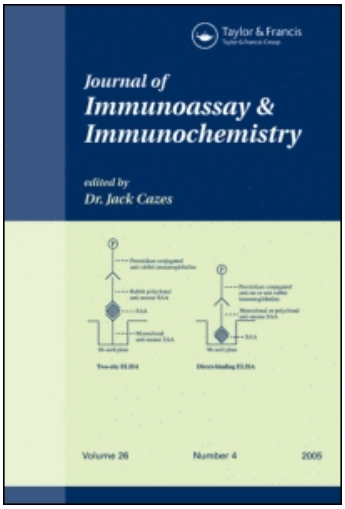

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A Sensitive Direct ELISA for Detection of Prostaglandin E_2

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A Sensitive Direct ELISA for Detection of Prostaglandin E_2

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Abstract: In order to improve the indirect ELISA for detection of PGE_2, a modified direct ELISA technique was developed to measure PGE_2 in cell culture supernatants. An evaluation of three types of coating buffer showed that PGE_2 was adsorbed efficiently to the solid phase using the gelatin phosphate buffer. The sensitivity of the assay was increased by employing polyclonal rabbit anti-PGE_2 antibody dilution of 1/100 and 1% skimmed milk as a blocking solution, with the detection limit of 7.8–500 ng/well. The within-run and between-run coefficients of variation (CV) ranges were 3.2–3.7% and 3.4–3.8%, respectively. A linear standard curve was observed over the range of 0.078–5 μg/mL with a coefficient of determination (r^2) of 0.99. Our results indicated that the developed direct ELISA was sensitive and suitable for a quick determination of PGE_2 levels from cell culture supernatants.

Keywords: PGE_2, Enzyme-linked immunosorbent assay, ELISA, Immunoassay, Anti-PGE_2, Ligand assay

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INTRODUCTION

Prostaglandin E_2 ($PGE_{2)}$ is the principle pro-inflammatory prostanoid that mediates inflammation in most tissues. Specifically, PGE_2 can cause pain and blood-vessel dilatation and, together with LTB_4, promote vascular leakage and the extravasation of fluid, creating redness, swelling, and discomfort in the affected area.[1–5] Therefore, the PGE_2 levels are presumably a reasonable indicator of sites with an on-going inflammatory process.

Several commercial ELISA kits specific for PGE_2 detection are readily available, but they are relatively expensive and, therefore, prohibitive for screening a large number of samples. Among commercially available methods, the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) offer the required specificity and sensitivity. However, because of the poor adsorption of PGE_2 antigen to polystyrene plates, many ELISA procedures use an indirect antibody-bound antigen phase.[6–8] This indirect technique requires highly purified, type-specific antibodies to decrease nonspecific background activity and increase sensitivity. We have developed a direct-coating ELISA for PGE_2 detection and have demonstrated its accuracy with a very low background activity. In addition, this method is more cost effective than a commercial indirect ELISA.

EXPERIMENTAL

Direct ELISA

PGE_2 (Sigma Chemical Co., St. Louis, Mo, USA) in DMSO (PGE_2-DMSO) was used to optimize the direct ELISA for PGE_2. The PGE_2-DMSO was serially diluted in 50 μL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco Invitrogen, Grand Island, NY, USA) as a standard. Ninety six-well ELISA plates (Corning Inc., Corning, NY) were coated with 50 μL of diluted PGE_2-DMEM and 50 μL gelatin phosphate buffer pH 5.0 (1 g of gelatin, 6.1 g of NaCl, 10.8 g of K_2HPO_4, 1.7 g of KH_2PO_4, and 0.7 g of NaN_3 in 500 mL of distilled H_2O, pH 5.0) at the final concentrations ranging from 0.156–5 μg/mL and incubated at 4°C for 72 h. The unbound antigens were removed by washing with phosphate-buffered saline pH 5.0 (PBS) containing 0.05% Tween-20 (PBST). The wells were then blocked with 100 μL of 1% skimmed milk in PBS per well. One-hundred microliters of rabbit PGE_2 antiserum (Sigma Chemical Co, St. Louis, MO), diluted 1/100 with PBS containing 0.05% skimmed milk, was then added and incubated for 2 h at 37°C in a humidified chamber. The unbound antibodies were washed five times with PBST, and subsequently reacted with biotinylated goat anti-rabbit IgG (H + L) conjugate at 1/5,000 dilution and incubated for 1 h. Plates were washed five times with PBST, and HRP-streptavidin conjugate at 1/10,000 was added. Both biotin and streptavidin

conjugates were added at dilutions recommended by the manufacturer (Zymed Laboratories Inc., San Francisco, CA, USA). Following 1 h of incubation, the plates were washed five times with PBST, and 100 μL of TMB single solution (Zymed Laboratories Inc.) was added to each well. After 45 min of incubation, the addition of 2 M sulfuric acid (100 μL per well) stopped the reaction, and absorbance was determined at 450 nm with an automated plate reader (Tecan U.S., Inc., Durham, NC, USA). In each experiment, a triplicate was performed for each sample and the mean value was calculated for evaluation. The anti-PGE_2 antiserum in our hands had negligible cross-reactivity.[9]

Optimizing ELISA Conditions

The assay optimization was performed using standard PGE_2-DMEM. Experimental parameters, including type of coating buffer, gelatin concentration in coating buffer, length of coating period, concentration of blocking solution, anti-PGE_2 diluting ratio and pH of working solution were varied systematically to achieve maximum sensitivity.

In order to achieve an optimal absorption efficiency of PGE_2 to the ELISA plate, the volume ratio of gelatin phosphate buffer (GPB) to DMEM was varied in the range of 10:90, 20:80, 30:70, 40:60, and 50:50. PGE_2 in DMSO (initial PGE_2 concentration = 5000 ng/mL) was serially diluted two-fold with different GPB pH 5.0 and DMEM solutions before coating on to the plates, and PGE_2 levels were subsequently determined by the developed ELISA method. In addition, the interference of fetal bovine serum (FBS) used in the experimental design was also determined. The serially diluted PGE_2 with DMEM medium alone or DMEM with 0.5%, 1%, 2.5%, and 5% fetal bovine serum were also evaluated.

Validation of the ELISA Procedure

Within-run and between-run precision was assessed using three different dilutions of a cell culture supernatant (IL-1β-stimulated human gingival cells) (PGE_2 concentrations: 0.105, 0.525, and 1.050 μg/mL), as well as using three dilutions of purified PGE_2 preparation (PGE_2 concentrations: 0.095, 0.475, and 0.950 μg/mL). For each sample, 5 determinations were performed independently and the mean and standard deviation of each were calculated.

To measure the recovery rate, different amounts of purified PGE_2 (0, 0.05, 0.15, 0.5, and 1.0 μg) were added to cell supernatants containing different concentrations of PGE_2 (1.01, 2.54, and 5.08 μg/mL). PGE_2 concentrations were then measured and the percentage of recovery rates was calculated.

Sample Detection

PGE_2 levels in the supernatant of IL-1β-treated human gingival fibroblast culture were also determined by the developed ELISA method and the commercial PGE_2 Biotrak (EIA) system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). The Biotrak (EIA) system is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody (mouse anti-human PGE_2). The final PGE_2 concentration in the induced cells was calculated after subtracting the baseline level from the uninduced cells.

Cost Analysis

Costs were calculated based on the costs of commercial reagents in US dollars at standard commercial prices, excluding labor.

Data Analysis

Linear regression analysis was carried out using the Sigmaplot software package (Jandel Scientific, San Rafael, CA, USA). PGE_2 levels were expressed as mean concentration in ng/well $\pm$ S.D. The statistical analysis was performed with the SPSS software. A one-way ANOVA and/or an unpaired, two-tailed Student's *t*-test were used to determine the significant differences between the means. A *p*-value of less than 0.05 was accepted as a significant difference.

RESULTS

Optimization of Direct ELISA

To evaluate coating buffer suitable for promoting direct attachment of PGE_2-DMEM to an ELISA plate, 50 mM carbonate (pH 9.6); gelatin phosphate buffer (pH 5.0) and 10 mM PBS (pH 7.2) were used. The condition was evaluated using PGE_2 at the concentrations of 1.25 and 5 μg/mL. The average absorbance value from triplicate at each concentration showed that the gelatin phosphate buffer allow the best attachment of PGE_2 to plates in a dose dependent manner (Table 1). As shown previously, that the gelatin phosphate buffer had been used in radioimmunoassay to prevent nonspecific binding and stabilized PGE_2, the amount of gelatin in coating buffer was also evaluated for optimal condition. We found that 0.2% gelatin provided the lowest background level when compared to other concentrations

Table 1. Optimization of coating buffer for direct ELISA

Sample	Mean optical density at 450 nm		
	Carbonate-bicarbonate buffer, pH 9.6	Gelatin phosphate buffer, pH 5.0	PBS buffer, pH 7.2
Blank	0.036 ± 0.007	0.137 ± 0.027	0.080 ± 0.010
PGE_2 1.25 μg/ml	0.062 ± 0.003	0.510 ± 0.069	0.063 ± 0.011
PGE_2 5.0 μg/ml	0.063 ± 0.007	1.773 ± 0.007	0.149 ± 0.021

(0.2–2% gelatin in gelatin phosphate buffer) under the same condition (data not shown). The amount of gelatin we used is considered much less than the amount of gelatin (5% w/v) employed in the previously described radioimmunoassay.[10,11]

The coating time was subsequently determined with varying concentrations of PGE_2. The results indicated that, at concentrations of PGE_2 from 0.078 to 0.625 μg/mL, the coating time between 24 and 72 h yielded similar detection levels (Fig. 1).

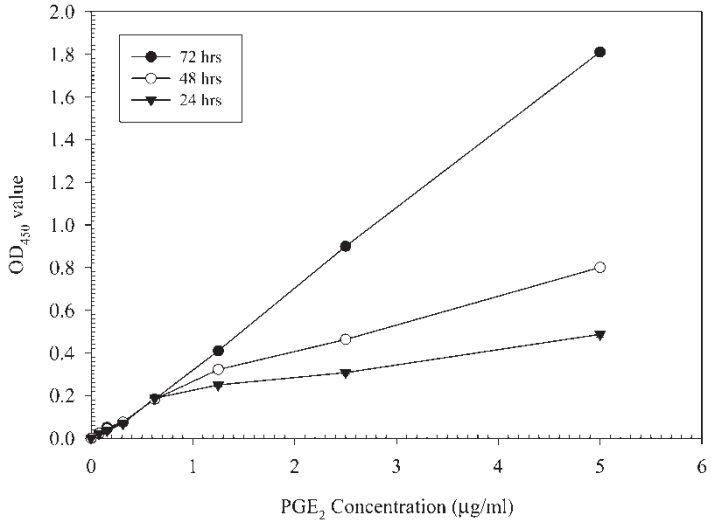

Figure 1. Optimal coating time. PGE_2 in gelatin phosphate buffer was attached to the polystyrene plates for 24, 36, and 72 h at 4°C. The plates were washed and reacted with 1:100 dilution of rabbit anti-PGE_2 serum. They were then reacted with enzyme-labeled anti-human IgG and, subsequently, with the enzyme substrate.

Three blocking solutions, 1%, 2.5%, and 5% skimmed milk, were compared; the results indicated that 1% skimmed milk was the optimal blocking buffer for PGE_2 ELISA (Fig. 2). Some background was observed when 2.5% and 5% skimmed milk were used. To determine the optimal level of rabbit anti-PGE_2 polyclonal antibody, different concentrations of the polyclonal anti-PGE_2 antibody were added at dilutions of 1/100, 1/200 and 1/500. The graph provided the highest absorbance values up to 5 μg/mL of PGE_2 using the polyclonal PGE_2 antibody at the dilution of 1/100 (Fig. 3). The maximum antigen concentration which gave a measurable change in optical density was 0.078 μg/mL.

Due to varied absorbance values observed when PBST solutions with different pH were employed, we chose PBST with pH 5.0 and pH 7.0 as washing solutions in our method. Under this condition, a standard curve of an experiment using pH 5.0 PBST demonstrated a good correlation between PGE_2 concentration and absorbance values with $r^2 = 0.99$ (Fig. 4).

Our data indicated that PGE_2 adsorbed effectively to the ELISA plates when GPB was employed as a coating buffer (Table 1). The ratio of GPB to DMEM at 50:50 (v/v) gave the highest absorbance value, indicating that this ratio provided the most efficient binding of PGE_2 to the plates (Fig. 5). In addition, the optical density decreased if the cell supernatants contained 2.5–5% FBS (Fig. 6).

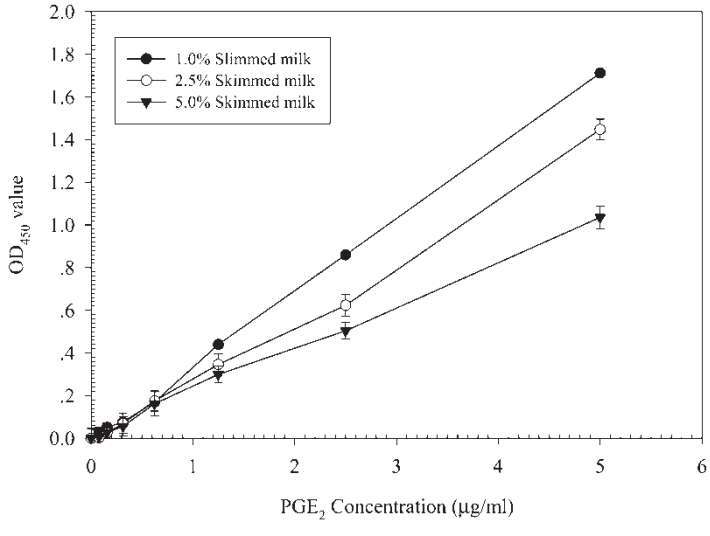

Figure 2. Optimal blocking solution for direct PGE_2 ELISA. Coating PGE_2 antigen was used at concentrations from 0.078–5 μg/mL. Plates were blocked for 1 h with 1%, 2.5%, or 5% skimmed milk. Plates were washed and reacted with 1/100 dilution of rabbit anti-PGE_2 serum. They were then reacted with enzyme-labeled anti-human IgG and, subsequently, with the enzyme substrate.

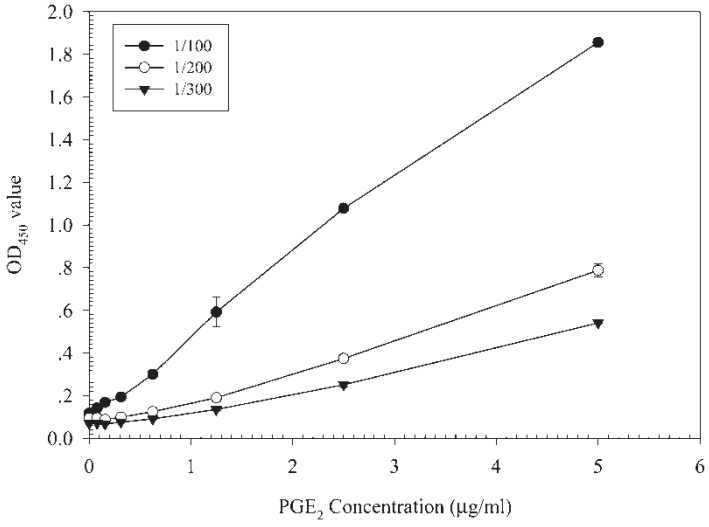

Figure 3. Optimal antiserum. PGE_2 in gelatin phosphate buffer was attached to the polystyrene plates for 72 h at 4°C. Plates were washed and reacted with various dilution of rabbit anti-PGE_2; 1/100, 1/200, and 1/500. They were then reacted with enzyme-labeled anti-human IgG and, subsequently, with the enzyme substrate.

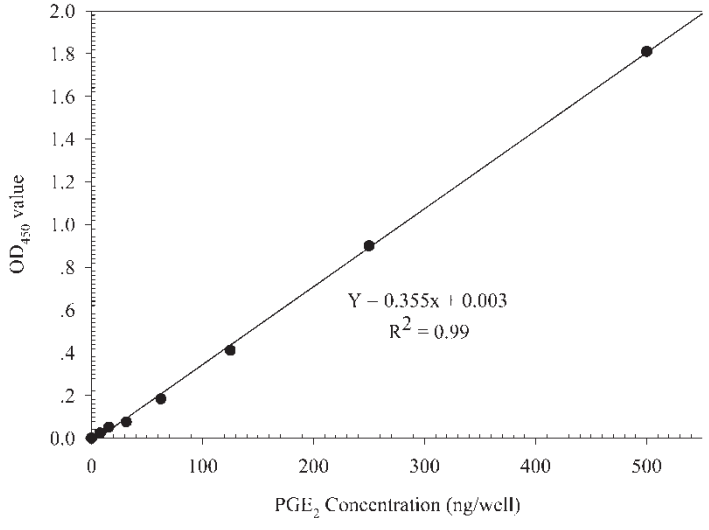

Figure 4. Standard curve constructed for PGE_2 by the direct ELISA method. PGE_2 antigen in gelatin phosphate buffer was allowed to attach to plates at 4°C for 72 h, followed by blocking with 1% skimmed milk and detected with rabbit anti-PGE_2 (dilution 1/100). The curve is linear over a concentration range of 7.8–500 ng/well.

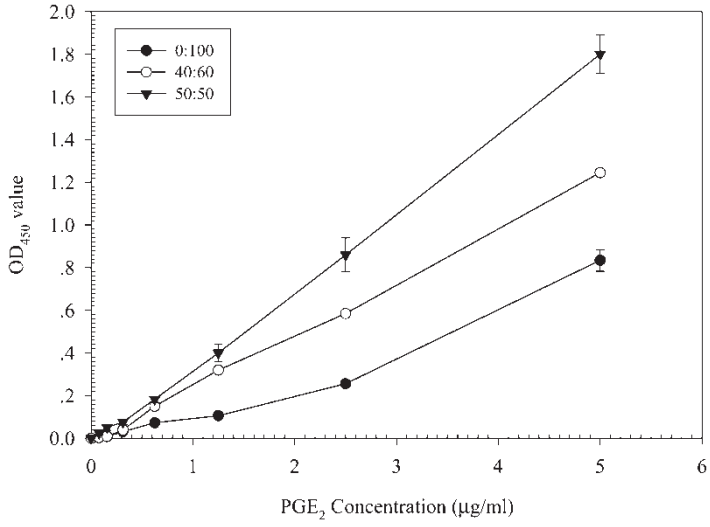

Figure 5. Detection of PGE_2 in cell culture supernatant. PGE_2 antigen in a mixture of DMEM and GPB at different volume ratios (0:100, 40:60, and 50:50) were detected using direct ELISA procedure.

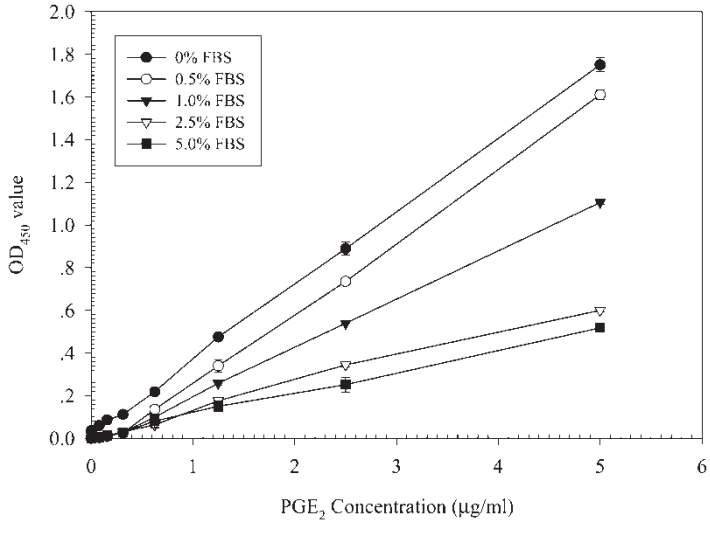

Figure 6. Detection of PGE_2 in cell culture supernatant containing FBS. PGE_2 antigen in a mixture of DMEM without or with FBS at different concentration (0.5%, 1.0%, 2.5%, and 5%) were detected using the direct ELISA procedure.

Validation of the ELISA Procedure

Data obtained from within-assay and between-assays were assessed using different dilutions of a cell supernatant sample (PGE_2 concentrations of 0.107, 0.535, and 1.070 μg/mL). The values of the coefficient of variation range (expressed as the ratio of the standard variation/mean value from 5 replicates) were from 3.2 to 3.7% for within-assay precision and from 3.4 to 3.8% for between-assay precision.

When purified PGE_2 was used in a similar manner (PGE_2 concentrations of 0.097, 0.485, and 0.970 μg/mL), the coefficient of variation values were 2.9–3.4% for within assay precision and 3.4–3.5% for between-assay precision.

Recovery tests were performed by adding purified PGE_2 (0, 0.03, 0.1, 0.2, and 0.3 μg/mL) to supernatant samples containing various amounts of PGE_2 (0.253, 0.507, and 1.014 mg/mL). The percentage of recovery ranged from 94% to 106%, and was comparable for all PGE_2 concentrations tested between 0.253 and 1.314 μg/mL.

Comparison of the Commercial EIA Kit with Direct ELISA

To directly compare our developed method with a commercial kit, different concentrations of standard PGE_2 standard and unknown supernatants were assayed by both the direct ELISA and the commercial EIA kit. The results indicated that the values obtained from both methods were very similar and closely represented the actual PGE_2 levels; the coefficient of variation (%CV) (Table 2) of the commercial ELISA kit was 6.70–8.02 (within the range that illustrated in the manufacturer's instructions), but the developed PGE_2 ELISA gave a lower coefficient of variation (%CV = 3.38–6.16).

Using the assay we developed, the cost per sample is estimated to be between 1.19–1.34 US dollars, as calculated from the commercial prices of all reagents and plasticware used. The commercial Biotrak (EIA) system

Table 2. Comparison of PGE_2 levels from supernatant from IL-1β-stimulated cells using two different methods

Sample	Direct ELISA method (ng/mL)[a]	CV (%)	Commercial EIA kit (ng/mL)	CV (%)
Sample 1	198.1 ± 6.7	3.38	233.4 ± 16.4	7.02
Sample 2	29.2 ± 1.8	6.16	37.3 ± 2.5	6.70
Sample 3	92.0 ± 5.1	5.54	139.8 ± 11.1	7.93
Sample 4	117.6 ± 4.1	3.48	189.2 ± 16.7	8.82
Sample 5	49.5 ± 2.6	5.25	55.8 ± 4.4	7.88

[a]Values are means ± standard deviation.

costs 4.69 US dollars per sample. The cost estimated by direct ELISA is 3–4 times lower than those quoted by the commercial ELISA kit.

DISCUSSION

Previous determination of prostaglandin levels from bodily fluids or plasma were conducted mostly using gas chromatography–mass spectrometry.[12–18] Recent determination employed radioimmunoassay using a gamma-labelled ligand[8] and competitive immunoassay or enzymatic method[6] to measure submicrogram quantities of prostaglandins. These methods are not suitable for assaying a large number of samples and are not applicable to less well equipped laboratories. We developed a protocol to detect PGE_2 in solutions or cell culture supernatants based on direct adsorption of PGE_2 antigen onto the ELISA plate. By varying the types of coating buffer, we found that gelatin phosphate buffer (0.2% gelatin) was more suitable than PBS and carbonate buffers. The difference in observed intensity may reflect a denaturing effect of PGE_2 epitopes caused by the carbonate buffer and the insolubility effect of PGE_2 in PBS buffer. The gelatin buffer had been previously used to diluted PGE_2 as a standard radioimmunoassay.[10,11,19] Five percent gelatin was previously used to block non-specific binding and to stabilize PGE_2.[10] Our results indicated that 0.2% gelatin phosphate buffer was the optimal condition for our direct PGE_2 ELISA and had no effect on the background of the detection.

This newly developed assay offers a good sensitivity at concentrations as low as 7.8 ng/well. A good dose-response curve was further obtained between 0.078 and 5 μg/mL, with a coefficient of determination (r^2) of 0.99. The sensitivity of the assay was also influenced by the type and concentration of blocking solution. Optimal results were obtained when employing the blocking solution of 1% skimmed milk; however, some background was shown with 5% skimmed milk. A background signal was also observed if the cell supernatant sample contained 2.5–5% FBS. This may be due to interference from proteins in FBS. Therefore, this newly developed technique is suitable only with the samples containing low levels of FBS.

Using PGE_2 as the antigen and a rabbit polyclonal antibody raised against purified PGE_2 as the antibody, it was, thus, possible to rapidly quantify PGE_2 in cell supernatant culture with a good sensitivity (7.8 ng/well) and a detection range of approximately 7.8–500 ng/well. Irrespective of the initial concentration of the cell supernatant samples used to test the validity of the present direct PGE_2 ELISA, the assay showed good accuracy with a coefficient of variation ranging from 3.2 to 3.7% and a high recovery, from 94% to 106%. A good dose response was obtained between 0.078 and 5 μg/mL with a slope of 0.355 and a coefficient of determination (r^2) of 0.99.

When known levels of PGE_2 were determined using our method and the commercial kit, there was no significant difference between the two methods.

Both could be used efficiently to determine PGE_2 levels, but with the advantage in cost using our direct ELISA method. Nevertheless, our developed ELISA is a accurate, highly reproducible, and inexpensive method; this may be useful for the routine quantification of PGE_2 in cell culture supernatants. Therefore, a large number of samples could be tested at the same time with lower cost; all reagents could be kept for longer time than with other ELISA kits.

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