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METABOLISM OF DRUGS—LXIII

THE METABOLIC FATE OF ^{14}C-PHENACETYLUREA (PHENURONE) IN MICE

KIYOSHI TATSUMI, SHIN'ICHI YOSHIHARA, HIDETOSHI YOSHIMURA and HISAO TSUKAMOTO

Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

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Abstract—In a study of the metabolism of ^{14}C-phenacetylurea in mice, the urinary excretory products have been found to include 4-hydroxyphenacetylurea, phenaceturic acid, phenylacetic acid, glucuronides of 4-hydroxyphenacetylurea and 4-hydroxyphenylacetic acid, as well as unidentified metabolites and the unchanged compound. However, unlike rabbits, mice apparently do not convert phenacetylurea to 3-methoxy-4-hydroxyphenacetylurea.

The formation of the 4-hydroxy metabolite of phenacetylurea has been demonstrated as not resulting from the action of intestinal bacteria.

IN OUR PREVIOUS paper, (this issue, p. 279) it was shown that phenacetylurea administered to rabbits is metabolized to 4-hydroxyphenacetylurea, 3-methoxy-4-hydroxyphenacetylurea, phenylacetic acid and phenaceturic acid. These results led to the suggestion that the metabolism of phenacetylurea follows two different pathways. The first pathway involves successive hydroxylation at the 4-position and then at the 3-position of the benzene ring, followed by methylation of the 3-hydroxyl group. The second pathway involves hydrolysis of the ureide group and is followed by conjugation with glycine.[1] Among these metabolites, particular interest was directed to the methoxyhydroxy metabolite, since the formation *in vivo* of such a metabolite has been reported in the intermediary metabolism of phenylalanine[2, 3] and of estrogens,[4] but never in the metabolism of foreign compounds possessing an aromatic ring. The latter compounds are usually metabolized by hydroxylation and subsequent conjugation. It seemed worthwhile, therefore, to determine whether 3-methoxy-4-hydroxyphenacetylurea is also formed in an animal species other than rabbits after the administration of phenacetylurea.

The present report explores such a metabolic pathway for phenacetylurea in mice utilizing the ^{14}C-labeled compound. Further experiments have been undertaken to investigate the influence of intestinal bacteria on the metabolism of this drug. For these purposes a routine method which permits the quantification of the metabolites was established by a combination of thin-layer and paper chromatography.

METHODS

Materials. Phenylacetic acid-1-^{14}C was purchased from the Radio-Chemical Centre, Amersham, Bucks (England). Hyamine hydroxide was obtained from

Packard Instrument Ltd., Wembley (England). PPO (2,5-diphenyloxazole) and POPOP (1,4-*bis*-2(5-phenyloxazolyl)benzene) were purchased from Wako Pure Chemical Industries, Ltd., Osaka (Japan). Protease "Nagarse" was purchased from Nagase & Co., Ltd., Osaka (Japan). The β-glucuronidase preparation was obtained from preputial glands of adult female rats[5] and its activity (116,000 units/ml) was determined by using *p*-nitrophenyl-β-D-glucuronide as the substrate according to the method of Kato *et al.*[6] Pure unlabeled phenacetylurea was kindly supplied by Dainippon Pharmaceutical Inc., Osaka (Japan).

Synthesis of ^{14}*C-phenacetylurea.* Phenylacetic acid-1-^{14}C (0·2 g, 0·1 mc) was converted to methyl phenylacetate-^{14}C by treatment with CH_2N_2 by the usual method. To a solution of urea (0·14 g) in a mixture of 25% sodium ethoxide (2·5 ml) and pyridine (0·9 ml) was added the methyl phenylacetate-^{14}C (0·23 g). This mixture was allowed to stand for 7 hr at room temperature, was poured into 8% acetic acid (20 ml) while stirring, and was then extracted with ethyl acetate (10 ml × 3). The ethyl acetate extract was successively washed with dilute HCl and H_2O, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*, leaving a crystalline material. Recrystallization from ethanol gave colorless crystals (84 mg) with an overall radiochemical yield of 25 per cent. The purified product was subjected to TLC with systems 3 and 4 (see below); this revealed only one spot at an R_f value which corresponded to that of an authentic sample of phenacetylurea. The location of the spots was determined by spraying with either H_2SO_4 or sodium nitroprusside reagent; a radio-chromatogram scan also indicated only one radioactive peak and this coincided with the same spot. An infrared absorption spectrum of the labeled compound was identical with that of authentic phenacetylurea.

Administration of ^{14}*C-phenacetylurea.* Male mice of CF-1 strain weighing 20–25 g were used in this study. The animals were fasted overnight prior to administration of the drug. ^{14}C-phenacetylurea was suspended in 10% gum arabic and administered orally in a dose of 160 mg/kg (sp. act., 0·29 μc/mg) in all experiments unless otherwise indicated. After medication, each group, which consisted of two animals, was housed in a Roth metabolism cage designed for the quantitative collection of urine and feces and of expired $^{14}CO_2$. Food and water were available at all times.

Radioisotopic methods of analysis. The radioactivity of all samples was measured with a Packard Tri-Carb liquid scintillation spectrometer (model 314 EX-2). Urine samples were filtered through filter paper (Toyoroshi no. 2, Toyoroshi Co., Tokyo, Japan) and diluted to 25 ml. Aliquots of urine (0·1 – 0·4 ml) were counted in a *p*-dioxane phosphor consisting of 60 g naphthalene, 4 g PPO, 0·2 g POPOP, 100 ml methanol, 20 ml ethylene glycol and *p*-dioxane to make 1 l. Expired CO_2 was trapped in 2-methoxyethanol—monoethanolamine (2:1). Aliquots (3 ml) of the above solution were counted in a phosphor containing 0·55% of PPO in 2-methoxyethanol—toluene (1:2). Feces (50 mg, wet weight) were digested in 2 ml Hyamine hydroxide and prepared for counting according to the method of Herberg.[7] The material was then counted in a toluene phosphor prepared by solubilizing 4 g PPO and 0·1 g POPOP in 1 l. of toluene.

Paper and TLC. Paper chromatography was carried out by the ascending technique with filter paper (Toyoroshi no. 51 A, Toyoroshi Co., Tokyo, Japan). The solvent systems used were: 1) benzene:propionic acid:H_2O (2:2:1); 2) *iso*-propanol: NH_4OH:H_2O (8:1:1); 3) *n*-butanol saturated with 1·5 N NH_4OH. Thin-layer plates

of silica gel (Kieselgel G, Merck; activated at 110° for 60 min) were used with solvent system 3 (described above) and with another solvent system: 4) benzene: chloroform: acetic acid (2:1:1). The solvent systems and R_f values of the reference compounds are given in Table 1.

TABLE 1. R_f VALUES OF THE REFERENCE COMPOUNDS

Compound	PPC			TLC	
	Solvent system			Solvent system	
	1	2	3	3	4
Phenacetylurea	0·99	0·84	0·77	0·68	0·70
4-Hydroxyphenacetylurea	0·68	0·64	0·65	0·73	0·37
3-Methoxy-4-hydroxy-phenacetylurea	0·84	0·57	0·59	0·67	0·49
Phenylacetic acid	0·99	0·53	0·42	0·38	0·80
Phenaceturic acid	0·72	0·56	0·31	0·24	0·30
4-Hydroxyphenylacetic acid	0·57	0·35	0·14	0·26	0·54
3-Methoxy-4-hydroxy-phenylacetic acid	0·75	0·28	0·12	0·21	0·66

Detection and determination of the metabolites. Ultraviolet light was used for nonspecific detection after the thin-layer plates were sprayed with 20% H_2SO_4 followed by heating. Phenolic compounds were visualized by spraying a 0·2% solution of diazotized sulfanilic acid in 10% Na_2CO_3 solution. Phenacetylurea was also visualized by spraying successively with a 5% sodium nitroprusside solution and a 30% NaOH solution. Bromthymol blue reagent (40 mg bromthymol blue in 100 ml alkaline ethanol) and a slightly modified Ehrlich reagent[8] were used for detection of phenyl acetic acid and phenaceturic acid, respectively, on the paper chromatograms. Radioactivity on paper chromatograms was determined by cutting the papers into 1- to 2-cm strips, placing these in vials containing 10 ml of a toluene scintillation mixture and counting the radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. Radioactivity on TLC's was determined by using a thin-layer radio-chromatogram scanner (Japan Radiation & Medical Electronics, Inc., Tokyo, Japan).

Since it was not possible to separate all of the metabolites from one another by either single thin-layer or paper chromatography, the quantitative determination of each urinary metabolite was carried out by a combination of the two processes. The recoveries of radioactive metabolites, namely phenacetylurea, 4-hydroxyphenacetylurea, phenaceturic acid and phenylacetic acid, were demonstrated to be more than 95 per cent by this method.

RESULTS

Excretion of metabolites. Assay of the excreta of mice showed that most of the radioactivity was present in the urine; only about 1·4 – 4·8 per cent of the administered ^{14}C was recovered in feces. The expired carbon dioxide contained negligible radioactivity. Although only 62–68 per cent of the dose was accounted for, most of the radioactivity appeared during the first 24 hr (Table 2).

TABLE 2. PERCENTAGE OF ADMINISTERED RADIOACTIVITY RECOVERED IN URINE, FECES AND CO_2*

Time (hr)	Group I			Group II			Group III		
	Urine	Feces	CO_2	Urine	Feces	CO_2	Urine	Feces	CO_2
0–24	60·0	1·34	0·01	59·2	4·70	0·02	62·9	1·72	0·03
24–48	0·3	0·02	trace	0·2	0·07	trace	1·9	0·28	0·01
48–72	0·2			0·1	0·02	0	0·7	0·08	0·01
72–96				0·05	0·03	0	0·2	0·04	trace
96–144				0·01	trace	0	0·04	0·05	0
Total	60·5	1·36	0·01	59·65	4·82	0·02	65·74	2·17	0·05

* Each group, consisting of two animals which were housed in a Roth metabolism cage, was given an oral dose of 160 mg/kg of phenacetylurea.

Detection of the urinary metabolites. The 24-hr urine from two mice which had received ^{14}C-phenacetylurea was adjusted to pH 1 with HCl and was extracted five times with an equal volume of ethyl acetate. After centrifugation, the organic phase was removed and evaporated to dryness *in vacuo*. The residue was redissolved in a small volume of ethyl acetate and was then examined by thin-layer chromatography with solvent system 4. The remaining aqueous phase was also evaporated to a small volume *in vacuo* and submitted to the same chromatographic examination. As seen in Table 3,

TABLE 3. THIN-LAYER CHROMATOGRAPHY OF THE ETHYL ACETATE EXTRACT AND REMAINING AQUEOUS PHASE OF URINE

Fraction	Peak	R_f value (TLC, system 4)	Percent radioactivity
Ethyl acetate extract	A	0·10	14
	B	0·35	15
	C	0·75	30
Aqueous phase	D	0·02	41

three radioactive peaks (A, B and C) were separated in the chromatogram of the ethyl acetate extract, whereas in the chromatogram of the aqueous phase only one peak (D) was located and it was close to the origin.

These peaks were extracted separately from the silica gel with methanol and treated as described below.

Ethyl acetate extract. The methanol extracts from peaks A, B and C were rechromatographed respectively on paper using system 3. By this treatment, peak A was further separated into three radioactive peaks with R_f values of 0·03, 0·18 and 0·48; however, none of these R_f values was in agreement with those of known compounds. These unidentified materials are tentatively designated herein as unknowns I, II and III respectively.

The paper chromatogram of peak B revealed four distinct radioactive peaks having R_f values of 0·03, 0·18, 0·31 and 0·65. The first two peaks were identical with those obtained from peak A, namely unknowns I and II. The contamination of peak B by unknowns I and II indicated incomplete separation of peaks A and B. The peaks with R_f values of 0·31 and 0·65 corresponded to those of authentic phenaceturic acid and

4-hydroxyphenacetylurea respectively. Each area having these R_f values on the paper chromatogram was eluted from the paper and was subsequently rechromatographed on paper in solvent systems 1 and 2. In both systems the radioactivity had the same mobility as that of the corresponding authentic sample.

The radioactive zone of peak C exhibited three radioactive peaks on paper with R_f value of 0·42, 0·53 and 0·77. Of these, the first peak (R_f 0·42) and the third peak (R_f 0·77) were identified as phenylacetic acid and unchanged phenacetylurea respectively; however, the second peak had an R_f value (0·53) which did not correspond to that of any known compound. The identity of the peaks of R_f 0·42 and 0·77 as phenylacetic acid and phenacetylurea was further confirmed by chromatography in solvent systems 1 and 2.

The aqueous phase. The methanol eluate from peak D described above was evaporated to dryness *in vacuo* and the residue was treated with β-glucuronidase in the absence or presence of saccharo-1,4-lactone in 0·1 M acetate buffer (pH 4·5) at 37° for 24 hr. This was done in order to obtain information about glucuronides which might be present in this polar fraction. In the experiment without inhibitor, the radioactive scan of the thin-layer chromatogram of this incubation mixture, using solvent system 4, revealed the presence of two small peaks having R_f values which corresponded to those for 4-hydroxyphenacetylurea and 4-hydroxyphenylacetic acid. Further identification of these new peaks was also performed by paper chromatography with solvent system 3. Most of the original activity, however, was retained close to the origin, as before (Peak D′, Table 4).

TABLE 4. TLC OF THE HYDROLYSATE OF PEAK D WITH β-GLUCURONIDASE

Peak	R_f value (TLC, system 4)	Percentage of radioactivity
4-Hydroxyphenylacetic acid	0·54	2
4-Hydroxyphenacetylurea	0·37	5
D′	0·02	93

From the above results, it was evident that the aqueous phase contained a small amount of the β-glucuronides of 4-hydroxyphenacetylurea and 4-hydroxyphenylacetic acid. The remaining radioactive peak (D′), the mobility of which was not affected by β-glucuronidase, was also not hydrolyzed by Takadiastase, which contains arylsulfatase. However, when this peak, after elution from the silica gel, was refluxed in 1 N HCl for 30 min and then submitted to TLC in system 4, a new radioactive peak appeared at an R_f (0·54) corresponding to that of 4-hydroxyphenylacetic acid. The radioactivity of this peak accounted for about 73 per cent of that of peak D′. This compound, when eluted from the silica gel, also had the same mobility as that of authentic 4-hydroxyphenlyacetic acid (paper chromatography, system 2).

Determination of the urinary metabolites. The composition of the first 24-hr urine was investigated. When the drug was given orally, about 60 per cent of the urinary radioactivity was extractable into an ethyl acetate solution and about 40 per cent remained in the aqueous phase. Unchanged phenacetylurea accounted for 20·8

per cent of the excreted radioactivity; 4-hydroxyphenacetylurea, 12·7 per cent; phenaceturic acid, 2·4 per cent; phenylacetic acid, 9·3 per cent; the glucuronide of 4-hydroxyphenacetylurea, 1·8 per cent; the glucuronide of 4-hydroxyphenylacetic acid, 0·5 per cent; and unknown, 52·5 per cent.

Detection of metabolites in tissues. Two mice were sacrificed 1 hr after drug administration. The liver and kidney of each animal were then removed, homogenized and dried *in vacuo*. The intestine was washed with water to remove the contents and was then treated the same as the liver and kidney. The dried tissues were suspended in water, heated for 3 min on a boiling water bath and hydrolyzed with Nagarse at 37° for 24 hr. The hydrolysates were adjusted to pH 1 with HCl and extracted four times with equal volumes of ethyl acetate. Each of the combined ethyl acetate solutions was evaporated to a small volume and submitted to TLC with system 4 in order to identify the metabolites present. It was found that the liver, kidney and intestine contained predominantly 4-hydroxyphenacetylurea as a metabolite in addition to unchanged compound. On the other hand, the intestinal contents contained only the unchanged compound.

Metabolism of phenacetylurea administered intraperitoneally as compared to that administered orally. Fifteen mice were divided into three groups containing five animals each. ^{14}C-phenacetylurea was given orally to one group and i.p. to another in a dose of 200 mg/kg. The third group was used as control. The urine samples used for detection of phenolic metabolites were collected for 24 hr from each group. The urine of each group was treated as follows. A 20-ml portion of the 24-hr urine was adjusted to pH 2·5 with 20% H_2SO_4 and extracted three times with an equal volume of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*. The residue was redissolved in 3 ml ethyl acetate and shaken twice with 2 ml of 0·5 N NaOH solution. The alkaline phase was adjusted to pH 1 with N HCl and then extracted three times with 3 ml ethyl acetate. The organic solvent layers were combined and evaporated to dryness *in vacuo*. The residue was redissolved in a small quantity of ethyl acetate, applied to a thin-layer plate, 20 × 20 cm, and developed with solvent system 4. The areas on the chromatogram which corresponded to authentic 4-hydroxyphenacetylurea and 3-methoxy-4-hydroxyphenacetylurea were scraped off and extracted with ethyl acetate. Each residue remaining after evaporation of the ethyl acetate from the extract was redissolved in a small quantity of ethyl acetate and rechromatographed on a thin-layer plate with solvent system 4. In both orally or i.p. dosed animals, one of two fractions which was obtained from the area corresponding to 4-hydroxyphenacetylurea, when sprayed with diazotized sulfanilic acid reagent, showed an orange color on the plate and had an R_f value the same as that of authentic 4-hydroxyphenacetylurea, but no 3-methoxy-4-hydroxyphenacetylurea was detected in the other fraction. It was shown that the urine sample from the control group did not give spots on the plates corresponding to those of any of the metabolites.

DISCUSSION

As shown herein, the development of a method employing TLC in conjunction with paper chromatography has made possible the quantitative identification of the radioactive metabolites of phenacetylurea. The results have shown that in mice the radioactivity derived from this compound is excreted almost entirely in the urine, a small amount appears in the feces and a negligible amount is excreted as $^{14}CO_2$. The total

recovery of the radioactivity in the excreta during the 6 days was only 65 per cent of the dose. The fate of the remaining radioactivity is uncertain at the present time.

About 60 per cent of the radioactivity in the 24-hr urine after administration of ^{14}C-phenacetylurea was extractable with ethyl acetate and it was composed of unchanged phenacetylurea, 4-hydroxyphenacetylurea, phenylacetic acid, phenaceturic acid and unidentified metabolites. The radioactivity remaining in the aqueous residue after ethyl acetate extraction accounted for about 40 per cent of the total urinary activity the metabolites in this fraction were demonstrated to be the glucuronides of 4-hydroxyphenacetylurea and of 4-hydroxyphenylacetic acid and a fraction which seemed to consist of one or more metabolites.

Previous work has demonstrated the conversion *in vivo* of phenacetylurea to 3 methoxy-4-hydroxyphenacetylurea in rabbits and has suggested that this metabolite may be formed via the 3,4-dihydroxy derivative of phenacetylurea,[1] since it is well known that methylation of the catecholamines does produce the analogous m-methylether. However, in the present study, it was found that the mouse did not excrete any detectable amount of this ether; rather it excreted only the monohydroxy-metabolite. On the basis of these results, it seemed reasonable to assume that the enzyme responsible for the formation of the dihydroxy derivative is either lacking or its activity in mice is very low.

Among the metabolites remaining in the aqueous phase after ethyl acetate extraction, two glucuronides were identified, although the amount of each was very small. One glucuronide may be the ether glucuronide of 4-hydroxyphenacetylurea, since it is known that *N*-glucuronides such as those of amines[9–12] and carbamates[13] are usually resistant to hydrolysis with β-glucuronidase. The other glucuronide is either an ether or ester glucuronide of 4-hydroxyphenylacetic acid, but the structure of this glucuronide has not yet been definitely determined. Most of the metabolites remaining in the aqueous phase were essentially unaffected by β-glucuronidase and Takadiastase. However, when these metabolites were subjected to acid hydrolysis, 4-hydroxyphenylacetic acid was formed as a product. Since in the earlier experiments it was shown that

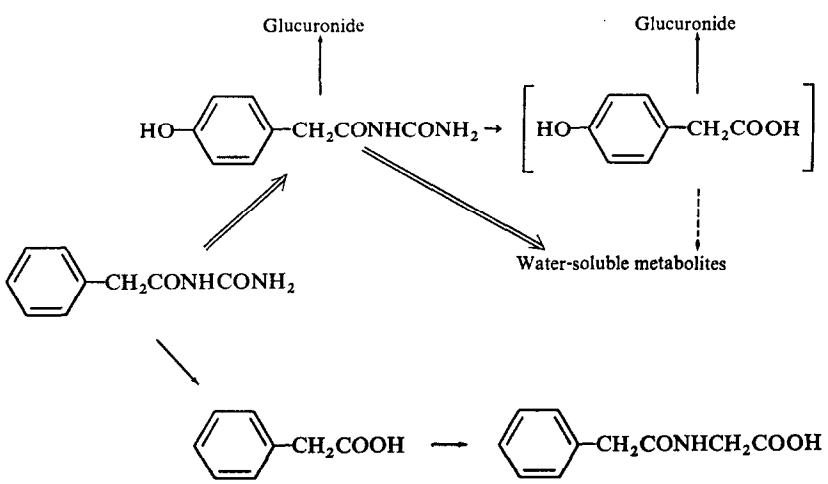

FIG. 1. The proposed metabolic pathways of phenacetylurea in mice.

4-hydroxyphenacetylurea splits to produce the corresponding acid under equivalent conditions, most of the water-soluble metabolites are believed to be conjugates either of 4-hydroxyphenacetylurea or of the corresponding acid.

On the basis of our present knowledge, it appears that the oxidative pathway is preponderant over the hydrolytic pathway in the biotransformation of phenacetylurea in mice. These possible metabolic pathways of phenacetylurea are illustrated in Fig. 1.

In order to examine any possibility that ring hydroxylation of phenacetylurea might be attributed to intestinal bacteria, additional experiments were performed and this possibility was essentially excluded, since phenacetylurea, even when administered i.p., was metabolized to 4-hydroxyphenacetylurea.

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