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Studies on Drug Metabolism by Use of Isotopes. XXII.[1] Identification of Human Urinary Metabolites of 3-Phenylpropyl Carbamate by an Ion Cluster Technique[2]

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The human urinary metabolites of 3-phenylpropyl carbamate (I) were analysed by using an ion cluster technique. After oral administration of an equimolar mixture of non-labeled I and deuterium labeled I, neutral metabolites were extracted with ethyl acetate at pH 7.0, trimethylsilylated and subjected to gas chromatograph-mass spectrometer. In the case of the analysis of acidic metabolites, the urine of human receiving deuterium labeled I alone was used. The obtained acidic metabolites were methylated with diazomethane and subjected to gas chromatograph-mass spectrometer. As neutral metabolites, 3-hydroxy-3-phenylpropyl carbamate, 2,3-dihydroxy-3-phenylpropyl carbamate and 3,4′-dihydroxy-3-phenylpropyl carbamate were identified. Unchanged I, however, was not detected. Benzoic and hippuric acids originated from I could be clearly distinguished from the corresponding compounds endogenously produced.

Keywords——3-phenylpropyl carbamate; ion cluster; deuterium; metabolism; stable isotope; gas chromatography-mass spectrometry

A central muscular relaxant, 3-phenylpropyl carbamate (I) was synthesized by Surber *et al.*[4] To clarify the metabolic fate of this drug by a stable isotope tracer technique, the drug was labeled with deuterium and with carbon-13. In the previous paper,[1] the physicochemical properties of the labeled drug were reported. An ion cluster technique has been used in the metabolic studies of nortryptyline,[5] propoxyphen,[6] mydocalm,[7] mepyrizole,[8] 1-butyryl-4-cinnamylpiperazine,[9] *etc.* In the present study, this technique was successfully applied for the structural elucidation of the metabolites derived from I.

Experimental

Chemicals——I[arom.-d_5] (I-d_5) was the same as the sample previously prepared from benzoic acid [arom.-d_5] in our laboratory.[1] 3-Hydroxy-3-phenylpropyl carbamate was prepared by carbamylation[10] of 3-hydroxypropiophenone,[11] followed by reduction with hydrogen gas on palladium charcoal. 2,3-Dihydroxy-3-phenylpropyl carbamate was prepared by carbamylation of cinnamyl alcohol, followed by epoxidation with *m*-chloroperbenzoic acid and hydrolysis in tetrahydrofuran–1% H_2SO_4 (1 : 1 v/v).

Instrumentations and Conditions——Gas chromatography-mass spectrometry (GC-MS) analyses were carried on Shimadzu LKB-9000 GC-MS connected with an OKITAC 4300 computer. A column packed with 1.5% OV-1 on 80—100 mesh Shimalite W in a coiled glass tube (2 m × 3 mm i.d.) was used under the

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2) A part of this work was presented at 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
3) Location: *1432–1, Horinouchi, Hachioji-shi, Tokyo, 192–03, Japan.*
4) W. Surber, T. Wagner-Jauregg, and M. Häring, *Arzneimittel Forsch.*, **9**, 143 (1959).
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following conditions; injection temperature: 120°, column temperature: 100—230° (programmed rate at 5°/min), separator temperature: 230°, ion source temperature: 250°, carrier gas: He (20 ml/min). After injecting the sample, mass spectra of gas chromatographic effluents were recorded every 7 sec at 20 eV during a period of 5 min to 30 min in the case of the neutral metabolites and 1 min to 30 min in the case of the acidic metabolites.

Administration of the Drug and Separation of Metabolites——A male volunteer, aged 26 years and weighing 55 kg, orally received an equimolar mixture of I and I-d_5 (200 mg), and for the following 24 hr, urine was collected. One-twentieth of the urine (total volume: 1400 ml) was concentrated to a small volume and deprotenized with acetone. After centrifugation the supernatant was evaporated to about 20 ml. The remaining water solution was adjusted to pH 7.0 and shaken with 20 ml of AcOEt three times and the combined AcOEt layer was dried over anhydrous magnesium sulfate. The AcOEt layer was distilled off and the residual neutral metabolites were trimethylsilylated with 10 μl of pyridine and 20 μl of N, O-bis-(trimethylsilyl)-acetamide (Tokyo Kasei Kogyo, Co., Ltd.) at 50° for 1 hr.

The same subject orally received I-d_5 (200 mg), and for the following 24 hr, urine was collected. After completion of extraction of the neutral metabolites in the urine by above mentioned procedure, the remaining water solution was acidified to pH 1.0 with HCl and extracted with 20 ml of AcOEt three times, and the combined AcOEt was dried over anhydrous magnesium sulfate. The AcOEt was distilled off and the residual acidic metabolites were chromatographed on a thin-layer plate of Wakogel B-5 F (developing solvent system: benzene: dioxane: AcOH=90: 25: 4). After the development, the plate was divided into four zones and metabolites were eluted from each zone with 5 ml of MeOH. The MeOH was evaporated to dryness under a nitrogen gas stream and the residual metabolites were methylated with diazomethane. Under the thin-layer chromatographic conditions used, the acidic compounds which were reported as I urinary metabolites[12] were developed to the following zones: zone-2 (*Rf* 0.25—0.50): hippuric and *p*-hydroxyphenylacetic acids, zone-3 (*Rf* 0.50—0.75): *p*-hydroxycinnamic and *p*-hydroxybenzoic acids, zone-4 (*Rf* 0.75—1.00): benzoic, phenylpropionic and phenylacetic acids.

Confirmation of Position of Phenolic Hydroxyl Group——A neutral fraction from the urine of human receiving an equimolar mixture of I and I-d_5 was developed on a thin layer plate (developing solvent system: benzene: MeOH=10: 1) A band corresponding to the phenolic metabolite (*Rf* 0.07) was scraped off from the plate and the metabolite was eluted from silica gel with MeOH. The MeOH was evaporated to dryness under a nitrogen gas stream and the residual metabolite was oxidized to hydroxybenzoic acid according to the methods of Chafetz[13] and Pearl.[14] The obtained hydroxybenzoic acid was trimethylsilylated with N, O-bis-(trimethylsilyl)-acetamide.

Results and Discussion

The metabolism of I was investigated by using thin layer chromatography. Unchanged I, benzoic, hippuric, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *p*-hydroxyphenylcinnamic, phenylacetic and phenylpropionic acids were detected as urinary metabolites of I. The quantitative analysis of the acidic metabolites originated from I was performed on the basis of the difference in the amounts of the metabolites before and after administration of I. Since there is a large daily variation in the amounts of excreted acidic metabolites such as hippuric acid, accurate determination of these metabolites derived from the drug can not be carried out by using a method mentioned above. Moreover, the present study was performed under the conditions of over dose and the results obtained here may not reflect the normal metabolic behavior.

Mass spectra of I and trimethylsilylated I (I-TMS) are shown in Fig. 1. I did not give rise to the detectable molecular ion (*m/e* 179) and the highest mass ion appeared at *m/e* 136, which may be due to the elimination of NHCO group with the rearrangement of proton of the amide group from the molecular ion. I-TMS also did not give rise to the detectable molecular ion (*m/e* 251) and the highest mass ion was observed at *m/e* 236, which may be resulted from the mono-demethylation of the molecular ion.

The gas chromatogram of trimethylsilylated neutral metabolites obtained by using a total ion monitoring is shown in Fig. 2. The ion clusters each having five mass units apart were

12) Von F. Schatz and U. Jahn, *Arzneimittel Forsch.*, **16**, 866 (1966).
13) L. Chafetz, *J. Pharm. Sci.*, **52**, 1193 (1963).
14) I.A. Pearl, *J. Org. Chem.*, **12**, 85 (1947).

observed in mass spectra of peak A', A, B' and B. On the other hand, the ion clusters of four mass units apart were observed in mass spectra of peak C' and C (Fig. 3).

In the spectrum of peak A, ion clusters having about one-to-one intensity ratio appeared at *m/e* 324/329 and 179/184 and an abnormal ion cluster whose peak intensity ratio was not

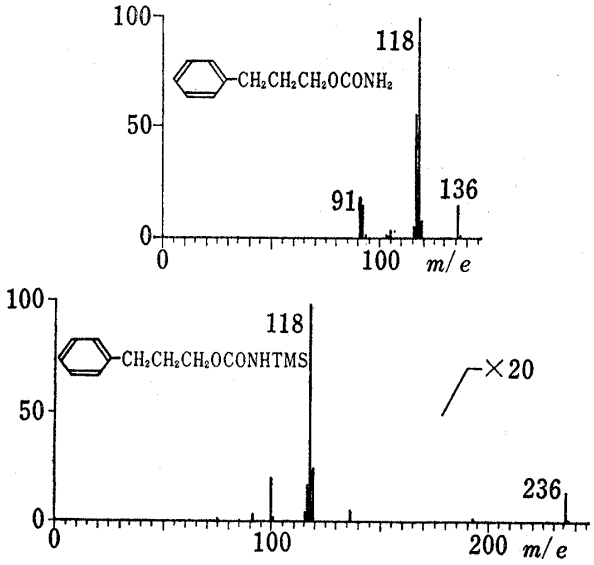

Fig. 1. Mass Spectra of 3-Phenylpropyl Carbamate(Upper) and Trimethylsilylated 3-Phenylpropyl Carbamate(Lower)

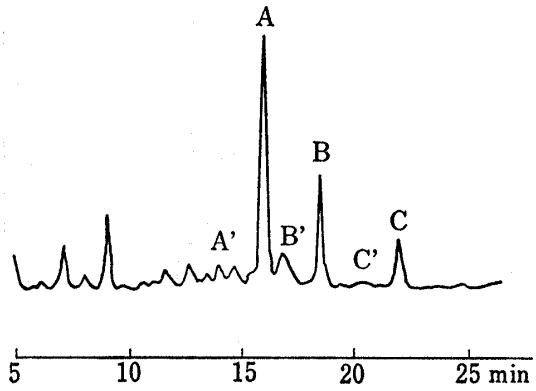

Fig. 2. Gas Chromatogram of Trimethylsilylated Neutral Metabolites

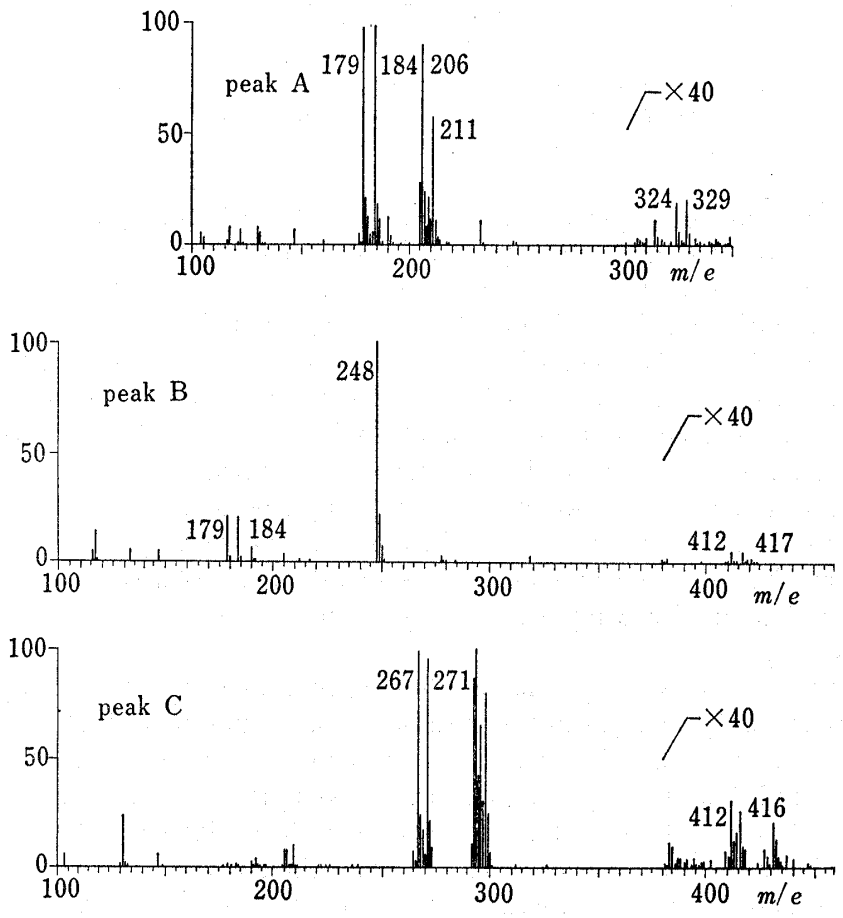

Fig. 3. Mass Spectra of Peak A(Upper), Peak B(Middle) and Peak C(Lower)

one-to-one appeared at *m/e* 206/211. By comparing the spectrum of I–TMS with that of peak A, an increase of 88 a. m. u. (*m/e* 236 to *m/e* 324) was observed in the highest mass region. This mass difference corresponded to the mass of trimethylsilyl ether group (–OTMS). As a mass of an ion at *m/e* 179 coincided with a mass of benzyl ion (*m/e* 91) plus 88 a. m. u., it was concluded that a hydroxyl group was attached to the C-3 position. This metabolite was assigned as 3-hydroxy-3-phenylpropyl carbamate by comparing the spectrum of this metabolite with that of an authentic sample (Fig. 4). The ions at *m/e* 206/211 may be pro-

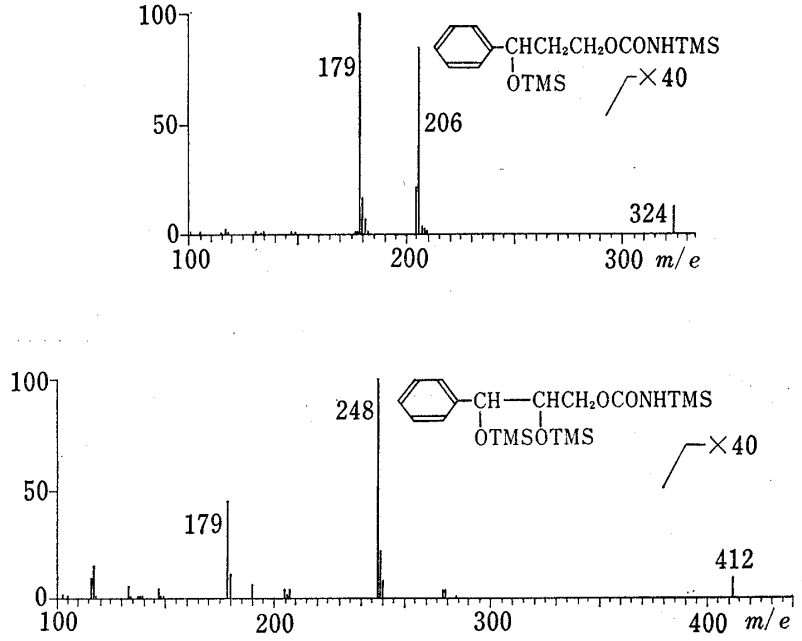

Fig. 4. Mass Spectra of Authentic Trimethylsilylated Derivatives of 3-Hydroxy-3-phenylpropyl Carbamate(Upper) and 2,3-Dihydroxy-3-phenylpropyl Carbamate(Lower)

duced through the following steps; 1) elimination of CONTMS group with the rearrangement of proton of the amide group, 2) dehydration. This dehydration process may proceed with two different pathways as in the case observed in the fragmentation of I.[1)]

In the spectrum of peak B, the ion clusters having original mass unit apart appeared at *m/e* 412/417 and 179/184. By comparing the spectrum of I–TMS with that of peak B, an increase of 176 a. m. u. (*m/e* 236 to *m/e* 412) was observed in the highest mass region. This mass difference corresponded to the mass of two trimethylsilyl ether groups. In addition to these ion clusters, a strong singlet ion appeared at *m/e* 248. It was concluded that the doublet ions at *m/e* 179/184 were fragments containing a benzene ring, while the singlet ion at *m/e* 248 was the fragment of the side chain moiety. These fragments were produced by fission of the bond between C-2 and C-3. This metabolite was assigned as 2,3-dihydroxy-3-phenylpropyl carbamate by comparing the spectrum of this metabolite with that of an authentic sample (Fig. 4).

In the spectrum of peak C, ion clusters having four mass units apart appeared at *m/e* 412/416, 294/298 and 267/271. As in the case of the spectrum of peak B, appearance of the highest mass ion at *m/e* 412 implied that this metabolite also possessed two hydroxyl groups which were trimethylsilylated. A change in the mass unit of the ion clusters from five to four indicated that one of hydroxyl groups was introduced into the benzene ring. From the fact that the ion at *m/e* 267 was 88 a. m. u. higher than an ion at *m/e* 179 observed in the spectra of peak A and B, it was concluded that the other hydroxyl group was in the C-3 position. The observation that retention time and mass spectrum of trimethylsilylated

hydroxybenzoic acid obtained by oxidation of this metabolite were the same as that of trimethylsilylated *p*-hydroxybenzoic acid indicated that the position of hydroxyl group on the benzene ring was in the *para* position.

There was observed to be no peak corresponding to the I–TMS (retention time: 12.5 min) on a gas chromatogram of the trimethylsilylated neutral metabolites of the urine sample.

Peaks A', B' and C' were found to be derived from the incomplete trimethylsilyl derivatization, that is, trimethylsilylation occurred only at the hydroxyl group of the metabolites mentioned above but not the amide group.

For the analysis of the acidic metabolites, the urine from a subject receiving I-d_5 alone was used, because the acidic metabolites originated from the drug were diluted with the corresponding endogenous compounds. In the spectra of methyl benzoate and methyl hippurate, ions resulted from deuterated species were clearly observed as shown in Fig. 5. However, in the mass spectra of other acidic metabolites such as *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *p*-hydroxycinnamic, phenylacetic and phenylpropionic acids, the ions resulted from protio species appeared, but the ions resulted from deuterated species were not detected. From these results, it was clarified that these acidic compounds were not produced from I.

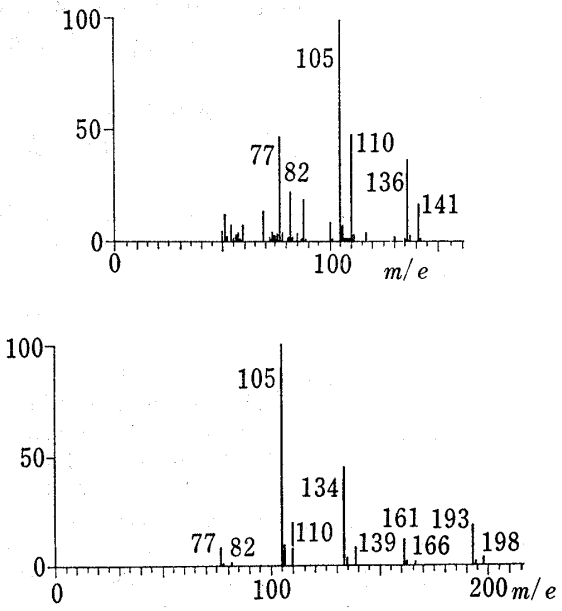

Fig. 5. Mass Spectra of Methyl Benzoate (Upper) and Methyl Hippurate (Lower) Recoverd from Human Urine after Administration of 3-Phenylpropyl Carbamate [arom.-d_5]

By using an ion cluster technique, the neutral metabolites such as 3-hydroxy-3-phenylpropyl carbamate, 2, 3-dihydroxy-3-phenylpropyl carbamate and 3, 4'-dihydroxy-3-phenylpropyl carbamate were newly identified as the urinary metabolites of I Furthermore, it was possible to differentiate benzoic and hippuric acids originated from I from the corresponding endogenous compounds. The structures of the urinary metabolites of I are shown in Fig. 6.

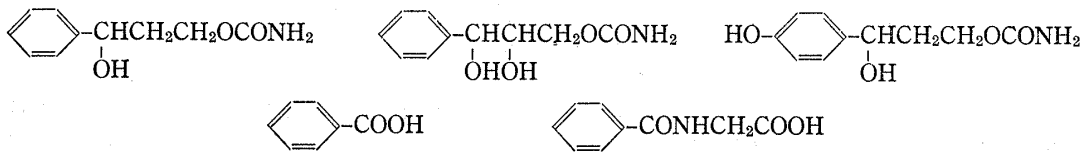

Fig. 6. Structures of Identified Urinary Metabolites of 3-Phenylpropyl Carbamate