DEVELOPMENT OF QUANTITATIVE ANALYSIS
OF PLASMA THROMBOXANE B$_2$
BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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In order to diagnose patients in thrombotic state, it is quite important to
detect increased concentration of plasma thromboxane B$_2$ (TXB$_2$), a stable
catabolite of TXA$_2$. To determine plasma TXB$_2$ levels with high sensitivity
and selectivity, we employed gas chromatography-mass spectrometry
(GC/MS). The trimethylsilyl (TMS) ether derivatives conventionally employ-
ed in GC/MS analysis of prostanoids are not suitable for quantitation of
plasma prostanoids, because the mass spectra are deficient in ions with high
intensity in the high mass range and TMS ether derivatives are sensitive to
moisture. To solve these problems we employed tert-butyldimethylsilyl (t-
BDMS) ether derivatives, based on the observation that t-BDMS ether deriva-
tives afforded abundant ions at [M-57]$^+$ and showed good hydrolytic stability.
The reaction conditions of tert-butyldimethylsilylation were also examined to
optimize the selected ion monitoring response. The t-BDMS ether derivatives
of prostanoids were successfully analyzed with a short capillary column with
a relatively large diameter, with maintaining good separation. In conjunction
with the use of reversed-phase high performance liquid chromatography as
purification procedure, a sensitive and reproducible stable isotope dilution
assay of plasma TXB$_2$ was developed. The values obtained by this method
correlated well with those obtained by the radioimmunoassay we have
developed.

ARAChIDONATE metabolites (prostanoids)
exert a wide range of biological activities
and modulate cell functions in various tissues.
Among them, thromboxane A$_2$ (TXA$_2$) has been
found to exhibit potent vasoconstricting activity
and the ability to increase aggregation of
platelets.$^1$ Because of these actions, TXA$_2$ is
considered to be closely related to the patho-
gnosis of circulatory disorders, such as throm-
bosis and vasospasm.$^2$

To clarify the role of this prostanoid in thromb-
otic disorders, it is quite important to quantitate
it in biological fluids. Among various assays
available for prostanoids, including bioassay,$^3$
receptor assay,$^4$ and enzyme assay,$^5$ combined gas
chromatography-mass spectrometry (GC/MS)
with the use of stable isotopes, affords superior
accuracy which is based upon the high specificity
of mass spectrometry. However trimethylsilyl
(TMS) ether derivatives, conventionally employ-
ed in GC/MS analysis of prostanoids$^6$ were found
to be inadequate for use in biological fluids since

Key Words:
Thromboxane B$_2$
Thrombotic disorders
Gas chromatography-mass spectrometry
Deuterated thromboxane B$_2$

(Received June 11, 1984; accepted October 25, 1984)
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This work was supported by research grants from the Ministry of Education, Science and Culture of Japan and a
grant-in-aid from the Muscular Dystrophy Association of America.
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such derivatives do not provide ions in the high mass range.

The present study reports that tert-butyl-dimethylsilyl (t-BDMS) ether derivatives of prostanoids are more adequate than TMS ether derivatives in terms of stability and sensitivity. Employing such a derivative with the use of specifically deuterated TXB₂ as an internal standard, we attempted to quantitate TXB₂, a stable catabolite of TXA₂, in human blood plasma by GC/MS.

MATERIALS AND METHODS

Materials

Prostaglandin D₂ (PGD₂), PGE₂, PGF₂α, TXB₂ and 6-keto-PGF₁α were supplied by Ono Pharmaceutical Co., Ltd. 3,3',4,4'-Tetradeutero-TXB₂ was kindly supplied by Dr. S. Terao and Dr. K. Kato of Takeda Chemical Industries, Ltd. Tiritated PGF₂α, TXB₂ and 6-keto-PGF₁α were purchased from New England Nuclear Corporation. Diazomethane was prepared from N-nitrosomethylurea (Maruwa Kagaku Kogyo Co., Ltd.). O-methylhydroxylamine hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. The silylation reagents, tert-butyldimethylcholorosilane (t-BDMS) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), were purchased from Tokyo Kasei Kogyo, Ltd. and GasuKuro Kogyo Inc., respectively.

Extraction Procedure

The subject population was as follows: thirty patients with angina pectoris (23 males and 7 females; mean age 55 years, range 31–70 years). Fourteen patients had effort angina, two had angiographically documented stenosis (more than 75%) in at least one of the major coronary arteries, and two of them had old myocardial infarction. Twelve patients had angina at rest, in nine cases of whom ergonovine-induced coronary vasospasm was documented during coronary angiography. The other four patients had unstable angina.

Blood was collected from the coronary sinus of patients with angina pectoris through a 7# coronary sinus catheter (Webster Co.) during cardiac catheterization.

Immediately after withdrawal into heparinized syringes, collected blood was transferred into polypropylene tubes containing 1 mM EDTA (ethylene diamine tetraacetic acid) and 0.1 mM indomethacin (in final concentration). After blood plasma was separated by centrifugation at 1,500g for 20 min, it was divided into nine groups, with each group serving as one sample. Deuterated TXB₂ was added to plasma samples (5 ng/ml plasma) as an internal standard. Twenty ml of plasma sample was deproteinized with 60 ml acetonitrile cooled to −20°C according to the method described by Grén et al, and neutral lipids were removed with 20 ml n-hexane. After the aqueous phase was acidified to pH 3–4 by 1N hydrochloric acid, TXB₂ was extracted with 60 ml ethyl acetate. The extract was dehydrated with anhydrous sodium sulphate, and ethyl acetate was removed with a rotary evaporator at reduced pressure.

Sample Purification

We employed reversed-phase column chromatography to purify the samples prior to derivatization for GC/MS analysis. To roughly separate prostanoids from other contaminants, we used gradient solution. The gradient was achieved by increasing the concentration of methanol in water acidified by acetic acid.

To examine elution pattern of prostanoids in a reversed-phase column (Sep-Pak C₁₈ cartridge, Waters Assoc.), tritiated prostanoids (PGF₂α, TXB₂ and 6-keto-PGF₁α) were applied to this column and eluted prostanoids were detected by liquid scintillation spectrometry. Further separation and purification was achieved by reversed-phase high performance liquid chromatography (HPLC) according to the method described by Whorton et al. The fractions in which TXB₂ eluted were collected and TXB₂ was extracted with 4 ml ethyl acetate.

Derivatization

PGF₂α and TXB₂ were subsequently converted with o-methylhydroxylamine hydrochloride and PGD₂, PGE₂ and 6-keto-PGF₁α were converted to methyl oxime (MO), Me, t-BDMS ether derivatives, respectively, according to the method described below.

Methyl oximation: The samples were reacted with o-methylhydroxylamide hydrochloride in anhydrous pyridine (Tokyo Kasei Kogyo Co., Ltd.) (1 mg/ml, 200 μl) at room temperature, overnight, and pyridine was evaporated in vacuo.

Methyl esterification: Fifty μl methanol was added to samples, which were reacted with freshly prepared ethereal diazomethane at room temperature for 5 min.

Fig. 1. Fractionation of a mixture of tritiated 6-keto-PGF₁α (0.005 μCi), TXB₂ (0.005 μCi) and PGF₂α (0.005 μCi) in a Sep-Pak C₁₈ cartridge (Waters Assoc.). The column was eluted sequentially with solvent mixtures containing increased concentrations of methanol in water acidified by acetate, as shown in the figure. Sequential aliquots (1 ml) were assayed for radioactivity, shown as percentage of the total radioactivity applied to the column. The ordinate indicates elution volume and the abscissa indicates % radioactivity eluted in each fraction.

**Silylation:** t-BDMS ether derivatives of prostanoids were prepared by treatment with 200 μl of t-BDMCS (1 mM) and imidazole (Wako Pure Chemical Industries, Ltd.) (2.5 mM) in anhydrous N,N-dimethylformamide. To remove excess amount of silylating reagents, reaction mixture was applied to Sephadex LH-20 (Pharmacia Fine Chemicals) column (5 x 0.5 cm) and t-BDMS ether derivatives of prostanoids were eluted with 2 ml chloroform-n-hexane-methanol (10:10:1, v/v/v)³. TMS ether derivatives were prepared by treatment with 100 μl BSTFA at 40°C for 1h. Prior to GC/MS analysis, the solvent was evaporated in vacuo, and all samples were dissolved in n-hexane after evaporation.

**Instrumentation and Conditions**

**Gas liquid chromatography:** The glass column (2 m x 2 mm i.d.) packed with SE-30 (1%) on Chromosorb W (80–100 mesh) was used. The gas chromatographic analysis was achieved on a Nihon-Denshi JGC 20K equipped with a flame ionization detector under the following conditions: the temperature of column oven, 260°C; the temperature of injection port, 280°C; the flow rate of carrier gas (N₂), 30 ml·min⁻¹.

**Comparison between Radioimmunoassay and GC/MS**

TXB₂ levels in 9 plasma samples were determined by the radioimmunoassay (RIA) process we have developed, and GC/MS, separately. The values estimated by the RIA were compared with those estimated by the GC/MS developed in this study.

**RESULTS**

**Isolation and Purification of Prostanoids**

Reversed-phase column (Sep-Pak C₁₈ cartridge) chromatogram of tritiated PGF₂α, TXB₂ and 6-keto-PGF₁α is shown in Fig. 1. Polar contami-
Determination of Plasma Thromboxane B₂ Levels by GC/MS

Fig. 3. Stability of t-BDMS ether derivative of prostanoioid. PGF₂α (50 μg) was derivatized to Me-TMS ether and Me-t-BDMS ether, separately. These derivatives were dissolved in 50 μl n-hexane and stored at room temperature. One μl of this solution was applied to GC every day after derivatization. The ratio of peak areas of PGF₂α-Me-t-BDMS ether (t-BDMS) and PGF₂α-Me-TMS ether (TMS), was measured. The ordinate indicates the ratio of peak areas and the abscissa indicates days after derivatization. Conditions: 1% SE-30 on Chromosorb W (80–100 mesh); glass column (2 m × 2 mm i.d.) at 260°C; nitrogen flow rate = 30 ml·min⁻¹; FID detection.

Fig. 4. Mass chromatogram of PGD₂, PGE₁, PGF₂α, TXB₂ and 6-keto-PGF₁α as Me-t-BDMS ether or Me-MO-t-BDMS ether derivatives in EI mode. The main fragments of each compound resulting from the loss of the tert-butyl radical from the molecular ion, were monitored; they were m/z 566 for PGD₂ and PGE₁, m/z 653 for PGF₂α, m/z 669 for TXB₂, and m/z 698 for 6-keto-PGF₁α. This profile was obtained on a SE-30 fused-silica capillary column (12.5 m × 0.35 mm i.d.) at 270°C; helium flow rate = 2.4 ml·min⁻¹; injector temperature, 280°C. 1, 6 = PGE₁, 2 = PGD₂, 3 = PGF₂α, 4 = TXB₂, 5 = 6-keto-PGF₁α.

Fig. 5. EI mass spectra of (a) Me-MO-TMS ether and (b) Me-t-BDMS ether derivatives of TXB₂ (70 eV). (a) The ion at m/z 301 was the base peak. In the high mass range over 350, only the intensity at m/z 366 represented only 10% of the base peak intensity. (b) The ion at m/z 669 resulting from the loss of the tert-butyl radical from the molecular ion was the base peak.

nents were eluted with mobile phase A (water-methanol-acetate, 80:20:0.4, v/v/v) and B (water-methanol-acetate, 60:40:0.3, v/v/v). Trinitiated prostanoioids were eluted with mobile phase C (water-methanol-acetate, 40:60:0.2, v/v/v) and D (methanol). The recoveries of tritiated PGF₂α, TXB₂ and 6-keto-PGF₁α were 78.7%, 88.1% and 88.0%, respectively. Elution pattern of TXB₂ and PGF₂α had two peaks. This was mainly because TXB₂ and PGF₂α were not fully eluted with mobile phase C and the residual amounts were eluted with mobile phase D, through their lesser polarity than 6-keto-PGF₁α. The fractions eluted with mobile phase C and D were collected and after extraction of prostanoioids with ethyl acetate, the extracts were subsequently applied to reversed-phase HPLC according to the method described by Whorton et al. As a result, PGF₂α, TXB₂ and 6-keto-PGF₁α were effectively separated (data not shown).

Derivatization Kinetics and Stability of the Derivatives

After TXB₂ was derivatized to Me, we examined the reaction condition of tert-butyltrimethylsilylation, to optimize the GC/MS/selected ion monitoring (SIM) response of the fully tert-butyltrimethylsilylated TXB₂. Figure 2 shows kinetic curves of the time course of tert-butyltrimethylsilylation of TXB₂-Me. The yield of
TABLE I COMPARISON BETWEEN PLASMA TXB₂ LEVELS DETERMINED BY GC/MS AND THOSE DETERMINED BY RIA

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>GC/MS (pg/ml)</th>
<th>RIA (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>190</td>
<td>210</td>
</tr>
<tr>
<td>2</td>
<td>230</td>
<td>358</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>324</td>
</tr>
<tr>
<td>4</td>
<td>360</td>
<td>370</td>
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<tr>
<td>5</td>
<td>230</td>
<td>224</td>
</tr>
<tr>
<td>6</td>
<td>1060</td>
<td>1070</td>
</tr>
<tr>
<td>7</td>
<td>600</td>
<td>1184</td>
</tr>
<tr>
<td>8</td>
<td>800</td>
<td>946</td>
</tr>
<tr>
<td>9</td>
<td>380</td>
<td>924</td>
</tr>
</tbody>
</table>

The correlation coefficient of the values determined by the two methods was 0.833 (p < 0.05).
Abbreviations: TXB₂ = thromboxane B₂; GC/MS = gas chromatography-mass spectrometry; RIA = radioimmunoassay.

PGE₂, PGE₂α, and 6-keto-PGF₁α were also fully tert-butyldimethylsilylated in ca. 2 h at 65°C.

The stability of the derivatives formed was also examined. The relative stability of PGE₂α-Me-t-BDMS to that of PGE₂α-Me-TMS was evaluated by calculating the ratio of peak areas on gas chromatogram of these derivatives. When the ratio immediately after derivatization was assigned to be the control value (100%), this value gradually decreased and reached to 56% two weeks after derivatization (Fig. 3).

Gas Chromatographic Separation and the EI Mass Spectra of the Derivatives

When a standard mixture of PGD₂-Me-MO-t-BDMS, PGE₂-Me-MO-t-BDMS, PGE₂α-Me-t-BDMS, TXB₂-Me-t-BDMS and 6-keto-PGF₁α-Me-MO-t-BDMS was applied to capillary GC, these prostanoids were effectively separated as shown in Fig. 4. The syn- and anti- isomers of PGE₂ and 6-keto-PGF₁α were delineated as shown in Fig. 4 (peak 1,1’ and peak 5,5’, respectively).

Figure 5-(a) and Fig. 5-(b) show the EI mass spectra of TXB₂-Me-MO-TMS and TXB₂-Me-t-BDMS. In TXB₂-Me-t-BDMS, an ion at [M-57]⁺ (m/z 669), which derived from the loss of tert-butyl radical from the molecular ion, exhibited large abundance compared with the ion in the high mass range of the respective TMS ether.

derivative. When TXB₂ was derivatized to Me-MO-t-BDMS ether, the base peak appeared at m/z 385, which resulted from cleavage of C8-C12 bond. The ion at [M-57]\(^+\) had weak intensity (data not shown).

Quantitation of Plasma TXB₂ by GC/MS and Comparison with RIA

To construct a calibration curve for TXB₂, we injected a known amount of mixtures of the unlabelled and deuterated derivatives of TXB₂ into GC/MS and calculated the peak area ratios of the ions at [M-57]\(^+\) in the protium and deuterium form (m/z 669 and m/z 673). The calibration curve is shown in Fig. 6. When known amounts of unlabelled and deuterated TXB₂ were added to plasma extracts and subjected to GC/MS measurement through these methods, a linear relationship was observed between the peak ratios of the ions at [M-57]\(^+\) and the amount of TXB₂ added in the range 2–10 ng. The lower limit of detection was 50 pg with a signal-to-noise ratio of 5:1. When 200 pg of TXB₂ was injected on the column, the coefficient of variation was 10.8%. TXB₂ levels in 9 human blood plasma samples were determined through the procedures described above. The typical SIM chromatogram for these measurements is shown in Fig. 7. The values were compared with those obtained by the RIA (Table I). They correlated well with each other, except for sample 7 and sample 9, the values of which were estimated to exist in the high value range, with a correlation coefficient of 0.833 (p < 0.05).

DISCUSSION

In trace analysis of prostanoids in biological fluids by GC/MS, it is essential to solve all the methodological problems. The conventional GC/MS methods, employing packed columns followed by quantitation of prostanoids derivatized to TMS ethers, are limited both in specificity and sensitivity. In the present study, we devised a sensitive and specific GC/MS assay procedure of TXB₂ employing capillary GC in conjunction with the use of t-BDMS ether derivative and reversed-phase HPLC as purification procedure. This procedure enabled us to quantitate plasma levels of TXB₂ in human blood samples.

Since prostanoids are low molecular weight compounds (MW ≈ 400) and exist in extremely small amounts in biological materials, their quantitation by selected ion monitoring (SIM), used in the present study, could be easily interfered with by numerous substances such as fatty acids and unknown contaminants. Under these conditions, it is desirable, therefore, that the prostanoids maintain their molecular structures as intact as possible in order not to be affected by contaminants at lower mass range. The t-BDMS ether derivatives have abundant ions at [M-57]\(^+\) which derive from the loss of tert-butyl radicals from the molecular ions. Since Corey and Venkateswarlu first reported the utility of the t-BDMS ether derivatives, these derivatives have been widely employed in the GC/MS analysis of biological compounds such as steroids, organic acids, phospholipids, acylglycerols and nucleosides. In the present study, Me-MO-t-BDMS derivatives of PGD₂, PGE₂, and 6-keto-PGF₁α, and Me-t-BDMS derivatives of PGF₂α and TXB₂ were found to afford abundant ions at [M-57]\(^+\). These results agreed with the data reported by other investigators. This mass spectral characteristic is superior to that of the TMS ether derivative conventionally employed in the GC/MS analysis of prostanoids. When the TMS ether derivatives are analyzed under EI, they do not provide abundant ions in the high mass range on mass spectra, because fragmentation follows the loss of trimethylsilanol group from the molecular ion. The stability of the derivative, especially its hydrolytic stability, is also one of the important factors in GC/MS measurement of compounds in biological materials. As shown in Fig. 3 the degradation rate of t-BDMS ether derivative was much lower than that of TMS ether derivative. In this aspect, t-BDMS ether derivatives of prostanoids are found to be superior to TMS ether derivatives.

There are two problems to be solved when t-BDMS ether derivatization is applied to prostanoids. First, t-BDMCS cannot easily react with a sterically crowded hydroxyl group at C9 of TXB₂, owing to its structural bulkiness. Second, t-BDMS ether derivatives have large retention indices in GC analysis which oblige us to spend more time in GC/MS measurement. As for the former problem, we found that all hydroxyl groups of TXB₂ were fully tert-butylidimethylsilylated in ca. 2 h at 65°C (Fig. 2). Indeed, under these conditions, the ratio of peak areas on gas chromatogram of bis-silylated TXB₂ (the hydroxyl groups at C11 and C15 are silylated) and trisilylated TXB₂ (all hydroxyl groups are silylated) was found to be near zero. We counteracted the latter problem by employing a short
capillary column with a relatively large diameter (12.5 mm x 0.35 mm i.d.), thus all the 1-BDMS ether derivatives of prostanoids eluted within 15 min, with maintaining good separation.

Plasma TXB₂ levels estimated by the present method correlated well with those estimated by the RIA we have previously developed. In sample 7 and sample 9, the values estimated by the GC/MS is lower than those estimated by the RIA. The reason for these differences may be that in the high value range the coefficient of variation of the values estimated by the RIA tend to be large because the slope of standard curve is not steep in the high value range. From the standpoint of practical use in routine clinical examination, the RIA we have developed, requires less time than the present method employing GC/MS, and is also highly sensitive (10 pg per plasma sample). A slight relative cross-reactivity (less than 1%) was seen only with PGD₂. However, other arachidonate metabolites such as PGI₂ and metabolites via lipooxygenase pathway (hydroxy-, hydroperoxy-icosatetraenoic acids and leukotrienes) are also known to exhibit potent physiological actions on vascular tissue and platelets. Therefore, in order to clarify the pathophysiologial roles of arachidonate metabolites in circulatory disorders, it is quite important to quantitate them simultaneously in body fluids. For this purpose, GC/MS is more suitable than RIA, because GC/MS enables us to perform profile analysis of these compounds.

It should be noted, however, that low basal levels of TXB₂ have been reported. Taking account of variation in materials, it is impossible to compare the values estimated in the present study with these lower ones, but there do exist variations of plasma TXB₂ levels estimated by varying methods. Although factors affecting such differences are not as yet fully defined, processing of samples has been pointed out. Difficulties in extraction and purification may also partly contribute to such variations. It remains to be seen whether further methodological improvements could lead to more accurate determination of plasma levels of TXB₂ and to accurate definition of metabolic alterations of vasoactive substances derived from arachidonate cascade in circulatory disorders.

Acknowledgements
The authors are greatly indebted to Dr. S. Terao and Dr. K. Kato for valuable guidance and discussions. We also wish to thank Mr. K. Nakano and Ms. M. Okada for skillful technical assistance.

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Japanese Circulation Journal Vol. 49, March 1985