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Note

Determination of 4-cyano-5,5-bis (4-methoxyphenyl)-4-pentenoic acid in human plasma and platelets by gas chromatography–mass spectrometry

YOSHIAKI YAMANO*, HIROMU NAKAI, TADASU OGAWA, TAMOTSU KANAZAWA and NOBUMICHI MORISHITA

Department of Clinical Chemistry, Tokyo Research Laboratories, Eisai Co., Ltd , 4-6-10 Koishikawa, Bunkyo-ku, Tokyo 112 (Japan)

and

KOUJI YAMADA and YOUJI YAMAGISHI

Tsukuba Research Laboratories, Eisai Co , Ltd , 5-1-3 Tohkohdai, Tsukuba, Ibaraki 300-26 (Japan)

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4-Cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid (E5510, I) is a new potential platelet aggregation inhibitor. This compound antagonizes platelet activation by inhibiting phospholipase C and/or A_2, resulting in the suppression of both phosphatidylinositol breakdown and arachidonic acid release from phospholipids, as well as by inhibiting cyclooxygenase [1].

Solid-phase extraction of drugs combined with gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS) has been demonstrated to yield very sensitive and specific analytical methods for the determination of drugs at low levels in biological fluids. Prostaglandins in plasma have been quantified with high sensitivity by GC–NICI-MS analysis of the pentafluorobenzyl (PFB) derivatives. For example the limit of detection of Iloprost, a stable prostaglandin analogue, was 5 pg/ml [2].

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This paper reports a highly sensitive and specific GC–NICI-MS analytical technique for the determination of I in human plasma and platelets.

EXPERIMENTAL

Apparatus

Kubota Models KS-5000, KS-5200C and KR-1500 were used as centrifugal separators. UR-2000P (Tomy Seiko) was employed as an ultrasonic disruptor. The GC–MS system consisted of a JEOL DX-300 gas chromatograph–mass spectrometer equipped with a post-accelerated detector and connected to a JOEL JMA-DA5000 data system. The vacuum manifold for the Bond Elut column was from Analytichem International (Harbor City, CA, U.S.A.), and the disposable extraction columns were filled with aminopropyl packing, 500 mg per 2.8 ml (Bond Elut NH_2, Analytichem International, Cat. No. 611 303).

Reagents and standards

Citral (Yamanouchi Pharmaceutical) was used as 3.8% sodium citrate solution, and a mixture of SH200 oil and SH550 oil (1:6) (Toray Silicone) as silicone oil. The standard solution of I was prepared in methanol. The internal standard solution (deuterium-labelled compound of I, 4-cyano-5,5-bis(4-[$^2H_3$]methoxyphenyl)-4-pentenoic acid, II) was prepared in acetonitrile. When these solutions were stored at −20°C, there was no deterioration for at least three months. The molecular structures of I and II are shown in Fig. 1. Pentafluorobenzyl bromide (PFBBr) from Tokyo Kasei and N,N-diisopropylethylamine from Aldrich were used for derivatization.

Synthesis of internal standard

Both 850 mg of 4-cyano-5,5-bis(4-hydroxyphenyl)-4-pentenoic acid and 2300 mg of anhydrous potassium carbonate were suspended in 20 ml of anhydrous N,N-dimethylformamide. To the suspension, 1 ml of iodo[$^2H_3$]methane was added and the mixture was stirred at room temperature for two days. The reaction mixture was extracted with ethyl acetate, washed with water and concentrated. Then it was dissolved in 20 ml in 1,4-dioxane, and 1 ml of 5 *M* aqueous solution of sodium hydroxide was added. The mixture obtained was stirred at 70°C for 5 h. The reaction mixture was acidified and extracted with

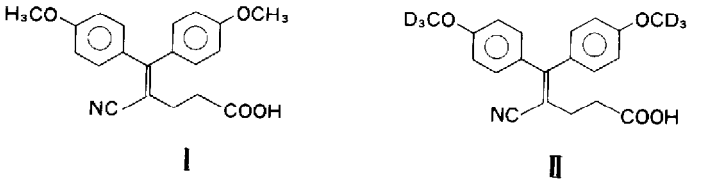

Fig. 1. Structures of E5510 (I) and the internal standard (II).

ethyl acetate, and then washed, dried and concentrated to give a residue. This was purified using silica gel column chromatography (methanol–chloroform) and by recrystallization from diethyl ether–*n*-hexane to give 570 mg of deuterated internal standard.

Administration of I

The healthy male volunteers were randomly divided into two groups of five. Each member of the first group was administered 0.3 and 3.0 g of I, and the second group each received 1.0 and 10.0 mg of the drug with a two-week washout period. The drug was given orally with 120 ml of water under fasting conditions.

Preparation of platelets from blood

Blood (4.5 ml) was collected in tubes containing 0.5 ml of 3.8% sodium citrate solution and centrifuged at 1100 *g* for 5 s and then at 100 *g* for 10 min. The platelet-rich plasma (PRP) was separated from the upper layer. After being suspended in 1% EDTA saline solution, platelets were obtained from the residual blood by centrifugation at 1500 *g* for 5 min, and silicone oil and 1% EDTA were used to purify the PRP.

Extraction of samples

The platelets were suspended in distilled water using an ultrasonic disruptor. To each 1.00-ml aliquot of plasma or 1.5 ml suspension of platelets, 0.5 ml of distilled water, 0.25 ml of 1 *M* hydrochloric acid, 100 μl of a solution of II and 5 ml of diethyl ether were added, and the resulting liquid was vigorously shaken for 10 min and then centrifuged at ca. 1500 *g* for 5 min. The organic solvent layer was separated and evaporated to dryness at 50 °C under a gentle stream of nitrogen gas.

The dried residue was then dissolved in 200 μl of chloroform. After the extraction column (Bond Elut NH_2) had been washed twice with 3 ml of methanol and then twice with 3 ml of chloroform, the mixture was applied onto the column. The column was then washed again with 3 ml of chloroform and with 3 ml of 0.2% acetic acid in methanol, and I and II were eluted with 3 ml of 10% acetic acid in methanol. The effluent was evaporated to dryness at 50 °C under a gentle stream of nitrogen gas.

Derivatization

Dried residues from extracted samples and standards were dissolved in 100 μl of acetone. To the acetone solution, 20 μl of PFBBr in acetone (1:3, v/v) and 15 μl of N,N-diisopropylethylamine in acetone (1:19) were added, and the reaction vessel was securely capped. The solution was allowed to stand at room temperature for 20 min. The reactants were diluted with 1 ml of 0.01 *M* citric

acid solution and extracted with 4 ml of *n*-hexane. The organic phase was washed with 1 ml of distilled water.

GC–NICI-MS analysis

The samples were analysed on a gas chromatograph–mass spectrometer by selective monitoring of $[M-C_6F_5CH_2COO]^-$, *m/z* 292 (I) and *m/z* 298 (II). The operating conditions of the mass spectrometer were as follows: ion source temperature, 240°C; ionization voltage, 200 V; ionization current, 300 μA. Isobutane was used as the reagent gas.

A cross-linked methylsilicone capillary column (Hewlett-Packard Ultra 1, 25 m×0.32 mm I.D., 0.52 μm film thickness) was directly interfaced with the ion source of the mass spectrometer. The carrier gas used was helium. Samples were injected by splitless injection at 305°C, and the temperature of the column oven was maintained at 295°C.

RESULTS

Extraction of I from plasma samples

Diethyl ether, ethyl acetate and ethanol–*n*-hexane were tested as extraction solvents, and diethyl ether was selected for its superior properties in recovering and purifying plasma samples.

Several columns were tested for Bond Elut extraction. Separation with reversed-phase columns (C_8, C_{18}, CN) was unsuccessful because of insufficient purification. Accordingly, the normal-phase and ion-exchange columns (Si, Diol, CN, NH_2) were tried for purification of plasma samples. The best results for both recovery and purification were obtained by using the Bond Elut NH_2 column. The extraction recovery was determined by analysing replicate plasma samples containing 0.6 ng/ml I. The extraction recovery was 89.6±3.6% (mean±S.D., $n=4$).

PFBBr derivatization

The reaction conditions, such as reaction time, temperature and solvents, were tested with plasma extracts. The reaction was carried out for 10 min at room temperature. A reaction time over 30 min and a temperature over 40°C were undesirable, because the peak of I was interfered with by endogenous substances. As the solvent, acetonitrile and acetone were tested, and the latter was selected because of its superior reaction efficiency.

Mass spectra

The effect of the reagent gases on NICI mass spectral fragmentation was investigated using methane and isobutane. Two reagent gases gave similar NICI mass spectra and the characteristic negative ions $[M-C_6F_5CH_2COO]^-$ and $[M-C_6F_5CH_2]^-$. Subsequently, we attempted to investigate the effect of re-

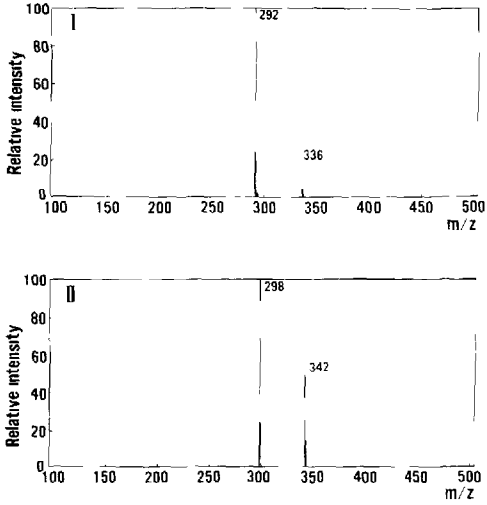

Fig. 2. NICI mass spectra of the PFB derivatives of I and II.

agent gases on the sensitivity of selected-ion monitoring (SIM) using $[M-C_6F_5CH_2COO]^-$ at *m/z* 292. When isobutane was used, the peak intensity provided a sensitivity two or three times greater than that of methane.

When isobutane was employed as a reagent gas, the NICI mass spectra of PFBBr derivatives of I and II were as shown in Fig. 2.

Selected-ion monitoring

As fragment ions for SIM determination, we used *m/z* 292 for I and *m/z* 298 for II. Fig. 3 shows a typical NICI-SIM mass spectrum of the PFBBr derivatives in the plasma and platelets, the interference by endogenous substances in the plasma and platelets was eliminated by the purification and derivatization procedures.

Precision and accuracy

The intra-day precision and accuracy were determined by analysing replicate plasma samples containing 0.7 or 1.2 ng/ml I. The inter-day precision and accuracy were also determined at various concentrations over two weeks ($n=4$). The results were presented in Table I and II.

Calibration graphs

Plasma. A 1-ml volume of drug-free plasma was spiked with 2.5 ng of II and with amounts of I in the range 0.2–15 ng. The linear regression equation was $y=0.608x-0.082$ ($r=0.9999$).

Platelets. A 1.5-ml volume of drug-free platelets suspension was spiked with 2.5 ng of II and amounts of I in the range 0.2–4 ng. The linear regression equa-

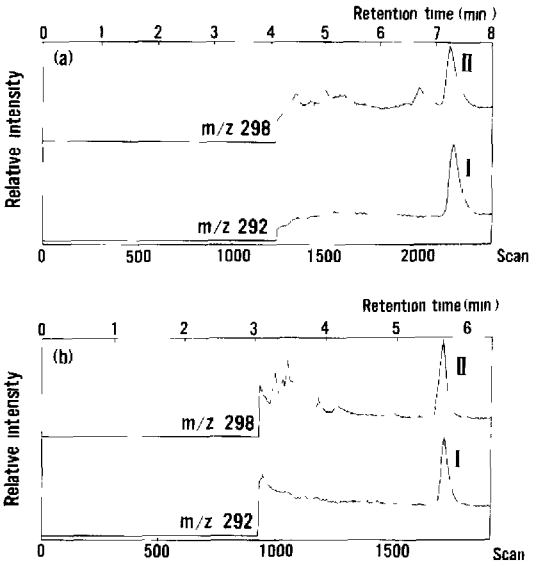

Fig. 3. Selected-ion monitoring of the PFB derivatives of (a) plasma extract and (b) platelets extract.

TABLE I

INTRA-DAY VARIATION IN PLASMA

Plasma standards containing I were prepared from stock methanolic solutions.

Intra-assay	n	Concentration (ng/ml)		Accuracy[a] (%)	Coefficient of variation (%)
		Spiked	Found		
Trial 1	4	0 700	0.703	100.4	4.0
Trial 2	7	1.200	1.227	102.2	5.2

[a]Accuracy = (found concentration/spiked concentration) × 100.

TABLE II

INTER-DAY VARIATION IN PLASMA AND PLATELETS

Plasma or platelets standards containing I were prepared from stock methanolic solutions ($n = 4$).

Plasma				Platelets			
Concentration (ng/ml)		Accuracy (%)	Coefficient of variation (%)	Concentration (ng/ml of PRP)		Accuracy (%)	Coefficient of variation (%)
Spiked	Found			Spiked	Found		
0.791	0 842	106.4	1.3	0.791	0.794	100.5	2.8
1.580	1.530	96.8	0.7	1.580	1.557	98.6	1.7
5.925	5.959	100.6	1.1	3.720	3.742	100 6	0.9

tion was $y=0.481x-0.001$ ($r=0.9994$). For the determination of drugs at low levels, the suspension was spiked with 0.05 ng of II and amounts of I in the range 0.02–0.2 ng. The linear regression equation was $y=0.0329x-0.1259$ ($r=0.9990$). The limit of detection of I was 20 pg/ml of PRP.

Stability in plasma

The plasma samples containing I (1 ng/ml) were stored at −20°C. I in plasma was stable over a period of fourteen weeks.

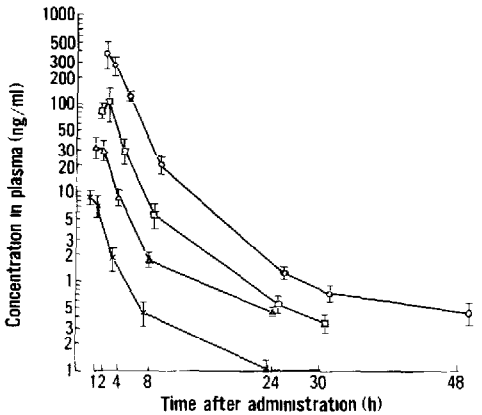

Fig. 4. Time courses of mean (±S.E.) plasma concentrations of I after a single oral administration of various amounts of I to healthy volunteers: (○) 10 mg; (□) 3.0 mg; (△) 1.0 mg; (×) 0.3 mg.

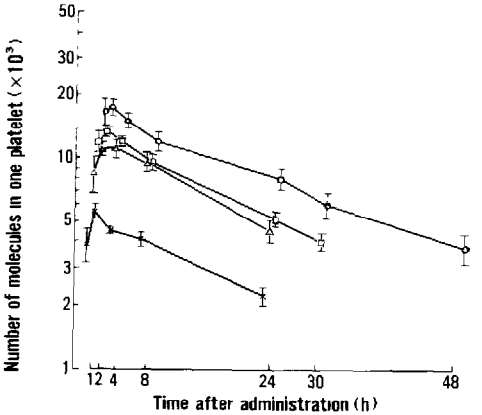

Fig. 5. Time courses of the mean (±S.E.) number of molecules of I in platelets after a single oral administration of various amounts of I to healthy volunteers: (○) 10 mg; (□) 3.0 mg; (△) 1.0 mg; (×) 0.3 mg. The number of molecules in one platelet is given by the concentration of I (ng/ml of PRP) $\times 6.02\times 10^{23}\times 10^{-9}$/MW of I/platelets count in 1 ml of PRP.

Stability of plasma samples repeatedly frozen and thawed

The stability of I (1 ng/ml) in plasma after three repetitions of freezing at −20 °C and thawing at room temperature was tested. Compound I was found to be stable under these conditions.

Drug concentration in plasma

The concentrations of I in plasma after oral administration to healthy male volunteers are shown in Fig. 4. The maximum concentrations in plasma were 12.9 ± 2.0, 57.9 ± 9.5, 185.4 ± 49.9 and 622.3 ± 120.1 ng/ml after doses of 0.3, 1.0, 3.0 and 10.0 mg, respectively.

The concentrations in the plasma increased proportionally to the dose.

Amount of drug in platelets

The amounts of I in platelets after oral administration are shown in Fig. 5. The apparent half-life of I in platelets seems to be much longer than that in plasma. The maximum amounts of I in platelets after oral administration of doses of 0.3, 1.0, 2.0 and 10.0 mg were 5.6 ± 0.4, 11.5 ± 0.8, 13.3 ± 0.7 and 18.7 ± 1.9 ($\times 10^3$) molecules in one platelet, respectively.

DISCUSSION

A method of determining the PFB derivative of I in plasma and platelets by GC–MS in the NICI mode was developed, and I levels in plasma and platelets after oral administration were determined. The high sensitivity of GC–NICI-MS is very attractive since it enables the minute amounts of I in platelets to be analysed.

Effective derivatizations for high-sensitivity determinations of many biologically important compounds by GC–NICI-MS have been discussed in a large number of papers. Prostaglandins substituted at their carboxyl moieties with PFB derivatives have shown high sensitivity in GC–NICI-MS analysis [3–6] and GC with electron-capture detection [7–10]. The method was successfully applied to the measurement of I in biological fluids. The samples were esterified with PFBBr and purified with Bond Elut NH_2. The PFBBr reaction was performed in order to avoid selective adsorption in the capillary column and to let the compound have electron affinity. NICI-MS had a sensitivity ten times greater than that of the positive-ion mode for I. The electron affinity inherent in the halogenated derivatives, such as the PFB ester, may be the most important factor in highly sensitive determinations [11–13].

The NICI mass spectrum of the PFB derivative consists of characteristic fragment anions, which were assigned to $[M-C_6F_5CH_2COO]^-$ and $[M-C_6F_5CH_2]^-$.

A deuterium-labelled compound is usually used as an internal standard in GC–MS because of the close similarities of its chemical and physical properties

to those of the non-labelled compound. In this study, hexadeuterated I was used as the internal standard for the GC–MS determination of I, which is undoubtedly the most specific method. However, selected purification procedures have been performed prior to the final analysis.

The disposable Bond Elut NH_2 columns, which feature both ion-exchange and adsorption, were very efficient for the purification of biological fluids. By means of this techniques, compound I in human plasma and platelets was sufficiently purified for chromatograms without interference to be obtained in subsequent GC–MS analysis.

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