Urinary Excretion Levels of Carcinogenic Glutamic Acid Pyrolysis Products and Their N-Acetyl Derivatives in Humans

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Introduction

Recent investigations have revealed a new series of mutagenic and carcinogenic heterocyclic amines in pyrolysates of amino acid and proteins. Some of them have been demonstrated to be present in various food stuffs, cigarette smoke condensate, airborne particles and rain water, and they are believed to be distributed widely in the environment. 2-Amino-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2) were originally isolated as potent mutagens from pyrolysates of glutamic acid and subsequently detected in cooked foods and cigarette smoke condensate. When fed with the diet, Glu-P-1 and Glu-P-2 are carcinogenic to mice and rats, causing carcinomas of various organs such as liver and intestines. Recently, our observations showed that Glu-P-1 and Glu-P-2 were present in human samples such as dialysis fluid of patients with uremia, as well as in cataractous lenses, urine, bile and metabolic organs, indicating that humans are actually exposed to these carcinogens.

Most studies on the in vivo fate of carcinogenic heterocyclic amines have been performed on mice and rats, and demonstrated that they are excreted into bile and urine with their metabolites. Negishi et al. showed that more than 90% of radioactivity administered to rats was recovered in bile and urine within 24h after a single dose of [14C]Glu-P-1. They further demonstrated that Glu-P-1 was excreted in bile with several metabolites and that most of the mutagenicity excreted in the bile of Glu-P-1 injected rats was due to unmetabolized Glu-P-1 and a mutagenic metabolite, 2-acetylamino-6-methylpyrido[1,2-a:3',2'-d] imidazole (N-acetyl-Glu-P-1). In vivo N-acetylation of Glu-P-1 was confirmed by our recent study showing that Glu-P-1 was detected with its N-acetyl derivative in Glu-P-1-injected rats. In humans, we observed that Glu-P-1 and Glu-P-2 were accumulated in the plasma of patients with uremia and that plasma levels of these carcinogens were decreased by hemodialysis treatment, indicating that one of the excretory pathways of these carcinogens in humans may be via the kidney. In a previous investigation, we analyzed a large amount of urine to search for carcinogenic glutamic acid pyrolysis products in human urine, and isolated and characterized Glu-P-1, Glu-P-2 and their N-acetyl derivatives. In the same study, N-acetylation of these carcinogens was further confirmed by in vitro experiments using the cytosolic fraction from a human liver specimen.

In the present investigation, we applied a sensitive high-performance liquid chromatography (HPLC) method and measured Glu-P-1, Glu-P-2 and their N-acetyl derivatives in 24-h urine of individual subjects. We demonstrated that Glu-P-1, Glu-P-2 and their N-acetyl derivatives in human urine can be suitable indicators for monitoring the exposure levels of carcinogenic glutamic acid pyrolysis products. Moreover, we investigated the importance of extrahepatic tissues such as kidney in the N-acetylation of these carcinogens.

Materials and Methods

Chemicals and Reagents

Glu-P-1, Glu-P-2, N-acetyl-Glu-P-1 and 2-acetylaminodipyrido[1,2-a:3',2'-d]imidazole (N-acetyl-Glu-P-2) were provided by Dr. Yuichi Hashimoto, Institute of Applied Microbiology.
University of Tokyo. Diethyl ether, HPLC-grade acetonitrile, methanol and chloroform were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetyl-CoA was from Sigma Chemicals (St. Louis, MO) and dithiothreitol and trichloroacetic acid were from Wako Pure Chemical Industries. All other chemicals were of analytical grade.

Extraction Procedures

The 24-h urine (1.12-1.85 l) was collected from 6 healthy male volunteers (26-38 years old) and stored for <3 days at 4°C without addition of antibacterial agents. Urine was extracted twice with the same volume of diethyl ether after adjusting to pH 10 with 28% ammonia water. The extract was evaporated to dryness under a nitrogen stream. The material obtained on evaporation was dissolved in 1ml of 20mM NH₄H₂PO₄/20mM H₃PO₄/acetonitrile (85/5/10, V/V/V) and subjected to HPLC analyses.

Reversed-phase HPLC

Reversed-phase HPLC analyses were performed using a Shimadzu LC-6A chromatograph (Shimadzu, Tokyo, Japan) equipped with Shimadzu RF-535 fluorometric detector (Shimadzu). The method described previously was used with some modifications. Partial purification was carried out on an Asahipack ES-502C column (9.0 µm particle size, 7.6 x 100mm; Asahi Chemical Industries, Kawasaki, Japan) under the following conditions: mobile phase, 20mM NH₄H₂PO₄/20mM H₃PO₄/acetonitrile (85/5/10, V/V/V); flow rate, 1.0 ml/min; column temperature, 40°C. Fluorescence was monitored at 416nm when excited at 259nm. The retention times of authentic N-acetyl-Glu-P-2, Glu-P-2, N-acetyl-Glu-P-1 and Glu-P-1 were 16.0min, 18.8min, 19.3min and 23.1min, respectively. Fractions corresponding to N-acetyl-Glu-P-2, Glu-P-2 plus N-acetyl-Glu-P-1 and Glu-P-1 were collected separately and acetonitrile in the eluate was evaporated off. The second purification step was carried out on a Nucleosil 5C₈ column (10µm particle size, 4 x 150mm; Union Co. Ltd, Takasaki, Japan). The mobile phase was 20mM NH₄H₂PO₄/20mM H₃PO₄/acetonitrile (70/25/5, V/V/V). A flow rate of 1.0ml/min at 40°C was used. Fluorescence was monitored at 416nm when excited at 259nm. The retention times of authentic N-acetyl-Glu-P-2, Glu-P-2, N-acetyl-Glu-P-1, and Glu-P-1 were 8.6min, 10.5min, 14.2min and 25.7min, respectively. The fraction corresponding to each authentic compound was collected separately and acetonitrile in the eluate was evaporated off. Final HPLC analysis was performed on a Kaseisorb LC ODS-300-5 column (5 µm particle size and 300 A pore size, 7.5 x 250 mm; Tokyo Chemical Industries, Tokyo). The mobile phase was a linear gradient (0-9%, V/V) of acetonitrile in 10mM H₃PO₄ over 30min. The flow rate was 3.0 ml/min at 50°C. The fluorescence was monitored at 445nm for Glu-P-1 (excited at 377nm), at 454nm for Glu-P-2 (excited at 385nm), at 413nm for N-acetyl-Glu-P-1 (excited at 260nm) and at 419nm for N-acetyl-Glu-P-2 (excited at 258nm). Characterization of fractions corresponding to Glu-P-1, Glu-P-2 and their N-acetyl derivatives was performed by spectrometric analyses. Thirty liters of pooled urine was processed as described above and the contents of the fractions corresponding to Glu-P-1, Glu-P-2 and their N-acetyl derivatives were extracted with chloroform after the acetonitrile in the fractions was evaporated off, and the pH was adjusted to 10 with 28% ammonia water. Fluorescence emission and mass spectra were measured as described previously.

Determination of N-acetylation activity in vitro

Autopsy liver and Kidney specimens, obtained about 6-10 h after death and kept frozen at -80°C in plastic bags, were used for assays for N-acetylation in vitro. The kidney specimens used in this investigation were part of the whole kidney and consisted of cortex and medulla. Preparation of the cytosolic fraction and the in vitro N-acetylation assay were done using the method of Shinohara et al., with some modifications. After quickly being defrosted with warm water, autopsy specimens were homogenized with three volumes of 50mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1mM dithiothreitol. The cytosolic fraction was prepared by sequential centrifugation at 9000 x g for 20min and 105000 x g for 30min at 4°C. The incubation mixture consisted of 100mM Tris-HCl buffer (pH 7.4), 0.2mM dithiothreitol, 0.2mM acetyl-CoA, 2-3mg/ml prpotein of cytosolic fraction and a 0.4mM substrate (Glu-P-1 or Glu-P-2) with a volume of 150 µl. After preincubation at 37°C for 3min, the reaction was started by adding the cytosolic fraction. The reaction was terminated by adding 15 µl of 20% trichloroacetic acid. The mixture was then centrifuged to precipitate the protein. The
supernatant was subjected to HPLC with a Kaseisorb LC ODS-300-5 column under the conditions described above. Livers and kidneys of male wistar rats (95-115 g in weight, Charles River Japan, Inc.) were processed as described above for human samples.

Results

Analysis of urine

Typical HPLC chromatograms for the final-step analysis of human urine are shown in Fig. 1. Sharp peaks that co-migrated with authentic Glu-P-1, Glu-P-2 and their N-acetyl derivatives were clearly identifiable (Fig. 1). The fraction corresponding to each compound was collected and examined by spectrometric analysis. The fluorescence emission peak of the Glu-P-1 fraction (excited at 377 nm in methanol) was 445 nm and that of the Glu-P-2 fraction (excited at 384 nm) was 454 nm. The fluorescence emission peaks of the N-acetyl-Glu-P-1 fraction (excited at 260 nm) were 392, 413 and 435 nm, and those of the N-acetyl-Glu-P-2 fraction (excited at 258 nm) were 397, 420 and 444 nm. These values were virtually identical to those of authentic compounds. The N-acetyl-Glu-P-2 fraction was further characterized by the mass spectrum, which revealed molecular ion peaks at m/z 79, 184 and 226, identical to the peaks of authentic N-acetyl-Glu-P-2. Peak heights in the final chromatograms obtained using an ODS-300-5 column showed linear relationships with the amounts of authentic compounds (the minimum detection limit was several tens of femtomoles for each compound). The amounts of Glu-P-1, Glu-P-2 and their N-acetyl derivatives in human urine were calculated on the basis of such linear relationships. In order to estimate the recovery rates of Glu-P-1, Glu-P-2 and their N-acetyl derivatives, human urine (1.5 ml), to which authentic Glu-P-1, Glu-P-2 and their N-acetyl derivatives (each 5 pmol) had been added, was treated by the same procedure as for the analytical samples. The percentages of Glu-P-1, N-acetyl-Glu-P-1, Glu-P-2 and N-acetyl-Glu-P-2 recovered by this method were 70.9±4.2%, 66.5±3.2%, 51.9±2.9% and 42.6±2.8% (mean±SD, n=4), respectively. Table 1 shows the data corrected for the recoveries.

N-Acetylation in vitro

Reaction mixtures were analyzed by HPLC using an ODS-300-5 column after precipitating proteins with trichloroacetic acid. The chromatograms revealed peaks corresponding to N-acetyl-Glu-P-1 and N-acetyl-Glu-P-2. The fraction corresponding to each peak was characterized by UV-absorbance, fluorescence-emission and the mass spectrum. The amounts of the compounds were determined by peak heights based on their linear relationships with the amounts of authentic compounds. Linear conditions of the reaction with respect to incubation time and protein concentration were first evaluated. The results of a representative experiment with the cytosolic fraction from a human autopsy liver

![Fig. 1 HPLC chromatograms of final-step analyses of a 24-h urine sample (subject 1) on an ODS-300-5 column. (A), (B), (C) and (D) correspond to chromatographic profiles of Glu-P-1, N-acetyl-Glu-P-1, Glu-P-2 and N-acetyl-Glu-P-2, respectively.](image-url)
Table 1  Amounts of daily excretion of Glu-P-1, Glu-P-2 and their N-acetyl derivatives into urine.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Glu-P-1</th>
<th>N-acetyl-Glu-P-1</th>
<th>Glu-P-2</th>
<th>N-acetyl-Glu-P-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.93</td>
<td>0.88</td>
<td>4.22</td>
<td>10.12</td>
</tr>
<tr>
<td>2</td>
<td>0.74</td>
<td>0.38</td>
<td>4.01</td>
<td>6.26</td>
</tr>
<tr>
<td>3</td>
<td>0.66</td>
<td>0.50</td>
<td>2.32</td>
<td>5.43</td>
</tr>
<tr>
<td>4</td>
<td>0.61</td>
<td>0.42</td>
<td>1.54</td>
<td>3.39</td>
</tr>
<tr>
<td>5</td>
<td>0.11</td>
<td>0.17</td>
<td>0.39</td>
<td>1.57</td>
</tr>
<tr>
<td>6</td>
<td>0.10</td>
<td>0.11</td>
<td>0.22</td>
<td>0.85</td>
</tr>
</tbody>
</table>

mean±   0.53±  0.41±  2.12±  4.60±  
S.D.    0.31    0.25    1.58    3.13

* Values were corrected for recoveries. The recoveries of Glu-P-1, N-acetyl-Glu-P-1, Glu-P-2 and N-acetyl-Glu-P-2 were 70.9%, 66.5%, 51.9% and 42.6%, respectively.

specimen are shown in Fig. 2. Table 2 summarizes the results of the cytosolic N-acetyltransferase activities with Glu-P-1 and Glu-P-2.

Discussion

In the present investigations, we confirmed that the carcinogenic glutamic acid pyrolysis products, Glu-P-1 and Glu-P-2 are excreted in human urine together with their N-acetyl derivatives after being partially N-acetylated. Furthermore, we demonstrated that daily excretion of these carcinogens and their N-acetyl derivatives into urine can be determined by using a sensitive HPLC method. In this study, Glu-P-1, Glu-P-2 and their N-acetyl derivatives were measured in 24-h urine of individual subjects. In the urine of all the subjects analyzed, Glu-P-1, Glu-P-2 and their N-acetyl derivatives were detected, and the amounts of N-acetyl-Glu-P-2 were greater than those of Glu-P-2, while the amounts of N-acetyl-Glu-P-1 were less than those of Glu-P-1. These results were comparable with those shown by using tons of human urine in our previous experiments. The fact that the N-acetylation ratio in Glu-P-2 is higher than that in Glu-P-1 suggests that Glu-P-2 is a better substrate for N-acetylation.

Fig. 2  Effects of incubation time (A) and protein concentration (B) on N-acetylation of Glu-P-2 by cytosolic fraction from a human liver (Liver I). A. The protein concentration of the reaction mixture was 2.8mg/ml. B. Synthesis of N-acetyl-Glu-P-2 (nmol/min) in 150 µl of reaction mixture was plotted against the protein concentration of the reaction mixture. Incubation time was 20min.
Table 2  Rate of N-acetylation of Glu-P-1 and Glu-P-2 by cytosolic fractions from autopsy specimens and rat organs.

<table>
<thead>
<tr>
<th></th>
<th>Rate of N-acetylation (pmol acetylated/mg protein/min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Glu-P-1</td>
</tr>
<tr>
<td>Human autopsy specimen a</td>
<td></td>
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<tr>
<td>Liver I</td>
<td>75.2</td>
</tr>
<tr>
<td>Liver II</td>
<td>56.3</td>
</tr>
<tr>
<td>Liver III</td>
<td>98.9</td>
</tr>
<tr>
<td>Liver IV</td>
<td>82.4</td>
</tr>
<tr>
<td>Liver V</td>
<td>37.6</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>70.1±21.2</td>
</tr>
<tr>
<td>Kidney I</td>
<td>1.47</td>
</tr>
<tr>
<td>Kidney II</td>
<td>0.75</td>
</tr>
<tr>
<td>Kidney III</td>
<td>1.18</td>
</tr>
<tr>
<td>Kidney IV</td>
<td>1.10</td>
</tr>
<tr>
<td>Kidney V</td>
<td>0.74</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>1.05±0.28</td>
</tr>
<tr>
<td>Rat organ</td>
<td></td>
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<tr>
<td>Liver</td>
<td>17.7±3.5 b</td>
</tr>
<tr>
<td>Kidney</td>
<td>9.1±2.1</td>
</tr>
</tbody>
</table>

a Liver I and Kidney I were obtained from a 66-year-old male subject who died of cerebral hemorrhage. Liver II and Kidney II, a 73-year-old female who died of colon cancer; Liver III and Kidney III, a 69-year-old female who died of cerebral hemorrhage; Liver IV and Kidney IV, a 56-year-old male who died of myocardial infarction; Liver V and Kidney V, a 68-year-old male who died of esophageal cancer.
b Mean±S.D. of 4 separate experiments.

Table 2 presents the rate of N-acetylation of Glu-P-1 and Glu-P-2 by cytosolic fractions from autopsy specimens and rat organs. The data show that the human liver specimens had significantly higher N-acetyltransferase activity compared to kidney specimens, while rat liver specimens had higher activity than kidney specimens.

N-Acetylation is known as one of the principal metabolic mechanisms of foreign compounds such as arylamines and arylhydrazides in most mammalian species. This process involves the transfer of the acetyl moiety of acetyl-CoA to substrates by N-acetyltransferase located in the soluble portion of liver and other organs. In our previous investigation, we showed that Glu-P-1 and Glu-P-2 were acetylated to form N-acetyl derivatives by the cytosolic fraction from a human-autopsy liver. In this study, we reconfirmed N-acetyltransferase activity with Glu-P-1 and Glu-P-2 in the cytosol of human livers. Furthermore, we tested N-acetylating activity of kidney cytosol from rats and human autopsy specimens and found that kidney cytosol had N-acetyltransferase activity with Glu-P-1 and Glu-P-2, suggesting that extrahepatic tissues may also play significant roles in the N-acetylation of these carcinogens. The acetylating activity of human kidney autopsy specimens was 50-100 times less than that of human liver specimens, while rat kidney N-acetylating activity was about half of that of rat liver cytosol. The N-acetyltransferase activity of the human-autopsy kidneys tested in the present investigation was approximately one tenth of that of the rat kidney. It has been demonstrated that N-acetyltransferase activity varies extensively in different species and different organs. Hears and Weber showed the existence of more than one N-acetyltransferase in the rabbit and that the enzymes differ in such properties as tissue distribution, substrate specificity, stability and pH characteristics. They further demonstrated that one of the enzymes was primarily associated with liver and catalyzed the acetylation of a wide range of substrates, while other enzymatic activities found in extrahepatic tissues such as kidney and intestine showed higher substrate specificity. Such differences in the properties...
of N-acetyltransferases might explain the differences between humans and rats observed in the present investigation, although a possible difference in enzyme stability between liver and kidney remains to be considered, especially in autopsy specimens.

In [\(^{14}\text{C}\)] Glu-P-1-administered rats, Negishi et al. \(^{13}\) showed that elimination of Glu-P-1 from the rat body was very rapid and that the radioactivity excreted into the urine within 24 h was about 35% of the administered dose. In humans, we observed in the previous investigations that Glu-P-1 and Glu-P-2 accumulated in the plasma of patients with uremia, although these carcinogens are excreted not only into urine but also into bile\(^{8,11}\). Therefore, it seems that the excretory pathway via the kidney into urine is crucial to the excretion of carcinogenic glutamic acid pyrolysis products. These facts suggest that urine is suitable for monitoring levels of carcinogenic glutamic acid pyrolysis products in humans.

Negishi et al. \(^{13}\) also observed that most of the mutagenicity excreted in the bile of Glu-P-1-administered rats was due to unmetabolized Glu-P-1 and N-acetyl-Glu-P-1. Although the specific mutagenic activity of Glu-P-1 was reported to be decreased by N-acetylation, it was suggested that N-acetyl derivatives of Glu-P-1 and Glu-P-2, as in the case of 2-acetylaminofluorene, may be activated through further N-hydroxylation and esterification reactions and be responsible for carcinogenesis\(^{15,18,21,22}\). Therefore, both the amounts of the N-acetyl derivatives and the unmetabolized forms excreted in urine should be considered in order to estimate the risks of these muta-carcinogens to humans.

Epidemiological studies have shown that dietary factors and cigarette smoking are among the most important environmental risk determinants for human carcinogenesis\(^{23}\). Glu-P-1 and Glu-P-2 were detected in cooked foodstuffs and cigarette smoke condensate\(^{1,3,7}\). In order to evaluate the significance of these carcinogens in human carcinogenesis, extensive epidemiological studies are required to elucidate the correlation between cancer risk and levels of exposure to these carcinogens. Many investigations have demonstrated that urinary mutagenicity, excreted mutagenic compounds and/or their metabolites reflect the levels of environmental and occupational exposure to mutagenic chemicals\(^{24,25,26}\). Glu-P-1, Glu-P-2 and their N-acetyl derivatives measured in urine can be suitable indicators for monitoring the exposure levels.

**Summary**

The carcinogenic glutamic acid pyrolysis products 2-amino-6-methyldipyrido [1, 2-a: 3', 2'-d]imidazole (Glu-P-1) and 2-amino-dipyrido [1, 2-a: 3', 2'-d] imidazole (Glu-P-2), and their N-acetyl derivatives were measured in 24-h urine of individual subjects by high-performance liquid chromatography. These compounds were detected in all urine samples analyzed, although the contents varied widely among subjects. The mean levels of Glu-P-1, N-acetyl-Glu-P-1, Glu-P-2 and N-acetyl-Glu-P-2 in 24-h urine were 0.53, 0.41, 2.12 and 4.60 pmol, respectively. In vitro experiments revealed N-acetyltransferase activity with Glu-P-1 and Glu-P-2 in the cytosolic fractions from rat kidneys and human autopsy kidney specimens as well as those from liver specimens, suggesting that extrahepatic tissues may also play significant roles in the N-acetylation of these carcinogens. These results show that Glu-P-1 and Glu-P-2, after being partially N-acetylated in metabolic organs such as liver and kidney, are excreted into urine together with their N-acetyl derivatives. It is suggested that daily excretion of carcinogenic glutamic acid pyrolysis products and their N-acetyl derivatives into urine can be a suitable biological monitor for exposure to these carcinogens.

**Abbreviations:** Glu-P-1, 2-amino-6-methyldipyrido [1, 2-a: 3', 2'-d] imidazole; Glu-P-2, 2-aminodipyrido [1, 2-a: 3', 2'-d] imidazole; N-acetyl-Glu-P-1, 2-acetylamino-6-methyldipyrido [1, 2-a: 3', 2'-d] imidazole; N-acety-Glu-P-2, 2-acetylaminodipyrido[1,2-a:3',2'-d]imidazole; HPLC, high-performance liquid chromatography.

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(Meabashi, Japan), for autopsy specimens. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References


Key words: Carcinogenic glutamic acid pyrolysis products, N-Acetylation, Urinary excretion level, Exposure level monitor

発癌性グルタミン酸熱分解物及びそのN-アセチル化物のヒト尿中排泄量の測定

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Glu-P-1, Glu-P-2は、グルタミン酸熱分解物から単離精製された変異原発癌性複素環状アミンであり、これまでに加熱食品、タバコ等に検出されている。本研究では、高速液体クロマトグラフを用い、Glu-P-1, Glu-P-2, 及びそのN-アセチル化物の尿中への一日排泄量の測定を行った。排泄量には個体間でのばらつきが認められたが、これらの物質は全検体中に検出された。Glu-P-1, N-acetyl-Glu-P-1, Glu-P-2, N-acetyl-Glu-P-2の尿中一日排泄量の平均値は、それぞれ、0.53, 0.41, 2.12, 4.60pmolであった。組織におけるN-アセチル化能を検討する目的で、さらにin vitroの実験を行った。その結果、肝及び腎由来の細胞可溶性画分に、Glu-P-1, Glu-P-2のN-アセチル化活性が示され、肝のみでなく、それ以外の組織もこれらの物質のN-アセチル化に関与していることが示唆された。以上の結果は、Glu-P-1, Glu-P-2が、肝や腎などの代謝機器において一部N-アセチル化を受け、N-アセチル化物とともに尿中に排泄されていることを示すものである。Glu-P-1, Glu-P-2, 及びそのN-アセチル化物の尿中への一日排泄量は、人体への暴露量を評価する際の有用な指標となると思われる。

Key words: Carcinogenic glutamic acid pyrolysis products, N-Acetylation, Urinary excretion level, Exposure level monitor

発癌性グルタミン酸熱分解物、N-アセチル化、尿中排泄量、暴露指標

（受付 1991年1月22日 受理 1991年3月20日）