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GAS CHROMATOGRAPHIC—MASS FRAGMENTOGRAPHIC DETERMINATION OF HOMOPANTOTHENIC ACID IN PLASMA

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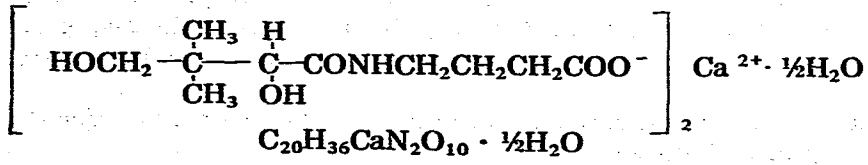
SUMMARY

A gas chromatographic—mass fragmentographic method was developed for the determination of homopantothenic acid in plasma. Acidified plasma was deproteinized by extraction with chloroform and subsequently the aqueous layer was extracted with ethyl acetate. The organic layer containing homopantothenic acid was reduced to dryness, and the resulting residue was redissolved in N,O-bis(trimethylsilyl)trifluoroacetamide—pyridine solution to allow trimethylsilylation. Aliquots of this solution were injected into the gas chromatograph—mass spectrometer and analyzed by the selected ion monitoring method using L-ascorbic acid as an internal standard. The detection limit for homopantothenic acid was 5 ng/ml of plasma.

A precise and sensitive assay for the determination of homopantothenic acid in plasma was established.

INTRODUCTION

Homopantothenic acid, which is a derivative of γ-aminobutyric acid, was first discovered in nature by Biserte et al. in 1955 [1]. It is known that homopantothenic acid improves the metabolism of glucose in brain and the higher functions of the brain [2—7]. This compound has been used clinically as a calcium salt, calcium hopantenate [calcium D-(+)-4-(2,4-dihydroxy-3,3-dimethylbutyramino)butyrate hemihydrate].



$$\left[HOCH_2-\underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{C}}-\underset{OH}{\overset{H}{C}}-CONHCH_2CH_2CH_2COO^- \right]_2 Ca^{2+} \cdot \tfrac{1}{2}H_2O$$

$$C_{20}H_{36}CaN_2O_{10} \cdot \tfrac{1}{2}H_2O$$

A colorimetric method for the assay of homopantothenic acid has been reported [8] in which homopantothenic acid is hydrolyzed to γ-aminobutyric acid in an alkaline solution and reacted with sodium 1,2-naphthoquinone-4-sulfate, then determined colorimetrically. This method, however, was not found to be sensitive enough for the determination of homopantothenic acid in plasma after administration of calcium hopantenate.

Therefore, we investigated the determination of homopantothenic acid in plasma by two methods, gas—liquid chromatography (GLC) with a flame-ionization detector and gas chromatography—mass fragmentography (GC—MF). The former method was not adequate because of poor sensitivity, while the second method provided high precision and sensitivity. This paper describes these results.

EXPERIMENTAL

Materials

Calcium hopantenate was synthesized and purified in our laboratory [9, 10]. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine for the silylating solvent were purchased from Pierce (Rockford, IL, U.S.A.). L-Ascorbic acid and the other chemicals used were obtained from Wako Pure Chemicals (Osaka, Japan). Ethyl acetate and chloroform were liquid-chromatography grade materials.

The samples of blood were collected from healthy men.

Instrumental

For GC—MF, a JEOL JMS D-300 mass spectrometer with an electron-impact ion source connected to a JEOL JGC-20kP gas chromatograph (Tokyo, Japan) was used.

The coiled glass column (1.0 m × 2.0 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80—100 mesh) (Gaschro Kogyo Co., Tokyo, Japan) and conditioned at 280°C for 24 h. The injector, column and ion source temperatures were 250°C, 220°C and 230°C, respectively. After homopantothenic acid and its internal standard had been detected, the temperature of the column was raised to 280°C for 3 min to burn out the remaining materials and then was returned to the operational temperature before the next analysis. The carrier gas was helium and the flow-rate was 30 ml/min.

The mass spectrometer was used under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3.0 kV; ion multiplier voltage, 1.4 kV. The fragment ions selected for mass fragmentographic analysis were the ions at *m/z* 434 and 449, which are produced from the molecular ion by the loss of a methyl group, $[M-CH_3]^+$, for the respective trimethylsilyl derivatives of homopantothenic acid and the internal standard, L-ascorbic acid.

The mass spectra of these derivatized compounds were recorded under the same conditions as described above.

For GLC, a Shimadzu GC4-CM gas chromatograph with a flame-ionization detector (Kyoto, Japan) was used.

The conditions of the gas chromatograph were as follows: a coiled glass column (1.0 m × 3.0 mm I.D.) packed with 3% OV-17 on Chromosorb W AW (80—100 mesh); injector and detector temperatures of 280°C; an initial column temperature of 170°C and a temperature rise of 6°C/min to 290°C; flow-rate of nitrogen as carrier gas, 60 ml/min.

Analytical procedure

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

The plasma (1.0 ml) was diluted to 2.0 ml with distilled water, adjusted to pH 2.0 with 5 *N* HCl and shaken vigorously with 20 ml of chloroform at room temperature for 10 min. The aqueous layer was separated, neutralized with sodium hydroxide solution followed by the addition of 1.0 ml of distilled water, and centrifuged at 2000 *g* for 10 min to remove proteins. The supernatant was readjusted to pH 2.0 with 5 *N* HCl and was shaken vigorously with 40 ml of ethyl acetate for 20 min. The ethyl acetate layer containing homopantothenic acid was separated, evaporated at 30°C, transferred to a 1.0-ml reaction vial by washing with methanol and concentrated to dryness in a water-bath under nitrogen gas. Then the residue was mixed with methanol containing 10.0 μg of L-ascorbic acid as internal standard and concentrated to dryness again under the same conditions as described above. The residue was dried thoroughly over phosphorus pentoxide under reduced pressure and subjected to trimethylsilylation at 70°C for 20 min by the addition of a freshly prepared solution of 100 μl of pyridine containing 20% BSTFA. After cooling, 1—2 μl of this solution were injected into the GC—MF apparatus.

Calibration curve

A calibration curve for homopantothenic acid analyzed by the GC—MF method was prepared by adding known amounts of calcium hopantenate (0.05, 0.10, 0.50, 1.00, 2.00, 5.00 and 10.0 μg/ml) to plasma (1.0 ml) and then analyzing by the same extraction procedure.

The calibration curve was obtained by plotting the ratio of the peak height of the trimethylsilyl derivative of homopantothenic acid to that of the trimethylsilyl derivative of L-ascorbic acid as an internal standard against concentration. This calibration curve was linear.

RESULTS AND DISCUSSION

The investigation of the extraction procedure for calcium hopantenate from the aqueous solution using several kinds of organic solvents showed that this compound could be recovered quantitatively from aqueous solution acidified with hydrochloric acid with ethyl acetate, in its free form — homopantothenic acid. Therefore, the following procedure was employed. The samples of plasma were deproteinized by extraction with chloroform under acidic conditions and the supernatant was extracted with ethyl acetate. This procedure was found to be the simplest and most rapid, and to be the most reliable with the highest recovery of homopantothenic acid. Furthermore, it was found that homopantothenic acid was not tranferred to the chloroform layer under the

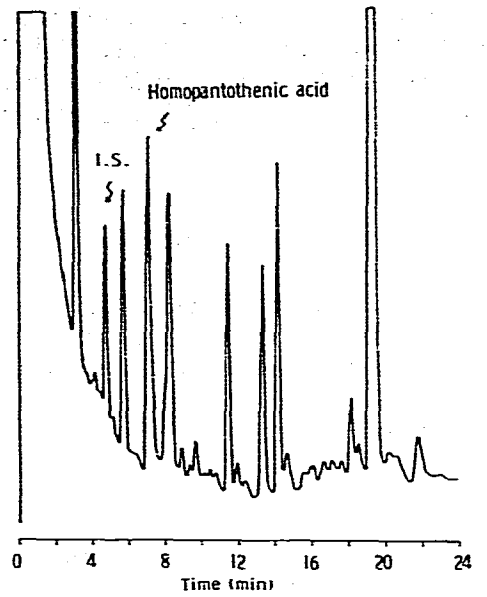


Fig. 1. Gas chromatogram showing the separation of homopantothenic acid extracted from human plasma with L-ascorbic acid as internal standard (I.S.). The result is for the trimethylsilyl derivative.

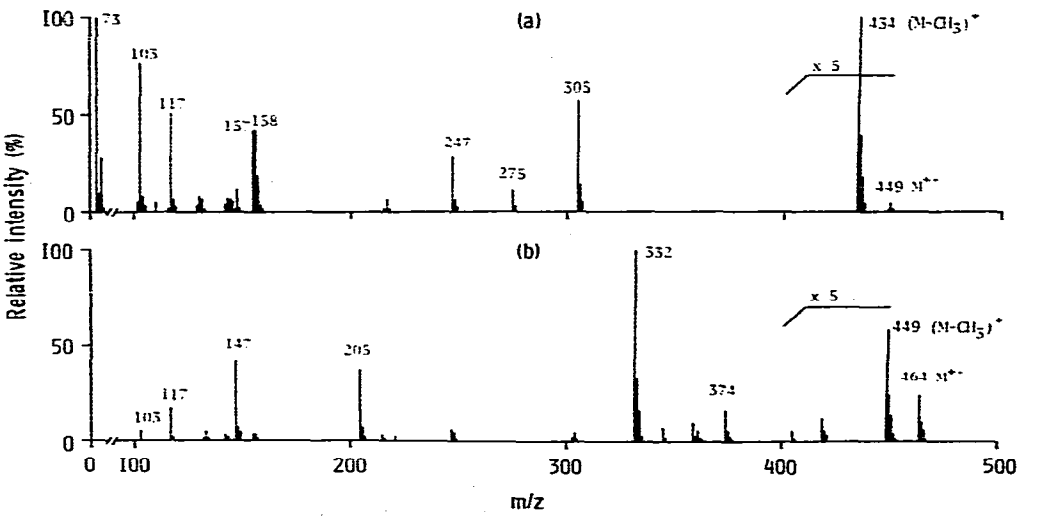


Fig. 2. Mass spectra of the trimethylsilyl derivatives of (a) homopantothenic acid and (b) L-ascorbic acid.

acidic condition described above.

On the basis of these results, a method for assay of homopantothenic acid was investigated for the subsequent experiments.

The GLC method was examined first for the determination of homopantothenic acid extracted from human plasma. To obtain a derivative of homopantothenic acid suitable for GLC, the procedures of trimethylsilylation

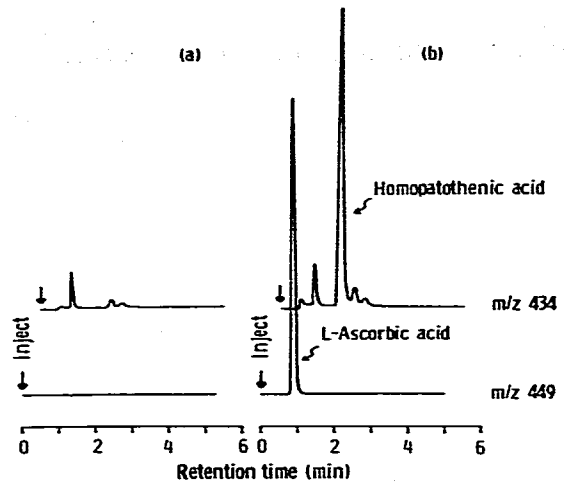


Fig. 3. Mass fragmentograms showing the separation of (a) control and (b) homopantothenic acid extracted from human plasma with L-ascorbic acid as internal standard. Results are for the trimethylsilyl derivatives.

with BSTFA or N,O-bis(trimethylsilyl)acetamide, and those of methylation with diazomethane or an on-column methylating agent (2% phenyltrimethylammonium hydroxide in methanol) were compared. The trimethylsilylation procedure with BSTFA was found to result in a quantitative reaction and a higher detection sensitivity. On the other hand, L-ascorbic acid was chosen as internal standard since it can be trimethylsilylated in the same way as homopantothenic acid. These trimethylsilyl derivatives were found to be stable at 5°C for 1—2 days.

The GLC method based on trimethylsilylation gave a good separation of homopantothenic acid from human plasma components, as shown in Fig. 1. The retention times of the trimethylsilyl derivatives of homopantothenic acid and the internal standard L-ascorbic acid were 7.2 and 4.8 min, respectively. The detection limit of homopantothenic acid in this GLC method was 1.0 μg/ml of plasma. This sensitivity is poor and not suitable for the assay of the plasma levels of homopantothenic acid found after administration of calcium hopantenate.

Next, the method for assay of trimethylsilyl homopantothenic acid by GC—MF was investigated. L-Ascorbic acid was also used as internal standard for the determination using the multiple-ion detection technique. The mass fragment ions detected for GC—MF were the $[M—CH_3]^+$ ion at *m/z* 434 and 449 in the mass spectra of the trimethylsilyl derivatives of homopantothenic acid and L-ascorbic acid, respectively (Fig. 2), since their ions at *m/z* 305 and 332, which are present in higher intensity than each $[M—CH_3]^+$ ion, were not separated clearly in some samples. In addition, the ions at *m/z* 449 are present in the spectra of L-ascorbic acid and homopantothenic acid, but since the two compounds were well separated by GC, there is no interference in the quantitation.

The GC—MF separation pattern of the trimethylsilyl derivative of homo-

TABLE I

RECOVERIES ON EXTRACTION OF HOMOPANTOTHENIC ACID FROM PLASMA

Each value is the mean of three determinations.

Added (μg/ml)	Recovery from plasma (%)
0.05	90.8
0.10	89.9
0.50	88.7
1.00	90.1
2.00	89.8
5.00	89.6
10.00	89.6
Mean ± S.D. (%)	89.7 ± 3.2

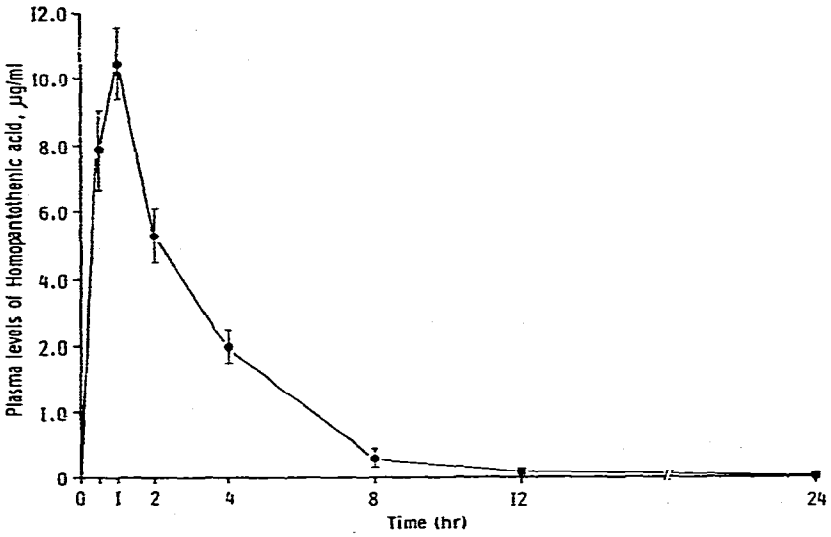


Fig. 4. Plasma levels of homopantothenic acid after oral administration of calcium hopantenate in a dose of 750 mg to ten healthy volunteers. Each point is the mean ± S.D. of ten men.

pantothenic acid extracted from human plasma following addition of 0.10 μg/ml calcium hopantenate and that of a control human plasma extract are shown in Fig. 3. The retention times of homopantothenic acid and the internal standard as their trimethylsilyl derivatives were 2.2 and 0.8 min, respectively.

The detection limit for homopantothenic acid in this GC—MF method was 5 ng/ml of plasma, which was sufficiently high. The reproducibility of the method was ± 3.1%. Thus, the present method appears to be satisfactory for the determination of homopantothenic acid in plasma.

In addition, γ-aminobutyric acid had no appreciable influence on the measurement of homopantothenic acid with the present method, because the retention time of γ-aminobutyric acid (0.6 min) was different from that of

homopantothenic acid. On the other hand, homopantothenic acid was not decomposed to γ-aminobutyric acid under the procedure described above.

Known amounts of calcium hopantenate were added to human plasma, and then the recovery of homopantothenic acid was determined. As shown in Table I, the overall recovery of homopantothenic acid was 89.7 ± 3.2%.

Furthermore, the following experiments were conducted. The stability of calcium hopantenate in the freezed-stocked plasma and that of homopantothenic acid in the cooled-stocked extract of plasma were examined. No significant decomposition was observed.

Finally, a 750-mg dose of calcium hopantenate was administered orally to healthy volunteers, and the concentration of homopantothenic acid in the plasma was determined by the present GC—MF method. The results obtained are shown in Fig. 4.

The present method also can be applied to plasma of animals. The results obtained for the chromatographic separation, recovery, precision and sensitivity were in good agreement with those obtained with human plasma.

The present GC—MF method is precise and has a higher sensitivity than the colorimetric or GLC method.

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