Talanta 79 (2009) 1478–1483

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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

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ARTICLE INFO

Article history:
Received 21 April 2009
Received in revised form 6 June 2009
Accepted 8 June 2009
Available online 16 June 2009

Keywords:
Pravastatin
Polyclonal antibody
Competitive binding
Enzyme immunoassay

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.

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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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0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.06.021

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

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