[Chem. Pharm. Bull. 28(6)1704—1710(1980)]

Studies on the Biotransformation of Paeonol by Isotope Tracer Techniques. II.[1] Species Differences in Metabolism[2]

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(Received November 8, 1979)

Species differences in the metabolism of paeonol (2-hydroxy-4-methoxyacetophenone: I) in mice, rats, guinea pigs and rabbits were studied by the RI tracer technique.

After the administration of a single dose of 20 mg (6—70 μCi)/kg paeonol[carbonyl-^{14}C] (I-^{14}C), radioactive metabolites excreted in the urine were analyzed. The excretion of I-^{14}C was rapid in all species; that is, about 83—98% of administered radioactivity was excreted in the urine and feces during two days after the administration. The ^{14}C-activity excreted in feces was 10.6% in mice, 4.7% in rats, 2.9% in guinea pigs, and 0.8% in rabbits. It is clear that the urine is the major excretion route in all species.

2,5-Dihydroxy-4-methoxyacetophenone (II), resacetophenone (III) and unchanged I were detected in all species as urinary metabolites. These metabolites were found as the free form, β-glucuronide, sulfate and enzyme-resistant conjugate. The major metabolite in each species was as follows; mice, II 43.1%; rats, II 62.0%; guinea pigs, III 58.1%; rabbits, III 42.7% and II 42.2%.

All the metabolites were conjugated in rats, while the total amount of unconjugated metabolites was 50—60% of total metabolites in other species. Although β-glucuronide and sulfate in rats accounted for 19.9% and 8.2%, respectively, only small amounts of these conjugated forms were excreted in other species. Enzyme-resistant conjugate accounted for 60.2% in rats, but only 35—43% in other species.

Keywords——excretion rate; metabolism; paeonol; species differences; RI tracer technique

Investigation of the metabolic fate of drugs in man is important for the evaluation of their safety.

In order to undertake reliable toxicological studies on a drug, it is necessary to select an animal species in which the metabolism of the drug in question is similar to that in man. Because of wide species differences in the metabolism of drugs,[4] however, the selection of appropriate species is difficult to rationalize.

In recent years, there have been many reviews[5] and articles[6] regarding studies of drug metabolism in man using a stable isotope (SI) tracer technique. However, there are many problems to be taken into consideration in this technique. It seems likely that stable and radioactive isotope tracer techniques will be used as complementary methods for comparative studies of the metabolic fate of a drug in experimental animals and man.

1) Part I: K. Mimura and S. Baba, *Radioisotopes*, **28**, 739 (1979).
2) A part of this work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August, 1979.
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4) H.B. Hucker, *Ann. Rev. Pharmacol.*, **10**, 99 (1970).
5) S. Baba, *Rinsho Yakuri*, **4**, 279 (1973); H. Miyazaki, M. Ishibashi, and H. Takayama, *ibid.*, **4**, 288 (1973); K. Hasegawa, *ibid.*, **4**, 310 (1973); M. Iio, *ibid.*, **4**, 318 (1973); H. Shindo, "*Kagaku No Ryoiki*, Zokan" Vol. 107, Nankodo, Tokyo 1975, p. 101.
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Paeonol (2-hydroxy-4-methoxyacetophenone; I) is a main component of *Botan-pi*,[7] the root bark of Paeony (*Paeonia moutan* Sims), which has been shown to exhibit antipyretic, analgesic, sedative, hemostatic, anti-inflammatory[8] and antibacterial[9] actions. Yokoyama *et al.*[10] investigated the metabolism of I in rats by ^{14}C tracer experiments in which 2,5-dihydroxy-4-methoxyacetophenone (II), resacetophenone (III) and unchanged I were identified as urinary components. However, no report has appeared on the metabolism of I in other experimental animals or in man.

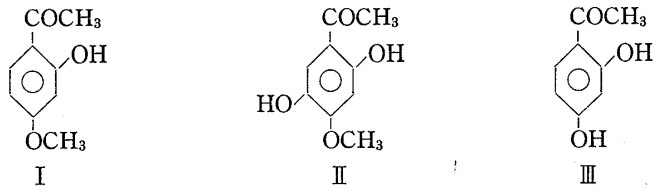

Fig. 1. Chemical Structures of 2-Hydroxy-4-methoxyacetophenone (I) and Its Metabolites

The present authors sought to clarify the metabolic fate of I in man by the SI tracer technique. Before comparing the metabolism of I in man with that in experimental animals, experiments were carried out by the RI tracer technique in order to investigate species differences in metabolism among various experimental animals. This communication deals with studies on the species differences in the metabolism of I in mice, rats, guinea pigs and rabbits.

Experimental

Labeled Compounds——2-Hydroxy-4-methoxyacetophenone[carbonyl-^{14}C] (I-^{14}C) was prepared by the method described in a previous paper,[1] with a minor modification as follows. Resacetophenone[carbonyl-^{14}C] (III-^{14}C) was obtained from resorcinol and acetic acid [1-^{14}C] (5 mCi, New England Nuclear, specific activity 4 mCi/mmol) using 265 mg of acetic acid as a carrier. I-^{14}C (210 mg, specific activity 0.522 mCi/mmol) was synthesized from III-^{14}C and methyl iodide. The overall radiochemical yield was 12.7% based on acetic acid [1-^{14}C]. The radiochemical purity determined by reverse dilution analysis, by thin-layer radio chromatography and by radio gas chromatography was more than 98%.

Nonlabeled Compounds——2,5-Dihydroxy-4-methoxyacetophenone (II) was synthesized in this laboratory by the method described in a previous paper.[1] 2-Hydroxy-4-methoxyacetophenone (I: Aldrich Chemical, U.S.A.) and resacetophenone (III: Wako Pure Chemical Industries, Tokyo) were used after recrystallization from EtOH, and from hot dilute (1: 11) hydrochloric acid, respectively.

Animal Studies——1) Metabolism and Excretion: Eighteen ddY male mice weighing 20—25 g, four Wistar male rats weighing 200—220 g, four Hartley male guinea pigs weighing 300—320 g, and four white male rabbits weighing 3.0—3.2 kg were used in one experimental group. The animals were placed individually in metabolic cages equipped with urine-feces separators. The mice were divided into six groups and a group of three mice was placed in one metabolic cage. Before administration all the animals were given only tap water for twelve hours, while food and water were given *ad libitum* after administration. Single doses of I-^{14}C in 50% propyleneglycol solution were administered orally: 20 mg (70 μCi)/kg for mice, 20 mg (30 μCi)/kg for rats and guinea pigs, and 20 mg (6 μCi)/kg for rabbits. The urine and feces were collected daily for four days and kept at −5°.

2) Acute Toxicity: Seventy of male mice weighing 15—20 g were used in one experimental group. II dissolved in polyethyleneglycol 300 was injected intraperitoneally at doses between 426 and 2056 mg/kg after 12-hr fasting. Overt signs of toxicity were observed for 48 hours after administration. The LD_{50} was calculated by the Litchfield-Wilcoxone method. The LD_{50} of II was 958 mg/kg and the 95% confidence limits were 753 to 1219 mg/kg.

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Thin-Layer Chromatography (TLC)——TLC of I, II and III with benzene–$CHCl_3$–AcOH (10: 3: 2, by vol., solvent system A), benzene–MeOH–AcOH (45: 8: 4, by vol., B), $CHCl_3$–AcOH (5: 1, by vol., C) and *n*–hexane–$CHCl_3$–AcOH (5: 3: 1, by vol., D) on silica gel plates (Kieselgel $60F_{254}$, Merck, 0.25 mm thickness) gave single spots, detecting by UV irradiation.

Measurement of Radioactivity——The ^{14}C-activity in urine and in MeOH solution of the ether extracts of treated or untreated urine was measured with an Aloka LSC-502 liquid scintillation counter (LSC) in the manner described in a previous paper.[11] The ^{14}C-activity in feces was measured with the LSC machine after oxidation[12] of the feces in an Aloka ASC-112 automatic combustion system. The ^{14}C-activity on thin-layer plates were scanned with an Aloka TLC-2B thin-layer radiochromatoscanner; the radioactive spot was scraped off and eluted with MeOH. An aliquot of the eluate corresponding to a radioactivity of 1×10^3—5×10^4 dpm was introduced into the hydrophilic scintillation fluid[11] for subsequent liquid scintillation counting.

Radio gas chromatography was performed with an Aloka radio gas chromatograph equipped with a synchronized accumulating radioisotope detector[13] developed in this laboratory. MeOH solutions of I-^{14}C and of the ether extracts of acid-hydrolyzed urine were used for the analysis. GC was done on a stainless steel column (1 m × 3 mm i.d.) packed with 1.5% silicone SE-30 on Shimalite w (80/100 mesh). Helium was used as a carrier gas (60 ml/min). The column temperature was 180° for I-^{14}C solution and 100—260° (10°/min) for the ether extracts of urine. The injection temperature was 310°. The sampling time and detector scale were 6 seconds and 10 k, respectively.

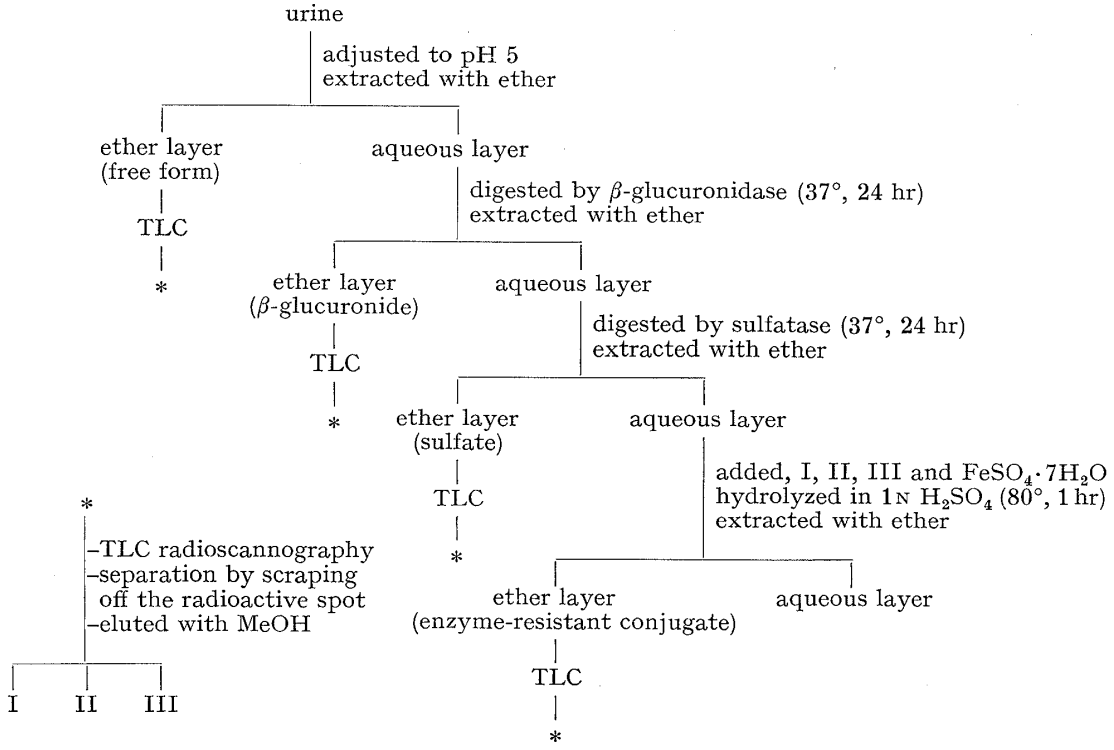

Chart 1. Separation of the Metabolites of Paeonol-^{14}C from the Urine of Various Animal Species

Analysis of Urinary Metabolites——A portion of 24-hr urine sample (in case of rabbits, 48-hr urine) corresponding to about 0.1 μCi of radioactivity was quantitatively analyzed by the method summarized in Chart 1. Rat or mouse urine to which the same volume of 0.2 M acetate buffer (pH 5.0) had been added was extracted with ether. In the case of guinea pig and rabbit, the urine was neutralized with 1 N HCl solution before adding the buffer. The residual aqueous layer was incubated with β-glucuronidase (Tokyo Zoki Co., bovine liver) for 24 hr at 37° then extracted 3 times with 2 volumes of ether. The aqueous layer was incubated with aryl sulfatase (Sigma Chemical Co., type V) for 24 hr at 37°, and then extracted 3 times with 2 volumes of ether. I, II, III and $FeSO_4$ were added to the residual aqueous layer then the solution was hydrolyzed

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with 1 N H_2SO_4 for 1 hr at 80°. Portions of the ether extracts of the free form, β-glucuronide form, sulfate form and enzyme-resistant conjugate[10] form fractions were subjected to TLC with solvent system A, which could separate the metabolites. TLC radio scanning was done, and the radioactivity of the spot was measured in the manner described above.

Results and Discussion

Excretion in Urine and Feces

The excretions of radioactivity during every 24-hr period for four days after oral administration of I-^{14}C to mice, rats, guinea pigs, and rabbits are shown in Table I. In all animals the rate of excretion was rapid: that is, the total activity excreted in the urine and feces in two days after administration was 83% of the administered radioactivity in mice, 90% in guinea pigs, 95% in rats, and 98% in rabbits. The excretion of radioactivity in feces in four days after administration was 11% in mice, while in the other animals it was less than 5%;

Table I. Excretion of ^{14}C- Activity by Various Animals after Oral Administration of Paeonol-^{14}C

Day	Rabbits[a]		Guinea pigs[a]		Rats[a]		Mice[b]	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	73.81± 10.42	0.59± 0.20	86.20± 5.25	2.41± 0.80	89.31± 1.99	4.29± 2.27	69.58± 3.20	8.27± 1.73
2	23.05± 9.61	0.15± 0.08	1.07± 0.57	0.42± 0.47	0.74± 0.63	0.20± 0.14	3.40± 1.76	2.05± 0.81
3	0.14± 0.24	0.02± 0.01	0.25± 0.10	0.02± 0.02	0.33± 0.18	0.17± 0.13	0.69± 0.16	0.23± 0.15
4	0.02± 0.04	0.01± 0.00	0.02± 0.01	0.00± 0.01	0.01± 0.01	0.00± 0.01	0.03± 0.05	0.02± 0.01
Total	97.02± 1.13	0.76± 0.21	87.53± 4.83	2.86± 0.53	90.39± 2.52	4.67± 2.18	73.70± 0.05	10.56± 1.51

a) Recovered radioactivity (%) in each day; the data are expressed as means±S.D. (n=4).
b) Recovered radioactivity (%) in each day; the data are expressed as means±S.D. (n=6).

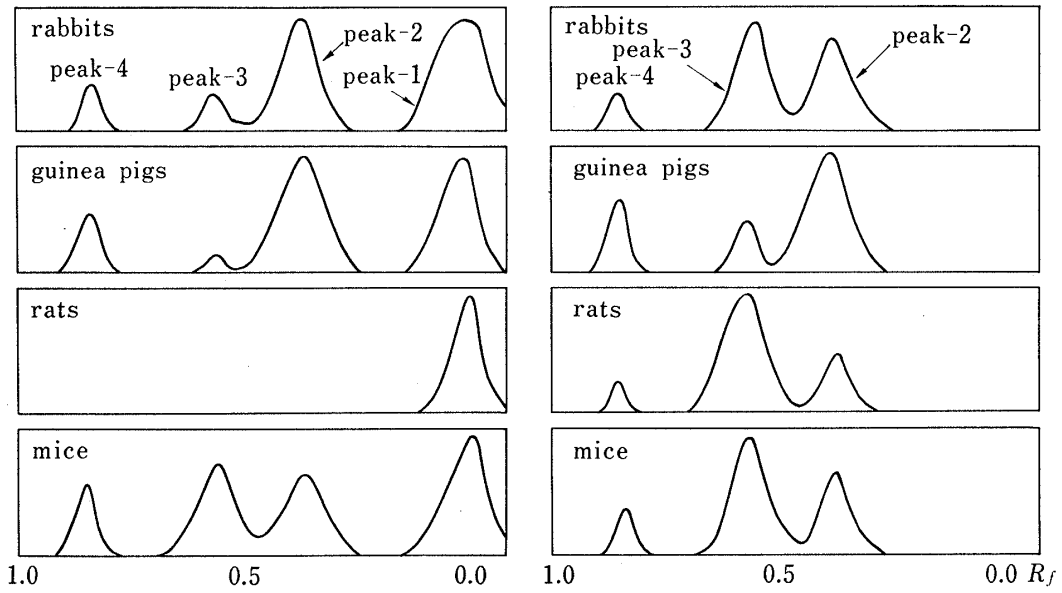

Fig. 2. TLC Radioscannograms of Urine from Animals Administered Paeonol-^{14}C
left: untreated urine; right: ether extracts of urine after acid hydrolysis.

in rabbits only 0.8% of the radioactivity was excreted. For the subsequent quantitative analysis of the metabolites, the 24-hr urine of mice, rats and guinea pigs, and the 48-hr urine of rabbits were used, because rabbits and the other animals excreted the bulk of the radioactivity within 48 hr and 24 hr, respectively.

Metabolites in Urine

Fig. 2 shows TLC radioscannograms of the untreated urine samples and the ether extracts of acid-hydrolyzed urine samples after developing with solvent system A. Each peak was named in order of its R_f value, *i. e.*, peak-1 (R_f 0.0), peak-2 (R_f 0.3—0.4), peak-3 (R_f 0.5—0.6), and peak-4 (R_f 0.8—0.9). By comparison with the R_f values of authentic samples (Table II) theses peaks were assigned as follows. peak-2: III, peak-3: II, and peak-4: I. It was suggested that peak-1 represented the conjugated forms of I, II and III, as indicated by the TLC behavior after extraction with ether followed by acid hydrolysis.

Table II. R_f Values of Authentic Samples

	Developing system	R_f value Authentic sample		
		I	II	III
A	Benzene–$CHCl_3$–AcOH (10: 3: 2, by vol.)	0.84	0.55	0.37
B	Benzene–MeOH–AcOH (45: 8: 4, by vol.)	0.86	0.69	0.64
C	$CHCl_3$–AcOH (5: 1, by vol).	0.89	0.76	0.52
D	*n*–Hexane–$CHCl_3$–AcOH (5: 3: 1, by vol.)	0.67	0.32	0.17

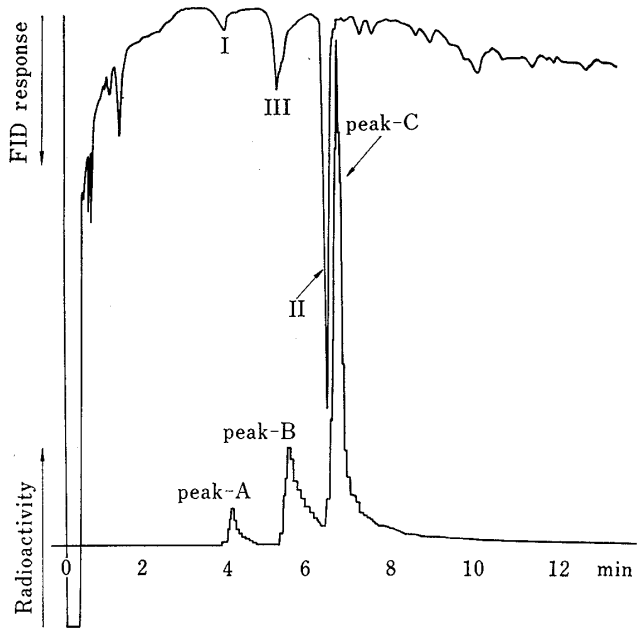

Fig. 3. Radio Gas Chromatogram of Urinary Metabolites of Paeonol-^{14}C after Oral Administration in Rats

A radio gas chromatogram of the ether extracts of acid-hydrolyzed rat urine is shown in Fig. 3. Although many peaks were found by flame ionization detection, only three peaks (A, B and C) with t_R values of 4.2, 5.7, and 6.8 min were detected by radio gas chromatography. By comparing the retention times of the peaks with those of authentic samples under various analytical conditions, it was confirmed that peak-A, peak-B, and peak-C corresponded to compounds I, III, and II, respectively. Moreover, the peak intensity ratios of the peaks in the radio gas chromatogram agreed well with those in the TLC radioscannogram. This indicates that by using radio gas chromatography the metabolites of a drug can be identified and determined quantitatively from biological samples: this is a simple and rapid analytical procedure.

Quantitative determination of the metabolites of I-^{14}C in the urine of various animal species was carried out in the manner described above. Yokoyama *et al.*[10] separated the metabolites in 7-hr rat urine without any carrier or antioxidant. However, we found that the metabolites were partly decomposed during the separation procedure, especially in the

process of acid hydrolysis, because I and its identified metabolites were all phenolic compounds. Therefore, 100 mg of ferrous sulfate heptahydrate and 0.1 ml of EtOH solution containing 50 mg/ml of I, II and III were added to the urine just before the acid hydrolysis step.

The results shown in Table III suggest that no other major metabolites were present in any animal species, because the total radioactivity excreted in the urine accounted for 88—98% of the administered radioactivity.

Table III. Urinary Metabolites of Paeonol-^{14}C in Various Animals

Metabolite	Rabbits[d]	Guinea pigs[e]	Rats[e]	Mice[e]
Free and Conjugated I	10.7	25.3	8.9	17.6
I	9.5±2.5	16.8±2.3	0.0±0.0	17.2±2.1
I-G[a]	0.5±0.2	1.1±0.4	7.5±1.0	0.3±0.2
I-S[b]	0.1±0.1	0.1±0.0	1.4±0.5	0.1±0.1
I-RC[c]	0.6±0.1	7.3±0.8	0.0±0.0	0.0±0.0
Free and Conjugated II	42.2	14.9	62.0	43.1
II	8.8±0.9	2.5±0.4	0.0±0.0	18.2±2.1
II-G	0.5±0.2	0.1±0.0	2.9±0.8	0.4±0.1
II-S	0.2±0.0	0.1±0.0	2.0±0.4	0.1±0.1
II-RC	32.7±1.6	12.2±3.0	57.1±2.3	24.4±3.8
Free and Conjugated III	42.7	58.1	17.5	29.9
III	31.3±0.9	42.2±2.6	0.0±0.0	16.2±3.3
III-G	1.1±0.3	0.4±0.2	9.5±1.8	0.8±0.4
III-S	0.7±0.4	0.3±0.1	4.9±0.9	0.8±0.4
III-RC	9.6±3.1	15.2±3.3	3.1±0.5	12.1±2.3
Total	95.6±1.6	98.3±3.4	88.4±3.9	90.6±1.9
^{14}C-Activity in Urine[f]	96.9	86.2	89.3	69.6

The data are expressed as means±S.D. (n=4 for rabbits, guinea pigs, rats; n=6 for mice)
a) β-glucuronide of I,
b) sulfate of I,
c) enzyme-resistant conjugate of I.
d) percentage of the ^{14}C-activity in the 48-hr urine.
e) percentage of the ^{14}C-activity in the 24-hr urine.
f) percentage of the administered ^{14}C-activity.

Table IV. Percentages of Various Groups of Metabolites

Group of Metabolites	Rabbits[a]	Guinea pigs[b]	Rats[b]	Mice[b]
Free form	49.6±2.7	61.4±3.6	0.0±0.0	51.6±3.4
β-Glucuronide	2.0±0.6	1.6±0.7	19.9±2.5	1.4±0.7
Sulfate	1.0±0.4	0.5±0.2	8.2±0.6	1.0±0.5
Enzyme-resistant conjugate	42.8±3.6	34.7±4.2	60.2±2.5	36.5±4.2

The data are expressed as means±S.D. (n=4 for rabbits, guinea pigs, rats; n=6 for mice)
a) percentage of the ^{14}C-activity in the 48-hr urine.
b) percentage of the ^{14}C-activity in the 24-hr urine.

Species Differences in Metabolism

In order to clarify the species differences in metabolic patterns among these animal species, metabolites were classified into three groups according to chemical type, and into four groups according to the type of conjugation, as shown in Tables III and IV. The total amount of II and its conjugated form accounted for 62.0% in rats, 43.1% in mice, and 42.2% in rabbits, but only 14.9% in guinea pigs. This suggests that a metabolic pathway involving hydroxylation of the benzene ring is predominant in rats, and is also significant in mice and

rabbits. However, the main metabolic route in guinea pigs was considered to be different. A similar result was obtained in the metabolism of *l*-ephedrine.[14]

In the case of III and its conjugated form, the total amounts were calculated to be 58.1% in guinea pigs, 42.7% in rabbits, 29.9% in mice, and only 17.5% in rats, suggesting that the metabolic pathway through O-demethylation is predominant in guinea pigs, while in rats it is not significant.

On the other hand, the total amount of unchanged I and its conjugated form reached 25.3% in guinea pigs, 17.6% in mice, 10.7% in rabbits, and 8.9% in rats. These results suggest that the metabolic patterns of I are quite different in guinea pigs and rats.

In the metabolic study of 3-*tert*-butyl-4-hydroxyanisole and 2-*tert*-butyl-4-hydroxyanisole in rabbits,[15] the major metabolite of the former was glucuronide, and that of the latter was sulfate, and only a small amount of the O-demethylated metabolite was observed. It is interesting that a large amount of O-demethylated or hydroxylated metabolites were found in this study.

Animal species could be classified into two groups according to the metabolic patterns of conjugation: the first group consisted of mice, guinea pigs and rabbits, and the second group of rats. That is, free form metabolites were not found in the urine of rats, while free form metabolites found in the urine of other species reached 50—60% of the total metabolites. In addition, significant quantities of β-glucuronide and sulfate were found in the urine of rats, while the total amount of these conjugates were less than 2 % in other species.

The acetyl group of I was not metabolized in any animal species. A similar result was reported in a metabolic study of 2,4,5-trihydroxybutyrophenone[16] by rats and dogs. That is, the butyryl group was not transformed, while O-glucuronide and sulfate were excreted in the urine, and unchanged compound was also excreted in the feces.

It is well known that phenolic compounds are generally transformed to glucuronide and sulfate.[17] However, in the metabolism of I among these animal species, β-glucuronide and sulfate were found only in small amounts, while a large amount of enzyme-resistant conjugate was found in all the species.

The results of the study on the acute toxicity of I[8a] and its metabolites showed that the LD_{50} values of I in the mouse were 3430 mg/kg (*p. o.*) and 781 mg/kg (*i. p.*), while those of II and III[18] in the mouse were 958 mg/kg (*i. p.*) and 2830 mg/kg (*p. o.*), respectively. It is suggested, therefore, that the toxicity of I in the rat was less than that in the rabbit, guinea pig and mouse, because unconjugated metabolites (considered to be more toxic than conjugated metabolites) were not excreted into the rat urine. Comparison of the LD_{50} and the amount of unconjugated metabolites excreted into the urine suggests that the species differences in metabolism observed here might not lead to remarkable species differences in toxicological response in experiments using rabbit, guinea pig and mouse.

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