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Identification of Isopropylantipyrine Metabolites in Rat and Man by Using Stable Isotope Tracer Techniques

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The metabolites of isopropylantipyrine (IPA) were identified in urine of rats by gas chromatography-mass spectrometry (GC-MS) combined with stable isotope tracer techniques. After the oral administration of an equimolar mixture of IPA and IPA-1-C_6D_5, the urinary metabolites were extracted with chloroform before or after hydrolysis with β-glucuronidase. The extracts were subjected to GC-MS after trimethylsilylation.

Characteristic doublet peaks in the mass spectra indicated the presence of 13 metabolites in the urine. The metabolized positions were determined on the basis of the retained numbers of deuterium atoms after the administration of various deuterated IPAs, *i.e.*, IPA-2-CD_3, IPA-3-CD_3 and IPA-4-$CH(CD_3)_2$. The identified metabolites were oxidation products of the phenyl, 2-methyl, 3-methyl and isopropyl groups and of the C-4 position of the pyrazolone ring. In human urine, one major metabolite, hydroxyphenyl-IPA, and four minor metabolites were detected after administration of IPA.

Keywords——isopropylantipyrine; isopropylantipyrine metabolism; urinary metabolite; deuterium label; stable isotope tracer; GC-MS analysis

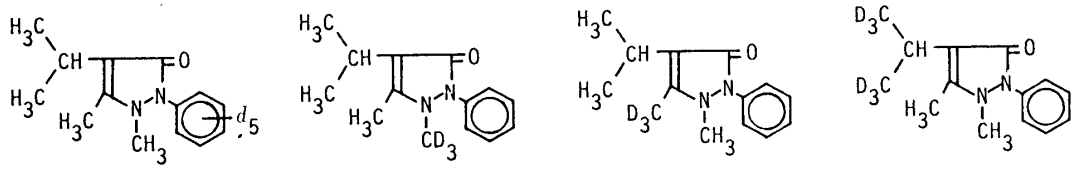
Isopropylantipyrine (4-isopropyl-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, IPA) has been used as an analgesic, but the metabolism of this drug has not yet been clarified. Recently, Tateishi *et al.*[1,2] investigated the metabolism of IPA in rats and humans using thin layer chromatography. They found a major metabolite, the enol glucuronide of *N*-desmethyl-IPA, and eight other minor metabolites which were oxidation products of the phenyl or isopropyl group or the C-4 position, without the *N*-methyl group of the parent drug. In spite of the structural similarity between IPA and other pyrazolones, such as antipyrine[3] and aminopyrine,[4] oxidation at the 3-methyl group of IPA was not observed.

In the present study, we attempted to detect and identify the urinary metabolites of IPA in rats and humans by using stable isotope tracer techniques. IPA was labeled with deuterium on the phenyl, 2-methyl, 3-methyl or 4-isopropyl group. The doublet peaks in the mass spectra were used to detect the IPA metabolites and the shifts of the mass numbers of the molecular ions were used to identify the metabolized position.

Experimental

Chemicals——JP X grade IPA was obtained from Kongo Chemical Co. Four kinds of deuterium -labeled IPA, 4-isopropyl-2,3-dimethyl-1-pentadeuterophenyl-3-pyrazolin-5-one (IPA-1-C_6D_5), 4-isopropyl-3-methyl-1-phenyl-2-trideuteromethyl-3-pyrazolin-5-one (IPA-2-CD_3), 4-isopropyl-2-methyl-1-phenyl-3-trideuteromethyl-3-pyrazolin-5-one (IPA-3-CD_3) and 4-hexadeuteroisopropyl-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one [IPA-4-$CH(CD_3)_2$], were used (Fig. 1).

IPA-1-C_6D_5 was prepared by condensation of phenylhydrazine-d_5 [prepared from aniline-d_5 (Merck,



IPA-1-C_6D_5 (97.5 atomD%) IPA-2-CD_3 (99.6 atomD%) IPA-3-CD_3 (96.4 atomD%) IPA-4-$CH(CD_3)_2$ (83.6 atomD%)

Fig. 1. Structures and Deuterium Contents of Deuterated Isopropylantipyrines

99 atom%D)][5] and ethyl 2-isopropylacetoacetate, followed by methylation.[6] IPA-2-CD_3 was prepared by methylation of *N*-desmethyl-IPA[7] with dimethyl sulfate-d_6 (Merck, 99 atom%D). IPA-3-CD_3 was prepared by the base-catalyzed hydrogen exchange reaction of IPA.[8] IPA-4-$CH(CD_3)_2$ was prepared by condensation of acetone-d_6 (Merck, 99 atom%D) and 3-methyl-1-phenyl-3-pyrazolin-5-one (Tokyo Chemical Industry), followed by reduction and methylation.[7]

Animal and Human Experiments——Male Wistar rats weighing 250—300 g were used. Fifty mg/kg of an equimolar mixture of IPA and IPA-1-C_6D_5 (IPA : IPA-1-C_6D_5), IPA : IPA-2-CD_3, IPA : IPA-3-CD_3 or IPA : IPA-4-$CH(CD_3)_2$ was orally administered to rats as a suspension in 0.5% carboxymethylcellulose. Urine was collected for 24 h after drug administration. The total collected urine was diluted to 50 ml with distilled water. Twenty ml of the urine was extracted twice with 20 ml of chloroform at pH 7.0 before or after hydrolysis with 5000 units of β-glucuronidase (from marine mollusc, P-L Biochemicals, Inc.) for 24 h at pH 5.0 at 37 °C. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue was trimethylsilylated by treatment with bis(trimethylsilyl)acetamide in pyridine at 60 °C for 30 min.

A healthy male volunteer (age 41, weight 60 kg) took 100 mg of IPA : IPA-1-C_6H_5 orally in the form of a powder with 180 ml of water. Urine samples were collected at 0—4, 4—8 and 8—12 h after dosing. Twenty ml of the urine was extracted with 20 ml of chloroform after hydrolysis with β-glucuronidase and the extract was trimethylsilylated as described above for the extract from rat urine.

Gas Chromatography-Mass Spectrometry (GC-MS)——GC-MS was carried out in a JEOL model JMS D-300 gas chromatograph-mass spectrometer-computer system. A coiled glass column (1 m × 3 mm i.d.) packed with 1.5% OV-17 on Chromosorb W (80—100 mesh) was used. The helium gas flow rate was 20 ml/min. The column temperature was programmed from 170 to 270 °C at 10 °C/min. The temperatures of the injection port, separator and ion source were 270, 280 and 280 °C, respectively.

Mass spectrometer conditions were as follows: accelerating voltage, 3 kV; ionizing energy, 20 eV for electron impact ionization (EI) or 200 eV for chemical ionization (CI). Iso-butane was used as the reagent gas in CI measurement.

Results and Discussion

In order to detect the urinary metabolites of IPA by the ion cluster technique, an equimolar mixture of IPA and IPA-1-C_6D_5 was administered to rats. The total ion chromatograms of the trimethylsilylated extract from urine before and after hydrolysis with β-glucuronidase are shown in Fig. 2. Ion clusters were observed in the mass spectra corresponding to the peaks a, b, c, d, e, f, g, h, i, j, k, l and m in the chromatogram obtained from the urine treated with β-glucuronidase. Four peaks, *i.e.*, b, f, l and m, were also detected in the urine before hydrolysis with β-glucuronidase. Therefore, these four metabolites were excreted as free forms and the other metabolites were excreted as conjugate forms. These conjugates were expected to be mainly glucuronides, but could also include sulfates since the β-glucuronidase used in this work was contaminated with aryl sulfatase.

In order to determine the metabolized position, IPA : IPA-2-CD_3, IPA : IPA-3-CD_3 or IPA : IPA-4-$CH(CD_3)_2$ was administered and the mass numbers of the molecular ions of the peaks, except peak 1, were determined. These m/z values are summarized in Table I. The metabolite corresponding to peak 1 was confirmed by CI measurement. The structural elucidation of the metabolites of IPA (peaks a—m) was carried out as follows.

Peak a——The partial mass spectra of peak a are shown in Fig. 3. The molecular ions

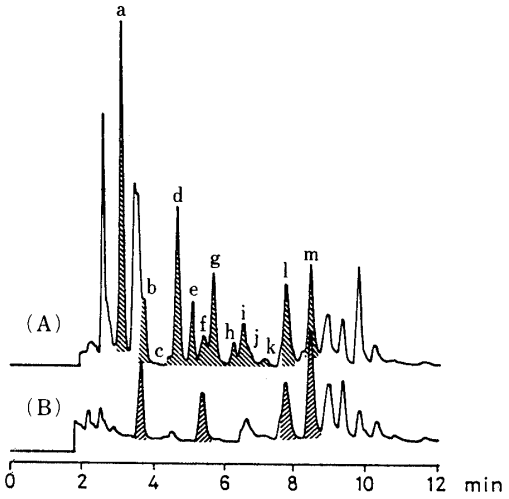


Fig. 2. Total Ion Chromatograms of the Trimethylsilylated Extract from Rat Urine with (A) or without (B) β-Glucuronidase Treatment after Administration of IPA : IPA-1-C_6D_5

TABLE I. Molecular Ions of the Metabolites after Administration of Various Deuterated Isopropylantipyrines

Peak	*m/z* value of molecular ion				
	Administered compound				
	IPA	1-C_6D_5	2-CD_3	3-CD_3	4-$CH(CD_3)_2$
a	288	293 (5)	288 (0)	291 (3)	294 (6)
b	232	237 (5)	232 (0)	235 (3)	238 (6)
c	392	396 (4)	392 (0)	394 (2)	398 (6)
d	376	381 (5)	376 (0)	378 (2)	382 (6)
e	376	381 (5)	376 (0)	379 (3)	381 (5)
f	392	396 (4)	392 (0)	395 (3)	398 (6)
g	376	380 (4)	376 (0)	379 (3)	382 (6)
h	320	324 (4)	320 (0)	323 (3)	326 (6)
i	318	323 (5)	321 (3)	320 (2)	324 (6)
j	422	425 (3)	422 (0)	425 (3)	428 (6)
k[a]	406	409 (3)	406 (0)	409 (3)	412 (6)
	464	468 (4)	464 (0)	466 (2)	470 (6)
l	391[b]	396 (5)	394 (3)	394 (3)	396 (5)
m	318	322 (4)	321 (3)	321 (3)	324 (6)

Numbers in parentheses are the numbers of retained deuterium atoms.
a) Includes two metabolites. *b*) The highest fragment ion $(M-15)^+$.

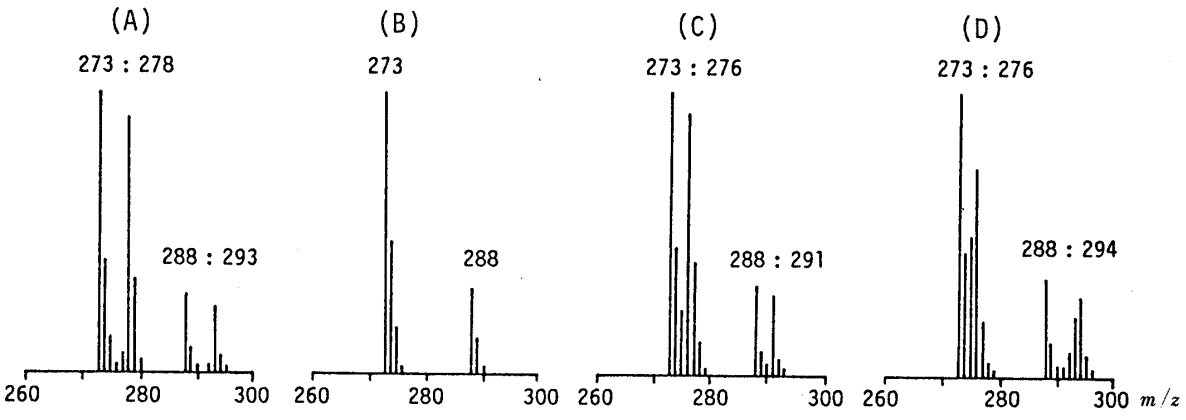


Fig. 3. Mass Spectra of the Metabolite in Peak a after Administration of IPA : IPA-1-C_6D_5 (A), IPA : IPA-2-CD_3 (B), IPA : IPA-3-CD_3 (C) or IPA : IPA-4-$CH(CD_3)_2$ (D)

appeared at *m/z* 288:293, 288:291 or 288:294 after administration of IPA:IPA-1-C_6D_5, IPA:IPA-3-CD_3 or IPA:IPA-4-$CH(CD_3)_2$, respectively. The presence of ion clusters separated by five, three and six mass units indicated that the 1-phenyl, 3-methyl and 4-isopropyl groups were not metabolized. No ion cluster was observed after administration of IPA:IPA-2-CD_3, and therefore the three deuterium atoms from the 2-methyl group were lost by *N*-demethylation. These results elucidated the structure of the metabolite in peak a as 4-isopropyl-3-methyl-1-phenyl-2-pyrazolin-5-one (A). The fragment ion at *m/z* 273 was formed by demethylation from the isopropyl group in view of the presence of the ion cluster separated by three mass units after administration of IPA:IPA-4-$CH(CD_3)_2$.

Peak b——The disappearance of three deuterium atoms was only observed after administration of IPA-2-CD_3. The molecular ion appeared at *m/z* 232, indicating the introduction of one oxygen atom into metabolite A but no hydroxyl group was present in this metabolite since it did not react with trimethylsilylacetamide. The fragment ions at *m/z* 216 $[(M-16)^+]$, 190 $[(M-\text{isopropyl})^+]$ and 174 $[(M-\text{isopropyl}-16)^+]$ suggested the presence of an *N*-oxide moiety. These results indicated that the metabolite in peak b was 2-*N*-oxido-4-isopropyl-3-methyl-1-phenyl-2-pyrazolin-5-one (B), which was formed by *N*-demethylation and subsequent *n*-oxidation.

Peak c——The disappearance of a deuterium atom from the 1-phenyl group, three deuterium atoms from the 2-methyl group and a deuterium atom from the 3-methyl group was observed. Thus, the metabolite in peak c was identified as 3-hydroxymethyl-1-hydroxyphenyl-4-isopropyl-2-pyrazolin-5-one (C), formed by the introduction of one oxygen atom at both 3-methyl and 1-phenyl group, and subsequent *N*-demethylation.

Peak d——The disappearance of deuterium atoms indicated that *N*-demethylation and hydroxylation at the 3-methyl group had occurred. These observations and the molecular ion at *m/z* 376 indicated that the metabolite in peak d was 3-hydroxymethyl-4-isopropyl-1-phenyl-2-pyrazolin-5-one (D).

Peak e——The disappearance of deuterium atoms indicated that *N*-demethylation and hydroxylation at the 4-isopropyl group had occurred. Thus, the metabolite in peak e was identified as 4-1-hydroxy-2-propyl-3-methyl-1-phenyl-2-pyrazolin-5-one (E).

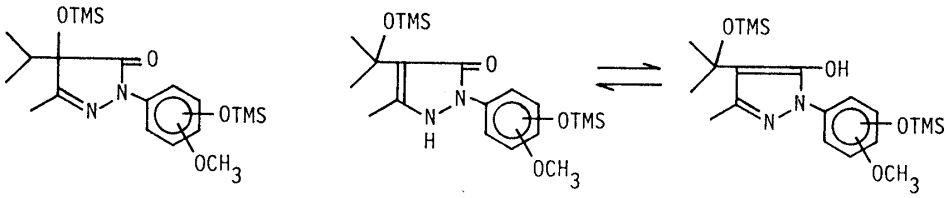
Peak f——The disappearance of deuterium atoms indicated that *N*-demethylation and hydroxylation at the phenyl group had occurred. The molecular ion appeared at *m/z* 392, indicating the presence of another hydroxyl group. The fragment ion at *m/z* 350 $[(M-\text{isopropyl})^+]$ suggested the introduction of a hydroxyl group at the C-4 position of the pyrazolone ring. These results indicated that the metabolite in peak f was 4-isopropyl-4-hydroxy-1-hydroxyphenyl-3-methyl-2-pyrazolin-5-one (F).

Peak g——The disappearance of deuterium atoms indicated that *N*-demethylation and hydroxylation at the phenyl group had occurred. Thus, the metabolite in peak g was identified as 4-isopropyl-1-hydroxyphenyl-3-methyl-2-pyrazolin-5-one (G).

Peak h——The disappearance of deuterium atoms indicated that *N*-demethylation and hydroxylation at the phenyl group had occurred. The fragment ions at *m/z* 304 $[(M-16)^+]$, 278 $[(M-\text{isopropyl})^+]$ and 262 $[(M-\text{isopropyl}-16)^+]$ suggested the presence of an *N*-oxide moiety. These results indicated that the metabolite in peak h was 2-*N*-oxido-4-isopropyl-1-hydroxyphenyl-3-methyl-2-pyrazolin-5-one (H).

Peak i——The disappearance of a deuterium atom was only observed after administration of IPA-3-CD_3. Thus, the metabolite in peak i was identified as 3-hydroxymethyl-4-isopropyl-2-methyl-1-phenyl-3-pyrazolin-5-one (I).

Peak j——The disappearance of deuterium atoms indicated that *N*-demethylation and introduction of two hydroxyl groups into the phenyl group had occurred. The molecular ion observed at *m/z* 422 suggested the presence of three hydroxyl groups, one in a methoxy form. Methyl conjugation commonly occurs at a phenolic hydroxyl group and therefore this



I II

Fig. 4. Possible Structures of the Metabolite in Peak j

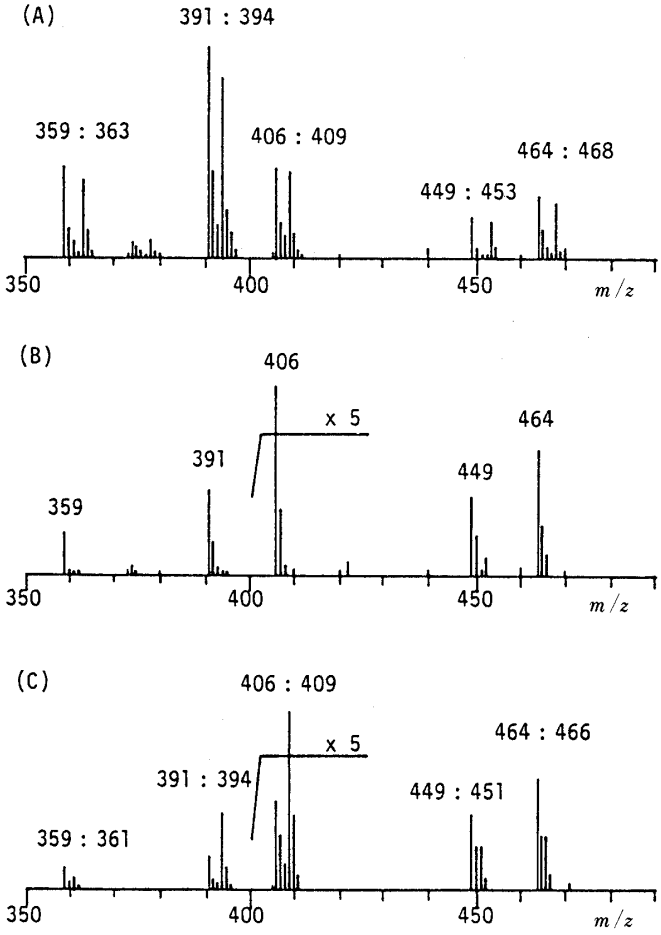


Fig. 5. Mass Spectra of the Metabolite in Peak k after Administration of IPA : IPA-1-C_6D_5 (A), IPA : IPA-2-CD_3 (B) or IPA : IPA-3-CD_3 (C)

compound was assumed to contain a hydroxy-methoxy-phenyl moiety. From these observations, the structure of this compound was speculated as either I or II (Fig. 4). If the structure is II, one more trimethylsilyl group should be introduced at the secondary amine or the enol hydroxyl group, and the molecular ion should appear at *m/z* 494. Thus, the metabolite in peak j was assigned as 4-hydroxy-1-(hydroxy-methoxy-phenyl)-4-isopropyl-3-methyl-2-pyrazolin-5-one (J).

Peak k——The mass spectra of peak k are shown in Fig. 5. The ions at *m/z* 464, 449 and 359 were shifted four or two mass units after administration of IPA-1-C_6D_5 or IPA-3-CD_3, respectively. The ions at *m/z* 406 and 391 were shifted three mass units after administration of both types of labeled IPA. These observations indicated that two compounds, k (molecular ion at *m/z* 406) and k′ (molecular ion at *m/z* 464), existed in peak k. In Fig. 5(C), irregular cluster ions at *m/z* 464 : 466, 449 : 451 and 359 : 361 might be formed as a result of the

deuterium isotope effect on the oxidation at the 3-methyl group, and "metabolic switching" was observed in the cluster ions at *m/z* 406 : 409 and 391 : 394. A similar isotope effect was observed in the case of aminopyrine-3-CD_3.[9] Compound k and k′ were elucidated as trimethylsilyl derivatives of 1-(hydroxy-methoxy-phenyl)-4-isopropyl-3-methyl-2-pyrazolin-5-one (K) and 3-hydroxymethyl-1-hydroxyphenyl-4-isopropyl-2-pyrazolin-5-one, respectively. Compound k′ is the same as the tris(trimethylsily)derivative of metabolite C.

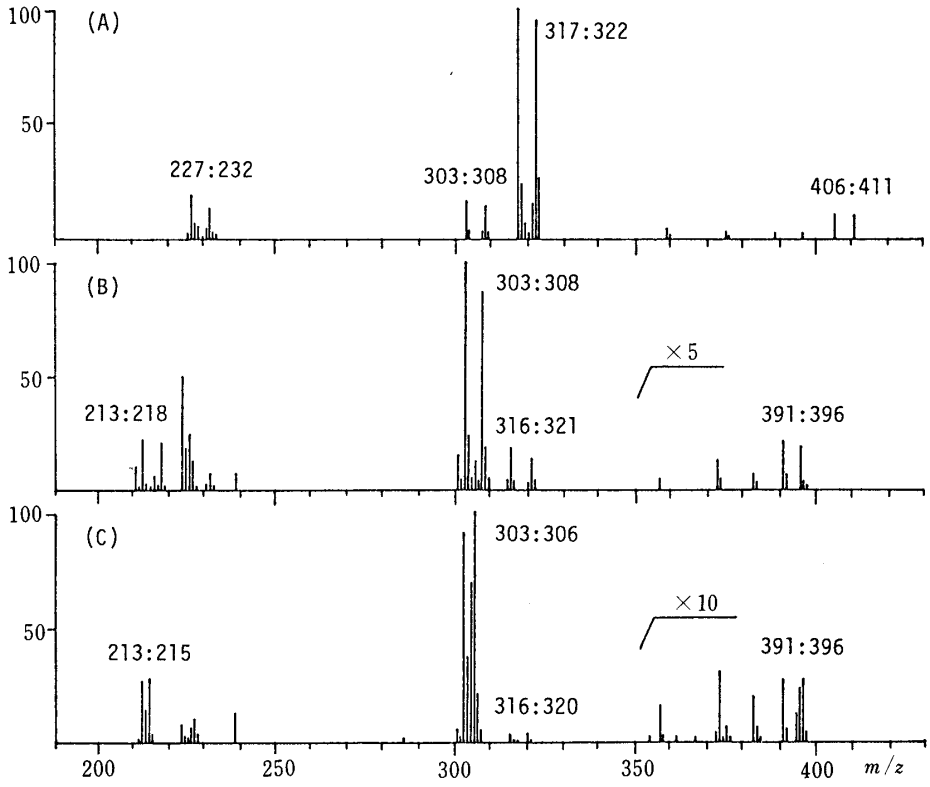


Fig. 6. Mass Spectra of the Metabolite in Peak 1 by CI Measurement (A) and EI Measurement (B) after Administration of IPA : IPA-1-C_6D_5 or by EI Measurement after Administration of IPA : IPA-4-$CH(CD_3)_2$ (C)

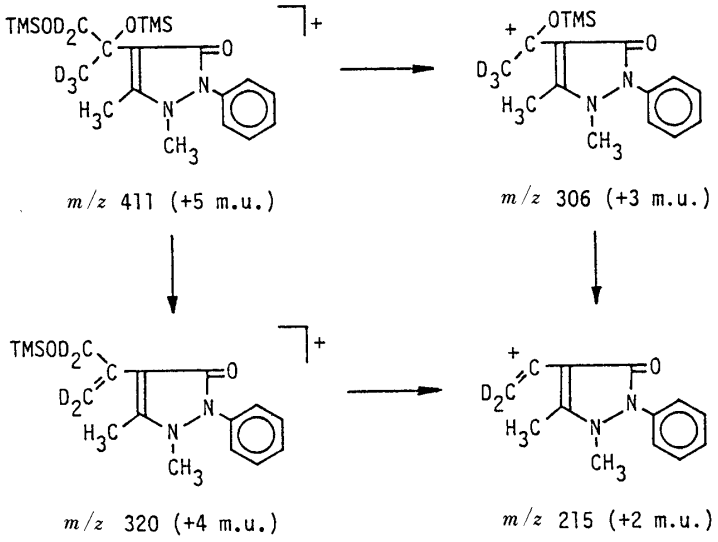


Fig. 7. Postulated Fragmentation Pathway of the Metabolite in Peak 1

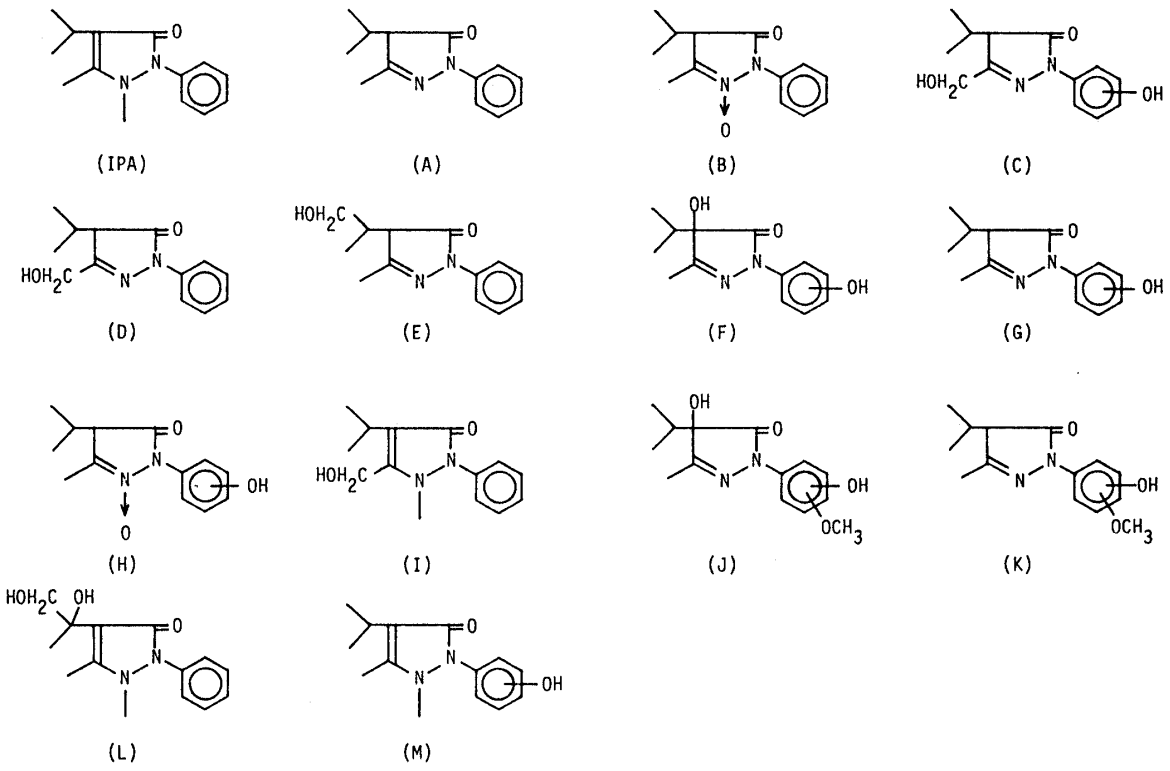


Fig. 8. Structures of Isopropylantipyrine Metabolites Established in This Study

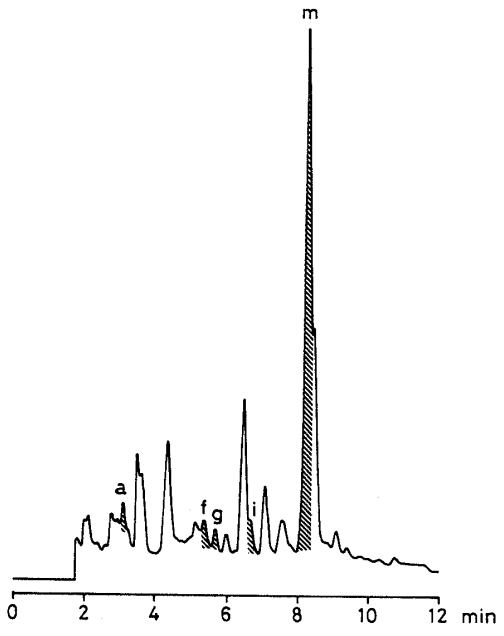


Fig. 9. Total Ion Chromatogram of the Trimethylsilylated Extract from Human Urine (with β-Glucuronidase Treatment) after Administration of IPA : IPA-1-C_6D_5

Peak l——The mass spectra of peak 1 are shown in Fig. 6. The molecular ions were observed at *m/z* 406 : 411 by CI measurement, but the highest cluster ions appeared at *m/z* 391 : 396 [$(M-15)^+$] by EI measurement after administration of IPA : IPA-1-C_6D_5. The mass number of the molecular ion and the disappearance of deuterium atoms indicated the metabolite in peak 1 to be 4-(1,2-dihydroxy-2-propyl)-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (L). This structure is supported by the following fragmentation (Fig. 7).

Peak m——The disappearance of a deuterium atom from the phenyl group indicated

that the metabolite in peak m was 1-hydroxyphenyl-4-isopropyl-2,3-dimethyl-3-pyrazolin-5-one (M).

The IPA metabolites established in this study are summarized in Fig. 8. Nine of these metabolites, B, C, D, H, I, J, K. L and M, are new metabolites and are oxidation products of the phenyl, 2-methyl, 3-methyl, or isopropyl group or the C-4 position of the pyrazolone ring. Oxidation of the 3-methyl group was observed in metabolites, C, D and I, in contrast to the study of Tateishi *et al.*[1] *N*-Demethylation is the main metabolic pathway of IPA in rats and most of the *N*-desmethyl metabolites, A, C, D, E, G and K, are excreted as enol-*O*-glucuronides, as described in the previous report.[1] The peak heights of the *N*-oxides, metabolites B and H, increased, whereas the peak heights of the *N*-desmethyl metabolites, A and G, decreased with increasing storage time after extraction until GC measurement. Therefore, it is likely that the *N*-oxides are formed from the *N*-desmethyl metabolites by air oxidation.

Finally, IPA metabolites were examined in man. The total ion chromatogram of the trimethylsilylated extract from 4—8 h urine after the administration of IPA : IPA-1-C_6D_5 is shown in Fig. 9. Metabolites A, F, G, I and M were detected by GC-MS. In this case, the main metabolite was hydroxyphenyl-IPA (metabolite M) rather than *N*-desmethyl-IPA (metabolite A) and oxidation of the 3-methyl group was also observed.

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