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High-performance liquid chromatographic assay of platelet-produced thromboxane B_2

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ABSTRACT

A method for the routine determination of platelet-produced thromboxane B_2 (TXB_2) from human serum is presented. To induce the secretion of thromboxane A_2 from the platelets, blood is kept at 37°C for 30 min before serum is separated. Serum is prepurified through small reversed-phase columns and TXB_2 is analysed by reversed-phase high-performance liquid chromatography. A column-switching technique is used to remove the interfering compounds present in serum. The detection limit with standard solution is 30 ng per injection. The method was applied to the measurement of platelet-produced TXB_2 serum from 1040 men. The mean TXB_2 was 247 ± 134 ng/ml in the serum of men who had not used prostaglandin inhibitors, and 208 ± 123 ng/ml in the serum of men who had used a prostaglandin inhibitor during a two-week period before blood sampling.

INTRODUCTION

Thromboxane B_2 (TXB_2) is a hydrolysis product of thromboxane A_2 (TXA_2), which acts as a potent vasoconstrictor and platelet activator. TXA_2 is an oxygenated metabolite of arachidonic acid and is mainly formed by platelets upon stimulation. It is quickly hydrolysed ($t_{1/2}$ = 30 s) to the more stable but inactive TXB_2 [1]. Measurement of TXB_2 from serum or blood has proved to be a useful index of platelet TXA_2 generation. Serum TXB_2 levels are usually very low (10–370 pg/ml) [2,3], and highly sensitive detection methods are required for the measurement of normal serum TXB_2 levels. Moreover, variations in blood sampling techniques and handling of the sample after blood drawing influence the release of thromboxanes from the platelets [3]. These difficulties can be avoided by measuring serum TXB_2 levels after incubation of blood at 37°C for 30 min, during which time platelets become activated [4] and release TXA_2. Serum TXB_2 levels after incubation indicate the capacity of the platelets to release thromboxanes and thus may indicate the capacity of platelets for the biosynthesis of TXA_2. This may be useful in studies of human diseases with elevated platelet activation, *e.g.* the pathogenesis of atherosclerosis [5], asthma [3] or diabetes mellitus [6].

Immunological methods for the determination of TXB_2 are widely used. Serum or plasma TXB_2 has been measured by radioimmunological methods [3,7]. Urinary TXB_2 as well as 2,3-dinorthromboxane B_2 has been prepurified by high-performance liquid chromatography (HPLC) and measured by radioimmunoassay [8]. Recently, enzyme immunoassays [9] and chemiluminescent immunoassays [10] have been developed for thromboxane determinations, but all these immunological methods are quite costly for routine

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work in population studies. Also, immunological reactions are more or less subject to cross-reactions that diminish accuracy and specificity.

Gas chromatographic–mass spectrometric (GC–MS) methods for the determination of TXB_2, 2,3-dinor-thromboxane B_2 and 11-dehydrothromboxane B_2 have been described [11–16]. GC–MS is sensitive and precise, but the methods need trained personnel and the equipment is very expensive. Thus the HPLC methods may be preferred as liquid chromatographic pumps and fluorescent or UV absorbance detectors nowadays are common devices in biochemical laboratories.

Fluorescence labelling of TXB_2 before the HPLC separation increases the sensitivity of the detection. Watkins and Peterson [17] used *p*-(9-anthroyloxy)phenacyl bromide (panacyl bromide) as a derivatizing reagent for TXB_2. The detection limit was 50 pg per injection using a fluorescence detector. However, in that method removal of the excess of non-reacted panacyl bromide was necessary before the HPLC separation, because in the reversed-phase mode retention times of eicosanoids were the same as that of the derivatizing reagent [18]. This required an additional step in sample prepurification. Bromomethyl-7-acetoxycoumarin has been described as a fluorescence labelling reagent for prostaglandins in human seminal fluid [19]. However, TXB_2 could not be detected with that method. Anthryldiazomethane (ADAM) labelling has been used for the determination of TXB_2 from stimulated platelets [20] and from plasma of bronchial asthma patients [2]. The detection limit was about 100 pg per injection for TXB_2 [2,20].

UV detection coupled with HPLC is rarely used for the measurement of thromboxanes from biological fluids. Terragno *et al.* [21] developed a method based on reversed-phase separation and UV detection, but they used it only for standard preparations. The detection limit was 30 ng. In a biological matrix there are many interfering compounds that clute together with the analyte in most reversed-phase systems [2,19] and are difficult to remove.

In this paper we describe a method for TXB_2 determination from human serum by HPLC with a column-switching technique and UV detection.

EXPERIMENTAL

Reagents

TXB_2 standard (Sigma, St. Louis, MO, USA) was prepared at a concentration of 1000 ng/ml in 0.1 mol/l potassium phosphate buffer, pH 6.8. $K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4 came from Merck (Darmstadt, Germany). Absolute ethanol was obtained from Oy Alko (Helsinki, Finland). Light petroleum (60–80°C) and citric acid were of analytical grade from BDH (Poole, UK). Methyl formate was obtained from Fluka (Buchs, Switzerland) and acetonitrile of HPLC grade from Rathburn (Walkerburn, UK).

Subjects

The subjects were from the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD) [22]. According to the study protocol the subjects were eastern Finnish men (aged from 42 to 60 years). They were asked to fast for 12 h before sampling. The patients were asked if they had used anti-inflammatory analgesics or any other prostaglandin generation inhibitors in the two weeks preceding blood sampling. Previous history of any ischaemic heart disease was checked.

Sample preparation

Blood was drawn from subjects after they had rested in a supine position for a half an hour. No tourniquet was used. Venous blood was collected into a vacuum tube (VT-050PZ, Terumo, Tokyo, Japan). The first 2 ml of blood were discarded. For the processing of platelet-produced TXB_2 the blood was allowed to clot on a water bath at 37°C for 30 min. After centrifugation at 500 *g* for 15 min serum was separated and frozen at −20°C until assayed.

Serum TXB_2 was extracted by the use of small C_{18} cartridges (Sep-Pak, Waters, Milford, MA, USA). Absolute ethanol (3.0 ml) was added to serum (1.0 ml). The mixture was centrifuged at 1000 *g* for 10 min and the supernatant was quantitatively decanted. To the supernatant 16.0 ml of 0.1 mol/l citric acid, pH 3.0 with 0.1 *M* sodium hydroxide, were added to acidify the mixture and to obtain a final concentration of 15% ethanol.

The Sep-Pak cartridges were prewashed with 8 ml of ethanol and water. The mixture was passed through a C_{18} Sep-Pak by means of a vacuum pump. The cartridge was washed with 20 ml of 15% ethanol, followed by 20 ml of light petroleum and then TXB_2 was eluted with 10 ml of methyl formate [23]. Methyl formate was evaporated rapidly under a stream of nitrogen and the remainder was dissolved in 0.6 ml of 0.1 mol/l potassium phosphate buffer, pH 6.8, and stored at −20°C until assayed. Sep-Pak cartridges were regenerated with methyl formate (10 ml) and 80% ethanol (20 ml). The same cartridges were used three times.

One standard preparation (1000 ng/ml) was extracted in each batch of samples to confirm the recovery of TXB_2.

HPLC system

The HPLC system involved two phases and it was an application of a column-switching technique (Fig. 1). The first phase of the system consisted of a Kontron 420 HPLC pump (Zürich, Switzerland), a Kontron 425 HPLC gradient former, a Beckman 507 autosampler (Beckman, Instruments, San Ramon, CA, USA) and a 7-μm Brownlee RP-18 column (15 mm × 3.2 mm I.D.) (Applied Biosystems, San Jose, CA, USA). The second phase consisted of another Kontron 420 HPLC pump, a Rheodyne Model 7010 sample injection valve (Rheodyne, Cotati, CA, USA), a 5-μm Brownlee Spheri-5 RP-18 (100 mm × 4.6 mm I.D.) analytical column and a Kontron HPLC UV detector. The wavelength was adjusted to 208 nm.

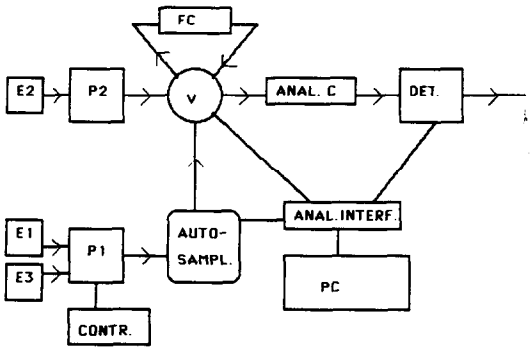

Fig. 1. HPLC system with a column-switching technique for TXB_2 analysis. E1, E2 and E3 = eluents 1, 2 and 3; P1 and P2 = pumps 1 and 2; V = valve combining the two phases of the system; CONTR. = pump 1 controller; AUTOSAMPL. = autosampler; FC = first column; ANAL. C = analytical column; ANAL. INTERF. = analog interface; PC = personal computer; DET. = UV detector.

An IBM personal computer with an 80386 processor and Beckman System Gold software was used to control the Beckman autosampler and Rheodyne injection valve via a Beckman Analog Interface Module 406, and to collect and analyse data from the detector (Fig. 1). TXB_2 was quantified by comparing the peak heights of the unknown samples with the height of the known standard.

In the first phase the sample (200 μl) was injected into the first column with an eluent composed of 10% (v/v) acetonitrile and 90% 0.1 mol/l potassium phosphate buffer, pH 6.8 (eluent 1). After 5 min the sample was eluted from the first column with 25% acetonitrile in phosphate buffer (eluent 2) into the analytical column. Between each sample the first column was washed with 75% (v/v) acetonitrile in phosphate buffer, pH 6.8 (eluent 3) for 30 min to remove serum impurities with long retention times.

Platelet counting

Platelets were counted from EDTA-blood in 3 h following sampling (Thrombocounter C, Coulter Electronics, Luton, UK).

RESULTS AND DISCUSSION

The recovery of TXB_2 was tested by analysing a standard solution both without extraction and after extraction through Sep-Pak cartridges. The recovery was 80 ± 6% (mean ± S.D., n = 10). The analytical recovery was measured by adding 625 ng and 312 ng of TXB_2 to 1.0 ml of serum. The analytical recoveries (mean ± S.D., n = 3) were 108 ± 12 and 112 ± 4%, respectively. The detection limit with the standard solution was about 30 ng per injection at a signal-to-noise ratio of 3.

The between-batch coefficient of variation was

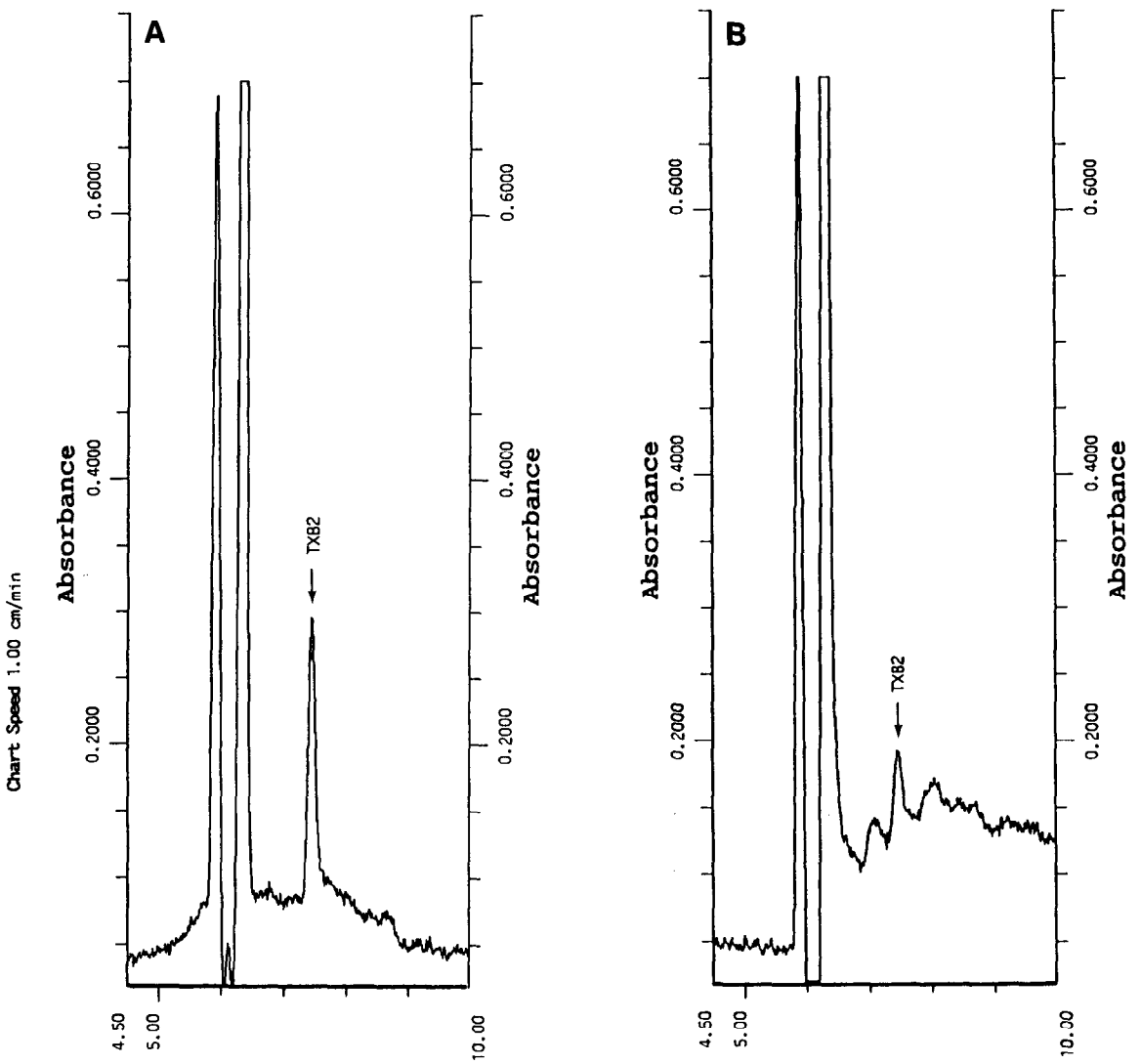

Fig. 2. Typical chromatograms of (A) the standard solution and (B) a serum sample after activation of platelets at 37°C for 30 min. Two peaks at the retention times of 6–7 min are from eluent 1, occurring when the running eluent is abruptly changed to eluent 2 by column switching. Flow-rate was 1.0 ml/min. The other conditions are described in the text.

11.7% (n = 9) for a serum sample with a TXB_2 level of 234 ng/ml. We have found that serum can be stored for at least four months at −20°C without any changes in TXB_2 concentration.

A typical chromatogram is shown in Fig. 2. TXB_2 is eluted at 7.7 min. The whole run time for one sample is 45 min, including washing and regeneration of the first column. Our HPLC system is fully automated, and the sample pretreatment process is rapid and the procedure does not require any derivatizing reagent. Interfering compounds that elute with the same retention time as TXB_2 and disturb the chromatogram have previously been removed by a gel permeation column [2] or by an additional purification procedure with a silica gel column [18]. In our HPLC system interfering compounds are simply removed by column switching. TXB_2 is retained by the first

column, in which it concentrates, but most of the interfering substances in the UV detector are eluted through the column to waste. TXB_2 is eluted with a less polar eluent to the analytical column.

We analysed several samples without heat activation of blood. TXB_2 was undetectable and no significant peak eluted with the same retention time (data not shown). Heat activation of platelets leads to increased levels of TXB_2 but does not increase other prostaglandins, *e.g.* prostaglandin $F_{2\alpha}$, which might interfere in reversed-phase mode [17,20,21].

Acetal formation of TXB_2 during the serum prepurification process often leads to peak broadening in reversed-phase systems, which probably represents an equilibrium condition between the two forms of TXB_2 [17,20]. This naturally reduces the sensitivity of the method. In our method peak broadening is minimal (Fig. 1) and sensitivity is good enough to detect normal levels of serum TXB_2 after platelet activation.

We measured serum TXB_2 from 1040 Finnish men (aged from 42 to 61 years) after platelet activation at 37°C. The mean TXB_2 level was 241 ± 133 ng/ml (Table I), which is in agreement with the levels reported by Patrono *et al.* [24] (274 ± 52 ng/ml, $n = 4$) after 1 h blood clotting. Because TXB_2 is released from platelets and thus depends on the platelet count in blood, we calculated the ratio of TXB_2 to the platelet count. TXB_2 was 97 ± 55 fg per 100 platelets in the whole study population. Prostaglandin inhibitors reduce TXA_2 generation from arachidonic acid and thus TXB_2 formation. The difference in TXB_2 levels between prostaglandin inhibitor users and non-users was small but significant ($p < 0.001$) (Table I). Prostaglandin inhibitor dose was not known, thus subjects who ingested small doses two weeks before sampling were also classified as drug users. This may have led to an increase in the mean and standard deviation. Inschaemic heart disease in patient history did not affect the levels of TXB_2 in our study population. Further studies are continuing to investigate the importance of platelet TXB_2 generation with regard to atherosclerosis.

Our HPLC system with a column-switching technique has proved to be a fast method for the determination of platelet-produced TXB_2 in human serum. The prepurification of a serum sample requires only one step before the HPLC analysis. The UV-interfering substances are removed automatically during the HPLC separation. This method may therefore be suitable for most laboratories that are involved in TXA_2 generation in platelets.

TABLE I

TXB_2 LEVELS IN SERUM OF 1040 FINNISH MEN, MEASURED BY HPLC

		n	TXB_2 (mean ± S.D.) (ng/ml)	TXB_2 per 100 platelets (mean ± S.D.) (fg)
Any ischaemic	No	659	246 ± 136	98 ± 56
heart disease?	Yes	381	233 ± 129	96 ± 53
Have used				
prostaglandin	No	886	247 ± 134	100 ± 55
inhibitors?	Yes	153	208 ± 123	84 ± 50
All		1040	241 ± 133	97 ± 55

REFERENCES

1 G.A. FitzGerald, C. Healy and J. Daugherty, *Fed. Proc.*, 46 (1987) 154.
2 M. Kurosawa, Y. Igarashi, H. Kobayashi, S. Kobayashi and S. Abe, *Ann. Allergy*, 64 (1990) 464.
3 N. Maurin, *Arzneim. Forsch.*, 36 (1986) 1174.
4 C. Cerletti, R. Latini, A. Del Maschio, F. Galletti, E. Dejana and G. de Gaetano, *Atheroscler. Rev.*, 13 (1985) 67.
5 D. J. Fitzgerald, L. Roy, C. Catella and G. A. FitzGerald, *N. Engl. J. Med.*, 315 (1986) 983.
6 P. Allessandrini, J. MacRae, S. Feman and G.A. FitzGerald, *N. Engl. J. Med.*, 319 (1988) 208.
7 K. Satoh, T. Imaizumi, H. Yoshida, M. Hiramoto, A. Konta and S. Takamatsu, *Acta Neurol. Scand.*, 83 (1991) 99.
8 C. Van Geet, J. Arnout, E. Eggermont and J. Vermylen, *Eicosanoids*, 3 (1990) 39.
9 M. Reinke, M. Piller and K. Brune, *Prostaglandins*, 37 (1989) 577.
10 A. Morello, S. Chang, A. Jacob, S. J. Law, M. Stastny, L. Martin, A. Pamadi and A. Kotake, *Prostaglandins*, 42 (1991) 55.
11 J. A. Lawson, A. R. Brash, J. Doran and G. A. FitzGerald, *Anal. Biochem.*, 150 (1985) 463.

12 C. Chiabrando, A. Benigni, A. Piccinelli, C. Carminati, E. Cozzi, G. Remuzzi and R. Fanelli, *Anal. Biochem.*, 163 (1987) 255.
13 R. Lorenz, P. Helmer, W. Uedelhoven, B. Zimmer and P. C. Weber, *Prostaglandins*, 38 (1989) 157.
14 C. Chiabrando, V. Pinciroli, A. Campoleoni, A. Benigni, A. Piccinelli and R. Fanelli, *J. Chromatogr.*, 496 (1989) 1.
15 K. Watanabe, K. Yamashita, M. Ishibashi, Y. Hayashi, S. Yamamoto and H. Miyazaki, *J. Chromatogr.*, 468 (1989) 383.
16 U. Justesen and G. Bojesen, *J. Chromatogr.*, 562 (1991) 59.
17 W. D. Watkins and M. B. Peterson, *Anal. Biochem.*, 125 (1982) 30.
18 W. Engels, M. A. F. Kamps, P. J. M. R. Lemmens, G. J. Van Der Vusse and R. S. Reneman, *J. Chromatogr.*, 427 (1988) 209.
19 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 231 (1982) 247.
20 K. Yamaki and S. Oh-ishi, *Chem. Pharm. Bull.*, 34 (1986) 3526.
21 A. Terragno, R. Rydzik and N. A. Terragno, *Prostaglandins*, 21 (1981) 101.
22 J. T. Salonen, R. Salonen, K. Seppänen, R. Rauramaa and J. Tuomilehto, *Circulation*, 84 (1991) 129.
23 W. S. Powell, *Methods Enzymol.*, 86 (1982) 467.
24 C. Patrono, G. Ciabattoni, F. Pugliese, A. Pierucci, I. A. Blair and G. A. FitzGerald, *J. Clin. Invest.*, 77 (1986) 590.