Journal of Chromatography, 525 (1990) 255–264
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5057

SIMULTANEOUS DETERMINATION OF PANTOTHENIC ACID AND HOPANTENIC ACID IN BIOLOGICAL SAMPLES AND NATURAL PRODUCTS BY GAS CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

KIYOSHI BANNO*, MASAYUKI MATSUOKA, SHINGO HORIMOTO and JYOJI KATO

Analytical Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., 16-89, Kashima 3-chome, Yodogawa-ku, Osaka (Japan)

(First received July 31st, 1989; revised manuscript received October 2nd, 1989)

SUMMARY

A method for the simultaneous determination of pantothenic acid and hopantenic acid in plasma samples was developed using gas chromatography–mass spectrometry with multiple ion detection. Plasma samples were directly purified without deproteinization on an ion-exchange resin, and the eluate was extracted with ethyl acetate under acidic conditions. The organic layer was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in an internal standard solution. Pantothenic and hopantenic acids were converted into their trimethylsilyl derivatives by treating with bis(trimethylsilyl)trifluoroacetamide. Aliquots of this solution were injected into the gas chromatograph–mass spectrometer, which was equipped with a wide-bore fused-silica column (DB-17) and analysed by the multiple ion detection method. The detection limits for pantothenic acid and hopantenic acid in plasma were 1 ng/ml each at a signal-to-noise ratio of 5. This method was applied to a study of the assay of pantothenic acid and hopantenic acid in biological samples and natural products.

INTRODUCTION

Hopantenic acid (HOPA), a natural homologue of pantothenic acid (PaA), was discovered in biological fluids by paper chromatography [1, 2]. Its calcium salt has been used to improve blood circulation and metabolism in the brain. There are many reports on the pharmacokinetics and assay of HOPA. However, no report exists on the investigation of the simultaneous determination of PaA and HOPA in biological substances and in natural products. Biserte

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and co-workers succeeded in identifying a hopantenic compound as a γ-aminobutyric acid (GABA) condensation product in human urine by paper chromatography [3], and also in renal and hepatic tissue of normal rats [4–6]. It is very important for pharmacokinetic studies to separate and to determine HOPA as its GABA condensation product and PaA as its β-alanine condensation product.

Gas chromatography–mass fragmentography (GC–MF) with a packed column has been used to investigate the assay of 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 hydrolysate of HOPA [8], the colorimetric determination of GABA with sodium 1,2-naphthoquinone-4-sulphate [9] and a high-performance liquid chromatographic method with 9-anthryldiazomethane as a fluorescent derivative [10]. The bioassay method using *lactobacillus arabinosus* [11], and the determination of trimethylsilylated pantoyllactone by GC–MF were developed for the assay of PaA [12]. The methods described above, however, were not developed for the simultaneous determination of PaA and HOPA, and are not sensitive enough.

This paper reports the results of an investigation of the simultaneous rapid microanalysis of PaA and HOPA in biological samples and natural products by GC–MF with a wide-bore column and multiple ion detection (MID). The internal standard (I.S.) was 5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl) amino]pentanoic acid calcium salt. PaA and HOPA were measured simultaneously in plasma samples of humans, monkeys, dogs, pigs, rabbits, mice, rats, chickens and soft-shelled turtles, in brain samples of chickens and soft-shelled turtles and also in natural products of rice, green tea and dried yeast.

EXPERIMENTAL

Materials

Calcium hopantenate was synthesized at Tanabe Seiyaku. Calcium pantothenate was a reagent of the pharmacopoeia of Japan. 5-[(2,4-Dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid calcium salt (I.S.) was synthesized in our laboratory [13]. Hydrochloric acid, pyridine and ammonium sulphate were obtained from Katayama Chemicals (Osaka, Japan). Ethyl acetate and chloroform were liquid chromatography grade. The silylation reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), was obtained from Gaskuro Kogyo (Tokyo, Japan). Plasma samples were collected from healthy men, monkeys, dogs, pigs, rabbits, mice, rats, chickens and soft-shelled turtles. Brain samples were taken from a chicken and a soft-shelled turtle. Rice, green tea and dried yeast were used to provide samples of natural products.

Apparatus

The gas chromatograph–mass spectrometer was a Hitachi M-80A equipped with an M-003 computer system. The wide-bore fused-silica column was coated with DB-17 (15 m×0.53 mm I.D.) (J & W Scientific, Rancho Cordova, CA, U.S.A.). The flow-rate of helium as carrier gas was 15 ml/min, and the injection port, column oven and separator temperatures were 250, 200 and 250°C, respectively. The mass spectrometer was operated in the electron-impact mode at 70 eV, the ionization current was 100 μA and the temperature of the ion source was 200°C. The stable fragment ions selected for MID were at *m/z* 420, 434 and 448; these are the fragment ions $[M-CH_3]^+$, produced from the trimethylsilyl derivatives of PaA, HOPA and I.S., respectively.

Internal standard synthesis

A mixture of calcium (0.52 g, 0.013 mol) and ethanol (10 ml) was refluxed under a stream of nitrogen for 2 h. 5-Aminovaleric acid (2.93 g, 0.025 mol) was added to the mixture at 60°C. After stirring at 70°C for 1 h, *p*-pantoyllactone (3.51 g, 0.027 mol) was added at 30°C and the mixture was stirred at 40±1°C for 16 h. Ethanol (15 ml) was added to the mixture, which was then warmed to 80°C. Active carbon (0.08 g) was added, and the mixture filtered at 40°C. Distilled water (2 ml) was added to the filtrate. It was stirred for 16 h at 40°C, and at room temperature for 24 h. The mixture was freeze-dried and a colourless compound (2.8 g) was then obtained.

Plasma samples

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

The plasma samples (1.0 ml) were diluted to 2.0 ml with distilled water, applied to 2.5 ml of the 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) was added to the eluate and the mixture was shaken vigorously for 5 min. The aqueous layer (5.0 ml) was taken, and after addition of hydrochloric acid (0.5 ml), ammonium sulphate (5.0 g) and ethyl acetate (20.0 ml), the mixture was shaken vigorously for 15 min and centrifuged at 1800 *g* for 5 min. The supernatant was taken and filtered through a filter paper. The filtrate was extracted with ethyl acetate (20.0 ml) again and centrifuged. The supernatant was filtered as described above. The ethyl acetate layer containing PaA and HOPA was collected and evaporated at 40°C under nitrogen gas to a volume of ca. 0.5 ml. The concentrate was 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 the I.S. The mixture was evaporated to dryness again under the conditions mentioned above. The residue was subjected to trimethylsilylation at 80°C for 60 min in screw-capped reaction vials by the addition

of 50 μl each of pyridine and BSTFA. The solution was allowed to stand at room temperature for 5 min and then 1 μl was subjected to GC–MF with MID.

Brain samples

The wet brain samples of a chicken (0.60 g) and a soft-shelled turtle (0.66 g) were homogenized with 0.005 *M* potassium hydroxide solution (2 ml). Each mixture was centrifuged at 21 000 *g* for 20 min. Each supernatant (1.0 ml) was taken, applied to 2.5 ml of the ion-exchange resin (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) was added to boiled rice (10.0 g), green tea (1.0 g) and dried yeast (0.1 g), and shaken vigorously at 70°C for 30 min. After centrifugation at 1800 *g* for 5 min, 2.0 ml of each supernatant were taken, applied to 2.5 ml of the ion-exchange resin (H type, MCI GEL CK08P, 17 cm×10 mm I.D.) and taken through the same procedure as the plasma samples.

Calibration curves

The calibration curves (5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml) for the determination of PaA and HOPA were prepared by addition of calcium hopantenate and calcium pantothenate, respectively. These aqueous solutions were extracted with ethyl acetate under acidic conditions and then analysed by the procedure described above.

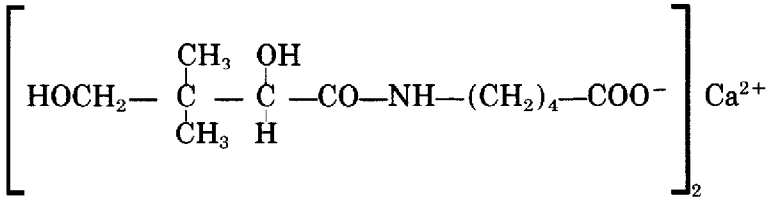
RESULTS AND DISCUSSION

Clean-up of PaA and HOPA in samples

The applicability of the direct purification procedure using the cation-exchange resin was investigated for biological samples and natural products. It was found that the basic interfering substances in biological samples and in natural products are rapidly eliminated almost entirely by the column of cation-exchange resin at a flow-rate of 0.5 ml/min. Furthermore, the eluate was purified by washing with chloroform. This procedure makes it possible to perform the test without adsorption on the cation-exchange resin and without transfer of PaA and HOPA to the chloroform layer. Calcium pantothenate and calcium hopantenate were extracted quantitatively with ethyl acetate from aqueous solutions acidified with hydrochloric acid in the presence of ammonium sulphate, in their calcium-free forms. This analytical procedure was found to be rapid and reliable, with the highest recoveries of PaA and HOPA.

Internal standard identification

The following chemical structure of the synthesized 5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid calcium salt was identified by elemental analysis, MS and ^1H NMR spectroscopy:



$C_{22}H_{40}CaN_2O_{10}$

This compound was chosen as the I.S. since it has the same chromatographic behaviour as PaA and HOPA, and it is not influenced by interfering substances. In addition, its trimethylsilyl derivative showed a stable intense ion suitable for MID.

Trimethylsilylation and mass chromatography

The derivatization of hydroxy and carboxyl groups with several types of trimethylsilylation reagent was investigated. It was found that the reaction proceeds quantitatively at 80°C for 60 min with BSTFA in a screw-capped reaction vial in the presence of pyridine. The best concentration of BSTFA in pyridine was found to be 50% (v/v) (Fig. 1). Pyridine was an effective catalyst for trimethylsilylation of hydroxy and carboxyl groups in PaA, HOPA and I.S. The trimethylsilyl derivatives of PaA, HOPA and I.S. showed a high detection sensitivity for the MID method and were completely separated by a wide-bore fused-silica DB-17 column (15 m×0.53 mm I.D.) (Fig. 2).

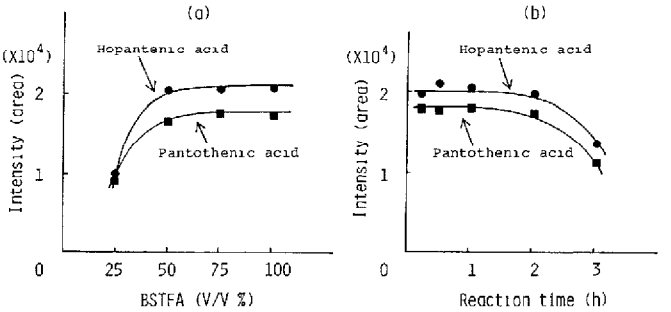


Fig. 1. Trimethylsilylation of (■) pantothenic acid and (●) hopantenic acid with BSTFA at 80°C. (a) Effect of BSTFA concentration; (b) effect of reaction time in pyridine.

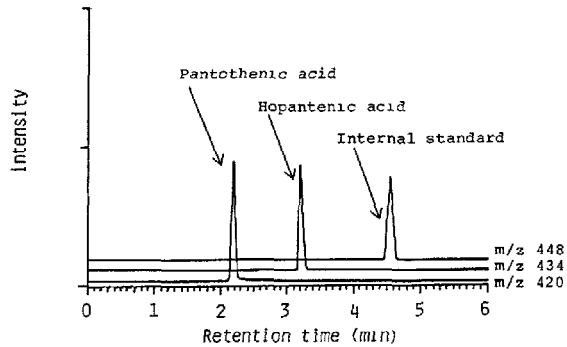


Fig. 2. Mass fragmentograms of the trimethylsilyl derivatives of pantothenic acid, hopantenic acid and internal standard using a wide-bore fused-silica column coated with DB-17 for gas chromatography.

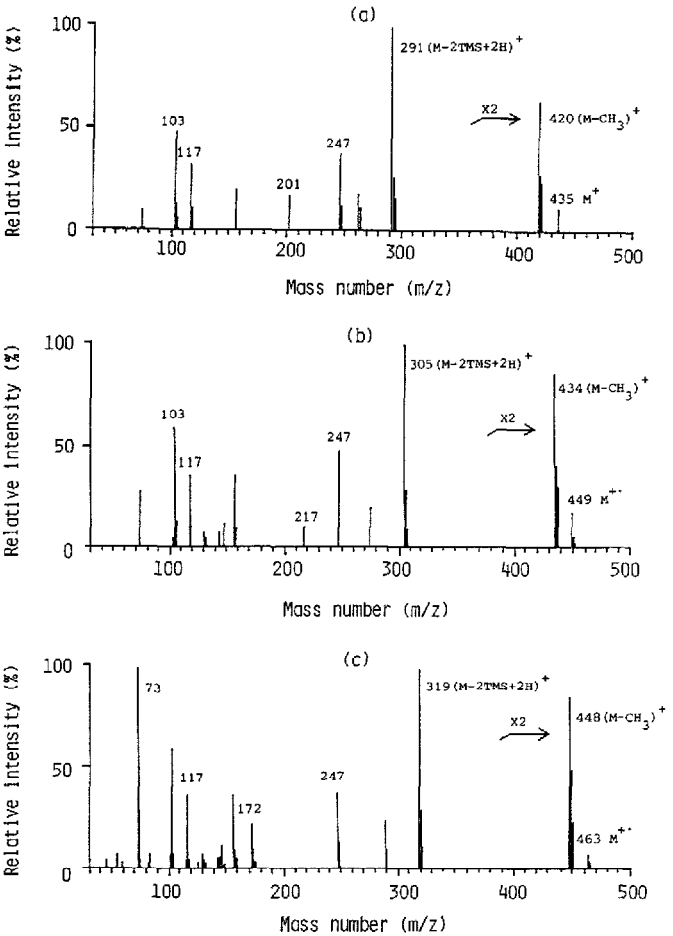


Fig. 3. Mass spectra of the trimethylsilyl derivatives of (a) pantothenic acid, (b) hopantenic acid and (c) internal standard.

Mass fragmentography with MID

The MID method for the simultaneous determination of trimethylsilyl PaA and trimethylsilyl HOPA was investigated in the constant volume of the trimethylsilyl I.S. solution. The stable mass fragment ions were detected at *m/z* 420, 434 and 448 as the characteristic $[M-CH_3]^+$ ions of the trimethylsilyl derivatives of PaA, HOPA and I.S., respectively (Fig. 3). These ions were selected for the simultaneous determination of PaA and HOPA by MID, because other ions did not interfere with the quantitation. On the other hand, as may be seen from Fig. 3, the base peak ions of trimethylsilyl derivatives of PaA, HOPA and I.S. were the $[M-2TMS+2H]^+$ ions at *m/z* 291, 305 and 319, respectively. It was found that base peak ions are not suitable for quantitation because they cannot be separated completely from the interfering substances in some samples.

Linearity and detection limit

The calibration curves were obtained by plotting the ratio of the peak intensity of the trimethylsilyl derivatives of PaA and HOPA to the intensity of the trimethylsilyl derivative of the I.S. against concentration. The calibration curves were linear in the range 5–100 ng/ml of plasma.

Because of no adsorption of trimethylsilyl PaA and HOPA and of a high theoretical plate number, the wide-bore fused-silica DB-17 column gave a sufficiently high sensitivity. As a result, the detection limits of PaA and HOPA using this method were ca. 1 ng/ml plasma samples.

Recovery from plasma samples

The recovery tests were performed with known amounts (10, 20, 30 ng) of PaA and HOPA added to human plasma (1 ml). The overall recoveries of PaA and HOPA were 92.9 ± 4.6 and $95.5 \pm 5.1\%$, respectively (Table I). It was suggested that the binding ratios of PaA and HOPA to plasma proteins are extremely low. These data suggest that the method described here is suitable for the simultaneous determination of PaA and HOPA. The recoveries of PaA and

TABLE I

RECOVERIES OF PANTOTHENIC ACID AND HOPATENIC ACID FROM HUMAN PLASMA ($n=4$)

Added (ng/ml)	PaA recovery (%)	HOPA recovery (%)
10.0	92.4	93.8
20 0	95.6	101.1
30.0	90.8	91.7
Mean ± S.D.	92.9 ± 4.6	95.5 ± 5.1

TABLE II

CONCENTRATIONS OF PANTOTHENIC ACID AND HOPATENIC ACID IN BIOLOGICAL SAMPLES AND NATURAL PRODUCTS

Samples	PaA	HOPA
Plasma samples (ng/ml)		
Human 1	23.2	7.4
Human 2	22.4	8.0
Human 3	25.1	5.4
Monkey	32 5	5.6
Rabbit	114.8	8.4
Mouse	234.3	15.7
Pig	74.8	2.4
Dog	30.4	11.6
Rat	403.3	9.6
Chicken	510.3	38.6
Soft-shelled turtle	267.9	38.1
Brain samples (ng/g)		
Chicken	$3.46 \cdot 10^4$	123.3
Soft-shelled turtle	$1.09 \cdot 10^4$	121.2
Natural products (ng/g)		
Dried yeast	$1.65 \cdot 10^5$	$8.5 \cdot 10^3$
Boiled rice	$1.11 \cdot 10^3$	21.2
Green tea	$1.26 \cdot 10^4$	52.3

HOPA were calculated by subtracting 23.2 ng/ml PaA and 7.4 ng/ml HOPA in human plasma (Table II).

Application and results

The measurements of PaA and HOPA in biological samples and natural products were carried out according to the analytical procedure described above. The experiments were applied to plasma samples from three humans, six kinds of animal, one species of fowl and one species of reptile. Brain samples from fowls and reptiles were also used. Natural products (boiled rice, green tea and dried yeast) were also investigated because we were interested in the content of PaA and HOPA in plants and microorganisms.

Typical examples of mass fragmentograms obtained from the assay of PaA and HOPA in samples of human plasma, soft-shelled turtle brain and green tea are presented in Fig. 4. 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 I.S. (m/z 449). The retention times of the trimethylsilyl derivatives of PaA, HOPA and I.S. in the samples were ca. 2.2, 3.2 and 4.5 min, respectively.

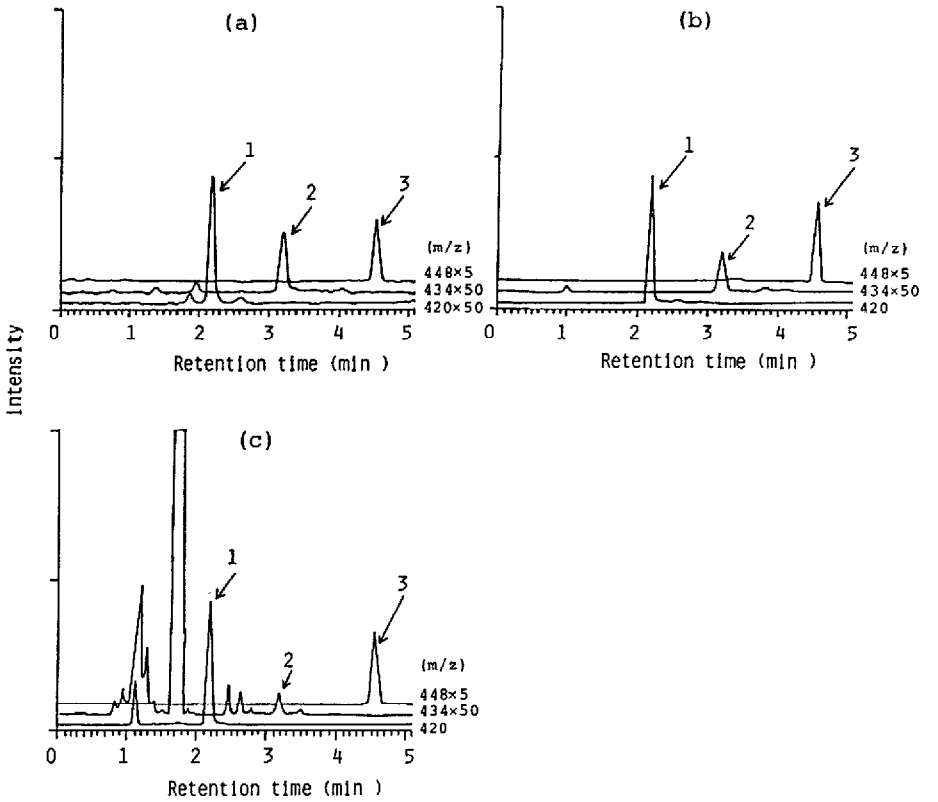


Fig. 4. Typical mass fragmentograms by the multiple ion detection method of (a) human plasma, (b) soft-shelled turtle brain and (c) green tea. Peaks: 1 = pantothenic acid; 2 = hopantenic acid; 3 = internal standard.

Both PaA and HOPA were identified in all the samples studied at the concentrations shown in Table II. It was found that an average of 23.6 ng/ml PaA and 6.9 ng/ml HOPA are 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. PaA and HOPA in plasma samples of other animals, such as fowls 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 a chicken brain was 280 times higher than that in plasma, and that in the brain of a soft-shelled turtle was ca. 90 times higher than that in plasma. The HOPA content was detected at a level only three times higher than that in plasma. On the other hand, the PaA content in natural products was determined to be much higher than that in biological samples, as may be seen from the experimental values. 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 is reported that PaA including β-alanine is widely distributed in every organ and that ingested PaA

has a tendency to accumulate in the brain [16]. It is also suggested that HOPA including GABA passes the blood–brain barier [13]. Our results show that PaA and HOPA are found in a wide range of concentrations in every animal studied, including fowls and reptiles, and every plant and microorganism. PaA is generally present in higher concentrations than HOPA in both biological samples and natural products. These results suggest that HOPA detected in animals is taken from foods such as plants, and that, unlike PaA, HOPA is not biosynthesized in animals.

CONCLUSIONS

The GC–MF technique with MID described here can be used for the simultaneous determination of PaA and HOPA in biological samples and natural products at the ng/g level. The recovery and sensitivity were higher than those of previous methods [7–12] because (i) the samples were directly purified without deproteinization by the ion-exchange resin and (ii) a wide-bore fused-silica DB-17 column was used instead of a packed column. The proposed simultaneous determination of PaA and HOPA is expected to be applicable to studies of bioavailability and pharmacokinetics, etc., after administration of calcium hopantenate.

ACKNOWLEDGEMENT

The authors thank Dr. Toshio Kakimoto, General Manager of our laboratory, for suggestions and discussion.

REFERENCES

1 P. Boulanger, G. Biserte and F. Courtot, Bull. Soc. Chim. Biol., 34 (1952) 366.
2 A.G. Moiseenok, V.M. Kopelevich, M.A Izraelit and L.M. Shmuilovich, Farmakal. Toksikol., 36 (1973) 489.
3 G Biserte, R. Plaquest and P. Boulanger, Bull. Soc. Chim. Biol., 37 (1955) 7.
4 G. Biserte, P. Boulanger, A. Finot, M Davril, E. Sacquet and H. Charlier, C.R. Acad. Sci. Paris, 260 (1965) 3219.
5 P. Boulanger, G. Biserte, M. Davril and M. Rache, C.R. Acad. Sci. Paris, 265 (1967) 157.
6 M Davril, G. Biserte and P. Boulanger, Biochimie, 53 (1971) 419.
7 Y. Umeno, K. Nakai, E. Matsushima and T. Marunaka, J. Chromatogr., 226 (1981) 333.
8 M. Anetai, T. Takahashi, H. Ogawa and H. Kaneshima, Hokkaidoritsu eisei kenkyushoho, 33 (1983) 138.
9 H. Terada, T. Hayashi, S. Kawai and T. Ohno, J. Chromatogr., 130 (1977) 281.
10 T. Fukuyama, T. Maki and M. Matsuoka, Jpn. Pat., 86 655 (1986) (unexamined).
11 H.R. Skeggs and L.D. Wright, J. Biol. Chem., 156 (1944) 21.
12 P. Tarli, Anal Biochem., 42 (1971) 8.
13 Y. Nishizawa and T. Kodama, Vitamins, 33 (1966) 589.
14 M. Hatano, J. Vitaminol., 8 (1962) 134.
15 I. Masugi, Vitamins, 46(5) (1972) 261.
16 T. Ariyama and S. Kimura, Vitamins, 22 (1961) 237.