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Note

Measurement of plasma thromboxane B_2 by gas chromatography–mass spectrometry using $^{18}O_2$-labelled thromboxane B_2 as the internal standard

DAVID A HEROLD*, B JANE SMITH, RICHARD M ROSS and FIONA MARQUIS

Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, VA 22908 (U S A)

JOHN SAVORY

Departments of Pathology and Biochemistry, University of Virginia Health Sciences Center, Charlottesville, VA 22908 (U S A)

MICHAEL R WILLS

Departments of Pathology and Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908 (U S A)

and

CARLOS R AYERS

Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908 (U S A)

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Thromboxane A_2 (TxA_2) is a labile naturally occurring lipid formed mainly by platelet metabolism or arachidonic acid which has a half-life of ~30 s in solution [1]. Its hydrolysis product, thromboxane B_2 (TxB_2), is more stable and can be measured by conventional methods such as radioimmunoassay (RIA) [2,3] and gas chromatography–mass spectrometry (GC–MS) [4–8] The problems with in vivo TxB_2 measurements are the technical difficulties of blood sample collection which can artifactually raise TxB_2 levels [9] However, TxB_2 is an ideal analyte to monitor TxA_2 production for in vitro studies, where other stable metabolites are not formed These analytes, 11-dehydro-

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TxB_2 and dinor-TxB_2 have been shown to give a more reliable picture of in vivo TxA_2 production than TxB_2 [10,11].

Since we are interested in applying our technique to early platelet aggregation effects in stop-flow experiments [12], we needed information concerning the endogenous TxB_2 level after blood sampling for preparation of platelet-rich plasma. Our future work would also follow the temporal generation of cyclooxyganase products, including TxB_2, as a function of platelet activation with different agents High sensitivity would be required to measure the initial endogenous levels (<10 pg/ml) of TxB_2, as well as the generation of TxB_2 throughout the stop-flow experiment A GC–MS method using stable isotope-labelled TxB_2 as an internal standard would be an excellent method for precise and accurate measurement of TxB_2 in this biological study

Stable isotope-labelled compounds used as internal standards are an integral part of the precise and accurate measurement of picogram quantities of prostaglandins in biological matrices by GC–MS. One serious limitation of this technique is the availability of the stable isotope-labelled species. At the present time, prostaglandin (PG) E_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ are the only labelled species commercially available. Two groups have synthesized tetradeuterated TxB_2 for use as an internal standard; Meese et al. [8] have synthesized [18,18,19,19-$^2H_4$]TxB_2 while Fitzpatrick et al [5] have synthesized [19,19,20,20-$^2H_4$]TxB_2 The preparation of these deuterated internal standards is expensive and reasonably difficult. Gleispach and Moser [7] have synthesized [$^{18}O_2$]TxB_2 using the $Li^{18}OH$ method introduced by Pickett and Murphy [13] and advanced by Strife and Murphy [14]. Gleispach and Moser [7] report that for TxB_2 four cycles resulted in a product containing <0 5% ^{16}O analogue with no reference to $^{18}O_2$ and $^{18}O^{16}O$ products formed. Hubbard et al. [15] have prepared ^{18}O-labelled TxB_2 using butyryl cholinesterase This exchange results in 70 6±2% [$^{18}O_2$]TxB_2 and 28.2±2% [$^{18}O^{16}O$]TxB_2. The results of Hubbard et al. [15] with porcine liver esterase for di- and mono-incorporation of ^{18}O in TxB_2 was 62 and 13 7%, respectively.

In an effort to implement this simple and inexpensive procedure for preparing an internal standard for TxB_2, we used a modification of the esterase-catalyzed carboxylic acid oxygen exchange method of Pickett and Murphy [13] The present report describes the preparation and characterization of [$^{18}O_2$]TxB_2 and its use as an internal standard for the measurement of endogenous TxB_2 in plasma using negative-ion chemical ionization (NICI) GC–MS

EXPERIMENTAL

Reagents

Porcine liver esterase was obtained from Sigma (St. Louis, MO, U.S A) and $H_2{}^{18}O$ from Cambridge Isotope Labs (Cambridge, MA, U.S A.). All solvents

used in the extraction, chromatographic separation and derivatization procedures were glass-distilled HPLC-grade reagents, obtained from Bodman (Media, PA, U.S.A.) and Curtin-Matheson (Beltsville, MD, U.S.A.). Sep-Pak C_{18} cartridges were supplied by Waters Assoc (Milford, MA, U S.A) and the silica HPTLC plates by Whatman (Clifton, NJ, U.S.A)

Reagents used in the preparation of prostaglandin derivatives were as follows: N,N-diisopropylethylamine (Aldrich, Milwaukee, WI, U.S A), pentafluorobenzyl bromide (Pierce, Rockford, IL, U.S.A.), methoxylamine hydrochloride (2% in pyridine, Pierce), and bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce). TxB_2 standard was obtained from Cayman (Ann Arbor, MI, U.S.A.) and its tritiated analogue from New England Nuclear (Boston, MA, U S.A) Both standards were stored at $-70°C$.

Preparation of glassware

Prior to each experiment, all glassware and Reacti-Vials® (Pierce) were cleaned and silylated as described previously [15].

Preparation of $[^{18}O_2]TxB_2$

The internal standard for TxB_2 was prepared based on the oxygen exchange method of Pickett and Murphy [13]. This reaction works equally well with either the free acid or its methyl ester. In the synthesis described, the free acid was chosen since it is readily available. Porcine liver esterase was obtained as a 1500 U/ml solution in $(NH_4)_2SO_4$ Prior to use, 1 ml of this solution was dialyzed against 2 l of 50 m*M* K_2HPO_4, pH 8.0, for 2 h at 4°C. The final volume of the dialyzed solution was measured and the specific activity calculated Then, 225 U of this esterase solution were transferred to a 5-ml Reacti-Vial and lyophilized. During each step of the procedure, vials were opened and sealed under argon to avoid any possible contamination To the lyophilized esterase, 150 μl of $H_2{}^{18}O$ were added and the solution was mixed well and lyophilized for 16 h. A second 150-μl aliquot of $H_2{}^{18}O$ was added and the sample lyophilized for 4 h. The esterase was reconstituted in 150 μl of $H_2{}^{18}O$ The exchange reaction was initiated by the addition of 1 mg of TxB_2 in 10 μl of methanol and the mixture incubated for 2 h at 37°C. The reaction was terminated by the sequential addition of 0 5 ml of methanol, 1 5 ml of anhydrous diethyl ether and 1.0 ml of water. The ether layer was discarded and the aqueous layer re-extracted with a second 1.5-ml aliquot of diethyl ether. The remaining aqueous solution was acidified with 10% formic acid to pH 3.0, and $[^{18}O_2]TxB_2$ was extracted with 1 ml of chloroform. The extraction was repeated and both chloroform layers were combined. Solvent was evaporated and the dried residue resuspended in 0.5 ml of acetone and stored at $-70°C$. The overall recovery was approximately 65% for the 1-mg reaction. The composition of the synthesized material was determined by GC–MS analysis of its pentafluorobenzyl–methoxime–trimethylsilyl (PFB–MO–TMS) derivative.

Extraction, separation and analysis of TxB_2 from plasma

Extraction TxB_2 was extracted from plasma using a modification of the procedure for PGE_2 developed in the authors' laboratory [16] Samples were obtained from eleven normal adult volunteers (23–48 years of age) using vacutainer tubes and nineteen gauge needles. A 40-ml volume of blood was collected in chilled 10-ml heparinized vacutainer tubes containing approximately 80 μg of indomethacin. The blood was centrifuged at 1500 *g* for 15 min at 4 °C yielding approximately 20 ml of plasma. The volume of each plasma sample was measured, then acidified to pH 3.5–4 0 with formic acid, and 3 1 ng of $[^{18}O_2]TxB_2$ were added. The sample was applied to a C_{18} reversed-phase Sep-Pak cartridge preconditioned with acetonitrile (10 ml) and HPLC-grade water (20 ml) The Sep-Pak was washed sequentially with 10 ml of water, 4 ml of 15% acetonitrile and 10 ml of hexane, and the prostaglandins were eluted with 10 ml of acetonitrile. The solvent was evaporated under argon, the residue resuspended in 1 ml of methanol, and the solvent evaporated again. The final residue was resuspended in 400 μl of methanol and the solution was transferred to a 1-ml Reacti-Vial. The centrifuge tube was rinsed with a second 200-μl aliquot of methanol which was also added to the reaction vial and the combined solvent fractions were evaporated and dried under vacuum.

Methoximation The plasma extract was converted to its methoxime derivative by dissolution in 50 μl of 2% methoxylamine hydrochloride in pyridine and heating at 70 °C for 1 h. Solvent was evaporated at room temperature under a stream of argon and the residue dried under vacuum for 5 min

Thin-layer chromatographic separation The methoximated extract was separated by thin-layer chromatography (TLC) according to a procedure developed in the authors' laboratory [17] with some modifications. Samples were chromatographed for 20 min and after drying scraped off the plate and eluted with 3 ml of acidified methanol (100 parts methanol/1 part acetic acid). The silica was removed by centrifugation at 1500 *g* at 4 °C for 15 min and the supernatant was transferred to a clean tube and evaporated. The residue was resuspended in 400 μl of methanol and transferred to a 1-ml Reacti-Vial, and the tube was then rinsed with another 200 μl of methanol which was transferred to the 1-ml Reacti-Vial. The solvent was evaporated.

Pentafluorobenzyl ester [14,18] The TLC extract was dissolved in 30 μl of acetonitrile and 10 μl of 35% pentafluorobenzyl bromide in acetonitrile and 10 μl of 10% diisopropylethylamine in acetonitrile were added. The vials were heated at 40 °C for 15 min and solvent was evaporated and dried as described above

Silylation The trimethylsilyl ether derivative was prepared by treating the residue with 50 μl of acetonitrile and 40 μl of BSTFA, and heating at 40 °C for 1 h. Solvent was then evaporated and the residue dried under vacuum

Gas chromatographic–mass spectrometric analysis The final residue from derivatization was dissolved in 20 μl of tetradecane, and a 2–3 μl aliquot was

injected onto the column of the GC–MS system. A Finnigan 4021 quadrupole mass spectrometer equipped with the INCOS data system and the pulsed positive-ion negative-ion chemical ionization (PPINICI) accessory (Finnigan, MAT, Sunnyvale, CA, U S.A.) was used. The gas chromatograph was equipped with a DB-1 methyl silicone bonded-phase fused-silica capillary column, 30 m×0.32 mm I.D. (J & W Scientific, Rancho Cordova, CA, U.S.A.). The mass spectrometer was operated in the negative-ion mode with the electron multiplier at 1200–1500 V and the conversion dynode at 3000 V. The source was maintained at 250 °C and pressurized to approximately 0.24 Torr with methane gas. Methane gas was also used as the GC carrier. The GC injector and separator oven were maintained at 290 °C and carrier gas inlet pressure at 400 Torr

Samples were injected using an on-column injector (OCI-3 Scientific Glass Engineering, Austin, TX, U.S A.). Injections were made at 200°C, then the oven temperature was programmed from 200 to 300°C at a rate of 20°/min. TxB_2 was quantified by selected-ion monitoring (SIM) in the negative-ion mode using fragment ions at *m/z* 614 (TxB_2) and *m/z* 618 ($[^{18}O_2]TxB_2$). Standard curves for TxB_2 were constructed from a plot of peak-height ratios (614/618) versus the concentration of exogenous TxB_2 added Plasma concentrations of TxB_2 were calculated from the amount $[^{18}O_2]TxB_2$ (internal standard) added to the sample.

RESULTS AND DISCUSSION

Preparation of $[^{18}O_2]TxB_2$ (internal standard)

The NICI mass spectra of the PFB–MO–TMS derivatives of TxB_2 and $[^{18}O_2]TxB_2$ are shown in Fig. 1A and B, respectively. The spectra of the TxB_2 derivative show a base peak at *m/z* 614 ($[M-C_7H_2F_5^{\bullet}]^-$) corresponding to the loss of the pentafluorobenzyl radical. In the spectra of the $[^{18}O_2]TxB_2$ derivative, the analogous peak has been shifted to *m/z* 618, indicating the exchange of two oxygen atoms. The composition of $[^{18}O_2]TxB_2$ (internal standard) determined from SIM was 95.8% $[^{18}O_2]TxB_2$ (*m/z* 618), 4% $[^{18}O^{16}O]TxB_2$ (*m/z* 616) and 0.2% unexchanged $[^{16}O_2]TxB_2$ (*m/z* 614) The contribution at *m/z* 614 and *m/z* 616 from the prepared $[^{18}O_2]TxB_2$ relative to the intensity of *m/z* 618 is demonstrated in Fig. 2. Standardization of the $[^{18}O_2]TxB_2$ was accomplished by comparison to known concentration of TxB_2 using ion masses at *m/z* 618 and *m/z* 614, respectively

Quantitation of TxB_2 in plasma

In the initial paper by Pickett and Murphy [13], the authors reported a considerable back-exchange of ^{18}O-labelled PGF_2 in plasma, with a half-life of 1 h. Subsequently Strife and Murphy [14] reported that the back-reaction could be quenched by precipitation of plasma proteins In the following studies, plasma proteins were always denatured by acidification of the sample prior to

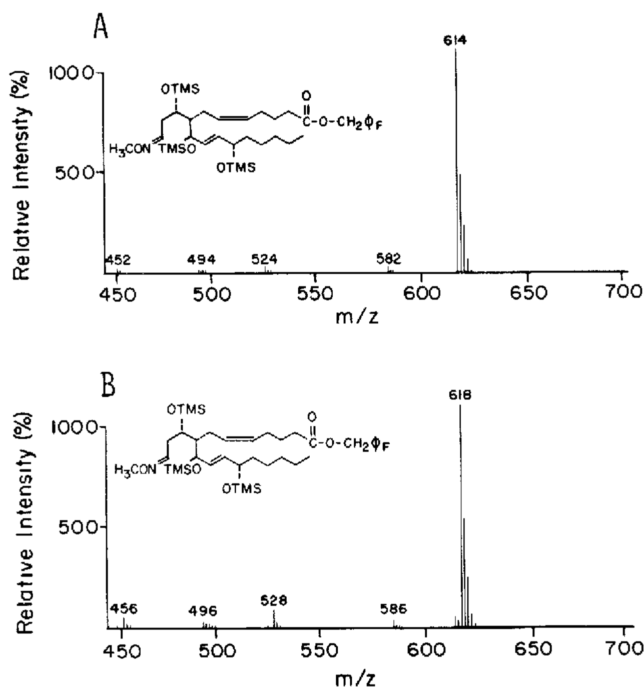

Fig 1 NICI mass spectra of the PFB-MO-TMS derivatives of (A) TxB_2 and (B) $[^{18}O_2]TxB_2$

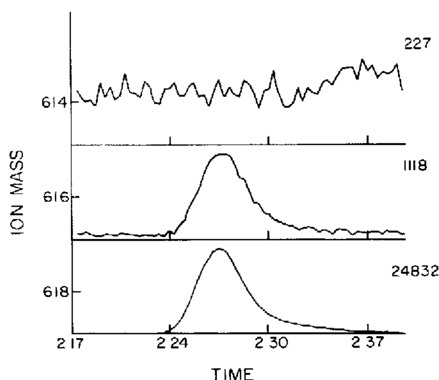

Fig 2 Limited mass chromatogram of $[^{8}O_2]TxB_2$ illustrating less than 0 2% contribution of *m/z* 614 and 4% at *m/z* 616 relative to *m/z* 618

the addition of $[^{18}O_2]TxB_2$. Fig 3 shows the lack of back-exchange of the isotope label when the internal standard is added to acidified 6% bovine albumin solution and extracted, chromatographed and derivatized as described. Attempts to use a water blank were unsuccessful due to poor recovery.

The response versus concentration curve was linear in the range of 100–600

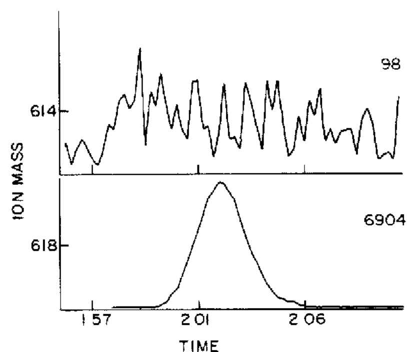

Fig 3 Limited mass chromatogram of $[^{18}O_2]TxB_2$ obtained from a 6% bovine albumin solution to demonstrate the lack of back-exchange of the ^{18}O in the internal standard

TABLE I

RECOVERY OF $[^3H]TxB_2$ ADDED TO PLASMA

Assay step	Recovery (%)
After C_{18} Sep-Pak	99 ± 5
After methoxime derivatization	88 ± 6
After TLC	51 ± 2

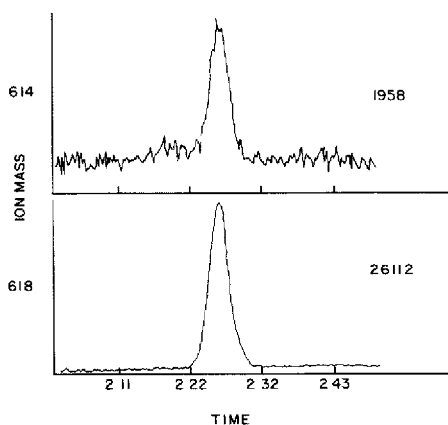

Fig 4 Limited mass chromatogram of endogenous TxB_2 (m/z 614) and $[^{18}O_2]TxB_2$ (internal standard) (m/z 618) obtained from a plasma extract using the purification and derivatization procedures described in the text The calculated TxB_2 from this normal volunteer was 10 6 pg/ml of plasma

pg of TxB_2 (equivalent to 5–30 pg/ml of plasma) using 3.1 ng of $[^{18}O_2]TxB_2$ (internal standard). The least-squares regression line had a correlation coefficient of 0.996.

Recoveries of TxB_2 through the extraction, methoximation and TLC separation steps were assessed by spiking five plasma samples with $[^3H]TxB_2$ (65 000 dpm) and $[^{18}O_2]TxB_2$ (3.1 ng). The results are shown in Table I. The excellent resolution of HPTLC has been previously reported with an illustration that shows the clear separation of TxB_2 from 6-keto-$PGF_{1\alpha}$ [17]. This separation eliminates the possibility of interference from 6-keto-$PGF_{1\alpha}$ which would have the same mass when derivatized as TxB_2 and a similar retention time

Endogenous plasma concentrations of TxB_2 were determined in eleven normal adults. A limited mass chromatogram obtained from the analysis of one plasma sample is presented in Fig. 4; the TxB_2 concentration calculated was 10.6 pg/ml. The mean ($\pm$S.D) plasma TxB_2 concentrations for all eleven individuals was 7.1 ± 1.7 pg/ml with a range of 5.1–10 6 pg/ml. The limit of detection of TxB_2 in plasma was 3 pg/ml at a signal-to-noise level greater than 2 1.

Patrono et al. [19] have estimated endogenous TxB_2 levels in plasma at 1–2 pg/ml. It has been noted that technical difficulties usually result in the activation of platelets during blood drawing [9,11] As Patrono et al. [19] pointed out, the slightest activation (0.1%) could result in an apparent TxB_2 plasma concentration of 200–400 pg/ml. However, when samples are drawn with large-bore needles into chilled tubes with a cyclooxygenase inhibitor followed by prompt centrifugation in a refrigerated centrifuge at 4°C activation could be minimized [2,3,6]. The results showed that this method provided the means to measure these endogenous levels of TxB_2 with minimal activation of platelets. Our results for plasma TxB_2 concentrations of 7.1 ± 1.7 pg/ml ($n=11$) are in good agreement with the plasma values of 11 ± 2 pg/ml ($n=24$) [2] and 17 ± 3 pg/ml ($n=8$) [3] obtained by RIA and 10.8 ± 6.0 pg/ml ($n=4$) measured by GC–MS [6]. All these values compare favorably to the estimated value of 1–2 pg/ml ($n=4$) reported by Patrono et al [19].

This modified esterase oxygen exchange method yielded a product with several desirable characteristics. The characteristics include (1) 95 8% $^{18}O_2$ incorporation, (2) a 4 mass unit difference form the naturally occurring analyte, (3) nearly identical chemical and physical properties as the material being measured and (4) extremely low isotope back-exchange under the conditions of extraction, derivatization and analysis The ease, inexpensive nature and general applicability of the procedure make it an attractive way to consider for preparing internal standards for eicosanoids

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