Journal of Chromatography, 338 (1985) 273—280
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2455

SIMULTANEOUS DETERMINATION OF PROSTANOIDS IN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

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(First received August 7th, 1984; revised manuscript received October 10th, 1984)

SUMMARY

A method for simultaneous determination of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and thromboxane B_2 (TxB_2) in plasma was developed. After acidification and addition of ^2H- and ^3H-labelled internal standards, plasma prostanoids were extracted by reversed-phase cartridges and purified by normal-phase high-performance liquid chromatography. The pentafluorobenzyl, methoxime, trimethylsilyl derivatives were formed. Negative-ion chemical-ionization mass spectra with methane as reagent gas show one intense peak at *m/z* (M — pentafluorobenzyl). This ion was used for selective-ion monitoring. Prostanoid plasma concentrations (pg/ml) in five healthy volunteers were: PGE_2 2.0—10.4, $PGF_{2\alpha}$ 2.2—9.8, 6-keto-$PGF_{1\alpha}$ 0.6—1.8, and TxB_2 3.0—45.3. However, there is evidence that the TxB_2 values may frequently be falsely high because of ex vivo production during the sampling procedure.

INTRODUCTION

For the measurement of prostanoids, several methods such as bioassay, radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), gas chromatography and gas chromatography—mass spectrometry (GC—MS) are used. Plasma levels from the low pg/ml to the ng/ml level have been reported [1—10]. Generally, GC—MS data are considered the most reliable [6, 11]. Furthermore, the low values are supported by infusion studies [3, 4]. Published negative-ion chemical-ionization mass spectral (NICI-MS) data are in the low pg/ml range [2, 5].

NICI mass spectra of the trialkylsilyl [trimethylsilyl (TMS) or dimethylpropylsilyl]-pentafluorobenzyl (PFB)-methoxime (MO) prostanoid derivatives contain $(M - PFB)^-$ as the most intense peak [11—13]. The detection limit of

0378-4347/85/$03.30

the TMS-PFB derivative of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is about 200 fg at a signal-to-noise ratio of 5; for the derivatives of the other prostanoids this value is in the range 0.5—1.0 pg. Sensitivity of NICI-MS is about 100-fold higher than electron-impact mass spectrometry (EI-MS).

In this paper we report a sensitive and specific assay suitable to determine $PGF_{2\alpha}$, PGE_2, thromboxane B_2 (TxB_2) and 6-keto-$PGF_{1\alpha}$ simultaneously in a single plasma sample.

EXPERIMENTAL

Apparatus

HPLC. Separation was carried out using a Waters (Eschborn, F.R.G.) HPLC system: a U6K injector, two M6000 pumps, a M660 solvent programmer and a μPorasil HPLC column (10-μm particles, 30 cm × 3.9 mm I.D.).

GC—MS. A modified Finnigan MAT 4021 (Bremen, F.R.G.) quadrupole gas chromatograph—mass spectrometer with a pulsed positive/negative-ion chemical-ionization (PPNICI) module was employed. The gas chromatograph was a Carlo Erba HRGC (Hofheim, F.R.G.) with a J&W on-column injector and a J&W fused-silica capillary column (DB-1, 30 m × 0.259 mm I.D., 0.25 μm film thickness; ICT, Frankfurt, F.R.G.).

Materials

Nanograde solvents were used (chloroform, methanol, and ethyl acetate: Promochem, Wesel, F.R.G.; acetonitrile and water: Baker, Gross-Gerau, F.R.G.; formic acid, p.a., E. Merck, Darmstadt, F.R.G.). Sep-Pak reversed-phase cartridges were obtained from Waters (Gross-Gerau, F.R.G.). O-Methylhydroxyammonium chloride was from Pierce (Günter Karl, Geisenheim-Johannisberg, F.R.G.), N-ethyldiisopropylamine and pentafluorobenzylbromide were from Fluka (Buchs, Switzerland), and bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Macherey, Nagel & Co. (Düren, F.R.G.). Sephadex LH-20 was supplied by Pharmacia (Uppsala, Sweden), Extrelut by E. Merck.

PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 and their 3,3′,4,4′-deuterated analogues were kind gifts from Drs. Udo Axen and John Pike (Upjohn, Kalamazoo, MI, U.S.A.). [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2, [5,6,8,9,11,12,14-15(*n*)-^{3}H]$PGF_{2\alpha}$, and [6-keto-(5,8,9,11,12,14,15(*n*)-^{3}H]$PGF_{1\alpha}$ were obtained from Amersham Buchler (Braunschweig, F.R.G.). [5,6,8,9,11,12,14,15(*n*)-^{3}H]TxB_2 was purchased from New England Nuclear (Dreieich, F.R.G.). Radiochemical purity of ^{3}H standards is about 90% as determined by HPLC.

Sample collection

Venous blood (18 ml) was collected without tourniquet into ice-cold tubes containing 0.5 mg of indomethacin in 2 ml of 3.8% sodium citrate solution and centrifuged immediately at 4°C and 1000 *g*. The plasma volume was determined and the plasma frozen at −80°C. Needles with different inner diameters (1.5 mm and 0.9 mm) were used for venipuncture.

Extraction and purification

Deuterated (1 ng each) and tritiated (25 000 dpm each) PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 were added to 10 ml of plasma. After acidification with formic acid to pH 3.2, samples were allowed to equilibrate at 0° C for 30 min. Extraction was carried out using a reversed-phase C_{18} Sep-Pak cartridge, which was pre-conditioned with methanol and water. Prostanoids were eluted with ethyl acetate. The solvent was evaporated, the residue dissolved in chloroform and the prostanoids were separated on a normal-phase HPLC column. The initial eluent was chloroform, the final eluent, after 20 min, was 96.8% chloroform, 2.7% methanol, and 0.5% formic acid and the flow-rate 1 ml/min. Fractions of 1 ml were collected. Aliquots of 25 µl were assayed for radioactivity by liquid scintillation spectrometry.

Derivatization

Esterification. The prostanoid-containing fractions were evaporated at room temperature under nitrogen. The PFB derivatives were prepared by dissolving the residue in acetonitrile (50 µl), adding 35% pentafluorobenzyl bromide in acetonitrile (15 µl), and N-ethyldiisopropylamine (15 µl). The mixture was allowed to react at 50° C for 30 min. After evaporation electron-capturing substances were separated by elution through a column (30 mm × 5 mm I.D.) of pre-swollen Sephadex LH-20 with dichloromethane. The solvent was removed as described above.

Methoximation. The fraction containing PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2 was dissolved in 100 µl of a O-methylhydroxyammonium chloride in pyridine (20 mg/ml). After reaction for 24 h at ambient temperature the solvent was evaporated. The residue was dissolved in water (0.1 ml) and applied to a short Extrelut column (30 mm × 5 mm I.D.) to remove the derivatization agent. The prostanoid derivatives were eluted with chloroform and the solvent was evaporated.

Silylation. The PFB-MO (PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2) and PFB ($PGF_{2\alpha}$) derivatives were silylated with 50 µl of BSTFA at 25° C for 24 h. After evaporation with nitrogen the samples were reconstituted in 10 µl of BSTFA. After 3 h the samples were ready for GC—MS analysis. The derivatives are stable at —20° C for some months. All glassware used in the extraction, purification, and derivatization steps were silanized.

GC—MS conditions

Temperature programme: initial temperature 140° C, increased at 25° C/min to 290° C, then at 7.5° C/min to 320° C, temperature held for 5 min. Carrier gas (helium) pressure: 100 kPa. Sample volume: 2 µl. Temperatures: interface, 300° C; ionizer, 280° C. CI gas (methane) pressure in source: 22 Pa. Electron energy: 70 eV. Emission current: 0.4 mA. Electron multiplier: 1100 V. Conversion dynode: ± 3000 V. Selective-ion monitoring (SIM) at *m/z*: 524.3 (PGE_2), 528.3 ([$^2H_4$]PGE_2), 569.4 ($PGF_{2\alpha}$), 573.4 ([$^2H_4$]$PGF_{2\alpha}$), 614.4 (TxB_2, 6-keto-$PGF_{1\alpha}$), and 618.4 ([$^2H_4$]TxB_2, 6-keto-[$^2H_4$]$PGF_{1\alpha}$).

RESULTS AND DISCUSSION

Our assay was designed to achieve simultaneous quantitation of several prostanoids in a single GC—MS run. This modification renders GC—MS analysis of prostanoids less labour-intensive, thus abolishing its major drawback.

HPLC purification of PGE_2, $PGF_{2\alpha}$, TxB_2 and 6-keto-$PGF_{1\alpha}$ was carried out on a silicic acid column. The chromatogram shows two peaks. The first one belongs to PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$, which were not separated [15], the second one to the more polar $PGF_{2\alpha}$ (Fig. 1). It is an advantage of this HPLC system that these three prostanoids co-elute allowing one to save time for derivatization and GC—MS quantitation. Recoveries after the extraction and purification steps are about 45—55%.

After derivatization the prostanoid concentrations in plasma were determined by GC—NICI-MS. Fig. 2 shows the SIM chromatogram of $PGF_{2\alpha}$. The retention time (t_R) is about 11.4 min (200 scans = 1 min). As observed in the other chromatograms the deuterated compound has a somewhat shorter retention time.

PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were determined in one GC—MS run. The PGF_2 PFB-MO-MTS derivative has the shortest t_R of these prostanoid derivatives. On the GC column the *syn*- and *anti*-oxime isomers were separated (Fig. 3). The concentration of the first eluting isomer (t_R 11.4 min) is about 40% of the second one (t_R 11.8 min). After elution of the PGE_2 derivatives we

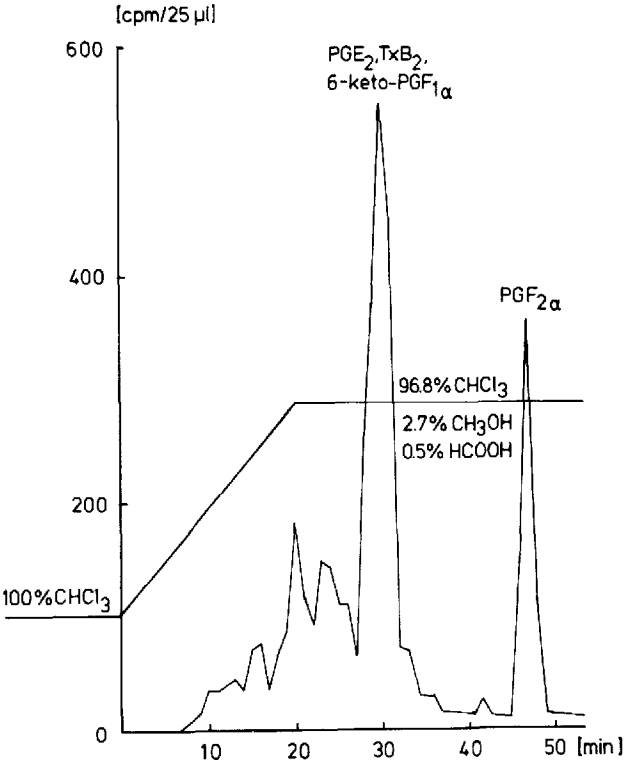

Fig. 1. HPLC radiochromatogram of tritiated PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$.

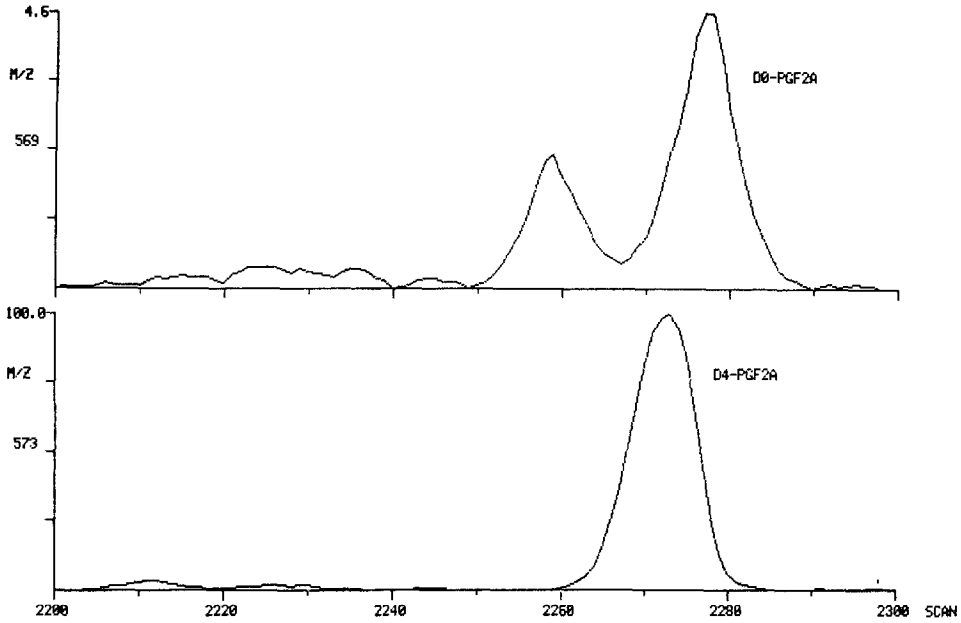

Fig. 2. SIM chromatogram of endogenous $PGF_{2\alpha}$ (m/z 569; relative intensity 4.6%) and deuterated $PGF_{2\alpha}$ (m/z 573; relative intensity 100%). Plasma concentration: 5.6 pg/ml.

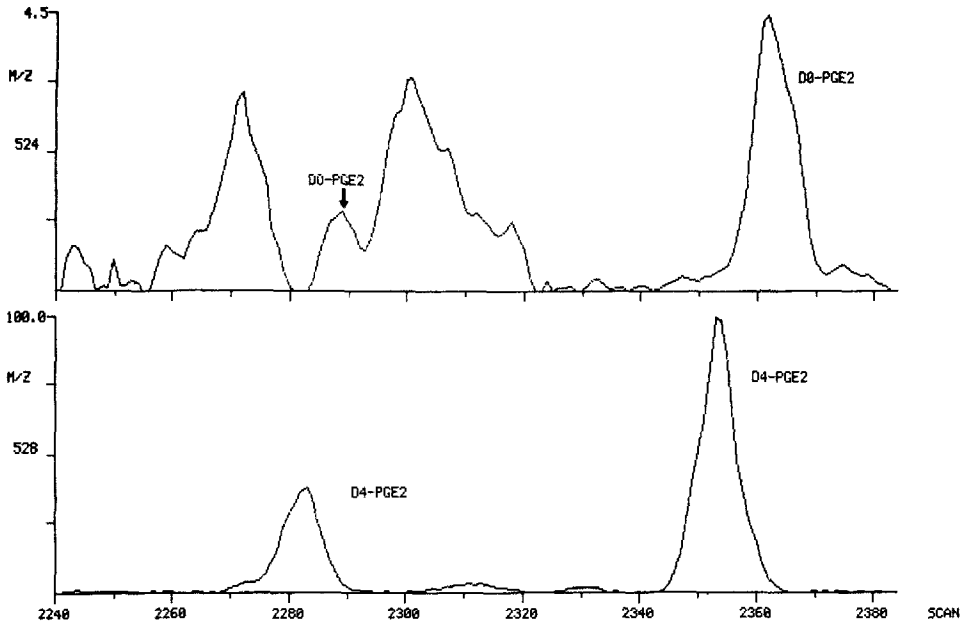

Fig. 3. SIM chromatogram of endogenous PGE_2 (m/z 524; relative intensity 4.5%) and deuterated PGE_2 (m/z 528; relative intensity 100%). Plasma concentration: 4.0 pg/ml.

switched from the SIM descriptor of PGE_2 to that of TxB_2 and 6-keto-$PGF_{1\alpha}$. The PFB-MO-TMS derivatives of these compounds have the same molecular weight. The TxB_2 derivative has a retention time of 12.1 min, the 6-keto-$PGF_{1\alpha}$

derivative elutes after 12.25 min (Fig. 4). The chromatogram shows no separation of the oxime isomers, but the TxB_2 peak is very broad.

For quantitation of endogenous prostanoids standard curves were prepared. They show linearity in the range from 10 pg to 1 ng of prostanoid using 1 ng of the deuterated compound as internal standard. The correlation coefficients (r) are shown in Table I. A blank caused by incomplete deuteration of the internal standards was observed in all investigated prostanoids (Table I). The inter-assay variations (n = 5) were between 8.6% (TxB_2) and 22.8% (6-keto-$PGF_{1\alpha}$) at the low pg/ml range (Table I).

Endogenous plasma concentrations in five healthy male volunteers were determined. The levels were as follows (see also Table II): $PGF_{2\alpha}$ 2.2—9.8 pg/ml, PGE_2 2.0—10.4 pg/ml, 6-keto-$PGF_{1\alpha}$ 0.6—1.8 pg/ml and TxB_2 3.0—45.3 pg/ml. These low values were also obtained in earlier studies using GC—NICI-MS [2, 5] by calculation of plasma levels from prostanoid concen-

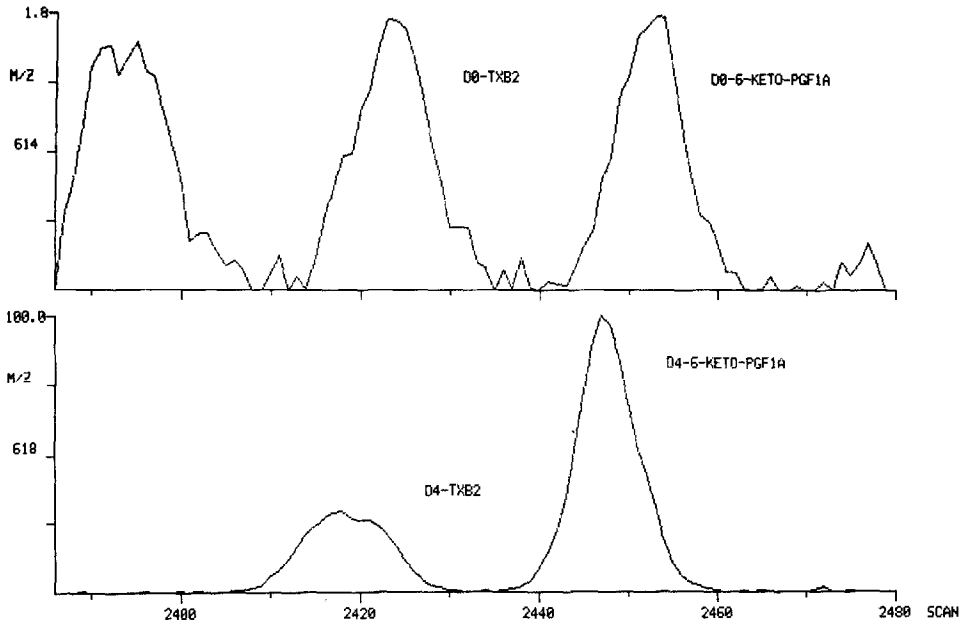

Fig. 4. SIM chromatogram of endogenous TxB_2 and 6-keto-$PGF_{1\alpha}$ (m/z 614; relative intensity 1.8%), and deuterated TxB_2 and 6-keto-$PGF_{1\alpha}$ (m/z 618; relative intensity 100%). Plasma concentrations: TxB_2 3.3 pg/ml, 6-keto-$PGF_{1\alpha}$ 1.0 pg/ml.

TABLE I

CORRELATION COEFFICIENTS (r), BLANKS AND PRECISION OF THE METHOD

Prostanoid	r	Blank (%)	Mean ± S.D. (pg/ml)	R.S.D. (%)
$PGF_{2\alpha}$	0.9998	0.2	4.84 ± 0.80	16.5
PGE_2	0.9999	0.1	2.16 ± 0.37	17.1
TxB_2	0.9990	0.2	5.14 ± 0.44	8.6
6-Keto-$PGF_{1\alpha}$	0.9995	0.5	0.92 ± 0.21	22.8

TABLE II

PROSTANOID CONCENTRATIONS IN PLASMA

Concentrations are expressed in pg/ml.

Volunteer (adult)	Large needle (1.5 mm I.D.)				Small needle (0.9 mm I.D.)			
	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TxB_2	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TxB_2
1	3.5	2.9	1.2	13.8	2.0	2.9	0.8	32.4
2	2.5	2.2	0.6	9.5	3.5	—	1.0	45.3
3	4.5	2.8	0.8	16.9	2.5	2.6	1.5	24.9
4	10.4	5.7	1.0	3.0	5.8	9.8	1.8	17.2
5					8.7	4.1	1.3	10.0

tration in urine after infusion of precursors [3, 4] and employing RIA after extraction and purification [7]. In contrast, other RIA investigations report very high levels of some hundred pg/ml [10].

For blood collection, needles with different inner diameters (0.9 and 1.5 mm) were used. The concentrations of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were not significantly affected, whereas TxB_2 levels were unpredictable higher when the smaller needle was used. The concentrations (n = 4) rose from 10.8 ± 6.0 pg/ml to 30.0 ± 12.0 pg/ml. This shows that TxB_2 levels are dependent on sampling conditions. Thrombocytes, endowed with an enormous capacity to synthesize and release TxB_2, are activated during the sampling procedure and increase TxB_2 levels by ex vivo production. The real TxB_2 concentrations in plasma may be much lower than the obtained values. Therefore, plasma TxB_2 levels should be interpreted with great caution unless sampling conditions are clearly described and accounted for.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Se 263/7-1). Dr. Seyberth is in receipt of a Heisenberg stipend from the Deutsche Forschungsgemeinschaft.

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