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DIMETHYLISOPROPYLSILYL ETHER DERIVATIVES IN GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF SOME PROSTANOIDS

APPLICATION TO THE MICRODETERMINATION OF 15-KETOPROSTAGLANDIN $F_{2\alpha}$ FORMED BY ENZYMATIC CONVERSION OF $PGF_{2\alpha}$ IN VITRO

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SUMMARY

Treatment of 15-ketoprostaglandin $F_{2\alpha}$ (15-keto-$PGF_{2\alpha}$) with ethereal diazomethane at −78°C provided only the expected methyl ester (ME), without the formation of the pyrazoline adduct. The electron-ionization mass spectrum of the dimethylisopropylsilyl (DMiPS) ether derivative of 15-keto-$PGF_{2\alpha}$ ME showed the molecular ion, and the characteristic fragment ion of $[M-43]^+$ was of high abundance. The appearance of these ions in the high-mass region made it possible to determine trace amounts of 15-keto-$PGF_{2\alpha}$ in biological specimens without any interference from endogenous substances. The detection limit for the DMiPS ether derivatives of 15-keto-$PGF_{2\alpha}$ ME was 12 pg (signal-to-noise ratio = 10). The method was applied to the analysis of 15-keto-$PGF_{2\alpha}$ obtained by the enzymatic conversion of $PGF_{2\alpha}$ with 15-hydroxyprostaglandin dehydrogenase in vitro.

INTRODUCTION

The primary prostaglandins (PGs) derived from arachidonic acid by cyclooxygenase are inactivated rapidly to the corresponding 13,14-dihydro-15-keto deriv-

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atives by oxidation with 15-hydroxyprostaglandin dehydrogenase and subsequent reduction with 15-ketoprostaglandin 13,14-reductase [1–3]. Since it has been recognized that 15-keto-$PGF_{2\alpha}$ possesses very potent bronchoconstrictor activity, attention has been focused on the elucidation of the physiological role of 15-keto-$PGF_{2\alpha}$, especially in relation to bronchial asthma [4]. It is therefore essential to develop a sensitive analytical method for the examination of the relationship between the pharmacological activity of 15-keto-$PGF_{2\alpha}$ and its distribution.

Although there have been reports of the identification of 15-keto-$PGF_{2\alpha}$ in biological specimens [5,6], details of its existence and distribution in mammalian tissues have not yet been investigated, because of rapid conversion to the corresponding 13,14-dihydro derivatives.

Analysis of 15-keto-$PGF_{2\alpha}$ by gas chromatography–mass spectrometry (GC–MS) has been demonstrated [7,8], but conversion of 15-keto-PGs into the methyl ester (ME) derivatives using ethereal diazomethane under the conventional reaction conditions is difficult, owing to rapid conversion of the $\Delta^{13,14}$-15-keto system into a non-volatile pyrazoline adduct [7]. It has been reported that brief exposure of 15-keto-$PGF_{2\alpha}$ to diazomethane results in the selective formation of the ME derivative without serious side-reactions [5,7]. However, we consider it undesirable to shorten the reaction time because of poor reproducibility, particularly with biological fluids.

During the course of our studies on the GC–MS of PGs and thromboxane B_2 (TXB_2), the dimethylisopropylsilyl (DMiPS) ether derivatives have been found to give excellent GC and GC–MS properties [9–14]. We have now attempted to extend the previous method for the trace analysis of 15-keto-PGs, including the selective methylation even of those having the $\Delta^{13,14}$-15-keto system, by means of controlled esterification with ethereal diazomethane.

EXPERIMENTAL

Samples and reagents

$PGF_{1\alpha}$ and $PGF_{2\alpha}$ were purchased from Funakoshi Yakuhin (Osaka, Japan). 15-Keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were kindly supplied by Ono Pharmaceutical (Osaka, Japan). 15-Keto-$PGF_{1\alpha}$ was synthesized in our laboratories by selective oxidation of $PGF_{1\alpha}$ with 2,6-dichloro-3,5-dicyano-*p*-benzoquinone (DDQ) [15]. Trimethylsilyl (TMS)-, dimethylethylsilyl (DMES)-, dimethyl-*n*-propylsilyl (DMnPS)- and DMiPS-imidazoles were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Trideuteromethyl iodide and 3,3,4,4-tetradeutero-$PGF_{2\alpha}$ were obtained from Merck Frosst Canada (Pointe Claire, Canada). Sephadex LH-20 (25–100 μm) and silica gel (Kieselgel 60, 70–230 mesh) were obtained from Pharmacia (Uppsala, Sweden) and E. Merck (Darmstadt, F.R.G.), respectively. Clin Elut 1003 was purchased from Analytichem International (Harbor City, CA, U.S.A.). 15-Hydroxoprostaglandin dehydrogenase obtained from bovine lung was purchased from Miles Labs. (Goodwood, South Africa). Other reagents and solvents were of the highest quality available.

Gas chromatography

A Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector was employed. An open-tubular glass capillary column coated with SE-30 (20 m × 0.3 mm I.D.) (LKB, Stockholm, Sweden) was used. Helium was used as the carrier and make-up gas. Construction of the all-glass solventless injector and regulation of the carrier gas were performed according to the method described previously [9]. The temperature of the column oven was maintained at 280°C and those of the injection heating block and detector at 320°C.

Gas chromatography–mass spectrometry

An LKB-2091 gas chromatograph–mass spectrometer equipped with a data processing system was employed. The column was a glass capillary coated with SE-30. A solventless injection system as described above was used for sample injection. The flow-rate of the carrier gas was maintained at 5 ml/min. An inlet pressure of 0.4 kg/cm^2 produced a linear gas velocity of 21 cm/s. The temperatures of the column oven and separator were kept at 290°C and that of the ionization source at 280°C. The ionization energy and the trap current were 22.5 eV and 100 μA, respectively. The accelerating voltage was 3.5 kV.

Preparation of [1-^{14}C] 15-keto-PGF$_{2\alpha}$

To a solution of [1-^{14}C]PGF$_{2\alpha}$ (40 mCi/mmol, 0.5 μCi) in dioxane (0.1 ml) was added DDQ (1 mg), and the resulting solution was stirred at room temperature overnight. The reaction mixture was acidified to pH 3 with 0.5 *M* hydrochloric acid and then extracted with diethyl ether. The organic layer was washed with saturated sodium chloride solution and water, and dried over anhydrous sodium sulphate. After evaporation of the solvent, the resulting crude product was purified by preparative thin-layer chromatography (TLC) using chloroform–methanol (9:1) as the solvent. Elution of the area corresponding to the zone of radioactivity (R_F=0.45) with ethyl acetate gave [1-^{14}C]15-keto-PGF$_{2\alpha}$ (0.3 μCi) (60% radioactive yield). GC and GC–MS (ME–DMiPS ether derivative): MU (methylene unit values) = 31.01; *m/z* 566 ([M]$^{+\cdot}$, *I* (relative intensity) = 0.1%), 523 ([M−43]$^+$, *I*=24.9%), 422 {[M−(CH$_2$=CHODMiPS)]$^+$, *I*=30.5%}.

Preparation of 15-keto-PGF$_{2\alpha}$ ME

To a solution of PGF$_{2\alpha}$ ME (10 mg) in dioxane (0.5 ml) was added DDQ (15 mg), and the resulting solution was stirred at room temperature overnight. After dilution with diethyl ether, the organic layer was washed with 0.1 *M* sodium hydroxide solution and then with water. The solvent was evaporated to dryness and the crude product obtained was purified by preparative TLC with chloroform–methanol (9:1) as the developing solvent. Elution of the area corresponding to the spot (R_F=0.65) with ethyl acetate gave 15-keto-PGF$_{2\alpha}$ ME (8 mg) as a colourless oil. GC and GC–MS (ME–DMiPS ether derivative): MU=31.02, *m/z* 566 ([M]$^{+\cdot}$, *I*=0.1%), 523 ([M−43]$^+$, I=25.8%), 422 {[M−(CH$_2$=CHODMiPS)]$^+$, *I*=31.8%}.

Preparation of 15-keto-$PGF_{1\alpha}$

To a solution of $PGF_{1\alpha}$ (2 mg) in dioxane (0.5 ml) was added DDQ (5 mg), and the resulting solution was stirred at room temperature overnight. After work-up as described for the preparation of [1-^{14}C]15-keto-$PGF_{2\alpha}$, the crude product obtained was purified by preparative TLC using chloroform–methanol (9:1) as the developing solvent. Elution of the area corresponding to the spot (R_F=0.42) with ethyl acetate gave 15-keto-$PGF_{1\alpha}$ (1.1 mg) as colourless oil. GC and GC–MS (ME–DMiPS ether derivative): MU=31.44, *m/z* 568 ($[M]^{+\cdot}$, I=0.1%), 525 ($[M-43]^+$, I=57.5%), 424 {$[M-(CH_2{=}CHODMiPS)]^+$, I=100%}.

Preparation of 15-keto-$PGF_{2\alpha}$ [$^2H_3$]ME

To a solution of $PGF_{2\alpha}$ (10 mg) in dichloromethane (2 ml) were added sodium hydrogencarbonate (50 mg), [$^2H_3$]methyl iodide (0.05 ml) and tetra-*n*-butylammonium hydrogensulphate (20 mg), and the resulting solution was stirred at room temperature for 2 h. The reaction mixture was diluted with dichloromethane. The organic layer was washed with water and dried over anhydrous sodium sulphate. After evaporation of the solvent, the crude product obtained was purified by preparative TLC using dichloromethane–methanol (9:1) as the developing solvent. Elution of the area corresponding to the spot (R_F=0.50) gave $PGF_{2\alpha}$ [$^2H_3$]ME (5 mg) as a colourless oil. To a solution of $PGF_{2\alpha}$ [$^2H_3$]ME (5 mg) in dioxane (0.5 ml) was added DDQ (7 mg), and the resulting solution was stirred overnight at room temperature. Extraction and purification of the reaction product were carried out essentially as described for the preparation of 15-keto-$PGF_{2\alpha}$ ME to give 15-keto-$PGF_{2\alpha}$ [$^2H_3$]ME (4 mg) as a colourless oil. GC and GC–MS (ME–DMiPS ether derivative): MU=30.97, *m/z* 569 ($[M]^{+\cdot}$, I=0.1%), 526 ($[M-43]^+$, I=24.0%), 425 {$[M-(CH_2{=}CHODMiPS)]^+$, I=30.5%}.

Preparation of 3,3,4,4-tetradeutero-15-keto-$PGF_{2\alpha}$

To a solution of 3,3,4,4-tetradeutero-$PGF_{2\alpha}$ (0.1 mg) in dioxane (0.1 ml) was added DDQ (5 mg), and the resulting mixture was stirred at room temperature overnight. Extraction and purification of the reaction product as described for the preparation of [1-^{14}C]15-keto-$PGF_{2\alpha}$ afforded 3,3,4,4-tetradeutero-15-keto-$PGF_{2\alpha}$ (0.08 mg). GC and GC–MS (ME–DMiPS ether derivative): MU=30.95, *m/z* 570 ($[M]^{+\cdot}$; I=0.1%), 527 ($[M-43]^+$, I=20.5%), 426 {$[M-(CH_2{=}CHODMiPS)]^+$, I=24.2%}.

Determination of time course of esterification

To a solution of 15-keto-$PGF_{2\alpha}$ (0.1 mg) in diethyl ether (1.0 ml) was added freshly distilled ethereal diazomethane (0.5 ml), and esterification was carried out at 25, 0 and −78°C. Part of each reaction mixture (0.1 ml) was added to cooled acetic acid–diethyl ether (1:99) (1 ml) containing 15-keto-$PGF_{2\alpha}$ [$^2H_3$]methyl ether (0.05 mg). After decomposition of excess diazomethane, the solvent was evaporated to dryness below 30°C under reduced pressure. The residue was dissolved in pyridine (0.05 ml) and then silylated with DMiPS-imidazole (0.05 ml). The reaction mixture was allowed to stand at room temperature

for 1 h and then chromatographed over Sephadex LH-20 as described previously [9,16]. The purified derivatives were dissolved in *n*-hexane (0.1 ml) containing 0.5% (v/v) pyridine and then subjected to GC–MS.

Derivatization of 15-keto-PGs

To a solution of PGs in methanol (0.1 ml) cooled to −78°C, pre-cooled (−78°C) ethereal diazomethane (1.0 ml) was added and the resulting solution was allowed to stand at −78°C for 1 h. The reaction mixture was carefully added to a cooled solution of acetic acid–diethyl ether (1:99, 2 ml). After decomposition of the excess diazomethane, the reaction mixture was evaporated to dryness below 30°C under reduced pressure. The residue was dissolved in pyridine (0.05 ml) and then silylated with TMS-, DMES-, DMnPS- and DMiPS-imidazoles (0.05 ml). The reaction mixture was allowed to stand at room temperature for 1 h and then chromatographed over Sephadex LH-20. With samples obtained from in vitro experiments, the ME of PGs was purified by silica gel column chromatography prior to silylation as follows. The residue of the methylated sample was dissolved in *n*-hexane–ethyl acetate (1:1) (2 ml) and subjected to silica gel column chromatography (5 cm×0.8 cm I.D.). The column was eluted with ethyl acetate–methanol (99:1) (20 ml) and the solvent was evaporated to dryness.

Enzymatic conversion of $PGF_{2\alpha}$ in vitro

To a solution of 10 m*M* NAD^+ in 0.05 *M* phosphate buffer (pH 7.5) (0.2 ml) was added 15-hydroxyprostaglandin dehydrogenase (0.01 U), and the mixture was pre-incubated at 37°C for 2 min. To this solution was added $PGF_{2\alpha}$ (0.01 mg) suspended in 0.05 *M* phosphate buffer, and then the mixture was incubated at 37°C for 30 min. After addition of internal standard (3,3,4,4-tetradeutero-15-keto-$PGF_{2\alpha}$, 0.005 mg), the reaction mixture was diluted with water and then acidified to pH 3 with 0.1 *M* hydrochloric acid. The resulting solution was applied to Clin Elut 1003 and then eluted with ethyl acetate (20 ml). The solvent was evaporated and the residue obtained was purified and derivatized as described above.

RESULTS AND DISCUSSION

In the profile analysis of prostaglandins, it is essential to protect the carboxyl group as the corresponding methyl ester or as another volatile alkyl, aryl or silyl ester. For this purpose, ethereal diazomethane has been widely used owing to its excellent reactivity towards carboxyl groups, but with the $\Delta^{13,14}$-15-keto system in PGs rapid formation of the pyrazoline adduct is a serious side-reaction under the usual reaction conditions [7]. Therefore, our initial efforts were directed to the quantitative conversion of 15-keto-$PGF_{2\alpha}$ into the corresponding ME with ethereal diazomethane. Fig. 1 shows the relationship between reaction time and recovery of 15-keto-$PGF_{2\alpha}$ ME when esterification with ethereal diazomethane was carried out at various temperatures, and the reaction products were determined by GC–selected-ion monitoring (SIM) using the deuterated ME of 15-keto-$PGF_{2\alpha}$ as an internal standard. A considerable decrease in yield was ob-

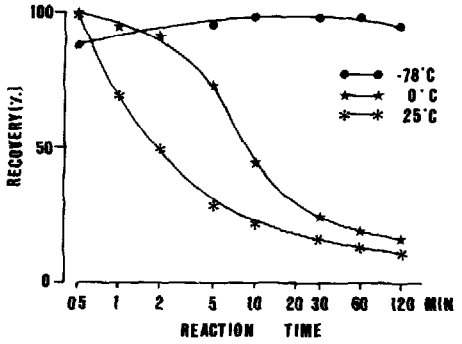

Fig. 1. Relationship between the reaction time and the recovery of 15-keto-$PGF_{2\alpha}$ ME, determined by GC–SIM after silylation with DMiPS-imidazole.

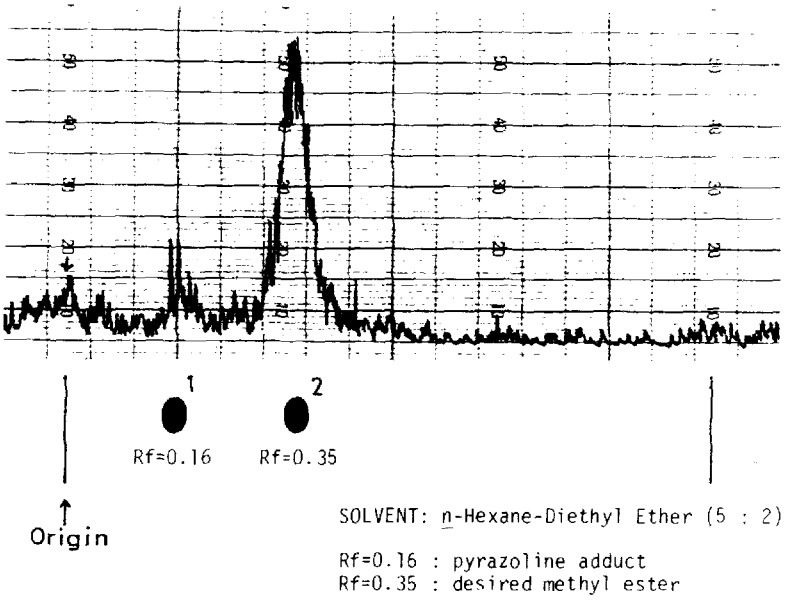

Fig. 2. TLC radioscannogram of the reaction product obtained by treating [1-^{14}C]15-keto-$PGF_{2\alpha}$ with diazomethane at −78 °C for 30 min and then with DMiPS-imidazole.

served with increasing reaction time at reaction temperatures of 25 and 0 °C. Reaction of 15-keto-$PGF_{2\alpha}$ at −78 °C with ethereal diazomethane gave only the desired ME derivative, without formation of a pyrazoline adduct. As shown in Fig. 1, the amount of ME formed by reaction of 15-keto-$PGF_{2\alpha}$ with diazomethane at 25 °C decreased by a factor of 2 within 2 min.

In order to determine the absolute recovery of methylation, TLC was used with [1-^{14}C]15-keto-$PGF_{2\alpha}$ as a tracer. Fig. 2 shows the thin-layer radioscannogram of the reaction product obtained by treating [1-^{14}C]15-keto-$PGF_{2\alpha}$ with diazomethane at −78 °C for 30 min and then with DMiPS-imidazole. It is obvious that most of the radioactivity was located at an R_F value of 0.35, which corresponds to the DMiPS ether derivative of authentic 15-keto-$PGF_{2\alpha}$ ME. This result indicates that 15-keto-$PGF_{2\alpha}$ is converted smoothly and quantitatively into the ex-

TABLE I

GAS CHROMATOGRAPHIC DATA FOR THE DMiPS ETHER DERIVATIVES OF FIVE KINDS OF PROSTAGLANDIN ME OR MO-ME

Compound	MU value	
	ME-DMiPS	ME-MO-DMiPS
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	30.65	30.40
15-Keto-$PGF_{1\alpha}$	31.44	30.81
15-Keto-$PGF_{2\alpha}$	31.02	30.42
$PGF_{1\alpha}$	32.41	—
$PGF_{2\alpha}$	31.91	—

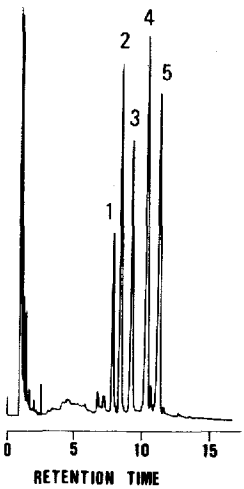

Fig. 3. Gas chromatographic separation of PGs as their ME-DMiPS ether derivatives, derivatives of three 15-keto metabolites, using a high-performance glass capillary column coated with SE-30 (20 m×0.3 mm I.D.) at 275°C under isothermal conditions. Peaks: 1 = 13,14-dihydro-15-keto-$PGF_{2\alpha}$; 2 = 15-keto-$PGF_{2\alpha}$; 3 = 15-keto$PGF_{1\alpha}$; 4 = $PGF_{2\alpha}$; 5 = $PGF_{1\alpha}$.

pected ME using diazomethane at −78°C. The DMiPS ether of 15-keto-$PGF_{2\alpha}$ ME exhibited a single and well shaped GC peak using a glass capillary column coated with SE-30.

Table I summarizes the GC data for the DMiPS ether derivatives of five kinds of ME or methoxime (MO)-ME derivatives of PGs. It can be seen that the DMiPS ether derivative of PG MEs provides excellent GC properties in comparison with those of the corresponding ME-MO derivatives in the separation between 15-keto-$PGF_{1\alpha}$, 15-keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$. Fig. 3 shows a typical GC separation of PGs as their ME-DMiPS ether derivatives, including the derivatives of three kinds of 15-keto metabolites, using a glass capillary column at 275°C under isothermal conditions. All PGs were separated completely within 13 min.

Fig. 4 shows the mass spectra of the DMiPS ether derivatives of 15-keto-$PGF_{1\alpha}$

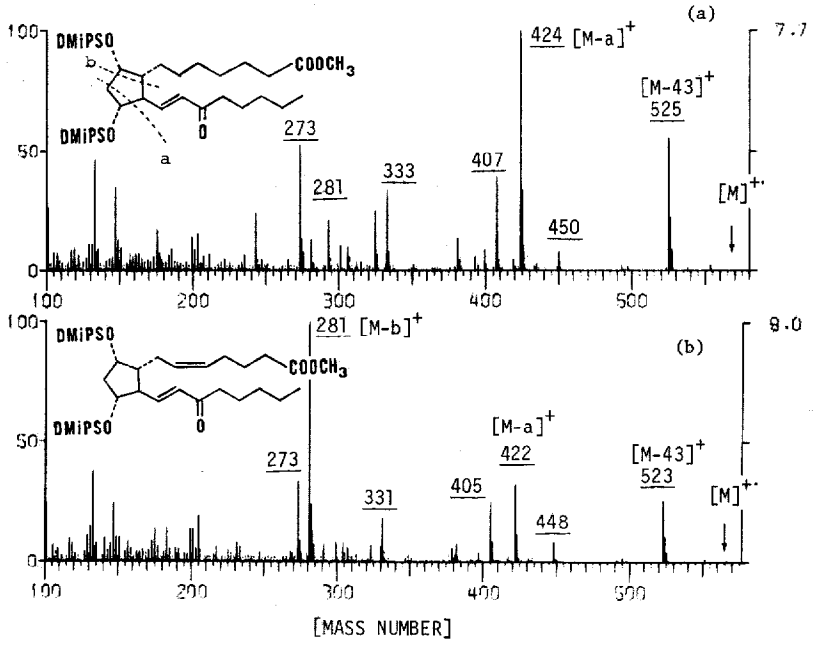

Fig. 4. Mass spectra of the DMiPS ether derivatives of (a) 15-keto-$PGF_{1\alpha}$ ME and (b) 15-keto-$PGF_{2\alpha}$ ME.

and 15-keto-$PGF_{2\alpha}$ ME. The molecular ions of these derivatives were observed at m/z 568 and 566, respectively, indicating that the expected derivatives were obtained by the present derivatization. Although the relative intensities of the molecular ions were very low, the appearance of the molecular ions was sufficient to confirm the structures of the derivatives. The characteristic ions of $[M-43]^+$ were observed at m/z 523 for 15-keto-$PGF_{2\alpha}$ and m/z 525 for $PGF_{1\alpha}$ with relative intensities of 25.8 and 57.5%, respectively. The ions of m/z 422 and 424 were assigned to be ions which might be produced by the loss of $[CH_2=CHODMiPS]^+$

TABLE II

MASS SPECTRAL DATA OF THE ME-DMiPS ETHER DERIVATIVES AND ME-MO-DMiPS ETHER DERIVATIVES OF THREE KINDS OF 15-KETO DERIVATIVES

Compound	Derivative*	Relative intensity (I, %)				
		$[M]^{+\cdot}$	$[M-43]^+$	Other ions		
13,14-Dihydro-	A	568(0.1)	525(6.3)	450(13.5)	407(100)	219(24.4)
15-keto-$PGF_{2\alpha}$	B	597(2.1)	554(73.9)	479(14.5)	448(66.7)	281(100)
15-Keto-$PGF_{2\alpha}$	A	566(0.1)	523(25.8)	422(31.8)	405(24.2)	281(100)
	B	595(4.2)	552(9.4)	446(14.6)	420(31.3)	310(100)
15-Keto-$PGF_{1\alpha}$	A	568(0.1)	525(57.5)	424(100)	407(39.4)	273(53.0)
	B	597(1.0)	554(11.7)	448(12.5)	422(100)	322(26.8)

*A, ME-DMiPS ether derivative; B, ME-MO-DMiPS ether derivative.

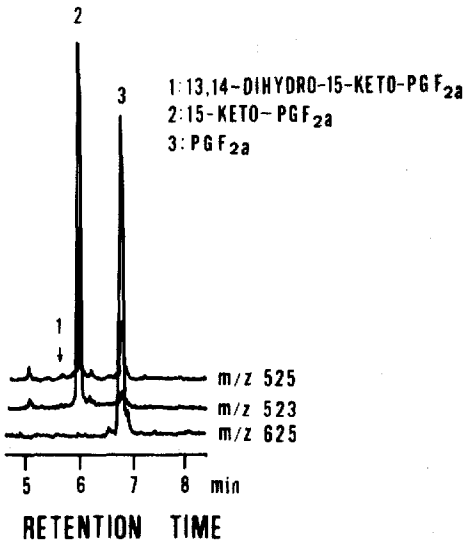

Fig. 5. Selected-ion recording of $PGF_{2\alpha}$ and its principal metabolites in the extract obtained by incubation of $PGF_{2\alpha}$ with 15-hydroxyprostaglandin dehydrogenase from bovine lung when monitoring their characteristic ions of $[M-43]^+$.

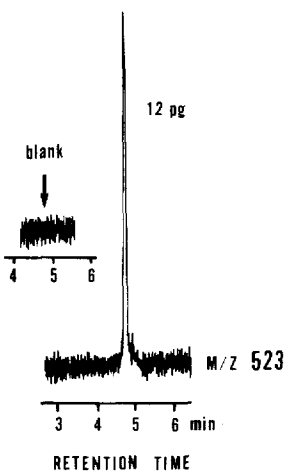

Fig. 6. Selected-ion recording of 15-keto-$PGF_{2\alpha}$ ME-DMiPS ether derivative when monitoring its characteristic ion of $[M-43]^+$ at m/z 523.

in the cyclopentane ring from the molecular ion. As shown in Table II, the relative intensities of the ions of $[M-43]^+$ in the ME-DMiPS ether derivatives of 15-keto-PGs except for 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were greater than that of the corresponding ME-MO-DMiPS ether derivatives. The high relative intensity of ions of $[M-43]^+$ in the high-mass region was very useful for the microdetermination of 15-keto-$PGF_{1\alpha}$ and 15-keto-$PGF_{2\alpha}$ in biological fluids by GC-SIM without any interference from endogenous substances.

Table II summarizes the mass spectral data of the ME-DMiPS and ME-MO-DMiPS ether derivatives of three kinds of 15-keto derivatives. Because

of their excellent GC separation and MS properties, we selected the ME–DMiPS ether derivatives for the microdetermination of 15-keto-$PGF_{2\alpha}$.

For the determination of 15-keto-$PGF_{2\alpha}$ in biological fluids, we synthesized deuterated 3,3,4,4-tetradeutero-15-keto-$PGF_{2\alpha}$ for use as an internal standard. Good linearity between the peak-area ratio and weight ratio for 15-keto-$PGF_{2\alpha}$ was observed in the range 2–20 ng/ml. The detection limit of the DMiPS ether derivative of 15-keto-$PGF_{2\alpha}$ ME was approximately 12 pg (injected amount) at a signal-to-noise ratio of 10 when monitoring the characteristic ion of $[M-43]^+$ at m/z 523, as shown in Fig. 5.

The method was applied to the analysis of 15-keto metabolites of $PGF_{2\alpha}$ in biological specimens. $PGF_{2\alpha}$ was incubated with partially purified 15-hydroxyprostaglandin dehydrogenase obtained from bovine lung. The metabolites were extracted from the reaction mixture, purified and then derivatized as described under Experimental. Fig. 6 shows the selected-ion recording of $PGF_{2\alpha}$ and its expected principal metabolites, 15-keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$, when monitoring the characteristic ions of $[M-43]^+$. Peak 1 (trace for m/z 523) was identified as 15-keto-$PGF_{2\alpha}$ and peak 2 (trace for m/z 625) corresponds to the substrate $PGF_{2\alpha}$. The metabolic intermediate 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (m/z 525) was not detected in the reaction mixture.

CONCLUSION

Esterification of PGs having the $\Delta^{13,14}$-15-keto system with diazomethane at $-78\,^{\circ}C$ made it possible to obtain only the expected ME derivative without formation of the pyrazoline adduct. Use of the DMiPS ether rather than the TMS ether derivatives enabled us not only to improve the GC separation but also to determine simultaneously trace amounts of the metabolites in biological samples using SIM, without any interference from other endogenous substances. The development and application of the method to the profile analysis of PGs, including β-ketols such as PGD_2 and PGE_2, are in progress and will be reported elsewhere.

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