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DETERMINATION OF PROSTAGLANDINS BY GAS-LIQUID CHROMATOGRAPHY

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SUMMARY

Two procedures are described for the determination of prostaglandins B, E and F by gas-liquid chromatography. In the first, keto prostaglandins are quantitatively dehydrated, esterified, and acetylated to give products stable to gas chromatography, while F prostaglandins are rapidly analyzed as trimethylsilyl ether-trimethylsilyl esters. In the second procedure, a mixture of prostaglandins is separated into B, E and F class fractions by column chromatography. The B class of prostaglandins, both that present originally and that formed from E prostaglandins by base treatment, is resolved according to degrees of unsaturation by gas chromatography of their acetylated methyl esters. The F prostaglandins are similarly resolved by gas chromatography of their trimethylsilyl ether-methyl esters.

INTRODUCTION

Prostaglandins, pharmacologically active derivatives of prostanoic acid ($C_{20}H_{38}O_2$) have been determined by thin-layer chromatography (*e.g.* refs. 1-3), paper chromatography[4], enzymatic assay[5], fluorescence[6], optical rotatory dispersion and absorption spectroscopy[7], as well as by their effects on smooth muscle preparations *in vitro* and/or blood pressure *in vivo* (ref. 8, review). The trihydroxy (F) prostaglandins have been analyzed by gas chromatography of their trimethylsilyl ether-methyl esters[9], but keto prostaglandins of the E type have not been successfully gas chromatographed without decomposition[2].

Those methods above which are most sensitive, *e.g.*, fluorescence and enzymatic assay, are also least selective in that they do not distinguish between prostaglandins of differing degrees of unsaturation. The highly selective techniques of reversed-phase and thin-layer chromatography lack the desired sensitivity, while the spectroscopic methods suffer interference from many biological materials. The bioassay methods have extreme sensitivity, but complete selectivity requires highly elaborate procedures. The present report is concerned with attempts to develop a sensitive and selective gas chromatographic method for the determination of a variety of classes of prostaglandins.

METHODS

Materials

Prostaglandins E_1, E_2 and $F_{1\alpha}$ were provided through the courtesy of Dr. JOHN PIKE, the Upjohn Company, Kalamazoo, Mich. Prostaglandins B_1, B_2, $F_{2\alpha}$ and $F_{2\beta}$ were synthesized from the appropriate E prostaglandins by base treatment[7] or sodium borohydride reduction[10], respectively.

Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide, distilled into ether and used immediately under nitrogen. Pyridine was distilled from barium oxide and stored over NaOH pellets. Acetic anhydride and dioxane were redistilled shortly before use.

Gas chromatography liquid phases and solid supports were obtained from Applied Sciences Laboratories, State College, Pa., as were 14% BF_3 in methanol, *n*-tetracosane and methoxyamine hydrochloride. Anhydrous diethyl ether was Mallinckrodt AR grade. Silylating reagents were from Pierce Chemical Company, Rockford, Ill. Isopropenyl acetate was obtained from Aldrich Chemical Company, Cedar Knolls, N.J. All other chemicals were the best grades available from Fisher Scientific Company, Raleigh, N. C.

A Perkin-Elmer Model 900 instrument equipped with hydrogen flame ionization detectors, a 1 mV recorder and adapted for direct on-column injection into glass columns was used for the gas chromatography. Mallinckrodt SilicAR CC-4 silicic acid (100–200 mesh) was washed with chloroform, activated at 105° for 2 h, and stored in glass bottles in a desiccator over Drierite until used for column chromatography. Silica Gel GF plates (Analtech), 250 μ thickness, were activated at 105° for 1 h shortly before use, and were developed in lined tanks.

Prostaglandin concentrate

The starting material for the fractionations to follow was an ethyl acetate solution of reference prostaglandins intended to simulate fraction "h" of ÄNGGÅRD[11]. The mixture was loaded onto a column of silicic acid[1] and washed with 20 ml of *n*-hexane–ethyl acetate (7:1) per gram of adsorbent. The prostaglandin "concentrate" was then eluted *in toto* with 40 ml of ethyl acetate–acetone (95:5) per gram of silicic acid. The two fractions were examined by thin-layer chromatography in system D-IV[1].

Preliminary screening of concentrate by gas chromatography

A rapid, qualitative or semi-quantitative procedure was needed for the detection of minute amounts of E and/or F prostaglandins in a variety of samples. Although this could be accomplished by a combination of thin-layer chromatography and bioassay[3], the following approach was developed because of lack of facilities for bioassay.

An aliquot of concentrate from the above procedure was dried with a nitrogen stream, dissolved in 0.5 ml of anhydrous methanol, and treated with 0.5 ml of 14% BF_3 in methanol at 60° in a closed tube for 10 min. The mixture was cooled, diluted with 5 ml of water, and extracted twice with ethyl acetate. The ethyl acetate solution was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated to dryness with a stream of nitrogen.

The residue was treated for 10 min at room temperature with 0.1 ml of a freshly prepared solution of 0.1% 2,4-dinitrobenzenesulfonic acid in isopropenyl acetate. A few grains of anhydrous potassium carbonate were added, and after 15 min aliquots of the solution were examined by gas chromatography as described below.

A second aliquot of the original concentrate was taken to dryness under nitrogen and treated with 0.1 ml of trimethylsilylimidazole–bis(trimethylsilyl)trifluoroacetamide (2:1) for 20 min at room temperature. Aliquots of this reaction mixture were also examined by gas chromatography.

Gas–liquid chromatography of both of the above reaction mixtures was accomplished on a 2 m × 0.5 cm column packed with 3% OV-1 silicone on 100–120 mesh Gas-Chrom Q. The injection port, column, and detector manifold temperatures were 240°, 230° and 280°, respectively. The helium flow rate was 60 ml/min.

Column fractionation of prostaglandins into classes

If the above screening procedures indicated the probable presence of prostaglandins, the remaining concentrate was separated into three fractions. Up to 200 µg of total prostaglandins were loaded onto a column of 1 g of SilicAR CC-4 slurried in *n*-hexane–ethyl acetate (2:1).

A and B prostaglandins (if present) were eluted with 25 ml of *n*-hexane–ethyl acetate (2:1), giving Fraction I. Type E prostaglandins were eluted with 30 ml of *n*-hexane–ethyl acetate (2:3) (Fraction II), and F prostaglandins were eluted with 30 ml of ethyl acetate–acetone (95:5) (Fraction III). This procedure was evaluated with 50 µg each of PGB_1, PGE_1 and $PGF_{1\alpha}$; each fraction was collected in 5 ml portions and examined by thin-layer chromatography in solvent A-I[2].

Derivatization of Fraction I

The dry residue from Fraction I was esterified with diazomethane and the reagent was removed with a stream of nitrogen. The methylated derivative was treated overnight at room temperature with 100 µl of acetic anhydride–pyridine (3:5). One milliliter of water was added, and the mixture was extracted three times with diethyl ether (total 1.5 ml); the ether extract was washed with water, dried over anhydrous sodium sulfate, and filtered. The ether was removed with a nitrogen stream and the residue was dissolved in a few microliters of methylene chloride for gas chromatographic examination.

Derivatization of Fraction II

The dry residue was dissolved in 0.5 ml of ethanol and treated with 0.5 ml of 1 *N* aqueous NaOH at 40° for 30 min. The solution was cooled to room temperature, diluted with 2.5 ml of water, and acidified with HCl. The reaction products were extracted into ethyl ether, washed once with water and taken to dryness under nitrogen, then esterified and acetylated as described for Fraction I.

Derivatization of Fraction III

The dry residue was esterified with diazomethane, the reagent was blown off, and 10 µl of methylene chloride containing 0.30 µg of *n*-tetracosane were added as an internal reference. Forty microliters of trimethylsilylimidazole–bis(trimethylsilyl)-

trifluoroacetamide (2:1) were added and the mixture was let stand for 20 min at room temperature.

If it was desired to remove the unreacted reagent, 1 ml of methylene chloride was added. The solution was washed first with 2 ml of 0.1 *M* potassium bicarbonate buffer pH 7.5, then with 2 ml of water. The methylene chloride phase was dried over anhydrous sodium sulfate, filtered, and concentrated under nitrogen.

Gas–liquid chromatography of methyl esters

The derivatives from Fractions I, II and III were chromatographed on a 2 m by 0.2 cm glass column packed with 3% cyclohexanedimethanol succinate (CHDMS) on 100–120 mesh Gas-Chrom Q. The helium flow rate was 45 ml/min and the detector manifold was at 280°. The column and injection port temperatures were 230° and 240°, respectively, for the acetylated fractions and 185° and 195°, respectively, for the trimethylsilyl ethers. Peak areas were measured as the product of peak height times the width at half the height.

RESULTS

The screening procedure

Thin-layer chromatography in systems A-I and M-I[2] of the products from BF_3–CH_3OH treatment of 100 μg samples of PGE_1 and PGE_2 revealed no detectable starting material remaining. Using the methyl esters of PGE_1, PGE_2 and PGB_1 produced with diazomethane[2] as reference compounds, it was found that the only detectable products from BF_3–CH_3OH treatment co-chromatographed with methyl-PGB_1 and were separated from authentic methyl-PGE_1 and methyl-PGE_2. The methanolysis products gave a positive reaction with the hydroxylamine–ferric chloride spray[12] indicative of esters. The BF_3–CH_3OH treatment thus apparently dehydrated the PGE as well as esterifying it. The dehydration product expected from

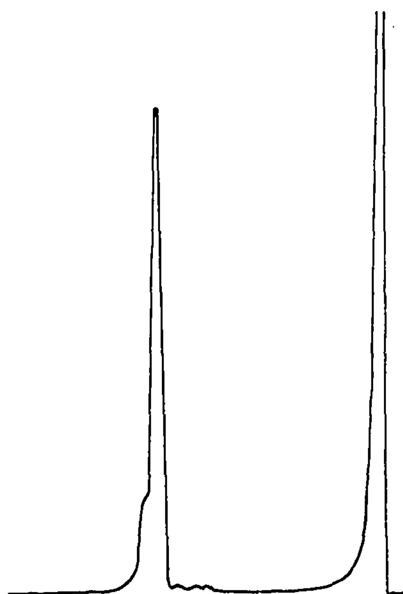

Fig. 1. Gas chromatography of PGE_1 and PGE_2 derivatives on OV-1 at 230°.

acid conditions is PGA[7], but PGA and PGB are not resolved in these solvent systems[1] and the product was not unequivocally identified.

Gas chromatography of the acetylated products on OV-1 gave a single major peak. However, the derivatives of PGE_1 and PGE_2 were not resolved on this column (Fig. 1). $PGF_{1\alpha}$ gave two peaks eluting well after the PGE derivatives under these conditions; accordingly, the F prostaglandins were determined as silylated derivatives.

Prostaglandins $F_{1\alpha}$, $F_{2\alpha}$ and $F_{2\beta}$ each gave a single peak on GLC of their

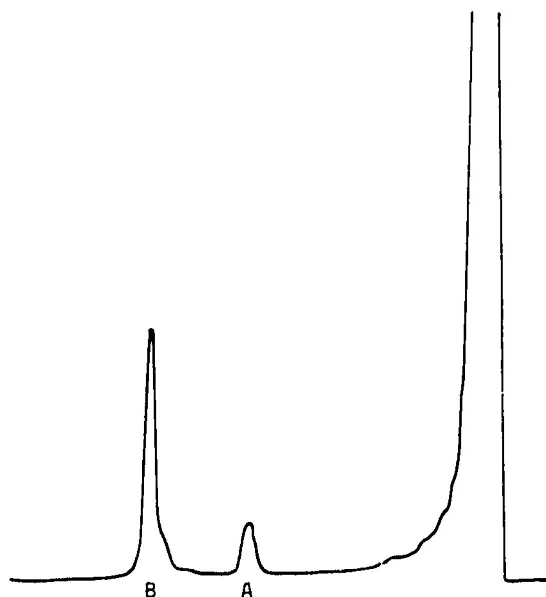

Fig. 2. Gas chromatography of silyl ether-silyl esters on OV-1 at 230°. Peak A = $PGF_{2\beta}$, B = $PGF_{1\alpha}$ and $PGF_{2\alpha}$.

silylation products. While the silyl ether-silyl ester of $PGF_{2\alpha}$ was separated from that of $PGF_{2\beta}$, no separation of silylated $PGF_{1\alpha}$ and $PGF_{2\alpha}$ was achieved (Fig. 2). Prostaglandins E_1 and E_2 did not give stable derivatives with the silylation reagent, and hence did not interfere with detection of F prostaglandins. It is not known whether the E prostaglandins failed to react under these conditions or the derivatives did not elute from the column.

Column fractionation

Complete separation of the three prostaglandin classes was routinely achieved through use of the volumes of eluting solvents indicated earlier. However, recovery of the E class in Fraction II free of contamination by dehydration products required that the ethyl acetate eluant be initially free of acetic acid. This was easily insured by passing it through a small column of F-20 Alumina (Applied Sciences) shortly before use. If this was done, the recoveries of PGE reported for SilicAR CC-4 by ANDERSEN[1] were readily achieved.

The recovery of $PGF_{1\alpha}$ was estimated by gas chromatography, after methylation and silylation of identical aliquots of a $PGF_{1\alpha}$ solution. Half of the aliquots were first chromatographed on SilicAR CC-4 as above, and half were not. On this basis the recovery of $PGF_{1\alpha}$ in Fraction III varied from 92–97%.

Prostaglandins of the B type

The acetylated methyl esters of PGB_1 and PGB_2 were apparently stable under the conditions used and gave resolvable, single peaks on CHDMS (Fig. 3). Acetylated methyl-PGB_1 was stable in dry methylene chloride for several weeks at 0°, and was used as a comparison standard for quantitation of the PGB obtained from Fractions I and II. No significant difference between the detector response to acetylated methyl esters of PGB_1 and PGB_2 was observed, and the limit of detectability for either was approximately 10 ng (for 1 sq. cm peak area).

Prostaglandins of the F type

Tetracosane was included as an internal standard as it was found difficult to store the trimethylsilyl ethers of PGF for long periods without hydrolysis. If this

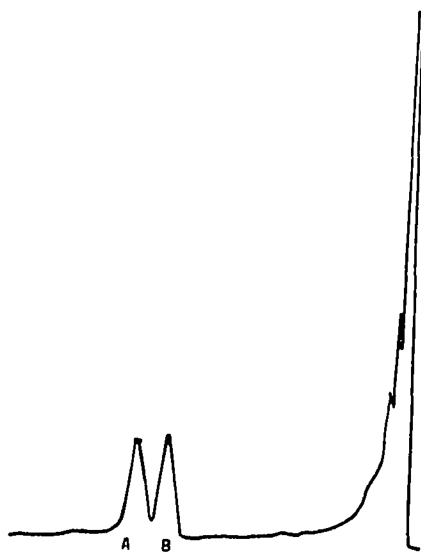

Fig. 3. Gas chromatography of acetylated methyl-PGB on CHDMS at 230°. Peak A = PGB_2; B = PGB_1.

were not the case, the silyl ether of methyl-$PGF_{1\beta}$ or of methyl-$PGF_{2\beta}$ might be used advantageously as internal standards, since they have not been found occurring naturally[8]. Under the conditions used in this study, the detector response per microgram of $PGF_{1\alpha}$ derivatized was found to be 0.75 ± 0.03 (S.D., three comparisons) times the response per microgram of *n*-tetracosane. The separation achieved is illustrated in Fig. 4.

DISCUSSION

A wide variety of procedures was tested in attempts to form a silylated derivative of PGE suitable for gas chromatography. Hexamethyldisilazane and trimethylchlorosilane in pyridine, bis(trimethylsilyl)acetamide with and without trimethylchlorosilane, trimethylsilylimidazole, bis(trimethylsilyl)trifluoroacetamide and Tri-Sil Z, alone and in various combinations, were applied to PGE_1 or methyl-PGE_1 but in every case the products either did not elute from OV-1 and CHDMS or gave multiple peaks. Attempts were also made to protect the keto group of methyl-PGE_1

by reacting it with methoxyamine in pyridine[13], but the methyl oxime decomposed on gas chromatography. The decomposition of the acetylated methyl ester of PGE_1 reported previously[2] was also observed in the present study; an example of this decomposition is shown in Fig. 5.

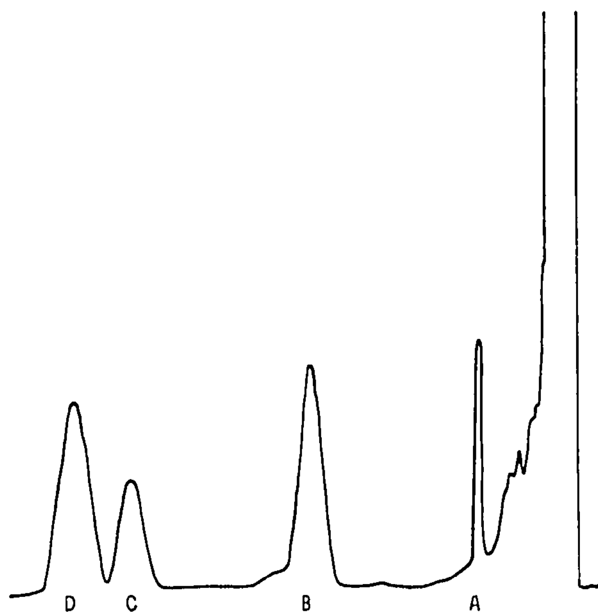

Fig. 4. Gas chromatography of silyl ether-methyl esters on CHDMS at 185°. Peak A = *n*-tetracosane internal standard; B = $PGF_{2\beta}$; C = $PGF_{2\alpha}$; D = $PGF_{1\alpha}$.

The conditions used for the screening procedure permit the detection of approximately 5–10 ng of either E or F prostaglandins. The preliminary steps leading to preparation of a prostaglandin concentrate require varying amounts of time depending on the nature of the starting material; thus, a tissue extract (*e.g.*, ref. 11) requires a more elaborate work-up than a sample of semen (*e.g.*, ref. 9). Starting from the concentrate, the complete derivatization and gas chromatography procedure for the keto prostaglandins requires about 90 min, while that for F prostaglandins in-

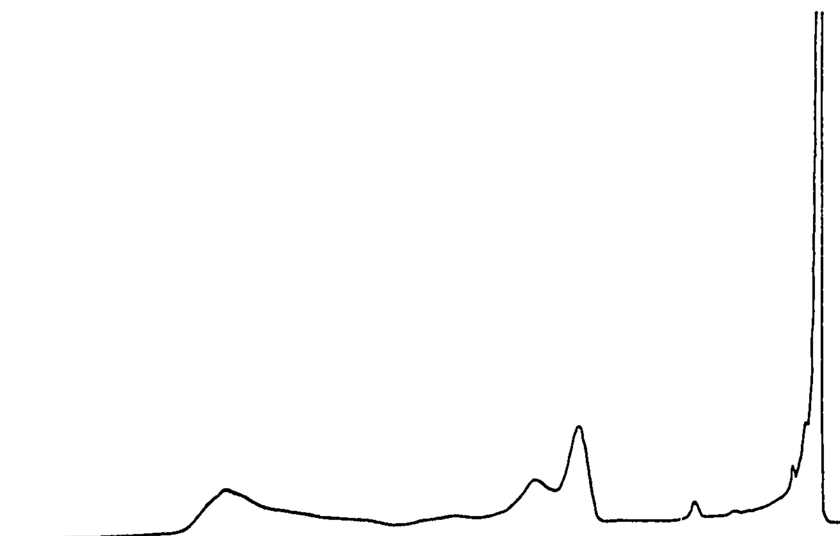

Fig. 5. Gas chromatography of methyl-PGE_1 acetate on CHDMS at 230°.

volves less than 1 h. The lack of selectivity toward unsaturation shown by OV-1 under these conditions is not a disadvantage for screening purposes, as it results in an increased sensitivity to a mixture of prostaglandins of the same class.

Fraction I from the silicic acid column may contain both A and B mono-keto mono-hydroxy prostaglandins[1]. Authentic samples of PGA were not available during the present study, so no data on their gas chromatographic properties are given. There is a tendency for PGE to dehydrate to PGA on handling, so some PGA is to be expected in Fraction I. This could be determined by dividing Fraction I into two portions, gas chromatographing the acetylated methyl esters made before and after treatment with NaOH. The amount of PGB present after NaOH treatment minus the amount present originally would thus be a measure of PGA.

Acetylation of methyl-PGB was accomplished under mild conditions with acetic anhydride–pyridine rather than with isopropenyl acetate and acid as in the screening procedure. Under certain circumstances the latter reagent will react with ketones[14] to give an enol-acetate, and it was considered advisable to avoid this potentiality in the quantitative studies.

The procedure used for analysis of F prostaglandins is a modification of that described by BYGDEMAN AND SAMUELSSON[9]. They used hexamethyldisilazane and trimethylchlorosilane in pyridine to form the trimethylsilyl ethers, evaporating the excess solvent and reagents prior to gas chromatography. This results in a precipitate of ammonium chloride, which occasionally may adsorb small amounts of the derivative. The silylating reagent used in the present work was chosen because it does not "tail" badly on gas chromatography and hence need not be removed from the reaction products; in addition, there is no adsorptive precipitate formed, and the same reagent can be used to form the silyl ether-silyl ester derivatives in the screening procedure. In regard to the latter, it was found that this silylating reagent was less sensitive to traces of moisture than were the others tested.

The trimethylsilyl ether-methyl esters of PGF were chromatographed on CHDMS at a liquid phase loading of 0.5% by the previous workers[9]. The loading was increased to 3% in the present work in order to increase column life. However, the use of a 0.2 cm diameter column here in place of the 0.5 cm used previously[9] gave approximately the same total amount of liquid phase in a 2 m length, permitting a column temperature of 185° in both studies.

The much greater retardation of the acetylated PGB than of trimethylsilylated PGF by CHDMS was not unexpected. This stationary phase has a very high affinity for keto groups (methyl heptadecyl ketone elutes after methyl stearate); moreover, trimethylsilyl groups tend to interfere with the interaction between the ester groups in the stationary phase and the oxygenated functions in the sample[15].

It was somewhat surprising that the more unsaturated prostaglandin F derivatives eluted from a polyester liquid phase ahead of their less unsaturated analogues. Under the conditions used, London dispersion interactions appear to outweigh dipole-induced dipole interactions. This phenomenon may be related to the temperature used, since resolution of PGB_1 from PGB_2 and of $PGF_{1\alpha}$ from $PGF_{2\alpha}$ was found to be diminished by lowering the temperature. A possibly related effect is seen with the methyl esters of ω-3 eicosatrienoic and ω-6 eicosatetraenoic acids on diethylene glycol succinate. At 220° the tetraene elutes after the triene, but at 170° the order is reversed[16].

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