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Analytical studies on the chiral separation and simultaneous determination of pantothenic acid and hopantenic acid enantiomers in rat plasma by gas chromatography–mass fragmentography

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ABSTRACT

The chiral separation and simultaneous determination of D- and L-pantothenic acids and D- and L-hopantenic acids in rat plasma using gas chromatography–mass fragmentography are described. The method is based on deproteinization by ion-exchange resin, extraction with ethyl acetate under acidic conditions, and derivatization to form several interesting compounds. After methyl esterification for carboxylic acid of D- and L-pantothenic acids, D- and L-hopantenic acids could be derivatized to trifluoroacetate, cyclic sulphite, and cyclic *n*-butylboronate for hydroxy groups. D- and L-forms of these derivatives were completely separated by mass fragmentographic technique with quasi-molecular ions. Calcium salt of D-5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid was used as an internal standard for the determination of DL-pantothenic acids and DL-hopantenic acids. The detection limits of DL-pantothenic acids and DL-hopantenic acids in this method were 5 and 12 ng, respectively. This method could be applied to the study of plasma levels of D-, L-pantothenic acids and D-, L-hopantenic acids in rat.

INTRODUCTION

Pantothenic acid (PaA) exists in natural products and biological substances, and hopantenic acid (HOPA) was detected in biological fluids by paper chromatography [1–2] as a natural homologue of PaA. A calcium salt of HOPA has been used for improving blood circulation and metabolism in the brain. Biserte *et al.* [3] succeeded in identifying a hopantenic compound as γ-aminobutyric acid (GABA) by means of paper chromatography. This hopantenic compound is a condensation product in human urine and is also found in normal rat renal and hepatic tissue [4–6].

A gas chromatographic–mass fragmentographic (GC–MF) system equipped with a packed column was used for the assay of HOPA in plasma or urine after the administration of calcium hopantenate [7]. Moreover, other assay methods have been reported for HOPA, such as the GC–MF determination of pantoyllactone which is a hydrolysate of HOPA [8], the colorimetric determination of GA-

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BA with sodium 1,2-naphthoquinone-4-sulphate [9] and the high-performance liquid chromatographic (HPLC) method with 9-anthrylidiazomethane as a fluorescent derivative [10]. The bioassay method using *Lactobacillus arabinosus* [11] and the determination of trimethylsilylated pantoyllactone by GC–MF were found as the assay of PaA [12].

Chiral separation of D- and L-PaA was reported by Koenig and Sturm [13], using the chiral polysiloxane phases XE-60–L-Val-(*S*)- or (*R*)-α-phenylethylamide, coated on Pyrex glass of fused-silica capillary columns. However, no report exists on the analytical studies of the chiral separation and simultaneous determination of D-, L-PaA and D-, L-HOPA in rat plasma. It is very important to determine the optical antipode in the development of new drugs.

We have already developed a rapid microanalysis for PaA and HOPA in biological substances and natural products using GC–MF [14]. This paper describes an analytical procedure for the chiral separation and simultaneous determination of D-, L-PaA and D-, L-HOPA in rat plasma using GC–MF with a fused-silica Chirasil–D-Val capillary column, after deproteinization and derivatization.

EXPERIMENTAL

Materials

D-HOPA was synthesized at Tanabe Seiyaku. D-, L- and DL-PaA used the pharmacopoeia of Japan XI material. L- and DL-HOPA were synthesized in the same manner as in the reaction with 5-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-amino]pentanoic acid calcium salt (internal standard) described in a previous paper [14]. Hydrochloric acid, methanol, dichloromethane, chloroform, ethyl acetate, pyridine and trifluoroacetic anhydride were obtained from Katayama Chemical (Osaka, Japan). Dichloromethane and thionyl chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). *n*-Butylboronic acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Plasma samples were collected from rats. A quantitative filter paper (No. 5C type) was obtained from Toyo Roshi (Tokyo, Japan).

Apparatus

GC–mass spectrometric (MS) measurements were made by a Shimadzu QP-1000 gas chromatograph–mass spectrometer equipped with a data processing system and by a Hitachi M-80A gas chromatograph–mass spectrometer equipped with an M-003 computer system. A fused-silica capillary column Chiralsil–D-Val (20 m × 0.22 mm I.D., film thickness 0.12 μm) coated with L-valine-*tert.*-butylamide (Gasukuro Kogyo, Tokyo, Japan) was connected to the GC–MS system. Helium was used as the carrier gas at 1.0 kg/cm^2. A split injection system CLH-702 (Shimadzu, Kyoto, Japan) was used with a split flow-rate of 27.5 ml/min (split ratio 1:30). The temperatures of the injection port, ion source and

separator were 180, 250, and 250°C, respectively. The oven temperature was maintained at 130°C.

Deproteinization

The plasma sample (1.0 ml) was charged to 2.5 ml of the anion-exchange resin (Cl type, MCI GEL CAO8P, 170 mm × 10 mm I.D.). After washing the resin with distilled water several times, PaA and HOPA were eluted twice with 2.5 ml of 1 *M* sodium chloride solution. Hydrochloric acid (6 *M*, 0.5 ml), ammonium sulphate (5.0 g) and ethyl acetate (20.0 ml) were added to the eluate and the mixture was shaken vigorously for 15 min and centrifuged at 1800 *g* for 5 min. The supernatant was withdrawn and filtered through a filter paper with a pore size of 1 μm.

The filtrate was shaken with ethyl acetate (20.0 ml) again and centrifuged. The supernatant was filtered as decribed above. The ethyl acetate layer was evaporated at 60°C under a stream of nitrogen. Then, the residue was derivatized (see *Derivatization*). After derivatization, the sample solution (2.0 μl) was injected into the GC–MS system.

Derivatization

Methyl esterification. DL-PaA, DL-HOPA and the internal standard were esterified with 1.5 *M* hydrochloric acid in methanol at room temperature for 1 h. Then, the solvents were removed under a stream of nitrogen. The residue was derivatized by the following three derivatizations.

Trifluoroacetylation. Dichloromethane (400 μl) and trifluoroacetic anhydride (100 μl) were added to the methyl esters, and the mixture was kept at room temperature for 30 min. After the removal of the excess reagent at 60°C under a stream of nitrogen, the sample was dissolved in dichloromethane (100 μl).

Cyclic sulfination. Dry pyridine (1 μl) and ethanol-free chloroform (200 μl) were added by stirring, followed by thionyl chloride (10 μl) and the mixture was kept at room temperature for 1.5–2 h. After the removal of the solvents at 60°C under a stream of nitrogen, the sample was dissolved in dichloromethane (100 μl).

Cyclic n-butylboronation. A solution of *n*-butylboronic acid (0.1 mg/ml, 1 ml) in ethyl acetate was added and heated at 60°C for 10 min. After the removal of solvents under a stream of nitrogen, the sample was dissolved in dichloromethane (100 μl).

The derivatization scheme described above is shown in Fig. 1.

Calibration curves. The calibration curves (50, 100, 500 ng/ml, 1 and 2 μg/ml) for the determination of DL-PaA and DL-HOPA were prepared by the addition of calcium pantothenate and calcium hopantenate, respectively. After the addition of the internal standard (2 μg), these aqueous solutions were extracted with ethyl acetate under acidic conditions and then analysed by the procedure described above.

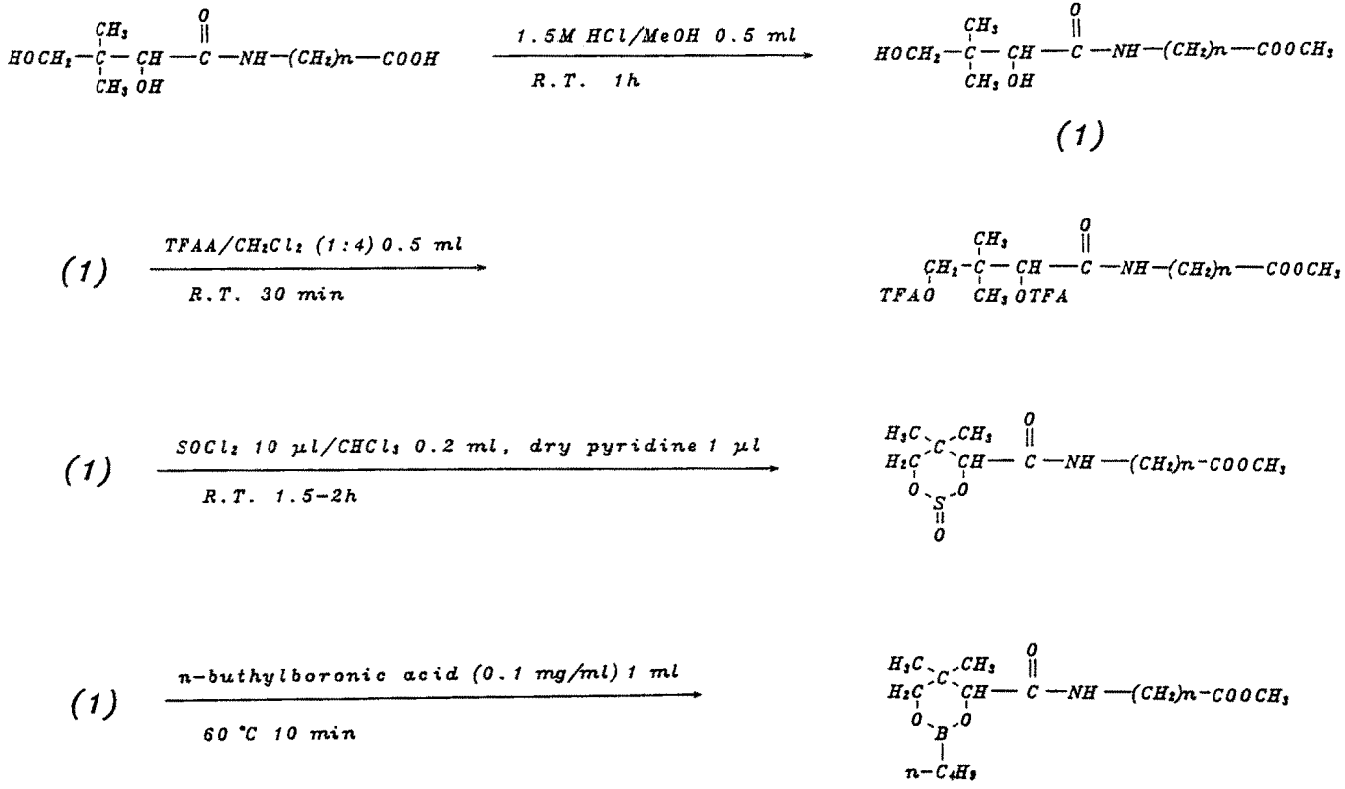

Fig. 1. Derivatization scheme for DL-PaA ($n=2$), DL-HOPA ($n=3$) and the internal standard ($n=4$).

RESULTS AND DISCUSSION

Purification for plasma samples

The applicability of the direct purification procedure using the anion-exchange resin was investigated for plasma samples. It was found that the basic interfering substances in the biological samples were rapidly almost entirely eliminated by employing the anion-exchange column at a flow-rate of 0.5 ml/min. Then, PaA and HOPA were extracted quantitatively with ethyl acetate from the aqueous solutions acidified with 6 *M* hydrochloric acid in the presence of ammonium sulphate.

Derivatization and mass chromatography

For GC–MS analyses, the column temperature must be decreased when compared with the usual GC system because of the reduced pressure. Thus, the samples must be converted into volatile derivatives. Esterification of the carboxylic group, and acetylation, cyclic sulphination and cyclic *n*-butylboronation of the two hydroxy groups were tried in order to obtain volatile derivatives. The mass spectrum of each derivative was identified using a Hitachi M-80A gas chromatograph–mass spectrometer (Figs. 2–4). After several investigations, it was found that the enantiomers of D-, L-PaA and D-, L-HOPA could be completely separated with Chirasil–D-Val (20 m × 0.22 mm I.D.); methyl esterification for the carboxylic group, and trifluoroacetylation, cyclic sulphination or cyclic *n*-butylboronation for the two hydroxy group (Figs. 2–4). Because the two hydroxy groups were in 2- and 4-positions, PaA and HOPA could be derivatized to cyclic sulphinates and cyclic *n*-butylboronates, giving six-membered rings. In this case, the volatility

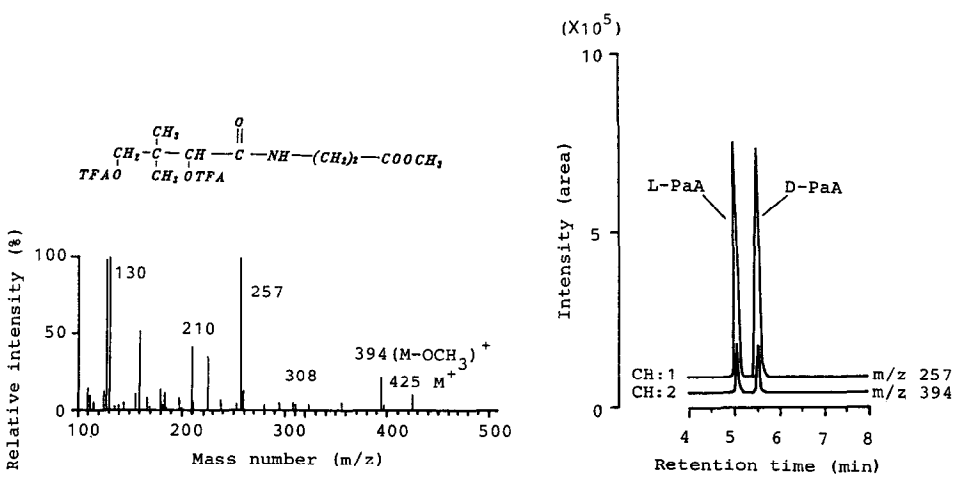

Fig. 2. Mass spectrum and mass fragmentogram of trifluoroacetate derivatives of DL-PaA (injection level, 20 ng per 2 μl).

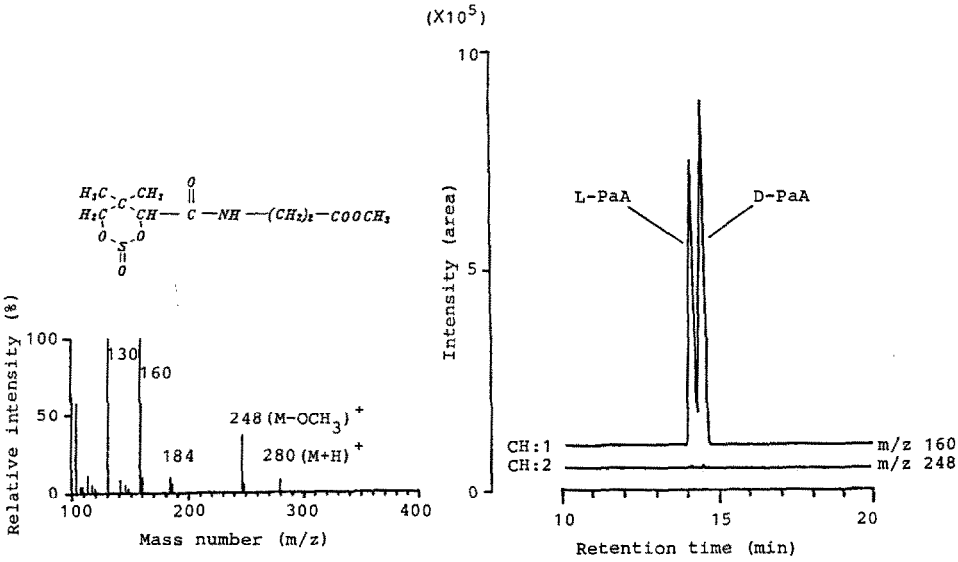

Fig. 3. Mass spectrum and mass fragmentogram of cyclic sulphinate derivatives of DL-PaA (injection level, 20 ng per 2 μl).

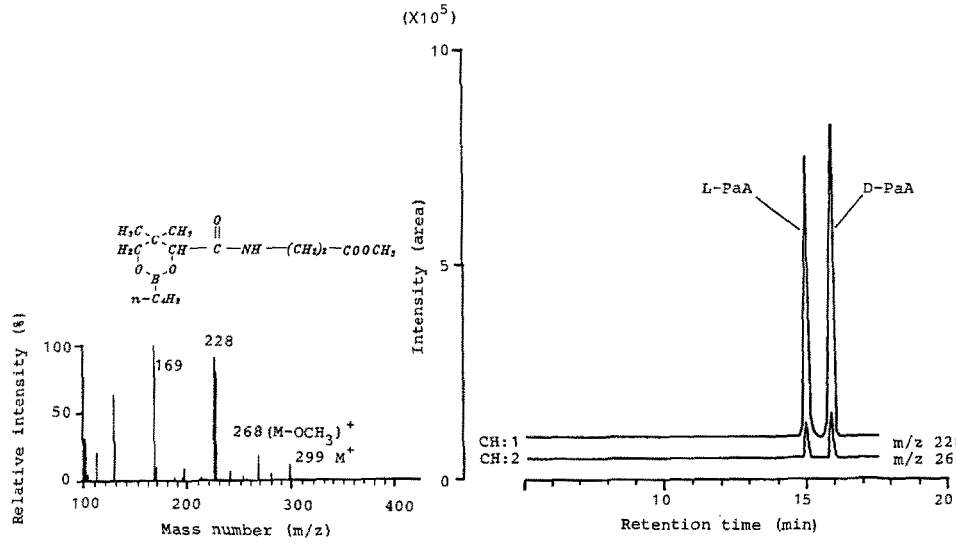

Fig. 4. Mass spectrum and mass fragmentogram of cyclic *n*-butylboronate derivatives of DL-PaA (injection level, 20 ng per 2 μl).

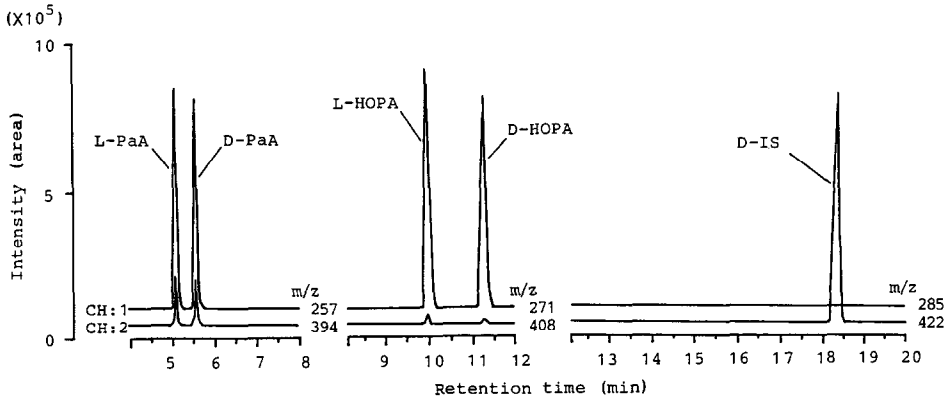

Fig. 5. Mass fragmentograms of the trifluoroacetates of DL-PaA, DL-HOPA and D-internal standard using a Chirasil–D-Val capillary column (injection level, 20 ng each per 2 μl).

of the PaA and HOPA with cyclic sulphinate and cyclic derivatives was decreased in comparison to that of the trifluroacetyl derivative.

These three derivatives could be simply prepared in a short period of time, and they were found to be stable in a tight container for one month. The resolution factors between D-PaA and L-PaA were 1.60 (trifluoroacetates), 1.16 (cyclic sulphinates) and 1.55 (cyclic boronates), respectively. All resolution factors were sufficiently high, but trifluoroacetylation resulted in the best resolution factor and was used for the measurement of samples in the shortest time period. The mass fragmentogram of trifluoroacetates for DL-PaA, DL-HOPA and the D-internal standard is shown in Fig. 5. The ratio of area intensities of L-PaA and D-PaA is 1.04.

MF with selected-ion monitoring

The MF method for the simultaneous determination of DL-PaA and DL-HOPA was investigated in the constant volume of the internal standard solution. The

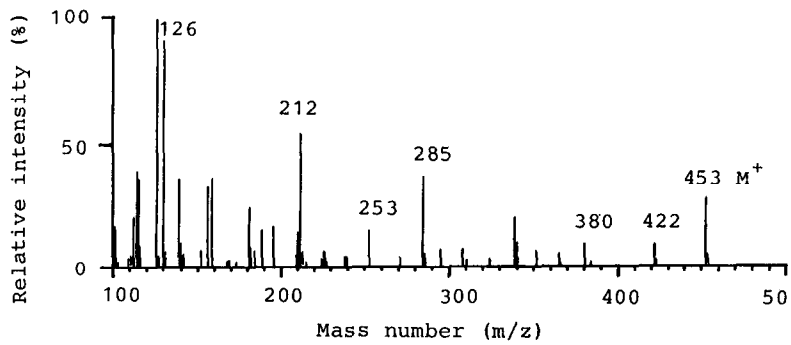

Fig. 6. Mass spectrum of the trifluoroacetate derivatives of the internal standard.

stable mass fragment ions were detected at *m/z* 257 and 271 as being the characteristic ions of the PaA and HOPA derivatives, respectively (Fig. 2–4). A stable mass fragment ion was detected at *m/z* 422 as being the characteristic ion ($[M-OCH_3]^+$) of the D-internal standard derivative, and a molecular ion (M^+) was detected at *m/z* 453 (Fig. 6). In this case, the *m/z* 422 ion was selected in preference to the *m/z* 453 ion having twice the intensive signal, since the peak intensity of the *m/z* 422 ion was stable compared with that of the *m/z* 453 ion. These ions were selected for the simultaneous determination of DL-PaA and DL-HOPA by MF, because other ions did not interfere with the quantitation.

Linearity

The calibration curves were obtained by plotting the ratio of the peak intensities of the derivatives of DL-PaA and DL-HOPA to the intensity of the internal standard against concentration. The calibration curves were linear in the 50–2000 ng/ml range.

Determination of D- and L-PaA and D- and L-HOPA in rat plasma

The applicability of the method to the simultaneous determination of DL-PaA and DL-HOPA was examined. Known amounts (5, 20 μg) of DL-PaA and DL-HOPA were added to rat plasma and the recoveries were determined. The recoveries of L-PaA and L-HOPA were 93.5 ± 5.5 and 92.4 ± 8.3% ($n=3$), respectively. Accuracy and reproducibility were found to lie within acceptable limits for the proposed method (Table I). This demonstrates that the proposed method is applicable to the quantitation of PaA and HOPA enantiomers in plasma since it is satisfactorily accurate and precise. The detection limits of DL-PaA and DL-HOPA were 5 and 12 ng/ml, respectively, under the measurement conditions of the injection sample volume (5 μl), the split ratio (1:20) and the signal-to-noise ratio of 4.

Using this method the D-PaA levels in rat plasma were found to be 400 ± 15 ng/ml ($n=3$) (Fig. 7). However, the levels of L-PaA, D- and L-HOPA in rat plasma were below the limits of detection. These results suggest that D-PaA in rat plasma is derived from foods such as plants.

TABLE I

ACCURACY OF DL-PaA AND DL-HOPA DETERMINATIONS ($n=7$)

Compound	Concentration added (ng/ml)	Calculated mean level (ng/ml)	C.V. (%)
DL-PaA	10	8.26	7.2
	20	18.11	6.0
DL-HOPA	20	17.95	8.7
	40	37.23	6.9

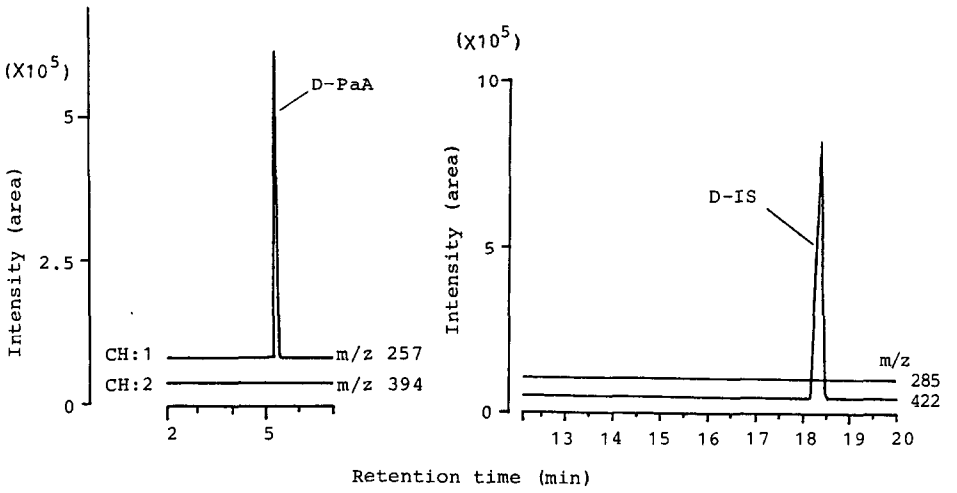

Fig. 7. Typical mass fragmentogram of rat plasma.

CONCLUSIONS

GC–MF can be used for the chiral separation and simultaneous determination of DL-PaA and DL-HOPA in rat plasma at ng/ml levels. The proposed chiral separation and simultaneous determination of D-, L-PaA and D-, L-HOPA are expected to be useful for the pharmacokinetic studies of enantiomeric drugs.

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