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Technical Notes

Determination of Prostacyclin in Plasma through a Bioluminescent Immunoassay for 6-Keto-prostaglandin $F_{1\alpha}$: Implication of Dosage in Patients with Primary Pulmonary Hypertension

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This work describes a solid-phase immunoassay for 6-keto-prostaglandin $F_{1\alpha}$, the stable hydrolysis product of prostacyclin (prostaglandin I_2). Prostacyclin, a potent vasodilator with antiplatelet and antiproliferative properties is an effective treatment for primary pulmonary hypertension and pulmonary arterial hypertension associated with scleroderma and scleroderma-like syndrome. Levels of 6-keto-prostaglandin $F_{1\alpha}$ can be directly correlated with levels of prostacyclin. Therefore, 6-keto-prostaglandin $F_{1\alpha}$ has become the indicator of choice to measure prostacyclin levels. The single-step immunoassay for 6-keto-prostaglandin $F_{1\alpha}$ reported here was developed using the bioluminescent protein aequorin as a label. Analyte−label conjugates were constructed by linking the carboxyl group of 6-keto-prostaglandin $F_{1\alpha}$ and lysine residues of aequorin by chemical conjugation methods. The binding properties of 6-keto-prostaglandin $F_{1\alpha}$ toward its antibody and the bioluminescent properties of aequorin were retained in the conjugate, which was then used to generate a dose−response curve for the analyte in a convenient microtiter plate format. The concentration of 6-keto-prostaglandin $F_{1\alpha}$ after extraction from plasma showed good correlation with the concentration of 6-keto-prostaglandin $F_{1\alpha}$ obtained without prior extraction of the same plasma sample. This measurement demonstrated that the assay allows the measurement of 6-keto-prostaglandin $F_{1\alpha}$ directly in plasma without any pretreatment of the samples, which results in a much simpler method with a faster assay time.

Primary pulmonary hypertension (PPH) is a rare disease of the pulmonary vasculature, resulting in abnormally high pressure in the pulmonary artery, right ventricular failure, and death. Although the pathogenesis of PPH is not clearly understood, the increased pulmonary vascular resistance observed is attributed to three main factors: (1) vasoconstriction, (2) thickening of the vessel wall due to vascular remodeling, and (3) in situ thrombosis.[1,2] There is no cure for PPH, and the treatment options are limited.[3] The survival rate is less than a year in patients presented with New York Heart Association functional class IV symptoms. Although lung transplantation is a possible treatment option, there are obvious drawbacks, such as shortage of donor organs and chronic rejection as well as the possibility of infection.[4] Long-term intravenous treatment with epoprostenol (also known as prostacyclin or PGI_2) has greatly increased the survival rate.

Prostacyclin or PGI_2 (prostaglandin I_2) is a major cyclooxygenase metabolite of arachidonic acid and is mainly produced by vascular endothelial cells. Prostacyclin is a prostaglandin, which occurs along with other eicosanoids, such as thromboxanes and leukotrienes. Prostacyclin is a potent vasodilator with platelet-inhibitory, antiproliferative, and fibrinolytic activities.[5] The exact levels of prostacyclin in human plasma are unknown; however, like most eicosanoids, it occurs in very low amounts (pg/mL). It might be worthwhile to mention the levels of certain other important eicosanoids here. Thromboxane B_2 (metabolite of thromboxane A_2, a vasoconstrictor) and its metabolites occur in very low amounts, such as 1−10 pg/mL, in plasma. Leukotrienes generally function as vasoconstrictors and bronchoconstrictors and occur in varying amounts in plasma and urine. Leukotriene B_4 occurs between 50 pg/mL and 50 ng/mL, leukotriene E_4 occurs between 80 pg/mL and 1000 ng/mg of creatinine. Prostaglandin $F_{2\alpha}$ concentrations are found between 10 and 100 pg/mL, prostaglandin D_2 is generally found at 5−500 pg/mL, and prostaglandin $F_{1\alpha}$ occurs at 1−100 ng/mL.

Currently, intravenous epoprostenol is indicated for the chronic treatment of PPH in New York Heart Association (NYHA) functional class III and class IV patients who do not respond to

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conventional therapy.[6,7] There is no set dose of prostacyclin for PPH, and the starting dose varies between 4 and 8 ng/kg/min. Following the initiation of intravenous prostacyclin, most patients require an automatic dose increase of 2 ng/kg/min every two weeks until the rate of administration reaches 20 ng/kg/min. A further dose increase of 0.5−1 ng/kg/min per week is needed only upon the return of symptoms. Common side effects of intravenous prostacyclin include flushing, muscle pain, and abdominal cramps, and these symptoms are not necessarily dose-dependent. Thus, a method to quantify and correlate the plasma levels of prostacyclin with the onset of the untoward side effects or functional improvement would be useful in determining the optimal level of plasma prostacyclin for an individual patient.

A method to quantify the plasma levels of prostacyclin would be useful in determining the optimal level of plasma prostacyclin for an individual patient. Prostacyclin is very unstable, with a half-life of only 60 min in plasma and 2−3 min in buffer. The immediate and stable product of hydrolysis of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), is quantifiable and can be used as a surrogate marker of the level of serum prostacyclin. The detection method for prostacyclin or its breakdown product has to be extremely sensitive and accurate, since either molecule is present in very low amounts (picograms-per-milliliter) in the body. Moreover, the existing methods are time-consuming, mainly due to the necessity to extract 6-keto-$PGF_{1\alpha}$ from biological samples.[8,9] In an effort to develop a rapid and sensitive detection method for prostacyclin, we now report a single-step, solid-phase immunoassay for 6-keto-$PGF_{1\alpha}$ using the bioluminescent protein aequorin.

Aequorin has been proven to be a highly sensitive label in the development of binding assays for the determination of large and small biomolecules.[10−14] The bioluminescent protein aequorin is native to the jellyfish *Aequorea victoria*. Aequorin is a bioluminescent photoprotein that is composed of an apoprotein (apoaequorin, 189 amino acid residues), a chromophoric unit (coelenterazine), and molecular oxygen. Aequorin has three Ca^{2+} binding sites. When Ca^{2+} binds to aequorin, it undergoes a conformational change that results in the oxidation of coelenterazine to coelenteramide, and this produces CO_2 and emits a flash of light at 469 nm. The bioluminescent signal produced by aequorin has virtually no background signal, and the quantum efficiency is between 11 and 17.5%, allowing it to be detected down to attomole levels.[13−15] We have used a cysteine-free mutant of aequorin in this work, because it shows enhanced activity.

The strategy that was employed here to develop an assay for 6-keto-$PGF_{1\alpha}$involved first the production and purification of recombinant aequorin. The compound 6-keto-$PGF_{1\alpha}$ was conjugated to pure aequorin by conventional covalent attachment to the free amine groups of the lysine residues in aequorin. Aequorin contains 15 lysine residues. The effect of choosing different initial ratios of analyte to the apoprotein on the bioluminescence properties of the protein and the binding ability of an anti-6-keto-$PGF_{1\alpha}$ antibody was investigated. Moreover, the effect of the antibody for 6-keto-$PGF_{1\alpha}$ on the luminescence signal was evaluated. A sensitive bioluminescent binding assay was then developed for 6-keto-$PGF_{1\alpha}$. The assay was then optimized for detection of the analyte in plasma. Several plasma samples from actual PPH patients were analyzed by using them directly in the assay without any extraction of 6-keto-$PGF_{1\alpha}$.

EXPERIMENTAL SECTION

Reagents. Luria Bertani (LB) broth and agar were purchased from DIFCO Laboratories (Detroit, MI). Sodium dodecyl sulfate (SDS), sodium hydroxide, sodium chloride, calcium chloride, bovine serum albumin (BSA), kanamycin, ethylenediaminetetraacetic acid (EDTA) disodium salt, and 6-keto-prostaglandin $F_{1\alpha}$ were obtained from Sigma Laboratories (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL). HQH, quaternized polyethyleneimine anion exchanger, was purchased from PerSeptive Biosystems (Cambridge, MA). The anti-6-keto-prostaglandin $F_{1\alpha}$ antibody and the 96-well microtiter plates coated with anti-sheep-IgG were purchased from Assay Designs Inc. (Ann Arbor, MI). Tris(hydroxymethyl)amino methane (Tris) was purchased from Research Organics (Cleveland, OH). Coelenterazine was purchased from Biosynth International (Naperville, IL). Bradford assay kits were obtained from Bio-Rad Laboratories (Hercules, CA). Patient samples as well as control samples were provided by Dr. Michael Poon (Mount Sinai School of Medicine, New York). All solutions were prepared using deionized (Milli-Q Water Purification System, Millipore, Bedford, MA) distilled water. All chemicals were analytical reagent grade or better, with >95% purity, and were used as received.

Apparatus. Cell cultures were grown in an orbital shaker, Forma Scientific (Marrietta, OH), and were centrifuged using a Beckman J2-MI centrifuge (Palo Alto, CA). The fractions were lyophilized using a VirTis Bench Top III freeze-dryer (Gardiner, New York). Bioluminescence measurements for the assay were made on MLX Microtiter Plate Luminometer Dynex (Chantilly, VA). For all of the measurements, four replicates were performed, and three of the closest were selected. All luminescence intensities reported are the average of three replicates, and have been corrected for the contribution of the background.

METHODS

Expression and Purification of Apoaequorin. The plasmid pSD110 containing the cysteine-free mutant (mutant S) of aequorin was constructed and initially transformed in competent *Bacillus subtilis* cells in our laboratory.[16] For protocols for the expression and purification of apoaequorin and the conversion of apoaequorin to the photoprotein, please see ref 16.

Conjugation of Aequorin to 6-Keto-prostaglandin $F_{1\alpha}$. A volume of 1 mL of 1×10^{-6} M aequorin was allowed to incubate

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with coelenterazine for 3 h at 4 °C. The *N*-hydroxysuccinimide ester of 6-keto-prostaglandin $F_{1\alpha}$ was prepared by reacting with the water-soluble analogue of *N*-hydroxysuccinimide (NHS), sulfo-NHS, and the dehydrating agent EDC.[17] The analyte 6-keto-prostaglandin $F_{1\alpha}$ (1 mg/mL) was allowed to react with ~ 5 mM sulfo-NHS and ~2 mM EDC (final concentrations) for 30 min at room temperature. The reaction was quenched by adding β-mercaptoethanol to a final concentration of 20 mM. This NHS ester of the analyte was then conjugated to aequorin with bound coelenterazine in three different mole ratios: 1:50, 1:200, and 1:500 of aequorin/6-keto-$PGF_{1\alpha}$. The conjugates were passed through a size-exclusion column to remove any unconjugated NHS ester of 6-keto-$PGF_{1\alpha}$ and any residual reactants or reducing agent. The fractions were tested for activity of aequorin by taking 100 μL of each fraction and then adding 3 μL of 2.36×10^{-4} M coelenterazine and measuring the intensity of light emitted, as described before. The fractions showing activity were combined and lyophilized. The lyophilized conjugates were resuspended in 30 mM Tris−HCl buffer, pH 7.0, containing 2 mM EDTA, 0.15 M NaCl, and 1 mg/mL BSA (assay buffer) and stored in aliquots at −80 °C.

Calibration Study. Calibration curves were generated for each of the conjugates (1:50, 1:200, 1:500) by first converting the apoaequorin conjugate to aequorin with coelenterazine as described before. Standard solutions were prepared by serially diluting the stock solutions of the conjugates with the assay buffer containing. A volume of 100 μL of these solutions was placed in a microtiter plate, and the luminescence activity of aequorin was measured. The luminescence signal was integrated over a 3-s time period. All points were obtained in triplicate.

Binder Dilution Study. A volume of 100 μL of different concentrations of serially diluted anti-6-keto-$PGF_{1\alpha}$ antibody produced in sheep and 50 μL of 1×10^{-10} M 6-keto-$PGF_{1\alpha}$−aequorin conjugate were placed simultaneously in microtiter plates precoated with a secondary antibody, donkey anti-sheep IgG. All dilutions were performed in the assay buffer. The plates were incubated in the dark at room temperature for 2 h with shaking at 300 rpm. Then the plates were washed with wash buffer (30 mM Tris−HCL buffer, pH 7.0, containing 2 mM EDTA, 0.15 M NaCl, and 0.1% Tween 20) three times to remove any unbound primary antibody or any unbound conjugate. Finally, the light intensity was measured as described earlier for the calibration plot.

Dose−Response Curve. A dose−response curve was generated for 6-keto-$PGF_{1\alpha}$ by simultaneously incubating 100 μL of 6-keto-$PGF_{1\alpha}$ antibody (1:10 000 dilution); 50 μL of different concentrations of the serially diluted analyte, 6-keto-$PGF_{1\alpha}$; and 50 μL of 1×10^{-10} M coelenterazine-bound 6-keto-$PGF_{1\alpha}$−aequorin conjugate (1:500) on microtiter plates coated with the secondary antibody. The plate was incubated in the dark at room temperature for 2 h with shaking at 300 rpm. The plate was washed three times with the wash buffer to remove any unbound components, and the light intensity was measured as described before.

Dose−Response Curve for 6-Keto-$PGF_{1\alpha}$ in Human Plasma. The dose−response curve for 6-keto-$PGF_{1\alpha}$ in plasma was generated by preparing serially diluted standard solutions of 6-keto-$PGF_{1\alpha}$ in human plasma (previously diluted 1:200 times with deionized water) and adding them simultaneously with 6-keto-$PGF_{1\alpha}$−aequorin conjugate and the 6-keto-$PGF_{1\alpha}$ antibody to the wells with the immobilized secondary antibody. The incubation and washing steps were performed in the same manner as previously described in the generation of dose−response curve. The bioluminescence was triggered and measured as described before.

Comparative Study between Analysis of 6-Keto-$PGF_{1\alpha}$ with and without Extraction from Plasma. To extract 6-keto-$PGF_{1\alpha}$ from plasma, a fixed volume of the plasma sample was first acidified with 2M HCl to pH 3.5. The sample was allowed to stand for 15 min at 4 °C, after which it was centrifuged for 2 min to remove any precipitate. Meanwhile, a C_{18} reversed-phase column was washed with 10 mL of ethanol, followed by 10 mLof deionized water. The sample was then applied under a slight positive pressure to obtain a flow rate of 0.5 mL/min. The column was washed with 10 mL of water, followed by 10 mL of 15% ethanol and, finally, 10 mL of hexane. The sample was eluted using 10 mL of ethyl acetate. Elution was carried out at a flow rate of 2 mL/min. The ethyl acetate in the samples was evaporated under a stream of nitrogen. The dried samples were first dissolved in 50 μL of ethanol and then in the same volume of the assay buffer as that of the plasma sample initially used for extraction. These samples were used in the dose−response curve performed in buffer.

The analysis of 6-keto-$PGF_{1\alpha}$ directly in plasma without extraction was performed by diluting the samples 1:200 times and using 50 μL in the dose−response curve along with 100 μL of 6-keto-$PGF_{1\alpha}$ of antibody and 50 μL of 6-keto-$PGF_{1\alpha}$−aequorin conjugate. The analysis was performed in the same manner as the dose−response curve described before. The amount of 6-keto-$PGF_{1\alpha}$ in the samples was calculated from the standard dose response curve generated in plasma. The appropriate dilution factor was taken into consideration when calculating the amount of 6-keto-$PGF_{1\alpha}$ present in each sample.

In Vivo Analysis. Plasma samples were obtained from five patients with severe pulmonary arterial hypertension and seven normal controls. Written informed consent was obtained from all patients; the protocol was approved by the Institutional Review Board at Mount Sinai School of Medicine. Of the five patients, three were on chronic intravenous prostacyclin. All patient samples were analyzed in the same manner as the dose−response curve in plasma. The samples were diluted 1:200 times to minimize matrix effects, and 50 μL of the sample was used in the assay. The amount of 6-keto-$PGF_{1\alpha}$ in the samples was calculated as described before for sample analysis without extraction. All plasma samples were obtained and stored frozen at −20 °C in aliquots until use.

To obtain recovery data, further analysis of the samples was performed. Two of the normal plasma samples and two patient plasma samples were first analyzed using the assay without extraction. Then the normal samples were spiked with 5 pg/mL of 6-keto-$PGF_{1\alpha}$, and the patient samples were spiked with 10 pg/mL of 6-keto-$PGF_{1\alpha}$, and the samples were used once again in the assay. In each case, the samples were diluted 1:200 times to minimize matrix effects, and 50 μL of the sample was used in the assay. The amount of 6-keto-$PGF_{1\alpha}$ in the samples was calculated as described before for sample analysis without extraction. The samples were analyzed in triplicate.

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RESULTS AND DISCUSSION

Various types of detection methods have been reported in the literature for prostacyclin using its stable hydrolysis product, 6-keto-$PGF_{1\alpha}$, as a measure of prostacyclin levels. For example, the methods include radioimmunoassays, enzyme immunoassays, and detection using gas liquid chromatography/mass spectrometry (GLC/MS) or gas chromatography-selective ion monitoring (GC-SIM).[9,18,19] Radioimmunoassay is a highly sensitive method, but it has the disadvantages associated with the use of radioactivity. Moreover, all the immunoassays or chromatographic techniques reported for 6-keto-$PGF_{1\alpha}$ require extraction of 6-keto-$PGF_{1\alpha}$ from plasma or serum samples, making the process laborious and time-consuming.[9,18,19] We have developed a sensitive bioluminescent immunoassay for 6-keto-$PGF_{1\alpha}$ in which the plasma samples can be analyzed directly in the assay without requiring the extraction of 6-keto-$PGF_{1\alpha}$ from plasma. This results in a simpler, faster method for detection of this analyte in plasma samples. The assay for 6-keto-$PGF_{1\alpha}$ was developed in a microtiter plate format using the photoprotein aequorin as the label. Colorimetric and fluorescent assays could possibly suffer from background interference, limiting their use in direct plasma analysis. Aequorin, however, being a bioluminescent label, has virtually no background associated with its use and works at physiological pH. Furthermore, as shown in our laboratory and elsewhere, aequorin is a highly sensitive label.[10−14]

The plasmid containing the cysteine free mutant of aequorin was previously constructed in our laboratory.[16] The cysteine-free mutant of aequorin was chosen for the development of this assay, because it showed enhanced activity, as compared to wild-type aequorin. The cysteine-free mutant of aequorin was expressed from plasmid pSD110. Purification of aequoin was carried out using an HQH anion-exchange column. The fractions containing bioluminescence activity were lyophilized with glucose and later resuspended in deionized water. SDS−PAGE analysis was carried out to check the efficiency of purification. A single band was observed at ~20 kDa in the lyophilized fraction for pure aequorin. The yield of the protein was found to be 1.8 mg/L of culture.

Before conjugating 6-keto-$PGF_{1\alpha}$ to aequorin, the lyophilized fraction was dialyzed against bicarbonate buffer to remove any residual Tris−HCl salt, which might interfere with the conjugation. The dialyzed aequorin was then charged with a three times molar excess of coelenterazine so that the analyte would not conjugate in the coelenterazine binding site, which might cause loss in activity of aequorin.

The conjugation method used involved a two-step coupling method using EDC (1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride) and sulfo-NHS, which is a water-soluble analogue of NHS (*N*-hydroxysuccinimide). EDC, a dehydrating agent, reacts with the carboxyl group of 6-keto-$PGF_{1\alpha}$ and forms an amine-reactive, unstable, *O*-acylisourea intermediate of 6-keto-$PGF_{1\alpha}$. NHS reacts with the *O*-acylisourea intermediate to form an NHS ester to stabilize this intermediate. The NHS ester then reacts with the amine groups on aequorin. The advantage of the two-step procedure is the prevention of the modification of the protein carboxyl groups as a result of exposure to EDC.[17] The reaction is quenched by a thiol-containing compound, such as β-mercaptoethanol. Three different conjugates were prepared (1:50, 1:200, 1:500) using this method by increasing the initial molar concentration of the NHS ester of 6-keto-$PGF_{1\alpha}$ while keeping the concentration of aequorin constant. The conjugates were passed through a size-exclusion column to remove any unconjugated ester of 6-keto-$PGF_{1\alpha}$ and unreacted β-mercaptoethanol. They were then lyophilized and later resuspended in the assay buffer.

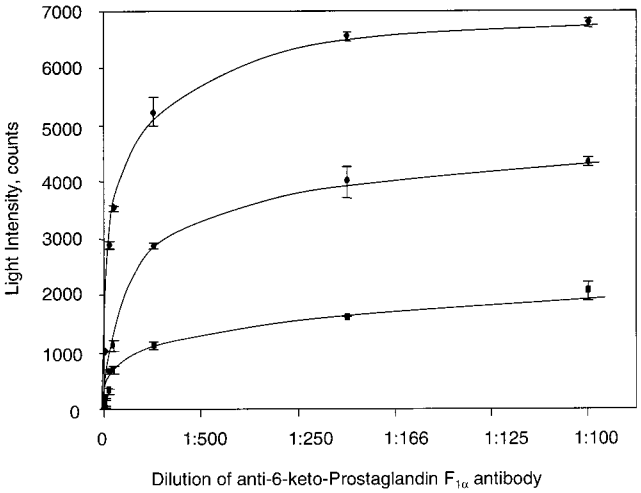

Figure 1. Binder dilution curves obtained by incubating a volume of 100 μL of varying concentrations of 6-keto-$PGF_{1\alpha}$ antibody and 6-keto-$PGF_{1\alpha}$−aequorin conjugates with secondary antibody coated plates. Data are an average of three points ± the standard deviation. (■) 1:50 conjugate, (●) 1:100 conjugate, (◆) 1:500 conjugate.

To study the effect of conjugation on the bioluminescence activity of aequorin and to select an appropriate conjugate concentration for the assay, calibration plots were constructed for each of the conjugates (data not shown). The conjugates could be detected down to 1×10^{-12} M. A concentration of 1×10^{-10} M of the three conjugates was selected for the binder dilution study. This concentration was low enough to be able to detect aequorin at low levels with high sensitivity, and at the same time, it showed much higher activity than the background.

The next step was to study the interaction between the anti-6-keto-$PGF_{1\alpha}$ antibody and the conjugates. For this, binder−dilution curves were generated for all three conjugates, as shown in Figure 1. The 6-keto-$PGF_{1\alpha}$ antibody was incubated with a concentration of 1×10^{-10} M of the conjugates in the secondary antibody-coated microtiter plate for 2 h at room temperature. After washing the plate to remove any unbound antibody or conjugate, the luminescence was measured. As the concentration of antibody increased, the light intensity increased until it reached a concentration after which there was no significant increase. It was observed that the 1:500 conjugate showed maximum binding; therefore, it was used for the remaining experiments in the assay. The amount of antibody to be used in the assay was selected such that the light intensity emitted is sufficient to measure a signal above the background, while keeping the amount of available binding sites to a minimum. The antibody dilution selected for developing the assay was 1:10 000 dilution.

The effect of increased or decreased amounts of 6-keto-$PGF_{1\alpha}$ antibody or conjugate on the dose−response curve was also evaluated. In the first two cases, the conjugate concentration was kept constant at 1×10^{-10} M, whereas the antibody concentration

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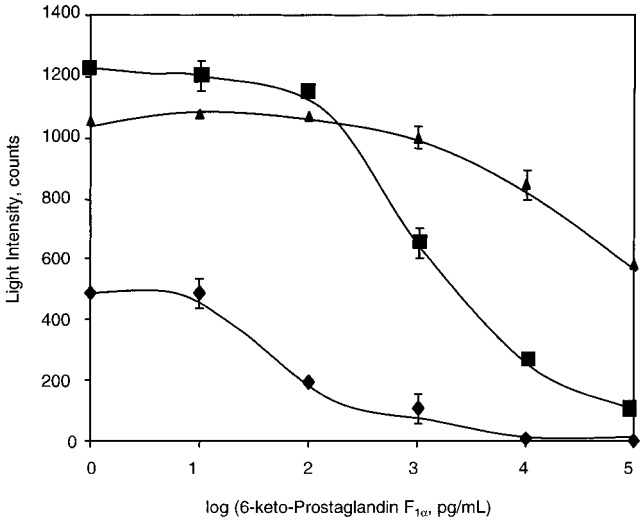

Figure 2. Dose−response curves for 6-keto-$PGF_{1\alpha}$ generated by incubating varying concentrations of 6-keto-$PGF_{1\alpha}$ with 1×10^{-10} M conjugate and (■) 1:5000 dilution of 6-keto-$PGF_{1\alpha}$ antibody, or (◆) 1:50 000 dilution of 6-keto-$PGF_{1\alpha}$ antibody and 1:10 000 dilution of 6-keto-$PGF_{1\alpha}$ antibody with (▲) 1×10^{-9} M 6-keto-$PGF_{1\alpha}$−AEQ conjugate. Data are an average of three points ± the standard deviation.

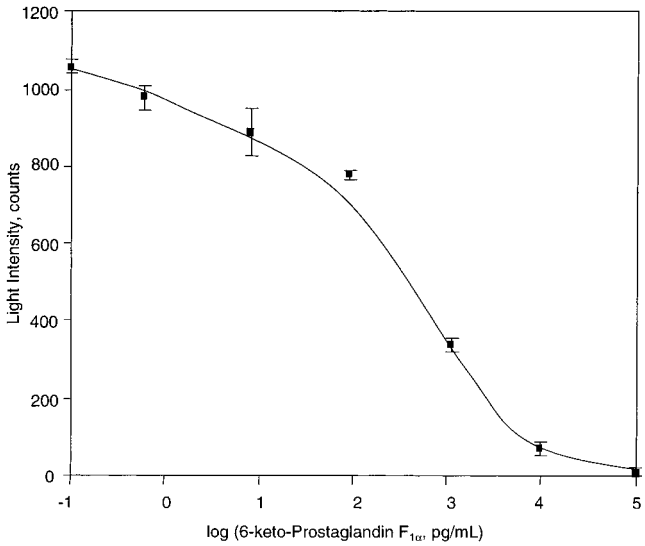

Figure 3. Dose−response curve for 6-keto-$PGF_{1\alpha}$ obtained by incubating 100 μL of 6-keto-$PGF_{1\alpha}$ antibody, a 50-μL volume of 1×10^{-10} M 6-keto-$PGF_{1\alpha}$−AEQ conjugate, and varying concentrations of 6-keto-$PGF_{1\alpha}$. Data are an average of three points ± the standard deviation.

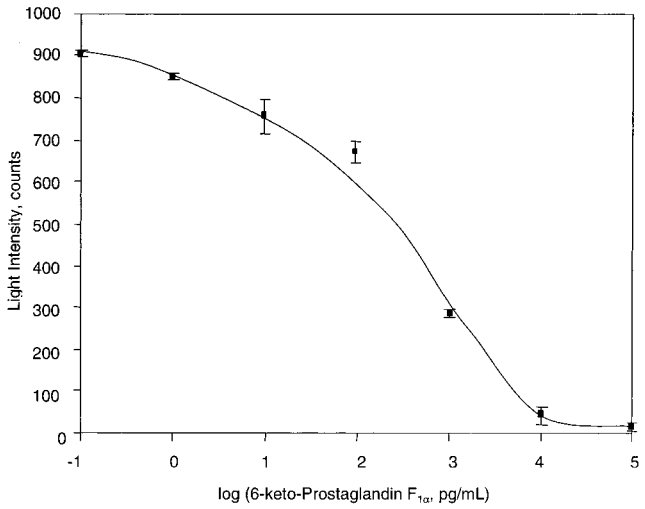

Figure 4. Dose−response curve for 6-keto-$PGF_{1\alpha}$ in plasma of a healthy person obtained by incubating 100 μL of 6-keto- $PGF_{1\alpha}$ antibody, a 50-μL volume of 1×10^{-10} M 6-keto-$PGF_{1\alpha}$−AEQ conjugate, and varying concentrations of 6-keto-$PGF_{1\alpha}$ in 1:200 diluted plasma. Data are an average of three points ± the standard deviation.

was either increased or decreased by a factor of 5. In the third experiment, the antibody concentration used was a 1:10 000 dilution, and the conjugate concentration was increased to 1×10^{-9} M. As shown in Figure 2, when a higher antibody concentration is used, the sensitivity improves, but the detection limit is compromised. When a more diluted amount of antibody is used, the dynamic range narrows significantly, and the sensitivity is reduced. Finally, when a higher concentration of the conjugate is employed, both the detection limit and the dynamic range worsen. Therefore, on the basis of these data, the optimum antibody dilution for the assay was determined to be 1:10 000, and the conjugate concentration, 1×10^{-10} M (Figure 2). Next, a dose−response curve was generated by simultaneously mixing varying concentrations of free 6-keto-$PGF_{1\alpha}$, 1×10^{-10} M of the 1:500 conjugate, and 1:10 000 dilution of the 6-keto-$PGF_{1\alpha}$ antibody and incubating them in microtiter plates immobilized with the secondary antibody. The dose−response curve obtained for 6-keto-$PGF_{1\alpha}$ as depicted in Figure 3 is sigmoidal in shape, with the luminescence intensity being lower at high concentrations of 6-keto-$PGF_{1\alpha}$ and increasing as the concentration of 6-keto-$PGF_{1\alpha}$ decreases. The working range of the curve ranges for concentrations of 10 000−1 pg/mL. The detection limit for 6-keto-$PGF_{1\alpha}$ was determined to be 1 pg/mL, which corresponds to a 2×10^{-12} M concentration of 6-keto-$PGF_{1\alpha}$.

Since the goal of this work was to detect 6-keto-$PGF_{1\alpha}$ in plasma, it was necessary to validate this assay in plasma and to study any possible matrix effects on the performance of the conjugate or the binding capabilities of the 6-keto-$PGF_{1\alpha}$ antibody in plasma. The assay was, therefore, performed in 1:200 diluted human plasma spiked with known concentrations of 6-keto-$PGF_{1\alpha}$. The dose−response curve obtained (Figure 4) was almost identical to that obtained for 6-keto-$PGF_{1\alpha}$ in buffer. According to this dose−response curve, and as expected for 6-keto-$PGF_{1\alpha}$, at high concentrations of free 6-keto-$PGF_{1\alpha}$, most of the binding sites are occupied by 6-keto-$PGF_{1\alpha}$, and therefore, a low bioluminescence signal in the solid phase was obtained. As the concentration of free analyte decreases, the bioluminescence intensity reaches a maximum, since most of the binding sites on the antibody are occupied by the conjugate. The detection limit of this dose−response curve was also found to be 1 pg/mL, and the limit of quantitation was found to be 5.11 pg/mL. This demonstrates that even low levels of 6-keto-$PGF_{1\alpha}$ could be detected in the assay and also that aequorin is an excellent label for the detection of biomolecules in biological fluids.

The interassay precision was determined by generating the dose−response curve in plasma for five consecutive days in exactly the same manner as described earlier. The percent coefficient of variation for samples containing different concentrations of 6-keto-

Table 1. Cross-Reactivities of Related Prostaglandins Measured by Using Varying Concentrations of 50 μL of the Cross Reactant in the Dose−Response Curve Instead of 6-Keto-$PGF_{1\alpha}$[a]

analyte	bound %	av std dev
6-keto-prostaglandin $F_{1\alpha}$	100	1.71
prostaglandin $F_{1\alpha}$	5.4	4.65
prostaglandin $F_{2\alpha}$	3.41	3.21
13,14-dihydroxy-15-keto-prostaglandin $F_{1\alpha}$	0.45	1.01
2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$	10.1	5.78
prostaglandin D_2	7.75	2.11
prostaglandin E_1	6.75	3.87
6,15-diketo-13,14-dihydro-prostaglandin $F_{1\alpha}$	1.83	0.92

[a] The percent cross-reactivities were calculated at 50% B/B_0

Table 2. In Vivo Analysis[a]

plasma sample	prostacyclin dose (ng/kg/min)	6-keto-prostaglandin $F_{1\alpha}$ concn measured without extraction (ng/mL)	std dev
av of 3 normal samples		1.7	0.01
PPH patient 1	28.5	9.9	0.2
PPH patient 2	10	6.7	0.05
PPH patient 3	8	4.1	3.1
PPH patient 4	0	2.2	1.5
PPH patient 5	0	1.5	0.05

[a] Plasma samples of the normal subjects were used to produce standard dose−response curves and values of 6-keto-$PGF_{1\alpha}$ in patient samples were obtained using those curves. The measurements were performed in triplicate.

$PGF_{1\alpha}$ was calculated. The average coefficient of variation was found to be 4.5%. The intraassay precision was determined by doing four replicate measurements of the same sample. The average coefficient of variation was found to be 1.25%.

The presence of various prostaglandins other than 6-keto-$PGF_{1\alpha}$ in the blood can cause nonspecific binding with the 6-keto-$PGF_{1\alpha}$ antibody and interfere with the detection of 6-keto-$PGF_{1\alpha}$, giving extremely high values of 6-keto-$PGF_{1\alpha}$. Therefore, it becomes important to confirm that the amount of 6-keto-$PGF_{1\alpha}$ obtained from our assay is due to 6-keto-$PGF_{1\alpha}$ only and no other prostaglandin. The cross-reactivities of other prostaglandins similar in structure to 6-keto-$PGF_{1\alpha}$ were determined by preparing standard solutions of the compounds in the assay buffer ranging from 100 000 to 100 pg/mL and using them in this assay for 6-keto-$PGF_{1\alpha}$. The measured 6-keto-$PGF_{1\alpha}$ concentration at 50% B/B_0 was then calculated. The percent cross-reactivities were calculated by comparison of measured cross-reactant concentration and the actual concentration (known amount) of the cross-reactant in the sample (Table 1). It must be noted that the prostaglandins occur at different times in the arachidonic acid pathway and in different concentrations. In addition, 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ is found in urine and does not occur in plasma; however, since it is the immediate metabolite of 6-keto-$PGF_{1\alpha}$, we decided to analyze the cross-reactivity of 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, as well. From the table, it is clear that the prostaglandins do not show any significant amount of cross-reactivity. This indicates that the assay is specific only for 6-keto-$PGF_{1\alpha}$, and there is no interference from other prostaglandins that might be present in the plasma samples. The assay can, therefore, be used to measure 6-keto-$PGF_{1\alpha}$ directly from plasma.

Finally, in vivo sample analysis was performed with this assay. The PPH patient and control samples were provided by Dr. Michael Poon of the Mount Sinai Pulmonary Hypertension Program. Five PPH patient plasma samples were diluted 1:200 times with buffer and used directly in the assay. The concentration of 6-keto-$PGF_{1\alpha}$ in the samples was measured using the standard dose−response curve obtained in plasma by diluting plasma of a normal subject 1:200 to measure the amount of 6-keto-$PGF_{1\alpha}$. It was observed that the levels of 6-keto-$PGF_{1\alpha}$ increased with an increase in the prostacyclin dose. Three normal control samples were used to produce the standard dose−response curves, and the curves were found to be similar. The values of 6-keto-$PGF_{1\alpha}$ in the samples of patients obtained with each of the standard curves were also similar. Table 2 shows the 6-keto-$PGF_{1\alpha}$ values in normal and patients' samples.

To verify the concentration of 6-keto-$PGF_{1\alpha}$ obtained from plasma using the assay, 6-keto-$PGF_{1\alpha}$ was extracted from the plasma samples of seven normal subjects and the five PPH patients using the C_{18} Sep-Pak columns, and these values were compared with the values obtained when plasma samples were used directly in our assay without extraction. In the extraction method, the 6-keto-$PGF_{1\alpha}$ was obtained in the ethyl acetate fraction. All of the ethyl acetate was evaporated under N_2, and the dried 6-keto-$PGF_{1\alpha}$ was dissolved first in ethanol, followed by the assay buffer. This 6-keto-$PGF_{1\alpha}$ was used in the assay performed in buffer. The concentrations of 6-keto-$PGF_{1\alpha}$ obtained using the aequorin assay in plasma samples without pretreatment were observed to be highly correlated with the measured 6-keto-$PGF_{1\alpha}$ after extraction. The equation for the straight line can be given as $y = 0.79x + 418$, $r = 0.988$, $n = 12$.

Further in vivo analysis was performed to determine the recovery of 6-keto-$PGF_{1\alpha}$ from samples using this assay. Two of the normal plasma samples and two patient plasma samples were first analyzed using the assay as described before and for determining the concentration of 6-keto-$PGF_{1\alpha}$ present in the samples by using the standard curve in 1:200 diluted plasma. A known amount of 6-keto-$PGF_{1\alpha}$ was spiked into the samples, and the samples were analyzed once again to determine the concentration of 6-keto-$PGF_{1\alpha}$. Percent recovery data was obtained using the concentration of 6-keto-$PGF_{1\alpha}$ in unspiked samples and the concentration obtained after spiking. It is clear from the data (Table 3) that this assay for 6-keto-$PGF_{1\alpha}$ gives recoveries within the range error.

In conclusion, this immunoassay is the first direct assaying technique for quantification of plasma 6-keto-$PGF_{1\alpha}$ without the process of extraction. The 6-keto-$PGF_{1\alpha}$ concentrations obtained from patient samples by extraction and without extraction show good correlation. In addition, the antibody we employed for the assay, and hence the assay itself, does not show significant binding to other related prostaglandins. Therefore, the possibility of obtaining falsely high values of 6-keto-$PGF_{1\alpha}$ due to the presence of other similar prostaglandins is eliminated. The assay has a wide working range and a low detection limit of 1 pg/mL, which enables the measurement of the creation of distribution curves of plasma prostacyclin levels in normal and disease states. This assay also characterizes the application of aequorin in quantification of

Table 3. Percent Recovery Data of Normal Samples and Patient Samples[a]

subject	prostacyclin dose (ng/kg/min)	weight (kg)	concn of 6-keto-$PGF_{1\alpha}$ found in unspiked samples (ng/mL)	amt of 6-keto-$PGF_{1\alpha}$ spiked into samples (ng/mL)	concn of 6-keto-$PGF_{1\alpha}$ found in spiked samples (ng/mL)	recovery %
normal sample	0		2.05	5	7.53	106.8
normal sample	0		2.91	5	8.01	109
PPH patient 1	10	95	8.32	10	20.2	115
PPH patient 2	28.5	78.5	9.96	10	21.7	117.4

[a] The measurements were performed in triplicate.

important components present in the body by directly measuring them in the biological matrix without requiring any pretreatment of the biological fluids. This assay may allow high-throughput analysis of the levels of plasma prostacyclin in patients with primary pulmonary hypertension and the clinical implications of various levels of plasma prostacyclin in the pathogenesis and clinical management of this devastating disease.

Abbreviations. 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin$F_{1\alpha}$; PPH, primary pulmonary hypertension; EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)-amino methane; EDTA, ethylenediaminetetraacetic acid.

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