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Note

Negative-ion chemical-ionization mass spectrometry of eicosanoids and its application to quantitation of prostacyclin synthesis in vascular tissue

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The cascade of arachidonic acid metabolites (eicosanoids) is complex and each eicosanoid exhibits various potent physiological activities [1]. To clarify the roles of these compounds under various pathophysiological conditions, many kinds of methods have been developed for measurement of endogenous amounts of several eicosanoids in biological materials [2]. Among various methods, radioimmunoassay techniques have been most widely employed for this purpose [3]. However, the structural similarity of eicosanoids and the variety of metabolites in any particular cellular situation demand extraordinarily high chromatographic separation and resolution followed by structural verification and quantitation by means of gas chromatography—mass spectrometry (GC—MS).

In conventional GC—MS analysis of eicosanoids in biological materials, electron impact (EI) has been widely employed as the ionization mode. Although the EI mode is reproducible, it has limited sensitivity because of the

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lack of ions with high intensity in the high mass range, which hinders quantitation of extremely small amounts of eicosanoids in biological materials. Several attempts have been made in recent years to improve the limited sensitivity of conventional GC—MS, such as employment of *tert.*-butyldimethylsilyl (*t*-BDMS) ether derivatives [4], capillary column GC and the chemical-ionization mode [5]. Among these attempts, the negative-ion chemical-ionization (NICI) mode has been successful in trace analysis of eicosanoids, employing pentafluorobenzyl (PFB) ester derivatives [6–8].

In the present study, we adopted the NICI mode, which yields prominent ions in the high mass range. Employing this mode, we examined mass spectral characteristics of eicosanoids derivatized to PFB esters and we attempted to estimate prostacyclin (PGI_2) synthesis in canine coronary arterial strips, in conjunction with the use of capillary column GC. We also examined the acute effect of indomethacin on PGI_2 synthesis in vascular tissue.

EXPERIMENTAL

Reagents

Analytical-grade reagents were used and solvents were redistilled immediately before use. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. Silica gel (Kieselgel 60, 70—230 mesh AMTS) was purchased from Merck (Darmstadt, F.R.G.). *o*-Methylhydroxylamine hydrochloride was purchased from Wako. PFB bromide, diisopropylethylamine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Tokyo Kasei Kogyo. Standard prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, thromboxane (TX) B_2 and 6-keto-$PGF_{1\alpha}$ were supplied by Ono Pharmaceutical. [3,3′,4,4′-$^2H_4$]-6-keto-$PGF_{1\alpha}$ was kindly synthesized and supplied by Dr. S. Terao of Takeda. Tritiated 6-keto-$PGF_{1\alpha}$ ([^{3}H]-6-keto-$PGF_{1\alpha}$, 120 Ci/mmol) was purchased from New England Nuclear.

Incubation and extraction procedure

Isolated canine coronary arterial strips (wet weight 98—156 mg; mean 128 mg) served as materials. They were incubated with 10 μM sodium arachidonate (Sigma) in 1 ml of Hanks' balanced salt solution (Sigma) containing 3 m*M* sodium bicarbonate (pH 7.4) and 20 n*M* hematin at 37°C for 1 h. Bovine thrombin (1 U/ml) (Sigma) was added to incubation media 10 min before the end of incubation. PGI_2 synthesis in coronary arterial strips was estimated by quantitating 6-keto-$PGF_{1\alpha}$, a stable catabolite of PGI_2.

After incubation was finished, a definite amount of deuterated 6-keto-$PGF_{1\alpha}$ (250 ng/ml) was added to the incubation media as an internal standard, and 6-keto-$PGF_{1\alpha}$ was extracted by the method of Powell [9]. When the effect of indomethacin was examined, we incubated coronary arterial strips with this drug for 5 min prior to addition of sodium arachidonate. The incubation media were acidified to pH 3—4 using 1 *M* hydrochloric acid, and applied to octadecylsilyl columns (Sep-Pak C_{18} cartridges). After these columns were washed with 10 ml of ethanol—water (15:85) to remove polar compounds, 6-keto-$PGF_{1\alpha}$ was eluted with 10 ml of ethyl acetate. Ethyl acetate was evaporated to dryness at reduced pressure.

To determine the recovery through the extraction procedure, ca. 0.005 μCi of [^{3}H]-6-keto-PGF$_{1\alpha}$ was added to other 1-ml aliquots of the incubation media and processed using the extraction procedure described above. Final recovery of [^{3}H]-6-keto-PGF$_{1\alpha}$ was determined by scintillation counting and compared to the counts of [^{3}H]-6-keto-PGF$_{1\alpha}$ originally added.

Derivatization of standard eicosanoids and samples

Standard eicosanoids possessing carbonyl groups (PGD$_2$, PGE$_2$, TXB$_2$ and 6-keto-PGF$_{1\alpha}$) and extracts from coronary arterial strips were converted to methyl oxime (MO), PFB ester and trimethylsilyl (TMS) ether derivatives. The other standard eicosanoid (PGF$_{2\alpha}$) was converted to the PFB-TMS derivative.

Methoximation. The MO derivatives were prepared by treating the samples with 100 μl of *o*-methylhydroxylamine hydrochloride in anhydrous pyridine (1 mg/ml) at room temperature, overnight [10]. Pyridine was removed in vacuo.

Esterification. To obtain the PFB esters of eicosanoids, samples were treated with PFB bromide (10%) in acetonitrile (80 μl) and diisopropylethylamine (20 μl) at 40°C for 1 h. After incubation, the reaction mixture was applied to a silica gel column (5 × 0.8 cm I.D.) to remove excess amounts of derivatizing reagents. PFB ester and MO-PFB ester derivatives of eicosanoids were eluted with 10 ml of ethyl acetate—methanol (99:1) according to the method of Miyazaki et al. [11]. The solvent was evaporated to dryness at reduced pressure.

Silylation. The residue was treated with 100 μl of BSTFA and 100 μl of acetonitrile at 40°C for 1 h. After evaporation of the solvent, the residue was dissolved in *n*-hexane (50 μl) and 1-μl aliquots were applied to the GC–MS.

Instrumentation and conditions

The NICI mass spectra and selected-ion chromatograms of eicosanoids were recorded on a Nihon-Denshi DX-303 mass spectrometer. A glass column (2 m × 2 mm I.D.) packed with SE-30 (1%) on Chromosorb W (80–100 mesh) was used for recording the NICI mass spectra of standard eicosanoids. A 25-m Ultra No. 2 fused-silica capillary column with 5% phenyl silicone (Hewlett-Packard) was used for constructing the calibration curve for 6-keto-PGF$_{1\alpha}$ and for recording selected-ion chromatograms of 6-keto-PGF$_{1\alpha}$ from incubation media, employing the splitless injection method. For NICI-MS, methane was used as the reagent gas with an ion source pressure of $8 \cdot 10^{-5}$ Torr. GC conditions for a packed column: temperature of column oven, 270°C; temperature of injection port, 290°C; flow-rate of carrier gas (helium), 22.5 ml/min. GC conditions for a fused-silica capillary column: column temperature, 80°C maintained for 1 min, then programmed to 280°C at 32°C/min; temperature of injection port, 300°C; flow-rate of helium, 1.0 ml/min. MS conditions: ionization voltage, 70 eV; emission current 370 μA; ionization current, 300 μA; selected-ion monitoring was performed at 2.4 kV ion multiplier voltage. The data were processed using a Nihon-Denshi JMA DA 5000 data system.

RESULTS

NICI mass spectra of eicosanoids

The methane NICI mass spectra of derivatized standard eicosanoids, PGD_2-MO-PFB-TMS, PGE_2-MO-PFB-TMS, $PGF_{2\alpha}$-PFB-TMS, TXB_2-MO-PFB-TMS and 6-keto-$PGF_{1\alpha}$-MO-PFB-TMS derivatives, were recorded on a packed column GC—NICI-MS, as shown in Table I. In all these derivatized eicosanoids, the ions at $[M - 181]^-$ (*m/z* 524 for PGD_2 and PGE_2, *m/z* 569 for $PGF_{2\alpha}$ and *m/z* 614 for TXB_2 and 6-keto-$PGF_{1\alpha}$), which derived from the loss of PFB radicals from the molecular ions, were observed to be the base peaks. The PGE_2-MO-PFB-TMS derivative showed two peaks on an SE-30 packed-column GC, which were derived from *syn*- and *anti*-isomers of its methoxime derivative. The retention times for these two peaks were 1.30 and 1.48 min, respectively, under the GC conditions described previously. Of the two peaks, the second peak was the major peak and, in this peak, the ion at $[M - 181]^-$ was the base peak. The relative abundance of the ions at $[M - 181]^-$ to the total-ion intensity in the mass range over *m/z* 50 was 52% for PGD_2, 55% for PGE_2 (major peak), 50% for $PGF_{2\alpha}$, 42% for TXB_2 and 52% for 6-keto-$PGF_{1\alpha}$.

TABLE I

METHANE NICI MASS SPECTRAL DATA OF EICOSANOIDS AS PFB-TMS AND MO-PFB-TMS DERIVATIVES

Relative intensities (%) are shown in parentheses.

Eicosanoid	Molecular weight	Major fragment ions over *m/z* 50		
		$[M - 181]^{-*}$	$[M - (181 + 90)]^{-**}$	Other ions
PGD_2	705	524 (100)	434 (16.2)	—
PGE_2***	705	524 (100)	434 (3.0)	315 (10.8)
$PGF_{2\alpha}$	750	569 (100)	479 (2.4)	—
TXB_2	795	614 (100)	524 (1.9)	585 (21.6) 405 (5.4)
6-keto-$PGF_{1\alpha}$	795	614 (100)	524 (1.8)	126 (5.4)

*Loss of PFB group.
**Loss of PFB and trimethylsilanol groups.
***The major isomer of the methoxime derivative.

Quantitation of 6-keto-$PGF_{1\alpha}$ synthesized in canine coronary strips

The recovery from the extraction procedure was determined using five aliquots of incubation media; the recovery of [^{3}H]-6-keto-$PGF_{1\alpha}$ was 88 ± 9.8% (mean ± S.D.).

According to the NICI mass spectral data, we selected the ion at $[M - 181]^-$ as the monitored ion of selected-ion monitoring (SIM) in quantitation of 6-keto-$PGF_{1\alpha}$ in incubation media. To examine the accuracy and precision of determination of 6-keto-$PGF_{1\alpha}$ in incubation media by the present method, known concentrations of mixtures of unlabelled and 250 ng of deuterated derivatives of 6-keto-$PGF_{1\alpha}$ were added to 1-ml aliquots obtained from pooled incubation media. These samples were then processed through the present method and injected into the GC—MS system. The peak-area ratios of the ions

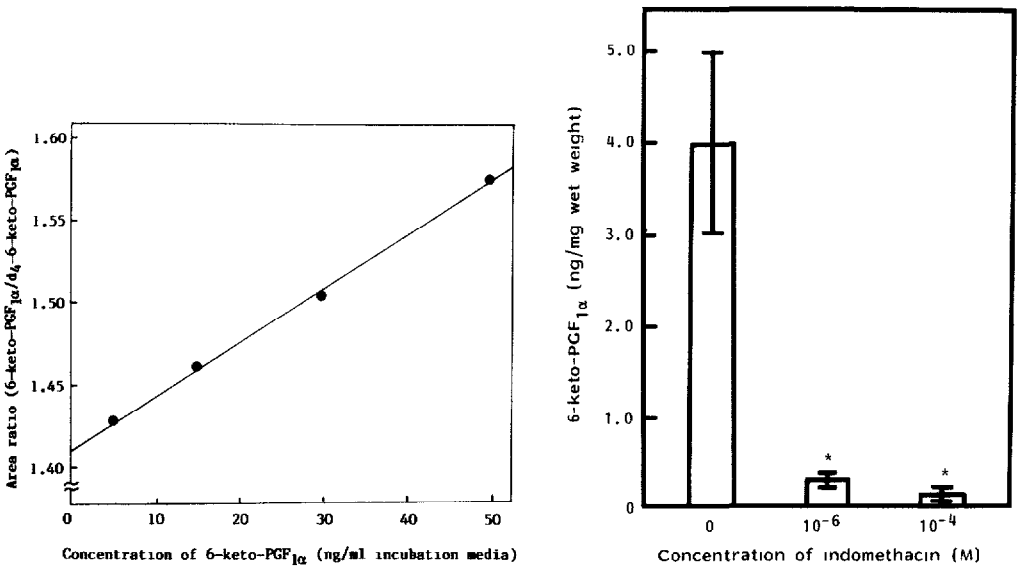

Fig. 1. Calibration curve for 6-keto-$PGF_{1\alpha}$. 6-keto-$PGF_{1\alpha}$ was chromatographed as the MO-PFB-TMS ether. The ratio of peak areas of the protium form (*m/z* 614) and tetradeutero form (*m/z* 618) was plotted against the concentration of protium form added to 1-ml aliquots obtained from pooled incubation media. The linear regression equation was $y = 0.0033x + 1.41$. The regression coefficient (r^2) was 0.998.

Fig. 2. Effect of indomethacin on the amount of 6-keto-$PGF_{1\alpha}$ synthesized by coronary arterial strips. The amounts of 6-keto-$PGF_{1\alpha}$ were calculated from the peaks obtained from *m/z* 614 and *m/z* 618 recorded on selected-ion chromatograms. (*) Significance of difference from control: $p < 0.05$.

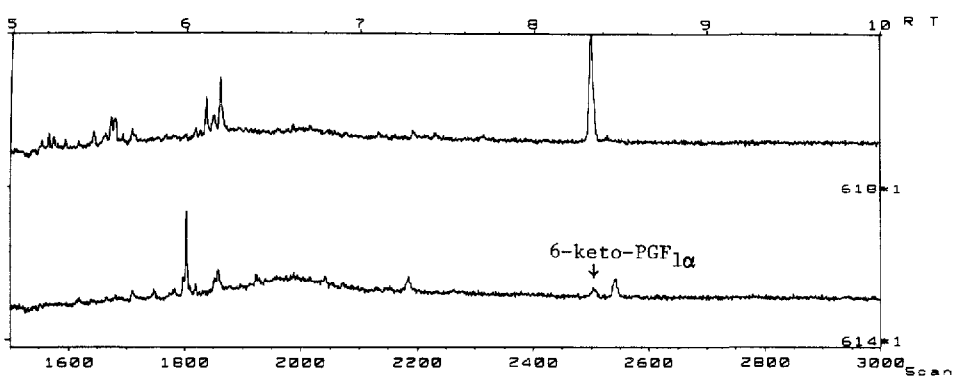

Fig. 3. Selected-ion chromatogram obtained from incubation media in the presence of 10^{-6} *M* indomethacin. The recorded ion pairs for MO-PFB-TMS ether of protonated and deuterated 6-keto-$PGF_{1\alpha}$ were *m/z* 614 and *m/z* 618.

at $[M - 181]^-$ in the protium and deuterium form (*m/z* 614 and *m/z* 618) were calculated. The calibration curve constructed by such a procedure is shown in Fig. 1. A linear relationship was observed between the peak-area ratios of the ions at $[M - 181]^-$ and the concentration of 6-keto-$PGF_{1\alpha}$ added in the range 5—50 ng/ml incubation media. The lower limit of detection was

10 pg, with a signal-to-noise ratio of 10:1. Deuterated 6-keto-$PGF_{1\alpha}$ used in the present study was shown to contain a 1.4% impurity of unlabelled 6-keto-$PGF_{1\alpha}$ by calculating the peak-area ratio of the ions at $[M - 181]^-$ (*m/z* 614 and *m/z* 618) on the selected-ion chromatogram. This value was taken into account when the calibration curve was constructed, and the endogenous amounts of 6-keto-$PGF_{1\alpha}$ were calculated.

The amount of 6-keto-$PGF_{1\alpha}$ synthesized in canine coronary strips under the present experimental conditions was 4.0 ± 1.1 ng/mg wet weight (mean ± S.D., $n = 5$). The amount of 6-keto-$PGF_{1\alpha}$ of 0.30 ± 0.10 and 0.16 ± 0.13 ng/mg wet weight at an indomethacin concentration of 10^{-6} and 10^{-4} *M*, respectively, was significantly lower than the above control value ($p < 0.05$) (Fig. 2). A typical selected-ion chromatogram is shown in Fig. 3.

DISCUSSION

Since eicosanoids exist in extremely small amounts in biological materials, it is desirable to employ the GC—MS method yielding ions with high intensity in the high mass range in order to be free from interference by numerous co-existing substances. Therefore, the conventional GC—MS employing TMS ether derivatives [10] is not suitable for this purpose, because fragmentation follows after elimination of the trimethylsilanol group. To improve the sensitivity of this method, several kinds of derivatives have been developed [12]. Among them, *t*-BDMS ether derivatives have been widely employed because of their hydrolytic stability and because they yield ions with high intensity [13]. But even these derivatives are limited in sensitivity when they are applied to samples containing eicosanoids in picogram orders.

Under these circumstances, improvement of the ionization mode, i.e. a softer form of ionization, is expected to reduce considerable fragmentation in the EI mode and provide an ion with high intensity in the high mass range. Suzuki et al. [14] employed a positive-ion CI mode and reported that 40 pg of 6-keto-$PGF_{1\alpha}$ were detectable. But, recently, the NICI mode has been found to be more useful in trace analysis of eicosanoids in conjunction with the use of derivatives having good electron-capture ability. Min et al. [15] achieved an increase in the electron-capture ability of the 6-keto-$PGF_{1\alpha}$ molecule by esterification with PFB bromide. When this derivative was used under NICI mode, the ion derived from the loss of PFB radical from the molecular ion (*m/z* 614) was observed to be the base peak with high intensity, and it has become possible to improve the limit of sensitivity. Blair et al. [16] reported NICI mass spectra of the 6-keto-$PGF_{1\alpha}$-MO-PFB-TMS derivative and applied it to quantitative analysis of 6-keto-$PGF_{1\alpha}$ in the low pg/ml range. The advantageous characteristic of PFB ester under NICI mode was also demonstrated for other eicosanoids. NICI mass spectra of MO-PFB-TMS [6- 8, 16] or MO-PFB-dimethyl-*n*-propylsilyl ether derivatives [11] of primary prostaglandins 6-keto-$PGF_{1\alpha}$ and TXB_2 were shown to contain $[M - PFB]^-$ as the most intense peak. In the present study, we ascertained that the ions derived from the loss of PFB radical from the molecular ion ($[M - 181]^-$) were observed to be the base peaks and other ions were scarcely yielded, as observed in the previous works mentioned above. The relative abundance of these ions was larger than that of the ions at $[M - 57]^+$ of *t*-BDMS ether derivatives of eicosanoids [17]. In

accordance with the larger intensities of the ions at $[M - 181]^-$, the lower limit of detection of the NICI method is lower than that of the EI method employing *t*-BDMS ether derivatives [18]. When 10 pg of 6-keto-$PGF_{1\alpha}$ were injected into the GC—MS system, the ion at $[M - 181]^-$ was detected by SIM with a signal-to-noise ratio of 10:1. In addition, employing deuterated 6-keto-$PGF_{1\alpha}$ as an internal standard, the linear relationship between the concentration of 6-keto-$PGF_{1\alpha}$ added to incubation media and the ratio of peak areas of the ions at $[M - 181]^-$ was observed. These data demonstrated the validity of the present method in quantitation of 6-keto-$PGF_{1\alpha}$ in incubation media. By employing the present method, we have highlighted the acute suppressive effect of indomethacin on PGI_2 synthesis at concentrations higher than 10^{-6} *M*. GC—SIM, using PFB derivatives, will allow us to quantitate small amounts of eicosanoids and to clarify their pathophysiological significance in various disorders.

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