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USE OF A NOVEL COLOUR REACTION IN THE CHROMATOGRAPHY OF 6-OXO-PROSTAGLANDIN $F_{1\alpha}$ AND RELATED PROSTAGLANDINS

F. B. UBATUBA

Department of Prostaglandin Research, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS (Great Britain)

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SUMMARY

6-Oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), derivatized by methylation, methoxylation and silylation, emerged from OV-1 and OV-17 silicone gas–liquid chromatographic columns as one major peak (equivalent chain length = 25.1). A standardization curve, constructed using pure 6-oxo-$PGF_{1\alpha}$ enabled quantification down to 5 ng. Silicic acid column chromatography effectively separated 6-oxo-$PGF_{1\alpha}$ (free acid) from all other major prostaglandins. On thin-layer chromatographic silica gel plates, 6-oxo-$PGF_{1\alpha}$ was resolved as discrete spots, well separated from other prostaglandins when developed in 5 out of 7 solvent mixtures. The difference in rate of resolution between 6-oxo-$PGF_{1\alpha}$ and the PGFs in the various solvents afforded another criterion for identification. 6-Oxo-$PGF_{1\alpha}$ produced a distinctive yellow chromogen on thin-layer chromatographic silica gel plates sprayed with an anisaldehyde–sulphuric acid reagent; the colour reaction is specific to 6-oxo-$PGF_{1\alpha}$ and some structurally related prostaglandins.

INTRODUCTION

6-Oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) was first described in 1976[1], as the main metabolic product formed *in vitro* when arachidonic acid (AA) is incubated with homogenates of rat stomach fundus.

Its importance became apparent when it was shown[2] that this prostaglandin is the stable product of the spontaneous degradation of prostacyclin (PGI_2) (Fig. 1) the platelet anti-aggregating factor produced in all vascular tissues so far studied, such as guinea-pig and rabbit aorta[3], rabbit coeliac artery[4], human colic and gastric arteries[5], bovine coronary artery[6] and guinea-pig coronary vascular bed[7].

Owing to the instability of PGI_2, much of the information about its formation has been obtained by bioassay; the cascade technique of Vane[8] is particularly useful in this respect.

In quantitation of 6-oxo-$PGF_{1\alpha}$ in biological fluids, tissue extracts and organ perfusates by the rather expensive gas chromatographic–mass spectrometric (GC–

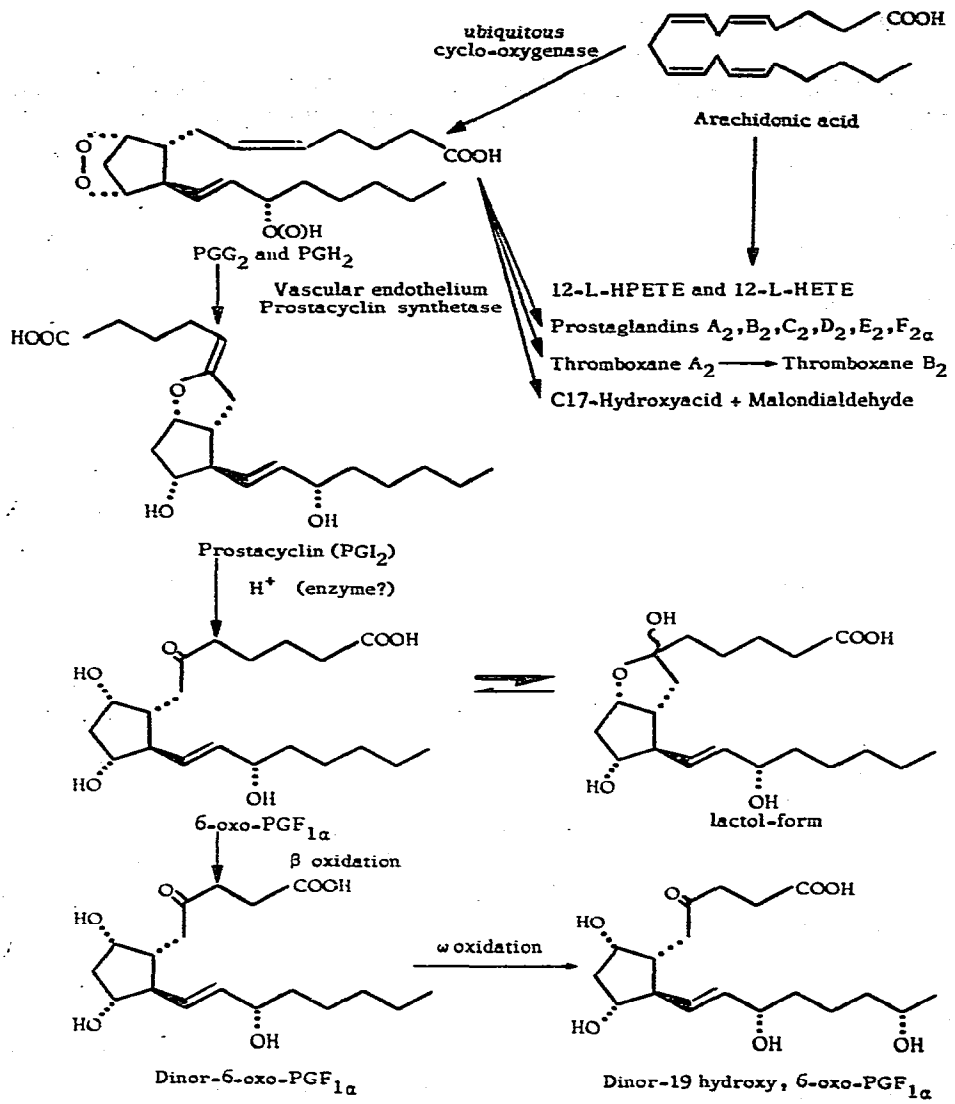

Fig. 1. Metabolic pathways of arachidonic acid (eicosa 5,8,11,14-tetraenoic acid).

MS) isotope method or by the radiochromatography on thin-layer chromatographic (TLC) silica gel plates, afford indirect ways of assessing PGI_2 formation. However, the lack of more simple chemical or physico-chemical methods for the quantitation of PGI_2 and 6-oxo-$PGF_{1\alpha}$ constitutes a limiting factor in the study of their physiological roles and metabolism.

This report deals with the chromatographic behaviour of 6-oxo-$PGF_{1\alpha}$ and some related compounds, as compared with other prostaglandins, both in silicic acid columns and thin-layer silica gel plates. A gas–liquid chromatographic (GLC) method

was devised which in its present form, was able to measure nanogram amounts of 6-oxo-$PGF_{1\alpha}$.

A novel sensitive colour reaction, useful for the detection of 6-oxo-$PGF_{1\alpha}$ and related compounds on TLC silica gel plates, is also described.

EXPERIMENTAL

Materials

Prostaglandins. The following prostaglandins were used: PGA_1, PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$, 6-oxo-$PGF_{1\alpha}$·methyl ester, PGI_2·sodium salt, PGI_2·methyl ester and dihydro-PGI_2. PGB_2 was prepared as shown below.

Solvent and chemicals. Diethyl ether, solvent grade from Macfarlane Smith (Edinburgh, Great Britain), was dried and preserved from peroxide formation with sodium wire. Ethyl acetate (AnalaR; BDH, Poole, Great Britain) was distilled in a Dufton fractionation column, stored in the dark at 4° and used within one week. Pyridine was silylation grade from Pierce & Warriner (Chester, Great Britain). All other solvents were commercial C.P. grade and used without further treatment. Other chemicals were: silicic acid (Unisil, 100–200 mesh, acid washed; Clarkson, Williamsport, Pa., U.S.A.); methoxyamine–HCl (Applied Science Labs., State College, Pa., U.S.A.); N-methyl-N-nitroso-*p*-toluenesulfonamide (Diazald from Ralph N. Emanuel, Wembley, Great Britain); N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA, Pierce & Warriner); Chromosorb AW DMCS HP (100–120 mesh; Analabs, North Haven, Conn., U.S.A.); silicones OV-1 and OV-17 (Analabs) and 2,4-dinitrophenylhydrazine (BDH).

Spray reagents were prepared with the following aromatic aldehydes. Anisaldehyde (4-methyoxybenzaldehyde) BDH-27231; salicylaldehyde (2-hydroxybenzaldehyde) BDH-30035; resorcylaldehyde (2,4-dihydroxybenzaldehyde) BDH-29157; cinnamaldehyde (3-phenylpropenal) BDH-27772. Vanillin (4-hydroxy-3-methoxybenzaldehyde), veratraldehyde (3,4-dimethyoxybenzaldehyde) and gallaldehyde-3,4,5-trimethylether were from the Department of Chemistry (Wellcome Research Labs., Beckenham, Great Britain). Fatty acid methyl esters mixture (H-108, Applied Science Labs.) consisting of five saturated acids ($C_{17:0}$ to $C_{21:0}$) was used as GLC standard.

METHODS

Preparation of PGB_2

PGB_2 was prepared from PGE_2 by alkali treatment according to the method of Pike *et al.*[9], slightly modified as follows. A mixture of 10 mg PGE_2, 3 ml of absolute ethanol and 3 ml of 1 *N* NaOH was stirred under nitrogen at 35° for 1 h. The clear solution was concentrated *in vacuo* at room temperature (20–22°) to remove ethanol, acidified with 4 ml of 1 *N* HCl and extracted three times with 1 volume of ethyl acetate. The extracts were combined, washed to neutrality with small portions of distilled water, dried (Na_2SO_4) and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 30% ethyl acetate in toluene and applied onto a column of 4 g silicic acid prepared in the same solvent mixture. The column effluent was monitored by UV absorption at 278 nm. All fractions containing PGB_2 were combined and evaporated to dryness in a stream of nitrogen. The uncrystallized residue was dissolved in a small volume of ethanol, analysed by UV spectrophotometry in

a Unicam SP-500 spectrophotometer using the molar absorption coefficient $\varepsilon =$ 26,800 (ref. 10), and then diluted with ethanol to 1 mg/ml.

Derivatization

Methyl esters of the prostaglandins (free acids) were prepared in micro scale using the diazomethane method*. Excess of diazomethane solution was added to the dry prostaglandin in Reacti-vials (Pierce & Warriner) left for 20 min at room temperature. After removal of the reagent and solvent in a stream of nitrogen, the methylated residue was either dissolved in absolute ethanol (1 mg/ml) or processed further when necessary.

6-Oxo-$PGF_{1\alpha}$ was derivatized as follows: a sample (5–500 μg) was methylated in a Reacti-vial as above. The methyl ester was then left to react overnight with excess of methoxyamine in dry pyridine (5 mg/ml) and the pyridine removed *in vacuo*. Finally, the trimethylsilyl (TMS) derivative was formed in a measured volume of BSTFA. The reaction was always completed at the end of 2 h at room temperature. The final solution was protected by a double seal cap (Mininert valve, Pierce & Warriner) and stored at −20° till used. Aliquots (μl) of this solution were injected directly into the GLC column using a micro-syringe.

Column chromatography

A glass chromatographic column (Quick-fit CR 12/10) fitted with an extension tube (200 ml) and a small vibrator was filled with 100 ml EM-I (30% ethyl acetate in toluene). A slurry of 4 g silicic acid was made in the same EM-I mixture and properly degassed before being transferred rapidly into the columns. The adsorbent was uniformly packed under constant vibration while 100 ml of EM-I ran throughout the column. The silicic acid columns were always operated by gravity, at room temperature and with a flow-rate of 0.5–1 drops per sec. Pure prostaglandins, alone or in mixture, and tissue extracts were dissolved in a measured small volume (0.5–3 ml) of ethyl acetate and then toluene was added in the right proportion. The solution was transferred quantitatively into the column and several 1-ml quantities of EM-I were used to rinse the vial. Elution was carried out by successive changes of an adequate volume of the elution mixtures which contained increased amounts of ethyl acetate in toluene (40% for EM-II, 60% for EM-III and 80% for EM-IV). Generally, 10-ml fractions were collected. According to the activity of the silicic acid batch, a small volume of methanol was added to the elution mixtures (0.1 to 0.8%) in order to obtain a uniform elution pattern.

Gas–liquid chromatography

A Pye-Unicam Series 104 Model 84 gas chromatograph fitted with a flame ionization detector (FID) operated at 300° was used. Glass columns (6 mm O.D. × 1.5 m long) were packed with 3% OV-17 silicone on Chromosorb and then conditioned at 280° for 24 h before use. The carrier gas was nitrogen (flow-rate 30–40 ml/min) and the column was operated isothermically.

* Diazomethane, generated from Diazald in an all-glass apparatus fitted with clear joints (Quick-fit) and constantly flushed with dry nitrogen, was trapped into ice-cold ether–methanol (9:1). This solution was stable for several days at −20°.

6-Oxo-$PGF_{1\alpha}$–Me–MO–TMS (Me = methyl, MO = methyloxime) peaks were identified by the equivalent chain length (ECL) of the ester. A fatty ester mixture containing estearic methyl ester ($E_{18:0}$–Me) and arachidic methyl ester ($C_{20:0}$–Me) was used as a standard in checking column performance and in calculating the ECL of the 6-oxo-$PGF_{1\alpha}$ derivative by the formula[11]:

$$ECL = 2\left[\frac{\log RT_x - \log RT_n}{\log RT_{n+2} - \log RT_n}\right] + n$$

where RT_x, RT_n and RT_{n+2} are the retention times of the unknown ester and of saturated esters of chain lengths n and $n + 2$.

A standard curve relating the detector responses (areas under the peaks) and the amounts of 6-oxo-$PGF_{1\alpha}$ derivative was constructed. The column was operated at 264°.

Gas–liquid chromatography–mass spectroscopy

The 6-oxo-$PGF_{1\alpha}$ sample used was characterised by MS in an LKB-9000 GC–MS apparatus. The 6-oxo-$PGF_{1\alpha}$–Me–MO–TMS derivative was chromatographed in a glass column 3 mm × 3 m, packed with 3% OV-1 silicone on Supelcoport and operated at 248°, using helium (flow-rate 35 ml/min) as the carrier gas.

Thin-layer chromatography

TLC silica gel plates Merck 60F254 precoated, 0.25 mm thick were used. Solutions were spotted over a line 3 cm from the bottom of the plate. A gentle blow of cold air was used to keep the substance in a small circle, 1.5–3 mm diameter. All solutions were applied with calibrated Microcaps (Drummond, Broomall, Pa., U.S.A.). The layer of silica was cut as a thick line, 2 cm from the top edge of the plate, limiting the solvent run to 15 cm. TLC plates (5 × 20 cm) were developed in cylindrical tanks containing 30 ml of solvent. Large plates (20 × 20 cm) were developed in rectangular tanks (Shandon, London, Great Britain) containing 200 ml of solvent. Solvent mixtures (Table I) were used only once and after 1–2 h equilibration in the tanks.

After development, the TLC plates were left for a few minutes in a fume cupboard to remove excess solvent and were then evenly sprayed with the reagent for 5–10 sec. Sprayed plates were again left for at least 15 min at room temperature before heating with hot air (about 90°) until full colour spots developed. The plates were inspected for fluorescence in a UV cabinet (Chromato-Vue Model CC-20, Ultraviolet Products, San Gabriel, Calif., U.S.A.).

Spray reagents

Aromatic aldehyde–sulphuric acid reagents[19]. The main colour reagent used to spray TLC silica gel plates was prepared as follows. To 20 ml ice-cold absolute ethanol, 2 ml of concentrated sulphuric acid was slowly added, mixing after each addition to dissipate the heat developed (ice-bath). Anisaldehyde (2 ml) was added in the same way, resulting in a practically colourless reagent ready for use. If stored at −20° the reagent is not discoloured for several days. Similar reagents were prepared with

TABLE I

SOLVENT MIXTURES USED IN TLC OF PROSTAGLANDINS

Code	*Composition*
SM-I	Ethyl acetate–acetone–acetic acid (90:10:1, v/v). Monophasic system F-VI by Andersen[21].
SM-II	Benzene–1,4-dioxane–acetic acid (20:20:1, v/v). Monophasic system A-I by Gréen and Samuelsson[13].
SM-III	Ethyl acetate–methanol–acetic acid (100:10:1, v/v). Monophasic system by Millar[14].
SM-IV	Chloroform–methanol–acetic acid–water (90:9:1:0.65, v/v). Monophasic system by Pace-Asciak and Wolfe[15].
SM-V	Ethyl acetate–acetic acid–2,2,4-trimethylpentane–water (90:20:50:100, v/v). Organic phase used after 2 h equilibration at toom temperature. System A-IX by Hamberg and Samuelsson[16].
SM-VI	Benzene–chloroform–*n*-butanol–ethanol (4:10:5:1, v/v). Monophasic system by Eastman and Dowsett[17].
SM-VII	Chloroform–methanol–acetic acid (90:5:5, v/v). Monophasic system C-I by Ramwell and Daniels[18].

other liquid aromatic aldehydes (resorcylic, cinnamic, salicylic) in the same proportions. Solid aldehydes (vanillic, veratric and gallalic) were used in reduced proportions (0.25–0.5 g).

Dinitrophenylhydrazine reagent[20]. A 100-mg amount of 2,4-dinitrophenylhydrazine was dissolved in 90 ml of ethanol containing 10 ml concentrated hydrochloric acid.

Copper acetate–phosphoric acid[21]. Cupric acetate (1.5 g) was dissolved in 50 ml of 15% aqueous orthophosphoric acid solution.

All reagents were sprayed in a fume cupboard using a spray gun (Shandon).

Solutions of prostaglandins

All prostaglandins and their methyl esters were dissolved in absolute ethanol (1 mg/ml) and stored at −20° in 2 ml screw-cap glass vials.

RESULTS

6-Oxo-$PGF_{1\alpha}$–Me–MO–TMS emerged as the main peak from the GLC OV-1 column (at 248°) characterised by an ECL value of 25.1. The mass spectrum was typical for this derivative of 6-oxo-$PGF_{1\alpha}$, 17 major fragment ions being at the following *m/e* values (Fig. 2): 629 (M^+), 614 ($M^+ - CH_3$), 598 ($M^+ - OCH_3$), 558 ($M^+ - C_5H_{11}$), 539 (M^+ − TMSOH), 508 (M^+ − TMSOH − OCH_3), 468 (M^+ − TMSHO − C_5H_{11}), 449 ($M^+ - 2 \times$ TMSOH), 418 ($M^+ - 2 \times$ TMS − OCH_3), 412 ($M^+ - C_4H_8 \cdot CO_2CH_3 - C_5H_{11} - OCH_3$), 378 ($M^+ - C_5H_{11} - 2 \times$ TMSOH) 353 (M^+ − TMSOH − $CH_2 \cdot C(=N \cdot OCH_3) - (CH_2)_4CO_2CH_3$, 321 ($C_4H_8 \cdot CO_2CH_3$ + $TMSO^+ = CHOTMS$), 217 ($TMSO^+ - C = CH \cdot CH_2$ − $TMSO^+$), 191 ($TMSO^+ = CHOTMS$), 173 ($TMSO^+ = CH \cdot C_5H_{11}$) and 115 ($C_4H_8 \cdot CO_2CH_3$). The most prominent peaks were at *m/e* 378 (100%), 598, 508 and 418 (80.5%), 449 (51.2%), 173 (48.8%), 468 (41.5%), 115 (39%), 191 (36.6%) and 558 (26.8%).

Minor fragment ions were found at *m/e* 524, 482, 443, 426, 392, 327, 321, 300, 276, 204, 133 and 129 (Fig. 2).

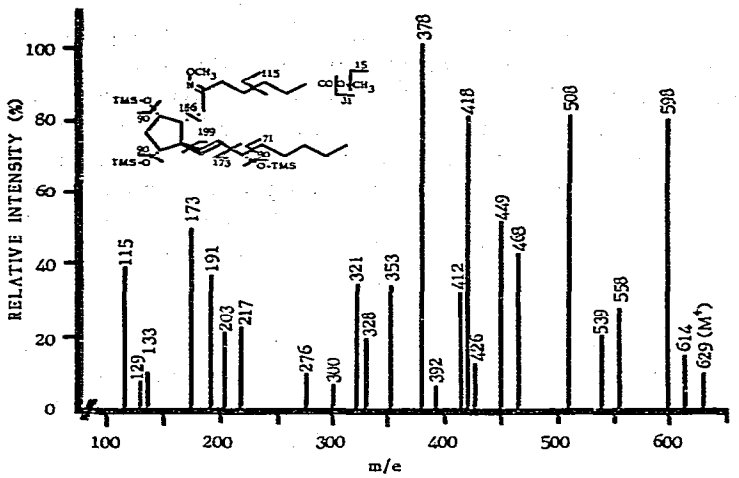

Fig. 2. Mass spectrum of 6-oxo-$PGF_{1\alpha}$–Me–MO–TMS.

In the GLC OV-17 columns the same derivative of 6-oxo-$PGF_{1\alpha}$ emerged as a very well constituted peak with a retention time of about 9 min at 264°. The calculated ECL value in this column was 25.1 (see Fig. 3).

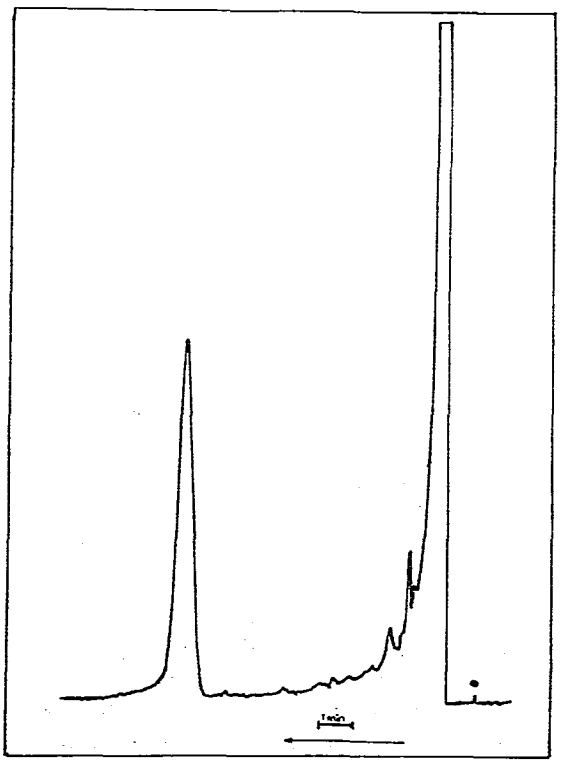

Fig. 3. Peak (5 ng) of 6-oxo-$PGF_{1\alpha}$–Me–MO–TMS. RT = 8.8 min. 3% OV-17 on Chromosorb W HP glass column (6 mm O.D. × 1.5 m). Temperature 264°, FID 300°. Pye-Unicam 104-84 GLC. Nitrogen carrier gas flow-rate 37 ml/min. Injection (0.2 μl in BSTFA) at the dot.

Silicic acid column chromatography was found to be effective in separating 6-oxo-$PGF_{1\alpha}$ (free acid) from all other major prostaglandins. 6-Oxo-$PGF_{1\alpha}$ was firmly bound in the adsorbent, nothing being eluted before pure ethyl acetate and methanol were percolated through the column bed. A typical experiment is shown in Fig. 4. A relatively highly loaded (1.8 mg total fatty acids) column of silicic acid (4 g) was able to resolve the mixture (>95% for pure prostaglandins) almost quantitatively. PGB_2, PGE_2 and $PGF_{2\alpha}$ were eluted in a total of 450 ml ethyl acetate–toluene mixtures. Only about 8% of 6-oxo-$PGF_{1\alpha}$ was eluted in 50 ml pure ethyl acetate. Methanol was very efficient in removing 6-oxo-$PGF_{1\alpha}$ from silicic acid columns. Pigments from tissue extracts, also retained in the columns were eluted with methanol, but they did not interfere when 6-oxo-$PGF_{1\alpha}$ was subsequently analysed as the Me–MO–TMS derivative by GLC or by radiochromatography on TLC silica gel plates. Table II summarizes the R_F values found for 19 prostanglandins chromatographed on TLC silica gel plates developed in the 7 solvent mixtures tested.

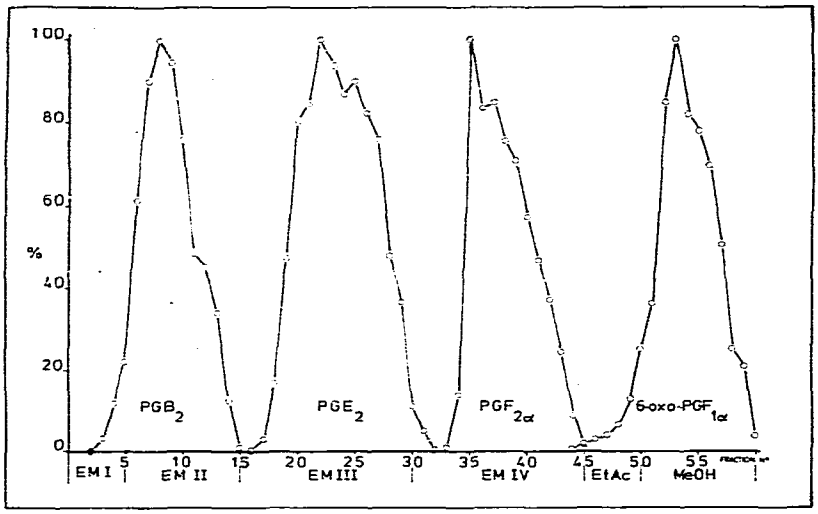

Fig. 4. Fractionation of a mixture of prostaglandins B_2 (500 μg), $F_{2\alpha}$ (400 μg), E_2 (400 μg) and 6-oxo-$PGF_{1\alpha}$ (500 μg) in a column (10 × 130 mm) of silicic acid (4 g). The column was eluted sequentially with solvent mixtures containing increased concentrations of ethylacetate in toluene. Fractions (10 ml) were collected at room temperature by gravity (0.5–1 drops per sec) and then individually monitored by a spot test with the anisaldehyde–H_2SO_4 reagent and analysed for PGB_2 (UV absorption at 278 nm), PGE_2 and $PGF_{2\alpha}$ (bioassay on rat stomach strips) and 6-oxo-$PGF_{1\alpha}$ (GLC of the Me–MO–TMS derivative).

A mixture of 8 prostaglandins (PGA_1, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$) spotted on TLC silica gel plates (10 μg each) was completely resolved into its components when developed in SM-VII ($CHCl_3$–MeOH–HAc, 90:5:5, v/v) (Fig. 5). The R_F for 6-oxo-$PGF_{1\alpha}$ (0.29) was very different from that shown by the closest prostaglandins, $PGF_{2\alpha}$ (0.19) and PGE_2 (0.36). Unless smaller amounts of prostaglandins were used, 6-oxo-$PGF_{1\alpha}$ could not be clearly separated from dihydro-PGI_2 (R_F = 0.32).

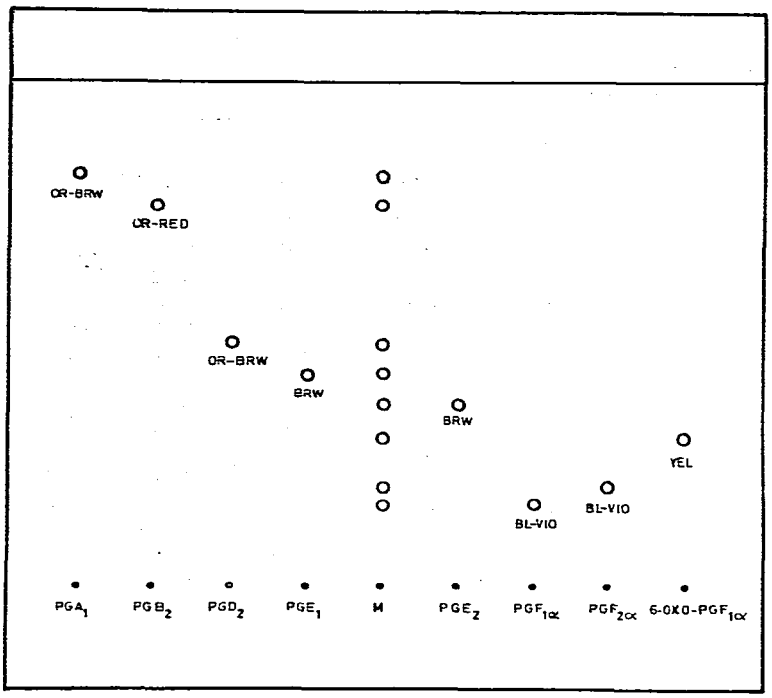

Fig. 5. Relative mobility of 6-oxo-$PGF_{1\alpha}$ and some major prostaglandins in a TLC silica gel plate developed in SM-VII ($CHCl_3$–MeOH–HAc, 90:5:5, v/v) at room temperature (20–22°). The mixture (M) containing 10 μg of each prostaglandin was completely resolved into its components. Anisaldehyde–sulphuric acid reagent. The characteristic yellow spot given by 6-oxo-$PGF_{1\alpha}$ developed in less than 30 sec at toom temperature. After heating (about 85°) the other prostaglandins displayed typical coloured spots: orange-brown (OR-BRW) for PGA_1 and PGD_2, orange-red (OR-RED) for PGB_2, brown (BRW) for PGE_1 and PGE_2, and blue-violet (BL-VIO) for $PGF_{1\alpha}$ and $PGF_{2\alpha}$.

The usefulness of SM-VII in TLC of the prostaglandins studied is to be stressed, not only because of its ability to resolve mixtures of protaglandins but also because in this solvent mixture PGI_2–Me was chromatographed at room temperature without decomposition, which occurred in all other 6 solvent mixtures employed. Furthermore, PGI_2–Me ran with $R_F = 0.65$, very well separated from PGD_2 ($R_F = 0.48$) and PGB_2 ($R_F = 0.75$). The small tail observed when PGI_2–Me was chromatographed in SM-VII offered no identification problems. PGI_2–Na could not be chromatographed in any solvent mixture at room temperature without decomposition. Also, as expected from their polarities, all prostaglandin methyl esters ran much faster in SM-VII than did the corresponding free acids. This actually occurred, with slight discrepancies, in all other solvent mixtures employed.

The only major problem in the identification of mixtures of prostaglandins by TLC mobility occurred between $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ where an excess of material in the plate produced large spots and sometimes considerable tailing. However, the reversal in mobility observed when SM-I, SM-V and SM-VI were used, always assisted in the separation of $PGF_{2\alpha}$ from 6-oxo-$PGF_{1\alpha}$.

In SM-III, 6-oxo-$PGF_{1\alpha}$ ran in two spots, as occurred with its methyl ester in SM-I, SM-II, SM-III, SM-IV and SM-V.

TABLE II

R_F VALUES OF 19 PROSTAGLANDINS

T = Tailing, D = decomposition on the plate.

Prostaglandin	R_F						
	SM-I	*SM-II*	*SM-III*	*SM-IV*	*SM-V*	*SM-VI*	*SM-VII*
PGA_1	0.75	0.88	0.73	0.75	0.53	0.85T	0.81
PGA_1–Me	0.91	0.97	0.93	0.97	0.59	0.93	0.97
PGB_2	0.69	0.81	0.78	0.52	0.53	0.82	0.75
PGB_2–Me	0.89	0.96	0.90	0.94	0.59	1.00	0.96
PGD_2	0.61	0.95	0.80	0.56	0.31	0.59T	0.48
PGD_2–Me	0.78	0.95	0.91	0.63	0.41	0.98	0.84
PGE_1	0.31	0.78	0.66	0.51	0.19	0.55T	0.42
PGE_1–Me	0.47	0.73	0.81	0.59	0.31	0.92	0.65
PGE_2	0.35	0.75	0.78	0.35	0.20	0.51T	0.36
PGE_2–Me	0.53	0.79	0.78	0.57	0.33	0.89	0.68
$PGF_{1\alpha}$	0.20	0.41	0.49	0.24	0.11	0.29T	0.16
$PGF_{1\alpha}$–Me	0.35	0.53	0.65	0.47	0.17	0.72	0.44
$PGF_{2\alpha}$	0.19	0.53	0.49	0.22	0.13	0.21T	0.19
$PGF_{2\alpha}$–Me	0.34	0.73	0.67	0.35	0.19	0.65	0.31
6-Oxo-$PGF_{1\alpha}$	0.13	0.53	0.41–0.67	0.33	0.06	0.12T	0.29
6-Oxo-$PGF_{1\alpha}$–Me	0.54–0.61	0.61–0.87	0.59–0.81	0.46–0.73	0.07–0.14	0.94	0.76
PGI_2–Me	D	D	D	D	D	D	0.65T
Dihydro-PGI_2	0.25	0.77	0.55	0.25	0.15	0.34T	0.32
Dihydro-PGI_2–Me	0.43	0.78	0.75	0.69	0.25	0.91	0.69

Dihydro-PGI_2 and its methyl ester ran at room temperature as a single spot in all solvent mixtures employed, no sign of decomposition being observed.

6-Oxo-$PGF_{1\alpha}$ developed an intense yellow colour on a white back-ground in TLC silica gel plates sprayed with the anisaldehyde–sulphuric acid reagent. The coloured spot could easily be seen in diffuse daylight less than 30 sec after spraying when the amount of prostaglandin was 0.1 μg or more. The chromogen was developed at room temperature (20–22°) and the limit of detection was found to be about 50 ng/0.25 cm^2 (0.14 nmoles). The yellow spot remained stable in the plates for more than 1 month. The reaction is unique in that only 6-oxo-$PGF_{1\alpha}$ and some of its structurally related prostaglandins (PGI_2–Na and PGI_2–Me) produced the yellow chromogen at room temperature. All other prostaglandins so far tested, including dihydro-PGI_2 and its methyl ester produced coloured spots only after heating the plate at about 85° for several minutes and under no circumstances was a yellow chromogen formed. The following coloured spots were observed: orange-red (PGA_1, PGB_2), orange-brown (PGD_2), brown (PGE_1, PGE_2) and blue-violet ($PGF_{1\alpha}$, $PGF_{2\alpha}$ dihydro-PGI_2 and dihydro-PGI_2–Me). Esterification of the carboxy group (methylation) did not alter the colour given by the corresponding free acid.

After heating, the yellow spots shown by 6-oxo-$PGF_{1\alpha}$ and the structurally related prostaglandins was reinforced but there was no change of colour. When other aromatic aldehydes were substituted for anisaldehyde, essentially similar results were obtained with 6-oxo-$PGF_{1\alpha}$ and related compounds.

When TLC silica gel plates were sprayed with the anisaldehyde–sulphuric acid reagent, heated and then observed under UV light (long wavelength), different

fluorescent spots were given by prostaglandins: ochre (6-oxo-$PGF_{1\alpha}$), bright orange (PGI_2–Me), beige (dihydro-PGI_2) and green-yellowish (PGI_2–Na). 6-Oxo-$PGF_{1\alpha}$ produced different fluorescence when other aromatic aldehydes were used: beige (salicylaldehyde) or dark yellow (cinnamaldehyde).

No colour at all was observed with 6-oxo-$PGF_{1\alpha}$ on plates sprayed with the acidic 2,4-dinitrophenylhydrazine reagent, even after heating and observing the plate for several days. Other carbonylated prostaglandins, such as PGE_1, PGE_2 and PGD_2, progressively developed a yellow-orange chromogen at room temperature, the colour development being accelerated by heating. PGD_2 was definitely much more reactive than the PGEs.

Although coloured compounds were also formed with the major prostaglandins after spraying TLC silica gel plates with the cupric acetate–phosphoric acid reagent and heating at 85°, 6-oxo-$PGF_{1\alpha}$ did not produce similar chromogens, only a pale gray spot which was seen after prolonged heating. This reagent produced a bluish spot with dihydro-PGI_2, and PGI_2–Me showed an intense brown-reddish spot rapidly formed.

DISCUSSION

When 6-oxo-$PGF_{1\alpha}$ was derivatized by methylation, methoxymation and silylation, the product emerged from OV-1 and OV-17 silicone GLC columns as only one major peak (ECL = 25.1). The mass spectrum of the derivative showed that apart from the molecular ion (M^+) at *m/e* 629, several other fragment ions supported the structure first proposed by Pace-Asciak[1]. Actually, there was remarkable agreement between our values and those reported in the literature for 6-oxo-$PGF_{1\alpha}$–Me–MO–TMS[1,12,22].

The GLC characteristics of the peak which emerged at 264° in about 9 min from an OV-17 on Chromosorb column, permitted not only the identification of the compound but its quantification as well, even in samples of 6-oxo-$PGF_{1\alpha}$ extracted and purified from tissue extracts. Reagent impurities, especially in the methoxyamine–pyridine mixture and from the tissue extracts, produced peaks which came off in less than 4 min. With pure 6-oxo-$PGF_{1\alpha}$, a standardization curve was constructed. Good linearity was observed between the detector response and concentration using the minimum attenuation (100 or 50) compatible with a stable base line. Under these conditions, 5 ng of 6-oxo-$PGF_{1\alpha}$ could be estimated.

Efficient purification of 6-oxo-$PGF_{1\alpha}$ was obtained by column chromatography on silicic acid. The use of a particular brand of commercial SiO_2 (Unisil) is strongly recommended, not only because of the uniform pattern of prostaglandin elution, but also because of the advantage in running the solumn by gravity without the risk of air entering the adsorbent bed.

6-Oxo-$PGF_{1\alpha}$ was resolved in discrete spots in TLC silica gel plates developed in most of the solvent mixtures used. 6-Oxo-$PGF_{1\alpha}$ ran faster in SM-III than in the original Andersen's mixture F-VI, which is potentially better due to the expected presence of biological pigments in the final extracts. Because 6-oxo-$PGF_{1\alpha}$ is resolved in two separated spots (R_F 0.41 and R_F 0.67) in SM-III, distinction from other prostaglandins is more difficult.

Very good separation between 6-oxo-$PGF_{1\alpha}$ and the PGFs was effected in 5

of the 7 solvent mixtures used (Table I). The peculiarity that 6-oxo-$PGF_{1\alpha}$ ran faster than the PGFs in SM-IV and SM-VII but slower in SM-I, SM-V and SM-IV, afforded another criterion of identification for these prostaglandins in TLC silica gel plates. In terms of resolution of mixtures in to their components, SM-VII gave the best results. In this solvent mixture, even the methyl ester of the unstable PGI_2 was chromatographed at room temperature without decomposition.

Colour reagents useful in the identification of prostaglandins in TLC plates have frequently been described[20,23,24], although they have not often been used. Nevertheless, the combination of chromatographic mobility and specific colour reactions can be considered a powerful tool for the identification of prostaglandins

sulphuric acid reagent. The reaction is apparently specific for 6-oxo-$PGF_{1\alpha}$ and its metabolic precursor PGI_2. Although the nature of the yellow chromogen is so far unknown it seems that hydroxy groups are important for its formation. The anisaldehyde–sulphuric acid reagent was originally used for the identification of sugars[19] and hydroxylated steroids[25]. The possibility of a chalcone formation with the aromatic aldehyde seems unlikely for the yellow chromogen formed had a very poor absorption in the UV.

The absence of reaction with 2,4-dinitrophenylhydrazine, a well known reagent for carbonylated compounds, was in contrast with the reactivity of prostaglandins carbonylated at the pentagonal ring (PGEs and PGD_2). This constitutes further evidence that 6-oxo-$PGF_{1\alpha}$ exists mainly in its lactol form[1], (see Fig. 1).

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