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High-performance liquid chromatography–thermospray mass spectrometry of prostaglandin and thromboxane acetyl derivatives

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ABSTRACT

A quantitative analysis of prostaglandin-related substances has been developed. Hydroxyl groups of prostaglandin and thromboxane were acetylated by acetic anhydride, the mixture was partially purified on a Sep-Pak C_{18} cartridge and analysed by high-performance liquid chromatography combined with thermospray mass spectrometry. Using this method, twenty kinds of prostaglandin derivative could be detected simultaneously within 11 min on a selected-ion monitoring detection chromatogram without a gradient system. Generally, the base ion, $[M + H - n(60)]^+$, is produced through elimination of acetic acid (n = number of the hydroxyl group of prostaglandin or thromboxane). The detection limit for these derivatives was *ca.* 0.2 pmol at the levels of prostaglandin-related substances prior to derivatization. They could be analysed in the range 0.5–10 pmol. The assay was successfully applied to prostaglandin-related substances in human seminal fluid and rat brain.

INTRODUCTION

Prostaglandins (PGs) and thromboxanes (TXs) have been assayed by high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) and radioimmunoassay (RIA). With HPLC, quantitation is generally conducted following pre-column fluorescence derivatization using 9-anthryldiazomethane [1,2]. However, with this technique, disturbance of fluorescence caused by the by-product produced from 9-anthryldiazomethane is usually a problem that is difficult to eliminate. At present, GC–MS is the most reliable method, although its operation is complicated because carboxyl, hydroxyl and carbonyl groups all have to be derivatized [3–6]. RIA methods still suffer from problems of cross-reaction with other sample components and, in most cases, a commercial antibody is not available, particularly in the cases of new or more unstable metabolites [7–9]. High-performance liquid chromatography–thermospray mass spectrometry (HPLC–TSP-MS) has recently come to be used for the analysis of trace compounds. Analysis of arachidonic acid metabolites using this technique has been reported [10–12]. However, this procedure is not

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satisfactory owing to the considerable time required for HPLC and quantification using a deuterium-labelled internal standard (I.S.).

This paper describes a newly developed method in which the time for HPLC is only 11 min, owing to use of a single mobile phase and acetylation of PG-related substances. During this short period, twenty kinds of PG-related substance can be detected simultaneously by selected-ion monitoring (SIM). A TSP interface of high temperature and a mobile phase composed of acidic buffer are used, resulting in greatly improved sensitivity. The single mobile phase facilitates quantitative analysis, in which a deuterium-labelled I.S. is used.

EXPERIMENTAL

Standards and reagents

PG or TX standards (PGA_1, PGA_2, PGB_1, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2) were obtained from Sigma (St. Louis, MO, USA). Other PG or TX standards (PGA_3, PGE_3, $PGF_{2\alpha}$, $PGF_{3\alpha}$, PGJ_2, 6-keto-PGE_1, 15-keto-PGE_2, 15-keto-$PGF_{1\alpha}$, 15-keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15-keto-PGD_2, 11-dehydro-TXB_2 and [3,3,4,4-$^2H_2$]$PGF_{2\alpha}$) were obtained from Cayman (Ann Arbor, MI, USA). The water for the HPLC eluent was of Milli-Q grade (Waters Assoc., Milford, MA, USA), that for preparing 15% ethanol, 5% acetonitrile, 2 *M* HCl and 10% acetic acid, and for washing and equilibrating the Sep-Pak C_{18} cartridge (Waters Assoc.) was prepurified by a Sep-Pak C_{18} cartridge. The other solvents and reagents were of analytical-reagent or chromatographic grade.

Extraction from biological samples

To each 100 mg of biological sample were added 139 pmol of [3,3,4,4-$^2H_2$]$PGF_{2\alpha}$, as the I.S., and 1 ml of ethanol cooled to -20°C. After homogenization for 1 min at 0°C with a Physcotron homogenizer (Nichion, Tokyo, Japan), the mixture was centrifuged at 1500 *g* for 10 min at 4°C. The supernatant was evaporated under reduced pressure, and 1.0 ml of 15% ethanol and 5 µl of 2 *M* HCl were added to the residue. The mixture was adjusted to *ca.* pH 3 and applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed successively with 5 ml of 5% acetonitrile, 10 ml of water and 5 ml of *n*-hexane. PGs and TXs in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure.

Derivatization

PGs and TXs were derivatized using acetic anhydride in pyridine to obtain the acetic ester. PGs and TXs were dissolved in 0.2 ml of pyridine, and 0.08 ml of acetic anhydride was added. The reaction mixture was left overnight under argon at 5°C. The mixture was acidified to pH 4–5 by addition of 3.5 ml of 10% acetic acid and 0.5 ml of acetonitrile. The mixture was then immediately applied to a

Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed successively with 3 ml of 5% acetonitrile and 10 ml of water, and the acetyl derivatives of PGs and TXs in the cartridge were eluted with 5 ml of acetonitrile. The eluent containing the PG and TX derivatives was stored at −20°C until analysis by HPLC–TSP-MS.

HPLC–TSP-MS

A Shimadzu (Kyoto, Japan) LC/GC/MS-QP1000S provided with Vestec (Houston, TX, USA) Model 750B HPLC–TSP-MS interface, a Shimadzu LC-9A HPLC pump and a Rheodyne injector fitted with a 20-μl loop were used. HPLC separation was carried out on a Nucleosil 100-5C_{18} (5 μm particle size, Macherey-Nagel, Duren, Germany) 150 mm × 4.6 mm I.D. column, with a mobile phase of 0.1 *M* ammonium acetate–0.1 *M* acetic acid–acetonitrile (4:6:15, v/v) at a flow-rate of 1.0 ml/min.

The TSP interface temperature was optimized for maximum detection sensitivity for the acetyl derivatives. In the positive-ion mode, the optimal vaporizer control, vaporizer tip, vapour, block and tip heater temperatures were maintained at 163, 302, 325, 349 and 352°C, respectively, on an electron beam-off or electrical discharge-off.

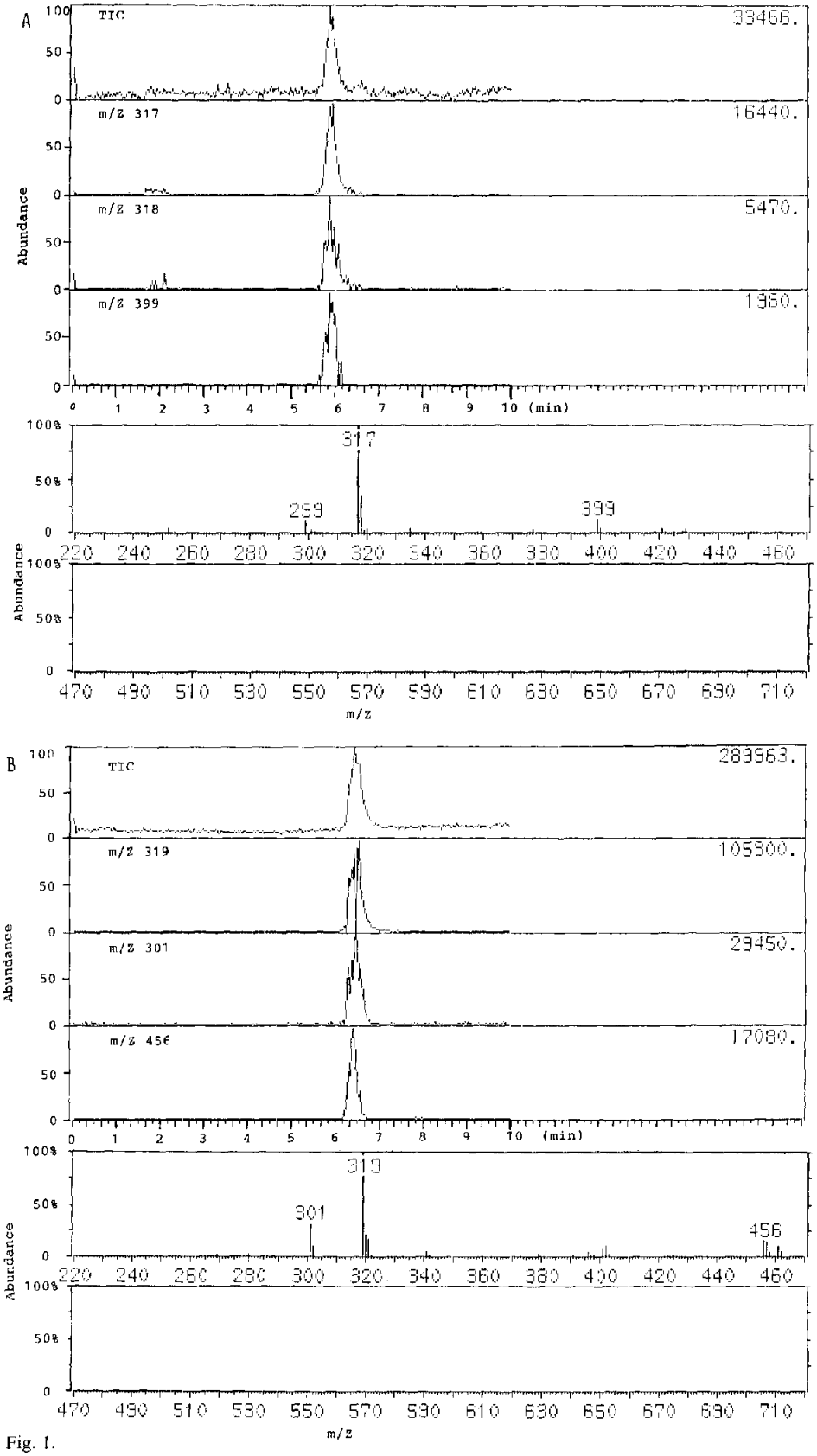
RESULTS AND DISCUSSION

The MS patterns of the derivatives showed a characteristic base ion (Fig. 1). As shown in Table I, the observed base ion is $[M+H-n(60)]^+$, based on the elimination of acetic acid (60 mass units) from the molecular ion. However, this was not noted for 15-keto-$PGF_{2\alpha}$, 19*R*-hydroxy-PGE_1 or 19*R*-hydroxy-PGE_2. With 19*R*-hydroxy-PGE_1 or 19*R*-hydroxy-PGE_2, the base ion became $[M+H-(n-1)\cdot 60]^+$, possibly owing to the stability of acetyl groups introduced at the 19 position.

The relationship between the TSP interface temperature and the $[M+H-60]^+$ ion intensity of the PGB_2 acetyl derivative is shown in Fig. 2. The best temperature conditions for detecting the base ion were 165°C (control), 303°C (tip) and 325°C (vapour). Chemical ionization due to an electric beam (filament on 150 μA) or electrical discharge caused no increase in the ion intensity of the PG acetyl derivatives (Fig. 2, data for electrical discharge not shown).

Most acetyl derivatives of PGs and TXs could be easily detected on the SIM detection chromatogram at *ca.* 5 pmol of PG-related substances prior to derivatization (Fig. 3). The derivatives of TXB_2, PGJ_2 and 11-dehydro-TXB_2 showed lower sensitivity than the others, possibly owing to the low yields of their acetylation, but this was not confirmed.

In our method, most PGE-type derivatives were converted into the corresponding PGA-type derivatives during acetylation and gave the same base ions (Table I). The dehydration of PGE type may be prevented by methylation of the


A
TIC
33466.
m/z 317
16440.
m/z 318
5470.
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1960.
Abundance
0 1 2 3 4 5 6 7 8 9 10 (min)
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299
388
220 240 260 280 300 320 340 360 380 400 420 440 460
470 490 510 530 550 570 590 610 630 650 670 690 710
m/z
B
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283963.
m/z 319
105800.
m/z 301
29450.
m/z 456
17080.
Abundance
0 1 2 3 4 5 6 7 8 9 10 (min)
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301
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220 240 260 280 300 320 340 360 380 400 420 440 460
470 490 510 530 550 570 590 610 630 650 670 690 710
m/z

Fig. 1.

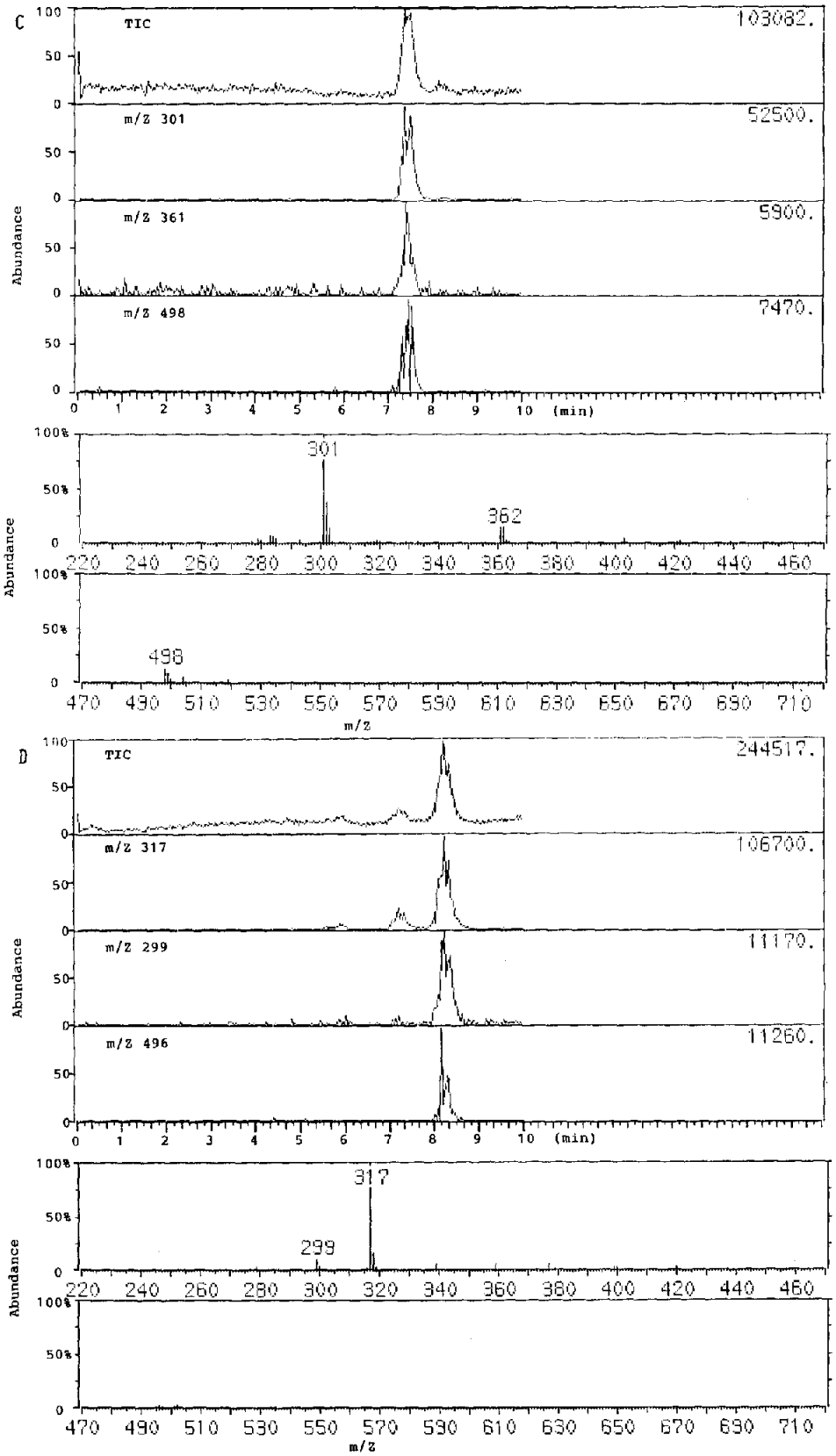

Fig. 1.

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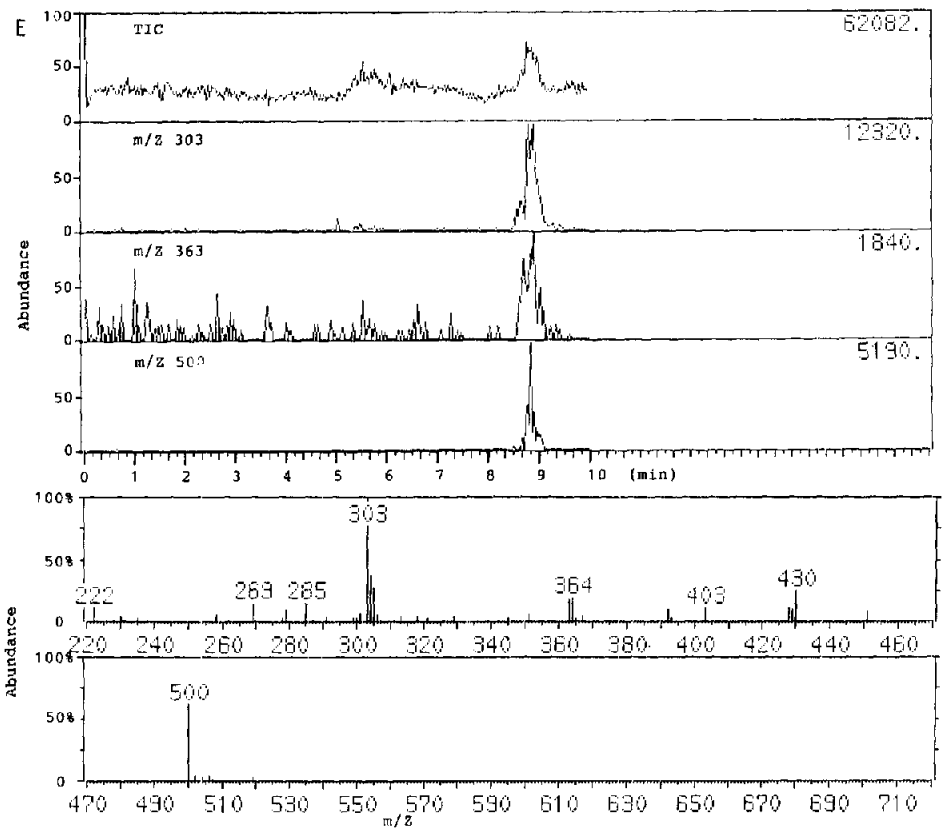

Fig. 1. Total ion chromatogram (TIC) profile, mass chromatogram profile on each *m/z* number and mass spectra obtained from PG acetyl derivatives. HPLC and TSP conditions as described in Experimental. Scan speed, 1.0 scan/s from *m/z* 220 to 520. Filament off. Spectral patterns corresponding to the main peak on the TIC are shown below. (A) PGB_2 acetyl derivative; (B) PGE_1 acetyl derivative; (C) $PGF_{2\alpha}$ acetyl derivative; (D) PGD_2 acetyl derivative; (E) $PGF_{1\alpha}$ acetyl derivative. The number in the upper right-hand corner of each chromatogram is ion count.

carboxylate group [12], but this has yet to be confirmed. 15-Keto-$PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, which overlap in the SIM detection chromatogram using ion 317, could be detected by the $[M+H-60]^+$ ion (*m/z* 377, 100%) and $[M+NH_4]^+$ ion (*m/z* 514, 33%), respectively.

Fig. 4 shows the SIM detection chromatogram of PG-related substances prior to derivatization of *ca.* 0.2 pmol. This amount is considered the limit of detection. The relationship between the peak area on SIM detection and the amount of PG-related substances prior to derivatization is given in Fig. 5. Determination was carried out from 0.5 to 10 pmol of PG-related substances prior to derivatization.

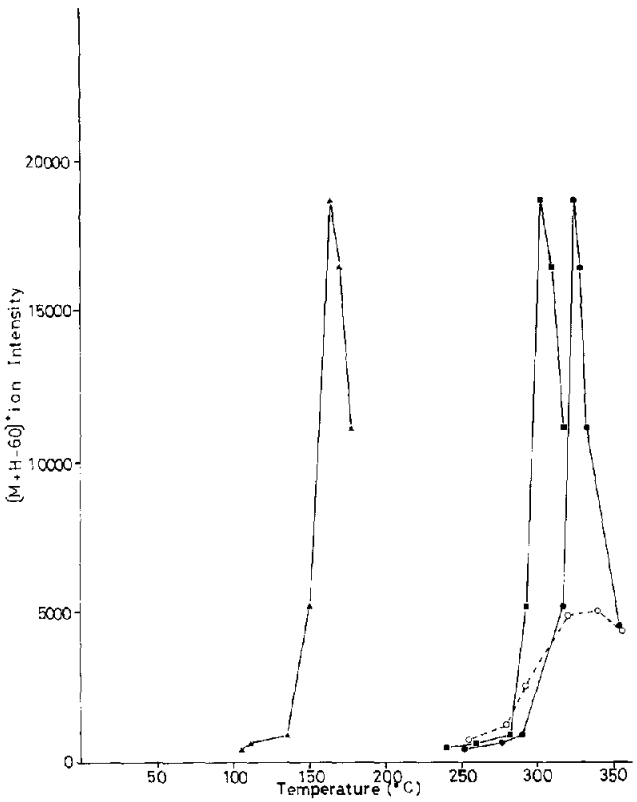

Fig. 2. TSP interface temperature and $[M + H - 60]^+$ ion intensity of the PGB_2 acetyl derivative. Conditions as in Fig. 1, except for TSP interface temperature. (▲) Control temperature; (■) tip temperature; (●) vapour temperature; (○) vapour temperature at filament-on (150 μA).

SIM detection chromatograms for the acetyl derivatives of human seminal fluid extract containing some PGs are shown in Fig. 6. On the chromatograms, peaks due to the derivatives of $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, PGA_2, PGD_2, 15-keto-$PGF_{1\alpha}$, PGA_1, PGE_1, 19*R*-hydroxy-PGE_1 and 19*R*-hydroxy-PGE_2 can be seen. Similarly, PGE_2, PGD_2, 6-keto-PGE_1 and 11-dehydro-TXB_2 from rat brain were noted and are shown in Fig. 6. However, no other peaks could be identified.

HPLC–TSP-MS techniques have been used previously for the determination of PG-related substances, but the sensitivity was not adequate because different fragment ions appeared following elimination of hydroxy groups [10]. The proton affinity (PA) of PG-related substances is less than that of the conjugate base of the reagent ion. PG-related substances are thus not efficiently ionized through chemical ionization or in the presence of ammonium acetate. Methods that change the PA or the polarity of compounds by diethylaminoethylation or meth-

TABLE I

RELATIVE ABUNDANCES OF IONS IN THE TSP SPECTRA OF PROSTAGLANDIN AND THROMBOXANE ACETYL DERIVATIVES

PG or TX	Acetyl group	M	$[M+H]^+$	$[M+NH_4]^+$	$[M+H-60]^+$	$[M+H-120]^+$	$[M+H-180]^+$	$[M+NH_4-60]^+$
PGA_1	1	378	–	13	100	–	–	–
PGA_2	1	376	–	22	100	–	–	–
13,14-Dihydro-15-keto-PGA_2	0	334	100	–	–	-	–	–
PGA_3	1	374	–	-	100	–	-	-
PGB_1	1	378	-	–	100	–	–	–
PGB_2	1	376	4	–	100	–	–	–
PGD_2	2	436	–	–	5	100	–	–
13,14-Dihydro-15-keto-PGD_2	1	394	13	–	100	–	–	–
PGE_1	2	438	–	16	2	100	–	5
6-Keto-PGE_1	2	452	–	–	–	100	–	–
19*R*-Hydroxy-PGE_1	3	496	–	-		100	24	-
PGE_2	2	436		10	–	100	–	–
15-Keto-PGE_2	1	392	44	–	100	–	–	–
19*R*-Hydroxy-PGE_2	3	494	–	5	10	100	9	–
13,14-Dihydro-15-keto-PGE_2	1	394	23	21	100	–	–	–
PGE_3	2	434	–	18	–	100	–	–
$PGF_{1\alpha}$	3	482	42	–	–	14	100	–
6-Keto-$PGF_{1\alpha}$	3	496		33		–	100	-
15-Keto-$PGF_{1\alpha}$	2	438	–	–	88	100	–	–
$PGF_{2\alpha}$	3	480	–	14	–	11	100	–
[3,3,4,4-$^2H_2$]$PGF_{2\alpha}$	3	484	–	12	–	10	100	–
15-Keto-$PGF_{2\alpha}$	2	436	–	–	100	26	–	–
$PGF_{3\alpha}$	3	478	–	10	–	16	100	–
PGJ_2	1	376	–	–	100	–	–	–
TXB_2	3	496	-	20		–	100	-
11-Dehydro-TXB_2	2	452		66	–	100	–	–

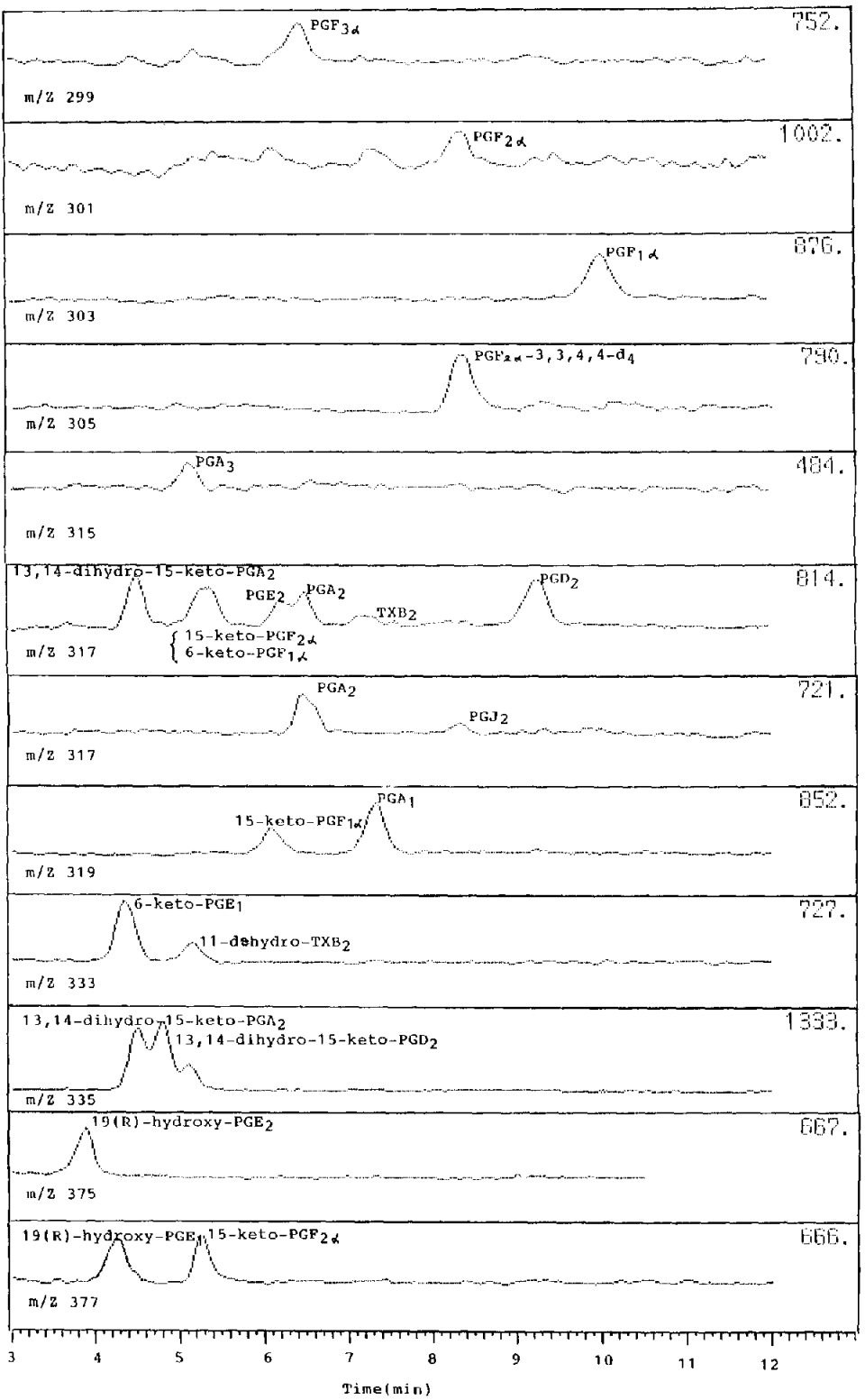

Fig. 3. SIM detection chromatogram of the acetyl derivatives of PGs or TXs at *ca.* 5 pmol of PG-related substances prior to derivatization. HPLC and TSP conditions as described in Experimental.

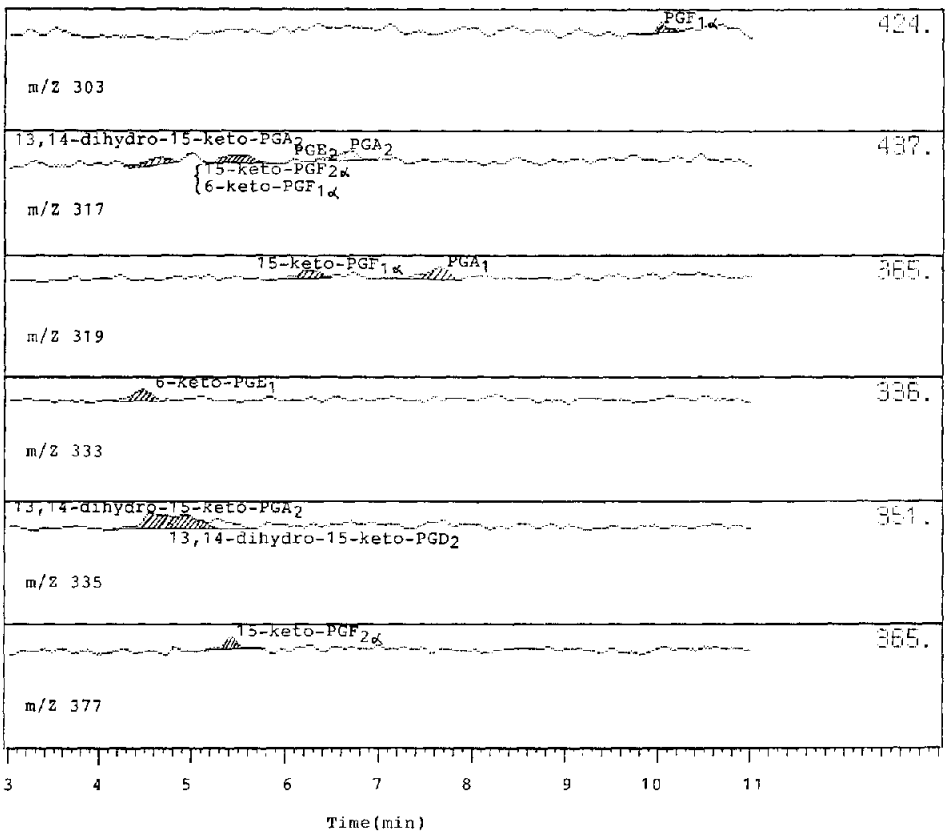

Fig. 4. SIM detection chromatogram for the acetyl derivatives of PGs and TXs at *ca.* 0.2 pmol of PG-related substances prior to derivatization. HPLC and TSP conditions as described in Experimental.

ylation, thereby improving the sensitivity, have been reported [11,12]. In these methods, since PG-related substances or derivatives have various hydroxy groups and are characterized by a wide range of polarities, gradient elution is used. To minimize overlap in the SIM detection chromatogram, measurement requires as long as 20–30 min.

However, in our method, the hydroxy groups of PG-related substances are acetylated and the derivatives are characterized by a limited range of polarities. Consequently, the retention time required for HPLC of the derivatives of PG-related substances is only 11 min when a single mobile phase is used and detection is by TSP-MS. Also, *ca.* twenty kinds of PG-related substance can be detected simultaneously by SIM of these base ions.

In quantitation using MS, the deuterium-labelled compound of a sample is best as the I.S., but not all deuterium-labelled analogues of PG-related substances

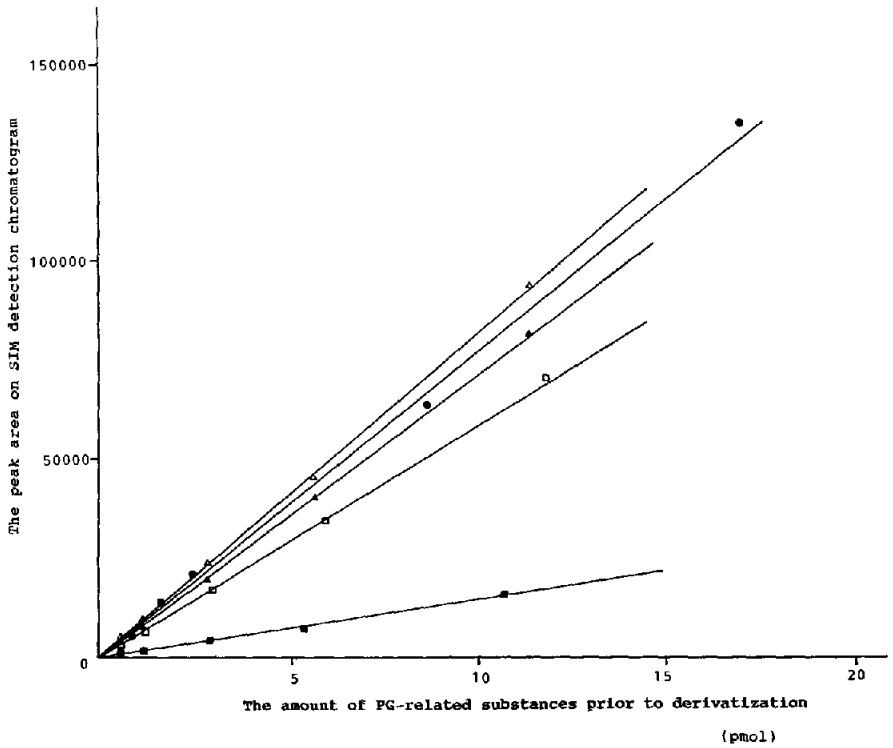

Fig. 5. Relationship between peak area on the SIM detection chromatogram and amount of PG-related substances prior to derivatization. HPLC and TSP conditions as described in Experimental. (△) PGE_2; (●) $PGF_{2\alpha}$; (▲) PGD_2; (□) PGA_1; (■) TXB_2.

are available. To determine PG-related substances corresponding to unavailable deuterium-labelled compounds, some other deuterium-labelled compounds must be used. In HPLC–TSP-MS methods using a gradient elution, the composition of the mobile phase on the TSP interface changes owing to differences in the retention times of samples and the I.S., and such changes affect the sensitivity. Consequently, reproducibility is a problem in quantitative analysis.

However, in our method, the composition of the mobile phase on the TSP interface is fixed because a single mobile phase is used, thus lessening the variation in sensitivity and improving the reproducibility.

At present, our method is being used for monitoring the biosynthesis of PG-related substances from various unsaturated fatty acids in homogenates of rat organs, and significant results have been obtained (unpublished data). This method is thus useful for studying the biosynthesis or metabolism of PG-related substances.

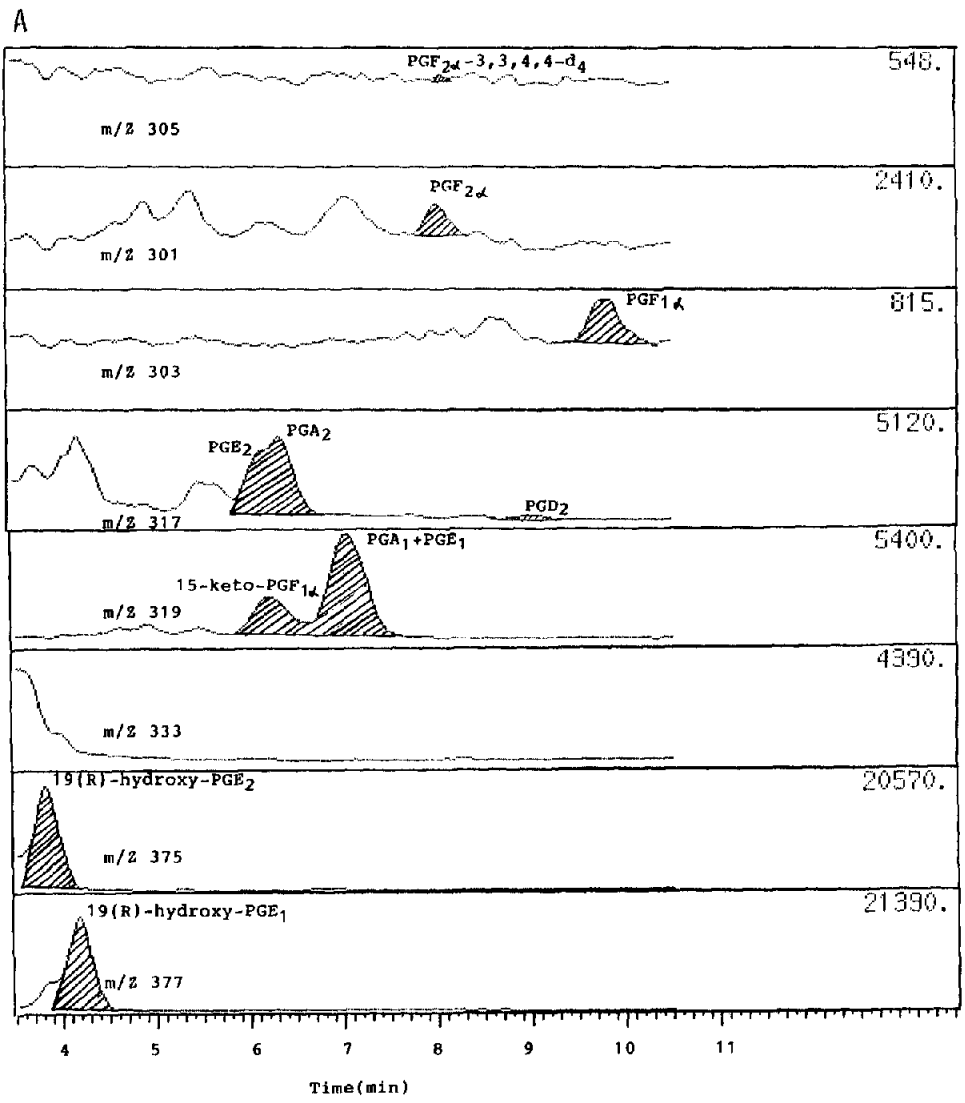
A
PGF2α-3,3,4,4-d4
548.
m/z 305
PGF2α
2410.
m/z 301
PGF1α
815.
m/z 303
PGE2
PGA2
PGD2
5120.
m/z 317
PGA1+PGE1
15-keto-PGF1α
5400.
m/z 319
4390.
m/z 333
19(R)-hydroxy-PGE2
20570.
m/z 375
19(R)-hydroxy-PGE1
21390.
m/z 377
4
5
6
7
8
9
10
11
Time(min)

Fig. 6.

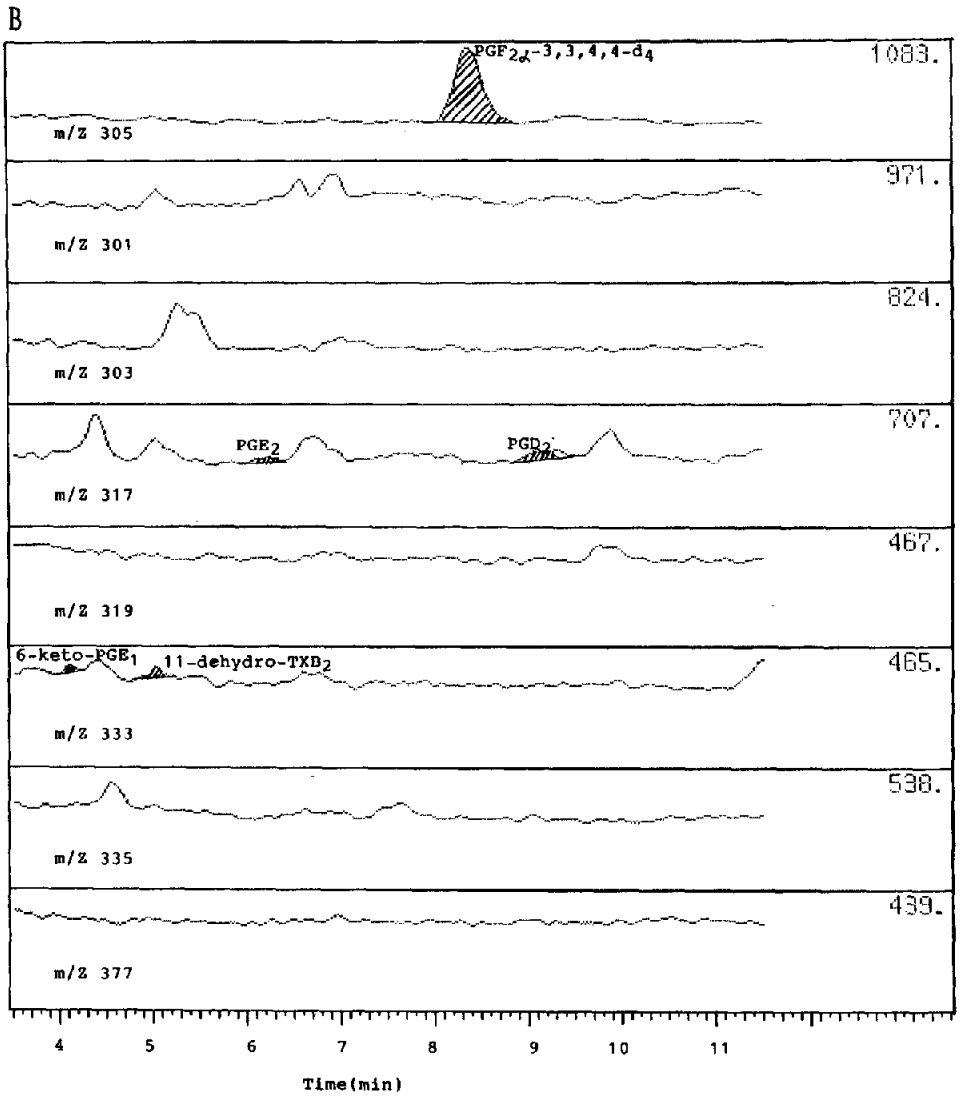

Fig. 6. SIM detection chromatograms of the acetyl derivative of extracts from biological samples. (A) Human seminal fluid; (B) rat brain. Derivatization, HPLC and TSP conditions as described in Experimental.

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