Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 792 (2003) 131–140
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of endocannabinoids (arachidonylethanolamide and 2-arachidonylglycerol) and isoprostane (8-epiprostaglandin F2α) by gas chromatography–mass spectrometry-selected ion monitoring for medical samples

Toru Obata[a,*], Yoshio Sakurai[b], Youichi Kase[b], Yasumasa Tanifuji[b], Toru Horiguchi[b]

[a]*Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, 3-25-8 Nishishimbashi, Minato-ku, Tokyo 105-8461, Japan*
[b]*Department of Anesthesiology, Jikei University School of Medicine, 3-25-8 Nishishimbashi, Minato-ku, Tokyo 105-8461, Japan*

Received 8 November 2002; received in revised form 17 March 2003; accepted 25 March 2003

Abstract

This article describes the overall procedure for the simultaneous determination of endocannabinoids (arachidonylethanolamide and 2-arachidonyglycerol) and isoprostane by gas chromatography–mass spectrometry in the selected-ion monitoring SIM mode (GC–MS-SIM) for medical samples. It also describes the general points of this method which a scientist who wants to assay a new, unidentified prostanoids and related compounds in medical samples would need to be clarified. The similar structures of prostaglandins, thromboxane, their metabolites, isoprostane, and arachidonyl compounds, allow them to be assayed after the simultaneous preparation of a single sample. The dimethyl isopropylsilyl ether forms of derivatized compounds are suitable for multiple GC–MS-SIM assay because of their molecular stability, and because they produce positive, strong, and large fragments on MS.

Keywords: Septic shock; Endocannabinoids; Anandamide; 2-Arachidonyglycerol; Isoprostane

1. Introduction

Prostaglandins (PGs) and thromboxane (TX) have similar molecular structures and molecular masses but exhibit different biological activities and medical reactions in various tissues. Our laboratory has developed a simultaneous assay for PGs and TX by means of gas chromatography–mass spectrometry in the selected-ion monitoring SIM mode (GC–MS-SIM) and the stable-isotope dilution method. This simultaneous assay can also be used for other types of arachidonate peroxides and related analogues (TXB2, 6-keto-PGF1α, PGF2α, 8-epi-PGF2α, 9α,11β-PGF2, PGE1, PGE2, 8-epi-PGE2, PGD2, PGD3, DK-PGD2, 2-AG, anandamide, Δ-PGJ2) to analyze new functions and physiological roles in biomedical research ([1–7], unpublished data).

The physiological roles of newly discovered arachidonate peroxide compounds, such as isoprostane, are also of considerable interest [8,9]. Con-

*Corresponding author. Tel.: +81-3-3433-1111; fax: +81-3-3435-1922.
E-mail address: obatat@jikei.ac.jp (T. Obata).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00311-8

centrations of isoprostane, also known as 8-epi-PGF2α, increases in specimens during long-term storage [10], but it is now accepted that active oxygen or free radicals are oxidized to arachidonate and that the D, E, and F forms of isoprostane are generated under physiological conditions [11,12]. For these reasons, isoprostane generation is one of the most direct markers of oxidative stress [13–15]. In addition, these isoprostanes might play other physiological roles, such as lipid mediators by affecting TX receptors [16,17].

Environmental factors, such as low-dose radiation from color cathode ray tubes, low-energy electromagnetic waves from cellular phones, air pollution, smoking, and hazardous chemical compounds, produce oxidative stress in the human body [1,4]. Isoprostane might play a role in hypersensitivity syndromes, inflammatory diseases, and arteriosclerosis that have recently become more prevalent [18].

On the other hand, endogenous cannabinoid receptor ligands, such as opioid receptors that bind to morphine, have recently been discovered. These endogenous ligands—arachidonyl-ethanolamide (anandamide) and 2-arachidonylglycerol (2-AG)—are derived from arachidonate [19,20]. These mediators have multiple physiological functions, such as sustaining pregnancy, mediating immune reactions, stimulating oncogene expression, stimulating apoptosis, preventing cell damage, and producing hypotension in septic shock [21–25]. Anandamide is thought to derive from macrophages, and 2-AG to derive from platelets; these similar mediators may play different roles in maintaining homeostasis [26,27]. The reasons for these contradictory reactions have not been clarified.

Especially, evidence is accumulating that these new arachidonate peroxide and arachidonate analogues are physiologically essential molecules which elicit septic shock.

To analyse and/or evaluate of these PGs and endocannabinoids in septic shock patients, simultaneous determination should be examined. They could be derivatized to the methyl esters of the dimethyl isopropylsilyl (DMiPSi) ether form with the same preparations for GC assay [7,28].

A method for the simultaneous determination of isoprostane and endocannabinoids in a plasma of septic shock with the stable isotope dilution method and GC–MS-SIM has applied to evaluate clinical status. In the present report, this simultaneous analysis method for the assay of additional PG-related compounds and endocannabinoids is verified for application and its possible practicible checkpoints are discussed.

2. Materials and methods

The strategy and principle of sample preparation and derivatization procedures for simultaneous analysis of PGs with GC–MS-SIM are as previously described [1,4,7].

2.1. Materials

All organic solvents (Nacalai Tesque, Kyoto, Japan) were of high-performance liquid chromatography or analytical-reagent grade. DMiPSi imidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). An ethereal solution of diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Tokyo Kasei Kogyo) under alkaline conditions.

Deuterated and nondeuterated PGs and endogenous cannabinoids were purchased from Cayman (Ann Arbor, MI, USA). Cartridges of octadecyl silica (Sep-Pak C_{18}, 500 mg per cartridge) were purchased from Waters (Milford, MA, USA). Silicic acid (silica gel 60 extra pure, 70–230 mesh, E. Merck, Darmstadt, Germany) was purchased from Nacalai Tesque. Silica gel 60 powder was immersed in organic solvent for at least 24 h. Sephadex LH20 (Amersham Pharmacia Biotech, Uppsala, Sweden) was purchased from Nacalai Tesque. Resin powder was immersed in organic solvent (chloroform–hexane–methanol, 10:10:1) for at least 24 h. Other reagents were of analytical-reagent grade and commercially available.

2.2. Sample collection

Blood samples were obtained from human subjects and animals. Venous blood from patients was gently drawn from the antecubital vein into plastic syringes containing 3.8% (w/v) trisodium citrate and 3 m*M* indomethacin. Blood from animals was collected

from the heart directly into identical plastic syringes. Blood samples were immediately centrifuged at 2000 *g* and 4 °C for 15 min, and the resulting plasma was stored at −80 °C until assay.

2.3. Extraction

Aliquots of 0.2 to 5 ml of plasma were diluted with distilled water (final volume, 10 ml). Deuterated PGs (d_4-8-epi-PGF2α, 1 ng; d_8-anandamide, 10 ng; d_8-2-AG, 5 ng) were added as internal standards. Solutions were acidified with hydrochloric acid (2 *M*) to pH 3.0, then ethanol was added to produce a final concentration of 15% (v/v). Solutions were applied to an octadecyl silica minicolumn (Sep-Pak C_{18}; preactivated with 10 ml of methanol and equilibrated with 15%, v/v, ethanol). The minicolumn was washed with 10 ml of 15% (v/v) ethanol (pH 3.0) and 10 ml of petroleum ether. PGs and TX were eluted with 10 ml of ethyl acetate. The eluate was evaporated to dryness in vacuo at less than 40 °C.

2.4. Purification and derivatization

2.4.1. Methyl ester formation

Crude extracts were dissolved in 0.05 ml of methanol and added to 0.4 ml of diazomethane ethereal solution. The mixture was allowed to stand in the dark at room temperature for 30 min, then evaporated to dryness in vacuo at less than 40 °C.

2.4.2. First purification by silicic acid column chromatography

The methyl esters of PGs and TX were dissolved in hexane–ethyl acetate (2:1); the resulting solutions were applied to a silicic acid column (5×0.5 cm I.D.) prewashed with the same solvent. The column was washed with 10 ml of the same solvent, and PGs and TX were eluted with 30 ml of ethyl acetate containing 1% (v/v) methanol. The effluent from the silicic acid column was evaporated to dryness in vacuo at less than 40 °C.

2.4.3. Methyloxim (MOX) formation and dimethylisopropyl (DMiP) silylation

The methyl esters of PGs and TX were dissolved in 0.05 ml of pyridine containing 4% (w/v) *o*-hydroxymethylamine. The resulting mixtures were allowed to stand for 1 h at 50 °C. After MOX formation, the reaction mixtures were added to 0.05 ml of DMiPSi imidazole and left to stand for 60 min at room temperature. Excess reagent was removed with a Sephadex LH-20 column (5×0.5 cm I.D.) and an organic solvent comprising hexane–chloroform–methanol (10:10:1, v/v) as eluate to obtain the derivatized PGs and TX. The effluent from the Sephadex LH-20 column was evaporated to dryness in vacuo at less than 30 °C.

2.5. GC–MS conditions

A GC–MS system (JMS-DX 303, Jeol, Tokyo, Japan) equipped with a data processing system (JMA-DA 5000, Jeol) was used. The column was a 30 m×0.317 mm I.D. fused-silica capillary (DB-1, film thickness, 0.1 μm; J&W Scientific, Folsom, CA, USA). The temperature of the column oven was programmed to be raised in two steps. In the first step, the oven temperature was 100 °C for 1 min, then raised to 250 °C at 32 °C/min; in the second step, the temperature was raised from 250 °C to 300 °C at 4 °C/min. An all-glass, solventless injector was mounted horizontally in the injection block of the gas chromatograph. Helium was used as the carrier gas at a liner velocity of 21 cm/s. The temperature of the injection port and the separator block was 300 °C, and the ionization source was maintained at 220 °C. The ionization energy was 70 eV. The selected ions monitored are described at a mass spectral resolution of 3000.

3. Results and discussion

Isoprostane and endocannabinoids were extracted with organic solvents; purification by means of solid-phase extraction with an octadecyl silica column and a silicic acid column was done three times with different organic solvent components between steps for extraction and derivatization for GC–MS assay. Derivatization of carbonyl groups of PGs and TX was performed to produce methyl esters, of the hydroxyl group to produce DMiPSi ether forms, and of the ketone group to produce MOX forms [1,7,28].

To perform a simultaneous preparation in purifica-

tion and derivatization, it was studied whether any procedure had interfered with another derivatization of each PG. For example, some PGs do not contain active groups, such as hydroxyl groups or carbonyl groups (Table 1). Anandamide, and 2-AG have no carbonyl or ketone groups. The methylation and MOX treatments do not interfere with derivatization of these endocannabinoids.

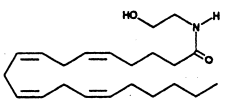
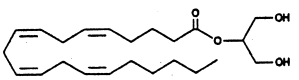
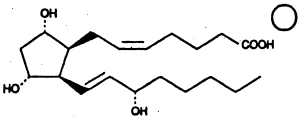
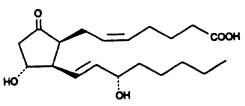
The DMiPSi ether forms of derivatives are extremely stable and can be stored at −20 °C or lower for more than 6 months after derivatization. This stability is an important advantage when many samples are continuously compared with other silylation reagents, such as trimethylsilyl. Another advantage is that these DMiPSi-derivatized PGs produce similar fragments [M−43], which are isolated isopropyl groups with high signal intensity. This fragment is large enough to contain whole molecular structures, including deuterated parts, and is useful for identification and assay in the SIM mode.

Mass spectra of simultaneous derivatized endocannabinoids and isoprostanes are shown in Fig. 1. Many compounds showing strong and specific fragments [M−43] were observed.

The DMiPSi ether forms of anandamide (Fig. 1A) and 2-AG (Fig. 1B) produced [M−43] fragments (*m*/*z* 404.30, 535.36, respectively).

In simultaneous preparation, 8-epi-PGF2α is the derivatized Me ester of the DMiPSi ether form (Fig. 1C), and 8-epi-PGE2 is the derivatized Me ester of the MOX-DMiPSi ether form (Fig. 1D). Methylation and methoxim preparation do not interfere with endocannabinoids derivatization. Although anandamide and 2-AG are derivatives of arachidonic acid, their DMiPSi ether forms are stable for more than 1 week longer than their original molecular

Table 1
Overview of derivatized PGs for simultaneous analysis

Name/ formula	Structure	Derivatization			Fragment (*m*/*z*)	
		Me	MOX	DMiPSi	[M+]	[M−43] [*M−31]
Arachidonyl ethanolamide (Anandamide) $C_{22}H_{37}NO_2$		—	—	○	447.35	404.30
		—	—	○○		
2-Arachidonyl glycerol (2-AG) $C_{23}H_{38}O_4$					578.42	535.36
8-epi-PGF2α $C_{20}H_{34}O_5$		○	—	○○○	668.47	625.41
8-epi-PGE2 (8-iso-PGE2) $C_{20}H_{32}O_5$		○	○	○○	595.41	552.35

○: Number of derivatized sites in PG structure. [M+]: Whole structure after derivatization. [M−43]: Liberated isopropyl group from the [M+] structure. Me, methyl; MOX, methyloxin; DMiPSi, dimethylisopropylsilyl.

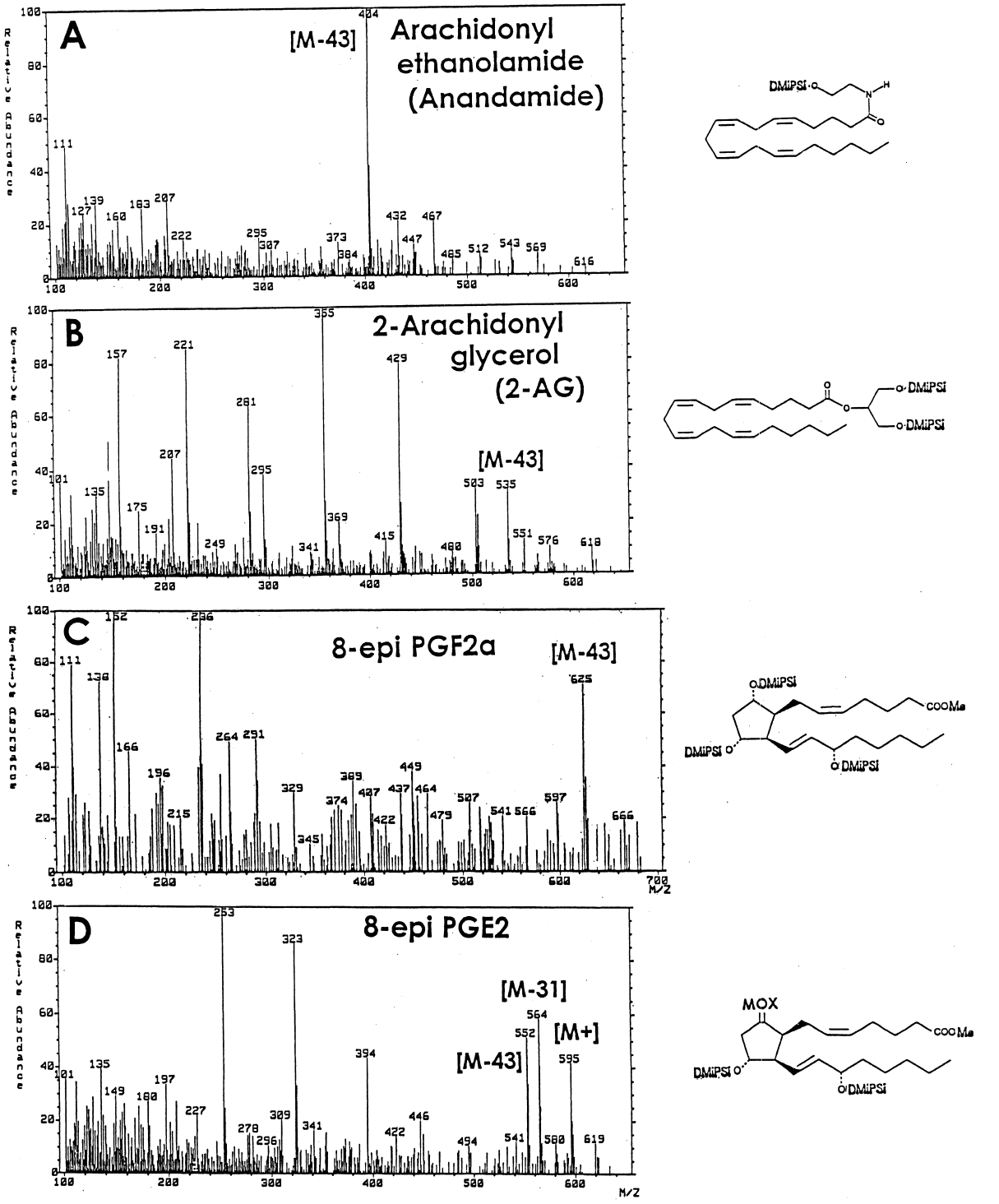


Fig. 1. Mass spectra of derivatized, authentic PGs and arachidonate peroxide. Compounds as in Table 1. Me: Methyl, DMiPSi: dimethylisopropylsilyl, MOX: methyloxim. (A) Arachidonyl ethanolamide (anandamide). (B) 2-Arachidonyl glycerol (2-AG). (C) 8-epi-PGF2α. (D) 8-epi-PGE2.

forms when stored at −20 °C, even when dissolved in organic solvent. These [M−43] fragments were stronger than the trimethylsilyl ether form fragments [24]. Therefore, a more sensitive assay could be performed with this derivatization.

To establish an assay system, the [M−43] mass fragments were selected for the SIM assay (anandamide, 2AG, 8-epi-PGF2α for m/z 404.30, 535.36, 625.41, respectively).

An overview of the chromatographic profile of these PGs by this simultaneous assay is shown in Fig. 2. Only derivatized, deuterated PGs, TX, and endocannabinoids are shown in this SIM chromatogram for indicating the distribution of each native compounds in this method. Of course, deuterated compounds can be used for assays of each native one. In addition, 9α,11β-PGF2 can be assayed with d_5-PGF2α at the same m/z fragment of PGF2α. In the same way, PGE1, PGD2, PGD3, 8-epi-PGE2 can be assayed with d_4-PGE2 at the m/z fragment of PGE2.

Endocannabinoids were also identified on the basis of the distances of their retention times from deuterated internal standards. Each deuterated internal standard appeared 1 to 3 s before the corresponding native compounds. This finding suggests that each compound was accurately identified and that calculating the efficiency of derivatization or the recovery rate of extraction for each one is unnecessary. Deuterated and native compounds should behave identically during simultaneous purification and derivatization.

The most important procedure in simultaneous assay preparation is chromatographic separation in the first silicic acid column [1]. After methylation, PGs are purified in this column elution. Endocannabinoids (anandamide and 2-AG) were eluted quickly as were the PGD2 group and TXB2. The E forms of PGs, included 8-epi-PGE2, eluted moderately quickly, and of the PGs examined, the F forms, including 8-epi-PGF2α, had the highest affinity to silicic acid on this organic solvent (Fig. 3). With a sufficient volume of solvent, all compounds can be recovered in a single fraction.

Each isoprostane and endocannabinoid can fit to a linear calibration curve from 5 pg to 100 ng (anandamide: $y=0.12x+0.006$, $r=0.99998$; 2-AG: $y=0.28x+0.006$, $r=0.99995$; y=8-epi-PGF2α: $y=0.78x+0.055$, $r=0.99994$). The minimum detectable concentrations of these compounds with this simultaneous assay method are 10 to 20 pg in plasma, 1 to 10 pg in cerebrospinal fluid (CSF) or cell-culture supernatant. These minimum concentrations are dependent on the amount of contaminants in each sample [1–7].

Fig. 4A shows the PG spectrum determined with the simultaneous assay of 8-epi-PGF2α, 2-AG, and anandamide (10 ng each). Anandamide and 2-AG were well separated and could be identified on the basis of their distances from the deuterated internal standard. Fig. 4B shows the results of an assay of plasma from a patient with septic shock. This method can also be used to assay 10 pg to 100 ng of anandamide and 2-AG from medical specimens with a single preparation, as for 8-epi-PGF2α.

Endocannabinoids (anandamide and 2-AG) and isoprostane (8-epi-PGF2α) were assayed in healthy volunteers, monkey, and mouse (Table 2). In patients with a variety of neoplastic diseases or inflammation or both, plasma levels of 2-AG are increased (manuscript in preparation).

Owing to limitations of instrument performance, large numbers of PGs and TX cannot be assayed simultaneously in the SIM mode. The chief limitation is that SIM switching requires time to switch channels for each PG fragment and to measure intensity. The measurement of too many channels can take a considerable time, decreasing the frequency at which each fragment is measured and lowering the efficiency with which fragment intensity is assayed.

In practice, the final derivatized sample of this simultaneous assay preparation is dissolved in 30 μl of ethylacetate and used in 2–10-μl aliquots for injection into the gas chromatograph using a solventless injector. Theoretically, the sample can be assayed two or more times. If all deuterated internal standards are added at the beginning of sample preparation, the final derivatized sample would be assayed in all PGs and TX, separately.

For example, the PG spectrum in plasma from rhesus monkeys was assayed with SIM chromatography to examine oxidative stress due to endotoxin treatment. Two groups divided on the basis of molecular mass (8-epi-PGF2α, PGF2α, and 9α,11β-PGF2; and TXB2, 6-keto-PGF1α) were observed (Fig. 5A). After 1 week, the remaining sample aliquots were injected into a gas chromatograph

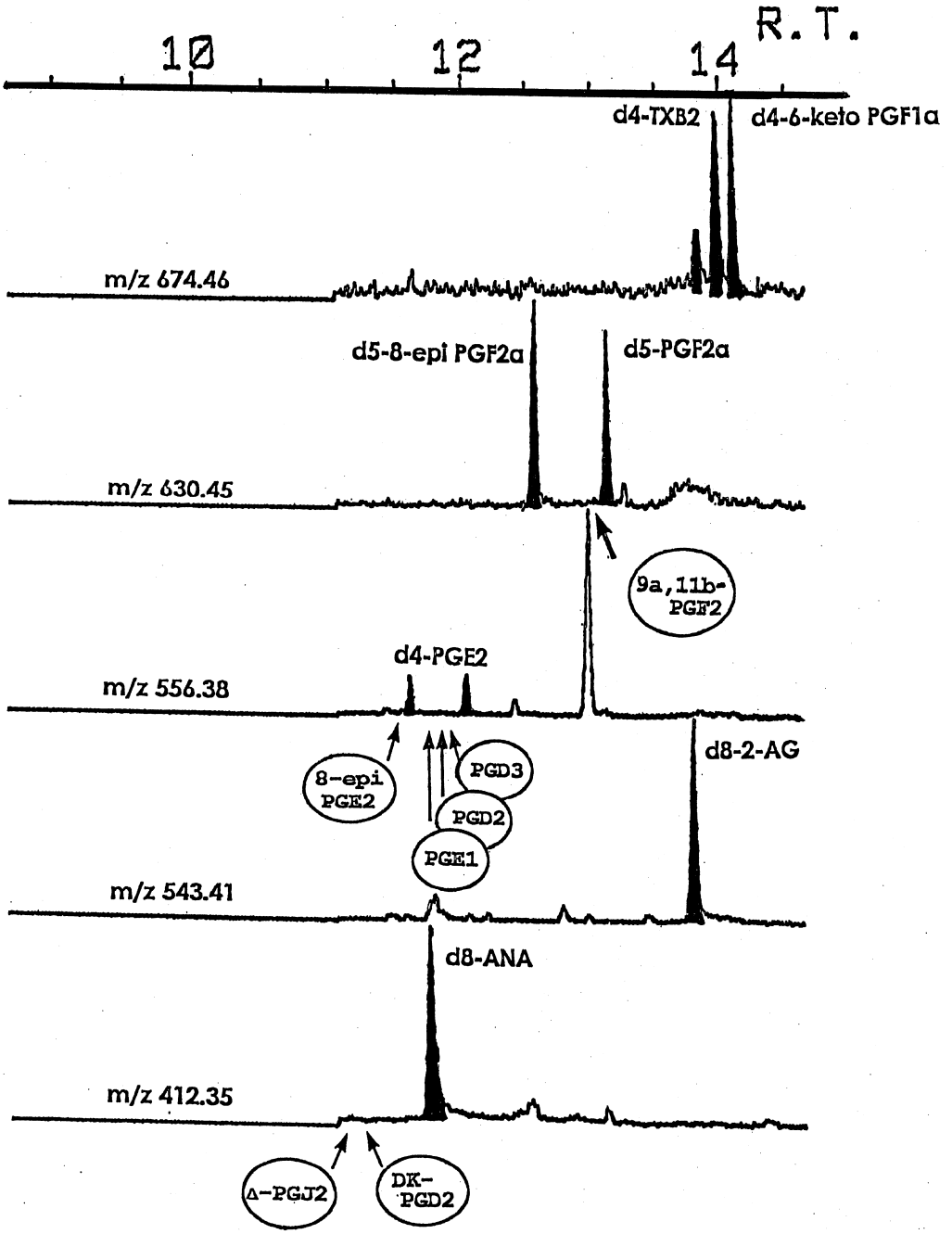


Fig. 2. SIM chromatograms of deuterated PGs, TX, and endocannnabinoids with estimated positions of other PGs. Deuterated PGs and TX (1 ng each), and endocannabinoids (10 ng each) were derivatized.

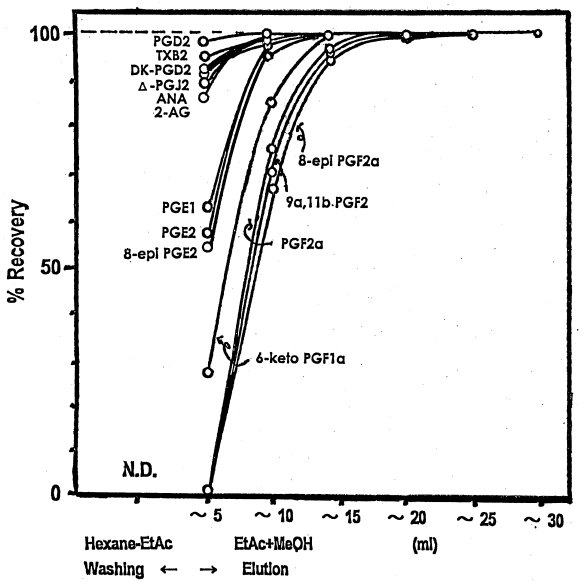


Fig. 3. Elution profile of various PGs and TX from the first silicic acid column chromatography. Each 5-ml fraction was assayed with GC–MS-SIM. Recovery was estimated by comparing the intensity of each sample with that of the original sample.

under different SIM conditions (Fig. 5B). With the second injections in a different channel, three groups (PGE2 and PGD2; 2-AG; and anandamide) can be assayed.

On the basis of assay priority, the SIM channel was chosen, and the derivatized sample can be assayed repeatedly. The DMiPSi ether form is suitable for repeated assay because of its stability, even if the second assay is performed 1 month after the first assay.

Using these derivatizations and simultaneous procedure, we have established an assay for PGs and endocannabinoids in one sample preparation in medical samples.

Acknowledgements

We would like to thank all the collaborators for providing important samples and comments on the results. T.O. thanks Dr. T. Nagakura and Dr. K. Yamashita for work during this and many other

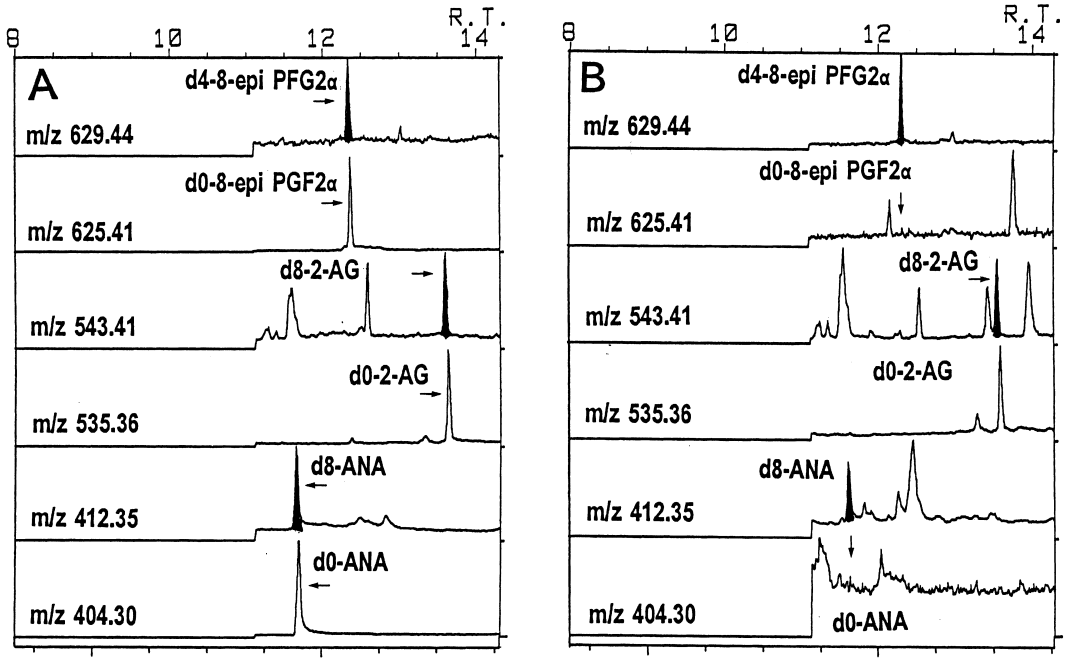


Fig. 4. PG spectra of anandamide and 2-AG. (A) Authentic anandamide, 2-AG, and 8-epi-PGF2α (10 ng each) with d_8-anandamide, d_8-2-AG (10 ng each), and d_4-8-epi-PGF2α. (B) Plasma sample from patients with septic shock showing deuterated internal standards as above.

Table 2
Level of endocannabinoids and isoprostane in human, monkey, and mouse

Species	Anandamide (pg/ml)	2-AG (ng/ml)	8-epi-PGF2α (pg/ml)
Human ($n=11$)	242.35±270.15	1.85±0.79	169.46±49.76
Monkey ($n=4$)	760.62±427.18	34.63±19.82	136.30±60.63
Mouse ($n=20$)	646.56±48.68	16.43±3.01	238.34±20.78

Mean±SD.

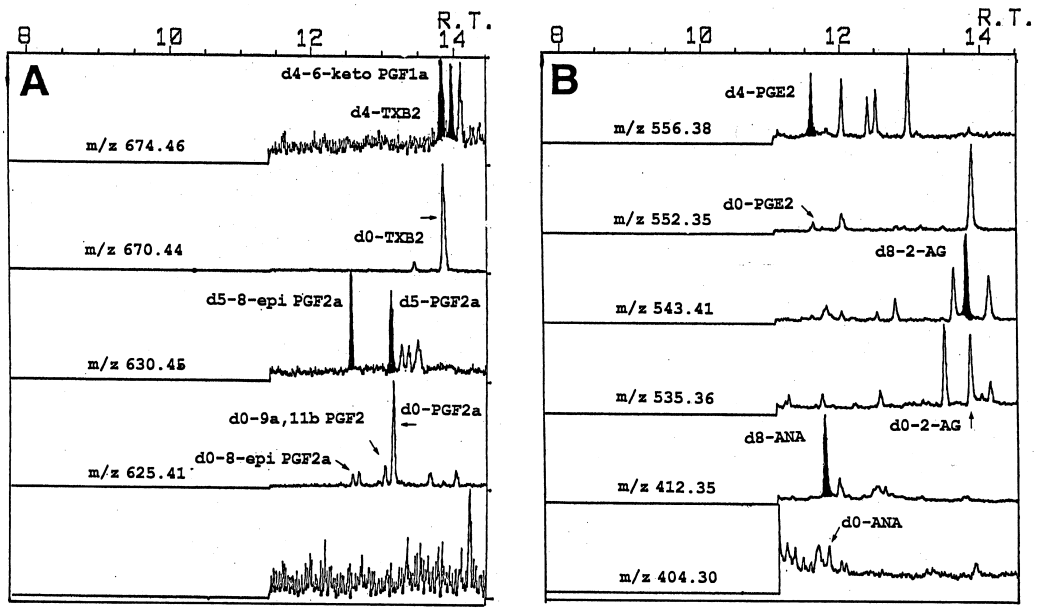


Fig. 5. PG spectrum of monkey plasma for analyzing oxidative stress. (A) Assay for TXB2, 6-keto-PGF1α, PGF2α, 9α,11β-PGF2, and 8-epi-PGF2α to analyze oxidative stress. (B) Remaining samples from (A): assay for PGE2, PGD2, 2-AG and anandamide.

studies and for their valuable advice and comments during the development of this method, and also thanks Dr. Okazaki who helped to prepare this manuscript.

References

[1] T. Obata, T. Nagakura, H. Maeda, K. Yamashita, K. Maekawa, J. Chromatogr. B 731 (1999) 73.
[2] T. Obata, T. Nagakura, M. Kammuri, T. Masaki, K. Maekawa, K. Yamashita, J. Chromatogr. B 655 (1994) 173.
[3] T. Obata, T. Nagakura, M. Kambe, T. Masaki, K. Maekawa, K. Yamashita, Biochem. Biophys. Res. Commun. 225 (1996) 1015.
[4] K. Yamaoka, T. Obata, Y. Hoshi, K. Iriyama, I. Iwasaki I, Radiat. Res. 149 (1998) 103.
[5] T. Obata, T. Nagakura, T. Masaki, K. Maekawa, K. Yamashita, Clin. Exp. Allergy 29 (1999) 1129.
[6] T. Obata, K. Tomaru, T. Nagakura, Y. Izumi, T. Kawamoto, J. Chromatogr. B 746 (2000) 11.
[7] T. Obata, Anal. Chim. Acta 465 (2002) 379.
[8] C.A. Opere, S.O. Awe, L.C. Harris, A.M. LeDay, S.E. Ohia, Free Radic. Res. 35 (2001) 257.
[9] T. Klein, K. Neuhaus, F. Reutter, R.M. Nusing, Br. J. Pharmacol. 133 (2001) 643.
[10] J.D. Morrow, T.M. Harris, L.J. Roberts II, Anal. Biochem. 184 (1990) 1.
[11] S.M. Lynch, J.D. Morrow, L.J. Roberts II, B. Frei, J. Clin. Invest. 93 (1994) 998.
[12] D.F. Taber, J.D. Morrow, L.J. Roberts II, Prostaglandins 53 (1997) 63.

[13] L.J. Roberts II, J.D. Morrow, Free Radic. Biol. Med. 28 (2000) 505.
[14] P. Montuschi, M. Corradi, G. Ciabattoni et al., Am. J. Respir. Crit. Care Med. 160 (1999) 216.
[15] M. Banerjee, K.H. Kang, J.D. Morrow, L.J. Roberts II, J.H. Newman, Am. J. Physiol. 263 (Heart Circ. Physiol. 32) (1992) H660.
[16] L.J. Roberts II, J.F. Reckelhoff, Biochem. Biophys. Res. Commun. 287 (2001) 254.
[17] G. Davi, G. Di Minno, A. Coppola, G. Andria, A.M. Cerbone, P. Madonna, A. Tufano, A. Falco, P. Marchesani, G. Ciabattoni, C. Patrono, Circulation 104 (2001) 1124.
[18] L. Oliveira, N.A. Stallwood, D.J. Crankshaw, Br. J. Pharmacol. 129 (2000) 509.
[19] C.C. Felder, E.M. Briley, J. Axelrod, J.T. Simpson, K. Mackie, W.A. Devane, Proc. Natl. Acad. Sci. USA 90 (1993) 7656.
[20] W. Van der Kloot, Brain Res. 649 (1994) 181.
[21] M. Maccarrone, T. Bisogno, H. Valensise, N. Lazzarin, F. Fezza, C. Manna, V. Di Marzo, A. Finazzi-Agro, Mol. Hum. Reprod. 8 (2002) 188.
[22] D. Panikashvili, C. Simeonidou, S. Ben-Shabat, L. Hanus, A. Breuer, R. Mechoulam, E. Shohami, Nature 413 (2001) 527.
[23] Y. Wang, Y. Liu, Y. Ito, T. Hashiguchi, I. Kitajima, M. Yamakuchi, H. Shimizu, S. Matsuo, H. Imaizumi, I. Maruyama, Anal. Biochem. 294 (2001) 73.
[24] K. Varga, J.A. Wagner, D.T. Bridgen, G. Kunos, FASEB J. 12 (1998) 1035.
[25] Y.H. Chang, S.T. Lee, W.W. Lin, J. Cell Biochem. 81 (2001) 715.
[26] H.H. Hansen, P.C. Schmid, P. Bittigau, I. Lastres-Becker, F. Berrendero, J. Neurochem. 78 (2001) 1415.
[27] J. Kurihara, M. Nishigaki, S. Suzuki, Y. Okubo, Y. Takata, S. Nakane, T. Sugiura, K. Waku, H. Kato, Jpn. J. Pharmacol. 87 (2001) 93.
[28] H. Miyazaki, M. Ishibashi, K. Yamashita, I. Ohguchi, H. Saitoh, H. Kurono, M. Shimono, M. Katori, J. Chromatogr. 239 (1982) 595.