terest and must absorb in the UV region in which the detector is sensitive. Although unlikely, if a problem is suspected, then minor changes in the solvent program should be investigated in an attempt to adequately resolve the peaks. Alternately, another chromatographic technique such as TLC (*3*) may be necessary for those samples which contain interfering materials which cannot be satisfactorily resolved.

In conclusion, the HSLC method presented in this paper offers improved precision, greater simplicity, general applicability, and a significantly lower limit of detection for the determination of isocyanates in the working atmosphere.

LITERATURE CITED

(1) Kalman Marcali, *Anal. Chem.*, **29**, 552 (1957).
(2) K. E. Grim and A. L. Linch, *Am. Ind. Hyg. Assoc. J.*, **25**, 285 (1964).
(3) Jurgen Keller, K. L. Dunlap, and R. L. Sandridge, *Anal. Chem.*, **46**, 1845 (1974).

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High Performance Liquid Chromatographic Determination of Prostaglandins $F_{2\alpha}$, E_2, and D_2 from In Vitro Enzyme Incubations

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The high performance liquid chromatographic separation of prostaglandins $F_{2\alpha}$, E_2, and D_2 as *p*-bromophenacyl esters on a microparticulate, bonded, reversed phase column is described. Detection and simultaneous quantitation of less than 3 μg of each prostaglandin is possible. The method has been applied to monitor prostaglandins produced from sheep seminal vesicle enzyme preparations and to evaluate antiinflammatory drugs on the basis of their inhibition of the synthetase enzyme. Advantages of this technique over other inhibitor screening techniques are discussed.

High performance liquid chromatography (HPLC) has only recently been applied to the problems of prostaglandin separation and analysis. Mikes et al. (*1*) compared HPLC and TLC separations of epimeric prostaglandins. Morozowich (*2*) resolved prostaglandins A_2 and B_2 on a triethylaminoethyl cellulose ion exchange column and studied the base catalyzed conversion of prostaglandin E_2 to B_2. Andersen and Leovey (*3*) separated closely related prostaglandins on a pellicular silica support and determined the degree of epimerization of prostaglandin $F_{2\alpha}$ using a refractive index detector. Except for a comparative study (*4*) of prostaglandin E_2 levels in rat kidney papillas by HPLC and bioassay, little work has been reported on the development of quantitative high performance liquid chromatographic assays for prostaglandins in biological specimens. Lack of sensitivity with a fixed wavelength detector has been the major obstacle in this area; however, recently reported work by Morozowich and Douglas (*5*) on the formation and separation of prostaglandins as *p*-nitrophenacyl esters has provided a solution to this problem.

HPLC appeared to offer several advantages for monitoring in vitro systems containing enzymes which actively synthesize prostaglandins from precursor fatty acids. Current methods of assessing prostaglandin synthetase activity include polarographic measurement of oxygen uptake (*6*), spectrophotometric measurement of prostaglandin B_2 (*7*), and radiometric thin layer chromatography (*8*). The primary drawbacks to the first two approaches are the lack of specificity and sensitivity, except in the most enzymatically active tissues. Radiometric thin layer chromatography is tedious, and accurate quantitation is difficult. In contrast to these methods, HPLC is rapid and can supply accurate quantitative results on several compounds in a single chromatographic run.

Described here are the reversed phase liquid chromatographic separation of several closely related prostaglandins as their *p*-bromophenacyl esters and the simultaneous analysis of micromolar levels of prostaglandins $F_{2\alpha}$, E_2, and D_2 formed from in vitro synthetase incubations.

EXPERIMENTAL

Apparatus. A Chromatec Model 5100 (Tracor Instruments, Austin, Texas) high performance liquid chromatograph with a pneumatic drive, positive displacement pump, and a single wavelength (254 nm) ultraviolet detector was used. Chromatographic columns were operated at ambient temperatures (25 ± 3 °C), and injections were made into a stopped-flow injection port with a Hamilton Model 701 10-μl syringe. Chromatograms were recorded on a Varian A-25 single-pen recorder, 0 to 1 mV full-scale.

Chromatographic Columns. Corasil C18 and Phenylcorasil (Waters Associates, Milford, Mass.) superficially porous, reversed phase column materials were packed into 1-m by 2-mm internal diameter (i.d.) stainless steel columns. A 2-m by 2-mm i.d. column of Permaphase ODS (Du Pont Instruments, Wilmington, Del.) was prepared by coupling two 1-m columns via a minimum dead volume fitting. A pre-packed, pre-tested, microparticulate, reversed phase column (μ Bondapak C18, Waters Associates) was used as received. Low dead volume fittings were easily constructed to adapt this column to the stopped-flow injection port of the chromatograph used.

Reagents. Distilled-in-glass acetonitrile, methanol, and methylene chloride (Burdick and Jackson, Muskegon, Mich.) were used as received. 2,4′-Dibromoacetophenone and *p*-nitrophenacylbromide (Eastman Kodak, Rochester, N.Y.) were used as received. Diisopropylethylamine (Aldrich) was redistilled (b.p. 127 °C) before use. All prostaglandins were supplied by the Experimental Chemistry Laboratories of The Upjohn Company. Arachidonic acid, 99% pure (NuChek Prep, Elysium, Ill.) was examined by TLC before use.

Procedure. *Derivatization.* Prostaglandin *p*-bromophenacyl and *p*-nitrophenacyl esters were prepared according to Morozowich and Douglas (*5*). Briefly, the prostaglandin (500 μg or greater) is dissolved in 1.0 ml of anhydrous acetonitrile containing a threefold molar excess of *p*-bromo- or *p*-nitrophenacylbromide. Two μl of diisopropylethylamine are added to catalyze the reaction, which is >95% complete in 1 h at 25 °C. For 1 to 100 μg of prostaglandin, the volume of acetonitrile should be reduced to 0.1–0.2 ml, and the amount of diisopropylethylamine should be reduced to 0.5 μl. A threefold molar excess of reagent must be maintained.

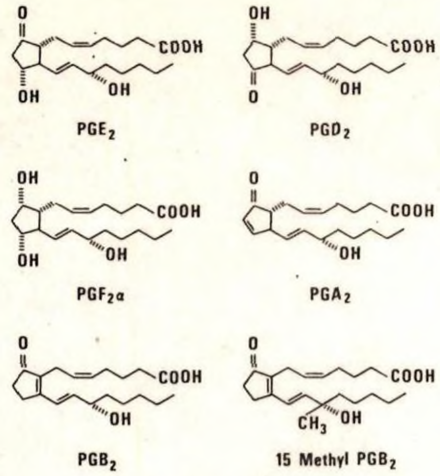

Figure 1. Structures of typical prostaglandins

For amounts of prostaglandin of 500 ng to 1 μg, the reaction time should be extended to 2.5–3.0 h. Heating to 45 °C accelerates the reaction, but the possibility of dehydration of prostaglandins E_2 or D_2 exists under anhydrous conditions at these temperatures. Tetrahydrofuran and dimethylformamide are suitable solvents in which to carry out the reaction if they are pure and anhydrous. For extracts from enzymatic incubations, an amount of derivatizing reagent corresponding to a threefold molar excess of fatty acid substrate should be added.

Enzyme Incubation. Sheep seminal vesicle synthetase enzyme was kindly supplied by D. Wallach, Experimental Biology Unit of The Upjohn Company. The preparation of this enzyme as a lyophilized acetone powder, and factors affecting the incubation have been described (*7*).

The lyophilized powder is homogenized in Tris buffer, pH 8.0, to give a concentration of 20 to 50 mg/ml depending on the enzyme activity. Then 0.5 μmol of hydroquinone are added to suppress oxidation of the arachidonic acid substrate. The enzymatic reaction is initiated by adding 400 μg of substrate to a 2-ml portion of homogenate which is being stirred vigorously at 35 °C. To quench the reaction, 2 ml of methanol are added, and the mixture is centrifuged to remove proteins. An internal standard can be conveniently contained in the methanol. The supernatant is then decanted into a separatory funnel containing 2 ml of 0.1 M citric acid and about 500 mg of sodium sulfate. The aqueous phase is extracted twice with 5 ml of methylene chloride. The pooled organic phase is dried over anhydrous sodium sulfate, transferred to a 12 × 75 mm disposable tube, and evaporated under nitrogen. The residue, concentrated in the bottom of the tube, is derivatized in 0.1 to 0.2 ml of acetonitrile, and the samples are chromatographed on a microparticulate, reversed phase column.

RESULTS AND DISCUSSION

Chromatography. Structures of prostaglandins A_2, B_2, D_2, E_2, $F_{2\alpha}$, and 15-methyl B_2 are shown in Figure 1. Despite elegant separations (*5*) of several prostaglandin *p*-nitrophenacyl esters on microparticulate silica gel columns, the development of reversed phase chromatographic separations became necessary for two principal reasons:

1) The separations on microparticulate silica gel were actually too refined for practical extension to quantitative analysis of prostaglandins in biological samples. For example, PGE_2 and its four geometric or epimeric isomers are resolved by adsorption chromatography. Quantitation of their total content in an enzymatic incubation might then have to take into account the contribution of five different peaks, with an increased probability of interferences from non-prostaglandin peaks in the extract. For simultaneous determination of $PGF_{2\alpha}$, PGE_2, and PGD_2, the problem would be compounded.

2) While adsorption columns are often used for quantitative analysis, good results depend on maintenance of a constant surface activity and precise duplication of mobile phase composition from day to day. Cumulative deposition of polar components ordinarily carried along in biological extracts rapidly depletes the number of active sites on the column, with corresponding adverse changes in elution time and peak shape. Column regeneration is often difficult. Reversed phase HPLC with a permanently bonded column is less prone to these problems and, if contamination occurs, column regeneration is easily accomplished by flushing with isopropanol or dimethylformamide.

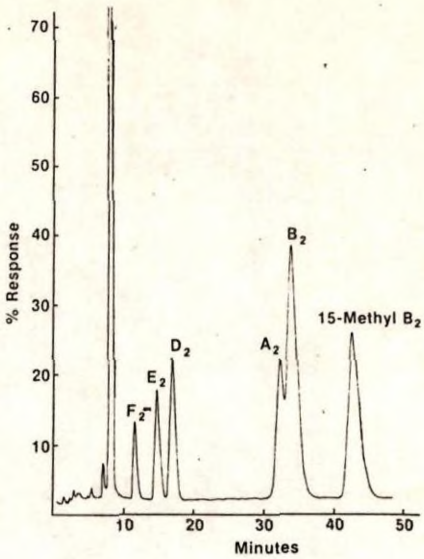

Figure 2. Separation of prostaglandins $F_{2\alpha}$, E_2, D_2, A_2, B_2, and 15-methyl B_2

Conditions: 50/50 acetonitrile/H_2O eluent, 1200 psig pressure, 1.2 ml/min flow rate, 25 cm × 4 mm i.d., μ Bondapak C18, UV 254 nm, 0.32 AUFS, 5–10 μg injected of each prostaglandin

Attempts to separate $PGF_{2\alpha}$, PGE_2, and PGD_2 as *p*-nitrophenacyl esters on superficially porous, bonded, reversed phase columns (Corasil C18, Phenylcorasil, and Permaphase ODS) were unsuccessful. Although retention volumes were different for the compounds injected separately, a mixture of all three could not be resolved. The column efficiencies of 200–240 plates/meter were typical for superficially porous columns; however, the results were inadequate. Because of an expected increase in efficiency, a totally porous, microparticulate, reversed phase column was examined. Figure 2 shows the separation of the prostaglandins of interest as their *p*-bromophenacyl esters. Separations of the *p*-nitrophenacyl esters were similar. *p*-Bromophenacyl esters were chosen for routine determination of levels less than 100 μg/ml, since subsequent experiments revealed an interfering reagent blank, coincident in retention time to $PGF_{2\alpha}$, when *p*-nitrophenacylbromide was

Table I. Derivatization Reproducibility

	Micrograms derivatized[a]	Peak height[b]	Rel std dev, %
PGE_2	4.43	17.0 ± 1.3	±7.6
	8.86	34.7 ± 1.1	±3.2
	13.29	53.4 ± 1.9	±3.6
	17.72	69.1 ± 2.4	±3.5
$PGF_{2\alpha}$	6.09	31.2 ± 4.9	±15.7
	12.18	59.1 ± 1.2	±2.0
	18.27	90.4 ± 4.3	±4.8
	24.36	114.0 ± 4.6	±4.0
PGD_2	3.35	13.9 ± 1.2	±8.6
	6.70	25.4 ± 1.0	±3.9
	10.05	36.5 ± 2.0	±5.5
	13.40	44.7 ± 0.6	±1.4

[a] Triplicate derivatizations. [b] Millimeters.

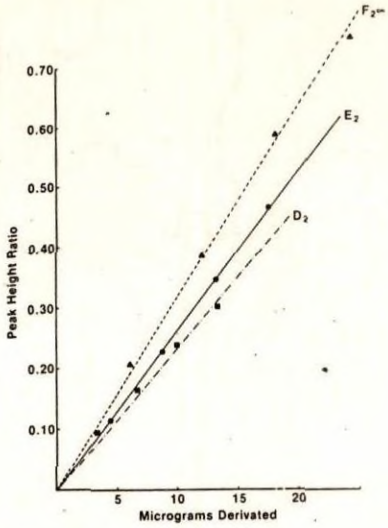

Figure 3. Calibration curve

(peak height prostaglandin $F_{2\alpha}$, E_2, or D_2)/(peak height prostaglandin 15-methyl B_2) vs. µg $F_{2\alpha}$, E_2 or D_2 derivatized

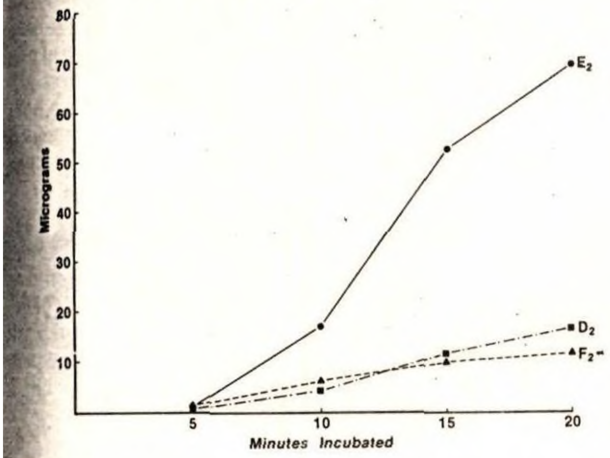

Figure 4. Levels of prostaglandin $F_{2\alpha}$, D_2, and E_2 vs. incubation time

Enzyme powder concentration 20 mg/ml, pH 8.0, Temperature 35 °C, GSH absent

used. Efforts to eliminate the blank by purification of reagents and substitution of different solvents were unsuccessful.

Calibration. Mixtures of prostaglandins $F_{2\alpha}$, E_2, and D_2 containing levels from 3 to 20 µg were derivatized in triplicate. Table I shows the results of these reaction reproducibility experiments, based on chromatographic peak height measurements. The reaction volume in each case was 100 µl, and 5 µl were injected onto the column. Fifty ng injected on-column from the derivatization of 500 ng produced a 5% response at the 0.04-absorbance unit full-scale setting of the detector. This represents the limit of quantitation. Although 5 ng should be detectable by extrapolation to the minimum detector sensitivity, in practical terms, this amount is impossible to quantitate because of noise and base-line drift.

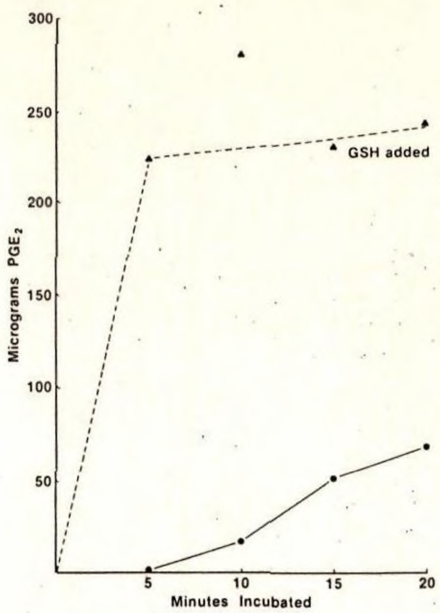

Figure 5. Influence of reduced glutathione (GSH) on PGE_2 production

Enzyme powder concentration 20 mg/ml, pH 8.0, Temperature 35 °C, GSH = 2 µmol/ml

An alternate approach to calibration which eliminates the need for quantitative sample handling during transfer steps is to use an internal standard. 15-Methyl PGB_2, a prostaglandin analogue, is ideal for this purpose. Not only is its chemistry similar to the compounds being determined, but it can be separated from PGA_2 and PGB_2, the principal degradation products due to the dehydration of PGE_2. Figure 3 shows calibration curves resulting from derivatization of each prostaglandin with 80 µg of internal standard. The ordinate in the figure represents the peak height ratio of each prostaglandin of interest relative to 15-methyl PGB_2. Quantitative precision was generally improved by the internal standard method, but each chromatographic run was approximately twice as long. Where throughput considerations are important, an external standard calibration might be favored. In both cases, the calibration curves were linear over the range tested, 1 to 80 µg.

Sample Analysis. Enzyme homogenates spiked with varying levels of prostaglandins were carried through the procedure to check recovery. Results are shown in Table II. Recoveries were quantitative for PGE_2 and PGD_2. Recovery of $PGF_{2\alpha}$ was near 90% because of its more hydrophilic nature but, since it was reproducible, the slight loss could be accounted for. Figure 4 shows the time course of action of the enzyme based on HPLC analysis. Figure 5 shows the marked increase in the % conversion of PGE_2 when 2 µmol/ml of reduced glutathione (GSH) was added to the incubation mixture. This result has been previously identified (7), and served to verify the utility of the assay.

One of the uses of synthetase enzyme preparations in

Table II. Recovery of Prostaglandins from Seminal Vesicle Enzyme Preparations[a]

$PGF_{2\alpha}$		PGE_2		PGD_2	
Added, µg	Recovery, %	Added, µg	Recovery, %	Added, µg	Recovery, %
121.8	86.0 ± 1.4	88.6	102.0 ± 0.7	67.0	94.8 ± 1.8
60.9	94.2 ± 3.3	44.3	100.0 ± 1.6	33.5	104.0 ± 2.8
24.0	86.4 ± 4.3	17.7	99.4 ± 3.2	13.4	96.1 ± 1.7

[a] Volume of homogenate spiked = 2.0 ml. Triplicate determinations.

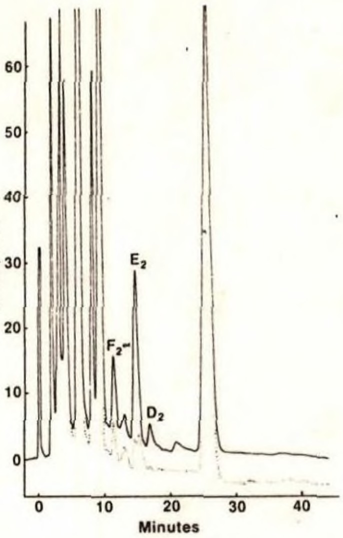

Figure 6. Influence of indomethacin on in vitro prostaglandin synthesis

(——) upper trace = incubation control, (. . .) lower trace = pre-incubated 3 min with 10^{-5} M indomethacin. Enzyme powder concentration 20 mg/ml, pH 8.0, Temperature 35 °C, GSH absent, 50/50 acetonitrile/H_2O eluent, 1200 psig pressure, 1.2 ml/min flow rate, 25 cm × 4 mm i.d. µ Bondapak C18, UV 254 nm, 0.08 AUFS

prostaglandin research is for identification of antiinflammatory drugs which act by suppressing prostaglandin synthesis. Incubations for this purpose are typically carried out in the presence of reduced glutathione and inhibitor, then the suppression of PGE_2 is measured. The glutathione is added to force the reaction to produce enough PGE_2 so that it can be conveniently measured spectrophotometrically after conversion to PGB_2. The physiological significance of glutathione in vivo has not been established. The HPLC method eliminates the necessity of tailoring the incubation conditions to compensate for an analytical deficiency. A typical chromatogram showing the influence of 10^{-5} M indomethacin, a known synthetase inhibitor incubated without GSH, is shown in Figure 6. Marked suppression of prostaglandin synthesis is evident.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) F. Mikes, V. Schurig, and E. Gil-Av, *J. Chromatogr.*, **83**, 91 (1973).
(2) W. Morozowich, *J. Pharm. Sci.*, **63**, 800 (1974).
(3) N. Andersen and E. M. K. Leovey, *Prostaglandins*, **6**, 361 (1974).
(4) E. W. Dunham and M. W. Anders, *Prostaglandins*, **4**, 85 (1973).
(5) W. Morozowich and S. Douglas, *Prostaglandins*, in press.
(6) W. L. Smith and W. E. Lands, *J. Biol. Chem.*, **246**, 6700 (1971).
(7) D. P. Wallach and E. G. Daniels, *Biochim. Biophys. Acta*, **231**, 445 (1971).
(8) F. T. Chasalow and B. B. Phariss, *Prostaglandins*, **1**, 107 (1972).

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Aqueous Phase High Speed Liquid Chromatographic Separation and Atomic Absorption Detection of Amino Carboxylic Acid–Copper Chelates

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The high speed liquid chromatographic separation of the copper chelates of EDTA, NTA, EGTA, and CDTA has been demonstrated using a weak anion exchange resin column and an aqueous 0.05 M $(NH_4)_2SO_4$ mobile phase. The chelates were selectively detected by an atomic absorption spectrophotometer interfaced to the high speed liquid chromatograph and set to measure copper concentration. The chelates are retained by the column as a function of charge and elute in the order $Cu_2(EGTA)$, $Cu(NTA)^-$, $Cu(EDTA)^{2-}$, $Cu(CDTA)^{2-}$. The method is applicable to the analysis of individual chelating agents such as those used in industrial water treatment, food processing, metal cleaning, and pharmaceuticals.

Naturally occurring and pollutant chelating agents may be important components of natural waters and wastewaters (*1*). They are of particular concern because of their potential role in the solubilization and transport of heavy metals (*1–3*). In addition to transport of heavy metals in natural waters and through sewage and water treatment processes, chelating agents also affect the availability of toxic and nutrient metals to aquatic biota (*4*). Synthetic chelating agents are widely used for industrial water treatment, metal cleaning, food preparation and preservation, and as components of pharmaceuticals. In nature, chelating agents, particularly fulvic acids, are produced as exudates from aquatic plants and by decaying vegetation. Lower rank coals may yield similar chelating compounds when exposed to alkaline waters or in the water used in slurry pipelines. Because of the widespread occurrence of chelating agents and because of their effects in water systems and on biota, it is important for environmental scientists, pollution control personnel, and biological scientists to have sensitive and selective analytical methods available for chelating agents.

Some methods for the analysis of chelating species have been published. Spectrophotometry has been employed (*5–9*) including the standard EPA procedure for the analysis of NTA (*9*). Electrochemical procedures have been developed (*10–15*), most of them designed for the determination of NTA. Several kinetic and catalytic analyses have been described (*16–19*). Ion-selective electrodes have been used in two procedures for the analysis of chelating detergent builders (*20, 21*). Various chromatographic methods