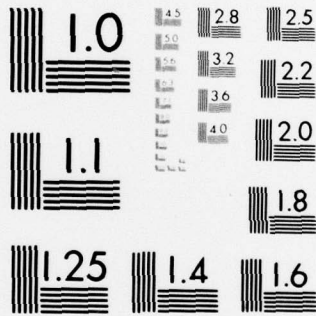


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6 THE HIGH PERFORMANCE MOLECULAR EXCLUSION  
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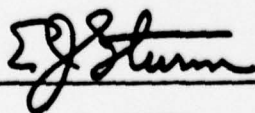
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This report describes a high performance molecular exclusion chromatographic (HPMEC) separation of PGB <sub>x</sub> that yields information concerning the distribution of components according to molecular size. It should be useful in describing the homogeneity of PGB <sub>x</sub> preparations. <i>a prostaglandin derivative</i>		

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## INTRODUCTION

In 1973, Polis *et al*<sup>1</sup> first synthesized PGB<sub>x</sub> from 15-diketo PGB<sub>1</sub> under rigidly controlled conditions of alkalinity, temperature and time. Even under these controlled conditions, the PGB<sub>x</sub> product that was isolated was found to be a complex mixture of polymers of varying molecular size and configurations. Because of the importance of PGB<sub>x</sub> in the reversal of the effects of myocardial ischemia<sup>2</sup> and cerebral ischemia<sup>3</sup> in animals and its possible use in humans, it was deemed necessary to purify PGB<sub>x</sub> to avoid possible side effects from contaminants. Studies have been under way in this laboratory to separate the active component from the PGB<sub>x</sub> complex. At the present time, the purification of PGB<sub>x</sub> is followed by the *in vitro* assay, which is based on the recovery of oxidative phosphorylation upon the addition of PGB<sub>x</sub> to aged degraded rat liver mitochondria<sup>4</sup>. Unfortunately, this type assay yields no information concerning the homogeneity of the PGB<sub>x</sub> preparations.

## EXPERIMENTAL

PGB<sub>x</sub> was prepared according to Polis *et al*<sup>4</sup> and dissolved as the sodium salt in the carrier buffer. The concentration was 1.0 mg/ml.

High performance molecular exclusion chromatography (HPMEC) was carried out with the Hewlett-Packard Liquid Chromatograph Model 1084A (Avondale, PA). The ME column was 4.6 mm I.D. X 250 mm long packed with Lichrospher S1-300 (Brownlee Laboratories, Santa Clara, CA). The first column used in this study eventually became clogged with sample and buffer impurities to the point where solvent flow stopped. To overcome this, a guard column (2 mm I.D. X 100 mm long) packed with Controlled Pore Glass, pore size 1250Å (Corning Glass Co., Corning, NY) was then placed between the injector and the S1-300 column in order to prevent clogging of the column packing.

Chromatography was carried out isocratically in 0.05 M KH<sub>2</sub>PO<sub>4</sub> + K<sub>2</sub>HPO<sub>4</sub> buffer, containing 0.001 M NaN<sub>3</sub> as a preservative to prevent mold growth. The pH of the carrier buffer was adjusted by varying the mixtures of two buffers stored in the solvent delivery reservoirs. Reservoir A contained pH 6.4 buffer and reservoir B contained pH 7.8. The pH of the buffer mixture was determined by measuring the pH of the solvent stream emerging from the detector. The separation was followed with a UV detector (λ254 nm) and a refractive index monitor in series. The following instrumental parameters were used for the chromatography: flow-rate, 1.0 ml per minute; column pressure, 140 bar; column temperature, 30°; chart speed, 1.0 cm per minute; UV detector, zero setting, 10 percent; UV detector attenuation, 64 x 10<sup>-4</sup> AU per cm. When an RI detector was used (LDC Refracto-Monitor, LDC Corp., Riviera Beach, FL) it was set at attenuation "8" and connected to a Hewlett-Packard Integrator set at attenuation "16."

## RESULTS AND DISCUSSION

Previously, it was reported from this laboratory<sup>5</sup> that the PGB<sub>x</sub> complex could be separated by gel filtration on Sephadex G-100 or G-150 (Pharmacia Inc., Piscataway, NJ) using aqueous neutral phosphate buffers as carrier solvents. Unfortunately, these Sephadex packings compress under pressure making it mandatory that solvents be delivered through the column by gravity. This packing shortcoming results in excessive and nonreproducible retention times. For this reason, this type of gel filtration is only practical for preparative applications. In order to apply the gel filtration principle to analytical chromatography, studies were undertaken to evaluate commercial molecular exclusion packings suitable for high performance liquid chromatography<sup>5</sup>.

Recently the Lichrospher family of highly porous silica spheres became available commercially in packed columns. These were evaluated and found suitable for the analysis of PGB<sub>x</sub> preparations in terms of molecular size. According to the manufacturer's specifications, S1-300 has an exclusion limit for polystyrenes between 150,000 and 300,000 when chromatographed in chloroform. Since no information was available as to the exclusion limits in aqueous carrier solvents, the S1-300 column was calibrated with known standards in 0.05 M, pH 6.85 buffer. These results are listed in table I.

Table I

Calibration of Aqueous MEC on Lichrospher S1-300

<u>Standard</u>	<u>M.W.</u>	<u>R.T. (minutes)</u>
Dextran Blue 2000*	2 x 10 <sup>6</sup>	2.7
Stachyose	666.6	6.0
Adenylic Acid	347.23	5.7
EDTA**	292.24	5.7
Sucrose	342.30	5.8
KCl	74.55	5.9
PGB <sub>x</sub>		{ 2.8 4.2

\*Pharmacia, Inc., Piscataway, NJ

\*\*N,N'-1,2-ethane diylbis (N-(carboxy-methyl)glycine)

Unfortunately, water soluble standards in the range of 1000 - 5000 daltons are not available so that the column calibration is not complete. Nevertheless, the data for Dextran Blue 2000 indicates the retention time of large

molecular weight substances, while the other standards define the retention time of small molecules; therefore this analytical system can separate only the large molecules from the small molecules. The retention time of  $PGB_x$  shown in table I indicates the separation of two components, one emerging after 2.8 minutes that exhibits a large molecular weight and the other emerging after 4.2 minutes suggesting an intermediate molecular weight between the completely excluded standard and the nonexcluded standard.

In order to determine the optimum pH for the MEC separation of  $PGB_x$ , the pH was varied by changing the "percent B" command of the solvent delivery system. The column was first equilibrated with the new buffer, and then the pH of the solvent flow-to-waste was measured. The samples were then injected and MEC performed. Usually three chromatographic runs were made in order to be sure that the column was equilibrated with the carrier buffer. The results of these experiments are shown in figure 1. The pH ranges used in this study were limited on the acid side by the insolubility of  $PGB_x$  at low pH (below pH 6.2), and the chemical instability of  $PGB_x$  at alkaline pH (above pH 8.5), and the instability of the S1-300 packing at alkaline pH (pH 8.0). At pH 7.45,  $PGB_x$  chromatographs as a single component with a retention time equivalent to the lower molecular weight standards. From pH 7.0 down to pH 6.6,  $PGB_x$  chromatographed as a two-component molecular weight system. In addition, the areas of the two components changed as the pH was lowered. Figure 1 shows a progressive increase in the large molecular weight component with lowered pH, while the area of the small molecular weight component decreases.

In a previous study<sup>5</sup>, it was reported that gel filtration of  $PGB_x$  on Sephadex packings using methanol as a carrier solvent yields only one chromatographic peak. Therefore,  $PGB_x$  MEC on S1-300 with methanol was also tried. Table II lists the retention time of molecular weight standards and  $PGB_x$  when chromatographed on S1-300 with methanol. These results show that  $PGB_x$ , when chromatographed in this system, emerges in the void volume suggesting that the molecular weight of the complex is above 5000.

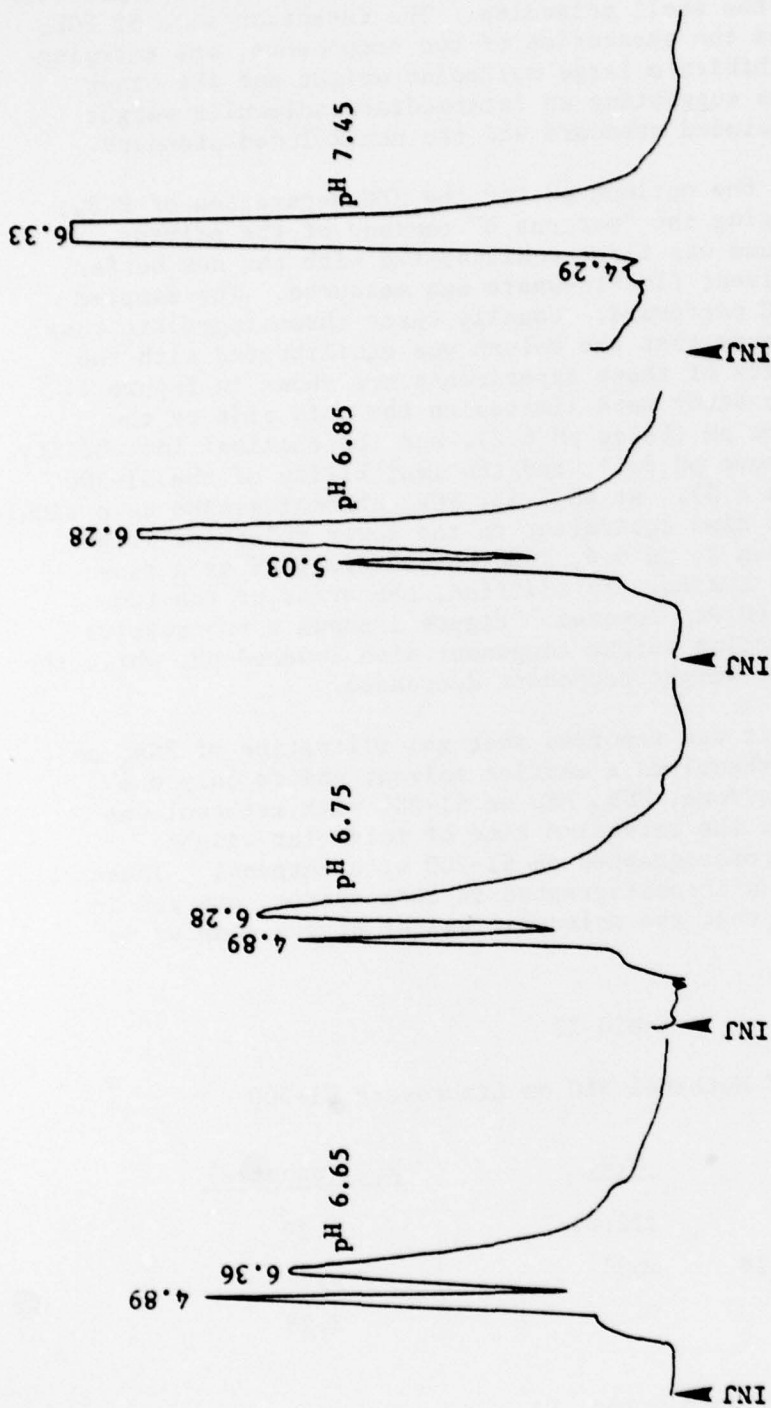
Table II

## Calibration of Methanol MEC on Lichrosorb S1-300

<u>Standard</u>	<u>M.W.</u>	<u>R.T. (minutes)</u>
Diethyl phthalate	222.24	4.29
Polypropylene glycol*	4000	4.27
$PGB_x$		2.28

\*Polysciences, Inc., Warrington, PA

The HPMEC of  $PGB_x$  in aqueous neutral phosphate buffer, described in this report, is similar to that reported for  $PGB_x$  gel filtration on



10  $\mu$ g of PGB<sub>x</sub> was injected and chromatography performed as described under "Experimental." The numbers at each peak indicate peak retention time in minutes. The time of sample injection is indicated on each chromatogram.

Figure 1 - THE EFFECT OF pH ON HIGH PERFORMANCE MOLECULAR EXCLUSION CHROMATOGRAPHY OF PGB<sub>x</sub>

Sephadex in a similar buffer system<sup>5</sup>. The advantage of the HPMEC over gel filtration is the use of a noncompressible packing. This type of packing is compatible with pressure delivery of the solvent, which in turn permits the chromatography to proceed at a predetermined constant flow-rate resulting in a rapid separation with reproducible retention times: two criteria for good analytical chromatography. With this method, it is now possible to determine the relative concentrations of high molecular and low molecular weight components in the PGB<sub>x</sub> complex, thus giving some indication of its homogeneity.

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