Identification of the Diastereomers of Pentobarbital *N*-Glucosides Excreted in Human Urine

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A study was undertaken to determine if humans excreted pentobarbital N-glucosides as urinary metabolites following oral administration of pentobarbital. (1'RS,5RS)-1-(β-D-Glucopyranosyl)pentobarbital ((1'RS,5RS)-PTBG) was isolated from the urine of one subject. The two diastereomers, (1'RS,5R)-PTBG and (1'RS,5S)-PTBG were separated and found to be identical to synthetic standards when compared using HPLC retention times coupled with UV (with and without post-column ionization) and mass spectrometry (HPLC/MS). A HPLC method was developed for detecting and quantifying (1'RS,5R)-PTBG, (1'RS,5S)-PTBG and pentobarbital in urine. Following a single oral dose of sodium pentobarbital to male subjects (n = 6), 1.6–6.2% of the pentobarbital dose was excreted as (1'RS,5S)-PTBG over 60 hours. (1'RS,5R)-PTBG was also detected in one subject and accounted for 0.3% of the pentobarbital dose. Using a modified HPLC system, the four pentobarbital N-glucosides were resolved and analysis of a partially purified pentobarbital N-glucoside extract from one subject indicated that only (1'R,5R)-PTBG and (1'S,5S)-PTBG could be detected as urinary excretion products. These results indicate that the side chain chirality of pentobarbital may influence the observed enantioselectivity for the formation and/or urinary excretion of the pentobarbital N-glucosides.

KEY WORDS: barbiturate; N-glycosylation; product enantioselectivity; pentobarbital; urinary excretion; phase II metabolism.

INTRODUCTION

N-Glucosylation has been proposed to be a general pathway for the metabolism of barbiturates in humans.[1,2] Extensive chemical and spectroscopic evidence have been provided to show that amobarbital and phenobarbital N-glucosides are excreted in human urine following oral administration of the parent drug to humans.[3–6] A prior study on pentobarbital metabolism in humans had proposed that pentobarbital also underwent N-glucosylation, however, the identification of the metabolite was based on limited mass spectral and chromatographic data.[7,8] In initial studies on the structural requirements necessary for the formation of the D-glucose conjugates of barbiturates, it became apparent that pentobarbital required further study. Since pentobarbital is used as a racemate, coupling D-glucose to one of the ring nitrogens will also confer asymmetry to C-5 of the barbiturate ring and four pentobarbital N-glucoside diastereomers ((1'RS,5RS)-PTBG) are possible[9], as shown in Figure 1. The identification of the pentobarbital N-glucoside metabolites in urine would provide information concerning the chirality associated with their formation and/or urinary excretion. In this report the pentobarbital N-glucosides were isolated and identified, and an isocratic HPLC method was developed for detection and quantification of (1'RS,5R)-PTBG and (1'RS,5S)-PTBG in urine. From one subject further investigations were carried out to study the absolute configuration of the pentobarbital N-glucosides excreted in the urine.

EXPERIMENTAL

Materials

Pentobarbital ((1'RS)-5-ethyl-5-(1'methylbutyl)barbituric acid) and butalbital (5-allyl-5-(2-methylpropyl)barbituric acid) were purchased from Sigma Chemical Co. (St. Louis, MO). (1'RS,5R)-, (1'RS,5S)-, (1'R,5R)-, (1'S,5R)-, (1'R,5S)-, and (1'S,5S)-5-ethyl-5-(1'-methylbutyl)-1-(β-D-glucopyranosyl)barbituric acid were available from a prior study.[9] Acetonitrile (MeCN), ethyl acetate (EtOAc), methanol and monobasic and dibasic sodium phosphate were HPLC grade. All other chemicals were reagent grade.

Chromatographic Analysis

The HPLC equipment used for the semipreparative purification of the pentobarbital N-glucosides has been previously described[4]. The LC/UV and LC/MS analysis were carried out using equipment described for the identification of phenobarbital N-glucosides in mouse urine.[10]

The HPLC equipment used for quantification of the pentobarbital N-glucosides has been previously described[11]. The analytical HPLC method used for quantifying the pentobarbital N-glucosides in urine used an Econosphere C_{18} column (5 μm, 250 × 4.6 mm i.d.; Alltech Associates, Deerfield, IL). The mobile phase was a solution of 19.4% (v/v) MeCN in 0.025 sodium phosphate buffer, pH 6.5. The injection volume was 20 μl, the flow rate was 1.4 ml min^{-1}, and the eluate was monitored at 198 nm. The analysis was carried out at 25°C.

Sample Preparation and Collection

Individual stock solutions of pentobarbital (0.27 mM), (1'RS,5S)-5-ethyl-5-(1'-methylbutyl)-1-(β-D-glucopyranosyl)barbituric acid ((1RS,5R)-PTBG, 0.87 mM) (1'RS,5S)-5-ethyl-5-(1'-methylbutyl)-1-(β-D-glucopyranosyl)barbituric acid ((1RS,5S)-PTBG, 0.90 mM), and butalbital (0.53 mM) were prepared in methanol and stored at −20°C. The procedure used for the preparation of sample standards in urine and extraction of urine samples was identical to that used for the analysis of barbital N-glucoside in urine[13].

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NOTATIONS: (1'RS,5RS)-1-(β-D-Glucopyranosyl)pentobarbital ((1'RS,5RS)PTBG) (1'RS,5S)-5-ethyl-5-(1'-methylbutyl)-1-(β-D-glucopyranosyl)barbituric acid ((1RS,5R)-PTBG) (1'RS,5S)-5-ethyl-5-(1'-methylbutyl)-1-D-glucopyranosyl)barbituric acid ((1RS,5S)-PTBG.

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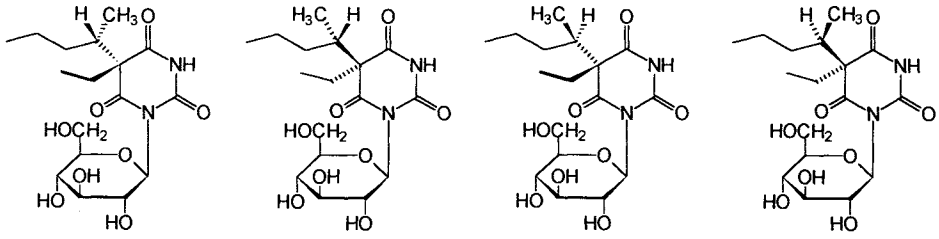

Fig. 1. Structures of pentobarbital N-glucoside diastereomers.

Six adult male volunteers were enrolled in the study. Four subjects were caucasian and two subjects were asian 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,[11] amobarbital[12] and barbital[13] had been evaluated. The same protocol was followed as described in the previous studies. All subjects received a 100 mg oral dose of sodium pentobarbital (403 μmole, capsule, Abbott Pharmaceuticals, Inc., North Chicago, IL) just prior to retiring for the night. The total urine was collected as individual samples at natural periods for 60 hr.

Partial Purification of (1RS,5R)-PTBG and (1RS,5S)-PTBG Conjugates from Urine

To confirm the presence of (1RS,5R)-PTBG and (1RS,5S)-PTBG in urine, approximately 1600 ml of urine (obtained 8 to 20 hours after dosing) from Subject #4 was extracted using techniques comparable to those described for phenobarbital N-glucosides[10]. The urine extract was partially purified by HPLC using a single C_{18} reversed-phase column (mobile phase: 30% MeCN/70% 0.1M NH_4OAc buffer (v/v), flow rate of 2.5 ml/min). The fractions containing (1RS,5R)-PTBG and (1RS,5S)-PTBG (retention volume 18–50 ml) were pooled, the solution was concentrated to apparent dryness, then dissolved in 600 μl of 0.1N HCL and extracted 5 times with 3 ml of EtOAc. A portion of the combined EtOAc extracts was retained for LC/MS analysis. The EtOAc fractions were dried over anh Na_2SO_4, evaporated to dryness and dissolved in 600 μl of HPLC mobile phase and purified again by semipreparative HPLC using two C_{18} reversed phase columns in series (mobile phase: 20% MeCN/80% water (v/v), flow rate of 4.0 ml/min). The fractions with retention volume of 36 to 60 ml contained (1RS,5R)-PTBG and (1RS,5S)-PTBG. The fractions were evaporated to dryness under reduced pressure to give 0.3 mg of a white powder that was used in the LC/UV analysis.

The mobile phase for the LC/UV characterization was 19% (v/v) MeCN in 0.025 M sodium phosphate buffer, pH 6.5. For the synthetic standard the t_R was 15.6 min for (1RS,5R)-PTBG and 16.6 min for (1RS,5S)-PTBG. The UV maximum was 196 nm for both (1RS,5R)-PTBG and (1RS,5S)-PTBG. Following post-column ionization two UV maxima were observed at 198 and 238–242 nm.

The conditions used to obtain the thermospray mass spectra (LC/MS) have been previously described[10]. The chromatography for the LC/MS used a mobile phase of MeCN:0.1 M ammonium acetate (15:85) at a flow rate of 1.2 ml/min. For the synthetic standards the t_R was 17.7 min for (1RS,5R)-PTBG and 18.1 min for (1RS,5S)-PTBG. The chemical ionization mass spectrum exhibited a *m/z* 389 (11%, $(M + H)^+$) and 406 (100%, $(M + NH_4)^+$) ion for both (1RS,5R)-PTBG and (1RS,5S)-PTBG.

RESULTS

Identification of Pentobarbital N-Glucosides Isolated from Urine

The partially purified urine extract from subject #4 was analyzed by LC/MS using single ion monitoring (SIM) for ions at *m/z* 406 (base ion, $M + NH_4^+$) and 389 (parent ion, $M + H^+$). When analyzing the urine concentrate, the base ion at *m/z* 406 was observed at 17.7 and 18.1 min for both (1RS,5R)-PTBG and (1RS,5S)-PTBG, however, the parent ion at *m/z* 389 was detected only for (1RS,5S)-PTBG.

In the LC/UV analysis of the partially purified urine concentrate, absorbances eluted at 15.6 and 16.6 min with λ_{max} at 196 nm (pH 6.5) and λ_{max} at 198–200 and 240 nm (pH 10.0), identical to synthetic standards. The relative percentage of the diastereomers present in the partially purified sample was 12% (1RS,5R)-PTBG and 88% (1RS,5S)-PTBG (based on the cut and weigh method).

Analytical Methodology and Assay Validation

All samples were run in duplicate. A chromatogram of (1RS,5R)-PTBG, (1RS,5S)-PTBG, pentobarbital and butalbital (IS) extracted from 200 μl of urine is shown in Fig. 2a. A chromatogram of an extract of blank urine containing butalbital is shown in Fig. 2b. A chromatogram of a urine extract from subject #1 following oral administration of pentobarbital is shown in Fig. 2c. In the blank urine potential interfering absorbances were observed to elute just prior to (1RS,5R)-PTBG. When this interference was observed, this necessitated use of a modified HPLC system (mobile phase of 18% (v/v) MeCN in 0.25 sodium phosphate buffer, pH 6.5) which enabled resolution of (1RS,5R)-PTBG from the interfering absorbance, but was not suitable for quantifying (1'RS,5S)-PTBG.

Butalbital was initially used as an internal standard to quantify (1RS,5R)-PTBG, (1RS,5S)-PTBG, and pentobarbital. The analysis of butalbital was found to be highly variable and it was necessary to estimate the amount of the pentobarbital urinary excretion products using the area normalization method. The ordinate was the peak area obtained for the analytes following a 20 μl injection. The abscissa was

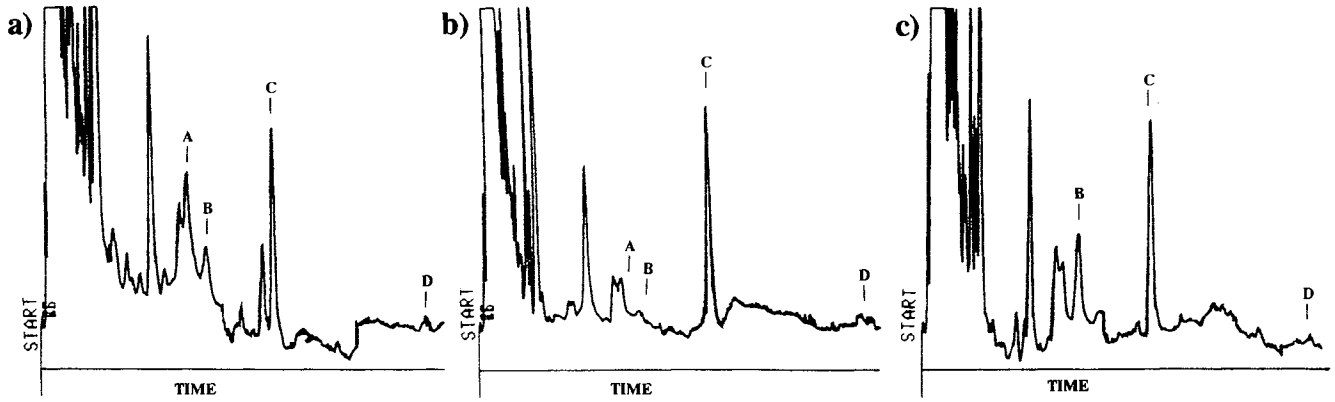

Fig. 2. Chromatograms of (a) the standards of (1'RS,5R)-PTBG (A, 4.3 μM, 16.3 min), (1'RS,5S)-PTBG (B, 4.5 μM, 17.4 min), butalbital (C, 27 μM, 30.4 min), and pentobarbital (D, 2.7 uM, 46.6 min) extracted from 200 μl of urine; (b) a 400-μl blank urine extract that was acidified with citric acid (butalbital was added as a standard prior to extraction, 31.5 min); (c) urine obtained 10 h after subject #1 had taken a 100 mg oral dose of sodium pentobarbital (A. (1'RS,5R)-PTBG-Not detected; B. (1'RS,5S)PTBG-6.7 μM, 17.5 min, D. pentobarbital- Not detected. Chromatographic conditions are described in text.

metabolite concentration (μM). A standard calibration curve was obtained by extraction of compounds from blank urine and plotting peak-area ratios of drug or metabolite to internal standard as a function of drug or metabolite concentrations. At concentration ranges of 1 to 45 nmol/ml, the y-intercept for (1RS,5R)-PTBG, was 8.5 ± 1.1 × 10^4 area units/μM (slope = 3.7 ± 1.8 × 10^4, r^2 = 0.961), (1RS,5S)-PTBG was 1.3 ± 1.0 × 10^5 (4.0 ± 1.4 × 10^4, 0.959) and pentobarbital was 1.9 ± 3.6 × 10^5 (1.1 ± 1.0 × 10^5, 0.872), respectively. The lower limit of detection was 0.8 nmole/ml. In a single run of 43 analyses the retention time for (1RS,5R)-PTBG was 16.3 ± 0.2 min, (1RS,5S)-PTBG was 17.5 ± 0.3 min and pentobarbital was 46.6 ± 0.5 min. (1RS,5S)-PTBG was present at a concentration of 30.4 nmole/ml in one urine sample, however, the concentration of (1RS,5S)-PTBG usually ranged from 3–8 nmole/ml during the first 24 hours.

Recovery of (1RS,5R)-PTBG, (1RS,5S)-PTBG, and pentobarbital was determined by analysis of standards prepared and extracted from blank urine. The recovery was determined by direct injection of equivalent quantities of compound dissolved in mobil phase. The mean recovery of (1RS,5R)-PTBG at 21.5 and 4.3 nmole/ml was 94 ± 10 (n = 13) and 72 ± 30 (n = 14), respectively. The mean recovery of (1RS,5S)-PTBG at 22.5 and 4.5 nmole/ml was 98 ± 13 and 84 ± 27 (n = 14), respectively. The mean recovery of pentobarbital at 13.3 and 2.7 nmole/ml was 136 ± 10 and 76 ± 43 (n = 13), respectively.

The within run precision was evaluated by analyzing urine samples spiked with (1RS,5R)-PTBG, (1RS,5S)-PTBG, and pentobarbital. The within run precision for (1RS,5R)-PTBG at 21.5 nmole/ml was 23.4 ± 2.6 nmole/ml (n = 13) and at 4.3 nmole/ml was 6.8 ± 3.4 nmole/ml (n = 14). The within run precision for (1RS,5S)-PTBG at 22.5 nmole/ml was 23.9 ± 3.5 nmole/ml and at 4.5 nmole/ml was 6.9 ± 3.1 nmole/ml. The within run precision for pentobarbital at 13.3 nmole/ml was 15.9 ± 2.9 nmole/ml and at 2.7 nmole/ml was 2.0 ± 2.4 nmole/ml.

Quantities of PTB Glucosides Excreted

The results of the analyses are shown in the Table. In the 0–60 hour urine following administration of sodium pentobarbital, (1RS,5S)-PTBG accounted for 2.6 ± 0.7% (n = 6) of the dose and (1RS,5R)-PTBG (0.3% of dose) was detected in only one individual.

Characterization of Pentobarbital N-glucosides in Subject #6

Material from subject #6 was partially purified as described above for identification of the individual diastereomers. Using two C_{18} reversed-phase columns (Econosil C-18 column, 5 μm, 250 × 4 mm i.d.) connected in series, the four diastereomers could be partially resolved using a mobile phase of 10% ACN/90% 0.25M sodium phosphate buffer, pH 6.5 (v/v) at a flow rate of 1.4 ml/min. The t_R of (1R,5R)-PTBG and (1R,5S)-PTBG was 138.2 and 163.3 min, respectively; and for (1S,5R)-PTBG and (1S,5S)-PTBG was 141.7 and 159.7 min, respectively. In the partially purified urine extract from subject #6, strong UV absorbances were observed at 138.6 and 160.2 min. These retention times indicate that (1R,5R)-PTBG and (1S,5S)-PTBG, respectively, are the major pentobarbital N-glucoside metabolites present in the sample.

Table I. Urinary Excretion of Pentobarbital Detected as Pentobarbital and Pentobarbital N-Glucosides

Subj	Race	(1'RS,5R)-PTBG μmoles (%)	(1'RS,5S)-PTBG μmoles (%)	Pentobarbital μmoles (%)
1	C	ND	11.6 (2.9)	1.1 (0.3)
2	C	ND	6.4 (1.6)	ND
3	C	ND	13.7 (3.4)	ND
4	C	ND	9.8 (2.4)	3.0 (0.8)
5	A	ND	12.6 (3.1)	ND
6	A	1.4 (0.3)	24.8 (6.2)	1.0 (0.3)
Average			10.4 (2.6)	1.0 (0.6)
std dev			2.7 (0.7)	1.2 (0.3)

C = Caucasian A = Asian

100 mg (403 μM) – M.W. 248.26, 60 hour collection

DISCUSSION

Pentobarbital is still used clinically for both its sedative and hypnotic properties, and is currently being evaluated for its control of intracranial pressure following severe head injury,[14,15] yet its metabolic profile in humans is still not well understood. Prior metabolism studies in humans have shown that pentobarbital is extensively metabolized in the human body, with approximately 1% of the ingested drug excreted unchanged in the urine.[16,17] The metabolite, 5-ethyl-5-(3′-hydroxy-1′-methylbutyl)barbituric acid formed by oxidation of the penultimate carbon (ω-1) of the methylbutyl side chain, is the major metabolite of pentobarbital, and accounts for 35–50% of the administered dose.[7,16–20] Other metabolites formed by humans include 5-ethyl-5-(1′-methyl-3′-oxobutyl)-barbituric acid (7–14% of the administered dose)[20], 5-ethyl-5-(4′-hydroxy-1′-methylbutyl)barbituric acid(0.1–1% of the administered dose)[16], and 5-ethyl-5-(1′-methyl-3′-carboxypropyl)barbituric acid (10–15% of the administered dose)[16,20]. The prior studies on pentobarbital biodisposition in which metabolites have been identified have accounted for approximately 65% of the ingested drug.[14,18]

Pentobarbital N-glucoside (referred to in the original literature as N-hydroxypentobarbital) was reported to account for 11–15% of the administered dose of pentobarbital[7,8], but a subsequent study was unsuccessful in isolating or detecting this metabolite.[20] The results from this study confirm that pentobarbital N-glucosides, **(1RS,5R)-PTBG** and **(1RS,5S)-PTBG**, are metabolites of pentobarbital. **(1RS,5R)-PTBG** and **(1RS,5S)-PTBG** isolated from urine following an oral dose of pentobarbital were found to be identical to synthetic standards when compared using HPLC retention times coupled with UV (with and without post-column ionization) and mass spectrometry (LC/MS). To determine the quantity of pentobarbital N-glucosides present as metabolites in human urine, an assay was developed for their detection and quantification. Due to the hydrophilic nature of the conjugates and the ability to resolve the pentobarbital N-glucoside diastereomers using a reverse-phase C-18 column, HPLC was the preferred analytical method. However, a major disadvantage of HPLC was its limited sensitivity for detecting the pentobarbital N-glucosides in urine. This is due to the weak chromophore associated with the barbiturate ring and the numerous interfering substances routinely present in urine. The assay that was ultimately developed for this study was useful in differentiating **(1RS,5R)-PTBG** and **(1′RS,5S)-PTBG** and for detection and quantification of **(1′RS,5S)-PTBG**. However, it can be seen in the Table that **(1′RS,5R)-PTBG** was produced at levels which could not be quantified even though its presence was verified in the semi-preparative preparation. **(1′RS,5R)-PTBG** appears to be present at approximately 1/10 the concentration of **(1′RS,5S)-PTBG**, below the lower limit of detection for the pentobarbital N-glucosides using this assay. Pentobarbital when detected, was present near its limits of detection. The low level of pentobarbital measured in the urine was consistent with that observed in comparable metabolism studies.[7,16,17] Using this HPLC assay, these results confirm that pentobarbital N-glucosides are metabolites of pentobarbital and that they are excreted in the urine. The pentobarbital N-glucosides accounted for 2.6% (range 1.6–6.5%) of the administered dose. It would appear that the pentobarbital N-glucosides excreted in the urine can account for only a small percentage of the biodisposition of pentobarbital and that 30–35% of the biodisposition of pentobarbital remains to be characterized.[16,20]

Little is known concerning the quantitative importance of the glucosylation pathway for drug disposition in man, however, some trends are beginning to develop for the barbiturates. Both the caucasian and asian subjects in this study had been previously documented as excreting amobarbital N-glucosides (3.1–22.9% of administered dose)[12] and phenobarbital N-glucosides (4.1–10.6% of administered dose)[11], but not a barbital N-glucoside[13]. It would appear that quantitatively, the relative importance of the glucosylation pathway in the biodisposition of the barbiturates is amobarbital $>$ phenobarbital $\gtrsim$ pentobarbital $>>$ barbital.

Metabolic processes which create additional chiral centers, such as aliphatic oxidation or N-glucosylation, results in the formation of diastereomers. Prior metabolism studies have shown that the biodisposition of racemic pentobarbital in humans exhibit a product enantioselectivity[21] and possibly a substrate enantioselectivity.[17] The N-glucosylation of pentobarbital exhibited a product enantioselectivity for the formation and/or excretion of the pentobarbital N-glucoside. The major pentobarbital N-glucoside diastereomer excreted had the S configuration at C-5 of the barbiturate ring, the same as what was observed for amobarbital and phenobarbital N-glucosylation. In addition, this study suggests that a substrate enantioselectivity could be occurring, since subject #6 appeared to excrete primarily the N-glucoside of (1′S)-pentobarbital. However, urinary excretion studies are not capable of proving substrate enantioselectivity, since once the pentobarbital N-glucosides are formed, additional stereoselective metabolism and excretion could be occuring. Substrate enantioselectivity for the biodisposition of pentobarbital is important since in humans it is reported that the S(-) isomer of PTB produces a longer period of sedation, has a smaller volume of distribution, a slower clearance, a longer half-life and is protein bound to a greater extent than the R(+) isomer.[17,22] However, the extent to which product and substrate enantioselectivity occurs during the metabolism of pentobarbital can best be resolved by *in vitro* studies using human tissues.

In conclusion, this study has shown that pentobarbital, like amobarbital and phenobarbital, are substrates for N-glucosylation in humans. These three barbiturates have shown a product enantioselectivity in the formation and/or excretion of these conjugates and the S diastereomer (C-5 of the barbiturate ring) is the major barbiturate N-glucoside conjugate excreted in human urine. The presence of structural asymmetry in pentobarbital may also be influencing the formation and/or excretion of the N-glucoside conjugates. Studies are now needed in which absorption, distribution, additional metabolism and excretion are minimized so that this unusual metabolic pathway in humans can be better characterized.

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