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MICRODETERMINATION OF 11-DEHYDROTHROMBOXANE B_2 IN HUMAN URINE BY GAS CHROMATOGRAPHY–SELECTED-ION MONITORING[a]

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SUMMARY

Simple and effective purification for quantitation of trace amounts of 11-dehydrothromboxane B_2 (11-dehydro-TXB_2) in human urine by gas chromatography–selected-ion monitoring (GC–SIM) was achieved. The procedure is based on stepwise elution of the methyl ester derivative from a silica gel column with *n*-hexane–ethyl acetate (1:1) after washing the column with *n*-hexane–ethyl acetate (2:1). After the methyl ester has been converted into the corresponding dimethylisopropylsilyl ether, GC–SIM is carried out by monitoring the ion at *m/z* 539.32 for 11-dehydro-TXB_2 and that at *m/z* 543.33 for its $^{18}O_2$-labelled variant as an internal standard. The detection limit is 2 pg per injection with a signal-to-noise ratio of 5:1. The method was applied to the determination of 11-dehydro-TXB_2 in human urine.

INTRODUCTION

Thromboxane A_2 (TXA_2), one of the cyclooxygenase products of arachidonic acid, is a potent vasoconstrictor and platelet activator. In order to measure the TXA_2 production *in vivo*, thromboxane B_2 (TXB_2) has been widely used as a stable hydrolysis product. However, as the level of TXB_2 in plasma is readily confounded by platelet activation *ex vivo*, the measurement of the plasma concentration of this compound does not reflect exactly the circulating TXA_2 level. In response to this

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significant problem, attention has been focused on searching for more stable metabolites as indices of thromboxane biosynthesis. Thus, 11-dehydro-TXB_2, which is one of the major metabolites of TXB_2 in plasma and urine[1,2], has been considered to reflect the TXA_2 release in the human circulation[3,4]. In addition, the urinary excretion of 11-dehydro-TXB_2 is higher than that of the other TXA_2 metabolites, such as 2,3-dinor-TXB_2 and TXB_2 [5,6]. Several methods for the microdetermination of 11-dehydro-TXB_2 in biological specimens by using radioimmunoassay or gas chromatography–selected-ion monitoring (GC–SIM) have been reported[1,3,5]. These methods require complicated clean-up by thin-layer and/or high-performance liquid chromatographic (HPLC) techniques in order to eliminate interfering substances in biological specimens. We have reported that purification by silica gel column chromatography after methylation of prostaglandins (PGs) with ethereal diazomethane is very effective for the determination of PGs by GC–SIM[7,8]. This method was applied to the purification of 11-dehydro-TXB_2 in urine. This paper deals with a convenient method for the determination of 11-dehydro-TXB_2 in urine without the use of tedious purification procedures and/or special mass spectrometric techniques.

EXPERIMENTAL

Sample and reagents

11-Dehydro-TXB_2 was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Dimethylethylsilyl(DMES)-, dimethyl-*n*-propylsilyl(DMnPS)- and dimethylisopropylsilyl(DMiPS)imidazoles were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, U.S.A.). Sephadex LH-20 and silica gel were obtained from Pharmacia (Uppsala, Sweden) and E. Merck (Darmstadt, F.R.G.). A Chem-Elut cartridge was purchased from Analytichem International (Harbor City, CA, U.S.A.). A Sep-Pak C_{18} cartridge was purchased from Waters Assoc. (Milford, MA, U.S.A.). Other solvents and reagents used were of the highest quality available.

Capillary gas chromatography

A Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector and data processing system was employed. An open-tubular capillary column (Ultra 1, 25 m × 0.3 mm I.D.) (Hewlett-Packard, Avondale, PA, U.S.A.) was used. Helium was used as the carrier gas and make-up gas. An all-glass VandenBerg-type solventless injector[9] was used for sample injection. The temperature of the injection port and detector was kept at 320°C and that of the column oven at 280°C.

Gas chromatography–mass spectrometry

A Hitachi (Ibaragi, Japan) M-80B gas chromatograph–mass spectrometer equipped with an electron-ionization source and data processing system and a VG (Manchester, U.K.) 70-SE gas chromatograph–mass spectrometer equipped with an electron-ionization source and data processing system were employed. An Ultra 1 open-tubular capillary column (25 m × 0.3 mm I.D.) was used. The flow-rate of the carrier gas was maintained at 10 ml/min. An inlet pressure of 0.1 kg/cm^2 produced a linear gas velocity of 25 cm/s. The temperature of the column oven was kept at 280°C, the injector at 320°C and the ionization source at 200°C. The ionization energy and

accelerating voltage were 20 eV and 3 kV for the Hitachi M-80B and 35 eV and 8 kV gor the VG 70-SE, respectively. SIM was carried out with a mass spectrometric resolution of 3000, 4000 and 12 000 ($M/\Delta M$) by monitoring the mass number of the ion corresponding to a particular elemental composition.

Preparation of ^{18}O-labelled 11-dehydro-TXB_2

^{18}O-labelled 11-dehydro-TXB_2 was prepared according to a modification of the procedure described by Strife and Murphy[10]. Ethereal diazomethane (2 ml) was added to a solution of 11-dehydro-TXB_2 (0.2 mg) in methanol (0.2 ml), and the resulting solution was allowed to stand at room temperature for 30 min. The reaction mixture was evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in 0.2 *M* $Li^{18}OH$ (0.1 ml) and the resulting solution was sonicated for 2 h. The solution was acidified to pH 2 with 0.5 *M* hydrochloric acid and then extracted with ethyl acetate. The organic layer was washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate and evaporated. The above operation was repeated five times. The final product was dissolved in acetone (0.2 mg/ml) and stored at −20°C.

Derivatization procedure

To a solution of 11-dehydro-TXB_2 in methanol (0.1 ml) was added freshly prepared ethereal diazomethane (0.5 ml), and the resulting solution was allowed to stand at room temperature for 1 h. After evaporation of the solvent, the residue was silylated with BSTFA, DMES-, DMnPS- and DMiPS-imidazole (20 μl). The resulting mixture was kept at 60°C for 1 h, then the excess of the reagent except for BSTFA was removed by Sephadex LH-20 column chromatography[11,12].

Collection and storage of urine samples

Urine was collected over a period of 24 h. During the collection period, the samples were kept in a refrigerator. The volume was determined and the urine was stored at −40°C until assayed.

Extraction and purification of 11-dehydro-TXB_2 from human urine

After addition of the ^{18}O-labelled 11-dehydro-TXB_2 (5 ng) as an internal standard, human urine (1–5 ml) was acidified to pH 2 with 0.5 *M* hydrochloric acid, allowed to stand at room temperature for 1 h and then transferred to a Chem-Elut column (No. 1003, 1005 or 1010). 11-Dehydro-TXB_2 was eluted with ethyl acetate (24–50 ml) and the eluate was evaporated to dryness. The residue was dissolved in 15% aqueous ethanol (10 ml) and acidified to pH 2 with 0.5 *M* hydrochloric acid. The resulting solution was applied to a Sep-Pak C_{18} cartridge, and the cartridge was washed with 15% aqueous ethanol (10 ml) and *n*-hexane (10 ml). 11-Dehydro-TXB_2 was eluted with ethyl acetate (10 ml). This eluate was evaporated to dryness and the residue was dissolved in methanol (0.5 ml). To this solution freshly prepared ethereal diazomethane (2 ml) was added, and the mixture was allowed to stand at room temperature for 1 h. After evaporation of the solvent, the residue was dissolved in *n*-hexane–ethyl acetate (2:1) (3 ml) and then transferred to a silica gel column (5 × 0.5 cm I.D.). The column was washed with *n*-hexane–ethyl acetate (2:1) (8 ml) and then the methyl ester of 11-dehydro-TXB_2 was eluted with *n*-hexane–ethyl acetate (1:1) (20 ml).

The eluate was evaporated to dryness and the residue was silylated with DMiPS-imidazole as described above.

RESULTS AND DISCUSSION

Gas chromatography–mass spectrometry

In the GC–MS analysis of prostaglandins (PGs) and thromboxanes (TXs), the corresponding methyl ester (ME)–alkyloxime (RO)–dimethylalkylsilyl (DMAS) ether derivatives have been widely used. The TMS, DMES, DMnPS and DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester were prepared by treatment with ethereal diazomethane and then with the corresponding silylating reagents. The resulting derivatives were used for the investigation of their GC–MS properties. Each of the reaction products exhibited a well shaped, single gas chromatographic peak. When analysed on a methylsilicone cross-linked fused-silica capillary column, the methylene unit values of these 11-dehydro-TXB_2 derivatives increased in the order TMS, DMES, DMnPS, and DMiPS ether derivatives, as listed in Table I.

The mass spectra of the TMS, DMES, DMnPS and DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester are shown in Fig. 1. The mass fragmentation patterns of the DMES, DMnPS and DMiPS ether derivatives were closely related to that of the TMS ether derivative, except for a 14 or 28 a.m.u. shift for each hydroxy group. For instance, the fragment ion of $[M-71]^+$, produced by cleavage of the C-15–C-16 bond, implies the presence of two silanoxy bonds by the shift from m/z 455 in the TMS ether derivative to m/z 483 ($455 + 14 \times 2$) in the DMES ether derivative and to m/z 511 ($455 + 28 \times 2$) in the DMnPS and DMiPS ether derivatives. In general, an increase in the carbon number of the silylating agent has a tendency to yield the $[M-\text{alkyl}]^+$ ion with high abundance in the high-mass region[13]. Of these DMAS ether derivatives, the relative intensities of the $[M-\text{alkyl}]^+$ ions in the DMES and DMnPS ether derivatives of 11-dehydro-TXB_2 ME were not increased in comparison with that of the $[M-CH_3]^+$ ion in the corresponding TMS ether derivative, whereas the DMiPS ether derivative gave the $[M-C_3H_7]^+$ ion as a base peak and more than 10% of total ion current was concentrated in this base-peak ion. The appearance of the base peak in the high-mass region may be useful for the specific and sensitive detection of 11-dehydro-TXB_2 in biological specimens by GC–SIM.

The mass spectrum of the corresponding DMiPS ether derivative exhibited the series of ions which were determined to be characteristic of the expected structure. The molecular ion was not observed. The loss of the isopropyl radical from a DMiPS group in the molecular ion gave rise to the ion of $[M-43]^+$ at m/z 539 as a base peak,

TABLE I

METHYLENE UNIT VALUES OF TMS, DMES, DMnPS AND DMiPS ETHER DERIVATIVES OF 11-DEHYDRO-TXB_2 METHYL ESTER

Derivative	*Methylene unit value*
TMS	28.16
DMES	31.18
DMnPS	32.33
DMiPS	32.55

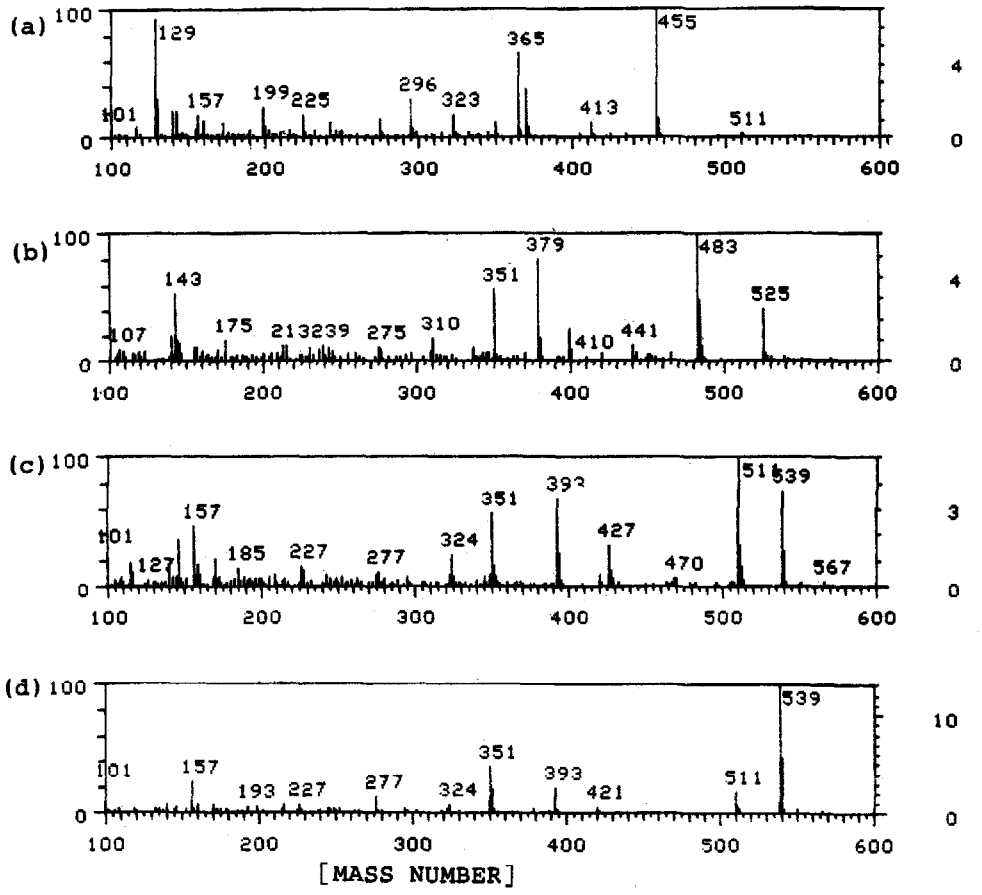
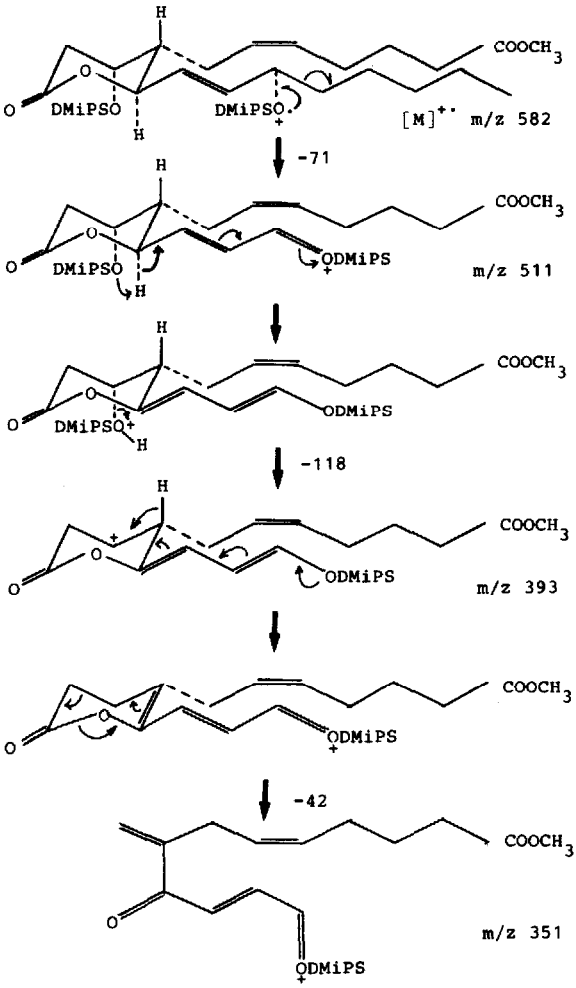


Fig. 1. Mass spectra of the (a) TMS, (b) DMES, (c) DMnPS and (d) DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester.

indicating the incorporation of two DMiPS groups into the 11-dehydro-TXB_2 methyl ester. This ion produced the ion at *m/z* 421 by the loss of dimethylisopropylsilanol (DMiPSOH: 118 a.m.u.). The radical loss of a C_{16}–C_{20} hydrocarbon fragment from the molecular ion, which is a typical mass fragmentation of prostanoids, produced the characteristic ion at *m/z* 511. This ion produced the ion at *m/z* 393 by the loss of a DMiPSOH molecule, initiated by a migration of the C-9 DMiPSO group to the hydrogen atom at C-12. The ion at *m/z* 393 further fragmented to that at *m/z* 351 by the migration of the hydrogen atom at C-8 to C-9, followed by the loss of a CH_2O group, as shown in Scheme 1. The resulting mass spectral data revealed that the ME–DMAS ether derivatives were in the lactone form.

In order to examine the applicability of the ME–DMiPS ether derivative of 11-dehydro-TXB_2, selected-ion monitoring was carried out, using the characteristic ion of $[M-43]^+$ at *m/z* 539 with a mass spectral resolution of 4000. The detection limit of this derivative was about 2 pg per injection with a signal-to-noise ratio of 5:1.



Scheme 1.

Internal standard of ^{18}O-labelled 11-dehydro-TXB$_2$

Fig. 2 shows the mass spectrum of the ^{18}O-labelled 11-dehydro-TXB$_2$ methyl ester–bis-DMiPS ether derivative, which is closely related to that of the corresponding non-labelled 11-dehydro-TXB$_2$ derivative, except for the obvious shift produced by the substitution of oxygen-18 atoms. The mass spectrometric analysis revealed that the product was a mixture of multi-^{18}O-labelled 11-dehydro-TXB$_2$ and the enrichments of the $^{18}O_3$-, $^{18}O_2$- and $^{18}O_1$-labelled variants were found to be 47.7, 43.6 and 8.5%, respectively. On the other hand, the content of the non-labelled 11-dehydro-TXB$_2$ derivative was calculated to be less than 0.3%.

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 weight

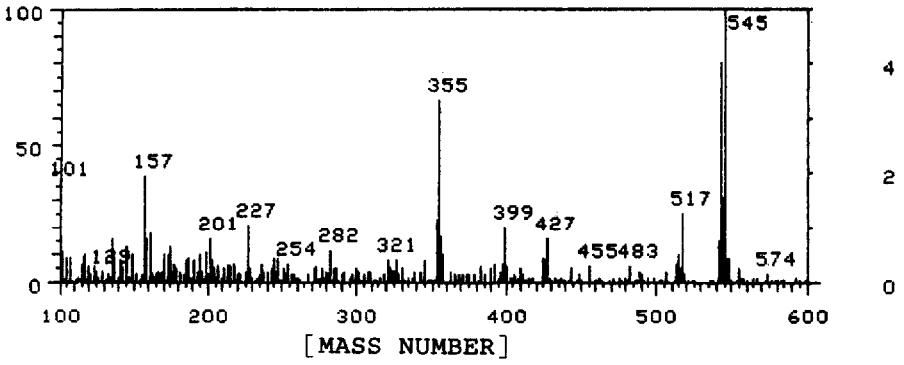


Fig. 2. Mass spectrum of ^{18}O-labelled 11-dehydro-TXB_2 methyl ester–bis-DMiPS ether derivative.

ratios. Good linearity was observed between the peak-area ratio and weight ratio of 11-dehydro-TXB_2 and its ^{18}O-labelled variant in the range 0–100 pg.

pH-dependent equilibrium between the lactone and open forms of 11-dehydro-TXB_2

It has been reported that there is a pH-dependent equilibrium between the lactone form of 11-dehydro-TXB_2 and its open form, and that the open form is obtained more easily at a higher pH, but the lactone is slowly formed at a lower pH[1]. Therefore, a time course study of this pH-dependent equilibrium between the open and lactone forms of 11-dehydro-TXB_2 was carried out to test the validity of the use of the ^{18}O-labelled variant as an internal standard, because the endogenous 11-dehydro-TXB_2 in human urine is presumably in the open form whereas the ^{18}O-labelled variant is in the lactone form.

The following urine samples were prepared. (1) A human urine sample was adjusted to pH 9 with 1 *M* sodium hydroxide solution and allowed to stand for 3 h at room temperature. After the above urine sample had been adjusted to pH 2 with 0.5 *M* hydrochloric acid, an aliquot of the ^{18}O-labelled variant was added to the sample as

TABLE II

TIME COURSE OF THE pH-DEPENDENT EQUILIBRIUM BETWEEN THE OPEN AND LACTONE FORMS OF 11-DEHYDRO-TXB_2

Experiment	*Lactone ring-opening conditions*			*Lactonization conditions*			*Found (ng/ml)*
	Addition of I.S.[a]	*pH*	*Standing time (h)*	*Addition of I.S.*[a]	*pH*	*Standing time (h)*	
I_1	–	9.0	3	+	2.0	1	0.683
I_2	–	9.0	3	+	2.0	3	0.686
I_3	–	9.0	3	+	2.0	Overnight	0.638
II_1	+	9.0	3	–	2.0	1	0.649
II_2	+	9.0	3	–	2.0	3	0.626
II_3	+	9.0	3	–	2.0	Overnight	0.673
III_1	–	–	–	+	2.0	1	0.662

[a] I.S. = internal standard (^{18}O-labelled 11-dehydro-TXB_2).

an internal standard. The resulting sample was allowed to stand for 1 h, 3 h and overnight. (2) An aliquot of the internal standard was added to the human urine prior to sample preparation. The urine sample was adjusted to pH 9 with 1 *M* sodium hydroxide solution and allowed to stand for 3 h at room temperature. The sample was adjusted to pH 2 with 0.5 *M* hydrochloric acid and allowed to stand for 1 h, 3 h and overnight at room temperature. (3) The urine sample to which the internal standard had been added was adjusted to pH 2 with 0.5 *M* hydrochloric acid and allowed to stand for 1 h at room temperature.

The determination of 11-dehydro-TXB_2 in the above urine samples was carried out. There was no statistically significant difference in the amounts of 11-dehydro-TXB_2 in these samples, as shown in Table II. This indicates that the formation of the lactone from the open form proceeds rapidly. This finding is different from the results of a time course study reported previously[1].

Sample preparation

The purification of the prostanoids was performed by silica gel column chromatography with ethyl acetate–methanol (99:1) as eluent, as described in previous papers[8,14]. This method was applied to the purification of 11-dehydro-TXB_2 from human urine, but it was difficult to find the peak of 11-dehydro-TXB_2 owing to interference of endogenous substances with the selected-ion recording. Therefore, an HPLC purification step was added to the above procedure before methylation, according to the method of Powell[15]. However, the separation of the peak in the selected-ion recording was not improved, as shown in Fig. 3a. The peak of 11-dehydro-TXB_2 could be observed by the use of temperature programming, holding the temperature at 200°C for 1 min and then increasing it at 5°C/min to 300°C, and high-resolution SIM ($M/\Delta M$ = 12 000), as shown in Fig. 3b.

The above facts required the development of a novel procedure for the purification of 11-dehydro-TXB_2 for GC–SIM. Schweer *et al.*[5] and Chiabrando *et al.*[6] have pointed out the importance and necessity of purification for the quantitation of trace amounts of prostanoids in biological specimens, even when an MS–MS technique was used. Therefore, the pattern of elution of 11-dehydro-TXB_2 methyl ester from a silica gel column was examined in detail by stepwise elution, using mixtures of *n*-hexane and ethyl acetate in various ratios[16]. This experiment showed that the 11-dehydro-TXB_2 methyl ester was more lipophilic than the other prostaglandins, such as $PGF_2\alpha$ and PGE_2, and was eluted with *n*-hexane–ethyl acetate (1:1) after washing the column with *n*-hexane–ethyl acetate (2:1). There was little interfering material in this fraction. The reproducibility of the elution of the 11-dehydro-TXB_2 methyl ester from a silica gel column was extremely good, and the mean and standard deviation of the recovery of 11-dehydro-TXB_2 from human urine through the present purification procedure were 72.6 ± 3.5% (n = 5).

Fig. 4 shows a typical selected-ion recording, obtained by analysing an aliquot of the urine extract. The peaks appearing in this selected-ion recording correspond to *ca.* 10 pg of 11-dehydro-TXB_2 and *ca.* 25 pg of its $^{18}O_2$-labelled variant, used as an internal standard. The selcted-ion recording was obtained with an extremely good signal-to-noise ratio when the ion monitored was $[M-43]^+$ at m/z 539.32 for the ME–DMiPS ether derivative of 11-dehydro-TXB_2 and at m/z 543.33 for its $^{18}O_2$-labelled variant at a mass spectrometric resolution of 4000. The interfering

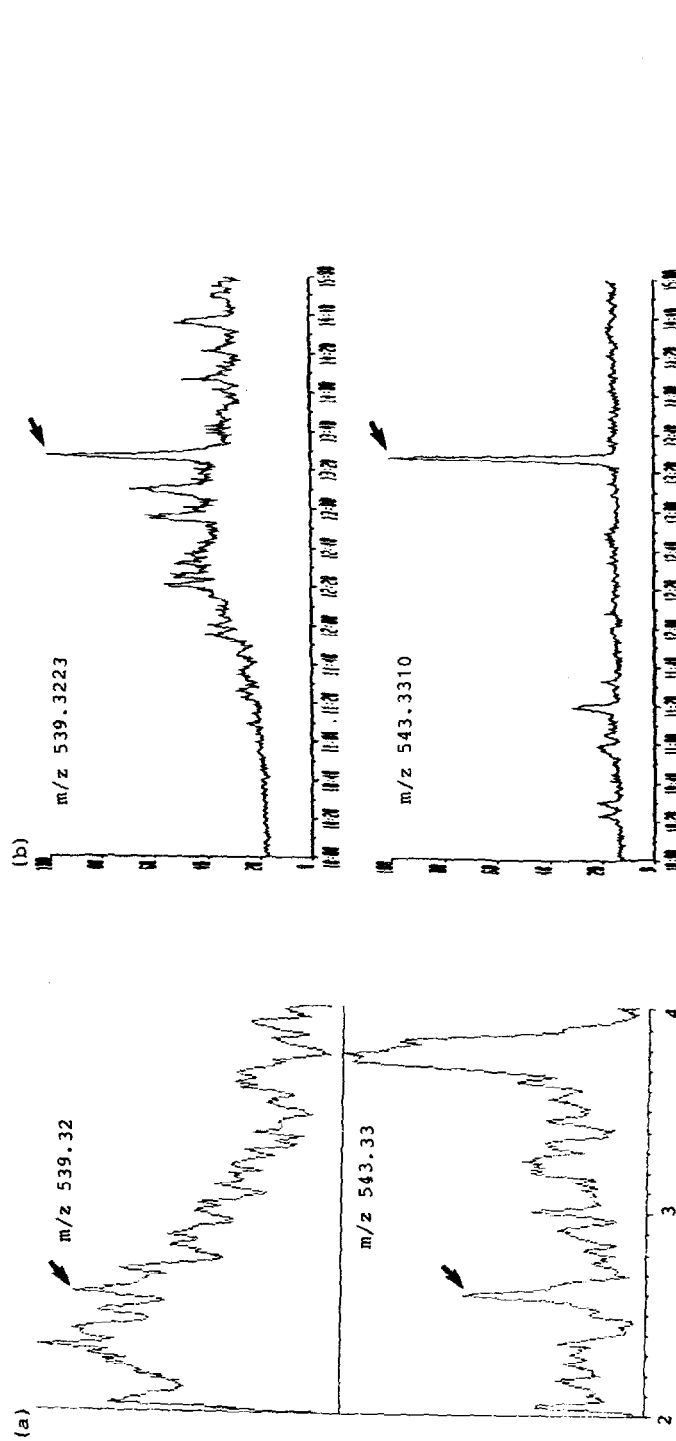


Fig. 3. Selected-ion recordings of human urinary 11-dehydro-TXB_2, purified by HPLC, monitoring the characteristic ion of $[M-43]^+$ in (a) a low-resolution mode ($M/\Delta M = 3000$) and (b) a high-resolution mode ($M/\Delta M = 12\,000$) with temperature-programmed GC.

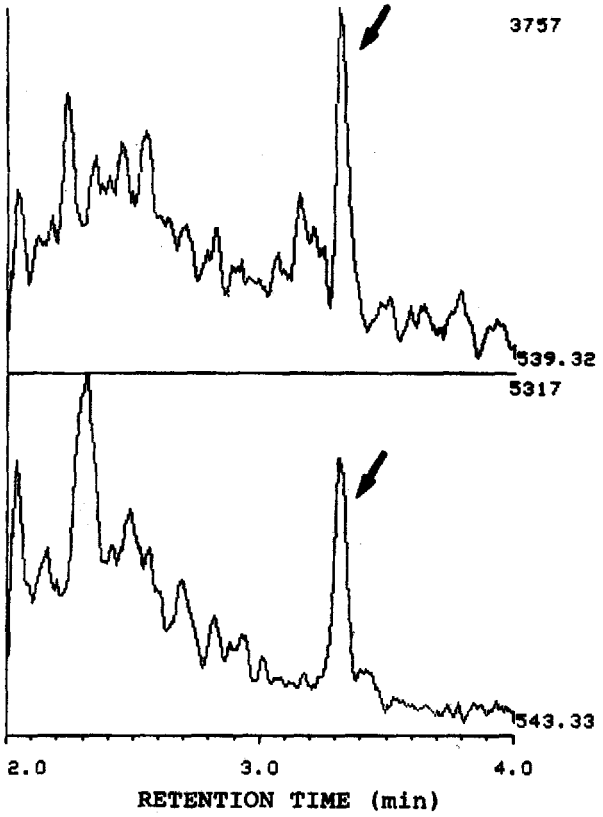


Fig. 4. Selected-ion recordings of the ME–DMiPS ether derivatives of 11-dehydro-TXB_2 (*m/z* 539.32) and its $^{18}O_2$-labelled variant (*m/z* 543.33) in the extract from human urine, monitoring the characteristic ion of $[M-43]^+$ at a mass spectrometric resolution of 4000.

substances in the extract from the urine were almost eliminated by the new purification procedure.

Reproducibility

In order to examine the accuracy and precision of the present method, four urine samples spiked with 11-dehydro-TXB_2 at concentrations of 0.25, 0.50, 0.75 and 1.00 ng/ml were prepared. These concentrations corresponded to approximately 50, 100, 150 and 200% of endogenous 11-dehydro-TXB_2, found in the urine of the healthy male volunteers. The results are given in Table III. The mean and standard deviation of the recovery of 11-dehydro-TXB_2 added were 101.5 $\pm$ 2.4% ($n = 8$). Statistical analysis was carried out according to a two-way layout in order to divide the analytical errors between the three sources of sample preparation, 11-dehydro-TXB_2 concentrati on and GC–SIM. The analysis indicates that there was no significant difference among sample preparation and 11-dehydro-TXB_2 concentration, and that almost all of the total error in this experiment could be attributed to the GC–SIM, because the errors between sample preparation and 11-dehydro-TXB_2 concentration were negligible. The coefficient of variation in GC–SIM was 4.5%.

TABLE III

RECOVERY OF 11-DEHYDRO-TXB_2 ADDED TO HUMAN URINE

Sample	*Added (ng/ml urine)*	*Found (ng/ml urine)*		*Mean recovery (%)*
1a	0	0.525	0.484	
1b	0	0.515	0.562	—
2a	0.25	0.786	0.844	
2b	0.25	0.840	0.765	104.1
3a	0.50	1.084	0.968	
3b	0.50	1.016	1.016	99.5
4a	0.75	1.290	1.218	
4b	0.75	1.303	1.268	99.5
5a	1.00	1.597	1.544	
5b	1.00	1.579	1.581	103.3

According to the orthogonal polynomial equation, the estimated urinary levels and their 95% confidence limit were calculated to be 0.527 $\pm$ 0.073, 0.544 $\pm$ 0.106 and 0.090 $\pm$ 0.033 ng/ml, and were in good agreement with the concentrations of endogenous 11-dehydro-TXB_2 in non-spiked urine (0.522, 0.557 and 0.084 ng/ml, respectively), as shown in Table IV. These facts suggest that the present method makes it possible to determine picogram levels of 11-dehydro-TXB_2 in urine with high reliability. The urinary levels of 11-dehydro-TXB_2 in normal subjects and patients are under investigation and these results will be discussed elsewhere.

TABLE IV

DETERMINATION OF 11-DEHYDRO-TXB_2 IN HUMAN URINE

Subject	*Urine volume (ml per 24 h)*	*11-Dehydro-TXB_2 concentration (ng/ml)*		*Urinary excretion (μg per 24 h)*
		Present method (mean)	*Orthogonal polynomial equation method (mean $\pm$ 95% confidence limit)*	
A	1120	0.522	0.527 $\pm$ 0.073	0.590
B	745	0.557	0.544 $\pm$ 0.106	0.405
C	2810	0.084	0.090 $\pm$ 0.033	0.253

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