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Simultaneous assay of prostaglandins and thromboxane in the cerebrospinal fluid by gas chromatography–mass spectrometry–selected ion monitoring

Toru Obata[a,*], Toshikazu Nagakura[b], Hiroko Maeda[c], Kowa Yamashita[d], Kihei Maekawa[b]

[a]*Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, Minato, Tokyo 105-8461, Japan*

[b]*Department of Pediatrics, Jikei University School of Medicine, Tokyo, Japan*

[c]*Division of Pediatrics, Kamada Hospital, Tokyo, Japan*

[d]*Research Laboratories of Pharmaceutical Group, Nippon Kayaku Co. Ltd., Tokyo, Japan*

Abstract

A method of simultaneous analysis of prostaglandins (PGs) and thromboxane (TX) B_2 in cerebrospinal fluid (CSF) with GC–MS–SIM was established. Deuterated PGs and TXB_2 were used as internal standards: tetra-deuterated PGE_2 (d_4-PGE_2) for PGE_2, PGE_1 and PGD_2; d_5-$PGF_2\alpha$ for $PGF_2\alpha$ and 9α,11β-PGF_2 and 8-epi $PGF_2\alpha$; d_4-TXB_2 for TXB_2; and d_4-6-keto $PGF_1\alpha$ for 6-keto $PGF_1\alpha$. The PGs and TXB_2 were derivatized to the methyl ester of the methoxim dimethyisopropylsilyl (DMiPSi) ether form or the methyl ester of the DMiPSi ether form with simultaneous preparation. Samples were extracted with octadecyl silica gel and purified in two steps with silisic acid gel chromatography between derivatization steps. The calibration curve of each PG and TXB_2 was linear from 10 pg to 10 ng with the isotope dilution method. The levels of the seven types of PG and of TXB_2 were assayed simultaneously in the cerebrospinal fluid (CSF) from patients with aseptic meningitis. The CSF pattern of the PG and TXB_2 concentrations in mumps meningitis differed from those in other types of aseptic meningitis and in disease controls.

Keywords: Prostaglandins; Thromboxane

1. Introduction

Prostanoids, which include prostaglandins (PGs) and thromboxanes (TXs), have similar molecular structures and molecular masses but may exhibit diverse biological and therapeutic effects. For example, PGI_2 is a vasodilator and TXA_2 is a vasoconstrictor which together regulate local blood pressure. Therefore, the relative levels of these lipid mediators have physiological significance and may be helpful in the diagnosis of various conditions. For this reason, a method is needed for measuring the relative and absolute contents of PGs and TXs in biomedical samples.

In our previous study, we assayed 9α,11β-PGF_2 (the primary metabolite of PGD_2) in the urine of asthma patients after exercise by gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM) with the stable isotope dilution method using d_5-$PGF_2\alpha$ [1–3]. Because PGs (PGD_2, PGE_2, PGE_1, $PGF_2\alpha$, 9α,11β-PGF_2, 8-epi $PGF_2\alpha$

*Corresponding author. Tel.: +81-3-3433-1111 ex. 2365; fax: +81-3-3435-1922.

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and 6-keto $PGF_1\alpha$) and TXB_2 have similar molecular structures, they might be prepared, purified, derivatized and assayed with a single procedure. They could be derivatized to the methyl (Me) esters of the methoxim-dimethyisopropylsilyl (MO-DMiPSi) ether form or of the DMiPSi ether at the same preparations for GC assay [4,5]. We have established a method for the simultaneous analysis of PGs and TXs in biomedical fluid samples, such as cerebrospinal fluid (CSF) with the stable isotope dilution method by GC–MS–SIM.

2. Materials and methods

Sample preparation and derivatization were modified for simultaneous analysis as described by Obata and co-workers [1–3].

2.1. Materials

All organic solvents (Nacalai Tesque, Kyoto, Japan) were of high-performance liquid chromatography or analytical-reagent grade. DMiPSi-imidazole and *O*-hydroxymethylamine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). An ethereal solution of diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Tokyo Kasei Kogyo). Deuterated PGs, excluding d_5-$PGF_2\alpha$ and TXB_2, were purchased from Cayman (Ann Arbor, MI, USA). Deuterated $PGF_2\alpha$ was synthesized in our laboratory from Corey's lactone and [$^2H_8$]tetrahydrofuran according to the method of Green et al. [6]. Cartridges of octadecyl silica (Sep-Pak C_{18}, 500 mg per cartridge) were purchased from Waters (Milford, MA, USA). Silica gel (Silica gel 60 extrapure, 70–230 mesh, E. Merck, Darmstadt, Germany) was purchased from Nacalai Tesque. Other reagents were of analytical-reagent grade and commercially available.

2.2. Sample collection

Samples of CSF were collected from 14 children (mean age, 6.2±2.5 years; eight boys and six girls) with mumps meningitis and 18 other children (mean age, 6.2±3.0 years; 10 boys and eight girls) for the purpose of diagnosis at admission; second samples were obtained seven to 10 days later when symptoms had resolved. Samples from four healthy children (mean age, 7.2±2.8 years; two boys and two girls) were obtained as control specimens. Informed consent was obtained in accordance with hospital guidelines from the parents of each subject. After collection, all samples were centrifuged to remove cellular contamination and stored at −40°C until assay.

2.3. Extraction from CSF

Aliquots of CSF (1 to 5 ml) were diluted with distilled water (final volume, 8.5 ml). One nanogram of each deuterated PG (d_4-PGE_2, d_5-$PGF_2\alpha$, d_4-6-keto $PGF_1\alpha$) and of TXB_2 (d_4-TXB_2) were added as internal standards. The solution was acidified with 2 *M* hydrochloric acid to pH 3.0; the resulting solution was added to 1.5 ml of ethanol (final concentration 15%, v/v) and applied to a octadecyl silica mini-column (previously activated with methanol and 15%, v/v ethanol, Sep-Pak Cartridge C_{18}, 500 mg, Waters). The mini-column was washed with 10 ml of 15% (v/v) ethanol (pH 3.0) and 10 ml of petroleum ether. The PGs and TXB_2 were then eluted with 10 ml of ethyl acetate. The eluate was evaporated to dryness in vacuo below 40°C.

2.4. Clean-up and derivatization

Samples were purified and derivatized as follows.

(1) Me ester formation: The crude extract was dissolved in 0.05 ml of methanol and added to 0.2 ml of a diazomethane ethereal solution. The mixture was allowed to stand in the dark at room temperature for 30 min, then evaporated to dryness in vacuo below 40°C.

(2) First clean-up by silica column chromatography: The Me esters of the PGs and TXB_2 were dissolved in hexane–ethyl acetate (2:1) and applied to a silica column (Silica gel 60, E. Merck, 5×0.5 cm I.D.) prewashed with the same solvent. The column was washed with 10 ml of the same organic solvent and the PGs and TXB_2 were eluted with 30 ml of ethyl acetate containing 1% (v/v) methanol. The effluent from the silica column was evaporated to dryness in vacuo below 60°C.

(3) Methoxim (MO) formation and dimethylisopropyl (DMiP) silylation: Ketone groups were de-

rivatized to methoxication by *O*-hydroxymethylamine. If a PG (such as PGF2a) did not possess a ketone group, it was not affected by the derivatization procedure. The Me groups of the PGs and TXB_2 were dissolved in 0.05 ml of pyridine containing 4% (w/v) of *O*-hydroxymethylamine, and the resulting mixture was allowed to stand for 60 min at 60°C. After MO formation, the reaction mixture was added to 0.05 ml of dimethylisopropylsilyl imidazole and allowed to stand for 30 min at 60°C. The excess reagent was removed with a Sephadex LH-20 column (Pharmacia, Uppsala, Sweden, 5×0.5 cm I.D.) and 3 ml of hexane–chloroform–methanol (10:10:1, v/v/v) as eluates to obtain the derivatized PGs and TX. The effluent from the Sephadex LH-20 column was evaporated to dryness in vacuo below 30°C.

(4) Second purification by silica column chromatography: The derivatized PGs and TX fractions were dissolved in 0.025 ml of ethyl acetate and then diluted with 1.25 ml of hexane and applied to a silica column (5×0.5 cm I.D.), prewashed with hexane. After the column was washed with 5 ml of hexane, the derivatized PGs and TXB_2 were eluted with 10 ml of hexane–diethyl ether (9:1, v/v). The eluate was concentrated in a centrifugal concentrator under reduced pressure at 25 to 30°C. The precipitate was dissolved with ethyl acetate and subjected to GC–MS.

2.5. GC–MS conditions

A JMS-DX 303 GC–MS system (Jeol, Tokyo, Japan) equipped with a JMA-DA 5000 data processing system was employed. The column was a 30× 0.317 mm I.D. fused-silica capillary (DB-1; film thickness, 0.1 μm; J&W Scientific, Folsom, CA, USA). The temperature of the column oven was programmed in a two-step gradient. In the first step, the temperature was 100°C for 1 min, then raised to 220°C at 32°C/min; in the second step, the temperature was raised from 220°C to 300°C at 4°C/min. An all-glass solventless injector was mounted horizontally in the injection block of the gas chromatograph. Helium was used as the carrier gas at a liner velocity of 21 cm/s. The temperature of the injection port and the separator block was 300°C, and that of the ionization source was kept at 220°C. The ionization energy was 70 eV. The selected ions monitored are described in the text at a mass spectral resolution of 3000.

2.6. Data analysis

Concentrations of the PGs and TXB_2 in patients with mumps or other types of aseptic meningitis and in disease controls are expressed as pg/ml CSF. When indicated, these data are presented as the mean and 95% confidence limits after logarithmic transformation. Differences between samples obtained when symptoms were present and those obtained after symptoms had resolved were tested for statistical significance with the paired *t*-test, and differences between the meningitis and control groups were analyzed with the unpaired *t*-test.

3. Results

The extraction, derivatization and clean-up of lipid mediators from CSF, once with a octadecyl silica column and twice with a silisic acid column, were performed with different organic solvent components for the extracting and derivatizing preparations for GC–MS assay. A flow chart of sample preparation, as described in Materials and Methods is shown in Fig. 1.

Because the PGs and TXB_2 have similar molecular structures and molecular masses, the same purification method and derivatization process produced similar *m/z* fragments. Fig. 2 shows the authentic PGs and TXB_2 mass spectra of the Me-MO-DMiPSi and Me-DMiPSi derivatives. PGs and TXB_2 derivatives produce strongly positive [M−43] (liberate isopropyl group) fragments. For the sake of convenience, the SIM assay was used to monitor [M−43] fragments for the PGs and TXB_2 assay because of their strength and molecular size near [M+]. The selected ions monitored were *m/z* 552.35 for PGE_2 and PGD_2, 554.37 for PGE_1, 556.38 for d_4-PGE_2, 625.41 for 8-epi $PGF_2\alpha$, 9α,11β-PGF_2 and $PGF_2\alpha$, 630.45 for d_5-$PGF_2\alpha$, 670.44 for TXB_2 and 6-keto $PGF_1\alpha$, 674.46 for d_4-TXB_2 and 6-keto $PGF_1\alpha$, respectively.

As internal standards, 1 ng of each of the deuterated PGs and TXB_2 were used for assay: d_4-PGE_2

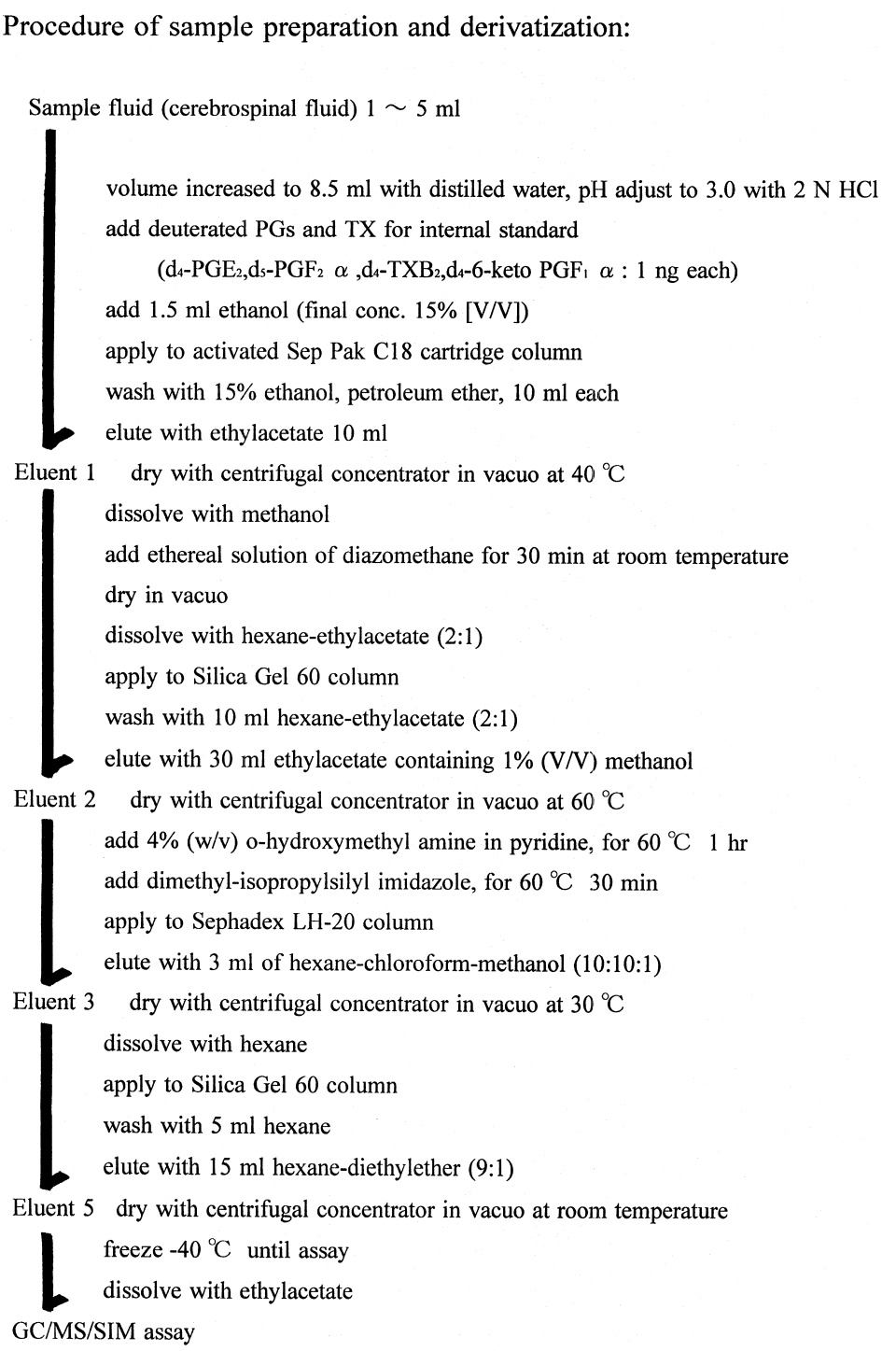

Fig. 1. Flow chart of the GC–MS sample preparation for the extraction, derivatization and purification of PGs and TXB_2.

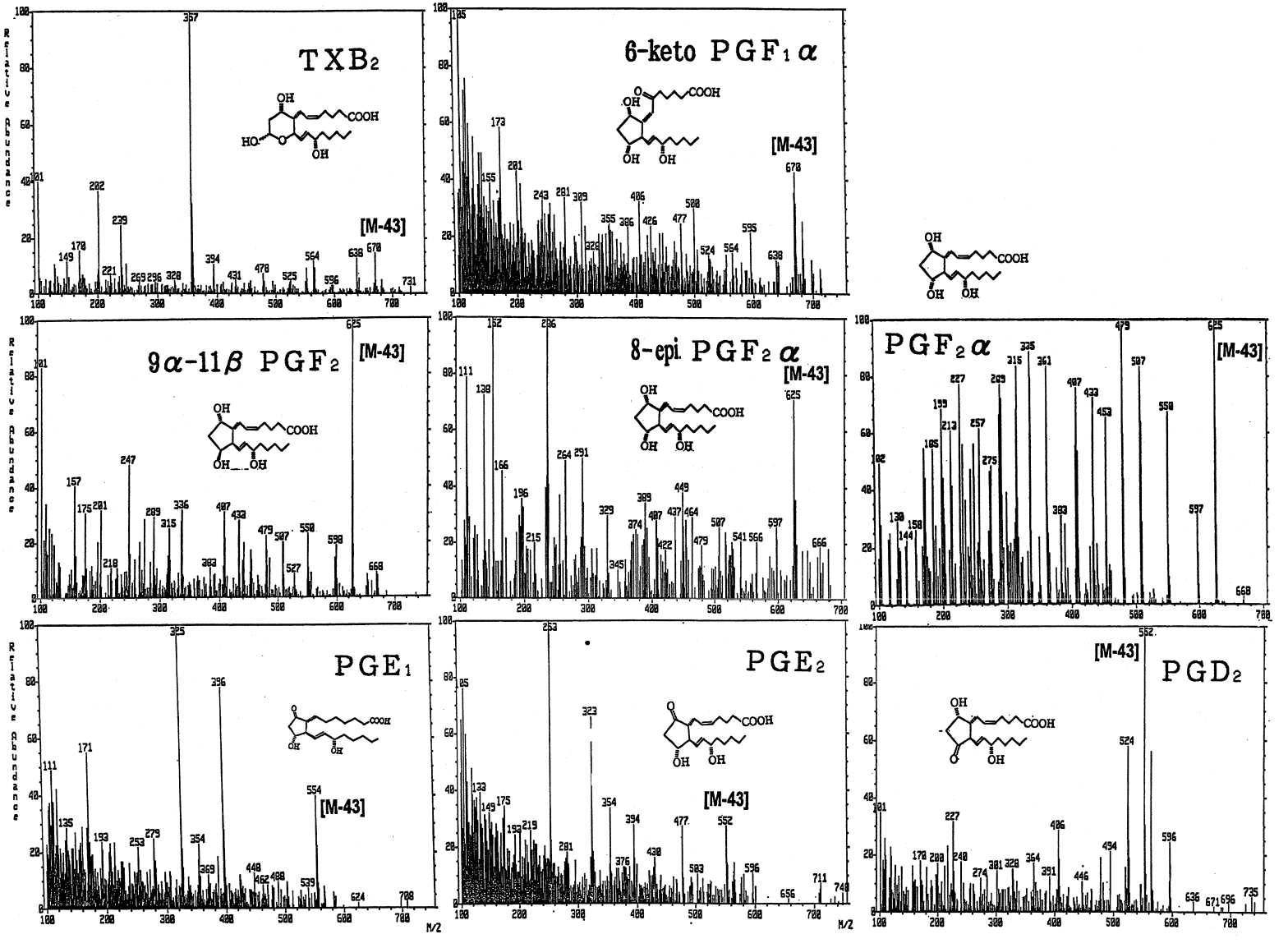

Fig. 2. Mass spectra of authentic prostaglandins and thromboxane. One nanogram of each PG and of TXB_2 was derivatized to the Me ester of the MO-DMiPSi ether or the Me ester of the DMiPSi ether form.

for PGE_2, PGE_1 and PGD_2; d_5-$PGF_2\alpha$ for $PGF_2\alpha$, 9α,11β-PGF_2 and 8-epi $PGF_2\alpha$; d_4-TXB_2 for TXB_2; and d_4-6-keto $PGF_1\alpha$ for 6-keto $PGF_1\alpha$.

During the derivatization and purification procedures, the chromatographic separation might be performed during the clean-up step of silica column chromatography. Fig. 3 shows the Me esters of PGs and TXB_2 eluted from the first clean-up step of the silica column with the organic solvent. To avoid differential recovery of the Me esters of PGs and TXB_2, the column was eluted with an excess volume of the solvent to compare the column bed-volume (1.5 ml). These close separation patterns indicate the reason for the co-usage of the deuterated internal standards (d_4-PGE_2 for PGE_2, PGE_1 and PGD_2 and d_5-$PGF_2\alpha$ for 8-epi $PGF_2\alpha$, 9α,11β-PGF_2 and $PGF_2\alpha$), and also the reason for the use of separate deuterated internal standards for TXB_2 and for 6-keto $PGF_1\alpha$ for different elution profiles. Because the separation in the second clean-up step of the silica column with the organic solvent was very small (data not shown), a smaller elution volume was needed than for the first clean-up step.

Table 1
Summary of calibration curves: correlation coefficients of PG calibration

PG	γ
6-keto $PGF_1\alpha$	0.999
TXB_2	0.988
PGF_2	0.994
9α,11β-PGF_2	0.985
8-epi $PGF_2\alpha$	0.997
PGE_2	0.998
PGE_1	0.995
PGD_2	0.999

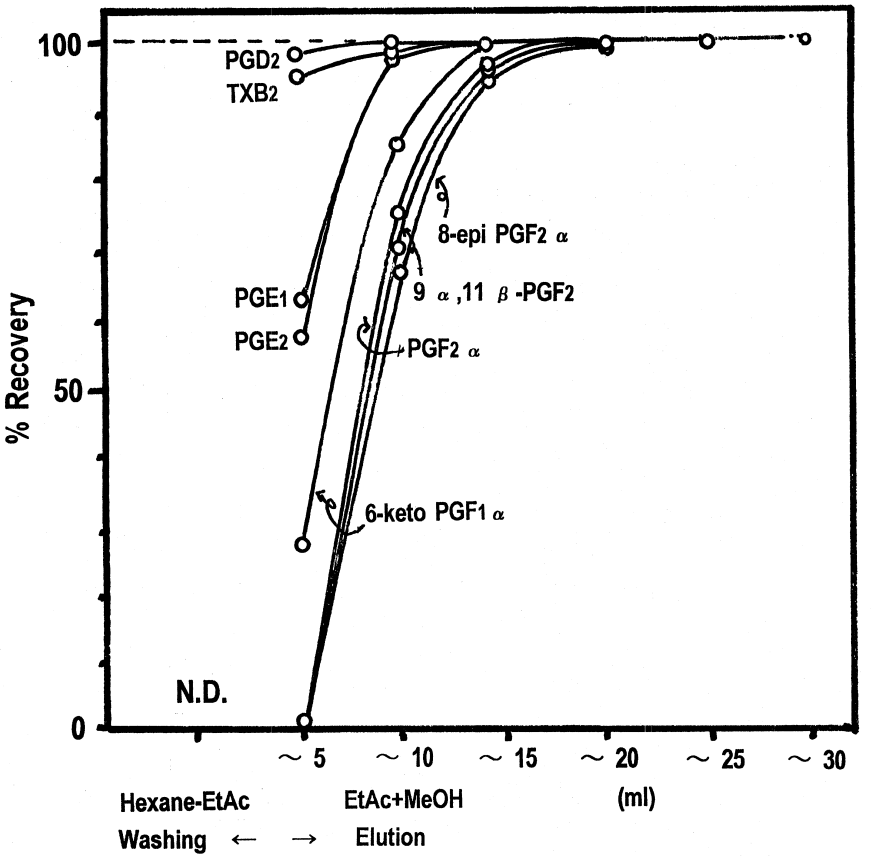

Fig. 3. Elution pattern of first silisic acid column of Me ester of PGs and TX. After methylation, the PGs and TXB_2 were purified with silisic acid column chromatography.

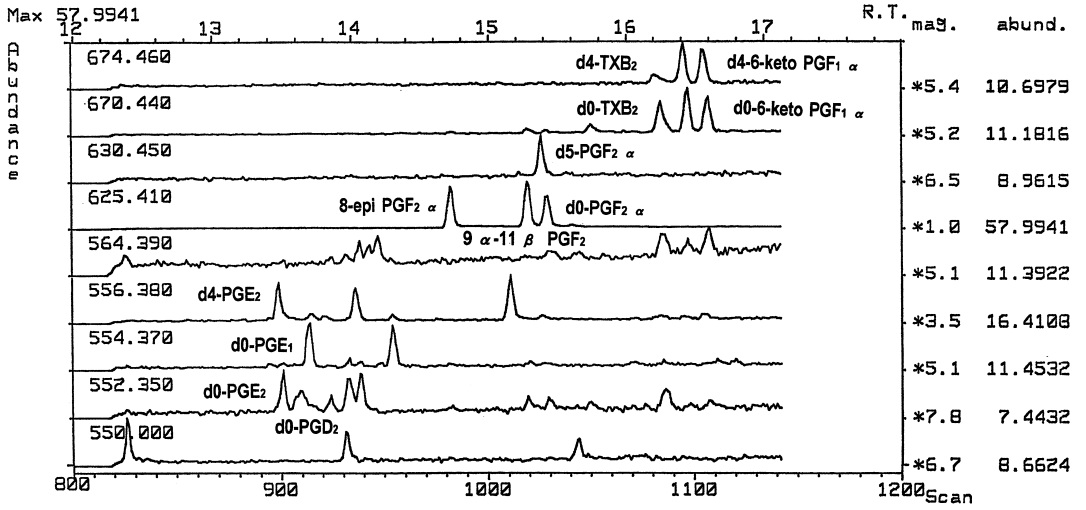

Fig. 4. Simultaneous mass chromatography of PGs and TXB_2. One nanogram of each PG and of TXB_2 with 1 ng of deuterated internal standard was derivatized and assayed with GC–MS–SIM.

Fig. 4 shows typical results of simultaneous SIM mass chromatography. Three groups were chromatographically separated: (1) PGE_2, PGE_1 and PGD_2; (2) 8-epi $PGF_2\alpha$, 9α,11β-PGF_2 and $PGF_2\alpha$; and (3) TXB_2 and 6-keto $PGF_1\alpha$.

The calibration curve of TXB_2 was linear from 10 pg to 10 ng with authentic samples with the isotope dilution method (Fig. 5). The correlation coefficients of calibration curves of all PGs and TX are shown in Table 1.

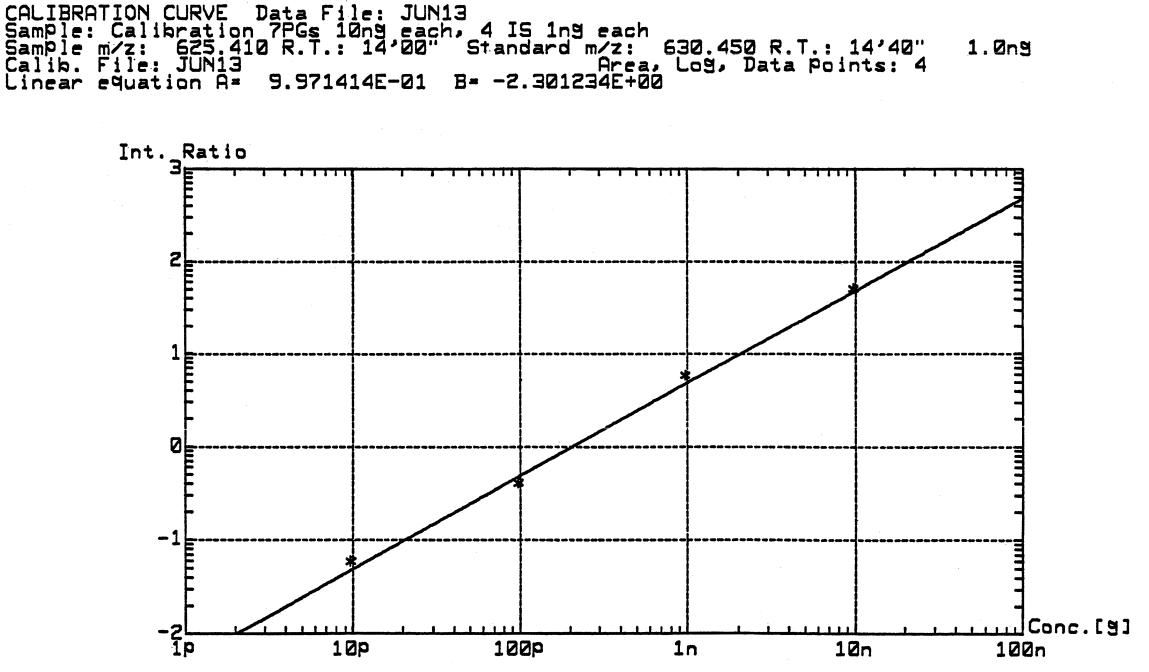

Fig. 5. Calibration curve of TX.

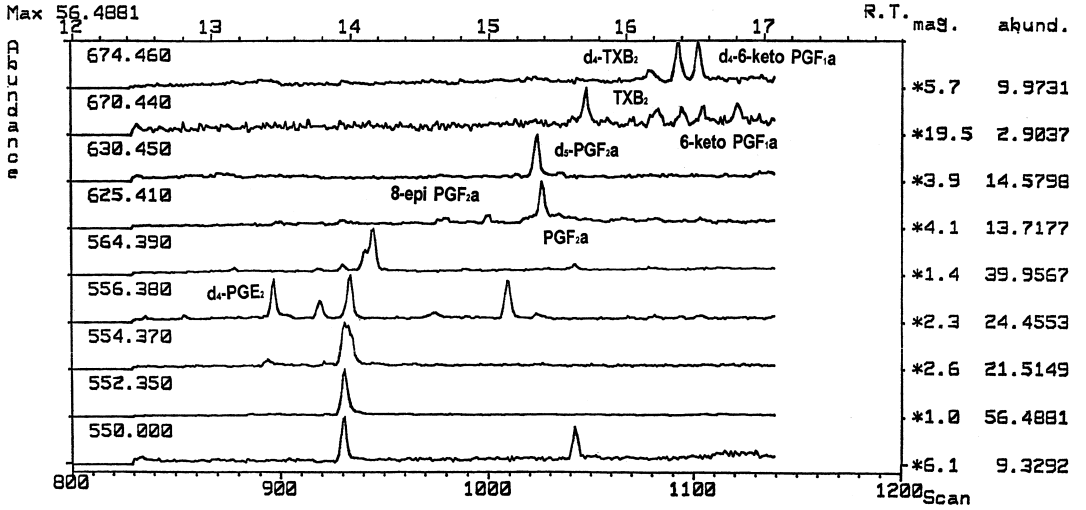

Fig. 6. Simultaneous assay of SIM mass chromatogram of PGs and TX of CSF in mumps meningitis patients. Sample was prepared with 1 ng of four deuterated internal standards for GC–MS derivatization and purification as described in Sections 2.4 and 2.5.

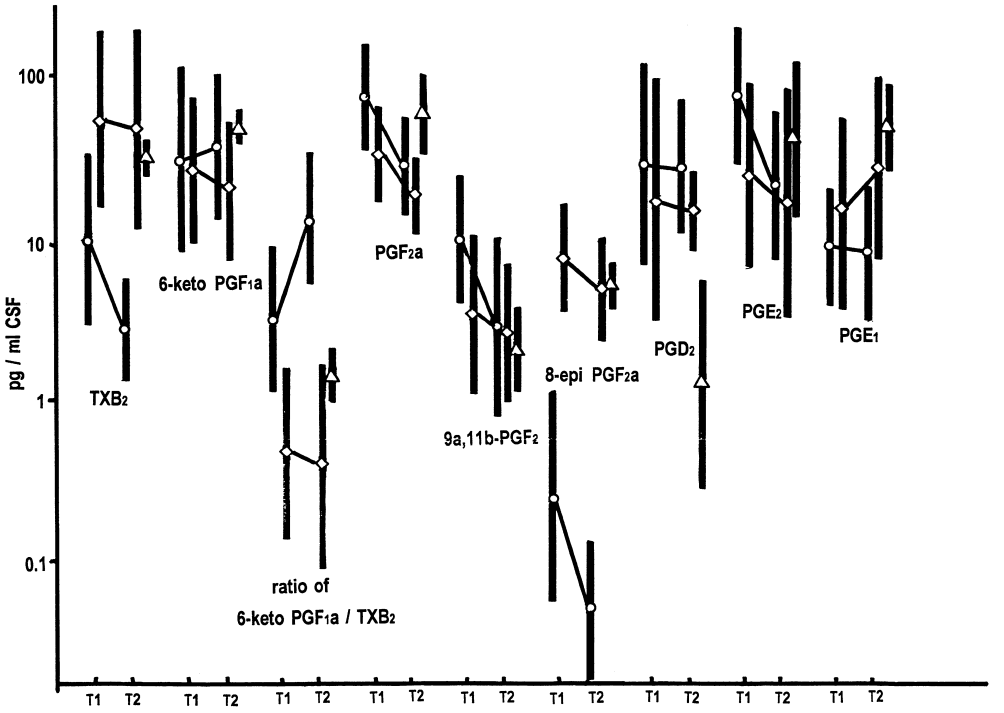

Fig. 7. The change in PGs and TXB_2 during and after meningitis. Circle: mumps meningitis ($n=14$); rectangle: aseptic meningitis excluding mumps meningitis ($n=18$); triangle: disease control ($n=4$); symbol: mean of sample group after logarithmic transformation; vertical bar: 95% confidence limits of each sample group; T1: at admission with meningitis; T2: 7 to 10 days later when symptoms had resolved.

With this method, the PG and TXB_2 contents were measured simultaneously in samples of CSF from patients with aseptic meningitis and from disease controls. Fig. 6 shows a typical mass chromatographic pattern of PGs and TXB_2 in the CSF from a patient with mumps meningitis.

Fig. 7 summarizes the content of PGs and TXB_2 in the CSF from patients with mumps or other types of aseptic meningitis and control patients. Although the 6-keto $PGF_1\alpha$ and TXB_2 contents were not significantly decreased in the CSF from patients with aseptic meningitis other than mumps meningitis or from control patients, the TXB_2 content in the CSF of a patient with mumps meningitis was decreased. Furthermore, the ratio of 6-keto $PGF_1\alpha$ and TXB_2 in the CSF changed as the condition of a patient with mumps meningitis improved.

The PGD_2 levels in all cases of aseptic meningitis other than mumps meningitis were higher than in control cases, and 9α,11β-PGF_2 levels decreased as meningitis resolved.

Levels of 8-epi $PGF_2\alpha$ (isoprostan) in cases of mumps meningitis were markedly lower than in cases of other types of aseptic meningitis or in control cases.

4. Discussion

The simultaneous assay of PGs with GC–MS–SIM is a useful method for examining the relationship of eicosanoids in vivo. It is the first choice to identify which PG would act in the physiological phenomenon related lipid mediator. Because PGs and TX have similar molecular structures, they can be simultaneously prepared and analyzed with GC–MS–SIM. The Me esters of the DMiPSi ether and of the MO-DMiPSi ether of PGs and TXB_2 produce strongly positive fragments [M−43] in electron impact ionization. These fragments separated with GC are convenient for the qualification and quantification of eicosanoids. We have assayed PGs and TXB_2 in the CSF from cases of aseptic meningitis with this simultaneous assay of GC–MS–SIM with the stable isotope dilution method.

Our results demonstrate different eicosanoid profiles in the CSF from patients with mumps meningitis and other types of aseptic meningitis and from control subjects. The decrease in the TXB_2/6-keto $PGF_1\alpha$ ratio in mumps meningitis suggests that the CSF stream may be regulated by PGI_2 and TXA2.

The concentration of the PGD_2 in patients with aseptic meningitis (including mumps meningitis) was higher than that in control subjects. In addition, levels of 9α,11β-PGF_2, the primary metabolite of PGD_2, decreased as the patients recovered from meningitis. These findings suggest that mast cell activation is involved in the primary immunological defense against viral infection of the brain [7].

Isoprostans are generated by the peroxidation of arachidonic acid [8]. X-Ray irradiation increases isoprostan levels in mouse plasma [9]. The low levels of isoprostan (8-epi $PGF_2\alpha$) in mumps meningitis suggest that peroxidation activity is lower than in other types of aseptic meningitis or in disease controls.

Further investigation of the significance of changes in CSF levels of PGs and TX in meningitis will require additional patients and longer follow-up after recovery.

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