Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8315–8319, December 1985
Biochemistry

Femtomole analysis of prostaglandin pharmaceuticals

(icosanoids/microcolumn liquid chromatography/laser-fluorescence detection)

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Contributed by Richard N. Zare, July 30, 1985

ABSTRACT **An analytical method is described whereby the major classes of prostaglandins are fully resolved by microcolumn liquid chromatography and detected at the subfemtomole level by laser-induced fluorescence. The prostaglandins are labeled with the fluorescent reagent 4-bromomethyl-7-methoxycoumarin and are subsequently separated on a high-efficiency fused-silica microcolumn (0.2 mm i.d., 1.06 m length, 150,000 theoretical plates). The optimal chromatographic conditions consist of a 3-μm octadecylsilica packing material and an isocratic mobile phase of 47.6% methanol, 23.8% acetonitrile, and 28.6% water. The prostaglandin derivatives are detected directly on the microcolumn by laser fluorimetry, using a helium/cadmium laser (325 nm, 15 mW) as the excitation source together with a simple filter/photomultiplier optical detection system. In real sample matrices, the prostaglandin $PGF_{2\alpha}$ is readily quantifiable from the detection limit (0.3 fmol) to the formulation strength of the therapeutic agent Lutalyse (Upjohn), spanning more than six orders of magnitude in concentration. The simplicity and general applicability of the present analytical methodology and instrumentation suggest that this technique can be used to attack a wide variety of biomedically important problems with exceptional sensitivity and selectivity.**

Prostaglandins constitute one of several classes of biologically active metabolites of arachidonic acid, an unsaturated fatty acid produced by the enzymatic degradation of cell membrane phospholipids (1, 2). Together with the closely related thromboxanes and leukotrienes, prostaglandins are present at trace levels in nearly all mammalian fluids and tissues and appear to be biosynthesized as required. They have diverse and potent pharmacological properties: some induce contraction and others relaxation of smooth muscle tissue, some promote while still others inhibit blood platelet aggregation. Present and potential uses of prostaglandins as therapeutic agents include the induction of labor, the healing or prevention of gastric ulcers, the treatment of angina, and the control of asthma (3). Unfortunately, the identification and quantitation of prostaglandins present a severe challenge to the analyst because of three factors: the structural similarity between molecules having radically different biological activity (see Fig. 1), the low concentrations at which prostaglandins occur in physiological fluids and tissues, and the shortness of their biological half-lives.

A variety of analytical methods, most importantly radioimmunoassay (RIA), combined gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC), have been used to determine prostaglandins in pharmaceutical and physiological samples. Although not yet widely employed, HPLC with fluorescence detection appears to be gaining popularity for this application because of the high sensitivity and specificity that can be achieved. Because prostaglandins are not naturally fluorescent, they must be derivatized with an appropriate fluorescent label prior to analysis. Yamada *et al.* (4) were able to detect and quantitate less than 10 ng of prostaglandin E_2 labeled with dansyl hydrazine. Turk *et al.* (5) found that 20 ng of prostaglandin E_2 could be detected after derivatization with 4-bromomethyl-7-methoxycoumarin, whereas Wintersteiger and Juan (6) were able to reduce the detection limit to 100 pg of the PGE_2 derivative by using the same method. A related fluorescent label, 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin, was reported by Karlsson *et al.* (7) to give detection limits on the order of 250 pg for prostaglandin derivatives. Comparable sensitivity (50–100 pg) has also been achieved by Hatsumi *et al.* (8), using the fluorescent reagent 9-anthryldiazomethane, and by Watkins and Peterson (9), using *p*-(9-anthroyloxy)phenacyl bromide. With the latter reagent, Cox and Pullen (10) recently reported a quantitation limit of 10 pg of E-type prostaglandins using automated column-switching HPLC with fluorescence detection. The highest sensitivity to date was reported by Tsuchiya *et al.* (11, 12), using the reagent 4-bromomethyl-7-acetoxycoumarin, which formed stable prostaglandin esters that were separated by HPLC and subsequently were hydrolyzed to a more highly fluorescent coumarin analog prior to fluorescence detection. With this method, prostaglandins could be determined in the range 1 nmol–5 pmol, with a detection limit of $\approx$10 fmol.

In these previous studies, conventional packed HPLC columns of 4.6 mm inner diameter and 25 cm length were employed, having a typical figure of merit of 10,000–20,000 theoretical plates. However, HPLC columns of reduced inner diameter and increased length have several advantages for clinical and biochemical applications, including the reduction of injection volume for samples of limited availability, the improvement in detectability due to reduced sample dilution on the chromatographic column, and the potential for higher chromatographic efficiency to improve resolution of complex samples (13). We report here the determination of prostaglandins in therapeutic agents by using HPLC microcolumns of capillary dimensions (0.2 mm i.d. and 1.06 m length), which routinely achieve chromatographic efficiencies of $\geq$150,000 theoretical plates. The prostaglandins are derivatized with the fluorescent label 4-bromomethyl-7-methoxycoumarin and detected with high sensitivity by laser-induced fluorescence. The combination of microcolumn HPLC and laser-fluorescence detection permits unparalleled resolution of the major classes of prostaglandins with detection limits at the subfemtomole level. This method offers much promise for the determination of other enzymatic oxidation products of arachidonic acid, including the leukotrienes and thromboxanes.

MATERIALS AND METHODS

Reagents. The standard prostaglandins, shown schematically in Fig. 1, were obtained from Sigma. Lutalyse, a

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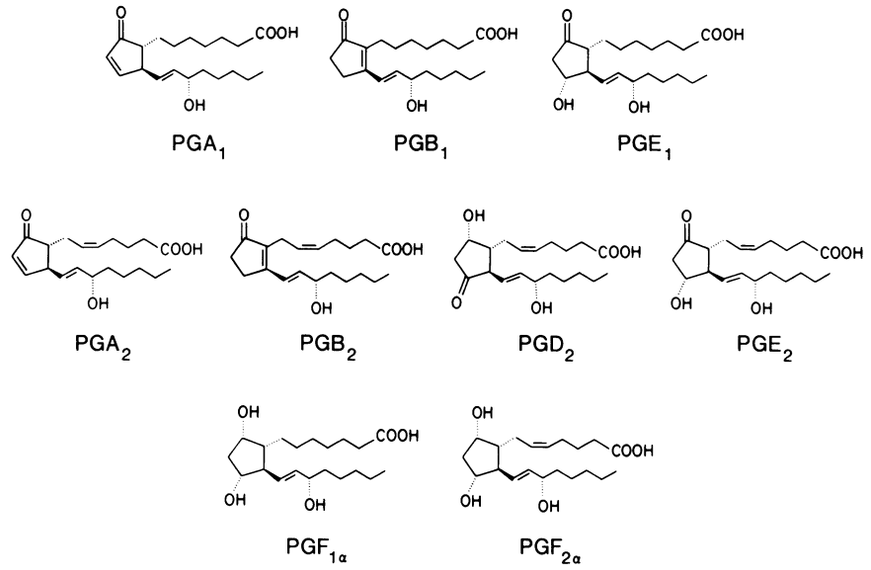

FIG. 1. Structures of prostaglandin standards.

pharmaceutical formulation of the tromethamine salt of prostaglandin $F_{2\alpha}$ used for the synchronization of equine estrus cycles, and Arbacet, an investigational therapeutic formulation of 15(*R*)-15-methylprostaglandin E_2, were kindly provided by R. A. Johnson (Upjohn). The reagents for derivatization, 4-bromomethyl-7-methoxycoumarin and dibenzo-18-crown-6, were purchased from Sigma and used without further purification. Organic solvents employed in this investigation were high-purity, distilled-in-glass grade (Burdick & Jackson, Muskegon, MI); water was deionized and doubly distilled in glass (Mega-Pure System, Corning).

Analytical Methodology. A 0.1 mM solution of prostaglandin standards was prepared in dry acetone and derivatized with 4-bromomethyl-7-methoxycoumarin under conditions similar to those of Dünges and Seiler (14, 15). An aliquot of this solution was added with stirring to a powdered, anhydrous mixture (1:1) of sodium sulfate and potassium bicarbonate (5 g) and dibenzo-18-crown-6 (2.7 mg, 10 μmol). The 4-bromomethyl-7-methoxycoumarin reagent (2.7 mg, 10 μmol) was added and the derivatization was allowed to proceed in the dark at 37°C for 6 hr. Elevated temperatures were avoided in order to minimize dehydration of the prostaglandins, which occurs readily in nonaqueous solvents. The Lutalyse and Arbacet pharmaceutical samples were diluted to an appropriate concentration level (5 ng/ml to 5 mg/ml) and derivatized in a similar manner. Hexanoic, heptanoic, and octanoic acids were introduced as internal standards prior to derivatization of all samples and standards in order to improve the accuracy of quantitation and to facilitate identification of the prostaglandins.

Apparatus. The microcolumn HPLC system with laser-induced fluorescence detection has been described (16, 17). A prototype HPLC micropump (Brownlee, Santa Clara, CA), constructed of two 10-ml stainless-steel syringe pumps, permitted pulse-free solvent delivery at flow rates of 1–1000 μl/min at pressures up to 500 atmospheres (506.5 MPa). By splitting the pump effluent between the microcolumn and a restricting capillary, flow rates as low as 5 nl/min were reproducibly achieved for isocratic separations. Samples (0.5–50 nl) were introduced by a split-injection technique using a 1-μl valve injector (Model ECI4W1., Valco Instruments, Houston, TX). The injection valve, restricting capillary, and microcolumn were maintained at constant temperature (35.0 ± 0.3°C) in a thermostatically controlled water bath.

Microcolumns having 150,000 or more theoretical plates were fabricated from fused-silica tubing (0.2 mm i.d., 1–2 m length) and packed under moderate pressure with a slurry of the chromatographic packing material in an appropriate solvent (18). To optimize the separation of prostaglandins, a variety of packing materials from several manufacturers was examined, including octadecyl, octyl, and cyano stationary phases. The best selectivity for this class of compounds was achieved on 3-μm spherical silica packing material with a heavily loaded octadecylsilane stationary phase without end-capping of the residual silanol groups (Micro-Pak SP-18, Varian). The mobile phase composition was systematically optimized by examining the retention and resolution of the prostaglandins as a function of the concentration of binary methanol/water and acetonitrile/water mixtures and subsequently employing a computer algorithm to predict the optimal ternary mixture. The separation of the standard prostaglandins was found to be optimal when a mobile phase of 47.6% methanol, 23.8% acetonitrile, and 28.6% water was used.

The laser-fluorescence detector employed as the excitation source a helium/cadmium laser (model 4240B, Liconix, Sunnyvale, CA) with 15 mW of continuous-wave output at 325 nm. The UV laser radiation was isolated with a dielectric mirror and focused on the flow cell, which was simply an extension of the fused-silica capillary from which the protective polyimide coating had been removed (17). Sample fluorescence, collected perpendicular to and coplanar with the excitation beam, was spectrally isolated by appropriate interference filters centered at the emission maximum of the 4-bromomethyl-7-methoxycoumarin label (430 nm) and then was focused on a photomultiplier tube (Centronic model Q 4249 B, Baily Instruments, Saddle Brook, NJ). The resulting photocurrent was amplified with a picoammeter (model 480, Keithly, Cleveland, OH) and displayed on a strip-chart recorder (model 585, Linear Instruments, Reno, NV).

RESULTS AND DISCUSSION

In previous studies, we employed the combination of microcolumn HPLC and laser-fluorescence detection for the determination of derivatized carboxylic acids (16) and amino acids (17). These studies showed that this analytical system was capable of sensing attomole amounts of a model analyte, coumarin 440, and that the response was linearly related to

concentration from the detection limit (0.5 nM) to the solubility limit (20 mM) of coumarin 440 in methanol.

In the present study, we applied this combination of analytical techniques for the separation and detection of prostaglandins. Because of the chemical similarity of prostaglandins, complete resolution has not been achieved previously in a single analysis by liquid chromatographic methods. Through the use of very high efficiency microcolumns, in conjunction with thorough and systematic optimization of the stationary and mobile phases, the major classes of prostaglandins have been fully resolved. The optimal separation of standard prostaglandins is illustrated in the lower trace of Fig. 2, which is compared with a blank containing only the internal standards and the by-products of derivatization. It is evident that no significant interferences are coeluted with the standard prostaglandins, which in the separation illustrated in Fig. 2 were detected at the level of 40–125 fmol per component. This high-efficiency separation further reveals the presence of a substantial impurity in the prostaglandin B_2 standard. Additional data obtained by mass spectrometry and combined GC/MS of the original standard after methylation indicate that this impurity may be an analog of PGB_2, but these results were not conclusive.

This analytical methodology has been employed to identify and quantitate prostaglandins in therapeutic agents, as illustrated in Fig. 3. For comparison, the lower trace shows the retention of standard prostaglandins, while the middle and upper traces, respectively, show the chromatograms obtained for Lutalyse, a pharmaceutical formulation of the tromethamine salt of prostaglandin $F_{2\alpha}$, and Arbacet, a formulation of 15(*R*)-15-methylprostaglandin E_2. These samples were diluted to $\approx$5 μg/ml before derivatization with the fluorescent coumarin label, and a 11-nl aliquot corresponding to 120–150 fmol of the prostaglandin derivative was injected onto the chromatographic column. We determined that prostaglandin $F_{2\alpha}$ was readily detectable at femtomole and subfemtomole levels in real sample matrices (Fig. 4). The detector response was linearly related to concentration from the detection limit (0.3 fmol injected, 5 ng/ml of sample derivatized) to the formulation strength of the therapeutic agent Lutalyse (5 mg/ml), spanning more than six orders of magnitude in concentration. This broad linear range facili-

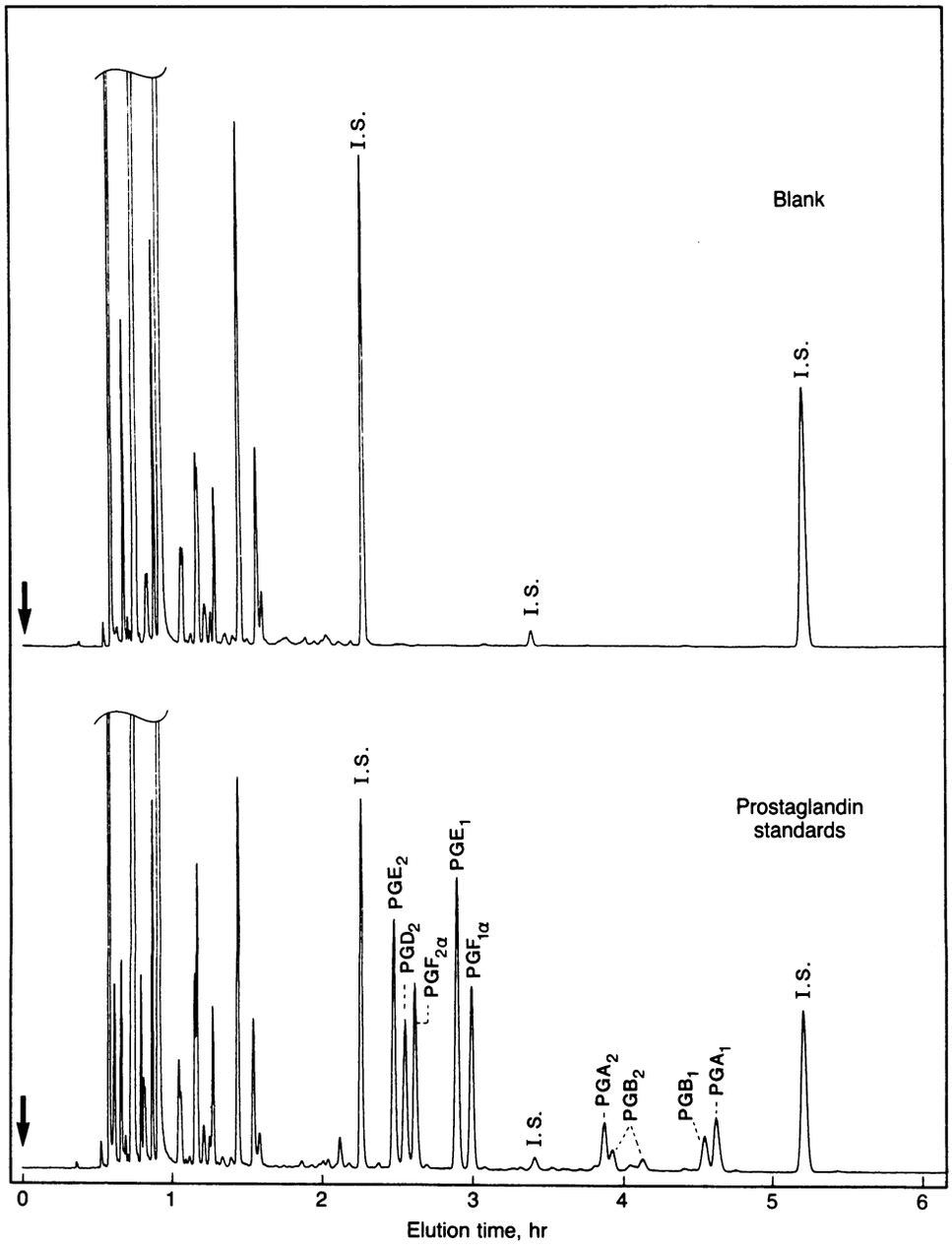

FIG. 2. High-efficiency separation of prostaglandin standards derivatized with 4-bromomethyl-7-methoxycoumarin. The fused-silica microcolumn (0.2 mm i.d., 1.06 m long) was packed with Micro-Pak SP-18 (3-μm particle size). The mobile phase was 47.6% methanol/23.8% acetonitrile/28.6% water, at 35°C and a flow rate of 0.6 μl/min. The injected sample volume was 11 nl, containing 40–125 fmol per component. I.S., internal standard.

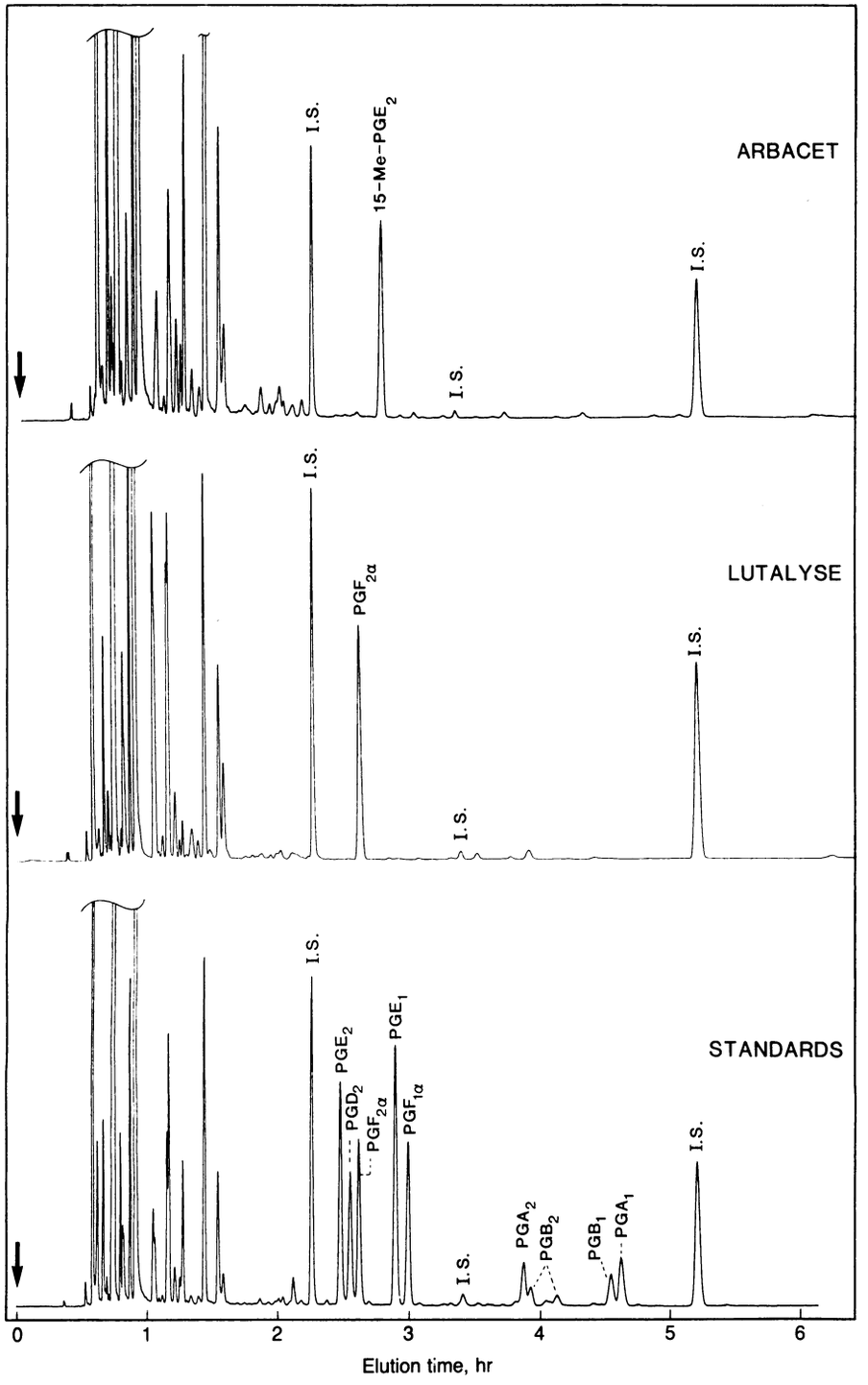

FIG. 3. Identification and quantitation of prostaglandins at the subpicomole level in therapeutic agents. Chromatographic conditions are described in the legend to Fig. 2. I.S., internal standard.

tated the quantitation of both major and minor components in complex sample matrices. Quantitative reproducibility, when measured as the peak height ratio with respect to the internal standard, was better than 4% relative standard deviation both within a single run and between runs, for three sets of five samples containing prostaglandin $F_{2\alpha}$ at 5 μg/ml.

The internal standards were also employed as a retention-index system to facilitate identification of the prostaglandins. Each standard fatty acid was assigned a retention index corresponding to the number of carbon atoms (z) in the molecule multiplied by 100: for example, hexanoic acid has a retention index of 600. The retention index (I) of each prostaglandin then was calculated from its retention time (t_{Ri}) according to the following equation (19, 20):

$$I = 100\left[z + \frac{\log(t_{Ri}/t_{Rz})}{\log(t_{R(z+1)}/t_{Rz})}\right],$$

where t_{Rz} is the retention time of the standard fatty acid immediately preceding the prostaglandin and $t_{R(z+1)}$ is that of the fatty acid immediately following. The retention indices of the derivatized prostaglandins are summarized in Table 1, and their reproducibility was better than 0.15% relative standard deviation. Under these chromatographic conditions, all prostaglandins had retention indices between 600 and 800. Those prostaglandins containing a single hydroxyl

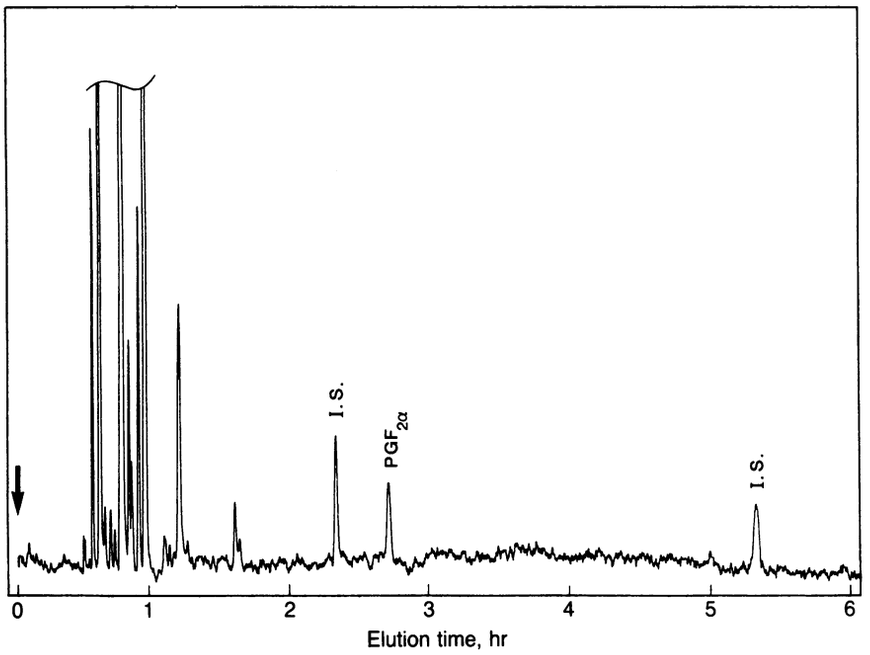

FIG. 4. Detection of 1.8 fmol of prostaglandin $PGF_{2\alpha}$ in Lutalyse pharmaceutical formulation. Chromatographic conditions were the same as described for Fig. 2, except that the fluorescence detector output was amplified 20-fold. I.S., internal standard.

and 800. Those prostaglandins containing a single hydroxyl group had retention indices between 700 and 800, whereas those containing multiple hydroxyl groups had retention indices between 600 and 700. Futhermore, prostaglandins with unsaturation at the C-5 positions (denoted by the secondary subscript; e.g., PGE_2) had retention indices that were notably smaller than those of prostaglandins lacking this unsaturation (denoted by the unitary subscript; e.g., PGE_1). This retention index system may aid in the structural identification of unknown prostaglandins in complex physiological and pharmaceutical samples.

The subfemtomole analysis of prostaglandins in real sample matrices demonstrated herein appears to be the most sensitive detection of such compounds by any chromatographic method reported to date. Moreover, the separation power of microcolumn HPLC is clearly apparent in the ability of this technique to resolve closely related structural isomers, indeed all major classes of prostaglandins, in a single analysis. The present combination of microcolumn HPLC with on-column laser-fluorescence detection is both simple and versatile, offering extremely high sensitivity (a few femtograms of analyte detected), a remarkable linear range (greater than six orders of magnitude), and high resolving power (150,000 theoretical plates). We suggest that this combination of analytical techniques can be applied to a wide variety of challenging problems of clinical and biomedical importance, of which the analysis of prostaglandins is only a representative example.

Table 1. Retention indices of prostaglandins and internal standards derivatized with 4-bromomethyl-7-methoxycoumarin

Species	Retention index
Internal standards	
Hexanoic acid	600
Heptanoic acid	700
Octanoic acid	800
Prostaglandins	
PGA_1	772
PGA_2	731
PGB_1	758
PGB_2	734,745
PGD_2	629
PGE_1	661
PGE_2	622
15-methyl-PGE_2	654
$PGF_{1\alpha}$	669
$PGF_{2\alpha}$	636

Chromatographic conditions are described in the legend to Fig. 2. Retention index is defined in the text.

R.N.Z. thanks E. J. Corey for introducing him to the problems associated with the analysis of arachidonic acid metabolites. This work was supported by Beckman Instruments.

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