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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR PROSTAGLANDINS USING A FLUORESCENCE REAGENT, 4-BROMOMETHYL-7-ACETOXYCOUMARIN

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SUMMARY

High-performance liquid chromatography of prostaglandins is developed in which a fluorescence reagent, 4-bromomethyl-7-acetoxycoumarin is used to perform the high-sensitivity detection. The reagent reacts with prostaglandins and related compounds to form the ester derivatives, which are separated using a reversed-phase system. Each labeled compound eluted from the column is successively hydrolyzed to the fluorescent coumarin derivative, and this fluorophore is introduced into a flow-through fluorometer. Prostaglandins can be determined in the range of at least 1 nmol to 5 pmol, and the detection limit is about 10 fmol. This system is applied to the analysis of prostaglandins in human seminal fluid.

INTRODUCTION

A number of analytical methods [1] have been developed for the detection and determination of prostaglandins (PGs) and related compounds to study their physiological or pharmacological effects. Among these methods, bioassay [2], radioimmunoassay (RIA) [3] and gas chromatography—mass spectrometry (GC—MS) [4] have been widely used for the analyses of PGs. Bioassay has the possibility of detecting unstable compounds such as thromboxane A_2 (TXA_2) and PGI_2, etc. This method, however, provides approxi-

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mate quantitation and low specific results [5]. RIA has been generally used for the routine analyses of PGs and seems to be better than the other methods in terms of sensitivity. The disadvantage of RIA is the use of radioactive isotopes. Furthermore, time-consuming separative pretreatment such as thin-layer chromatography or column chromatography, etc., is necessary for the measurement of each PG present in a single sample, because it is difficult otherwise to obtain the antibody with little cross-reactivity and high affinity [5]. GC—MS can provide not only specific and sensitive results, but also structural information. However, the cost of the equipment is high and skilled personnel are needed [5], and only relatively few samples can be analyzed using this method.

High-performance liquid chromatography (HPLC) provides specific and reproducible results, and offers the possibility to perform the simultaneous analysis of multiple components with closely similar structures such as PGs. The cost of the equipment is also relatively low compared with GC—MS.

PGs do not show sufficient strong absorption or fluorescence in the UV or visible region; therefore, most HPLC methods [6—11] for their determination are too insensitive to be applied to most biological samples. Several HPLC methods using some reagents which react with PGs to form UV-absorbing [12—15] or fluorescent [16] compounds have been reported, but no method shows sufficient sensitivity.

In a previous paper [17] we reported a highly sensitive HPLC system for the determination of carboxylic acids using a fluorescence labeling reagent, 4-bromomethyl-7-acetoxycoumarin (Br-Mac). This present paper deals with the sensitive detection and determination of PGs by HPLC using this system.

EXPERIMENTAL

Reagents and chemicals

Standard PGs and TXB_2 were kindly supplied by Ono Pharmaceutical (Osaka, Japan). Arachidonic acid (AA) was obtained from Wako (Osaka, Japan). Br-Mac was prepared according to the method reported previously [17]. Dibenzo-18-crown-6 was purchased from Aldrich (Milwaukee, WI, U.S.A.). Redistilled water was used for all investigations. All other reagents and solvents used in this study were of reagent grade.

Apparatus

The apparatus for the HPLC system was constructed as reported previously [17]. All parts were obtained from Japan Spectroscopic Co., (Tokyo, Japan). For the separation of labeled PGs, a Model Tri Rotor I high-performance liquid chromatograph equipped with a Model GP-A 30 solvent programmer was used. HPLC separation was carried out with a stainless-steel column (250 × 4 mm) packed with LiChrosorb RP-18 (particle size 5 μm; Merck, Darmstadt, G.F.R.) by a balanced density slurry packing method. The eluate from a column was mixed with 0.1 *N* sodium hydroxide solution using a Model LCP 150 liquid chromatographic pump and hydrolysis was performed through a coil made of stainless-steel tube (10 m × 0.5 mm). Fluorescent hydrolysate was introduced into a Model FP-110 fluorescence spectrofluorometer (ex-

citation 365 nm, emission 460 nm) connected to a Model RC strip-chart recorder.

The operating conditions for HPLC are given in Fig. 2.

Procedure for the determination of PGs

Human seminal fluid (1—5 μl) was deproteinized with 100 μl of methanol containing 16-methyl-PGF$_{1\alpha}$ (500 pmol) as an internal standard. To the supernatant was added 3 ml of water and PGs were extracted with 7 ml of ethyl acetate after acidifying with 0.1 *N* hydrochloric acid (pH 3—4). The ethyl acetate layer was evaporated to dryness and the residue was dissolved in a small amount of methanol. This solution was transferred to a glass ampoule and methanol was evaporated to dryness. About 10 mg of the finely powdered mixture of $KHCO_3$ and Na_2SO_4 (1:1) were added to this ampoule. Then, after adding 50 μl each of an acetone solution of Br-Mac (20—50 nmol) and dibenzo-18-crown-6 (10 nmol), the derivatization was performed by heating at 80°C for 1 h in the dark. After cooling, an aliquot (20—40 μl) of the reaction solution was injected into the HPLC column.

Calibration curves of PGs were prepared using methanol solutions of authentic PGs which were derivatized under the same conditions as those of the human seminal fluid sample.

RESULTS AND DISCUSSION

A scheme for the reaction of Br-Mac with PGs (represented by PGE$_2$) and the principle of the detection system are shown in Fig. 1. PGs are derivatized

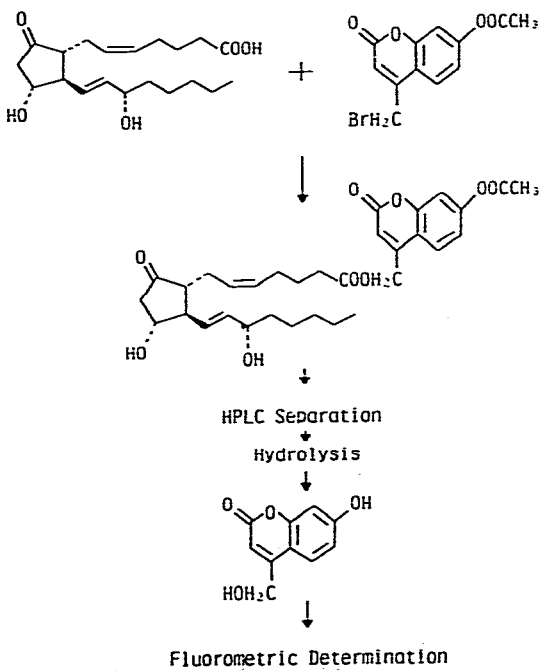


Fig. 1. Scheme of the determination system for prostaglandin E$_2$ using Br-Mac.

to PG-Mac esters and separated by HPLC. As soon as a mixture of PG-Mac esters is separated on a column, each ester is hydrolyzed to the fluorescent coumarin derivative which is introduced into the spectrofluorometer. Turk et al. [16] used 4-bromomethyl-7-methoxycoumarin (Br-Mmc) as a fluorescence labeling reagent for HPLC of PGs. It was suggested, however, that Br-Mmc had some disadvantages [18]; that is, the fluorescence quantum yields of the derivatives depended on the kind of carboxylic acids and were subject to the solvent effect. On the other hand, whatever the kind of PG, the fluorophore to be detected is common to all PGs in this system. Therefore, the fluorescence quantum yields are hardly effected by the PG moieties. Furthermore, compared with Br-Mmc, a gradient elution technique can be used more effectively, because the fluorescence quantum yield of the hydrolysate is not influenced by the mobile phase constitution (the variation of acetonitrile concentration in water), as reported previously [17].

Compounds prepared as described in the procedure for derivatization were subjected to HPLC and it was found that Br-Mac had good reactivity with all PGs and related compounds examined in this work such as $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2, PGD_1, PGD_2, PGB_1, PGB_2, PGA_1, PGA_2, 6-keto-$PGF_{1\alpha}$, TXB_2 and AA.

A mixture of PGs was subjected to derivatization and then HPLC separation. Fig. 2 shows the separation of derivatives of PGH_2 metabolites and

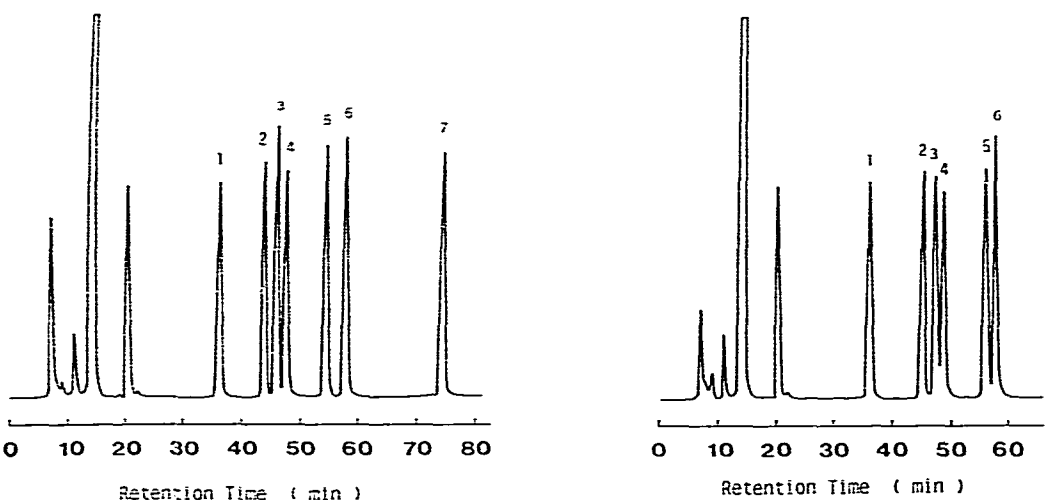


Fig. 2. High-performance liquid chromatographic separation of a mixture of Br-Mac derivatives of prostaglandin H_2 metabolites and arachidonic acid. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = $PGF_{2\alpha}$; 3 = PGE_2; 4 = PGD_2; 5 = PGB_2; 6 = 2-chlorothioxanthone (internal standard); 7 = arachidonic acid. Chromatographic conditions: column, 250 × 4 mm LiChrosorb RP-18 (5 μm); column and mixing coil temperature, 50°C; mobile phase, aqueous acetonitrile solution 30% (0)—90% (99). The gradient was prepared using a Model GP-A30 solvent programmer (Concave 1, 64 min); flow-rate, 1.0 ml/min; 0.1 *N* sodium hydroxide solution flow-rate, 0.4 ml/min; detector, spectrofluorometer (excitation 365 nm, emission 460 nm).

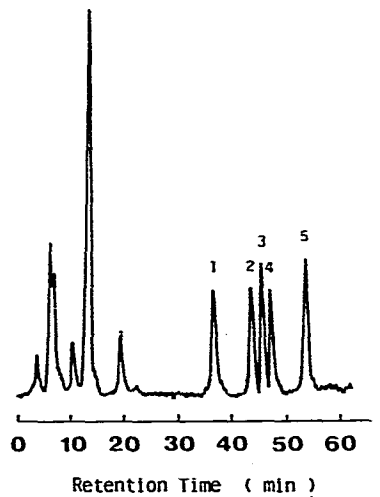
Fig. 3. High-performance liquid chromatographic separation of a mixture of Br-Mac derivatives of prostaglandin 1 groups. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = $PGF_{1\alpha}$; 3 = PGE_1; 4 = PGD_1; 5 = PGB_1; 6 = 2-chlorothioxanthone (internal standard). Chromatographic conditions as in Fig. 2.

AA using a gradient elution technique. Also, a typical chromatogram of PG_1 derivatives is shown in Fig. 3. Excess reagent and its decomposition products eluted faster than PG- and AA-Mac esters. Five PGs could be chromatographed within about 60 min and a good separation was obtained, as shown in Figs. 2 and 3.

The alkali hydrolysis conditions used in this system are suitable for producing a very strong fluorescence of the hydrolysate as described in the previous paper. Due to this property of Br-Mac, a highly sensitive detection of PGs is expected using this system. Each peak shown in Fig. 4 corresponds to 200 fmol of PG. The detection limit might be at about the 10-fmol level, considering the signal-to-noise ratio. Compared with the HPLC method using Br-Mmc in which the detection limit was reported to be about 70 pmol [16], a much higher sensitivity could be obtained by this system.

A typical result of this method is shown in Fig. 5, which is a plot of the peak height of PG-Mac ester relative to 2-chlorothioxanthone (internal standard), versus the amount of the ester converted into the molarity of unlabeled PG. The plot gave a straight line from at least 1 nmol to 5 pmol (more than 200 pmol is not shown in Fig. 5). These results indicate that the derivatization procedure is suitable for quantitative purposes. PGs other than those shown in Fig. 5 also showed approximately the same results. It seems that the smaller the number of hydroxy groups in the five-membered ring, the higher the reactivity of PGs with Br-Mac.

It has been reported that several PGs were contained in human seminal fluid [19—24] and that PG levels in semen might be related to male infertility [25]. Attempts were made to apply this system to the analysis of PGs in human seminal fluid. After deproteinization with methanol, the supernatant was acidified and then extracted with ethyl acetate. The extract was



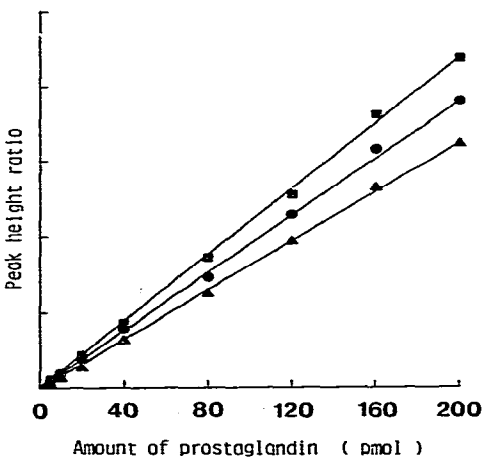


Fig. 4. High-performance liquid chromatogram obtained from a mixture of Br-Mac derivatives of prostaglandin H_2 metabolites. Chromatographic conditions as in Fig. 2. Peak number as in Fig. 2.

Fig. 5. Calibration curves of prostaglandins. ■, PGB_2; ●, PGE_2; ▲, $PGF_{2\alpha}$.

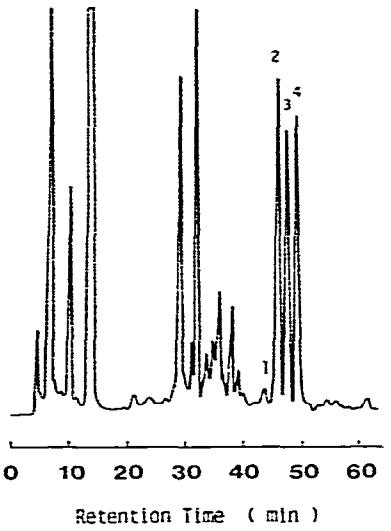


Fig. 6. High-performance liquid chromatogram obtained from human seminal fluid sample. Peaks: 1 = $PGF_{2\alpha}$; 2 = PGE_2; 3 = PGE_1; 4 = 16-methyl-$PGF_{1\alpha}$ (internal standard). Chromatographic conditions as in Fig. 2.

allowed to react with Br-Mac and an aliquot of the reaction solution was injected on to the HPLC column. The results are shown in Fig. 6. Each peak on the chromatogram was identified by comparing the retention time with that of each authentic PG-Mac ester as shown in Fig. 7.

The extract from seminal fluid was also treated with sodium hydroxide solution or acetic acid according to the method of Pike et al. [26]. With alkali treatment, the peaks related to PGE_2 and PGE_1 disappeared and two peaks at the retention times corresponding to PGB_2 and PGB_1 appeared. Conversion of the PGE series to the PGA series was also observed with acid treatment.

Analytical recovery of this method was estimated by adding 100 pmol of $PGF_{2\alpha}$ and 300 pmol each of PGE_2, PGE_1, PGB_2 and PGB_1 to 5 μl of seminal fluid (n = 8). The percentage recoveries and coefficients of variation (C.V., %), using 16-methyl-$PGF_{1\alpha}$ as internal standard, were as follows: $PGF_{2\alpha}$ = 100.0 (8.9), PGE_2 = 104.5 (4.2), PGE_1 = 69.8 (5.2), PGB_2 = 64.8 (9.6), PGB_1 = 66.2 (5.9).

In this study, peaks of $PGF_{2\alpha}$, PGE_2 and PGE_1 were found on the chromatogram obtained from human seminal fluid. It was also found in every experiment that two major peaks eluted faster than the PGE series. These peaks may correspond to those of 19-hydroxy-PGE series, the occurrence of which has been suggested by some workers [23, 24]. It was reported [21, 22] that human seminal fluid contains PGB and PGA series, and their 19-hydroxy analogues in high concentration. In this investigation, seminal fluid was frozen immediately after ejaculation and the chromatogram was obtained after relatively simple preparation. However, no prominent peaks corresponding to the above compounds could be observed on the chromatogram. These results seem to agree with the suggestion by Jonsson et al. [24] that some,

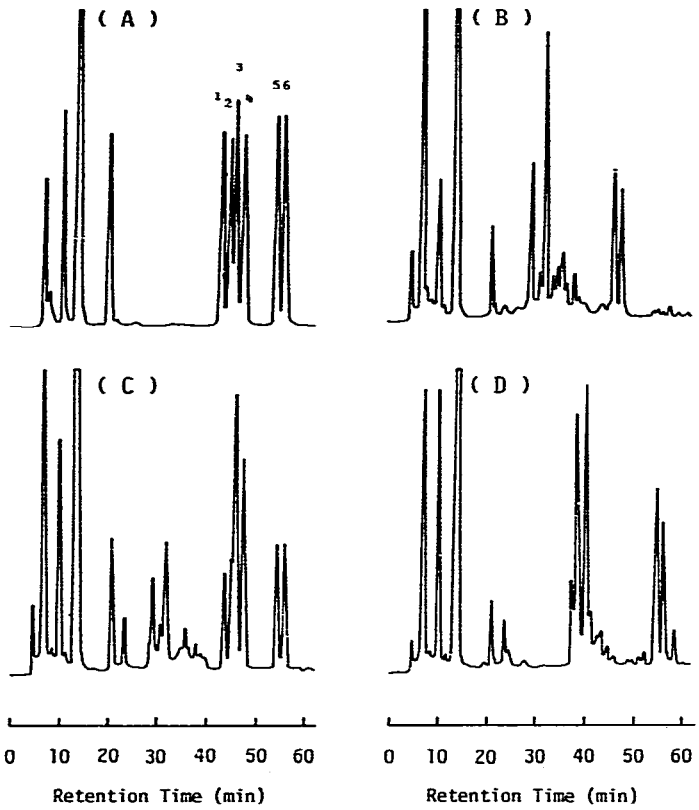


Fig. 7. Identification of the peaks on high-performance liquid chromatograms obtained from human seminal fluid sample. (A) Chromatogram of a mixture of standard PG-Mac esters; Peaks: 1 = $PGF_{2\alpha}$; 2 = $PGF_{1\alpha}$; 3 = PGE_2 ; 4 = PGE_1 ; 5 = PGB_2 ; 6 = PGB_1 . (B) Chromatogram obtained from human seminal fluid sample. (C) B spiked with standard PG-Mac esters. (D) B treated with alkali. Chromatographic conditions as in Fig. 2.

if not all, of the PGB and PGA series and their 19-hydroxy analogues are artifacts.

It was found that HPLC using Br-Mac was useful for the detection and the simultaneous determination of several PGs. Compared with other HPLC methods reported previously, this system offered higher sensitivity in the detection and determination of PGs and related compounds. This system was not, however, sufficiently sensitive to be effectively used for the analysis of other biological samples such as plasma and urine, in which the concentrations of PGs have been described to be very low. As described above, the detection limit of the fluorescent hydrolysate of PG-Mac ester was at the low fmol level. But, the reactivity of Br-Mac with PGs was not too good in concentrations lower than pmol. Therefore, it may be necessary to introduce a more reactive group into the reagent for the determination of subpicomoles of PGs using an HPLC system like this.

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