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PICOGRAM DETECTION OF EICOSANOIDS BY ULTRAVIOLET ABSORBANCE AFTER NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

COMPARISON WITH CONVENTIONAL-BORE COLUMN

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SUMMARY

Mobile-phase variations were employed to achieve optimal separation by narrow-bore reversed-phase high-performance liquid chromatography of eleven eicosanoids. Separation and quantitation by ultraviolet absorbance at 190 nm using conventional-bore ODS columns were compared. Using the improved sensitivity obtained by means of the narrow-bore column, i.e. 250-pg detection limits of a standard solution, analysis of eicosanoids in kidney medulla was achieved. Parallel quantitation by radioactivity, using [1-^{14}C]arachidonic acid as substrate, was applied.

INTRODUCTION

The involvement of eicosanoids has been implicated in numerous physiological and pathological processes. Consequently, several attempts to quantitate eicosanoids directly after high-performance liquid chromatographic (HPLC) separation have been made. Most of these studies have involved derivative formation to achieve sensitivity in the nanogram range of the eicosanoids by elec-

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trochemical [1,2], ultraviolet (UV) [3—6], or fluorescence [6—9] detection. Although picogram detection with some fluorescent derivatives has been accomplished [6,7], the derivatization reaction is often not quantitative at the picogram level [7]. Others have used UV absorbance directly for detection in the nanogram or microgram range [10—17]. After HPLC separation, quantitation in biological samples was accomplished by subsequent radioimmunoassay [18,19], gas chromatography—mass spectrometry [20], or radioactivity of converted radiolabeled substrate [5,16,17,21—24]. Previously, we reported quantitation of 6-ketoprostaglandin $F_{1\alpha}$ ($6KPGF_{1\alpha}$), the stable hydrolysis product of prostacyclin (PGI_2), in plasma by direct UV absorbance [25]. However, detection sensitivity precluded quantiation of other eicosanoids.

With the advent of small-diameter columns, there have been reports of increased sensitivity of detection [26,27]. This increased sensitivity is due to the higher concentration of solute reaching the detector cell, since there is less dilution by the mobile phase. In this report, an HPLC separation using a narrow-bore C_{18} column was accomplished for eleven eicosanoids: $6KPGF_{1\alpha}$; 6-keto-prostaglandin E_1 ($6KPGE_1$); 6,15-diketoprostaglandin $F_{1\alpha}$ ($6,15DiKPGF_{1\alpha}$); thromboxane B_2 (TxB_2); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$); prostaglandin E_2 (PGE_2); 15-ketoprostaglandin $F_{2\alpha}$ ($15KPGF_{2\alpha}$); prostaglandin E_1 (PGE_1); prostaglandin D_2 (PGD_2); and 15-ketoprostaglandin E_2 ($15\ KPGE_2$). In addition, the UV detection sensitivity of a narrow-bore column (2.0 mm) was compared with that of conventional-bore column (4.6 mm). Subsequently, narrow-bore HPLC separation, with direct absorbance quantitation at 190 nm, of the HPLC effluent was applied to detect several eicosanoids from the kidney medulla. Correlative radioactive quantitation of the HPLC effluent as well as the use of a cyclo-oxygenase inhibitor was used as a verification of the method.

EXPERIMENTAL

Reagents and materials

Methanol, chloroform, ethyl acetate, acetonitrile, water and orthophosphoric acid were HPLC grade (Fisher Scientific). Kreb's solution salts were all ACS grade (Baker Chemical). Prepacked, 1-g LH-20 columns (Isolabs) were used for sample purification. Meclofenamic acid (Warner-Lambert) and eicosanoid standards (Upjohn) were kind gifts of Dr. Roger Westland and Dr. John Pike, respectively. The specific activity of [1-^{14}C] arachidonic acid (AA) (New England Nuclear) was 51.6 mCi mmol^{-1}.

Chromatographic apparatus

Mobile phase, in the solvent reservoir, passed through a 2-μm, 1.5-mm solvent inlet filter (Alltech) by way of PTFE tubing (1.5 mm × 0.8 mm I.D.) to a six-position, 0.8-mm bore, PTFE low-pressure Rheodyne rotary valve. A Beckman Model 112 reciprocating pump delivered the selected solvent to a Rheodyne Model 7125 injector equipped with a 10-μl sample loop. Either a 250-mm conventional-bore (4.6 mm) or narrow-bore (2.0 mm) Altex Ultrasphere 5-μm particle size, C_{18} column, protected by a 2-μm precolumn filter (Upchurch), was used for sample separation. Absorbance detection at 190 nm of the HPLC effluent was accomplished using a Kratos Model 773 variable-

wavelength UV—Vis spectrophotometer equipped with an 8-μl flow cell having a 1-cm pathlength. Sensitivity on the detector was adjusted between 0.007 and 2.500 absorbance units full scale (a.u.f.s.). Absorbance changes, resulting in the chromatogram, were recorded on a single-pen recorder (Kipp-Zonen, Model BD 40/04) set at 1 mV full scale deflection (f.s.d.) and operated at a chart speed of 5 mm min^{-1}. Radioactive quantitation was done by collecting 1-min fractions of the detector effluent, mixing with 4 ml scintillation cocktail (Scintiverse II, Fisher Scientific) followed by ^{14}C-counting for 2 min in a scintillation counter (Beckman, Model LS 7500).

Mobile phase systems were prepared by mixing proportions of acetonitrile with 0.0025 *M* phosphoric acid. Eicosanoid standards (25 pg/μl—25 ng/μl) as well as purified biological extracts were dissolved in water—acetonitrile (70:30).

Biological sample preparation

New Zealand white rabbits (3—4 kg) were killed by cervical dislocation and their kidneys quickly removed. The kidney medulla was dissected free from the cortex, finely cut and incubated in 2.5 ml Kreb's solution per 500 mg tissue wet weight containing 0.5 μCi/ml [1-^{14}C] AA for 1 h at 37°C under an oxygen—carbon dioxide (95:5) atmosphere as previously described [17]. Meclofenamic acid was dissolved in Kreb's solution at a concentration of 1 mg/ml for inhibition studies [28].

After incubation, the medium was acidified to pH 3.5 with 0.8 *M* phosphoric acid and extracted once by vigorous shaking using 4 vol. ethyl acetate. The organic phase was dried under nitrogen, reconstituted in 0.5 ml chloroform—ethyl acetate (85:15) and applied onto a chloroform pre-equilibrated LH-20 column. After sequential washing of the column with an additional 3.5 ml chloroform—ethyl acetate (85:15), 10 ml chloroform, and 2 ml methanol, the eicosanoid fraction was collected with 1.5 ml methanol. The methanol fraction was dried under nitrogen and stored until the HPLC separation. Recoveries using this purification scheme were 51%, 81% and 86% for $6KPGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2, respectively, as previously reported [25].

RESULTS AND DISCUSSION

Separation

By ionic suppression of the carboxyl group with acidification of the mobile phase by phosphoric acid, separation of eicosanoids by reversed-phase chromatography can be governed by mobile-phase polarity, i.e. water—acetonitrile ratios [11]. Fig. 1 shows the effect of changing the percentage from 27.5% to 35.0% of acetonitrile in 0.0025 *M* phosphoric acid on the capacity factors for the eleven eicosanoids using a 250 × 2.0 mm ODS column. A similar profile is seen by conventional-bore ODS columns [11], with the curves shifted slightly to the right in Fig. 1. That is, a higher percentage of acetonitrile was necessary to give the same k' value. Likewise, the optimal solvent strength for the narrow-bore column was a 69:31 ratio of 0.0025 *M* orthophosphoric acid—acetonitrile compared to a 67.2:32.8 ratio for a conventional bore column. At optimal solvent strengths for both columns, migration of the various eicosanoids was governed by their hydrophobicity. Therefore, the more polar two series,

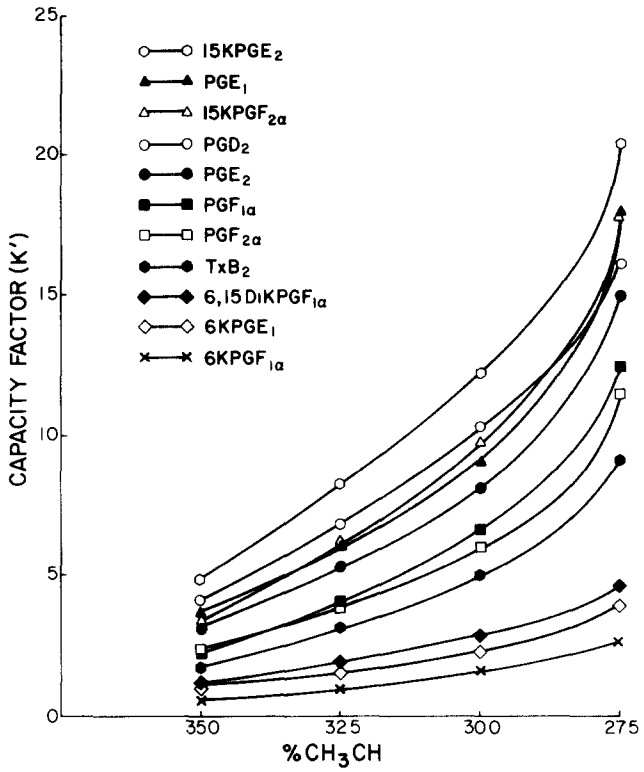

Fig. 1. Relationship between capacity factor (k') and amount of acetonitrile in the mobile phase. Column: 5 μm ODS Ultrasphere, 250 × 2.0 mm; mobile phase: various percentages of 0.0025 *M* orthophosphoric acid—acetonitrile, flow-rate: 0.3 ml/min.

eicosanoids derived from arachidonic acid, having two carbon—carbon double bonds eluted before the one series, products of eicosatrienoic acid which contain only a single alkene group, i.e., $PGF_{2\alpha}$ eluted before $PG_{1\alpha}$ and PGE_2 prior to PGE_1. Furthermore, the addition of either an hydrophilic hydroxyl or carbonyl group lowered the capacity factor, i.e. $6KPGF_{1\alpha}$ and $6KPGE_1$ eluted before $PGF_{1\alpha}$ and PGE_1, respectively. Substitution of an oxygen for the hydrogen atom and hydroxyl group at a given position, however, lengthened the retention time. This was illustrated by PGE_2 eluting after $PGF_{2\alpha}$, due to the substitution at C-9, and 6,15 $DiKPGF_{1\alpha}$ after $6KPGF_{1\alpha}$ by the oxygen substitution at position 15.

Quantitation

Table I gives the regression analysis, i.e. $y = mx + b$ where m is the slope and b the intercept, for both diameter columns using peak height in mm normalized at 0.01 a.u.f.s. on the detector. Analysis was done in triplicate for each point over a range of concentrations of 25 pg/μl—25 ng/μl for the 2.0-mm I.D. column and 0.1 –25 ng/μl for the 4.6-mm I.D. column. By comparing the slopes of the regression lines, (m_1/m_2), the increase in sensitivity with the narrow-diameter column is seen to be 3—3.5 fold that of the conventional-diameter column. The higher concentration of eicosanoid reaching the detector cell after

TABLE I

REGRESSION ANALYSIS OF PEAK HEIGHT VERSUS CONCENTRATION OF STANDARD EICOSANOIDS USING NARROW-BORE AND NORMAL-BORE COLUMNS

Eicosanoid	Narrow-bore column			Normal-bore column			m_1/m_2
	b_1	m_1	r_1^2	b_2	m_2	r_2^2	
6KPGF$_{1\alpha}$	85.74	0.081	0.999	98.91	0.028	0.999	2.9
6KPGE$_1$	77.35	0.100	0.999	60.62	0.033	0.999	3.0
6,15DiKPGF$_{1\alpha}$	25.38	0.022	0.999	56.33	0.007	0.995	3.1
PGF$_{2\alpha}$	27.87	0.080	0.999	27.23	0.025	0.999	3.2
PGF$_{1\alpha}$	93.69	0.052	0.999	74.15	0.015	0.998	3.5
PGE$_{2\alpha}$	97.25	0.082	0.999	39.30	0.026	0.999	3.2
15KPGF$_2$	1.63	0.037	0.999	11.67	0.011	0.999	3.4
PGE$_1$	73.50	0.042	0.999	65.03	0.012	0.998	3.5
PGD$_2$	−65.61	0.049	0.999	−61.73	0.014	0.998	3.5
15KPGE$_2$	44.89	0.037	0.999	27.59	0.011	0.999	3.4
Conditions							
Column:	ODS 5 μm Ultrasphere, 250 × 2.0 mm			ODS 5 μm Ultrasphere, 250 × 4.6 mm			
Flow-rate:	0.3 ml/min			1.5 ml/min			
Mobile phase:	0.0025 *M* orthophosphoric acid—acetonitrile (69:31)			0.0025 *M* orthophosphoric acid—acetonitrile (67.2:32.8)			
Detection:	UV 190 nm, 5 sec rise time peak height (mm) normalized to 0.01 a.u.f.s.			UV 190 nm, 5 sec rise time peak height (mm) normalized to 0.01 a.u.f.s.			
Recorder:	1 mV f.s.d.			1 mV f.s.d.			
Sample loop:	10 μl			10 μl			
Concentration:	25 pg/μl—25 ng/μl			0.1—25 ng/μl			

the narrow-bore HPLC separation is illustrated by the chromatograms in Fig. 2. Only 2 ng of each eicosanoid were injected onto the narrow-bore column compared to 8 ng on the conventional-bore column. Both mobile phases were of optimal solvent strength, with all other conditions, except flow-rate, constant.

Biological application

Having realized the improvement in sensitivity using a narrow-bore column, the separation was applied to biological samples. Because of the end absorption of many substances at 190 nm, sample clean-up was necessitated. Purification by LH 20 chromatography, after acidic organic extraction, offers multiple separation mechanisms by solvent alterations [29]. Since impurities in the extract also show a high distribution coefficient for ethyl acetate, separation by partition chromatography is seen by using the chloroform—ethyl acetate mixture with a chloroform-rich gel as the stationary phase and ethyl acetate as the mobile phase. Using chloroform alone, the structural characteristics of eicosanoids are exploited by absorption of their hydroxyl and carboxyl groups, by hydrogen bonding, to the glucose hydroxyls within the LH-20 gel. Therefore, some of the coextracted impurities, having affinity for the chloroform, are removed. Gel permeation with methanol alone as the mobile phase, elutes the eicosanoids by their molecular weight.

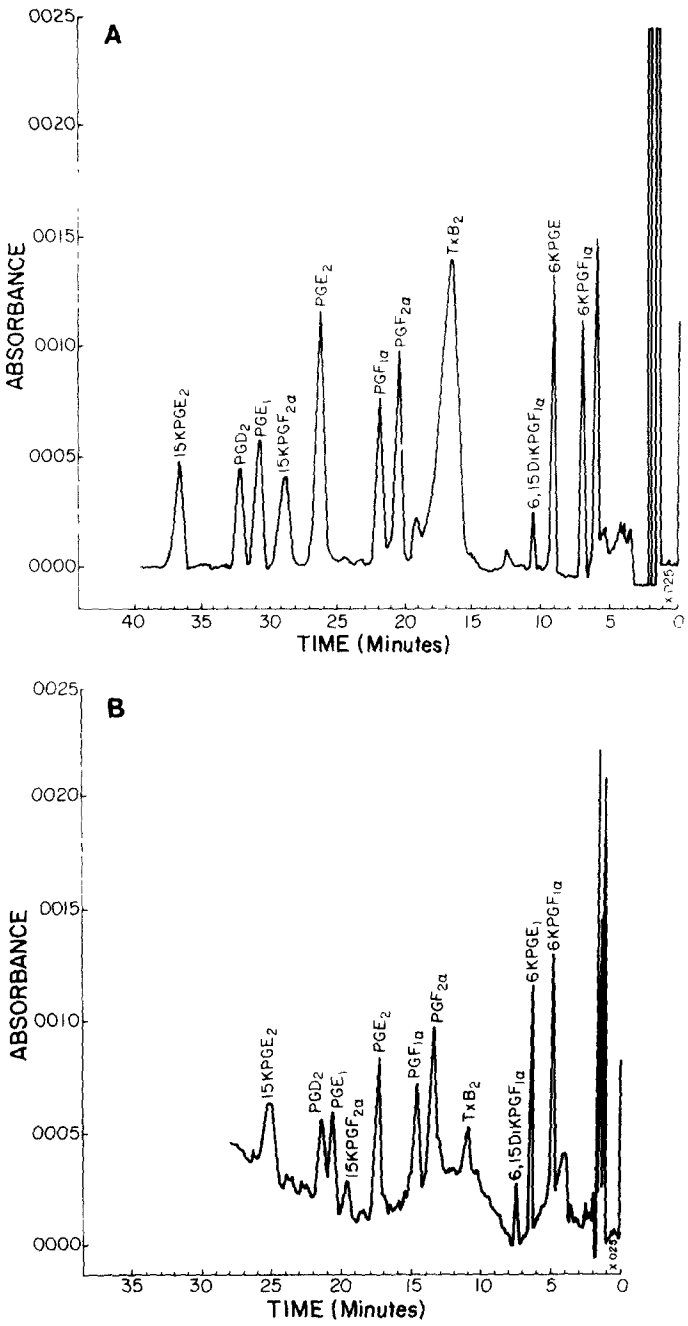

Fig. 2. Chromatograms of eicosanoid standards. (A) Column 250 × 2.0 mm Ultrasphere ODS; flow-rate: 0.3 ml/min; mobile phase: 0.0025 *M* orthophosphoric acid—acetonitrile (69:31); amount: 2 ng of each eicosanoid. (B) Column: 250 × 4.6 mm Ultrasphere ODS; flow-rate: 1.5 ml/min; mobile phase: 0.0025 *M* orthophosphoric acid—acetonitrile (67.2:32.8); amount: 8 ng of each eicosanoid. Detection: UV at 190 nm, 0.025 a.u.f.s.; recorder 1 mV.

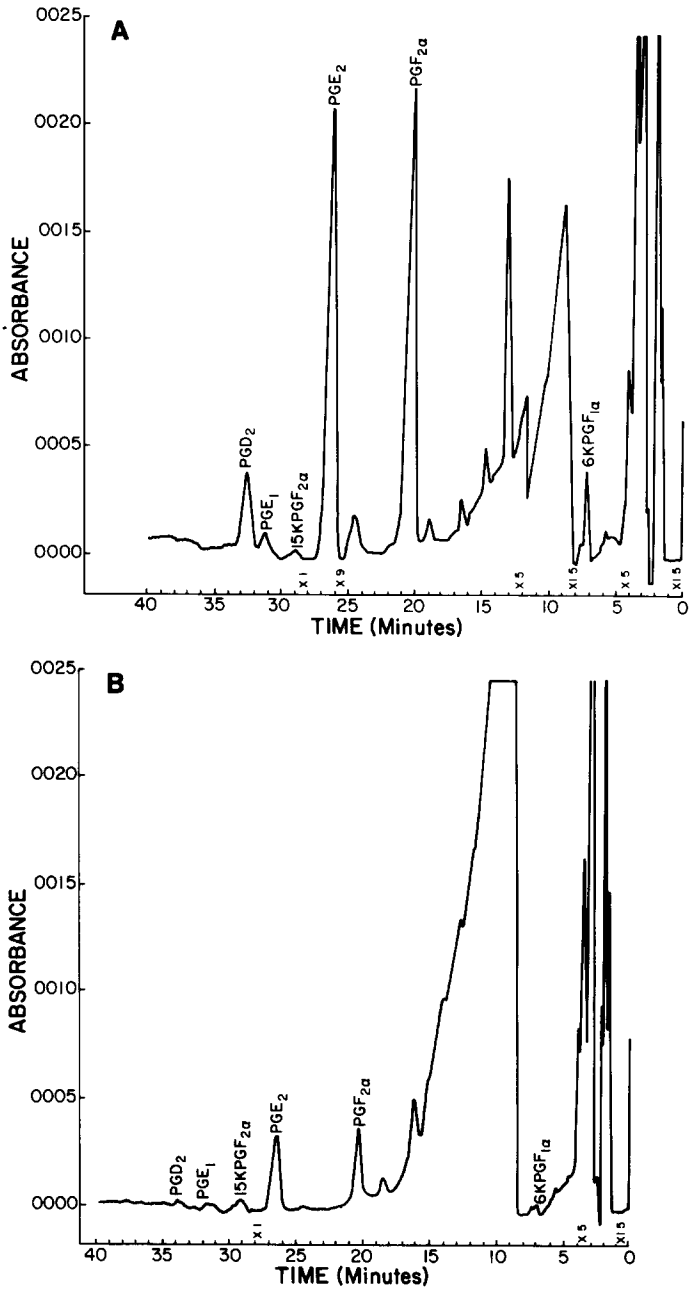

Fig. 3. Chromatogram of kidney medulla extract using narrow-bore HPLC. (A) Control: 56 mg tissue (wet wt.); (B) meclofenamic acid: 1 mg/ml, 67 mg tissue (wet wt.). Conditions: same as Fig. 2A.

Fig. 3. shows the UV absorbance of eicosanoids produced by approximately 60 mg of kidney medulla tissue. When the same sample incubate was injected at twice the concentration, the UV absorbance peaks corresponding to the eicosanoids, doubled. The kidney medulla was selected because of the wide variety

of eicosanoids produced, its pivotal role in maintaining physiological homeostasis, as well as its function in the elimination of potentially toxic substances. As illustrated in Fig. 3A, several eicosanoids were readily detected. Meclofenamic acid, a cyclo-oxygenase inhibitor, showed a marked reduction of eico-

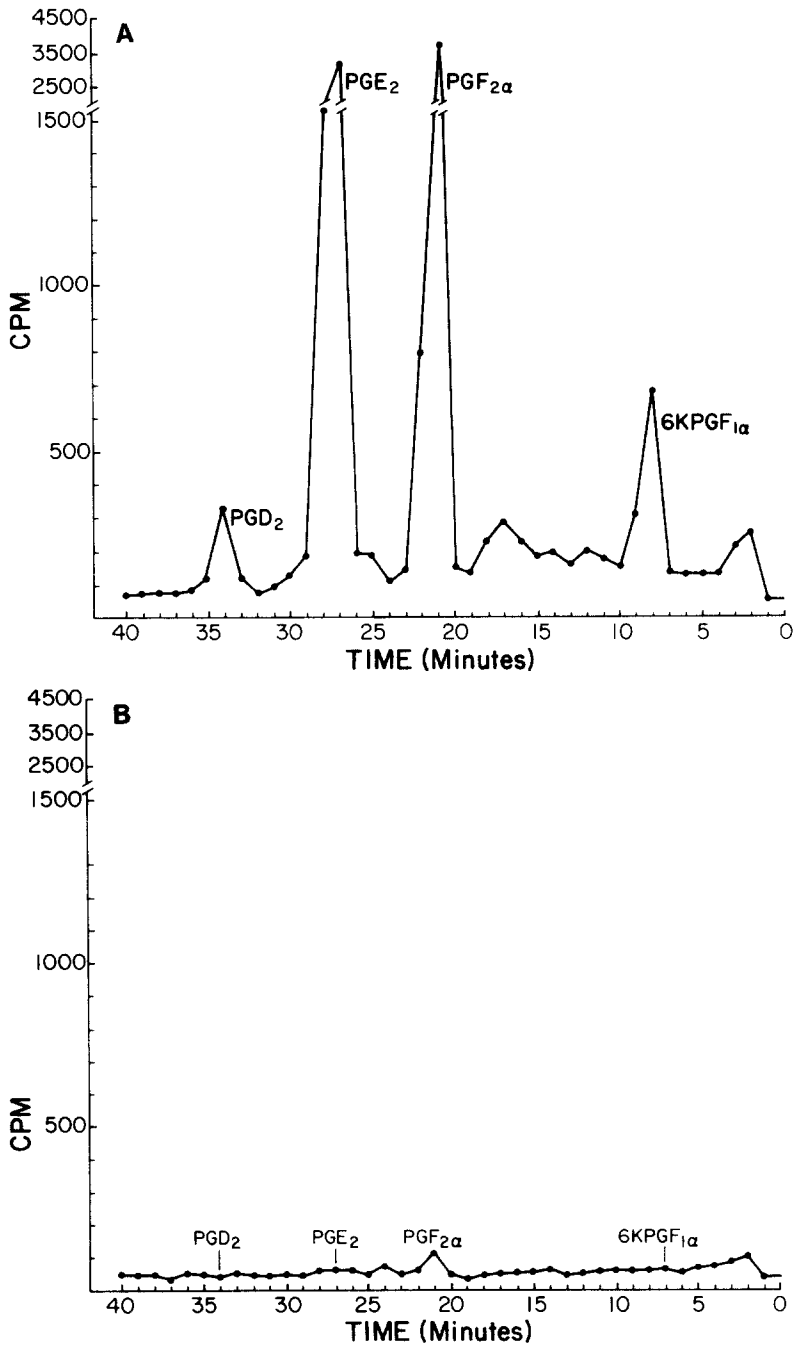

Fig. 4. Radioactivity patterns of HPLC effluent from Fig. 3. (A) Control; (B) meclofenamic acid.

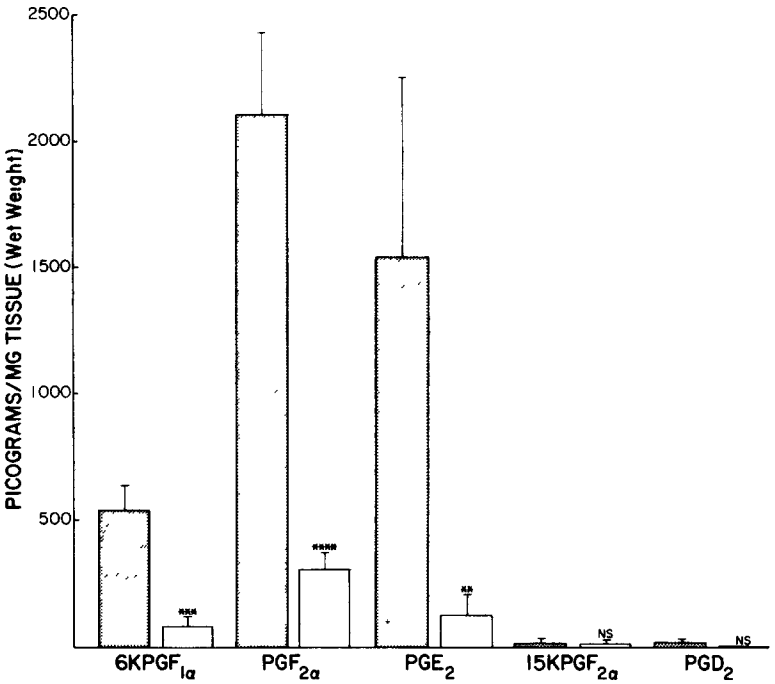

Fig. 5. Pattern of eicosanoids produced by kidney medulla, absorbance quantitation. Mean pg/mg tissue wet weight ± S.E.M. (□) Control (n=5); (▣) meclofenamic acid (n=3). (****) $p<0.005$, (***) $p<0.010$, (**) $p<0.050$, (NS) $p>0.10$.

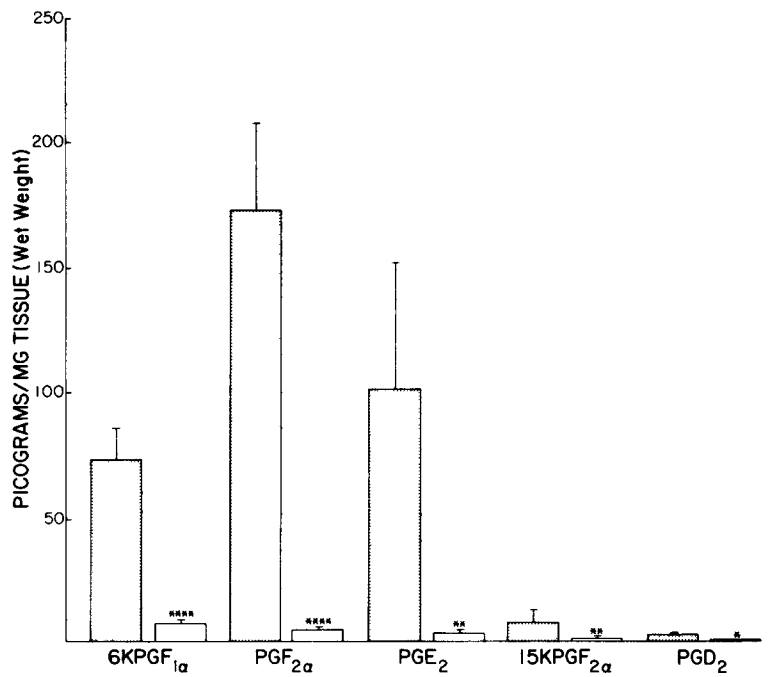

Fig. 6. Pattern of eicosanoids produced by kidney medulla as determined by radioconversion of [1-^{14}C]arachidonic acid. Mean pg/mg tissue wet weight ± S.E.M. (□) Control (n=5), (▣) meclofenamic acid (n=3). (****) $p<0.005$, (**) $p<0.050$, (*) $p<0.10$.

sanoids produced (Fig. 3B). Correlation by conversion of [1-^{14}C] AA into radiolabeled products collected from the HPLC effluent and counted shows a similar production pattern, Fig. 4, with the radioactivity coincident with the absorbance peak.

A one-tailed t=statistic (n=8) showed a significant reduction in eicosanoid levels for both absorbance and radioactive quantitiation after meclofenamic acid, respectively (Figs. 5 and 6). The radioconversion pattern of eicosanoids produced by the kidney medulla is typical of that shown by others [30] as well as being similar to the absorbance detection profile. An approximate ten-fold increase in eicosanoid production is given by absorbance quantitation, reflecting the endogenous release of arachidonic acid by phospholipase A_2, the rate limiting step, as well as that produced by the addition of [1-^{14}C)AA.

CONCLUSIONS

This study demonstrates a method for detecting picogram quantities of eicosanoids using narrow-bore HPLC. Application of this method to kidney medulla tissue shows several chromatographic peaks, although not structurally elucidated by mass spectrometry, identified as eicosanoids by: (1) retention times identical to standard eicosanoids; (2) absorbance, i.e. peak height, proportional to concentration; (3) radioactivity in the HPLC effluent of the absorbance peak; (4) inhibition by a cyclo-oxygenase inhibitor, meclofenamic acid.

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