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A QUANTITATIVE SOLID-PHASE ENZYMEIMMUNOASSAY FOR 13,14-DIHYDRO-15-KETO-PROSTAGLANDIN $F_{2\alpha}$ IN PLASMA[1]

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ABSTRACT

Enzymeimmunoassays (EIA) can be viable alternatives to radioimmunoassays (RIA). Indeed, from an environmental perspective, EIA are preferable to RIA. Therefore, the purpose of this project was to develop a quantitative EIA for 13,14-dihydro-15-keto-prostaglandin $F_2\alpha$ (PGFM) in bovine plasma. Acetylcholine esterase bound covalently to PGFM, rabbit anti-PGFM, mouse monoclonal anti-rabbit IgG, and PGFM were the principle reagents used for the EIA. Validation experiments indicated that: 1) PGFM standard curves, with doses ranging from 391 to 200,000 fg per microtiter well, were linear; 2) assay sensitivity averaged 391 fg per well; 3) for satisfactory results, PGFM had to be extracted from plasma; 4) content of PGFM in ethyl ether extracts of aliquots from serial dilutions of whole plasma with unknown amounts of PGFM and charcoal-stripped plasma supplemented with known amounts of PGFM did not deviate from parallelism with PGFM standard curves in buffer; 5) correlation between EIA and RIA measurements of PGFM in the same plasma samples was .95; 6) the regression of EIA data on RIA data was linear ($Y = .93\ X + 83.9$; $r^2 = .91$); 7) intra- and inter-assay coefficients of variation were 3.3 and 10.6 %, respectively. The EIA developed in this project is a valid and reliable method for quantitating PGFM in extracts of bovine plasma.

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INTRODUCTION

Bioassays, chromatography, radioimmunoassays (RIA) and, most recently, enzymeimmunoassays (EIA) have been used to measure prostaglandins and other arachidonic acid metabolites. Methodological innovations have usually made prostaglandin assays more sensitive and simpler to perform. Thus, RIA has become, perhaps, the most popular method for measuring prostaglandins. Prostaglandin RIA are usually rapid, sensitive and simple, but RIA generate radioactive wastes that, because of environmental concerns and disposal costs, have become problems in many institutions. Enzymeimmunoassays have the analytical advantages of RIA, and EIA eliminate the problems associated with radioactive wastes.

Plasma concentrations of 13,14-dihydro-15-keto-prostaglandin $F_2\alpha$ (PGFM) are frequently measured in many physiological, endocrinological and clinical studies. In the bovine, measuring blood plasma PGFM has been used by numerous investigators to monitor uterine prostaglandin $F_2\alpha$ production in postpartum cows (1,2,3). Significant correlations have been found between the duration of elevated PGFM and the time required for complete uterine involution as well as the interval from parturition to first ovulation followed by a normal luteal phase (4).

The purpose of this project was to develop a sensitive, simple, quantitative EIA for PGFM in bovine plasma. Enzymeimmunoassays for PGFM have been reported (5,6), but those PGFM EIA did not have the combination of sensitivity and simplicity that we believe is desirable for routine use.

MATERIALS AND METHODS

EIA reagents.

Acetylcholine esterase (AChe; EC 3.1.1.7, molecular form = G4) from electric eels (Electrophorus electricus) was bound covalently to PGFM (3), and the PGFM-AChe conjugate (Cayman Chemical Co.; Ann Arbor, Michigan) was used as EIA tracer. Acetylcholine esterase was chosen because previous work indicated that the combination of sensitivity and simplicity of AChe-based EIA was superior to that for EIA developed with other PGFM-enzyme conjugates (5,6,7). Other reagents used for the EIA were: rabbit anti-PGFM (WS 4468-4-1) from Dr. W.J. Silvia (University of Kentucky); mouse monoclonal anti-rabbit IgG (MAB; Cayman Chemical Co.); Ellman's reagent (750 μM 5,5'-dithio-bis(2-nitrobenzoic acid) and 500 μM acetyl thiocholine iodide; Cayman Chemical

Co.); authentic PGFM for standards (Sigma Chemical Co.; St Louis, Missouri). The PGFM-AChe conjugate, MAB and Ellman's reagent were stored lyophilized until just before they were used. Rabbit anti-PGFM was received lyophilized. Crossreactivity of anti-PGFM lot WS 4468-4-1 was identical to that for WS 4468-3 (W.J. Silvia, personal communication), which was < .1% with $PGF_2\alpha$, PGE_2, PGA_2 and 6-keto-$PGF_1\alpha$ (8).

EIA buffers.

Four primary buffers were used for this EIA; they were potassium phosphate buffer (1 M), EIA buffer, saturation buffer and wash buffer. All buffers were prepared with deionized, organic-free, reagent-grade water. Potassium phosphate buffer (1 M) contained 174.18 g of potassium phosphate, dibasic and 136.09 g of potassium phosphate, monobasic per liter, and 10 M potassium hydroxide was used to adjust pH to 7.4. Potassium phosphate buffer (1 M) was used to prepare other buffers. A liter of EIA buffer contained 23.4 g sodium chloride, 370 mg tetrasodium EDTA, 1 g fraction V bovine serum albumin (BSA; 98 to 99 % albumin; Sigma Chemical Co.), 100 mg sodium azide and 100 ml of 1 M potassium phosphate buffer; pH was adjusted to 7.4 with 10 M potassium hydroxide. Saturation buffer was the same as EIA buffer, except saturation buffer contained 3 g BSA and 300 mg sodium azide per liter. A liter of wash buffer contained 10 ml of 1 M potassium phosphate buffer and .5 ml Tween 20 (Sigma Chemical Co.). All buffers were stored at 4°C.

Reagent dilutions.

The PGFM-AChe in each vial from the supplier was reconstituted with 60 ml of EIA buffer; PGFM-AChe was packaged as 500 determinations per vial. Aliquots (6 ml) of PGFM-AChe solution (master dilution) were stored at -20°C. For use in the EIA, aliquots of the master dilution of PGFM-AChe were diluted with EIA buffer (2:1 PGFM-AChe solution:EIA buffer). Rabbit anti-PGFM was reconstituted with water, diluted 1:100 with EIA buffer, and stored at -20°C in 1 ml aliquots. For use in the EIA, aliquots of anti-PGFM were thawed and diluted to 1:800,000 with EIA buffer. Mouse monoclonal anti-rabbit IgG in each vial from the supplier was reconstituted with 100 ml of .05 M potassium phosphate buffer; MAB was packaged as 500 determinations (1000 μg) per vial. Ellman's reagent (250 determinations per vial) was reconstituted with 50 ml of water. Authentic PGFM was diluted with 100 % ethanol to a concentration of 10 ng/ml (master dilution) and stored at -20°C until it was used to prepare EIA standards. Ethanol

was evaporated from aliquots of the master dilution of PGFM, and assay standards in doubling doses from 391 to 200,000 fg/50 µl were prepared in EIA buffer. This allowed for the measurement of 7.8 to 4000 pg/ml using a 50 µl sample volume.

PGFM extraction.

Because initial results indicated that whole blood plasma interfered with the EIA, PGFM was extracted from plasma. Anhydrous ethyl ether was used to extract PGFM from acidified (25 µl of 1 N HCl/200 µl plasma) bovine plasma (200 µl). The ratio of ether to plasma was 15:1. Freezing was used to separate ether and aqueous phases. Ether phases were decanted into polypropylene tubes, and the ether was evaporated at room temperature with a stream of air. In previous work with RIA and high-performance liquid chromatography, we found that air did not damage PGFM appreciably; however, evaporating solvents with air, rather than nitrogen, damaged PGE_2 and $PGF_2\alpha$. Ether extracts were reconstituted with 200 µl of EIA buffer. Efficiency of extracting [^{3}H]PGFM from bovine plasma averaged 91%.

Equipment.

Essential equipment for the EIA included: polystyrene microtiter plates and self-adhesive polyester sealing film (Nunc Maxisorb type II-F96; Thomas Scientific, Swedesboro, New Jersey); microtiter plate spectrophotometer (Titertek Multiskan; Flow Laboratories Inc., McLean, Virginia); water purification system (Corning Mega-pure MP-190 LC, Corning, New York); orbital shaker.

EIA procedures.

To coat wells in microtiter plates, 200 µl of MAB solution (10 µg/ml) were pipetted into each well; MAB binds to sites on the surface of the wells. Plates were covered with sealing film and incubated for 18 to 20 hr at room temperature. To block binding sites remaining on the surface of the wells after incubation with MAB, 100 µl of saturation buffer were added to MAB solution in each well. Plates were covered with sealing film and incubated at 4°C. After at least 18 hr at 4°C, plates could be used immediately for EIA, or they could be stored for several weeks at 4°C. Plates were stored with MAB and saturation buffer in the wells.

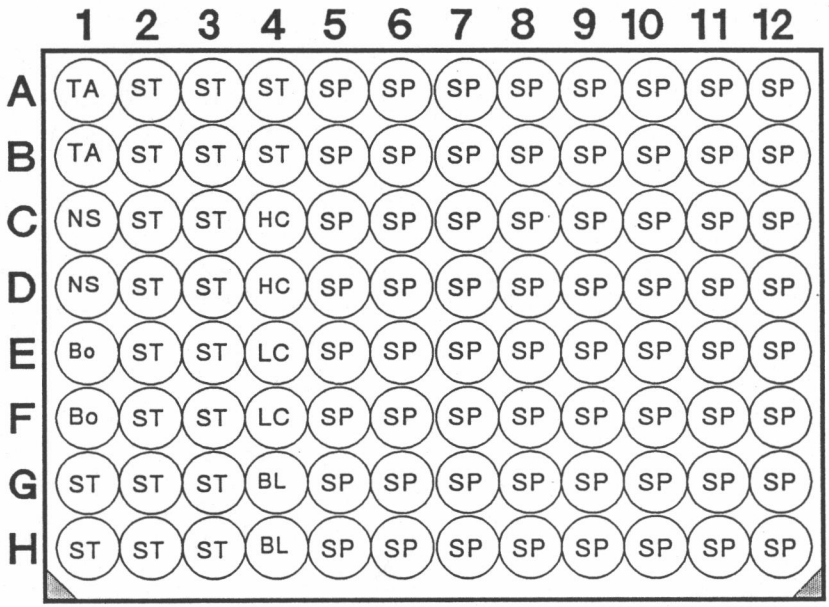

Figure 1. Outline of a microtiter plate showing the arrangement of total activity (TA), non-specific binding (NS), total binding (B_o), PGFM standard (ST), high (HC) and low (LC) PGFM concentration control samples, solvent blank (BL) and sample (SP) wells.

For EIA, microtiter plates were warmed to room temperature, and solutions were emptied from the wells. Wells were rinsed three times with wash buffer. Wash buffer was allowed to drain from the plates for about 5 min after the last rinse.

Standards and reconstituted extracts from plasma samples were pipetted (50 μl) into microtiter wells; the format is depicted in Figure 1. Initial trials indicated that position in the plate did not affect standard curves. So for convenience, standard curves were positioned at the front of each plate. Except for total activity (TA) wells, PGFM-AChe (50 μl) solution was added to each well. Except for TA and nonspecific binding (NSB) wells, anti-PGFM (50 μl) was added to each well. EIA buffer was used to adjust NSB and total binding (B_o; PGFM-AChe and anti-PGFM only) wells to 150 μl. Plates were incubated at room temperature.

After 18 to 20 hr of incubation, solutions were emptied from wells, and wells were rinsed five times with wash buffer. PGFM-AChe (5 µl) was added to TA wells, and 200 µl of Ellman's reagent were added to all wells. Plates were covered with sealing film and placed in the dark on an orbital shaker. Optical density (OD; measured at 414 nm) of solutions in microtiter wells was measured periodically. When OD for B_0 wells was between .3 and .5 units, which was about 6 h after addition of Ellman's reagent, OD was measured in all wells in a plate.

Because the volume of PGFM-AChe added to TA wells was 10% of that added to other wells, OD for TA wells was multiplied by 10. Adjusted OD for TA wells was used to calculate percent binding. Equations derived from the regression of logit of percent binding on log of concentration were used to calculate the amount of PGFM in extracts of plasma. Content of PGFM per well was not corrected for extraction efficiency.

PGFM radioimmunoassay.

Procedural details and validation statistics for the RIA used in this project have been reported (3,9).

EIA validation procedures.

The Statistical Analysis System (SAS; 10) was used for all data analyses. Regression analysis was used to derive the equation that best described the shape (i.e., linear, quadratic, etc.) of standard curves. To determine if plasma PGFM curves and standard PGFM curves in EIA buffer deviated from parallelism, bovine plasma samples (n=15) with unknown amounts of PGFM and charcoal-stripped bovine plasma (CSP) supplemented with known amounts of PGFM (n=5 lots) were diluted serially with CSP. PGFM was measured in extracts from aliquots of diluted plasma. Analyses of variance and tests for heterogeneity of residual variances were used to determine if plasma PGFM curves and standard PGFM curves deviated from parallelism.

Enzymeimmunoassay and RIA were used to measure PGFM in 40 bovine plasma samples in which PGFM concentrations were expected to range from low to high. Analysis of variance was used to determine if PGFM concentrations differed between EIA and RIA. Pearson product-moment correlation was used to determine the degree of association between EIA and RIA data. The regression of EIA data on RIA data was used to derive the equation that best described the mathematical relationship between PGFM measurements made with the two

analytical procedures. Regression equations usually indicate cause and effect relationships. However, the regression equation derived from this analysis of EIA and RIA data is only descriptive and not indicative of a cause and effect relationship.

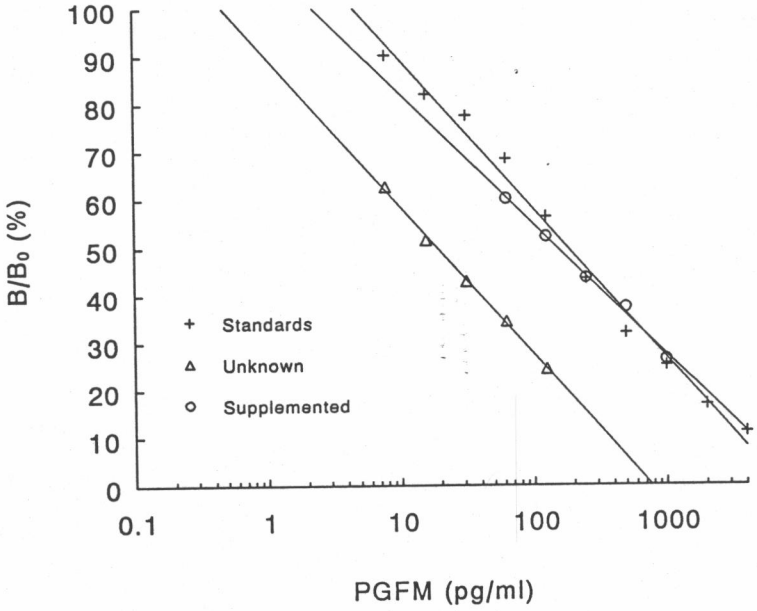

Figure 2. Relationship between plasma PGFM curves and standard PGFM curves in buffer. Charcoal-stripped bovine plasma (CSP) supplemented with known amounts of PGFM and bovine plasma samples with unknown amounts of PGFM were diluted serially with CSP, and PGFM was measured in extracts of aliquots of diluted plasma. Curves did not deviate ($P > .10$) from parallelism.

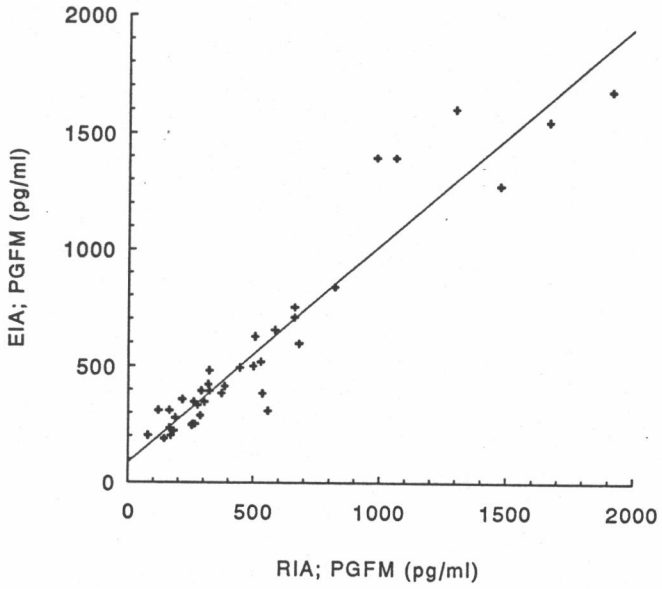

Figure 3. Relationship between concentrations of PGFM measured with enzymeimmunoassay (EIA) and radioimmunoassay (RIA) in extracts of the same bovine plasma samples (n = 40). The equation, $Y = .93\ X + 83.9$ ($r^2 = .91$), best described the mathematical relationship between EIA and RIA data. Correlation (.95) between EIA and RIA data was significant ($P < .0001$).

RESULTS

Standard curves were linear over doses ranging from 391 to 200,000 fg of PGFM per microtiter well. At 93 % relative binding (calculated from 23 assays), EIA sensitivity was 391 fg/well, which was equivalent to 7.8 pg/ml plasma.

Plasma PGFM curves and standard PGFM curves in buffer did not deviate ($P > .10$) from parallelism (Figure 2). Concentrations of PGFM measured with EIA did not differ ($P > .10$) from those measured with RIA. The correlation between EIA and RIA data was .95 ($P < .0001$). The equation, $Y = .93\ X + 83.9$ ($r^2 = .91$), best described the mathematical relationship between EIA and RIA measures of PGFM in

extracts of the same plasma samples (Figure 3). Based upon data from extracts of control plasma samples included in 23 assays, intra- and inter-assay coefficients of variation were 3.3 and 10.6 %, respectively.

DISCUSSION

Results indicate that the EIA developed in this project is a valid and reliable method for quantitating PGFM in extracts of bovine plasma. Our EIA is considerably more sensitive (391 fg/well) than is our RIA (10,000 fg/tube) for PGFM or the RIA developed with the same PGFM antibody used in our EIA (77,000 fg/ml; 8). In addition, our EIA can be completed more rapidly and is inherently more environmentally responsible than is our RIA. Replacing sodium azide (LD 50 = 27 mg/kg in rats; 11) in buffers with another less toxic preservative such as thimerosal (LD 50 = 98 mg/kg in rats; 12), which should not affect assay validity, could enhance the overall safety of our EIA. Reagent costs per plasma sample for the EIA and RIA are comparable, but our estimates did not include the cost of disposing of radioactive wastes from the RIA.

Based upon the literature, our assay for PGFM is more sensitive than one EIA, which used a ß-galactosidase-PGFM conjugate (391 vs. 3,540 fg/well; 5), and less sensitive than another, which used a biotin-streptavidin amplified peroxidase system (391 vs. 57 fg/well; 6). The PGFM EIA with the biotin-streptavidin amplified peroxidase system appears to be more complicated and less rapid than our assay. Thus, we believe that our EIA combines the sensitivity and simplicity needed for making routine measurements of PGFM in large numbers of bovine plasma samples.

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