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FLUORIMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROSTAGLANDINS AND ITS APPLICATION TO THEIR DETERMINATION IN HUMAN SEMINAL FLUID

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SUMMARY

A highly sensitive and simple high-performance liquid chromatographic method with fluorescence detection for the determination of prostaglandins is described. Prostaglandins are converted into the corresponding fluorescent derivatives by reaction with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone in the presence of potassium hydrogen carbonate and 18-crown-6 in acetonitrile. The derivatives are separated simultaneously within 34 min (the total run time per injection, 56 min) on a reversed-phase column (YMC Pack C_8) by a stepwise elution with mixtures of acetonitrile, methanol and water and detected fluorimetrically. The detection limits are 10—15 fmol at a signal-to-noise ratio of 5 in a 10-μl injection volume. Prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ in human seminal fluids are measured by this method.

INTRODUCTION

Many kinds of prostaglandins (PGs) are present in biological materials, and play physiologically important roles at trace levels in the living body. Thus, a

highly sensitive and simple method for the simultaneous determination of PGs is required in their clinical and biological investigations.

Various methods including bioassay [1], gas chromatography (GC) [2], GC—mass spectrometry [3—5] and radioimmunoassay [6] have been widely used for the detection and determination of PGs. Several high-performance liquid chromatographic (HPLC) methods with electrochemical [7, 8] and spectrophotometric [9—16] detections have also been developed for the determination of PGs. The electrochemical and spectrophotometric detections have limited sensitivities. Recently, HPLC methods with fluorescence detection utilizing 4-bromomethyl-7-methoxycoumarin (Br-MMC) [17], 9-anthryldiazomethane (ADAM) [18], 4-bromomethyl-7-acetoxycoumarin (Br-MAC) [19] and p-(9-anthroyloxy)phenacyl bromide (panacyl bromide) [20] as fluorescence derivatization reagents for carboxylic acids, including PGs, have been reported. Of these methods, the fluorimetric HPLC methods with ADAM, Br-MAC and panacyl bromide are fairly sensitive. However, the derivatization reactions of PGs with these reagents do not proceed quantitatively at fmol levels of PGs. More recently, improved methods with panacyl bromide have been reported for the quantitation of fmol levels of PGs [21, 22] and applied to the determination of endogenous PGs in some biological materials [23].

We have developed 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (Br-DMEQ) as a highly sensitive fluorescence derivatization reagent for carboxylic acids [24, 25]. The reagent has then been applied to the determination of free fatty acids including polyunsaturated fatty acids, precursors of prostaglandins, in human serum [26, 27]. This paper aims to establish a sensitive and simple HPLC method utilizing Br-DMEQ for the microanalysis of eleven PGs. In order to ascertain practical applicability of the method to biological samples, the quantification of PGs in a minute amount of human seminal fluid is investigated. 16-Methyl-PGF$_{1\alpha}$, which is not present in human physiological fluids, is used as an internal standard.

EXPERIMENTAL

Reagents and materials

All chemicals and solvents were of analytical-reagent grade, unless stated otherwise. Deionized and distilled water was used. Eleven PGs (PGA$_1$, PGA$_2$, PGB$_1$, PGB$_2$, PGD$_2$, PGE$_1$, PGE$_2$, PGF$_{1\alpha}$, PGF$_{2\alpha}$, 6-keto-PGF$_{1\alpha}$ and 16-methyl-PGF$_{1\alpha}$) were kindly supplied by Ono Pharmaceutical (Osaka, Japan). Br-DMEQ was prepared as described previously [25]. Br-DMEQ (10 mM) and 18-crown-6 (5.7 mM) solutions were prepared in acetonitrile. The Br-DMEQ solution could be used for more than one week when stored in a refrigerator at 4°C. 16-Methyl-PGF$_{1\alpha}$ [2.5 μM; internal standard (I.S.)] solution was prepared in methanol and stored at —18°C in the dark; the solution was stable for at least two months.

Reversed-phase columns, YMC Pack C$_8$ (150 × 6 mm I.D.; 10 μm particle size), LiChrosorb RP-8 (150 × 4 mm I.D.; 10 μm particle size) and Radial-Pak cartridges C$_{18}$, C$_8$, phenyl and CN (all 100 × 8 mm I.D.; 10 μm particle size) were purchased from Yamamura Chemical Labs. (Kyoto, Japan), Merck (Tokyo, Japan) and Waters Assoc. (Tokyo, Japan), respectively.

HPLC apparatus and conditions

A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow-cell operating at 370 nm excitation and 455 nm emission was used. The column was a YMC Pack C_8. This column can be used for more than 1000 injections with only a small decrease in the theoretical plate number when washed with methanol at a flow-rate of 2 ml/min for ca. 20 min everyday after analyses. The column temperature was ambient (20—27°C). Stepwise elution with acetonitrile—methanol—water mixtures (35:10:55 and 35:30:35; mobile phases A and B, respectively) was carried out with a Hitachi 833A solvent gradient device; the flow-rate was 2.0 ml/min (ca. 100 kg/cm^2). Mobile phase A was first run for 26 min, and mobile phase B for the next 10 min; then the column was equilibrated successively with mobile phase A for 20 min before the start of the next sample.

When uncorrected fluorescence excitation and emission spectra of the eluates needed to be measured, a Hitachi 650-60 fluorescence spectro-photometer fitted with a 20-μl flow-cell was used; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Procedure for the preparation of seminal fluid sample solution

Human seminal fluid specimens were obtained from healthy volunteers in our laboratories. The fluid was frozen immediately after ejaculation and stored at −40°C in the dark until just before use. A 5-μl aliquot of the seminal fluid was diluted with 50 μl of the 16-methyl-PGF$_{1\alpha}$ solution and allowed to stand for ca. 2 min to coagulate the protein. The resulting solution was mixed with 1 ml of dilute hydrochloric acid (pH 3.0—3.5) and 2.5 ml of ethyl acetate. The mixture was vortexed for ca. 2 min and centrifuged at 1000 g for 5 min. The organic layer (ca. 1 ml) was evaporated to dryness in vacuo and the residue was dissolved in 200 μl of acetonitrile. The final solution was used as the sample solution.

Derivatization procedure

A 100-μl portion of the sample solution was placed in a screw-capped 10-ml vial, to which were added 2.5—5.0 mg of potassium hydrogen carbonate and 50 μl each of the Br-DMEQ and 18-crown-6 solutions. The vial was tightly closed and warmed at 50°C for 15 min in the dark. After cooling, 10 μl of the resulting mixture were injected into the chromatograph.

The calibration graphs were prepared according to the procedures for sample preparation and derivatization, except that 50 μl of the 16-methyl-PGF$_{1\alpha}$ (I.S.) solution was replaced with the I.S. solution containing 20 pmol—10 nmol each of the ten PGs. The net peak-height ratios of the individual PGs and 16-methyl-PGF$_{1\alpha}$ were plotted against the concentration of the spiked PGs.

RESULTS AND DISCUSSION

HPLC conditions

The simultaneous separation of the DMEQ derivatives of the eleven PGs tested (Fig. 1) was studied on reversed-phase columns described in the
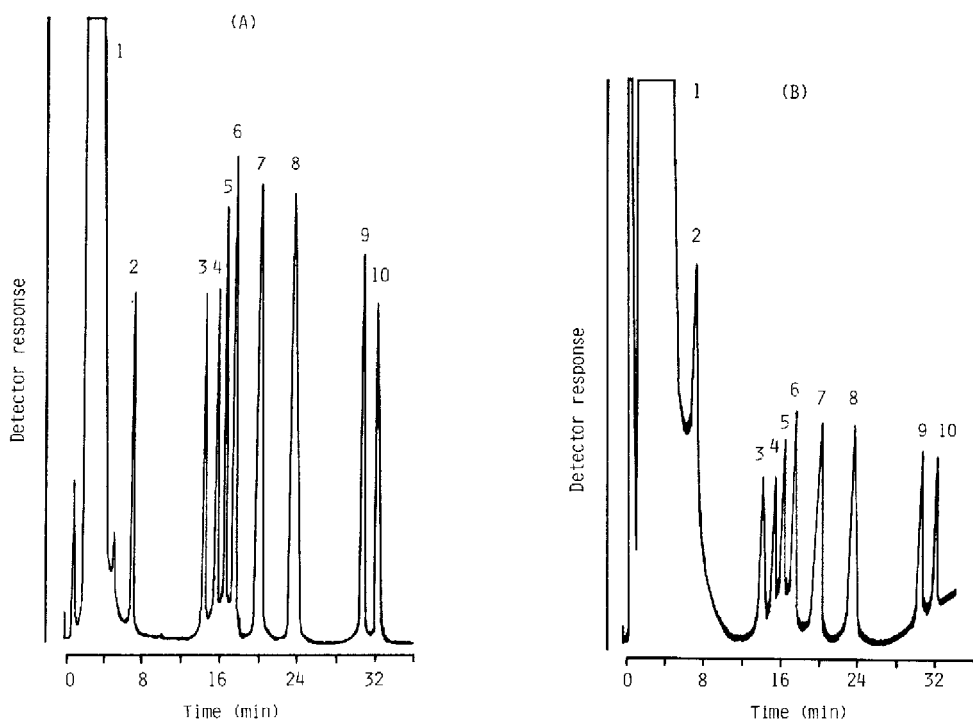
Fig. 1. Chromatograms of the DMEQ derivatives of prostaglandins. Portions (100 μl) of standard mixtures of PGs in acetonitrile were treated according to the derivatization procedure. Peaks: 1 = Br-DMEQ, 2 = 6-keto-PGF$_{1\alpha}$, 3 = PGF$_{2\alpha}$, 4 = PGF$_{1\alpha}$, 5 = PGD$_2$, 6 = PGE$_2$, 7 = PGE$_1$, 8 = 16-methyl-PGF$_{1\alpha}$, 9 = PGA$_2$ and PGB$_2$, 10 = PGA$_1$ and PGB$_1$. Concentrations of PGs: (A) 2—8 = 10 pmol/100 μl; 9 and 10 = 5 pmol/100 μl (total, 10 each, respectively) (B) 2—8 = 500 fmol/100 μl; 9 and 10 = 250 fmol/100 μl (total, 500 each, respectively). Detector sensitivity: (A) 1; (B) 10.

Experimental section using methanol, acetonitrile, water and their mixtures as a mobile phase. The best separation of the derivatives was achieved on a YMC Pack C$_8$ column with an isocratic elution using acetonitrile—water (35:65), but the derivatives were eluted late (retention times for PGA$_1$ and PGB$_1$ were both ca. 100 min) with broadening of the peaks. The addition of methanol served to minimize the retention times and also to sharpen the peaks. However, at methanol concentrations of more than 15%, the peaks for PGF$_{1\alpha}$, PGD$_2$ and PGE$_2$ overlapped. Thus, a stepwise elution was employed for suitable separation. However, the peaks for PGA$_1$ and PGB$_1$, and PGA$_2$ and PGB$_2$ could not be resolved successfully by any proportions of the solvents. Typical chromatograms obtained with standard solutions of the PGs at the concentrations of 10 pmol and 500 fmol/100 μl are shown in Fig. 1A and B, respectively. The PG derivatives other than the PGA and PGB series were completely separated within 25 min. The reproducible retention times were obtained for all the PGs tested under the present HPLC conditions (Table I). The change in methanol and acetonitrile concentrations actually had no effect on the fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra and intensities of the DMEQ derivatives of all the PGs.

TABLE I

RETENTION TIMES FOR PGs

PG	Retention time, min (mean ± S.D., n = 10)
6-Keto-PGF$_{1\alpha}$	6.8 ± 0.2
PGF$_{2\alpha}$	14.0 ± 0.2
PGF$_{1\alpha}$	15.2 ± 0.2
PGD$_2$	16.4 ± 0.2
PGE$_2$	17.6 ± 0.2
PGE$_1$	20.4 ± 0.2
16-Methyl-PGF$_{1\alpha}$	23.2 ± 0.2
PGA$_2$ and PGB$_2$	30.8 ± 0.3
PGA$_1$ and PGB$_1$	32.8 ± 0.3

Derivatization conditions

Br-DMEQ gave the most intense peaks at concentrations greater than ca. 8 mM in the solution; 10 mM was used as a sufficient concentration. Maximum and constant peak heights were attained at 18-crown-6 concentrations in the solution in the range 5—25 mM; 5.7 mM was selected in the procedure. Although the peak heights for PGF$_{1\alpha}$ and PGF$_{2\alpha}$ reached almost maxima in the amount of potassium hydrogen carbonate of 0.5—1.0 mg, constant peak heights were observed in the 2.2—7.5 mg range (Fig. 2). The same result was also obtained for all the other PGs. Thus, an amount of 2.5—5.0 mg of potassium hydrogen carbonate was selected in the procedure. When potassium carbonate was used, constant peak heights were not obtained (Fig. 2). When triethylamine or N,N-diisopropylethylamine [15] was used as a catalyst of the reaction, an unknown large and broad peak occurred at the retention time of 30—40 min.
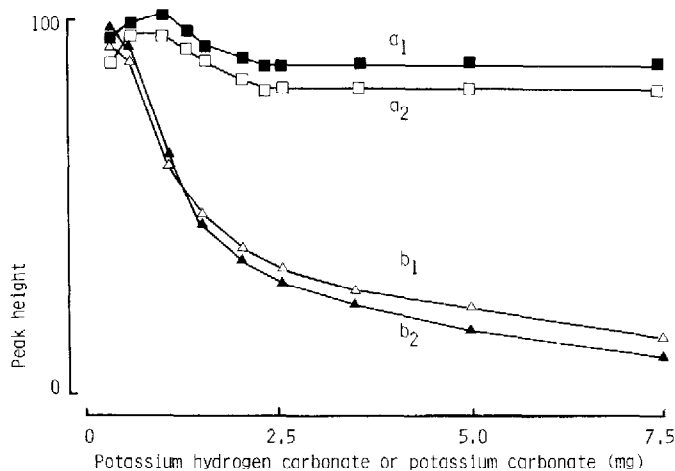

Fig. 2. Effect of the amounts of potassium hydrogen carbonate (a) and potassium carbonate (b) on the fluorescence derivatization of PGF$_{1\alpha}$ (a$_1$, b$_1$) and PGF$_{2\alpha}$ (a$_2$, b$_2$). Portions (100 μl) of PGF$_{1\alpha}$ or PGF$_{2\alpha}$ solution in acetonitrile (100 pmol/ml) were treated as in the derivatization procedure in various amounts of potassium hydrogen carbonate or potassium carbonate. Peak height in arbitrary units.
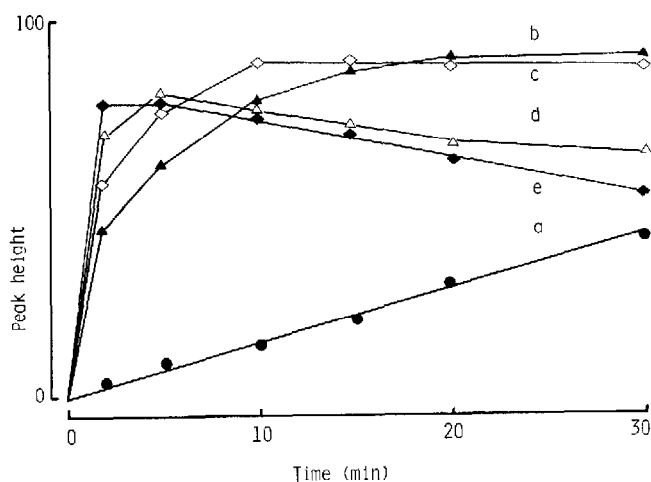
Fig. 3. Effect of reaction time and temperature on the fluorescence derivatization of PGE_1. Portions (100 μl) of PGE_1 solution in acetonitrile (100 pmol/ml) were treated as in the derivatization procedure at various temperatures for various periods. Temperatures: a, 20°C; b, 37°C; c, 50°C; d, 80°C; e, 100°C. Peak height in arbitrary units.

The derivatization reaction of PGs with Br-DMEQ apparently occurred even at moderately low temperatures; higher temperatures allowed the derivatization to proceed more rapidly. An example for PGE_1 is shown in Fig. 3. However, at 80—100°C, peak heights were decreased for prolonged heating time (10—30 min). At 50°C, the peak heights for all the PGs reached almost maxima after warming for 10 min; 15-min warming at 50°C was recommended in the procedure. The DMEQ derivatives of all the PGs were stable for at least 2 h in daylight and for at least 72 h in the dark at room temperature.

The within-day precision was examined by performing ten separate analyses using standard mixtures of PGs (20 and 0.5 pmol/100 μl each); the coefficients of variation for all the PGs did not exceed 2.5 and 5.8%, respectively. The detection limits for the PGs were 10—15 fmol in a 10-μl injection volume at a signal-to-noise ratio of 5 (Fig. 1B). The sensitivity is higher than those of the methods with Br-MMC, ADAM and Br-MAC, and comparable to that of the method with panacyl bromide [21, 22]. The relationships between the peak heights and the amounts of the eleven individual PGs were linear from 2 fmol to at least 10 nmol per injection volume (10 μl). The linear correlation coefficients were 0.995 or better for all the PGs. This indicates that the reactivity of Br-DMEQ with PGs is good even at extremely low concentrations (fmol levels). On the other hand, the relationships in the methods with ADAM [18] and Br-MAC [19] were not linear at concentrations lower than 5 pmol per 20-μl injection volume.

Determination of PGs in human seminal fluid

PGs in human seminal fluid were extracted with ethyl acetate in the usual manner [19].

Fig. 4A shows a typical chromatogram obtained with normal human seminal fluid. PGs in the seminal fluid were identified on the basis of their retention
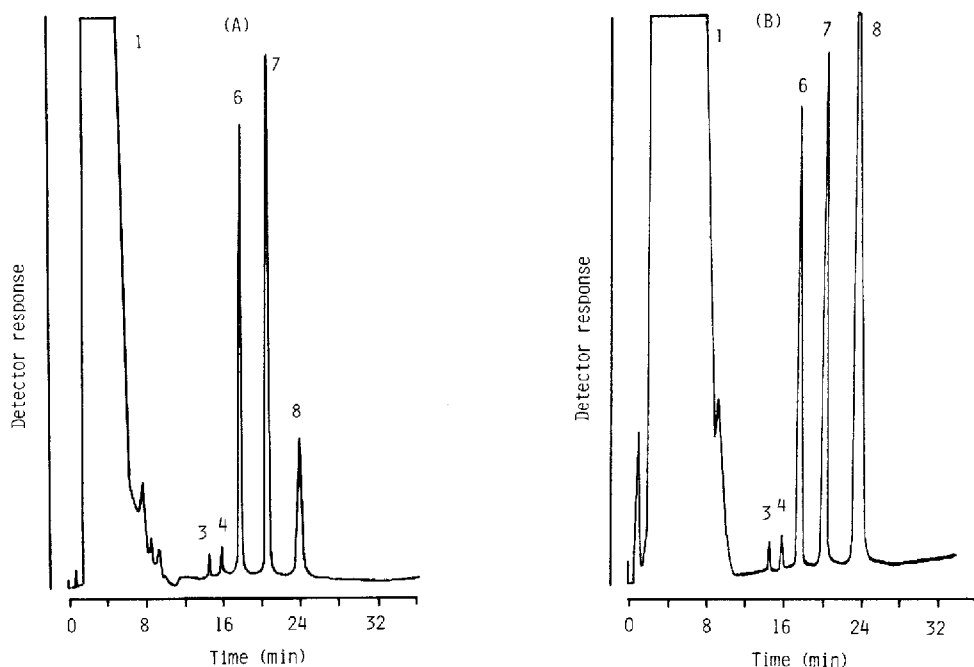
Fig. 4. Chromatograms obtained with human seminal fluid (A) and its ten-fold water-diluted fluid (B). Portions (5 μl) of the samples were treated according to the procedure. Peak 1 = Br-DMEQ and endogenous carboxylic acids in the sample. For the other peaks, see Fig. 1. Concentration of PGs in the seminal fluid: $PGF_{2\alpha}$, 5.5 (2.0); $PGF_{1\alpha}$, 8.2 (2.9); PGE_2, 84.1 (29.6); PGE_1, 94.1 (33.3) nmol (μg)/ml. Detector sensitivity: (A) 0.1; (B) 1.

times and fluorescence excitation and emission spectra of the eluates by comparison with the standard compounds, and also by co-chromatography of the standards and the seminal fluid with aqueous 50—100% acetonitrile or methanol as the mobile phase. Authentic samples of 19-hydroxy-PGE_1 and E_2, of which occurrence has been suggested by some workers [28, 29], were not available, and so could not be investigated.

Br-DMEQ reacts with biogenic carboxylic acids such as dicarboxylic (oxalic, malonic, succinic and adipic acids), hydroxycarboxylic (lactic and malic acids) and long-chain saturated and unsaturated fatty acids (C_{12}—C_{22}) to produce fluorescent derivatives [24—27]. However, the DMEQ derivatives of the dicarboxylic and hydroxycarboxylic acids were co-eluted with Br-DMEQ under the HPLC conditions. The derivatives from the fatty acids were strongly retained on the column and not eluted. Thus, these compounds did not interfere with the determination of the PGs even when they were spiked at unusually high concentration in the seminal fluid (20 nmol/5 μl each). However, since the DMEQ derivatives of the fatty acids in human seminal fluid may slowly alter the retention properties of the column, the column was washed with methanol every day after analyses to remove the derivatives.

For calibration graphs, linear relationships were observed between the ratios of the peak heights of the ten PGs to that of 16-methyl-$PGF_{1\alpha}$ and the amounts of the PGs added in the range of 20 pmol—10 nmol (corresponding to ca.

400 fmol—200 pmol per injection volume) each to 5 μl of seminal fluid, and no change of the slopes in the graphs of the relationships was observed, depending on the seminal fluid used. These facts indicate that the present internal standard method permits the determination of the PGs over wide ranges of their concentrations.

The within-day precision was established by repeated determination ($n = 20$) using normal human seminal fluid. The coeffficients of variation were 3.5, 3.8, 2.8 and 2.2% for mean concentrations (nmol/ml) of 3.3 for $PGF_{1\alpha}$, 1.8 for $PGF_{2\alpha}$, 48.3 for PGE_1 and 53.1 for PGE_2, respectively. The between-day precision was obtained by performing the analyses ($n = 3$ each day) using the calibration graphs prepared on that day for ten days with the same fluid kept frozen at $-40°C$. The coefficients of variation were 4.2, 3.9, 5.1 and 4.5% for PGF_1, PGF_2, PGE_2 and PGE_1, respectively.

The recoveries (%, mean ± S.D., $n = 10$ each) of the PGs (0.2 nmol/5 μl each) added to a seminal fluid were 101.2 ± 2.8 for 6-keto-$PGF_{1\alpha}$, 94.0 ± 3.1 for $PGF_{2\alpha}$, 100.0 ± 1.9 for $PGF_{1\alpha}$, 97.3 ± 2.2 for PGD_2, 94.0 ± 3.5 for PGE_2, 104.8 ± 3.6 for PGE_1, 98.7 ± 2.6 for PGA_2, 97.2 ± 2.9 for PGB_2, 97.5 ± 2.3 for PGA_1 and 97.5 ± 1.9 for PGB_1, when the recovery of 16-methyl-$PGF_{1\alpha}$ was taken as 100. These results show that 16-methyl-$PGF_{1\alpha}$ can be used as an internal standard.

The amounts of PGs in normal human seminal fluids were determined by this method (Table II). The mean values for the individual PGs in the seminal fluids were not too different from those obtained by a spectrophotometric method [30]. 6-Keto-$PGF_{1\alpha}$ and PGD_2 could not be detected in human seminal fluid. In addition, since the seminal fluids were frozen immediately after ejaculation and treated immediately by the procedure described, the PGA and PGB series (artifacts from the PGE series), were not observed in the seminal fluid. These results were also reported by other workers [19, 28].

TABLE II

CONCENTRATIONS OF PGs IN HUMAN SEMINAL FLUIDS FROM HEALTHY VOLUNTEERS

Age	Prostaglandin, nmol (μg/)/ml			
	$PGF_{2\alpha}$	$PGF_{1\alpha}$	PGE_2	PGE_1
59	0.9 (0.3)	2.9 (1.0)	62.8 (22.1)	40.4 (14.3)
37	3.3 (1.2)	2.7 (1.0)	31.0 (10.9)	27.2 (9.6)
35	1.8 (0.6)	3.3 (1.2)	53.1 (18.7)	48.3 (17.1)
28	8.8 (3.1)	4.4 (1.6)	198.3 (69.8)	114.1 (40.4)
24	0.3 (0.1)	0.6 (0.2)	15.0 (5.3)	26.6 (9.4)
24	7.2 (2.6)	12.0 (4.3)	82.1 (28.9)	102.8 (36.4)
23	0.3 (0.1)	0.5 (0.2)	33.5 (11.8)	38.7 (13.7)
23	0.3 (0.1)	0.3 (0.1)	33.5 (11.8)	14.7 (5.2)
23	1.9 (0.7)	0.8 (0.3)	31.8 (11.2)	19.5 (6.9)
23	5.5 (2.0)	8.2 (2.9)	84.1 (29.6)	94.1 (33.3)
22	3.5 (1.2)	1.0 (0.3)	64.5 (22.7)	55.9 (19.8)
22	5.9 (2.1)	4.2 (1.5)	110.0 (38.6)	59.9 (21.2)
Mean	3.3 (1.2)	3.4 (1.2)	66.6 (23.4)	53.5 (18.9)
S.D.	2.8 (1.0)	3.4 (1.2)	47.7 (16.6)	32.0 (11.3)

The present fluorimetric HPLC method using Br-DMEQ gives a satisfactory sensitivity. In this study, 5 μl of human seminal fluid were used for precise sampling. A chromatogram (Fig. 4B) obtained with a ten-fold water-diluted human seminal fluid indicates that if the seminal fluid is accurately taken, the method may permit the determination of PGs in only 0.5 μl or less. This method is also rapid and simple enough to assay ten samples within a day and should therefore be useful for physiological and pharmacological investigations of PGs.

ACKNOWLEDGEMENT

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