Western Michigan University
ScholarWorks at WMU

Master's Theses | Graduate College

12-1986

High Performance Liquid Chromatographic Determination of Thromboxane B_2 in Human Serum as a Methoxime-Panacyl Ester Derivative

Robert H. Pullen

Follow this and additional works at: https://scholarworks.wmich.edu/masters_theses

 Part of the Analytical Chemistry Commons

Recommended Citation

Pullen, Robert H., "High Performance Liquid Chromatographic Determination of Thromboxane B_2 in Human Serum as a Methoxime-Panacyl Ester Derivative" (1986). *Master's Theses*. 1358.
https://scholarworks.wmich.edu/masters_theses/1358

This Masters Thesis-Open Access is brought to you for free and open access by the Graduate College at ScholarWorks at WMU. It has been accepted for inclusion in Master's Theses by an authorized administrator of ScholarWorks at WMU. For more information, please contact wmu-scholarworks@wmich.edu.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THROMBOXANE B_2 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THROMBOXANE B_2 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 B_2 (TxB_2) 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 $F_{1\alpha}$, 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 TxB_2 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 TxB_2 levels in human serum. Results were consistent with previously published values when drug-free serum was analyzed to assess ex vivo TxB_2 formation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGEMENTS

I would like to thank Dr. James A. Howell and Dr. Jeffrey W. Cox for their guidance throughout the course of this research. I would also like to thank Drs. Frank A. Fitzpatrick and Edward G. Daniels for their helpful discussions, and the management of Drug Metabolism Research, The Upjohn Company, for their financial support of this work. A special thanks to my wife, Gini, for her constant patience and encouragement.

Robert H. Pullen, Jr.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again–beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International

300 N. Zeeb Road
Ann Arbor, MI 48106

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1329454

Pullen, Robert H.,Jr.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THROMBOXANE B(2) IN HUMAN SERUM AS A METHOXIME-PANACYL ESTER DERIVATIVE

Western Michigan University M.A. 1986

University
Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1986

by

Pullen, Robert H.,Jr.

All Rights Reserved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Copyright by
Robert H. Pullen, Jr.
1986

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table of Contents--Continued

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER I

INTRODUCTION

Biochemistry and Physiology

Thromboxane B_2 (TxB_2, Figure 1) is the stable hydrolysis product of thromboxane A_2 (TxA_2), a smooth muscle contractant and potent platelet aggregator. These molecules are products of the arachidonic acid cascade. Regulation of TxA_2 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 PGH_2. PGH_2 can be converted by thromboxane synthetase to TxA_2 or it can be converted by prostacyclin synthetase to prostacyclin (PGI_2). When TxA_2 is formed in the platelet, Ca^{2+} 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. PGI_2 acts to antagonize the effects of TxA_2 by stimulating adenylate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

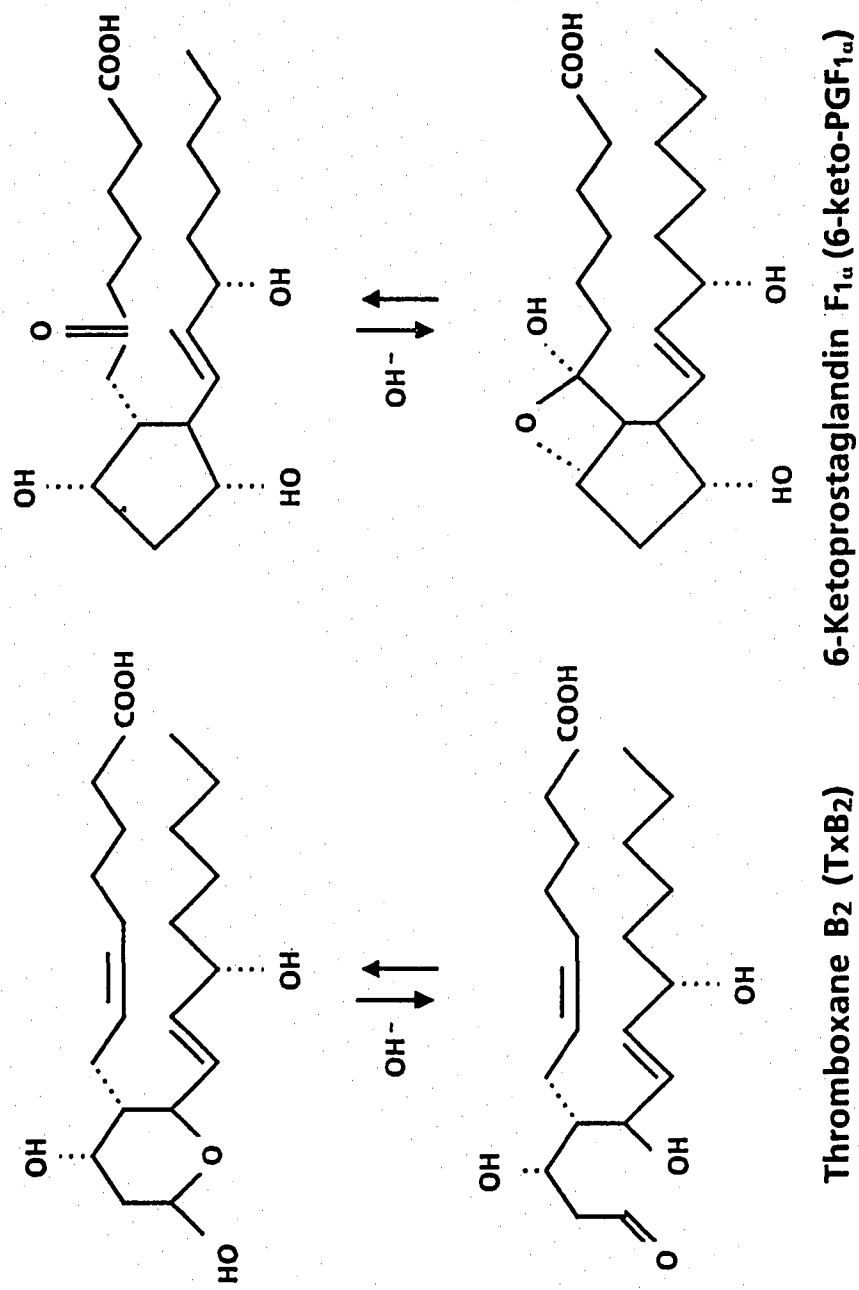

Figure 1. Structures of the Two Forms of TxB_2 and 6-Keto-$PGF_{1\alpha}$.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

have inherent disadvantages. Selectivity in RIA is dependent upon the degree of cross reactivity of the TxB_2 antiserum with other arachidonic 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 instrumentation. Another serious problem is due to the limited sample capacity necessitated by involved experimental procedures and manual sample injection.

Ideally, HPLC could represent a less costly, sensitive and specific physiochemical alternative to GC/MS with comparable sample capacity to RIA. Detection of TxB_2 by HPLC is hampered by the absence of strong ultraviolet (UV) chromophores or natural fluorescence. Additionally, TxB_2 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 solutions of TxB_2 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 derivatization with 4-bromomethyl-7-methoxycoumarin. Their application was limited to the qualitative identification of TxB_2 produced by human platelets. Fitzpatrick et al. (11) prepared the p-nitrobenzyloxime of the TxB_2 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)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

to measure underivatized eicosanoids. Detection in this region is subject to a large number of interferences and a biological application for TxB_2 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, TxB_2 exhibited a broad, double-peaked chromatographic elution pattern and was not resolved from prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). 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 TxB_2 in serum involved doubly derivatizing the eicosanoid molecule. Serum TxB_2 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 TxB_2 concentrations in the presence and absence of a specific thromboxane synthetase inhibitor.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER II

EXPERIMENTAL

Materials

Thromboxane B_2 (TxB_2), 6-ketoprostaglandin $F_{1\alpha}$, the internal standard (IS) (6-keto-$PGF_{1\alpha}$), PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, 11-dehydro-TxB_2, PGA_2, PGB_2, 13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$, 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 B_2 (TxB_2-^3H) (specific activity of 120 Ci/mmol) amd 6-[5,8,9,11,12,14,15-$^3H(N)$]-ketoprostaglandin-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$-^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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Sample Preparation Procedure

Standard Preparation

An accurately weighed amount of TxB_2 was dissolved in acetonitrile to produce a concentration of 50 µg/mL. This solution was further diluted to produce another TxB_2 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 TxB_2 standard solutions. Eight 3% BSA TxB_2 standards were prepared over a concentration range of 0-500 ng/mL. Pooled 3% BSA TxB_2 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-$PGF_{1\alpha}$ 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

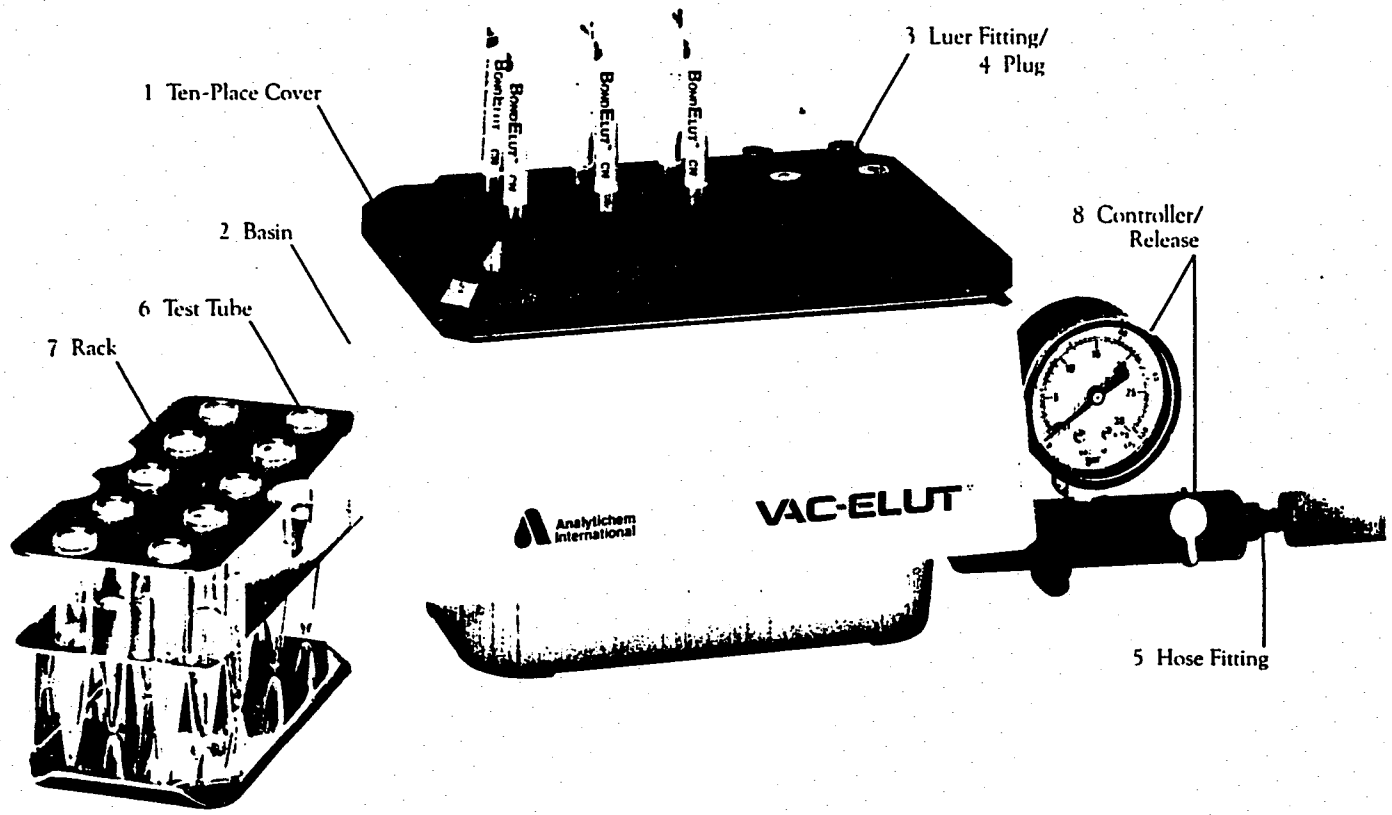

Figure 2. Example of a Solid Phase Extraction System.

Source: Analytichem Internaional. (1985). Sample preparation and separation science. (Available from Analytichem International, Harbor City, CA 90710)

solution (aq.). The samples were vortexed then centrifuged at 1500 x g for 15 min a 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 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). 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 µL of an 80 µg/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 µg in tetrahydrofuran-acetonitrile, 1:4, and 3 µl 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 TxB_2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

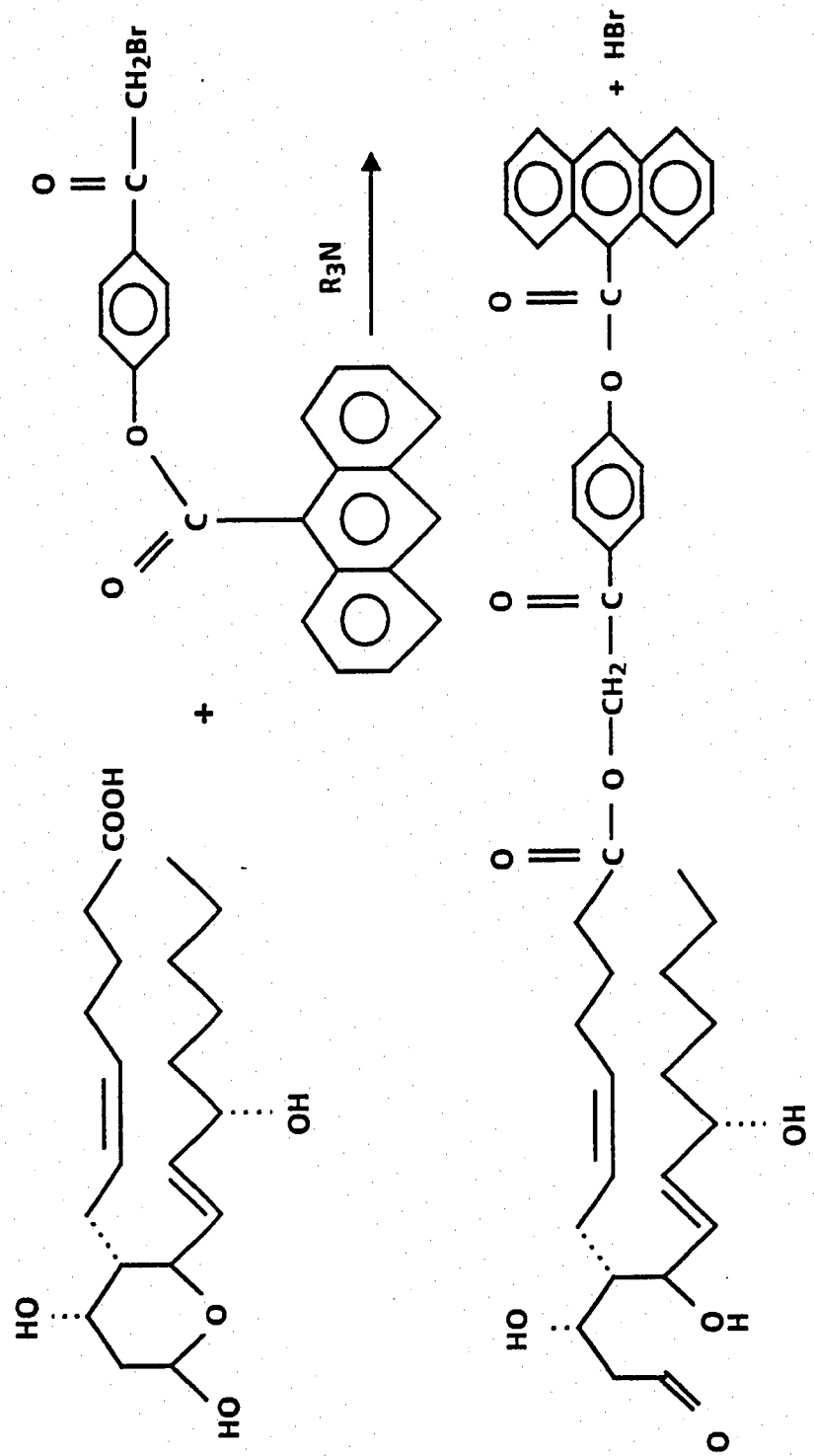

Figure 3. Panacyl Bromide Derivatization of TxB_2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 TxB_2 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

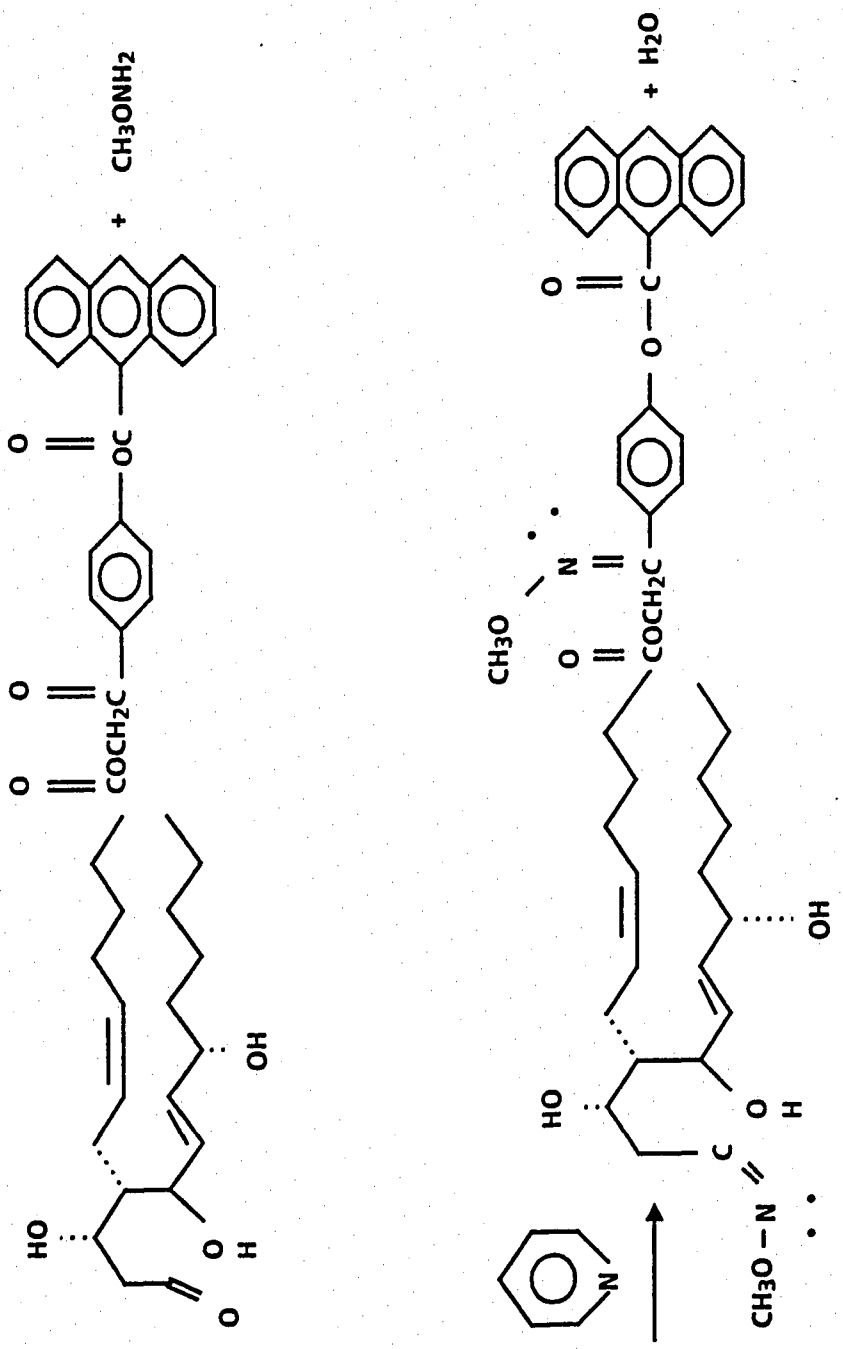

Figure 4. Methoximation Reaction Showing the Syn/Syn Orientation of the TxB_2 Derivative.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 columm 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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

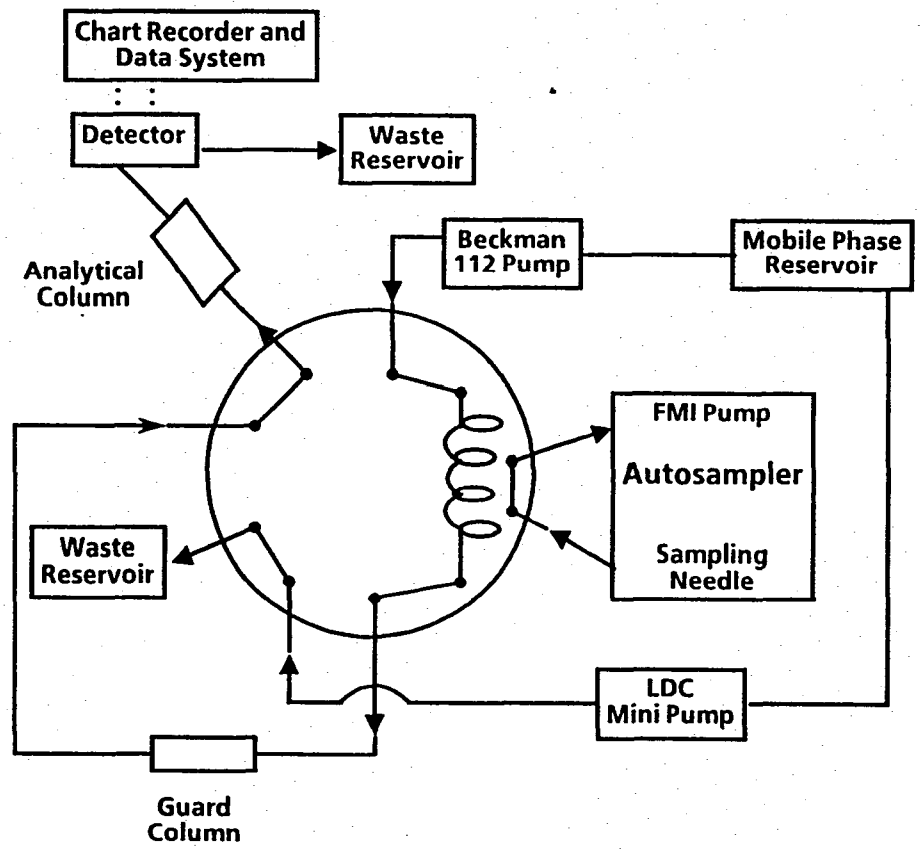

Figure 5. Schematic Diagram of the HPLC System in the Inject Position.

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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

As a consequence, the IS was added after the solid phase extraction. When 6-keto-$PGF_{1\alpha}$-^3H was added to the silica column eluant, procedural recovery was 57.1% with a RSD = 6.7%. Procedural recovery of TxB_2-^3H (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 µg 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 µg of panacyl bromide. All other reaction conditions remained unchanged. Using 250 µg of panacyl bromide did not increase the peak height of the two eicosanoids compared to the 25 µg samples, but the amount of interference peaks increased significantly. The 2.5 µg 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 TxB_2 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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-panacyl 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 panacyl 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 panacyl 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 panacyl ester chromatographs as two broad unresolved peaks (13). As a consequence, Watkins and Peterson were unable to separate the panacyl 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

support (PRP-1, Hamilton) for the determination of underivatized TxB_2-^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 TxB_2 molecule in the ring open position. However, the TxB_2 peak was unresolved from $PGF_{2\alpha}$. Work in this laboratory indicated that in the absence of $PGF_{2\alpha}$ the panacyl ester of TxB_2 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 $PGF_{2\alpha}$ 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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

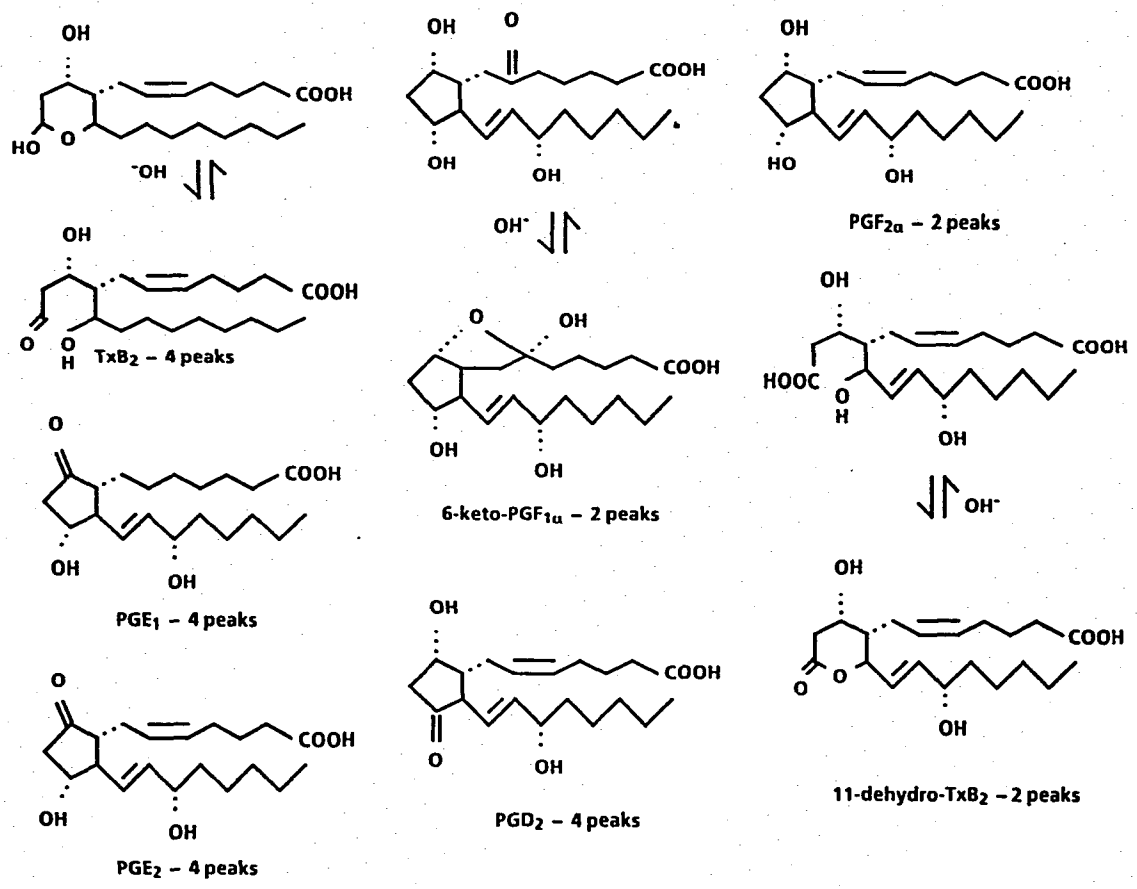

Figure 6. Eicosanoid Structures and the Number of Resultant Methoxime-Panacyl Ester Peaks After Derivatization Under Basic Conditions.

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

Eicosanoid	Retention times (min)[a]
6-keto-$PGF_{1\alpha}$	9.9, 11.6*
$PGF_{2\alpha}$	11.7, 13.7*
TxB_2	13.3, 14.0, 15.6*, 16.5*
11-dehydro-TxB_2	14.3, 16.8*
PGD_2	18.8, 20.5, 22.1, 23.9*
PGE_1	20.1, 21.6, 24.2, 25.6*
PGE_2	21.0, 21.5, 24.4, 25.3*

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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

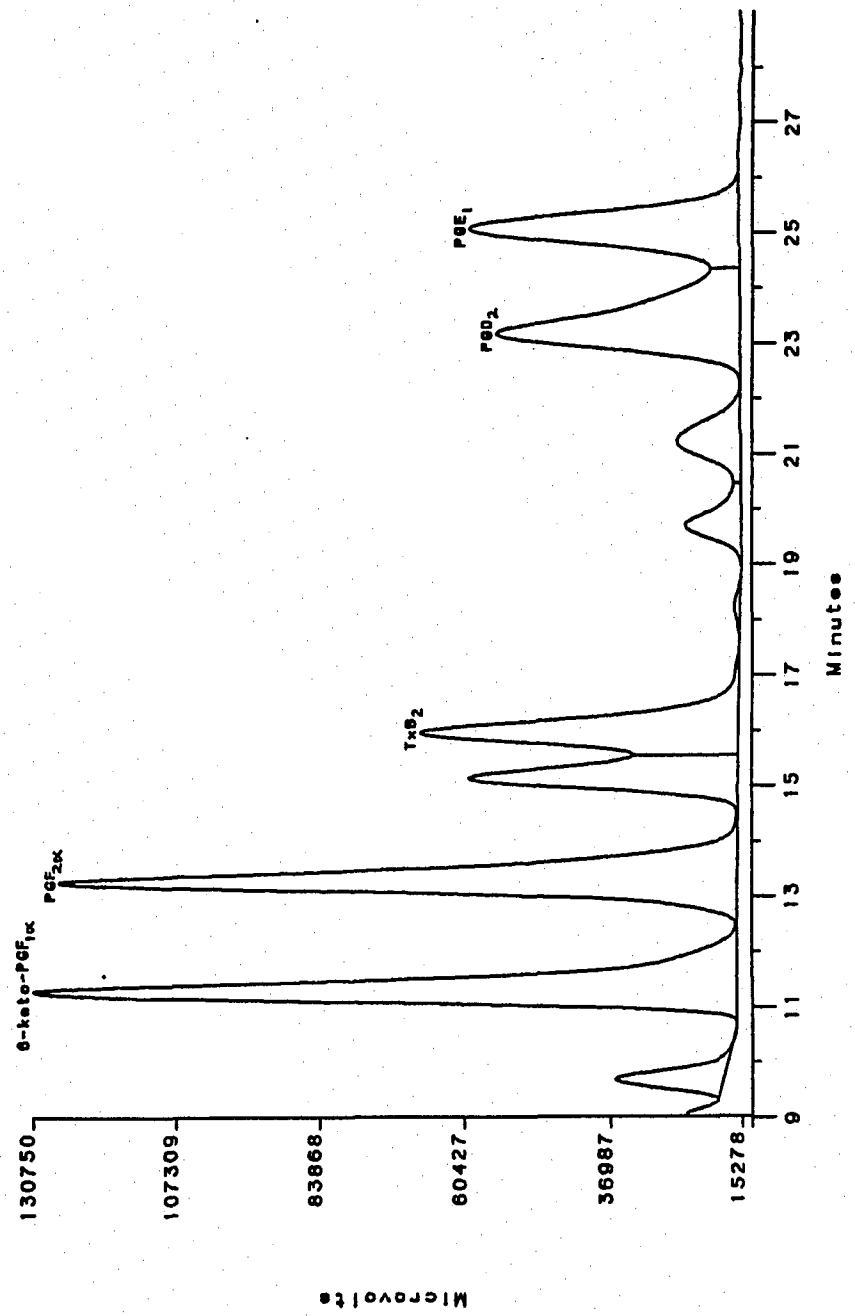

Figure 7. Chromatogram of Methoxime-Panacyl Ester Derivatives of Some Relevant Eicosanoids.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 $\lesssim$ 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 ($\lesssim$0.3 min). Interday differences in mobile phase composition, as well as chromatography using three

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 TxB_2. Although human plasma did not contain a detectable amount of endogenous TxB_2, 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 TxB_2 derivative peak was chosen for quantitative purposes to provide the maximum signal-to-noise ratio. Plots of peak height ratio (TxB_2/IS) versus TxB_2 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:

$$RF = \frac{[(\text{peak height ratio } TxB_2/IS) \times (\text{IS concentration})]}{TxB_2 \text{ concentration}}$$

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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 2

Linearity of Representative 3% BSA TxB_2 Standard Calibration Curves

Day 1		Day 2		Day 3	
Concentration (ng/mL)	Response factor[a]	Concentration (ng/mL)	Response factor	Concentration (ng/mL)	Response factor
510	0.632	502	0.661	525	0.607
408	0.551	402	0.652	420	0.524
-	-	301	0.756	315	0.562
204	0.623	201	0.564	210	0.571
102	0.566	100	0.707	105	0.600
51.0	0.595	50.2	0.718	52.5	0.541
25.5	0.545	25.1	0.742	26.3	0.647
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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

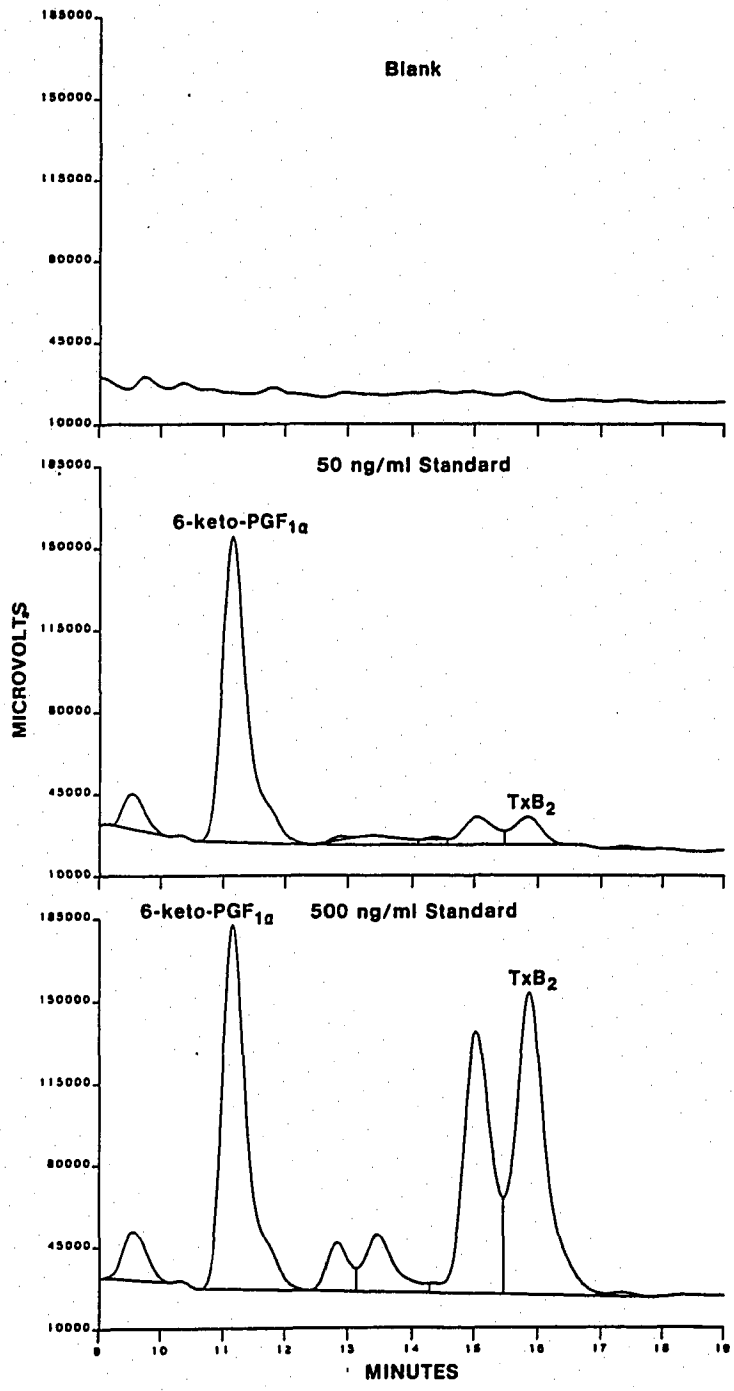

Figure 8. Chromatograms of 3% BSA Calibration Standards.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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 µg/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 µg/mL versus 2 µg/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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 3

Intraday and Interday Assay Accuracy and Precision for Pooled 3% BSA TxB_2 Standards

Actual concentration (ng/mL)		Mean assay results (ng/mL)[a] Day 1	Day 2	Day 3	Pooled estimate of RSD and accuracy[b]
75.2		72.7	76.2	78.5	75.8
	SD	5.12	3.57	6.63	5.21
	RSD	7.0%	4.7%	8.4%	6.9%
	Error	-3.3%	+1.4%	+4.4%	+0.8%
226		210	225	213	216
	SD	12.9	9.3	14.4	12.5
	RSD	6.1%	4.1%	6.8%	5.8%
	Error	-6.9%	-0.6%	-5.6%	-4.4%
376		382	410	356	382
	SD	42.3	25.0	24.7	36.0
	RSD	11.1%	6.1%	6.9%	9.4%
	Error	+1.6%	+8.9%	-5.4%	+1.6%

[a] $\underline{n} = 3$ 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 4

Assay Results from a Study of Ex Vivo TxB_2 Production

U-63557A concentration (μg/mL)[a]	Subject	Serum TxB_2 concentration (ng/mL)
0	PL	366, 362
	RP	379, 389
	JC	178, 183
	MB	339, 386
2	PL	108, 113
	RP	100, 101[b]
	JC	69.5, 71.6[b]
	MB	86.6[b]
20	PL	60.5, 58.9
	RP	60.4, 57.4[b]
	JC	33.7, 47.0[b]
	MB	54.1[b]

[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 artifically 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).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

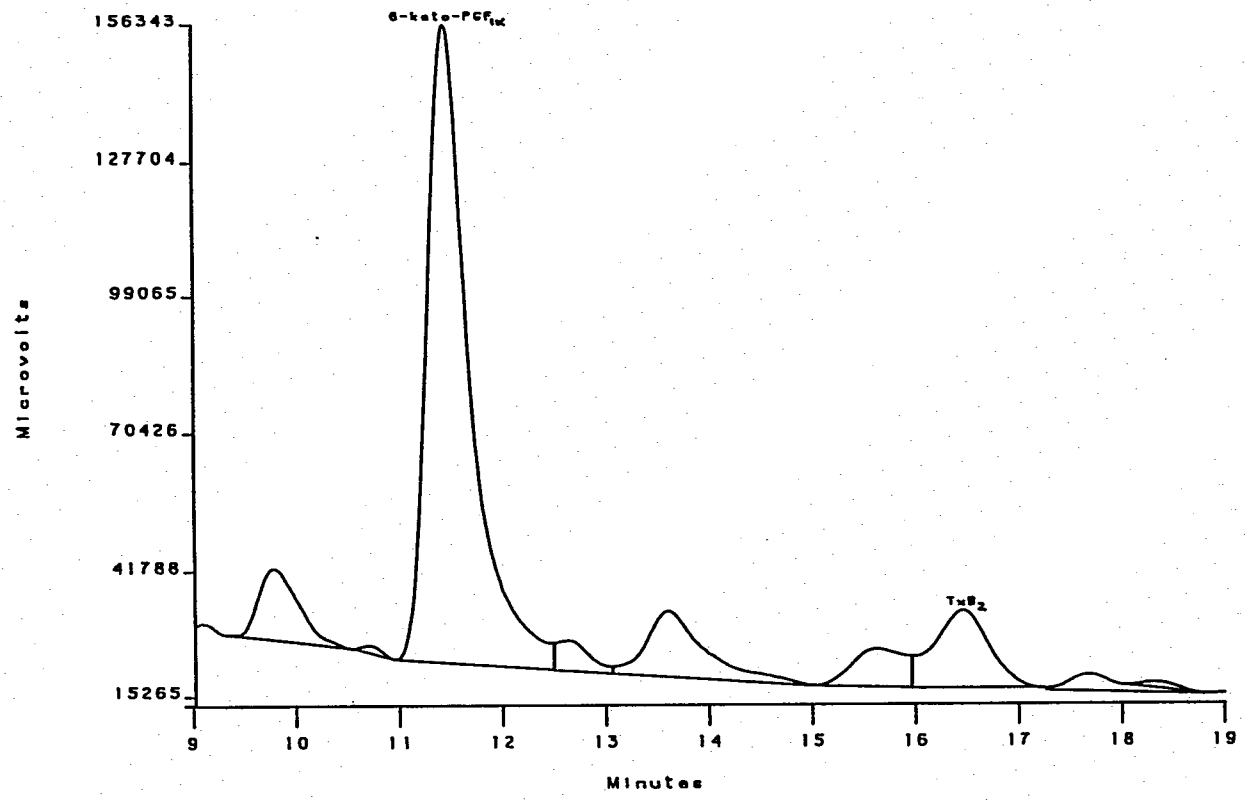

Figure 9. Chromatogram of a Sample Containing 2 µg/mL of U-63557A Displaying the Altered TxB_2 Doublet Peak Height Ratio Due to an Interference.

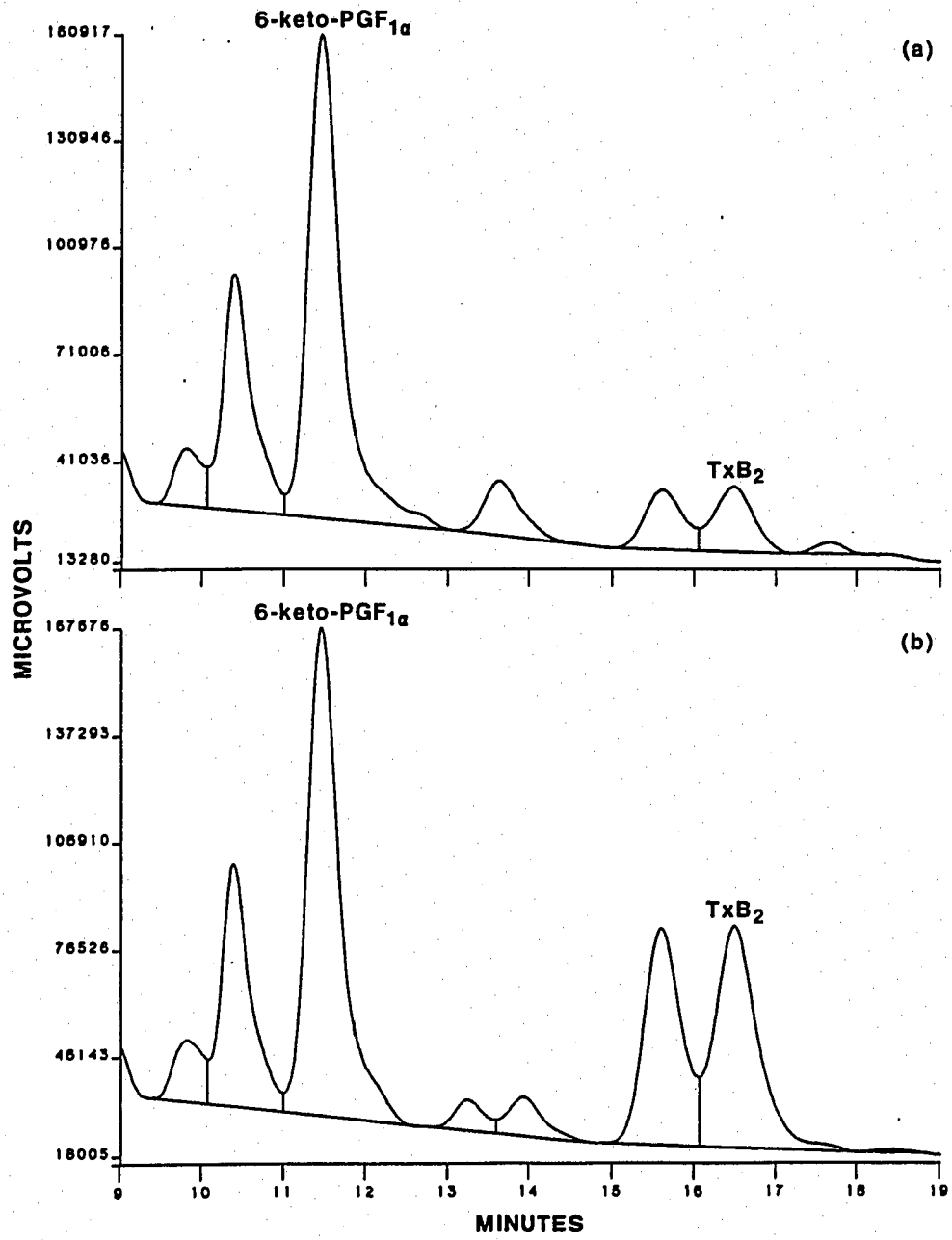

Figure 10. Chromatograms From Subject PL for U-63557A Concentrations of (a) 2 μg/mL and (b) 0 μg/mL.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER IV

CONCLUSIONS

The applicability of an HPLC technique employing UV detection (254 nm) was demonstrated for the quantitative analysis of TxB_2 in human serum. TxB_2 was extracted from serum using a mode sequencing (C18/silica) solid phase extraction. The internal standard, 6-keto-$PGF_{1\alpha}$, was spiked into the eluant from the serum extraction. The extracts were derivatized with panacyl 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 TxB_2 in the ring opened form. The methoxime-panacyl 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 TxB_2. The method was linear over a concentration range of 25 to 500 ng/mL when processing a 2 mL sample ($r \geq 0.994$, 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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

results for the analysis of human serum to assess ex vivo production of TxB_2 during clot formation. In the presence of a thromboxane synthetase inhibitor, three out of four subjects contained an interference which coeluted with TxB_2. This introduced a positive assay bias but did not mask the trend of decreased TxB_2 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 TxB_2. 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 PGH_2 converted to $PGF_{2\alpha}$, 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-TxB_2 serum (approximately $200/mL).

The only other physicochemical method with the demonstrated capability of quantitating TxB_2 in serum is GC/MS (8). The procedure described in this report provides an alternative physicochemical method for the analysis of TxB_2 without the high instrumentation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cost and limited sample throughput associated with GC/MS. Serum TxB_2 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 TxB_2 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 $PGF_{2\alpha}$, PGD_2 and PGE_2 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 TxB_2 inhibition contained a component with a peak pattern and retention times identical to the PGE_2 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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

REFERENCES

1. Gorman, R. R., & Marcus, A. J. (1981, June). Prostaglandins and cardiovascular disease. (Available from The Upjohn Company, Kalamazoo, MI)

2. Pedersen, A. K., Watson, M. L., & FitzGerald, G. A. (1983). Inhibition of thromboxane biosynthesis in serum: Limitations of the measurement of immunoreactive 6-keto-$PGF_{1\alpha}$. Thrombosis Research, 33, 99-103.

3. Chiabrando, C., Castagnoli, M. N., Noseda, A., Fanelli, R., Rajtar, G., Cerletti, C., & de Gaetano, G. (1984). Comparison of radioimmunoassay and high-resolution gas chromatography mass spectrometry for the quantitative determination of serum thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ after pharmacological blockade of thromboxane synthetase. Prostaglandins Leukotrienes and Medicine, 16, 79-88.

4. Morris, H. G., Sherman, N. A., & Shepperdson, F. T. (1981). Variables associated with radioimmunoassay of prostaglandins in plasma. Prostaglandins, 21(5), 771-788.

5. FitzGerald, G. A., Brash, A. R., Oates, J. A., & Pedersen, A. K. (1983). Endogenous prostacyclin biosynthesis and platelet function during selective inhibition of thromboxane synthase in man. Journal of Clinical Investigation, 71, 1336-1343.

6. Orchard, M. A., Blair, I. A., Dollery, C. T., & Lewis, P. J. (1983). Blood can synthesise prostacyclin. The Lancet, 2, 565.

7. Salmon, J. A. (1983). Measurement of eicosanoids by bioassay and radioimmunoassay. British Medical Bulletin, 39(3), 227-231.

8. Blair, I. A. (1983). Measurement of eicosanoids by gas chromatography and mass spectrometry. British Medical Bulletin, 39(3), 223-226.

9. Moonen, P., Klok, G., & Keirse, M. J. N. C. (1983). An improved method for separation of thromboxane B_2 by reversed phase liquid chromatography. Prostaglandins, 26(5), 797-803.

10. Turk, J., Weiss, S. J., Davis, J. E., & Needleman, P. (1978). Fluorescent derivatives of prostaglandins and thromboxanes for liquid chromatography. Prostaglandins, 16(2), 291-309.

11. Fitzpatrick, F. A., Wynalda, M. A., & Kaiser, D. G. (1977). Oximes for high performance liquid and electron capture gas

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

chromatography of prostaglandins and thromboxanes. Analytical Chemistry, 49(7), 1032-1035.

12. Terragno, A., Rydzik, R., & Terragno, N. A. (1981). High performance liquid chromatography and UV detection for the separation and quantitation of prostaglandins. Prostaglandins, 21(1), 101-112.

13. Watkins, W. D., & Peterson, M. B. (1982). Fluorescent/ultraviolet absorbing ester derivative formation and analysis of eicosanoids by high-pressure liquid chromatography. Analytical Biochemistry, 125, 30-40.

14. Rydzik, R., Terragno, A., & Tackett, R. (1984). Picogram detection of eicosanoids by ultraviolet absorbance after narrow-bore high-performance liquid chromatography. Journal of Chromatography, 308, 31-41.

15. Cox, J. W., & Pullen, R. H. (1984). Determination of E prostaglandins by automated heteromodal column switching high-performance liquid chromatography with fluorescence detection. Analytical Chemistry, 56(11), 1866-1870.

16. Pullen, R. H., & Cox, J. W. (1985). Determination of arbaprostil [(15 R)-15-methyl-prostaglandin E_2] and its (15S) epimer in human plasma with picogram per milliliter sensitivity by column switching high-performance liquid chromatography. Journal of Chromatography, 343, 271-283.

17. Fitzpatrick, F. A. (1978). Separation of prostaglandins and thromboxanes by gas chromatography with glass capillary columns. Analytical Chemistry, 50(1), 47-52.

18. Fitzpatrick, F. A., Stringfellow, D. A., Maclouf, J., & Rigaud, M. (1979). Glass capillary gas chromatography with electron-capture detection. Separation of prostaglandins. Journal of Chromatography, 177, 51-60.

19. Daniels, E. G. (1976). Chromatography of prostaglandins. In G. V. Marinetti (Ed.), Lipid chromatographic analysis: Vol. 2 (pp. 611-662). (2nd ed.). New York: Marcel Dekker.

20. Ross, B. D. (1972). Perfusion techniques in biochemistry. London: Oxford University Press.

21. DuPont Specialty Diagnostics. (1984). Instruction manual for RIANEN™ 6-keto-prostaglandin $F_{1\alpha}$ [^{125}I] RIA kit. (Available from DuPont Specialty Diagnostics, North Billerica, MA 01862)

22. O'Leary, I. A. (1985). [Ether extraction of prostaglandin methoximes]. Unpublished raw data, The Upjohn Co., Kalamazoo, MI.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

23. Morozowich, W., & Douglas, S. L. (1977). Detection of prostaglandins by HPLC after conversion to p-(9-anthroyloxy) phenacyl esters. Unpublished manuscript, The Upjohn Co., Kalamazoo, MI.

24. Pullen, R. H. (1985). [Chromatography of TxB_2 panacyl ester derivative using a PRP-1 column]. Unpublished raw data.

25. Westlund, P., Kumlin, M., Nordenström, A., & Granström, E. (1986). Circulating and urinary thromboxane B_2 metabolites in the rabbit: 11-Dehydro-thromboxane B_2 as parameter of thromboxane production. Prostaglandins, 31(3), 413-443.

26. Cox, J. W., Pullen, R. H., & Royer, M. E. (1985). Isolation of plasma components by double antibody precipitation and filtration: Application to the chromatographic determination of arbaprostil [(15R)-15-methyl-prostaglandin E_2]. Analytical Chemistry, 57(12), 2365-2369.

27. Cox, J. W., & Pullen, R. H. (in press). Determination of prostaglandin E_1 in plasma with picogram per milliliter sensitivity by double antibody extraction and column switching high performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis.

28. Krause, W., Jakobs, U., Schulze, P. E., Nieuweboer, B., & Humpel, M. (1985). Development of antibody-mediated extraction followed by GC/MS (antibody/GC/MS) and its application to iloprost determination in plasma. Prostaglandins Leukotrienes and Medicine, 17(2), 167-182.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.