METABOLITES OF PIPERIDINE IN RAT URINE

Yoshiro OKANO, Takeshi MIYATA, Shiang-Huey HUNG,
Toshiro MOTOYA, Mikiko KATAOKA, Kazuo TAKAHAMA,
and Yoshitoshi KASÉ

*Department of Chemico-Pharmacology, Faculty of Pharmaceutical Sciences,
Kumamoto University, Kumamoto 862, Japan*

Accepted August 29, 1977

Abstracts—Piperidine is one of pharmacologically active biogenic amines. While two pathways for piperidine production have been reported, little is known about metabolism of the compound. In the present study, piperidine and its hydroxylated, conjugated and unknown metabolites were detected in rat urine by radiochromatographic analysis. Using GC-MS technique, it was confirmed that 3-hydroxypiperidine and 4-hydroxypiperidine are major metabolites of piperidine of either exogenous or endogenous origin. The findings substantiate the existence of a mechanism which inactivates piperidine in the living body, since both metabolites lack the potent pharmacological activities as those induced by piperidine.

Piperidine (Pip) is a normal constituent of brain (1, 2, 3, 4, 5, 6, 7), skin (8, 9) and urine (3, 10) of mammals, and of the brain (11), cerebrospinal fluid (12), and urine (3, 10, 13) of humans, and of cerebral ganglia of molluscs (14). Pip shows potent nicotine-like actions on the peripheral (15, 16, 17) and central nervous systems (CNS) (18, 19). Pip acts on the autonomic ganglia, neuromuscular junctions and chemoreceptors, i.e., it elicits stimulation in small doses and depression in large doses. In the CNS, it produces synaptic stimulation followed by depression (19), exerts tranquilizing effects on schizophrenic patients (20) and counteracts experimentally induced aggressiveness in mice and rats (19). The findings obtained from chemical stimulation of the brain by Pip suggest that the amine itself affects neural mechanisms governing regulation of emotional behaviour, sleeping and extrapyramidal function (21, 22).

Pip can be produced *in vitro* (23) as well as *in vivo* (24, 25) from pipecolic acid, which is an intermediate of lysine metabolism and found in serum (26), urine (27) and the brain (28) of mammals. On the other hand, another possible pathway of Pip production from cadaverine has been reported in invertebrates (29).

A study using tritiated Pip on the subcellular distribution of the amine shows that ³H-Pip is mostly localized in the nerve ending particles after subfractionation of the rat brain (30). It is also suggested that there are uptake and storage mechanisms for Pip in nerve endings (31).

Thus, Pip plays an important role in neural mechanism of the brain, and the present authors (22) and others (32) have assigned a modulator role to Pip in the CNS. On the

other hand, there is little information on the metabolism of Pip. In the present study, metabolites of Pip in the rat urine were studied in order an attempt to elucidate the mechanism of inactivation of the amine in living body.

MATERIALS AND METHODS

Detection of metabolites using ^3H -labelled piperidine

^3H -Pip hydrochloride (specific activity, 300 $\mu\text{Ci}/\text{mg}$) was prepared by New England Nuclear Corp. according to the method of Wilzbach (33). Unstable tritium was removed by repeated crystallization to a constant specific activity and pure crystals of the salt were used. Glusulase used for the present experiment was prepared by Endo Laboratories Inc. and contains 100,000 units of β -glucuronidase and 50,000 units of aryl-5-sulfatase per ml.

Twenty-four male Wistar rats, weighing 180 ± 20 g, were divided in two groups. ^3H -Pip HCl (50 $\mu\text{Ci}/\text{animal}$) was given i.p. to each of 12 rats of one group (Pip group). Rats of the other group (control group) were given the same volume of saline. Total urine of each group was collected over a 72 hour period under toluene layer (5 ml) with 1N HCl (10 ml) in a flask. Each pooled urine was extracted by a mixture of cyclohexane and benzene (1:1). After evaporation, the residue was dissolved in a small volume of ethanol, and the solution was spread in a narrow band on a chromatograph paper (Toyo-Roshi, No. 51, 3×57 cm). Analysis by paper chromatography was performed as follows: developing solvent, *n*-butanol : acetic acid : water = 4:1:2, ascending method, for 24 hours. After drying, the chromatograms were scanned on a radiochromatography scanner (RCS, Aloka-type JPC 102).

In order to detect the presence of conjugated Pip, a part of the urine described above was adjusted to pH 6.5 with 2N NaOH. Both phosphate and sulfate ions were removed from the urine by adding an excess of saturated BaCl_2 solution and by centrifuging the precipitate. The supernatant was incubated with 0.1 ml of β -glusulase for 24 hours at 37°C (34), extracted and subsequently treated by the same procedure as described above.

Prior to this experiment, R_f -values of authentic Pip and its putative metabolites such as 3-hydroxypiperidine (3-OH-Pip), 4-hydroxypiperidine (4-OH-Pip), N-acetylpiperidine, N-methylpiperidine and piperidone-2 on paper chromatogram were determined by using ninhydrin and Dragendorff's reagents.

Identification and quantitation of metabolites

Four groups each including nine rats were used. Pip HCl (1.0 mg/animal) was given i.p. to each rat of two groups (pip groups) and the same volume of saline to each rat of the other two groups (control groups). Urine from each group was collected over a 72 hour period. The pooled urine was treated with the same volume of 5% trichloroacetic acid solution (TCA). After centrifugation, the supernatant was adjusted to pH 12 with 2N NaOH and the amines in the solution were converted to dinitrophenyl (DNP) derivatives according to the procedures previously described (23, 24, 25).

The DNP derivatives produced were extracted with 50 ml of cyclohexane and then with the same volume of ethyl acetate. After evaporation, the residue was dissolved in a

small volume of mixed solvents of benzene, cyclohexane and ethyl acetate (1:1:1). The extract was spread in a narrow band on a thin-layer chromatograph (TLC, 20×20 cm glass plate, 100 μ m thick, Wakogel B-5) and developed with chloroform, using the ascending method, for 30 min. Authentic samples of DNP-Pip, DNP-3-OH-Pip and DNP-4-OH-Pip were also run parallel with the samples described above. Each spot, corresponding to each of R_fs of authentic DNP-derivatives, was scraped off and eluted with a mixture of benzene and ethyl acetate (1:1). After evaporation *in vacuo*, the residue was dissolved in a fixed volume of ethanol. A part of the solution (0.4–2.0 μ l) was analyzed with a gas chromatograph-mass spectrometer.

Gas chromatography-mass spectrometry (GC-MS): Following the gas chromatographic separation, mass spectrometry was utilized for identification, and selected ion monitoring technique was also adopted for quantitation of Pip metabolites.

Gas chromatographic separation was performed by using a 2 mm×1 m glass column packed with 1.5% OV-17 on Shimalite W (80 to 100 mesh). Chromatographic conditions: column temp., 200°C; injection port temp., 300°C; ion source temp., 230°C; He flow rate, 40 ml/min. Mass spectral studies were carried out with a double focussing mass spectrometer (JEOL GC-MS D-100). Mass spectral conditions: electron energy, 25–75 eV; trap current, 300 μ A; accelerating voltage, 3 KV; ion multiple voltage, 1.2–1.75 KV (35, 36). The quantitation of hydroxylated Pip was performed by selected ion monitoring (SIM), and the ion employed was m/e 250. Quantities were read from a calibration curve which had been prepared by plotting intensities of the fragment ions of authentic DNP-3-OH-Pip and DNP-4-OH-Pip against concentration of both compounds.

RESULTS

Detection of metabolites

As shown in the upper record of Fig. 1, five peaks appeared on the radiochromatogram of samples obtained from rat urine. By comparing the R_f-value of each peak with that of corresponding authentic compounds, the peaks were identified as follows. The highest peak (M₄) was identical with Pip itself, and the peak M₂ with 3-OH-Pip or/ and 4-OH-Pip.

FIG. 1. Radiochromatograms of the sample obtained from rat urine. Upper: Before treatment with β -glucosylase. M₄ represents a peak for 3 H-Pip itself and the remaining four peaks represent the metabolites of 3 H-Pip. Lower: After treatment with β -glucosylase. Both M₃ and M₅ disappeared.

However, the remaining three peaks such as M_1 , M_3 and M_5 were identical with none of authentic compounds such as N-acetylpiperidine, N-methylpiperidine or piperidone-2. After treatment with β -glucosylase, the peaks M_3 and M_5 disappeared as shown in the lower record of Fig. 1.

Identification and quantitation of metabolites

Authentic DNP-3-OH-Pip and DNP-4-OH-Pip were applied to GC-MS and peaks appeared on chromatograms of total ion monitor (TIM) were analyzed by mass spectrometry. Mass spectra of both authentic amines are shown in Fig. 2. Major fragment ions of both amines were as follows. 1) DNP-3-OH-Pip : a parent ion peak at m/e 267, a base ion peak at m/e 232 and another intensive fragment ion peak at m/e 250. 2) DNP-4-OH-Pip : a parent ion peak at m/e 267, a base ion peak at m/e 232 and another intensive fragment ion peaks at m/e 250 and 186.

The sample obtained from the urine of the Pip group was then applied to GC-MS. Two peaks having retention times equal to those of authentic DNP-3-OH-Pip and DNP-4-OH-Pip (9.9 and 12.1 minutes, respectively) appeared on the TIM chromatogram. Mass

FIG. 2. Mass spectra of authentic DNP-3-hydroxypiperidine (upper) and DNP-4-hydroxypiperidine (lower). The pattern of mass spectrum of the sample obtained from the Pip group or the control group was identical with that of authentic DNP-3-OH-Pip (upper). In other words, 3-OH-Pip was present not only in the urine of the Pip group but also in that of the control group. The same holds true in the case of DNP-4-OH-Pip (lower).

TABLE 1. Quantities of hydroxylated piperidines in rat urine

	3-OH-Pip		4-OH-Pip	
	$\mu\text{g/ml}$	Mean	$\mu\text{g/ml}$	Mean
Pip group	3.58	4.15	2.49	3.15
	4.71		3.80	
Control group	1.80	1.84	0.57	1.01
	1.87		1.45	

spectra of the peaks were quite identical with those of DNP-3-OH-Pip and DNP-4-OH-Pip (Fig. 2). The sample from the control group also showed the same pattern of spectra as those of Pip group, that is, both 3-OH-Pip and 4-OH-Pip were found in the urine of the control group.

The quantities of hydroxylated Pip measured are shown in Table 1. The quantities of 3-OH-Pip and 4-OH-Pip in the Pip group were 2.26 and 3.12 times larger than those of corresponding compounds in the control group, respectively.

DISCUSSION

Pip is one of biogenic amines possessing potent pharmacological and physiological actions (15–22). The present study was undertaken to determine the metabolic fate of Pip in the living body.

Five peaks (M_1 – M_5) appeared on the radiochromatogram of the sample obtained from urine of rats administered ^3H -Pip. Two were identified with Pip (M_4) and hydroxylated piperidine (M_2), respectively. GC-MS analysis revealed that the major metabolite (M_2) consisted of two components identical with 3-OH-Pip and 4-OH-Pip. Another two (M_3 and M_5) were assumed to be conjugated compounds of Pip or those of metabolites because they disappeared after treatment with β -glucosylase. However, further elucidation of the compounds and identification of the other small peak (M_1) were not carried out.

Thus, it is presumed that hydroxylated piperidine derivatives such as 3-OH-Pip and 4-OH-Pip should be major candidates of the Pip metabolites excreted into urine. Both 3-OH-Pip and 4-OH-Pip were found not only in the urine of the Pip group but also in the urine of the control group. This is the first documentation that 3-OH-Pip and 4-OH-Pip are normal constituents in rat urine.

The study on pharmacological properties of 3-OH-Pip and 4-OH-Pip showed that both hydroxylated metabolites are pharmacologically different from Pip itself. As shown in Table 2, they do not have synaptotropic properties which constitute the most characteristic pharmacological feature of Pip (17, 19), but rather simulate the pharmacological actions of long-chained amino alcohols.

These findings suggest that Pip of either endogenous or exogenous origin is, at least in part, inactivated by being metabolized to its hydroxylated compounds and conjugated

TABLE 2. Comparison of pharmacological actions of piperidine and 3- or 4-hydroxypiperidine

	Pip	3- or 4-OH-Pip
Respiratory stimulation	++	—
Blood pressure rise	++	—
Contraction of smooth muscle	++	—
Contraction of skeletal muscle	++	—
Prolongation of sleeping time	++	+
Decrease of spontaneous movements	++	+
Vomiting	++	—

ones and is then excreted into the urine.

Acknowledgements: This work was supported by grant 044072 from the Ministry of Education, Science and Culture, Japan. Thanks are due to Drs. I. Matsumoto and T. Shinka, Kurume University School of Medicine, for their pertinent advice concerning the GC-MS technique.

REFERENCES

- 1) HONEGGER, C.G. AND HONEGGER, F.: Volatile amines in brain. *Nature* **185**, 530-532 (1960)
- 2) PERRY, T.L., HANSEN, S., FOULKS, J.G. AND LING, G.M.: Aliphatic and aromatic amines of the cat brain. *J. Neurochem.* **12**, 397-405 (1965)
- 3) KASÉ, Y., KATAOKA, M. AND MIYATA, T.: An improved method for determination of micro-amounts of piperidine in living materials. *Japan. J. Pharmacol.* **19**, 354-362 (1969)
- 4) KATAOKA, M., KASÉ, Y., MIYATA, T. AND KAWAHITO, E.: Piperidine in the cerebellum of the dog. *J. Neurochem.* **17**, 291-292 (1969)
- 5) NIXON, R.: Volatile amines in mouse brain: A radioassay with picogram sensitivity. *Analyt. Biochem.* **48**, 460-470 (1972)
- 6) SEILER, N. AND SCHNEIDER, H.H.: Estimation of picomole quantities of piperidine in tissues. *Biomed. Mass Spectrom.* **1**, 381-385 (1974)
- 7) STEPITA-KLAUČO, M., DOLEZALOVA, H. AND FAIRWEATHER, R.: Piperidine increase in the brain of dormant mice. *Science* **183**, 536-537 (1974)
- 8) STUETTGEN, G.: Detection of biogenic amines in animal and human skin and skin tumor by thin-layer chromatography according to Seiler's method. *Arch. klin. exp. Dermatol.* **230**, 349-360 (1967)
- 9) STUETTGEN, G.: Der Gehalt der Haut an basischen niedermolekularen Aminen. *Fette. Seifen. Anstrich.* **70**, 667-669 (1968)
- 10) VON EULER, U.S.: Identification of a urine base with nicotine-like action. *Nature* **154**, 17 (1944)
- 11) PERRY, T.L., HANSEN, S. AND MACDOUGALL, L.: Amines of human whole brain. *J. Neurochem.* **14**, 775-782 (1967)
- 12) PERRY, T.L., HANSEN, S. AND JENKINS, L.C.: Amine content of normal human cerebrospinal fluid. *J. Neurochem.* **11**, 49-53 (1964)
- 13) BLAU, K.: Chromatographic methods for the study of amines from biological materials. *Biochem. J.* **80**, 193-200 (1961)
- 14) DOLEZALOVA, H., GIACOBINI, E., SEILER, N. AND SCHNEIDER, H.H.: Determination of piperidine in snail brain (*Helix pomatia*). *Brain Res.* **55**, 242-244 (1973)
- 15) VON EULER, U.S.: The occurrence and determination of piperidine in human and animal urine. *Acta Pharmacol. Kbh.* **1**, 29-59 (1945)
- 16) LOCKETT, M.F.: Some pharmacological actions of piperidine, pyrrolidine and of pressor concentrates from dog urine. *Brit. J. Pharmacol.* **4**, 111-119 (1949)
- 17) KASÉ, Y., MIYATA, T. AND YUIZONO, T.: Pharmacological studies on alicyclic amines. I. Comparison of pharmacological activities of piperidine with those of other amines. *Japan. J. Pharmacol.* **17**, 475-490 (1967)
- 18) ABOOD, L.G., RINALDI, F. AND EAGLETON, U.: Distribution of piperidine in the brain and its possible significance in behavior. *Nature* **191**, 201-202 (1961)
- 19) KASÉ, Y., MIYATA, T., KAMIKAWA, Y. AND KATAOKA, M.: Pharmacological studies on alicyclic amines. II. Central actions of piperidine, pyrrolidine and piperazine, *Japan. J. Pharmacol.* **19**, 300-314 (1969)
- 20) TASHER, D.C., ABOOD, L.G., GIBBS, F.A. AND GIBBS, E.L.: Introduction of a new type of psychotropic drug; cyclopentimine. *J. Neuropsychiat.* **1**, 266-273 (1960)
- 21) MIYATA, T., KAMATA, K., NISHIKIBE, M., KASÉ, Y., TAKAHAMA, K. AND OKANO, Y.: Effects of intracerebral administration of piperidine on EEG and behavior. *Life Sci.* **15**, 1135-1152 (1974)
- 22) KASÉ, Y. AND MIYATA, T.: Neurobiology of piperidine: Its relevance to CNS function.

- Advances in Biochemical Psychopharmacology*. Edited by COSTA, E., GIACOBINI, E. AND PAOLETTI, R., Vol. 15, p. 5-16, Raven Press, New York (1976)
- 23) KASÉ, Y., KATAOKA, M. AND MIYATA, T.: In vitro production of piperidine from pipecolic acid in the presence of brain tissue. *Life Sci.* 6, 2427-2431 (1967)
- 24) KASÉ, Y., OKANO, Y., YAMANISHI, Y., KATAOKA, M., KITAHARA, K. AND MIYATA, T.: In vivo production of piperidine from pipecolic acid in the rat. *Life Sci.* 9, 1381-1387 (1970)
- 25) KASÉ, Y., OKANO, Y., MIYATA, T., KATAOKA, M. AND YONEHARA, N.: The production of piperidine from pipecolic acid in the rat brain. *Life Sci.* 14, 785-791 (1974)
- 26) YORITAKA, I. AND HIWAKI, C.: The biochemistry of pipecolic acid and 5-hydroxypipecolic acid. *Nagasaki Igakukai Zasshi* 34, 277-280 (1959) (in Japanese)
- 27) TOMINAGA, F. AND HIWAKI, C.: New amino acids in rat urine. *Nagasaki Igakukai Zasshi* 33, 1435-1438 (1957) (in Japanese)
- 28) KASÉ, Y., KATAOKA, M., MIYATA, T. AND OKANO, Y.: Pipecolic acid in the dog brain. *Life Sci.* 13, 867-873 (1973)
- 29) DOLEZALOVA, H., STEPITA-KLAUCO, M. AND FAIRWEATHER, R.: An elevated cadaverine content in the brain of dormant mice. *Brain Res.* 77, 166-168 (1974)
- 30) KASÉ, Y., YONEHARA, N., OKANO, Y., MIYATA, T. AND TAKAHAMA, K.: Subcellular localization of ³H-piperidine in the rat brain. *Life Sci.* 15, 1197-1202 (1974)
- 31) KASÉ, Y., YONEHARA, N., OKANO, Y., MIYATA, T. AND TAKAHAMA, K.: Pharmacological studies on alicyclic amines XVIII: Studies on the uptake system for ³H-Piperidine in rat brain. *Folia pharmacol. japon.* 71, 52P (1975) (in Japanese)
- 32) GIACOBINI, E.: Piperidine: A new neuromodulator or a hypnogenic substance? *Advances in Biochemical Psychopharmacology*, Edited by COSTA, E., GIACOBINI, E. AND PAOLETTI, R., Vol. 15, p. 17-56, Raven Press, New York (1976)
- 33) WILZBACH, K.E.: Tritium-labeling by exposure of organic compounds to tritium gas. *J. Am. Chem. Soc.* 79, 1013 (1957)
- 34) SHIMIZU, H. AND LABROSSE, E.H.: Metabolism of catecholamines—Identification and quantification of 3-methoxy-4-hydroxyphenylglycol glucuronide in human urine. *Biochem. Pharmacol.* 18, 1643-1654 (1969)
- 35) MATSUMOTO, I., SHINKA, I., KASÉ, Y. AND OKANO, Y.: Quantitative determination of piperidine in the rat brain by selected ion monitoring following dinitrophenyl derivatization. *Quantitative Mass Spectrometry in Life Sciences*. Edited by DE LEENHEER, A.P. AND RONCUCCI, R.R., p. 245-253, Elsevier Scientific Pub. (1977)
- 36) MIYATA, T., OKANO, Y., MURAO, K., TAKAHAMA, K. AND KASÉ, Y.: Pharmacological studies on alicyclic amines XXI: Application of selected ion monitoring (SIM) for quantitative determination of piperidine in the brain. *Japan. J. Pharmacol.* 27, Suppl. 47P (1977)