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Determination of 9α,11β-prostaglandin F_2 in human urine and plasma by gas chromatography–mass spectrometry

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Abstract

Sensitive and specific assay methods for 9α,11β-prostaglandin F_2 (9α,11β-PGF_2) by gas chromatography–mass spectrometry with electron impact ionization are described. The mass spectrometric assay for 9α,11β-PGF_2 was based on the use of the methyl ester–dimethylisopropylsilyl ether derivative, and pentadeuterated $PGF_{2\alpha}$ as a convenient internal standard. The calibration graph for 9α,11β-PGF_2 was linear from 5 pg to 100 ng for both the standard and spiked biological samples. The limit of detection was 50 pg/ml for urine and 25 pg/ml for plasma (signal-to-noise ratio = 2.3). The method was applied to the determination of 9α,11β-PGF_2 in urine and plasma samples from patients with bronchial asthma.

1. Introduction

It is useful to assess the endogenous production of prostaglandin D_2 (PGD_2) because it has a number of biological properties in human health and disease. PGD_2 is, however, rapidly transformed into 9α,11β-prostaglandin F_2 (9α,11β-PGF_2) [1]. Hence PGD_2 can be determined by measuring 9α,11β-PGF_2, which not only is a primary metabolite of PGD_2 but also has specific biological activities itself as one of the markers of mast cell activation [2]. However, there are some difficulties in assaying endogenous 9α,11β-PGF_2 because of its low concentration in humans. For large samples of urine and plasma, 9α,11β-PGF_2 has been determined as its pentafluorobenzyl ester–*tert*-butyldimethylsilyl ether derivative by negative-ion chemical ionization GC–MS with an [^{18}O]9α,11β-PGF_2 stable isotope dilution method [1] or as its methyl ester–O-methoxyloxime–trimethylsilyl ether derivative by electron impact ionization GC–MS [3,4]. However, for biomedical applications, a more sensitive, small-scale and simple assay is needed.

To study the changes in the levels of 9α,11β-PGF_2 in many human diseases, we have tried to develop an assay method for 9α,11β-PGF_2 using GC–MS with selected-ion monitoring (SIM) and pentadeuterated $PGF_{2\alpha}$ as a stable isotope dilution internal standard. This paper describes the GC–MS determination of 9α,11β-PGF_2 using a stable dimethylisopropylsilyl (DMiPS) ether de-

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rivative of the methyl ester and its assay in small amounts of urine and plasma from patients with bronchial asthma, in which PGD_2 is thought to be one of the pathogenetic candidates.

2. Experimental

2.1. Materials

All organic solvents (Nacalai Tesque, Kyoto, Japan) were of HPLC or analytical-reagent grade. DMiPS-imidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). An ethereal solution of diazomethane was prepared from N - methyl - N - nitroso - *p* - toluenesulphonamide (Tokyo Kasei Kogyo). Cartridges of octadecasilylsilica (Sep-Pak C_{18}, 500 mg per cartridge) were purchased from Waters (Milford, MA, USA). Silica gel (Kieselgel 60, 70–230 mesh) (Merck, Darmstadt, Germany) was purchased from Nacalai Tesque. A Chem-tube hydromatrix (10-ml capacity) was purchased from Varian (Harbor City, CA, USA). Other reagents were of analytical-reagent grade and commercially available. The deuterated internal standard was synthesized in our laboratories from Corey's lactone and [$^2H_8$]tetrahydrofuran according to the method of Green *et al.* [5].

2.2. Sample preparation

Urine collection

Urine samples were obtained from hospitalized children with or without acute asthma (age: 6–15 yr). Urine was collected in a bottle containing 3 ml of toluene, which prevents oxidation and bacterial growth, at 4°C for 24 h. Volumes of 10 ml of the urine were withdrawn from the bottle on the following day and kept frozen at −70°C until assayed.

Blood sampling

Blood samples were obtained from children with or without acute asthma (age: 10–16 yrs). A volume of 9 ml of venous blood was gently withdrawn from the antecubital vein of the patient into a plastic syringe containing 1 ml of 3.8% (w/v) trisodium citrate and 3 m*M* indomethacin. The blood was immediately centrifuged at 2000 *g* at 4°C for 15 min and stored at −70°C until assayed.

Extraction

Aliquots of 5–10 ml of urine and 2–5 ml of plasma were diluted with distilled water (final volume 10 ml). Pentadeuterated $PGF_{2\alpha}$ (10 ng) was added as an internal standard. The solution was acidified with hydrochloric acid (2 *M*) to pH 3.0, then the resulting solution was applied to a Chem-tube column (purified Kieselgel). After being allowed to stand for 5 min to allow adsorption of the aqueous phase on the matrix, PGs were eluted with 40 ml of ethyl acetate. The organic solvent fraction was then evaporated in a centrifugal concentrator under reduced pressure below 40°C. The residue was dissolved in 10 ml of 15% (v/v) ethanol (pH 3.0) and the solution was applied to a Sep-Pak C_{18} cartridge [6]. The cartridge was washed with 10 ml of 15% (v/v) ethanol (pH 3.0) and 10 ml of petroleum ether. PGs were eluted with 10 ml of ethyl acetate. The eluent was evaporated to dryness *in vacuo* below 40°C.

2.3. Clean-up and derivatization

Methyl ester formation

The crude extract was dissolved in 50 μl of methanol and 400 μl of diazomethane ethereal solution were added. The mixture was allowed to stand at room temperature for 30 min and evaporated to dryness *in vacuo* below 40°C.

First clean-up by silica gel column chromatography

The methyl ester of PGs was dissolved in hexane–ethyl acetate (2:1) and the solution was applied to a silica gel column (5 × 0.5 cm I.D.), pre-washed with the same solvent [7]. The column was washed with 10 ml of the same solvent and PGs were eluted with 30 ml of ethyl acetate containing 1% (v/v) of ethanol. The effluent from the silica gel column was evaporated to dryness *in vacuo* below 40°C.

Silylation

The methyl esters (Me) of PGs were dissolved in 50 μl of pyridine, 50 μl of DMiPS-imidazole were added and the resulting mixture was allowed to stand for 60 min at room temperature [8]. The excess of the reagents was removed on a Sephadex LH-20 column (5 × 0.5 cm I.D.) with hexane–chloroform–methanol (10:10:1, v/v/v) as eluent to obtain the Me-DMiPS derivatives of PGs [9]. The effluent from the Sephadex LH-20 column was evaporated to dryness *in vacuo* below 30°C.

Second purification by silica gel column chromatography

The Me-DMiPS ether of the PGs fraction was dissolved in 25 μl of ethyl acetate and then diluted with 1.25 ml of hexane and applied to a silica gel column (5 × 0.05 cm I.D.), pre-washed with hexane). After washing the column with 5 ml of hexane, the Me-DMiPS ether derivatives of PGs were eluted with 10 ml of hexane–diethyl ether (9:1, v/v). The eluate was concentrated in a centrifugal concentrator under reduced pressure at 25–30°C.

2.4. GC–MS conditions

A JMS-DX 303 gas chromatograph–mass spectrometer (JEOL, Tokyo, Japan) equipped with a JMA-DA 5000 data processing system was employed. The column was a 30 m × 0.317 mm I.D. fused-silica capillary (DB-1, film thickness 0.1 μm) (J&W Scientific, Folsom, CA, USA). The temperature of the column oven was programmed in a two-step gradient. The first step was initially 100°C for 1 min, then raised to 220°C at 32°C/min, and in the second step the temperature was raised from 220 to 300°C at 4°C/min. An all-glass solventless injector was mounted horizontally in the injection block of the gas chromatograph [10]. Helium was used as the carrier gas at the linear velocity of 21 cm/s. The temperatures of the injection port and separator block were 300°C and the ionization source was kept at 270°C. The ionization energy was 70 eV. The selected ions monitored were *m/z* 625.40 and 630.43 or 479.23 and 484.33 at a mass spectral resolution of 1500.

3. Results and discussion

9α,11β-PGF_2 is formed enzymatically from PGD_2 [1]. The molecular formula is identical with that of $PGF_{2\alpha}$ (9α,11α-PGF_2) except for the fact that they are epimers. Our initial effort was directed towards the separation of 9α,11β-PGF_2 from $PGF_{2\alpha}$. When 9α,11β-PGF_2 and $PGF_{2\alpha}$ were converted into the Me-DMiPS ether derivative, 9α,11β-PGF_2 was completely separated from $PGF_{2\alpha}$ by GC (Fig. 1). This labelled molecule may be useful as an internal standard for the simultaneous determination of 9α,11β-PGF_2 and $PGF_{2\alpha}$.

The two PGs showed almost identical chromatographic behaviour during the derivatization and purification from urine and plasma; we can treat them in the same extraction, clean-up and derivatization of the DMiPS ether of the Me ester for the two PGs.

The Me-DMiPS ether derivative of 9α,11β-

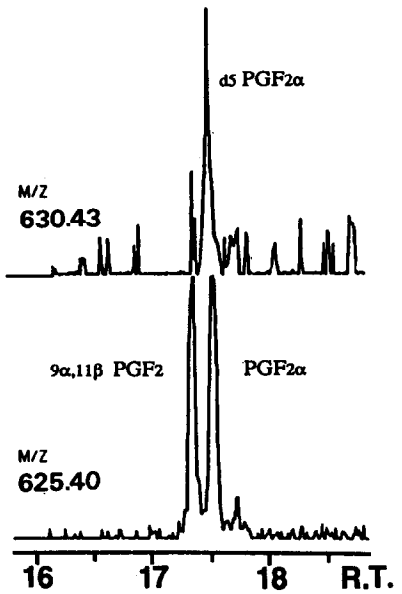

Fig. 1. Typical mass chromatogram recording of 9α,11β-PGF_2, $PGF_{2\alpha}$, and d_5-$PGF_{2\alpha}$ obtained from Me-DMiPS derivatives.

PGF_2 exhibited a mass spectral pattern similar to that of the Me-DMiPS ether derivative of $PGF_{2\alpha}$ (Fig. 2).

The characteristic ion $[M-43]^+$ was observed at m/z 625. Subsequent elimination of the C_5H_{11} moiety of the ω-side-chain and dimethylisopropylsilanol from the molecular ion provided the ion at m/z 479. Then, to enhance the sensitivity in SIM, we chose intense ions of m/z 625.40 and 479.23. We constructed a calibration graph by using authentic 9α,11β-PGF_2 and d_5-$PGF_{2\alpha}$ as an internal standard with two different fragment ions (Fig. 3). Good linearity was exhibited between the two calibration graphs calculated from two different fragment intensities. This indicates that the ratios of the fragment forma-

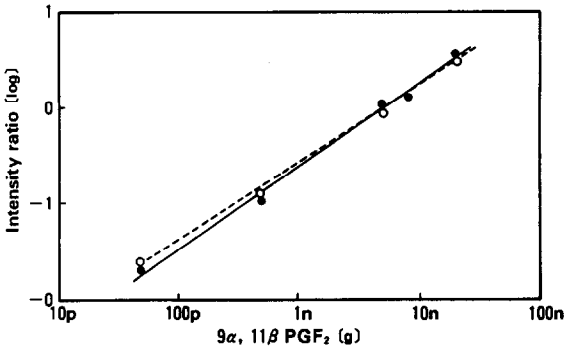

Fig. 3. Calibration of 9α,11β-PGF_2 using the stable isotope (d5-$PGF_{2\alpha}$) dilution method from two different fragments. Intensity ratio calculated, using SIM recording of different fragments [m/z (●) 479/484 and (○) 625/630] on 9α,11β-PGF_2 with d_5-$PGF_{2\alpha}$ (10 ng) as internal standard.

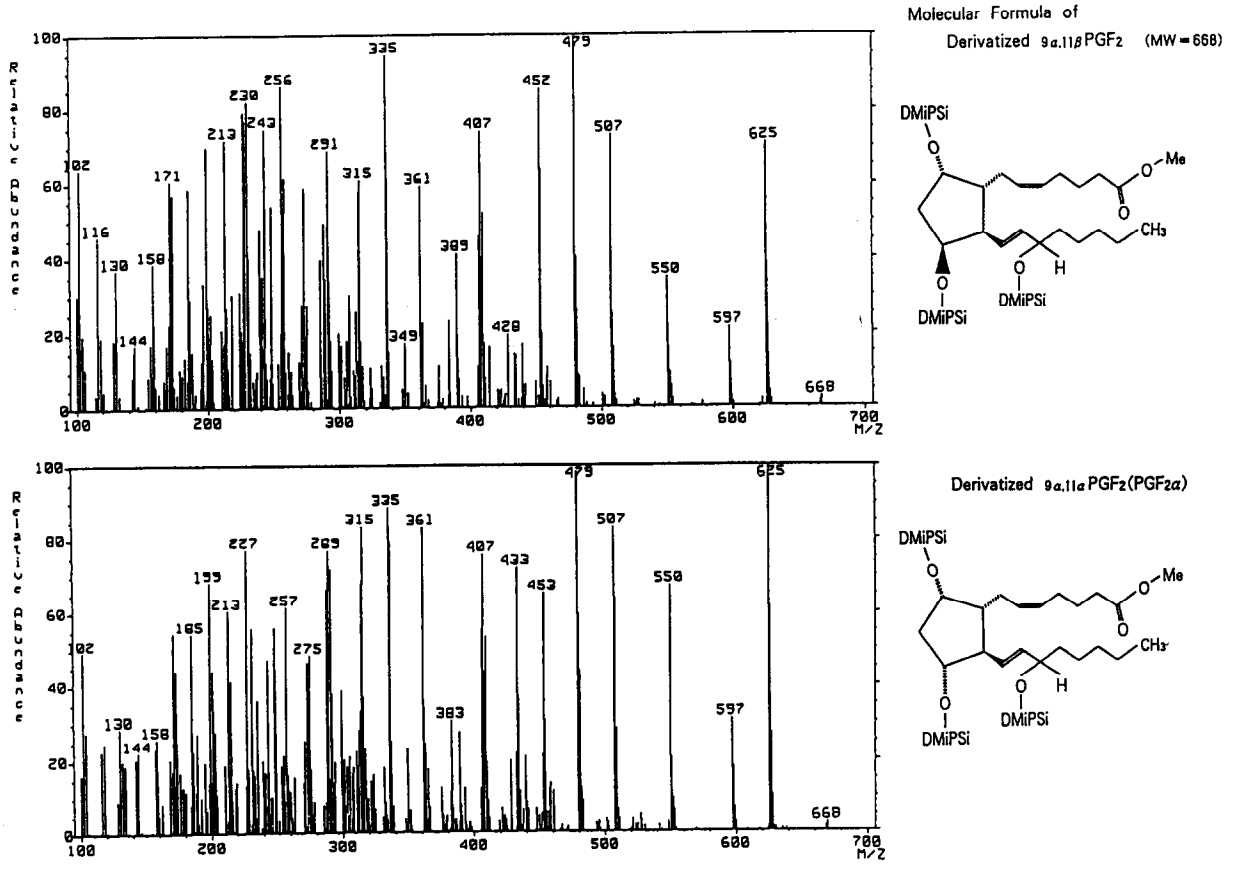

Fig. 2. Comparison of mass spectra and molecular formulae of 9α,11β-PGF_2 and 9α,11α-PGF_2 ($PGF_{2\alpha}$) Me-DMiPS derivatives.

tion for 9α,11β-PGF_2 and d_5-$PGF_{2\alpha}$ are not different. This means that d_5-$PGF_{2\alpha}$ can be used as an internal standard for the determination of both 9α,11β-PGF_2 and $PGF_{2\alpha}$. Routinely, we recorded the intensity ratio of the *m/z* 625.40/630.43 ions for the quantification of 9α,11β-PGF_2.

To avoid experimental errors due to differences in the efficiency of extraction and clean-up procedures for different sample sizes, we took the samples for assay on an equal-volume basis. In the urine samples especially, there are large differences in the creatinine content and many drug contaminants in various clinical conditions. Therefore, we developed a sample extraction, clean-up and derivatization procedure based on equal volumes of various samples (details as described under Experimental).

We checked the recovery and sensitivity of the quantification procedure by comparing the direct ion intensity of 9α,11β-PGF_2 after the complete procedure or after only the clean-up and derivatization procedure. The efficiency of the clean-up and derivatization procedure was 82%. Also, the sample extraction from the Chem-tube hydromatrix was 36%, and the total efficiency was *ca.* 30% (data not shown). These recoveries are similar to those reported previously [1,2,6].

The calibration graph for 9α,11β-PGF_2 was linear from 5 pg to 100 ng using a pure standard of 9α,11β-PGF_2 with d_5-$PGF_{2\alpha}$ (10 ng), as shown in Fig. 4 (control). However, if we added the pure 9α,11β-PGF_2 to the biological sample (*e.g.* urine or plasma), the linearity of the calibration graph became poor in the lower concentration range, because of the noise due to the urine and plasma matrix, as shown in Fig. 4. This tendency depends on the sample quality (and/or contaminant) and amount. The limit of detection for 9α,11β-PGF_2 in biological samples was 50 pg/ml (urine) and 25 pg/ml (plasma) at a signal-to-noise ratio of 2.3.

The precision of the quantification procedure is shown in Table 1. The intensity ratio of d_0-9α,11β-PGF_2/d_5-$PGF_{2\alpha}$ recovered was between 98 and 117% on the calibration graph. The intra-assay coefficient of variation (C.V.) of the procedure ($n = 5$) was 5–10% and the correlation

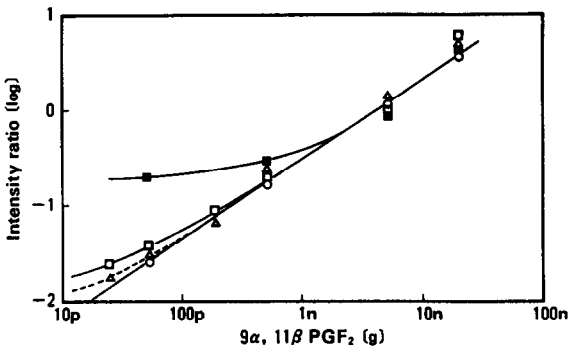

Fig. 4. Calibration of 9α,11β-PGF_2 by addition to various medical samples. Various concentrations of 9α,11β-PGF_2 and 10 ng of d_5-$PGF_{2\alpha}$ were added to urine or plasma. (○) Control; (□) 5 ml of urine; (■) 10 ml of urine; (△) 2 ml of plasma.

coefficient was 0.9979 (equation of the control: $y = 0.213x + 0.078$). We also checked the inter-assay variation of the procedure using the Friedman test [11]. There were no significant differences in the calibration graphs among the five independent experiments ($n = 5$, chi-squared value >12%, data not shown).

The method was applied to the determination of 9α,11β-PGF_2 in samples obtained from patients with bronchial asthma. Urine and plasma samples were obtained from children with acute asthmatic attacks on admission and at the time of discharge. Table 2 shows levels of 9α,11β-PGF_2 obtained in urine samples from four asthmatics and in plasma samples from three asthmatics. The urine and plasma levels of 9α,11β-PGF_2 were lower at the time of discharge compared with those on admission.

9α,11β-PGF_2 is known to be a potent bronchoconstrictor generated in mast cells. Liston and Roberts [1] reported that by using GC–MS

Table 1
Recovery and instrumental precision in a typical calibration

9α,11β-PGF_2 (ng per injection)	Recovery (mean ± S.D., $n = 5$) (%)	C.V. (%)
20	98.8 ± 5.2	5.3
5	117.9 ± 9.7	11.2
0.5	110.2 ± 9.2	8.3
0.05	104.0 ± 10.0	9.6

Table 2
Urinary and plasma 9α,11β-PGF_2 in asthmatic children

No.	Case	Age (yr)	Sex	Acute asthmatic attack	No asthmatic attack
Urinary 9α,11β-PGF_2 in asthmatic children (ng per 24 h)					
1	M.K.	15	F	342.0	230.0
2	Y.M.	16	F	1073.0	170.0
3	H.Y.	10	M	116.0	0.32
4	F.J.	6	M	214.7	40.0
Plasma 9α,11β-PGF_2 in asthmatic children (ng/ml)					
1	M.K.	15	F	0.94	0.15
2	Y.M.	16	F	0.71	0.47
3	H.Y.	10	M	0.85	0.05

with negative-ion chemical ionization, the levels of 9α,11β-PGF_2 were 6634 ng/ml in urine and 490.09 ng/ml in plasma from a patient with systeic mastocytosis. Our results support the view that 9α,11β-PGF_2, one of the mast cell-derived arachidonic acid metabolites, is involved in acute asthmatic attacks.

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