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Improved assay for the quantification of 11-dehydrothromboxane B_2 by gas chromatography–mass spectrometry

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ABSTRACT

Endogenous thromboxane production is best assessed by the measurement of its excreted metabolites, of which 11-dehydrothromboxane B_2 (11-dehydro-TxB_2) is most abundant. Gas chromatographic–mass spectrometric assays have been developed for this compound but suffer from the presence of co-eluting impurities which make the measurement of 11-dehydro-TxB_2 difficult. Furthermore, these assays are often time-consuming. We now report a modified assay for the measurement of this compound employing gas chromatography–mass spectrometry which alleviates the problem of co-eluting impurities primarily through modification of extraction and chromatographic methods. Furthermore, the time to complete the assay is significantly shortened. It is adaptable to both urine and plasma. Precision of the assay is ± 7% and accuracy is 90%. The lower limit of sensitivity in urine is approximately 20 pg/mg creatinine. Normal levels of urinary excretion of this metabolite were found to be 370 ± 137 pg/mg creatinine (mean ± 1 S.D.) and normal plasma levels were found to be 1.5 ± 0.4 pg/ml (mean ± 1 S.D.). Urinary excretion of 11-dehydro-TxB_2 is markedly altered in situations associated with abnormalities in thromboxane generation when quantified using this assay. Thus, this assay provides a sensitive and accurate method to assess endogenous thromboxane production and to further explore the role of this compound in human disease.

INTRODUCTION

Thromboxane A_2 is the major cyclooxygenase product of platelets. It is an unstable compound which quickly undergoes degradation to the biologically inactive hydration product, thromboxane B_2 (TxB_2) [1]. Although quantification of TxB_2 in plasma has been used to assess endogenous thromboxane generation, it is well recognized that artifactual generation of this substance by platelets occurs upon blood drawing [2]. Furthermore, quantification of TxB_2 in urine as a marker of systemic thromboxane production is confounded by the fact that urinary TxB_2 arises, to a large extent, from the kidney [3]. As a result, endogenous thromboxane production is better assessed by measuring its metabolites [4]. TxB_2 is metabolized *in vivo* via two major pathways; first, by β-oxidation leading to 2,3-dinor-TxB_2 and, second, by dehydrogenation of the alcohol group to yield 11-dehydro-TxB_2 [5]. Measurement of either compound provides an accurate index of systemic thromboxane production and these metabolites have been shown to be increased in disorders associated with increased platelet activation such as severe atherosclerosis and unstable angina [4]. Of the two major metabolites, 11-dehydro-TxB_2 is produced in greatest amounts [6]. A variety of methods over the last several years have been developed to measure 11-dehydro-TxB_2 [7–13]. Most rely on either immunoassay

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techniques or gas chromatography mass spectrometry (GC–MS). Assays of 11-dehydro-TxB_2 utilizing immunoassay techniques are generally facile and applicable to large sample numbers but often suffer from a lack of specificity [4]. On the other hand, mass spectrometric assays, while often cumbersome, are more specific for the measurement of this compound. Several GC–MS assays have been developed differing primarily with regards to purification and derivatization techniques or the use of single *versus* tandem MS [7–10]. One of the first GC–MS assays developed was reported by Lawson *et al.* [7] and it has subsequently been used extensively to assess the role of thromboxane in human disease. The assay employs an initial extraction step using a C_{18} Sep-Pak cartridge with subsequent thin-layer chromatographic (TLC) purification and derivatization to the pentafluorobenzyl ester, trimethylsilyl ether for negative ion chemical ionization (NICI) MS quantitation. While relatively simple to perform, we have found that analysis of 11-dehydro-TxB_2 using this technique is occasionally confounded by co-eluting impurities. This observation has also been noted by others using modifications of this assay [9,10]. Further, the method, as originally reported, requires a considerable amount of time to complete. Thus, in an effort to overcome these problems, we report modifications of this assay which make the analysis of 11-dehydro-TxB_2 by GC–MS simpler and more accurate.

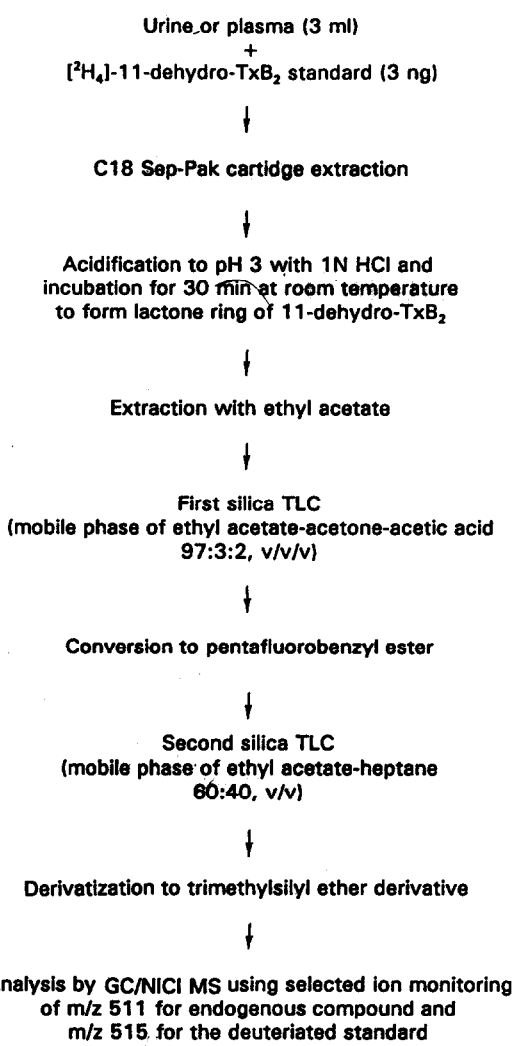

Fig. 1. Summary of method employed for the quantification of 11-dehydro-TxB_2.

EXPERIMENTAL

Chemicals and reagents

11-Dehydro-TxB_2, [$^2H_4$]-11-dehydro-TxB_2, and prostaglandin D_2 (PGD_2) were purchased from Cayman (Ann Arbor, MI, USA). All other chemicals and reagents were obtained from sources previously described [7,14].

General extraction and purification procedure

The general method for the quantification of 11-dehydro-TxB_2 is briefly summarized in Fig. 1 and is based on that outlined by Lawson *et al.* [7]. Differences between the two methods are discussed in detail in the Results section. The assay is equally adaptable to urine and plasma.

Clinical studies and sample collection

To determine normal urinary levels of 11-dehydro-TxB_2, 24-h urine collections were obtained from healthy volunteers ($n = 10$) taking no medications. They were processed immediately. To determine normal circulating levels of 11-dehydro-TxB_2, 100 ml of blood were obtained from each of six healthy volunteers taking no medications. Blood was drawn from an antecubital vein in each individual using a large-bore needle and

collected into syringes containing EDTA ($5 \cdot 10^{-3}$ *M* final concentration) and indomethacin (10^{-6} *M* final concentration) to prevent platelet aggregation and artifactual generation of eicosanoids. The blood was cooled on ice and centrifuged at 800 *g* for 10 min to obtain platelet-poor plasma which was then processed immediately. In another study, four normal volunteers were administered aspirin, 325 mg orally, for five days. A pre-administration 24-h urine collection and a 24-h collection beginning on the fifth day were obtained and processed for 11-dehydro-TxB_2. Informed consent was obtained prior to all clinical studies.

RESULTS

Assay modifications

The assay reported herein for the quantification of 11-dehydro-TxB_2 differs in several important ways from that originally reported by Lawson *et al.*[7]. Firstly, lactone ring formation of 11-dehydro-TxB_2 is accomplished by addition of hydrochloric acid rather than formic acid. We found that the use of formic acid, in our hands, often made samples difficult to dry after ethyl acetate extraction and resulted in a viscous residue. Furthermore, this residue created a large degree of variability in the migration of the solvent front from sample to sample on the subsequent TLC. These problems are entirely eliminated with the use of hydrochloric acid.

Secondly, as originally reported, a 2-h acid incubation was recommended to ensure complete lactone ring formation of the 11-dehydro-TxB_2 molecule [4,7]. We have found, however, that the degree of cyclization of the molecule after 30 and 60 min is essentially as complete as after 120 min (94% at 30 min and 90% at 60 min compared to 100% at 120 min, $n = 3$). Thus, we believe 30 min to be adequate to ensure lactone ring formation of 11-dehydro-TxB_2. It should be pointed out that decreasing the time of this step significantly shortens the overall time (by about 25%) to complete the assay.

Thirdly, we have altered the solvent system in the second silica TLC. As originally reported, Lawson *et al.* [7] had used ethyl acetate as the mobile phase and subsequently modified this to ethyl acetate–heptane (75:25, v/v) [6]. When chromatographed in these solvents, the pentafluorobenzyl ester of 11-dehydro-TxB_2 has an $R_F = 0.52$ and $R_F = 0.40$, respectively, and we observed that 11-dehydro-TxB_2 migrated in the region of other, presumably contaminating, substances in biological fluids that are visible to the eye. Further, when we analyzed samples chromatographed in these solvent systems by GC–MS, we occasionally obtained co-eluting or closely eluting substances which made the quantification of 11-dehydro-TxB_2 difficult. In an effort to separate 11-dehydro-TxB_2 from other substances,we decreased the polarity of the chromatographing solvent even further with heptane and found that a mixture of ethyl acetate–heptane (60:40, v/v) significantly decreased the migration of 11-dehydro-TxB_2 ($R_F = 0.20$). Subsequent analysis of urine and plasma samples by GC–MS showed no interfering chromatographic peaks.

Finally, we have substituted PGD_2 and its pentafluorobenzyl ester as standards in the first and second TLCs, respectively, rather than using 11-dehydro-TxB_2 and its pentafluorbenzyl ester as originally described by Lawson *et al.* [7]. The reason for this is to avoid possible contamination of biological samples which contain picogram quantities of 11-dehydro-TxB_2 with microgram quantities of an identical TLC standard. Although PGD_2 and its pentafluorobenzyl ester display nearly identical chromatographic characteristics in the TLC systems outlined in Fig. 1 compared with 11-dehydro-TxB_2 and its pentafluorobenzyl ester, respectively, they are not detected when analyzed by GC–MS employing selected ion monitoring.

A representative chromatographic tracing of 11-dehydro-TxB_2 obtained from the analysis of urine from a normal individual utilizing the assay presented herein is depicted in Fig. 2. At the bottom is the *m/z* 515 chromatogram representing the [$^2H_4$]-11-dehydro-TxB_2 internal standard and at the top is the *m/z* 511 chromatogram representing endogenous 11-dehydro-TxB_2. As is

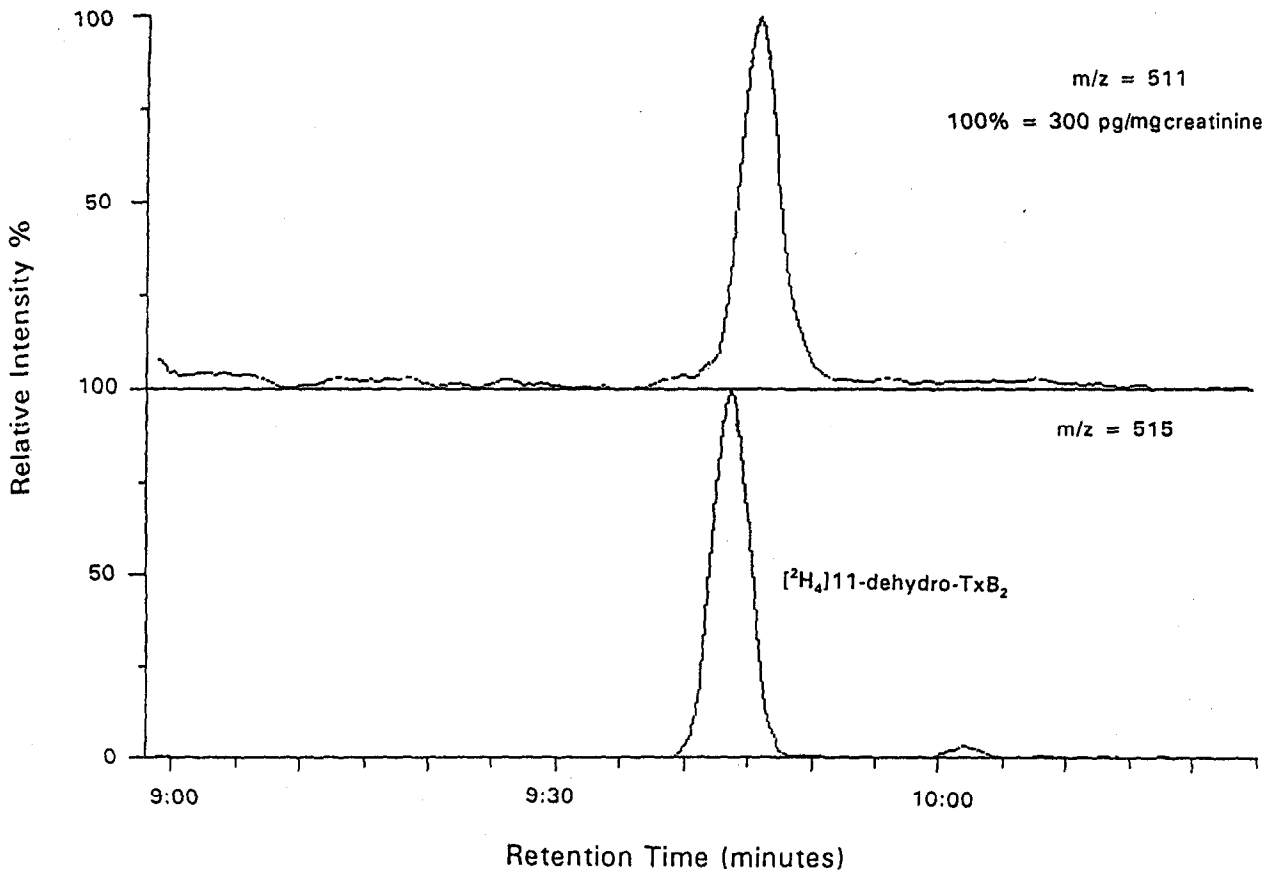

Fig. 2. Analysis of urine from a normal human volunteer for 11-dehydro-TxB_2. Endogenous 11-dehydro-TxB_2 is represented by the peak in the *m/z* 511 chromatogram and the deuterated standard by the peak in the *m/z* 515 chromatogram.

evident, the chromatographic peaks are entirely free of interfering impurities. Tracings of similar quality are obtained from plasma using this assay method (*vide infra*).

Assay parameters and validation

Employing this assay, the lower limits of detection (signal-to-noise ratio of approximately 4:1) of 11-dehydro-TxB_2 in urine is in the range 20 pg/mg creatinine using 3 ng of a commercially available standard with a blank of 3–5 parts per thousand.

Several procedures were performed to establish the accuracy of this assay. Initially a standard curve was constructed by adding varying amounts of unlabeled 11-dehydro-TxB_2 to a fixed quantity of 3 ng of [$^2H_4$]-11-dehydro-TxB_2 and the measured ratio of *m/z* 511 to *m/z* 515 to the expected ratio was compared. The standard curve was found to be linear over a concentration range of 100-fold (data not shown).

Experiments were then carried out to establish precision and accuracy of this assay. Precision was measured by analyzing six 3-ml aliquots of urine obtained from a normal volunteer. The mean of three replicate measurements of the ratio of *m/z* 511 to *m/z* 515 was determined for each sample. The precision was found to be ±7%. Accuracy was assessed using the same urine. For this 1 ng of unlabeled 11-dehydro-TxB_2 was added to another four 3-ml aliquots of the urine and reassayed. The amount of endogenous 11-dehydro-TxB_2 measured in the precision experiment was subtracted from the total, and the accuracy of the assay to measure the added 11-dehydro-TxB_2 was calculated. The accuracy was found to be 90%.

Urinary and plasma 11-dehydro-TxB_2 levels in normal humans

To establish the normal range of the urinary excretion of 11-dehydro-TxB_2 using this assay, aliquots of urine from 24-h collections were obtained and analyzed from ten healthy individuals. Normal levels were 370 ± 137 pg/mg creatinine (mean ± 1 S.D.). These values are similar to, though somewhat lower than, levels reported by other groups [6,9,10].

Circulating plasma levels of 11-dehydro-TxB_2 have been reported to be very low (not more than a few pg/ml) [15]. Thus, to determine normal plasma levels utilizing this assay, and to overcome the blank of the internal standard, 50 ml of plasma from six healthy individuals were assayed for 11-dehydro-TxB_2. Normal plasma levels were found to be 1.5 ± 0.4 pg/ml (mean ± 1 S.D.), in agreement with other reports [15].

11-Dehydro-TxB_2 levels in clinical situations associated with alterations in thromboxane production

We next examined the ability of this assay to assess alterations in endogenous thromboxane production in humans. Initially, we examined the effect of aspirin on urinary 11-dehydro-TxB_2 excretion in normal individuals. Aspirin is known to significantly reduce thromboxane generation *in vivo* [4]. In these studies, four individuals were administered aspirin 325 mg per day for 5 days. Pooled urine samples (24 h) were collected before initiation of aspirin and on the fifth day of treatment. Levels before and after initiation of dosing are shown in Table I. Aspirin administration was associated with a 75 ± 10% (mean ± 1 S.D.) decrease in 11-dehydro-TxB_2 excretion when quantified by this assay.

In an effort to determine the ability of this assay to detect increases in endogenous thromboxane generation, we measured plasma and urinary 11-dehydro-TxB_2 levels in a patient with documented increases in eicosanoid production. A chromatographic tracing of her plasma analyzed for 11-dehydro-TxB_2 is shown in Fig. 3. Circulating levels of this compound were 342 pg/ml, 224-fold above the normal mean. In addition to the marked increase in circulating 11-dehydro-TxB_2, there was also a 102-fold increase in the urinary level of this compound.

DISCUSSION

Thromboxane A_2 is believed to be an important pathophysiological mediator or marker in a number of human diseases including atherosclerosis, pregnancy-induced hypertension and unstable angina [4,16]. Assessment of systemic thromboxane production *in vivo* is best undertaken by measuring long-lived metabolites of this compound, either 2,3-dinor-TxB_2 or 11-dehydro-TxB_2 [4]. Previously, Lawson *et al.* [7] reported assays for both metabolites, and these methods have been used extensively to examine the role of

TABLE I

EFFECT OF ASPIRIN (325 mg PER DAY) FOR FIVE DAYS ON THE EXCRETION OF 11-DEHYDRO-TXB_2 IN FOUR VOLUNTEERS

Volunteer	Urinary 11-dehydro-TxB_2 (pg/mg creatinine)		Percent reduction
	Before treatment	During treatment	
1	508	99	81
2	218	46	79
3	180	39	79
4	132	54	60
Mean	259 ± 169	60 ± 27	75 ± 10[a]

[a] $p < 0.05$, before treatment compared to during treatment using Student's *t*-test.

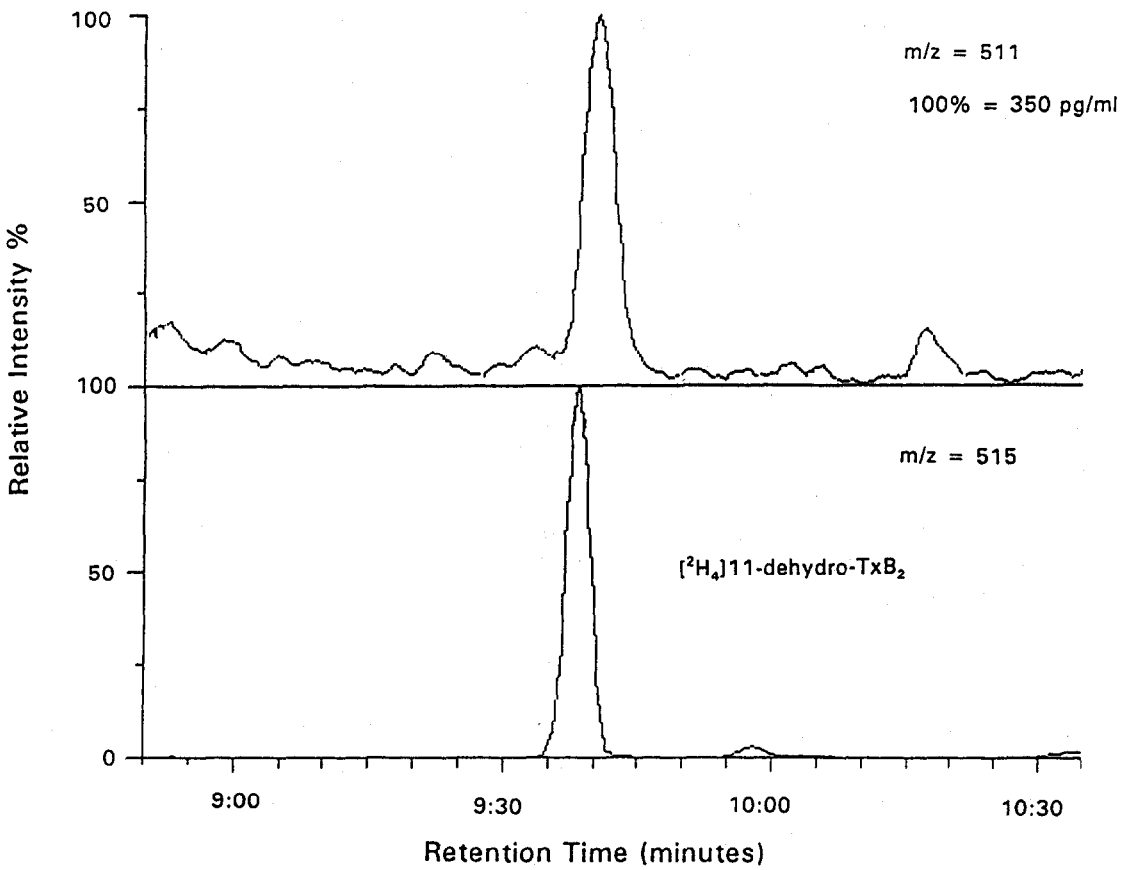

Fig. 3. Analysis of plasma (3 ml) from an individual producing excessive amounts of eicosanoids. Endogenous 11-dehydro-TxB_2 is represented by the peak in the *m/z* 511 chromatogram and the deuterated standard by the peak in the *m/z* 515 chromatogram.

thromboxane in pathophysiological situations [4,7]. In our hands, however, we have found the assay for 11-dehydro-TxB_2 lengthy and, on occasion, analysis of 11-dehydro-TxB_2 has been confounded by co-eluting impurities. Thus, we sought to modify the assay to make it more efficient and accurate. This was accomplished by introducing four modifications. Firstly, the use of hydrochloric acid rather than formic acid during the lactonization of 11-dehydro-TxB_2 in biological samples alleviates formation of a viscous residue which interferes with the subsequent chromatographic step. Secondly, changing the solvent system in the second TLC effectively separates 11-dehydro-TxB_2 from co-eluting impurities. Thirdly, the use of PGD_2 and its pentafluorobenzyl ester as TLC standards alleviates the risk of sample contamination. Finally, reducing the acid cyclization step from 2 h to 30 min significantly shortens the time of the assay.

As a result, the modified assay reported herein is both sensitive and accurate. As illustrated in Figs. 2 and 3, the chromatographic tracings are free of co-eluting impurities, a problem encountered not only by us but by other groups [9,10]. Recently, however, several researchers have reported that assays employing tandem MS render 11-dehydro-TxB_2 free of co-eluting impurities [8–10]. These instruments, on the other hand, are very expensive and are not widely available. Thus, it would appear that because of the quality of the chromatographic tracings obtained with the assay reported herein, this method is a suitable alternative to assays employing tandem instruments for the quantification of 11-dehydro-TxB_2.

Although GC–MS assays for eicosanoids are generally not as efficient as other methods of analysis (*i.e.*, immunoassays), approximately fifteen to twenty samples can be processed and analyzed for 11-dehydro-TxB_2 per day using this method. In this regard, it is as equally facile as other GC–MS assays reported for the quantification of this compound [7–9]. Thus, the improvements in techniques reported herein should make this assay for 11-dehydro-TxB_2 a valuable tool to further study the role of thromboxane in human disease.

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