FEASIBILITY OF USING [$^{18}O_2$]11-DEHYDROTHROMBOXANE B_2 AS AN INTERNAL STANDARD OF IMMUNOAFFINITY PURIFICATION FOLLOWED BY GAS CHROMATOGRAPHY/SELECTED ION MONITORING

Masataka ISHIBASHI,*,[a] Yoshiharu OHYAMA,[b] Keiko WATANABE,[a] Yoko HAYASHI,[c] Wataru TAKASAKI,[d] Akihiko NAKAGAWA,[d] and Michinao MIZUGAKI,[b]

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co.,Ltd.,[a] Kita-ku, Tokyo 115, Japan, Department of Pharmaceutical Science, Tohoku University Hospital,[b] Aoba-ku, Sendai 980, Japan, Department of Biochemistry, Tokushima University School of Medicine,[c] Kuramoto-cho, Tokushima 770, Japan, and Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,[d] Shinagawa-ku, Tokyo 140, Japan

[1,1-$^{18}O_2$]11-Dehydrothromboxane B_2 (11-dehydro-TXB_2) was prepared by repetitive base-catalyzed hydrolysis of the lactone ring of its [1,1,11-$^{18}O_3$]-analogue, and evaluated for its suitability as an internal standard in gas chromatography/selected ion monitoring (GC/SIM) of 11-dehydro-TXB_2. Use of the present [$^{18}O_2$]-analogue as an internal standard may make it a suitable candidate for specific immunoaffinity purification followed by GC/SIM.

KEYWORDS 11-dehydrothromboxane B_2; [$^{18}O_2$]11-dehydrothromboxane B_2; ^{18}O-labelled analogue; internal standard; immunoaffinity purification; dimethylisopropylsilyl ether; mass spectrum; GC/MS; GC/SIM

With the advances in thromboxane A_2 (TXA_2) metabolism, 11-dehydrothromboxane B_2 (11-dehydro-TXB_2), one of the major enzymatic metabolites of thromboxane B_2, has been the object of much interest for elucidation of the physiological roles of TXA_2.[1,2] Microanalytical methods for determination of 11-dehydro-TXB_2 with high sensitivity and high specificity have been developed using gas chromatography/selected ion monitoring (GC/SIM).[3-5] Choosing stable isotopes as a suitable internal standard (IS) is important in trace analysis to compensate for loss of the compounds of interest caused during the sample preparation. ^{18}O-Labelled analogues have been synthesized by repetitive base-catalyzed hydrolysis of the methyl ester derivatives in [^{18}O]-water and have been widely adopted as an IS for the GC/SIM analysis of arachidonic acid metabolites because of their simple, rapid and efficient preparation.[6]

The immunoextraction (immunoaffinity extraction) technique provides a rapid and convenient procedure for the selective extraction of the trace amounts of the compounds of interest from biological fluids. Yamamoto and co-workers[7] described an immunoextraction of 11-dehydro-TXB_2 in human urine, permitting

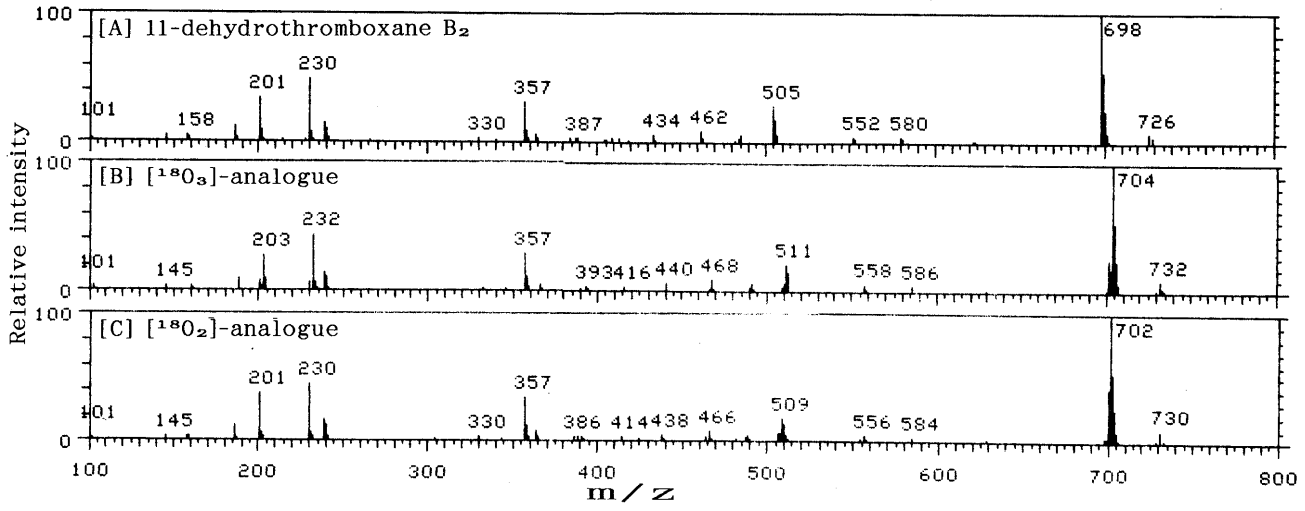

Fig. 1. Mass Spectra of the Methyl Ester-n-propylamide-dimethylisopropylsilyl Ether Derivatives of (A) 11-Dehydro-TXB_2 and Its (B) [1,1,11-$^{18}O_3$]- and (C) [1,1-$^{18}O_2$]-Analogues

direct analysis of the extracts by radioimmunoassay. 11-Dehydro-TXB_2 may be absorbed by a lactone of either cyclic or acyclic (ring opened) form, depending on the property of the antibody employed. The above immunoextraction method was carried out at a physiological pH of 7.4 where the monoclonal antibody used for immunoaffinity-extraction of anti-11-dehydro-TXB_2 recognized the acyclic form. In fact, pre-incubation was carried out for 2 h at pH 10.5 prior to immunoaffinity-extraction so the 11-dehydro-TXB_2 could be completely hydrolyzed to the acyclic form.

^{18}O-Labelled 11-dehydro-TXB_2 was prepared and was used for GC/SIM analysis of urinary levels of 11-dehydro-TXB_2.[5] The oxygen atoms inbuilt for 11-dehydro-TXB_2 were not limited to the carboxylic acid moiety but extended also to the oxygen at the lactone ring moiety. In the process of immunoextraction, the [$^{18}O_3$]-analogue is also hydrolyzed to the acyclic form, which upon acidification to pH 2.0 re-lactonized with a loss of half of the ^{18}O-atoms at C-11 (mass spectrum not shown). Therefore, application of the [$^{18}O_3$]-analogue as an IS for the above immunoextraction purification causes a serious problem of overestimation of 11-dehydro-TXB_2 during GC/SIM analysis of urinary levels. Considering the necessity of an IS for the above immunoextraction, the [1,1-$^{18}O_2$]-analogue was prepared by repetitive base-catalyzed hydrolysis of the lactone ring moiety. This paper deals with the feasibility of using [$^{18}O_2$]-analogue as an IS for the GC/SIM analysis of 11-dehydro-TXB_2.

11-Dehydro-TXB_2 and its ^{18}O-labelled analogues were converted to the methyl ester (ME)-n-propylamide-dimethylisopropylsilyl (DMIPS) ether derivatives by treating them with diazomethane, n-propylamine and DMIPS-imidazole successively.[8] Fig. 1 shows the mass spectra of these derivatives. The mass spectra of the derivatives of the ^{18}O-labels were closely related to that of the non-labelled compound except for an obvious mass shift produced by substitution of the ^{18}O-atoms. The fragmentation mechanisms of this derivative were discussed in detail elsewhere.[9] In the mass spectrum of the [$^{18}O_2$]-analogue (Fig. 1-c), the shift of the base peak ion of $[M-C_3H_7]^+$ from m/z 698 to 702 represents incorporation of two ^{18}O-atoms, and the fragmentation products by simple bond fission mechanisms such as the ions of $[M-(DMIPSOH)_n]^+$, $[M-C_3H_7-(DMIPSOH)_n]^+$ and $[M-C_5H_{11}-(DMIPSOH)_n]^+$ were also observed with a shift of four mass units, the same as the base peak ion. In turn, the $[M-(DMIPSOH)_2-\alpha\text{-chain}]^+$ ion (m/z 364) and the ions containing the protected 11-carboxylic acid moiety, such as m/z 158, 186, 201 and 230, remained as they were. But the corresponding ions observed in the mass spectrum of the [$^{18}O_3$]-analogue were shifted with two mass units (Fig. 1-b). The characteristic fragment ions, however, which yield information about the position of the incorporated ^{18}O-atoms, are not prominent. In response to this problem, another derivative was prepared. The ME-DMIPS ether derivative[5] of the [$^{18}O_2$]-analogue gave a characteristic mass spectrum in which informative ions for the lactone ring moiety are obtained with prominent intensity. Characteristic fragmentation by lactone ring fission gave rise to the ion at m/z 355 which was shifted four mass units from that of the original compound, supporting directly the proposition that two ^{18}O-atoms were incorporated into 1-carboxylic acid moiety. Consequently, this observation led to a prediction that two ^{18}O-atoms remained in the 1-carboxylic acid moiety and the third one was eliminated by back-exchange under repetitive base-catalyzed hydrolysis of the lactone ring moiety. The mass spectrometric analysis revealed that the resulting compound was a mixture of a [$^{18}O_1$]- and a [$^{18}O_2$]-analogue, and their contents were 29.1 and 66.7%, respectively. The content of the non-labelled 11-dehydro-TXB_2 was calculated to be about 2.4%. The intensity ratio of the ions at m/z 698 to 702 in the [$^{18}O_2$]-analogue derivative was about 3.2%.

Table I. Parameters from Calibration Graphs Obtained by Using of the [$^{18}O_2$]-Analogue as an Internal Standard

	Hydrolysis (pH 10.5, 2h)	Lactonization (pH 2.0, 1 h)	Parameters	
			Slope	Coefficient of correlation
A (n=4)	+	+	3.246 +/- 0.024	0.9969 +/- 0.0021
B (n=4)	-	+	3.306 +/- 0.051	0.9994 +/- 0.0004

To examine the suitability of the [$^{18}O_2$]-analogue as an IS, calibration graphs were prepared from GC/SIM analysis of increasing amounts of 11-dehydro-TXB_2 (0, 2, 4, 6, 8, and 10 ng/tube) and added to constant amounts of an IS (about 5 ng). The calibration graph would correspond to the observed range covering the concentration found in 10 ml of healthy human urine. The prepared samples were hydrolyzed at pH 10.5 for 2 h, followed by acidification to pH 2.0 for 1 h. In parallel with these samples, another series of samples without the hydrolysis procedure were prepared. 11-Dehydro-TXB_2 and an IS extracted from the samples with a reverse-phase octadecylsilica cartridge of BondElut C_{18} were derivatized and analyzed by GC/SIM monitoring their base peak ions of [M-C_3H_7]$^+$. The parameters from the calibration graphs are listed in Table I. Each of the coefficients of correlation indicates that there was a good linearity between the peak area ratio of 11-dehydro-TXB_2 and the IS and the amounts of 11-dehydro-TXB_2 in the range of 0-10 ng/tube. The slopes of these calibration curves also seemed to agree well with each other. So, data from the slopes were submitted to statistical analysis according to the one way-lay out. This indicated that there was no significant difference by introduction of the hydrolysis reaction and that almost all of the total error in this experiment was attributed to the GC/SIM analysis (coefficient of variation: 1.2 %). These results support the selection of [$^{18}O_2$]11-dehydro-TXB_2 as an IS suitable for specific immunoextraction followed by GC/SIM.

Fig. 2 illustrates a typical SIM result from the urine sample, and shows that interfering substances from urine matrix were nearly eliminated during the microanalysis. Peaks appearing at a retention time of about 7.2 min on the traces of 698.47 and 702.48 from the urine extract sample corresponded to about 250 pg for 11-dehydro-TXB_2 and 165 pg for an internal standard of [$^{18}O_2$]11-dehydro-TXB_2.

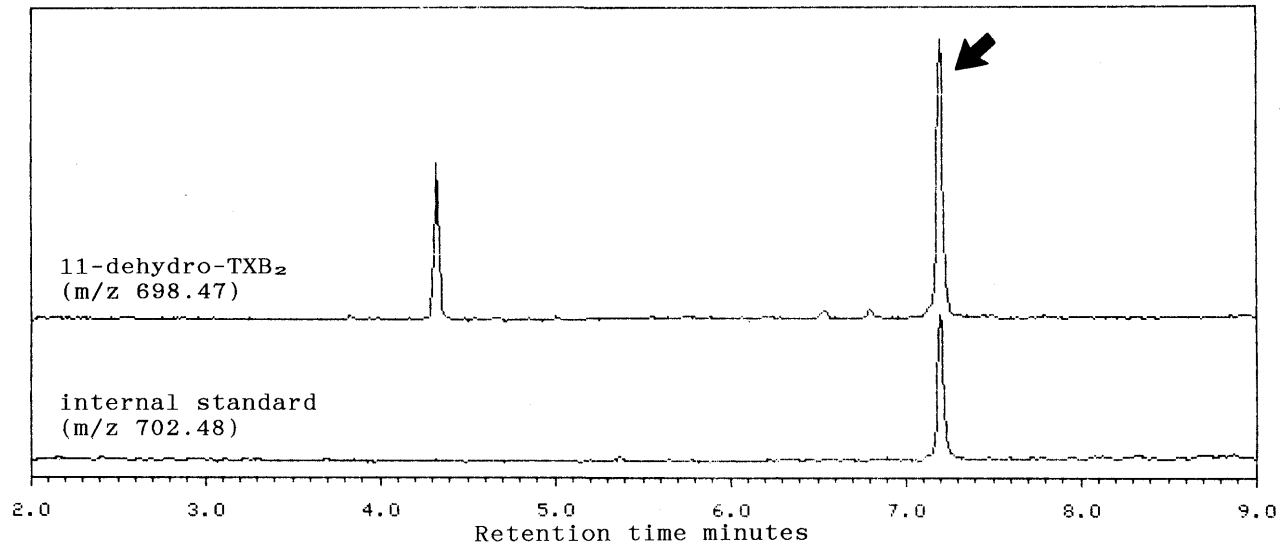

Fig. 2. GC/SIM Result of the 11-Dehydro-TXB_2 Methyl Ester-n-propylamide-DMPIS Ether Derivative in the Immunoextract from Healthy Male Adult Urine over a GC Retention Time Range of 2-9 min

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