High Performance Liquid Chromatographic Determination of Thromboxane B$_2$ in Human Serum as a Methoxime-Panacyl Ester Derivative

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THROMBOXANE B₂ IN HUMAN SERUM AS A METHOXIME-PANACYL ESTER DERIVATIVE

by

Robert H. Pullen, Jr.

A Thesis Submitted to the Faculty of The Graduate College in partial fulfillment of the requirements for the Degree of Master of Arts Department of Chemistry

Western Michigan University Kalamazoo, Michigan December 1986
HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THROMBOXANE B₂ IN HUMAN SERUM AS A METHOXIME-PANACYL ESTER DERIVATIVE

Robert H. Pullen, Jr., M.A.
Western Michigan University, 1986

A high performance liquid chromatographic (HPLC) method was developed to measure thromboxane B₂ (TXB₂) levels in human serum. Serum samples (2 mL) were extracted using solid phase extraction columns in a C18/silica mode sequencing approach. The internal standard, 6-ketoprostaglandin F₁α, was added to the serum extracts. The eicosanoids were doubly derivatized, first with panacyl bromide, then with methoxyamine to form methoxime-panacyl ester derivatives. The eicosanoid derivatives were chromatographed using a reverse phase HPLC system with UV detection (254 nm).

Assay linearity was demonstrated with fortified TXB₂ standards in 3% bovine serum albumin over a range of 25 to 500 ng/mL ($r \geq 0.994$). There was no significant interday difference or bias in assay results for pooled standards at 75, 226 and 376 ng/mL concentrations ($p > 0.05$). Pooled estimates of precision at these levels indicate an assay relative standard deviation of 6-9%.

The HPLC assay was used to quantitate TXB₂ levels in human serum. Results were consistent with previously published values when drug-free serum was analyzed to assess ex vivo TXB₂ formation.
ACKNOWLEDGEMENTS

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Robert H. Pullen, Jr.
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CHAPTER 1

INTRODUCTION

Biochemistry and Physiology

Thromboxane B₂ (TxB₂, Figure 1) is the stable hydrolysis product of thromboxane A₂ (TXA₂), a smooth muscle contractant and potent platelet aggregator. These molecules are products of the arachidonic acid cascade. Regulation of TXA₂ synthesis has been implicated as an important factor in cardiovascular disease and the biochemistry and physiology of prostaglandins (PG) in this regard has been reviewed by Gorman and Marcus (1).

Mechanical or chemical stimulation of the platelet results in release of arachidonic acid from the cell membrane phospholipids. Some of the free arachidonate is converted via the platelet cyclooxygenase enzyme to the endoperoxide PGH₂. PGH₂ can be converted by thromboxane synthetase to TXA₂ or it can be converted by prostacyclin synthetase to prostacyclin (PGI₂). When TXA₂ is formed in the platelet, Ca²⁺ mobilization is stimulated. This results in the release of adenosine 5'-diphosphate (ADP) from platelet granules and the inhibition of platelet adenylate cyclase. Adenylate cyclase inhibition causes adenosine-3', 5'-cyclic phosphoric acid (cyclic AMP) levels to fall. The released ADP activates platelets so that they become sticky and aggregate other platelets in the presence of fibrinogen. PGI₂ acts to antagonize the effects of TXA₂ by stimulating adenylate...
Figure 1. Structures of the Two Forms of TxB₂ and 6-Keto-PGF₁₅α.

Thromboxane B₂ (TxB₂)

6-Ketoprostaglandin F₁₅α (6-keto-PGF₁₅α)
cyclase which elevates cyclic AMP levels. The increased cyclic AMP levels result in Ca$^{2+}$ sequestration, and inhibit phospholipase and cyclooxygenase activities.

A balance between TxA$_2$ and PGI$_2$ could therefore control platelet aggregability in vivo. Too much TxA$_2$ or too little PGI$_2$ may cause thromboembolic disease. The therapeutic pharmacological control of this balance could theoretically be achieved by the selective inhibition of thromboxane synthetase or stimulation of the prostacyclin synthetase enzyme.

The Problem

In order to monitor the influence of various pharmacological and biological factors on the TxA$_2$/PGI$_2$ balance, sensitive and specific analytical assays have been developed to measure TxB$_2$ in blood serum and plasma. In a situation where maximal TxA$_2$ production is stimulated, such as harvesting serum from clotted blood, the level of TxB$_2$ is a valid measure of total TxA$_2$ biosynthesis. Work was undertaken to develop and validate a high performance liquid chromatography (HPLC) method for the measurement of TxB$_2$ in human serum.

Background

Because TxB$_2$ levels in serum and plasma are low (<550 ng/mL) (2-6), current techniques with the demonstrated capacity to measure the eicosanoid at these concentrations are limited to radioimmunoassay (RIA) (7) and combined gas chromatography-mass spectrometry (GC/MS) (8). Although RIA and GC/MS are highly sensitive techniques, both
have inherent disadvantages. Selectivity in RIA is dependent upon
the degree of cross reactivity of the TxB₂ antiserum with other arachi­
donic acid metabolites and sample matrix components. As a consequence,
RIA methods should be cross-validated with a physiochemical technique,
such as GC/MS or HPLC, to confirm assay accuracy. The most obvious
problem with GC/MS is the prohibitive cost of the sophisticated instru­
mentation. Another serious problem is due to the limited sample capac­
ity necessitated by involved experimental procedures and manual
sample injection.

Ideally, HPLC could represent a less costly, sensitive and spe­
cific physiochemical alternative to GC/MS with comparable sample
capacity to RIA. Detection of TxB₂ by HPLC is hampered by the absence
of strong ultraviolet (UV) chromophores or natural fluorescence. Addi­tionally, TxB₂ chromatographs as an asymmetrical, broad peak due to an
equilibrium between ring opened and closed forms of the molecule (9)
(Figure 1).

Several reports have described the detection of standard solu­
tions of TxB₂ using HPLC (10-14) but none have been successfully
applied to the measurement of the eicosanoid in serum or plasma.
Turk et al. (10) prepared fluorescent eicosanoid esters by derivati­
zation with 4-bromomethyl-7-methoxycoumarin. Their application was
limited to the qualitative identification of TxB₂ produced by human
platelets. Fitzpatrick et al. (11) prepared the p-nitrobenzylloxime
of the TxB₂ methyl ester for detection of a solution standard using
UV absorbance (254 nm, 7.5 μg injected). Terragno et al. (12, 14)
employed detection in the short wavelength UV region (190-192.5 nm)
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to measure underivatized eicosanoids. Detection in this region is subject to a large number of interferences and a biological application for TxB₂ was not given. Watkins and Peterson (13) described the formation of eicosanoid ester derivatives with p-(9-anthroyloxy) phenacyl bromide (panacyl bromide). The esters were both strongly fluorescent and UV absorbant (254 nm). Unfortunately, TxB₂ exhibited a broad, double-peaked chromatographic elution pattern and was not resolved from prostaglandin F₂α (PGF₂α). More recently, Cox and Pullen have extended the work of Watkins and Peterson to permit the analysis of some E-type prostaglandins in human plasma by HPLC with picogram per milliliter sensitivity (15-16).

The Approach Taken

The approach to the problem of developing an HPLC assay for the quantitation of TxB₂ in serum involved doubly derivatizing the eicosanoid molecule. Serum TxB₂ extracts were first converted to panacyl esters to permit detection by UV absorbance or fluorescence. The panacyl esters were converted to methoximes to fix the ring in an open position and impart satisfactory chromatographic properties similar to those obtained in GC applications (17-18). The assay was validated by performing triplicate assays of pooled 3% bovine serum albumin (BSA) standards on three separate days using independently prepared calibration standards. The results were evaluated to confirm linearity, accuracy, and precision. The utility of the method was investigated by determining human serum TxB₂ concentrations in the presence and absence of a specific thromboxane synthetase inhibitor.
CHAPTER II

EXPERIMENTAL

Materials

Thromboxane B₂ (TXB₂), 6-ketoprostaglandin F₁α, the internal standard (1S) (6-keto-PGF₁α), PGD₂, PGE₁, PGE₂, PGF₂α, 11-dehydro-TXB₂, PGA₂, PGB₂, 13,14-dihydro-6,15-diketo-PGF₁α, U-63557A (a thromboxane synthetase inhibitor), and panacyl bromide [p-(9-anthroyloxy)phenacyl bromide] were supplied by the Pharmaceutical Research and Development Laboratories of the Upjohn Company. [5,6,8,9,11,12,14,15-³H(N)]-Thromboxane B₂ (TXB₂-³H) (specific activity of 120 Ci/mmol) and 6-[5,8,9,11,12,14,15-³H(N)]-ketoprostaglandin-F₁α (6-keto-PGF₁α-³H) (specific activity of 113 Ci/mmol) were purchased from New England Nuclear. The samples were purified before use by thin layer chromatography using a mobile phase of toluene-dioxane-acetic acid (20:20:1, v/v) (19). All solvents were UV or HPLC grade and were purchased from Burdick & Jackson Laboratories. N,N-Diisopropylethylamine, 98%, and formic acid, 95-97%, were obtained from Aldrich Chemical Company. Phosphoric acid, 85%, and sodium hydroxide pellets were obtained from Mallinckrodt, Inc. Silylation grade pyridine and methoxyamine hydrochloride were purchased from Pierce Chemical Company.
Sample Preparation Procedure

Standard Preparation

An accurately weighed amount of TxB₂ was dissolved in acetonitrile to produce a concentration of 50 μg/mL. This solution was further diluted to produce another TxB₂ standard with a concentration of 10 μg/mL. All dilutions were performed using polypropylene materials. The solutions were prepared fresh for each experiment. The calibration standard curve was prepared by spiking 2.0 mL aliquots of 3% BSA in Krebs-Henseleit buffer (pH 7.4) (20) with the TxB₂ standard solutions. Eight 3% BSA TxB₂ standards were prepared over a concentration range of 0-500 ng/mL. Pooled 3% BSA TxB₂ standards were prepared to assess interday and intraday assay precision and accuracy. They were prepared at 75, 226, and 376 ng/mL by spiking an appropriate amount of standard solution into a polypropylene tube containing 19 mL of 3% BSA, then aliquoting 2.0 mL samples to tubes for storage at -70°C. Blank, pooled controls were also aliquoted.

Serum Extraction

The serum extraction was a modification of a procedure described in a radioimmunoassay (RIA) kit designed to measure 6-keto-PGF₁₀ in plasma (21). Bond Elut® C18 solid phase extraction columns (Analytichem International) (200 mg/3 mL) were placed in a vacuum manifold (J. T. Baker) (Figure 2) and prepared for the serum extraction by washing with 4 mL of methanol followed by 4 mL of water. The serum samples were acidified to pH 4 by adding 0.2 mL of a 5% formic acid.
Figure 2. Example of a Solid Phase Extraction System.

solution (aq.). The samples were vortexed then centrifuged at 1500 \( \times \) g for 15 min at 4°C. The serum samples were applied to the extraction columns and vacuum was applied (660 mm Hg internal manifold pressure). The columns were washed with 2 mL of water, twice, 2 mL of 10% methanol in water, twice, and 2 mL of toluene.

The C18 columns were removed from the vacuum manifold after the toluene wash and replaced with Bond Elut silica columns (500 mg/3 mL). The silica extraction columns were conditioned by washing with 5 mL of toluene-ethyl acetate (8:2). The C18 columns were placed on top of the silica columns by means of an adaptor and \( \text{TxB}_2 \) was eluted onto the silica column with 1 mL of ethyl acetate. The C18 columns and adaptors were removed and the silica columns were washed sequentially with 1 mL of toluene-ethyl acetate (6:4), toluene-ethyl acetate-methanol (60:40:2), and toluene-ethyl acetate-methanol (6:4:1). \( \text{TxB}_2 \) was eluted from the silica columns with 2 mL of toluene-ethyl acetate-methanol (6:4:3) and the eluent was collected in 4 mL polypropylene tubes. The samples were spiked with IS (10 \( \mu \)L of an 80 \( \mu \)g/mL solution of 6-keto-PGF\(_{1\alpha} \) in acetonitrile) and evaporated under a stream of nitrogen in a 40°C water bath.

**Panacyl Bromide Derivatization**

The derivatization conditions were as described previously (15). Briefly, the samples were vortexed 1 min to reconstitute in 0.25 mL of panacyl bromide solution, 25 \( \mu \)g in tetrahydrofuran-acetonitrile, 1:4, and 3 \( \mu \)L of N,N-diisopropylethylamine was added to initiate the reaction. The samples were capped and incubated for 1 hr at 40°C. The reaction is depicted in Figure 3 for \( \text{TxB}_2 \).
Figure 3. Panacyl Bromide Derivatization of TxB₂.
Panacyl Ester Purification

Excess derivatizing reagent was removed using a procedure previously described by Watkins and Peterson (13). Bond Elut silica columns (500 mg/3 mL) were placed in the vacuum manifold and washed sequentially with 5 mL of tetrahydrofuran-water (95:5), acetonitrile, and methylene chloride. The derivatization samples were applied and pulled through the column under vacuum. The columns were washed with 2.8 mL of methylene chloride three times. The eicosanoids were eluted into 4 mL polypropylene tubes in 2.8 mL of acetonitrile-methanol (85:15). The samples were placed in a 40°C water bath and evaporated under nitrogen.

Methoxyamine Derivatization

Methoximes were prepared as previously described by Fitzpatrick et al. (18). The reaction of the TxB₂ panacyl ester is depicted in Figure 4. The residue from the panacyl ester purification was reconstituted in 0.2 mL of a saturated solution of methoxyamine hydrochloride in pyridine by vortexing for 1 min. The samples were incubated overnight (16 hr) at room temperature. After incubation the pyridine was evaporated under a nitrogen stream in a 40°C water bath.

Extraction and Preparation for HPLC

The methoximes were extracted using a procedure employed by O'Leary for PG derivatives prepared for GC/MS analysis (22). The dried samples were removed from the water bath and 2.0 mL of diethyl ether and 1.0 mL of water were added to the tubes. The tubes were
Figure 4. Methoximation Reaction Showing the Syn/Syn Orientation of the TxB2 Derivative.
capped and vortexed for 1 min followed by a five min centrifugation. The ether layer was drawn off using a disposable polyethylene transfer pipet (Bio-Rad) and placed in another tube. Another 2.0 mL of diethyl ether was added to the remaining aqueous fraction and the process was repeated. The ether layers were pooled and evaporated. The dried samples were reconstituted by adding 680 µL of acetonitrile and vortexing for 1 min. The reconstituted samples were mixed with 320 µL of water and filtered (Nylon 66 filters, 3 mm membrane, 0.45 µm pore size, Micron Separations) into 2 mL polypropylene injection vials (Sarstedt). The samples were loaded onto the autosampler for analysis by HPLC.

High Performance Liquid Chromatography

Instrumentation and Conditions

A schematic of the HPLC system is presented in Figure 5. The mobile phase was acetonitrile-10 mM phosphoric acid in water (pH 7.2) (68:32). Mobile phase was filtered (0.2 µ nylon-66 filter, Rainin) prior to use. The two HPLC pumps were a Beckman 112 and a Laboratory Data Control (LDC) miniPump operated at flow rates of 2.0 and 1.0 mL/min, respectively. Samples (200 µL) were injected with an Upjohn µP autosampler equipped with a Valco ten-port air-actuated switching valve and an FMI sampling pump. The guard column was a Brownlee RP-8 30 x 4.6 mm I.D. octylsilane bonded-phase column and the analytical column was a Supelcosil LC-18 (Supelco) 250 x 4.6 mm I.D. octadecylsilane bonded-phase column (both packing materials are spherical particles, 5 µm in diameter). The detector was an LDC UV Monitor III equipped with a mercury lamp to monitor absorbance at 254 nm.
Figure 5. Schematic Diagram of the HPLC System in the Inject Position.
The switching valve position was controlled by the autosampler (shown in the inject position in Figure 5). In the inject position the Beckman 112 pump routed the injection loop contents onto the guard column. The analytes were eluted from the guard column and directed onto the analytical column. The switching valve remained in the inject position for 81 seconds at which time it switched to the load position. In the load position, solvent from the Beckman pump flowed directly into the analytical column and solvent from the miniPump was directed through the guard column into waste. The total chromatography time was 25 min per sample.

Data Acquisition and Quantitation Method

Collection and analysis of the detector output (1V/A.U.) was performed with the UPACS II automated chromatography system (Upjohn) on a Harris 1000 computer. Output from the detector was also recorded on a Linear strip chart recorder. Data were analyzed by an unweighted linear regression forced through the origin of peak height ratio (major TxB$_2$ peak/IS) versus concentration.

Ex Vivo TxB$_2$ Production Studies

Human blood was allowed to clot in the presence and absence of a thromboxane synthetase inhibitor (U-63557A, Upjohn) and the serum was harvested and analyzed to determine TxB$_2$ levels. Six 10 mL tubes of blood were collected from four volunteers. Duplicate tubes contained 0, 20, and 200 µg of U-63557A dissolved in 100 µL of 0.9% saline solution. The tubes were incubated at 37°C for 1 hr after collection.
then centrifuged for 20 min at 1500 x g and 4°C. Serum from duplicates was pooled and stored in 2 mL aliquots at -70°C. Samples were analyzed within one week of collection.
CHAPTER III

RESULTS AND DISCUSSION

Serum Extraction

The serum extraction was a modification of a previously reported procedure for extracting 6-keto-PGF$_{1\alpha}$ from plasma (21). The extraction technique involved C18/silica mode sequencing employing Bond Elut solid phase extraction columns. In the first phase of the procedure, serum (2 mL) was acidified to pH 4 with 200 µL of 5% formic acid (aq.) and passed through the C18 columns. The columns were washed with water and 10% methanol (aq.) to elute polar components; then toluene to elute neutral lipids and to remove residual water from the columns. TXB$_2$ was eluted from the C18 columns onto the silica columns with ethyl acetate.

In the second phase of the extraction the silica columns were washed sequentially with toluene-ethyl acetate (6:4), toluene-ethyl acetate-methanol (60:40:2), and toluene-ethyl acetate-methanol (6:4:1). TXB$_2$ was eluted in toluene-ethyl acetate-methanol (6:4:3). The effect of washing with increasingly polar solvent mixtures was to remove components less polar than TXB$_2$ and leave components with greater polarity on the columns.

Procedural recovery for the IS in plasma and serum was determined by adding 6-keto-PGF$_{1\alpha}$-³H (26,500 dpm) to a 400 ng/mL cold standard. The modified mixed mode extraction resulted in unacceptably low recovery (20.5%) with a large amount of variability (21% RSD, n = 6).
As a consequence, the IS was added after the solid phase extraction. When 6-keto-PGF$_1$-$^3$H was added to the silica column eluant, procedural recovery was 57.1% with a RSD = 6.7%. Procedural recovery of TxB$_2$-$^3$H (20,000 dpm) from standards containing 400 ng/mL of cold TxB$_2$ and IS was 66.3% with a RSD = 5.3% (n = 6). An attempt to simplify the procedure by performing only the C18 extraction step was unsuccessful due to increased interferences in the HPLC elution window of TxB$_2$.

Derivatizations and Purification

**Panacyl Bromide Derivatization**

Samples were derivatized as described previously (15) using 25 $\mu$g of panacyl bromide. To ensure that the optimum amount of reagent was being used, triplicate solution standards containing 800 ng of both TxB$_2$ and IS were derivatized using 2.5, 25 and 250 $\mu$g of panacyl bromide. All other reaction conditions remained unchanged. Using 250 $\mu$g of panacyl bromide did not increase the peak height of the two eicosanoids compared to the 25 $\mu$g samples, but the amount of interference peaks increased significantly. The 2.5 $\mu$g samples contained barely detectable peaks.

**Panacyl Ester Purification**

Excess derivatizing reagent was removed using a procedure described by Watkins and Peterson (13) for silica Sep-Pak cartridge columns (Waters Associates). The Bond Elut columns were partially
deactivated by rinsing with tetrahydrofuran-water (95:5) and any column impurities soluble in tetrahydrofuran were removed. This was followed by washes with acetonitrile and methylene chloride. The derivatization solution was applied and the unreacted panacyl bromide was eluted in 3 x 2.8 mL washes of methylene chloride. The panacyl esters were eluted with acetonitrile-methanol (85:15).

Methoxyamine Derivatization

The panacyl esters were converted to methoximes by reacting with methoxyamine (18). The reaction was allowed to take place overnight (16 hr) at room temperature. The reaction could have been completed in 2 hr at an elevated temperature (45°C) but this proved to be a convenient stopping point in the procedure. To confirm that the optimum amount of methoxyamine was being added (5 mg), triplicate 800 ng solution standards of TXB₂ and IS were reacted with 0.5, 5 and 10 mg of methoxyamine. The 10 mg samples did not display increased peak heights but the evaporation time was lengthened considerably. The 0.5 mg samples had significantly reduced peak heights.

Ether Extraction and Preparation for HPLC

Unreacted methoxyamine and the methoxime-panacyl ester derivatives were separated by partitioning between ether and water. The pooled ether extracts containing the methoxime-panacyl esters were evaporated then reconstituted in 680 μL of acetonitrile. The samples were diluted with 320 μL of water to make the solvent strength of the injection solution equal to that of the mobile phase. A sample
filtration step was instituted after HPLC column back pressure build up was detected with unfiltered samples and mobile phase.

**High Performance Liquid Chromatography**

The HPLC system was operated under isocratic conditions [mobile phase: acetonitrile-10 mM phosphoric acid in water (pH 7.2) (68:32)] with detection at 254 nm and a ten-port switching valve to permit a guard column wash step. It was necessary to prepare methoxime-panaclyl ester derivatives of the serum extracts to impart the TxB$_2$ molecule with favorable absorbance/fluorescence and chromatographic properties. TxB$_2$ does not contain any conjugated double bonds or aromatic rings and therefore does not exhibit innate absorbance at 254 nm. Previous reports have demonstrated the use of panaclyl bromide to prepare strongly UV absorbant and fluorescent derivatives of prostaglandins and TxB$_2$ (13, 15, 23). As an example, the molar absorptivity of the panaclyl ester of PGE$_2$ in acetonitrile is 163,480 at 254 nm and fluorescence detection is approximately 7-10 times more sensitive than UV (23). In the current study, no gain in assay sensitivity was achieved using fluorescence detection because baseline interferences were increased proportionately.

Because of the tendency of TxB$_2$ to exist in an equilibrium between ring open and closed forms of the molecule, the panaclyl ester chromatographs as two broad unresolved peaks (13). As a consequence, Watkins and Peterson were unable to separate the panaclyl esters of TxB$_2$ and prostaglandin F$_2\alpha$ (PGF$_2\alpha$) (13). A more recent report described the use of a reverse phase column with a polymer-based
support (PRP-1, Hamilton) for the determination of underivatized TxB₂⁻³H using a radioactivity monitor (9). The authors operated with a mobile phase of acetonitrile-1.2% triethylamine in water (pH 7.5) (21:79) and reported an increase in the number of theoretical plates over silica-based columns, presumably because the alkaline pH fixed the TxB₂ molecule in the ring open position. However, the TxB₂ peak was unresolved from PGF₂α. Work in this laboratory indicated that in the absence of PGF₂α the panacyl ester of TxB₂ still exhibits tailing with a PRP-1 column and a mobile phase of acetonitrile-1.2% triethylamine in water (pH 10.0) (68:32) (24). This tailing would have been obscured by PGF₂α in the work by Moonen et al. (9).

The chromatography problem was solved by converting the panacyl ester to a methoxime, thus fixing the molecule in the ring opened form. A methoxime was formed for each carbonyl carbon in an aldehyde or ketone functionality. Two symmetrical peaks were obtained for each methoxime, corresponding to the syn and anti isomers of the molecule (Figure 4). All compounds had at least two peaks resulting from methoximation of the ketone functionality in the panacyl group. Eicosanoids that retained ketone or aldehyde functionalities under the basic reaction conditions produced four chromatographic peaks corresponding to the syn/syn, syn/anti, anti/syn and anti/anti isomer pairings of the two methoximes. The relative peak heights that were produced reflected the extent of formation of the particular configurational isomer. Structures of seven eicosanoids that were derivatized and the number of chromatographic peaks they produced are presented in Figure 6.
Figure 6. Eicosanoid Structures and the Number of Resultant Methoxime-Panacyl Ester Peaks After Derivatization Under Basic Conditions.
The chromatographic specificity is demonstrated by the data in Table 1 and the chromatogram in Figure 7. In addition to TxB$_2$ and 6-keto-PGF$_{1\alpha}$, eight relevant eicosanoid standard solutions were derivatized and chromatographed. All were resolved from TxB$_2$ and 6-keto-PGF$_{1\alpha}$ with the exception of 11-dehydro-TxB$_2$ which coeluted with two of the peaks produced with TxB$_2$.

### Table 1

HPLC Retention Times of Methoxime-Panacyl Ester Derivatives of Some Relevant Eicosanoids

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Retention times (min)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-keto-PGF$_{1\alpha}$</td>
<td>9.9, 11.6*</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>11.7, 13.7*</td>
</tr>
<tr>
<td>TxB$_2$</td>
<td>13.3, 14.0, 15.6*, 16.5*</td>
</tr>
<tr>
<td>11-dehydro-TxB$_2$</td>
<td>14.3, 16.8*</td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>18.8, 20.5, 22.1, 23.9*</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>20.1, 21.6, 24.2, 25.6*</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>21.0, 21.5, 24.4, 25.3*</td>
</tr>
</tbody>
</table>

Note: PGA$_2$, PGB$_2$ and 13,14-dihydro-6,15-diketo-PGF$_{1\alpha}$ were not detected after derivatization and injection under these chromatographic conditions.

$^a$Major peak(s) indicated with an asterisk.
Figure 7. Chromatogram of Methoxime-Panacyle Ester Derivatives of Some Relevant Eicosanoids.
Studies on thromboxane metabolism in the rabbit have indicated that 11-dehydro-TxB$_2$ is formed by tissue bound enzymes (25) and thus would not be manufactured during ex vivo clot formation. Levels of 11-dehydro-TxB$_2$ would reflect in vivo thromboxane release and therefore approximate plasma TXB$_2$ levels of < 100 pg/mL (4). 11-Dehydro-TxB$_2$ would not represent an interference problem given the quantitation limit (25 ng/mL) of the current assay. PGF$_{2\alpha}$, which is formed in ng/mL concentrations in serum (6), was baseline resolved from the major peaks for TXB$_2$. Previously, underivatized TXB$_2$ (9) and the panacyl ester of TXB$_2$ (13) could not be resolved from the corresponding forms of PGF$_{2\alpha}$ by HPLC.

A ten-port switching valve was employed in the chromatography system to permit guard column washing while the analytical separation was taking place. The elution volume of the methoxime-panacyl ester derivatives of TXB$_2$ was determined by using a set switching time and varying the flow rate of the HPLC pump. Complete recovery was achieved in a 2.2 mL elution volume. To allow for variations in mobile phase strength and column retention properties, a 2.7 mL elution window was injected onto the analytical column (2 mL/min for 1.35 min) before switching.

The chromatography system appeared to display satisfactory reproducibility. Within-run retention times (and % RSD) for the IS and TXB$_2$ were 11.27 (0.14%) and 16.06 (0.28%) minutes, respectively (n = 15). Injection of more than 200 derivative samples over a two-month period produced only a small increase in analytical column backpressure (20 psi, 1.2%) and retention times (< 0.3 min). Interday differences in mobile phase composition, as well as chromatography using three
different Supelcosil LC-18 analytical columns did not significantly alter chromatographic peak resolution.

Assay Linearity

The matrix chosen for the calibration standards was 3% BSA. Standards could not be prepared in human serum because of the presence of endogenous TxB₂. Although human plasma did not contain a detectable amount of endogenous TxB₂, it did contain a component which coeluted with the IS.

Representative linearity data from three standard calibration curves is presented in Table 2. The major TxB₂ derivative peak was chosen for quantitative purposes to provide the maximum signal-to-noise ratio. Plots of peak height ratio (TxB₂/IS) versus TxB₂ serum concentration had correlation coefficients of 0.994 or greater over a range of 25-500 ng/mL (25, 50, 100, 200, 300, 400 and 500 ng/mL). A response factor (RF) was calculated at each concentration using the following equation:

\[ RF = \frac{[\text{peak height ratio } \text{TxB}_2/\text{IS}] \times \text{(IS concentration)}}{\text{TxB}_2 \text{ concentration}} \]

Response factors for individual standards were scattered randomly about the mean indicating that extraction recovery and derivatization efficiency was constant over the entire concentration range. The relative standard deviation about the mean RF ranged from 6 to 10%. The y-intercept was not different than zero at the 95% significance level and therefore data was analyzed using an unweighted linear regression forced through the origin. Chromatograms of blank, 50, and 500 ng/mL calibration standards are presented in Figure 8.
Table 2

Linearity of Representative 3% BSA TxB$_2$
Standard Calibration Curves

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Response factor$^a$</th>
<th>Concentration (ng/mL)</th>
<th>Response factor</th>
<th>Concentration (ng/mL)</th>
<th>Response factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>510</td>
<td>0.632</td>
<td>502</td>
<td>0.661</td>
<td>525</td>
<td>0.607</td>
</tr>
<tr>
<td>408</td>
<td>0.551</td>
<td>402</td>
<td>0.652</td>
<td>420</td>
<td>0.524</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>301</td>
<td>0.756</td>
<td>315</td>
<td>0.562</td>
</tr>
<tr>
<td>204</td>
<td>0.623</td>
<td>201</td>
<td>0.564</td>
<td>210</td>
<td>0.571</td>
</tr>
<tr>
<td>102</td>
<td>0.566</td>
<td>100</td>
<td>0.707</td>
<td>105</td>
<td>0.600</td>
</tr>
<tr>
<td>51.0</td>
<td>0.595</td>
<td>50.2</td>
<td>0.718</td>
<td>52.5</td>
<td>0.541</td>
</tr>
<tr>
<td>25.5</td>
<td>0.545</td>
<td>25.1</td>
<td>0.742</td>
<td>26.3</td>
<td>0.647</td>
</tr>
</tbody>
</table>

Mean RF  0.585  0.686  0.579
SD    0.037  0.066  0.042
RSD  6.3%  9.6%  7.3%
Slope$^b$  0.00151  0.00167  0.00144
y-intercept$^c$ -0.00429  0.00290  -0.00208
r    0.996  0.994  0.996

Note: A 300 ng/mL standard was not prepared on day 1.

$^a$Response factor (RF) = [(Peak height ratio TxB$_2$/IS) x (IS concentration)]/TxB$_2$ concentration.

$^b$Slope obtained from a best-fit unweighted linear regression analysis of a plot of peak height ratio (TxB$_2$/IS) versus the concentration of TxB$_2$.

$^c$The y-intercept was not different than zero at the 95% significance level in all cases.
Figure 8. Chromatograms of 3% BSA Calibration Standards.
Assay Precision and Accuracy

The intraday and interday assay precision and accuracy was evaluated at 75, 226 and 376 ng/mL using pooled 3% BSA standards. Triplicate assays of the pooled standards were repeated on three successive days using independently prepared calibration standards (Table 3). There was no significant interday difference in assay results ($p>0.05$) so the data at each concentration were pooled. The estimate of the assay RSD at all three concentrations was between 6 and 9%. The pooled assay results showed no significant bias (within the limits of assay precision, $p>0.05$) for any of the three pooled standards (error $\leq 4\%$).

Ex Vivo $TxB_2$ Production

The utility of the method was tested by analyzing serum samples obtained from four human subjects. The experiment was designed to evaluate ex vivo production of $TxB_2$ during clot formation in the presence and absence of thromboxane synthetase inhibitor U-63557A. Results from the analysis of serum samples with whole blood concentrations of U-63557A of 0, 2 and 20 $\mu$g/mL are presented in Table 4. All but two of the samples were assayed in duplicate.

The assay results verify that U-63557A inhibits ex vivo production of $TxB_2$, and to a greater extent at 20 $\mu$g/mL versus 2 $\mu$g/mL. Serum $TxB_2$ concentrations in the absence of inhibitor ranged from 340 to 390 ng/mL in three of the four subjects. This is well within the range reported in the literature (220-550 ng/mL) for serum $TxB_2$. 

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Table 3
Intraday and Interday Assay Accuracy and Precision for Pooled 3% BSA TxB\(_2\) Standards

<table>
<thead>
<tr>
<th>Actual concentration (ng/mL)</th>
<th>Mean assay results (ng/mL)(^a)</th>
<th>Pooled estimate of RSD and accuracy(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75.2</td>
<td>72.7</td>
<td>76.2</td>
</tr>
<tr>
<td>SD</td>
<td>5.12</td>
<td>3.57</td>
</tr>
<tr>
<td>RSD</td>
<td>7.0%</td>
<td>4.7%</td>
</tr>
<tr>
<td>Error</td>
<td>-3.3%</td>
<td>+1.4%</td>
</tr>
<tr>
<td>226</td>
<td>210</td>
<td>225</td>
</tr>
<tr>
<td>SD</td>
<td>12.9</td>
<td>9.3</td>
</tr>
<tr>
<td>RSD</td>
<td>6.1%</td>
<td>4.1%</td>
</tr>
<tr>
<td>Error</td>
<td>-6.9%</td>
<td>-0.6%</td>
</tr>
<tr>
<td>376</td>
<td>382</td>
<td>410</td>
</tr>
<tr>
<td>SD</td>
<td>42.3</td>
<td>25.0</td>
</tr>
<tr>
<td>RSD</td>
<td>11.1%</td>
<td>6.1%</td>
</tr>
<tr>
<td>Error</td>
<td>+1.6%</td>
<td>+8.9%</td>
</tr>
</tbody>
</table>

\(^a\) \(n = 3\) on each day at each concentration.

\(^b\) There was no interday difference in means or assay bias in the pooled means at the 95% significance level in all cases.

levels using GC/MS and RIA techniques (2-6). Subject JC was taking allergy medication during the study which may be responsible for the depressed TxB\(_2\) concentrations.

In the presence of U-63557A, three out of four subjects contained an obvious interference which coeluted with TxB\(_2\). The interference was easily detected because of the increased assay selectivity.
Table 4
Assay Results from a Study of Ex Vivo TxB$_2$ Production

<table>
<thead>
<tr>
<th>U-63557A concentration (µg/mL)$^a$</th>
<th>Subject</th>
<th>Serum TxB$_2$ concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>PL</td>
<td>366, 362</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>379, 389</td>
</tr>
<tr>
<td></td>
<td>JC</td>
<td>178, 183</td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>339, 386</td>
</tr>
<tr>
<td>2</td>
<td>PL</td>
<td>108, 113</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>100, 101b</td>
</tr>
<tr>
<td></td>
<td>JC</td>
<td>69.5, 71.6b</td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>86.6b</td>
</tr>
<tr>
<td>20</td>
<td>PL</td>
<td>60.5, 58.9</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>60.4, 57.4b</td>
</tr>
<tr>
<td></td>
<td>JC</td>
<td>33.7, 47.0b</td>
</tr>
<tr>
<td></td>
<td>MB</td>
<td>54.1b</td>
</tr>
</tbody>
</table>

$^a$ Whole blood concentration of thromboxane synthetase inhibitor U-63557A.

$^b$ Contained an interference peak which coeluted with TxB$_2$.

afforded by the doublet peak pattern for TxB$_2$. In subjects containing this unidentified serum component the doublet peak height ratio was significantly altered (Figure 9). The TxB$_2$ peak used for quantitation was artificially increased, thus creating a positive bias in assay results. Chromatograms of samples from subject PL did not contain a TxB$_2$ interference peak (Figure 10).
Figure 9. Chromatogram of a Sample Containing 2 µg/mL of U-63557A Displaying the Altered TxB₂ Doublet Peak Height Ratio Due to an Interference.
Figure 10. Chromatograms From Subject PL for U-63557A 
Concentrations of (a) 2 μg/mL and (b) 0 μg/mL.
CHAPTER IV

CONCLUSIONS

The applicability of an HPLC technique employing UV detection (254 nm) was demonstrated for the quantitative analysis of TxB₂ in human serum. TxB₂ was extracted from serum using a mode sequencing (C₁₈/silica) solid phase extraction. The internal standard, 6-keto-PGF₁α, was spiked into the eluant from the serum extraction. The extracts were derivatized with panacetyl bromide to impart UV absorbant/fluorescent properties to the molecules. Excess derivatizing reagent was removed by a silica solid phase purification. The samples were then converted to methoximes to provide satisfactory chromatographic properties by fixing TxB₂ in the ring opened form. The methoxime-panacetyl ester derivatives were extracted into ether and filtered before injection into the HPLC system in acetonitrile-water (7:3, v/v).

Assay linearity, precision and accuracy was evaluated using 3% BSA fortified with TxB₂. The method was linear over a concentration range of 25 to 500 ng/mL when processing a 2 mL sample (r²=0.994, eight separate analysis days). The precision of the method at 75, 226 and 376 ng/mL ranged from 6-9% RSD with no assay bias at any level. The sample preparation time for one person to process twenty samples was 8 hr (excluding an overnight incubation). The HPLC run time for twenty samples was 8.3 hr (unattended).

HPLC assay results were consistent with previously published
results for the analysis of human serum to assess *ex vivo* production of *Tx*B₂ during clot formation. In the presence of a thromboxane synthetase inhibitor, three out of four subjects contained an interference which coeluted with *Tx*B₂. This introduced a positive assay bias but did not mask the trend of decreased *Tx*B₂ levels as the amount of inhibitor was increased.

The source of this positive bias is unknown since chromatographic specificity experiments did not reveal any substances present in ng/mL amounts that would coelute with *Tx*B₂. Given the selectivity of the current sample preparation procedure, it is reasonably safe to speculate that the interference is most likely a structurally similar metabolite of the arachadonic acid cascade. *Ex vivo* thromboxane synthetase inhibition increases the amount of *PG*H₂ converted to *PGF*₂α, *PGD*₂, *PGE*₂, and *PGI*₂ (6). The interference may be a metabolite of one of these compounds that is not produced in detectable amounts in the absence of U-63557A. Elimination of this interference would require a highly selective procedure, such as an immunoextraction approach that has been used in the isolation and analysis of prostaglandins in plasma by HPLC (26, 27) and GC/MS (28). Such extractions however, are best suited to substances present in pg/mL amounts due to the high cost of commercially available rabbit anti-*Tx*B₂ serum (approximately $200/mL).

The only other physicochemical method with the demonstrated capability of quantitating *Tx*B₂ in serum is GC/MS (8). The procedure described in this report provides an alternative physicochemical method for the analysis of *Tx*B₂ without the high instrumentation...
cost and limited sample throughput associated with GC/MS. Serum TxB₂ levels from three drug-free human subjects ranged from 340-390 ng/mL by HPLC analysis. This is well within the range reported in the literature (220-550 ng/mL) using both GC/MS and RIA techniques to analyze serum TxB₂ levels (2-6).

Two areas for further investigation include the extension of the assay to the analysis of other components of the arachadonic acid cascade and the improvement of assay sensitivity. The investigation of chromatographic specificity indicated that solution standards of PGF₂α, PGD₂ and PGE₂ could be derivatized and detected using the current procedure. All three compounds are present in serum in ng/mL amounts (6) and could possibly be quantitated during thromboxane synthetase inhibition with only minor procedural modifications. In fact, serum samples collected with TxB₂ inhibition contained a component with a peak pattern and retention times identical to the PGE₂ standard. The sensitivity limit of the current assay could possibly be extended to the pg/mL range by employing a more selective extraction procedure combined with fluorescence detection. Successful modifications in this regard would permit the application of the assay to the measurement of eicosanoid levels in plasma.
REFERENCES


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