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New highly sensitive enzyme immunoassay for the determination of pravastatin in human plasma

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ABSTRACT

New highly sensitive enzyme immunoassay (EIA) has been developed and validated for the determination of pravastatin (PRV) in human plasma samples. PRV was coupled to keyhole limpt hemocyanin (KLH) and bovine serum albumin (BSA) via its terminal carboxylic acid group by carbodiimide reagent. PRV–KLH conjugate was used as an immunogen for raising anti-PRV polyclonal antibody in rabbits. The generated anti-PRV antibody recognized PRV with high affinity and selectivity. PRV–BSA conjugate was immobilized onto microwell plates and used as a solid phase. The assay involved a competitive binding reaction between PRV, in plasma sample, and the immobilized PRV–BSA for the binding sites on a limited amount of the anti-PRV antibody. The anti-PRV antibody bound to the plate wells was quantified with horseradish peroxidase-labeled anti-immunoglobulin second anti-rabbit IgG antibody and 3,3′,5,5′-tetramethylbenzidine as a substrate for the peroxidase enzyme. The concentration of PRV in the sample was quantified by its ability to inhibit the binding of the anti-PRV antibody to the immobilized PRV–BSA and subsequently the color development in the assay wells. The conditions of the proposed EIA were investigated and the optimum conditions were employed in the determination of PRV in plasma samples. The assay limit of detection was 0.2 ng mL^{-1} and the effective working range at relative standard deviation (RSD) of $\leq$5% was 0.5–20 ng mL^{-1}. The mean analytical recovery of PRV from spiked plasma was 100.9 ± 2.98%. The precision of the assay was satisfactory; RSD was 2.61–3.70 and 3.96–4.17% for intra- and inter-assay precision, respectively. The analytical procedure is convenient, and one can analyze ~200 samples per working day, facilitating the processing of large-number batch of samples. The proposed EIA has a great value in the routine analysis of PRV in plasma samples for its therapeutic monitoring and pharmacokinetic studies.

1. Introduction

Pravastatin (PRV); hexahydro-6-hydroxy-2-methyl-8-(2-methylbutyryloxy)-1-naphthyl-3,5-dihydroxyheptanoate is a 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase inhibitor, which reduces the cholesterol biosynthesis. It exerts its action by competitive inhibition of the microsomal HMG-CoA reductase enzyme, which catalyses the conversion of HMG-CoA to mevalonate, a critical intermediary in the pathway of cholesterol biosynthesis. Because of the hydroxyl group attached to the decalin ring of PRV, it is characterized by its greater hydrophilicity than other HMG-CoA reductase inhibitors [1,2].

Pravastatin is administered orally in the active form, rapidly absorbed, and its peak plasma level is attained 1–1.5 h following ingestion. PRV plasma concentration is directly proportional to the administered dose, however it, like other HMG-CoA reductase inhibitors, has variable bioavailability. Because of the small doses of PRV, its concentrations in plasma samples are expected to be very low [3]; in a typical single dose of 40 mg, the PRV level in plasma after 16 h is ~0.5 ng mL^{-1} [4]. Therefore, a sensitive method is required for its quantitative determination in plasma. The analytical methodologies that have been developed for the determination of PRV in plasma are mostly high performance liquid chromatography [4–11] and gas chromatography [12]. These methods were associated with major drawbacks such as decreased sensitivity (limits of detection were 1–5 ng mL^{-1}), multiple laborious purification steps for the samples, pre-derivatization with critical derivatizing reagents, in addition to the use of expensive detectors (e.g. tandem mass spectrometry) that are not available in most laboratories [8–11]. Therefore, the development of an alternative highly sensitive and less instrumental intensive analytical methodology for the determination of PRV in plasma was necessary.

Enzyme immunoassay (EIA) has been widely used in pharmaceutical and clinical analysis because of its inherent specificity, applicability for a wide range of analytes, high-throughput, and

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low cost [13–15]. EIA is remarkably quick, easily performed yielding information that would be difficult to obtain by the chromatographic methods, and also offers great sensitivity when an appropriate enzyme label is used [15–17]. As well, immunoassays as they use analyte-specific antibodies do not require pretreatment for the samples and they are well suited for screening of large number of samples [18–20]. For these reasons, the development of EIA was considered to be the alternative approach as it can offer significant advantages over the reported instrument-intensive methods for the determination of PRV in plasma.

The development of EIA for the determination of PRV in plasma has been reported by Muramatsu et al. [21], however the detection limit of this assay was 0.5 ng mL^{-1}. The present study describes the development of new highly sensitivity EIA for the determination of PRV at concentrations as low as 0.2 ng mL^{-1} in human plasma samples.

2. Experimental

2.1. Apparatus

FLX808 microplate reader (Bio-Tek Instruments Inc., USA). FLX50 microplate washer (Bio-Tek Instruments Inc., USA). EM-36N microtube shaker (Taitec, Japan). Biofuge Pico centrifuge (Heraeus Instruments, Germany). Model Mini/18 incubator (Genlab Ltd., UK). Water purification system (Milli-Q Labo, Millipore Ltd., Bedford, USA).

2.2. Materials

Pravastatin (PRV), horseradish peroxidase-labeled goat anti-rabbit IgG (HRP-IgG), bovine serum albumin (BSA), 2,4,6-trinitrobenzene sulfonic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and Tween-20 were purchased from Sigma Chemical Co. (St. Louis. MO, USA). Keyhole limpet hemocyanin (KLH) was purchased from Novabiochem Co. (La Jolla, CA, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) peroxidase substrate was obtained from Kirkegaard-Perry Laboratories (Gaithersburg, MD, USA). ELISA high-binding microwell plates were a product of Corning/Costar, Inc. (Cambridge, MA, USA). Centricon-30 filter (Amicon, Inc., Beverly, MA, USA). BCA reagent for protein assay and protein A column were obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). All water was purified by filtration through a water purification system.

2.3. Procedures

2.3.1. Preparation of PRV–protein conjugates

PRV was conjugated with keyhole limpt hemocyanin (KLH) and bovine serum albumin (BSA) according to the method described by Darwish et al. [22]. Briefly, EDC (150 mg) was added to 10-mL PRV solution of (5 mg mL^{-1}) in 12.5 mmol L^{-1} phosphate buffer (PB) of pH 5, and the pH was maintained at pH 5–5.5 using 0.01 mol L^{-1} HCl for 5 min. Five milliliters of protein solution (5 mg mL^{-1}, in 50 mmol L^{-1} PB of pH 7.2) was added, and the pH of the reaction mixture was rapidly adjusted to pH 6.4 and maintained constant for 90 min. The reaction was left to proceed overnight in dark at 4 °C. The uncojugated PRV was removed from the PRV–protein conjugates by buffer exchange using a Centricon-30 filter. Protein content of each conjugate was determined by BCA reagent kit and the extent of conjugation was then estimated by spectral analysis of the conjugates and by estimation of the free amino groups on the proteins and on PRV–protein conjugates according to the procedure described by Habeeb [23].

2.3.2. Immunization of animals and purification of antibody

The immunogen used was PRV–KLH protein conjugate. Four female 8-weeks old New Zealand white rabbits were injected subcutaneously with 1 mg, of PRV–KLH emulsified in Freund's complete adjuvant, divided in different sites for each rabbit. The same immunization procedure was repeated 6 times with 2 weeks interval, however incomplete adjuvant was used instead. Antibody response in each rabbit was determined by direct enzyme immunoassay described by Darwish et al. [24]. The sera of the rabbit that showed the highest response and gave best affinity to PRV were collected as crude anti-PRV polyclonal antibody sample.

The sera (~20 mL) was kept overnight at 4 °C and then centrifuged at 4 °C for 10 min. To 5 mL of supernatant, an equal volume of a saturated ammonium sulfate solution was gradually added and gently mixed. For complete precipitation of the IgG, the solution was kept over ice for 3 h. The precipitate was collected by centrifugation at 10,000 × *g* at 4 °C for 30 min. The precipitate was resuspended in 10 mL phosphate buffered saline (PBS; 137 mmol L^{-1} NaCl, 3 mmol L^{-1} KCl, and 10 mmol L^{-1} sodium phosphate, pH 7.4) followed by reprecipitation with ammonium sulfate. After repeating this step three times, the precipitate was dissolved in 10 mL of PBS. The produced antibody solution was purified by protein A column chromatography. A 1 mL aliquot of the solution was mixed with an equal volume of the binding buffer (1.5 mol L^{-1} glycine–NaOH containing 3 mol L^{-1} NaCl, pH 8.7) and the mixture was applied to the protein A column and the eluent was monitored for protein by measuring the absorbance of the eluted fractions at 280 nm. The column was washed with 50–60 mL of binding buffer, and the bound immunoglobulin was eluted with 0.1 mol L^{-1} sodium citrate buffer (pH 3.0). The eluate was collected in 1.5 mL fractions into tubes containing 100 μL of 1 mol L^{-1} Tris–HCl buffer (pH 9.0), and mixed. The pooled fractions were dialyzed overnight against five changes of PBS (~4 h intervals). The protein content of the dialyzate was determined by BCA reagent kit, and used as the pure anti-PRV antibody sample.

2.3.3. Determination of the optimum concentrations of antibody and coating conjugate

The optimum PRV–BSA concentration required for coating onto the microwell plates and the best working concentration of the anti-PRV antibody were determined by checkerboard titration [25]. Different concentrations (0.1–5 μg mL^{-1}) of PRV–BSA in 50 μL of PBS were coated onto microwell plates in triplicate rows for 2 h at 37 °C with gentle agitation by microtube shaker. After incubation, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T) using microplate washer. The wells were blocked with 200 μL of 3% BSA in PBS by incubation at 37 °C for 1 h. Fifty microliters of different concentrations (0.5–4 μg mL^{-1}) of anti-PRV antibody solution (in PBS) was added in columns across the microwell plates. After 1.5 h incubation at 37 °C, the plates were washed with PBS-T, and 50 μL of HRP-IgG (1/5000 in PBS) was added to each well. After similar incubation and washing step, 50 μL of TMB substrate solution was added and the reaction was allowed to proceed for 10 min at 37 °C for color development. The absorbance of each well was measured at 630 nm using a microplate reader. Concentrations of PRV–BSA conjugate and anti-PRV antibody that yielded 1–1.5 absorbance units were considered as reference binding conditions for further testing.

2.3.4. Competitive EIA procedures and data analysis

Plasma samples were centrifuged at 3500 rpm at 4 °C for 10 min and the supernatants were collected. The supernatants were used directly for the analysis by EIA. Samples of PRV (50 μL) were mixed with equal volumes of anti-PRV antibody (2 μg mL^{-1}). Aliquot (50 μL) of the mixture was added to each well of the microwell plate that had been previously coated with 1 μg mL^{-1} of PRV–BSA

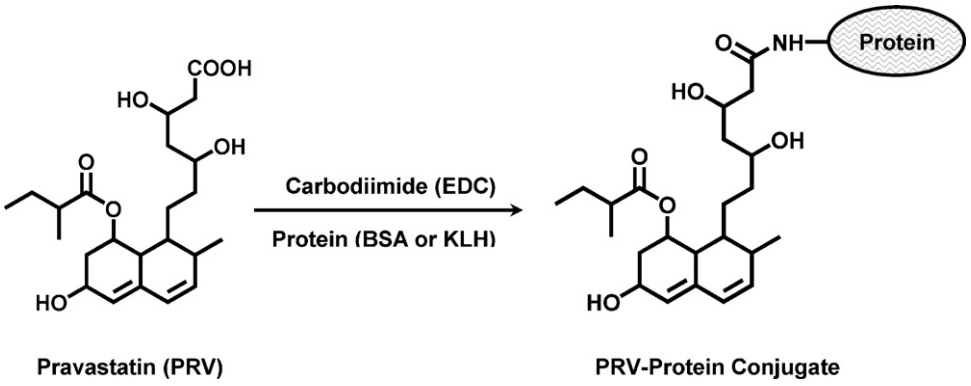

Fig. 1. Preparation of PRV–protein (BSA and KLH) conjugates.

conjugate and blocked with 3% BSA. After 1.5 h incubation at 37 °C, the plates were washed with PBS-T, and 50 μL of HRP-IgG (1/5000 in PBS) was added to each well. After 1.5 h incubation, the plates were washed with PBS-T and the amount of the bound HRP-IgG was quantified using TMB microwell substrate as described above. The data were acquisitioned by KC Junior software (Bio-Tek Instruments Inc., USA), and transformed to a four-parameter curve fitting using Slide Write software, version 5.011 (Advanced Graphics Software, Inc., USA). Values for IC_{50} were those that gave the best fit to the following equation:

$$A = A_0 - \left\{ \frac{(A_0 - A_1)[\mathrm{PRV}]}{\mathrm{IC}_{50} + [\mathrm{PRV}]} \right\}$$

where A is the signal at a definite known concentration of PRV, A_0 is the signal in the absence of PRV, A_1 is the signal at the saturating concentration of PRV, and IC_{50} is the PRV concentration that produces a 50% inhibition of the signal. The concentrations of PRV in the samples were then obtained by interpolation on the standard curve.

3. Results and discussion

3.1. Preparation and characterization of PRV–protein conjugates

Since PRV is a small molecule, it is not naturally immunogenic. In order to produce antibody specific to PRV, immunogenic conjugate (immunogen) must be first prepared by covalently linking to a carrier protein. PRV contains reactive group (COOH) through which conjugation with protein could proceed directly. Although the introducing of a "spacer group" between the hapten molecule and the carrier protein usually increases the specificity of the antibody aimed to be produced [26], however the reactive terminal COOH group of PRV is adequately spaced (6 carbon atoms) from the characteristic epitopic moiety of the PRV molecule. Therefore, PRV was directly linked to the carrier proteins (BSA and KLH) by carbodiimide reagent (Fig. 1).

In order to ascertain the extent to which PRV was conjugated to the proteins, spectral analysis of the PRV–protein conjugates were conducted. Fig. 2 illustrates the UV absorption spectra of BSA and PRV–BSA conjugate under the same pH conditions. Obviously, the absorbance that has exhibited by PRV–BSA conjugate was higher than that of an equal concentration of unconjugated BSA. This hyperchromic effect was evident for the successful conjugation of the PRV with BSA. Similar results were obtained for PRV–KLH conjugate. The extent of conjugation was determined by the procedures described by Habeeb [23]. The percentages of PRV residues in PRV–BSA and PRV–KLH conjugates were found to be 22.25 and 45.66%, respectively.

3.2. Preparation and characterization of anti-PRV antibody

Because of the high immunogenicity of KLH [27], PRV–KLH was selected as an immunogen for immunization of animals and PRV–BSA was selected for immobilization onto the microwell plates in the EIA. To monitor the progress of the immune response of

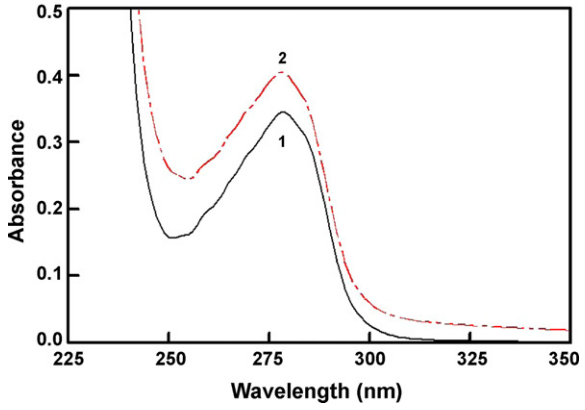

Fig. 2. Absorption spectra of equal protein concentration (0.5 mg mL^{-1}) of BSA (1) and PRV–BSA conjugate (2).

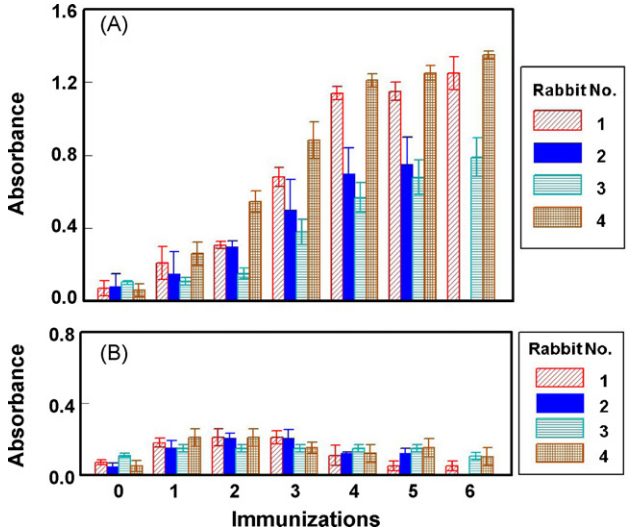

Fig. 3. Monitoring the immune response of rabbits immunized with PRV–KLH. Micowell plates were coated with PRV–BSA (panel A) and BSA (panel B). Antiserum samples after different consecutive immunizations were allowed to bind to the immobilized antigen (BSA and PRV–BSA). Signals were generated as described in Section 2.

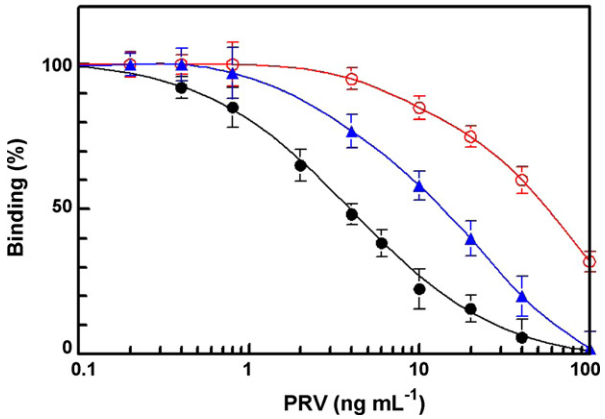

Fig. 4. Affinity of antisera from rabbits no. 1 (▲), no. 3 (○), and no. 4 (●) for PRV. PRV–BSA was coated onto the microwell plates. Antiserum samples were mixed with PRV and transferred into the microwells. Competitive binding reaction was allowed to proceed and the signals were generated as described in Section 2.

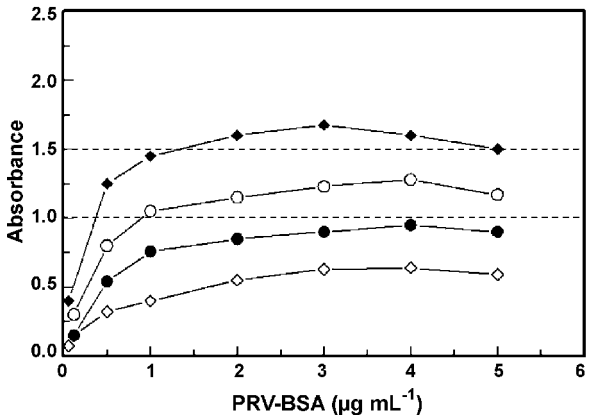

Fig. 6. Checkerboard titrations for PRV–BSA versus anti-PRV antibody. Varying concentrations (0.1–5 μg mL^{-1}) of PRV–BSA conjugate were coated onto the microwell plates. Varying concentrations of anti-PRV antibody were allowed to bind to the immobilized PRV–BSA. These concentrations were 0.5 μg mL^{-1} (◊), 1 μg mL^{-1} (●), 2 μg mL^{-1} (○), and 4 μg mL^{-1} (♦). Signals were generated as described in Section 2. Absorbance values were plotted as a function of PRV–BSA concentrations.

the rabbits to immunization and to confirm that they were sufficiently immunized, the sera collected after each immunization were analyzed by direct EIA. As shown in Fig. 3A, the titers of the antisera increased with the repetitive immunizations. As well, it was observed that the reactivity of the produced antibodies to the immobilized protein (BSA) was comparable to that before immunization (Fig. 3B). These data indicated the specificity of the raised antibodies to the PRV residues in the immobilized PRV–BSA, but not BSA molecules. Rabbit no. 2 was lost after the fifth immunization. Although, all the rabbits responded to the repetitive immunizations, however in order to select the most appropriate animal for scarifying and collecting the total sera, the affinity of these antisera from the animals was checked by competitive EIA as described above. As shown in Fig. 4, the sera from rabbit no. 4 had the highest affinity (lowest IC_{50}) for PRV. Therefore this rabbit was scarified and its total serum was collected as anti-PRV antibody. The purified antibody was used in the development of the proposed EIA; Fig. 5 illustrates the features of this assay.

3.3. Optimization of assay variables

3.3.1. Choice of conjugate and antibody concentrations

To determine the optimum concentration of PRV–BSA required for immobilization onto the microwell plates and the concentration of anti-PRV antibody for an effective competitive binding reaction, checkerboard titration of anti-PRV antibody versus PRV–BSA was carried out. The conjugate and antibody concentrations that gave 1–1.5 absorbance unit (in the direct EIA), and lowest IC_{50} (in the competitive EIA) were considered as a reference optimum binding

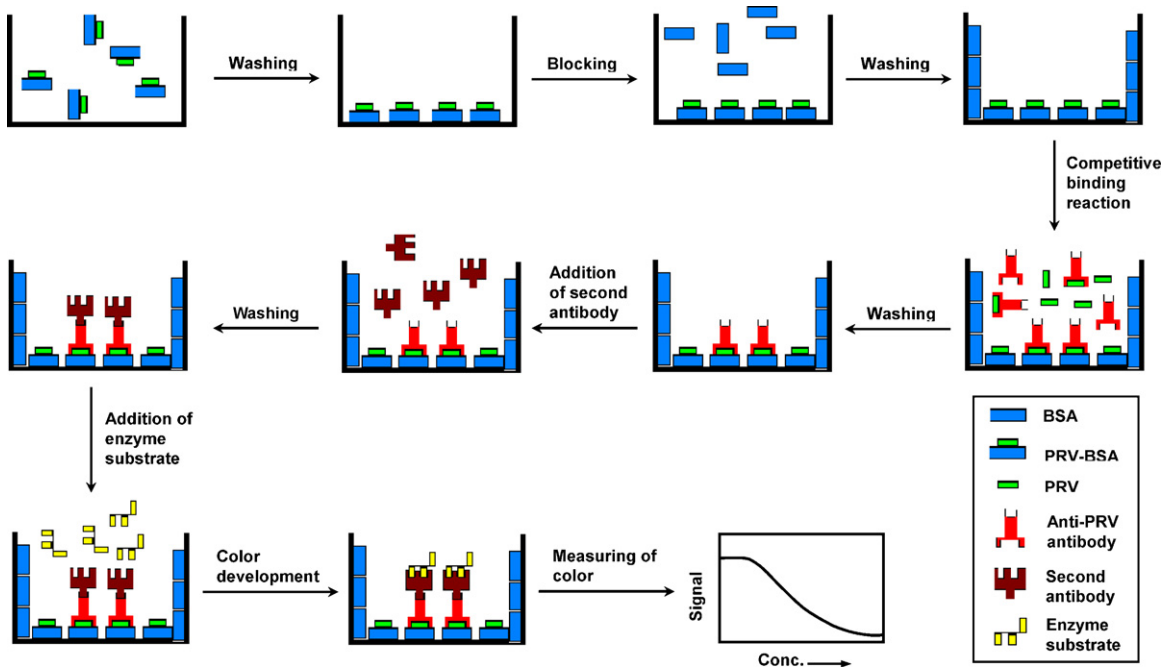

Fig. 5. Schematic diagram of the competitive EIA for PRV. Microwell plates were coated with PRV–BSA and blocked with BSA. After blocking, competitive binding reaction was performed between PRV (in the sample) and immobilized PRV–BSA (coated on the plate wells) for a limiting amount of the anti-PRV antibody. After a wash step to remove any antibody bound to the soluble PRV, an enzyme labeled secondary antibody was added. A second wash step removes unbound secondary antibody, and a signal is generated by the addition of TMB as a colorimetric substrate. Signals were correlated with the PRV concentrations for generating the calibration curve for the determination of PRV.

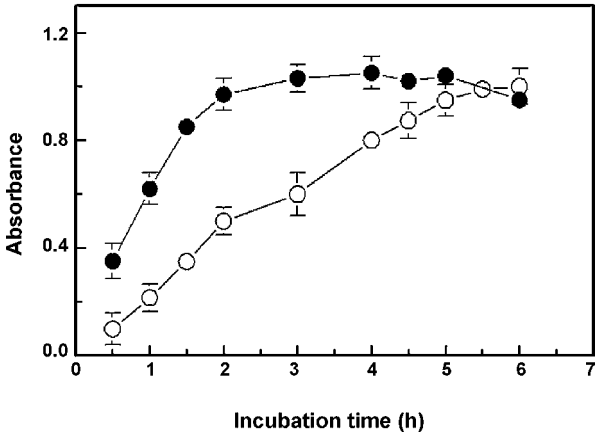

Fig. 7. Effect of temperature and incubation time on the immobilization of PRV–BSA onto the microwells. Incubations were carried out at 37 °C (●), and room temperature (25 ± 5 °C) (○).

conditions. The optimum concentrations of PRV–BSA and anti-PRV antibody were 1 and 2 μg mL^{-1}, respectively (Fig. 6). Therefore, these concentrations were used in all further testing.

3.3.2. Binding of PRV–BSA to microplate wells

Optimum binding of the PRV–BSA conjugate to the microwells was attained when the incubation time was 2 h at 37 °C, and more than 6 h at room temperature (25 ± 5 °C) (Fig. 7). For more convenience in clinical testing, it was important to check the lifespan of the conjugate after its immobilization onto the microwell plates. Plates were coated with the PRV–BSA and stored for varying periods of time at 4 and −20 °C, and then were analyzed for the amount of immunoreactive conjugate remaining on the microwells. The results indicated that the plates could be stored for at least 4 and 6 weeks at 4 and −20 °C, respectively (Fig. 8). This gives an advantage that the plates could be kept, after coating with the conjugate and blocking with BSA, until the assay time, and consequently reduction of 3 h from the total assay time.

3.4. Validation of the assay

3.4.1. Calibration curve and detection limit

The calibration curve of PRV using the proposed EIA is shown in Fig. 9. This curve was generated using PRV at concentrations from 0.02 to 200 ng mL^{-1}, prepared in PBS. The data showed good correlation coefficient (r = 0.995) on the four-parameter curve fit. The limit of detection of the proposed EIA, defined as the lowest PRV

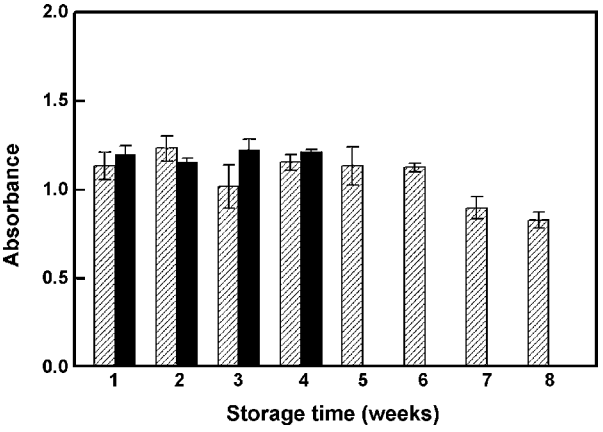

Fig. 8. Life span of PRV–BSA immobilized onto the microwell plate at 4 °C (solid bars) and −20 °C (stripped bars). Values are mean of 10 determinations.

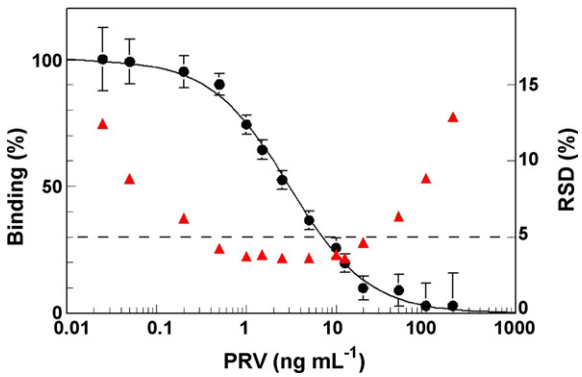

Fig. 9. Calibration curve (●) and precision profile (▲) of the proposed EIA for PRV. Varying concentrations of standard PRV were mixed with anti-PRV antibody (2 μg mL^{-1}). The reaction mixtures were further manipulated as described in Section 2. The values plotted are mean ± SD of three determinations.

concentration significantly different from zero concentration at 95% confidence limit (mean of 0 ± 4.65 SD) was determined [28]. Based on the basis of 8 replicate measurements, the limit of detection was found to be 0.2 ng mL^{-1}. This high sensitivity enables the determination of PRV in plasma without any sample pre-concentration procedures.

3.4.2. Precision profile

The assay precision profile obtained from the results of calibration standard samples, assayed in triplicate, is shown in Fig. 9. From this profile, the working range of the assay at values of relative standard deviation (RSD) less than 5% was derived. This range was found to be 0.5–20 ng mL^{-1}. The RSD at the detection limit of the assay (0.2 ng mL^{-1}) was found to be 6.25%. The intra- and inter-assay precision was tested at three varying levels of PRV. The intra-assay precision was assessed by analyzing 8 replicates of each sample in a single run and the inter-assay precision was assessed by analyzing the same sample, as duplicates, in 4 separate runs. According to the recommendation of immunoassay validation [29], the assay gave satisfactory results; the RSD was 2.61–3.70 and 3.96–4.17% for intra- and inter-assay precision, respectively (Table 1).

In general, the precision in competitive immunoassays depends mainly upon the uniformity in the quantity of the coated reagent from well to well in a microwell plate. Any interference in this uniformity could arise from the experimental protocol and other manipulations; change in the temperature of incubation and dispensing the reagents. The good precision observed in the proposed EIA was attributed to the facts that all the incubation steps of the assay were carried out at the same temperature.

3.4.3. Analytical recovery

Recovery of the assay was assessed by adding known concentrations (0.5–16 ng mL^{-1}) of PRV to the PRV-free plasma, and the samples were analyzed for their PRV content by the proposed EIA. The analytical recovery values were 97.5–104.1% with

Table 1
Precision of the proposed EIA for the determination of PRV.

PRV (ng mL^{-1})	Intra-assay		Inter-assay	
	Mean ± SD[a] (ng mL^{-1})	RSD[b] (%)	Mean ± SD[a] (ng mL^{-1})	RSD[b] (%)
0.5	0.54 ± 0.02	3.70	0.48 ± 0.02	4.17
2.0	2.05 ± 0.06	2.93	2.02 ± 0.08	3.96
10.0	9.58 ± 0.25	2.61	10.22 ± 0.42	4.11

[a] Values are mean of 8 determinations ± SD.
[b] RSD is the relative standard deviation.

Table 2
Analytical recovery of PRV spiked into plasma sample.

Added PRV (ng mL^{-1})	Found PRV (ng mL^{-1})[a]	Recovery (% ± RSD)[a]
0.5	0.52	104.0 ± 4.85
1.0	0.98	98.0 ± 4.52
2.0	1.95	97.5 ± 3.92
4.0	3.98	99.5 ± 4.56
8.0	8.21	102.6 ± 4.82
16.0	16.65	104.1 ± 4.95
	AV	100.9 ± 2.98

[a] Values are mean of three determinations ± SD.

Table 3
Comparison of the proposed enzyme immunoassay (EIA) with HPLC for analysis of plasma samples spiked with PRV.

Spiked PRV (μg mL^{-1})	Found PRV (μg mL^{-1})[a]	
	EIA	HPLC
0.50	0.48 ± 0.04	0.52 ± 0.03
1.00	1.20 ± 0.05	0.98 ± 0.02
2.00	1.98 ± 0.16	2.02 ± 0.04
2.50	2.46 ± 0.22	2.54 ± 0.15
4.00	4.05 ± 0.25	3.86 ± 0.22
8.00	8.25 ± 1.09	7.87 ± 0.52
16.00	15.95 ± 1.28	16.05 ± 1.02

[a] Values are mean of triplicate determinations ± SD.

RSD 2.98–4.95%, and the overall mean recovery was 100.9 ± 2.8% (Table 2). This indicated the accuracy of the proposed method for the determination of PRV in plasma samples, and absence of endogenous interfering substances in the plasma samples.

3.4.4. Application of the proposed EIA method and comparison with HPLC

In order to compare the proposed EIA with HPLC, plasma samples were spiked with PRV at concentrations of 0.5–16 μg mL^{-1} and analyzed by both HPLC [7] and the proposed EIA. As the proposed EIA has higher sensitivity, the samples were diluted with PBS to get their concentrations within the working range of the proposed EIA method. The values obtained by both the methods correlated well with each other (Table 3). The regression analysis of the results showed a good agreement between the results obtained by the two methods:

$$\text{EIA} = -0.0845 + 1.0018\,\text{HPLC} \quad (r = 0.9994)$$

This indicated the accuracy of the proposed EIA for the determination of PRV in plasma.

4. Conclusions

The present study described the development and validation of a new simple and highly sensitive EIA for the determination of PRV in plasma. Since the assay produces a colored read-out, only a colorimetric plate reader is required. The entire protocol of the present assay is very easy to perform in a 96-well plate and permits an operator to analyze a batch of 200 samples, in triplicate, per day when the plates have been previously coated with PRV–BSA and blocked with BSA. This facilitates the processing of large number of samples. Unlike HPLC, the marked specificity of the proposed EIA eliminates the need for pretreatment of plasma samples by affinity chromatography or other sophisticated equipment. For these reasons, the proposed EIA is expected to contribute to the therapeutic monitoring and pharmacokinetic studies of PRV.

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