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ENZYMATIC AND CHEMICAL LABELLING OF PROSTAGLANDIN E_1 AND 13,14-DIHYDRO-15-KETO-PROSTAGLANDIN E_1

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Summary

Chemical and enzymatic tools were employed to synthesize [1,1-$^{18}O_2$]-prostaglandin (PG) E_1, [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE_1 and [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1 starting from the corresponding unlabelled compounds and [5,6-$^3H_2$]-PGE_1, respectively. Reaction products were purified by high-performance liquid chromatography and their isotopic purity was determined by gas chromatography-mass spectrometry (GC-MS). The utility of [1,1-$^{18}O_2$]-PGE_1 and [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE_1 as internal standards for GC-MS analysis of the corresponding endogenous compounds in biological fluids is demonstrated. [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1 was found to be useful in developing extraction and purification procedures for the quantitation of endogenous 13,14-dihydro-15-keto-PGE_1 in human plasma by GC-tandem MS.

Key words

Enzymatic and chemical labelling; [1,1-$^{18}O_2$]-PGE_1; [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE_1; [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1; gas chromatography-tandem mass spectrometry

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Introduction

Prostaglandin E_1 (PGE_1), a dihomo-γ-linolenic acid metabolite formed via the cyclooxygenase pathway, exerts various biological activities such as peripheral vasodilatation, inhibition of platelet aggregation and proliferation of smooth muscle cells [1]. It also affects immune and inflammatory responses [2]. These properties of PGE_1 are pharmacologically utilized mainly in the treatment of peripheral arterial occlusive diseases (AOD) [3]. Since almost a decade ago Alprostadil® (Schwarz Pharma AG, Monheim, FRG) is an approved drug for the treatment of AOD. PGE_1 is rapidly metabolized in humans [4-6]. Amongst its metabolites 13,14-dihydro-15-keto-PGE_1 (15-keto-PGE_0) represents the major metabolite in human blood [7]. For pharmacokinetic studies this circulating metabolite is a reliable index metabolite for PGE_1 [8]. Gas chromatography-mass spectrometry (GC-MS) and GC-tandem MS (GC-MS/MS) are widely used techniques to quantitate prostaglandins and their metabolites in biological fluids [9]. Application of these techniques to quantitative measurements requires stable isotope-labelled analogs. Both for PGE_1 and 15-keto-PGE_0 there are so far no stable isotope-labelled analogs available. Also, no radioactive labelled 15-keto-PGE_0 is currently available. The aim of this study was therefore to synthesize [1,1-$^{18}O_2$]-PGE_1, [1,1-$^{18}O_2$]-15-keto-PGE_0 and [5,6-$^{3}H_2$]-15-keto-PGE_0 and to test their applicability in GC-MS. GC-MS and GC-MS/MS were applied to identify the reaction products, determine their isotopic purity and quantify PGE_1 and 15-keto-PGE_0 in biological fluids.

Experimental

Materials

PGE_1 and 15-keto-PGE_0 were a generous gift of Dr. Samel and Prof. Lille (Tallin, Estonia). [5,6-$^{3}H_2$]-PGE_1 (55 Ci/mmol) was obtained from DuPont de Nemours (Bad Homburg, Germany). Pig liver esterase (PLE; 130 units/mg) was from Boehringer Mannheim (Mannheim, Germany). $H_2{}^{18}O$ (97.8 atom% ^{18}O) was purchased from MSD Isotopes Merck Frosst Canada (Montreal, Canada). $Li^{18}OH$ (0.36 N) was prepared by dissolving the appropiate amount of Li in $H_2{}^{18}O$. 5 wt.% Rh/Al_2O_3 was obtained

from Fluka (Neu-Ulm, Germany). Pyridinium dichromate (PDC) and 2,3,4,5,6-pentafluorobenzyl (PFB) bromide were obtained from Aldrich (Steinheim, Germany). Methoxyamine hydrochloride and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, Illinois, USA). N,N-Diisopropylethylamine was obtained from Sigma (Munich, Germany). Acetonitrile and all other chemicals were from Merck (Darmstadt, Germany). 10 wt% Pd/C and 5 wt% Rh/C were obtained from Merck Schuchardt (Hohenbrunn, Germany). Octadecyl silica (ODS) cartridges (500 mg, 3 ml) were purchased from J.T. Baker (Deventer, The Netherlands).

Reverse-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analyses were performed on an LKB solvent delivery system model 2150 coupled with a variable UV-VIS LKB detector model 2151 (Bromma, Sweden), a Waters autosampler model WISP 710B (Hamburg, Germany), and a Shimadzu integrator model C-R3A (Kyoto, Japan). The stationary phase consisted of a column (250 mm x 4.6 mm I.D.) packed with ODS Hypersil, 5 μm particles size (Bischoff, Solingen, Germany). Two RP-HPLC systems were employed. System A: Underivatized compounds were analyzed isocratically using 35 % (v/v) acetonitrile in 10 mM sodium phosphate buffer (pH adjusted to 3.5 with phosphoric acid) at a flow-rate of 1.5 ml/min. The effluent was detected at 190 nm. The retention times of PGE_1 and 15-keto-PGE_1 and 15-keto-PGE_0 in this system were 9.5 min, 11.7 min and 17.5 min, respectively. System B: The PFB-MO derivatives were analyzed isocratically using acetonitrile/isopropanol/water (55/15/30, v/v/v) as the mobile phase at a flow-rate of 1.5 ml/min. The effluent was monitored at 210 nm.

Derivatization procedures

The PFB ester of the compounds were prepared by addition to the residue of 100 μl of acetonitrile, 10 μl of N,N-diisopropylethylamine used as the catalyst and 10 μl of a PFB bromide solution in acetonitrile (30 vol. %) and heating at 30 °C for one hour. Excess of reagents and solvents were evaporated under nitrogen and the residue was treated with 100 μl of a 2 g% methoxyamine (MO) hydrochloride solution in pyridine. Methoximation was completed by heating at 60 °C for one hour. Thereafter pyridine was evaporated under nitrogen, the residue treated with 200 μl of water and the PFB-MO derivatives were extracted by vortex-mixing with 1 ml of diethylether. The

solvent was evaporated to dryness under nitrogen, the residue was treated with 10 or 20 µl of BSTFA and allowed to react at 60 °C for one hour; 1-2 µl aliquots thereof were injected into the GC-MS/MS system in the splitless mode.

Gaschromatography-mass spectrometry and gas chromatography-tandem mass spectrometry

GC-MS and GC-MS/MS analyses were performed on a Finnigan MAT TSQ 45 triple-stage quadrupole mass spectrometer equipped with a Finnigan gas chromatograph model 9611 (San Jose, CA, USA). A fused silica capillary column OV-1 (25 m x 0.25 mm I.D., 0.25 µm film thickness) from Macherey-Nagel was used (Düren, Germany). Helium was the carrier gas at a pressure of 55 kPa. For negative-ion chemical ionization (NICI) methane was used as reagent gas at a pressure of 65 Pa. In GC-MS/MS argon was applied to collisionally activated dissociation (CAD) at a pressure of 3 mTorr, a collision and electron energy of 14 eV and 90 eV, respectively, and an emission current of 200 µA. Injector, interface and ion source were kept at 280, 290 and 130 °C, respectively. The column was held at 100°C for 2 min, then programmed to 280°C at a rate of 25°/min followed to 320°C at a rate of 4°/min.

Chemical synthesis of [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE$_1$

[5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE$_1$ was prepared from [5,6-$^3H_2$]-PGE$_1$ via [5,6-$^3H_2$]-15-keto-PGE$_1$. Selective oxidation of [5,6-$^3H_2$]-PGE$_1$ to [5,6-$^3H_2$]-15-keto-PGE$_1$ was performed using PDC as described by Doehl and Greibrokk [10]. Briefly, 50 µg of PGE$_1$ in preliminary experiments or 20 µCi of [5,6-$^3H_2$]-PGE$_1$ were treated with 100 µl of a 7 mM solution of PDC in acetonitrile (10 equiv.), vigorously mixed and kept for 8 min at room temperature. After addition of 2 ml of 0.5 M sodium phosphate buffer (pH 3.5) the reaction products were extracted twice with each 2 ml of ethyl acetate-hexane (50/50, v/v). The organic phase was decanted, the solvents removed under nitrogen, the residue redissolved in methanol and aliquots thereof analyzed by RP-HPLC and GC-MS.

The RP-HPLC isolated reaction product from the PDC oxidation with the retention time of 15-keto-PGE$_1$ was further catalytically hydrogenated in order to reduce the

double-bond at position 13,14. [5,6-$^3H_2$]-15-keto-PGE_1 or 50 μg of 15-keto-PGE_1 were diluted with 1.5 ml of methanol and treated with 2.5 mg and 5 mg of 5 wt% Rh/Al_2O_3, respectively. The suspensions were gently shaken, allowed to stand for 5 min on ice, and hydrogen gas was bubbled through the suspensions for 5 min at 0° C. Thereafter the samples were centrifuged (2000 g, 5 min), methanol was taken up, the catalyst washed with 1 ml of methanol and after re-centrifugation the combined methanol supernatants were further treated for RP-HPLC and GC-MS analysis.

Enzymatic synthesis of [1,1-$^{18}O_2$]-PGE_1 and [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE_1

[1,1-$^{18}O_2$]-PGE_1 was synthesized applying the following procedure. PGE_1 (50 μg in experiment A and 1 mg in experiment B) was converted to its methyl ester using a freshly prepared etheral solution of diazomethane. An aliquot of the PLE suspension (130 units) was dried at reduced pressure and the residue was resuspended in 100 μl of ^{18}O-water and this suspension was used to dissolve PGE_1 methyl ester. The pH of the resulting suspension was adjusted to 7.4 by treating with 10 μl of 0.36 N $Li^{18}OH$ and the mixture was incubated at 37° C for 5 min. The reaction was stopped by addition of 500 μl of ice-cold ethanol and the mixture was allowed to stay at -20° C for 30 min. Following centrifugation (2000 g, 5 min) the supernatant was taken, diluted with 1 ml of water and analysed by RP-HPLC using system A. RP-HPLC fractions with the retention time of unlabelled PGE_1 were collected, the solvents evaporated and an aliquot of the residue was treated for GC-MS analysis. An aliquot of the remainder was subjected to oxidation by PDC followed by reduction using 5 wt.% Rh/Al_2O_3 as described above for 13,14-dihydro-15-keto-PGE_1 and [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1.

Results and discussion

Chemical synthesis of [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1

For the catalytic reduction of [5,6-$^3H_2$]-15-keto-PGE_1 to [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE_1 the experimental conditions were optimized using unlabelled 15-keto-PGE_1. From the catalysts tested (5 wt% Rh/Al_2O_3, 10 wt% Pd/C, 5 wt% Rh/C) the use of 5 wt% Rh/Al_2O_3 gave the highest recovery which was found to depend upon the

catalyst amount used and the hydrogenation time (Table I). Recovery was determined by RP-HPLC (system A) using the reference compound 13,14-dihydro-15-keto-PGE1 for calibration of peaks. The highest recovery was achieved using 5 mg of the catalyst and a hydrogenation time of 5 min. The reaction product was identified by GC-MS as 13,14-dihydro-15-keto-PGE1 following RP-HPLC separation (system A) and derivatization.

Table I

Total recovery of 13,14-dihydro-15-keto-PGE1 from PGE1 (50 μg) via 15-keto-PGE1 as a function of hydrogenation time and amount of the catalyst 5 wt% Rh/Al_2O_3.

time / Rh/Al_2O_3	5 min	10 min	30 min
5 mg	39%	30%	4%
20 mg	20%	3%	<1%

For the synthesis of [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE1 the hydrogenation time was 5 min while 2.5 mg of 5 wt% Rh/Al_2O_3 was applied. RP-HPLC analysis (system A) of the reaction product followed by counting of collected 0.25 ml-RP-HPLC fractions showed a peak with identical retention time to unlabelled 13,14-dihydro-15-keto-PGE1. To an aliquot thereof 13,14-dihydro-15-keto-PGE1 was added (1 μg) and subsequently derivatized by PFB esterification followed by methoximation. A partial chromatogram from RP-HPLC analysis of the reaction product applying system B is shown in Fig. 1. As can be seen in this figure the UV profile as well as the radioactivity profile are identical. The appearance of radioactivity as well as UV absorbance as four not completely resolved peaks is further evidence for the formation of [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE1 as methoximation of two keto groups gives four syn/anti isomers. The total recovery for [5,6-$^3H_2$]-13,14-dihydro-15-keto-PGE1 was determined to be 32%.

Enzymatic synthesis of [1,1-$^{18}O_2$] PGE1 and [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE1

Enzymatic conversion of PGE1 in oxygen-18 labelled water (experiment A) resulted in the incorporation of two O^{18}-atoms in the carboxylic group to the extent of 80.4% and

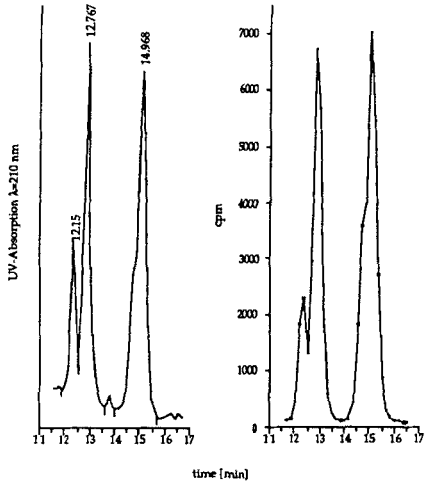

Figure 1: RP-HPLC analysis (system B) of chemically synthesized 15-keto-[5,6-$^{3}H_2$]-PGE_0 spiked with unlabelled 15-keto-PGE_0 followed by derivatization to the PFB-MO derivatives. Left, UV-chromatogram; right, radiogram

one O^{18}-atom to the extent of 17.8% while 1.8% of PGE_1 remained unlabelled as determined by integration of the peak areas of the major GC-MS peak (Fig. 2; scan number 1512). Calibration of the isolated [1,1-$^{18}O_2$] PGE_1 by co-derivatization of an amount from the [1,1-$^{18}O_2$] PGE_1 preparation with known amounts of synthetic unlabelled PGE_1 (0-10 ng), GC-MS analysis of the PFB-MO-TMS derivatives in the selected ion monitoring mode on m/z 526 for [1,1-$^{16}O_2$] PGE_1 and m/z 530 for [1,1-$^{18}O_2$] PGE_1 and regression analysis of the ratio m/z 526 to m/z 530 (y) vs. m/z 526 (x) gave a straight line with the equation $y = 0.105 + 1.058x$, $r>0.996$. Based on this experiment the recovery with respect to [1,1-$^{18}O_2$] PGE_1 was calculated to be 24%.

In experiment B starting from 1 mg PGE_1 its enzymatic labelling with ^{18}O resulted in the formation of a preparation with the following distribution: 54.8 % [1,1-$^{18}O_2$] PGE_1, 41.8% [1,1-$^{18}O^{16}O$] PGE_1 and 4.4% [1,1-$^{16}O_2$] PGE_1 as determined by selected ion monitoring as shown in Fig. 2. Further oxidation of this preparation followed by catalytic hydrogenation, RP-HPLC separation (system A), derivatization and GC-MS/MS analysis (Fig. 3) gave ^{18}O-labelled 15-keto-PGE_0 with the following

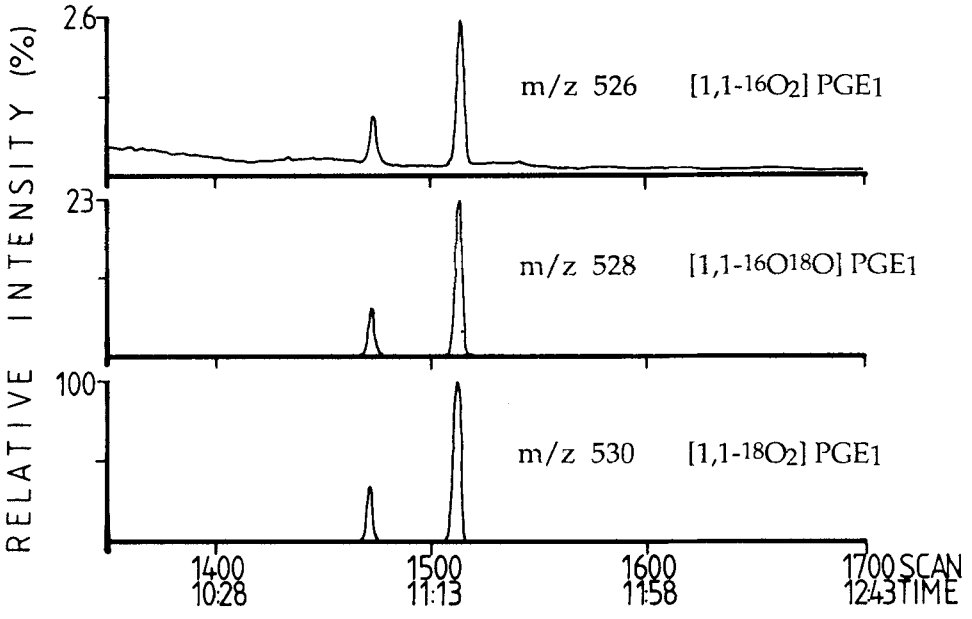

Figure 2: Partial GC-MS chromatogram from the analysis of the PFB-MO-TMS derivative of enzymatically synthesized [1,1-$^{18}O_2$] PGE_1. Selected ion monitoring of the anions $[M\text{-}PFB]^-$ at m/z 526 for [1,1-$^{16}O_2$] PGE_1, m/z 528 for [1,1-$^{18}O^{16}O$] PGE_1 and m/z 530 for [1,1-$^{18}O_2$] PGE_1.

distribution: 53.4 % [1,1-$^{18}O_2$]-15-keto-PGE_0, 41.1% [1,1-$^{18}O^{16}O$]-15-keto-PGE_0 and 5.5% [1,1-$^{16}O_2$]-15-keto-PGE_0. This distribution is very similar to the starting material [1,1-$^{18}O_2$] PGE_1 (see above).

The [1,1-$^{18}O_2$]-15-keto-PGE_0 preparation was standardized by co-derivatization of six 10-µl aliquots taken from a stock solution assummed to contain 5 ng of [1,1-$^{18}O_2$]-15-keto-PGE_0 by RP-HPLC with known amounts (0 to 10 ng) of 15-keto-PGE_0, GC-MS/MS analysis in the selected reaction monitoring (SRM) mode at m/z 331 and m/z 335 and regression analysis of the ratio m/z 331 to 335 (y) vs. m/z 331 (x) gave a straight line with the equation $y = 0.053 + 0.171x$, $r>0.999$. From this equation, the concentration of [1,1-$^{18}O_2$]-15-keto-PGE_0 in the ethanolic stock solution was estimated to be 5.84 ng/10 µl. The overall recovery for [1,1-$^{18}O_2$]-15-keto-PGE_0 was calculated to be 18%.

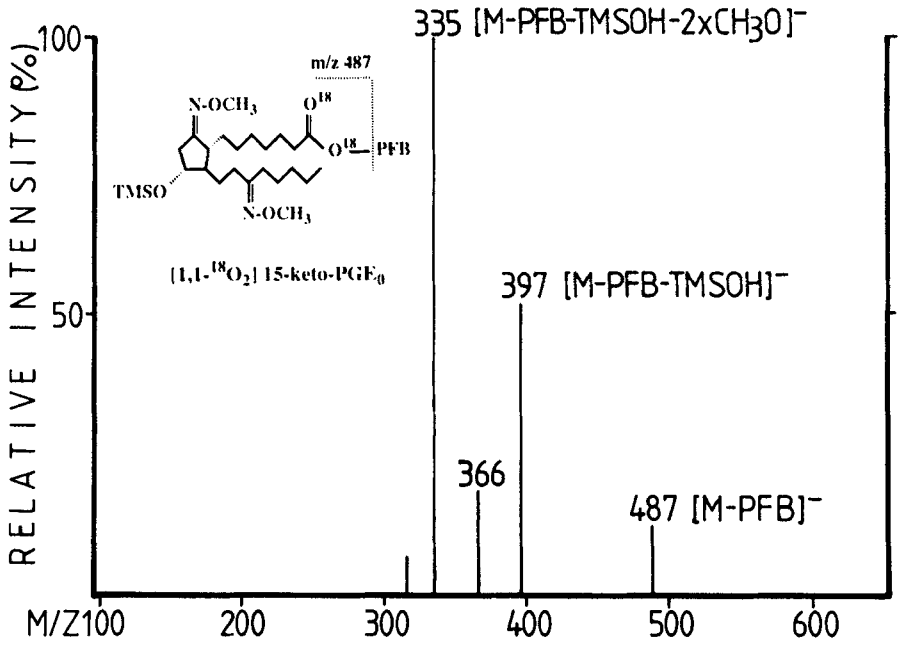

Figure 3: Daughter mass spectrum of the PFB-MO-TMS derivative of enzymatically synthesized [1,1-$^{18}O_2$]-15-keto-PGE$_0$ generated by collisionally activated dissociation from the parent ion at m/z 487 ([1,1-$^{18}O_2$]-15-keto-PGE$_0$). All mass fragments obtained were increased by four Da with respect to the signals generated from the parent ion at m/z 483 for unlabelled 15-keto-PGE$_0$

The applicability of [1,1-$^{18}O_2$]-15-keto-PGE$_0$ as internal standard for the quantitative determination of endogenous 15-keto-PGE$_0$ in human plasma by GC-MS/MS is shown in Fig. 4. The concentration of 15-keto-PGE$_0$ in this human plasma was determined to be 36 pg/ml at a coefficient of variation of 0.928 from triplicate injection. The detection limit of the method was below 4 pg of 15-keto-PGE$_0$ injected onto the column.

Conclusions

PGE$_1$ and its metabolites are acid- and base-labile compounds. The chemical and enzymatic methods described in this paper for the synthesis of [1,1-$^{18}O_2$]-PGE$_1$, [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE$_1$ and [5,6-$^{3}H_2$]-13,14-dihydro-15-keto-PGE$_1$ are easily

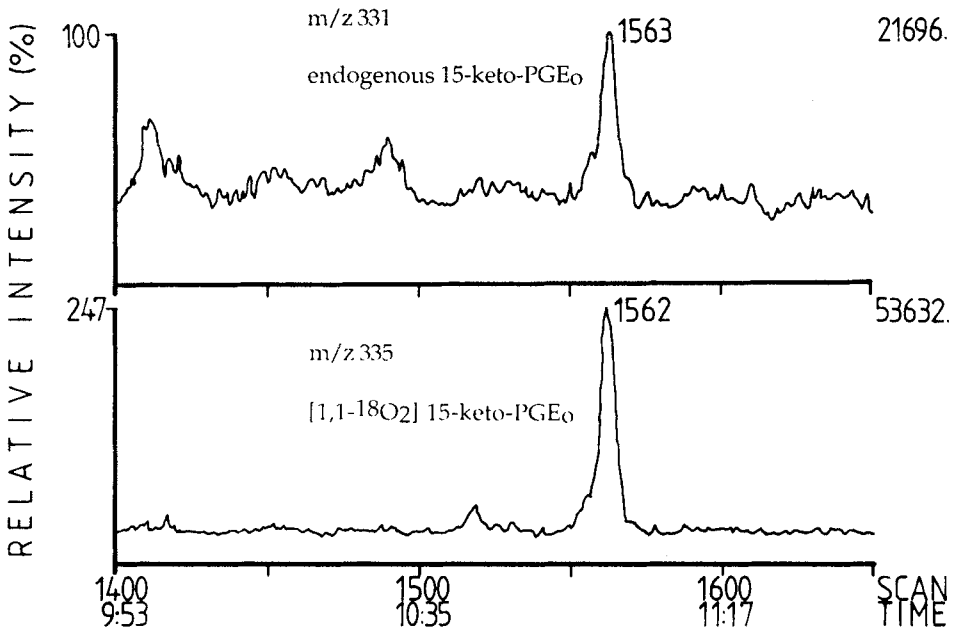

Figure 4: **Partial chromatogram from the GC-MS/MS analysis of a blood plasma sample taken from a patient. 8 ml of plasma was spiked with 5000 cpm of 15-keto-[5,6-$^3H_2$] PGE_0 and 1.2 ng of [1,1-$^{18}O_2$]-15-keto-PGE_0. SRM was performed at m/z 331 for endogenous 15-keto-PGE_0 and m/z 335 for [1,1-$^{18}O_2$]-15-keto-PGE_0**

performed at neutral pH and under mild conditions, and give reaction products of high isotopic purity at relative good recoveries. No total synthesis is necessary as the starting materials and the reagents needed are commercially available. [1,1-$^{18}O_2$]-PGE_1 and [1,1-$^{18}O_2$]-13,14-dihydro-15-keto-PGE_1 are suitable internal standards for quantitative determination in biological fluids by GC-MS.

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