Fluorometric Determination of Prostaglandins and Thromboxane in Plasma by High-Performance Liquid Chromatography

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INTRODUCTION

It is well known that prostaglandins (PGs) and thromboxane (TX) have various physiological effects at trace levels. For the detection of PGs and TX, radioimmunoassays (RIA) have been used in clinical and experimental medicine[1~8]. However, the development of multiple micro-analysis for PGs and TX has long been awaited. From this point of view, high-performance liquid chromatography (HPLC) is a useful method. Although there have been some reports[9~11] on the HPLC analysis of PGs and TX using 9-anthryldiazomethane (ADAM), these methods have less sensitivity than HPLC for the quantitative determination of PGs and TX in human plasma.

The purpose of this investigation was to establish an HPLC method for the determination of PGs and TX in biological samples. For the analysis of PGs and TX at higher sensitivities, we investigated the optimal detection method for ADAM derivatives of PGs and TX . Previous reports have not paid attention to the Raman spectrum of the eluent in the HPLC system in the fluorometric analysis of PGs-ADAM[9~11]. In this study, we have demonstrated that ADAM derivatives of PGs and TX should be monitored at 412 nm with excitation at 350 nm. Our data also indicate that partial purification of ADAM derivatives of PGs and TX after esterification with ADAM is necessary to obtain sufficient sensitivity for the quantitative determination of PGs and TX in plasma.

MATERIALS AND METHODS

Materials

6-Keto-$PGF_{1\alpha}$, PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$ and TXB_2 were obtained from Seragen Inc. (Boston, Mass., U.S.A.). ADAM (9-anthryldiazomethane) was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). HPLC-grade methanol and chloroform were obtained from Wako Pure Chemical Industries (Osaka, Japan). Ethylacetate and tetrahydrofuran for HPLC were purchased from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were of analytical grade.

Extraction procedure

Venous blood (10 m*l*) was collected from five healthy male volunteers [31.2±5.3 years (Mean±S. D.)]. Fourteen mg of EDTA disodium salt and 5 mg of indomethacin were used for preventing coagulation and the further metabolism of arachidonate. The blood was first centrifuged at 1,500×g for 20 min to obtain plasma. The plasma sample was used for the extraction of PGs and TX. Three m*l* of plasma was diluted with the same volume of 10 mM H_3PO_4 (pH 2.2) and the diluted plasma sample was ultra-filtered through an Amicon membrane (Centriflo CF-25) at 1,000×g for 20 min at 4°C to remove the major parts of the plasma protein, after which 3 m*l* of chloroform was added to the filtrate and mixed well. The organic layer was then collected and evaporated to dryness under the nitrogen stream.

Derivatization with ADAM

In order to select the optimal solvent for esterification with ADAM, ADAM was dissolved in various organic solvents including chloroform, ethylacetate, methanol, and tetrahydrofuran, followed by mass

spectrometry using a Hitachi M-80B mass spectrometer system (Hitachi, Tokyo, Japan), and ionization by the field desorption (FD) method. Chloroform and ethylacetate seem to be suitable solvents for ADAM, because large parts of ADAM are present as monomers (Fig. 1). However, ethylacetate cannot be applied to a GEL PACK-A110 column because it easily destroys the column. Therefore, ADAM was dissolved in chloroform. Authentic compounds or the extracts of plasma dissolved in 10 μl of chloroform were added to 200 μl of 0.2% ADAM in chloroform, and incubated at room temperature. After incubation, ADAM derivatives of arachidonate metabolites were separated from free ADAM and its degradation products by using HPLC (described in "HPLC Analyses").

Fluorometric analyses of PGs-ADAM

To determine the optimal conditions for fluorometric detection of PGs-ADAM, fluorescence determinations of PGs-ADAM were carried out using a Hitachi F-3000 Fluorescence Spectrophotometer (Hitachi, Toyko, Japan). As shown in Fig. 2 (B), PGE_2-ADAM derivatives had a fluorescence excitation spectrum with peaks at 350, 367, and 388 nm when monitored at 412 nm. However, fluorescence of the ADAM derivative purified using HPLC (described below) was not detected when excited at 388 nm. Therefore, fluorescence when excited at 388 nm seemed to be derived from the degradation products of ADAM and its derivatives. Moreover, the analytical wavelength (412 nm) when excited at 367 nm fell on the Raman spectrum as shown in Fig. 2 (A) and Fig. 3. Almost the same results were obtained from ADAM derivatives of other arachidonate metabolites, including 6-Keto-$PGF_{1\alpha}$, PGD_2, $PGF_{2\alpha}$, and TXB_2. Therefore, PGs-ADAM were monitored at 412 nm with excitation at 350 nm.

HPLC Analyses

The determination was performed using a Hitachi 655A HPLC equipped with an F-1000 fluorometric

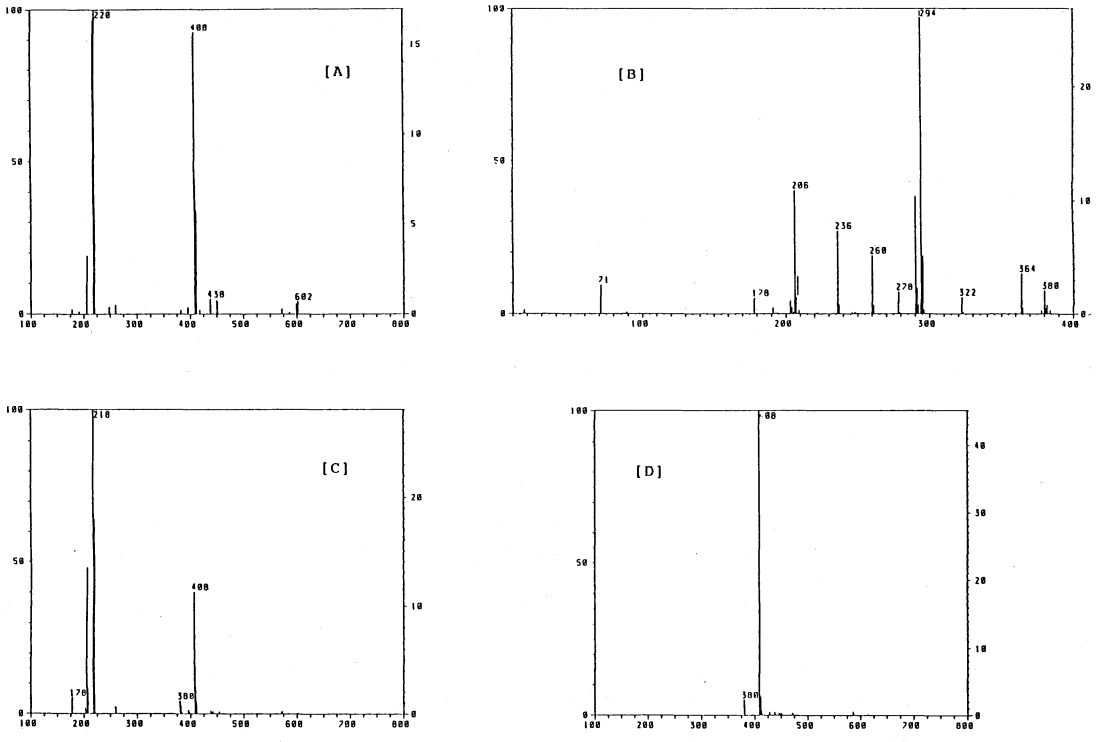
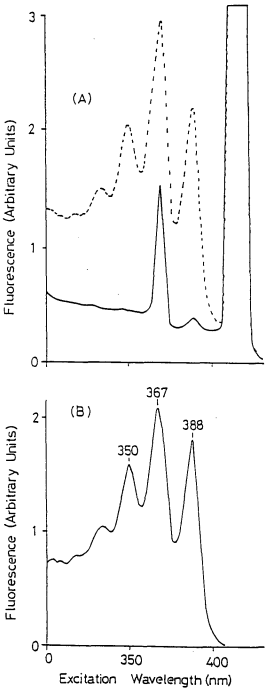


Fig. 1 Mass spectra of 9-anthryldiazomethane (ADAM) in various organic solvents. (A) chloroform (B) tetrahydrofuran (C) ethylacetate (D) methanol.



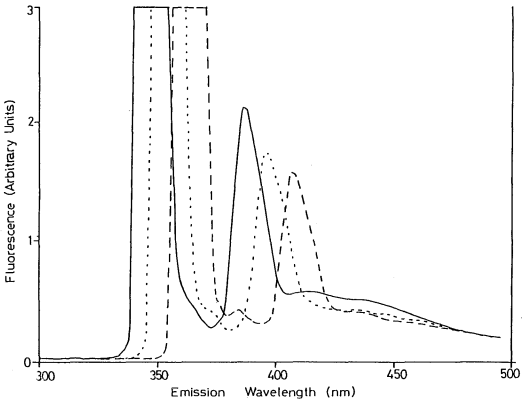


Fig. 3 Raman spectra of the eluent used in HPLC analysis when excited at 350 nm (——), 356 nm (------) and 365 nm (---). The Raman spectrum of the eluent when excited at 365 nm fell at around 412 nm, which is the analytical wavelength used in this assay system.

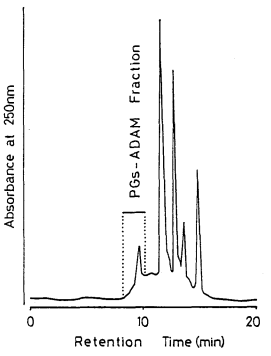


Fig. 4 Partial purification of ADAM derivatives of PGs and TX by using a GEL PACK-A110 column.

Fig. 2 Fluorescence excitation spectra of ADAM derivatives of PGE_2 without purification by HPLC when monitored at 412 nm. (A) Fluorescence excitation spectra of PGE_2-ADAMs (-----) and the solvent for PGE_2 and ADAM (——). (B) Difference spectrum of PGE_2-ADAM.

detector, a 655-21 spectrophotometric detector and a data processor (all from Hitachi).

Partial purification of ADAM derivatives of arachidonate metabolites was carried out by using a GEL PACK-A110 (8×500 mm) column (Hitachi) under the following conditions: mobile phase, chloroform; flow rate, 1.0 m*l*/min at room temperature; absorbance, 250 nm. The fraction corresponding to ADAM derivatives of PGs and TX was collected (Fig. 4) and evaporated to dryness.

The partially purified sample was further analysed using a Hitachi ODS column (3 μm particle size, 4.0×150 mm, Hitachi). The solvent system was methanol/distilled water/acetic acid (75/25/0.5, by vol.). A flow rate of 0.7 m*l*/min was used. The fluorescence of ADAM derivatives of arachidonate metabolites was monitored at 350 nm (excitation) and 412 nm (emission), as described above.

RESULTS

HPLC analyses of ADAM derivatives of authentic PGs and TX

When the reaction mixture containing ADAM, PGs and TX was directly applied on an ODS column and eluted with the solvent system, many interfering peaks appeared on the chromatogram, as shown in Fig. 5 (A). However, when PGs-ADAM derivatives which had been partially purified using a GEL PACK-A110 (Fig. 4) were injected onto the ODS column, these interfering peaks disappeared from the chromatogram, as shown in Fig. 5 (B).

The chromatographic behavior of ADAM derivatives of PGs and TX was investigated in various concentrations of methanol in the presence of 0.5 % acetic acid, and it was found that 75 % methanol was the most suitable eluent (Table 1). However, we could not separate PGD_2 from PGE_2 under our assay conditions.

The reaction of ADAM with PGs and TX was almost completed within 4 hours at room temperature (not shown), as described in the previous paper[11]. The peak height of PGs- and TX-ADAM derivatives

Table 1 Retention times of arachidonate metabolites via cyclooxygenase : Dependence of retention times upon the percentage of methanol used as the eluent.

Percent Methanol	Retention time (min)				
	TXB_2	PGD_2&E_2	$PGF_{2\alpha}$	6-Keto-$PGF_{1\alpha}$	PGE_1
80	12.4	13.0	14.1	15.0	16.2
77	18.7	19.5	21.3	22.3	24.4
75	27.3	28.5	31.5	32.8	36.6
72	41.6	43.5	48.5	48.6	57.2
70	57.8	60.2	67.2	67.1	78.2
68	71.6	74.7	83.6	82.3	98.6

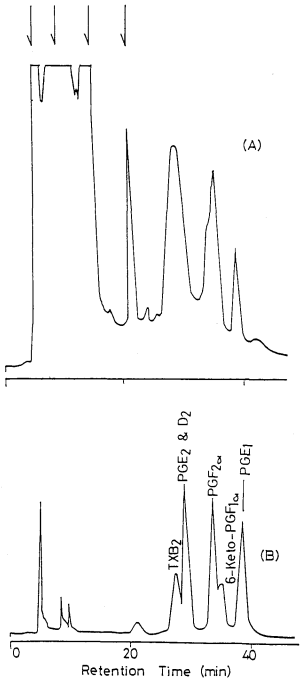


Table 2 Recoveries of authentic standards from the incubation mixture containing human plasma in the presence of EDTA and indomethacin.

Amount added	Amount recovered (pg)			
(pg)	6-Keto-$PGF_{1\alpha}$	PGE_1	PGE_2	TXB_2
10	5.5	6.0	4.9	5.5
100	65	62	55	58
1000	850	900	860	790
2000	1600	1850	1800	1750

Fig. 5 Effect of partial purification with a GEL PACK-A110 column on the HPLC profiles of ADAM derivatives of PGs and TX. A Hitachi ODS column was used for the analyses. (A) A reaction mixture of authentic compounds with ADAM was applied without partial purification of PGs-ADAM. (B) The chromatogram of the same sample (A), applied after partial purification through a GEL PACK-A110 column to remove interfering substances. Other conditions are the same as those in (A).

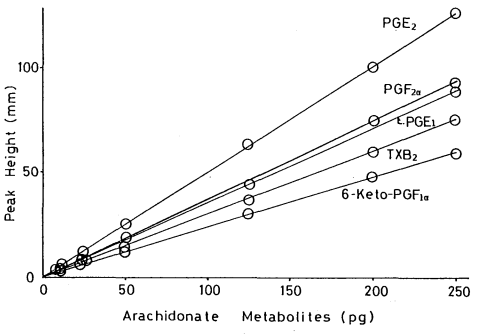


Fig. 6 Standard curves of ADAM derivatives of authentic PGs and TX. Reaction was performed at room temperature overnight.

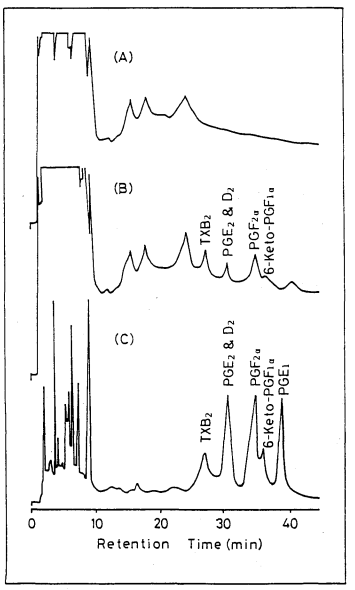


Fig. 7 HPLC profiles of ADAM derivatives of arachidonate metabolites in human plasma with (A) and without (B) boiling, and of authentic compounds (C).

on the chromatogram had a good correlation in the amounts of authentic PGs and TX (10-250 pg) (Fig. 6).

Recoveries of authentic PGs and TX

Recoveries for 6-Keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and TXB_2 were determined with authentic compounds added to human plasma in the presence of indomethacin and EDTA. Recoveries are shown in Table 2.

HPLC analyses of PGs and TX in plasma

Figure 7 (A) shows the chromatogram obtained by subjecting human plasma samples boiled for 30 min to the analytical procedure. There were not any peaks which corresponded to ADAM derivatives of authentic PGs and TX (Fig. 7 (A) and (C)). However, several peaks corresponding to ADAM derivatives of 6-Keto-$PGF_{1\alpha}$, PGE_2 (D_2), $PGF_{2\alpha}$ and TXB_2 could be detected in ADAM derivatives of arachidonate metabolites in the human plasma samples, as shown in Fig. 7 (B).

By using our HPLC method, the plasma levels of arachidonate metabolites via cyclooxygenase in normal subjects were determined simultaneously (Table 3).

Table 3 Plasma levels of arachidonate metabolites via cyclooxygenase in normal subjects.

Subject No.	Plasma level (pg/m*l*)			
	TXB_2	PGE_2+PGD_2	6-Keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$
1	20.8	5.6	50.0	6.7
2	25.0	4.2	11.1	22.9
3	26.5	2.8	29.2	18.8
4	18.8	N.D.*	33.3	5.0
5	29.2	7.8	45.6	21.5
Mean±S.D.	24.1±4.3	5.1±2.1**	33.84±15.3	14.98±8.49

*: Not detected. **: N=4.

DISCUSSION

It has been reported that the derivatization of PGs and TX with ADAM provides a sensitive HPLC method for PGs and TX[9~11]. In these methods, however, the minimal detection limit for PGs and TX was about 100 pg. Because of lower sensitivity, these HPLC methods using ADAM derivatization seem to have some limitations for the determination of the PGs and TX in plasma. In these procedures[9~11], the fluorescence of the analytical wavelength (412 or 418 nm) was apparently interfered with by the Raman spectrum when excited at 367 or 365 nm (Fig. 2 and 3). In the present study, we modified the previous methods to get a higher sensitivity. Our findings that partial purification of PGs-ADAM with GEL PACK -A110 enabled us to remove interfering substances on the chromatogram (Fig. 4 and 5) and that the Raman spectrum of the eluent used for HPLC analysis when excited at 350 nm did not interfer with the analytical wavelength (412 nm) (Fig. 2 (A) and 3) led to modifications in the methods for the determination of PGs and TX in human plasma.

Using the RIA method, Hennam et al.[1] reported that the mean level of $PGF_{2\alpha}$ in normal plasma was approximately 24 pg/m*l*. Dray et al.[2] showed that the normal plasma level of PGE_2 was 4.5±1.0 (mean± S.D.) pg/m*l*. In our HPLC system, PGD_2 was not separated from PGE_2 (Fig. 7). However, PGD_2 levels in plasma have not been reported, presumably because of their low concentrations. Therefore, the PGE_2 fraction which might contain PGD_2 in our assay system was presumed to represent PGE_2. It is assumed that the PGE_2 value determined by HPLC is similar to that previously reported. TXB_2 in normal plasma was reported to be 21.1±4.0 pg/m*l* when the plasma extract was used for RIA[3]. These data seem to be consistent with our results (Table 3). However, normal plasma 6-Keto-$PGF_{1\alpha}$ levels determined by RIA were more than five-fold compared to our value, which is presented in Table 3. Though the number of subjects tested is smaller, our present data, except for those on 6-Keto-$PGF_{1\alpha}$, seem to be consistent with

the data reported previously using RIA[1~5]. In order to compare the degree of accuracy of our method with that of RIA, further studies are needed.

It has been proposed that PGs and TX may be influenced by certain pathological conditions[5~8], and recently, plasma levels of PGs and TX are reported to be increased in various diseases[6~8]. Thus, an evaluation of PGs and TX levels in plasma may be of interest to biochemists and clinicians. By the use of our method, 6-Keto-$PGF_{1\alpha}$, PGE_2 & D_2, $PGF_{2\alpha}$ and TXB_2 in plasma can be detected simultaneously. Moreover, the use of radioactive materials and special facilities for the assay is unnecessary omitted in our method. Therefore, our HPLC method is useful for determing PGs and TX levels in biological specimens.

SUMMARY

The high-performance liquid chromatography (HPLC) method for detecting prostaglandins (PGs) and thromboxane (TX) which was reported previously was found to have a serious problem in the detection of PGs and TX. In the present study, we tried to solve this problem by developing a sensitive HPLC method for determining PGs and TX in human plasma. An analytical wavelength (412 nm when excited at 350 nm) which was not interfered with by the Raman spectrum was found to be suitable for the detection of PGs- and TX-ADAM (9-anthryldiazomethane) derivatives. Moreover, partial purification of 9-anthyryldiazomethane (ADAM) derivatives by using a GEL PACK-A110 column enabled us to remove interfering substances from the chromatogram. The levels of 6-Keto-PGF_1, PGE_2 & D_2, PGF_2 and TXB_2 in plasma were detected simultaneously using our method. The present HPLC method is useful for determining the PGs and TX levels in biological samples.

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高速液体クロマトグラフィーによる血漿中プロスタグランディン・トロンボキサンの螢光的検出

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従来報告されている高速液体クロマトグラフィー（HPLC）によるプロスタグランディン（PG）およびトロンボキサン（TX）の測定は，それらの検出方法にいくつかの問題点があることを見いだした。我々は，その問題点を解決することを試み，HPLCによる検出感度の向上と共に，ヒト血漿中のPGおよびTXを検出することを可能とした。

本測定法の検出波長（励起光350 nm，測光412 nm）は，ラマン光の妨害を受けずPGおよびTXの9-アンスリルジアゾメタン（ADAM）誘導体の検出に適していることを明らかにした。さらにゲルパックA110カラムを用いたADAM誘導体の部分精製は，クロマトグラム上の妨害物質の除去に有効であった。以上のような改良により，高感度検出法を確立することができた。

我々の方法では，血漿中の6-Keto-$PGF_{1\alpha}$，PGE_2（D_2），$PGF_{2\alpha}$，TXB_2の同時測定が可能であり，生体試料中のPGやTXの測定に有用であると思われる。

Key words: Prostaglandin, Thromboxane, High-performance liquid chromatography, Fluorescence.

プロスタグランディン，トロンボキサン，高速液体クロマトグラフィー，螢光

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