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Note

Reversed-phase high-performance liquid chromatography of prostaglandins — biological applications

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The isolation and purification of prostaglandins is increasingly important to studies of their physiologic or pharmacologic effects as the number of known compounds and variety of their actions increase. In the more commonly used thin-layer systems, resolution and recovery of prostaglandins is seldom optimal. The application of high-performance liquid chromatography (HPLC) to the isolation and purification of a large number of related prostaglandins in high yields has been shown to be uniquely suited to problems in this field of research from the view point of high resolution, short retention times, and good sample recovery [1, 2].

We recently reported HPLC methods using silicic acid columns for isolation of prostaglandins of purity suitable for gas chromatographic—mass spectrometric (GC—MS) analysis [1]. However, these methods are not suitable for analysis of a wide spectrum of prostaglandins since we have found that compounds such as PGE_1, PGE_2, 6-keto $PGF_{1\alpha}$, and thromboxane B_2 co-chromatograph in the solvent system reported.

Conventional reversed-phase chromatography has previously been applied to prostaglandin analysis [3], but adequate resolution has been hard to achieve and retention times are excessively long. Reversed-phase HPLC, however, offers both excellent resolution and short retention times. We have therefore extended the usefulness of HPLC methods by using reversed-phase chromatography in combination with adsorption chromatography for the isolation of several prostaglandins from the same biological matrix. These techniques have allowed us to investigate more thoroughly the products of arachidonic acid metabolism in various tissues and to analyze a wider range of prostaglandins in a single sample.

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MATERIALS AND METHODS

All solvents were glass distilled reagents from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). HPLC was done using two Waters Assoc. (Milford, Mass., U.S.A.) solvent delivery systems (Model 6000A) coupled to a solvent flow programmer (Model 660). The reversed-phase column and silicic acid columns were pre-packed μBondapak Fatty Acid Analysis and μPorasil columns respectively manufactured by Waters Assoc. (both column materials were 10-μm particles). The packing material for the Fatty Acid Analysis column is proprietory information (Waters Assoc.).

Unlabeled prostaglandins were obtained from Upjohn (Kalamazoo, Mich., U.S.A.). Labeled prostaglandins other than [^{3}H]6-keto-PGF$_{1\alpha}$, [^{3}H]PGD$_2$ and [^{3}H]thromboxane B$_2$ (TxB$_2$) were obtained from Amersham Searle (Arlington Heights, Ill., U.S.A.). Tritiated 6-keto-PGF$_{1\alpha}$, PGD$_2$ and TxB$_2$ were synthesized in our laboratory from tritiated arachidonic acid obtained from Amersham Searle [4]. [1-^{14}C]Arachidonic acid (55 mCi/mmole) was obtained from New England Nuclear (Boston, Mass., U.S.A.).

Liquid scintillation spectrometry was done using a Mark III instrument from Searle Analytic (Des Plaines, Ill., U.S.A.). GC—MS analysis was done on a Hewlett-Packard dodecapole Model 5980A mass spectrometer as previously described [5, 6] using glass columns (3 ft. × 2 mm I.D.) packed with 3% OV-1 (Supelco, Bellefonte, Pa., U.S.A.) operated isothermally at 250°. Prostaglandins were derivatized as previously described [6].

The PGE$_2$ methyl ester methoxime trimethylsilyl ether and PGF$_{2\alpha}$ methyl ester trimethylsilyl ether were quantitated by selected ion monitoring (SIM), using tetradeutero internal standards (Upjohn), at *m/e* 508 vs. 512 or *m/e* 494 vs. 498 respectively. PGE$_1$ was quantitated against hexadeutero-PGE$_1$ (Upjohn) as previously reported [7]. For analysis of 6-keto-PGF$_{1\alpha}$, the methylester methoxime trimethylsilyl ether was prepared and measured by SIM at *m/e* 598 vs. 605 against heptadeutero-6-keto-PGF$_{1\alpha}$ synthesized from 5,6,8,9,11,12,14,15-octadeuteroarachidonic acid [4].

Prostaglandin synthesis was studied in renal papillary slices prepared from normal male rats. The tissue was incubated with [^{14}C]-20:4 (Na^+ salt, 1 μCi, 5.5 μg) in 2 ml of Krebs—Ringer bicarbonate buffer (pH 7.4) containing 2 g/l of glucose under an atmosphere of oxygen—carbon dioxide (95:5). After 30 min the incubation mixture plus tissue was acidified and homogenized in 20 ml of chloroform—methanol (1:2). The mixture was filtered, evaporated to dryness (room temperature), redissolved in chloroform and purified for GC—MS by HPLC using both adsorption and reversed-phase chromatography.

For analysis of urinary prostaglandins, normal female Sprague Dawley rats were placed in metabolic cages. The urine was collected in vessels maintained at 0° and immediately a known amount of internal standards for SIM analysis was added. Prostaglandins were extracted from the acidified urine with chloroform, purified by HPLC (adsorption and reversed-phase), derivatized and quantitated by SIM.

RESULTS AND DISCUSSION

The silicic acid (Fig. 1) and reversed-phase (Fig. 2) columns were standardized using a mixture of tritiated prostaglandin standards (1—2 μg of each plus 0.05—0.1 μCi of the tritiated compound). The separation achieved is primarily due to differences in polarity of the prostaglandins; however, other effects are important on the silicic acid column since prostaglandins of dissimilar polarity co-elute in the organic solvents used. Fractions containing unresolved prostaglandins from the silicic acid column were subsequently run on the reversed-phase column which allowed the complete separation and isolation of a wide range of prostaglandins. Through both columns the recovery is normally 70–80% of the initial sample. Table I gives a summary of the average retention volumes of prostaglandins on each column.

These chromatographic techniques were employed in experiments designed to study prostaglandin synthesis in rat renal papillary slices. In Fig. 3A, the chromatographic pattern of ^{14}C-labeled compounds synthesized from [^{14}C]-20:4 is shown. In these experiments approximately 5% of the labeled arachidonic acid was converted to prostaglandins by 50 mg of papillary slices. Again

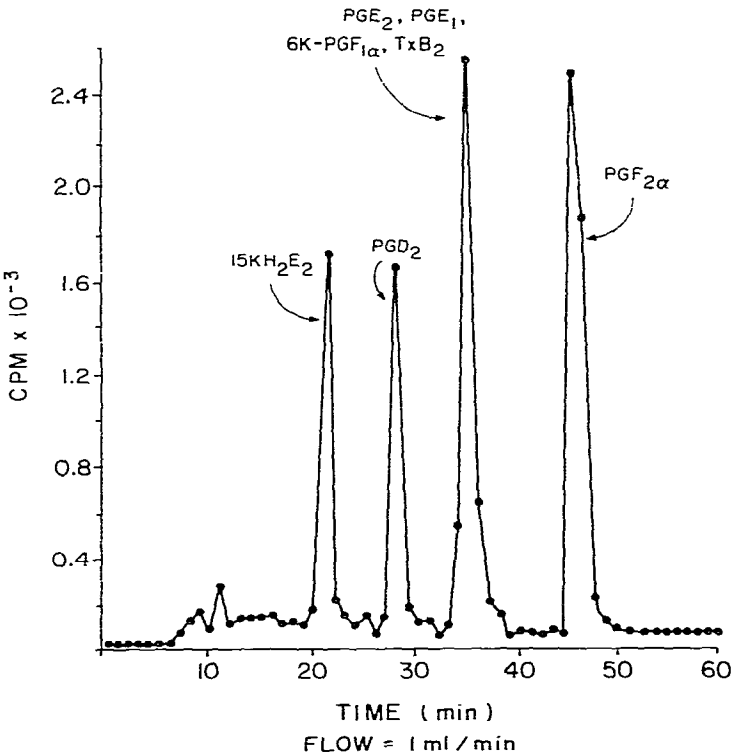

Fig. 1. Chromatogram produced by HPLC of prostaglandins (1—2 μg or 0.05—0.1 μCi of tritiated compound) as free acids on a silicic acid column (μPorasil). The sample was dissolved in 0.25 ml of chloroform, applied to the column and eluted by a 60-min linear gradient from chloroform to 6% methanol and 0.6% acetic acid in chloroform. The flow-rate was 1 ml/min and 1-min fractions were collected and assayed for radioactivity. In other experiments, the identity of eacn compound was confirmed by GC—MS.

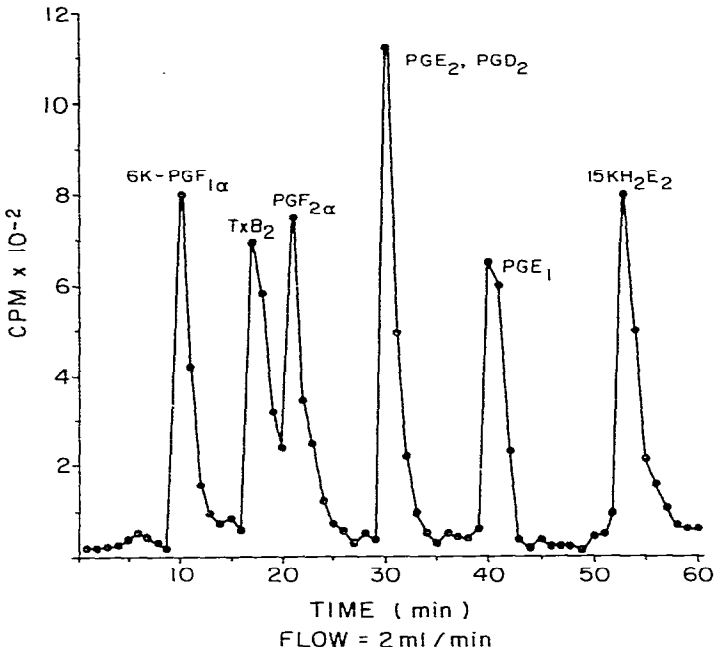

Fig. 2. Chromatogram produced by HPLC of prostaglandins (1—2 μg of 0.05—0.1 μCi of tritiated compound) as free acids on a reversed-phase column (μBondapak Fatty Acid Analysis). The sample was dissolved in 0.5 ml of the column solvent and eluted isocratically using a mixture composed of water—acetonitrile—benzene—acetic acid (76.7:23:0.2:0.1). The flow-rate was 2 ml/min and 1-min fractions were collected and assayed for radioactivity. In other experiments, the identity of each compound was confirmed by GC—MS.

TABLE I

AVERAGE RETENTION VOLUME OF PROSTAGLANDINS ON HPLC

Prostaglandins were chromatographed on silicic acid (μPorasil) columns as free acids or methyl esters using a linear gradient of chloroform to 6% methanol and 0.6% acetic acid in chloroform in 60 min or a linear gradient of chloroform to 5% methanol in chloroform in 50 min respectively, with a flow-rate of 1 ml/min in both cases. Reversed-phase (Fatty Acid Analysis column) chromatography of prostaglandins as the free acid was done isocratically using the solvent system acetonitrile—benzene—acetic acid—water (23:0.2:0.6:76.7) and the methyl esters were run isocratically using acetonitrile—benzene—water (28:0.2:71.8). Flow-rate for reversed-phase chromatography was 2 ml/min. Data represent peak center mean ± range of 3 runs. The peak center for compounds chromatographed on silicic acid varied less than ± 1 fraction while the center varied less than ± 2 fractions on the reversed-phase column.

Prostaglandin	Average retention volume (ml)			
	Silicic acid		Reversed-phase	
	Free acid	Methyl ester	Free acid	Methyl ester
15-keto-H_2E_2	22	19	106	106
PGD_2	28	29	60	60
PGE_2	35	38	60	60
PGE_1	35	38	82	82
TxB_2	35	38	36	36
6-keto-$PGF_{1\alpha}$	35	38	20	20
$PGF_{2\alpha}$	45	49	42	42

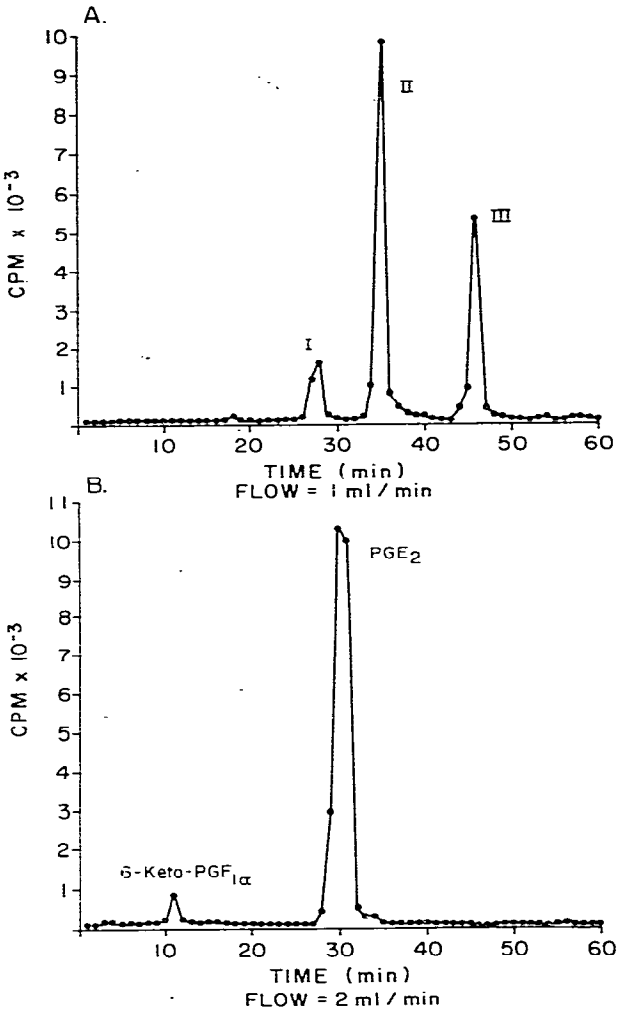

Fig. 3. (A) Silicic acid HPLC of labeled prostaglandins isolated from incubations of [^{14}C]-20:4 with rat renal papillary slices. Incubations were as described in the text. Labeled prostaglandins were isolated and chromatographed as in Fig. 1. The fractions (34—36) containing prostaglandin E_2 were collected and re-chromatographed as in Fig. 3B. (B) Reversed-phase HPLC of the peak containing prostaglandin E_2 from the silicic acid column (Fig. 3A). Fractions 29—32 contained prostaglandin E_2 and fractions 9—11 contained 6-ketoprostaglandin $F_{1\alpha}$. Conditions were as in Fig. 2.

the fractions containing PGE_2 were further analyzed for 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 on the reversed-phase column (Fig. 3B). Under these experimental conditions 54 ± 4% of the prostaglandins produced was PGE_2, 30 ± 3% was $PGF_{2\alpha}$, 13 ± 1% was PGD_2, and 2 ± 1% was 6-keto-$PGF_{1\alpha}$ (mean ± S.D., n = 4). No thromboxane B_2 was detected in these experiments. The combination of adsorption and reversed-phase chromatography using these methods allows the complete resolution of all the prostaglandins studied.

Analysis of biological samples requiring isolation and derivatization of prostaglandins is improved with the combination of these two chromatographic

columns, particularly for the analysis of PGE_2 and $PGF_{2\alpha}$. Fig. 4A shows a SIM tracing at *m/e* 508 vs. 512 for PGE_2 isolated from normal rat urine by silicic acid alone. As can be seen, several other peaks are present in addition to those due to urinary PGE_2. Another aliquot of the same sample was run over both silicic acid and reversed-phase columns. Only the major and minor isomers of the PGE_2 derivative are seen in the SIM tracing of the latter sample (Fig. 4B). demonstrating the improved selectivity of these methods as judged by GC—MS. With these techniques, normal female rat urines were analyzed for PGE_2, $PGF_{2\alpha}$, PGE_1 and 6-keto-$PGF_{1\alpha}$. Using the silicic acid column, the

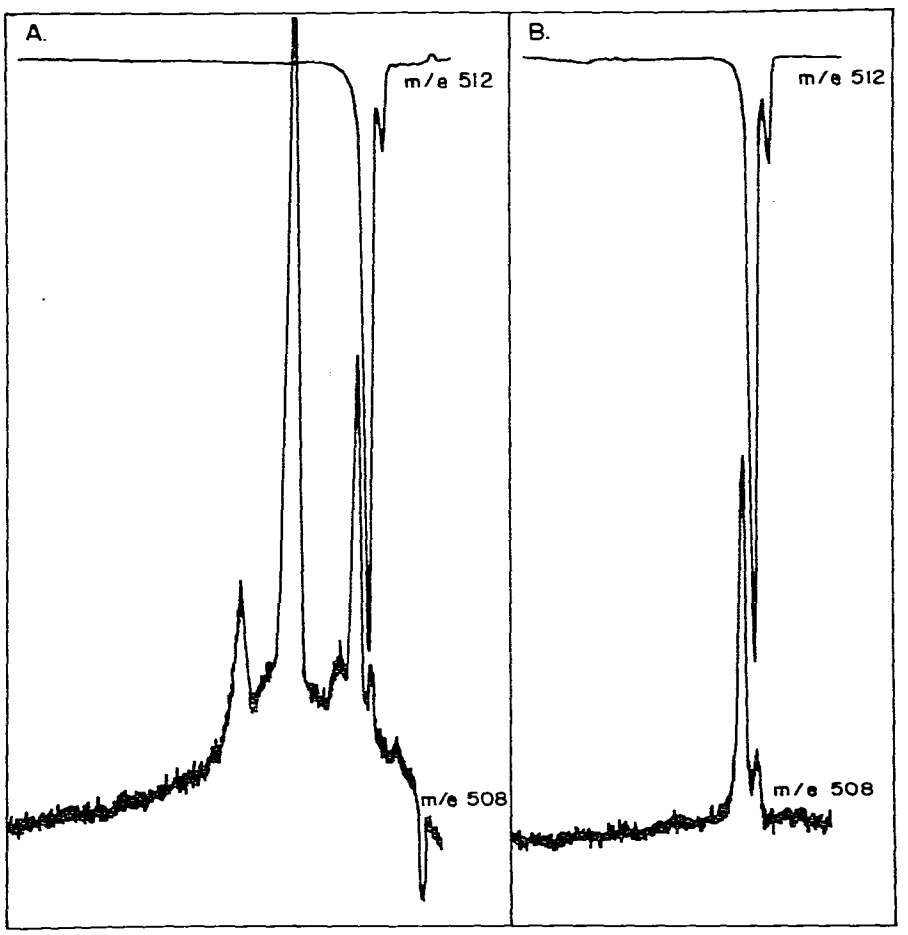

Fig. 4. SIM tracings of PGE_2 methyl ester methoxime trimethylsilyl ether from 6 ml of rat urine. Upper tracings represent the intensities of the ion *m/e* 512 (M^+ —31 for the tetradeutero-PGE_2 derivative) and the lower tracings represent the intensities of the ion at *m/e* 508 (M^+ —31 for the endogenous PGE_2 derivative). Tracings run from right to left with the *m/e* 508 channel being 20-fold more sensitive than the 512 channel. The intensity of the peak *m/e* 512 corresponds to 1.86 μg of deuterated PGE_2; thus, the 6-ml urine sample contained 53 ng of PGE_2. (A) SIM tracing of the urine sample chromatographed on a silicic acid column as in Fig. 1. (B) SIM tracing of another aliquot of the identical sample chromatographed on a silicic acid column (Fig. 1) followed by re-chromatography on a reversed-phase column (Fig. 2).

urine extract (tritiated and deuterated internal standards added) gave two peaks of radioactivity. One contained endogenous $PGF_{2\alpha}$ plus the labeled internal standard, while the other peak contained endogenous PGE_2, PGE_1 and 6-keto-$PGF_{1\alpha}$ plus their corresponding labeled internal standards. Both peaks were collected and re-chromatographed on the reversed-phase column from which individual prostaglandins were collected, derivatized and quantitated by SIM. Measurable levels of each of these prostaglandins were found in this group of rats and are given in Table II. The major prostaglandins in rat urine were PGE_2 and $PGF_{2\alpha}$. Smaller amounts of PGE_1 and 6-keto-$PGF_{1\alpha}$ were also measured.

TABLE II

URINARY PROSTAGLANDINS FROM NORMAL FEMALE RATS

Urine was collected at 0° from normal female rats. Deuterated and tritiated prostaglandin standards were added and the sample was extracted, purified by HPLC and quantitated by SIM. Data represent mean ± S.E.M.

Prostaglandin	*n*	ng/24 h
PGE_2	9	130 ± 15
$PGF_{2\alpha}$	6	71.9 ± 3.5
6-keto-$PGF_{1\alpha}$	6	15.0 ± 2.6
PGE_1	8	7.5 ± 2.7

The importance of these combined techniques to the analysis of a variety of prostaglandins from biological samples can be readily seen. Processing samples through both columns results in the complete resolution of all prostaglandins studied. Methylation of the prostaglandins results in a greater difference in polarity between the prostaglandin of interest and interfering compounds. Isolation of prostaglandins based on the differing characteristics of these two chromatographic materials results in a relatively pure sample, free from the overwhelming quantity of contaminants frequently found after adsorption chromatography. Thus, these methods are particularly applicable to quantitative analysis by GC—MS and should improve quantitation by radioimmunoassay techniques by removing compounds which may interfere.

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