *O*-glucoside of pantothenic acid [25], a very hydrophilic compound, is formed. Since barbital is a relatively hydrophilic barbiturate [26], it may not be sufficiently lipophilic to be a substrate for the enzyme(s) that is responsible for formation of the *N*-glucosides of amobarbital and phenobarbital.

The second hypothesis is that the barbital *N*-glucoside, once formed, is a substrate for additional biotransformation pathways, including enzymatic release of barbital. It was shown that following intraperitoneal administration of either epimer of phenobarbital *N*-glucosides to mice, that phenobarbital was present in the urine [27]. This suggested that the release of phenobarbital was due to an enzymatic reaction. Both epimers of amobarbital and phenobarbital *N*-glucosides are excreted in urine by humans, and a product enantioselectivity has been observed for their excretion. This product enantioselectivity could be because the major epimer excreted in the urine is a poor substrate relative to the other epimer for additional biotransformation pathways. This fact would account for its much higher concentration in the urine. Since both substituents at the 5 position of the barbital *N*-glucoside are ethyl, a regioselective metabolism would not occur. The barbital *N*-glucoside could undergo additional biotransformation reaction and would not be detected in the urine. Unfortunately, if either of these mechanisms or a combination of these mechanisms is occurring, urinary excretion studies are not capable of resolving this question. In the only study on formation of barbiturate *N*-glucosides using human liver samples, the product enantioselectivity for formation of the amobarbital *N*-glucosides was not addressed [2]. Elaborate *in vitro* studies using human tissues are necessary to determine if *N*-glucosylation is a general metabolic pathway for all barbiturates used clinically in humans. In conclusion, barbital is the first example of a barbiturate in which its *N*-glucoside was not detected as a significant urinary metabolite in man when studies were specifically designed to detect this novel drug conjugate.

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