Journal of Chromatography, 562 (1991) 613 624
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5535

Novel derivatization and immunoextraction to improve microanalysis of 11-dehydrothromboxane B_2 in human urine

MASATAKA ISHIBASHI* and KEIKO WATANABE

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co. Ltd., 3-31 Shimo, Kita-ku, Tokyo 115 (Japan)

YOSHIHARU OHYAMA and MICHINAO MIZUGAKI

Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980 (Japan)

YOKO HAYASHI

Department of Biochemistry, Tokushima University School of Medicine, 3-18-15 Kuramoto-machi, Tokushima 770 (Japan)

and

WATARU TAKASAKI

Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiro-machi, Shinagawa-ku, Tokyo 140 (Japan)

ABSTRACT

A new, highly selective procedure for the determination of 11-dehydrothromboxane B_2 (11-dehydro-TXB_2) in human urine is described. Following the addition of [19,19,20,20-$^2H_4$]11-dehydro-TXB_2 as an internal standard, samples were extracted with an affinity column of *anti*-11-dehydro-TXB_2. Conversion of the immunoextracted 11-dehydro-TXB_2 into its 1-methyl ester-11-*n*-propylamide-9,12,15-tris-dimethyl-isopropylsilyl ether derivative was followed by gas chromatography–selected-ion monitoring. The mass spectrum of the 11-dehydro-TXB_2 derivative was dominated by the base peak ion of $[M-C_3H_7]^+$ at *m/z* 698, which accounted for more than 10% of the total ion current. A typical result showed that the immunoaffinity purification procedures provided an extremely clean alternative to more conventional methods of chromatographic fractionation, and that interfering substances from the urine matrix were almost entirely eliminated during the microanalysis.

INTRODUCTION

11-Dehydrothromboxane B_2 (11-dehydro-TXB_2), one of the major enzymic metabolites of thromboxane B_2 (TXB_2), has been the object of much interest for the elucidation of the physiological roles of thromboxane A_2 (TXA_2). 11-Dehydro-TXB_2 has recently been considered to be a more reliable index of thromboxane biosynthesis than TXB_2, which has been monitored as the stable hydrolysed product of TXA_2 [1,2]. In response to advances in the knowledge of TXA_2

0378-4347/91/$03.50

metabolism, microanalytical methods for the 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].

11-Dehydro-TXB_2 can occur in a lactone form and lactone ring-opened (acyclic) form. The methyl ester (ME)–trimethylsilyl (TMS) ether derivative of 11-dehyro-TXB_2, obtained by treating it with diazomethane followed by trimethylchlorosilane–hexamethyldisilazane–pyridine was prepared, and GC of the reaction product revealed the formation of two different derivatives, the expected lactone form and the lactone ring-opened form of methyl 8-[2-methoxycarbonyl-1-(trimethylsilyloxy)ethyl]-9,12-bis-trimethylsilyloxy-5,10-heptadecadienoate [6].

It is essential for the microanalysis of 11-dehydro-TXB_2 to keep the derivative in one form during sample preparation. An alternative solution to this problem is to convert the unfavorable lactone ring directly into a stable acyclic form. Use of the lactone ring-opening reaction with alkylamine for derivatization enables preparation of specific derivatives that reflect more structural information about the compound of interest and that can be separated more efficiently and easily from other compounds in a mixture of related homologues. Additionally, the acyclic product has a characteristic spectrum in which significant ions are prominent [7,8].

It has been established that the sensitivity and specificity of using GC–SIM in the electron impact mode for microanalysis may be improved by choosing a derivative with a mass spectrum in which the ion current is concentrated in characteristic ions in the high mass region. The GC–MS properties of dimethylisopropylsilyl (DMIPS) ether derivatives have been found to be superior to those of other silyl ether derivatives in enhancing GC separation and improving chemical stability for purification by column chromatography, in addition to improving the intensity of characteristic ions for quantification by GC–SIM. For this reason, the DMIPS ether derivatives have been examined exclusively in our laboratory for quantification of prostanoids [9,10].

The combined use of this lactone ring-opening reaction with *n*-propylamine and dimethylisopropylsilylation was adopted for 11-dehydro-TXB_2 to obtain a chemically stable derivative. The 11-dehydro-TXB_2 ME-11-*n*-propylamide (PA) 9,12,15-tris-DMIPS ether derivative [methyl 8-(1-dimethylisopropylsilyloxy-2-N-*n*-propylcarbamoyl)ethyl-9,12-dimethylisopropylsilyloxy-5,10-heptadecadienoate] was prepared and used to evaluate the feasibility of using the present derivative for detecting 11-dehydro-TXB_2 in human urine [11].

The 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. Hayashi *et al.* [12] described an immunoaffinity extraction of 11-dehydro-TXB_2 in human urine, which permitted direct analysis of the extracts by radioimmunoassay. This paper deals with the microanalysis of 11-dehydro-TXB_2 in human urine using purification by chromatography on an affinity column of anti-11-dehydro-TXB_2 monoclonal antibody and GC–SIM with the above ME-PA-DMIPS ether derivative.

EXPERIMENTAL

Samples and reagents

11-Dehydro-TXB_2 was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). DMIPS-imidazole, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *n*-propylamine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Bond Elut Si and Bond Elut C_{18} cartridge were obtained from Analytichem International (Harbor City, CA, U.S.A.). Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluenesulphonamide. Other solvents and reagents used were of analytical grade.

[19,19,20,20-$^2H_2$]Thromboxane B_2 was kindly donated by Upjohn Pharmaceuticals Japan. [19,19,20,20-$^2H_4$]11-Dehydro-TXB_2 was prepared from [19,19,20,20-$^2H_4$]TXB_2 by enzymic oxidation according to the method of Lawson *et al.* [3]. Solid phase-coupled *anti*-11-dehydro-TXB_2 monoclonal antibody was prepared in the Analytical and Metabolic Research Laboratories, Sankyo (Tokyo, Japan) [12].

Gas chromatography

A Shimadzu GC-9A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, an all-glass VandenBerg-type solventless injector [13] and a data-processing system was employed. An MS fused-silica capillary column (25 m × 0.25 mm I.D., 0.10 μm film thickness, Quadrex, New Haven, CT, U.S.A.) was used. Helium was used as the carrier gas and make-up gas. The temperature of the injection port and detector was kept at 320°C, and the column oven temperature was 280°C.

Gas chromatography–mass spectrometry

A Hitachi M-80B GC–MS system (Hitachi, Ibaragi, Japan) with an all-glass Vandenberg-type solventless injector [13] interfaced with an M-0101 data-processing system (Hitachi) was employed. An MS fused-silica capillary column (25 m × 0.25 mm I.D., 0.10 μm film thickness, Quadrex) was used. The temperature of the column oven was maintained at 200°C for 1 min, then programmed to 320°C at 10°C/min. The carrier gas was helium, with a linear velocity of *ca.* 25 cm/s. The temperature of the injector port and transfer line was kept at 300°C and that of the ion source at 250°C. The ionization energy and trap current were 20 eV and 200 μA, respectively. The accelerating voltage was 3 kV. The mass spectrum of each of the 11-dehydro-TXB_2 derivatives was recorded by repeatedly scanning over the mass range m/z 0–800 (cycle time *ca.* 3 s) with dynamic resolution of 2000.

GC–SIM

GC–SIM was performed using the Hitachi M-80B GC–MS system under the same conditions as used in GC–MS with a dynamic resolution of 2000. The base

peak ions of $[M-C_3H_7]^+$ at *m/z* 698.47 for 11-dehydro-TXB_2 and 702.48 for an internal standard of $[^2H_4]$11-dehydro-TXB_2 were monitored.

Derivatization procedure

To a solution of 11-dehydro-TXB_2 in methanol (0.5 ml) freshly prepared ethereal diazomethane (1 ml) was added, and the resulting solution was allowed to stand at room temperature for 30 min. After evaporation of the solvent under reduced pressure, the residue was dissolved with *n*-propylamine (0.3 ml) and allowed to stand for 1 h at room temperature. After evaporation under reduced pressure, the resulting ME-PA derivative was treated with DMIPS-imidazole (50 μl) for 30 min at 60°C. The reaction mixture was dissolved with *n*-hexane (1 ml) and transferred onto Bond Elut Si cartridge, and the cartridge was washed with *n*-hexane–ethyl acetate (95:5, 6 ml). The ME-PA-DMIPS ether derivative was then eluted with *n*-hexane–ethyl acetate (10:1, 6 ml). After evaporation of the solvent, the residue was dissolved in *n*-hexane–pyridine (99:1, v/v; 100 μl) and used for GC–MS and GC–SIM.

Extraction of 11-dehydro-TXB_2 from human urine

Immunoextraction for 11-dehydro-TXB_2 was carried out according to the method of Hayashi *et al.* [12] with some modification. The urine sample (5–10 ml), to which was added 10 ng of $[^2H_4]$11-dehydro-TXB_2 as an internal standard, was made alkaline to pH 10.5 with 0.5 *M* NaOH and then allowed to stand for 2 h at room temperature. The pH was re-adjusted with 0.5 *M* HCl to *ca.* 7.4 and the sample was diluted with 0.1 *M* phosphate buffer (pH 7.4) and applied to an affinity column of *anti*-11-dehydro-TXB_2 monoclonal antibody. Thereafter, the gel was washed with water (10 ml). Excess water was removed by applying pressurized nitrogen gas to the top of the column. 11-Dehydro-TXB_2 was eluted with methanol–water (95:5; v/v; 10 ml). The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in ethanol–water (15:85, v/v; 10 ml), acidified with 0.5 *M* HCl to pH 2, and allowed to stand for 1 h at room temperature. The resulting solution was applied to a Sep-Pak C_{18} cartridge and washed with ethanol–water (15:85, v/v; 10 ml) and *n*-hexane (10 ml). 11-Dehydro-TXB_2 was eluted with ethyl acetate (10 ml). Immunoextracted 11-dehydro-TXB_2 was converted into its ME-PA-DMIPS ether derivative by the method described above, and the product was used for GC–SIM.

RESULTS AND DISCUSSION

Gas chromatography

The 11-dehydro-TXB_2 ME-PA-DMIPS ether derivative was prepared by treating 11-dehydro-TXB_2 with diazomethane, *n*-propylamine and then DMIPS-imidazole. The gas chromatogram of the reaction product showed a well shaped peak when a fused-silica capillary column cross-linked with methylsilicone was

used. This suggested that the stepwise derivatization had proceeded smoothly and quantitatively. The methylene unit value of the 11-dehydro-TXB_2 derivative was 35.29, which was *ca.* 4.5 higher than that of the corresponding TMS ether derivative and 5.8 higher than that of 9,15-bis-TMS ether derivative of 11-dehydro-TXB_2 methyl ester. When the 11-dehydro-TXB_2 methyl ester was allowed to stand in *n*-propylamine medium at room temperature, only the lactone moiety was susceptible to this reaction to give an *n*-propylamide group and a hydroxyl group within 30 min.

Mass spectrometry

The mass spectrum of the 11-dehydro-TXB_2 ME-PA-DMIPS ether derivative is shown in Fig. 1A. It exhibits a series of ions characteristic of the expected derivative. The molecular ion was not observed, whereas the ion of $[M-15]^+$ at *m/z* 726 was observed with low intensity. Loss of an isopropyl radical at the DMIPS group from the molecular ion gave rise to the ion of $[M-43]^+$ at *m/z* 698 as the base peak. The appearance of these ions in the spectrum, typical of DMIPS ether derivatives, together with the absence of the molecular ion indicates the incorporation of three DMIPS groups into the 11-dehydro-TXB_2 ME-PA derivative.

Successive losses of the dimethylisopropylsilanol molecule (DMIPSOH; 118 a.m.u.) gave rise to the ions at *m/z* 623, 505 and 387 (nucleic fragment ion) from the molecular ion and ions at *m/z* 580 and 462 from the $[M-43]^+$ ion, respectively. The ions at *m/z* 552 and 434 were formed by the elimination of the C-16–C-20 hydrocarbon fragment (71 a.m.u., typical of the prostanoid series 1 and 2) from the ions at *m/z* 623 and 505. The ion at *m/z* 506, accompanied by that at *m/z* 505, was considered to arise by loss of a $(CH_3)_2SiO$ group (74 a.m.u.) from the

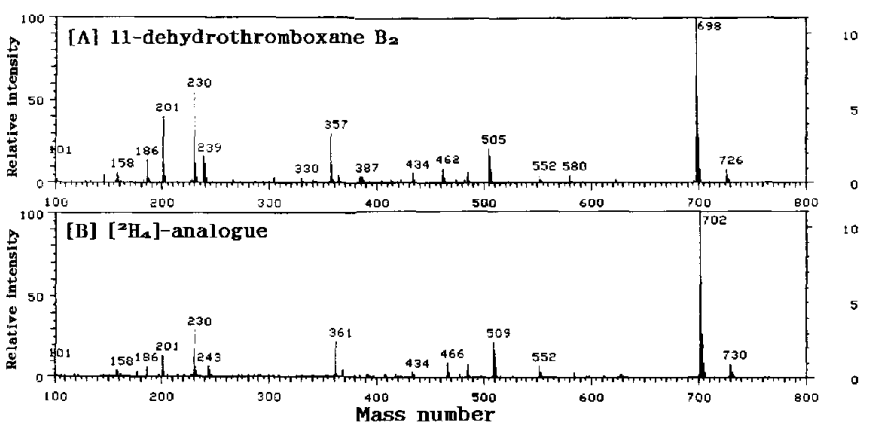

Fig. 1. Mass spectra of the ME-PA-DMIPS ether derivatives of (A) 11-dehydrothromboxane B_2 and (B) its $[^2H_4]$11-dehydrothromboxane B_2.

ion at *m/z* 580. A similar fragmentation was observed for the formation of the ion at *m/z* 624 from *m/z* 698 and that at *m/z* 388 from *m/z* 462. Cleavage of the C-8–C-12 bond in the molecule resulted in a characteristic ion at *m/z* 357 and this ion further fragmented to form the ion at *m/z* 239 by loss of DMIPSOH molecule. The structures of these two ions were identical with those determined in the tris-DMIPS ether derivative of TXB_2 ME-methyloxime [9]. The observation of fragmentation products of a simple bond-fission mechanism is sufficient to con-

TABLE I

MASS SPECTRAL DATA OF THE ION STRUCTURES ASSIGNED AND THEIR ELEMENTAL COMPOSITIONS FOR THE 11-DEHYDROTHROMBOXANE B_2 ME-PA-DMIPS ETHER DERIVATIVE

m/z	Fragmentation[a]		Elemental composition				
			C	H	N	O	Si
741	$[M]^+$		39	79	1	6	3
726	$[M-15]^+$	M – CH_3	38	76	1	6	3
710	$[M-31]^+$	M – OCH_3	38	76	1	5	3
698	$[M-43]^+$	M – C_3H_7	36	72	1	6	3
623	$[M-118]^+$	M – DMIPSOH	34	65	1	5	2
580	$[M-43-118]^+$	M – C_3H_7 – DMIPSOH	31	58	1	5	2
552	$[M-71-118]^+$	M – C_5H_{11} – DMIPSOH	29	54	1	5	2
506	$[M-43-118-74]^+$	M – C_3H_7 – DMIPSOH – $(CH_3)_2Si=O$	29	52	1	4	1
505	$[M-118\times2]^+$	M – DMIPSOH × 2	29	51	1	4	1
485		C_1/C_{11} + DMIPS	25	51	1	4	2
462	$[M-43-118\times2]^+$	M – C_3H_7 – DMIPSOH × 2	26	44	1	4	1
434	$[M-71-118\times2]^+$	M – C_5H_{11} – DMIPSOH × 2	24	40	1	4	1
388	$[M-43-118\times2-74]^+$	M – C_3H_7 – DMIPSOH × 2 – $(CH_3)_2Si=O$	24	38	1	3	0
387	$[M-118\times3]^+$	M – DMIPSOH × 3	24	37	1	3	0
364	$[M-118\times2-141]^+$	M – DMIPSOH × 2 – α-chain	21	38	1	2	1
357		C_{12}/C_{20}	19	41	0	2	2
340		C_1/C_{11} – C_3H_7 – H	17	30	1	4	1
239	$[357-118]^+$	357 – DMIPSOH	14	27	0	1	1
230		C_9/C_{11}: DMIPSO–CH–CH_2–CONH–C_3H_7	11	24	1	2	1
201		C_{15}/C_{20}	11	25	0	1	1
201		DMIPSO–C(=CH_2)–NH–C_3H_7	10	23	1	1	1
186		CH_3–(C_3H_7–)Si=O–C(=CH_2)–NH–C_3H_7	9	20	1	1	1
186		$(CH_3)_2Si$=O–CH=CH–CONH–C_3H_7	8	16	1	2	1
175		$(CH_3)_2Si$=O–DMIPS	7	19	0	1	2
158	$[201-43]^+$	$(CH_3)_2Si$=O–C(=CH_2)–NH–C_3H_7	7	16	1	1	1
145	$[201-56]^+$	DMIPS–OH–CH=CH_2	7	17	0	1	1
133	$[175-42]^+$	$(CH_3)_2Si$=O–SiH(=$(CH_3)_2$)	4	13	0	1	2
101		DMIPS	5	13	0	0	1

[a] DMIPS = dimethylisopropylsilyl; DMIPSOH = dimethylisopropylsilanol.

firm formation of the expected derivative. The abundant fragmentation products in the low-mass region were ions containing silicon atoms. These may have been produced via a six-membered ring transition state or by the formation of a cyclic dimethylsilylene ring system, whereas many of the ions were explained by simple bond-fission mechanisms. Table I lists the mass spectral data of the ion structures assigned and their elemental compositions for the 11-dehydro-TXB_2 derivative. Details of these fragmentation mechanisms have been described in our previous paper [14].

The present 11-dehydro-TXB_2 derivative concentrated more than 10% of the total ion current into the base peak ion of *m/z* 698. This indicates that this characteristic ion in the high-mass region, with its relatively high abundance, may be a suitable candidate for the specific and sensitive analysis of 11-dehydro-TXB_2 by GC–SIM.

Preparation of deuterium-labelled 11-dehydro-TXB_2 as an internal standard

[19,19,20,20-$^2H_4$]11-Dehydro-TXB_2 was prepared from [19,19,20,20-$^2H_4$]-TXB_2 by the enzymic oxidation according to the method of Lawson *et al.* [3]. Fig. 1B shows the mass spectrum of the [$^2H_4$]11-dehydro-TXB_2 derivative, which is very similar to that of the corresponding non-labelled 11-dehydro-TXB_2 derivative except for the obvious shift produced by the substitution of deuterium atoms. The shift of base peak ion from *m/z* 698 to 702 represents the incorporation of four deuterium atoms, and the fragmentation products of simple bond-fission mechanisms, such as the ions $[M-(DMIPSOH)_n]^+$ and $[M-C_3H_7-(DMIPSOH)_n]^+$, are also observed to be shifted by four mass units. However, the $[M-C_5H_{11}-(DMIPSOH)_n]^+$ ions of *m/z* 434 and 552 are unmoved. The ions containing the C-16–C-20 hydrocarbon fragment, such as *m/z* 243 and 361, are shifted by four mass units. Consequently, these observations led to a prediction that the four deuterium atoms remained in the C-16–C-20 moiety. The MS analysis revealed that the resulting compound was a mixture of a $^2H_3$- and $^2H_4$-labelled analogue, and their contents were 6.5 and 92.2%, respectively. The intensity ratio of the ions at *m/z* 698 to 702 in the derivative of the internal standard was *ca.* 0.6%.

Calibration graph

The calibration graph for 11-dehydro-TXB_2 was obtained by plotting the peak-area ratio of 11-dehydro-TXB_2 to an internal standard against their mass ratios. Good linearity was observed in the range 0–10 ng per tube, which would cover the concentrations found in healthy human urine.

Sensitivity

Fig. 2 shows the GC–SIM result from 11-dehydro-TXB_2 using 5 pg of the ME-PA-DMIPS ether derivative, obtained by monitoring the base peak ion at *m/z* 698, specific for the structural integrity of the 11-dehydro-TXB_2 derivative,

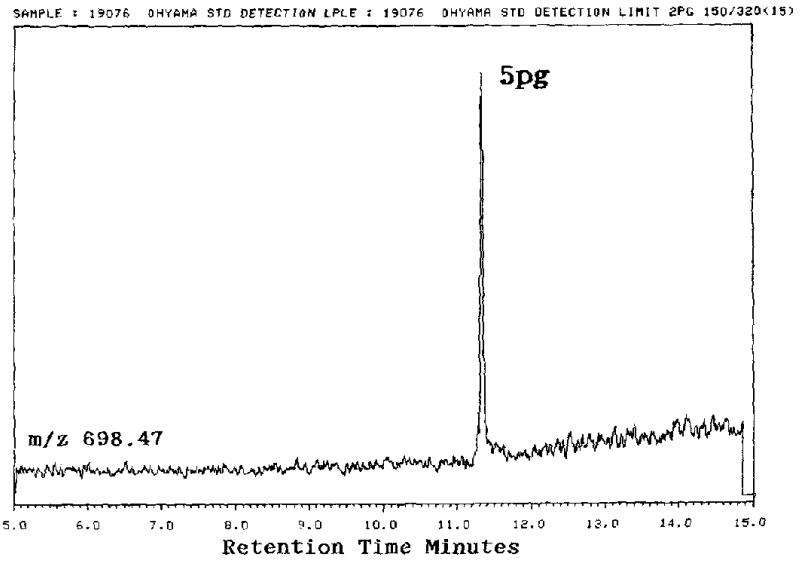

Fig. 2. Selected-ion recording of 11-dehydrothromboxane B_2, using 5 pg of the ME-PA-DMIPS ether derivative obtained by monitoring the base peak ion at *m/z* 698.47.

and it shows a well shaped peak with a signal-to-noise ratio of *ca.* 35:1. We previously reported [11] that, when 5 pg of the 11-dehydro-TXB_2 ME-PA-DMIPS ether derivative were injected, high-resolution SIM showed a well shaped peak with a signal-to-noise ratio of more than 100:1, a response factor three times greater than that of the present result. This discrepancy may be explained by the fact that high-resolution SIM is less susceptible to interference by impurities [10]. Consequently, the sensitivity in the low-resolution mode was considered to be reasonable.

Immunoextraction and purity of the peak corresponding 11-dehydro-TXB_2

The conditions for the immunoextraction of 11-dehydro-TXB_2 have already been established for human urine by Hayashi *et al.* [12]. The immunoextraction procedures described here are expected to provide an extremely clean alternative to more conventional methods of chromatographic fractionation. The excellent analytical specificity achieved during GC–SIM of a derivatized immunoextract is illustrated in Fig. 3, which shows a typical SIM result from a urine sample, and indicates that interfering substances from the urine matrix were almost eliminated during the microanalysis. Peaks appearing on the selected-ion recording traces of *m/z* 698.47 and 704.48 from the urine extract corresponded to *ca.* 290 pg for 11-dehydro-TXB_2 and 250 pg for [$^2H_4$]11-dehydro-TXB_2. The intensity-matching technique [15] was adopted to make certain that there was no other urine

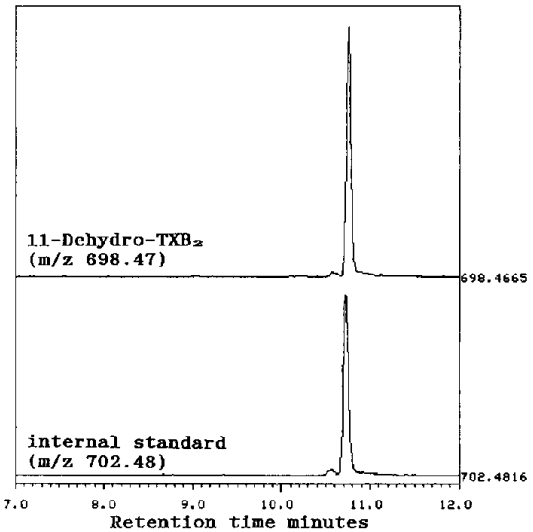

Fig. 3. Selected-ion recordings of the ME-PA-DMIPS ether derivatives of 11-dehydrothromboxane B_2 (*m/z* 698.47) and [$^2H_4$]11-dehydrothromboxane B_2 (*m/z* 702.48) in the immunoextract obtained from healthy male adult urine over a GC retention time range of 7–12 min.

constituent with the same retention time contributing to the recorded intensity on the conventional selected-ion recording. In order to use this technique, the two characteristic ions in the high-mass region were used to enhance the selectivity of the detection and to avoid interference from contaminants that give rise to the same ions as those being monitored. The coefficient, which is defined as a reciprocal of the ion-intensity ratio of *m/z* 726 to 698 (base peak ion), is calculated to be 12.9 from the GC–SIM result of the authentic compound, and then the ion intensity of *m/z* 726 is multiplied by the above coefficient. The reconstructed ion profile shown in Fig. 4 could be illustrated as a single peak. Therefore, it was recognized that the peak appearing on the conventional selected-ion recording was not only confirmed beyond a doubt to be the derivative of 11-dehydro-TXB_2, but also to contain no contaminant at all. This indicates that the immunoextraction without a complementary chromatographic purification may be considered to permit direct GC–SIM of urine extract. On the other hand, no peak was detected at the retention time of [$^2H_4$]11-dehydro-TXB_2 following GC–SIM analysis of the immunoextract.

Reproducibility

In order to examine the accuracy and the precision of the present method, five urine samples spiked with 11-dehydro-TXB_2 at a concentration of 0.7 ng/ml were prepared and analysed in duplicate by GC–SIM. This spiked concentration corresponded to *ca.* 45–180% of endogenous 11-dehydro-TXB_2 found in the urine of the healthy male volunteers, from the GC–MS–MS result reported by Schweer

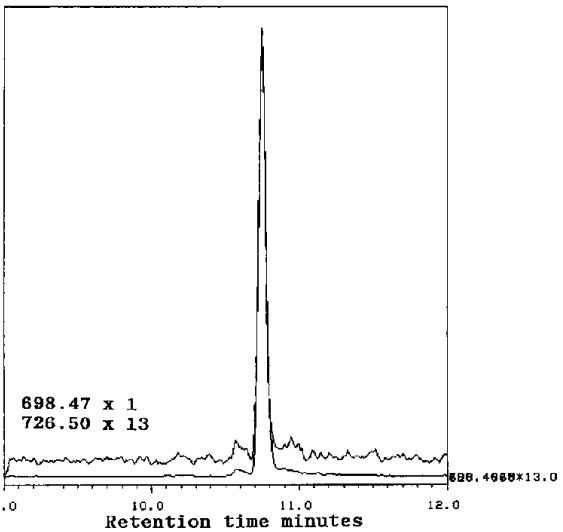

Fig. 4. Selected-ion recordings by use of the intensity-matching technique of the peak corresponding to 11-dehydrothromboxane B_2 in the immunoextract obtained from healthy male adult urine. Intensity ratio of the ion at m/z 726 to 698 (base peak ion) was obtained from a standard sample, and the reciprocal of the intensity ratio was used for reconstructing the ion profile by multiplying the corresponding ion intensity.

et al. [4] that the urinary 11-dehydro-TXB_2 concentrations in five male volunteers ranged from 0.39 to 1.50 ng/ml. The results are shown in Tables II and III. The recoveries of added 11-dehydro-TXB_2 ranged from 99.5 to 107.6%, with a mean and a coefficient of variation of 103.8 ± 2.7%.

TABLE II

RECOVERY OF 11-DEHYDROTHROMBOXANE B_2 SPIKED INTO HUMAN URINE: ANALYTICAL DATA

Sample	Urinary levels (ng/ml)		Recovered (ng/ml)		Recovery (%)	
Non-spiked urine						
1	0.853	0.853				
2	0.829	0.859				
3	0.841	0.793				
Mean	0.838					
Spiked urine (+0.7 ng/ml)						
1	1.540	1.566	0.703	0.729	100.4	104.1
2	1.575	1.534	0.738	0.697	105.4	99.5
3	1.561	1.576	0.724	0.739	103.4	105.5
4	1.583	1.538	0.746	0.701	106.6	100.1
5	1.576	1.590	0.739	0.753	105.5	107.6
Mean (± C.V.)	1.564		0.727		103.8 ± 2.7	

TABLE III

ANALYSIS OF VARIANCE FROM THE RECOVERY TEST

$F(4,5,0.05) = 5.19$; S = residual sum of squares; f = number of degree of freedom: $f_1 = f_{\text{sample preparation}}$; $f_2 = f_{\text{error}}$; V = unbiased variance; F_0 = observed value following F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); $F(f_1 f_2, \alpha)$ = density function of F distribution with f_1 and f_2 degrees of freedom.

Source	S	f	V	F_0
Sample preparation	24.824	4	6.206	0.623
Error (GC–SIM)	49.875	5	9.957	
Total	74.785	9		

The statistical analysis was carried out according to one way lay out [17] in order to divide the analytical errors between the two sources of sample preparation and GC–SIM. The result indicated that there was no significant difference in sample preparation, and that almost all of the total error in this experiment was attributable to the GC–SIM, because the error between sample preparation was negligible. The coefficient of variation in GC–SIM was 3.2%.

Recently, we have succeeded in identifying one enzymatic metabolite of thromboxane B_3, 11-dehydrothromboxane B_3 [16]. This could demonstrate directly the endogenous formation of thromboxane A_3 after oral administration of eicosapentaenoic acid. Applications of the present derivatization and immunoaffinity purification will be described elsewhere, as will the simultaneous determination of 11-dehydro-TXB_2 and 11-dehydrothromboxane B_3 in human urine.

ACKNOWLEDGEMENTS

The authors are grateful to Professor S. Yamamoto, Tokushima University School of Medicine, and Drs. M. Tanaka and A. Nakagawa, Sankyo Co. Ltd., for valuable advice on immunoaffinity extraction. M. M. was supported by Grants-in-Aid for Scientific Research in Analysis of Body-Modulating Functions of Foods (No. 01 636 501) from Ministry of Education, Japan.

REFERENCES

1 P. Westlund, M. Kumlin, A. Nordenström and E. Granström, *Prostaglandins*, 31 (1986) 413.
2 P. Westlund, E. Granström, M. Kumlin and A. Nordenström, *Prostaglandins*, 31 (1986) 929.
3 J. A. Lawson, C. Patrono, G. Ciabattoni and G. A. FitzGerald, *Anal. Biochem.*, 155 (1986) 198.
4 H. Schweer, C. O. Meese, O. Fürst, P. G. Kühl and H. W. Seyberth, *Anal. Biochem.*, 164 (1987) 156.
5 K. Watanabe, K. Yamashita, M. Ishibashi, Y. Hayashi, S. Yamamoto and H. Miyazaki, *J. Chromatogr.*, 468 (1989) 383.
6 M. Kumlin and E. Granström, *Prostaglandins*, 32 (1986) 741.
7 K. Watanabe, M. Ishibashi, H. Harima and S. Krolik, *Chem. Pharm. Bull.*, 39 (1989) 140.

8 M. Ishibashi, K. Watanabe, Y. Ohyama, M. Mizugaki and N. Harima, *Chem. Pharm. Bull.*, 39 (1989) 539.
9 H. Miyazaki, M. Ishibashi, K. Yamashita, Y. Nishikawa and M. Katori, *Biomed. Mass Spectrom.*, 8 (1981) 521.
10 M. Ishibashi, K. Yamashita, K. Watanabe and H. Miyazaki, in S. J. Gaskell (Editor), *Mass Spectrometry in Biomedical Research*, Wiley, Chichester, 1986, p. 423.
11 M. Ishibashi, K. Watanabe, N. Harima and S. Krolik, *Biomed. Environ. Mass Spectrom.*, 17 (1988) 133.
12 Y. Hayashi, F. Shono, S. Yamamoto, W. Takasaki, A. Nakagawa, K. Watanabe, K. Yamashita and H. Miyazaki, *Anal. Biochem*, 187 (1990) 151.
13 P. M. J. VandenBerg and T. P. Cox, *Chromatographia*, 5 (1971) 301.
14 M. Ishibashi, Y. Ohyama, K. Watanabe, M. Mizugaki and N. Harima, *Chem. Pharm. Bull.*, 39 (1989) 3015.
15 H. Miyazaki, M. Ishibashi and K. Yamashita, *Biomed. Mass Spectrom.*, 5 (1978) 469.
16 M. Ishibashi, Y. Ohyama, M. Mizugaki and N. Harima, *Biomed. Environ. Mass Spectrom.* 19 (1990) 387.
17 G. Taguchi, *Experimental Designs*, Maruzen, Tokyo, 1962.