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Determination of 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, thromboxane B_2, 2,3-dinor-thromboxane B_2, PGE_2, PGD_2 and $PGF_{2\alpha}$ in human urine by gas chromatography–negative ion chemical ionization mass spectrometry

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ABSTRACT

A method for quantification of 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, TXB_2, 2,3-dinor TXB_2, PGE_2, PGD_2 and $PGF_{2\alpha}$ in human urine samples, using gas chromatography–negative ion chemical ionization mass spectrometry, is described. Deuterated analogues were used as internal standards. Methoximation was carried out in urine samples which were subsequently applied to phenylboronic acid cartridges, reversed-phase cartridges and thin-layer chromatography. The eluents were further derivatized to pentafluorobenzyl ester trimethylsilyl ethers for final quantification by gas chromatography–mass spectrometry. The overall recovery was 77% for tritiated 6-keto-$PGF_{1\alpha}$ and 55% for tritiated TXB_2. Urinary levels of prostanoids were determined in a group of six volunteers before and after intake of the thromboxane synthase inhibitor Ridogrel, and related to creatinine clearance.

INTRODUCTION

Thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), the predominant cyclooxygenase metabolites of arachidonic acid in the blood platelet and the vessel wall respectively, have potent and opposing effects on vascular tone and blood platelet functions. TXA_2 is a potent stimulator of platelet aggregation and a vasoconstrictor, whereas PGI_2 is a vasodilator and an inhibitor of platelet aggregation. The balance between their opposing effects is believed to be important for the homeostasis of the cardiovascular system [1,2].

Experimental as well as clinical studies suggest a role for TXA_2 in several pathogenic conditions of platelet activation, including myocardial infarction, unstable coronary diseases, pregnancy-induced hypertension and coronary throm-

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bolysis [3,4]. Furthermore, prostaglandins and TXA_2 are involved in the modulation of various renal functions, under normal as well as pathological conditions [5].

One important factor in the understanding and elucidation of the pathophysiological role of prostanoids is the quantitative determination of prostaglandins and their metabolites in biological fluids, particularly after application of thromboxane synthase inhibitors, for example. Owing to the extensive and rapid metabolism of prostanoids *in vivo* and the high risk of inducing artifacts during the invasive sampling of blood, measurements of plasma levels of prostanoids are often not very reliable for detecting changes in the endogenous biosynthesis of prostanoids *in vivo* [6]. Therefore, determination of prostanoids and their metabolites in urine has been used as an alternative non-invasive approach [3,4,6–9]. Furthermore, urinary prostanoids from renal and extrarenal (systemic) sources can be distinguished. In fact extrarenal (systemic) production of TXA_2 and PGI_2 is reflected by the urinary levels of 2,3-dinor-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$, respectively [6], whereas urinary TXB_2 and 6-keto-$PGF_{1\alpha}$ predominantly reflect renal TXA_2 and PGI_2 generation under physiological conditions [5,6]. Similarly, urinary PGE_2, $PGF_{2\alpha}$ and PGD_2 levels are generally a measure of renal prostaglandin synthesis, although extrarenal sources (*e.g.* seminal fluid) can substantially contribute to the urinary PGE_2 levels [5].

Urine appears to be the most difficult matrix to purify with regard to prostanoids, especially in humans [8]. This paper describes a new and simple method for the determination of renal (6-keto-$PGF_{1\alpha}$, TXB_2, PGE_2, PGD_2 and $PGF_{2\alpha}$) and systemic (2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2) metabolites of prostanoids in a single urine sample with high rates of recovery.

Quantification of the above-mentioned compounds was achieved after methoximation of prostanoids in urine, subsequent isolation and purification of the methoximated prostanoids, and formation of pentafluorobenzyl ester trimethylsilyl ether derivatives (Fig. 1). The method was applied for the determination of urinary prostanoids before and during the oral intake of Ridogrel, a new specific inhibitor of the thromboxane synthase [10–12].

EXPERIMENTAL

Reagents and solvents

[1-^{14}C]PGE_2, [5,6,8,9,11,14,15(*n*)-^{3}H]TXB_2 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.). TXB_2, PGE_2, PGD_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were purchased from Sigma (Munich, F.R.G.). 2,3-Dinor-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). [3,3,4,4-^{2}H]PGE_2 and [3,3,4,4-^{2}H]$PGF_{2\alpha}$ were obtained from IC Chemikalien (Munich, F.R.G.).

[18,18,19,19-^{2}H]TXB_2, [18,18,19,19-^{2}H]PGD_2, 6-keto[18,18,19,19-^{2}H]$PGF_{1\alpha}$, 2.3-dinor-6-keto[18,18,19,19-^{2}H]$PGF_{1\alpha}$, [18,18,19,19-^{2}H]TXB_2 and 2,3-di-

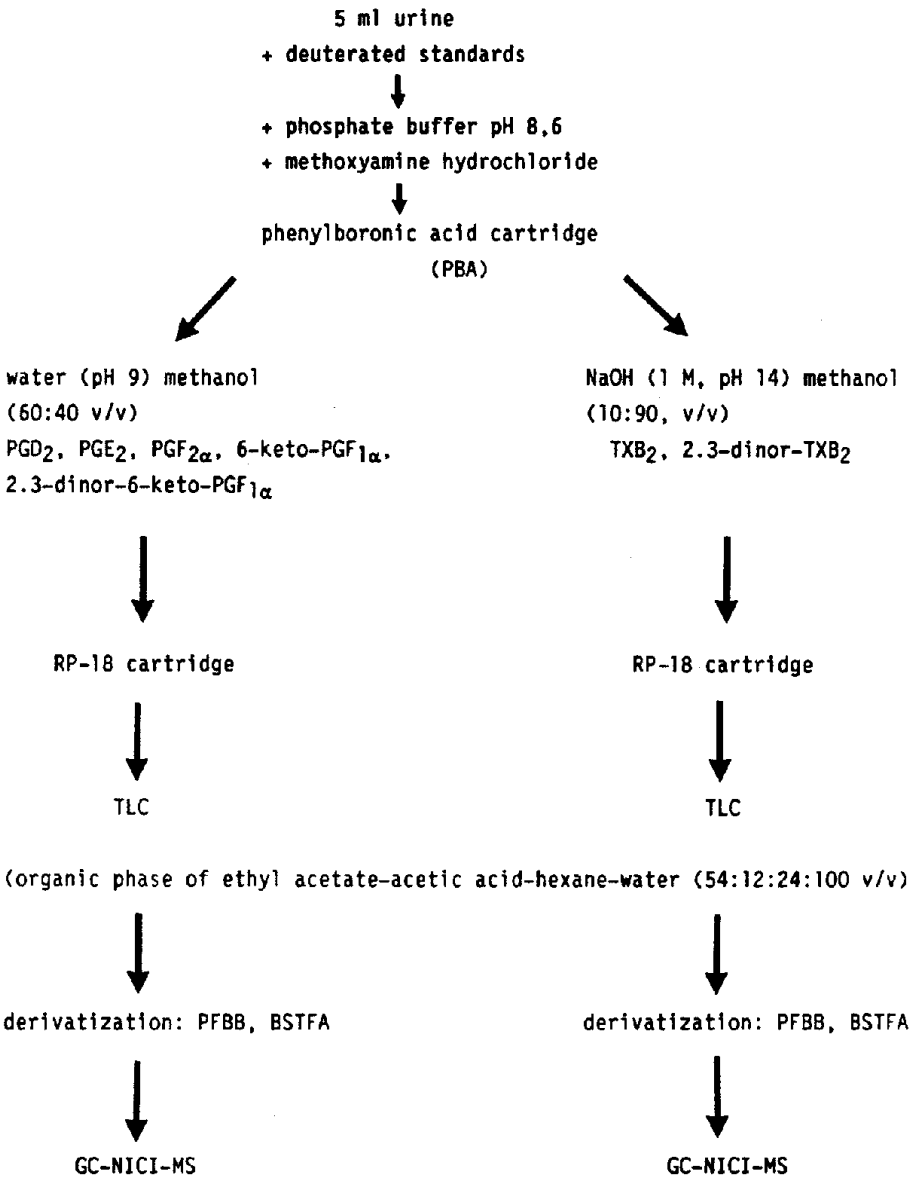

Fig. 1. Extraction and purification steps for prostaglandins and thromboxanes in urine prior to GC–MS analysis.

nor[18,18,19,19-^{2}H]TXB_2 were from Dr. C. O. Meese, Dr. Margarete-Fischer-Bosch-Institut (Stuttgart, F.R.G.). Bonded-phase phenylboronic acid (0.1 g) (PBA) and RP-18 (0.5 g) columns were purchased from ICT (Frankfurt, F.R.G.). Nineteen-channel silica glass plates were obtained from J. T. Baker (Groß-Gerau,

F.R.G.). O-Methylhydroxylammonium chloride was obtained from Merck (Darmstadt, F.R.G.) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Serva (Heidelberg, F.R.G.). All other reagents and solvents were of HPLC-grade and obtained from Merck.

Ultrapure demineralized water, obtained from a Milli-Q purification unit (Millipore, Eschborn, F.R.G.), was used. Glassware was silanized for 1 h with 2% (v/v) trimethylchlorosilane in toluene before use.

Biological samples

From six healthy volunteers, 24-h urine samples were collected before and after the intake of Ridogrel (R68070; Janssen Pharmaceutica, Beerse, Belgium). Volunteers were not allowed to take non-steroidal antiinflammatory drugs for at least ten days before and throughout the study. After a one-week control session, during which four 24-h urine collections were delivered, volunteers received 300 mg of Ridogrel orally twice daily (8 a.m., 8 p.m.), for two days. On the second day, another 24-h urine sample was collected (from 8 a.m. to 8 a.m. next day).

The total volume of urine was measured, and aliquots of 20 ml were immediately spiked with $^2H_4$-labelled standards: 10 ng of PGE_2, $PGF_{2\alpha}$, PGD_2, 6-keto-$PGF_{1\alpha}$, TXB_2 and 20 ng of 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2. After thorough mixing, the samples were stored at -70°C until analysis. Another aliquot (3 ml) was stored at -20°C for the determination of urinary creatinine levels.

Urinary levels of prostanoids were expressed as picograms of prostanoid per milligram of creatinine. Urinary creatinine was measured according to Kroll *et al.* [13] and used as a measure of renal excretion.

Extraction and purification procedure

Methoxyamine hydrochloride (0.5 g) and 1.5 ml of 2.4 *M* phosphate buffer (pH 8.6) were added to 5 ml of urine. The samples were mixed and allowed to react at 35°C for 30 min. The urine was applied to a PBA cartridge, which was prewashed with 3 ml of methanol and 3 ml of sodium hydroxide (pH 9). Prostaglandins were eluted first with 3 ml of water–methanol (60:40, v/v) adjusted to pH 9 with sodium hydroxide (PG fraction). Subsequently thromboxane and its dinor metabolite were eluted with a mixture of 1 ml of sodium hydroxide (1 *M*, pH 14) and 9 ml of methanol (TXB_2 fraction). The eluates were purified separately. Both fractions were diluted with water to a water–methanol ratio of 85:15 (v/v) and adjusted to a pH of 3.5 using formic acid. The two solutions were applied separately to RP-18 cartridges, which were prewashed with 10 ml of methanol, 10 ml of water and 5 ml of acidified water (pH 3.5; formic acid). The cartridges were flushed with 5 ml of methanol–water (pH 3.5; 15:85, v/v), and then prostaglandins and thromboxanes were eluted with 3 ml of ethyl acetate from each cartridge. The eluates were evaporated to dryness under a stream of dry nitrogen.

The residues were repeatedly dried with ethanol (500 μl, 200 μl and 50 μl) and afterwards redissolved in 25 μl of ethanol. Both fractions were applied to a nineteen-channel silica gel TLC glass plate (prewashed overnight with acetonitrile and dried at 60°C). The plate was developed (19 cm) with the organic phase of ethyl acetate–acetic acid–hexane–water (54:12:25:100, v/v). Corresponding zones of the eicosanoid-methoximes were localized by parallel development of 2 μg of a standard methoxime mixture. Visualization was achieved with 10% phosphomolybdic acid in ethanol [7]. The zones containing the prostanoids of interest were scraped out with a tolerance of ±0.2 cm or if possible with ±0.5 cm. After addition of 0.5 ml of water, the silica gel was extracted twice with 1.5 ml of ethyl acetate–acetic acid–hexane (54:12:25, v/v/v). The organic layers were combined and evaporated to dryness under nitrogen.

The dry residues of the methoxime derivatives (MO derivatives) were converted into their pentafluorobenzyl ester trimethylsilyl ether derivatives (PFB-esters-TMSi-ethers), as already described [14]. Samples were stored in 20 μl of BSTFA at 4°C until analysis.

Recovery, reproducibility and data analysis

For recovery studies, human urine (5 ml) was spiked with tritiated TXB_2 and 6-keto-$PGF_{1\alpha}$ and with ^{14}C-labelled PGE_2. Additionally, urine was spiked with 5 ng of each prostanoid for reproducibility studies ($n = 5$) and with 1, 2, 10, 15 and 20 ng of each prostanoid for validation of the accuracy of the method ($n = 1$). To each sample 2.5 ng of each deuterated prostanoid and 5 ng of each deuterated dinor metabolite were added. These samples underwent the whole purification procedure and GC–MS analysis. Data were calculated using a calibration curve in the range 0.2–20 ng of each prostanoid taken from standard solutions that did not undergo the extraction procedure [14].

Results were expressed as mean ± S.E.M. ($n = 6$). Statistical evaluation was performed using the Student's *t*-test for paired observations. *P*-values no greater than 0.02 were considered as being statistically significant, taking into consideration that day-to-day variations in the Ridogrel-treated group could not be compared with that in placebo-treated volunteers.

Gas chromatography–mass spectrometry

A Hewlett-Packard 5890 gas chromatograph equipped with a cooled injection system (Gerstel, Mühlheim, F.R.G.) was used. Helium was used as carrier gas and samples were injected in BSTFA (0.5 μl out of 20 μl) in the splitless mode. The injection port was temperature-programmed from 60°C to 250°C at 10°C/s and held at 250°C for 1.5 min. Chromatography was carried out with an Ultra 2 (12 m × 0.2 mm I.D., 0.33 μm film thickness) column for all eicosanoids except $PGF_{2\alpha}$. The GC oven temperature was programmed from 140°C to 290°C at 25°C/min. For determination of $PGF_{2\alpha}$ two columns were coupled: OV-17 (Weeke, Duisburg, F.R.G.; 30 m × 0.2 mm I.D.; 0.33 μm film thickness) and

DB-5 (J & W Scientific, ASS-Chem, Bad Homburg, F.R.G.; 30 m × 0.25 mm I.D.; 0.25 μm film thickness) and temperature-programmed from 140°C to 280°C at 25°C/min. The GC column was directly connected to the ion source of the mass spectrometer (MS 8230, Finnigan MAT). Analysis was carried out using negative ion chemical ionization (NICI) with ammonia as reagent gas. MS parameters were: ion source pressure, 0.2 Pa; ion source temperature, 200°C; emission current, 0.1 mA; electron energy, 92 eV. Registration and quantification of the $[M-PFB]^-$ ions were performed with multiple ion detection. (The resolution was 1000, and for $PGF_{2\alpha}$ it was increased to 2000.)

RESULTS AND DISCUSSION

Most methods for prostanoid methoximation in non-aqueous solutions require rather long reaction times or rather high reaction temperatures [8,15]. This is due to the fact that methoximation is limited by the rate of proton capture, which is restricted on an organic Lewis base with high proton affinity.

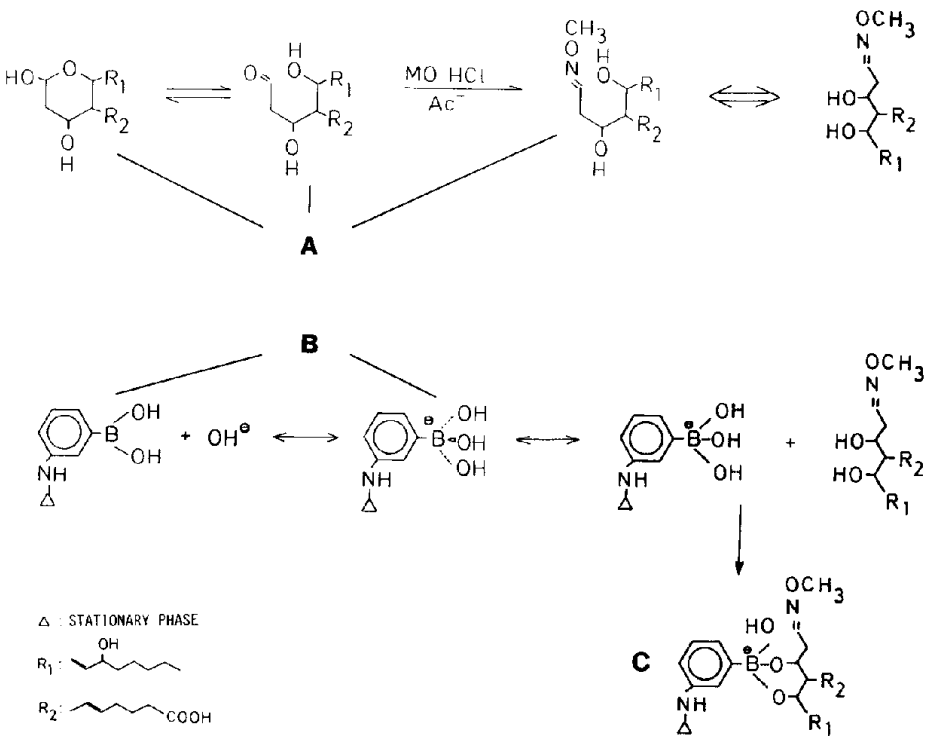

Fig. 2. (A) Methoximation of the open ring form of thromboxane. (B) Conditioning of phenylboronic acid towards boronates by alkalinization. (C) Chemical complex between 1,3-diol of thromboxane and boronate group of PBA cartridge.

TABLE I

RECOVERIES OF TRITIATED 6-KETO-$PGF_{1\alpha}$, TXB_2 and ^{14}C-LABELLED PGE_2

Method	Recovery (%, related to initial amount)		
	PGE_2	6-Keto-$PGF_{1\alpha}$	TXB_2
Bond Elut PBA			
MeOH–NaOH, pH 9 (40:60, v/v)	97	93	25
MeOH–NaOH, pH 14 (90:10, v/v)	–	–	60
Bond Elut RP-18	97	93	60 (TX fraction)
TLC	77	77	55 (TX fraction)

Furthermore, derivatization in organic phases is extremely sensitive to aqueous contaminants, since most organic Lewis bases suffer from a loss of potency in the presence of water. Moreover, from the analytical point of view, it is disadvantageous to extract prostanoids from an aqueous biological matrix into an organic system; it results in a low recovery and difficulties during derivatization. Therefore, it is worthwhile to carry out methoximation directly in urine samples in order to save time and increase the final recoveries. When methoximation is carried out directly in an aqueous solution, the solvent itself has the function of a very effective proton acceptor, if adequate buffering is provided. The completeness of prostanoid methoximation in urine was examined by radio-TLC using labelled TXB_2, PGE_2 and 6-keto-$PGF_{1\alpha}$. The reaction was quantitative at least after 30 min at 35°C.

The methoximated prostanoids were directly applied to PBA cartridges, which were conditioned at pH 9.0 in order to activate the tetrahedral anionic boronate. The result was a selective reaction with 1,2- and 1,3-diols and formation of covalent complexes [16].

Since thromboxanes are transformed into 1,3-diols during methoximation (Fig. 2), only the reaction products of these prostanoids were retained by chemical condensation on the PBA cartridge [16].

TABLE II

R_F VALUES OF TLC ZONES

Zone	R_F
6-Keto-$PGF_{1\alpha}$, dinor-6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$	0.35–0.50
TXB_2, dinor-TXB_2	0.54–0.65
PGE_2, PGD_2	0.79–0.90

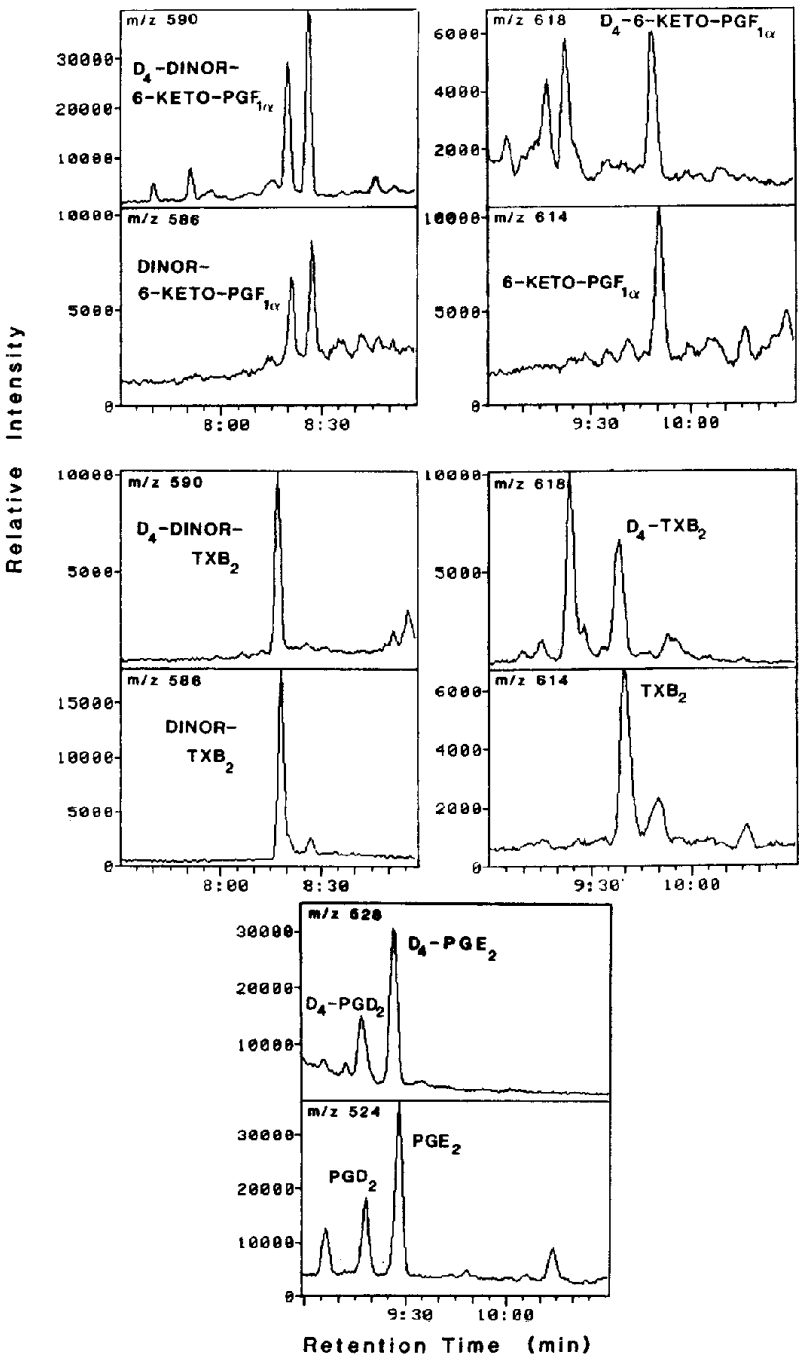

Fig. 3. GC–NICI-MS chromatograms of prostaglandins and thromboxanes in human urine using an Ultra 2 column (12 m × 0.2 mm I.D.; 0.33 μm film thickness).

The non-chemically bound prostaglandin methoximes retained by the cartridge could therefore be eluted almost quantitatively (Table I) with methanol–water (40:60, v/v) of pH 9.0. Unexpectedly, under these conditions thromboxane methoximes were coeluting already to an extent of 25%. Thromboxane methoximes that remained on the column after this step could be extracted quantitatively by applying methanol–water with a higher organic strength (90:10, v/v) and a pH of 14. This yielded the thromboxane derivatives in a very pure fraction, free from contaminants that interfere in final GC separation. This high purity was essential for the quantification of the very low urinary levels of thromboxane and dinor-thromboxane, particularly after thromboxane synthase inhibition.

Further purification of the prostaglandin methoxime and thromboxane methoxime fractions was achieved by C_{18} column chromatography without major loss of prostaglandins and thromboxanes (Table I). The subsequent TLC separation of both fractions resulted in the R_F values shown in Table II.

Elution of prostanoids from TLC plates for further analysis is one of the most wasteful steps, resulting in losses of up to 75% [5]. This is one major reason for the relatively low overall recoveries of prostanoids during sample pretreatment [5,13]. In this study, therefore, prostanoids were eluted from the plates with the same solvent used for the TLC itself. The recovery of this step was *ca.* 90% for all prostanoids investigated (Table I). The high recovery may be explained by the fact that the eluent is sufficiently polar to extract all the analytes of interest.

Chromatograms of the MO-PFB-esters-TMSi-ethers are shown in Fig. 3. In contrast to all other prostanoids, $PGF_{2\alpha}$ could not be separated from urinary contaminants on an Ultra 2 capillary column in GC–MS. However, quantification of $PGF_{2\alpha}$ could be achieved using a combination of OV-17 and DB-5 capillary columns for GC–MS analysis (Fig. 4). The detection limit was 10 pg/ml urine for all prostanoids. This sensitivity was high enough to detect the very low levels of TXB_2 and 2,3-dinor-TXB_2 resulting from thromboxane synthase inhibition. Although radioimmunoassay (RIA) is in general simple to perform and more sensitive than GC–MS, the success of the immunological method is crucially dependent on the specificity of the antibodies in terms of their cross-reactivity with various other structurally related compounds, and its non-immunological interaction with other material in the analytical matrix. In particular, in urine the presence of a large number of potentially cross-reacting enzymic derivatives of prostaglandins and TXB_2, excreted in an 10- to 100-fold excess over unmetabolized eicosanoids, and unidentified contaminants, differently interfering with different antibodies, represents a serious limitation on the specificity of such immunological measurements, even after extensive extraction and chromatographic clean-up of the sample [6,17,18].

The reproducibility for the whole extraction procedure and GC–MS analysis is given in Table III. The accuracy of the method is given in Table IV. Calibration curves obtained with urine samples resulted in correlation coefficients better than 0.994 (range from 40 pg/ml to 20 ng/ml).

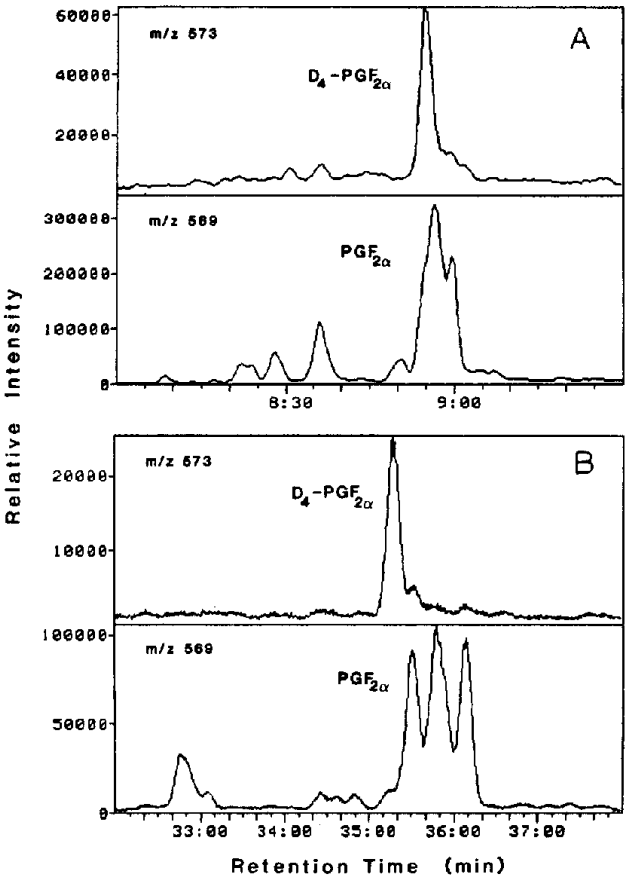

Fig. 4. GC–NICI-MS chromatogram of $PGF_{2\alpha}$ in human urine: (A) Ultra 2 column (12 m × 0.2 mm I.D.; 0.33 μm film thickness); (B) OV-17 (30 m × 0.2 mm I.D.; 0.33 μm thickness) connected with DB-5 (30 m × 0.2 mm I.D.; 0.33 μm film thickness).

TABLE III

REPRODUCIBILITY OF EXTRACTION PROCEDURE AND GC–MS ANALYSIS

A 5-ng amount of each compound was added to 5 ml of urine; S.D. data relate to the mean value (n = 5).

Compound	S.D. (%)
PGD_2	5.0
PGE_2	10.8
$PGF_{2\alpha}$	2.3
6-Keto-$PGF_{1\alpha}$	4.3
2,3-Dinor-6-keto-$PGF_{1\alpha}$	6.7
TXB_2	5.6
2,3-Dinor-TXB_2	9.9

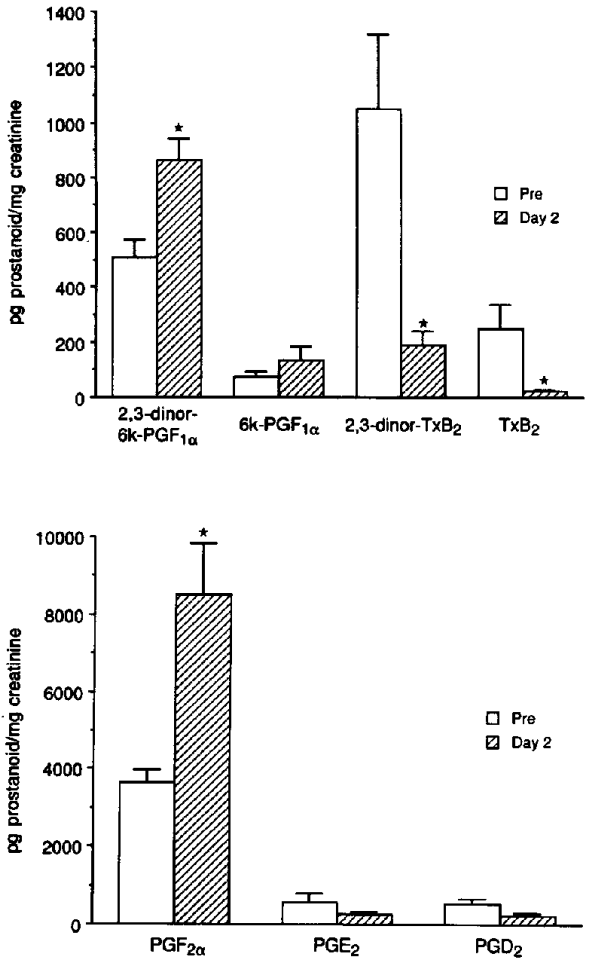

Fig. 5. Effect of Ridogrel (300 mg b.i.d. orally in six volunteers) on the urinary excretion of prostanoids and metabolites. Results are expressed as pg prostanoid/mg creatinine excreted (mean ± S.E.M., $n = 6$). Levels of prostanoids measured four times during a one-week control session in each volunteer were averaged and served as control level prior to Ridogrel treatment.

Urinary excretion of prostanoids in six healthy volunteers under basal conditions was compared with that during intake of Ridogrel (300 mg, b.i.d.), a specific inhibitor of thromboxane synthase [11,12]. The results are summarized in Fig. 5. Ridogrel intake resulted in a significant reduction of the systemic (measured as 2,3-dinor-TXB_2) as well as the renal (measured as TXB_2) levels of thromboxane (80 ± 3% and 84 ± 7% inhibition, respectively).

Concomitantly, systemic formation of PGI_2 (measured as 2,3-dinor-6-keto-$PGF_{1\alpha}$) was slightly but significantly increased, which suggests a reorientation of platelet-derived prostanoid endoperoxides towards prostacyclin biosynthesis af-

TABLE IV

CONCENTRATIONS OF EICOSANOIDS FOUND AFTER ADDITION OF DEFINED AMOUNTS TO HUMAN URINE AND SUBTRACTION OF THE BLANK VALUE

Values in ng/ml; urine sample size 5 ml (n = 1).

Compound	Amount added (ng/ml)				
	0.4	1	2	3	4
PGD_2	0.40	1.1	2.6	3.4	4.7
PGE_2	0.35	0.6	1.8	2.6	3.8
$PGF_{2\alpha}$	0.32	1.0	1.8	2.8	4.3
6-Keto-$PGF_{1\alpha}$	0.38	0.9	1.8	2.6	3.5
2,3-Dinor-6-keto-$PGF_{1\alpha}$	0.37	1.7	2.0	3.0	4.1
TXB_2	0.42	1.1	1.9	3.5	4.4
2,3-Dinor-TXB_2	0.46	0.9	1.3	3.0	3.6

ter thromboxane synthase inhibition, as also demonstrated by others [9]. No significant changes were observed in the renal production of 6-keto-$PGF_{1\alpha}$, PGE_2 and PGD_2, whereas the excretion of renal $PGF_{2\alpha}$ was significantly increased.

These results confirm our previous findings that Ridogrel is a specific inhibitor of the platelet thromboxane synthase after oral administration in humans [12]. Furthermore, the study demonstrates that Ridogrel is also a specific inhibitor of the renal thromboxane synthase, making it a suitable tool to analyse the role of the balance between TXA_2 and PGI_2 in vascular and renal pathologies.

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