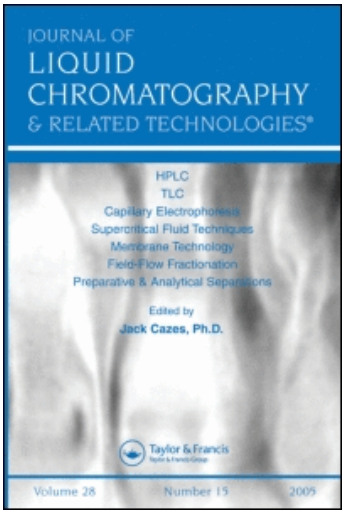

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Simultaneous Preparation and Extraction of Prostaglandins from Gastric Tissue with Separation and Quantification by High Pressure Liquid Chromatography

C. D. Palm[a]; B. A. Levine[a]
[a] Department of Surgery, University of Texas Health Science Center and Audie L. Murphy Memorial Veterans' Hospital, San Antonio, Texas

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SIMULTANEOUS PREPARATION AND EXTRACTION OF PROSTAGLANDINS FROM GASTRIC TISSUE WITH SEPARATION AND QUANTIFICATION BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

C. D. Palm and B. A. Levine
Department of Surgery
University of Texas Health Science Center
and Audie L. Murphy Memorial Veterans' Hospital
San Antonio, Texas 78284

ABSTRACT

A rapid and practical method utilizing High Pressure Liquid Chromatography (HPLC) for analyzing prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGE_1 extracted from gastric mucosa is described. Separation of prostaglandins using a reverse phase C_{18} column and ultraviolet (UV) spectrophotometry allows detection and simultaneous quantitation of as little as one nanogram. Sensitivity and reproducibility make this method particularly suitable for prostaglandin determination in gastric mucosa.

INTRODUCTION

The prostaglandins (PGs) constitute a group of closely related derivatives of prostanoic acid. Their potent biological properties have resulted in numerous efforts directed at the delineation of their physiological function. The ultimate

success of such efforts often depends on the application of a specific and sensitive detection method.

Various methods for detecting PGs in biologic fluids have been reported: bio-assay (1), radioimmunoassay (RIA) (1-6) and gas chromatography-mass spectrophotometry (GC/MS) (5) are the most frequently used.

Recently, HPLC was introduced to separate PGs (1,3,8,9) which were then quantified by RIA (2,3,5,6), or GC/MS (7,10). Fluorescence and UV spectophotometry have both been used to detect derivatives of prostaglandins (p-nitrobenzuloxine and p-bromophenacyl esters) which contain chemical groups that strongly absorb UV light (8,11,12,13).

Terragno et al. (14) reported the use of HPLC for the separation and quantitation of PGs without derivatization. Eleven standard PGs and metabolites were evaluated and a sensitivity of as little as 30 nanograms was obtained. However, greater sensitivity is needed if quantitation in biologic tissue is to be routinely carried out in physiologically meaningful experiments. We therefore endeavored to perfect HPLC methodology that would separate and quantitate 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGE_1 in gastric mucosa without derivatization at levels as low as 1 nanogram.

MATERIALS AND METHODS FOR SAMPLE PREPARATION

Hexane, 2-propanol, chloroform, methanol, formic acid, scinti-verse, and sodium phosphate were purchased from Fischer

Scientific (Fair Lawn, N.J.). All chemicals used were HPLC grade. SEP-PAK sample preparation cartridges were purchased from Waters Chromatography Division (Milford, Ma.). Hamilton glass syringes and 4mm filters were purchased from Millipore Corp. (Milford, Ma.). Vap-Ports were purchased from American Scientific Products (Houston, TX). Arachidonic Acid was purchased from New England Nuclear (Boston, Ma.). The rats were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.). The incubator used was National Appliance Model 3518 (Portland, Ore.). The freezer used in the storage of specimens at -100°C was Queue Systems Model 7140 (Parkersburg, W. Vir.). Cell disruption was accomplished with a Branson Sonic Power Sonifier Model 350 (Danbury, Conn.). Scinillation counter used was Beckman Model LS-7000 (Irving, Ca.).

Eight Sprague-Dawley rats (225-250 grams) were used for this study. The rats were fed a standard diet of commercial rat chow and were housed two rats per cage with wide wire bottoms to prevent coprophagia. The animals were deprived of food but not water for 48 hours prior to begin each experiment. Following the fast, the rats were randomly divided into two equal groups, and sacrificed on consecutive days. All rats were subjected to the same conditions.

Immediately after euthanasia, the stomach of each rat was removed and opened along the greater curvature according to the method of Basso et al (5). The lumen was rinsed with 0.9% saline at 4°C. Two samples of approximately 100mg of glandular mucosa

was dissected from the corpus of each rat with the use of microscissors under a surgical microscope. Each tissue sample was placed in a test tube filled with 2ml of saline. Within 10 minutes of tissue biopsy each sample was placed in a freezer at -100°C until ready for assay.

In preparation for incubation, the 16 tissue samples were allowed to thaw at room temperature, rinsed in ice cold saline, weighed, and placed in 2ml of 10 mM phosphate-buffered saline (pH 7.4). Eight of the samples were radiolabeled with a 10µl solution of 5 X 10^3 dpm [^{14}C]arachidonic acid for determination of extract recovery. All of the samples were then incubated for four hours at 37°C. These conditions were appropriate to permit prostaglandin synthesis (5).

At the end of the incubation period each tissue sample was removed from the media and placed in 1ml of 0.1M formic acid in order to halt prostaglandin synthesis (10). The incubation media, which did not undergo extraction, was filtered to remove particulate matter using a 1ml Hamilton glass syringe and a 4mm CTFE filter (.22µm pore size). It was then placed into a 2.0ml Wheaton style vial and kept at -20°C until HPLC determination were carried out.

Each tissue sample was homogenized for one minute using a cell disruptor. Eighteen microliters of Hexane:2-propanol (3:2) per mg of wet tissue weight (15) was added and homogenization was repeated for one minute. The portion of the cell disruptor horn that came in contact with the homogenate was rinsed with 2ml of

hexane:2-propanol (3:2). This rinse was kept in a test tube for future processing. The homogenate was then centrifuged for 30 minutes at 5000g. The upper layer was aspirated, and the interface was washed three times with 1ml of hexane:2-propanol (3:2). These washings along with the upper layer were also saved for future processing. The lower phase was taken to dryness under nitrogen and redissolved with 2 ml of chloroform:methanol (1:1).

The extracts were preparatively separated using SEP-PAK sample preparation cartridges. The entire extract volume including the previously saved rinses and aspirated upper layer, was injected into the cartridge and eluted with 10ml of each of the following; water (neutral lipids, fatty acids), 10% acetonitrile (glycolipids and phospholipids) and 20% acetonitrile (prostaglandins) at a flow rate of 400µl/min. The acetonitrile eluent was saved and evaporated under nitrogen using Vap-ports. Care was taken to ensure that all prostaglandin residue was rinsed from the walls with chloroform:methanol (1:1) and concentrated at the base of the vial. After the final evaporation the residue was resuspended in 100µl of methanol. Using a 1ml Hamilton glass syringe this suspension was filtered through a 4mm filter. The filtrate was placed into a 1.5ml Wheaton style sample vial containing a 100µl insert and kept at -20°C until High Pressure Liquid Chromatography. The filtrate of each sample that was radiolabeled with [^{14}C]arachidonic acid was then suspended in 2ml scinillation cocktail and subjected to

TABLE 1

Percent Recovery of [^{14}C]Arachidonic Acid

SAMPLE NO.	TISSUE EXTRACT	UNEXTRACTED MEDIA	TOTAL
1	27.24%	70.06%	97.30%
2	23.60%	74.62%	98.22%
3	28.30%	69.31%	97.61%
4	30.65%	68.26%	98.91%
5	20.87%	78.54%	99.41%
6	26.96%	70.56%	97.52%
7	25.69%	73.90%	99.59%
8	24.44%	73.75%	98.19%

Eight samples of mucosal tissue (100mg each) were radiolabeled with 5 x 10^3 dpm [^{14}C]arachidonic acid, a precursor of prostaglandins. This was added to the samples before incubation to trace extract recovery. The tissue was then processed in exactly the same manner as the tissue that was to be analyzed by HPLC. The counting efficiency for the eight samples was 98.34% ± 0.30% (n=8). (Efficiency = net cpm /dpm of sample) (Percent recovery = efficiency x 100)).

scinillation counting for ten minutes. The radiolabeled unextracted media was also counted in the same manner. The counting efficiency for the eight samples and their respective incubation media was 98.34% ± 0.30% (n=8) (Table 1).

MATERIALS AND METHODS FOR HPLC

A Rainin gradient solvent delivery system (Rainin Instrument Co. Woburn, Ma.) controlled by a Standard Brand 286 AT II (IBM-AT compatible) computer (Compu-Add Corp. Austin, Tx.), using Gilson 714 system controller software, interfaced to the HPLC system via a Gilson 621 Data Manager (Gilson Medical Electronics Middleton, Wis.). Sample injection was accomplished with a Kortec K65B HPLC Automated Injector (Kortec of Australia) with a variable loop

(1-100µl). To achieve separation, a Microsorb C_{18} 4.6mm X 15 cm reverse phase column (Rainin Instrument Co.) was used with 5 micron packing. Detection was made possible using a Knauer 87 variable wavelength UV-Vis detector (Dr. Herbert Knauer, Berlin, West Germany) adjusted to a wavelength of 193nm and 0.0025 AUFS. The mobile phase consisted of reagent grade orthophosphoric acid, HPLC grade water and HPLC grade acetonitrile (Burdick and Jackson Muskegon, Mi.). Prostaglandin standards 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 were purchased from Sigma Chemical Co. (St.Louis, Mo.).

QUANTITATIVE ANALYSIS

Analysis was carried out with a mobile phase of 0.017M orthophosphoric acid:acetonitrile (67.2:32.8) (14) at a flowrate of 2.0ml per minute. Standards of prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGE_1 were titrated into concentrations ranging from 20pg/µl to 2ng/µl. Fifty microliter samples of these standards were injected onto the column using the automated injector. An eleven point calibration curve for each prostaglandin was developed using peak area and peak height. The range of the calibration curve was from 1ng to 100ng (Figures 1 and 2). Detector output with a range of -10.00mV to 1200.00mV was read by the computer by way of the 621 Data Manager. Detector sensitivity was adjusted to .0025 AUFS.

A typical chromatogram of prostaglandins from gastric mucosa is shown in Figure 3. A good separation of 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 was obtained. Retention times of these compounds were

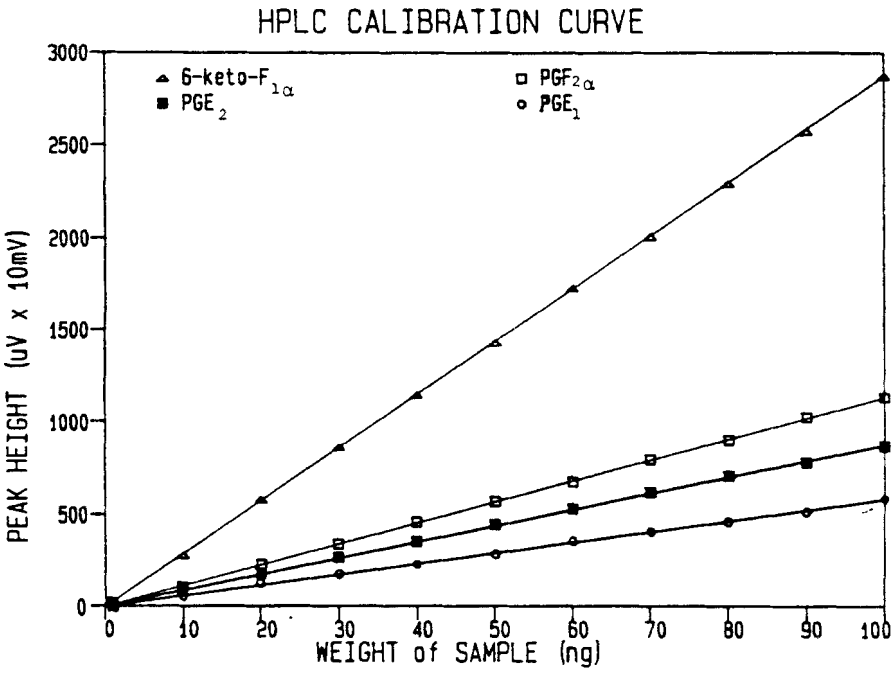

FIG. 1 Peak height vs. weight of each prostaglandin calibration graph. Microsorb C_{18} column 15 cm x 4.6 mm I.D.; mobile phase, .017M H_3PO_4: CH_3CN (67.2:32.8, v/v); flowrate 2.0 ml/min; UV detection at 193 nm/.0025 AUFS.

2.33, 5.04, 5.86, and 6.64 minutes repectively. PG peaks were identified on the basis of their absolute and relative retention times by analyzing known amounts of PG standards.

RECOVERY

Determination of the percent prostaglandin recovery from the column was obtained by the following procedure;

A known amount of a prostaglandin was injected onto the column bed. The resulting peak was collected as a fraction and

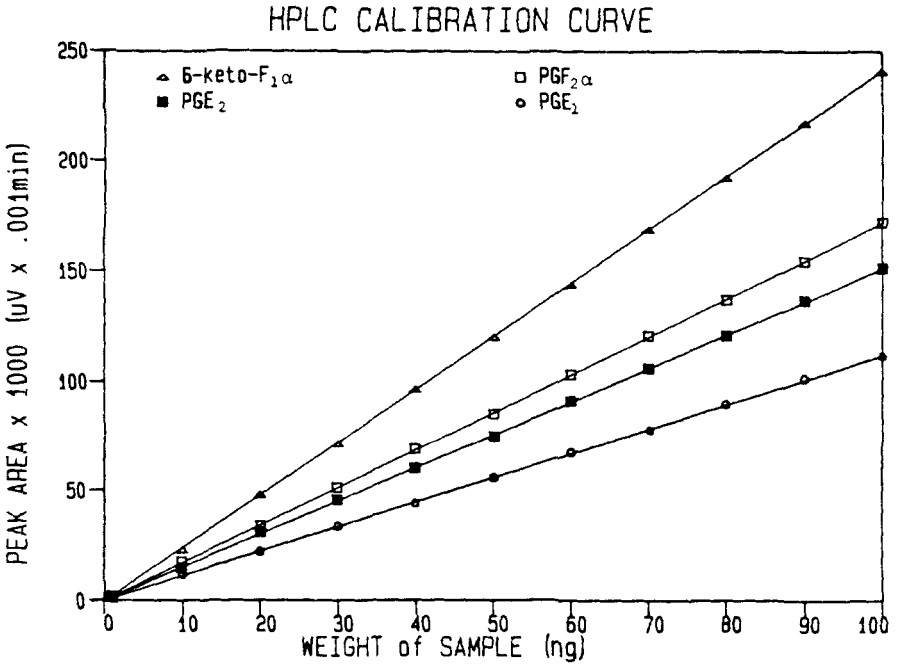

FIG. 2 Peak area vs. weight of each prostaglandin calibration graph. Microsorb C_{18} column 15 cm x 4.6 mm I.D.; mobile phase, .017M H_3PO_4: CH_3CN (67.2:32.8, v/v); flowrate 2.0 ml/min; UV detection at 193 nm/.0025 AUFS.

reinjected onto the column bed. The recovery was then calculated (area of second peak / area of the first peak x 100). (Table 2)

RESULTS OF SAMPLE ANALYSIS

In preparation for analysis of the prostaglandin content, the previously frozen samples of tissue and media were allowed to warm to room temperature. They were then subjected to HPLC analysis as previously described. Table 3 represents the level

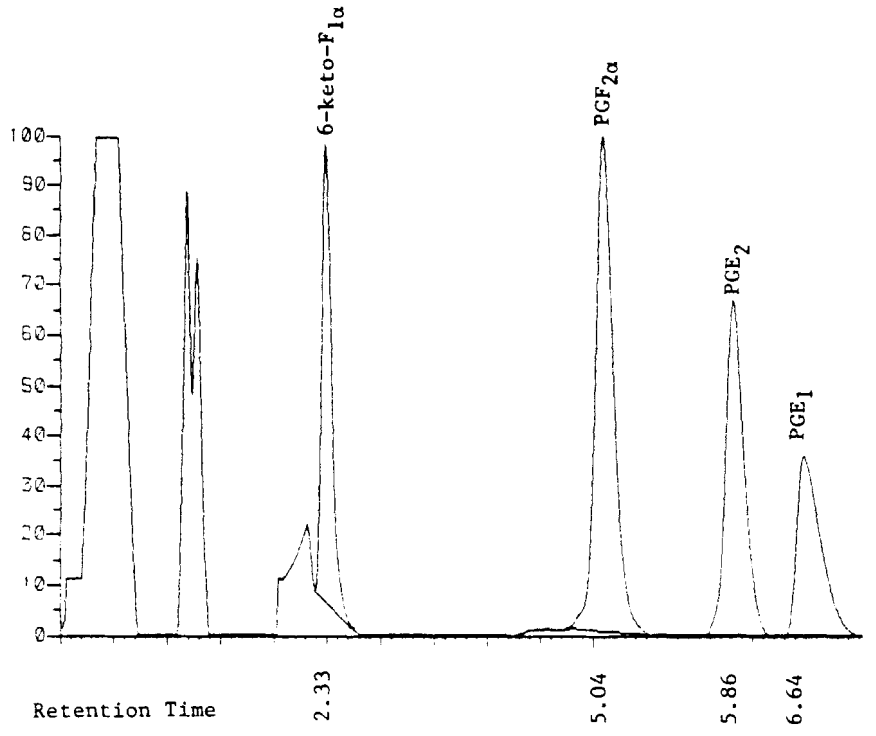

FIG. 3 This typical chromatogram demonstrates the elution order of prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 extract from gastric mucosa. Conditions for HPLC were; Microsorb C_{18} column 15 cm x 4.6 mm I.D.; mobile phase, .017M H_3PO_4: CH_3CN (67.2:32.8, v/v); flowrate 2.0 ml/min; UV detection at 193 nm/.0025 AUFS.

of prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 in each gastric mucosa sample as measured by this method.

DISCUSSION

Sample clean-up, HPLC sensitivity and reproducibility are critical for consistently quantifying prostaglandins as low as one nanogram. After extraction, Sep-Pak sample preparation

TABLE 2

Percent Recovery of Prostaglandins from HPLC Column

Sample wt		6-keto-$F_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	PGE_1
10 (ng)	mean	95.20	96.7	94.8	98.1
	±	2.1	0.7	0.6	1.4
20 (ng)	mean	96.1	96.8	95.6	97.8
	±	1.8	1.0	0.4	1.5
30 (ng)	mean	95.6	97.0	96.0	98.4
	±	1.6	0.5	1.0	0.7
40 (ng)	mean	96.4	97.1	94.9	96.9
	±	1.0	0.7	1.4	1.1
50 (ng)	mean	95.5	96.9	95.8	98.0
	±	0.7	1.1	0.8	0.6
60 (ng)	mean	96.0	96.4	97.9	98.2
	±	1.2	0.6	1.0	1.6
70 (ng)	mean	95.9	97.2	97.1	97.0
	±	0.6	0.6	0.8	1.2
80 (ng)	mean	96.2	96.9	96.8	95.9
	±	2.0	1.4	0.8	1.3
90 (ng)	mean	95.8	97.3	97.8	98.6
	±	1.1	1.2	0.5	0.4
100 (ng)	mean	95.4	96.8	96.7	97.7
	±	0.9	0.4	0.9	1.0

Ten known concentrations of prostaglandin standards 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 were evaluated.

TABLE 3

Prostaglandin Content of Gastric Mucosa
(pg/mg wet tissue weight)(n=8))

PROSTAGLANDIN		TISSUE	MEDIA	TOTAL
6-keto-$F_{1\alpha}$	mean	121.00	456.25	577.25
	±	2.02	7.60	9.56
$PGF_{2\alpha}$	mean	118.17	499.63	617.81
	±	6.66	11.11	14.21
PGE_2	mean	115.95	585.52	701.47
	±	4.33	6.22	4.19
PGE_1	mean	128.51	648.97	777.48
	±	3.51	3.76	5.57

The level of prostaglandins in incubated tissue were consistently 20 to 30% of the total prostaglandin synthesis. These levels were consistent with those reported by Basso et al. (5) and Rachmizewitz (6) with RIA.

cartridges were used as a preparative column for sample clean-up and trace enrichment. Sep-Pak cartridges are small disposable chromatographic columns packed with reverse phase C_{18} packing material. As the sample mixture passed through the cartridge, components (lipids, prostaglandins, etc) were selectively retained or eluted depending on the strength of the solvent used. Filtering the sample after clean-up is also essential for HPLC analysis.

The sensitivity and reproducibility in HPLC analysis is based on stability of the baseline and the ability of the column to separate prostaglandins of interest. The factors that affect baseline stability are; the purity of the solvents, pressure

fluctuations within the flow matrix, sample-to-sample carry over, choice of column, UV detection, and integration.

Our choice of acetonitrile for HPLC was based on the low UV Absorbance (0.1 AUFS at 188nm,). HPLC grade water was used to assure that no organic contamination was present. Purity of mobile phase solvents is crucial when using a wavelength of 193nm. To further eliminate baseline interference from the mobile phase, the solvents were simultaneously sparged with helium (a method of replacing the dissolved oxygen with helium), degassed, and filtered (0.45μm) immediately prior to HPLC.

To minimize pressure fluctuations, the solvent pump refill and compression rate was adjusted by using a pulse dampener sensitive to one psi. The flow was also limited to 2ml/min to minimize pressure fluctuations.

Sample injection was accomplished by an automated injector. The injector has a flow-through needle design. This design has an inherently low memory effect (less than 0.1%). This is critical when sampling trace amounts.

The column that we chose was 15cm x 4.6mm with reverse phase 5μm packing. The theoretical plate count was 42,000. This column offered extreme sensitivity and short retention times. To avoid sample dilution after injection and band broadening we elected not to use a guard column. This helped to reduce back pressure and the length of the stainless steel tubing needed between the injector and the head of the column.

Detection sensitivity was adjusted to .0025 AUFS. This was twenty times more sensitive than what had been previously reported (14). Electrical power for our detector was filtered through a Topaz power conditioner Model 70301 (Boston, Ma.). This eliminated most baseline electronic noise.

The integration was accomplished using a 80286 based computer, data acquisition software, and an analog to digital convertor. Although this method of integration is in its infancy, it is a major step forward in HPLC analysis when compared to other instruments used for integration.

Since prostaglandins were isolated and characterized in the early 1960s, quantitative assays of various types have been developed for a large number of prostaglandins. Of the various methods employed for the detection of prostaglandins, bioassay, spectrophotometric methods, gas-liquid chromatography, mass spectrometry, radioimmunoassay, and high pressure liquid chromatography are the most commonly used. Bioassay, although sensitive lacked specificity. Conversely, the spectrophotometric and gas chromatographic methods were considerably more specific, but were too insensitive for use in most biological studies. Mass spectrometry although extremely specific and sensitive requires large initial sample volumes and time-consuming purification prior to analysis.

Radioimmunoassay (RIA) is presently the most widely employed quantitative method used in this area of research. RIA offers the advantage of high sensitivity with large sample capacity and

rapid analysis. However, specificity is often questionable due to its' inability to differentiate the dienoic from the monoenoic prostaglandins. In comparison with RIA, HPLC determination of prostaglandins is not hindered by problems of poor specificity. The sensitivity of HPLC, although less than that obtained by RIA, is sufficient for detecting physiologic levels of prostaglandins. The ability to separate many compounds in a single chromatographic run, offers a rapid and reliable method for detecting and quantifying the physiologic levels of prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 and other arachidonic acid metabolites found in gastric mucosa.

HPLC, although still a young branch of chromatography, is a rapidly developing technique due to recent advances in instrumentation and column technology. The combination of high effiency columns and on-line UV detection is currently one of the most poverful analytical tools in the field of prostaglandin research.

CONCLUSIONS

HPLC, as described herein, produces analysis of prostaglandins 6-keto-$F_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1, in biologic tissue. By separating many compounds in a single chromatographic run, HPLC offers a reliable, highly specific, method for detecting and quantifying the physiologic concentrations of prostaglandins and arachidonic metabolites found in gastric mucosa.

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