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Simple Radioimmunological Method for Urinary 6-Keto-$PGF_{1\alpha}$ Measurement

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A radioimmunoassay for the determination of 6-keto-$PGF_{1\alpha}$ (stable metabolite of Prostacyclin) in urine using a specific antiserum, is described. The antibody used, showed no significant cross-reactivity with other PGS. (^{3}H)-6-keto-$PGF_{1\alpha}$ has been used as tracer and a 30 % Hydroxyapatite suspension was utilized for the separation of bound and free fractions. Standard curve ranges between 2 and 1000 pg thus allowing measurements in urine. Aliquots of 0.2 to 1 ml of 24 hours urinary samples were submitted to a preextraction step with Hexane in order to eliminate neutral fats. After acidifying with 0.1 N HCl to pH = 3.5 — 4, an Ethyl Ether extraction step was performed showing a recovery of 66 %. Repeated analysis of a rat urine pool, showed 7.7 intraassay and 9.8 interassay variation coefficients. Values obtained amounted to 38.3 ± 4.6 ng/24 h in normal rats and 133 ± 32 ng/24 h in 5 % NaCl loaded rats ($p < 0.001$). The values obtained in healthy men were 508 ± 94 ng/24 h and in women 375 ± 136 ($p < 0.05$).

Key words: Radioimmunoassay, Urine, 6-keto-$PGF_{1\alpha}$.

Prostacyclin (PGI_2) is the most potent inhibitor of platelet aggregation and has a powerful vasodilator activity on the peripheral, coronary and pulmonary vasculature (11, 13, 15).

It is generally believed that urinary excretion of PGE_2 and $PGF_{2\alpha}$ reflects renal formation of these prostaglandins (PGS) (18, 25). PGI_2 is also formed in the kidney in considerable amounts. It is difficult to develop a physico-chemical method for measuring PGI_2 directly due to its instability (11). Several methods (2, 3, 5, 7, 10, 12, 16, 19, 22, 27), have been described to evaluate the PGI_2 levels through its spontaneous hydrolysis product 6-keto-$PGF_{1\alpha}$. In this way, a simple radioimmunological method to estimate urinary 6-keto-$PGF_{1\alpha}$ as an indirect form for measuring PGI_2 renal production, has been developed.

Materials and Methods

Solvents and Reagents. Antiserum was purchased from Seragen. (^{3}H)-6-keto-$PGF_{1\alpha}$ diluted in Acetinotrile/water (9:1) was purchased from Amersham. Standard 6-keto-$PGF_{1\alpha}$ was supplied by Upjohn. Bovine serum albumin fraction V Cohn and Hydroxyapatite suspension in 0.001 M buffer phosphate pH 6.8 type I, were supplied by Sigma. PCS scintillation liquid was purchased from Amersham/Searle. Solvents (analytical grade) were purchased from Merck.

Buffer phosphate 0.01 M + 0.01 % bovine serum albumin pH=7.6 was used in preparing the working solutions of the antibody, tracer and hydroxyapatite suspension. Phosphate buffer 0.5 M pH=7.6 was used to break up the hydroxyapatite antibody complex in order to get an easier mixture of the tracer with the scintillation liquid.

Urine samples. Wistar male rats were housed in metabolic cages in order to collect 24 h urine samples. Likewise, 24 h urine samples were obtained from healthy subjects (men and women) under normal sodium intake.

Urine aliquots were stored at —20°C until their analysis.

Preextraction. Once the samples were thawed, approximately 1,500 cpm of (^{3}H)-6-keto-$PGF_{1\alpha}$ were added to 200 µl of rat urine or 1 ml of human urine to control the recovery percentage during the purification steps. After an equilibration period of 15 min, a preextraction step with 10 ml of hexane was carried out by slow rotative agitation. In order to separate the hexane and the urine phases, the mixture was frozen and then, hexane was decanted from the urinary phase.

Extraction. After acidifying, with 0.1 N HCl to pH = 3.5-4, 10 ml of diethyl ether were added to the samples and submitted to rotative agitation. The phase separation was also made by freezing, and the ether phase was collected in glass tubes for evaporation in a water bath at 37°C under current air. The residue was dissolved in 1 ml of assay buffer by vortex-mixing.

Standard curve. One mg of standard 6-keto-$PGF_{1\alpha}$ was dissolved in 10 ml of acetone obtaining a stock solution of 100 µg/ml. Successive solutions (1,000, 100, 10, 1, and 0.1 ng/ml) of the stock solution were made and stored at —20°C.

To construct the standard curve points (2, 5, 10, 20, 50, 100, 200, 500, 1,000 pg), appropriate volumes of the standard solutions were pipeted into glass tubes. The PGI_2-Acetone dilutions were evaporated to dryness in a water bath at 37°C under air current. One hundred µl of assay buffer were added to the dry residue and vortex-mixed to continue with the assay procedure.

Assay procedure. Aliquots of 100 µl in duplicate of the redissolved dry residue were pipeted into glass tubes. The antiserum was diluted (1:12,000) in assay buffer and aliquots of 100 µl were mixed with standard or sample. (^{3}H)-6-keto-$PGF_{1\alpha}$ in assay buffer 50 µl containing approximately 7,000 cpm), was immediately added to give a total reaction volume of 250 µl. Incubation was carried out for 16-24 h at 2-4°C.

Separation and counting. To remove bound and free fractions, 200 µl of 30 % hydroxyapatite suspension were added to each tube, vortex-mixed and centrifuged for 10 min at 3,000 rpm in a refrigerated centrifuge.

Results

Time and temperature dependence of the antigen-antibody reaction. In order

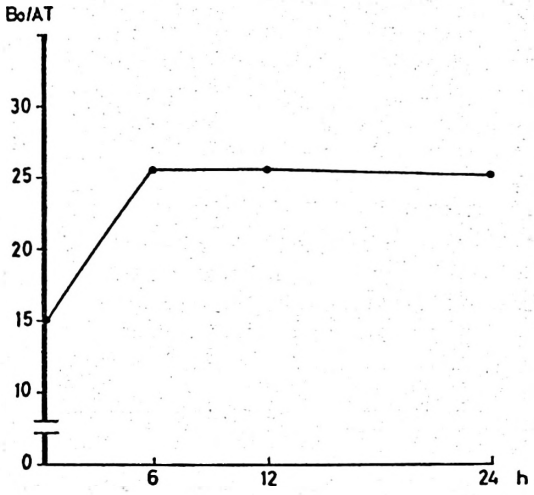

Fig. 1. *Time dependency of the antigen-antibody incubation.*

to find the optimal incubation conditions, the time and temperature dependence of the binding of (^{3}H)-6-keto-PGF$_{1\alpha}$ to the antibody was studied at 0-4°C and 37°C, and between 0-24 h. The highest binding rate was observed at 0-4°C. The equilibrium was reached after 6 h and remained without significant changes until 24 h (fig. 1).

Standard curve and sensitivity. Figure 2 shows a mean standard curve for synthetical 6-keto-PGF$_{1\alpha}$. Each point of the curve represents the B/Bo average and its standard deviation of the mean from nine different assays. The method is sensitive enough to appreciate 2 pg of 6-keto-PGF$_{1\alpha}$.

Effect of hydroxyapatite on the removal of bound and free fractions. In order to find the optimal quantity of hydroxyapatite for the separation of free and bound fractions, 200 µl of several suspensions of it (1 to 40 %) were added to the antigen-antibody mixture. The 30 % suspension yields the chosen binding and a small blank effect (less than 7 %).

Specificity. Table I shows the cross-reactivity at 50 % B/Bo of the 6-keto-PGF$_{1\alpha}$ antiserum used in the assay. The tested PGS do not cross-react significantly to interfere 6-keto-PGF$_1$ measurements.

Reproducibility. Urine samples from a pool of normal rats were measured in several assays to determine the variation coefficients. The mean intraassay variation of 58 samples in duplicate, divided in six assays during four months, was 7.7 % and the interassay variation of the six assays was 9.8 %. Neither an increase nor a decrease due to storage dependency was detected during the four months.

Linearity. To study the linearity of the method, successive volumes (100, 200, 300, 400 µl) of a normal rat pool were assayed. Figure 3 shows the linear relationship between increasing volumes of urine and the values calculated after the whole procedure.

Recovery and accuracy. The mean recovery percentage obtained after the preextraction steps was 66 %. The re-

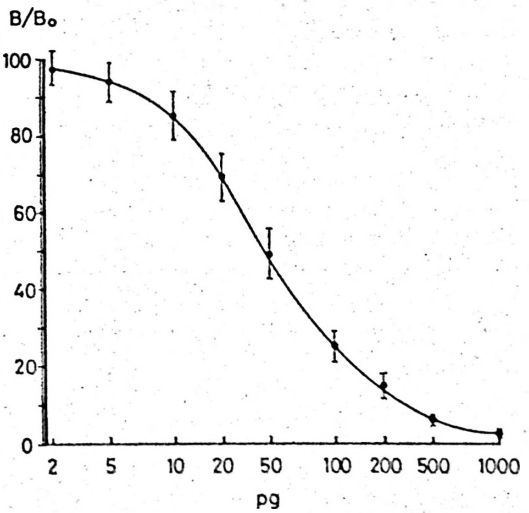

Fig. 2. *Synthetical 6-keto-PGF$_{1\alpha}$ standard curve.* (Means ± S.D.).

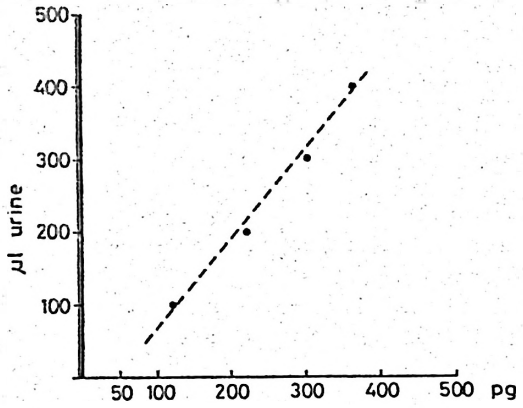

Fig. 3. *Linear relationship between increasing volumes of urine and values obtained.*

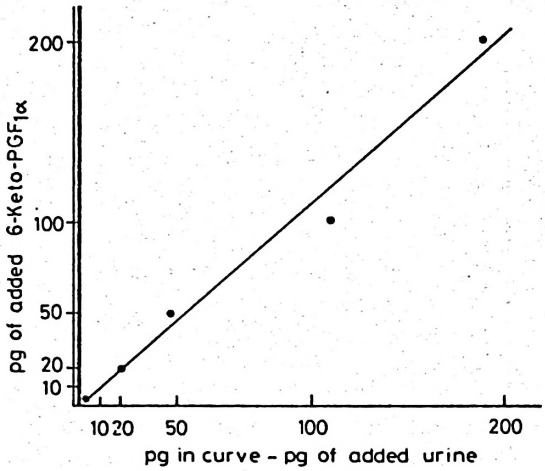

Fig. 4. *Linear relationship between amounts of synthetical 6-keto-PGF$_{1\alpha}$ added to urine and values obtained.*

covery percentage corresponding to the assay procedure was studied by adding progressive amounts of synthetical 6-keto-PGF$_{1\alpha}$ (10-200 pg) to urine samples of a normal rat pool. The percentage found was 96 %. Accuracy was likewise studied. Figure 4 shows the linear relationship between the amount of 6-keto-PGF$_{1\alpha}$ added to urine and the values obtained.

Urinary 6-keto-PGF$_{1\alpha}$ concentrations in normal and salt loaded rats. The urinary levels of 6-keto-PGF$_{1\alpha}$ in eight normal rats weighing 227 ± 10 g, were 38.3 ± 4.6 ng/24 h (mean ± SD).

The levels in eight rats submitted to 5 % of NaCl in the diet during one week were 133 ± 32 ng/24 h. The differences between both groups were statistically significant ($p < 0.001$).

Urinary 6-keto-PGF$_{1\alpha}$ concentrations in humans. The levels in seven normal men aged 34±14 years were 508±94 ng per 24 h, and in ten normal women of 33 ± 10 years of age were 357 ± 136 ng per 24 h. The differences between men and women were significant ($p < 0.05$).

Table I. *Cross reactivity (%) of the 6-keto-PGF$_{1\alpha}$ antiserum with other PGS at 50 % B/Bo.*

6-keto-PGF$_{1\alpha}$	100.00
PGF$_{1\alpha}$	10.70
PGF$_{2\alpha}$	2.30
6-keto-PGE$_1$	1.50
PGE$_1$	0.40
PGE$_2$	0.06
PGA$_2$	0.02
PGB$_2$	0.02

Discussion

To study the PGI_2 renal production, urinary measurements of 6-keto-PGF$_{1\alpha}$ may be a good indication of total renal synthesis, as it has been shown for PGE_2 and PGF$_{2\alpha}$ (6, 10, 18, 25).

Several radioimmunological methods to measure 6-keto-PGF$_{1\alpha}$ in plasma, different tissues, and urine (4, 7, 9, 10, 14, 22, 27) have been developed so far. Other methods as gas chromatography — mass spectrometry, gas chromatography with negative ion chemical ionization and

gas chromatography with electron capture (2, 3, 5) have also been described.

In spite of the various methods published, data obtained by different authors showed considerable variations (4, 16, 26). The radioimmunoassay described in this paper is in agreement with some of them (2, 19, 22, 27). The improvement of this method, consists of a more simple purification procedure. It avoids the use of classical steps like thin layer chromatography or silicic acid columns (16, 19) and modern ones like HPLC (22).

A preextraction step with hexane is used to eliminate neutral fats. The extraction procedure with diethyl ether has been used instead of other more commonly utilized solvents like ethyl acetate (16, 19). Although the recovery obtained with the first one is lower, it is compensated with its more clear extracts obtained and its faster evaporation. For this reason, this purifying methods is cheaper, since the use of chromatographical purifications is not neccesary, and faster than others described above.

The lowest detection limit of the standard curve is 2 pg, which is similar to others (19, 22), making possible its use in the measurement of biological samples.

The recovery percentage after the whole procedure, is similar and in some cases superior to those obtained in methods using more sofisticated methodologies (2, 12).

It is noteworthy mentioning that standard solutions used to construct the curve, have a limited life of less than six months, although they were stored at —20°C.

The cross-reactivity with other PGS is very low and similar to others previously published, allowing its use for urine sample measurements.

The inter and intraassay variations are better than others already published. During six months the interassay variation was never higher than 9.8 %.

When amounts of synthetical 6-keto-PGF$_{1\alpha}$ were added to urine samples, a linear relationship was observed, indicating the accuracy of the method.

In relation to temperature dependence, small differences between the two curves, incubated at 20°C and 2-4°C, were appreciated. The last one, showed a better fall than the other one representing more sensitivity. Due to this fact, the curve incubated at 2-4°C was chosen.

The use of hydroxyapatite suspension for separating bound and free fractions is slower than the use of dextran-charcoal separation, but this procedure allows to work at room temperature, in a time independent manner and without stripping effect. Another interesting characteristic is the fact of using the same small glass tubes (used in the radioimmunoassay) instead of scintillation vials. Thus, this method allows the saving of scintillation fluid, vials and their caps (23, 24). Consequentely, this separation method becomes cheaper and more simple than the dextran-charcoal one.

Several papers have reported levels of 6-keto-PGF$_{1\alpha}$ (1, 8, 17, 22). The values obtained in humans and rats with this method which avoids chromatographical steps, are in the same range as other works published, in spite of the different unities in which they are expressed.

In conclusion, this radioimmunological method is more simple, time-saving and cheaper than others previously published, yielding a good accuracy, specificity, reproducibility and sensitivity to be used for the evaluation of 6-keto-PGF$_{1\alpha}$ levels in urine.

Acknowledgements

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Resumen

Se describe un radioinmunoensayo para la determinación de 6-ceto-PGF$_{1\alpha}$ (metabolito es-

table de la prostaciclina) en orina utilizando un antisuero específico. El anticuerpo utilizado no presentó reacciones cruzadas significativas con otras PGS; como trazador se utilizó (^{3}H)-6-ceto-$PGF_{1\alpha}$ y una suspensión del 30 % de hidroxiapatita para separar las fracciones libre y unida. La curva patrón cubre una escala entre 2 y 1.000 pg, permitiendo por tanto medidas en orina. Las muestras de orina de 24 h, entre 0,2 y 1 ml, se sometieron a un proceso de preextracción con hexano con objeto de eliminar las grasas neutras. Después de acidificar con HCl 0,1 N, pH 3,5-4, se llevó a cabo un proceso de extracción con éter etílico obteniéndose una recuperación del 66 %. Mediante repetidos análisis de un «pool» de orina de rata se obtuvo un coeficiente de variación intraensayo del 7,7 % y un 9,8 % interensayo. Los valores obtenidos en ratas normales fueron 38,3 ± 4,6 ng/24 h y en ratas sometidas a sobrecarga del 5 % de NaCl 133 ± 32 ng/24 h ($p < 0,001$). En hombres sanos los valores obtenidos fueron 508 ± 94 ng/24 h y en mujeres 375 ± 136 ng/24 h ($p < 0,05$).

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