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Reversed-phase high-performance liquid chromatographic method for the determination of the 11-hydroxythromboxane B_2 anomers equilibrium

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Abstract

An improved reversed-phase HPLC method for the separation and detection of both hemiacetalic 11-hydroxy anomers of thromboxane B_2 (TXB_2) is described. Water–acetonitrile mixtures have served as mobile phases. By diminishing stepwise the temperature of the chromatographic system from 40 to 0°C, the UV-absorbance profile of TXB_2 changed from one broad peak to two clearly separated narrow peaks corresponding to the two anomers existing in equilibrium. Modification of the mobile phase pH from 1.6 to 6.9 (0°C) resulted in different concentration ratios of the anomers. The equilibrium constant and the Gibbs free energy were calculated. The intermediate open aldehyde form of TXB_2 is unstable and, therefore, cannot be observed either by HPLC or by ^1H NMR measurements.

Keywords: 11-Hydroxythromboxane B_2

1. Introduction

Arachidonic acid (AA) is a ubiquitous essential unsaturated fatty acid. It is metabolized by the cyclooxygenase enzyme system producing prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) [1–3]. PGH_2 is converted into several prostaglandins by specific enzymes [4] and into thromboxane A_2 (TXA_2) by the thromboxane synthase [5,6]. TXA_2 is an important platelet aggregation factor and a potent vaso-constrictor of large vessels [5]. TXB_2 ({5*Z*,9*a*,13*a*,15*S*}-9,11,15-trihydroxythromboxa-5,13-dienoic acid) is a stable product of thromboxane A_2 formed by non-enzymatic hydrolysis [5]. This metabolite is usually used for quantifying TXA_2. For the estimation of the PG-producing capacity in physiological systems, HPLC methods have been used for qualitative and quantitative analysis. In some methods, either a precolumn derivatization for converting PGs into fluorescent and UV-absorbing derivates [7–14], or radioactively marked precursors like [^{14}C]AA [15,16] or [^{3}H]AA [17,18] without derivatization have been used. Generally, reversed-phase columns – especially C_{18} – are capable of separating PGs. Unfortunately, the chromatograms showed partially coeluting peaks with extended shoulders as described for TXB_2 [19–21]. Platelet

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aggregation experiments in our own laboratory confirm this phenomenon (see Section 2 and Fig. 1).

The present studies were carried out to improve these methods. In order to sharpen the peaks we varied the chromatographic parameters, such as column temperature [22], mobile phase polarity and pH. Furthermore, the applied TXB_2-standard in the sample matrix has been investigated by ^1H NMR spectroscopy.

2. Experimental

2.1. Apparatus

The HPLC equipment consisted of a Rheodyne 7725i syringe-loading injector (Cotati, CA, USA), a pump system 322, a diode-array detector 440 (DAD), the Kroma 2000 HPLC software from Kontron Instruments (Neufahrn, Germany), and a fraction collector, Superrac 2211 from LKB (Bromma, Sweden). The temperatures of the RP column and reservoirs containing the two components of the mobile phase were adjusted in Precitherm PFV water bathes (Boehringer, Mannheim, Germany). Radioactivity of tritium-carrying fractions was measured by a liquid-scintillation counter (LSC) 1409 (Wallac, Turku, Finland). ^1H NMR spectroscopy was carried out by a Bruker, AM 360 (360 MHz; controlled by an Aspect 3000 computer; Bruker, Karlsruhe, Germany).

2.2. HPLC column and mobile phases

The separations were carried out on a 125×4.6 mm I.D. column and a 125×4.0 mm I.D. column packed with Nucleosil 120 C_{18} (particle size 5 μm) and a precolumn (26×6.0 mm I.D.) of the same material which was obtained from Bischoff (Leonberg, Germany). The aqueous solvent systems comprised of different percentages of acetonitrile (30–55%) and the pH was adjusted between 1.6 and 6.9 using trifluoracetic acid (TFA) and NaOH. The flow-rate was 1.0 ml/min, and the experiments were carried out at temperatures between 0 and 40°C.

2.3. Chemicals

Thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Sigma (St. Louis, MO, USA). 12-Hydroxyheptadecatrienoic acid (12-HHT) and 12-hydroxyeicosatetraenoic acid (12-HETE) as HPLC references were purchased from Cayman (Ann Arbor, MI, USA). Tritiated arachidonic acid ([^{3}H]AA, 200 Ci/mmol) was provided by ARC (St. Louis, MO, USA). Acetonitrile (ultragradient grade) and water (HPLC grade) were obtained from J.T. Baker (Deventer, The Netherlands). All salts and TFA (reagent grade) for the used buffers were from Merck (Darmstadt, Germany). 'Ultima Gold' from Camberra Packard (Groningen, The Netherlands) was used as scintillation cocktail. For the ^1H NMR spectroscopy D_2O (99.8 at.% D) and CD_3CN (99 at.% D) were purchased from Acros (Geel, Belgium), DCl and NaOD as solutions in D_2O (99 at.% D) from Aldrich (Milwaukee, WI, USA). All other chemicals were provided by Merck and were of reagent grade.

2.4. Sample preparation

2.4.1. Platelet aggregation procedure

Platelet-rich plasma (PRP) was prepared as described [23]. A 5-μl volume of an ethanolic solution of [^{3}H]AA was dried in a glass tube under a gentle stream of nitrogen and 2 ml of the PRP were added. After gentle shaking at 37°C for 60 min, the incubation was stopped by acidifying with 1.5 *M* citric acid. AA and its metabolites were extracted twice with 7 ml of ethyl acetate. Organic layers were decanted, pooled in a glass tube and dried under nitrogen. The remaining residue was redissolved in 200 μl of the mobile phase. Aliquots of 20 μl of these samples were used for the chromatography. Fractions of 20 s were collected in scintillation vials and counted in a β-counter.

2.4.2. TXB_2 HPLC elution profile experiments

Ethanolic solutions of TXB_2 were dried under a gentle stream of nitrogen in glass tubes and redissolved in 200 μl (300 μg TXB_2/ml) of the

necessary mobile phase (pH from 1.6 to 6.9). Samples of 20 μl were injected. All test runs were done in triplicate at temperatures between 0 and 40°C to calculate the peak areas detected at 192 nm with mean and standard deviation. The pH of the aqueous component of the mobile phase was measured before mixing with acetonitrile.

For ^{1}H NMR spectroscopy, TXB_2 was dissolved in deuterated water and acetonitrile adjusted to pH 5.6 with NaOD and DCl and measured at 6°C.

TXB_2–methyl ester was prepared from TXB_2 using diazomethane [24] and TXB_2–*N*-hydroxysuccinimide ester from TXB_2 as described in Refs. [25,26].

3. Results and discussion

As demonstrated in the chromatogram of the platelet aggregation experiment (Fig. 1), the TXB_2 peak was broad, while the peaks of HHT and 12-HETE were high and narrow.

In order to improve the shape of the TXB_2 peak for better separation and integration, the temperature of the chromatographic system was varied from 40 to 0°C (Fig. 2). Two peaks unexpectedly appeared by decreasing the temperature to 0°C. At the highest temperature of 40°C only one broad peak for a TXB_2 standard solution could be observed (Fig. 2f), detected at 192 nm by a DAD. By stepwise decrease of the temperature, the peak profile changed from a peak with a strong shoulder (Fig. 2d) to a peak with high tailing (Fig. 2c). At 17°C a second peak, appearing at the end of the tailing, could clearly be identified (Fig. 2b). At the lowest temperature (0°C) of the aqueous mobile phase, two well-separated narrow peaks were detected (Fig. 2a). Their UV-spectra measured in a range of 190–300 nm by the DAD were identical and showed one maximum at 192 nm indicating a similar structure. The separation factor α was calculated to be 2.0. The first eluted, more polar, peak was defined as peak A, and the second, less polar, one as peak B. In order to analyze the cause for this 'two-peak phenomenon' the stationary phase and the TXB_2 standard were examined.

To prove whether these two peaks result from

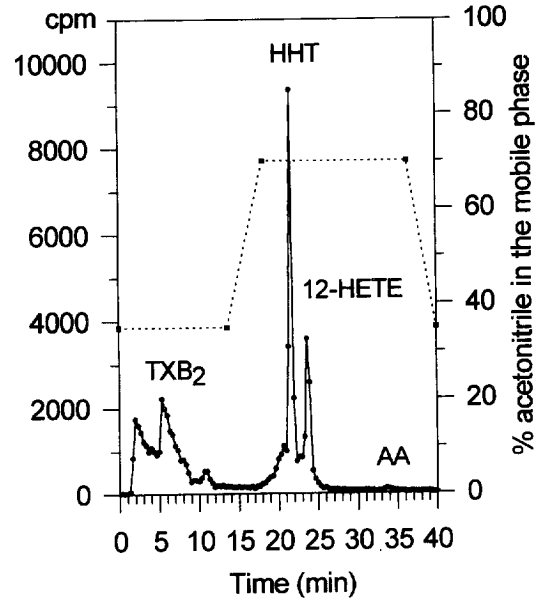

Fig. 1. Resolution of [^{3}H]AA metabolites produced by platelet aggregation. Samples were prepared as described in Section 2 and chromatographed on Nucleosil 120 C_{18} (125×4.6 mm I.D.), 5-μm particle size, at a flow-rate of 1.0 ml/min with an acetonitrile gradient from 35 to 70% (v/v) at 30°C. Fractions of 20 s (0.33 ml) were collected and radioactivity was measured in a LSC. TXB_2, thromboxane B_2; HHT, 12-*S*-hydroxyheptadecatrienoic acid; 12-HETE, 12-*S*-hydroxyeicosatetraenoic acid; AA, arachidonic acid.

different interactions of the analyte with the stationary phase, e.g. silanol groups, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ as model substances for related PGs were chromatographed on the same column. Both prostaglandins showed a characteristic peak independent of the chosen temperature. This fact clearly shows that the stationary phase used cannot be the reason for the elution phenomenon. The appearance of two peaks that could coalesce to one peak by changing the chromatographic temperature is therefore considered as an indication of the dynamic equilibrium of this compound [27]. Two equilibria for TXB_2 may occur by the dissociation of the carboxylic function at C1 and the ring opening at C11 to an open aldehyde form. As TXB_2 is a weak acid, we investigated whether peak A and B are the results of the dissociation equilibrium by chromatographing the methyl ester and the *N*-hydroxysuccinimide ester of

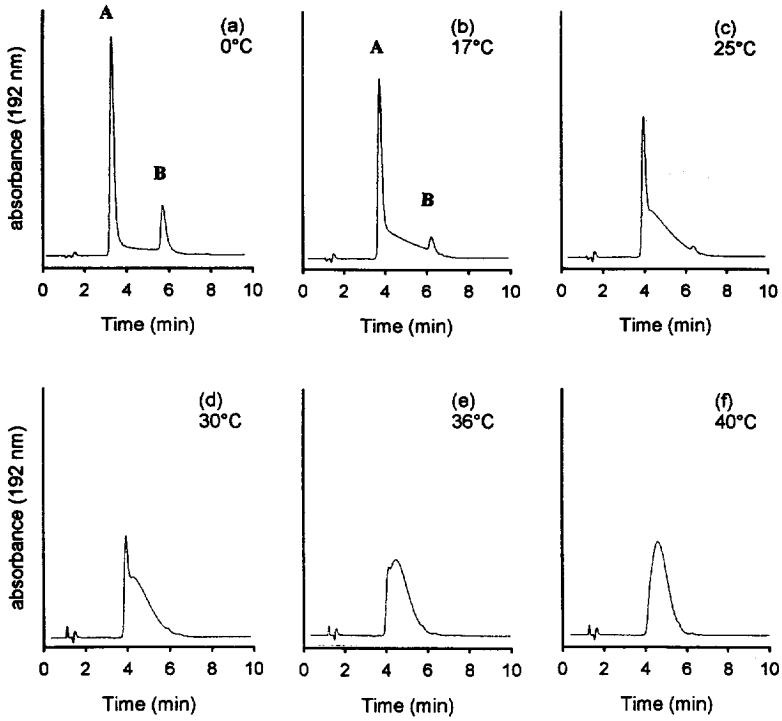

Fig. 2. Resolution of TXB_2 at different column temperatures. Chromatography was carried out on Nucleosil 120 C_{18} (125×4.0 mm I.D.), 5-μm particle size, with CH_3CN–H_2O (35:65, v/v, pH 3.5) as mobile phase. Flow-rate was 1.0 ml/min and it was detected at 192 nm by a DAD.

TXB_2 on the same column. Both substances exhibited temperature-dependent peak profiles very similar to those of TXB_2 (results not shown), although they do not possess a free –COOH function. From these results we conclude that the 'two-peak phenomenon' does not result from the protolysis of a carboxylic function.

Furthermore, TXB_2 exists also in an equilibrium of three forms: two hemiacetalic anomers of a closed ring with the equatorial position (11-β-OH-TXB_2) and the axial position (11-α-OH-TXB_2) of the 11-hydroxy function and an open aldehyde form (Fig. 3 and Fig. 5) [9,28–32] confirmed by derivatisation and NMR spectroscopy. Generally, the equilibrium of the closed hemiacetalic form and the open aldehyde form is pH dependent. In an acid solution the equilibrium is shifted towards the open aldehyde form [33,34]. To examine the influence of pH on the observed peaks, TXB_2 standards were chromatographed at 0°C with mobile phases of 43% acetonitrile with different pH. The percentage of organic solvent in the mobile phase causes its change in polarity and elution power for TXB_2. Table 1 shows the influence of different percentages of acetonitrile on the resulting polarity (Snyder index) [35] and retention (t_R) of TXB_2. The proportion of peak area B to peak area A was independent of the applied sample pH, but dependent on the pH of the mobile phase. The peak area of compound A decreases proportionally to the pH increase. Fig. 4 shows a linear correlation of the peak area ratios as a function of the increasing pH of the mobile phase ($\alpha = 1.8$). If the appearance of two peaks is the result of the equilibrium of two hemiacetalic and one open aldehyde form [9], it should be possible to detect the protons at C11 and to distinguish between the aldehyde and the hydroxy function in ^1H NMR spectroscopy. Fig. 5 shows the 360-MHz ^1H NMR spectrum of a TXB_2 solution in D_2O–D_3CCN (40:60, v/v, pH 5.6, 6°C) in the range from 4.9 to 5.7 ppm. No aldehyde proton could be detected (usually shifted highfield to 9–10 ppm). According

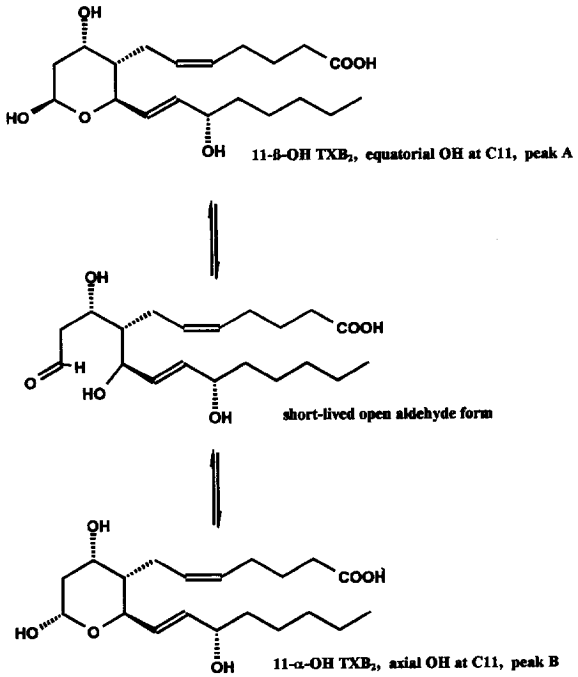

Fig. 3. Equilibrium of TXB_2.

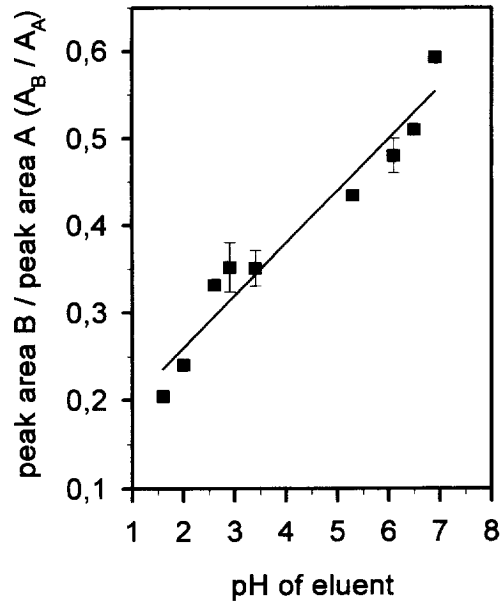

Fig. 4. Correlation of the peak area ratio of the compounds A and B (Fig. 2a) in comparison to the pH. TXB_2 was chromatographed on Nucleosil 120 C_{18} (125×4.0 mm I.D.), 5-μm particle size, with a mobile phase of CH_3CN–H_2O (43:57, v/v) at 0°C detected at 192 nm by a DAD. Every datapoint is the mean and the standard deviation of three runs (A_B/A_A=0.060×pH+0.14, r=0.943).

to Kotovych and Aarts [28], the signals between 4.9 and 5.2 ppm are the effects of the C11 vicinal proton–proton coupling to C10 caused by their α- (axial) or β- (equatorial) position at the closed hemiacetal ring of TXB_2. Under these conditions, 11-β-OH-TXB_2 is the dominant anomer. This experiment clearly demonstrates that the aldehyde form is unstable and very short-lived. Therefore, it is not possible to separate this product by using the HPLC method described in this work with a retention of close to 3 min. However, this aldehyde form may be the active compound for the transformation of TXB_2 by the 11-hydroxythromboxane B_2 dehydrogenase [32]. By comparing the proportion of the two anomers in the ^1H NMR spectrum (pH 5.6) and the HPLC results (Fig. 4) we conclude that peak A is the 11-β-OH-TXB_2 and peak B is the 11-α-OH-TXB_2. The explanation for the 'two-peak phenomenon' of TXB_2 may be simply the equilibrium of the mutarotation of the two hemiacetalic closed ring forms. Both anomers differ in the stereochemistry of one

Table 1
Retention of TXB_2 according to the mobile phase polarity

Acetonitrile in the mobile phase (%)	Polarity of mobile phase (Snyder)	Retention time of TXB_2 (min)
30	8.88	8.5±0.05
32	8.79	6.5±0.05
35	8.66	4.7±0.05
40	8.44	3.2±0.05
45	8.22	2.5±0.05
50	8.00	2.2±0.05
55	7.78	1.9±0.05

Retention of TXB_2 on a Nucleosil 120 C_{18}, (125×4.6 mm I.D.) column, 5-μm particle size, at 192 nm at 30°C. Data presented are the means and standard deviations of three runs. Polarity was calculated according to Snyder [35].

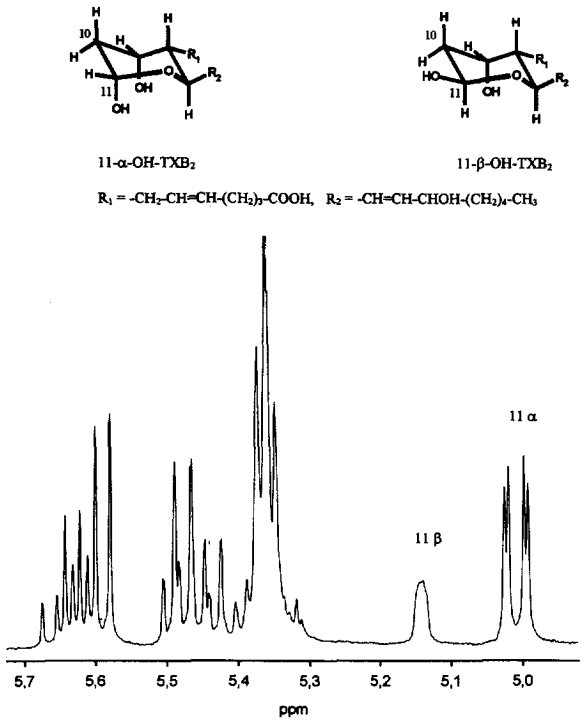

Fig. 5. Extract of the ^{1}H NMR spectrum of TXB_2. TXB_2 was dissolved in CD_3CN–D_2O (60:40, v/v, pH 5.6) and measured at 360 MHz at 6°C.

carbon atom causing a different polarity that makes possible the reversed-phase separation. As the hemiacetalic TXB_2 anomers exist as heterocyclic six-membered rings of the chair conformation [28], the different positions of the C11-hydroxy function (Fig. 5) result in different dipole moments of the anomers [33]. HPLC separations of enantiomers without chiral columns have already been described. Kikuchi et al. [36] reported an HPLC separation of arabinose and xylose, two glycosides differing in the stereochemistry of only two carbon atoms, on amino-bonded column material. Herrmann et al. [37] reported the separation of isomers of some lipoxins on an RP column.

Quantitative calculations for characterizing the kinetics of this anomer equilibrium can be done by integration of the areas of peaks A and B. The equilibrium constant, K, is defined by the law of mass action: $K=[B]/[A]$. As A and B seem to have the same molar absorptivity, ϵ, the detected areas of these compounds are proportional to their concentrations. This ratio is shown in Fig. 4. The calculated values of the Gibbs free energy (ΔG) are positive which indicates an endergonic reaction for the conversion of compound A to compound B. Based on this calculation ΔG decreases from 3.3±0.4 kJ/mol (pH 1.6) to 1.3±0.2 kJ/mol (pH 6.9) at 0°C. This endergonic process that may occur during passing the column is also substantiated by the reduction of the peak area A resulting from the rising temperature (Fig. 6).

Shifting the equilibrium quantitatively to one side is not possible on an RP column, since a pH lower than 1.6 can cause degradation of TXB_2 and damage to the column material. At pH values higher than 7.5 the carboxylic function of TXB_2 is deprotonated which results in lower retention.

The described HPLC method makes it possible to examine the 11-hydroxythromboxane B_2 anomer equilibrium including the calculation of thermodynamic data. This method might also be useful for quantification of TXB_2 in real samples.

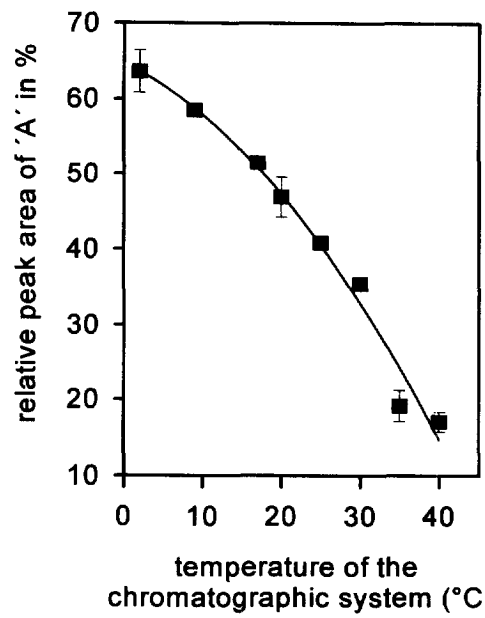

Fig. 6. Calculation of the relative peak area of compound A (Fig. 2a) dependent on the chromatographic temperature. Relative peak area was calculated by integration of peak A in the interval from maximum −0.5 min up to maximum +0.3 min and related to the total area of both peaks.

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