A PROSTAGLANDIN SYNTHASE INHIBITION ASSAY WITH DETECTION BY ELISA

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I. ABSTRACT

A simple, reliable method is described for the routine measurement of non-steroidal anti-inflammatory drugs (NSAIDs) as inhibitors of prostaglandin synthase. Appropriate concentrations of inhibitor are incubated with ram seminal vesical prostaglandin synthase, sodium arachidonate and reduced glutathione. Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) production is then quantitated by and enzyme linked immunosorbant assay (ELISA), in which anti-PGE\textsubscript{2} antibody is utilized in a binding competition between test sample and adsorbed conjugate of PGE\textsubscript{2}-BSA. The antibody which remains bound to the solid phase is quantitatively determined colorimetrically by incubation with horse radish peroxidase labeled goat anti-rabbit IgG followed by incubation with N,N,N',N',-tetramethylbenzidine.

The relative inhibitory potency of Indomethacin was determined by comparison of PGE\textsubscript{2} content in sample incubations, with that of controls containing no Indomethacin and was shown to inhibit prostaglandin synthase.

The combination of enzyme-inhibitor incubation assay with detection by ELISA permits the testing of other NSAID compounds for their ability to inhibit prostaglandin synthase activity expediently, and in a financially
feasible manner. Furthermore, the use of an ELISA system eliminates radioactive and toxic chemical waste generated by radioimmuno assay methods.

II. ABBREVIATIONS
Ram PG synthase, prostaglandin synthase from ram seminal vesicals; PGE₂, prostaglandin E₂; Na arachidonate, sodium arachidonate; ELISA, enzyme-linked immunosorbant assay; PGE₂-BSA, prostaglandin E₂ conjugated to bovine serum albumin; rb α PGE₂, rabbit anti-prostaglandin E₂ antibody; g α rb HRPO, horse-radish peroxidase labeled goat anti-rabbit Ig antibody.

III. INTRODUCTION
Approximately 20 years ago, Dr. John Vane, a medicinal chemist, suggested that non-steroidal anti-inflammatory drugs (NSAIDs) reduced fever, swelling and pain by inhibiting an enzyme called prostaglandin synthase. (1) These symptoms are caused in part by an excess biosynthesis of prostaglandins. Prostaglandins are a series of approximately 20 oxygenated, unsaturated compounds with a 7-(2-octylcyclopentyl) heptanoic acid skeleton. (1,2) (see Fig.1) Also, prostaglandins are extremely potent

![Prostaglandin](image)

Fig. 1 Prostaglandin
R may be OH, O or OCH₃
regulators of a sweeping range of normal functions, including blood pressure and flow, blood platelet aggregation, labor in childbirth and gastric acid secretion in addition to pathological pain, fever and inflammation. (1)

It is now known that the cascade of reactions that leads to biosynthesis of prostaglandins begins with the disruption of membrane lipids, by phospholipase A2, to yield a polyunsaturated fatty acid called arachidonic acid. (see Fig. 2)

Subsequent reactions can occur next. Among these is the reaction in which prostaglandin synthase (also called cyclooxygenase) catalyzes the addition of molecular oxygen across conjugated double bonds to form cyclic prostaglandin endoperoxides such as prostaglandin G2 (PGG2). This same enzyme molecule also possesses peroxidase activity, which then converts PGG2 to prostaglandin H2 (PGH2). Hydrolysis of PGH2 leads to mediators of inflammation like PGE2 and prostaglandin F2α (PGF2α). (2)
Membrane bilayer

Disrupted membrane

Arachidonic Acid

Prostaglandin Synthase

Lipoxygenase

Leukotrienes

PGH₂ → PGE₂ → PGF₂α

Fig. 2 Biochemical pathway of the formation of Prostaglandin E₂
Indomethacin, an NSAID, is a known inhibitor of the prostaglandin synthase pathway described above. (2) NSAIDs compete with arachidonic acid for the active site on the enzyme, prostaglandin synthase. (see Fig.3)

Fig. 3 Inhibition of prostaglandin synthase by Indomethacin.

The following report describes an assay suitable for the routine determination and estimation of compounds, such as NSAIDs, as inhibitors of prostaglandin synthase.

IV. MATERIALS AND METHODS

Materials

The following materials were purchased from the sources indicated. Catalog numbers are in parentheses. Na arachidonate (A8198), reduced glutathione (G4251), PGE₂ (P5640), g α rb HRP0 (P5164), 1-cyclohexyl-3-2-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (C1011), bovine serum albumin (A9647), sodium azide (S2002), ovalbumin (A5253), polyethylene sorbiten monolaurate (Tween 20) (P1379), Indomethacin (I7378), from Sigma Chemical Company, St. Louis, MO.; ELISA plates from Immulon I, Dynatech Lab. Inc., Alexandria, VA.; rb α PGE₂ from Dr. Harvey
Sheinkein, Medical College of Virginia, Richmond VA.; prostaglandin synthase from ram seminal vesicles (434738) from Cal Biochem Corp., San Diego, CA.

**Methods**

**Assay to produce PGE2:**

Ram PG synthase was stored in 0.1 M phosphate buffer (pH 7.5) as 1 unit/ml and kept in aliquots at -70°C until use.

For the estimation of ram PG synthase inhibition, dilutions [4] of Indomethacin in 0.1 M phosphate buffer (pH 7.5) were incubated in a standard assay mixture (total volume 150 µl) containing 3.84 x10^{-11} molar (13.3 ng/µl) Na arachidonate, 1.6 x10^{-9} molar (485.8 ng/µl) reduced glutathione as a cofactor and .24 µg ram PG synthase.

The incubation was begun by the addition of Na arachidonate, and carried out in a shaking water bath at 37°C. After 20 min. the reaction was stopped by adding 30 µl of 0.1 M citric acid. All reagents were maintained at 4°C prior to initiation of the assay to prevent loss of activity.

Complete hydrolysis of PGH to PGE2 was obtained by incubating with citric acid at room temperature for 10 min. Dilutions of the incubated assay mixture were made into phosphate buffer for estimation of PGE2 by ELISA.

**ELISA for PGE2**

Small haptens, such as PGE2, contain only one epitope and therefore are unsuitable candidates for "sandwich" ELISAs, which require that two molecules of antibody recognize the same antigen. Furthermore, adsorption of a small hapten directly to the ELISA plate
is usually unsuccessful because the hapten fails to bind, or because it loses antigenicity if binding occurs. This problem can be circumvented by adsorbing conjugates of hapten and protein. (3)

**Conjugation of PGE\(_2\) to Bovine Serum Albumin**

PGE\(_2\) was conjugated to BSA by an amphoteric carbodiimide method. (3) 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate was gradually added to PGE\(_2\) that had been dissolved in N,N dimethylformamide (DMF). The PGE\(_2\)-DMF-carbodiimide solution was added dropwise to a solution of bovine serum albumin. The reaction mixture was maintained at pH 5.0-5.2, stirred for 24 hrs., dialyzed twice against 20 volumes of 0.1 M phosphate buffer (pH 6.5) overnight at 4°C, then diluted in glycerol, aliquoted and stored at -20°C.

**PGE\(_2\) stock solutions**

PGE\(_2\) stock solutions were prepared by dissolving 5 mg PGE\(_2\) in 1.0 M phosphate buffer (pH 6.5) containing 0.10% ovalbumin to give a 10 mg/ml solution, which was aliquoted and stored at -20°C. (3) Standards were made containing 50 to 0.78 ng PGE in 100 µl phosphate buffer (pH 7.5).

**Assay Procedure**

PGE\(_2\)-BSA conjugate was diluted (4 µg/ml) in adsorption buffer, which was 54 mM NaCl, 45 mM glycine, and 0.05 % sodium azide, (pH 8.2). (3) Aliquots of 100 µl/well were adsorbed to 96-well ELISA plates. (Fig. 4, A a & b) After incubating for 2 hours at room temperature, the plates were washed four times with phosphate
buffered saline (pH 7.5) containing 0.05% Tween 20. A standard curve was derived by incubating with 100 µl of known PGE$_2$ concentrations with a 1:22k dilution of rb α PGE$_2$. (Fig. 4, Ba & Ca) Dilutions of the incubated assay mixture were also added to a 1:22k dilution of rb α PGE$_2$ (Fig. 4, Bb & Cb) and then incubated at room temperature for 40 min. After washing (Fig. 4, D a & b) as described above, the plate was incubated for 1 hour at room temperature with 100 µl of 1:1000 g α rb HRPO. (Fig. 4, E a & b) A TMB substrate solution was then made by adding 100 µl of a 10 mg/ml solution of N,N,N',N'-tetramethylbenzidine (dissolved in DMF), and 1.4 µl of 30% hydrogen peroxide to 10 ml of 0.1 M Na acetate buffer (pH 6.0). (CalBiochem, La Jolla, CA). After washing and rinsing the plate with deionized water, 100 µl of this solution was added to each well. (Fig. 4, F a & b) After incubation for 20 min, 25 µl of 2M H$_2$SO$_4$ was added to the plate and absorbance was read at 450 nm on a micro-ELISA auto reader (Dynatech).

**Data Reduction**

Absorbance readings at 450 nm were plotted against the log of PGE$_2$ input concentrations, generating a standard curve from which unknown concentrations were calculated. Values which fell outside the 15-85% inhibition range were generally disregarded.
**Competition ELISA to detect Prostaglandin E₂**

**A)** Coat with PGE₂-BSA

**B)** Addition of standards

**C)** Addition of rabbit α PGE₂

**D)** Wash

**E)** Addition of goat α rabbit HRPO

**F)** Addition of TMB substrate

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**Fig. 4**  
A) Coat with PGE₂-BSA  
B) a- Addition of standards  
b- Addition of samples  
C) Addition of rabbit α PGE₂  
D) Wash  
E) Addition of goat α rabbit HRPO  
F) Addition of TMB substrate
V. RESULTS

Enzyme Incubation Optimization

The enzyme incubation conditions used were based on the results of optimization studies which investigated the effects of increasing Na arachidonate concentration (Fig. 4), ram PG synthase concentration (Fig. 5), reduced glutathione concentration. (Fig. 6) In an effort to conserve materials used in the assay, Na arachidonate was used at 3.84 x 10^{-11} molar (13.3 ng/µl) instead of the optimum which was greater than 7.2 x 10^{-8} molar (166.7 ng/µl).

Fig. 4  Relationship between arachidonate concentration and PGE2 production by ram seminal vesicle prostaglandin synthase. (* Indicates arachidonate concentration (3.84 x 10^{-11}M, 13.3 ng/µl) selected for routine use.)
Fig. 5 Relationship between enzyme concentration and PGE₂ production by ram prostaglandin synthase. (* Indicates enzyme concentration (0.24 mg) selected for routine use.)

Fig. 6 Relationship between reduced glutathione concentration and PGE₂ production by ram seminal vesicle prostaglandin synthase. (* Indicates glutathione concentration (1.6 x 10⁻⁹ M, 485.8 ng/µl) selected for routine use.)
PGE$_2$ levels found in incubates containing [ram PG synthase only], [Na arachidonate only], [reduced glutathione only], [ram PG synthase and Na arachidonate], [ram PG synthase and reduced glutathione], [Na arachidonate and reduced glutathione] were significantly less than the PGE$_2$ produced in incubation assays containing ram PG synthase, Na arachidonate and reduced glutathione.

**ELISA Standardization**

**Coatings**

The optimum PGE$_2$-BSA conjugate titre was determined by coating the plate with 4, 5, and 10 µg/ml and incubating for 2 hours with standard solutions that also contained 1:1000, 1:2000, 1:4000, and 1:8000 dilutions of rb α PGE$_2$. No significant difference was seen between PGE$_2$-BSA coating dilutions, which meant the plate was saturated with conjugate at 4 µg/ml. (Data not shown)

Coating overnight with PGE$_2$-BSA greatly decreased the reliability of the plate. Coating with conjugate that had been frozen and thawed twice, had no effect on the assay.

**Rb α PGE$_2$ Concentration**

To determine optimum rb α PGE$_2$ concentration, dilutions of 1:2000, 1:4000, 1:6000, and 1:8000 were used in wells containing standard solutions of PGE$_2$. (Fig. 8) The linear regression r values were generally poor. The antibody was spun to remove lipids. This significantly increased r values, but absorbance values for less than 10 ng PGE$_2$ were off scale. Rb α PGE$_2$ dilutions of 1:22k and 1:26k were
then tested. A 1:22k dilution gave the greatest range in absorbance, and was selected for use in remaining ELISAs. (Fig. 9)

A sixfold increase in absorbance was typically seen between maximum and minimum standard PGE$_2$ concentrations. Concentrations $>100$ ng PGE$_2$ saturated the rb $\alpha$ PGE$_2$ antibody and inhibited its binding to PGE$_2$-BSA. Concentrations $<0.5$ ng were not detectable.

Rabbit $\alpha$ Zymosan, diluted 1:22k, was used as a control for non-specific binding to PGE$_2$-BSA in all ELISAs. Raw absorbances were generally $<0.08$, and were used as a blank.

No difference in standard curves was seen between stock solutions diluted directly on the ELISA plate and those diluted in separate tubes and transferred onto the plate.

Fig. 8 Standard curves using rb $\alpha$ PGE$_2$ at dilutions of 1:2000, 1:4000, 1:6000, and 1:8000, before spinning antibody to remove lipids.
Specificity of rb α PGE₂

Cross reactivity of rb α PGE₂ with materials used in the assay to produce PGE₂ was tested. Ram PG synthase, Na arachidonate, and reduced glutathione stock solutions were serially diluted from 1:10 to 1:640. No materials bound to the rb α PGE₂. In assays to produce PGE₂, the materials generally reached a final dilution of 1:50 to 1:150.
Time Dependance of rb α PGE2 Incubation

Initially rb α PGE2 was incubated with standards for 2 hours. (3) Binomial regression values fluctuated on a daily basis. We believed that this long incubation period allowed enough time for rb α PGE2 antibody originally bound to free PGE2, to unbind and then rebind to the PGE2-BSA solid phase. (see Fig. 10) This would result in an underestimate of the soluble (free) PGE2. An ELISA was run in which incubation periods with rb α PGE2 and standards were 60, 40, 20, and 10 min. (Fig. 11). Incubation for 40 min. gave a binomial regression of .95. Subsequent ELISAs were then incubated for 40 min.

Fig. 10 Effects of long incubation periods with samples and rb α PGE2.
Determination of ram PG synthase Inhibitors

Indomethacin was added to standard assay mixtures at concentrations of 100 to 1 ng/ml. Indomethacin showed 20-60% inhibition respectively (Fig. 12) using this method but further studies needs to be done in order to determine the IC$_{50}$ (concentration of inhibitor that decreases the activity of the enzyme by 50%).
VI. DISCUSSION

We have developed and validated an enzyme incubation-ELISA detection system for the determination of prostaglandin synthase inhibitors, such as NSAIDs, that is both simple and reliable. The elimination of time consuming extraction steps and radiochemical waste, used in previous inhibition-radioimmuno detection systems, (4,5,6) adds to the efficiency and safety of this method.

Detection of PGE$_2$ by ELISA has numerous advantages over radioimmunoassays (RIAs), such as a dramatic increase in testing
capacity, the elimination of radioactive waste, and an appreciable decrease in the time required for sample turnover.

Two features of the enzyme incubation-ELISA detection system worthy of further comment are the uses of ram seminal vesicle prostaglandin synthase (ram PG synthase) and rb α PGE₂ antibody.

Although the synthase enzyme from ram seminal vesicles is well characterized (7,8) and widely used in studies of prostaglandin synthesis, synthases from different tissues do exhibit differences in their sensitivities to pharmacological inhibitors and in their in vitro versus in vivo sensitivities. (9)

The rb α PGE₂ antibody used in the ELISA was an ammonium sulfate cut of rabbit IgG. Therefore, the concentration of antibodies specific for PGE₂ was extremely low. This resulted in higher backgrounds, (caused by other antibodies in the serum binding to the BSA on the plate), and a greater day to day fluctuation of standard curves. Further work to affinity purify the serum, (thereby increasing rb α PGE₂ concentration), or work in obtaining a monoclonal anti-PGE₂ antibody, would greatly increase the accuracy and precision of the ELISA.

Taking into account these considerations, we believe the enzyme incubation-ELISA detection system reported herein to be particularly suitable for further studies of potential prostaglandin synthase inhibitors such a nonsteroidal anti-inflammatory drugs (NSAIDs).
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VI. REFERENCES


