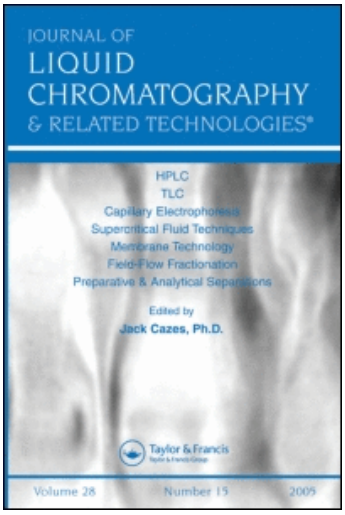

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A New Approach to the Extraction of Prostaglandins 6-Keto-$F_1\alpha$, $F_2\alpha$, E_2, and E_1 from Gastric Mucosa with Quantitative Analysis by Reverse Phase Liquid Chromatography

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A NEW APPROACH TO THE EXTRACTION OF PROSTAGLANDINS 6-KETO-$F_{1\alpha}$, $F_{2\alpha}$, E_2, AND E_1 FROM GASTRIC MUCOSA WITH QUANTITATIVE ANALYSIS BY REVERSE PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and reliable method is presented for the extraction of Prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2 and E_1 from gastric mucosa with quantitation by Reverse Phase Liquid Chromatography. Extraction is accomplished without the need for the harsh chemicals normally associated with tissue extraction. The efficiency of prostaglandin extraction is demonstrated utilizing radiolabeled prostaglandins. Quantitation of these compounds is compared to measurement by radioimmunoasay.

INTRODUCTION

Since their discovery by Goldblatt(1) and von Euler(2) in 1933, prostaglandins as well as other related metabolites of arachidonic acid have continued

to be discovered in increasing numbers, and also have demonstrated diversity of biological action. Concurrently, interest in the ability to analyze prostaglandins and related substances has also increased. Unfortunately, the analysis of prostaglandins from biological media remains a delicate procedure. This is mainly due to both instability of the compounds themselves and their low tissue concentrations.

Of the various methods available for detecting and quantifying prostaglandins in biologic fluids, radio-immunoassay (RIA) has been the most widely implemented(3,4,5). Although RIA is specific and sensitive, it is usually limited to single compound analysis. Multiple compounds can only be analyzed if they can first be preparatively separated from each other by chromatographic techniques. Until recently, Reverse Phase Liquid Chromatography (RPLC) has not possessed the sensitivity required for analytical quantitation of prostaglandins(6,7). But with recent technological advances, RPLC has been catapulted from an instrument of separation, into one with analytical capabilities.

The current problem facing researchers who would like to use RPLC for the analysis and quantitation of prostaglandins lies primarily with the compounds needed to acheive successful extraction from the biologic sample. Traditional extraction methods(8,9,10,11) involve the use of chemicals with high ultraviolet absorption as well as a wide range of strengths and polarities (i.e. chloroform, ethly ether, acetone, etc.). Although these solvents provide good extraction performance

when coupled with radioimmunoassay quantitation, they are unacceptable when used in conjunction with RPLC measurement. This is because samples undergoing RPLC must be free of any contaminants which may be visibly detected during the RPLC process.

This paper will describe a unique and reliable method for extraction of Prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2 and E_1 from gastric mucosa using solvents that do not interfere with quantitation by Reverse Phase Liquid Chromatography. As such, it is a significant evolution from our previously published methodology(12). Data will be presented demonstrating the efficiency of both the extraction and quantification processes. Also, comparison of tissue prostaglandin measurement techniques (RIA vs. RPLC) will be provided. Finally, a discussion will center on some of the finer technical points which must bear adherence for accurate, and reproduceable, determinations to result.

METHODS FOR SAMPLE EXTRACTION AND ANALYSIS

Materials Used:

HPLC grade acetonitrile, water and phosphoric acid were purchased from Burdick and Jackson (Muskegon, MI). Radiolabeled prostaglandins 6-keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2 and E_1 and RIA kits were purchased from New England Nuclear (Boston, Ma.). The Sprague-Dawley strain of rats were purchased from Harlan (Indianapolis, Ind.). SEP-PAK cartridges were purchased from Waters Chromatography Division (Milford, Ma.). Cell disruption was accomplished with a Branson Sonic Power

Sonifier Model 350 (Danbury, CT.). Scintillation counting was accomplished with LKB Model 1219 (Turku, Finland).

Subjects:

One hundred Sprague-Dawley rats, weighing between 225 and 250 grams, formed the basis for this study. The rats were fed commercial rat chow ad libitum. They were randomly assigned two rats per cage with wide wire bottoms to prevent coprophagia. The animals diet was restricted to water 48 hours prior to mucosal harvesting. All rats were subjected to equal conditions.

Collection of Mucosal Tissue:

Immediately after euthanasia, via a CO_2 chamber, the stomach of each rat was removed. An incision was made along the greater curvature(13). The lumen was rinsed with normal saline at 4°C. One sample of approximately 100 mg of glandular mucosa was sharply dissected from the corpus of each stomach from the same glandular area in each animal. Each sample was placed in a microcentrifuge tube filled with 0.5 ml normal saline and immediately frozen at -100°C to inhibit bioactivity.

Incubation of Gastric Mucosal Tissue and Radiolabeling:

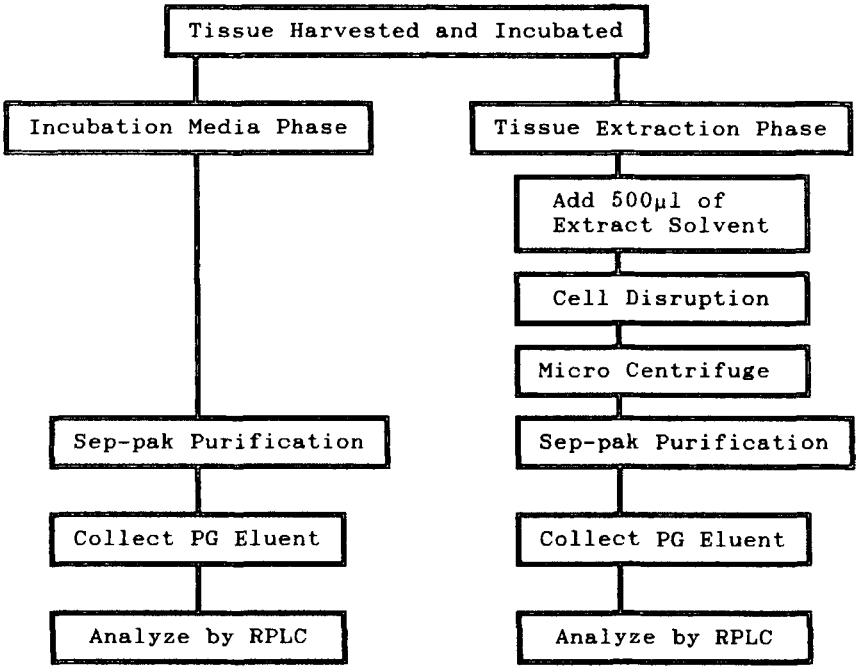
Immediately prior to incubation, the samples were allowed to thaw at room temperature. Each sample was rinsed in ice cold saline, weighed, and placed in a new microcentrifuge tube with 0.5 ml of a 10mM phosphate-buffered saline (pH 7.4). Each sample was radiolabeled with 7700 dpm of prostaglandins

6-Keto$F_{1\alpha}$, $F_{2\alpha}$, E_2, and E_1. The mucosal samples were then incubated for four hours at 37°C to permit prostaglandin synthesis. At the end of the incubation period each sample was centrifuged for five minutes at 10,150g. The media from each was removed and, of the 100 incubation media samples, 50 were saved for future prostaglandin quantitation with RPLC and 50 were counted on a scintillation counter to determine the radiolabeled prostaglandin content. The 100 tissue samples then underwent the extraction process.

Extraction of Prostaglandins from Gastric Mucosal Tissue:

In preparation for extraction, 0.5ml of extract solvent (which consisted of 32.8% acetonitrile and 67.2% aqueous phosphoric acid (pH 3.0)) was added to the tissue samples. The lowered pH was sufficient to stop further prostaglandin synthesis. Each tissue sample was homogenized for one minute using a cell disruptor with a micro tip at a power setting of 2, and a 30% duty cycle. Subsequent to cell disruption, the homogenate was centrifuged again for five minutes at 10,150g. The tissue extract was then collected, and the tissue interface rinsed twice with 0.5 ml of extract solvent. Both the extract and rinses combined (henceforth known as the tissue extract) were saved for future analysis. The tissue pellets and 50 tissue extract samples were then counted by a scintillation counter for determination of the radiolabeled prostaglandin content. The other 50 tissue extract samples then underwent sample purification. Table 1 demonstrates this simple yet efficient extraction process.

TABLE 1



This flowchart highlights the extraction process.

Sample Preparation and Purification:

The prostaglandins contained in the incubation media and tissue extract were isolated, enriched and preparatively separated using SEP-PAK preparation cartridges. These cartridges contain the same medium (octadecylsilane) as the column used for RPLC. The procedure for selectively eluting the prostaglandins of interest from either the incubation media or the tissue extract is as follows; The sample was injected onto the cartridge and the neutral lipids, phosolipids and fatty acids were eluted from the sample with

5ml of water at a flow rate of 2ml per minute and discarded. The prostaglandins of interest were then eluted with 0.5ml of 50% aqueous phosphoric acid (pH 3.0) and 50% acetonitrile (RPLC grade). This entire preparation eluent was saved for future analysis by RPLC.

RPLC METHODS AND RESULTS

The samples were analyzed by Reverse Phase Liquid Chromatography as previously described by Palm(12). The basic parameters were as follows; The mobile phase was mixed at a ratio of 32.8% acetonitrile and 67.2% aqueous phosphoric acid (pH 3.0). The solvent flow rate was limited to 1.7ml per minute. The column used was a microsorb C^{18} 4.6mm X 150mm. The ultraviolet detector was set at a wavelength of 193nm and the absorbance range was adjusted to 0.0025 Absorbance Units Full Scale.

A real-time chromatogram of prostaglandins from gastric mucosa is shown in Figure 1. Proper separation of Prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2, and E_1 was achieved. Retention times of these compounds were 2.54, 4.43, 4.77, and 5.36 minutes respectively. Mucosal prostaglandins were identified and quantified by peak area on the basis of their absolute and relative retention times (Table 2). Calibration was accomplished every third injection by analyzing external standards of known amounts of each specific prostaglandin of interest.

Efficacy of column:

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

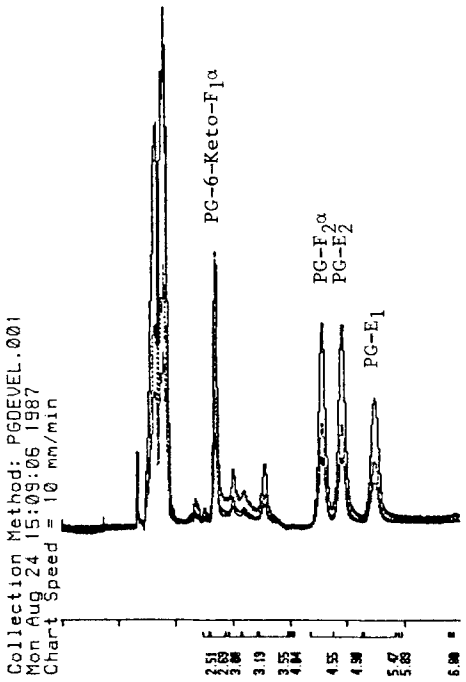


FIGURE 1: This real-time chromatogram demonstrates the elution order of prostaglandins 6-Keto-F_1-α, $F_2\alpha$, E_2, and E_1 extract from gastric mucosa. Retention times were 2.54, 4.43, 4.77, and 5.36 minutes respectively. Mobile phase was 32.8% acetonitrile and 67.2% aqueous phosphoric acid. Flow rate 1.7ml/minute.

TABLE 2
Prostaglandin Content of the Mucosal Samples.
(pg/mg wet tissue weight((n=50)

Prostaglandin	Media	Tissue	Total
6-Keto-$F_{1\alpha}$	646±13	154±21	800±23
$F_{2\alpha}$	514±9	139±14	653±17
E_2	468±19	123±11	591±18
E_1	480±23	128±10	608±26

This table reflects the physiologic levels of prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2, and E_1 found in Murine gastric mucosa. Incubation time was 4 hours.

samples of known amounts of H^3 labeled prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2, and E_1 were injected onto the column bed. The resulting peaks were identified in real time, collected as fractions, and counted. The recovery was then calculated ((counts recovered / counts injected) x 100). Table 3 demonstrates the consistently high recovery (97.5% to 99.5%) of H^3 labeled prostaglandins experienced when testing for sample recovery.

Tracer Analysis:

Of the 100 tissue samples that underwent extraction, 50 served as controls for H^3 tracer recovery along with their previously reserved media fractions. Tritium levels were monitored at post extraction, sample purification, pre-RPLC and post-RPLC. Intersample extraction levels were consistently greater than ninety five percent. The results in Table 4 confirm the stability of this extraction method.

RPLC versus RIA:

In a separate but related experiment, ten paired gastric mucosal samples were subjected to simultaneous quantification by both radioimmunoassay and RPLC. The RIA kits used for the determination of Prostaglandins 6-Keto-$F_{1\alpha}$ and E_2 were purchased from NEN Products (Boston, MA). RIA was performed as per enclosed instructions. The RPLC was done as previously described in the methods section above. Table 5 reflects the levels obtained from the assays. Statistical analysis was carried out using the Tukey analysis of variance test. No

TABLE 3
Percent Recovery of Prostaglandins from RPLC Column.

Amount Injected	Amount Recovered	Percent Recovery
1538 dpm	1521 dpm	98.9 %
3093 dpm	3068 dpm	99.2 %
4654 dpm	4630 dpm	99.5 %
6123 dpm	5973 dpm	97.5 %
7681 dpm	7607 dpm	98.9 %
9234 dpm	9064 dpm	98.1 %
10,727 dpm	10,542 dpm	97.8 %
12,386 dpm	12,147 dpm	98.6 %
13,960 dpm	13,776 dpm	99.4 %
15,400 dpm	15,076 dpm	97.9 %

Ten known amounts of H^3 labeled prostaglandins 6-Keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2, and E_1 were evaluated for column recovery. Counting time was 1 minute.

TABLE 4
Percent of H^3 Labeled PG's Recovered from Tissue Sample.
(n=50, mean and range)

Incubation Media	Tissue Extract	Tissue Pellet	Total
88.5% (84-92%)	5.7% (4-7%)	1.1% (1-2%)	95.3 %

50 samples were radiolabeled with 7700 dpm of H^3 PG-6-keto$F_{1\alpha}$, PG-$F_{2\alpha}$, PG-E_2, PG-E_1 and traced throughout the extraction process.

TABLE 5
Radioimmunoassay Versus Reverse Phase Liquid Chromatography.

Prostaglandin	RIA	RPLC
6-Keto-$F_{1\alpha}$	146±20.7	161±7.1
E_2	119±32.1	127±11.4

Comparison between prostaglandins 6-Keto-$F_{1\alpha}$ and E_2 as measured by radioimmunoassay and RPLC. ($P>.05$)

significant difference exists between the levels obtained with RIA and RPLC.

DISCUSSION

The need for a highly sensitive and simple method of separation and quantification has become obvious to researchers working with prostaglandins, where progress is hampered by time-consuming, tedious, or inadequate methodologies. Sensitivity, reproducibility, specificity, ease of use and precision remain of utmost importance in the measure of prostaglandins and other prostanoic acid metabolites in the submicrogram range. All of these critical factors can be met by Reverse Phase Liquid Chromatography providing certain guidelines are adhered to.

Sensitivity and Reproducibility

Baseline stability is of extreme importance when analyzing submicrogram levels of prostaglandins. The major factors that assure baseline stability are; sample purity, the purity of the mobile phase solvents, lack of pressure pulses, intersample overlap, and ultraviolet detection.

Importance of Sample Cleanup:

When preparing a sample for RPLC, special care must be taken to rid it of extraneous chemicals which may be absorbed during the RPLC process causing baseline drift and thus, reducing sensitivity and resolution. Due to the harsh solvents used during traditional RIA sample extraction methods(8,9,10,11), sample purification when using these

methods is difficult. The strong solvents containing the sample must be severely diluted before placing the sample onto the preparation cartridge. If this is not done properly, there is a risk of losing the compounds of interest even before the elution process commences. Since the method described herein uses the mobile phase solvent for the extraction procedure, sample purity is easily attained by passing the sample through a sample preparation cartridge and collecting only the compounds of interest.

Selection of Mobile Phase Solvents:

Purity of mobile phase solvents is crucial in RPLC. Therefore, Burdick and Jackson solvents were chosen. In preparation for RPLC, the mobile phase solvents were sparged with helium, degassed, filtered (0.45μm) four times, and placed into a specially coated bottle that filters out harmful ultraviolet light, thus eliminating UV degradation. This process reduces baseline noise and drift.

RPLC Pump Performance:

To minimize pressure pulses caused by the solvent pumps, their refill and compression rate was set to one psi. Solvent flow was also limited to 1.7ml/min to lessen pump strain. Inlet valve failure was also eliminated by keeping the mobile phase under constant positive pressure (7psi).

Sample Injection and Intersample Carryover:

Sample injection was accomplished by an automated injector with a memory effect of less than .1%. This is critical when analyzing small samples. Sample volumes as low as one

microliter can be injected with consistency, allowing extremely concentrated extracts to be analyzed. Additionally, the sample vials used contain a PTFE seal and a 100µl insert which minimizes sample loss.

Column Selection:

The column chosen contained a theoretical plate count of 42,000 with an internal diameter of 4.6mm and a length of 15cm. Packing was five micron octadecylsilane. This column configuration allows the proper sensitivity and minimal retention times needed for quantity sample analysis.

Column Temperature:

Because temperature fluctuations can also affect baseline stability, the column was placed in a water jacket and kept at a constant temperature of 30°C using a circulating pump. This allowed control over baseline fluctuations caused by our laboratory air conditioning system.

UV Detection:

Detector sensitivity was set at .0025 AUFS. A 3mm flow path was attained by using a microbore flow cell with a volume of 0.8µl. This flow path inhibits band broadening within the flowcell and causes peaks to have a greater height by keeping the sample volume to a minumum. A backpressure valve was attached to the outlet tubing and adjusted to 50lbs to reduce outgassing within the flowcell. To minimize electrical interference, all power to the RPLC system was conditioned and filtered (Topaz Model 70301 Boston, MA.).

Data Analysis and Integration:

Data integration was accomplished using an INTEL 80286 (Austin, Texas) based computer and data acquisition software. Four channels of data were collected simultaneously during analysis. This allowed detailed post-run analysis of all peaks detected. To monitor environmental conditions, standards of known concentrations were analyzed every third injection. This allowed complete confidence of peak quantification during all runs.

Specificity

The differential migration of compounds of interest during passage through the chromatographic column under proper conditions assures the specificity of these compounds during analysis.

Ease Of Use

With the wide availability of computerized pump controllers, integration systems, automated injectors, and programmable detectors, the ease and constistency of sample analysis is enhanced.

CONCLUSIONS

Advances in column technology and instrumentation, great operational simplicity, high efficiency, column stability, and the ability to simultaneously analyze a broad spectrum of compounds have made RPLC the analytical tool of choice. Adequate sample extraction plays a major role in the ultimate success of using RPLC for the analysis of prostaglandins and other arachidonic acid metabolites found in gastric mucosa.

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REFERENCES

1) Goldblatt, M. W., A depressor substance in seminal fluid, J. Soc. Chem. Ind. (London) 52, 1056, 1933.

2) von Euler, U.S., Zur Kenntnis der pharmakologishen Wirkungen von Nativeskreten und Extrakten mannlicher accessorischer Geschlechtsdrusen, Arch. Exp. Pathol. Pharmakol, (Naunyn-Schmeidebergs) 175, 78, 1934.

3) Jaffe, B., Behrman, H., Parker, C., Radioimmunosassy Measurement of Prostaglandins E, A, and F in Human Plasma, The Journal of Clinical Investigation, 852, 398, 1973.

4) Pradelles, S. and Dray, F., Analytical Methods For Thromboxane B_2 Measurement and Validation of Radioimmunoassay by Gas Liquid Chromatography-Mass Spectometry, Prostaglandins, 16(2), 277, 1978.

5) Rachmilewitz, D., Ligumsky, M., Fich, A., Goldin, E., Eliakim, A., and Karmeli, F., Role of Endogenous Gastric Prostanoids in the Pathogenesis and Therapy of Duodenal Ulcer, Gastroenterology, 90, 963, 1986.

6) Terragno, A., Rydzik, R., and Terragno, N., High Performance Liquid Chromatography and UV Detection for the Separation and Quantitation of Prostaglandins, Prostaglandins, 21, 101, 1981.

7) Saunders, R.D., Horrocks, L., Simultaneous Extraction and Preparation for High-Performance Liquid Chromatography of Prostaglandins and Phospholipids, Anal. Biochem., 143, 71, 1984.

8) Shaw, J.E., and Ramwell, P.W., Methods of Biochem. Analysis, 17, 325, 1969.

9) Green, K., Hamberg, M., Samuelsson, B., and Frolich, J.C., Adv. in Prostaglandin and Thromboxane Research, 5, 15, 1978.

10) Skrinska, V. and Lucas, F., Prostaglandins, 22, 365, 1981.

11) Robert, A., Nezamis, J., Lancaster, C., and Hanchar, A.,Cytoprotection by Prostaglandins in Rats, Gastroenterology, 77, 433, 1979.

12) Palm, C.D., Levine, B.A.,Simultaneous Preparation and Extraction of Prostaglandins from Gastric Tissue with Separation and Quantification by High Pressure Liquid Chromatography, Journal of Liquid Chromatography, 10(5), 881, 1987.

13) Basso, N., Materia, A., Forlini, A., and Jaffe, B., Prostaglandin Generation in the Gastric Mucosa of Rats with Stress Ulcer, Surgery, 94, 104, 1983.