for 30 min. The phenolic metabolites extracted from the hydrolyzed bile or incubation mixture were isolated by thin-layer chromatography (TLC).[5]

Chemical Hydroxylation——Oxidation of ^{2}H-3′-Me-DAB, 3′-Me-MAB, or 3-acetaminotoluene with such model systems as photolysis of pyridine-N-oxide or hydrogen peroxide, *m*-Cl-perbenzoic acid, and the Udenfriend or Hamilton system were carried out as described in the previous report,[8] except for the use of *tert*-BuOH as solvent of aminoazo dyes.

Retention of Isotopic Hydrogen in Hydroxylated Products——Percentage retention of deuterium or tritium in each product isolated with TLC was calculated by dividing its deuterium content determined by mass spectrometry or its specific radioactivity by that of the substrate.[5,7,8]

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Studies on Gas–Liquid Chromatography Separative Determination of 4′-Chloro-5-methoxy-3-biphenylylacetic Acid and Its Metabolites in Urine

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The amounts of 4′-Chloro-5-methoxy-3-biphenylylacetic acid (DKA-9) and its metabolites (DKA-9G, DKA-24, DKA-24G, DKA-24OG and DKA-24S, see Chart 1) in human urine could be determined separately by measuring the amounts of DKA-9 and DKA-24 generated after hydrolizing under various conditions with gas–liquid chromatography.

Keywords——gaschromatography; anti-inflammatory drug; separative determination; deconjugation; metabolite; derivatization

4′-Chloro-5-methoxy-3-biphenylylacetic acid (DKA-9) is a potent anti-inflammatory and analgesic agent developed in our laboratories.[2] Its biopharmaceutical behaviors, the ADME studies, in various animal species and human were already studied[3–5] and as shown in Chart 1, DKA-9 was mainly metabolized through two major pathways, glucuronide formation and demethylation followed by sulfate formation and about 73% of the dose was excreted in urine as metabolites in 24 hours after oral administration in human.

In order to design the dosage form and dosage schedule of a drug, the drug behaviors in body must be elucidated pharmacokinetically. And for these studies, the precise analytical method of the drug and its metabolites is necessary.

The present report describes about the separative determination of DKA-9 and its metabolites in human urine by measuring the amount of DKA-9 and 4′-chloro-5-hydroxy-3-biphenylylacetic acid (DKA-24)[6] with gas–liquid chromatography (GLC) after hydrolizing glucuronides and sulfate under various conditions.

1) Location: **14**, *Nishinosho-Monguchi-cho, Kisshoin, Minami-ku Kyoto, Japan.*
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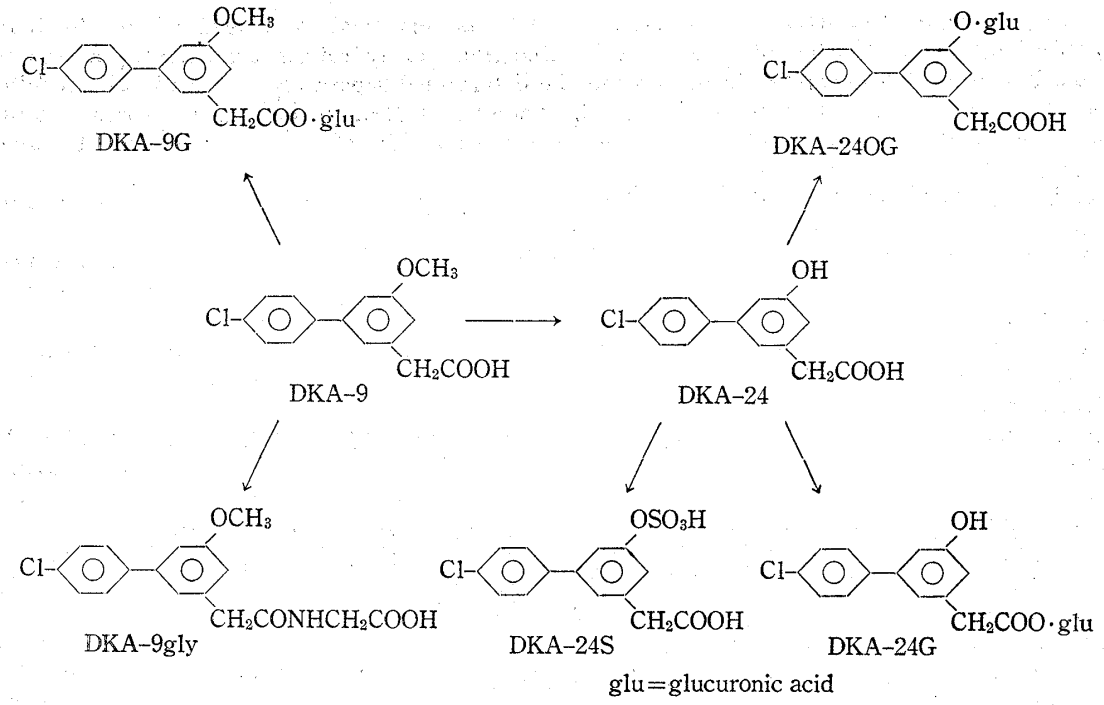

Chart 1. Possible Metabolic Pathways of DKA-9 in Human

Materials and Methods

Materials——DKA-9,[6] DKA-24,[6] potassium 4′-chloro-5-hydroxy-3-biphenylylacetic acid O-sulfate (DKA-24S),[4] ^{14}C-DKA-9[3] and 5-carboxymethyl-4′-chloro-3-biphenylyl β-D-glucopyranosiduronic acid (DKA-24 OG)[4] were synthesized in our laboratories. ^{14}C-DKA-24 was obtained by acidic hydrolysis of ^{14}C-DKA-9 and ^{14}C-DKA-24S was also obtained from rat urine after intravenous administration of ^{14}C-DKA-9. DKA-9G and DKA-24G were obtained from human urine[5] after oral administration of DKA-9. Lyophilized Helicase (I.B. Francaise) was purchased from Nakarai Chemicals and other chemicals were of analytical reagent grade. As an internal standard (IS), 1-phenyl-3-allyl-5-bromo-6-methyluracil was synthesized in our laboratories.

Gas Chromatograph and Conditions——Gas chromatographic measurements were made with a gaschromatograph (Shimadzu GC-6AM) equipped with a hydrogen flame-ionization detector. All chromatography was conducted on coiled glass column (1.0 m×3 mmφ) of 2% (w/w) SE-30 on 80—100 mesh Chromosorb W. The temperature of the column, the injection port and that of the detector block were maintained at 200°, 300° and 300°. Under these conditions, IS, trimethylsilylated DKA-9 (TMS-DKA-9), trimethylsilylated DKA-24 (TMS-DKA-24) and trimethylsilylated 2-(4′-chloro-5-methoxy-3-biphenylyl)-2-hydroxyacetic acid (TMS-α-OHDKA-9) have retention times 5.1, 7.0, 8.7 and 9.9 min, respectively. The chromatograms of these compounds are shown in Fig. 1.

Thin-Layer Chromatography (TLC)——Thin-layers (500 μm) of silicagel GF_{254} were developed in a solvent system composed of chloroform–acetone–acetic acid (16:3:1).

Separative Determination Method——As shown in Chart 1, oral administration of DKA-9 in human gives seven metabolites and four of which, DKA-9G, DKA-24G, DKA-24OG and DKA-24S, are in conjugated forms. And it was

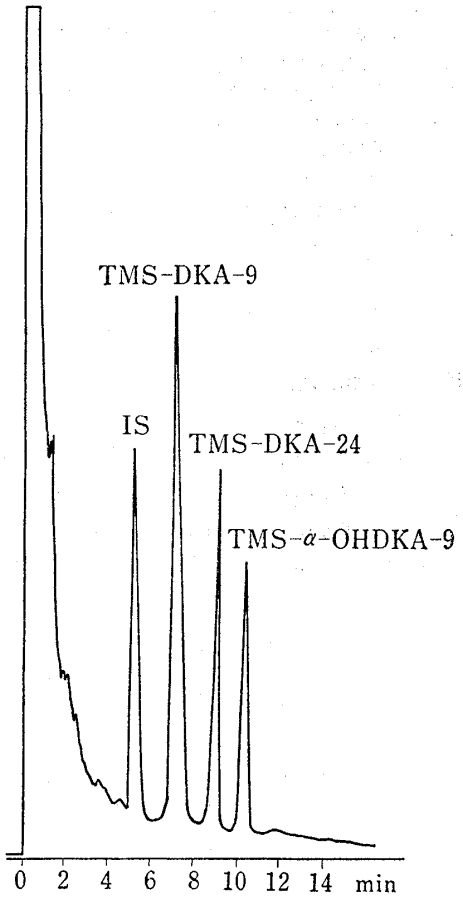

Fig. 1. Gaschromatograms of IS, TMS-DKA-9, TMS-DKA-24 and TMS-α-OHDKA-9

found that the amount of α-OHDKA-9 excreted in urine was very small and negligible.[5] So if it was possible to hydrolize each conjugate differentlly by changing the hydrolysis conditions, the amount of DKA-9 and its metabolites excreted in urine could be determined separately. Accordingly, if following conditions (1—4) are examined to be adequate, the amount of DKA-9 and its metabolites could be determined separately by treating the urine under several conditions (1′—4′) and measuring the amounts of DKA-9 and DKA-24 generated.

1. When the urine is adjusted pH 3 and extracted with benzene, the conjugates in urine are not decomposed.
2. When the urine is adjusted around pH 1 and extracted with ethylacetate, DKA-24S in urine is completely hydrolized to DKA-24 and extracted to ethylacetate layer whereas other conjugates in urine are not hydrolized.
3. Four conjugates in urine are completely hydrolized under Lyophilized Helicase treatment.
4. When the basic urine (pH 13) is incubated at 50° for 2 hours, DKA-9G and DKA-24G are completely hydrolized and DKA-24S and DKA-24OG are quite stable.

And the conditions of urine treatment are as follows: 1′. Non hydrolysis (A_1, B_1. the amount of DKA-9 and DKA-24 determined after various treatments are designated A_n, B_n, respectively). 2′. Basic hydrolysis at 50° for 2 hours (A_2, B_2). 3′. Basic hydrolysis at 50° for 2 hours and ethylacetate extraction of acidic urine (A_3, B_3). 4′. Lyophilized Helicase treatment (A_4, B_4).

By using the values obtained under these conditions (1′—4′), the amount of DKA-9 and its metabolites in urine can be calculated by following equations: DKA-9=A_1, DKA-24=B_1, DKA-9G=A_2-A_1, A_3-A_1 and A_4-A_1, DKA-24G=B_2-B_1, DKA-24OG=B_4-B_3 and DKA-24S=B_3-B_2.

Assay Procedure——Place 0.5—3 ml of the sample urine treated with each condition (1′, 2′ and 4′) in a glass stoppered test tube. Add 0.5 or 1.0 N HCl to 4 ml, 1 ml of IS benzene soln. (10 μg/ml) and 7 ml of benzene and shake for 30 min by shaker. After centrifugation, transfer 6 ml aliquot of benzene layer to a fresh flask and evaporate to dryness *in vacuo*. Add 5 μl of silylating agent [N,O-bis(trimethylsilyl)trifluoroacetamide: BSTFA, Tokyo Kasei Kogyo Co., Ltd.] and 100 μl of CS_2 to benzene extract residue. After heating the mixture on water bath at 50° for 20 min, 5 μl aliquot of reactant is injected onto gaschromatograph. In the case of the hydrolysis condition 3′, 4 ml of acidic urine is extracted with 8 ml of ethylacetate containing IS (10 μg) and operate in the same method described above thereafter.

Calculation——The peak heights for IS, TMS–DKA-9 and TMS-DKA-24 are measured. Peak height ratios are obtained by dividing the peak heights of TMS–DKA-9 and TMS–DKA-24 by the peak height of IS. Standard curves from known concentrations of DKA-9 and DKA-24 in urine are prepared by plotting peak height ratios *versus* free acid concentrations (μg/ml). Values for unknown concentration of DKA-9 and DKA-24 in urine specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curves.

Measurement of Radioactivity——The radioactivity was measured in a liquid scintilation counter (packard LS 2650). Quenching was corrected by the channel ratio method.

Results and Discussion

Determination of DKA-9 and DKA-24

In order to determine the amount of free DKA-9 and DKA-24 in urine accurately, it is necessary to extract them quantitatively and not to generate extra DKA-9 and DKA-24 from conjugates during extraction.

1. Extraction of ^{14}C-DKA-9 and ^{14}C-DKA-24——The extraction ratios at various pH and the time required for equilibration of DKA-9 and DKA-24 between urine and benzene

Table I. pH Profiles of Drug Extractability of DKA-9 and DKA-24
(concn.: 20 μg/ml, shaking time: 30 min, benzene)

pH	Extraction ratio	
	DKA-9	DKA-24
1.0	0.989	0.868
3.0	0.997	0.872
7.0	0.780	0.120
8.0	0.275	0.038
9.0	0.021	0.018
13.0	0.001	0.001

(1:2) were calculated by measuring the radioactivities distributed both layers and the results were shown in Table I. It was found that DKA-9 and DKA-24 were extracted quantitatively from urine adjusted pH 3 and shaking for 20 min was required for the attainment of equilibration.

2. Stability of Conjugates during Extraction——The stability of each conjugate was examined by measuring the amount of DKA-9 and DKA-24 liberated from conjugates when the urine solution containing 15—100 μg of each conjugate was extracted with benzene at pH 3. As shown in Table II, the deconjugation ratios of DKA-24OG and DKA-24S were

Table II. Deconjugation Ratios of Conjugates during Benzene Extract

	0—5°	18—22°
DKA-9G (50 μg[a])	0.8%	2.5%
DKA-24G (15 μg[b])	0.5	2.0
DKA-24S (100 μg[b])	—	<0.2
DKA-24OG (100 μg[b])	—	<0.2

a) Drug amount equiv. to DKA-9 in urine.
b) Drug amount equiv. to DKA-24 in urine.

found to be very small and negligible whereas those of DKA-9G and DKA-24G were considerably large and might cause the overestimation of free DKA-9 and DKA-24. So the extraction experiments of DKA-9G and DKA-24G at 0—5° were carried out and the deconjugation ratio decreased to below 1%. From these results, it was concluded that, by extracting the sample urine immediately after the sampling at 0—5°, the overestimation of free DKA-9 and DKA-24 could be made minimum.

3. Standard Curve——DKA-9 and DKA-24 urine solutions containing 2.5, 5.0, 10.0, 15.0 and 20.0 μg/ml respectively were analyzed with GLC and peak height ratios were calculated. The standard curves of both compounds were obtained as shown in Fig. 2 and there is a linearity between the peak height ratios and the amount of drugs in both cases.

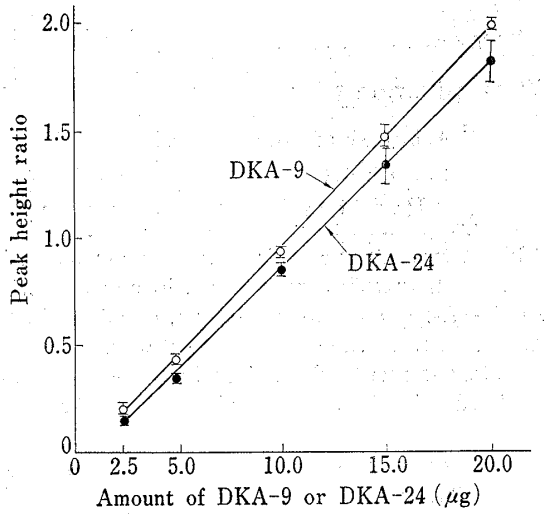

Fig. 2. Standard Calibration Curves for DKA-9 and DKA-24 in Urine

Each point represents the mean of 3–5 determinations and vertical bars indicate standard deviations.

Determination of DKA-9G and DKA-24G

Generally, it was recognized[7] that acyl glucuronides were unstable in alkaline medium and hydrolized to aglycone. So the amount of DKA-9G and DKA-24G in urine could be measured by subtracting the amounts of free DKA-9 and DKA-24 from total amounts of DKA-9 and DKA-24 after hydrolysis, if both DKA-9G and DKA-24G were hydrolized quantitatively and both DKA-24OG and DKA-24S were stable during hydrolysis (pH 13, 50°, 2 hours).

1. Alkaline Hydrolysis of DKA-9G and DKA-24G——Small amount of DKA-9G and DKA-24G sample were dissolved in 5 ml of urine and the urine soln. was alkalized with

7) "Fundamentals of Drug Metabolism and Drug Disposition," ed. by B.N. Ladu, H.G. Mandel, and E.L. Way, the Wilkins Company, Baltimore, 1972.

1 ml of 1.0 N NaOH soln. and was heated at 50°. The amounts of DKA-9 and DKA-24 generated were measured at given intervals after extraction of acidified soln. with benzene. As shown in Table III, the amounts of DKA-9 and DKA-24 generated became unchanged after 1 hour and the hydrolysis of DKA-9G and DKA-24G might be completed within 1 hour. So the time of alkaline hydrolysis was decided as 2 hours.

Table III. Generation of DKA-9 and DKA-24 after Heating DKA-9G and DKA-24G Urine Solution (pH 13, 50°)

Time (hr)	0.5	1.0	2.0	3.0
DKA-9 (μg/ml)	56.3	58.1	57.9	58.3
DKA-24 (μg/ml)	9.3	10.7	10.8	10.4

2. **Stability of DKA-24S and DKA-24OG during Alkaline Hydrolysis**——2 ml of urine soln. containing 100 μg equiv. of DKA-24/ml of the authentic DKA-24S and DKA-24OG were adjusted to about pH 13 by adding 1 ml of 0.5 N NaOH soln. and heated at 50° for 2 hours. The amounts of DKA-24 generated were measured and found to be negligible, 0.6% and 0.2% respectively. And no appreciable decomposition of DKA-9 and DKA-24 was observed in urine during alkaline hydrolysis.

Determination of DKA-24S

For the determination of DKA-24S, it must be hydrolized[8] quantitatively to DKA-24 in acidic medium during extraction with ethylacetate.

1. **Hydrolysis of DKA-24S**——In previous report,[4] it was found that DKA-24S was chemically stable and was hydrolized quantitatively with arylsulfatase (Sigma, Type-2).

The effect of shaking time on extraction ratio (as DKA-24) was studied by measuring the radioactivities of organic phase after shaking 0.25 N HCl soln. containing 1 μg/19000 dpm/ml of ^{14}C–DKA-24S with an equal volume of ethylacetate for 10, 20, 30 and 45 min. As shown in Table IV, the extraction ratios increased as the shaking time increased and at 45 min when the ratio reached approximately 1.0. The components extracted in ethylacetate layer were also investigated by TLC. TLC radio chromatograms of ethylacetate layer were shown in Fig. 3. Though there were two peaks corresponding to authentic DKA-24 and DKA-24S on TLC radio chromatograms of 10, 20 and 30 min extracts,

Table IV. Influence of Shaking Time on Extraction Ratio of DKA-24S ($n=4$)

Shaking time (min)	10	20	30	45
Extraction ratio (mean±S.D.)	0.901±0.004	0.924±0.005	0.964±0.010	1.036±0.005

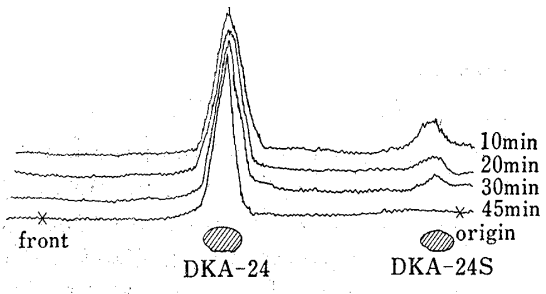

Fig. 3. TLC Radiochromatograms of Ethylacetate Layers after Extracting ^{14}C–DKA-24S Acidic Solution with Ethylacetate for Various Shaking Times

8) It was found that DKA-24S was hydrolized to DKA-24 while extracting with ethylacetate and extracted almost quantitatively as DKA-24 in preliminary experiment.[4]

only one peak corresponding to authentic DKA-24 could be recognized in 45 min extract. And the generating ratio of DKA-24 was found to be over 99% by measuring the radioactivities[9] of DKA-24 and DKA-24S on TLC.

In order to study the effect of DKA-24S concentration on extraction ratio (hydrolysis), 2 ml of urine containing 1, 50 and 100 μg of ^{14}C-DKA-24S (19000 dpm) were acidified with 2 ml of 0.5 N HCl soln. and those acidic urine samples were extracted with 8 ml of ethylacetate for 45 min. The extraction ratios were quantitative for all concentrations studied.

2. **Stability of DKA-24OG**——In this method, there was a possibility overestimating the amount of DKA-24S unless DKA-24OG was stable during operation. The stability of DKA-24OG was studied by measuring the amount of DKA-24 generated through alkaline hydrolysis and ethylacetate extraction. As a result the generating ratios were found to be very small, 0.05—0.2% which was negligible for the determination of DKA-24S.

3. **Standard Curve of DKA-24S**——DKA-24 urine solutions containing 5.0, 10.0, 15.0 and 20 μg/ml were analyzed with GLC after alkaline hydrolization and ethylacetate extraction. The standard curve obtained showed a good linearity.

Determination of DKA-24OG

In order to determine the amount of DKA-24OG correctly, DKA-24G, DKA-24OG and DKA-24S had to be hydrolized quantitatively to DKA-24 when they were incubated with Lyophilized Helicase at 37° for 24 hours in pH 5.5.

1. **Enzymatic Hydrolysis of DKA-24G, DKA-24OG and DKA-24S**——DKA-24G, DKA-24OG and DKA-24S urine solutions containing 10.0 μg/ml, respectively, were incubated with Lyophilized Helicase in the same condition and the amount of DKA-24 liberated were measured with GLC after extraction of acidified solution with benzene. As shown in Table V, it was found that DKA-24G, DKA-24OG and DKA-24S were hydrolized quantitatively.

Table V. DKA-24 Amount Liberated from DKA-24G, DKA-24OG and DKA-24S after Incubation with Lyophilized Helicase at 37°, pH 5.5 for 24 hr (n=4, urine: 1 ml, Helicase: 10 mg which contains 5600 units of β-glucuronidase and 84060 units of sulfatase)

	Added (μg)	DKA-24 (μg±S.D.)	% hydrolysis (±S.D.)
DKA-24G	10.0	10.3±0.4	102.8±4.2
DKA-24OG	10.0	9.8±0.4	98.1±3.8
DKA-24S	10.0	10.2±0.2	102.0±2.0

2. **Standard Curve**——The standard curve obtained after enzymatic incubation showed a good linearity.

Recovery Experiments and Stability of DKA-9G and DKA-24G

It was found, so far, that DKA-9 and its metabolites (DKA-24, DKA-24S, DKA-24OG, DKA-24G and DKA-9G) in urine could be determined separately by deconjugation of these conjugates. The recovery experiments were carried out by analyzing the urine containing known concn. of DKA-9 and its metabolites. The results were shown in Table VI and the recoveries of all substances were nearly 100%. Concerning the acylglucuronide, it was said that acylglucuronides were instable in acidic and alkaline medium.[7] In this determination method, as described before, there were possibilities to overestimate the amount of free DKA-9 and DKA-24 because of instability of their glucuronides. But it was found that the total amount of free drug and its acylglucuronide could be determined with considerable

9) The radioactivities were extracted by adding 1 ml of distilled water into the silicagel scratched off TLC plate.

accuracy (A_2 or B_2 value) and by using these values the pharmacokinetical analysis might be possible.

TABLE VI. Recovery of DKA-9 and Its Metabolites in Human Urine according to GLC Separative Determination Method Established ($n=4$)

	Added (μg/ml)	Found (μg/ml±S.D.)
DKA-9	5.0	5.08±0.32
DKA-24	5.0	5.10±0.17
DKA-24S	20.0	19.71±1.18
DKA-24OG	10.0	9.50±0.78
DKA-9G[a)]	50.0	48.28±1.52
DKA-24G[a)]	10.0	9.68±2.16

a) Recovery experiments were carried out according to the method of alkaline hydrolysis.

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Seasonal Variation in Urinary Excretion of Aminopyrine and Its Metabolites in Man[1)]

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The data of urinary excretion of aminopyrine and its metabolites were collected for two years using eight of male healthy volunteers. In conclusion, individual differences among subjects were remarkable. Besides, seasonal variations were demonstrated statistically.

Keywords——aminopyrine and its metabolites; human urinary excretion; individual difference; seasonal variation; data collected for 2 years

Since 1974, we have examined the fate of aminopyrine (AM) in man by means of gas chromatography (GC) for measuring concentration of AM and its metabolites in cooperation with male healthy volunteers in our laboratory. The previous papers indicated the individual variability for urinary and plasma levels of AM and its metabolites.[3,4)]

The present study provides the additional supporting evidence for inter- and intraindividual variations in AM metabolism and preliminary observations relative to a genetic factor in acetylation and/or environmental factor in oxidative metabolism. The data in the present report are very valuable from the standpoint of clinical pharmacy and pharmacology in order to establish a proper dosing schedule for a patient.

1) This work was partly presented at 8th Symposium of Drug Metabolism and Action, Hiroshima, November 1976.

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