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High-performance liquid chromatographic detection of myocardial prostaglandins and thromboxanes

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ABSTRACT

Reperfusion of ischemic myocardium is associated with the breakdown of membrane phospholipids and a corresponding increase in arachidonic acid, ultimately resulting in the production of prostaglandins (PGs) and thromboxanes (TXs). However, quantification of these arachidonic acid metabolites has been limited to radioimmunoassay because of their presence in extremely low amounts. In this report, we describe a method suitable to detect sub-picogram levels of 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_2 and TXB_2 in myocardial perfusates by high-performance liquid chromatography (HPLC) with a high-gain photomultiplier and a xenon–mercury arc lamp. Strong Raleigh scatter of the lamp was eliminated by both interference and long-pass cut-off filters. Improved sample clean-up and HPLC separation were achieved by an HPLC system with an Ultrasphere 3-μm C_{18} column.

INTRODUCTION

Arachidonic acid metabolites, prostaglandins (PGs), thromboxanes (TXs), and leukotrienes, commonly known as eicosanoids, play an important role in the regulation of a variety of physiological and biological functions [1,2]. These oxygenated fatty acids are not stored in tissues; rather, they are formed from the membrane phospholipids during cell membrane perturbation under certain pathophysiological conditions [3,4]. Recent studies demonstrated the formation of PGs and TXs in ischemic and reperfused tissues including heart [5,6]. Because of their powerful biological activity in the cardiovascular system, there is a growing body of interest in their isolation from and quantification in heart.

However, because of their presence in extremely low quantities, it has not been possible to accurately estimate PG and TX levels in biological tissues, such as heart. Sophisticated and time-consuming methods, such as gas chromatography–electron-capture detection [7], gas chromatography–mass spectrometry [8] and radioimmunoassay (RIA) [5,9,10], are used to detect picogram levels of these eicosanoids in tissues. Although RIA offers excellent sensitivity in the picogram range, this method is not suitable to the analysis of multiple components in a single experiment.

Recently, high-performance liquid chromatography (HPLC) methods have

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been described for the quantification of PGs and TXs [11–15]. Detection limits of these procedures vary from nanogram to 60 picogram levels of injected dose. Unfortunately, PGs and TXs occasionally are present in sub-picogram quantity in tissues, which make these methods unsuitable.

We report here a method for detection of picogram quantities of PGs and TXs in heart by HPLC. This method, based on several previously published methods [11,12,15], has been modified extensively with respect to extraction techniques as well as in HPLC detection. Using this method, we have been able to detect sub-picogram levels of PGs and TXs in heart.

EXPERIMENTAL

Materials

Radioactive PGE_2 was obtained from Amersham (Arlington Heights, IL, U.S.A.). The authentic standards of 6-keto-$PGF_{1\alpha}$, $PGE_{1\alpha}$, PGE_2 and TxB_2 were purchased from Serdary Research Labs. (Port Huron, MI, U.S.A.). Panacyl bromide was obtained from Molecular Probes (Eugene, OR, U.S.A.) and was purified by adding 10 mg of this compound to 10 ml of acetonitrile–water–acetic acid (55:45:0.1, v/v/v). The mixture was vortex-mixed and centrifuged at low speed at room temperature, and the supernatant was collected. This procedure was repeated three times. The pooled supernatant was applied to a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.) and washed with 10 ml of acetonitrile–water–acetic acid (55:45:0.1 v/v/v). The eluted panacyl bromide was extracted with equal volumes of diethyl ether and centrifuged, and the ether supernatant was dried under a stream of nitrogen at 40°C. All the solvents used in this experient were of HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Water was purified with a Milli-Q system. All other chemicals were of analytical grade.

Methods

Sample collection. Isolated rat heart was perfused by the Langendorff technique, using Krebs–Henseleit bicarbonate buffer (KHB) (pH 7.4) as described elsewhere [16]. Hearts were initially perfused for 15 min to allow stabilization and for collection of perfusate samples for baseline measurements. Global ischemia was then induced by terminating the coronary flow for 60 min, which was followed by 60 min of reperfusion. Perfusate samples were also withdrawn after the ischemic insult and during the reperfusion phase. Samples were collected in test tubes containing indomethacin (10 μg/ml).

Extraction. A 3-ml volume of perfusate sample was acidified to pH 3 with hydrochloric acid and applied to a preconditioned Sep-Pak C_{18} cartridge (Waters Assoc.) for extraction of PGs and TXs according to the method described by Powell [13,14]. The samples were washed with 20 ml of water, 15% aqueous ethanol, light petroleum (30–60°C) and finally with light petroleum–chloroform (65:35, v/v). PGs and TXs were eluted with 10 ml of methyl formate and then evaporated under nitrogen at 40°C.

Derivatization and sample clean up. The evaporated samples were derivatized with panacyl bromide according to the method described by Engels *et al.* [11] and Watkins and Peterson [15]. The dried extract was redissolved in 400 μl of acetonitrile

and allowed to react with 75 μl of panacyl bromide in tetrahydrofuran (0.1 mg/ml) and 2 ml of triethylamine (Fluka, Ronkonkoma, NY, U.S.A.) at 40°C for 2 h. The samples were then loaded onto a preconditioned silica Sep-Pak cartridge, washed with a mixture of 10 ml of dichloromethane–methanol (100:1, v/v), and eluted with 3 ml of acetonitrile–methanol (85:15, v/v). The fluorescent PGs and TXs were dried under nitrogen at 40°C, brought up in 0.5 ml dichloromethane–methanol (100:1, v/v), and re-extracted on a new preconditioned silica Sep-Pak cartridge.

The eluates were dried under nitrogen at 40°C and derivatized according to the method described by Pullen *et al.* [12]. A 200-μl volume of 2% methoxamine hydrochloride in pyridine (Pierce, Rockford, IL, U.S.A.) was added to the evaporated extract, vortex-mixed for *ca.* 1 min, and allowed to react for 16 h. The pyridine was evaporated at 40°C under nitogen, and 1 ml of water and 2 ml of diethyl ether were added, mixed for 1 min, and then centrifuged. The ether layer was saved, whereas the water layer was re-extracted with 2 ml of diethyl ether. The pooled ether layers were evaporated under nitrogen at 40°C and redissolved in 200 μl of acetonitrile.

HPLC. A 25-μl volume of the sample was injected into a Beckman Ultrasphere ODS C_{18} (3-μm particle size, 7.5 cm × 4.6 mm I.D.) column in a Waters chromatograph, equipped with a Model 820 full-control Maxima computer system, satellite Wisp Model 700 injector, Model 490 programmable multi-wavelength UV detector (set at 253 nm), two Model 510 pumps and a Bondapak C_{18} Guard-Pak pre-column. The fluorescent derivatives were detected by a McPherson Model FL-750 fluorescent detector with a high-gain photomultiplier and a xenon–mercury arc lamp. The fluorescent signal was increased due to a lamp emission maximum overlapping the panacyl bromide derivative absorption maximum at 365 nm. Strong Raleigh scatter of the lamp was eliminated by both interference and long cutoff filters to further improve the signal-to-noise ratio.

Radioactive [^{14}C]PGE_2 was used as internal standard. Radioactivity was detected by a Berthold LB506C radioactive monitor and NEC Powermate I computer.

The flow-rate was adjusted to 1 ml/min. Samples were eluted for 40 min with acetonitrile–water–acetic acid (55:45:0.1, v/v/v), and then a linear gradient to acetonitrile–water–acetic acid (75:25:0.1, v/v/v) was applied for the next 29 min. Hydroxy fatty acids were eluted with acetonitrile–acetic acid (100:0.1, v/v). The column was then equilibrated with acetonitrile–water–acetic acid (55:45:0.1, v/v/v) for 20 min.

RESULTS

Separation of prostaglandins and thromboxanes

The methoxamine panacyl derivatives of PG and TX standards were separated by HPLC as shown in Fig. 1. Each standard resulted in two methoxamine derivatives [12], with the retention times of 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_2, and TXB_2 being 24.5 and 29.0 min, 26.5 and 32.0 min, 45.0 and 52.0 min, and 63.5 and 65.5 min, respectively, making the analysis time *ca.* 105 min. In this chromatogram, 8 fmol of each of the above compounds were injected into the HPLC system.

Construction of calibration curve

Using the HPLC system and the fluorescence detector as described in the Experimental section, and employing the Maxima 820 software, a calibration curve for

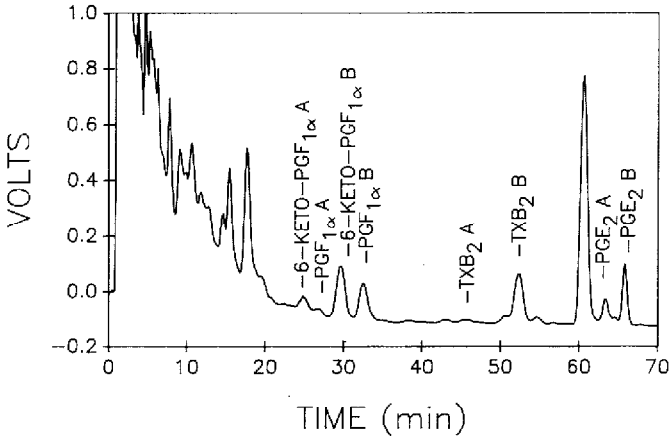

Fig. 1. Separation of methoxamine panacyl derivatives of 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, TXB_2 and PGE_2 standards by reversed-phase HPLC. A volume of 25 μl of a solution, containing 8 fmol of each standard, was injected into a C_{18} column and measured by fluorescent detection, as described in the Experimental section.

each of the following was produced: 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_2 and TXB_2. At least five different concentrations in the four- to ten-fold ranges for each standard were injected and chromatographed as described in the Experimental section. The concentrations of each standard, 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, TXB_2 and PGE_2, were plotted against the peak area obtained. The peak area for each standard was the total area for two methoxamine derivative peaks. In each case, the calibration line was linear, with all points having a small standard deviation and falling on the line. The r values were 0.9823 for 6-keto-$PGF_{1\alpha}$, 0.9828 for $PGF_{1\alpha}$, 0.9815 for TXB_2 and 0.9935 for PGE_2. The major peak seen eluting just before the two methoxamine panacyl derivative peaks of PGE_2 is a reagent peak.

Quantitative estimation of prostaglandins and thromboxanes in heart

Since PGs and TXs are known to be formed from the accumulated arachidonic acid during reperfusion of ischemic myocardium, we assayed these compounds in the perfusate, obtained from the control heart (A), ischemic heart (B) and reperfused heart (C), as shown in Fig. 2. The perfusates collected over indomethacin were quickly frozen and processed, as described in *Methods*, and the methoxamine panacyl derivatives of PGs and TX were loaded onto an HPLC column equipped with a fluorescence detector. As shown in Fig. 2, even though the amounts of TX and PGs were extremely low, they were still detected with reasonable peak heights enough to obtain accurate estimates. As expected, the values of these compounds were near zero in the preischemic control hearts, and they increased slightly after 60 min of ischemic insult. A significant increase in these values was obtained after 60 min of ischemia, followed by 60 min of reperfusion. The accuracy of this method was determined by standard addition technique. Addition of even 5 fmol of any of these compounds were accurately reflected in the peak heights. The limits of detection for 6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, TXB_2 and PGE_2 were 0.2, 0.33, 0.2 and 0.5 fmol injected, respectively. The

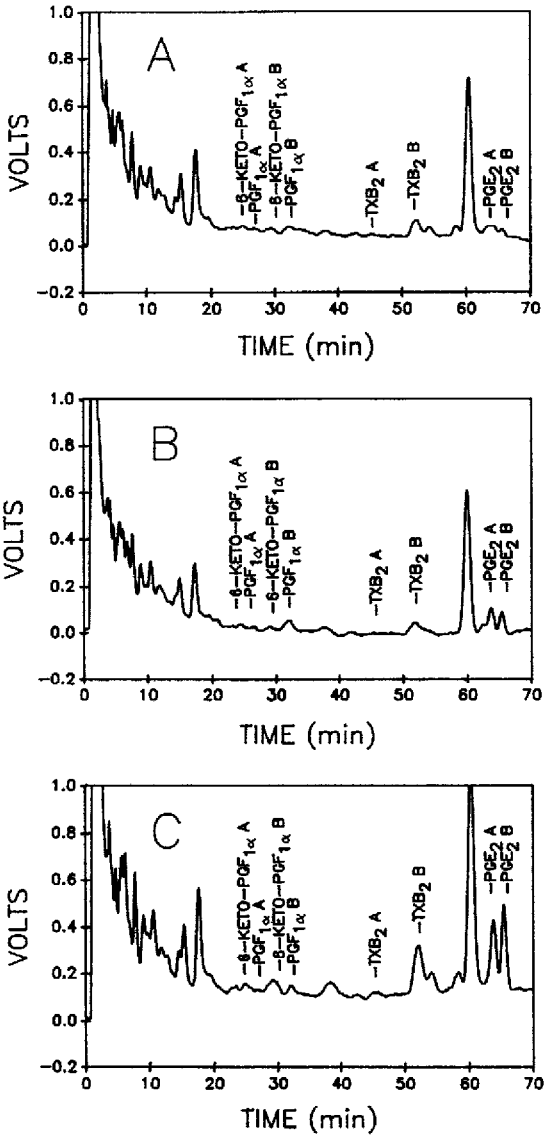

Fig. 2. Separation of derivatized prostaglandins and thromboxane from perfusates of isolated rat hearts. Rat heart perfusates were collected, derivatized, and chromatographed as described in the Experimental section. The injection volume was 25 μl, and the range of the fluorescent detector was 0.1 a.u.f.s. (A) Baseline perfusate; (B) after 60 min of ischemia; (C) after 60 min of reperfusion.

exact values of these compounds as a function of duration of ischemia and reperfusion are shown in Table I. Using [^{14}C]PGE_2 in the collected perfusates, recovery was found to be 17%.

TABLE I

RELEASE OF PROSTAGLANDINS AND THROMBOXANES FROM ISOLATED AND PERFUSED RAT HEART DURING ISCHEMIA AND REPERFUSION

	Basclinc	60 min ischemia (pmol/ml perfusate)	60 min reperfusion
6-Keto-$PGF_{1\alpha}$	0.87 ± 0.20	0.85 ± 0.18	2.80 ± 0.47[a]
$PGF_{1\alpha}$	0.19 ± 0.02	0.24 ± 0.04	0.18 ± 0.03
TXB_2	0.87 ± 0.16	0.63 ± 0.11	8.58 ± 0.78[a]
PGE_2	1.45 ± 0.32	2.99 ± 0.44	9.70 ± 0.92[a]

[a] $p < 0.05$ compared to baseline. Results (pmol/ml perfusate) are expressed as mean ± S.D. of six experiments in each group.

DISCUSSION

The method described in this report is capable of giving an estimate of virtually all the arachidonic acid metabolites of heart via cyclooxygenase pathway that are of major interest in picogram quantities. This method is based on several previously published methods. Several modifications enhance the detection limit so as to allow

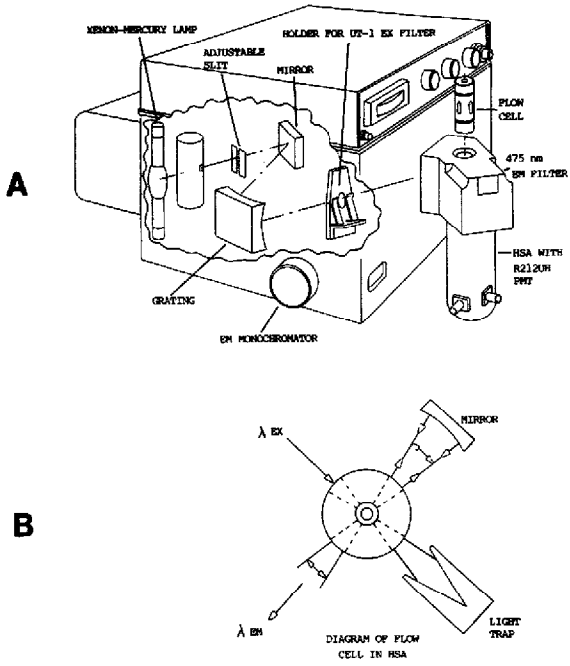

Fig. 3. Diagrams of McPherson FL-450 fluorescent detector with a high-sensitivity accessory (HSA). (A) Diagram of FL-450 fluorescent detector with its filters and HSA; (B) diagram of flow cell in HSA.

detection of PGs and TXs in the range present in the heart. For example, in contrast to the method described by Engels *et al.* [11], we performed fluorescent derivatization first, followed by methoxamination. The clean-up process was also modified using a second silica Sep-Pak chromatography prior to methoxamination. By these modifications, we were able to detect sub-picogram quantities of injected prostaglandins and thromboxanes compared to a minimum detection limit of about 60 pg, as reported previously [11,12,15]. In addition, we noticed some impurity in panacyl bromide, which interfered with the assay procedure by contaminating the derivatized PG peaks. We solved this problem by purifying the reagent as described in the *Methods* section.

The major modification performed was to enhance the sensitivity of the detection system by using a high-gain photomultiplier and a xenon–mercury arc lamp. High-pressure xenon–mercury arc lamp provided a high photon energy near the absorption maximum of the compound, *i.e.* 365 nm. Holographic excitation grating was used to tune the lamp emission maximum to 365 nm. The UV transmitting filter, UT-1, used in this experment can maximally transmit at 275–375 nm. This can eliminate the stray light and second-order effects resulting from grating (n), where $n = 1, 2, 3$, etc. Thus, without this stray light, the high-gain photomultiplier tube is likely to be able to eliminate excessive noise. In addition, this fluorescent detecting system is equipped with a excitation light trap and a reflecting mirror to double the collection efficiency. A larger angle of light collection is eliminated, since increased scatter noise relative to signal exists at angles other than 90°. Also, the long-pass emission filter was able to eliminate both Raleigh and Raman scatter. The photomultiplier tube in the high-sensitive accessory was a tube with high quantum efficiency at the emission wavelength of interest. The fluorescent detecting system used in this experiment (McPherson, Acton, MA, U.S.A.) is diagrammatically shown in Fig. 3. The output spectrum of the xenon–mercury lamp (Oriel Corp., Stratford, CT, U.S.A.) is shown in Fig. 4. This instrument system also improved the signal

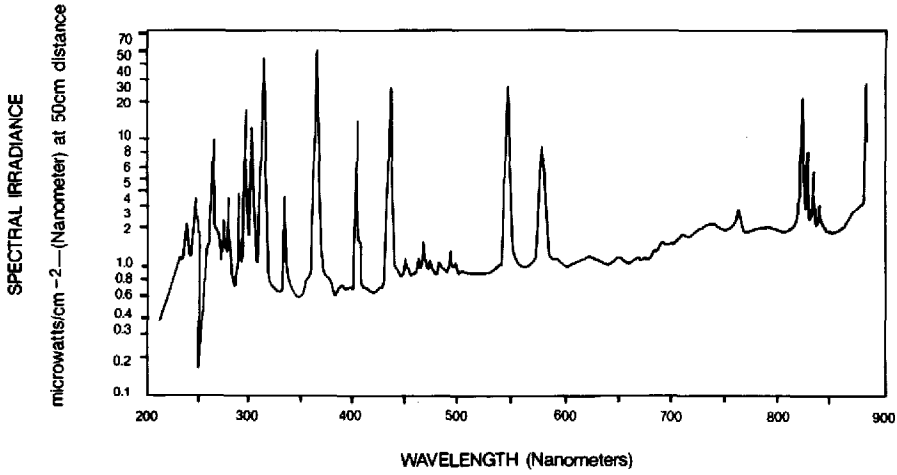

Fig. 4. Output spectra of a 200-W xenon–mercury lamp. Spectral irradiance of a xenon–mercury lamp is plotted against wavelength. Note the spike around 365 nm.

(S)-to-noise (N) ratio significantly, allowing us to measure very low levels of PGs and TXs as described below:

$$\frac{S}{N} = \frac{S}{\sigma_n} \frac{2.3\, I_0\, \theta F(\theta) g(\lambda) elc}{(\sigma^2 s + \sigma^2 f + \sigma^2 dc + \sigma^2 b)^{1/2}}$$

where

I_0 = source intensity: xenon–mercury lamp provides 100-fold excess energy
θ = photoluminescence efficiency: this was improved by improved clean-up technique
$F(\theta)$ = solid angle of fluorescence: this was improved by increasing light collection of high-sensitive accessory, because signal was collected only at 90° and 270° to the incident light (these angles have lowest scatter)
$g(\lambda)$ = detector efficiency at emission wavelength
e = extinction coefficient of the compound
l = path length
c = concentration
σ = standard deviation of the noise
σ^2 = variance
dc = dark current
f = flicker noise of lamp
s = shot noise of lamp
b = back ground noise of the mobile phase

Reperfusion of ischemic tissue is known to be associated with the accumulation of arachidonic acid released from the membrane phospholipids [5,6,17]. This is particuarly important in the heart, because the accumulated arachidonic acid leads to the formation of several arachidonic acid metabolites via the cyclooxygenase pathway [18]. Some of these eicosanoids are beneficial to the heart, whereas others may cause further injury to the heart by several different mechanisms. Despite many investigations the precise mechanisms of the myocardial reperfusion injury are not known [19]. It is quite possible some of these metabolites contribute to reperfusion injury either directly or indirectly through the formation of cytotoxic oxygen-derived free radicals. It has been extremely difficult to examine the pathogenesis of such injury because of the lack of proper methods for identifying these arachidonic acid metabolites in such a low amount. In this paper, we have described a method suitable for assaying picogram quantities of the four major PGs and TXs, generated during reperfusion of ischemic myocardium, which are likely to play a role in the pathogenesis of ischemic and reperfusion injury.

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