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DETERMINATION OF THE ANTI-ULCER AGENT GERANYLGERANYLACETONE IN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A highly specific and sensitive method for the determination of the anti-ulcer drug geranylgeranylacetone (GGA) in human serum is described. The extract from serum with hexane was saponified with potassium hydroxide and subjected to silica gel column chromatography to remove interfering substances. GGA in the partially purified extract was then reacted with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine and measured by selected ion monitoring using gas chromatography—mass spectrometry. A low detection limit (1 ng/ml) and high precision were obtained.

INTRODUCTION

Geranylgeranylacetone (6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraene-2-one) (GGA, Fig. 1), is a newly synthesized polyisoprenoid compound that has been shown to be effective on several experimentally induced ulcers in rats [1]. The evaluation of the clinical usefulness of GGA on gastric ulcers is now in progress.

In order to carry out pharmacokinetic studies, a specific and sensitive method for the determination of GGA in biological samples was required. In this work, gas chromatography—mass spectrometry (GC—MS) has been studied for the separation and determination of GGA in biological fluids.

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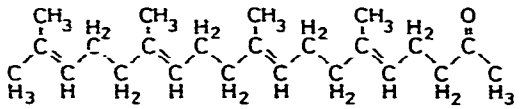

Fig. 1. Structure of all-*trans*-GGA.

The mass spectrum of GGA did not give fragment ions suitable for determination by GC—MS, while an oxime derivative of GGA [GGA-O-(2,3,4,5,6-pentafluorobenzyl)oxime] was found to give a fragment ion with the intensity and *m/z* suitable for the assay.

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA) has been developed as a novel derivatizing reagent for carbonyl compounds for electron-capture GC. Kobayashi et al. [2] reported the usefulness of PFBOA for the GC of low-molecular-weight carbonyl compounds. However, it was found that the resulting oxime of GGA (GGA-O-PFBO) shows four peaks on the gas chromatogram as GGA formed *syn*- and *anti*-oximes of *cis*- and *trans*-GGA.

This paper describes a highly specific and sensitive method using GC—MS which can overcome this disadvantage and is sensitive enough for the measurement of GGA in human serum.

EXPERIMENTAL

Reagents and chemicals

The purity of GGA used in this study was 98.7%, as checked by GC, and was a mixture of *cis*-5-*trans*-9,13,17- (35.3%) and *trans*-5,9,13,17- (64.7%) isomers. Pure *cis*- and *trans*-forms were also used, the purities of which were 95.0% and 98.3%, respectively.

PFBOA was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) according to the procedure of Nambara et al. [3] and Youngdale [4]. White leaflets (m.p. 115°C) were obtained. Elemental analysis: calculated for $C_7H_4NOF_5 \cdot HCl$, C 33.69, H 2.02, N 5.61; found, C 33.69, H 1.97, N 5.52%.

A stock PFBOA solution was prepared by dissolving PFBOA in distilled pyridine (25 mg/ml), and was stable for several weeks when stored in the dark in a refrigerator.

Cholestane used as internal standard, was obtained from Applied Science Labs. State College, PA, U.S.A.). Silica gel for column chromatography (70—230 mesh) was purchased from Merck (Darmstadt, G.F.R.). Hexane, ethyl acetate and pyridine were of analytical-reagent grade and distilled prior to use. All other reagents were of analytical-reagent grade and were obtained from commercial sources.

Standards and calibration samples

A stock standard GGA solution was prepared by dissolving 1 mg in 1 ml of ethanol containing 0.2% of α-tocopherol as an antioxidant. Dilute working standard solutions were prepared as follows: the GGA stock solution was diluted with ethanol to give ten batches covering the concentration range

5 ng/ml—5 μg/ml. Calibration samples were prepared as follows: to 1 ml of serum, 0.2 ml of ethanol solution from each batch was added and the mixture was allowed to stand for 30 min.

Glassware

The glassware was cleaned using normal laboratory washers and rinsed with acetone before use.

Extraction and partial purification of GGA from serum

To each 1 ml of serum, 1 ml of ethanol was added and mixed on a vortex mixer. After addition of 0.1 ml of 50% potassium hydroxide solution, the mixture was saponified for 10 min in a boiling water-bath. After cooling, the mixture was shaken with 5 ml of hexane for 10 min and centrifuged for 5 min at 1900 *g*. This extraction procedure was repeated twice and the combined organic layer was submitted to silica gel column chromatography. The silica gel, which had been allowed to swell for at least 2 h in hexane containing 5% of ethyl acetate, was packed in a glass column (7 cm × 1 cm I.D.) with continuous tapping, and washed with 6 ml of hexane. The hexane extract from serum was applied on the top of the column and adsorbed at a flow-rate of about 1 ml/min. After the column had been washed with 20 ml of hexane containing 1% of ethyl acetate, the eluent was changed to hexane containing 10% of ethyl acetate. The first 3 ml of the effluent was discarded and the next 6 ml of the fraction containing GGA was collected and evaporated to dryness under a gentle stream of nitrogen at 50°C and subjected to derivatization.

Derivatization and removal of excess of PFBOA

The residue was dissolved in 0.2 ml of PFBOA solution (25 mg/ml), heated at 60°C for 2 h and 2.5 ml of 1 *N* hydrochloric acid were added. The mixture was shaken mechanically with 5 ml of hexane for 10 min and centrifuged for 5 min at 1900 *g*. The organic layer was separated and 1 ml of an internal standard solution of cholestane (2 μg/ml) was added. After evaporation to dryness under a gentle stream of nitrogen at 50°C, the residue was dissolved in 0.1 ml of ethyl acetate and a 2—4-μl portion was injected into the GC—MS system.

Apparatus

Analyses were carried out on a Shimadzu-LKB 9000 GC—MS instrument with an electron impact source and a total ion current detector. A coiled Pyrex column (1 m × 3 mm I.D.) filled with 3% OV-17 on Gas-Chrom Q (100—120 mesh) was used. The column was operated isothermally at 270°C, the temperatures of the injection port, separator and ion source being kept at 300, 300 and 330°C, respectively. Helium was used as carrier gas at a flow-rate of 30 ml/min. The ionization energy was 25 eV and the trap current was 60 μA.

Calibration graph

The calibration graph was prepared by subjecting human serum calibration samples to the above procedures. The ratio of the peak height of GGA-O-

PFBO at *m/z* 320 to that of cholestane at *m/z* 357 was plotted against GGA concentration.

Serum samples

Serum samples were taken in syringes by venipuncture over a 48-h period after a single oral dose of 150 mg of GGA (as a capsule) to three male healthy volunteers 30 min after breakfast. The serum was obtained by letting the blood clot, followed by centrifugation, and was stored frozen until taken for analysis.

RESULTS AND DISCUSSION

Extraction and partial purification of GGA

As the hexane extract from serum was found to contain endogenous lipids that interfered in the GC—MS analysis, silica gel column chromatography was applied to remove these substances. Each fraction eluted from the column was examined by a thin-layer chromatographic system with flame ionization detection (TLC-FID) (Iatroscan TFG-10, Iatron Labs.), where the silica gel-coated glass rods (Chromarods) were developed in hexane—diethyl ether—formic acid (95:5:4) (Fig. 2). As shown in the TLC-FID trace (Fig. 2), lipids such as cholesterol (Ch), cholesterol esters (ChE) such as cholesterol palmitate and stearate, fatty acids (FA) such as oleic, stearic and palmitic acid and monoglycerides (MG) such as monopalmitate and monoolein in serum were removed by silica gel column chromatography, while triglycerides (TG) such as tristearin and triolein moved together with GGA on the column, and were removed by saponification of serum prior to hexane extraction. Both *cis*- and *trans*-isomers of GGA in serum were demonstrated to be stable during the saponification process.

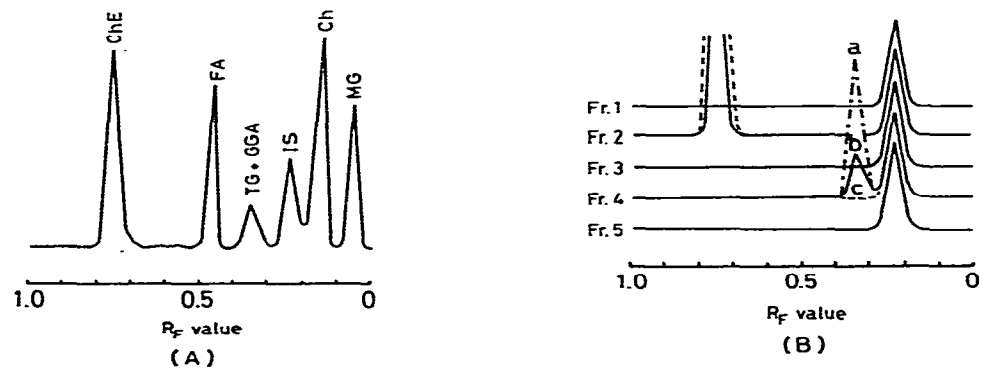

Fig. 2. TLC-FID traces of (A) authentic samples and (B) fractions from silica gel chromatography of hexane extracts from (a) serum spiked with GGA after saponification, (b) serum without saponification and (c) serum after saponification. ChE = cholesterol palmitate or stearate; FA = oleic, stearic or palmitic acid; TG = tristearin or triolein; IS = stearyl alcohol as internal standard; Ch = cholesterol; MG = monopalmitate or monoolein.

Derivatization of GGA to GGA-O-PFBO

GGA gave a complex spectrum under EI ionization (Fig. 3), in which frag-

ment ions with a mass higher than *m/z* 150 were not abundant and the molecular ion was not observed. The CI mass spectrum obtained on a Shimadzu-LKB 9000B GC—MS system using methane, isobutane and ammonia as reagent gas also showed that there was no prominent ion with sufficient intensity for GC—MS analysis.

Investigation of several kinds of derivatives of GGA revealed that GGA-O-PFBO was the most suitable, based on stability, volatility and the fragmentation pattern in the EI mass spectrum.

The influence of the concentration of PFBOA on the reaction in pyridine at 60°C is illustrated in Fig. 4, where it can be seen that the quantitative

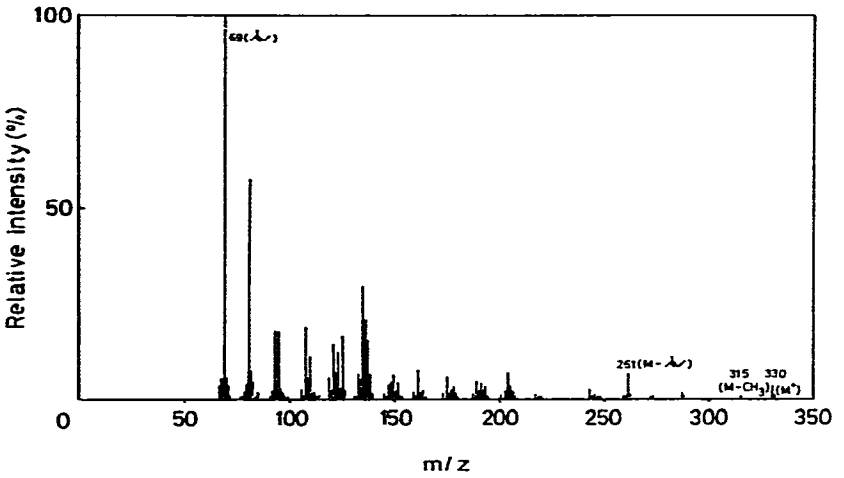

Fig. 3. Electron impact mass spectrum of GGA.

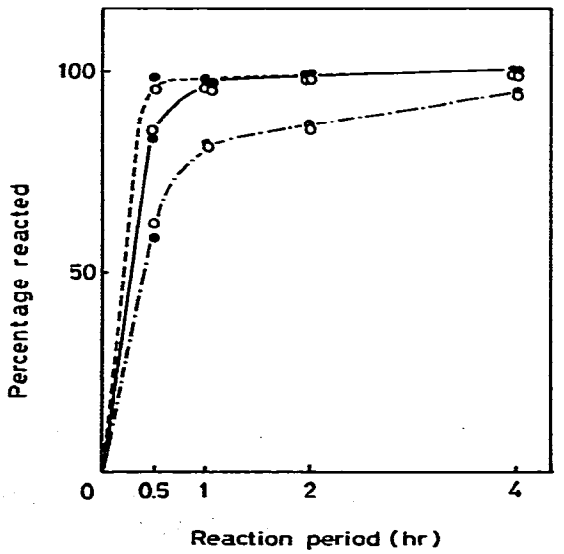

Fig. 4. Effect of the concentration of PFBOA on the formation of *cis*- (○) and *trans*-isomers (●) of GGA-O-PFBO at 60°C. Concentrations: (—•—) 2, (——) 5 and (— — —) 10 mg per 0.2 ml.

formation of GGA-O-PFBO was achieved in 2 h when 5 mg of PFBOA were used.

GC—MS measurement

When GGA-O-PFBO was subjected to GC—MS, four peaks were observed on total ion monitoring, as shown in Fig. 5. Derivatization of many compounds with carbonyl groups to the corresponding oximes has been reported to form *syn*- and *anti*-isomers and to give two peaks on the gas chromatogram [5]. As GGA itself is a mixture of *cis*- and *trans*-isomers, the four peaks of GGA-O-PFBO might be due to the *syn*- and *anti*-isomers of each *cis*- and *trans*-isomer.

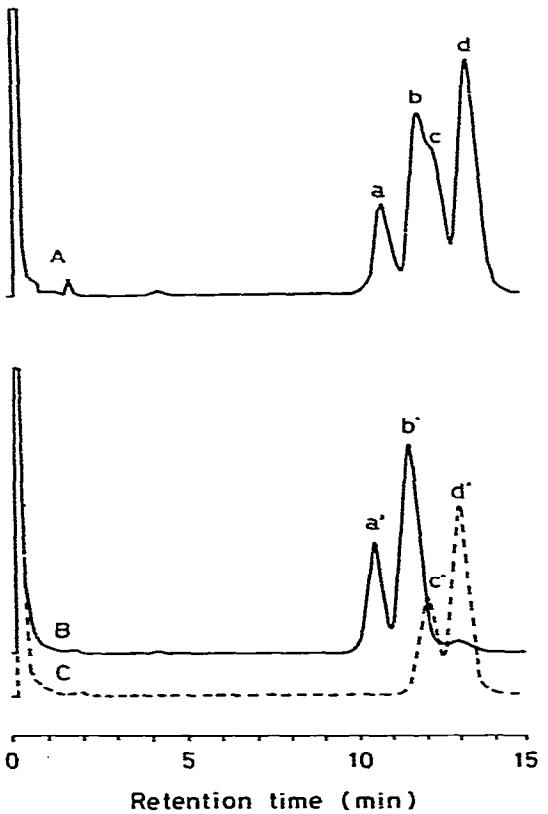

Fig. 5. Total ion current chromatograms of (A) GGA, (B) pure *cis*- and (C) pure *trans*-isomer of GGA after derivatization.

When pure *cis*- and *trans*-isomers of GGA were separately reacted with PFBOA and subjected to GC—MS, the pure *cis*-isomer gave two peaks (a′ and b′) and the pure *trans*-isomer gave two peaks (c′ and d′) (Fig. 5). The retention times and mass spectra of a′, b′, c′ and d′ were idential with those of a, b, c and d, respectively. Peaks a and b were tentatively identified as the *syn*- and *anti*-isomers of *cis*-GGA-O-PFBO, respectively, and peaks c and d as the *syn*- and *anti*-isomers of *trans*-GGA-O-PFBO, respectively [6].

The appearance of multiple peaks on the gas chromatogram is troublesome for GC analysis. However, as shown in Fig. 6, the peak intensity of the fragment ion of *m/z* 320, used as the monitoring ion for measurement of GGA, was very different between *syn*- and *anti*-forms of the *cis*-isomer.

Both spectra showed the molecular ion at *m/z* 525 and the base peak at *m/z* 69, which was the characteristic fragment ion of terpenoids [7]. However, in the *anti*-isomer of *cis*-GGA, an ion of *m/z* 320 was observed as an intense fragment ion, the intensity of which was about eight times that of the *syn*-isomer. Similar mass spectra were also obtained with *trans*-GGA-O-PFBO. This fragment ion was assigned to the structure shown in Fig. 6 with the aid of exact measurement; elemental compsition, $C_{15}H_{15}NOF_5$; accurate mass, 320.107; found, 320.105.

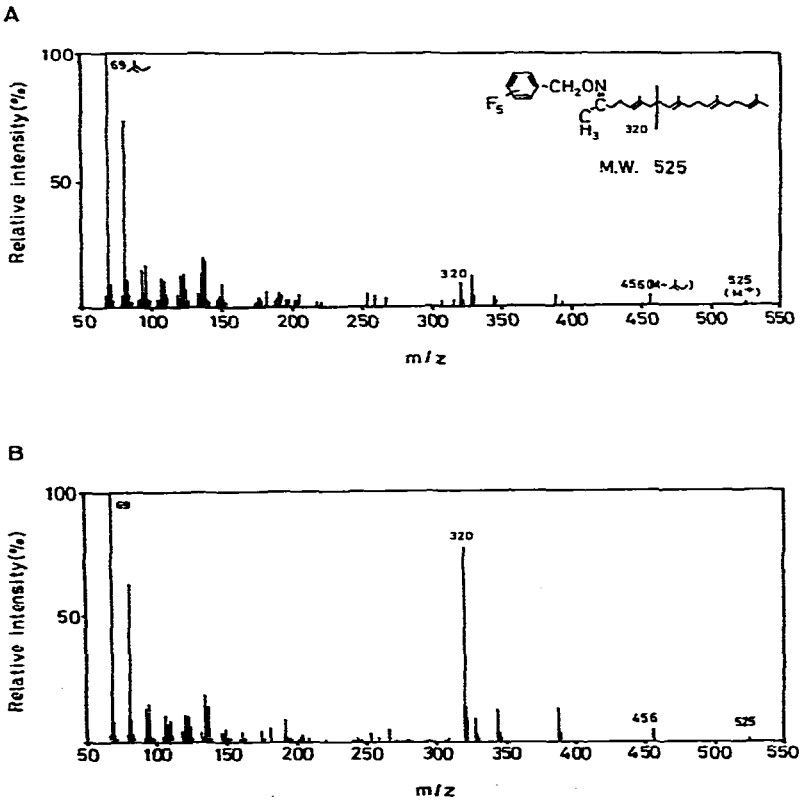

Fig. 6. Electron-impact mass spectra of (A) *syn*- and (B) *anti*-isomer of *cis*-GGA-O-PFBO.

Fig. 7 shows the selected ion monitoring of pure *cis*- and *trans*-GGA after derivatization. The peak height of the *syn*-isomer is 5% of that of *anti*-isomer, as expected from the intensity of the ion at *m/z* 320 in the mass spectrum of each isomer.

The ratio of the amount of *syn*-isomer to that of the *anti*-isomer formed by derivatization was almost constant (Table I).

Consequently, the difficulty with the determination of the concentrations of *cis*- and *trans*-GGA-O-PFBO caused by multiple peaks on the gas chromato-

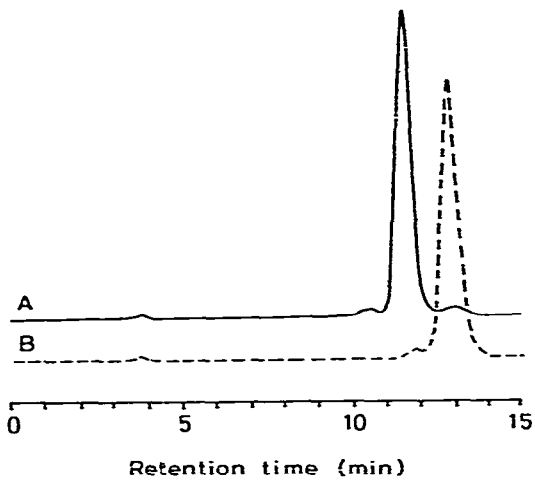

Fig. 7. Mass fragmentograms (*m/z* 320) of (A) *cis*- and (B) *trans*-isomers of GGA-O-PFBO.

TABLE I

RATIO OF AMOUNTS OF *SYN*- AND *ANTI*-ISOMERS FORMED BY DERIVATIZATION OF GGA

Figures represent mean ± S.D. from three measurements by GC with a flame ionization detector.

Isomer of GGA	Amount of GGA reacted (μg)	*Syn*-isomer	*Anti*-isomer
Pure *cis*-	1	32.1±2.3	67.9±2.2
	5	33.1±3.0	66.9±3.0
	10	32.2±2.5	67.8±2.4
Pure *trans*-	1	33.3±2.4	66.7±2.3
	5	32.5±2.8	67.5±2.8
	10	32.6±2.9	67.4±2.8

TABLE II

ACCURACY AND PRECISION OF THE DETERMINATION OF GGA IN SERUM

Isomer	Parameter	Amount of GGA added to serum (ng/ml)				
		1	5	25	100	1000
Cis-	Content (ng/ml)	0.353	1.77	8.83	35.3	353
	Amount found (n=3)	0.341	1.73	8.52	34.8	345
	Precision (C.V., %)	4.7	3.6	2.8	2.4	2.8
Trans-	Content (ng/ml)	0.647	3.24	16.2	64.7	647
	Amount found (n=3)	0.640	3.20	15.9	63.9	638
	Precision (C.V., %)	4.9	3.8	2.6	3.1	2.9

grams could be overcome by focusing on the fragment ion at *m/z* 320 of the *anti*-isomer.

Accuracy and specificity

The selected ion monitoring chromatograms of a control serum sample and the same sample spiked with 100 ng of GGA are shown in Fig. 8. Each serum sample contained 2 μg of cholestane as an internal standard.

The calibration graph was linear in the range 1—1000 ng/ml. The coefficient of variation was less than 5% in the concentration range tested (Table II).

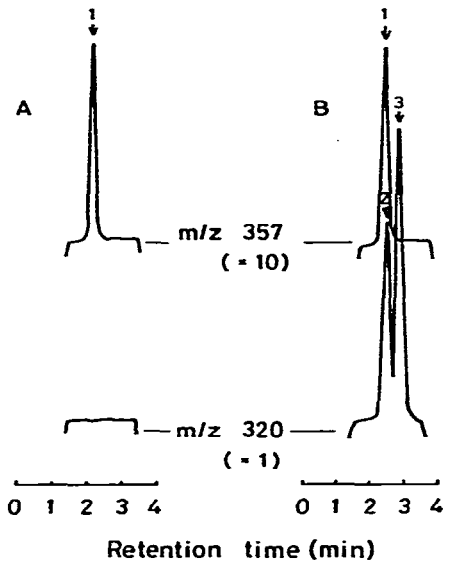

Fig. 8. Examples of chromatograms: (A) extract of blank serum to which 2 μg of cholestane alone had been added; (B) extract of serum to which 100 ng of GGA and 2 μg of cholestane had been added. Peaks 1, 2 and 3 are cholestane, *cis*-GGA and *trans*-GGA, respectively.

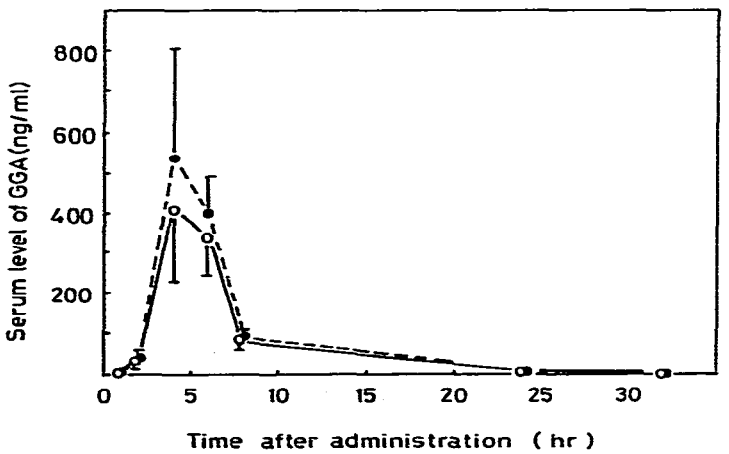

Fig. 9. Average serum levels of *cis*- (○) and *trans*-isomers (●) of GGA following an oral dose of 150 mg to three volunteers. Each bar represents the standard error of means.

Application of the method

The time course of serum GGA levels was examined after an oral dose of 150 mg to three volunteers (Fig. 9). The peak average serum level was observed after about 4 h and could be detected until 32 h after administration, indicating that this method would be appropriate for pharmacokinetic studies in humans.

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