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Studies on the Percutaneous Absorption of Paeonol by Using Stable Isotopes

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Studies on the percutaneous absorption, metabolism and excretion of paeonol (2-hydroxy-4-methoxyacetophenone, I) were carried out by using the stable isotope (SI) method, which is applicable to man. Radioactive tracer experiments in rats were also performed in order to evaluate the SI tracer technique. Hydrophilic ointment or absorptive ointment containing 5% I was topically applied under an occlusive dressing to man and rat for 6, 12 and 24 h. The amounts of metabolites (2,5-dihydroxy-4-methoxyacetophenone II, resacetophenone III, and unchanged substrate I) excreted in the urine were determined by selected ion monitoring assay using I [methoxy-d_3], II [methoxy-d_3] and III [acetyl-$^{13}C_2$] as internal standards.

After topical application of hydrophilic ointment for 6 h in man, the amounts of I, II and III in 0—24-h urine were 3.35, 16.28 and 5.17% of the applied dose, respectively. Urinary excretion of I metabolites continued for 6 d from the time of application, but the total amount of metabolites excreted in 0—24 h was as much as 89% of that excreted in 0—144 h.

The results indicate that the SI tracer technique described here has sufficient sensitivity and repeatability. Paeonol is easily absorbed through the skin, and the metabolites are excreted rapidly in urine.

Keywords——paeonol; percutaneous absorption; stable isotope; urinary excretion; metabolism; hydrophilic ointment; man; rat

Studies[2] on percutaneous absorption of drugs have been carried out by many investigators in dermatology and pharmaceutical sciences, because they are very important for evaluating the efficacy and safety of topical medicaments. Since the amount of penetrating drug is so small, good quantitative microtechniques are required. Accordingly, studies on percutaneous absorption of drugs have mostly been performed by radioactive tracer techniques in exprerimental animals. However, percutaneous absorbability of a drug in man is not readily predictable from the results of animal experiments, because the structure and physiological functions of the skin in man are different[3] from those in experimental animals.

In order to develop a new technique for studying percutaneous absorption by the use of the stable isotope (SI) method, which is applicable to man, we are carrying out a series of studies by using paeonol[4] (2-hydroxy-4-methoxyacetophenone, I) as a model drug. We have already reported the synthesis of SI-labeled compounds,[5] and the metabolism of I in experimental animals[6] and in man.[7]

In this paper, the metabolism and excretion of I after topical application to man and rat were studied by the use of stable isotopes. Radioactive tracer experiments in rats were also carried out to check the validity of the proposed technique.

Experimental

Labeled Compounds——2-Hydroxy-4-methoxy[d_3]acetophenone (I-d_3), 2,5-dihydroxy-4-methoxy[d_3]acetophenone (II-d_3), resacetophenone[acetyl-$^{13}C_2$] (III-$^{13}C_2$), and 2-hydroxy-4-methoxyacetophenone[carbonyl-^{14}C] (I-^{14}C) were prepared in our laboratory as described previously.[5,6]

Nonlabeled Compounds——2,5-Dihydroxy-4-methoxyacetophenone (II) was prepared by the method described above. 2-Hydroxy-4-methoxyacetophenone (Aldrich Chemical, U.S.A.; I) and resacetophenone (Wako Pure Chemical Ind., Tokyo; III) were used after recrystallization from hot ethanol, and from hot dilute (1 : 11) hydrochloric acid, respectively.

Instrumentation and Conditions——Gas chromatography-mass spectrometry-selected ion monitoring measurements were carried out in the same manner as described previously.[7] Quantitative determination of I in sample ointment was done by high-performance liquid chromatography (HPLC) as follows. The liquid chromatograph consisted of a double plunger pump (model 635s, Hitachi Seisakusho, Tokyo), an injection valve (type 7125, Rheodyne) equipped with a 20 μl loop, and a variable wavelength detector (Uvilog-5III, Oyobunko, Tokyo) set at 274 nm. The mobile phase, consisting of water (200 ml), acetonitrile (50 ml), and triethanolamine (0.1 ml), was used at a flow rate of 1 ml/min through a Lichrosorb RP-18 column (5 μ, 4.6 mm i.d. $\times$ 150 mm) which was held at 40 °C during analysis. The amount of I in radioactive ointment was determined by radio gas chromatography[5] using a flame ionization detector after dissolving the sample ointment and 2,4-dimethoxyacetophenone (internal standard; I.S.) in methanol. The radioactivity of the methanol solution of radioactive ointment was measured with a liquid scintillation counter in the same manner as described[5] previously. The ^{14}C-activity on thin-layer plates was measured as previously reported.[5]

Preparation of Sample Ointment——A mixture of 246.3 mg of I and 16.7 mg of I-^{14}C (52.5 μCi) was dissolved in 0.60 g of propyleneglycol, then 5.30 g of hydrophilic ointment[8] (ointment 1; o/w type) was prepared in the usual manner. Hydrophilic ointment (ointment 2) and absorptive ointment[8] (ointment 3; w/o type) each containing 5% I were prepared in the same manner.

Measurement of Release Rate of I from Ointment——The *in vitro* release rate of I from an ointment was determined by a modification of the method described in the previous report.[9] That is, about 31.4 mg of ointment 2 or 3 was applied to Toyo filter paper No. 2 (ϕ = 2 cm), then the filter paper was fixed in a diffusion apparatus as shown in Fig. 1. The lower compartment was filled with 0.2 M acetate buffer solution (pH 5.6). The temperature of the solution held at 30 °C, with stirring at150 rpm. An aliquot of 50 μl was taken up with an auto pipette and silicone tube through a sampling hole at 20, 40, 60, 90 and 120 min after initiation, and the amount of I released from the ointment was determined by HPLC as mentioned above.

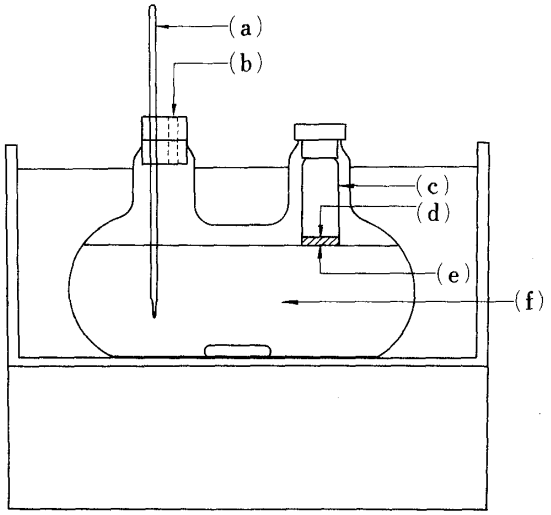

Fig. 1. Apparatus for Release Experiment

(a) thermometer; (b) sampling hole; (c) cylinder; (d) ointment; (e) filter paper; (f) buffer solution.

Percutaneous Absorption of I in Man——The upper forearms of three healthy male volunteers (subjects 1, 2, 3; 41, 37, 24 years of age; weighing 56, 64, and 62 kg, respectively) were used as test sites. If a test site was used more than twice, an interval of four weeks between applications was allowed, taking into consideration the turnover rate[10] of human skin.

Protocol 1: About 10 mg·cm^{-2} of ointment 2 was weighed and spread evenly on aluminum foil (10 $\times$ 3 cm, Nihon Seihaku, Tokyo), then the whole was placed on the test site of subjects 1 and 2, and the aluminum foil was fixed with an adhesive tape (12 $\times$ 5 cm, Johnson and Johnson, Inc., Dermicel hypoallergenic cloth tape). The aluminum foil was removed after 6 h, and the ointment remaining on the skin was wiped off with absorbent cotton soaked in water. The aluminum foil and the cotton were immersed in methanol, ultrasonicated for 1 h. An aliquot of 100 μl was taken up, and the amount of I in the methanol solution was determined by HPLC. The amount of I permeated into the skin (permeated dose; D_p) was calculated as follows;

$$D_p = D_a - D_r$$

where D_a = the amount of I applied to the skin (applied dose).

D_r = the amount of I recovered from the skin surface (skin surface residual dose).

After the application of ointment, urine was collected during 0—6, 6—12, 12—18, 18—24, 24—36, 36—48, 48—60, 60—72, 72—84, 84—96, 96—120, and 120—144 h; samples were kept at −5 °C until analysis.

Protocol 2: Subjects 1, 2 and 3 received ointment 2 or 3 topically in the same manner as mentioned above. Sample ointment was removed after 6, 12, or 24 h after the application, and the skin surface residual dose of I was determined. Urine was collected for 24 h after the application, and kept at −5 °C until analysis.

Percutaneous Absorption of I in Rat——Three or four male Wistar rats weighing 200—220 g were used in one experimental group. Hair on the back was clipped with an electric clipper taking great care to cause no skin damage. Ointment 1 or 2 was applied for 6, 12 or 24 h in the same manner as mentioned above. Skin surface residual dose of I-^{14}C or I was determined. Determination of I-^{14}C was carried out by liquid scintillation counting. Rats were kept in individual metabolic cages after topical application, and urine samples collected during 24 h were kept at −5 °C until analysis.

Quantitative Determination of Urinary Metabolites——Urinary metabolites of man or rat after topical application of ointment 2 or 3 were quantified by the isotope dilution method.[7] Urinary metabolites of rat after topical application of ointment 1 were quantified by thin-layer radio chromatography and liquid scintillation counting.[6]

Results and Discussion

In previous papers[6,7] we reported that 2,5-dihydroxy-4-methoxyacetophenone, resacetophenone, unchanged I, and their various conjugates were excreted mostly in urine after oral administration of I to man, rabbits, guinea pigs, rats, and mice, and that cumulative urinary excretions of these compounds during 48 h after administration reached 83% of the dose. Thus, the absorption, metabolism and excretion of I after oral administration in man or experimental animal can be sufficiently studied by the determination of I, II and III in urine.

As a preliminary experiment for studying the percutaneous absorption of I, I-^{14}C was topically applied to rats, and the ^{14}C-activity in urine or feces was determined. Thus, compounds I, II and III were identified in urine, and no other metabolites were detected. From these results it is suggested that I is absorbed through the skin, then unchanged substrate I and metabolites II and III were mostly excreted in urine. Therefore, we estimated the percutaneous absorption of I by quantifying I, II and III in urine after topical application of I to man and rat.

Specific Activity and/or Content of I in Ointment

The contents of I in ointment 2 and 3 were 5.04 ± 0.065 and 5.06 ± 0.051% ($\bar{X} \pm$S.D.; $n=5$), respectively. The specific activity and content of I in ointment 1 were 0.01016 ± 0.00064 μCi/mg and 4.97 ± 0.12% ($\bar{X} \pm$S.D.; $n=5$), respectively. It was clear that I and I-^{14}C were distributed quite homogeneously in each sample ointment, and that the quantification methods for I and I-^{14}C showed adequate reproducibility.

Release of I from Ointment Bases

Percutaneous absorption of a drug from an ointment involves two consecutive processes, that is, drug release from the ointment base and drug absorption through the skin barrier. Release of I from the sample ointment was determined in order to investigate whether the rate-limiting process in the percutaneous absorption of I is the drug release step or the drug absorption step. The release of I from ointments 2 and 3 is shown in Fig. 2. The experimental data were fairly well approximated by a straight line when the amount of I released was plotted *versus* $\sqrt{t}$, where t is the time. It seems that the time course of drug release fitted well to Higuchi's equation[11] concerning drug release from a heterogeneous system controlled by diffusion. The apparent release rate constants of I from ointments 2 and 3 calculated by means of Higuchi's equation were 2.17×10^6 and 1.87×10^6 mol·h$^{-(1/2)}$, respectively. The straight lines did not pass through the origin, and this suggests the existence of an induction period for the penetration of I through the filter paper.

As is mentioned later, the total amounts of metabolites and substrate excreted in 0—6-h urine after topical application of ointments 2 and 3 to man were about 13 and 10% of the

applied dose, respectively. The amount of a drug absorbed can be calculated from the urinary excretion data as follows.[12]

$$A_a = (K_{e1})^{-1} \times (dA_e/dt) + A_e$$

A_a : amount of drug absorbed.
K_{e1} : elimination rate constant.
dA_e/dt : urinary excretion rate.
A_e : amount of drug excreted in urine.

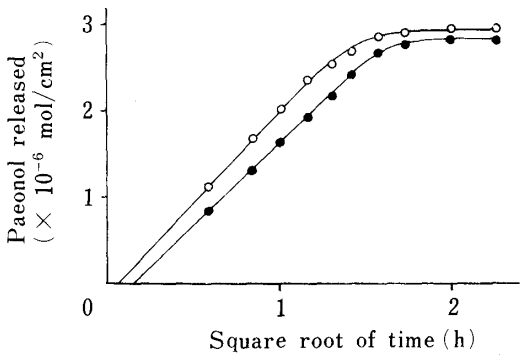

Fig. 2. Plot According to Higuchi's Equation for Ointments

○, ointment 2 (hydrophilic ointment); ●, ointment 3 (absorptive ointment).
Each symbol represents the mean value of 6 data.

Thus, the amount of I absorbed during the first 6 h after topical application of ointment 2 to man was 37% of the applied dose, while the amounts of I released from ointments 2 and 3 during 6 h were 52 and 44% of the applied dose, respectively. Consequently, it is suggested that drug release from the ointment is not the rate-limiting process in human percutaneous absorption of I.

Comparison of SI Tracer Technique with Radioactive Tracer Technique

In order to evaluate the reliability of the SI tracer technique in the study of percutaneous absorption of a drug, a comparative study was carried out in rats. Four rats were divided into two groups. An SI tracer experiment was performed with the first group, followed by a radioactive tracer experiment four weeks after the first application. Both experiments were carried out similarly but in the reverse order with the second group. That is, about 10 mg·cm^{-2} of ointments 1 or 2 was applied for 6 h on the rat skin (20 cm^2) by occlusive dressing treatment,[13] then the amounts of metabolites and substrate excreted in 0—24-h urine were determined. The results of quantitative determination of the urinary metabolites and substrate are shown in Table I. No significant difference was found between the data obtained by the two techniques. For example, the percentages (% of permeated dose) of II determined by SI and radioactive tracer experiments were 17.58±3.01% and 17.22±2.82%, respectively. Moreover, no significant difference was found between the data obtained by the two methods for the amount of each compound in 120—144-h urine.

Thus, it is considered that the SI tracer technique proposed here gives results comparable with those of the radioactive tracer technique in the study of percutaneous absorption of I with experimental animals and man.

Percutaneous Absorption of I in Rat

Ointment 2 was applied on the rat skin for 6, 12 and 24 h, then the amounts of I, II and III excreted in 0—24-h urine were determined by the SI tracer technique. As shown in Table I, the amount of I remaining on the skin decreased rapidly, and only 0.62% of I was recovered from the skin surface after 24-h application. Total excretion rates of I, II and III (TER) in 0—24-h urine, on the other hand, increased rapidly with increasing application time. That is, TER (% of applied dose) in 0—24-h urine was 24.2% for 6-h application, 36.6% for 12-h application, and 47.6% for 24-h application. TER (% of permeated dose) in 0—24-h urine was 27.5% for 6-h application, 38.3% for 12-h application, and 47.9% for 24-h application. Thus, TER based on applied dose and that based on permeated dose became increasingly similar as the application time increased (Fig. 3).

TABLE I. Percutaneous Absorption of I in Rat

Application[a]			Skin surface residual dose		Permeated dose (mg)	Amount of metabolite excreted in urine[b]				Total excretion rate[c]	
Tracer	Time (h)	Applied dose (mg)	(mg)	(%)		I (mg)	II (mg)	III (mg)	Total (mg)	Percent of permeated dose	Percent of applied dose
RI	6	10.02±0.25[d]	1.09±0.28	10.88±2.73	8.93±0.32	0.271±0.029	1.679±0.224	0.497±0.077	2.345±0.317	26.38±4.38	23.47±3.72
SI	6	10.13±0.23	1.18±0.37	11.65±3.49	8.94±0.30	0.293±0.076	1.718±0.261	0.538±0.145	2.447±0.471	27.46±5.76	24.18±4.70
SI	12	10.10±0.14	0.44±0.19	4.31±1.84	9.66±0.08	0.453±0.152	2.713±0.494	0.814±0.204	3.697±0.459	38.30±5.05	36.59±4.31
SI	24	10.24±0.23	0.06±0.03	0.62±0.30	10.18±0.23	0.562±0.035	3.373±0.673	1.134±0.143	4.877±0.788	47.93±8.00	47.62±7.80

a) Ointment 1 (RI) or ointment 2 (SI) was applied (10 mg/cm^2) on the skin (20 cm^2) for 6 (n=4), 12 (n=3) or 24 (n=3) h.
b) Urine was collected for 24 h from the beginning of ointment application. Total (mg) = I (mg) + II (mg) × (166/182) + III (mg) × (166/152).
c) Excretion rate of I, II and III in 0—24-h urine.
d) $\bar{X}$±S.D.

TABLE II. Percutaneous Absorption of I in Man

Application[a]			Skin surface residual dose		Permeated dose (mg)	Amount of metabolite excreted in urine[b]				Total excretion rate[c]	
Ointment	Time (h)	Applied dose (mg)	(mg)	(%)		I (mg)	II (mg)	III (mg)	Total (mg)	Percent of permeated dose	Percent of applied dose
Hyd	6	14.92±0.10[d]	2.71±0.67	18.2 ±4.6	12.21±0.76	0.50±0.08	2.66±0.31	0.71±0.07	3.70±0.44	30.48±5.53	24.77±3.09
Hyd	12	14.98±0.13	0.87±0.32	5.82±2.09	14.10±0.20	0.58±0.07	3.83±0.42	1.15±0.16	5.33±0.60	37.80±4.67	35.55±3.72
Hyd	24	15.10±0.12	0.15±0.07	0.99±0.46	14.95±0.05	0.67±0.16	4.35±0.32	1.63±0.26	6.41±0.72	42.88±4.70	42.44±4.45
Abs	6	15.01±0.14	3.89±0.52	25.93±3.27	11.11±0.42	0.27±0.03	1.67±0.17	0.55±0.05	2.39±0.24	21.58±2.98	15.93±1.49

a) Ointment 2 (hyd) or ointment 3 (abs) was applied (10 mg/cm^2) on the skin (30 cm^2) for 6, 12 or 24 h.
b) Urine was collected for 24 h from the beginning of ointment application. Total (mg) = I (mg) + II (mg) × (166/182) + III (mg) × (166/152).
c) Excretion rate of I, II and III in 0—24-h urine.
d) $\bar{X}$±S.D. (n=3).

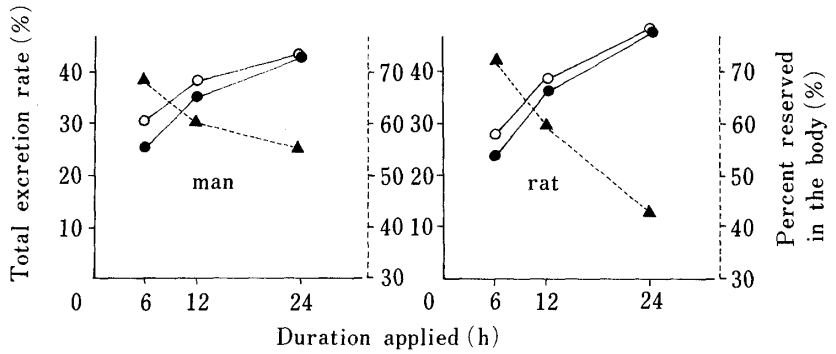

Fig. 3. Effect of Application Time on Percutaneous Absorption and Reservoir of I in Man and Rat

○, total excretion rate (% of permeated dose); ●, total excretion rate (% of applied dose); ▲, percent of I reserved in the body (% of applied dose).
Ointment 2 was applied (10 mg/cm^2) for 6, 12 and 24 h, then the amounts of I metabolites in 0—24-h urine were determined.

When I-^{14}C was administered orally to rats, guinea pigs, mice and rabbits,[6] fecal excretion of the ^{14}C-activity was small, and respiratory excretion was not found. It was also confirmed in animal experiments that the proportions of urinary excretion and fecal excretion of the ^{14}C-activity after topical application of I-^{14}C were very similar to those after oral administration. Therefore, the percentage of I reserved in the body can be calculated from permeated dose and excreted dose as follows;

$$R=\left[P-\left(UE_T\times\frac{UE_O+FE_O}{UE_O}\right)\right]\times\frac{100}{P}$$

R = Percentage of I reserved in the body (% of permeated dose).
P = Percentage of I permeated into the skin (% of applied dose).
UE_T = Cumulative[a] urinary excretion of I, II and III (% of applied dose) after topical application.
UE_O = Cumulative[a] urinary excretion of I, II and III (% of applied dose) after oral administration.
FE_O = Cumulative[a] fecal excretion of I, II and III (% of applied dose) affer oral administration.
a) Total amount of each compound excreted in the urine or feces during the period of their collection.

However, this equation is not applicable when elimination from the stratum corneum occurs. As shown in Fig. 3, the percentage of I reserved in the rat decreased rapidly with increasing application time.

Percutaneous Absorption of I in Man

Ointment 2 was applied for 6 h on the upper forearm of subjects 1 and 2, and the amounts of I, II and III excreted in urine were determined for 6 d. Urinary excretion rates of I, II, III, and TER are shown in Figs. 4 and 5. The excretion patterns of the compounds in both subjects were almost the same, and the proportions of I, II and III excreted after topical application agreed well with those[7] excreted after oral administration. The average TER equivalent to I in 0—12-h urine was more than 200 $\mu g\cdot h^{-1}$; those in 12—18 h and in 18—24 h were 115 and 41 $\mu g\cdot h^{-1}$, respectively; and that in 120—144 h was only 0.060 $\mu g\cdot h^{-1}$. Percentage TER values in 0—24 h and 0—144 h were 32.9% (% of permeated dose) and 37.0%, respectively; the ratio of 0—24-h excretion to 0—144-h excretion was as high as 89%.

From these results, it is considered that the percutaneous absorption of I in man can be adequately studied by the quantification of I, II and III excreted in 0—24-h urine after topical application. A metabolite with a concentration level of about 1 $ng\cdot ml^{-1}$ in urine could be

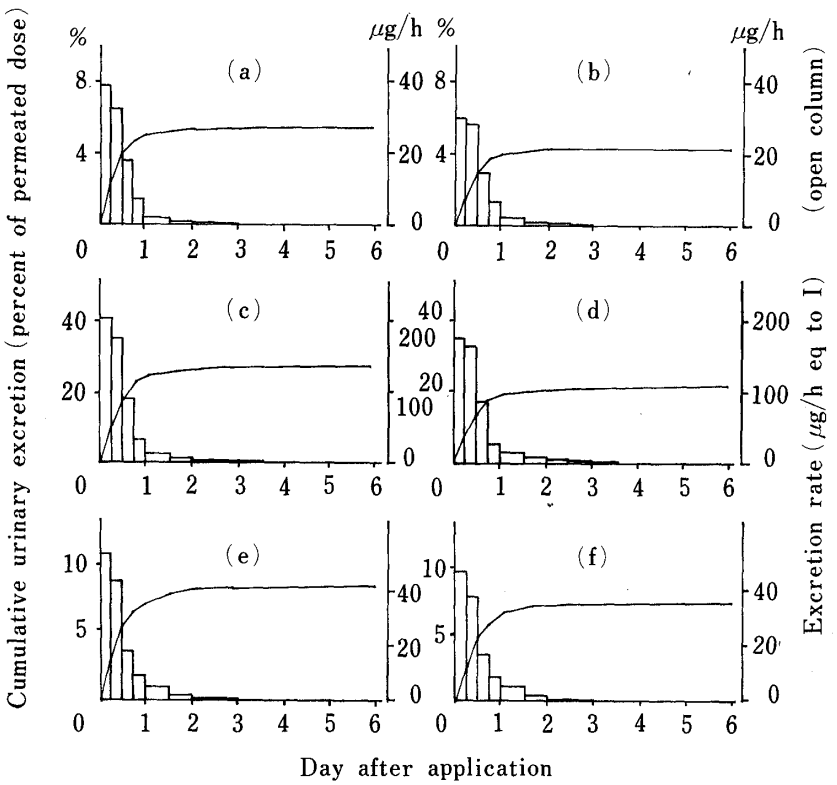

Fig. 4. Urinary Excretions of I, II and III after Topical Application of Ointment 2 for 6 h in Man

Subject 1: (a), (c) and (e). Subject 2: (b), (d) and (f).
Excretion of I and its conjugate, (a) and (b); excretion of II and its conjugate, (c) and (d); excretion of III and its conjugate, (e) and (f).

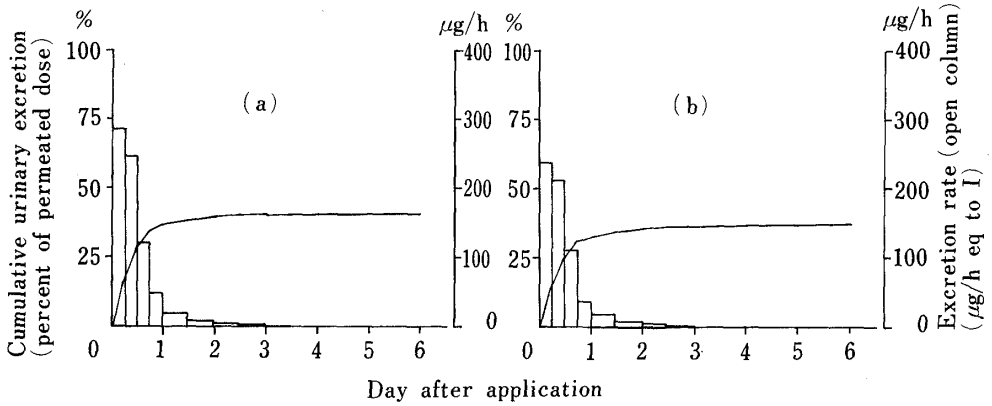

Fig. 5. Total Excretion Rate in Man after Topical Application of Ointment 2 for 6 h

Total amounts of I, II and III in urine were determined for 6 d. Subject 1: (a). Subject 2: (b).

determined by the isotope dilution method used here. It is suggested that the investigation of percutaneous absorption and metabolic fate of a drug is possible by using an SI tracer technique, even if the quantity absorbed is small. Excretion of I metabolites continued for 3 d after oral administration, but continued for 6 d after topical application. It may generally be said that the absorption of a drug after topical application continues for a longer period than after oral administration. The durability of percutaneous absorption must be caused by reserving[14] of the drug in the stratum corneum, stratum lucidum, stratum granulosum,

stratum spinosum, stratum basale and corium followed by slow diffusion in the tissues.

Secondly, ointment 2 or 3 was applied to subjects 1, 2 and 3 for 6, 12 or 24 h, and the amounts of I, II and III in each 0—24-h urine were determined. The results are shown in Table II. The permeated dose of I, and the excreted dose of I metabolites increased with increase of the application time. However, a sharp increase was found in the latter, as was the case in the rat. That is, the percentages permeated into the skin (% of applied dose) after topical application of ointment 2 for 6, 12 and 24 h were 81.8, 94.2, and 99.0%, respectively, while the excretion rates of I metabolites (% of applied dose) in 0—24-h urine for these application times were 24.8, 35.6, and 42.4%, respectively. The difference between excretion rate based on applied dose and that based on permeated dose became small with increasing application time; namely, the difference of 5.71% for 6-h application fell to only 0.44% for 24-h application (Fig. 3). These results indicate that the excess amount of a drug applied on the skin might not participate in percutaneous absorption during a short period after application. In such a case, the apparent percutaneous absorption rate, which is expressed as % of applied dose, shows a smaller value than the maximal value. Therefore, the percutaneous absorption rate should be calculated, if possible, based on permeated dose of the drug. As shown in Fig. 3, the relation between percentage reserved and application time in man was similar to that in rat. The reserved amount of I decreased with increase of the application time. However, 55% of I still remained in the body after 24-h application. Paeonol is a drug which is easily absorbed after topical application, but nevertheless, more than 50% of the drug permeated into skin was found remaining in the body. This suggests that a drug applied topically is easily reserved in skin tissue, especially in the stratum corneum, and that the proportion of the drug which is absorbed into the bloodstream after topical application is very much smaller than that after oral administration. The percentage of I reserved in man was far smaller than those in the cases of corticosteroids.[15] It appears that a chemical which is easily reserved in the tissue has a tendency not to be absorbed quickly through the skin. The urinary excretion rate of I after topical application in man was rather high compared with those of other drugs.[16] High absorbability of I must be a result of its low molecular weight, and suitable solubility in water and oils. Although percutaneous absorbability of I from hydrophilic ointment seemed to be somewhat higher than that from absorptive ointment, the drug release rates of I from these ointment bases were not so different. These results indicate that the percutaneous absorbability of a drug is influenced by many factors such as hydration of the stratum corneum[17] due to the occlusive effect[18] of the vehicle, partition coefficient of the drug between the vehicle and stratum corneum depending on HLB value[19] or solubility, and physiological effect of surfactants, polyols and vehicles on skin barrier functions.[20]

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