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DETERMINATION OF PROSTAGLANDIN $F_{1\alpha}$ AND $F_{2\alpha}$ BY GAS–LIQUID CHROMATOGRAPHY

MAMORU SUGIURA and KAZUYUKI HIRANO
Tokyo College of Pharmacy, Ueno-sakuragi, Taito-ku, Tokyo 110 (Japan)
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SUMMARY

A quantitative gas–liquid chromatographic method has been developed for the determination of prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ ($PGF_{1\alpha}$ and $PGF_{2\alpha}$). The method involves the conversion of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ into their trimethylsilyl methyl esters or heptafluorobutyryl methyl esters and their chromatography using 3% OV-1 on Gas-Chrom Z; their structures were established by combined gas chromatography–mass spectrometry.

The method can be applied to human semen plasmas with prostaglandin $F_{2\beta}$ ($PGF_{2\beta}$) as an internal standard.

INTRODUCTION

Prostaglandins (PGs) were first detected as a result of their biological activities[1,2], and such activities have frequently been employed as the basis for their measurement. Spectrophotometric analyses are not sufficiently sensitive for the determination of the very small amounts that exist in tissues[3,4], and an enzymatic procedure for measuring PGs[5–7] also lacks specificity and has limited sensitivity of detection when applied to biological samples. Radioimmunoassays for PGs[8–11] have the capacity to measure picogram amounts, but have incomplete specificity owing to immunological cross-reactions.

Gas–liquid chromatography (GLC) has the potential to provide simultaneous separations according to the extent of unsaturation of PGs. GLC can be employed with several PGF derivatives[12–17], but it is difficult to apply these techniques to biological samples. Recently, combined gas chromatography–mass spectrometry (GC–MS) measurements[18–20] have made it possible to identify and measure nanogram amounts of individual PGs. However, most laboratories probably cannot afford to purchase the necessary equipment.

In this paper, we describe our studies on the extraction of PGF from biological samples and the use of GLC to determine $PGF_{1\alpha}$ and $PGF_{2\alpha}$. This technique has been applied to assay $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasmas.

EXPERIMENTAL

Reagents

Methanol, ethanol, *n*-hexane, ethyl acetate and ether were distilled before use. Other solvents and reagents were purchased commercially and used as received.

Apparatus and conditions

A Jeol, Model JGC-20KPF, gas chromatograph equipped with a hydrogen flame ionization detector (HFID), and a Jeol, Model JGC-20KE, gas chromatograph equipped with an electron capture detector (ECD) were used. The 3 m×2 mm I.D. and 1 m × 2 mm I.D. glass columns were packed with 3% OV-1 on Gas-Chrom Z (80–100 mesh).

Gas chromatography–mass spectrometry (GC–MS). The mass spectra were measured with a Hitachi Model RMU-6E mass spectrometer. The operating conditions were: chamber voltage, 70 eV; ion source temperature, 220°; total emission, 80 μA; current, 70 μA. All the samples were introduced into the ionization chamber through a Hitachi K-53 gas chromatograph. A 2.0-m glass column packed with 3% OV-1 on Gas-Chrom Z (80–100 mesh) was used and the temperature was maintained at 210°.

Scintillation counter. A Beckmann Model LS 100 scintillation counter was used, and ^{3}H-labelled PGF was counted in a dioxan scintillator.

Standard procedure

Method A (preparation of $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$-TMS–Me derivatives). PGF was methylated with diazomethane in diethyl ether–methanol (9:1), allowed to stand for 10 min and then evaporated under a stream of nitrogen at room temperature. To the dried residue of PG–Me ester, 50 μl of dimethyl sulphoxide–ethyl acetate (1:5) or 50 μl of triethylamine–ethyl acetate (1:5) and 50 μl of bistrimethylsilyl trifluoroacetoamide (BSTFA) were addded. After 30 min, the excess of the reagents was evaporated under a stream of nitrogen at room temperature, and the dried residue was dissolved in a small volume of ethyl acetate and injected into the gas chromatograph (HFID).

Method B (preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives). Methylation of PGF was carried out according to Method A and 50 μl of ethyl acetate and 50 μl of heptafluorobutyryl imidazole (HFBI) were added to the dried residue. After 10 min, the reaction mixture was evaporated to dryness under a stream of nitrogen at room temperature. The dried residue was extracted with three 1-ml volumes of *n*-hexane, and the combined *n*-hexane extracts were concentrated and injected into the gas chromatograph (ECD).

Extraction of total PGs

The extraction of PGs from samples is shown in Fig. 1.

The sample was homogenized with 10 volumes of Bloor solution (ethanol–diethyl ether, 3:1) in a glass homogenizer, the homogenate was allowed to stand for 30 min and then filtered. The filtrate was evaporated to dryness under reduced pressure at 40°, and 5 ml of carbon tetrachloride and 10 ml of water were added to the residue. The mixture was shaken vigorously and centrifuged at 3,000 rpm

Sample: Bloor solution (1:10)
↓
Filtrate — Mixed and allowed to stand for 30 min, filtered and washed with Bloor solution
↓
Upper phase — Evaporated to dryness under reduced pressure at 40°, dissolved in 5 ml of CCl_4, 10 ml of water added and shaken vigorously, then centrifuged at 3,000 rpm for 10 min
↓
Upper phase — Adjusted at pH 3 with 1 *N* HCl, 10 ml of ethyl acetate added and shaken vigorously, then centrifuged at 3,000 rpm (800 *g*) for 10 min
↓
Residue (Total PGs) — 200 μl of 1 *N* ammonia solution and 2 ml of methanol added, then evaporated to dryness under reduced pressure at 40°

Fig. 1. Procedure for extraction of PGs from samples.

(800 *g*) for 10 min, then the upper layer (aqueous layer) was adjusted to pH 3 with 1 *N* hydrochloric acid and 10 ml of ethyl acetate were added. The mixture was shaken vigorously and centrifuged at 3,000 rpm (800 *g*) for 10 min and then 200 μl of 1 *N* ammonia solution was added to the upper layer (ethyl acetate layer) and the mixture was evaporated to dryness under reduced pressure at 40°.

RESULTS AND DISCUSSION

Preparation of $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives

The optimum reaction conditions for the standard procedure as described under Experimental was established from preliminary experiments. Various trimethylsilylating reagents and reactive solvent systems were examined, and it was found that the PGF–TMS derivatives were most easily produced by using BSTFA as the trimethylsilylating reagent in the above mixed solvent systems. The effects of temperature and time on the production of PGF–TMS–Me derivatives were examined relative to standard conditions of 30 min and room temperature. The $PGF_{2\beta}$–TMS–Me derivative was used as the internal standard and a GLC sepa-

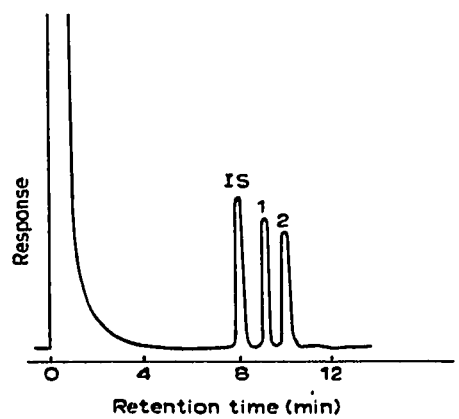

Fig. 2. Gas chromatogram of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ using $PGF_{2\beta}$ as the internal standard. Conditions: 3% OV-1; 3-m glass column; 230° (240°); HFID; nitrogen carrier gas, flow-rate 50 ml/min. IS, internal standard, $PGF_{2\beta}$–TMS–Me; (1) $PGF_{2\alpha}$–TMS–Me; (2) $PGF_{1\alpha}$–TMS–Me.

ration was achieved successfully by using a glass column, as shown in Fig. 2; the calibration curves passed through the origin for $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in the range 1–10 μg.

Structures of the $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives

The structures of the $PGF_{1\alpha}$–TMS–Me and $PGF_{2\alpha}$–TMS–Me derivatives were established by GC–MS and the spectral data are shown in Figs. 3 and 4.

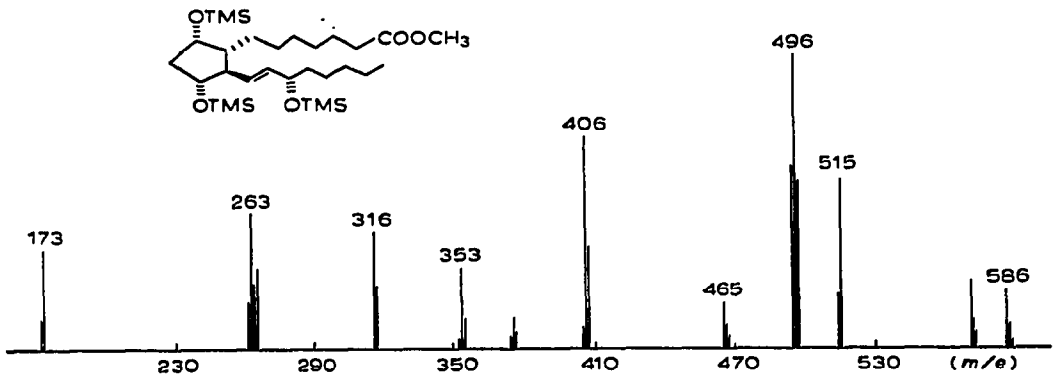

Fig. 3. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{1\alpha}$–TMS–Me derivative. GC–MS conditions: mass range, 750; chamber voltage, 70 eV; chamber temperature, 230°; column, 3% OV-1, 2 m; 210°; helium carrier gas, flow-rate 50 ml/min.

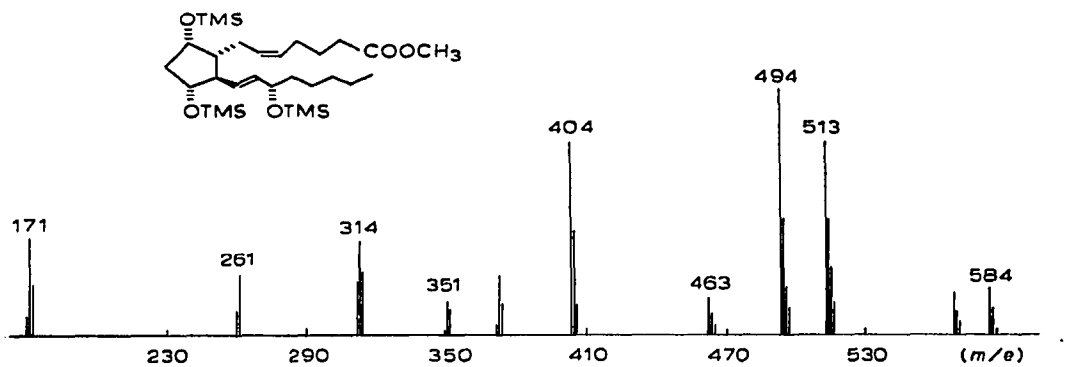

Fig. 4. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{2\alpha}$–TMS–Me derivative. GC–MS conditions: mass range, 750; chamber voltage, 70 eV; chamber temperature, 230°; column, 3% OV-1, 2 m; 210°; helium carrier gas, flow-rate 50 ml/min.

In Fig. 3, the ion at *m/e* 586 indicates the molecular weight and *m/e* 496 corresponds to the loss of one TMS group, *m/e* 406 corresponds to the loss of two TMS groups and the peak at *m/e* 316 corresponds to the loss of three TMS groups from the $PGF_{1\alpha}$–TMS–Me derivative. In Fig. 4, the ion at *m/e* 584 indicates the molecular weight and that at *m/e* 494 corresponds to the loss of one TMS group, the peak at *m/e* 404 corresponds to the loss of two TMS groups and the peak at *m/e* 314 corresponds to the loss of three TMS groups from the $PGF_{2\alpha}$–TMS–Me

derivative. It is concluded that three hydroxy groups in both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are substituted with three TMS groups.

Preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives

The optimum reaction conditions for the preparation of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives were as described under *Standard procedure*. PGs are unstable towards acids and bases and, as reagent for the formation of PGF–HFB–Me derivatives, it seems that HFBI is more desirable than heptafluorobutyric anhydride (HFBA), because heptafluorobyturic acid is produced from HFBA in the course of the preparation of PGF–HFB–Me derivatives. In fact, when HFBA is used, the PGF–HFB–Me derivatives give two peaks on the gas chromatogram, according to Levitt *et al.*[15], but when HFBI is used, PGF–HFB–Me derivatives give a single peak and can be separated successfully, as shown in Fig. 5.

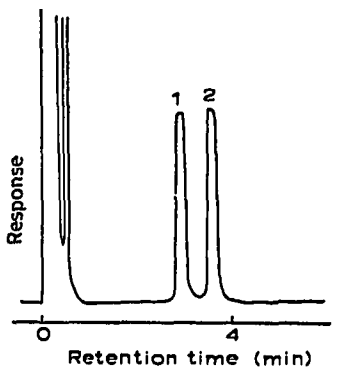

Fig. 5. Gas chromatogram of (1) $PGF_{1\alpha}$–HFB–Me and (2) $PGF_{2\alpha}$–HFB–Me derivatives. Conditions: 3% OV-1; 1-m glass column; 190° (260°); ECD (^{63}Ni); nitrogen carrier gas, flow-rate 60 ml/min.

The thermal decomposition of $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives was not observed during GLC under the experimental conditions used. The PGF–HFB–Me derivatives have suitable properties for the ECD gas chromatograph: they are volatile, adequately separated, have sufficient electron-capturing properties to be detectable at a low level and have adequate stability when stored in *n*-hexane at 0°. Calibration curves passed through the origin when $PGF_{2\beta}$–HFB–Me was used as the internal standard.

Structures of the $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives

The structures of the $PGF_{1\alpha}$–HFB–Me and $PGF_{2\alpha}$–HFB–Me derivatives were established by GC–MS and the spectral data are shown in Figs. 6 and 7.

In Fig. 6, a molecular peak at *m/e* 958 does not appear, the peak at *m/e* 744 corresponds to the loss of one HFB group, the peak at *m/e* 530 corresponds to the loss of two HFB groups and the peak at *m/e* 316 corresponds to the loss of three HFB groups from the $PGF_{1\alpha}$–HFB–Me derivative. In Fig. 7, a molecular peak at *m/e* 956 does not appear, the peak at *m/e* 742 corresponds to the loss of one HFB group, the peak at *m/e* 528 corresponds to the loss of two HFB groups and

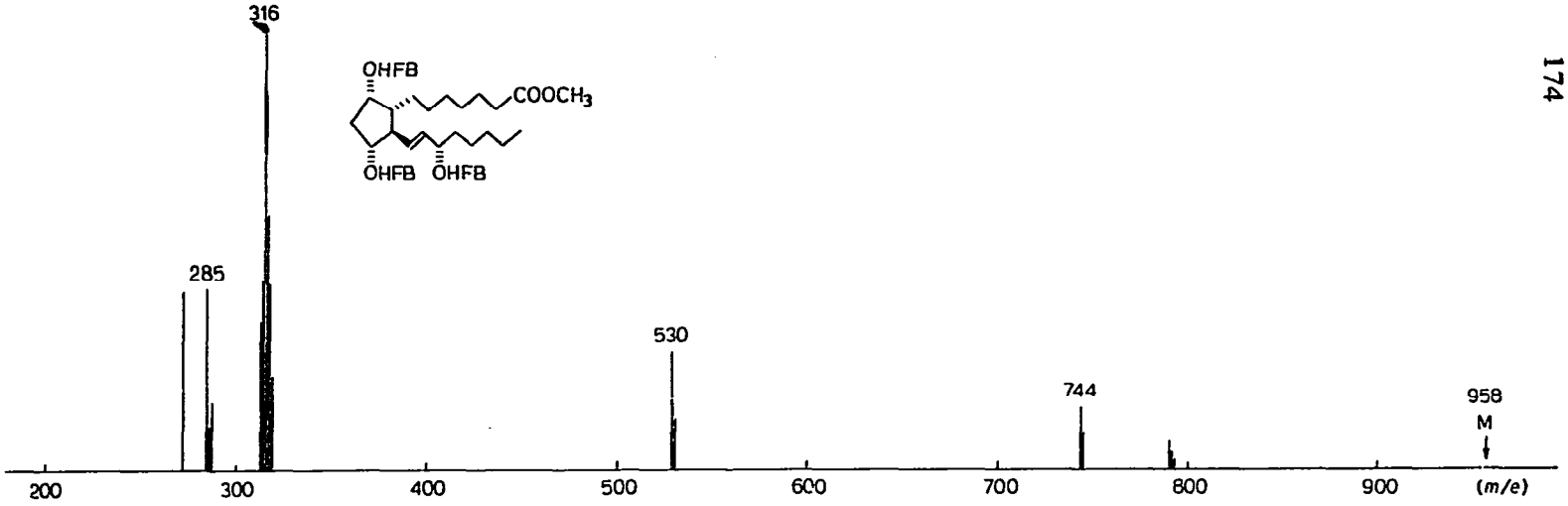

Fig. 6. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{1\alpha}$-HFB-Me derivative. GC–MS conditions: mass range, 1,000; chamber voltage, 70 eV; chamber temperature, 230°; column, 3% OV-1, 2 m; 210°; helium carrier gas, flow-rate 50 ml/min.

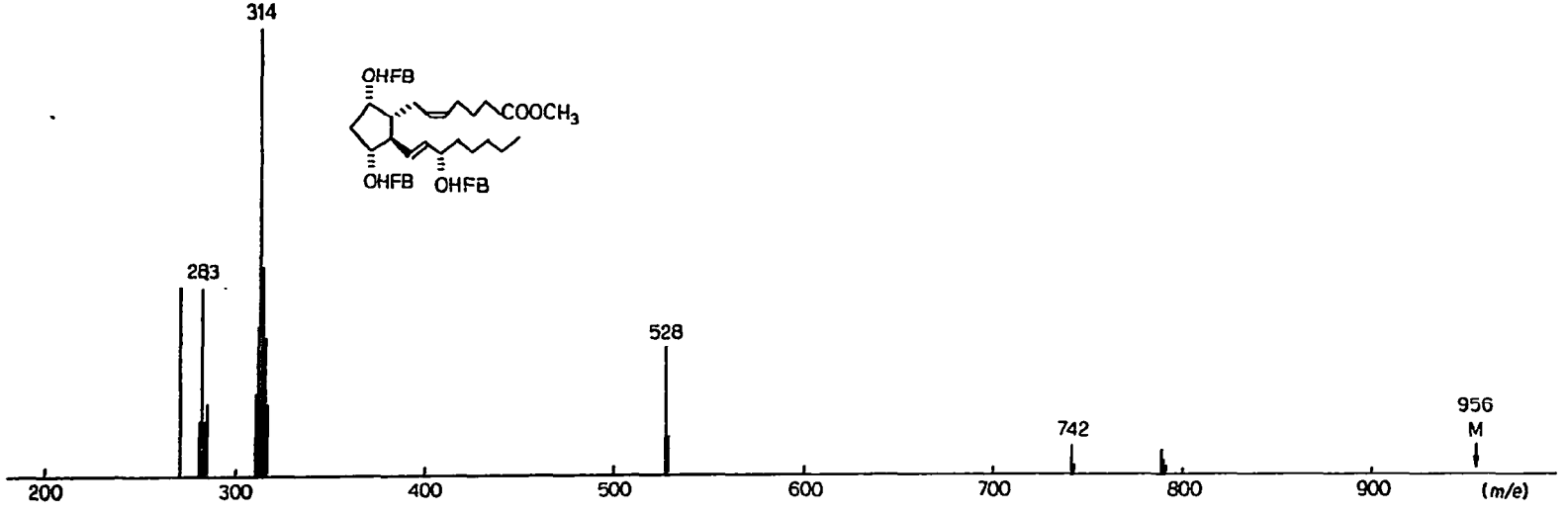

Fig. 7. Mass spectral data obtained on gas chromatographic effluent at a retention time corresponding to $PGF_{2\alpha}$-HFB-Me derivative. GC–MS conditions as in Fig. 6.

the peak at *m/e* 314 corresponds to the loss of three HFB groups from the $PGF_{2\alpha}$–HFB–Me derivative. From these results, it seems that three hydroxy groups in both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are substituted with three HFB groups.

Extraction of PGs

Recently, Inagawa *et al.* reported on the determination of PGs by means of radioimmunoassay[11], using Folch solution (methanol–chloroform, 1:2) for the extraction of all the lipids from the samples. In the gas chromatographic determination by ECD, the impurity peaks based on chloroform as the solvent appeared on the gas chromatograms and therefore, in this work, we used Bloor solution (ethanol–diethyl ether, 3:1) instead of Folch solution in the first step of the extraction of all the lipids from the samples and established the optimum procedure for the extraction of samples for GLC determination as shown in Fig. 1. The addition of 1 *N* ammonia solution to the ethyl acetate layer prevents the decomposition of PGF during evaporation at 40°, which would have occurred because PGs are unstable towards acids. The transfer of PGF from the carbon tetrachloride layer into the aqueous layer is specific for all lipids and, in this step, the lipids other than PGs can be removed.

Table I shows the recovery of 5 μg and 5 ng of PGF_2 according to Fig. 1 by use of tritium-labelled $PGF_{2\alpha}$, and the mean recoveries of $PGF_{2\alpha}$ were 88.7% and 84.3%, respectively. $PGF_{1\alpha}$ exhibits the same behaviour as $PGF_{2\alpha}$ in the extraction procedure.

TABLE I

RECOVERY OF PGs IN EXTRACTION PROCEDURE

No.	*PG*	*Amount*	*Recovery (%)*	
1	$PGF_{2\alpha}$*	5 μg		92.8
2				85.5
3				87.8
			average	88.7
4		5 ng		86.2
5				82.2
6				84.6
			average	84.3

* $PGF_{2\alpha}$ included ^3H-labelled $PGF_{2\alpha}$ (200 pg). Counted in a dioxan scintillator with a Beckman Model LS 100 scintillation counter.

Determination of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasma

$PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasmas were assayed by using method B. A chromatogram obtained from human semen plasma is illustrated in Fig. 8 using $PGF_{2\beta}$ as the internal standard and the results are listed in Table II.

The amounts of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ determined in human semen plasmas were mid-way between the results so far reported by radioimmunoassay and GLC.

We are planning to apply this technique to the GLC determination of other PGs.

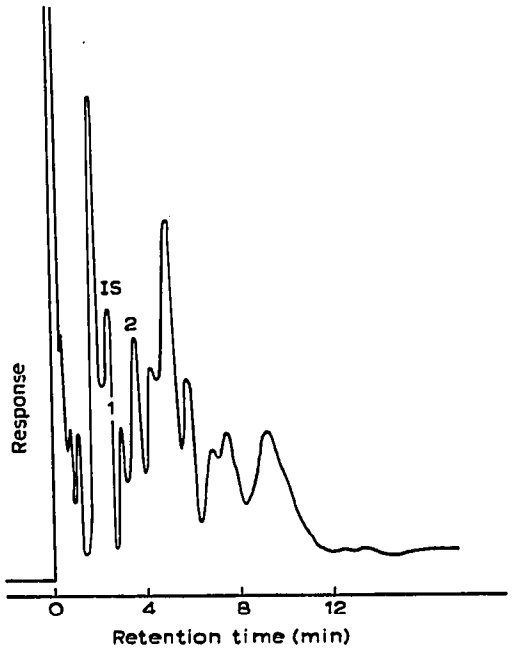

Fig. 8. Gas chromatogram of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in human semen plasma using $PGF_{2\beta}$ as the internal standard. Conditions: 3% OV-1; 1-m glass column; 190° (260°); ECD (^{63}Ni); nitrogen carrier gas, flow-rate 60 ml/min. IS, internal standard; (1) $PGF_{2\alpha}$-HFB-Me; (2) $PGF_{1\alpha}$-HFB-Me.

TABLE II

DETERMINATION OF PGF IN HUMAN SEMEN PLASMA

Sample No.	*Age (years)*	*$PGF_{1\alpha}$ (μg/ml)*	*$PGF_{2\alpha}$ (μg/ml)*
1	20	0.87	1.60
2	25	1.16	1.44
3	23	0.76	0.46

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