[23] Measurement of Pantothenic Acid and Hopantenic Acid by Gas Chromatography–Mass Spectroscopy

By KIYOSHI BANNO

Introduction

Hopantenic acid (HOPA), a natural homolog of pantothenic acid (PaA) containing γ-aminobutyric acid (GABA) in place of β-alanine, was first discovered in biological fluids by paper chromatography.[1,2] Its calcium salt and pharmacologic properties have been used to improve blood circulation and metabolism in the brain. There are many reports on the pharmacokinetics and assay of HOPA. Biserte and co-workers succeeded in identifying hopantenic acid as a GABA derivative by paper chromatography in human urine,[3] and also in renal and hepatic tissue of normal rats.[4–6] Because hopantenic acid is used pharmacologically it is important to be able to determine HOPA and PaA separately for pharmacokinetic studies.

Gas chromatography–mass fragmentography (GC–MF) has been used to assay HOPA after administration of calcium hopantenate.[7] Other methods for the assay of HOPA have also been reported, such as the GC–MF determination of pantoyllactone, which is a product of HOPA hydrolysis,[8] the colorimetric determination of GABA with sodium 1,2-naphthoquinone 4-sulfate,[9] and a high-performance liquid chromatographic (HPLC) method with 9-anthryldiazomethane as a fluorescent derivative.[10] A bioassay method using *Lactobacillus arabinosus*,[11] and the determination of trimethylsilylated pantoyllactone by GC–MF, were developed for the assay of

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[2] A. G. Moiseenok, V. M. Kopelevich, M. A. Izraelit, and L. M. Shmuilovich, *Farmakal. Toksikol.* **36,** 489 (1973).
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[5] P. Boulanger, G. Biserte, M. Davril, and M. Rache, *C.R. Acad. Sci.* **265,** 157 (1967).
[6] M. Davril, G. Biserte, and P. Boulanger, *Biochimie* **53,** 419 (1971).
[7] Y. Umeno, K. Nakai, E. Matsushima, and T. Marunaka, *J. Chromatogr.* **226,** 333 (1981).
[8] M. Anetai, T. Takahashi, H. Ogawa, and H. Kaneshima, *Hokkaidoritsu Eisei Kenkyushoho* **33,** 138 (1983).
[9] H. Terada, T. Hayashi, S. Kawai, and T. Ohno, *J. Chromatogr.* **130,** 281 (1977).
[10] T. Fukuyama, T. Maki, and M. Matsuoka, Japanese Patent Publication No. 86655 (1986). [Unexamined]
[11] H. R. Skeggs and L. D. Wright, *J. Biol. Chem.* **156,** 21 (1944).

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PaA.[12] The methods described here, however, were not developed for the simultaneous determination of PaA and HOPA, and are not sensitive enough for pharmacokinetic studies.

The simultaneous rapid microanalysis for PaA and HOPA in biological samples and natural products by GC–MF with a wide-bore column and multiple ion detection (MID) is described here. We use 5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid calcium salt as an internal standard (IS) to measure PaA and HOPA simultaneously in plasma samples from humans, monkeys, dogs, pigs, rabbits, mice, rats, chickens, and soft-shelled turtles; in brain samples of chicken and soft-shelled turtle; and also in natural products (rice, green tea, and dried yeast).

Sample Preparation

Materials

Calcium hopantenate and 5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid calcium[13] salt used as an internal standard are synthesized at Tanabe Seiyaku Co., Ltd. (Osaka, Japan). The calcium pantothenate used is a reagent of the Pharmacopoeia of Japan. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) for the silylation reagent is obtained from GL Sciences, Inc. (Tokyo, Japan). The ion-exchange resin (H type, MCI GEL CK08P) is obtained from Mitsubishi Chemical Corporation (Tokyo, Japan).

Plasma Samples

Blood samples are collected in heparinized containers and centrifuged to obtain the plasma.

The plasma samples (1.0 ml) are diluted to 2.0 ml with distilled water, applied to 2.5 ml of ion-exchange resin (H type, MCI GEL CK08P, 17 cm × 10 mm i.d.), and eluted several times with 1 ml of distilled water to make 6 ml. Chloroform (3.0 ml) is added to the eluate and the mixture is shaken vigorously for 5 min. The aqueous layer (5.0 ml) is taken, and after addition of 6 *M* hydrochloric acid (0.5 ml), ammonium sulfate (5.0 g), and ethyl acetate (20.0 ml), the mixture is shaken vigorously for 15 min and centrifuged at 1800 *g* for 5 min at 4°. The supernatant is taken and filtered through a filter paper. The filtrate is extracted with ethyl acetate (20.0 ml) again and centrifuged at 1800 *g* for 5 min at 4°. The supernatant

[12] P. Tarli, *Anal. Biochem.* **42,** 8 (1971).
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is filtered as described. The ethyl acetate layer containing PaA and HOPA is collected and evaporated at 40° under nitrogen gas to a volume of ca. 0.5 ml. The concentrate is transferred to a 3-ml reaction vial by washing with ethyl acetate (1.0 ml) and then mixed with ethyl acetate containing 100.0 ng of IS. The mixture is evaporated to dryness again, as described previously, before derivatization.

Brain Samples

Fresh brain samples of chicken (0.60 g) or soft-shelled turtle (0.66 g) are homogenized with 0.005 *M* potassium hydroxide solution (2 ml) and centrifuged at 21,000 *g* for 20 min at 4°. The supernatant (1.0 ml) is applied to 2.5 ml of ion-exchange resion (H type, MCI GEL CK08P, 17 cm × 10 mm i.d.) and taken through the same procedure as the plasma samples.

Natural Products

Distilled water (20.0 ml) is added to boiled rice (10.0 g), green tea (1.0 g), or dried yeast (0.1 g), and shaken vigorously at 70° for 30 min. After centrifugation at 1800 *g* for 5 min at 4°, 2.0 ml of supernatant is applied to 2.5 ml of ion-exchange resin (H type, MCI GEL CK08P, 17 cm × 10 mm i.d.) and taken through the same procedure as described for the plasma samples.

Derivatization

The derivatization of hydroxy and carboxyl groups with several types of trimethylsilylation reagent has been investigated. It has been found that the reaction proceeds quantitatively at 80° for 60 min with BSTFA in a screw-capped reaction vial in the presence of pyridine. The best concentration of BSTFA in pyridine has been found to be 50% (v/v). Pyridine is an effective catalyst for trimethylsilylation of hydroxy and carboxyl groups in PaA, HOPA, and IS. The trimethylsilyl derivatives of PaA, HOPA, and IS show a high detection sensitivity for the MID method and are completely separated by a wide-bore fused-silica DB-17 column (15 m × 0.53 mm i.d.) (Fig. 1).

Mass Fragmentographic Analysis

The gas chromatograph–mass spectrometer is a Hitachi M-80A equipped with an M-003 computer system. The wide-bore fused-silica column is coated with DB-17 (15 m × 0.53 mm i.d.) (J & W Scientific, Folsom,

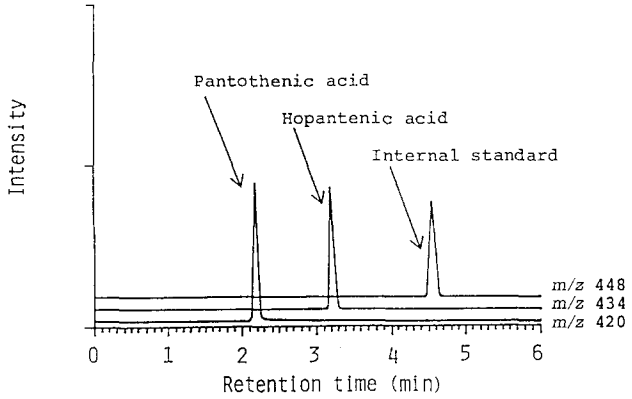

FIG. 1. Mass fragmentograms of the trimethylsilyl derivatives of pantothenic acid, hopantenic acid, and internal standard using a wide-bore fused-silica column coated with DB-17 for gas chromatography.

CA). The flow rate of helium as carrier gas is 15 ml/min, and the injection port, column oven, and separator temperature are 250, 200, and 250°, respectively. The mass spectrometer is operated in the electron-impact mode at 70 eV, the ionization current is 100 μA, and the temperature of the ion source is 200°.

The MID method for the simultaneous determination of trimethylsilyl-PaA and trimethylsilyl-HOPA has been investigated in a constant volume of the trimethylsilyl IS solution. The stable mass fragment ions are detected at *m/z* 420, 434, and 448 as the characteristic $[M - CH_3]^+$ ions of the trimethylsilyl derivatives of PaA, HOPA, and IS, respectively (Fig. 2). These ions have been selected for the simultaneous determination of PaA and HOPA by MID because other ions do not interfere with quantitation. However, as may be seen from Fig. 2, the base peak ions of trimethylsilyl derivatives of PaA, HOPA, and IS are the $[M - 2TMS + 2H]^+$ ions at *m/z* 291, 305, and 319, respectively. It has been found that base peak ions are not suitable for the quantitation because they cannot be separated completely from interfering substances in some samples.

The calibration curves are linear in the range of 5–100 ng/ml of plasma. The detection limits of PaA and HOPA using this method are ca. 1 ng/ml of plasma. The overall recoveries of PaA and HOPA are 92.9 ± 4.6% and 95.5 ± 5.1%, respectively.

Measurement in Natural Substances

The measurements of PaA and HOPA in a variety of biological samples and natural products were carried out as previously described. Samples

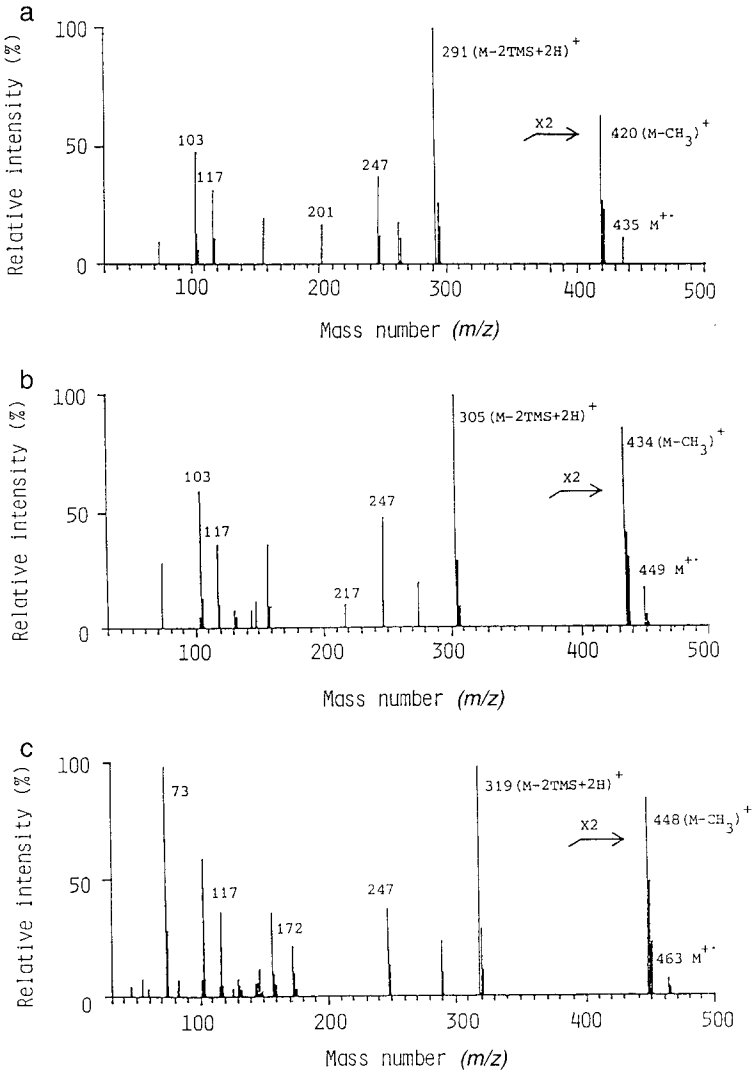

FIG. 2. Mass spectra of the trimethylsilyl derivatives of (a) panthothenic acid, (b) hopantenic acid, and (c) internal standard.

included the plasma from humans, fowl, and reptiles. Brain samples from fowl and reptiles were also analyzed, as were food products (boiled rice, green tea, and dried yeast).

Typical examples of mass fragmentograms obtained from the assay of PaA and HOPA in samples of human plasma, soft-shelled turtle brain, and

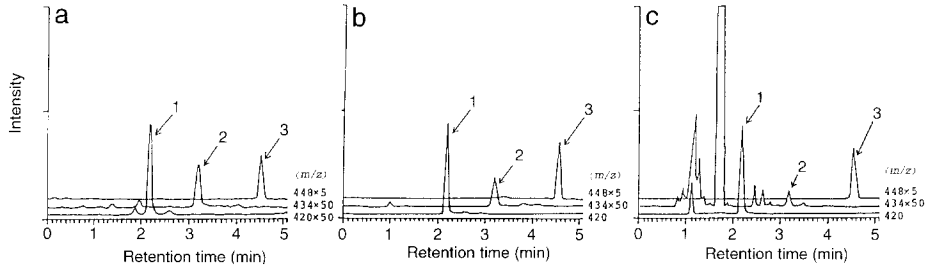

FIG. 3. Typical mass fragmentograms by the multiple ion detection method of (a) human plasma, (b) soft-shelled turtle brain, and (c) green tea. Peaks: 1, pantothenic acid; 2, hopantenic acid; 3, internal standard.

green tea are presented in Fig. 3. The contents of PaA and HOPA in samples were determined using previously obtained calibration curves with the peak intensity ratio of the trimethylsilyl derivatives of PaA (*m/z* 420) and HOPA (*m/z* 434) to that of the trimethylsilyl derivative of IS (*m/z* 449). The retention times of the trimethylsilyl derivatives of PaA, HOPA, and IS in the samples were ca. 2.2, 3.2, and 4.5 min, respectively.

TABLE I
LEVELS OF PANTOTHENIC ACID AND HOPANTENIC ACID IN NATURAL SUBSTANCES

Sample	PaA	HOPA
Plasma		
Human 1	23.2 ng/ml	7.4 ng/ml
Human 2	22.4 ng/ml	8.0 ng/ml
Human 3	25.1 ng/ml	5.4 ng/ml
Monkey	32.5 ng/ml	5.6 ng/ml
Rabbit	114.8 ng/ml	8.4 ng/ml
Mouse	234.3 ng/ml	15.7 ng/ml
Pig	74.8 ng/ml	2.4 ng/ml
Dog	30.4 ng/ml	11.6 ng/ml
Rat	403.3 ng/ml	9.6 ng/ml
Chicken	510.3 ng/ml	38.6 ng/ml
Soft-shelled turtle	267.9 ng/ml	38.1 ng/ml
Brain		
Chicken	3.46×10^4 ng/g	123.3 ng/g
Soft-shelled turtle	1.09×10^4 ng/g	121.3 ng/g
Natural products		
Dried yeast	1.65×10^5 ng/g	8.5×10^3 ng/g
Boiled rice	1.11×10^3 ng/g	21.2 ng/g
Green tea	1.26×10^4 ng/g	52.3 ng/g

Both PaA and HOPA were identified in all samples studied at concentrations shown in Table I. It was found that an average of 23.6 ng of PaA, and 6.9 ng of HOPA, per milliliter is contained in plasma obtained from healthy men ($n = 3$). The resulting value for the PaA content in human plasma agrees with the microbiological values reported previously.[14,15] Plasma levels of PaA and HOPA in monkeys were almost the same as those in humans. Pantothenic acid and HOPA in plasma samples of other animals, such as fowl and reptiles, ranged between 30.4 and 510.3 ng/ml and between 2.4 and 38.6 ng/ml, respectively. Furthermore, the PaA content in chicken brain was 280 times higher than that in plasma, and that in brain of soft-shelled turtle was ca. 90 times higher than that in plasma. The HOPA content of brain was detected at a level only three times higher than that plasma. In contrast, the PaA content in natural products was determined to be much higher than that in biological samples (Table I). It was found that the HOPA content in dried yeast is more than 400 times greater than that in boiled rice, and ca. 160 times greater than that in green tea. It has been reported that PaA is widely distributed in every organ and that ingested PaA has a tendency to accumulate in the brain.[16] It has also been suggested that HOPA passes the blood–brain barrier.[13] The experimental results show that both PaA and HOPA are found in a wide range of concentrations in every animal studied, including microorganisms. Pantothenic acid is generally present in higher concentrations than HOPA in both biological samples and in natural products.

Acknowledgments

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