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Note

Determination of a thromboxane A_2 receptor antagonist in human plasma by capillary gas chromatography with electron-capture detection

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Compound I (Fig. 1) is a specific antagonist of thromboxane A_2 (TXA_2) receptor, a major metabolite of arachidonic acid. As a potent platelet aggregator, vasoconstrictor and bronchoconstrictor, TXA_2 has been implicated in a number of disorders [1—4]. Compound I, developed by the Squibb Institute for Medical Research, is an investigational drug for therapeutic use in pathophysiology involving an imbalance in the levels of TXA_2*.

The method developed for the determination of I in plasma involves isolation of the drug from plasma by a liquid—liquid extraction followed by a

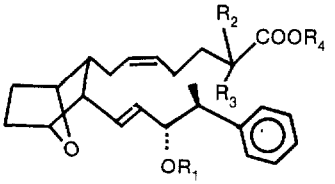

Fig. 1. Structures of I and II (the internal reference) and their derivatives.

Compound	R_1	R_2	R_3	R_4
I	H	H	H	H
II	H	CH_3	CH_3	H
Derivative of I (III)	$Si(CH_3)_3$	H	H	$C_6F_5CH_2$
Derivative of II (IV)	$Si(CH_3)_3$	CH_3	CH_3	$C_6F_5CH_2$

*Squibb designated name for I is SQ 28,668.

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solid-phase extraction, reacting the isolate to form the pentafluorobenzyl (PFB) ester and trimethylsilyl (TMS) ether (III, Fig. 1), and measuring the product by high-resolution capillary gas chromatography (GC) using splitless cold trapping injection.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5840 gas chromatograph, equipped with a constant-current electron-capture detector, was used. The fused-silica capillary column (10 m × 0.22 mm I.D., 0.11 μm film thickness) was coated with bonded cyanopropylphenylmethylpolysiloxane phase, CP-Sil 19 CB (Chrompack). The carrier gas was helium with an inlet pressure of 103 kPa (15 p.s.i.g.) and the make-up gas for the electron-capture detector was 5% methane in argon at a flow rate of 30 ml/min. The oven was operated isothermally at 180°C for 1.0 min after injection, then heated at 30°C/min, held at 230°C for 7 min, heated at 10°C/min to 270°C and held at this final temperature for 2.0 min. Injections were made by the splitless mode, with a split flow of 30 ml/min and a septum purge of 2.0 ml/min. The inlet purge was turned on 1.0 min after injection. The injector and detector temperatures were maintained at 280 and 320°C, respectively.

A Hewlett-Packard 5970A mass-selective detector was used for the identification of the derivatives of I. The detector was interfaced with a Hewlett-Packard 5790A gas chromatograph, with a capillary column inserted directly into the ion source. The detector was calibrated with the Autotune® program using perfluorotributylamine (PFTBA).

Silica Bond-Elut® 1-ml columns (Analytichem International) were used for solid-phase extraction. The columns were fitted into a ten-place Vac-Elut® (Analytichem International) vacuum manifold. Plastic syringe barrels (10 ml) (Fisher) were connected to the columns to act as reservoirs for larger volumes of the extraction solvent. Before use, the silica columns were activated by passing, dropwise, 5 ml of methanol followed by 5 ml of heptane.

Autosampler vials (1 ml) with PTFE-lined caps were obtained from Supelco. Glass scintillation vials (20 ml) with cork-backed tin foil were purchased from Fisher. A sample concentrator, Model SC-248, with a scintillation vial adapter (Brinkmann Instruments) and an SC-3 concentrator with DB-3 Dri-Block heating bath (Techne) were used.

Reagents and chemicals

Compounds I and II* were characterized pharmaceutical products (E.R. Squibb & Sons). The esterification reagent was prepared daily by diluting 0.1 ml of pentafluorobenzyl bromide (Aldrich) with 9.0 ml of acetonitrile. N,N-Diisopropylethylamine, a catalyst for the esterification reaction, was obtained from Aldrich. Bis(trimethylsilyl)acetamide (BSA) was supplied by Pierce. Control plasma, prepared from fresh citrated blood obtained commercially, was kept in a freezer. Acetonitrile, methanol, heptane and toluene, glass-distilled, were obtained from Burdick & Jackson Labs.

*Squibb designation for II is SQ 29,473.

Stock solutions for I and II, the internal reference, were prepared separately in methanol at a concentration of 250 μg/ml. Diluted stock solutions, prepared by dilution with methanol, were stable for at least three months when stored in a refrigerator. Calibration standards were obtained by adding to 1.0 ml of control plasma in culture tubes appropriate volumes of the diluted stock standard and a fixed amount of the diluted stock internal standard. A typical calibration set consisted of a zero and six other values ranging from 10 to 500 ng of I, each containing 250 ng of II.

Sample preparation

Purification of plasma samples and calibration standards was accomplished by a liquid–liquid extraction followed by a solid-phase extraction.

Liquid–liquid extraction. Plasma samples were thawed at room temperature or kept in a refrigerator overnight. A 1.0-ml volume of plasma was transferred into a culture tube containing 250 ng of II and 1.0 ml of 1.0 *M* hydrochloric acid. After mixing, 10 ml of heptane were added, the tubes shaken mechanically for 5.0 min and the phases separated by centrifugation at about 500 *g* for 5 min. The lower aqueous layer was then frozen completely by immersion in an acetone–dry ice bath.

Solid-phase purification. The upper heptane layer was transferred into a 10-ml syringe barrel attached to an activated 1.0-ml silica column. The heptane was passed through the column, dropwise, retaining compounds I and II. The column was then washed with 5.0 ml of heptane, followed by elution with 3.0 ml of methanol into a 20-ml scintillation vial. Methanol was removed by evaporation at 60°C in the Brinkmann sample concentrator. After adding 0.5 ml of methanol to the scintillation vial and mixing thoroughly, the methanol solution was quantitatively transferred into a 1-ml autosampler vial. The scintillation vial was rinsed with a second 0.5-ml volume of methanol, which was then combined with the first solution in the autosampler vial. The methanol was removed under a stream of nitrogen by placing the autosampler vial in the Techne sample concentrator at 60°C. The vial was then dried in a desiccator, under vacuum, for 15 min.

Derivatization and dissolution

Derivatization of the dried extracts was performed in a two-step reaction, esterification followed by trimethylsilyl ether formation. To each dried vial, 50 μl of pentafluorobenzyl bromide solution in acetonitrile were added, followed by 10 μl of N,N-diisopropylethylamine. The vial was sealed with PTFE-lined caps, vortexed and heated at 40°C for 20 min. The cooled vial was then uncapped and immediately placed in a desiccator and vacuum-dried for 30 min. To each vial, 0.1 ml of acetonitrile was added, mixed and the vial vacuum-dried for additional 30 min. Each vial was further treated by adding 50 μl of BSA, capping the vial and heating at 60°C for 1 h. After uncapping, the BSA was removed under a stream of nitrogen at 50°C. The dried sample was dissolved in 0.2 ml of toluene.

Method

The calibration graph was established at the beginning of the study. Daily,

a control sample, which represented the calibration point of 250 ng of I and 250 ng of II per ml of plasma, was processed together with the samples. The response of the control sample was used to correct the data for differences in response from the calibration slope [5].

The calibration graph was constructed by plotting peak-area ratios of the analyte III to those of the internal reference (IV) versus the ratios of the amounts of I and II, the amount being the total nanograms of I or II in each calibration standard. A typical calibration graph gave a correlation coefficient of 0.9995, a y-intercept of −0.00503 (standard deviation = 0.002, n = 7) and a slope of 0.804 (standard deviation = 0.043, n = 7).

RESULTS AND DISCUSSION

Extraction

Compounds I and II could be adsorbed from plasma diluted with water on C_2, C_8, C_{18}, phenyl and cyclohexyl solid-phase columns and then eluted from the columns with methanol, acetonitrile, acetone, ethyl acetate or *n*-butyl acetate. However, these extracts were not clean enough for GC analysis with electron-capture detection. The compounds could be extracted from acidified plasma with a number of solvents such as ethyl acetate, *n*-butyl acetate, diisopropyl ether, *tert.*-butyl methyl ether, toluene, benzene, cyclohexane, hexane, diethyl ether and heptane. The last was the extraction solvent of choice. For further purification, the compounds were back-extracted into 0.1 *M* sodium hydroxide and, after adjustment of the pH to neutral, were adsorbed on a C_{18} solid-phase column. After elution of the compounds with *n*-butyl acetate, relatively pure extracts were obtained. This extraction procedure was not suitable because of the lengthy procedure involved.

Of the C_2, C_8, C_{18}, phenyl, cyanopropyl, diol, silica and cyclohexyl solid-phase columns, only the silica column adsorbed compounds I or II completely from heptane. The compounds were then eluted efficiently from the silica column with methanol. After derivatization, the extracts were clean enough for GC with electron-capture detection. The overall extraction and derivatization efficiency was over 85%.

Derivatization

Esterification of the carboxyl groups of I and II is performed with pentafluorobenzyl bromide in the presence of N,N-diisopropylethylamine, with acetonitrile as solvent [6–8]. Esterification was complete in less than 20 min at 40°C. As little as 50 μg of pentafluorobenzyl bromide per vial were adequate for the esterification of standards I and II. An excess of the reagent was used for the extracted samples as some co-extracted impurities from plasma consume the reagent.

It was desirable to remove the unreacted pentafluorobenzyl bromide in order to reduce the adverse effects on the electron-capture detector. Attempts to remove the reagent by passing the reacted product through Sephadex LH-20 [6, 7], silica or silicic acid [8] failed because of the poor recovery. A procedure based on partitioning between water and hexane, benzene or ethyl acetate [7] proved to be impractical for the routine processing of a large number of

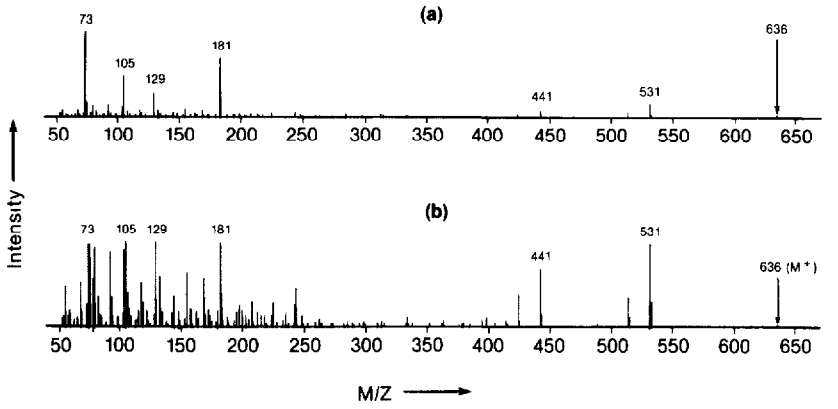

Fig. 2. Electron-impact mass spectra of PFB ester TMS ether of I, scanned from 50 to 700 a.m.u. (a) Spectrum normalized to *m/z* 73 (base peak); (b) spectrum normalized to *m/z* 531.

m/z	%	Fragment
73	100	TMS
105	47	Methylbenzyl
181	69	PFB
531	21	$M^+ - 105$
441	13	$M^+ - 105 - 90$
636	Not seen	M^+

samples. Removal of the reagent with a stream of nitrogen produced bulky precipitates. Removal of the reagent could be accomplished by passing a methylene chloride solution of the esterified product through C_1, C_2 or C_8 solid-phase columns, which retain the reagent while passing through the esters of I and II. However, the expedient procedure of vacuum-drying efficiently removed the excess reagent.

The dried esterified product was trimethylsilylated by reaction with BSA. Excess of BSA was removed by evaporation under nitrogen, to avoid adverse effects of BSA on the electron-capture detector. The injection solution in toluene was found to be stable for several days on storage in a refrigerator, and there was no degradation of the product in the chromatographic system.

The structures of the reacted products were confirmed by GC—electron-impact (EI) mass spectrometry. A typical EI spectrum of the PFB ester TMS ether derivative of I is presented in Fig. 2, with the diagnostic ions identified.

Gas chromatography

Typical chromatograms are shown in Figs. 3 and 4. It is of interest that the PFB ester TMS ether of I (III) elutes after that of II (IV), which is larger than I by two carbon units. This was also found to be true with lower polarity capillary columns such as CP-Sil 8 CB or CP-Sil 5 CB, where the resolution between the two compounds was lower. The methyl ester TMS ether of I and II eluted in the usual order. The unexpected order of elution of III and IV appears to be related to the relative bulkiness of the PFB group and the *gem*-dimethyl group in close proximity.

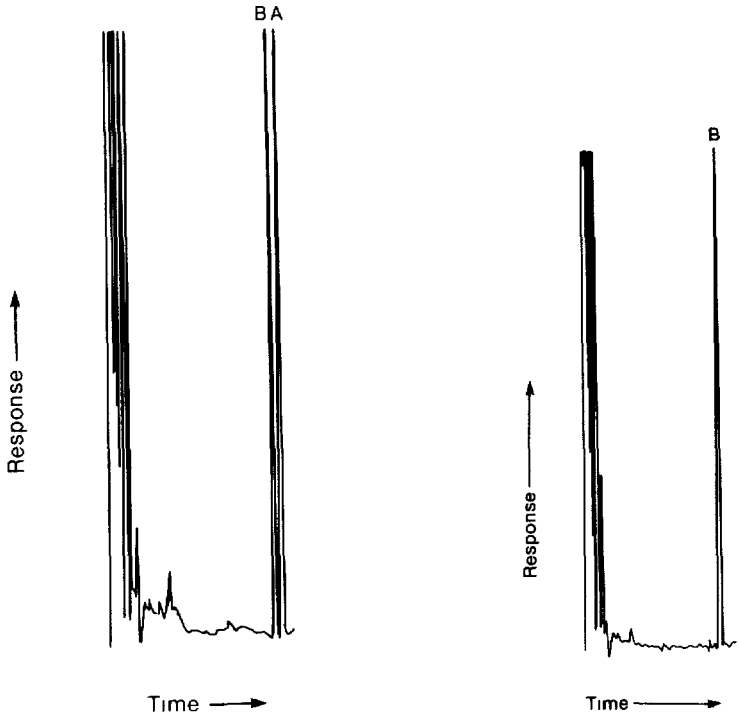

Fig. 3. Chromatogram of extract from 1.0 ml of sample plasma, fortified with 250 ng of II, the internal reference. (A) PFB ester TMS ether of I (13.30 min, 250 ng/ml I); (B) PFB ester TMS ether of II (12.97 min). Attenuation = 512; chart speed = 0.1 cm/min from 0 to 6.0 min, then 0.5 cm/min.

Fig. 4. Chromatogram of extract from 1.0 ml of blank plasma fortified with 250 ng of II. (B) PFB ester TMS ether of II (12.97 min). Attenuation = 512; chart speed = 0.1 cm/min from 0 to 6.0 min, then 0.5 cm/min.

Samples were dissolved in toluene for injection and the cold trapping splitless injection mode [9, 10] was employed, the components being initially cold-trapped at the head of the column, which was maintained at 180°C.

Compound III has a sufficient electron-capture response to produce a quantitative measurement from less than 20 pg injected. The limit of quantitation is less than 2 ng/ml in plasma, with a precision of better than 10% (relative standard deviation) (n = 5).

Clinical samples

The concentration of I in the plasma samples assayed ranged from less than 1 to more than 1000 ng/ml. For samples with levels above 500 ng/ml, the 0.2 ml of toluene solution for injection was diluted so that the concentration fell in the linear region of the detector response.

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