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Short Communication

Micro-quantitative determination of ciprostene in plasma by gas chromatography–mass spectrometry coupled with an antibody extraction

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ABSTRACT

A simple and highly sensitive method has been developed for the determination in plasma of ciprostene, 9β-methyl-6α-carbaprostaglandin I_2, using gas chromatography–mass spectrometry following solid-phase extraction on an immobilized antibody column. The anti-ciprostene antibody obtained from rabbit serum was coupled to an agarose support matrix, and the immobilized antibody thus prepared was used as an extraction phase for sample clean-up. The extracted drug was treated with pentafluorobenzyl bromide followed by bis(trimethylsilyl)trifluoroacetamide. The derivative was quantitatively analysed by negative-ion chemical ionization gas chromatography–mass spectrometry. The lower limit of quantitation was 50 pg/ml when 1 ml of human plasma was used. The plasma concentration of ciprostene in a dog treated with ciprostene at 2.5 μg/kg was determined successfully by this method.

INTRODUCTION

Ciprostene, 9β-methyl-6α-carbaprostaglandin I_2 (Fig. 1), is a chemically stable analogue of prostacyclin and shows a potent inhibitory effect on platelet aggregation. Owing to the extremely low clinical dosage and rapid metabolism, the concentration of ciprostene in human plasma is estimated to be less than 5 ng/ml even at its peak. Hence, quantitative determination of trace amounts of ciprostene in plasma is essential for clinical trials.

This paper describes a simple and highly sensitive method for the determination of ciprostene in plasma by negative-ion chemical ionization (NICI) gas chromatography–mass spectrometry (GC–MS) following solid-phase extraction on an immobilized antibody column.

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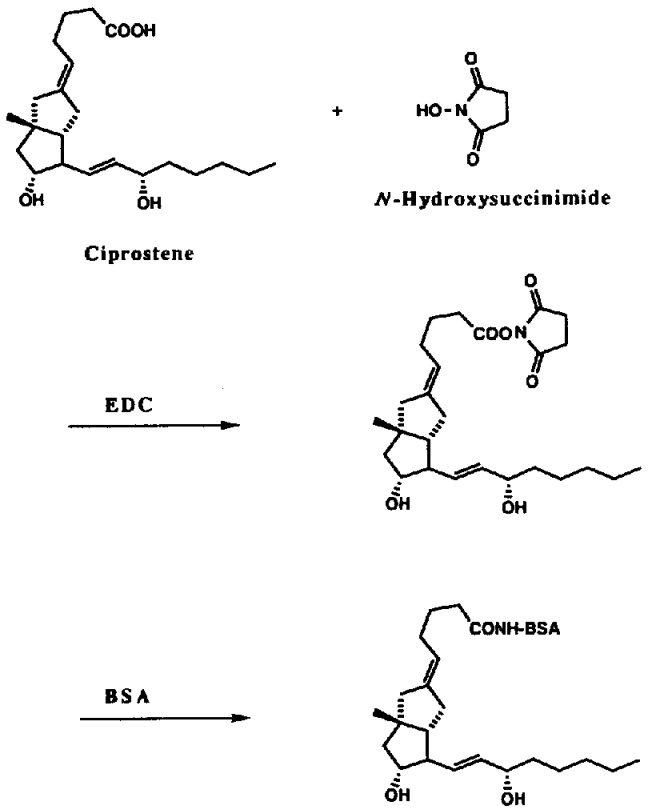

Fig. 1. Synthesis of the ciprostene–BSA conjugate.

EXPERIMENTAL

Materials and animals

The calcium salt of ciprostene and its $^{18}O_2$-labelled analogue (internal standard, I.S.) were supplied from The Upjohn Company (Kalamazoo, MI, USA). Ciprostene labelled with ^{3}H at 11β-position (specific activity, 3.799 mCi/mg) was supplied from Upjohn (Crawley, UK). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), pentafluorobenzyl bromide (PFBB), bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N,N-diisopropylethylamine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Freund's complete adjuvant (FCA) and bovine serum albumin (BSA) were obtained from Difco Labs. (Detroit, MI, USA) and Sigma (St. Louis, MO, USA), respectively. Columns of PD-10 Sephadex® G-25M and Sephadex® LH-20 were from Pharmacia (Uppsala, Sweden). Affi-Gel® Hz hydrazide gel, Hz oxidizer, Hz coupling buffer, and Protein-A MAPS®-II kit, which contained binding buffer and elution buffer, were

products of Bio-Rad Labs. (Richmond, CA, USA). All other chemicals were obtained commercially from Wako (Osaka, Japan) and were of HPLC or analytical-reagent grade. Male Japanese white rabbits (n = 9, *ca.* 2.7 kg, nine to eleven weeks old) and a male beagle (10 kg, one year old) were purchased from Ichikawaya (Tokyo, Japan) and Nihon Nosan Kogyo (Yokohama, Japan), respectively.

Instruments

A Hewlett Packard 5988A GC–MS system with HP 59970 MS ChemStation data systems (Palo Alto, CA, USA) was employed. GC was carried out on an HP-1 fused-silica capillary column (12 m × 0.2 mm I.D., film thickness 0.33 μm), which was directly attached to the ion source. Helium was used as carrier gas at an inlet pressure of 0.67 bar. The column temperature was linearly programmed from 200 to 290°C (held for 2 min) at 10°C/min. The temperatures of the interface and ion source were maintained at 280 and 200°C, respectively. Methane was used as reagent gas (ionizer pressure, 1.0 Torr). The operating conditions were as follows: electron energy, 240 eV; electron multiplier voltage, 3.0 kV; emission current, 0.3 mA. Selected-ion monitoring was performed at m/z 507 (ciprostene, [M − pentafluorobenzyl (PFB) moiety]$^-$) and m/z 511 ([$^{18}O_2$]ciprostene, [M − PFB]$^-$, I.S.).

Radioactivity was measured in a scintillation cocktail (Packard INSTA-GEL) with the Aloka liquid scintillation system (Model LSC-3600, Tokyo, Japan).

Preparation of antigen and immunization

Ciprostene was esterified with N-hydroxysuccinimide in the presence of EDC followed by conjugation with BSA (Fig. 1) [1]. Immunization was performed every two weeks by injecting the antigen mixed with FCA into rabbits. On the tenth day after each immunization, antibody production in the blood was evaluated by Ouchterlony's diffusion method [2] at first and then by radioimmunoassay with [^{3}H]ciprostene.

Purification and immobilization of antisera

On the tenth day after the fifth boost injection, blood was drawn from the carotid artery of rabbits carrying the antibody for ciprostene, under diethyl ether anaesthesia. To obtain antisera, the blood specimens were allowed to stand at room temperature for 15 min followed by centrifugation at 1500 g for 10 min. The immunoglobulin G (IgG) fraction was obtained from the antisera at 4°C with a protein-A affinity column (Affi-Gel Protein-A MAPS-II kit). The IgG was subsequently lyophilized and immobilized with the hydrazide gel kit (Affi-Gel Hz). The IgG-coupled gel thus prepared was poured into a column (0.5-ml bed volume) and washed with 2.5 ml of 20 mM phosphate-buffered saline (PBS), pH 7.4, followed by 10 ml of methanol. After being equilibrated with PBS, the column was stored at 4°C until use.

Sample preparation and derivatization

A human plasma sample (1.0 ml) spiked with 500 pg of [$^{18}O_2$]ciprostene (I.S.) was applied to the antibody column. The column was washed with 4 ml of PBS and 4 ml of water. The drug was subsequently eluted with 5.0 ml of methanol, and the organic solvent was evaporated to dryness under a stream of nitrogen.

To prepare the PFB ester of ciprostene, 15 μl of an acetonitrile solution of PFBB (35%) and 25 μl of N,N-diisopropylethylamine were added to 50 μl of on acetonitrile solution of the extracted sample. The mixture was allowed to stand for 1 h at room temperature, followed by evaporation. Excess reagent was removed by dichloromethane on a column of pre-swollen Sephadex LH-20. The organic solvent was evaporated to dryness, and 50 μl of BSTFA were added to the residue. After 1 h, an aliquot (1 μl) of the reaction mixture was injected into the GC–MS system.

Determination of ciprostene in dog plasma

Ciprostene dissolved in saline (25 μg/ml) was injected into a male beagle dog via the cephalic vein at 2.5 μg/kg. Concentrations of ciprostene in the plasma were determined at 1, 10 and 30 min , and 1, 3 and 6 h after dosing by the method described above.

RESULTS AND DISCUSSION

The ciprostene–BSA conjugate was successfully prepared by the method in which the N-succinimide ester was used as active ester (Fig. 1) [1]. Approximately thirty molecules of ciprostene were calculated to be bound to one molecule of BSA by using [^{3}H]ciprostene. Antibody production was evaluated on the tenth day after each boost injection by radioimmunoassay with [^{3}H]ciprostene and found to reach the plateau level on the tenth day after the second boost (data not shown). In this study, we collected the blood specimens after the fifth boost. The antiserum that possessed the highest antibody activity could be diluted more than 2000-fold for radioimmunoassay.

Activation of Sepharose by cyanogen bromide has been widely used for immobilization of proteins on solid supports [3]. However, the activity of the antibodies was lowered by this method in some cases, because at least a portion of the essential primary amino group at the active site(s) of antibodies might be utilized for binding during random coupling of antibodies to the support. Hence, in this study the antibodies were coupled to an agarose support via hydrazone bonds between oxidized sugar moieties of antibodies and hydrazine bound on agarose gel, by the method of Little *et al.* [4]. Indeed, the activity was not affected by the immobilization to any extent (data not shown).

Among the several solvents (methanol, ethanol, methanol containing acetic acid) examined as possible elution solvent, only methanol showed a high recovery of ciprostene without any interferences during GC–MS analysis. When 1 ml of

human plasma containing two different amounts of [^{3}H]ciprostene, *i.e.* 100 pg/ml and 5 ng/ml, was applied to the column and eluted with methanol, the recovery ratios were more than 90%.

To determine the antibody column capacity, 1 ml of human plasma spiked with known amounts (1–400 ng) of [^{3}H]ciprostene was applied to a 0.5-ml bed volume of the column, and the recovery was determined. Over 100 ng of ciprostene, the recovery decreased to less than 80%; on the basis of this result, the maximum amount of ciprostene that can be applied to this column was determined to be 100 ng (Fig. 2). The lifetime of the column was examined by consecutive measurement of the recovery with 1 ml of human plasma spiked with 20 ng of [^{3}H]ciprostene; the column showed sufficient recoveries even after ten analyses.

Since PFB esters yield a strong carboxylate anion in the NICI mode [5], we esterified ciprostene with PFBB in this study. The methane NICI mass spectrum of the PFB-trimethylsilyl derivative of ciprostene gave an intense base ion at m/z 507 $[M - 181]^-$, which was derived by the loss of a PFB radical from the molecular ion. An injected amount of 500 fg of ciprostene was detected with a signal-to-noise ratio of 3:1 by using the ion at m/z 507.

In control human plasma, no interfering peaks were detected at the m/z 507 and 511 channels at the retention time (*ca.* 9.9 min) of ciprostene. To examine the accuracy and precision of this procedure, 1 ml of human plasma spiked with

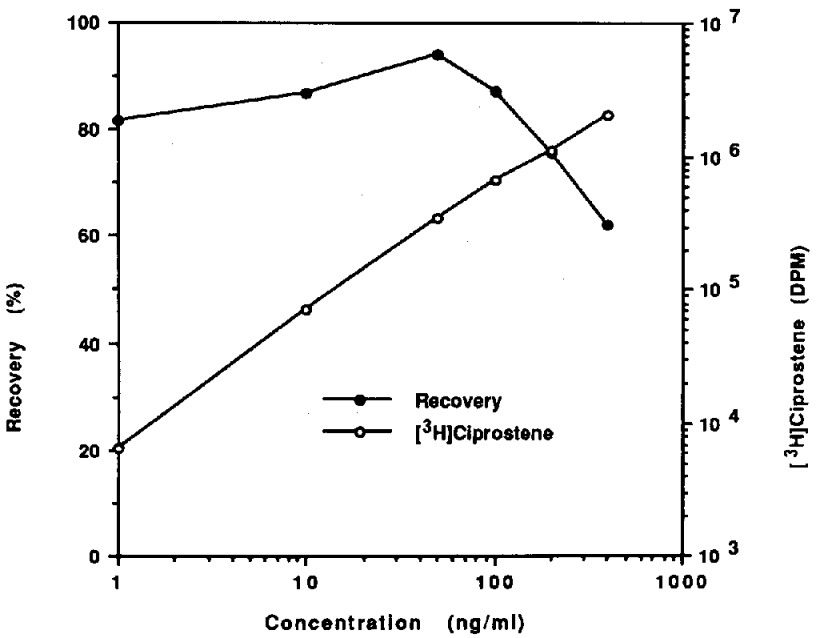

Fig. 2. Capacity of the antibody column. Human plasma spiked with known amounts (1–400 ng/ml) of [^{3}H]ciprostene was applied to a 0.5-ml bed volume of the column, and the recovery was determined by the method described in the text.

known amounts of ciprostene (50–5000 pg) and I.S. (500 pg) was processed by the present method and injected into the GC–MS system. Calibration curves were constructed by plotting the ratio of the peak area of ciprostene to that of the I.S. against the amount of ciprostene. Satisfactory linearity was observed over the range 50–5000 pg/ml (equation of a typical regression line: $y = mx + c$, where $m = 1.195 + 0.007$, $c = 0.167 \pm 0.016$ and $r^2 = 0.999$). Sufficient intra-assay reproducibility ($n = 10$) was obtained at the concentrations of 50, 500 and 5000 pg/ml with coefficients of variation of 7.4, 1.2 and 2.6%, respectively. The corresponding inter-assay ($n = 4$) data were found to be 2.5, 1.2 and 0.3%. Sufficient accuracy was observed in the deviation values: −6.0, −1.6 and −4.1% at the concentrations of 50, 500 and 5000 pg/ml, respectively. The limit of quantitation of 50 pg/ml could be lowered to 10 pg/ml, if 5-ml plasma samples were used.

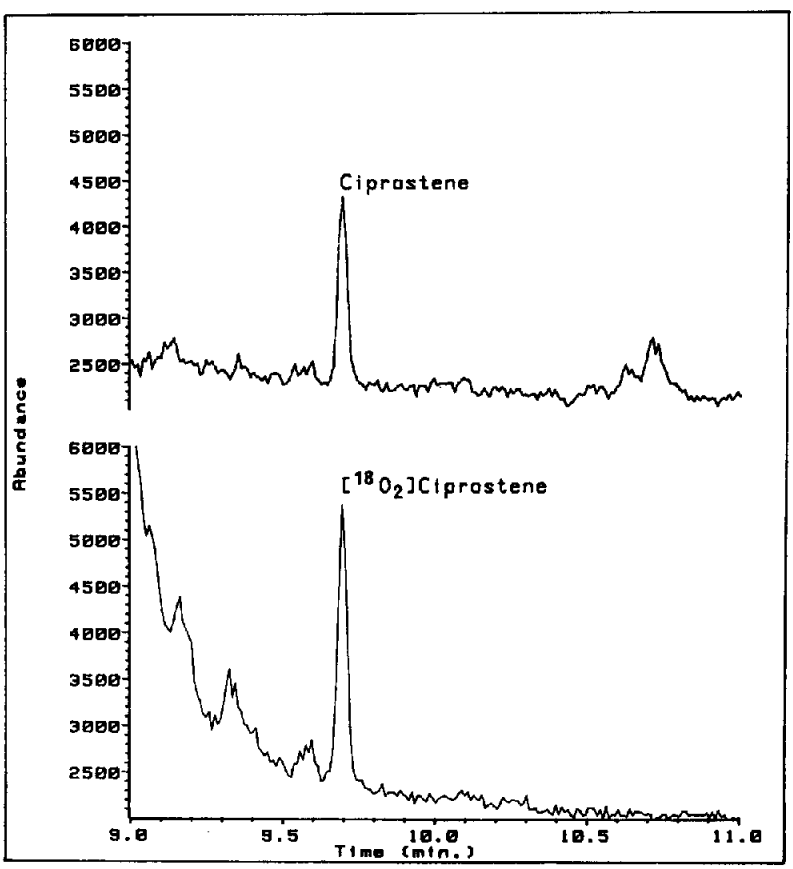

Fig. 3. NICI selected-ion recording obtained from dog plasma 30 min after intravenous injection with ciprostene (2.5 μg/kg). Selected-ion monitoring was performed at m/z 507 (ciprostene, [M − PFB moiety]$^-$) and m/z 511 ([$^{18}O_2$]ciprostene, [M− PFB]$^-$, I.S.).

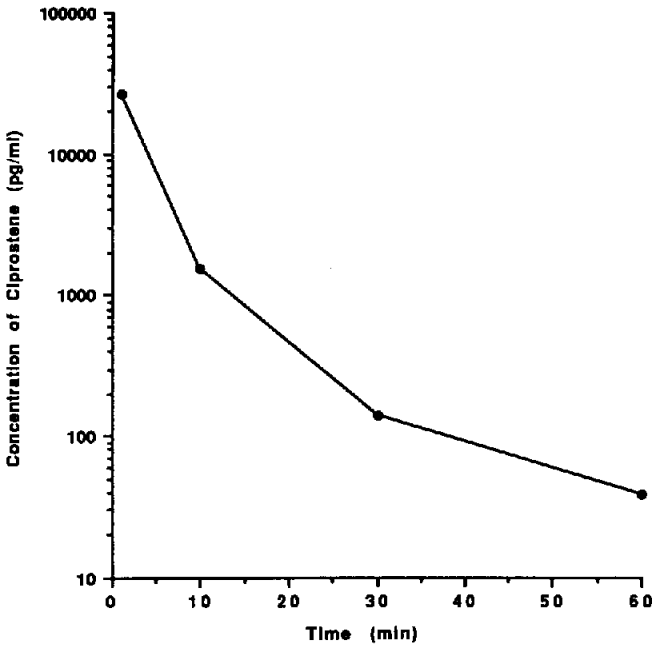

Fig. 4. Concentration of ciprostene in dog plasma after intravenous injection (2.5 μg/kg).

The concentration of ciprostene in dog plasma after intravenous injection was determined by the present method without any interference. A representative chromatogram is shown in Fig. 3. The concentrations decreased rapidly: 27, 1.5 and 0.15 ng/ml at 1, 10 and 30 min, respectively. At 1 h, the concentration was less than 50 pg/ml (Fig. 4).

In conclusion, the extraction method with immobilized antibody is much better than that of the conventional clean-up methods [6,7] with respect to specificity and simplicity. Thus, coupled with the clean-up method developed in this study, the present NICI GC–MS analysis is useful for determining trace amounts (no less than 50 pg/ml) of ciprostene in biological fluids.

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