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DETERMINATION OF 2,3-DINOR-6-KETOPROSTAGLANDIN $F_{1\alpha}$ IN URINE SAMPLES BY LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

JOSEFINA CASAS, JOAN ROSELLO and EMILIO GELPÍ*

Department of Neurochemistry, Centro de Investigación y Desarrollo (CSIC), Jorge Girona Salgado, 18–26, 08034 Barcelona (Spain)

FRANCISCO GUARNER*

Department of Medicine, Clinica Universitaria, University of Navarra, Pamplona (Spain)

CARLOS GUARNER

Gastroenterology Division, Hospital de la Santa Creu i Sant Pau, Barcelona (Spain)

and

JORGE QUIROGA, INMACULADA COLINA and JESUS PRIETO

Department of Medicine, Clinica Universitaria, University of Navarra, Pamplona (Spain)

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SUMMARY

A method for 2,3-dinor-6-ketoprostaglandin $F_{1\alpha}$ quantification based on high-performance liquid chromatography–radioimmunoassay is described. Samples are acidified to pH 3 and processed through C_{18} disposable cartridges. The prostanoids are eluted with methyl formate and further separated on a reversed-phase column using acetonitrile–acetic acid-triethylamine buffer (32:68). Studies of the effect of eluent pH were performed in order to optimize resolution and separation of 2,3-dinor-6-keto-$PGF_{1\alpha}$ from other prostanoids. Eluates were collected and assayed by radioimmunoassay using a heterologous system, with 6-keto-$PGF_{1\alpha}$ as radioligand and an antiserum with high cross-reactivity for 2,3-dinor-6-keto-$PGF_{1\alpha}$. Sensitivity, precision and accuracy of the assay procedure are reported together with the validation of its specificity. The proposed method has been applied to the determination of this prostacyclin metabolite in human urine.

*Present address: Department of Hepatology, Hospital Clinico Provincial, Barcelona, Spain.

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INTRODUCTION

Prostacyclin is the main cyclo-oxygenase product generated by blood vessels [1]. Its powerful anti-aggregatory and vasodilatory properties caused particular interest in its role in vascular physiology and in some pathological disorders. After its local synthesis, it is readily transformed into 6-ketoprostaglandin $F_{1\alpha}$ and then into further metabolites. Different prostacyclin metabolites have been detected in human urine, including 2,3-dinor-6-keto-$PGF_{1\alpha}$ as the major product [2,3]. Since plasma levels of 6-keto-$PGF_{1\alpha}$ are low and may be influenced by sampling-induced artifacts and ex vivo prostaglandin formation, quantification of 2,3-dinor-6-keto-$PGF_{1\alpha}$ has been proposed as a more reliable index of endogenous prostacyclin synthesis [4].

Quantitative analysis of dinor urinary metabolites of prostacyclin by gas chromatography–mass spectrometry (GC–MS) has been reported by several groups [5–7]. Extraction of samples, extensive purification and multi-step derivatization are required prior to GC–MS analysis. Furthermore, the necessary GC–MS instrumentation is not readily available in most clinical laboratories. In addition, the GC–MS method does not seem to be as accurate as expected, according to the substantial differences in estimates of normal levels [3,6,8,9].

The aim of the current study was the development of a combined high-performance liquid chromatography–radioimmunoassay (HPLC–RIA) alternative procedure for the determination of this urinary prostacyclin metabolite. Our approach is an attempt to capitalize on the advantages of RIA in terms of sensitivity, precision and compliance to a large number of samples. Specificity is increased by previous HPLC purification [10].

EXPERIMENTAL

Chemicals

Acetonitrile (ACN) HPLC grade was purchased from Koch-Light (Suffolk, U.K.) and methyl formate and petroleum ether from Fluka (Buchs, Switzerland). Phosphoric acid and triethylamine (TEA) were both from Scharlau (Barcelona, Spain). Polyethylene glycol 4000, N-butylamine, N-dibutylamine, formic acid (FA), acetic acid (AA), Tris and hydrochloric acid were from Merck (Darmstadt, F.R.G.). Bovine serum albumin and gamma-globulins were obtained from Sigma (St. Louis, MO, U.S.A.). Lysine-acetylsalicylic acid was from Almirall (Barcelona, Spain). Tritiated prostaglandins were purchased from New England Nuclear (Dreieich, F.R.G.). Scintillation fluid used was from Packard (Instagel). Prostaglandin standards were a generous gift from Dr. J.E. Pike of the Upjohn Co. (Kalamazoo, MI, U.S.A.).

Collection of samples

Eight healthy subjects (four males and four females) were included in the study. After two days on controlled sodium intake (40 mmol per day), oral water was given at 7 am (5 ml per kg body weight) and urine was collected from 7 to 9 am from subjects who were fasting and on bed rest. Lysine-acetylsalicylic acid was

added to the samples at a concentration of 0.5 mg/ml, and these were stored at −20 °C until assay. Five patients with diagnosed cirrhosis under controlled sodium intake were also studied, before and after a single 400-mg dose of sulindac given 4 h prior to urine collection.

Extraction

Aliquots of urine (5 ml) were adjusted to pH 3 with 50% FA, and applied to disposable Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) for prostaglandin extraction according to Powell [11]. The methyl formate eluates were evaporated to dryness under a stream of purified helium, and the residues were taken up in 1 ml of AA-TEA–ACN (68:32, pH 5.8). A 200-μl aliquot of this solution was analysed by HPLC.

Chromatography

Sample enrichment for RIA was carried out on a 30 cm×3.9 mm I.D. μBondapak, 10 μm, C_{18} reversed-phase column from Waters Assoc. Elution was carried out at 1.5 ml/min with various buffer–ACN (68:32) mixtures differing in the composition of the buffers AA-TEA or FA-TEA. The pH values of the buffers were adjusted with TEA in all cases. The instrumentation used consisted of two pumps, Model LC 414, a Series 200 solvent programmer from Kontron (Zürich, Switzerland), a Rheodyne 7125 injector and a variable-wavelength UV detector Uvikon 722 system, also from Kontron. Eluents were monitored at 192 nm. Alternatively, a diode array UV detector, Model 1040 from Hewlett Packard (Avondale, PA, U.S.A.), was used. All buffers were filtered through 0.45-μm Millipore (Bedford, MA, U.S.A.) membrane filters and degassed before use. Eluent fractions were collected at appropriate retention times, previously established for 2,3-dinor-6-keto-$PGF_{1\alpha}$, and lyophilized prior to RIA. The lyophilized fractions were stored at −40 °C until assay.

Production of antisera

Authentic 6-keto-$PGF_{1\alpha}$ was conjugated to bovine serum albumin, and three rabbits were immunized as described by Kirton et al. [12]. One of the antisera raised showed high cross-reactivity with 2,3-dinor-6-keto-$PGF_{1\alpha}$ (16%). Association constants at equilibrium were estimated as $2.4 \cdot 10^{10}\ M^{-1}$ for 6-keto-$PGF_{1\alpha}$ and $3 \cdot 10^{9}\ M^{-1}$ for 2,3-dinor-6-keto-$PGF_{1\alpha}$. Cross-reactivities with other prostanoids were as follows: thromboxaneB_2 (TXB_2), 5%; PGE_2, 4.7%; $PGF_{2\alpha}$, PGD_2, $PGF_{1\alpha}$, 2,3-dinor-6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$, 2,3-dinor-TXB_2 and arachidonic acid, all less than 0.1%.

RIA procedure

Immunoassays were performed using the method described by Salmon for 6-keto-$PGF_{1\alpha}$ [13], with tritiated 6-keto-$PGF_{1\alpha}$ as radioligand (11 pg, 120 Ci/mmol) and the above-mentioned antiserum at a final dilution of 1:40 000. A standard curve was prepared with authentic 2,3-dinor-6-keto-$PGF_{1\alpha}$ from 10 to 3000 pg (eleven points) diluted in Tris–HCl buffer 50 m*M*, pH 7.4. HPLC eluates were taken up in 1 ml of buffer and the assay was carried out with 100-μl aliquots.

Separation of bound and free fractions was performed by the polyethylene glycol method [14], and supernatants were counted on a LKB 1217 Rackbeta (LKB, Turku, Finland).

Radioimmunoassays for other prostaglandins were performed as described elsewhere [15]. Antiserum for TXB_2 cross-reacts less than 0.1% with PGE_2, $PGF_{2\alpha}$, PGD_2, 6-keto-$PGF_{1\alpha}$ and arachidonic acid (final dilution 1:24 000). Cross-reactivities of 6-keto-$PGF_{1\alpha}$ antiserum are 2% for PGE_2, 1.4% for 2,3-dinor-6-keto-$PGF_{1\alpha}$ and less than 0.1% for $PGF_{2\alpha}$, PGD_2, 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{1\alpha}$, TXB_2 and arachidonic acid (final dilution 1:36 000).

RESULTS AND DISCUSSION

HPLC fractionation

As previously reported by Desiderio et al. [16], 6-keto-$PGF_{1\alpha}$ can be effectively separated from TXB_2 and the major prostaglandins on reversed-phase HPLC columns eluted with 0.04 *M* TEA–FA buffer (65:35) at pH 3.15. In our hands, however, elution of TXB_2 was not satisfactory in terms of peak shape [17]. The equilibrium between open and closed oxane ring forms of TXB_2 may account for the irregular peak-shape observed with this compound [18]. The situation was even worse when injecting standard 2,3-dinor-6-keto $PGF_{1\alpha}$. Under these HPLC conditions a very wide and highly distorted peak, spread over a time span of ca. 10 min, was obtained [19]. This behaviour could be related to on-column interconversion of different structural configurations adopted by this compound as a function of pH. It is known that an equilibrium exists between the γ-lactone and its corresponding hemiketal form, the latter predominating at basic pH, where it is also in equilibrium with the open form of the free acid carrying both the free 6-keto and 9-hydroxy moieties [6,19]. Hence, a study of the effect of eluent pH was undertaken in order to optimize the response.

Fig. 1A and B illustrates the chromatographic profiles obtained at pH 4.8 and 5.2, respectively, after injection of TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$. While the dinor metabolite shows a rather well-defined peak, TXB_2 splits into two peaks interconnected by a significant tailing at pH 4.8. On the other hand, at 5.2 one of the peaks of TXB_2 would interfere with the elution of the dinor metabolite. This is prevented at pH 5.8, as shown in Fig. 2A. The HPLC profile showing the elution of other prostaglandins relative to the dinor metabolite is illustrated in Fig. 2B. Note that to adjust the eluent to pH 5.8 formic acid has been replaced by acetic acid in the aqueous buffer. Under these conditions, 6-keto-$PGF_{1\alpha}$, TXB_2 and PGE_2 elute well ahead of 2,3-dinor-6-keto-$PGF_{1\alpha}$. As mentioned above, these prostaglandins are known to cross-react with the antiserum used.

Substitution of TEA by other amines, such as N-butylamine and dibutylamine, did not have any effect. Likewise, phosphoric acid was tried instead of formic or acetic acid without any significant improvement. Although this buffer was used for prostaglandin separation [20], phosphoric acid is less compatible with the subsequent RIA procedure because of its involatility in the lyophilization step.

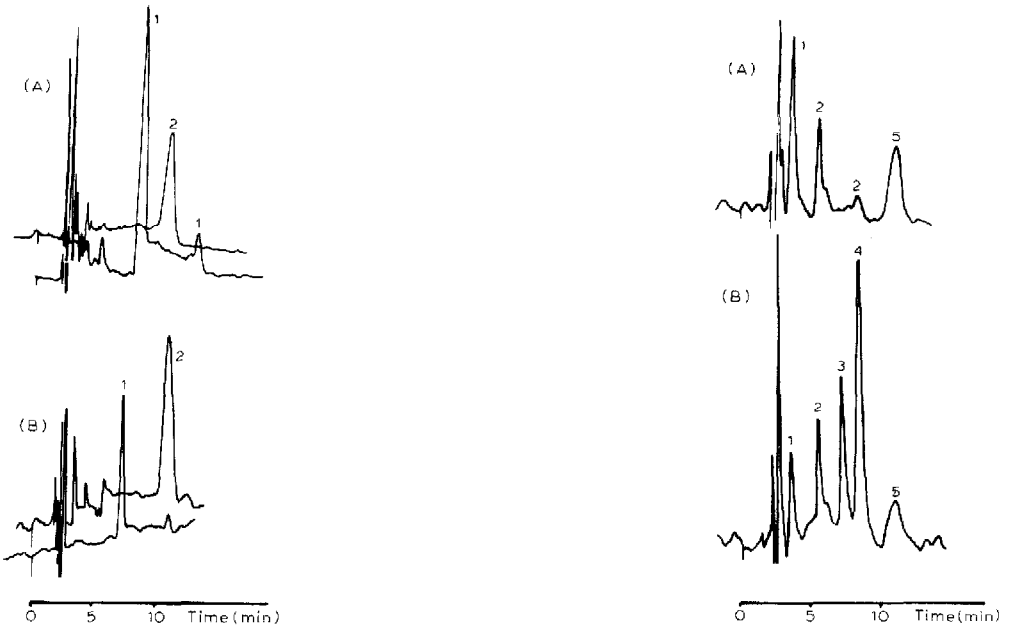

Fig. 1. HPLC profiles of samples of authentic TXB_2 (peak 1) and 2,3-dinor-6-keto-$PGF_{1\alpha}$ (peak 2). Column, 30 cm×3.9 mm I.D. μBondapak C_{18}; eluent, FA-TEA buffer–ACN (68:32) mixture adjusted to pH 4.8 (A) and 5.2 (B) with TEA; flow-rate, 1.5 ml/min; UV detection at 192 nm.

Fig. 2. (A) HPLC profile of a mixture of authentic 6-keto-$PGF_{1\alpha}$ (peak 1), TXB_2 (peak 2) and 2,3-dinor-6-keto-$PGF_{1\alpha}$ (peak 5). Column, 30 cm×3.9 mm I.D. μBondapak C_{18}; eluent, AA-TEA buffer–ACN (68:32) adjusted to pH 5.8 with TEA; flow-rate, 1.5 ml/min; UV detection at 192 nm. (B) Elution profile of the same mixture supplemented with PGE_2 (peak 4) and $PGF_{2\alpha}$ (peak 3).

Radioimmunoassay

The log/logit plot of the standard curve prepared with 2,3-dinor-6-keto-$PGF_{1\alpha}$ was linear. The correlation coefficient, r, was higher than 0.980 for all eleven dose levels. The slopes of the regression line varied from 1.5 to 1.8, as predicted by the different affinities of tracer and unlabeled ligand for the binding sites [21]. The detection limit of the assay was ca. 25 pg/ml, as determined by the amount of standard displacing 10% of the bound radioactivity, and by the mean plus two standard deviations of ten zero-standards. The intra-assay coefficient of variation (C.V.) was 10.8%, and the inter-assay C.V. was 25%. Urine samples of subjects participating in this study were assayed within the same analysis.

To test the accuracy of the procedure, known amounts of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (25–750 pg, seven samples) were added to HPLC eluate aliquots of extracted urine. A close linear correlation was found between the added and the measured amounts of the dinor metabolite ($r=0.99$, slope$=0.97$). Specificity was tested using serially diluted aliquots of blank and 2,3-dinor-6-keto-$PGF_{1\alpha}$-loaded urine subjected to the whole procedure, i.e. extraction, chromatography and immunoassay. Recoveries of both series of samples showed linear relationship and parallelism, as observed in Table I. In addition, no immunoreactivity

TABLE I

SERIAL DILUTIONS OF URINE AND 2,3-DINOR-6-KETO-$PGF_{1\alpha}$ LOADED URINE SUBJECTED TO ASSAY

Results are expressed as pg 2,3-dinor-6-keto-$PGF_{1\alpha}$ per ml of urine.

Dilution	Blank urine		Dilution	Loaded urine	
	Measured	Expected		Measured	Expected
1:1	320	–	1:1	541	–
1:2	146	160	1:2	289	270
1:3	107	107	1:3	174	180
1:4	58	80	1:4	128	135
1:8	23	40	1:8	77	68
1:12	19	27	1:12	45	45
Regression line	$n=5$ $r=0.988$ slope = 0.963		Regression line	$n=5$ $r=0.994$ slope = 0.942	

was found in HPLC eluates at the retention window for 2,3-dinor-6-keto-$PGF_{1\alpha}$ when a mixture of prostaglandins, including 6-keto-$PGF_{1\alpha}$, TXB_2, PGE_2, $PGF_{2\alpha}$, PGD_2 and PGE_1, was injected. After injections of urine extracts, eluates beyond the 2,3-dinor-6-keto-$PGF_{1\alpha}$ window did not show immunoreactivity nor non-specific binding. Taken together, these observations tend to support the specificity of the assay. However, no rigorous structural proof can be assumed.

Recovery of tritiated 6-keto-$PGF_{1\alpha}$ after extraction and chromatography was estimated as 79.3% in ten urine samples. Standard 2,3-dinor-6-keto-$PGF_{1\alpha}$ added in urine and subjected to the whole procedure was recovered at $84 \pm 11\%$ in six samples. Since the last estimation includes the within-assay error of the immunoassay, it is assumed that recoveries of both substances were nearly the same, but estimations based on direct counting of the tritiated compound are more precise. The results for samples from subjects studied were corrected for a 79% recovery.

Urinary levels of 2,3-dinor-6-keto-$PGF_{1\alpha}$

The results obtained with urine from normal individuals are given in Table II. Fisher et al. [7] reported 210 ± 190 pg per mg creatinine (mean $\pm$S.D.) as the levels observed in seven adult subjects. Fitzgerald et al. [9] found a range from 45 to 219 pg per mg creatinine, with a median of 149, in 54 healthy males. In our group, the median was 103 and the range 31–200 pg per mg of creatinine, which is in good agreement with the literature values.

Cirrhotic patients were found to excrete significantly higher amounts of 2,3-dinor-6-keto-$PGF_{1\alpha}$ than the normal subjects, as reported elsewhere [22]. Treatment with sulindac, known as a renal "sparing" prostaglandin inhibitor [23], significantly reduced 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion (see Table III). Like-

TABLE II

URINARY LEVELS OF 2,3-DINOR-6-KETO-$PGF_{1\alpha}$ IN HEALTHY SUBJECTS

Sex	Age	Concentration★	Excretion rate★★
F	50	75	42
F	57	31	24
F	60	200	238
F	61	119	117
M	30	141	138
M	47	35	63
M	57	81	93
M	62	103	100
Mean ± S.E.M.		98.1 ± 19.8	101.9 ± 23.6

★As pg per mg of urinary creatinine.
★★As pg per min.

TABLE III

EFFECTS OF SULINDAC ON RENAL AND SYSTEMIC PROSTAGLANDINS IN FIVE CIRRHOTIC PATIENTS

Results are mean ± standard error of the mean.

	Control	After sulindac	p★
Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ (pg/min)	259 ± 47	86 ± 41	<0.05
Serum TXB_2 (ng/ml)	76.6 ± 13.7	9.5 ± 5.4	<0.01
Urinary 6-keto-$PGF_{1\alpha}$ (pg/min)	966 ± 74	1134 ± 177	n.s.
Urinary TXB_2 (pg/min)	376 ± 111	345 ± 127	n.s.

★Student's t test; n.s. = non-significant.

wise, TXB_2 generated by platelets in serum was clearly inhibited. Nevertheless, urinary 6-keto-$PGF_{1\alpha}$ and TXB_2 excretion rates were not significantly influenced by the drug. Urinary excretion of both substances is believed to reflect intrarenal rather than systemic synthesis [4]. This observation suggests that estimations of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ by our procedure were not affected by interferences from prostanoids of renal origin.

In conclusion, HPLC–RIA may constitute a useful alternative to the GC–MS determination of prostacyclin metabolites in the urine. Its main advantage over GC–MS procedures is its relative simplicity, allowing clinical studies of large series of samples.

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