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Gas chromatographic–mass spectrometric determination of tiopronin in human blood using acrylic acid esters as a derivatization reagent for the thiol group

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ABSTRACT

A gas chromatographic–mass spectrometric method for determining tiopronin, which has a thiol group, in human blood has been described. To prevent the oxidative degradation of tiopronin in the blood, its thiol group was immediately protected by treatment with isobutyl acrylate, which reacted readily with tiopronin in a 0.1 *M* Na_2HPO_4 solution (pH 9.1). The reaction was quantitative within 30 min. The blood sample was deproteinized and purified by a combination of liquid–liquid extraction and solid-phase extraction. Finally, the carboxyl moiety of the ester adduct was derivatized to the pentafluorobenzyl ester. The derivatives of tiopronin and the internal standard were analysed with gas chromatography–mass spectrometry. The precision of the method was satisfactory, and the calibration curve had good linearity in the concentration range investigated. The limit of determination of tiopronin in blood was estimated to be *ca.* 1 ng/ml.

INTRODUCTION

Tiopronin, N-(2-mercaptopropionyl)glycine (Thiola), has been used for many years as a hepatoprotective agent [1,2], an antidote to heavy metal poisoning [3,4] and an anti-cataract agent [5,6]. Pharmacokinetic studies of tiopronin are important, but they have not been carried out satisfactorily because of the difficulty of determining tiopronin in blood. Tiopronin contains a thiol group, which is easily oxidized, so derivatization has to be carried out immediately after sampling. The determination of tiopronin in biological fluids has been accomplished by high-performance liquid chromatography (HPLC) using an N-substituted maleimide derivative for the derivatization of the thiol group [7,8].

N-Ethylmaleimide (NEM) and other maleimide derivatives have been used for the derivatization of many other compounds containing a thiol group [9–12], but the resulting maleimide adduct generates an asymmetric carbon in the C-2 position of the maleimide moiety. When the analytical compound has an asymmetric carbon, the derivatization with the maleimide derivative leads to the formation of two diastereoisomers, which are occasionally separable by chromatography. These diastereoisomers complicate the chromatograms [7,10].

Acrylic acid esters have been used as specific reagents for the modification of protein thiol groups in the biochemical field for a long time [13,14]. As acrylic acid esters have an α,β-unsaturated structure, like maleimide, it seems possible

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to derivatize a thiol group completely, without generating an asymmetric carbon by addition of the thiol group.

This paper describes a method for determining tiopronin in human blood with gas chromatography–mass spectrometry, using acrylic acid esters to derivatize the thiol group.

EXPERIMENTAL

Materials

Tiopronin and SA16 (internal standard, I.S.), which is a homologue of tiopronin, were synthesized in our laboratory. Methyl acrylate (MA), ethyl acrylate (EA), isobutyl acrylate (IA) and pentafluorobenzyl bromide (PFBBr) were purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan), diisopropylethylamine was purchased from Aldrich (Milwaukee, WI, USA), and a Bond Elut SI column was purchased from Analytichem International (Harbor City, CA, USA). All other chemicals were of analytical-reagent grade.

Gas chromatography–mass spectrometry

A Varian GC3400 (Walnut Creek, CA, USA) was used with a DB-1 fused-silica capillary column (13 m × 0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA). The column was directly connected to the ion source of the mass spectrometer. The carrier gas was helium at a linear velocity of 40 cm/s. The oven temperature was operated isothermally at 80°C for 1 min after injection, heated at 20°C/min to 195°C and then at 15°C/min to 295°C. The injection was made in the splitless mode, and the inlet purge was turned on 0.75 min after injection. The injection and the transfer line temperatures were maintained at 260 and 250°C, respectively. A Finnigan Mat INCOS500 mass spectrometer (San Jose, CA, USA) was used. The ion-source temperature was maintained at 180°C, and the electron multiplier voltage was set at 1400 V. The electron ionization (EI) mode was used, and the monitored ions were *m/z* 311 for the tiopronin-IA-PFB derivative and *m/z* 339 for the SA16-IA-PFB derivative. The sampling time was set at 100 ms per mass.

Investigation of reactivity of derivatization reagents

A 50-μl volume of 3.4 m*M* tiopronin aqueous solution was added to 0.5 ml of 0.1 *M* Na_2HPO_4 (pH 9.1), and then 50 μl of 5% (v/v) MA, EA or IA in acetonitrile were added. After vortex-mixing, the mixture was left at room temperature. The reaction product was measured at 5, 10, 15 and 30 min after the reaction started.

Investigation of concentration of derivatization reagent

A 50-μl volume of 3.4 m*M* tiopronin solution was added to each 0.25 ml of 0.1 *M* Na_2HPO_4 (pH 9.1) and blood, and then 50 μl of 0.1, 0.5, 2, 5 or 10% (v/v) IA in acetonitrile were added. The solution was mixed with a vortex mixer and left at room temperature for 30 min. The subsequent procedure was carried out as described below.

Sample preparation

A 50-μl volume each of 5% (v/v) IA–acetonitrile solution and of I.S. aqueous solution (SA16, 2 μg/ml) were mixed with 0.25 ml of human blood and 0.25 ml of 0.1 *M* Na_2HPO_4 (pH 9.1), and the reaction mixture was left at room temperature for 30 min. The resulting mixture was deproteinized by treatment with 1 ml of acetone, and the precipitate was removed by centrifugation at 1600 *g* for 10 min. The supernatant was evaporated under a stream of nitrogen to less than 0.2 ml. Water (0.75 ml) was added to the remaining solution, which was then washed twice with 1.5 ml of ethyl acetate. The aqueous layer was acidified with 0.2 ml of 1 *M* hydrochloric acid, and extracted twice with 1.5 ml of ethyl acetate. The organic layer was evaporated to dryness. A 0.1-ml volume of 3% (v/v) PFBBr–acetonitrile solution and 10 μl of diisopropylethylamine were added to the residue, and left at 50°C for 30 min. The reaction mixture was evaporated under a stream of nitrogen. The residue was dissolved in 1 ml of *n*-hexane–benzene (1:1) and applied to a Bond Elut SI column (sorbent mass: 100 mg), which had been activated by rinsing with 0.5 ml of *n*-hexane–benzene (1:1). The column was rinsed with 0.5 ml of *n*-hexane–benzene

(1:1) and then the analyte was eluted with 1 ml of *n*-hexane–acetone (9:1). The eluate was evaporated to dryness, the residue was dissolved in 0.1 ml of ethyl acetate, and a 1-μl aliquot of this solution was subjected to GC–MS.

RESULTS AND DISCUSSION

It has been demonstrated that immediate derivatization of the thiol group is required to prevent oxidative degradation of tiopronin in biological fluids. As derivatization with acrylic acid esters does not generate an asymmetric carbon, we first investigated the reactivity of acrylic acid esters towards tiopronin in 0.1 *M* Na_2HPO_4 (pH 9.1).

When treated with MA, EA or IA in 0.1 *M* Na_2HPO_4 (pH 9.1), tiopronin was converted into the corresponding S-alkyl derivatives. The time-courses for the derivatization of tiopronin are shown in Fig. 1. The derivatization proceeded readily at room temperature and quantitatively within 15 min with each acrylic acid ester. IA was used for the derivatization of tiopronin in this study, because it had an appropriate retention time on GC. The derivatization rates of tiopronin with different IA concentrations in the mixture of blood and 0.1 *M* Na_2HPO_4 (pH 9.1) are shown in Fig. 2. The peak area of the IA adduct increased with increasing IA concentration and reached a plateau at the concentration of 5% (v/v) of IA. Thus, the optimum IA concentration added to the blood solution was determined to be 5% (v/v). The IA adduct was stable at −80°C.

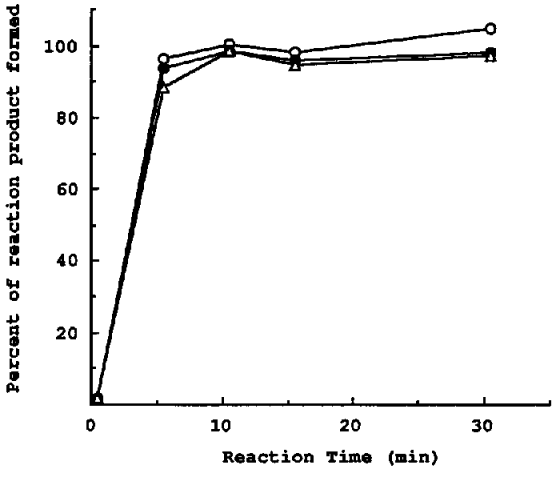

Fig. 1. Time-courses for the derivatization of tiopronin with methyl acrylate (△), ethyl acrylate (●) and isobutyl acrylate (○) in 0.1 *M* Na_2HPO_4 (pH 9.1).

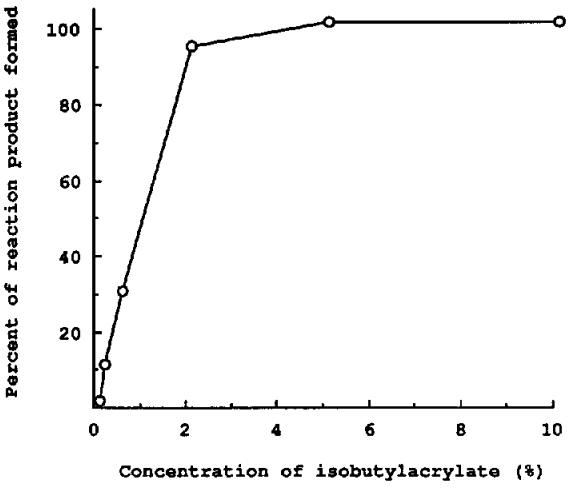

Fig. 2. Derivatization rates of tiopronin with different isobutyl acrylate concentrations in a mixture of blood and 0.1 *M* Na_2HPO_4 (pH 9.1).

After the derivatization of tiopronin with IA in the blood sample, the reaction mixture was deproteinized by treatment with acetone, and the precipitate was removed by centrifugation. The supernatant was reduced under a stream of nitrogen. The IA adduct was extracted by liquid–liquid extraction with ethyl acetate from the remaining solution. The IA adduct in the extract was derivatized to the pentafluorobenzyl ester [15,16]. Finally, the derivatized analytical compounds were purified with a Bond Elut SI column and subjected to GC–MS. The absolute recovery rate of this procedure was 78.6%.

Typical EI mass spectra of the tiopronin-IA-PFB ester and the SA16-IA-PFB ester (I.S.) are shown in Figs. 3 and 4, respectively. The EI mass spectrum of the tiopronin-IA-PFB ester showed the molecular ion at *m/z* 471 and the base peak ion at *m/z* 311, which resulted from loss of the S-isobutylacrylate moiety. The derivatization of the IA adduct to the pentafluorobenzyl ester improved the ion intensity in the mass spectrum

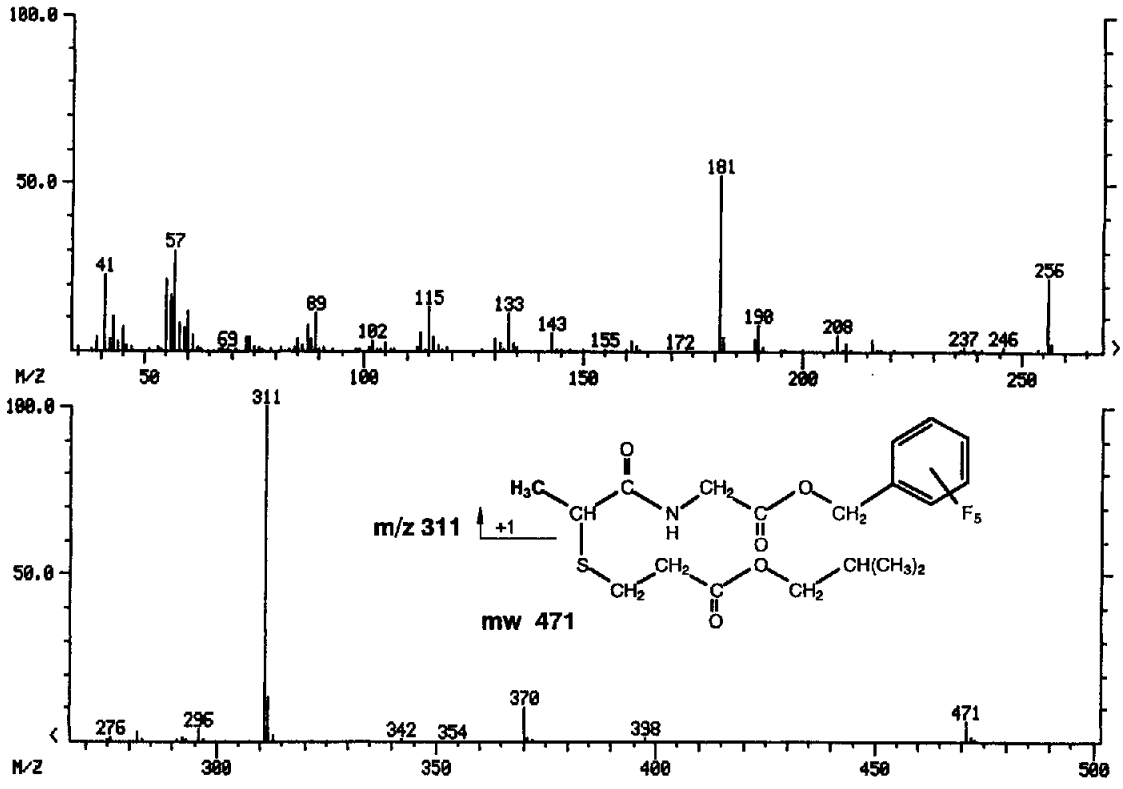

Fig. 3. EI mass spectrum of the tiopronin-IA-PFB derivative.

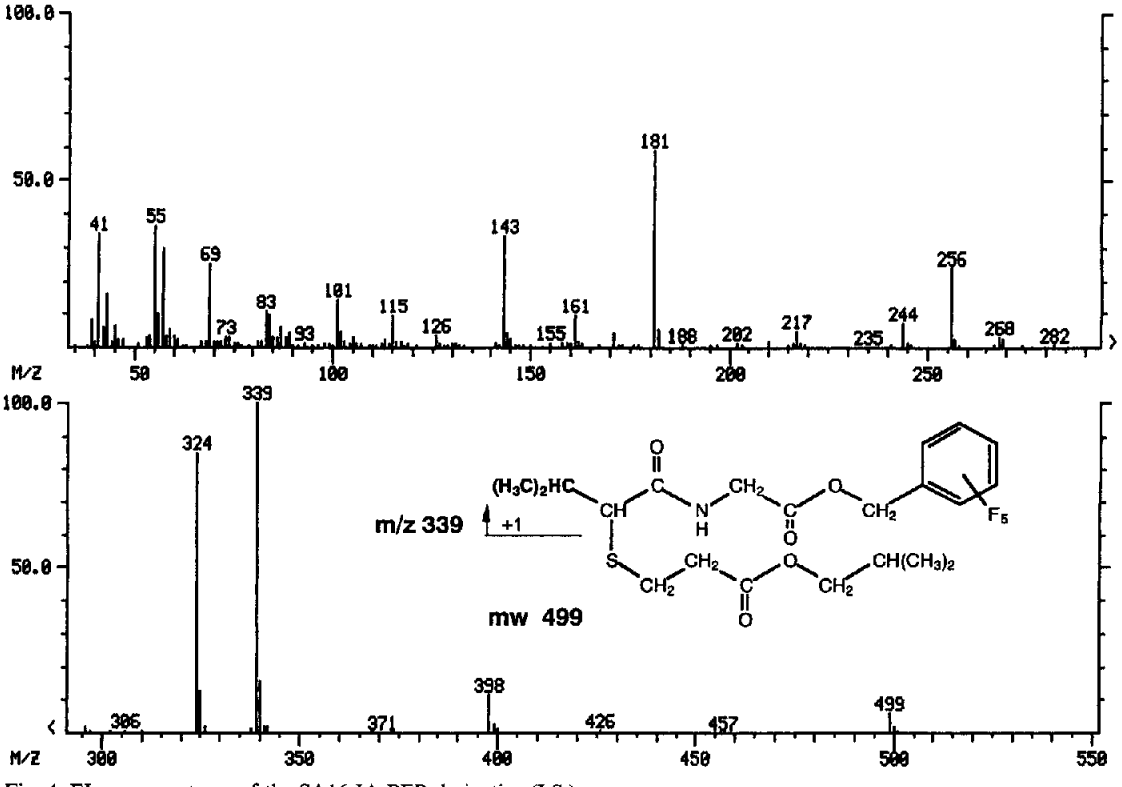

Fig. 4. EI mass spectrum of the SA16-IA-PFB derivative (I.S.).

compared with its methyl ester, and the base peak ion shifted to a high-mass region. The EI mass spectrum of the SA16-IA-PFB ester showed the molecular ion at m/z 499 and the base peak ion at m/z 339; the latter peak was chosen as the I.S. ion.

To examine the validity of the proposed procedure, known amounts of tiopronin corresponding to the expected blood concentrations were added to human blood, and their concentrations were determined (Table I). The accuracy and precision for each level of spiking were satisfactory. The limit of quantification was estimated to be *ca.* 1 ng/ml of blood concentration. When the ratio of the amount of tiopronin to that of the I.S. was plotted against the peak-area ratio, a linear

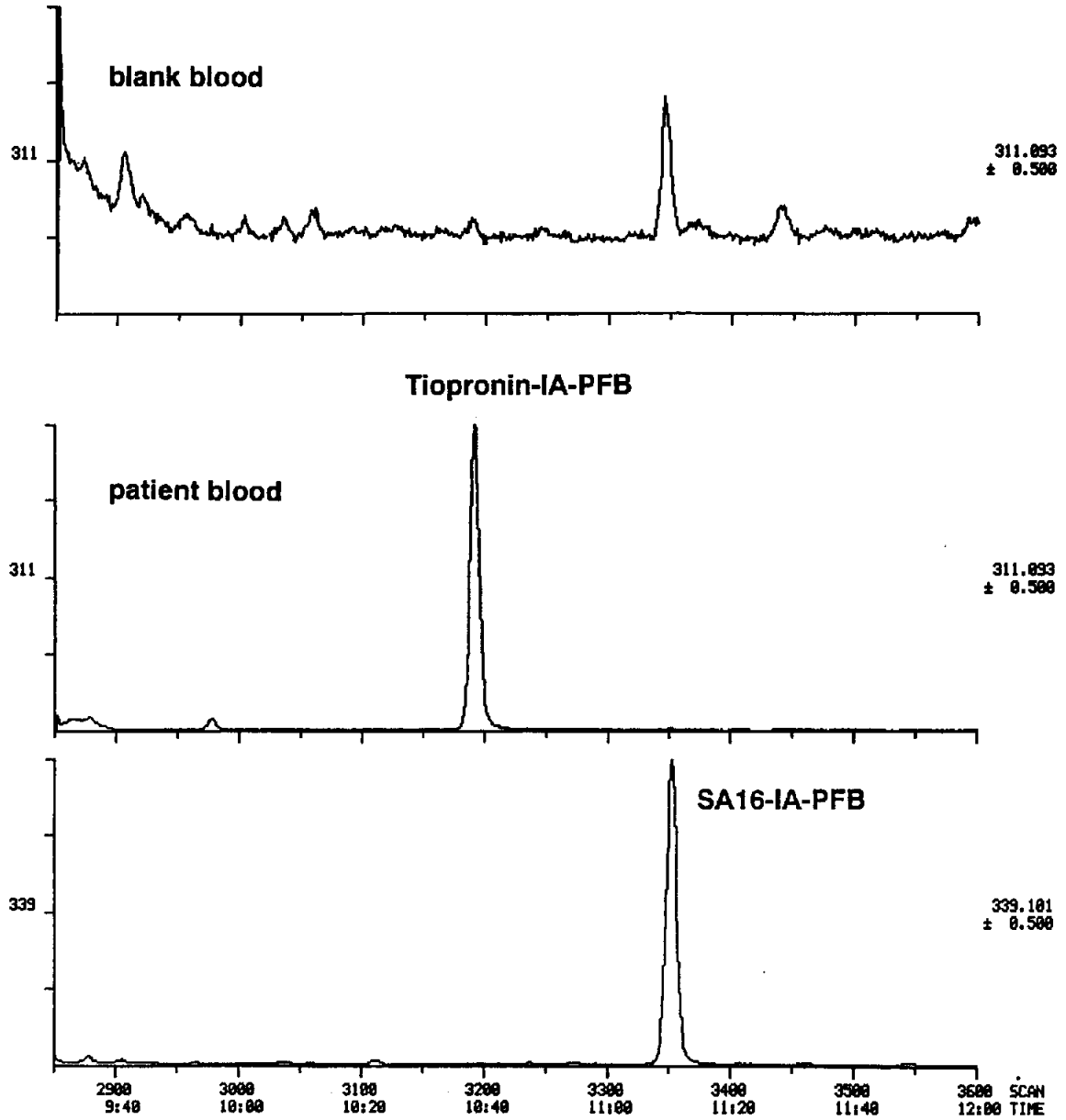

Fig. 5. Typical selected-ion mass chromatograms obtained from blank blood and a patient's blood taken 3 h after oral administration of a tiopronin tablet (100 mg).

TABLE I

ASSAY PRECISION OF QUANTIFICATION OF TIOPRONIN IN SPIKED BLOOD (n = 3)

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V. (%)
1.0	1.02 ± 0.15	14.7
4.0	3.68 ± 0.09	2.4
20.1	17.40 ± 0.58	3.3
200.8	202.8 ± 2.5	1.3
1004.0	1022.3 ± 6.4	0.6

relationship was observed in the range 1–1000 ng/ml (r = 0.99993), the regression equation being $y = 0.00278x - 0.00353$.

Typical selected-ion mass chromatograms obtained from the blank blood and the patient blood taken 3 h after oral administration of tiopronin tablet (100 mg) are shown in Fig. 5. There was no interfering peak in the blank blood sample, and the method was valid to determine the concentration of tiopronin in patient blood.

The proposed method has proved to be satisfactory with respect to sensitivity and reliability for the determination of tiopronin in blood samples. Acrylic acid esters are better derivatization reagents for the thiol group than maleimide because they do not generate an asymmetric carbon and do not raise the boiling temperature of the reaction product. Acrylic acid esters might thus be applicable to compounds with several thiol groups and asymmetric centres.

Acrylic acid esters have other advantages. If there are any interference peaks corresponding to the retention times of the analytes, the retention times could be changed by choosing a different ester in the ester moiety of the acrylic acid ester. Also, by incorporating the compound, which can be detected with a fluorometric detector or a UV detector, into the ester moiety of the acrylic acid ester, it might be detected by HPLC.

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