Journal of Chromatography, 417 (1987) 1–10
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3590

PURIFICATION AND QUANTITATIVE ANALYSIS OF URINARY PROSTANOIDS IN HUMAN AND IN RAT BY PACKED AND CAPILLARY GAS CHROMATOGRAPHY–NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

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(First received September 18th, 1986; revised manuscript received January 5th, 1987)

SUMMARY

We describe a method for the quantitative analysis of prostaglandin (PG) E_2 and the major urinary metabolites PGI_2 and thromboxane (Tx) A_2 in human and in rat by combined gas chromatography and negative-ion chemical-ionization mass spectrometry. The procedure is based on the sequential use of small columns with distinct properties combined with a thin-layer chromatography step, for the extraction and the purification of urinary prostaglandins. The compounds are then analysed as their pentafluorobenzyl ester-O-methyloxime-trimethylsilyl ether derivatives, using either packed or capillary columns. Deuterated analogues are used as internal standards. The method was established by using tritiated prostaglandins covering the extremes of polarity in order to optimize the recovery of prostanoids as well as the quality of the chromatograms and spectra. The overall recovery was 24%. Standard curves were obtained by the same procedure and found to be reproducible, with a maximal day-to-day variation of $\pm 5\%$. The relatively simple approach required for the sequential extraction and purification of prostaglandins on small columns of distinct properties, combined with the highly specific and highly sensitive method of detection, places this procedure among the most reliable method for measuring urinary prostanoids in both humans and animals. In addition, the procedure is faster than classical approaches and necessitates smaller amounts of samples and solvents.

INTRODUCTION

Prostaglandin (PG) I_2 and tromboxane (Tx) A_2, the major endoperoxide metabolites synthesized by the blood vesels and platelets, respectively, have sig-

0378-4347/87/$03.50

nificant biological properties: PGI_2 is a vasodilator substance and a potent inhibitor of platelet aggregation whereas TxA_2 induces platelet aggregation and vasoconstriction. The balance between the opposing effects of PGI_2 and TxA_2 is thought to be important for the homeostasis of the cardiovascular system [1–5]. PGE_2, the major prostaglandin synthesized in the renal medulla, is a vasodilator and a natriuretic substance which, together with PGI_2, is involved in the modulation of various renal functions, such as renal haemodynamics, renin secretion and sodium and water excretion [3,6,7].

One important approach for understanding the role of prostaglandins in the homeostasis of the cardiovascular and the renal systems includes the quantitative determination of prostaglandins and their metabolites in biological fluids. For many years, combined gas chromatography–mass spectrometry (GC–MS) with traditional electron-impact ionization was the method of reference for the quantitative analysis of eicosanoids. However, the relative lack of sensitivity of the method proved to be a serious drawback. Recently, electron-capture negative-ion chemical-ionization mass spectrometry (NICIMS) has gained popularity for the measurement, in biological fluids, of endogenous substances, including arachidonic acid metabolites, in the femtomole–picomole range [9–19]. Various methods have been described for the measurement of specific prostanoids in plasma or urine in humans and animals: urine appears, by far, to be the most difficult matrix to purify with regard to prostaglandins, especially in humans.

In this context, we have developed a method for the quantitative analysis of PGE_2 and the major metabolites of PGI_2 and TxA_2 in urine, in both human and rat. The method relies upon the sequential utilization of small columns and thin-layer chromatoraphy (TLC) for the extraction and the purification of prostaglandins in urine, followed by their analysis by electron-capture GC–NICIMS, using either packed or capillary columns.

EXPERIMENTAL

Materials

Tritiated 6-oxo-$PGF_{1\alpha}$ and PGE_2 of high specific activity were purchased from New England Nuclear, via Dupont Canada (Dorval, Canada). [$^2H_4$]PGE_2 and [$^2H_4$]-6-oxo-$PGF_{1\alpha}$ were obtained from Merck Sharp and Dohme (Pointe-Claire, Canada), [8,10,10-$^2H_3$]-2,3-dinor-6-oxo-$PGF_{1\alpha}$ was prepared as already described [20]. All other prostaglandins were kindly supplied by Dr. J.E. Pike from Upjohn (Kalamazoo, MI, U.S.A.). All solvents, from Caledon (Georgetown, Canada), were glass-distilled or HPLC-grade. Clin Elut columns were purchased from Analytichem (Harbor City, CA, U.S.A.). Silica TLC plates (J.T. Baker silica gel G) were purchased from Canlab (Montreal, Canada). A lipophilic dye (De Saga, Heidelberg, F.R.G.), used as reference for TLC, was obtained from Canlab. Pentafluorobenzyl bromide (PFBB) was purchased from Pierce (Rockford, IL, U.S.A.), and diisopropylethylamine (DIPEA) and N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Chromatographic Specialities (Brockville, Canada).

Biological samples

Twenty-four urinary collections were obtained from normal healthy volunteers or from rats. Aliquots corresponding to 1/30 (0.2–1 ml) of the total urinary volume in rats and 1/400 (1–5 ml) in humans were taken for analysis. Each aliquot was immediately spiked with the following internal standards: [8,10,10-$^2H_3$]-2,3-dinor-6-oxo-$PGF_{1\alpha}$ (5 ng), [3,3,4,4-$^2H_4$]-6-oxo-$PGF_{1\alpha}$ (10 ng), [19,19,20,20-$^2H_4$]TxB_2 (10 ng), [3,3,4,4-$^2H_4$]PGE_2 (20 ng) and [19,19,20,20-$^2H_4$]-2,3-dinor-TxB_2 (20 ng). The samples were stored at −80°C until further processing. In order to assess recoveries, some samples were also spiked with tritiated PGE_2 and 6-oxo-$PGF_{1\alpha}$.

Extraction and purification procedure

After overnight equilibration at 4°C, each sample is acidified at pH 3–3.5 with 4 *M* hydrochloric acid and poured over a Clin Elut column. Prostaglandins are eluted with ethyl acetate and further extracted in 50 m*M* Tris buffer (pH 8). 2,3-Dinor-6-oxo-$PGF_{1\alpha}$, which forms a lactone in acidic media, remains in the organic phase and is purified as previously described [21]. The aqueous phase is acidified at pH 3–3.5 and applied in a polypropylene syringe on a C_{18} Sep-Pak cartridge previously washed with ethanol and water. Prostaglandins are eluted with 7 ml of ethyl acetate–dichloromethane (75:25, v/v). The obtained solution is diluted with 13 ml of dichloromethane and applied on a silica Sep-Pak cartridge prewashed sequentially with methanol, ethyl acetate and hexane. After application of the sample, the cartridge is washed with 5 ml ethyl acetate–dichloromethane (50:50, v/v) and the prostaglandins are eluted with 5 ml of methanol–dichloromethane (60:40, v/v).

Derivatization and TLC purification

After evaporation of the solvent and transfer of each sample in a reacti vial, the prostaglandins are converted into their pentafluorobenzyl ester-O-methyloxime derivatives. The ester is made first, by letting the sample react with 10 μl of 35% PFBB in acetonitrile and 10 μl of DIPEA at 40°C for 30 min. The solvent is evaporated and the prostaglandins are then converted into their O-methyloxime derivatives by adding 50 μl of a solution of methoxyamine hydrochloride (MOX) in pyridine (5 mg/ml) and allowing the reaction to proceed overnight at room temperature.

Pyridine is then removed under a stream of nitrogen, and the sample is dissolved in dichloromethane and transferred on the preconcentration zone of a 19-channel silica gel G TLC plate prewashed twice with acetonitrile. The plate is developed with the organic phase of ethyl acetate–acetic acid–hexane–water (54:12:25:60, v/v) according to Blair et al. [9]. The zones containing the prostaglandins of interest are extracted from the silica with either ethyl acetate (2×2 ml) or 1 ml of methanol–ethyl acetate (20:80, v/v). The solvent is evaporated and the samples are stored in 25 μl of pyridine at −80°C. Prior to the analysis, the samples are further converted into their trimethylsilyl ether derivatives by adding 25 μl of BSTFA at room temperature for 15 min. After evaporation of the mixture, the samples are dissolved in dry hexane and analysed.

The same derivatization–TLC procedure is used for the preparation of the standard curves. Each standard is stored in pyridine at −80°C, at its pentafluorobenzyl ester-O-methyloxime derivative.

GC–NICIMS analysis

The samples are analysed on a Hewlett-Packard Model 5984B gas chromatograph–mass spectrometer by selectively monitoring the $[M-PFB]^-$ ion. A 3% SP-2100 packed column (1.2 m × 2 mm I.D.) is programmed from 270°C (1 min) to 300°C at 10°C/min (helium flow-rate, 30 ml/min). When a capillary column is used, a 12 m × 0.2 mm I.D. (0.33 μm film thickness) cross-linked methylsilicone capillary column (Hewlett-Packard, Pointe-Claire, Canada) is programmed from 100°C (1 min) to 290°C at 15°C/min. The injection port is held at 250°C and the samples are injected in splitless mode. Methane (at a source pressure of 0.9 mmHg) is used as the reagent gas and the source temperature maintained at 100°C.

RESULTS

Fig. 1 summarizes the various steps in the extraction and purification of the samples prior to their analysis. The method was established by using tritiated prostaglandins covering the extremes of polarity (PGE_2 and 6-oxo-$PGF_{1\alpha}$). The percentage recoveries obtained for these two prostaglandins at each step of the procedure are given in Table I. The overall recovery was in the range 20–25%, the major losses occurring at the silica Sep-Pak and the TLC steps.

The R_F values for the eicosanoids of interest (as their pentafluorobenzyl ester-O-methyloxime derivatives) are given in Table II. A lipophilic dye was added in separate channels to facilitate the location of the different compounds: the purple dye served as reference. Three zones (length 1–2.5 cm) were scraped off the plate: the first contained 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (in a separate channel), the next 6-oxo-$PGF_{1\alpha}$ and the last TxB_2, 2,3-dinor-TxB_2 and PGE_2.

The retention times of the different prostaglandins on either packed or capillary columns, together with the ions monitored, are summarized in Table III. As expected, a much better separation of the various prostaglandins was obtained on the capillary column. Interestingly, the isomers of the pentafluorobenzyl ester-O-methyloxime-trimethylsilyl ether derivative of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ could be resolved on the capillary column, whereas a single symmetrical peak is regularly observed on packed columns.

The GC–MS traces obtained from rat and human urine are illustated in Fig. 2. The deuterated analogues have slightly shorter retention times than their natural counterparts on the capillary column. In the rat, all the desired prostanoids could be easily quantitated on either packed or capillary columns except 2,3-dinor-TxB_2, which is absent from rat urine [26]. In human, capillary columns were optimal for monitoring 2,3-dinor-TxB_2 (which was often buried in matrix interferences on packed columns), PGE_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$, whereas packed columns were surprisingly efficient for monitoring TxB_2 and 6-oxo-$PGF_{1\alpha}$, following their prior separation by TLC. In our study, the prostanoids were analysed by groups

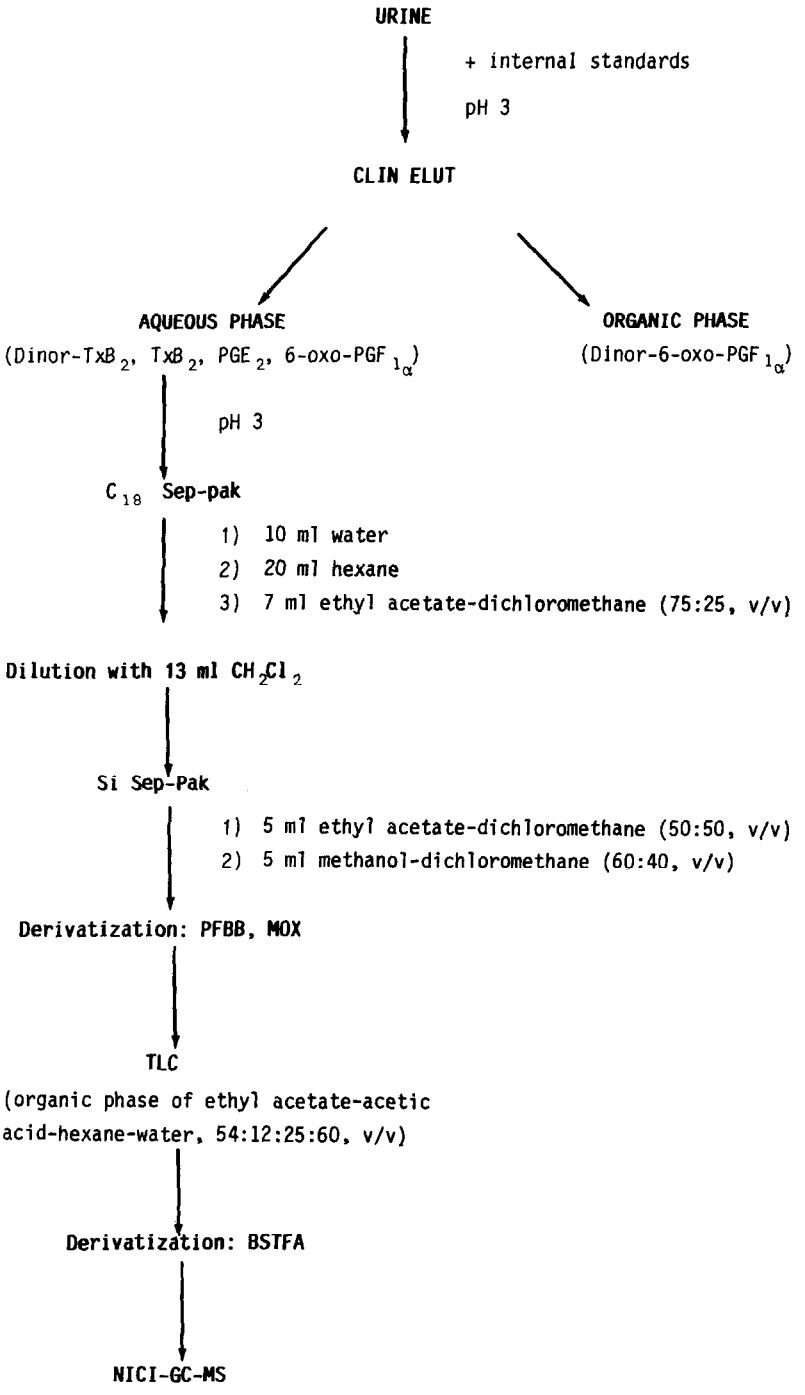

Fig. 1. Summary of the steps involved in the extraction and purification of prostaglandins prior to analysis.

TABLE I

RECOVERIES OF TRITIATED PGE_2 AND 6-OXO-$PGF_{1\alpha}$

Values are % cpm added to urine.

	Recovery (%)	
	PGE_2	6-Oxo-$PGF_{1\alpha}$
Clin Elut	100	95
Tris	90	86
C_{18} Sep-Pak	87	77
Silica Sep-Pak	61	59
TLC	24	22

TABLE II

R_F VALUES OF PROSTAGLANDINS AS THEIR PENTAFLUOROBENZYL ESTER-O-METHYLOXIME DERIVATIVES ON TLC

Solvent system: organic phase of ethyl acetate–acetic acid–hexane–water (54:12:25:60, v/v).

Compound	R_F
Purple dye	0.23
6-Oxo-$PGF_{1\alpha}$	0.27
Dinor-6-oxo-$PGF_{1\alpha}$	0.30
Dinor-TxB_2	0.45
TxB_2	0.47
PGE_2 minor isomer	0.46
PGE_2 major isomer	0.55

TABLE III

FRAGMENT IONS MONITORED FOR THE QUANTITATIVE ANALYSIS OF PROSTAGLANDINS AND THEIR RETENTION TIMES ON PACKED AND CAPILLARY COLUMNS (PENTAFLUOROBENZYL ESTER-O-METHYLOXIME-TRIMETHYLSILYL ETHER DERIVATIVES)

Compound	Fragment (M−181) (*m/z*)			Retention time (min)	
	$[^2H_0]$	$[^2H_3]$	$[^2H_4]$	Packed	Capillary
Dinor-TxB_2	586		590	2.10	15.39
Dinor-6-oxo-$PGF_{1\alpha}$	586	589		2.20	15.45
					15.59
PGE_2 minor	524		528	2.50	16.16
PGE_2 major	524		528	2.80	16.80
TxB_2	614		618	2.95	17.02
6-Oxo-$PGF_{1\alpha}$	614		618	3.10	17.23

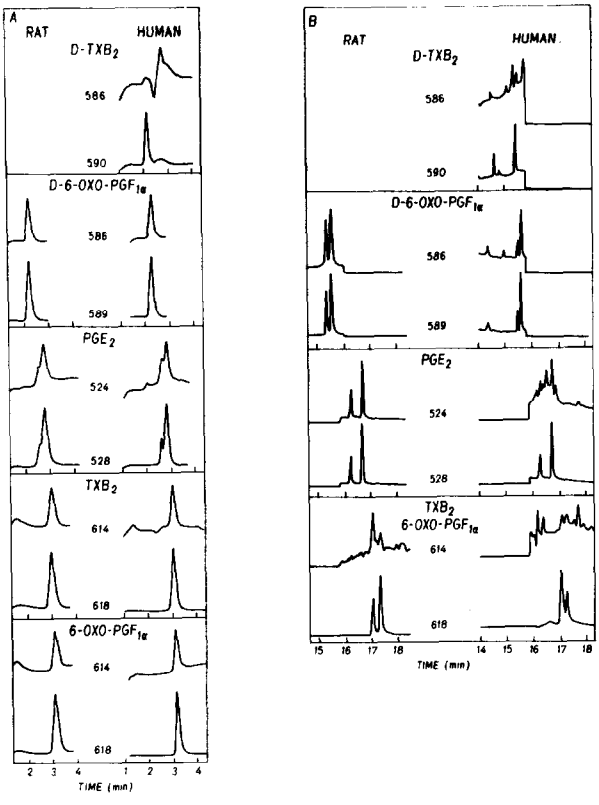

Fig. 2. GC–NICIMS traces of prostaglandins as their pentafluorobenzyl ester-O-methyloxime-trimethylsilyl ether derivatives in human and in rat using packed (A) and capillary (B) columns.

of two or three, since our program for analysis by selected-ion monitoring does not allow for the simultaneous analysis of more than four ions.

The sensitivity of the assay was regularly in the order of a few picograms of prostaglandins injected on-column (either packed or capillary). Standard curves (D_0/D_3 or D_0/D_4 in the range 0.05–0.40) were obtained by the same derivatization–TLC procedure and were linear. The stability of the derivatives was found to be adequate. Solutions for standard curves were prepared and injected several times: each time that the solutions were thawed, an aliquot was taken for further derivatization and analysis and the solutions frozen again at $-80\,^{\circ}C$. Similar curves were obtained for a period of at least two months, with a day-to-day variation of $\pm 5\%$. No loss of sensitivity could be detected, indicating that the pentafluorobenzyl ester-O-methyloxime derivatives are stable in pyridine at $-80\,^{\circ}C$.

DISCUSSION

The quantitative determination of urinary prostanoids is a well established approach for evaluating the renal and the systemic production of prostaglandins

in vivo [22–26]. However, a biological matrix such as urine contains a large amount of endogenous substances, which interfere with the analysis of prostaglandins that are found only in very low concentrations. Classically, the analysis of urinary prostaglandins is tedious, involving their extraction from the matrix, followed by several (at least two) steps of purification by column chromatography [open-bed or high-performance liquid chromatography (HPLC)] prior to their quantitative determination by either radioimmunoassay or GC–MS [8,27,28]. The rate-limiting step in the analysis of urinary prostanoids is their purification by standard column chromatography.

The sequential use of small columns with distinct properties, combined with a TLC step, for the extraction and purification of urinary prostaglandins provides a simple, comparatively inexpensive and relatively fast method for processing simultaneously several samples. This approach requires minimal amounts of solvents and is within the reach of all laboratories, since it obviates the need for an HPLC system or the use of classical time-consuming open-bed column chromatography.

Using tritiated PGE_2 and 6-oxo-$PGF_{1\alpha}$ as references, overall recoveries of 59–61% could be obtained for the three combined steps of column extraction and purification. Since this was associated with GC–MS traces of variable quality, the polarity of the extraction solutions was then adjusted in order to obtain a good compromise between high yields and reliable GC–MS traces. The overall final yield of 20–25% is adequate considering the high sensitivity of detection of prostaglandins by GC–NICIMS.

The TLC step was found to have several advantages. Firstly, it provides an additional necessary easy step of purification. Secondly, it allows for the separation of groups of prostaglandins, an essential step when using packed columns for GC–MS analysis. The excess reagents added to the sample before the TLC (MOX, PFBB) stay at the bottom of the plate (R_F 0–0.15), so that after TLC no further purification is needed. In the literature, the TLC step is usually done on the free acid O-methyloxime derivative [9]; this approach, however, requires a subsequent purification step in order to get rid of the excess reagent used for esterification of the prostanoids.

Compared with packed columns, capillary columns can achieve much better chromatographic resolution, with complete separation of the prostaglandins of interest. This advantage of capillary columns is particularly evident in the case of dinor-TxB_2, where contamination from the matrix often interferes with a proper analysis of the compound on packed columns. However, packed columns are still quite satisfactory for the analysis of dinor-6-oxo-$PGF_{1\alpha}$, TxB_2 and 6-oxo-$PGF_{1\alpha}$. The shorter analysis time provided by packed columns can be of interest when only one or two prostanoids have to be measured in a particular experiment: the lack of resolution associated with the use of packed columns is compensated by a good separation of the interfering prostaglandins on TLC.

In conclusion, the relatively simple approach required for the sequential extraction and purification of prostaglandins on small columns of distinct properties, combined with the highly specific and highly sensitive method of detection

conferred by GC–NICIMS, places this procedure among the most reliable methods for measuring urinary prostanoids in both humans and animals. In addition, the procedure is faster than classical approaches and necessitates smaller amounts of samples and solvents.

ACKNOWLEDGEMENTS

The authors thank Edith Bellemare and Michel Robillard for their expert technical assistance and Sylvie Ouellet for her precious secretarial assistance. This study was supported by the Medical Research Council of Canada (Grant No. MA-6753) and the Quebec Heart Foundation.

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