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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROFILING WITH NEGATIVE-ION CHEMICAL IONIZATION DETECTION OF PROSTAGLANDINS AND THEIR 15-KETO AND 15-KETO-13,14-DIHYDRO CATABOLITES IN RAT BLOOD

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SUMMARY

A method was set up in which the primary prostaglandins (PGF_{2α}, PGD₂, PGE₂, thromboxane B₂, 6-keto-PGF_{1α} and 6-keto-PGE₁) and their catabolites (15-keto and 15-keto-13,14-dihydro) could be analyzed in the same sample at the same time. The method makes use of long capillary columns (60 m) to resolve the complex mixture during gas chromatography and mass fragmentography to provide the specificity of detection of these products. Selectivity and sensitivity is provided through use of appropriate derivatives (pentafluorobenzyl esters) which allow detection by negative-ion chemical ionization in which high-abundance fragments in the high end of the mass spectrum (M-pentafluorobenzyl) are observed. A purification procedure of whole blood is described involving diethyl ether extraction, C₁₈ Sep-Pak chromatography, derivatization into the pentafluorobenzyl-O-methyloxime, C₁₈ Sep-Pak and silicic acid chromatography followed by final derivatization into trimethylsilyl ethers for gas chromatographic—mass spectrometric analysis. Recovery of added [³H]PGF_{2α} was 73.8 ± 2.2% (n=10). Sample workup and analysis takes ten days for six samples. The method is sufficiently sensitive for the profiling of a 10-ml sample of whole blood (limit approximately 1 pg/ml; 1-pg injection on column).

INTRODUCTION

Methods for the profiling of all prostaglandins (PGs) in the presence of their catabolites are mostly lacking. It is evident that neither radioimmunoassay nor bioassay are appropriate since the former would entail analysis of each product separately through use of specific antibodies rendering such a method expensive and cumbersome [1–3]; bioassay is also inappropriate as most prostaglandin catabolites lack biological activity [4–7]. Clearly, the problem

of measuring each product in such a mixture of closely related substances is ideally suited to mass spectrometry (MS) after appropriate resolution of the mixture is performed first by gas chromatography (GC) on long capillary columns. The GC-MS system described in this paper used in the negative-ion chemical ionization (NICI) mode for enhanced sensitivity provides the much needed analytical means to achieve this goal. We describe a method capable of simultaneous measurement of a mixture of the six primary prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites (total of eighteen products plus internal standard). Application of this method to the profiling of these products in normal rat blood is also described.

MATERIALS AND METHODS

[9-³H]PGF_{2α} of high specific activity (i.e. 13.7 Ci/nmol) was purchased from New England Nuclear. All prostaglandin standards used in this study were kindly supplied by Dr. J.E. Pike, Upjohn (Kalamazoo, MI, U.S.A.). The 15-keto and 15-keto-13,14-dihydro catabolites of 6-keto-PGE₁ were prepared in our laboratory [8]. Male adult Wistar rats (300 g) were purchased from Camm Research Laboratory Animals (Wayne, NJ, U.S.A.). All solvents were HPLC grade glass-distilled as supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Sample workup

Arterial blood (10 ml) was withdrawn from lightly anaesthetized (diethyl ether) rats by cardiac puncture into plastic syringes containing 1 ml sodium citrate (3.8%). After gentle mixing, the blood was transferred into glass centrifuge tubes containing 50 ml ethanol. The samples were mixed thoroughly, centrifuged at 1000 *g* for 15 min and 20 ng C22-PGF_{2α} as internal standard and 120,000 cpm [³H]PGF_{2α} (3.6 ng) were added. The ethanol supernatant was evaporated to complete dryness *in vacuo*. The residue was washed with dry ethanol and the ethanol-soluble fraction was transferred and evaporated again *in vacuo*. This procedure was repeated several times. The final residue after evaporation of the ethanol was extracted with diethyl ether and acidified (pH 3) water in centrifuge tubes. After neutral washing, the diethyl ether layer was evaporated to dryness and the residue was taken up in 10 ml water-methanol-acetic acid (80:20:0.4, v/v/v) and passed through a C₁₈ Sep-Pak cartridge (Waters) prewashed with methanol and water. The Sep-Pak was eluted with water-methanol-acetic acid mixtures of increasing amounts of methanol, i.e. 70:30:0.4 (10 ml), 35:65:0.4 (10 ml) and finally pure methanol (10 ml); the mixture of prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites were eluted with water-methanol-acetic acid (35:65:0.4, v/v/v). This fraction was taken to complete dryness *in vacuo* and derivatized by a modification of the method of Wickrema Sinha et al. [9] into the pentafluorobenzyl (PFB) esters as follows: the sample was taken up in 10 μl methanol and 50 μl acetonitrile, then 2 μl pentafluorobenzyl bromide (Pierce) and 1.5 μl diisopropylethylamine (Aldrich) were added, and the sample was heated at 60°C for 5 min. The solvents were evaporated with a fine stream of nitrogen and the residue was dissolved in 25 μl methoxylamine hydrochloride in pyridine (MOX reagent; Pierce). After standing overnight at room tempera-

ture, the solvent was evaporated with nitrogen and the residue was extracted with 200 μ l diethyl ether and 50 μ l water. The diethyl ether extract was dried and the residue was dissolved in 10 ml water—methanol (1:1, v/v). The sample was placed on a C₁₈ Sep-Pak cartridge prewashed with methanol, water and the same solvent mixture. The prostaglandin mixture was subsequently eluted with water—methanol (15:85, v/v). This fraction was taken to complete dryness in vacuo and the residue was dissolved in benzene and placed on a small Pasteur pipette column filled with a slurry of silicic acid (HA minus 325 mesh, Bio-Rad Labs.) in benzene. After washing the column with benzene, the prostaglandin mixture was eluted with chloroform—methanol (9:1, v/v). This fraction was taken to dryness, transferred to a micro vial (400- μ l volume) and 25 μ l TRISIL Z (Pierce) was added. After 5 min at 60°C, the solvent was evaporated with a fine stream of nitrogen and 50 μ l ice cold hexane and 20 μ l ice cold water were added. The sample was rapidly mixed and the water was frozen by immersing the sample in a dry ice—methanol bath. The sample was stored at -20°C until analysis. Recovery of [³H]PGF_{2 α} throughout the procedure was 73.8 \pm 2.2% (n=10) (see Fig. 1).

Citratated blood sample or tissue extract	
↓	
Addition of labelled PGF _{2α} (120,000 cpm) and C22-PGF _{2α} standard (20 ng)	Recovery (%) of ³ H-PGF _{2α} (n=10)
↓	
1. 5 vols. ethanol	
2. Diethyl ether-H ⁺ extraction	99.3 \pm 3.4
3. C ₁₈ Sep-Pak, water—methanol—acetic acid (35:65:0.4, v/v/v) fraction	97.8 \pm 2.8
4. Derivatization	
a. Pentafluorobenzyl	
b. O-Methylxime	
5. Diethyl ether—water extraction	90.4 \pm 1.9
6. C ₁₈ Sep-Pak, water—methanol (15:85, v/v) fraction	75.3 \pm 3.2
7. Silicic acid, chloroform—methanol (9:1, v/v) fraction	73.8 \pm 2.2
8. Derivatization	
a. Trimethylsilyl	
9. Assay by GC—ECD and GC—MS	

Fig. 1. Brief procedure for the purification of a mixture of the primary PGs and their catabolites indicating recoveries of added labelled PGF_{2 α} .

Instrumentation

Sample analysis was performed by two methods, GC with electron-capture detection (GC—ECD) and GC—MS in the NICI mode making use of single-ion monitoring (SIM) to increase the sensitivity and selectivity of the products under measurement. Both methods employed 60-m capillary columns of fused silica (DB-1, J. & W. Scientific) and hydrogen as carrier gas. The GC—ECD system had a make-up flow of argon—methane (98.35:1.65) while in GC—MS the NICI was operated with 100% methane as reactant gas. The carrier gas was passed through moisture and oxygen traps while detector gases were passed through moisture traps only. The GC conditions were: column

injection temperature 100°C maintained for 1 min, then programmed to 270°C at 30°C/min. This temperature was held for the rest of the run. Samples were injected through a Hewlett-Packard splitless injector into the GC-ECD system (HP 5700 Series) or the GC-MS system (HP 5987A Series). Inlet temperature was maintained at 300°C; detector temperature in GC-ECD was 350°C, in GC-MS 300°C. Source temperature in GC-MS was kept at 150°C. SIM was performed at 3000 electron multiplier voltage.

RESULTS

A sample purification scheme was devised which would permit purification of a mixture of known prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites as a group from contaminating substances in blood or other biological fluids. The efficient use of C₁₈ Sep-Pak cartridges was explored for this purpose. A procedural scheme was initially devised through use of products with polarity at the extreme end of the mixture of products to be analyzed, i.e. 15-keto-13,14-dihydro-PGE₂ as the least polar product and -PGF_{2α} as the most polar product. The procedure described in Fig. 1 satisfied our requirements for sample cleanliness and good recoveries. To test this scheme, a mixture of authentic prostaglandins and their catabolites (20 ng each) was passed through this purification process and assayed by GC-ECD. All products were recovered in a similar proportion to that in which they were made up (not shown) indicating that the "windows" chosen for each chromatographic step gave good recoveries of the least polar and most polar products of the group of PGs and PG catabolites.

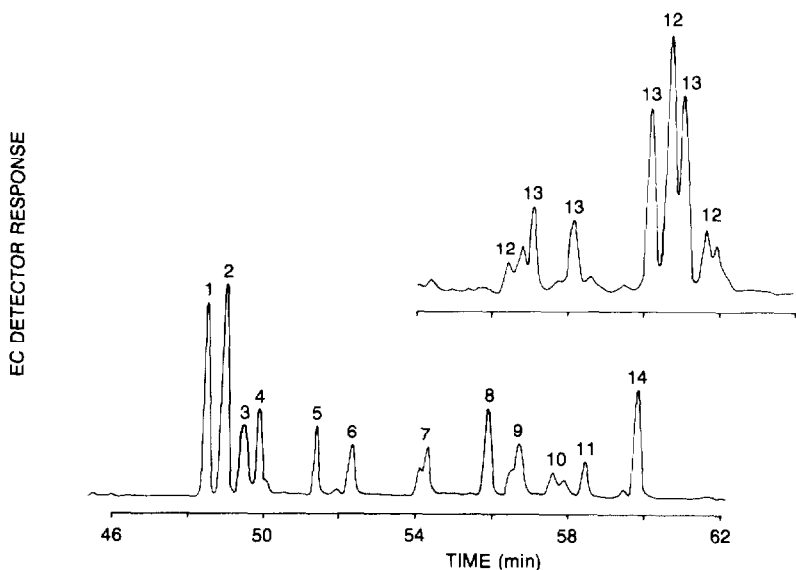


Fig. 2. GC-ECD profiles showing resolution of a standard mixture of primary PGs and their 15-keto and 15-keto-13,14-dihydro catabolites as pentafluorobenzyl-O-methyloxime-trimethylsilyl (PFB-MO-TMS) derivatives on a 60-m fused silica column of DB-1. Hydrogen was used as carrier gas (see Materials and methods for details). The retention times on the axis for the top chromatogram are the same as those for the bottom. For peak identification see Table I.

Fig. 2 shows the type of chromatographic resolution observed on a 60-m capillary column of DB-1 routinely used in our assay. Composite data are shown for GC-ECD analysis to show that the catabolites are easily resolved from the primary PGs making such PG profiling possible. Further specificity of this assay is derived through use of GC-MS for the detection and quantita-

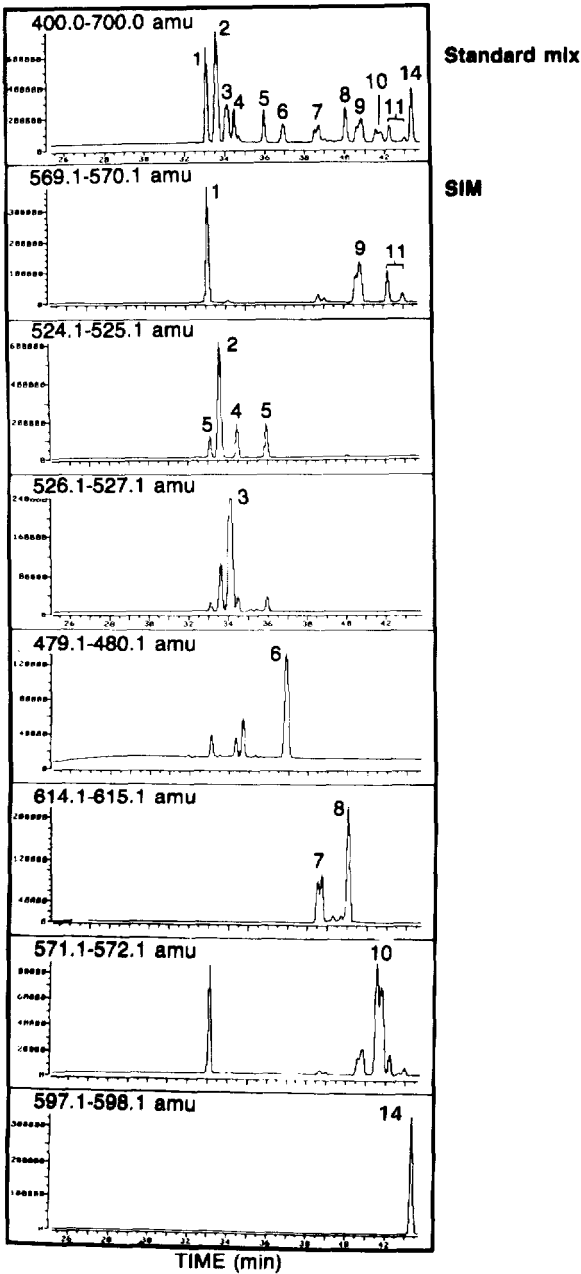


Fig. 3. GC-MS-SIM profiles showing analysis by NICI of a mixture of primary PGs and their catabolites as PFB-MO-TMS derivatives under GC conditions similar to those employed in Fig. 2. For peak identification see Table I.

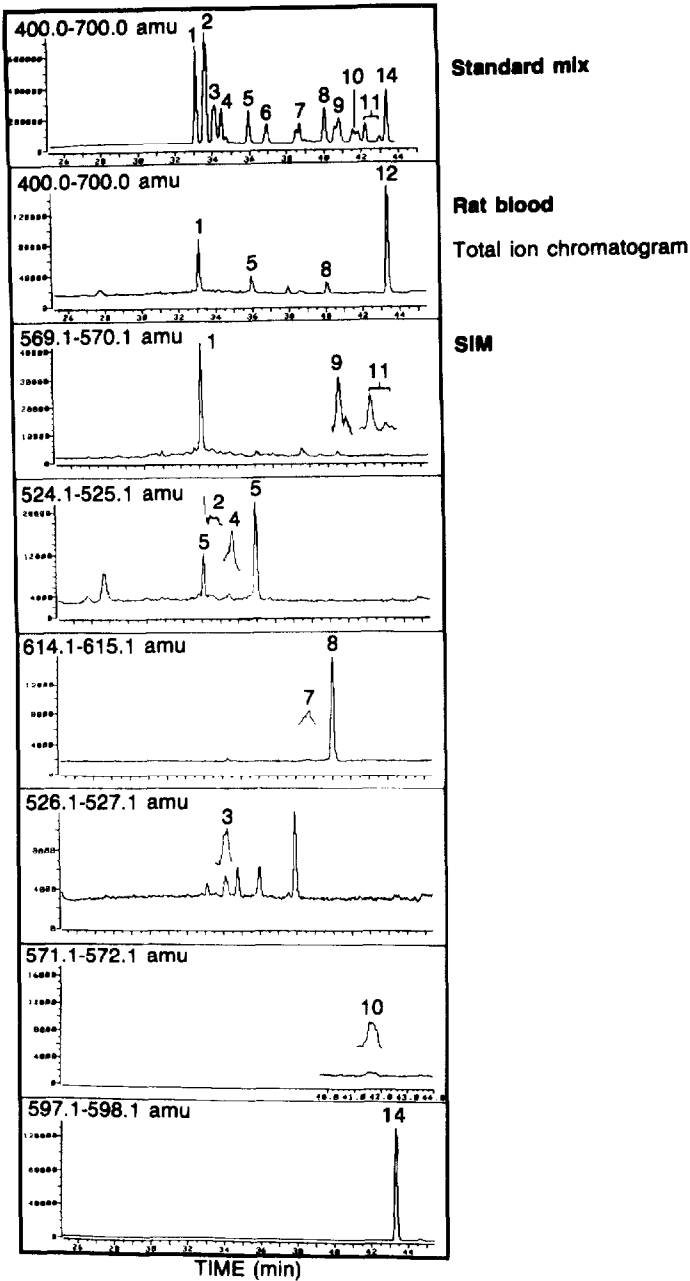


Fig. 4. Application of GC-MS-SIM-NICI to the profiling of PGs and catabolites in rat whole blood. Conditions employed were similar to those in Fig. 3. For peak identification see Table I.

tion of each product. Using NICI detection wherein the mass spectrum of each product is dominated by a single mass peak of high abundance ($>50\%$) due to the loss of the PFB group (M-181) [10], a high degree of specificity and sensitivity is obtained (Fig. 3). Undulations in baseline through minor contamination peaks in tissue extracts are often observed with non-specific assays

such as GC-ECD. The use of specific assays such as the GC-MS assay with SIM, described in this paper, eliminates to a large extent these interferences. The selectivity of GC-MS in the SIM mode with NICI detection is also of benefit in the separation of overlapping peaks. This can be seen by comparison of the $\text{PGF}_{2\alpha}$ ion chromatogram (which shows absence of PGE_2) with the PGE_2 ion chromatogram which shows an isomer of PGE_2 overlapping with $\text{PGF}_{2\alpha}$ (Fig. 3). Because m/z 569 is specific for $\text{PGF}_{2\alpha}$ (it is not present in the mass spectrum of PGE_2) and m/z 524 is specific for PGE_2 in that it is absent from that of $\text{PGF}_{2\alpha}$, a complete resolution of the two products (i.e. one isomer of PGE_2 with $\text{PGF}_{2\alpha}$) is obtained despite identical retention times on GC. In a similar fashion, several of the isomers of the 15-keto and 15-keto-13,14-dihydro catabolites of 6-keto- PGE_1 which overlap (see Fig. 2) with 6-keto- $\text{PGF}_{1\alpha}$ and its 15-keto and 15-keto-13,14-dihydro catabolites can be completely resolved by SIM with NICI detection since the catabolites of 6-keto- PGE_1 contain mass spectral fragments at m/z 524 and m/z 526 in contrast to 6-keto- $\text{PGF}_{1\alpha}$ (m/z 614), 15-keto-6-keto- $\text{PGF}_{1\alpha}$ (m/z 569) and 15-keto-13,14-dihydro-6-keto- $\text{PGF}_{1\alpha}$ (m/z 571). SIM profiles to demonstrate this have not been included here to avoid further cluttering of the data presented in Fig. 3.

Application of this method to PG profiling analysis in normal rat whole blood is shown in Fig. 4. Selected ion chromatograms are shown identifying most products searched for as listed in Table I. In most cases, clearly distinct peaks are observed for each product at the identical retention time as authentic standard.

Fig. 5 shows data from the analysis of four samples of rat whole blood. The low levels of thromboxane B_2 indicate the lack of clotting during with-

TABLE I

MAJOR MASS FRAGMENTS OBSERVED IN THE MASS SPECTRA OF PGs AND PG CATABOLITES AS PENTAFLUOROBENZYL-O-METHYLOXIME-TRIMETHYLSILYL DERIVATIVES USING NICI (METHANE REACTANT GAS) DETECTION

Compound	M-181 fragment (m/z)	Retention time* (min)
1. $\text{PGF}_{2\alpha}$	569	33.1
2. 15-Keto- $\text{PGF}_{2\alpha}$	524	33.6
3. 15-Keto-13,14-dihydro- $\text{PGF}_{2\alpha}$	526	34.1
4. PGD_2	524	34.4
5. PGE_2	524	35.9
6. 15-Keto-13,14-dihydro- PGE_2	479	36.9
7. Thromboxane B_2	614	38.5
8. 6-Keto- $\text{PGF}_{1\alpha}$	614	40.0
9. 15-Keto-6-keto- $\text{PGF}_{1\alpha}$	569	40.6
10. 15-Keto-13,14-dihydro-6-keto- $\text{PGF}_{1\alpha}$	571	41.7
11. 6-Keto- PGE_1	569	42.2
12. 15-Keto-6-keto- PGE_1	524	43.8**
13. 15-Keto-13,14-dihydro-6-keto- PGE_1	526	43.3, 44.1**
14. C22- $\text{PGF}_{2\alpha}$ (internal standard)	597	43.4

*60-m DB-1 capillary column (see Materials and methods for details).

**Retention times of major peaks.

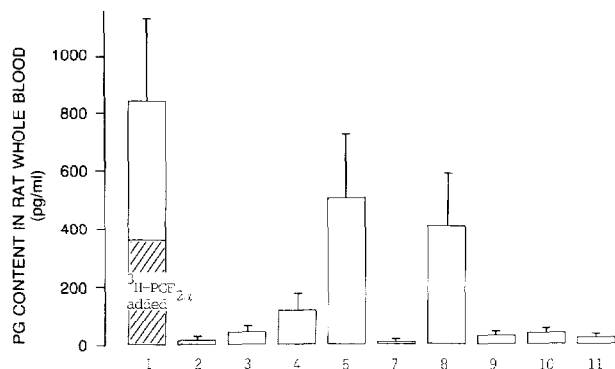


Fig. 5. Quantitative data derived by GC-MS-SIM-NICI of PGs and catabolites in whole blood samples from four rats. Values represent the mean \pm S.E.M. For compound identification see Table I.

drawal of blood in citrate. We have found considerable thromboxane B₂ in venous blood from man or rat arterial blood collected in 77 mM EDTA (not shown). It is interesting to note that the method is so sensitive that the [³H]PGF_{2α} added for recovery purposes (120,000 cpm = 3.6 ng) is easily detectable. Using authentic PGF_{2α}, we have observed that detection of 10 pg injected into the GC-MS instrument is easily possible.

DISCUSSION

We report a method whereby a mixture of prostaglandins and their primary catabolites, i.e. 15-keto and 15-keto-13,14-dihydro, can be purified as a group from a biological sample with subsequent analysis as a group by GC-MS. Analysis is possible through use of a 60-m capillary column where complete resolution of the products can be attained. We have chosen whole blood because we felt that this is most relevant to *in vivo* studies (physiologic, pharmacologic and pathologic stresses) and because this would provide a stringent test on our methods of purification since samples would have to be quite clean before injection on capillary columns can be made.

We have chosen as derivatives the PFB esters instead of methyl esters because (1) this permits an initial check of samples on a gas chromatograph in our own laboratory equipped with an electron-capture detector prior to analysis by GC-MS and (2) because PFB esters provide exceedingly simple fragmentation patterns (M-PFB represents over 50% of the total abundance) in NICI-MS [10] making these derivatives highly suitable for attaining a high degree of sensitivity when the GC-MS is used in the SIM mode. The additional benefit of the use of PFB esters is that all products containing a carboxyl group are detectable permitting analysis not only of known PGs and their known catabolites but also of hitherto unknown or uncharacterized products. For example, the method is highly suited to prostaglandin catabolism studies since the precursor and all of its products contain a carboxyl group making them visible by ECD with subsequent analysis by NICI-GC-MS. We have recently applied this method of analysis to the study of the catabolism of 6-keto-PGF₁ by rat kidney homogenates and to the analysis of rat blood for this PG and its 15-keto and 15-keto-13,14-dihydro catabolites [8].

The use of PFB esters or oximes in PG analysis by GC—ECD has been reported previously [11] although this method has not found much use because of limitations in sensitivity and selectivity in that mode of analysis. A previous report [12] has shown the suitability of GC—MS in the electron impact (EI) mode for the analysis on 'short' (21 m) columns of the primary prostaglandins of the "2" series despite the shortcomings of the EI mode of analysis in terms of sensitivity. Recently, Blair et al. [10] have shown that PFB esters have excellent properties in GC—MS especially in the NICI mode due to the facile loss of the PFB group. We have applied this method through use of long (60 m) capillary columns to the analysis of all PGs of the "2" series in the presence of their primary catabolites (15-keto and 15-keto-13,14-dihydro). We know of no other method presently employed in the prostaglandin area which allows the analysis of all primary PGs and their catabolites in a single sample. The benefits of profiling methods described in this paper are obvious since information on all products is derived from a single analysis. It is also obvious that analysis of all these products at the same time is required if the effects of specific PG synthesis or catabolism inhibitors are to be assessed *in vivo*, or in the investigation of drugs which divert the PG biosynthetic pathway into one or other PGs or in pathological conditions where one PG may be formed at the expense of others.

Although use of an internal standard, C22-PGF_{2α}, as described in this study for the quantification of PGs has its inherent limitations, this is unavoidable at present because appropriate deuterium-labelled products as internal standards are not commercially available. The purification method employs a "window" wide enough to retain all products including C22-PGF_{2α} for simultaneous analysis by GC—MS.

The sensitivity of the method described here makes it suitable for the quantitation of PGs in whole blood. Although in this study we have used 10 ml of citrated blood, workup of lesser amounts of blood is possible for PGF_{2α}, PGD₂, PGE₂ and 6-keto-PGF_{1α} and some of their catabolites. Further studies are in progress to determine the effect of physiologic stresses on levels of this group of PGs *in vivo*.

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