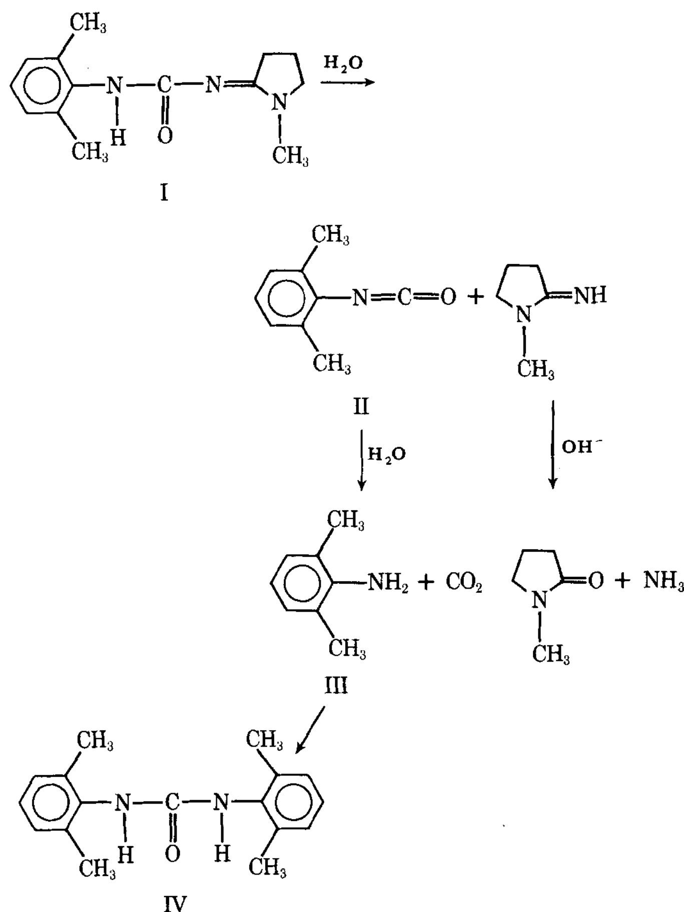

Scheme I—Proposed decomposition pathway for xilobam.

placed on stability. The tablets were stable at room temperature and 40° but decomposed at 40° and 80% RH and at 60° and decomposed rapidly at 80°. An odor of ammonia was noted from the decomposed samples. Tablets then were stored at 70° for 2 days in containers with and without closures. The tablets stored without closures were white and assayed satisfactorily. Tablets stored with closures were badly discolored, and

Table VI—Xilobam Tablet Stability

Days	Percent of Label Amount (200 mg/tablet)				
	25°	40°	60°	80°	With Molecular Sieves (80°)
Initial	98.7	98.7	98.7	98.7	98.7
19	—	—	—	—	96.1
39	98.4	97.9	24.5	5.4	—
109	98.1	96.2	—	—	77.4

the assays showed extensive degradation. The experiment was repeated, and the headspace was analyzed by GLC–mass spectrometry. The mass spectroscopic data showed the presence of ammonia.

Molecular sieves that absorb both water and ammonia vapors were added to bottles of xilobam tablets. The tablets in amber glass bottles with standard closures were stored at 80° for 109 days. Tablets stored with molecular sieves were much more stable at 80° (Table VI). Without molecular sieves, complete degradation was observed.

The tablets that were degraded extensively were ground, extracted with methanol, and chromatographed by TLC. The separated spots were scraped from the plates; sufficient quantities were obtained to run IR, NMR, and mass spectrometric spectra. The degradation products were *N*-methylpyrrolidone, 2,6-dimethylaniline, and *N,N'*-bis(2,6-dimethylphenyl)urea. The proposed degradation pathway is given in Scheme I.

Xilobam tablets were studied for up to 2 years at 25° (room temperature) and 40° in amber glass containers. No differences were observed at these conditions for tablets stored without molecular sieves compared to those stored with molecular sieves. However, at higher temperatures, the tablets with molecular sieves were much more stable than those without them.

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ACKNOWLEDGMENTS

The authors thank J. Korpi, E. L. Kelly, H. R. Almond, R. K. Gilpin, C. Y. Ko, J. S. Ranweiler, and F. A. Chrzanowski for technical assistance.

Studies on Drug Metabolism by Use of Isotopes XXVI: Determination of Urinary Metabolites of Rutin in Humans

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Abstract □ Determination of urinary metabolites of orally administered rutin and rutin-2',5',6'-d_3 in humans was carried out by TLC and GLC–mass spectrometry. In human urine, 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxytoluene, and β-*m*-hydroxyphenylhydracrylic acid were identified as rutin metabolites. Unchanged rutin and quercetin were not present in the urine.

Keyphrases □ Rutin—GLC–mass spectrometric analysis, urinary metabolites in humans □ GLC–mass spectrometry—analysis, urinary metabolites of rutin in humans □ Metabolites, urinary—of rutin, GLC–mass spectrometric analysis, humans

Rutin, a flavonol glycoside, has been used to treat disease states characterized by capillary bleeding associated with increased capillary fragility.

The metabolic fate of rutin has been studied extensively in animals (1–4), but there have been only a few studies in humans. Some investigators (5, 6) reported that unchanged

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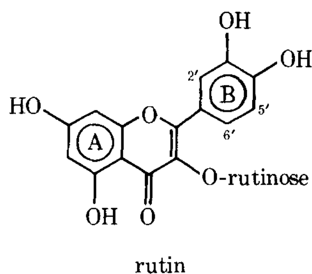


rutin

rutin and its metabolites were not present in human urine after oral administration. However, Booth *et al.* (7) identified 3-hydroxyphenylacetic acid (I), 3-methoxy-4-hydroxyphenylacetic acid (II), and 3,4-dihydroxyphenylacetic acid (III) as the urinary metabolites of rutin. The fundamental problem involved with using the method of Booth *et al.* (7) is that these compounds are excreted in the ordinary urine (8–13). Thus, an accurate determination of I–III derived from administered rutin is not possible.

A radioactive isotope tracer method is useful for metabolic studies. However, this method is inferior to a stable isotope tracer method in the structural elucidation of metabolites and in the safety in administration to humans.

This report identifies the urinary metabolites of rutin-2′,5′,6′-d_3 (rutin-*d*) (14) by GLC–mass spectrometry.

EXPERIMENTAL

Rutin and Authentic Compounds—Rutin[1] was purified by column chromatography using cross-linked dextran gel[2] with methanol and then was recrystallized from methanol–water (1:1). Quercetin[1], 3-hydroxyphenylacetic acid[1] (I), 3-methoxy-4-hydroxyphenylacetic acid[1] (II), 3,4-dihydroxyphenylacetic acid[1] (III), 3,4-dihydroxytoluene[3] (IV), 3-(3,4-dihydroxyphenyl)propionic acid[4], and α-*p*-hydroxyphenyllactic acid[5] were obtained commercially.

β-*m*-Hydroxyphenylhydracrylic acid (V) was synthesized from β-*m*-benzyloxyphenylhydracrylic acid (mp 95°) by reductive debenzylation under hydrogen gas on 5% palladium-on-charcoal and then was recrystallized from ethyl acetate, mp 158° dec. [lit. (15) mp 159°].

Anal.—Calc. for $C_9H_{10}O_4$: C, 59.34; H, 5.53. Found: C, 59.38; H, 5.43.

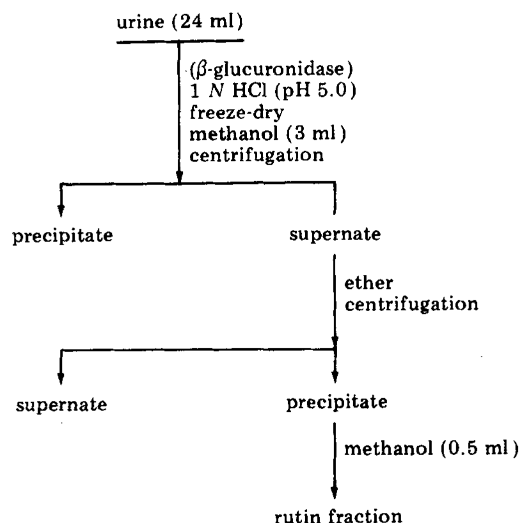
Rutin-*d* was prepared by hydrogen exchange according to the procedure of Hiraoka *et al.* (14). PMR spectra showed the isotopic ratio of d_1-, d_2-, and d_3-forms in deuterium-labeled rutin prepared in these laboratories was approximately 1:6:3.

GLC–Mass Spectrometry—A GLC–mass spectrometer[6] and a data processing system[7] connected to a minicomputer[8] were used. The GLC system employed a 2-m × 3-mm i.d. glass column packed with 2% OV-105 on 60–80-mesh Chromosorb W; it was packed with 1.5% OV-1 on 80–100-mesh Shimalite for quercetin analysis. The column, flash heater, and separator temperatures were 160, 180, and 280°, respectively, and the helium flow rate was 20 ml/min. The mass spectrometer employed an ionization-source temperature of 310° and ionization energy of 20 ev.

One minute following injection of the sample, automatic magnet scanning was initiated, covering an *m/z* range of 50–500 every 8 sec. All scanning data were stored in the data processing system. Mass chromatograms were obtained on a digital plotter for selected mass numbers.

Drug Administration—Two of four healthy male volunteers received 10 mg of rutin-*d*/kg po, and the other two subjects received 50 mg of nonlabeled rutin/kg po. The test subjects were not allowed fruit and vegetables for 4 days prior to and 2 days after drug administration.

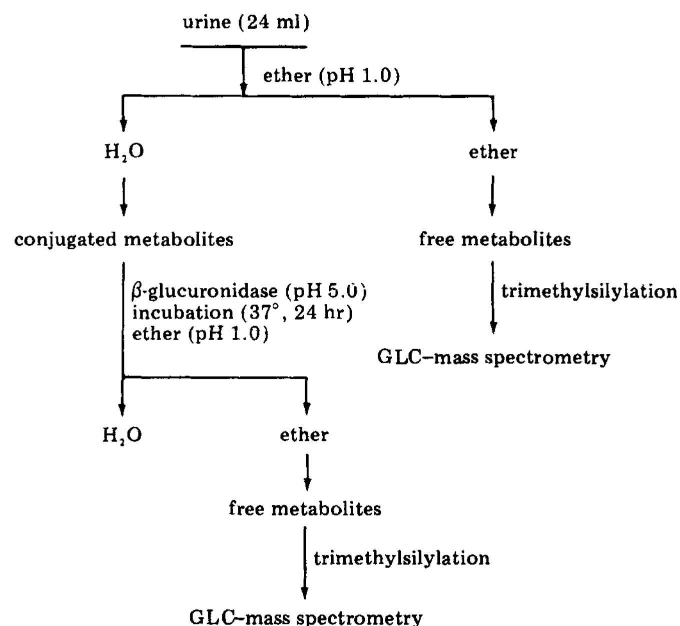
[1] Nakarai Chemicals Ltd., Kyoto, Japan.
[2] Sephadex LH-20, Pharmacia Fine Chemicals, Uppsala, Sweden.
[3] Wako Pure Chemical Industies, Tokyo, Japan.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Tokyo Chemical Industry, Tokyo, Japan.
[6] LKB-9000, Shimadzu Seisakusho Ltd., Kyoto, Japan.
[7] GCMSPAC-300, Shimadzu Seisakusho Ltd., Kyoto, Japan.
[8] OKITAC-4300, Oki Electric Industry Co., Tokyo, Japan.



Scheme I—Extraction and separation procedures of rutin in human urine prior to and after oral administration of rutin for TLC analysis.

TLC Analysis of Rutin—Urine collections were made at 0–24 hr prior to and at 0–48 hr after administration of nonlabeled rutin. The fractionation procedure of urine samples for TLC[9] is shown in Scheme I. Conjugated metabolites were hydrolyzed by incubation with β-glucuronidase[10] (30,000 units/24 ml of urine) at 37° for 24 hr. Each ether layer thus fractionated was concentrated to dryness under reduced pressure and then was dissolved in 5 ml of methanol. An aliquot (0.1 ml) of this solution was subjected to TLC. The solvent system was *n*-butyl alcohol–acetic acid–water (4:1:2). The spots were visualized by aluminum chloride (16); rutin had an R_f value of 0.45.

Identification of Metabolites by GLC–Mass Spectrometry—Urine was collected for 24 hr prior to and after administration of rutin-*d*. The fractionation procedure of urine samples is shown in Scheme II. The ether layer was concentrated to dryness under reduced pressure. The residue was dissolved in dry pyridine (0.1 ml) and trimethylsilylated with *N*,*O*-bis(trimethylsilyl)acetamide (0.05 ml) and then was subjected to the GLC–mass spectrometric computer system.



*Scheme II—Extraction and separation procedures of metabolites in human urine prior to and after oral administration of rutin-*d *for GLC–mass spectrometric analysis.*

[9] Merck.
[10] Type H-1, Sigma Chemical Co.

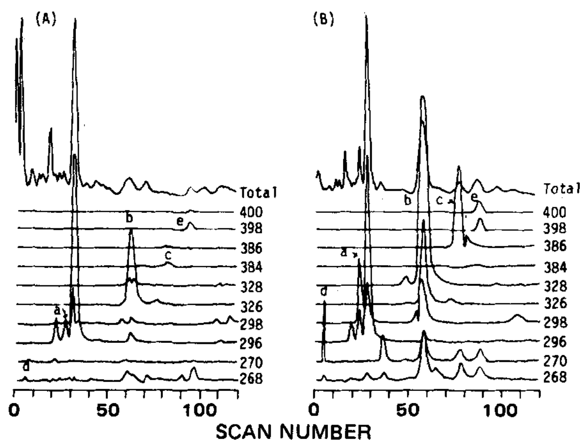


Figure 1—*Mass chromatograms of trimethylsilylated metabolites in human urine without β-glucuronidase hydrolysis prior to (A) and after (B) oral administration of rutin-*d. *Key: a, I [M^+: 296; $(M + 2)^+$: 298]; b, II [M^+: 326; $(M + 2)^+$: 328]; c, III [M^+: 384; $(M + 2)^+$: 386]; d, IV [M^+: 268; $(M + 2)^+$: 270]; and e, V [M^+: 398; $(M + 2)^+$: 400].*

RESULTS AND DISCUSSION

TLC Analysis of Rutin—The rutin analysis was not performed successfully by GLC under the various conditions tested with methyl and cyanoethyl silicone phase columns. The urine samples collected prior to and after rutin administration were subjected to TLC after the extraction and separation procedures shown in Scheme I. The TLC results revealed that rutin was not detected in the urine with or without β-glucuronidase treatment. To another urine sample collected prior to rutin administration was added rutin in an amount to give a concentration of 20 μg/ml, and this sample also was subjected to TLC. In this case, rutin was detected. Therefore, excretion of rutin into the urine after rutin administration is negligible or, at least, <20 μg/ml. These findings suggest that orally administered rutin might be metabolized completely.

Determination of Metabolites by GLC–Mass Spectrometry—The isotopic ratio of d_1-, d_2-, and d_3-forms in deuterium-labeled rutin (rutin-*d*) was 1:6:3. Particular attention was paid to the d_2-form to determine rutin metabolites by GLC–mass spectrometry. The masses of the molecular ions of the deuterated and nondeuterated trimethylsilylated derivatives of the metabolites derived from rutin are shown in Fig. 1.

The urine samples collected prior to and after administration of rutin-*d* were subjected to trimethylsilylation with *N,O*-bis(trimethylsilyl)-acetamide and analyzed by the GLC–mass spectrometric computer system. Mass chromatograms were obtained by monitoring the molecular ions of the d_0- (M^+) and d_2- [$(M + 2)^+$] forms of the trimethylsilylated derivatives of possible metabolites derived from rutin. A metabolite was identified by examining the presence of duplicate peaks at the same retention time in the mass chromatogram.

Typical mass chromatograms obtained from the urine without β-glucuronidase hydrolysis are shown in Figs. 1A (prior to) and 1B (after rutin-*d* administration). The ratio of peak intensities [$(M + 2)^+$:M^+] shown in Fig. 1A was practically the same as that estimated from the natural abundance. On the other hand, in peaks a, b, c, and d shown in Fig. 1B, the ratios of the peak intensities [$(M + 2)^+$:M^+] became larger than those shown in Fig. 1A, indicating the increment of the peak intensity in the respective $(M + 2)^+$ peaks. In addition, the comparison of the peak intensity ratios of M^+ and $(M + 2)^+$ in the mass chromatogram peak e (Figs. 1A and 1B) indicated a clear increment of the $(M + 2)^+$ peak in Fig. 1B. These findings make it clear that orally administered rutin-*d* was metabolized to five metabolites, which also were present in the urine as endogenous metabolites.

The mass spectra corresponding to the mass chromatogram peaks a, b, and c were the trimethylsilylated derivatives of 3-hydroxyphenylacetic acid (I), 3-methoxy-4-hydroxyphenylacetic acid (II), and 3,4-dihydroxyphenylacetic acid (III), respectively. Identification was made by comparing the mass spectra and the GLC retention times of the respective mass chromatogram peaks with those of the trimethylsilylated authentic compounds.

In the mass spectrum of the mass chromatogram peak d, the ion at *m/z* 268 appeared to be the molecular ion corresponding to the trimethylsilylated derivative of 3,4-dihydroxytoluene (IV) because it was 116 mass units [$COOSi(CH_3)_3 - H$] lower than the molecular ion of the trimethylsilylated derivative of III (*m/z* 384). The GLC retention time and mass spectrum of trimethylsilylated authentic IV confirmed the structure of Metabolite d as IV. The *m/z* values of the molecular ion and fragment ion in the mass spectrum of peak d after rutin-*d* administration were 2 mass units higher than those in the mass spectrum prior to rutin-*d* administration and thus were in mass spectra a, b, and c. These findings clearly indicated that these metabolites were derived from rutin-*d*.

In the mass spectrum of the mass chromatogram peak e after rutin-*d* administration, peak intensities at *m/z* 269, 282, and 400 were larger than those estimated from the natural abundance. The metabolite, although excreted in only a small amount, should originate from rutin-*d*. This metabolite was assigned as β-*m*-hydroxyphenylhydracrylic acid (V) and was identified by comparing the GLC retention time and mass spectrum of the trimethylsilylated authentic compound. The optical rotation and the absolute configuration of this metabolite could not be determined.

These five metabolites identified in urine without β-glucuronidase hydrolysis also were found in the urine treated with β-glucuronidase.

Recently, much attention has been directed toward organic acids in human urine by the GLC–mass spectrometric computer system called "metabolic profiling" (9–13, 17). Rutin was metabolized to give four phenolic acids in human urine. The observation showed that flavonoids in plants as a diet also must be metabolized to give these types of phenolic acids in the urine.

Other possible metabolites derived from rutin-*d*, such as 3,4-dihydroxyphenylpropionic acid, α-*p*- or α-*m*-hydroxyphenyllactic acid, and β-*p*-hydroxyphenylhydracrylic acid, were excluded by the mass spectra in one investigation (18). Quercetin, an aglycone of rutin, was not found in urine. It was impossible to follow the metabolic change occurring in the A ring on which no deuterium atoms are labeled.

In this study, five metabolites (I–V) derived from orally administered rutin-*d* were differentiated from these compounds endogenously present and were successfully identified by the mass chromatographic method. These metabolites occur in urine *via* the reduction of the double bond of the γ-pyron ring (I–V) and the dehydroxylation at the 4′-position of the B ring (I and V). A study concerning the reduction in this metabolism is now in progress.

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