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Metabolism and Excretion of Aminopyrine in Man[1,2]

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The metabolism and urinary excretion of aminopyrine in man were examined after an oral administration of drug solution. The metabolites were identified and measured quantitatively by gas chromatography and gas chromatography-mass spectrometry. For the microanalysis of metabolite, mass fragmentography was applied.

It was clarified that a new metabolite, 4-formylaminoantipyrine, was detected in all cases and it must be noticed as an important metabolite. It was also meaningful that the individual difference among subjects in their urinary excretion behavior of aminopyrine was remarkable beyond our expectations.

Aminopyrine is used frequently as an analgesic-antipyretic drug even now in Japan, and it is especially noticeable that the combination of aminopyrine with barbiturate has been recommended in order to obtain a synergetic effect.

Since the authors have been interested in this combination from a pharmaceutical point of view, the crystalline structure of the molecular complex formed between aminopyrine and barbiturate,[4,5] and the difference of dissolution behavior between the molecular complex and the simple mixture of aminopyrine and barbiturate[6] have been studied in our laboratory. Moreover, the absorption of these drugs in the gastrointestinal tract of animals has been also investigated.[7]

As an extention of these investigations, we intended to examine the metabolism and excretion of the drugs in man. Although the purpose of this study is to identify the latent biopharmaceutical problems after the simultaneous administration of these drugs, the case of oral administration of aminopyrine alone was examined first. It is surprising, however, that only a few studies on the behavior of aminopyrine have been made in humans.[8–11]

In our present investigation, the metabolites of aminopyrine from man's urine were first identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS), and then quantitative measurement of the main metabolites were performed periodically with some volunteers. As a result, it is reported in this paper that the individual difference of metabolic behavior among men was remarkable and a new metabolite, 4-formylaminoantipyrine, was excreted to an appreciable extent. A brief communication concerning this metabolite has already been reported.[1]

1) Preliminary communication: S. Iguchi, T. Goromaru, and A. Noda, *Chem. Pharm. Bull.* (Tokyo), **23**, 932 (1975).
2) This work was partly presented at the 6th Symposium on Drug Metabolism and Action, Pharmaceutical Society of Japan, Tokyo, November 1974. The work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education.
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4) S. Kiryu and S. Iguchi, *Yakugaku Zasshi*, **89**, 707 (1969).
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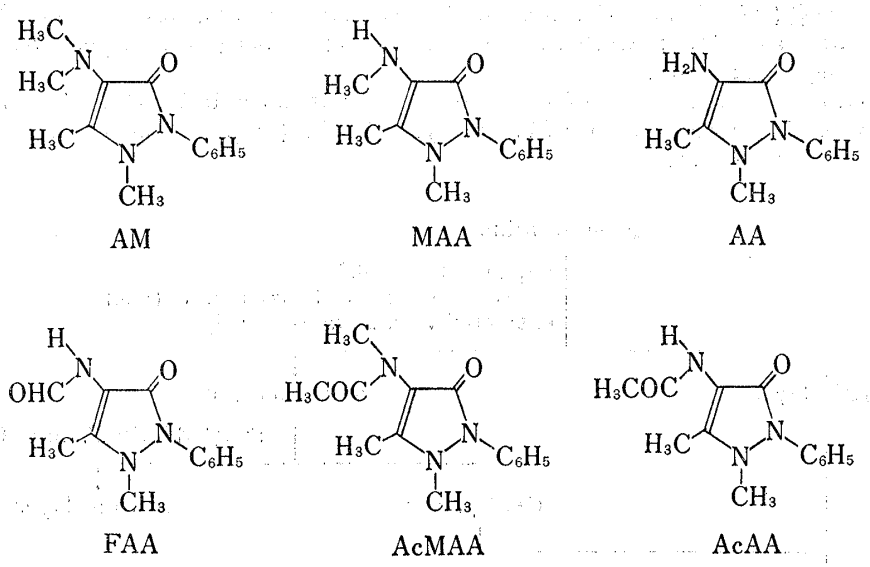

Chart 1. Metabolites of Aminopyrine in Man

Materials and Methods

Chemicals——J.P. VIII grade of aminopyrine (Daiichi Seiyaku Co.) and the reagent grade of 4-aminoantipyrine (Wako Pure Chemical Ind. Ltd.) were used. Bis-trimethylsilylacetamide was purchased from Tokyo Chemical Ind. Co. Ltd., β-glucuronidase (Marine Mollusc) from P-L Biochemicals Inc. 4-Methylaminoantipyrine, 4-acetylaminoantipyrine, 4-acetylmethylaminoantipyrine and 4-hydroxyantipyrine were prepared by the known methods.[12-15] Although 4-formylaminoantipyrine can be prepared *via* 4-nitrosoantipyrine,[16] it was synthesized from 4-aminoantipyrine as follows.

4-Formylaminoantipyrine: 4-Aminoantipyrine (20 g) was dissolved in 100 ml of 50% formic acid. The solution was stirred at room temperature for 10 hr after the addition of 35.5 g of zinc powder in small portions. The progress of the reaction was examined by TLC (ethyl acetate–methanol–ammonia, 7: 1.5: 0.5). When the disappearance of 4-aminoantipyrine was confirmed on TLC, zinc powder was filtered off and the filtrate was adjusted to pH 6.0 with sodium bicarbonate. The solution was extracted three times with chloroform. The extracts were combined, dehydrated with anhydrous sodium sulfate, and evaporated to dryness. The residue was recrystallized from methanol to give white prisms, mp 187—189°. *Anal.* Calcd. for $C_{12}H_{13}O_2N_3$: C, 62.43; H, 5.62; N, 18.34. Found: C, 62.33; H, 5.62; N, 18.18. IR ν_{max}^{KBr} cm^{-1}: 1690 and 1710 (C=O). NMR ($CDCl_3$) ppm: 2.23 (3H, s, 3-CH_3), 3.11 (3H, s, N-CH_3), 7.40 (5H, m, C_6H_5), 8.24 (1H, s, CHO) and 9.12 (1H, broad, NH). Mass Spectrum *m/e*: 231 (M^+).

Human Experiment——Healthy adult men with normal values in routine tests of clinical blood chemistry[17] participated in this study as volunteers. They had taken no other drugs at least for two weeks prior to the study and drinking alcohols was prohibited from 24 hr before the experiment to the end. Except for these restrictions, they were permitted to live as usual. They took aminopyrine in the form of solution orally in the morning, following a 12 hr fast. The ingestion of breakfast was permitted at 2 hr after drug administration. After obtaining a baseline sample, urine was collected at 0—2, 2—4, 4—6, 6—8, 8—12, 12—24, 24—36 and 36—48 hr following administration of aminopyrine. The subsequent administration was not performed within at least 2 months.

Gas Chromatography (GC)——Aminopyrine and its metabolites were assayed on a glass column (2 m×3 mm inner diameter) containing 1.5% OV-17 on Shimalite W (80—100 mesh) in Shimadzu Model GC-4BM-PF gas chromatograph equipped with a hydrogen flame ionization detector. Temperature of the injection port and the detector was 250°, while the column oven was 225°. Nitrogen was used as the carrier at flow rate of 20 ml/min. Flow rate of hydrogen and compressed air was adjusted to maximum response as possible.

Gas Chromatography-Mass Spectrometry (GC-MS)——JEOL Model JMS-D100 mass spectrometer was used with a JGC-20K gas chromatograph. A glass column (1 m×2 mm inner diameter) containing 4%

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17) BUN, serum alkaline phosphatase, serum total bilirubin, SGPT, SGOT, LDH, fasting blood sugar, and serum creatinine.

OV-17 on Gas Chrom Q (100—120 mesh) was used. The column oven temperature was 220°. Mass spectrometer conditions were as follows: Accelerating voltage, 3 kV; ionizing current, 300 μA; ionizing energy, 25 eV; separator temperature, 240°.

Assay Procedure——The procedure of sample preparation is shown in Chart 2. To 20 ml of urine was added an aqueous solution of 0.5 mg of benzydamine hydrochloride (supplied from Yoshitomi Pharmaceutical Ind. Ltd.) as an internal standard.

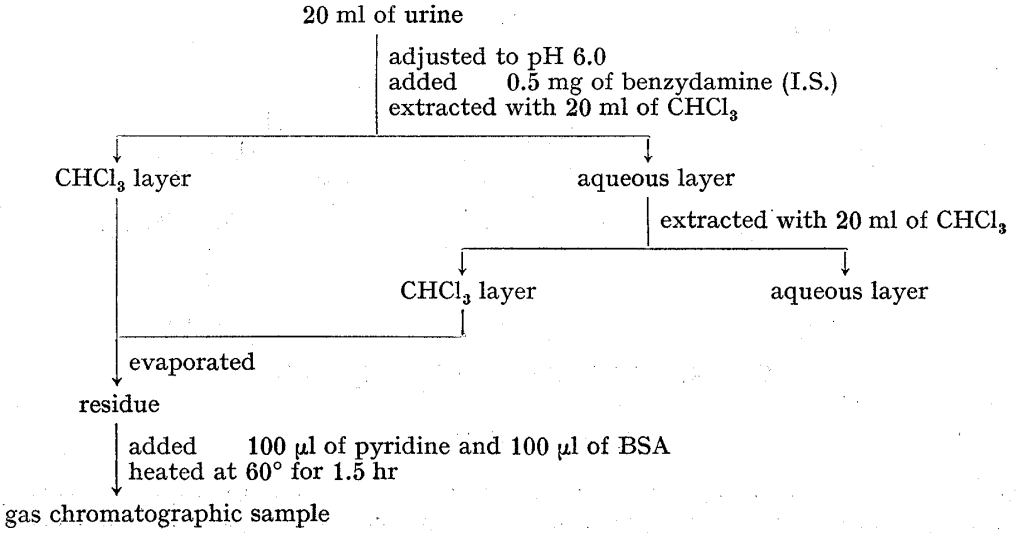

Chart 2. Preparation of Sample for Gas Chromatographic Determination of AM and Its Metabolites from Man's Urine

The solution was adjusted to pH 6.0 with hydrochloric acid or sodium hydroxide and extracted twice with 20 ml of chloroform. The combined extracts were dehydrated with anhydrous sodium sulfate, and evaporated to dryness. For the detection of conjugated metabolites from the aqueous layer, the hydrolysis was performed under two of the following conditions: (1) 20 ml of the solution containing 2 ml of conc. HCl was heated for 1 hr on a boiling bath. The chilled solution was shaken once with 40 ml of chloroform, (2) 20 ml of the solution was incubated at 37° for 12 hr with 10000 units of β-glucuronidase in acetate buffer (pH 4.7). After incubation, the fluid was extracted twice with 20 ml of chloroform. The each extract was dehydrated with anhydrous sodium sulfate, and evaporated to dryness. Just before GC, each extract was trimethylsilylated as follows; to each extract were added 100 μl of pyridine and 100 μl of bis-trimethylsilylacetamide. The solution was heated at 60° for 2 hr. Two μl of the solution was injected into the gas chromatograph.

Overall recovery from the initial extraction to the final analysis by GC was examined with the standard urine sample containing fixed amounts of aminopyrine and/or its metabolites (authentic samples). As a result, the overall recovery ratio of aminopyrine, 4-methylaminoantipyrine, 4-aminoantipyrine, 4-acetylaminoantipyrine, 4-(N-acetyl-N-methyl)aminoantipyrine and 4-formylaminoantipyrine were 95 (±2), 91 (±5), 79 (±5), 66 (±3), 97 (±4) and 47 (±3)%, respectively, under the condition of pH 6.0 urine sample.

The standard deviations were written in parentheses. The extraction ratio of 4-formylaminoantipyrine was the lowest, but it was ascertained that it could be measured at the concentration as low as 30 μg/ml (urine) by this procedure.

Results and Discussion

From the results of preliminary observation on the metabolites in the subjects' urine following single oral administration of aminopyrine (300 mg/body wt[18]), it was found that the main metabolites were 4-methylaminoantipyrine (MAA), 4-aminoantipyrine (AA), 4-acetylaminoantipyrine (AcAA), and besides a small amount of unchanged aminopyrine (AM). Furthermore, two of new peaks which could be the unknown metabolites I and II appeared on the gas chromatogram of a few subjects. 4-Hydroxyantipyrine (HA) could be detected from the urine of subjects after acid-hydrolysis or enzymatic treatment, but the levels were generally

18) The maximum dose (single: oral) of AM in the Pharmacopoeia of Japan.

Table I. Urinary Excretion of Metabolites following the Oral Administration of AM (300 mg) in the Preliminary Experiment

Subject	Age	Body weight (kg)	Metabolites			
			AM	MAA+AA[a)]	AcAA	AcMAA
S.I.	54	52	++	++	++	+
S.K.	41	58	+	++	++	−
T.G.	32	58	+	++	++	−
O.T.	32	60	+	+	++	+
F.H.	24	56	+	++	++	−
M.N.	23	63	+	++	++	−
T.O.	23	63	+	+	++	++
H.S.	23	64	+	+	++	++

−: undetected by GC +: detected by GC to an appreciable extent ++: detected markedly by GC
a) MAA and AA showed the same t_R without trimethylsilylation in this experiment.

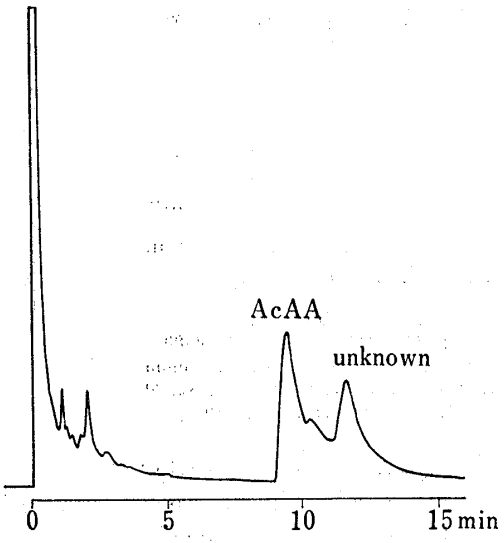

Fig. 1. Gas Chromatogram of Urine Extract (T.O., AM 300 mg)

* Unknown means metabolite I.
conditions: 1.5% OV-1 on Shimalite W (80—100 mesh), 3 mm (I.D.)×2 m, glass column, column temp: 190°, injection port temp: 230°, N_2: 20 ml/min, HFID, instrument: GC-4BM-PF

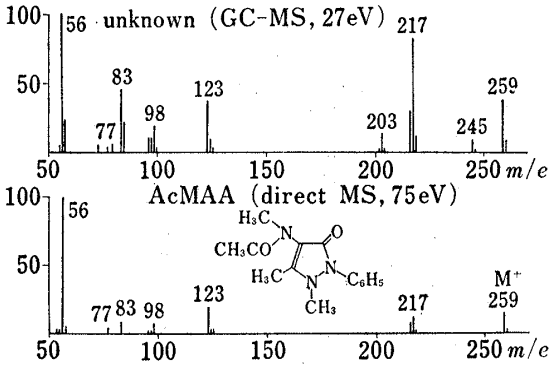

Fig. 2. Mass Spectra of Unknown and AcMAA (Authentic sample)

quite low. Table I shows the metabolites clearly found in the urine of each subject in this experiment qualitatively.

Metabolite I was excreted in the urine of two subjects (T. O., H. S.) during 36 hr following administration of AM (300 mg/body wt) as shown in Fig. 1. According to its mass spectrum by GC–MS shown in Fig. 2, the molecular ion peak appeared at *m/e* 259 and the other peaks were observed at *m/e* 216, 217, 245, and 203. The latter two peaks were also observed with AcAA. Thus, metabolite I is probably an acetyl compound whose structure is similar to AcAA. 4-(N-Acetyl-N-methyl)aminoantipyrine (AcMAA) with a molecular weight of 259 was synthesized as a possible metabolite, since this compound has already been detected in man's urine by Večerková using paper chromatography.[14)] There is exact coincidence of the retention time of metabolite I and AcMAA on the GC and mass spectrum. Although AcMAA can be analysed by GC, mass fragmentography which is more applicable for the microanalysis of metabolites was applied to AcMAA and the other coexisting metabolites of AM. When three fragment ion peaks at *m/e* 203, 217, and 231 were utilized for analysis, AM (M^+=231), MAA (M^+=217), AA (M^+=203), AcAA (M^+-42=203) and AcMAA (M^+-42=217) could be analysed independently as shown in Fig. 3.

Another new peak, possibly metabolite II, was first noticed in the gas chromatogram of the urine of subjects (N.T., S.I.) as shown in Fig. 4. This unknown peak which appeared just after the peak of AcAA in the early excreted urine following administration of AM (100 mg/

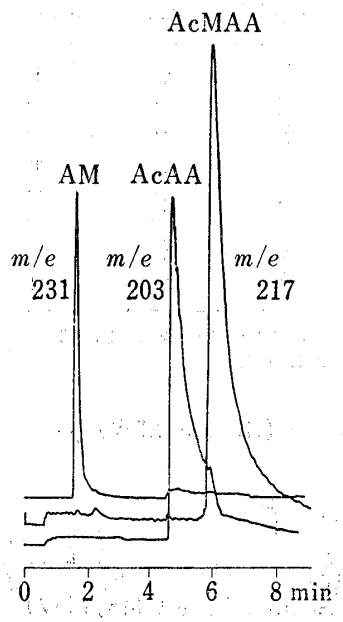

Fig. 3. Mass Fragmentogram of Urine Extract (T.O. AM 300 mg)

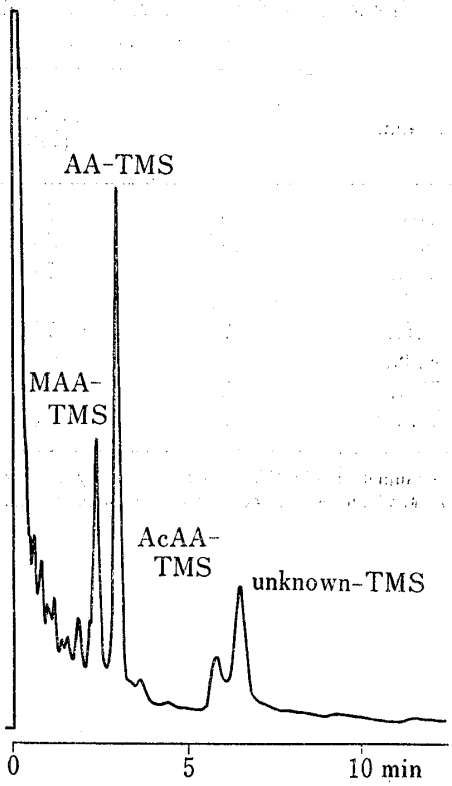

Fig. 4. Gas Chromatogram of Urine Extract (N.T. AM 100 mg)

* Unknown means metabolite II.
conditions: 1.5% OV-17 on Shimalite W (80—100 mesh), 3 mm×2 m, glass column, column temp: 225°, injection port temp: 250°, N_2: 20 ml/min, HFID, instrument: GC-4BM-PF

body wt) suggested the presence of a new metabolite. Comparing with an authentic sample by GC-MS, metabolite II was proven to be 4-formylaminoantipyrine[1] (Fig. 5). Recently, with high-pressure liquid chromatography (Dupont 830), metabolite II has been successfully identified as FAA. Finally, it was possible to detect FAA and AcAA separately by a more sensitive microdetermination using trimethylsilylation and mass fragmentography at *m/e* 303 (FAA-TMS, M^+) and 317 (AcAA-TMS, M^+) (Fig. 6).

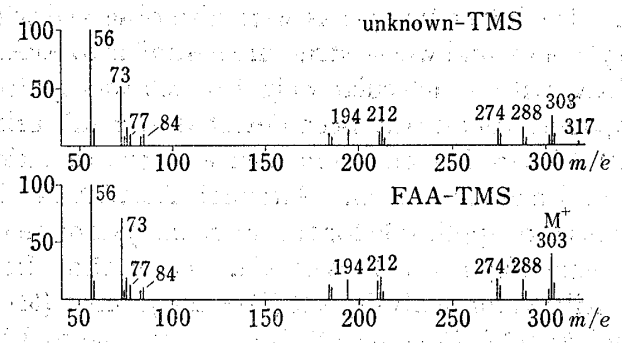

Fig. 5. Mass Spectra of Unknown-TMS and FAA-TMS (Authentic sample)

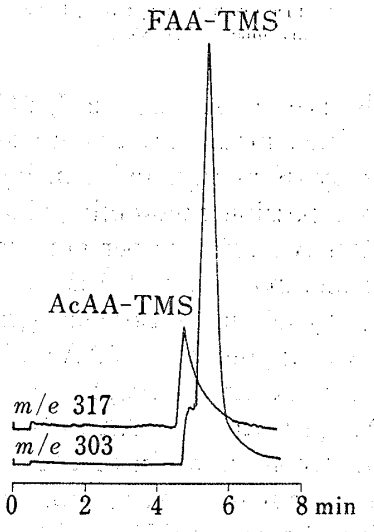

Fig. 6. Mass Fragmentogram of Urine Extract (N.T., AM 100 mg)

As an extention of general examination, the quantitative determination by GC and GC–MS was performed on six compounds, AM, MAA, AA, AcAA, AcMAA, and FAA, during 48 hr after the oral administration of AM(100 mg/body wt[19]). It is general that the excretion of the main metabolites of AM finishes almost within 48 hr after administration except a few cases. HA and rubazonic acid were excluded in this experiment because of their negligible amounts.

The results of experiments performed in summer (first experiment) and autumn (second experiment) are shown in Table II and III, which show the excreted amounts of metabolites in each of the subject's urine. The amounts of metabolites were calculated after correcting each value with the overall recovery ratio obtained from the repeated experiments with each metabolite (authentic sample) in the standard urine as mentioned above. Furthermore, the values obtained were all converted into AM quantity and listed in the table. These values represent directly the percentage to the dose of AM.

Table II. Urinary Excretion of Metabolites during 48 hr following the Oral Administration of AM (100 mg) in Summer, 1974

Subject	Age	Excreted amount (mg)					Total (mg, %)
		AM	MAA	AA	AcAA	FAA	
S.I.	54	0.8	1.2	1.0	38.1	6.7	47.8
T.G.	32	0.1	2.6	8.3	31.1	0.0	42.1
M.N.	24	0.0	6.6	1.2	32.0	0.0	39.8
K.M.	24	0.0	1.7	1.6	36.2	0.0	39.5
N.T.	23	0.3	3.0	5.5	9.2	3.2	21.2
O.T.	32	0.2	1.3	0.7	12.7	0.0	14.9

* All values were determined by calculation from the converted value into AM.

Table III. Urinary Excretion of Metabolites during 48 hr following the Oral Administration of AM (100 mg) in Autumn, 1974

Subject	Age	Excreted amount (mg)					Total (mg, %)
		AM	MAA	AA	AcAA	FAA	
K.M.	24	0.0	0.9	0.7	15.2	24.4	41.2
Y.K.	26	0.9	1.1	0.5	32.5	5.9	40.9
M.N.	24	0.1	4.8	4.2	28.6	2.4	40.1
T.G.	32	0.4	1.0	7.7	5.8	18.0	32.9
O.T.	32	0.2	1.8	1.7	26.5	0.6	30.8
S.I.	54	0.2	1.4	1.1	20.9	2.4	26.0
N.T.	23	0.1	1.8	7.9	11.0	3.9	24.7

All values were determined by calculation from the converted value into AM.

It is an interesting observation that FAA could be detected in all of subjects' urine in the second experiment, while only in two cases it was detected in the first time. The reason of FAA formation has not been clarified, but it is undoubted that FAA is one of the main metabolites from the fact that a few subjects excreted it in amounts as much as around 20% to the dose.

On the other hand, AcMAA which was detected in a few cases (T.O., H.S.[20]) on the preliminary experiment as shown in Table I could not be detected in any subject's urine this time.

19) The usual dose (single: oral) in the Pharmacopoeia of Japan.
20) They could not participate in the first and the second experiments because of their graduation from Kyushu University.

Unchanged AM was found very little in general, suggesting that AM was easy to be metabolized. The excreted amount of MAA and AA were generally small, but the amount of them were a little larger than that of unchanged AM. On the contrary, a few subjects excreted considerable amount of MAA and/or AA exceptionally.

It is natural that AcAA which is known as a final metabolite of AM was a predominant metabolite generally through all subjects. It is noticeable in some cases that the subjects (T.G., K.M.) excreted FAA much more than AcAA.

As is stated above, such a remarkable difference exists among only some subjects in their behavior of metabolism and urinary excretion following oral administration of AM. The extent and rate of drug absorption may be different from each other. But it is inconceivable that the absorption process played an important role in bringing about such the remarkable difference among subjects in their urinary excretion behavior of metabolites, since AM has been regarded as a well absorbable drug and the form of solution was used as a dosage form throughout a series of this experiment. Thus, the difference in metabolism and excretion of AM might be arising from the individual difference in activity of drug metabolizing enzyme.

As an approach to this problem, the extent of acetylation of AA which is an intermediate metabolite of AM was examined. The excreted amount of AcAA in urine was measured by GC as one of the metabolites following the oral administration of AA (44 mg/body weight[21]) in the form of solution.

High ranking of three subjects (K.M., S.I., Y.K.) in Table IV showed a considerable amount of total excretion (AA+AcAA) more than 50% to the dose, and a large amount of AcAA excretion more than 90% to the amount of total excretion.

Table IV. Urinary Excretion of AA and Its Metabolites during 48 hr following the Oral Administration of AA (44 mg)

Subject	Excreted amount (mg)			Recovery (%)	AcAA/Total (%)
	AA	AcAA	Total (AA+AcAA)		
K.M.	2.5	22.5	25.0	56.8	91.5
S.I.	2.1	21.3	23.4	53.2	92.4
Y.K.	1.8	21.3	23.1	52.5	92.0
T.G.	16.4	6.0	22.4	50.8	30.7
N.T.	11.6	3.0	14.6	33.2	24.0
O.T.	1.8	8.6	10.4	23.6	85.0

* All values were determined by calculation from the converted value into AA.

On the contrary, the other subjects (T.G., O.T., N.T.) in Table IV excreted a small amount of AcAA after the administration of AA as the same manner as in AM. But the metabolic behavior appeared to be quite different each other. In the case of subject T.G., the excreted amount of AA was much larger than that of AcAA, while the amount of total excretion was not less. Therefore, the subject seems to be a slow acetylator. In the case of subject N.T., the excretion behavior is similar to T.G. except for the less amount of total excretion. Subject O.T. excreted not only AcAA but also AA in a small amount, while the amount of total excretion was the lowest among all subjects. Thus, the decomposition of AA into the other types of metabolites due to a special activity of the oxidative enzyme might be probable in this case.

From the results of AA administration, it seems to be explicable to some extent why the excreted amounts of AA and AcAA are variable in each subject in the case of AA similar to AM. Unfortunately, the subject M.N. who excreted a large amount of MAA could not participate in

21) A half equivalent dose of AM 100 mg.

the experiment of AA administration. Thus, it is impossible to discuss his characteristics in AM metabolism. Furthermore, it can not be discussed from our data this time what the formation and excretion of FAA mean.

In this paper, it is the most interesting that a new metabolite FAA was detected first. It is also valuable that the real state of the individual difference in metabolism and excretion of AM was clarified to some extent. The difference might be arising chiefly from the individual difference in activity of drug metabolizing enzyme.

Therefore, it is very important to examine the activity of oxidizing enzyme as well as conjugation by an appropriate method. The problem of biliary excretion is also remaining to be solved. Furthermore, the most important problem is to examine the extent of variation among men not only in the metabolism and excretion but also in the action of the drug. In order to solve this problem, the active form of the drug and its concentration in the blood should be examined. This aspect of the problem is now under investigation.

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