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Note

Separation of six major prostacyclin metabolites by high-performance liquid chromatography

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Prostacyclin, (5-*Z*)-9-deoxy-6,9$_\alpha$-epoxy-Δ5-prostaglandin $F_{1\alpha}$ (PGI_2), a bicyclic ether derivative of arachidonic acid, is produced by blood vessels and endothelial cells [1, 2]. In addition to its antiplatelet aggregatory activity [1], PGI_2 relaxes vascular smooth muscles [3] and lowers systemic blood pressure [4]. PGI_2 has a relatively short half-life and its biological activity in aqueous solution disappears within a few minutes during the course of its transformation to a more stable product, 6-keto-$PGF_{1\alpha}$ [5]. Although PGI_2 is not metabolized during one passage across the lung [6], it is metabolized by 15-hydroxyprostaglandin dehydrogenase and 13,14-dihydroreductase in blood vessels and kidney to yield 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ [7, 8]. Recently, we demonstrated that, in the liver and platelets, PGI_2 is converted to a biologically active metabolite, 6-keto-PGE_1 [8], via 9-hydroxyprostaglandin dehydrogenase, and is also converted to pentanor-$PGF_{1\alpha}$ (γ-lactone) by β-oxidation followed by oxidative decarboxylation [9]. Although high-performance liquid chromatographic (HPLC) separation of PGI_2 [10, 11] and other classical prostaglandins [12, 13] has been described, there has been no method available for the separation of the various metabolites of PGI_2. This report describes a simple and rapid reversed-phase HPLC (RP-HPLC) method for the separation of up to six major metabolites of PGI_2. Concomitantly, the antiplatelet aggregatory activity of 6-keto-PGE_1, before and after RP-HPLC, is monitored, which offers a useful method for the detection of this biologically active metabolite of PGI_2 in the nanogram range in biological fluid.

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EXPERIMENTAL

Apparatus

HPLC was performed on a system incorporating dual pumps (Waters Assoc. Model 6000A, Milford, MA, U.S.A.) and Ultrasphere C_{18}-ODS (octadecylsilyl silica, 25 cm X 4.6 mm I.D., particle size 5 μm, Beckman, Palo Alto, CA, U.S.A.), a WISP 710B automatic injection system (Waters), and a Model SF770 Spectroflow monitor coupled with a Model GM770 variable-wavelength UV monochrometer (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.), operated at 192 nm. Chromatograms were recorded on a Waters Data Module operated at 0.5 cm/min. The ODS reversed-phase column was guarded with a pre-column packed with Bondapak C_{18} Corasil (2.2 cm X 4 mm I.D., Waters). All procedures were executed through a Waters System Controller, which is connected by an Inter-link system with the WISP pumps, detector and data module. Fractions of 0.4 ml were collected at room temperature with an on-line fraction collector (Gilson 800A), and simultaneously monitored for biological activity (e.g. antiplatelet aggregatory activity).

Reagents

HPLC grade water was obtained through a Milli-Q Water Purification System (Millipore, Milford, MA, U.S.A.). Phosphoric acid (H_3PO_4), acetonitrile and methanol (HPLC grade) were obtained from Fisher (Fairlawn, NJ, U.S.A.). Diluted phosphoric acid (pH 2.95) and acetonitrile were filtered through a Millipore filter (aqueous 0.45 μm and organic 0.5 μm, respectively), followed by sonication for 3—5 min to ensure the solvents were free of gas.

Procedure

A solution containing a microgram mixture of 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1, pentanor-$PGF_{1\alpha}$ (γ-lactone), 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1 as free acids (Upjohn, Kalamazoo, MI, U.S.A.) in acetonitrile, was injected and eluted isocratically with a solvent system of diluted phosphoric acid (pH 2.95)—acetonitrile (70:30, v/v). The flow-rate was set at 0.4 ml/min under a pressure of approximately 50 bar, and the eluent was monitored with a UV detector at 192 nm (0.1 a.u.f.s.). Subsequently, individual prostaglandins were injected and eluted under essentially identical situations (0.02 a.u.f.s.) to analyze and plot detection reproducibility in the nanogram range. Peak areas of each metabolite were recorded and identified by their retention time.

RESULTS AND DISCUSSION

A reversed-phase column was chosen over a silicic acid column to avoid co-chromatography of various prostaglandins as reported by Whorton et al. [12]. The chromatographic peaks for 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1, pentanor-$PGF_{1\alpha}$ (γ-lactone), 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1 were separated isocratically with retention times of 17.97 ± 0.35, 21.84 ± 0.41, 23.37 ± 0.30, 24.52 ± 0.53, 28.82 ± 0.87 and 31.20 ± 0.78 min (mean ± S.D.), respectively (average of 18 experiments). These experiments

were performed over the course of one month, and it appeared that the latter three metabolites (i.e. 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1) were more likely than the former three to be eluted at altered retention times in response to slight changes in diluted phosphoric acid pH (ca. 2.93—2.97) A typical HPLC run is presented in Fig. 1. Under our chromatographic conditions, the UV monitoring wavelength of 192 nm appeared to offer the best compromise between efficiency of the detector and maximum sensitivity for each prostaglandin, while avoiding interferences from the solvents. This HPLC method should prove invaluable in analyzing prostacyclin metabolites in in vitro biological samples (Fig. 2). The recovery of over 90% of antiplatelet aggregatory activity of 6-keto-PGE_1 after RP-HPLC indicated that there was no substantial loss of biological activity of 6-keto-PGE_1 during the HPLC separation (Fig. 3). In addition, 6,15-diketo-PGE_1, the metabolite of 6-keto-PGE_1 by the action of 15-hydroxyprostaglandin dehydrogenase in the kidney [14], was well separated by our chromatographic conditions. Nanogram levels of various metabolites can also be detected under this condition at 0.04 a.u.f.s. The possible interference by other polar prostaglandins (e.g. TXB_2 and $PGF_{2\alpha}$), which also absorb spectrophotometrically at 192 nm, was also investigated. Under identical conditions TXB_2 and $PGF_{2\alpha}$ were well separated from all six prostacyclin metabolites with retention times of 35.4 and 41.3 min, respectively. Since the data module stores the retention

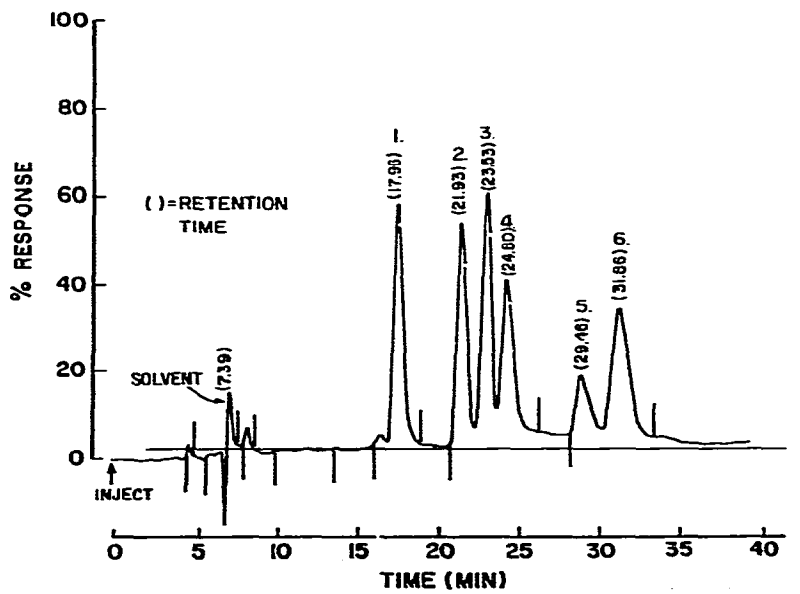

Fig. 1. HPLC separation of prostacyclin metabolites. Five μl of acetonitrile containing the following mixture: (1) 6-keto-$PGF_{1\alpha}$ (1.25 μg); (2) 6-keto-PGE_1 (0.625 μg); (3) pentanor-$PGF_{1\alpha}$ (γ-lactone 1.25 μg); (4) 6,15-diketo-$PGF_{1\alpha}$ (3.75 μg); (5) 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ (12.5 μg) and (6) 6,15-diketo-PGE_1 (5 μg) were injected into an Ultrasphere-ODS column (25 cm × 4.6 mm I.D.) by the use of an automatic injector (Waters Assoc. WISP 710B). Separation was performed isocratically at room temperature at a flow-rate of 0.4 ml/min using acetonitrile—water (pH 2.95) (30:70, v/v); UV detector at 192 nm at 0.1 a.u.f.s.; recorder chart speed 0.5 cm/min. Retention times (min) in parentheses.

time, peak area, peak height and response factor (RF) of all sample peaks formed during every run, it is possible to recall the previously stored peak area and RF values from the data module memory system when calculation of an unknown compound is needed [17].

Since thin-layer chromatography cannot resolve and separate all five metabolites from 6-keto-$PGF_{1\alpha}$ [9], this HPLC method is useful and convenient for the separation of large numbers of major PGI_2 metabolites, and it retains the biological activity (e.g. 6-keto-PGE_1). The latter inhibits platelet aggregation similar to PGI_2 [15], and was found to be four- to five-fold more potent than PGI_2 in the stimulation of renin release [16]. The biological activity from

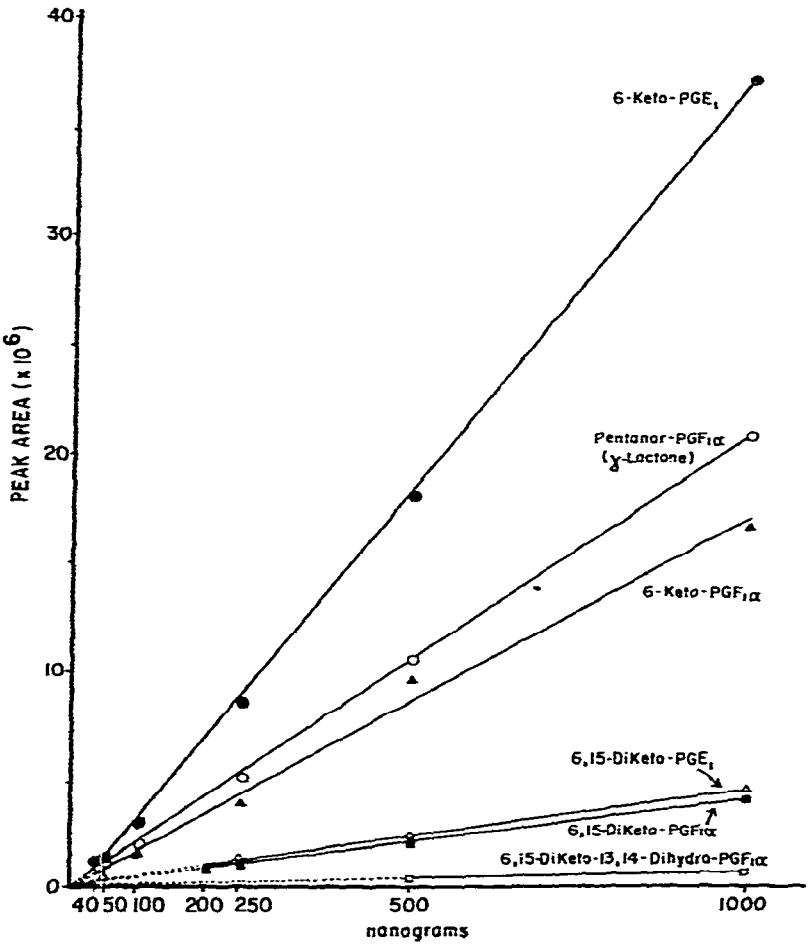

Fig. 2. Relationship of peak area and amount of various prostacyclin metabolites. Samples were dissolved in acetonitrile and injected into an Ultrasphere-ODS column (25 cm × 4.6 mm I.D.) by the use of an automatic injector (Waters Assoc. WISP 710B). Separation was performed isocratically at room temperature at 0.4 ml/min using acetonitrile—water (pH 2.95) (30:70, v/v); UV detector at 192 nm at 0.02 a.u.f.s.; peak area response equivalent to μm^2.

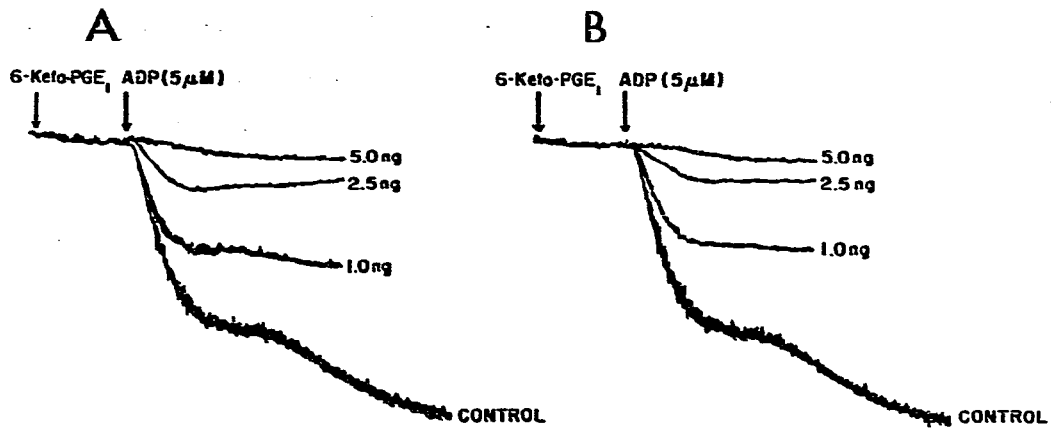

Fig. 3. Biological activity of 6-keto-PGE_1 as monitored by the inhibition of platelet aggregation. Nanogram amount of 6-keto-PGE_1 was injected into the Ultrasphere-ODS column and reported from other prostacyclin metabolites as described in Fig. 2. Fractions of 0.4 ml were collected with an on-line fraction collector (Gilson 800A). Fractions corresponding to 6-keto-PGE_1 were pooled and dried with nitrogen, and the residue was resuspended in Tris buffer (50 m*M*, pH 8.4) and tested for its anti-aggregatory activity on human platelets induced by ADP as described by Wong et al. [15]. (A) Anti-aggregatory activity before injection; (B) anti-aggregatory activity after HPLC separation.

the 6-keto-PGE_1 fractions collected by an on-line fraction collector will be useful for the quantitative in vitro assays for various PGI_2 metabolites isolated from biological samples.

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