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PROSTAGLANDINS E AND F, AND 19-HYDROXYLATED E AND F (SERIES I AND II) IN SEMEN OF FERTILE MEN

GAS AND LIQUID CHROMATOGRAPHIC SEPARATION WITH SELECTED ION DETECTION

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SUMMARY

Low concentrations of prostaglandins (PG) could be related to male clinical infertility although relevant experimental data are scarce. The aim of this work is to establish reliable seminal PG levels in fertile men by rigorous sample control, to prevent degradation, and by rapid and simple extraction and assay procedures.

Single semen samples from healthy fertile men were immediately centrifuged (within 30 min of ejaculation) adding $PGF_{2\alpha}$ D_4 to the seminal plasma as internal standard. The samples were next ultrafiltered and the PGs in the ultrafiltrate were derivatized with a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and piperidine (1:1) at 60° for 30 min. Optimum gas—liquid chromatographic separation of all of the peaks of interest was achieved on 4 m × 1/4 in. I.D. Dexsil 300 packed columns at 280°. The detection and quantitation of all the peaks of interest depends on the selected ion monitoring of specific masses. The values obtained (in μg/ml, range in parentheses) were: PGEs, 63.5 ± 49.3 (9—164); PGFs, 2.6 ± 1.92 (0.95—6.63); 19-OH PGEs, 592.6 ± 312.5 (142.1—1047); and 19-OH PGFs, 12.66 ± 5.21 (4—19). Individual values for members of both series I and II are also presented.

The sample collection and extraction procedures were further checked by high-performance liquid chromatography on a μPorasil column, with individual isolation and collection of all of the PGs, including the 19-OH PGs not previously separated by liquid chromatography.

INTRODUCTION

The possibility of a relationship between low prostaglandin (PG) levels in human semen and clinical male infertility has been considered in the literature by different authors [1—7]. However, at the present time it is not known either how or which of the prostaglandins (PGs) may promote human fertility.

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Nevertheless, there are various interesting effects to consider, both in relation to the female as well as the male reproductive tracts [7]. Likewise, it has been suggested that PGs may affect spermatozoal metabolism through stimulation of cAMP [8], a possibility as yet unproven. Thus, any research effort in this direction could be potentially very rewarding, providing that the problem is well defined not only in relation to the infertility but also as to the nature and levels of the likely influencing factors, such as the PGs. It is now known that the recent re-examination of the type and extent of prostaglandins that occur in human semen has led to a significant reduction in the number of primary PGs identified in these samples from a high of thirteen, of which eight have been shown to be artifacts, to just five plus the recently identified 19-hydroxylated PGs (19-OH PGEs) and PGFs (19-OH PGFs) [7].

Briefly, at the present time, only nine types of endogenously synthesized prostaglandins can be considered as positively identified in semen, of which PGE_1 and PGE_2 together with their corresponding 19-hydroxylated analogs [9,10] are quantitatively the most significant.

On the other hand, it has also been indicated that even storage of samples at −20° may not prevent the possible breakdown of PGEs and 19-OH PGEs into PGAs and PGBs [11]. However, this seems to be a variable and rather uncontrolled process which most likely could account for the remarkable discrepancies observed in the type as well as in the reported levels of seminal PGs [2,7,10,12], although there are reports in the literature which still attempt to establish the concentration of PGAs, PGBs, 19-OH PGAs and 19-OH PGBs in fresh human seminal plasma [13]. In contrast, there are very few descriptions of the endogenous levels of the 19-hydroxylated PGEs and PGFs in human semen [6].

For all of these reasons it would be interesting to establish on a firmer basis the number and concentrations of PGs in semen of fertile men. This has been attempted through strict control of the sampling and extraction procedures followed by a rapid simultaneous determination of all PGs and 19-OH PGs present in the extracts by a highly specific and accurate technique, combined gas chromatography—mass spectrometry (GC—MS) with multiple ion detection (MID) using a deuterated internal PGF standard for reliable quantitation. A parallel high-performance liquid chromatographic (HPLC) study of these major PGs in human semen demonstrated that no degradation took place under the experimental conditions described.

EXPERIMENTAL

Reagents, samples of seminal plasma and labelled reference compounds

Ethyl acetate, methanol, piperidine, chloroform, toluene, acetone, hexamethyldisilazane (HMDS), acetic acid and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (chromatography grades) were used as described below.

The prostaglandin samples (A, B, E and F series I and II and F_2 D_4) were kindly supplied by Dr. J. Pike, Upjohn, Kalamazoo, Mich., U.S.A.

^{3}H-Labelled prostaglandins Fs, Es, As and Bs were purchased from the Radiochemical Centre, Amersham, Great Britain.

The samples of human seminal plasma were from the Instituto Urologico

Puigvert in Barcelona, except for the samples from fertile men, which were provided by volunteers.

Gas—liquid chromatography

The glass columns (4 m or 2 m × 2.5 mm I.D.) were washed with acetone, methanol and water and silanized for 24 h with a 5% solution of HMDS in toluene. After washing with methanol they were dried and immediately packed with 3% OV-17 or 3% Dexsil 300 on Gas-Chrom Q (100—120 mesh). The columns were usually conditioned for at least 48 h at 260—330° and were silanized by repeated injections of BSTFA before use. The separations were carried out on a Perkin-Elmer Model 900 gas chromatograph equipped with dual flame ionization detectors. Samples were injected with Hamilton syringes Models 701 (10 μl capacity) and 7101 (1 μl capacity).

High-performance liquid chromatography

The prostaglandins were separated on a 30 cm × 4 mm I.D. straight stainless-steel column packed with μPorasil. The packing of 10 μm silica particles was obtained from Waters Assoc., Milford, Mass., U.S.A. The liquid chromatograph was equipped with two solvent delivery pumps (Model 6000, Waters), a U6K injector and a solvent programming system (Model 660) also from Waters. The eluates were collected in an LKB 7000 fraction collector and analyzed either by liquid scintillation on an Intertechnique counter (Model SL 32) when they contained tritiated standards, or by gas—liquid chromatography as described above.

The liquid chromatographic separations of PGAs, PGBs, PGEs, PGFs, 19-OH PGE_2 and 19-OH PGF_2 were performed by a sequence of two programs. In the first non-linear program using gradient No. 4, which lasted 50 min, the initial solvent was 100% chloroform and the concentration of the second solvent (10% methanol, 2% acetic acid in chloroform) was increased to 50%. This first program was followed by a period of 10 min holding the final concentration of the first program. After the 10 min a second linear program (Waters gradient No. 6) was started until a final concentration of 100% of the second solvent was reached in 30 min. The flow-rate was maintained at 1 ml/min.

Gas chromatography—mass spectrometry

The mass spectra and the multiple ion detection (MID) profiles were obtained with an Hitachi RMU-6H mass spectrometer coupled via a single-stage jet separator to a Perkin-Elmer gas chromatograph Model 3920. The MS operating conditions were: electron energy 70 eV, accelerating voltage 1800 V, emission current 80 μA, separator at 290° and ion source at 180°. The mass spectrometer was equipped with a four-channel monitoring system developed and built in our laboratory [14]. The GC parameters were adjusted so as to maximize the performance of the mass spectrometer. Thus, the retention times are not always directly comparable in absolute values to those measured on the gas chromatograph.

*Preparation of derivatives**

Amounts of 20—40 μg of synthetic PGEs, PGAs, PGFs, PGEs, 19-OH PGEs and 19-OH PGFs were simultaneously derivatized in a one-step reaction with 40 μl of BSTFA—piperidine (1:1) for 30 min at 60° [15] and directly injected into the gas chromatograph.

The dry residues obtained from the biological extracts were treated in the the same manner.

Extraction of prostaglandins from semen

The samples of human seminal plasma (1.5—2 ml) were deposited on the membrane of a 25 mm, stirred, 17 ml ultrafiltration cell (Millipore Cat. No. XX 4202510), equipped with a 25 mm diameter Pellicon PSAC membrane of approximately 39 cm^2. The nominal molecular weight limit of this molecular filter is 10^3. A filtration pressure of 8.5 kg/cm^2 was applied, to yield a very clear aqueous ultrafiltrate. The residual pellet left on the membrane was washed with 2 ml of methanol. The washings were taken to dryness under a gentle stream of purified helium and the residue was redissolved in the aqueous ultrafiltrate, which was then adjusted to pH 3 with 1 *N* HCl and extracted three times with an equal volume of ethyl acetate. The three extracts were combined and evaporated to dryness under a stream of purified helium. A detailed account of this simplified extraction procedure has been made [16].

RESULTS AND DISCUSSION

In a previous report [16] it was demonstrated that the simplified extraction process developed in this laboratory is remarkably efficient, with recoveries (referred to ^3H-labelled $PGF_{1\alpha}$) of the order of 88% in the ultrafiltrate and 99% in the ethyl acetate extract. It was also shown that, in practice, and judging from the reproducibility of the GC and MID profiles obtained from different extracts of semen samples of fertile men, these extractions are also sufficiently reliable from a qualitative standpoint, as illustrated in Fig. 1. A detailed examination of these three illustrative profiles shows that while there are the expected quantitative variations in the abundance of various components of these chromatograms, qualitatively all the samples contain the same major and even minor components, as reflected for instance in the twenty peaks labelled in the GC traces of Fig. 1. What needs to be known with sufficient certainty is which of these peaks are due to endogenous prostaglandins and their corresponding concentration levels.

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*All prostaglandin (PG) derivatives described in this work were the trimethylsilyl (TMS) esters (TMSO indicates the trimethylsiloxy group) of the following acids: PGE_1, 9 enol TMS-11,15-bis TMSO prost-13-enoic; PGE_2, 9 enol TMS-11,15-bis TMSO prosta-5,13-dienoic; $PGF_{1\alpha}$, 9,11,15-tris TMSO prost-13-enoic; $PGF_{2\alpha}$, 9,11,15-tris TMSO prosta-5,13-dienoic; PGD_2, 11 enol TMS-9,15-bis TMSO prosta-5,11,13-trienoic; 15 keto PGF_2, 15 keto-9,11-bis TMSO prosta-5,13-dienoic; PGB_1, 15 TMSO, 9-keto prosta-8(12),13-dienoic; PGB_2, 15 TMSO, 9-keto prosta-5,8(12),13-trienoic; 19-OH $PGF_{2\alpha}$, 9,11,15,19-tetrakis TMSO prosta-5,13-dienoic; PGA_1, 9-enol TMSO-11-piperidyl-15 TMSO prosta-10,13-dienoic; PGA_2, 9-enol TMSO-11-piperidyl-15 TMSO prosta-5,10,13-trienoic; 19-OH PGA_2, 9-enol TMSO-11-piperidyl-15,19 bis TMSO prosta-5,10,13-trienoic; 19-OH PGE_2, 9-enol TMSO-11,15,19-tris TMSO-prosta-5,13-dienoic.

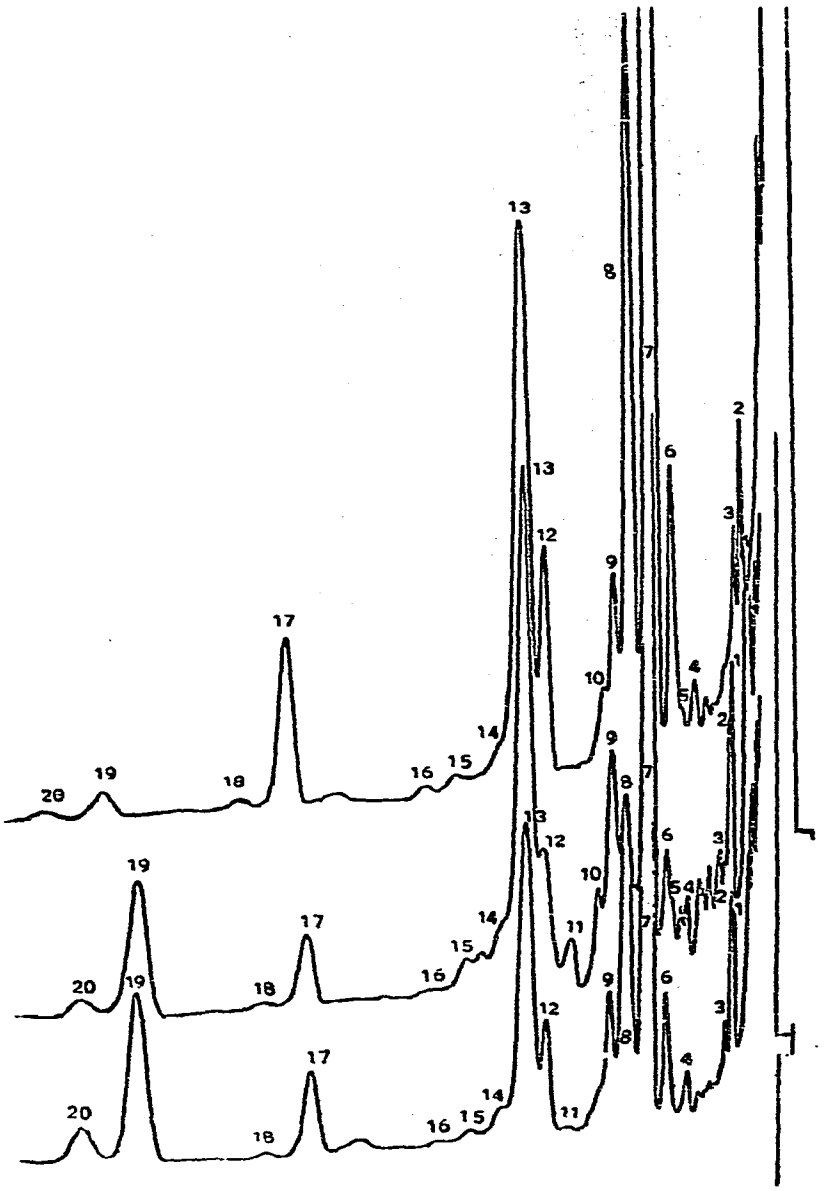

Fig. 1. GC profiles on Dexsil 300 at 280° obtained from the extracts of three different samples of seminal plasma.

Gas chromatographic separations

For GC purposes the dried extracts were derivatized in one step by direct silylation with BSTFA—piperidine (1:1). The structural identification of the persilylated derivatives obtained in this fashion has been described in detail [15]. It is interesting to note that like PGEs [17], the 19-OH PGEs also undergo an identical process of enolization with subsequent silylation of the ring keto function. The process is schematically illustrated in Fig. 2.

The GC retention indices of these silyl derivatives, calculated on two differ-

TABLE I

RETENTION INDICES OF SILYLATED PROSTAGLANDIN DERIVATIVES

The retention indices were calculated at 270° for the Dexsil 300 columns and at 260° for the OV-17 columns. Data given in parentheses correspond to theoretically calculated values. All PGs were derivatized by direct one-step silylation with a BSTFA—piperidine reagent mixture, obtaining the following derivatives (see footnote on p. 298). PGE_1, 9-enol PGE_1 $(TMS)_4$; PGE_2, 9-enol PGE_2 $(TMS)_4$; $PGF_{1\alpha}$, $PGF_{1\alpha}$ $(TMS)_4$; $PGF_{2\alpha}$, $PGF_{2\alpha}$ $(TMS)_4$; PGD_2, 11-enol PGD_2 $(TMS)_4$; 15-keto $PGF_{2\alpha}$, 15-keto $PGF_{2\alpha}$ $(TMS)_4$; PGB_1; PGB_1 $(TMS)_2$; PGB_2, PGB_2 $(TMS)_2$; 19-OH PGE_2, 19-OH PGE_2 $(TMS)_3$; PGA_1, 11-piperidyl PGA_1 $(TMS)_3$; PGA_2, 11-piperidyl PGA_2 $(TMS)_3$; 19-OH PGA_2, 11-piperidyl 19-OH PGA_2 $(TMS)_4$; 19-OH PGE_2, 9-enol 19-OH PGE_2 $(TMS)_5$.

	Series	PGEs	PGFs	PGDs	15-Keto PGFs	19-OH PGEs	PGBs	19-OH PGFs	PGAs	19-OH PGAs
Dexsil 300	I	2659	2730	—	—	(2845)	2880	(2929)	3026	(3241)
	II	2659	2693	2734	2827	2845	2872	2892	3038	3229
OV-17	I	2774	2807	—	—	(3002)	3006	(2982)	3185	(3371)
	II	2774	2780	2844	2908	3002	3006	2955	3185	3371

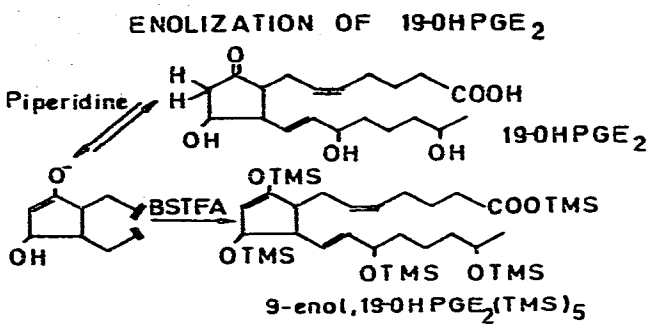

Fig. 2. Scheme of the enolization—silylation process for 19-OH PGE_2.

ent GC columns, are given in Table I. The conclusions that can be drawn from these data are that the Dexsil 300 GC column would elute all of these derivatives in less time than the OV-17 and, more importantly, with a better separation of PGEs and PGFs, which physiologically are the most significant prostaglandins. In other words, the separation of the two PGEs from $PGF_{2\alpha}$ is virtually impossible to achieve on these packed columns due to the small ΔI value of only six units (2780 vs. 2774), while the same ΔI is equivalent to 34 units on Dexsil (Table I). Likewise, the separation between both PGFs is much better on Dexsil (ΔI = 63 vs. 27). Furthermore, as Dexsil 300 is a high-temperature phase with a working range of 50—400°, it is specially suited for this application since the usual working temperature with persilylated PG derivatives is around 270°.

The practical benefits derived from the use of glass columns packed with Dexsil 300 is illustrated in Fig. 3. This profile shows the separation achieved at 270° on a 4 m × 1/4 in. I.D. column. In contrast to what happens on the OV-17 column, the peak of the $PGF_{2\alpha}$ $(TMS)_4$ derivative appears clearly resolved from the peak of the PGEs. Also the separation of both PGFs ($PGF_{1\alpha}$ and $PGF_{2\alpha}$) is better than on OV-17 [15].

Nevertheless, even with this improved GC system there is no possibility of resolving the tetrakis-TMS derivatives of PGE_1 and PGE_2, which would preclude in principle the simultaneous GC determination of their individual physiological levels in human semen samples. On the other hand, it is to be expected that the combined GC—MS technique in its selected ion detection mode of operation would allow, by proper selection of specific *m/e* ratios, the quantitation of these two prostaglandins as well as other PGs present in the samples analyzed.

Multiple ion detection

Table II gives the partial mass spectra of the TMS derivatives of the main PGs found in human semen. The table only includes the relative abundances of the molecular ions, as well as those of the structurally most significant fragment ions, whose origin has already been discussed in some detail in various publications [17—21]. In this respect, it is to be noted that the mass spectrum of the 19-OH PGE_2 $(TMS)_5$ derivative does not show, as predicted, fragment ions at M — 71 (loss of the C_{16}—C_{20} chain) of M — 161 (loss of the C_{16}—C_{20}

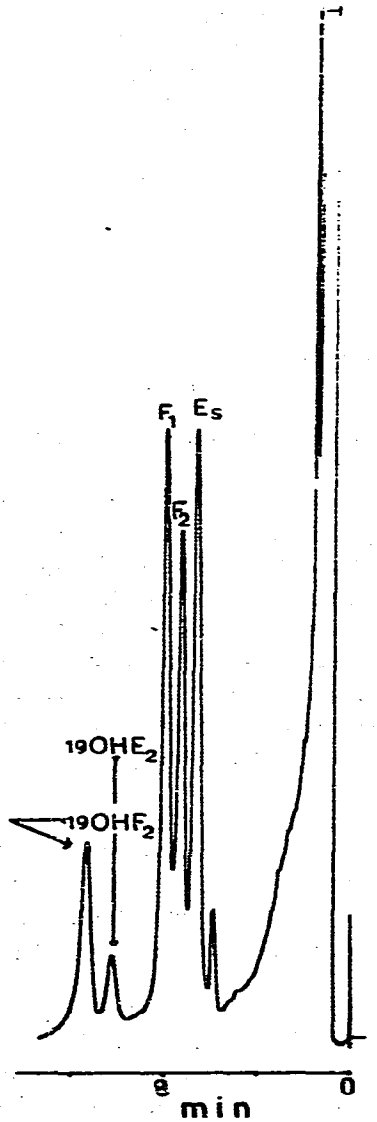

Fig. 3. GC separation of persilylated PG derivatives on a glass column packed with 3% Dexsil 300 at 280°. See Table I for retention indices.

chain plus the extra 19-HOSi$(CH_3)_3$ group) since, in this case, the terminal C_{16}—C_{20} chain is 89 mass units heavier due to the presence of the OTMS group on the C_{19} position. Also, the M — 131 ion is absent, as in the mass spectra of both PGE derivatives. The M — 249 ion, at *m/e* 479, would be the equivalent of the M — 161 fragment in the mass spectra of other PGs, considering that the loss of the C_{16}—C_{20} fragment entails here a loss of 249 a.m.u., corresponding to the C_{16}—C_{19} OTMS-C_{20} moiety. The mass spectra of the pentasilylated derivatives of 19-OH $PGF_{1\alpha}$ and 19-OH PGE_1 are not included as there were no reference standards available. In spite of this, a detailed comparative analysis of the MS patterns of the other related PGs indicates that the

TABLE II

PARTIAL MASS SPECTRA OF THE SILYLATED DERIVATIVES OF PGEs, PGFs, 19-OH PGEs AND 19-OH PGFs

See Table I for nomenclature of derivatives.

	PGF_2 and PGE_1			PGE_2		PGE_1		19-OH PGE_2		19-OH PGF_2	
	m/e	r.a.*	r.a.	*m/e*	r.a.	*m/e*	r.a.	*m/e*	r.a.	*m/e*	r.a.
M**	642	5	5	640	26	644	0.2	728	16	730	—
M − 15	727	11	31	625	36	629	6	713	21	715	—
M − 71	571	11	5	569	5	573	26	—	—	—	—
M − 90	552	32	100	550	86	554	39	638	100	640	64
M − 105	537	17	17	535	11	539	18	623	26	625	21
M − 131	511	10	—	—	—	513	18	—	—	—	—
M − 161	481	48	38	479	63	483	55	—	—	—	—
M − 180	462	33	59	460	100	464	21	548	73	550	55
M − 249	—	—	—	—	—	—	—	479	31	481	100

*r.a. = relative abundance.

**M = molecular ion; M − 15, loss of CH_3; M − 71, loss of C_{16}—C_{20}; M − 90, loss of $HOSi(CH_3)_3$; M − 105, loss of $HOSi(CH_3)_2$ and CH_3; M − 131, loss of $CH_2COOSi(CH_3)_3$; M − 161, loss of C_{16}—C_{20} and $HOSi(CH_3)_3$; M − 180, loss of two $HOSi(CH_3)_3$ groups (18—20); M − 249, in 19-OH PGs is equivalent to the M − 161 fragment of other PGs and corresponds to the loss of C_{16}—C_{19} OTMS-C_{20} fragment.

mass spectrum of the silylated derivative of 19-OH PGE_1 should contain a significant fragment at *m/e* 481, which would correspond to the mass of the M − 249 ion, the molecular weight in this case being 730 mass units. Accordingly, the mass spectrum of the 19-OH $PGF_{1\alpha}$ $(TMS)_5$ derivatives would show a major M − 249 ion at *m/e* 483 since the mass spectral patterns of its analog structure, the 19-OH $PGF_{2\alpha}$ $(TMS)_5$ derivative, is characterized by the appearance of a relatively abundant ion at *m/e* 481.

The data in Tables I and II provide the necessary information for selecting the most suitable, specific and characteristic ions to be monitored by the MID unit [14]. There is, however, a practical limitation to consider in the sense that this unit has only four channels available whereas the simultaneous determination of all PGEs and PGFs present in the samples would necessitate using a minimum of eight channels plus one for the monitoring of the deuterated internal standard. Nevertheless, this practical limitation could be obviated by a restrictive selection of as many common ions as possible for those derivatives that have been well separated by GC and of specific but different mass ions for those PGs not resolved by GC.

In this way, according to the scheme in Table III, the limitation imposed by the co-elution of PGE_1 and PGE_2, in terms of the simultaneous quantitation of these two prostaglandins, can be readily solved by monitoring the ions at *m/e* 481 for the quantitation of PGE_1 and at *m/e* 479 for the quantitation of PGE_2, as indicated in Table III. The same applies to the co-eluting 19-OH PGE derivatives, both monitored at the same masses but widely separated in retention times from the PGEs. The eight prostaglandins can be simultaneously detected and quantitated if the ions at *m/e* 483 and 485 are also monitored in the two other channels remaining. In the first case the silylated derivatives of $PGF_{1\alpha}$

TABLE III

IDENTIFICATION SCHEME OF SELECTED IONS VERSUS GC RETENTION TIMES

I values on Dexsil 300 at 270° (see Table I). In parentheses correction factor in percent due to the contribution of the isotopic ion cluster of the other co-eluting prostaglandin.

Recording channel	*m/e* selected						
I	485		F_2D_4				
II	483			$F_{1\alpha}$			19-OH $F_{1\alpha}$
III	481	E_1 (22.5)	$F_{2\alpha}$ (27)		19-OH E_1 (39.4)	19-OH $F_{2\alpha}$	
IV	479	E_2			19-OH E_2		
Retention index *I*		2659	2693	2730	2845	2892	
		PGEs	PGF_2	$PGF_{1\alpha}$	19-OH PGEs	19-OH PGF_2	19-OH $PGF_{1\alpha}$

and its 19-OH analog are detected, while in the second case the ion at *m/e* 485 is used for detecting the deuterated internal standard ($PGF_{2\alpha}$ D_4). $PGF_{2\alpha}$ and 19-OH $PGF_{2\alpha}$ are detected in the channel monitoring the ions of *m/e* 481.

The selected ion profiles obtained at 280° on the Dexsil 300 GC column with a derivatized mixture of standard prostaglandins of the E and F series plus the $PGF_{2\alpha}$ D_4 internal standard are illustrated in Fig. 4, showing that all of these PGs could be readily quantitated. However, taking into account the possible cross-channel contributions of the isotopic species of each of these selected ions, the only case that needs to be accounted for would be that due to the contribution of the isotopic cluster of the ion of *m/e* 479, selected for detection of PGE_2, to the profile of the ions at *m/e* 481, selected for detection of PGE_1, since both of these prostaglandins elute with the same retention time. This interchannel contribution, as well as that corresponding to the 19-OH counterparts, will be defined below.

The corresponding GC—flame ionization detection profile has been shown in Fig. 3. This sample also contained the TMS derivatives of the 19-OH PGE_2 and $PGF_{2\alpha}$, which appear well separated. This becomes a very important factor in the quantitative determination of these PGs in human semen.

The use of only one deuterated internal standard as reported by Perry and Desiderio [5] for the quantification of eight different PGs requires a preliminary study of the response curves corresponding to each one of the PGs relative to that of the internal standard. For this purpose, we carried out an appraisal of the response of the pentasilylated derivative of $PGF_{2\alpha}$ D_4 used as the internal standard, versus its corresponding protium form. Once the deuterated standard had been properly calibrated, we proceeded to check the relative responses of the PG derivatives relative to the internal standard by preparing three solutions of different concentrations for each PG and adding to them the same amount of deuterated internal reference. As an example, the graphical representation of the response ratio of $PGF_{2\alpha}/PGF_{2\alpha}$ D_4 vs. the corresponding concentration ratio gave a straight line of slope = 1.

Due to the unavailability of standard 19-OH $PGF_{1\alpha}$ and PGE_1, it was not possible to obtain the corresponding response curves relative to the deuterated

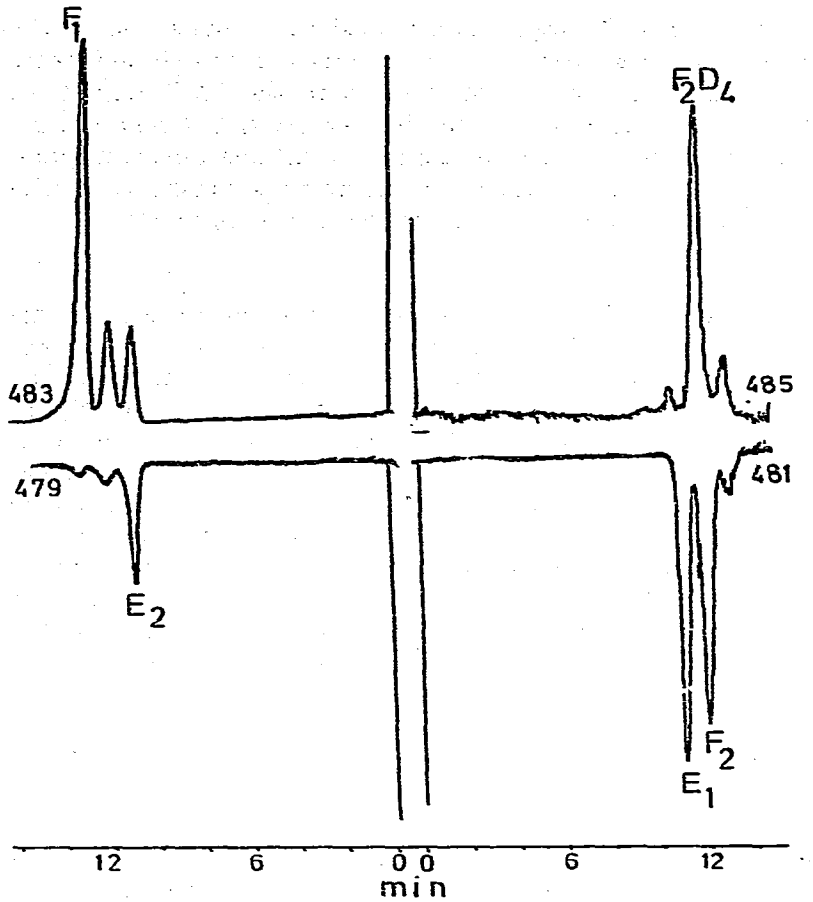

Fig. 4. Selected ion profiles of an authentic prostaglandin mixture derivatized according to the described procedure (see Tables I and II). As indicated in the text the two significant peaks eluting ahead of the PGF_1 derivative (*m/e* 483) are due to the response of the isotopic ion cluster from the *m/e* 481 ions of the PGE_1 and $PGF_{2\alpha}$ derivatives.

internal standard. However, it has been assumed that their chromatographic behaviour would be practically identical to that of their $PGF_{2\alpha}$ and PGE_2 analogs, in much the same manner as observed with the other series of PGs.

It has been already mentioned that the quantitative measurements taken on selected ion profiles, such as those shown in Fig. 4, may be influenced by the contributions of the isotopic clusters of neighbouring mass ions, themselves focused on other channels. For instance, as illustrated in Fig. 4, when the samples thus analyzed contain detectable amounts of the silylated derivative of PGE_1, monitored at *m/e* 481, there is also a response at the channel monitoring ions of *m/e* 483. In other words, the ions of *m/e* 483 are also natural components of the isotopic ion cluster of the fragment that appears at *m/e* 481 in the mass spectra of these PGE_1 derivatives. However, these responses will only have to be taken into account whenever the overlapping of GC retention times could cause a contribution of this nature to some of the ions monitored by the MID unit. Such would be the case, for instance, in relation to

PGE_2, whose response at *m/e* 479 would contribute to increase the actual height of the PGE_1 ion measured at *m/e* 481, to an extent that can be readily calculated as 22.5%. The same reasoning would apply to the TMS derivatives of the 19-OH PGEs where, as indicated in Table III, the contribution amounts to 39.4%. Also, since the deuterated internal standard contains a certain quantity of the corresponding protium form, its contribution to the response of the PGF_2 derivative would also have to be taken into account (see Table III).

HPLC separations

As the possibility of prostaglandin degradation is of great importance in this kind of study, a check was made to determine the level of degradation, if any, experimentally induced by the conditions under which we extract, derivatize and analyze these samples. For this purpose the ultrafiltrates were directly injected into a LC column and the various PGs eluted under the conditions described above. As indicated in Table IV, the combined non-linear/linear program herein described provides a good separation of all classes of authentic PGs, with the exception of PGAs and PGBs which elute together in cut No. 2. On the other hand, although the HPLC of free prostaglandins on silicic acid columns has been reported before [22,23], the elution parameters used in both cases (60 min linear gradient from chloroform to a chloroform—methanol—acetic acid mixture) are not adequate for the separation of the 19-OH PGEs and PGFs in a reasonable time. In contrast, and according to the cuts indicated in Table IV, the 19-OH PGE_2 can be readily isolated in cut No. 8 (52—57 ml) while the 19-OH PGF_2 can be recovered from cut No. 10 (81—87 ml).

The biological samples under study were subjected to the same LC separation system, and the identity of each of the compounds thus collected was confirmed by GLC analysis of the corresponding persilylated derivatives. For example, the gas chromatograms obtained from the cuts containing the 19-OH PGs from a sample of human semen are shown in Fig. 5. Quantitation was achieved by selected ion monitoring as described above. In this way it could be demonstrated that the cut that should contain the PGAs and

TABLE IV

HPLC SEPARATION OF FREE PROSTAGLANDINS

See text for experimental parameters.

Cut No.	Eluate fraction (ml)	PGs
1	5—9	—
2	10—16	As and Bs
3	19—22	—
4	23—28	Es
5	29—34	—
6	35—41	Fs
7	42—51	—
8	52—57	19-OH E_2
9	58—80	—
10	81—87	19-OH Fs

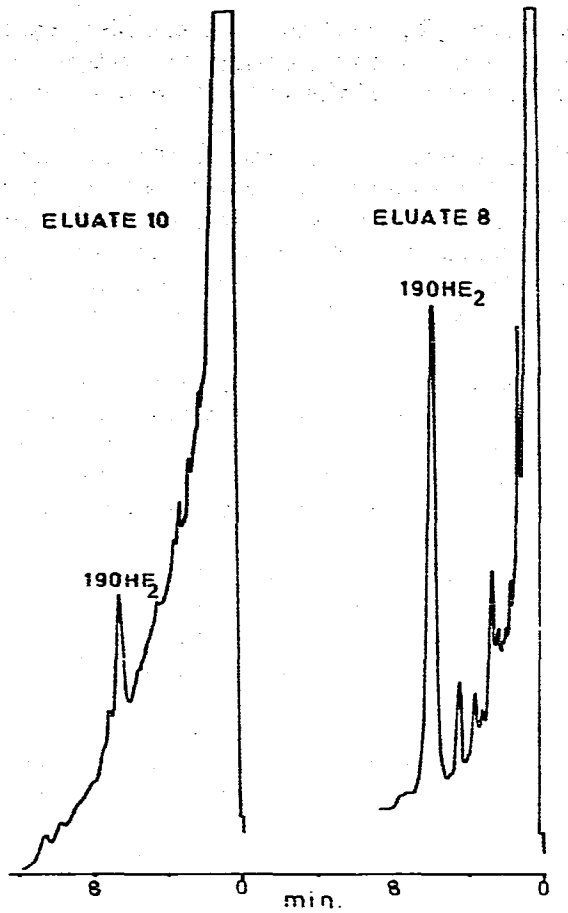

Fig. 5. Gas chromatograms of the persilylated HPLC eluate fractions (corresponding to the elution volume of 19-OH PGs) obtained from an extract of human semen. Same GLC conditions as in Fig. 3.

PGBs did not give any appreciable response with the ultrafiltered semen samples. The only cases where a relatively small GLC response was obtained for these two secondary PGs was in a few of the cuts containing the PGEs, thus demonstrating that they had been formed from PGEs upon derivatization and GLC analysis of these fractions. However, this can be prevented by careful handling of the collected eluates. A more thorough account of the HPLC of these PGs is given elsewhere [24].

Analysis of semen samples

Once the response parameters of these derivatives and their possible isotopic contributions to the MID traces had been established, the method was applied to a study of the physiological levels of the major prostaglandins in samples of human semen obtained from fertile donors. This study was undertaken under extremely controlled conditions, with regard to both sample collection and extraction procedures. The aim was to minimize any possibility of prostaglandin degradation leading to structural changes during the freezing, storage

with preservatives and defreezing procedures [7], and thus all samples were extracted without previously subjecting them to any freezing or storage. In fact, the time from ejaculation to the beginning of the extraction was never more than 30 min.

Single semen samples were obtained from seven volunteers, all of whom had been duly informed on the nature of the study and whose wives had recently given birth, except in one case whose wife was pregnant. All men were aged between 25 and 30 years. Six of them collected the sample by ejaculation into clean glass containers and one collected it by intercourse using a condom. Also, according to the responses given in a previously supplied questionnaire, this man was taking four aspirin tablets daily.

The extraction of the seven samples was carried out exactly under identical conditions and in the following manner: each sample of semen was centrifuged for 5 min at 17,500 *g*. The seminal plasma thus obtained was agitated and an aliquot of 1.5 ml was taken for ultrafiltration together with 50 μl of the solution containing the deuterated internal standard as described in the Experimental section. The final residue was dissolved in 40 μl of the BSTFA—piperidine mixture and allowed to react for 30 min at 60°. A total of five injections of each derivatized extract was made into the GC—MS combination, thus effectively obtaining five MID determinations for each sample. After subtracting the isotopic contributions to the response of the ions indicated in Table III, the mean of the five determinations was calculated.

Table V gives the prostaglandin concentrations found in the samples of semen from healthy fertile men. The mean concentrations and ranges shown are basically in good agreement with the data recently published by Templeton et al. [6], although these authors do not report the individual values of the two components (series I and II) of each family of prostaglandins. It must be noted, however, that the values corresponding to the sample obtained from the man taking daily doses of aspirin were in all cases remarkably low in relation to the other samples, an observation which can probably be explained considering the relationship between aspirin and the prostaglandins. In the last few years this relationship has been widely documented to the point where there seems to be a clear inhibitory effect of aspirin on the cyclooxigenase

TABLE V

PROSTAGLANDIN LEVELS (μg/ml) IN SEMEN OF HEALTHY FERTILE MEN

	$\bar{X}$ ± S.D. (*n*=7)	Range		$\bar{X}$ ± S.D. (*n*=7)	Range
PGE_2	33.26±28.68	5.02— 94.47	19-OH PGE_2	294.17±151.88	77.28— 498.60
PGE_1	29.63±20.55	3.99— 65.56	19-OH PGE_1	280.53±155.93	60.93— 515.01
PGEs	63.46±49.28	9.01—164.03	19-OH PGEs	574.70±302.71	138.21—1014.11
$PGF_{2\alpha}$	1.36± 0.83	0.75— 2.93	19-OH $PGF_{2\alpha}$	8.64± 3.87	2.5 — 12.46
$PGF_{1\alpha}$	1.22± 1.16	0.20— 3.7	19-OH $PGF_{1\alpha}$	5.48± 2.62	3.24— 9.97
PGFs	2.58± 1.92	0.95— 6.63	19-OH PGFs	14.12± 4.90	5.9 — 20.44

enzyme system catalyzing the formation of endoperoxides from arachidonic acid, these endoperoxides being the known precursors of all prostaglandins [25–27].

Under these strict conditions of sample collection and rapid analysis (samples extracted within 30 min of ejaculation), we did not detect any PGAs or PGBs, although in a recent publication from this laboratory [28] mass spectrometric proof was given on the identification in samples of human semen of PGA_1, PGA_2, 19-OH PGA_1 and 19-OH PGA_2. However, it was also acknowledged that no significant levels of PGAs had been detected in a few samples that had been frozen at −40° within 1 h of ejaculation [28]. In that report we did not take a stand on the "fact or artifact" issue regarding the presence of PGAs in semen [29,30], the aim at that time being just to demonstrate the advantages of the new piperidyl-TMS PGA derivatives for the detection of PGAs regardless of their origin. Nevertheless, our latest results obtained with recently ejaculated semen, as reported herein, would support the artifact theory [30]. Furthermore, in order to avoid even the minimum possible interconversion that may take place upon derivatization and/or on the GC injector or GC columns, some of the samples herein reported were previously qualitatively identified by HPLC and no PGAs or PGBs were found. The apparent contrast with a recent publication [13] reporting concentrations of PGAs, 19-OH PGAs and PGBs as well as PGEs and 19-OH PGEs in fresh semen, could be explained again by storage in cold ethanol at −20°, it having been reported that even at −20° there is the possibility of PGEs breaking down to PGAs [11].

Also, regarding the suggested relationship between PG levels in semen and male infertility, we tend to agree with Templeton et al. [6] as our results also show that the concentration ranges (Table V) are much too wide to allow for meaningful comparisons between fertile and infertile men. The values reported, for instance, for PGEs and 19-OH PGEs in men classified as azoospermic or oligospermic (see Table VI) would still fit very well within the normal range reported by Templeton et al. [6] and in this work.

Finally, it could be added that the data given in Table V can be considered very reliable because the process of extraction was carried out under exactly reproducible conditions in all seven cases, avoiding any freezing and/or storage of the samples and always within 30 min from ejaculation to sample extrac-

TABLE VI

PROSTAGLANDIN CONCENTRATIONS IN SEMEN FROM HEALTHY FERTILE MEN

Prostaglandin	Mean concentration (μg/ml) ± S.D. and range				
	Perry and Desiderio [5]			Templeton et al. [6]	This work
	Fertile	Azoospermic	Oligospermic	Fertile	Fertile
E_1—E_2	149± 91.7 (NR)*	113±114 (NR)	59.9±35.0 (NR)	73.2± 71.6 (2—272)	63.46± 49.28 (9—164)
19-OH E_1—19-OH E_2	526±367 (NR)	266±167 (NR)	163 ±63.6 (NR)	267 ±240 (53—1094)	574.7 ±302.7 (138—1014)

*NR = not reported.

tion. To our knowledge this is the first attempt at the direct extraction of semen immediately after its collection. Also to be noted is the possibility of determining simultaneously in a single extract all of the physiologically significant prostaglandins and 19-OH prostaglandins.

REFERENCES

1 D.F. Hawkins, in P.W. Ramwell and J.E. Shaw (Editors), Prostaglandin Symposium of the Worcester Foundation, Interscience, New York, 1968, p.1.
2 M. Bydeman, B. Fredricsson, K. Svanborg and B. Samuelsson, Fertil. Steril., 21 (1970) 622.
3 H.C. Brumer and A. Gillispie, Clin. Endocrinol., 1 (1972) 363.
4 J.G. Collier, R.J. Flower and S.L. Stanton, Fertil. Steril., 26 (1975) 868.
5 D.L. Perry and D.M. Desiderio, Prostaglandins, 14 (1977) 475.
6 A.A. Templeton, I. Cooper and R.W. Kelly, J. Reprod. Fertil., 52 (1978) 147.
7 R.W. Kelly, Int. J. Androl., 1 (1978) 188.
8 D.D. Hoskins and E.R. Casillas, in J.A. Thomas and R.L. Sighal (Editors), Advances in Sex Hormone Research, Vol. 1, University Park Press, Baltimore, Md., 1975, p. 283.
9 H.T. Jonsson, Jr., B. Middleditch and D.M. Desiderio, Science, 187 (1974) 1093.
10 P.L. Taylor and R.W. Kelly, Nature (London), 250 (1974) 665.
11 I. Cooper and R.W. Kelly, Prostaglandins, 10 (1975) 507.
12 M. Hamberg and B. Samuelsson, J. Biol. Chem., 241 (1966) 257.
13 K.C. Srivastava and V.K.S. Shukla, Z. Anal. Chem., 293 (1978) 45.
14 F. Artigas, E. Gelpí, M. Prudencio, J.A. Alonso and J. Ballart, Anal. Chem., 49 (1977) 543.
15 J. Roselló, J. Tusell and E. Gelpí, J. Chromatogr., 130 (1977) 65.
16 J.M. Tusell, J. Roselló and E. Gelpí, Prostaglandins, 15 (1978) 219.
17 J. Roselló, C. Suñol, J.M. Tusell and E. Gelpí, Biomed. Mass Spectrom., 4 (1977) 237.
18 J. Roselló, C. Suñol and E. Gelpí, in A. Frigerio (Editor), Recent Developments in Mass Spectrometry in Biochemistry and Medicine, Vol. 1, Plenum, New York, 1978, p. 229.
19 F. Vane and M.G. Horning, Anal. Lett., 2 (1969) 357.
20 J.T. Watson and B.J. Sweetman, Org. Mass Spectrom., 9 (1974) 39.
21 B.S. Middleditch and D.M. Desiderio, in A.R. West (Editor), Advances in Mass Spectrometry, Vol. 6, Applied Science Publishers, Barking, 1974, p. 173.
22 K. Carr, B.J. Sweetman and J.C. Frölich, Prostaglandins, 11 (1976) 3.
23 A.R. Whorton, K. Carr, M. Smigel, L. Walker, K. Ellis and J.A. Oates, J. Chromatogr., 163 (1979) 64.
24 J.M. Tusell and E. Gelpí, in Biological/Biomedical Applications of Liquid Chromatography III, Marcel Dekker, New York, 1980, in press.
25 M. Hamberg, Biochem. Biophys. Res. Commun., 49 (1972) 720.
26 A. Dembińska-Kieć, A. Zmuda and J. Krupińcka, in B. Samuelsson and R. Paoletti (Editors), Advances in Prostaglandin and Thromboxane Research, Vol. 1, Raven Press, New York, 1976, p. 99.
27 J.R. Vane, Hosp. Pract., 7 (1972) 61.
28 J. Roselló and E. Gelpí, Biomed. Mass Spectrom., 5 (1978) 531.
29 A. Attalah, L.J. Payakkapan, A. Carr and E. Brazelton, Prostaglandins, 5 (1974) 69.
30 B.S. Middleditch, Prostaglandins, 9 (1975) 409.