CHARACTERISTIC BIOTRANSFORMATION OF AMINOPYRINE IN RAT WHEN ADMINISTERED WITH BARBITAL

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After the administration of aminopyrine with or without barbital to rats, aminopyrine and its main metabolites were detected in plasma and the brain by means of gas chromatography–mass spectrometry. It was clarified that a marked increase of 4-monomethylaminopyrine was observed in the case of coadministration of aminopyrine and barbital, while the plasma level of aminopyrine decreased significantly compared with single administration.

Keywords — aminopyrine; 4-monomethylaminopyrine; barbital; coadministration; single administration; intravenous administration; oral administration; plasma concentration; brain concentration; GC–MS analyses

The combined administration of aminopyrine (AM) and barbital (BA) to patients had long been used frequently as an excellent analgesic in Japan. Since the authors have been interested in this combination from a pharmaceutical point of view, the crystalline structure of the molecular complex formed between AM and BA and the difference of dissolution behavior between the molecular complex and the simple mixture of AM and BA have been studied in our laboratory. Moreover, the absorption of these drugs in the gastro-intestinal tract of animals has also been investigated. With reference to the synergetic effect, Minami and Ohara studied on this combination from a pharmacological point of view. According to their reports, the coadministration of AM and BA improved the antipyretic and analgesic efficacies of AM and conversely weakened the acute toxicity of AM and the anesthetic effect of BA. In the previous paper, the metabolic behavior of AM in man was studied in detail after the oral administration of AM alone as the initial approach to the biopharmaceutical evaluation of the combination of these drugs.

But, an important problem about the mutual effect of the drugs on their metabolic behavior has remained unsolved till today. In the present paper, the plasma and the brain concentration of the intact drug and the main metabolites (4-monomethylaminopyrine, 4-aminopyrine, 4-formylaminopyrine and 4-acetylaminopyrine) were determined by gas chromatography–mass spectrometry (GC–MS) after the AM administration to rats with or without BA and the behavior of BA was also discussed.

MATERIALS AND METHODS

Chemicals — Aminopyrine (AM), 4-monomethylaminopyrine (MAA), 4-aminopyrine (AA), 4-formylaminopyrine (FAA), 4-acetylaminopyrine (AcAA), N,N-dibutylamides (BDA) and 3-aminopyrine (d3-AM) used in the experiment were obtained as described in the previous paper.

The Synthesis of Nitrogen-15 Labeled Barbital (15N-BA) — 15N-BA was prepared from nitrogen-15-urea (purchased from BOC Ltd. 99% 15N) and diethylmalonic acid diethylester

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Effect of Barbital on Aminopyrine

(purchased from Tokyo Chemical Ind. Co. Ltd.) in the presence of sodium ethylate by Fischer's method.

Animal Experiment — Male Wistar rats weighing 200—230g were used in the experiments. The rats which were kept with a commercial diet (Oriental Yeast Co., Ltd.) were fasted for 12 hr prior to experiments. Water was given ad libitum.

i) Oral Administration: In the case of single administration, 100 mg/kg of $d_3$-AM or 44 mg/kg of sodium barbital was orally administered to rats in an aqueous solution.

In the case of coadministration, the mixture of 100 mg/kg of $d_3$-AM and 44 mg/kg of sodium barbital was orally administered.

ii) Intravenous Administration: 1 ml of saline solution containing 35 mg/kg of $d_3$-AM or 15.4 mg/kg of sodium barbital was injected via tail vein within 1 min.

In the case of coadministration, a mixture of 35 mg/kg of $d_3$-AM and 15.4 mg/kg of sodium barbital in saline solution was injected.

Assay Procedure — i) Preparation of Plasma Sample: 5 ml of blood sample collected from rat carotid was immediately centrifuged at 3000 rpm. The supernatant was used for GC-MS following the sample preparation. A portion of plasma (2 ml) was gently shaken in a stoppered centrifuge tube with hand for 10 sec after the addition of 5 ml of phosphate buffer (pH 8.0) containing $^{15}$N-BA (60 µg), AM (10 µg), FAA (10 µg), AcAA (10 µg), MAA (40 µg) and AA (50 µg) as internal standards. 2 g of (NH$_4$)$_2$SO$_4$ was added to the mixture. After shaking the mixed solution was extracted twice with 20 ml of CHCl$_3$. The combined extract were used for GC-MS (Chart 1).

wet whole brain

- add 2 ml of phosphate buffer (pH 8.0) containing $^{15}$N-BA, AM and its metabolites as internal standards ($^{15}$N-BA; 30 µg, AM, FAA, AcAA; 5 µg, MAA; 20 µg, AA; 25 µg)
- homogenize the mixture
- add 4 ml of 10% trichloroacetic acid
- centrifuge at 3000 rpm for 10 min
- supernatant
- adjust to pH 8.0
- extract with 20 ml of CHCl$_3$ for 10 min twice
- CHCl$_3$ layer
- evaporate to dryness
- residue
- add 50 µl of CHCl$_3$
- GC-MS sample for determination of BA, AM and MAA
- evaporate CHCl$_3$ to dryness
- add 30 µl of acetone
- GC-MS sample for determination of AA
- evaporate acetone to dryness
- add 25 µl of pyridine and 25 µl of N,O-bis-(trimethylsilyl)acetamide (BSA)
- heat at 80° for 3 hr
- GC-MS sample for determination of AcAA and FAA

CHART 1. Sample Preparation for GC-MS Determination of Barbital, Aminopyrine and Its Metabolites in Plasma

2 ml of plasma

- add 5 ml of phosphate buffer (pH 8.0) containing $^{15}$N-BA, AM and its metabolites as internal standards ($^{15}$N-BA; 60 µg, AM, FAA, AcAA; 10 µg, MAA; 40 µg, AA; 50 µg)
- 2 g of ammonium sulfate
- centrifuge at 3000 rpm for 10 min
- supernatant
- extract with 20 ml of CHCl$_3$ for 10 min twice
- CHCl$_3$ layer
- evaporate to dryness
- residue
- add 50 µl of CHCl$_3$
- GC-MS sample for determination of BA, AM and MAA
- evaporate CHCl$_3$ to dryness
- add 30 µl of acetone
- GC-MS sample for determination of AA
- evaporate acetone to dryness
- add 25 µl of pyridine and 25 µl of N,O-bis-(trimethylsilyl)acetamide (BSA)
- heat at 80° for 3 hr
- GC-MS sample for determination of AcAA and FAA

CHART 2. Sample Preparation for GC-MS Determination of Barbital, Aminopyrine and Its Metabolites in the Brain
ii) Preparation of brain sample: A wet whole brain was homogenized by a Potter-Elvehjem homogenizer after the addition of 2 ml of phosphate buffer (pH 8.0) containing $^{15}$N-BA (30 μg), AM (5 μg), FAA (5 μg), AcAA (5 μg), MAA (20 μg) and AA (25 μg) as internal standards. The homogenate was centrifuged at 3000 rpm for 10 min after the addition of 4 ml of 10% trichloroacetic acid for deproteinization. The pH of the supernatant was adjusted to 8.0 by addition of 1 M sodium hydroxide. After the extraction twice with 20 ml of CHCl$_3$, the extracts were dried over anhydrous sodium sulfate followed by evaporation to dryness (Chart 2).

iii) Gas Chromatography–Mass Spectrometry (GC-MS): The GC-MS analyses of AM, MAA, AA, FAA, AcAA and BA were indicated in Table I. In order to measure the amount of BA, the strongest peak (base ion peak) height of BA at $m/z$ 156 was compared with the base ion peak height of $^{15}$N-BA at $m/z$ 158. In the case of AM and MAA, the molecular ion peaks ($m/z$ 231 and 234 for AM and $d_3$-AM, $m/z$ 217 and 220 for MAA and $d_3$-MAA) were utilized for the analyses. As for AA detection, derivatization with acetone was performed. The acetonide formed was analyzed at $m/z$ 243 and 246. In the case of FAA and AcAA, trimethylsilylation was necessary to obtain the appropriate peaks for GC, and the molecular ion peaks ($m/z$ 303 and 306 for FAA-TMS and $d_3$-FAA-TMS, $m/z$ 317 and 320 for AcAA-TMS and $d_3$-AcAA-TMS) were utilized.

Mass spectrometer conditions: JEOL Model JMS-D 100 mass spectrometer was used with a JGC-20 K gas chromatograph. A glass column (1 m×2 mm inner diameter) containing 1.5% OV-17 on Shimalite W (80—100 mesh) was used. Accelerating voltage, 3 kV; ionizing current, 300 μA; ionizing energy, 25 eV; separator temperature, 260°.

RESULTS AND DISCUSSION

Fig. 1 and 2 show plasma and the brain concentration of AM and four kinds of metabolites (MAA, AA, FAA and AcAA) following the oral administration of AM with or without BA. In general, the distribution of AM into plasma and the brain takes place immediately. The plasma level of AM in the case of single administration reached maximum within 15 min, while in the case of coadministration about 30 min. AM was metabolized so rapidly that the two metabolites, MAA and AA, were detected even at an early stage and that the plasma level of MAA was considerably higher than that of intact AM. Especially, it is noticeable that a marked increase of MAA was observed in the case of coadministration,

<table>
<thead>
<tr>
<th>Chemical</th>
<th>MID focus</th>
<th>Column</th>
<th>Oven Temp.</th>
<th>Derivative</th>
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<tr>
<td>AM</td>
<td>$m/z$ 231 (AM)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>230°</td>
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<tr>
<td></td>
<td>$m/z$ 234 ($d_3$-AM)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
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<td>MAA</td>
<td>$m/z$ 217 (MAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
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<td></td>
<td>$m/z$ 220 ($d_3$-MAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>250°</td>
<td>TMS</td>
</tr>
<tr>
<td>AA</td>
<td>$m/z$ 243 (AA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>250°</td>
<td>TMS</td>
</tr>
<tr>
<td>FAA</td>
<td>$m/z$ 246 ($d_3$-AA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>150°</td>
<td>—</td>
</tr>
<tr>
<td>AcAA</td>
<td>$m/z$ 303 (FAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>150°</td>
<td>—</td>
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<tr>
<td></td>
<td>$m/z$ 306 ($d_3$-FAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>150°</td>
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<tr>
<td>BA</td>
<td>$m/z$ 317 (AcAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>150°</td>
<td>—</td>
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<tr>
<td></td>
<td>$m/z$ 320 ($d_3$-AcAA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
<td>150°</td>
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<tr>
<td></td>
<td>$m/z$ 156 (BA)</td>
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<tr>
<td></td>
<td>$m/z$ 158 ($^{15}$N-BA)</td>
<td>1.5% OV-17/Shimalite W (80—100 mesh) 2 mmφ×1 m</td>
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</table>
while the plasma level of AM decreased significantly.

Naito\(^9\) reported that BA enhanced the absorption of AM to provide a considerable increase of AM in plasma level. According to his report, AM was determined colorimetrically after the reaction with ferric ion in solution. By the method AM, MAA and AA can not be determined separately. Therefore, the high concentration of AM obtained by Naito might include MAA and AA.

Concerning the plasma concentration of other metabolites (FAA, AcAA and AA), no significant differences between coadministration and single administration were observed.

The metabolic behavior of AM following the intravenous administration of AM with or without BA was examined. The coadministration caused a decrease in AM level at an initial stage and an increase in MAA level as shown in Fig. 3. This tendency was almost similar to the case of oral administration. The AM level at 15 min after the coadministration with BA decreased about a half as compared with that of single administration. On the contrary, the maximum level of MAA was increased more than twice. The same tendency was observed in the brain levels of AM and its metabolites (Fig. 4).

The reason why such phenomenon occurred has not been clarified yet. Although various kinds of factors could be considered, we speculate the inductive effect of BA as the most plausible factor. BA is thought to play an important role in the

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**Fig. 1. Time Concentration Curves of Aminopyrine and Its Metabolites in Plasma after Oral Administration of 100 mg/kg of Aminopyrine (Left Figure) and after Oral Coadministration of the Same Dose of Aminopyrine and 44 mg/kg of Sodium Barbital (Right Figure) to Rats**  
Each point represents the mean ± S.E. of 7—10 experiments.

**Fig. 2. Time Concentration Curves of Aminopyrine and Its Metabolites in the Brain after Oral Administration of 100 mg/kg of Aminopyrine (Left Figure) and after Oral Coadministration of the Same Dose of Aminopyrine and 44 mg/kg of Sodium Barbital (Right Figure) to Rats**  
Each point represents the mean ± S.E. of 7—10 experiments.
demethylation of AM in an early stage, when AM is coadministered with BA. In the past studies on the inductive effects of drugs, AM has been frequently used as an indicator. Since the metabolic amount of AM was only followed by the determination of HCHO derived from N-methyl group at 4 portion of AM, regardless of separating AM, MAA and AA, we can not obtain the correct data of AM determination from the past reports. Therefore, we are now planning the detailed examination concerning the effect of BA on AM demethylation.

FIG. 3. Time Concentration Curves of Aminopyrine and Its Metabolites in Plasma after Intravenous Administration of 35 mg/kg of Aminopyrine (Left Figure) and after Intravenous Coadministration of the Same Dose of Aminopyrine and 15.4 mg/kg of Sodium Barbital (Right Figure) to Rats. Each point represents the mean ± S.E. of 4 experiments.

FIG. 4. Time Concentration Curves of Aminopyrine and Its Metabolites in the Brain after Intravenous Administration of 35 mg/kg of Aminopyrine (Left Figure) and after Intravenous Coadministration of the Same Dose of Aminopyrine and 15.4 mg/kg of Sodium Barbital (Right Figure) to Rats. Each point represents the mean ± S.E. of 4 experiments.
Effect of Barbital on Aminopyrine

On the other hand, the behavior of BA was also examined by means of mass fragmentography using the stable isotope dilution method. Fig. 5 shows the time course of the plasma and brain concentration of BA in rats after the oral administration of BA with or without AM.

A significant difference was observed between the two cases. The coadministration gave rise to a decrease of the initial plasma level of BA and afterwards a prolongation of BA plasma level as compared with the single administration. The maximum plasma level of BA was obtained at 8 hr. Otherwise, in the case of BA alone it took about 2 hr to reach the maximum level. The same tendency was also observed in the brain concentration. The behavior of BA free from the gastrointestinal absorption process was investigated by the intravenous administration. There was no significant difference between single administration and coadministration (Fig. 6).

Considering the result described above, it seems that the delay of maximum plasma level of BA in the case of oral coadministration depends on a characteristic phenomenon caused by AM, i.e., the inhibition of the gastric emptying rate to BA as pointed out by Goro et al. As shown in Fig. 5, the behavior of BA after the coadministration with AM seems to be parallel with the pharmacological observation which suggested the decrease of the anesthetic potency and the prolongation of the sedative effect of BA.

As for the pharmacological properties of AM metabolites (MAA, AA, FAA and AcAA), AA was recognized to be only one substance which shows the antipyretic effect clearly. Brodie et al. reported that the effect of AA was stronger than acetanilide, antipyrine and acetylsalicylic acid, though somewhat less than AM. But, MAA and FAA were not tested because these metabolites could not be detected at that time.

Recently, examinations using AM metabolites, including MAA and AA, were performed. By using stretching, hot plate and Haffner's methods, Iguchi et al. found that the analgesic potency of MAA was about a half less than AM and 1.5 times higher than AA, while AcAA and FAA had little or no effect. Taking the fact described above into consideration, it seems that the characteristic biotransformation of AM, especially the increase of MAA observed in this experiment, well explains the characteristics of pharmacological observation in the past, i.e., the improvement of the

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**Fig. 5. Time Concentration Curves of Barbital in Plasma (Left Figure) and in the Brain (Right Figure) after Oral Administration of 44 mg/kg of Sodium Barbital (--- ○ ---) and Oral Coadministration of the Same Dose of Barbital and 100 mg/kg of Aminopyrine (--- ● ---) to Rats**

Each point represents the mean ± S.E. of 7 experiments.
antipyretic and analgesic effect and the decrease of the acute toxicity of AM.

Though the combination of AM and BA had been appreciated for a long time as described in the preface, it is regrettable that the oral administration of AM has been prohibited since 1977 by the Ministry of Health and Welfare in Japan, because a carcinogen, dimethylnitrosamine, might be formed more or less as a reaction product between AM and nitrite in the gastrointestinal tract.

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REFERENCES