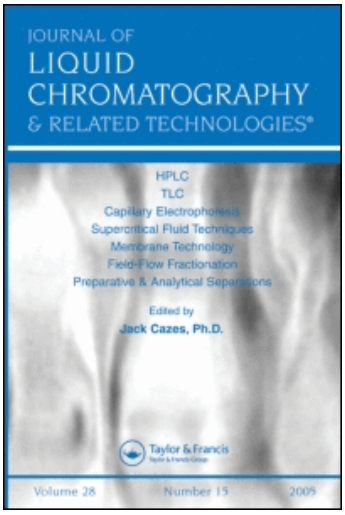


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# High Performance (Pressure) Liquid Chromatography Separation and Quantification of Picomole Amounts of Prostaglandins Utilizing a Novel Triethylamine Formate Buffer

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**To cite this Article** Desiderio, D. M. , Cunningham, M. D. and Trimble, J. A.(1981) 'High Performance (Pressure) Liquid Chromatography Separation and Quantification of Picomole Amounts of Prostaglandins Utilizing a Novel Triethylamine Formate Buffer', Journal of Liquid Chromatography & Related Technologies, 4: 7, 1261 — 1268

**To link to this Article: DOI:** 10.1080/01483918108068810

**URL:** http://dx.doi.org/10.1080/01483918108068810

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(7), 1261-1268 (1981)

# HIGH PERFORMANCE (PRESSURE) LIQUID CHROMATOGRAPHY SEPARATION AND QUANTIFICATION OF PICOMOLE AMOUNTS OF PROSTAGLANDINS UTILIZING A NOVEL TRIETHYLAMINE FORMATE BUFFER

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## ABSTRACT

A novel, volatile buffer system utilizing dilute triethylamine-formic acid is useful for high resolution HPLC separation and quantification of nanogram amounts of prostaglandins. Lyophilizability of the buffer facilitates subsequent mass spectral, bioassay or radioimmunoassay analyses.

## INTRODUCTION

An increasing number of metabolites continues to result from the continued study of arachidonic acid metabolic cascade (1). In addition to classical primary prostaglandins (PGs), other pathways yielding endoperoxide-hydroperoxide intermediates, prostacyclins, thromboxanes and leukotrienes are being studied and quantified and structures of metabolites are being elucidated. Because of the great number of structurally similar compounds and metabolites resulting from the arachidonic acid metabolic cascade, chromatographic separation techniques capable of high resolution of prostaglandins, metabolites, thromboxanes, prostacyclins and leukotrienes is required. Due to general molecular similarity of

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these compounds, radioimmunoassay (RIA) techniques, sensitive to picogram amounts, are not necessarily sufficiently structurally specific and unambiguous in compounds being quantified. It is generally considered that gas chromatographic-mass spectrometric (GC-MS) data provide the ultimate level of molecular specificity and confidence in quantification of these biologically important compounds.

A novel buffer system has been developed in our laboratory for separation and quantification of endogenous brain neuropeptides, and, as this report will show, is also amenable to separation of mixtures of prostaglandins. High performance (pressure) liquid chromatography (HPLC) separation of prostaglandins requires a buffer capable of high resolution, optimal speed of separation, high sensitivity of detection, and, because of subsequent GC-MS analysis, lyophilizability.



Previous workers utilized HPLC for separation (2-8) and quantification (9-10) of prostaglandins. Radioimmunoassay of prostaglandins was preceded by HPLC (11, 12). Prostacyclin (13, 14), thromboxanes (15) and leukotrienes (16, 17) have been analyzed. Chemical derivatives included both fluorescent coumarins (18) and UV-sensitive phenacyl esters (19, 20) in an effort to achieve ng sensitivity. An electrochemical detector analyzed hydroxy-anilide derivatives (21).

This manuscript reports preliminary results employing a novel, triethylamine: formic acid (TEAF) buffer system which is UV transparent at wavelengths below 200 nm, is volatile, buffers at pH 3 to protonate carboxylic groups and facilitates quantification of ng amounts of chemically underivatized PGs.

## EXPERIMENTAL

### Apparatus

Figure 1 contains a schematic illustrating the Waters (Milford, MA) HPLC apparatus employed. A U6K injector, guard column packed with

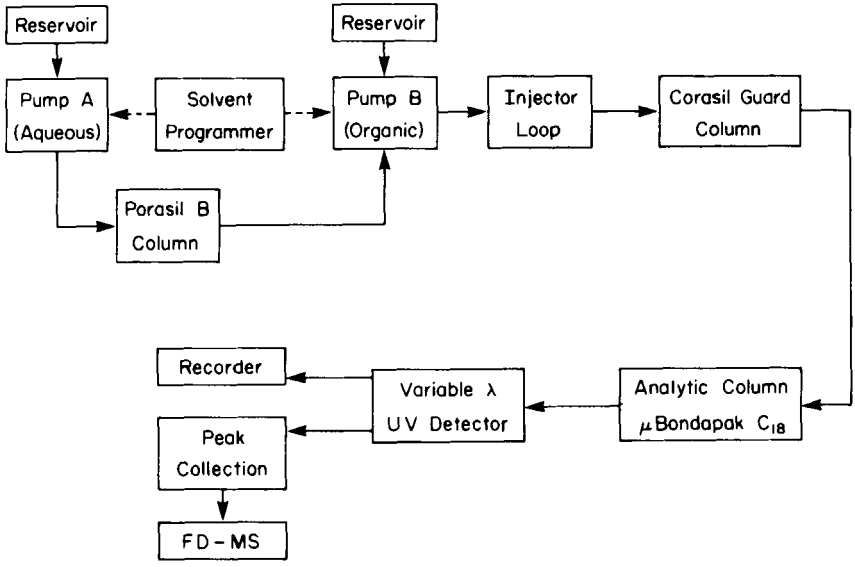


Figure 1: Schematic of HPLC apparatus.

Corasil B (50μ), Bondapak $C_{18}$ 10μ reverse phase (RP) analytic steel column, two model 6000A solvent delivery pumps, a model 660 solvent programmer, model 450 variable wavelength UV detector (set at 194 nm) were utilized. The variable wavelength UV detector is demanded for this type of study because wavelength can be set at $\lambda_{max}$ for the compound under investigation. The analog signal from the UV detector is recorded. Individual HPLC fractions can be collected for subsequent MS, bioassay or RIA analysis.

Buffer System

The buffer, 0.04M triethylamine: formate (TEAF), is made by titrating formic acid to pH 3.15 with distilled (87.5°C) triethylamine (Pierce Chemical Co., Rockford, IL). Acetonitrile (Burdick and Jackson, Muskegon, MI) and TEAF buffer were filtered and degassed through appropriate

Millipore (Bradford, MA) filters before use. Solvent flow rate was 1.5 ml $min^{-1}$; acetonitrile: 0.04M TEAF (45:55). A synthetic mixture containing two µg of each PG was injected onto the column. Peak height (mm) recorded for each peak injection was measured to construct the calibration curve. Straight line regression statistics were obtained with a Hewlett Packard (Cornwallis, OR) HP97 programmable calculator and pre-recorded program.

Prostaglandins were a generous gift of Dr. Udo Axen (Upjohn, Kalamazoo, MI).

## RESULTS AND DISCUSSION

No conversion of PGE to PGA occurred at pH 3.15 during the time required for sample preparation, injection and elution.

The HPLC chromatogram shown in Figure 2 illustrates isocratic elution of model compounds 6-keto-$PGF_{1\alpha}$, $PGF_{1\beta}$, $PGF_{2\alpha}$, $PGE_2$ and $PGE_1$ eluting according to increasing hydrophobicity of each prostaglandin.

The calibration curve for $PGE_2$ is shown in Figure 3. Serial dilutions ranging from 100 down to 20 ng $PGE_2$ were injected onto the column and peak heights measured. Regression analysis of data points yielded a straight line, $y = 0.98X + 2.81$, with a correlation coefficient ($r^2$) of 1.00 over the range of PG injected.

## CONCLUSIONS

High sensitivity utilizing UV absorbance is obtainable down to 20 ng for underivatized prostaglandins with TEAF buffer and reverse phase HPLC. This detection mode for underivatized PGs competes favorably with fluorescence derivatives utilizing coumarin derivatives, phenacyl derivatization utilizing ultraviolet detection and electrochemical detection of hydroxyanilides (see Table).

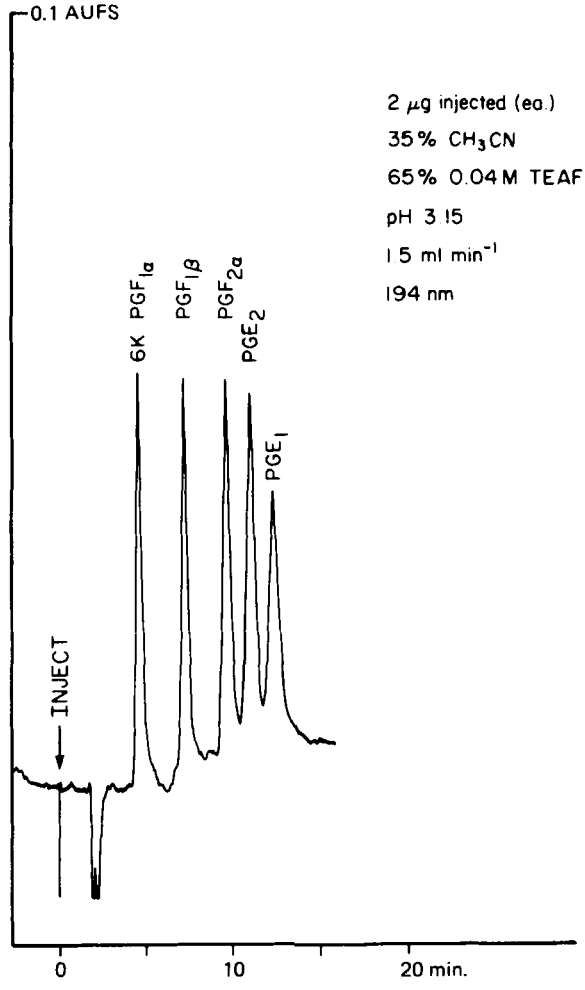


Figure 2: Isocratic elution of a mixture of synthetic prostaglandins: 6-keto $PGF_1$ , $PGF_1$ , $PGF_2$ , $PGE_2$ and $PGE_1$.

Isocratic resolution of five synthetic PGs is accomplished within 15 minutes. Quantification of $PGE_2$ at a higher percentage of the organic modifier acetonitrile is accomplished within minutes. Lyophilizability of buffer is required for subsequent GC-MS quantification or structure

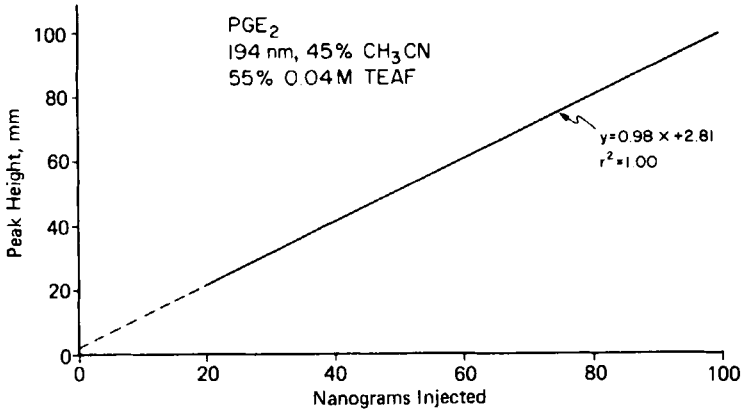


Figure 3: Regression analysis of data resulting from plotting peak height (mm) versus ng $PGE_2$ injected over the range 20-100 ng.



Table

Detection limits for PGs utilizing various detection modes.

| Detection Mode | Derivative | Detection Limit | | Reference |
|---|---|---|---|---|
| UV | None | 20ng | $PGE_2$ | This work. |
| Electrochemical | Hydroxyanilide | 20ng | $PGF_{2\alpha}$ | 21 |
| Fluorescence | Coumarin | 20ng | $PGE_2$ | 18 |
| U.V. | Phenacyl ester | ca.1 ng | $PGE_2$ | 20 |

elucidation or, on the other hand, when HPLC is followed by RIA quantification. This novel TEAF buffer system has been utilized to great advantage in our laboratory to quantify femtomolar (fmol) amounts of a neuropeptide somatostatin by HPLC (22) and to quantify pmol amounts of enkephalins by a new combination of RP-HPLC and field desorption mass spectrometry (23).

The high sensitivity of detection for underivatized $PGE_2$ is due to several critical factors:

1.) The molar extinction coefficient of PGE at 192.5 nm equals 14,200. (10).

2.) The novel dilute TEAF buffer system possesses optimal UV transparency properties.

3.) Because no chemical derivatization steps are required, no residual reagents interfere with the high sensitivity of detection.

This simplified, high sensitivity system provides levels of detection required for biologic samples (24).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge partial financial assistance from NIH (GM NS 26666) and typing assistance from K. Smith.



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