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Selective clean-up method using an immunoaffinity column following radioimmunoassay of prostaglandin $F_{2\alpha}$ in biological fluids

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ABSTRACT

A selective clean-up method using an immunoaffinity column followed by radioimmunoassay (RIA) was developed for determining prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in human urine and plasma. Polyclonal antibody raised against $PGF_{2\alpha}$, obtained from rabbits, was coupled to a tresyl-activated support based on a synthetic hydrophilic resin, TSKgel Tresyl-Toyopearl 650M, and used as the stationary phase for the immunoaffinity column. A human urine or plasma sample was introduced to this column, and $PGF_{2\alpha}$ was eluted with methanol–water (50:50, v/v) after the column had been washed. The eluate was subjected to competitive RIA for $PGF_{2\alpha}$. The cross-reactivities of the RIA to a number of endogenous prostanoids, except PGD_2, were negligible and the sensitivity was 4 pg/tube ($p < 0.05$), giving a detection limit of 40 pg/ml when 1 ml of plasma or urine was available. The recoveries of plasma and urine samples were 98–108% and 96–106%, respectively, and their assay variances were 7–23%. The concentrations of endogenous $PGF_{2\alpha}$ in plasma and urine used here were estimated to be 72 and 98 pg/ml, respectively. This method should be very useful for various biological samples because of its good specificity, sensitivity, reliability and reproducibility.

INTRODUCTION

Prostaglandins (PGs) play important roles in biological systems and can be determined by high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) and radioimmunoassay (RIA). Unfortunately, these highly sensitive analyses for biological samples require tedious clean-up procedures because PGs in biological fluids are present at less than the ng/ml level and must be separated from fatty acids and other substances. Several papers have reported the purification of PG-related compounds from biological fluids by immunoaffinity [1–5], but most of them include other extraction steps before the immunoaffinity purification and were developed for purification before determination by GC–MS. RIA is the most sensitive, simple and useful method for multiple sampling of small volumes and it is commercially available for the major metabolites of arachidonic acid. A precise assay which does not require previous extraction has been reported for enantioselective RIA of plasma S-145, a thromboxane (TX) A_2 receptor antagonist with a similar structure to PG [6]. However, most immunoassays for prostanoids, as is usual with other analyses, are tedious when used for analysing biological samples [7–11]. In this work, a selective one-step clean-up method using an immunoaffinity column following RIA was developed for the determination of $PGF_{2\alpha}$ in human plasma and urine.

EXPERIMENTAL

Materials

Prostaglandins used in this study were purchased from Funakoshi Pharmaceutical (Tokyo, Japan). [5,6,8,9,11,12,14,15-^{3}H]$PGF_{2\alpha}$ (6086.5 GBq/mmol, nominally labelled) and the liquid scintillation cocktail, Aquasol-2, were obtained from New England Nuclear (Boston, MA, USA). Freund's complete adjuvant (FCA) and gelatine were from Difco

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(Detroit, MI, USA). Crystallized bovine serum albumin (BSA), the Affi-Gel Protein A MAPS II kit and TSKgel AF-Tresyl Toyopearl 650M were purchased from Sigma (St. Louis, MO, USA), Bio-Rad (Richmond, CA, USA) and TOSOH (Tokyo, Japan), respectively. All other chemicals were of analytical-reagent grade.

Assay buffer

The assay buffer for RIA of $PGF_{2\alpha}$ was 0.01 *M* phosphate-buffered saline, pH 7.4, containing 0.05% NaN_3 and 0.1% gelatine.

Preparation of antisera

$PGF_{2\alpha}$ was coupled to BSA to prepare the immunogen according to the mixed anhydride method [12]. The number of $PGF_{2\alpha}$ molecules coupled to a BSA molecule was estimated to be 30 by the trinitrobenzenesulphonic acid procedure [13], which measures the free amino groups remaining on the $PGF_{2\alpha}$–BSA conjugate.

The immunogen was dissolved in saline to 2 mg/ml and emulsified with an equal volume of FCA. A 0.5-ml volume of emulsion containing 0.5 mg of immunogen was injected subcutaneously into three Japanese white female rabbits. Immunization was repeated seven times every 3 weeks. Whole blood of each rabbit was collected 10 days after the last immunization and centrifuged at 2000 *g* for 30 min to separate the serum after clotting. The antisera (F157-159) were stored at −80°C until further use.

Preparation of the immunoaffinity column

The immunoglobulin G (IgG) fraction was isolated from each antiserum using the Affi-Gel Protein A MAPS II kit and a protein A–agarose column. Twenty milligrams of the IgG were dissolved in 10 ml of a coupling buffer in the kit and mixed with 1 g of dried TSKgel AF-Tresyl Toyopearl 650M powder, which gave a volume of 5 ml in the swollen state. After gentle shaking at 4°C for 16 h, 20 ml of 0.1 *M* phosphate buffer (PB, pH 6.0) containing 0.5 *M* NaCl were added to the mixture. It was then filtered through a fritted disc funnel and the remaining active sites were inactivated with 0.1 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* NaCl. The coupling yield was calculated from the UV absorption at 280 nm of the IgG solutions before and after the coupling reaction. The antibody coupled to the supports was stored in 0.1 *M* PB (pH 6.0) containing 0.1% NaN_3 at 4°C. The immobilized antibody gel (containing *ca.* 2.3 mg of IgG) was packed into a polypropylene open column, Sepacol-Mini-PP (65 × 9 mm I.D.; Seikagaku Kogyo, Tokyo, Japan).

Clean-up procedure

Human plasma or urine, 1 ml, was applied to the immunoaffinity column after equilibration with 0.1 *M* PB (pH 6.0) and incubated for 15 min. The column was then washed with 6 ml of PB and 1 ml of distilled water at 0.2–0.5 ml/min. $PGF_{2\alpha}$ was eluted with methanol–water (50:50, v/v) and the eluate was evaporated to dryness.

Radioimmunoassay

A 100-μl portion of the diluted [^{3}H]$PGF_{2\alpha}$ (*ca.* 200 000 cpm/ml) and 100 μl of the diluted antiserum (F158; 1:5000) were added to an assay tube containing 100 μl of the sample in the assay buffer or standard solution (32–100 000 pg $PGF_{2\alpha}$/ml). The mixture was incubated at 4°C for 16 h and then 300 μl of a dextran (0.1%)-coated charcoal (1%) suspension was added. After further incubation at 4°C for 10 min the mixture was centrifuged at 2000 *g* for 5 min, and half of the supernatant (300 μl) was transferred into a counting vial. Ten millilitres of Aquasol-2 were added to the vial and the radioactivity was measured with an LSC-672 liquid scintillation counter (Aloka, Tokyo, Japan).

RESULTS

Characterization of the antiserum

The characteristic features of the antisera (F157-159) were evaluated by RIA. The titres, defined as the dilution ratio of antiserum with 50% bound of the tracer, were determined to be 1000, 4000 and 1500, respectively. Standard curves of the RIA for $PGF_{2\alpha}$ are shown in Fig. 1. The minimum detectable doses ($p<0.05$) of $PGF_{2\alpha}$ were 30, 4 and 20 pg/tube, respectively. The cross-reactivities of a number of endogenous prostanoids are shown in Table I. PGD_2 showed a high cross-reactivity, and $PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$ showed some cross-reactivities. There was a negligible cross-reaction with other prostanoids tested here. From these results, F158 was selected for the RIA and F159 for the immunoaffinity column.

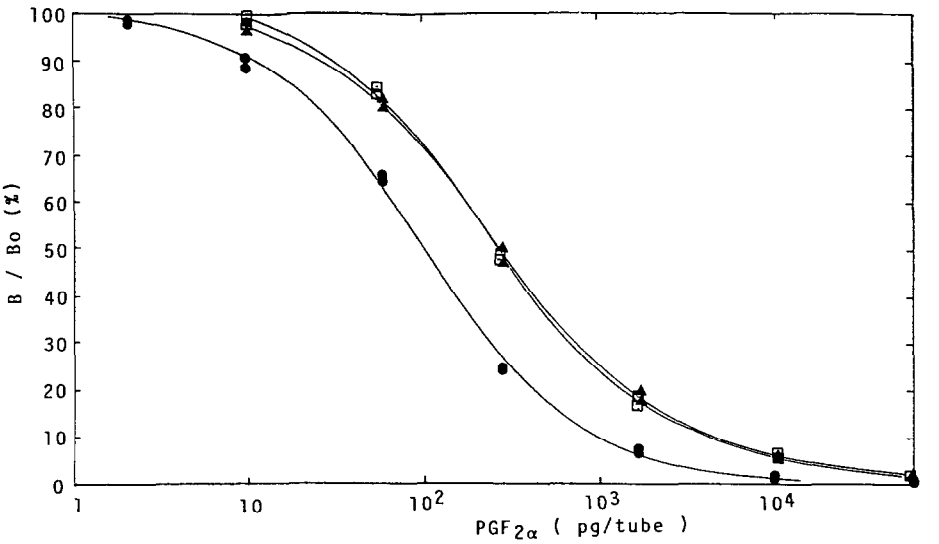

Fig. 1. Standard curves of the $PGF_{2\alpha}$ RIA using several antibodies. □ = F157; ● = F158; ▲ = F159. B = Bound count; B_0 = bound count in the zero concentration.

Recovery of [^{3}H]$PGF_{2\alpha}$ and binding capacity of the immunoaffinity column

Using [^{3}H]$PGF_{2\alpha}$ in the assay buffer, the effect of methanol concentration in the elution buffer on the recovery of $PGF_{2\alpha}$ was tested. On the basis of the data shown in Fig. 2, subsequent experiments were carried out using 50% methanol as the elution buffer. When different amounts of tracer were applied to the immunoaffinity column, the maximum capacity to retain the antigen was more than 50 ng of $PGF_{2\alpha}$, which was far in excess of the amounts in plasma and urine.

TABLE I

CROSS-REACTIVITIES OF VARIOUS PG-RELATED COMPOUNDS

	Cross-reactivity (%)		
	Antiserum No.		
	F157	F158	F159
$PGF_{2\alpha}$	100	100	100
PGE_2	0.08	2.40	0.38
6-Keto-$PGF_{1\alpha}$	0.52	4.50	4.12
$PGF_{1\alpha}$	3.86	5.51	4.25
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	0.36	0.83	0.16
PGA_2	<0.05	0.02	0.03
PGB_2	≪0.05	≪0.01	0.01
PGD_2	12.7	60.1	25.9
13,14-Dihydro-15-keto-PGD_2	0.05	0.11	0.06
TXB_2	0.42	0.28	0.34
15(S)-HETE[a]	0.12	0.05	0.07

[a] HETE = Hydroxyeicosatetraenoic acid.

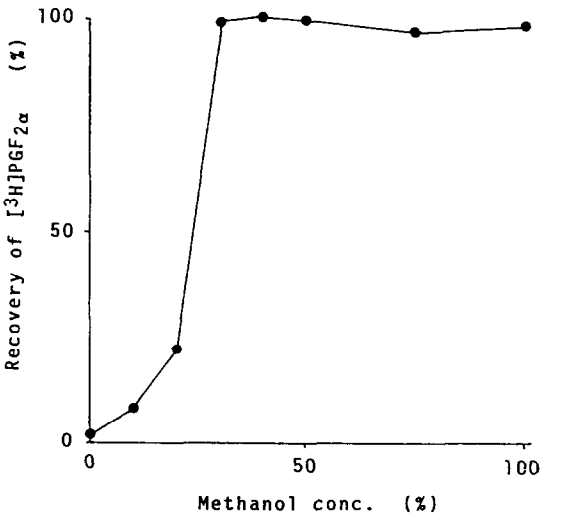

Fig. 2. Effect of methanol concentration in the elution buffer on [^{3}H]$PGF_{2\alpha}$ recovery from the immunoaffinity column. Sample, [^{3}H]$PGF_{2\alpha}$ in 1 ml of assay buffer (*ca.* 200 000 cpm; 0.44 ng); clean-up procedure, see text.

TABLE II

RESULTS OF THE ANALYSIS OF PLASMA SPIKED WITH VARIOUS AMOUNTS OF $PGF_{2\alpha}$

Concentration (pg/ml)		Recovery (%)	Relative standard deviation (%)
$PGF_{2\alpha}$ added	$PGF_{2\alpha}$ found[a]		
0	72.2 ± 16.5	–	22.9
250	343 ± 41.0	108	11.9
750	829 ± 80.3	101	9.7
1500	1530 ± 102	98	6.7

[a] Mean ± S.D.; $n = 4$.

RIA of $PGF_{2\alpha}$ in human plasma and urine samples after clean-up by the immunoaffinity column

Human plasma and urine spiked with different amounts of $PGF_{2\alpha}$ ranging from 0 to 5000 pg/ml were applied to the immunoaffinity column following the RIA of $PGF_{2\alpha}$. Each measured value corresponded to the added amount plus the endogenous $PGF_{2\alpha}$ concentration for a wide range of $PGF_{2\alpha}$ (Tables II and III). The relationship between the added (x, pg/ml) and measured (y, pg/ml) values were both linear (plasma sample, $y = 0.96x + 90$; urine sample, $y = 1.05x + 86$). The endogenous concentrations in the plasma and urine used here were estimated from the measured values of the original samples, 72 and 98 pg/ml, respectively.

The recoveries of plasma and urine samples were 98–108% and 96–106%, respectively, and the assay variances were 7–23%, and 10–18%, respectively. As the sensitivity of the RIA was 4 pg/tube, the detection limit of this method was 40 pg/ml when 1 ml of plasma was available. Fig. 3 shows the linearity between plasma volume up to 2.5 ml and the measured $PGF_{2\alpha}$, which indicates that the detection limit could be improved to 15 pg/ml by concentration onto the immunoaffinity column.

TABLE III

RESULTS OF ANALYSIS OF URINE SPIKED WITH VARIOUS AMOUNTS OF $PGF_{2\alpha}$

Concentration (pg/ml)		Recovery (%)	Relative standard deviation (%)
$PGF_{2\alpha}$ added	$PGF_{2\alpha}$ found[a]		
0	97.5 ± 18.0	–	18.4
500	576 ± 58.2	95.6	10.1
1000	1160 ± 113	106	9.7
5000	5340 ± 950	105	17.8

[a] Mean ± S.D.; $n = 4$.

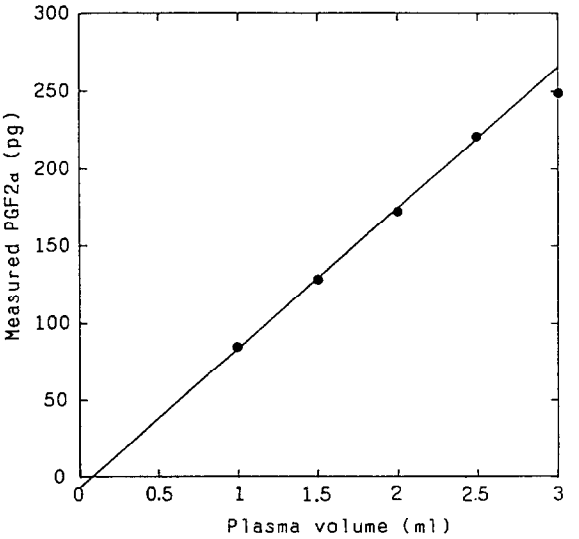

Fig. 3. Effect of plasma volume on the determination of $PGF_{2\alpha}$. Various amounts of plasma extracted by the immunoaffinity column were subjected to the RIA. A correlation was seen between the plasma volume (x, ml) and measured $PGF_{2\alpha}$ (y, pg/tube) ($y = 91.0x - 7.00$, $r = 1.00$). The slope represents the endogenous concentration of $PGF_{2\alpha}$ in this plasma (91 pg/ml).

DISCUSSION

Feasible immunoafffinity clean-up methods of PG-related compounds from biological fluids have been reported [1–5] for the preparation of conventional solvent extraction or chromatographic purifications, or both, which are tedious and time consuming and can lead to sample loss. In general, the most important requirements of an immunoaffinity clean-up method are specificity and reproducibility. The antibodies used in this study showed high specificity. It is assumed that they mainly recognize the five-membered ring of $PGF_{2\alpha}$ from the results of cross-reactivities (Table I) and the binding site of the hapten to BSA. The high cross-reactivity of PGD_2, however, suggests that the antisera weakly discriminate the oxidation of the 11-position on the

ring, compared with the negligible cross-reactivity of PGE_2, which results in oxidation of the 9-position on the ring. As endogenous PGD_2 is present in only trace amounts in plasma [14], the high cross-reactivity of PGD_2 is insignificant. In addition, an interfering compound with even a slightly weaker affinity to the antibody than the target antigen could be swept away from the assay system because a reaction system developed on an immunoaffinity column is open, unlike in immunoassay. Therefore, this combination of immunoaffinity purification and RIA should increase the specificity beyond that expected from the cross-reactivities to the interfering compound of each antibody tested. The values of endogenous $PGF_{2\alpha}$ in plasma and urine presented here are similar to those of previous reports [10,11,15,16]. To prevent inactivation of the antibody, mild clean-up conditions were used, for example, an elution buffer containing as little organic solvent (methanol) as possible. Therefore, the immunoaffinity column can be used repeatedly more than 50 times without any change in its clean-up capacity. The immobilized antibody was very stable as no change was found in the binding behaviour of the RIA from leakage of the antibody. These results suggest that this method is highly reliable with a wide linearity of recovery and good reproducibility.

$PGF_{2\alpha}$ is one of the primary PGs and is the metabolite of PGH_2, together with PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2. Their urinary concentrations are thought to be a good reflection of renal prostaglandin synthesis and the valance of their plasma components is of great importance in many physiopathological conditions [16]. The most common method used to determine $PGF_{2\alpha}$ in biological fluid is RIA [9–11,15–17]; in particular, commercially available RIA kits, such as Clinical Assays (Baxter Travenol Diagnostics, Cambridge, MA, USA) are often used [17,18]. However, there has been no report of the use of RIA for $PGF_{2\alpha}$ without tedious pre-treatment of each biological sample. Although the RIA described in this study also requires pre-treatment of the biological samples examined, it yields excellent results when combined with the selective clean-up method. Consequently, this method should be very useful when studying various biological samples because of its good specificity, sensitivity, reliability and reproducibility. In addition to the $PGF_{2\alpha}$ reported in this paper, highly specific antibodies against other primary PGs have been obtained (data not shown). Therefore, when packing multiple immobilized antibodies into the same immunoaffinity column, this method should allow extraction of the desired PGs in one step, *i.e.* group-specific purification, to allow further separation analysis, including RIA.

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