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Original paper

DEVELOPMENT OF IMMUNORADIOMETRIC ASSAY FOR QUANTITATIVE DETERMINATION OF FREE PROSTATE-SPECIFIC ANTIGEN

Miroslava M. Janković, Maja M. Kosanović,
Ljiljana D. Hajduković-Dragojlović, Snežana J. Golubović

Institute for the Application of Nuclear Energy – INEP,
Banatska 31b; Zemun, Serbia and Montenegro

Summary: In this study we reported the development and analytical validation of new assay for quantitative determination of free prostate-specific antigen, fPSA. It is formulated as one step, two-site »sandwich« immunoradiometric assay. Specificity of this assay was achieved by using epitope-1-reactive anti-fPSA antibody as tracer antibody. Assay was calibrated against first international standard 96/668, and its detection limit was determined as 0.08 μg/L. Intra- and inter-assay coefficients of variation were 3.42–7.53% and 7.04–8.33%, respectively. Measured concentrations of serially diluted serum samples were close to the calculated concentrations, indicating good linearity with recovery percentage ranging from 98.7–107.4%. Analytical performance characteristics of fPSA assay speaks in favour of its use as a reliable tool in laboratory diagnostics relating to prostate deseases.

Key words: free PSA, immunoassay, anti-PSA antibodies, standardization

Introduction

Prostate specific antigen, PSA, is a serine protease belonging to kallikrein multigene family (1, 2). Regarding its structural characteristics, it is extremely heterogenous molecule. This heterogeneity is related to its protein backbone properties as well as oligosaccharide chain composition, resulting in the existence of numerous glycoisoforms differing in activity and molecular mass (3–5). In addition, enzymatically active PSA molecule forms complexes with at least six different inhibitors, main of which are those with α_1-antichymotrypsin (ACT) and α_2-macroglobulin (5). PSA occurs in complex predominantly in sera, whereas in tissue, seminal plasma or urine, it is present mainly as free form (5). Term free PSA refers to enzymatically active form as well as enzymatically inactive form comprising: pro-PSA, mature PSA, and several types of clipped PSA forms.

Address for correspondence:

Miroslava Janković
Institute for the Application of Nuclear Energy – INEP
Banatska 31 b, 11080 Zemun, Serbia and Montenegro
tel: +381 11 199 949
fax: + 381 11 618 724
e-mail: miraj@inep.co.yu

Quantitative determination of PSA is widely used for early detection and monitoring of prostate cancer (6). Actually, only two forms of PSA are measurable by available serological immunoassay, major PSA-α_1ACT fraction and minor free PSA fraction. Determination of % free PSA i.e. free PSA/total PSA ratio was introduced, as one of the ways to increase specificity and sensitivity of cancer detection in »gray zone« of elevated concentrations of total PSA associated with both malignant and benign or inflammatory prostate conditions (6). The use in everyday laboratory practice of a number of commercially available assays for PSA determination, point out to problem of statistically significant differences in value obtained for the same sample when analyzing by different kits. Assay configuration, based on various monoclonal or polyclonal antibodies differing in their affinities and capacities to recognize distinct molecular forms of PSA in equimolar ratio, is identified as a main reason for observed discrepancies (7, 8). For instance, the decreased recognition of complex PSA is found with assays utilizing polyclonal antibodies, and some monoclonal antibodies also underestimate complexed PSA or do not recognize it at all. In addition, some of anti-PSA antibodies recognize highly homologous human kallikrein 2, influencing the assay accuracy (8, 9).

So far, several attempts were done to overcome these problems and increase the comparability. As a results of these endaveours, reference materials containing specified quantity of PSA antigen was defined and provided by National Institute for Biological Standards and Control (Hartfordshire, UK) for both total and free PSA assays standardization (10). Besides, threedimensional structure of PSA antigen with map of six epitopes have been proposed as a reference for characterization of PSA antibodies *i.e.* for selection of pairs of non-competitive antibodies (9).

In this study we describe the development of new immunoradiometric assay for quantitative determination of free PSA. Considering PSA microheterogeneity, i.e. the international guidelines for requirement in immunoassay production, special emphasis was placed on standardization and selection of antibodies. In addition, assay validation including: detection limit, precision profile, linearity and recovery, was performed using serum samples.

Material and Methods

Material

Mouse monoclonal anti-PSA antibody recognizing PSA-ACT and free PSA and mouse monoclonal anti-PSA antibody recognizing only free PSA were from Medix, Biochemica, Finland and Acris Antibodies, Germany. Radioiodine (^{125}I) was from Institute of Isotopes Co., Ltd., Budapest, Hungary. Free PSA first international standard NIBSC code 96/668 (1st IS 96/668) was from National Institute for Biological Standards and Control (Hartfordshire, UK). Bovine serum albumine (BSA) was from Sigma (St. Louis, USA). Molecular mass markers, Sephadex G-75 and Sephadex G-100 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Polystyrene tubes were from Spektar (Čačak, SCG). All other chemicals were reagent grade.

Coating of tubes

Anti-PSA antibody (1 μg/250 μL) was immobilized on polystyrene star tubes by physical adsorption, overnight at +4 °C. After washing (3×1 mL of 0.1 mol/L, PBS pH 7.2), 1% bovine serum albumine (1 mL) was added as blocking reagent and incubation proceeded for two hours at 37 °C. The tubes were washed and allowed to dry in vacuum-oven. They were stored at +4 °C, until used.

Labelling of tracer antibody

Anti-free PSA antibody was labelled with 125I, using chloramine T method (11). Free iodine was separated from labelled antibody, on Sephadex G-75 column equilibrated with 0.1 mol/L PBS pH 7.2 supplemented with 0.05 % BSA. The fraction volume was 1 mL. Elution was monitored by measuring radioactivity in each fraction. Fractions corresponding to peak of radioactivity were pooled and used for preparation of tracer antibody solution.

Gel filtration

Serum or urine samples (0.3 mL) were passed through a Sephadex G-100 column (bed volume 40 mL) equilibrated and eluted with 0.1 mol/L PBS, pH 7.2. The fractions (1 mL) were collected and the elution was monitored by measuring PSA-immunoreactivity by means of antibodies for total PSA or free PSA. The column was calibrated with molecular mass standards: bovine serum albumine 66 kDa; ovalbumin 43 kDa; chymotrypsinogen 25 kDa and ribonuclease 13.7 kDa.

Data evaluation

Statistical analysis (mean value, standard deviation and coefficient of variation) was performed using Microsoft Excell, and correlation using linear regression analysis created by means of Primer of Biostatistics for Windows, version 5.0 software.

Results

Immunoassay components – anti PSA antibodies

Optimal concentration of capture anti-PSA antibodies was estimated as 1 μg/tubes. No significant variation was observed between different runs in specific activity of tracer ^{125}I-anti-fPSA antibody. Recognition of serum and urinary PSA antigen based on direct »sandwich« design was represented on *Figure 1.* The results obtained indicated that, in both sources, fPSA – immunoreactivity was associated with 28–33 kD peak corresponding to molecular mass of free form. fPSA was found to be predominant PSA form in urine, whereas in serum, its ratio to PSA-ACT was low.

Immunoassay procedure – calibration curve

Kinetic (3 hour to 18 hours) of the binding of five fPSA standards (50 μL or 100 μL) to constant concentration of capture antibody in the presence (simultaneous incubation) of constant volume (100 μL) of tracer antibody, at room temperature (20–25 °C) was measured. Based on the results obtained, the optimal volumes and incubation time was assessed, *i.e.*, the following assay procedure was established. Thus, 100 μL of standards or samples to be assayed was added to the corresponding tubes. Then, 100 μL of tracer ^{125}I-anti-fPSA antibody was dispensed into each

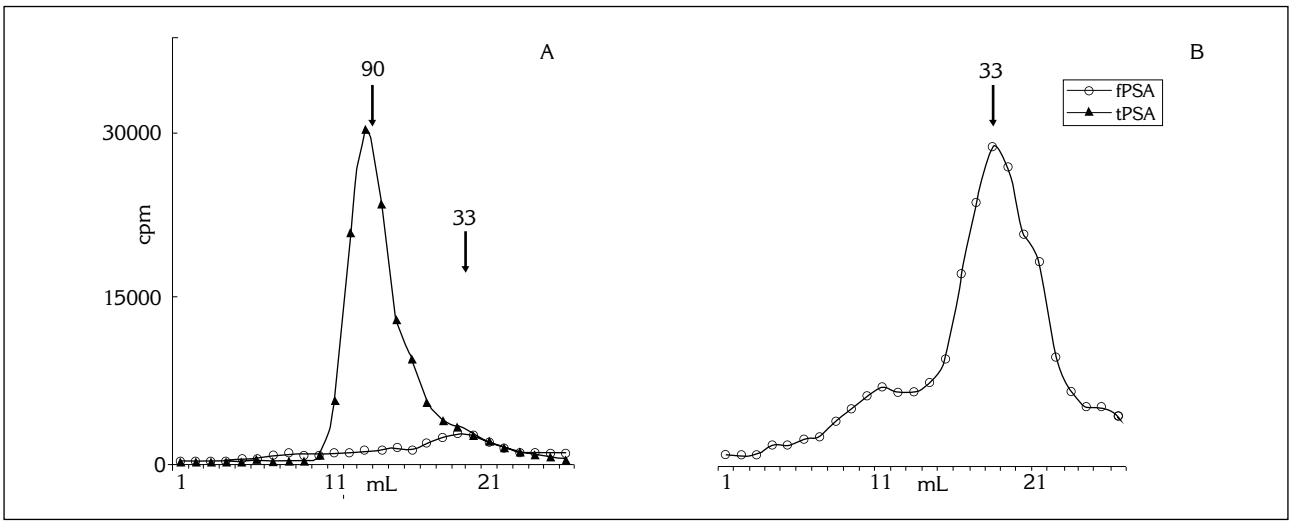

Figure 1. PSA immunogram
Serum (A) and urine (B) were subjected to gel filtration on Sephadex G-100 column (29 × 1.2 cm) equilibrated with 0.1 mol/L PBS buffer pH 7.2. Elution was monitored by detecting PSA – immunoreactivity in each fraction (1 mL). The number indicated molecular mass of corresponding peaks (kDa).

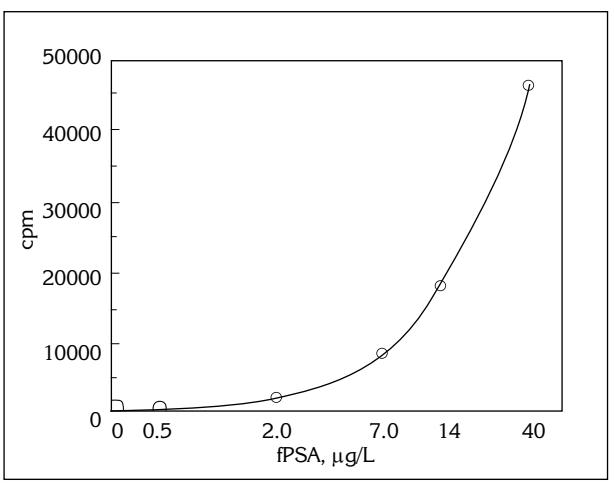

Figure 2. Representative calibration curve for fPSA
Concentrations of the standards for fPSA (0; 0.5; 2; 7; 14; 40 μg/L) were ploted on x axis and bound radioactivity (cpm) were ploted on y axis. Four parameter logistic weighted function is used for curve fitting.

tube, and incubation proceeded for 18 hours at room temperature with constant shaking. The contents of the tubes were aspirated and then 2 mL of washing solution was added to each tube and re-aspirated. The washing procedure was repeated twice. The remaining radioactivity bound to the tubes were measured, and the typical standard curve was obtained as represented on *Figure 2*. In this non-competitive assay, the bound radioactivity was directly proportional to concentration of fPSA in the sample examined. Subsequentlly, the assay was calibrated against 1st IS 96/668, based on concentration responses of serially diluted reference material. Correlation between expected and measured values was 99%.

Table I Precision profile of fPSA assay

	mean value	SD (μg/L)	CV(%)
intra-assay variations	2.1	0.07	3.42
	3.0	0.24	7.53
	7.0	0.30	4.36
inter-assay variations	1.6	0.14	8.33
	2.6	0.11	7.04
	14.0	1.10	7.87

Assay characteristics

Analytical validation of fPSA assay was based on determination of detection limit, precision profile, linearity and recovery. The detection limit, *i.e* the lowest detectable fPSA concentration, defined as the concentration associated with the mean signals of the zero calibrators +2SD (two standard deviations), were assessed as being 0.083 μg/L.

Intra-assay variations were determined by analyzing 3 serum samples at 2.1 μg/L 3.0 μg/L and 7.0 μg/L, with 10 replicates in the same run. Intra-assay variations were studied by analyzing 3 serum sample at 1.6 μg/L, 2.60 μg/L and 14.0 μg/L, in duplicate, in 10 different runs, during 10 days, one assay per day. The intra-assay coefficients of variation (CVs) of the measurements were 3.42–7.53% and inter-assay CVs of the mean concentrations of the duplicate were 7.04–8.33 % (*Table I*).

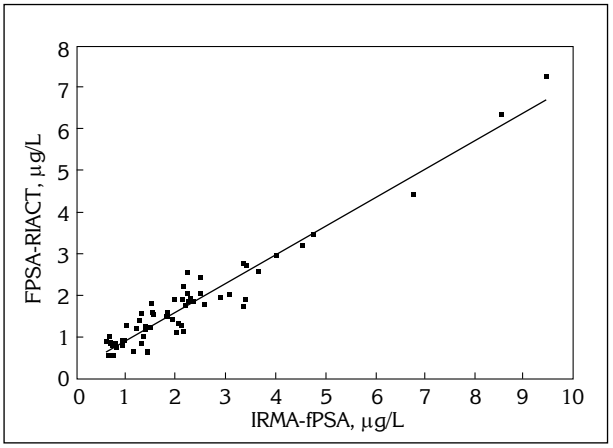

Figure 3. Correlation of the results of fPSA measurments Concentration of fPSA measured by IRMA fPSA (on the x axis) and FPSA RIACT (on the y axis) are compared: y = 0.679x + 0.258; r = 0.964; n = 59

Linearity of the assay was evaluated after serial dilutions of serum samples to the zero calibrator and by comparing the obtained results with expected values. The recovery percentage ranging from 98.7 –107.4%, indicated good linearity.

Effect of sample matrix was analyzed by addition a small volume of PSA reference material to male serum and comparing the measured concentrations with expected ones. The recovery was up to 72% of value with spiked male serum, indicating possible formation of complexes with serum proteins.

Comparison study

Serum samples were analyzed within one day by IRMA fPSA (INEP) and by FPSA-RIACT (CIS bio international – Schering, Germany). The measured values (n = 59) and their comparison using linear regression analysis indicated good correlation between assays (r = 0.964) for the concentration range 0.5 μg/L – 40 μg/L (*Figure 3*).

Discussion

Immunoradiometric assays, in general, are based on recognition of distinct analyte by means of combination of capture and tracer monoclonal antibodies specific for different epitopes (12). The described assay for quantitation of fPSA is formulated as one step, two-site »sandwich« immunoradiometric assay. Specificity of this assay was achieved by using fPSA antibody as tracer antibody. Selection of particular clone of fPSA antibody was a very important step in the assay development due to the demands dependent on the intristic biochemical characteristics of PSA as analyte, *i.e.* its structural heterogeneity. Anti-fPSA antibody used for the formulation of this assay was grouped to epitope 1-reactive antibodies. It recognized equimolarly mature PSA as well as pro-PSA and showed no cross-reaction to PSA-ACT complex (9). It was one of a number of anti-PSA antibodies (commercially available or not) characterized during ISOBM TD-3 Antibody workshop in 1999, and being suggested as appropriate for use for assay development (9).

Free PSA constituites up to 30% of total PSA in serum, whereas, in urine, it is found as predominant form. Serum PSA is common analyte in immunoradiometric assays, but urinary PSA also has diagnostic potential in follow up of prostate patients, although it is not widelly analyzed (13). Anti-fPSA antibody used in this assay recognizes both serum and urinary PSA, but differences in PSA immunograms were observed between these samples. This indicated indirectlly that assay has possible applicability to urine analysis but distinct features of urinary antigen deserves further consideration in terms of assay validation, especially matrix effect.

The second point of interest to consider in strategies of improving reliability of fPSA quantitation was the standardization. Standardization as quality control factor is generally performed aiming at improvement of comparability of results from various laboratories and in various technologies by increasing the accuracy (12, 14). We compared our assay for fPSA and FPSA-RIACT (CIS bio international – Schering, Germany) both of them being calibrated against 1st IS 96/668. Concentrations of fPSA measured by our assay in which expected value *i.e.* 1ng 1st IS 96/668 = 1 ng fPSA (measured value), and FPSA-RIACT in which 1ng 1st IS 96/668 = 12 ng fPSA (measured value) were in good correlation (r= 0.964). PSA is glycoprotein exsisting in various glycoisoforms and it belongs to the group of heterogenous analytes (15). Thus, except for the possible differences due to the specific characteristics of particular antibodies, standardization of immunoassays for heterogenous analytes is associated with problems relating to the fact that reference material can not truly reflects heterogeneity of analytes present in biological fluids (12, 14). That is why the recommendation that follow-up of patients must be based on the results obtained using test of same producers is important, *i.e.* the results obtained in different assays are not interchangeable.

It is accepted that the results of fPSA assay are expressed as %fPSA *i.e.* as fPSA/total PSA ratio. Although, its lower ratio is associated with higher risk of prostate cancer, there is no consensus about the reference values for % fPSA (16). There is generall suggestion of producers of assay, that each laboratory should established its own range of reference values. Preliminary results obtained on the limited number of serum samples from patients with benign prostatic hyperplasia and prostate cancer, in our study

which is still in progress, indicated that cut-off of 15% are applicable to this assay.

All these results, taken together with the results of fPSA assay evaluation which indicate good linearity and precision profile with acceptable CVs lower than 10%, encourage its use as tool in laboratory diagnostic. However, in spite of good assay format, the drawbacks relating to the biochemical properties of free PSA in terms of its stability as well as the influence of age and different medical treatments on its serum concentration, must be kept in mind and general guidelines for assay interpretation should be followed.

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RAZVOJ IMUNORADIOMETRIJSKOG TESTA ZA ODREĐIVANJE SLOBODNOG PROSTATA-SPECIFIČNOG ANTIGENA

Miroslava M. Janković, Maja M. Kosanović, Ljiljana D. Hajduković-Dragojlović, Snežana J. Golubović

Institut za primenu nuklearne energije – INEP, Banatska 31b Zemun, Srbija i Crna Gora

Kratak sadržaj: U ovom radu su saopšteni rezultati razvoja i analitičke validacije novog testa za određivanje slobodnog prostata-specifičnog antigena, fPSA. Test je formulisan kao »sendvič« imunoradiometrijska metoda u jednom koraku. Specifičnost testa je postignuta korišćenjem monoklonskog antitela prema epitopu-1 na slobodnom PSA, koje je korišćeno kao antitelo za detekciju. Test je kalibrisan prema prvom međunarodnom standardu 96/668, a njegov detekcioni limit je iznosio 0.08 μg/L. Koeficijenti varijacije za test iznose 3,42–7,53%, a između testova 7,04–8,33%. Izmerene koncentracije serijski razblaženih uzoraka seruma bile su bliske izračunatim koncentracijama, što je ukazivalo na dobru linearnost sa procentom povraćaja od 98,7–107,4%. Analitičke karakteristike testa za fPSA govore u prilog njegovog korišćenja kao pouzdanog sredstva u laboratorijskoj dijagnostici oboljenja prostate.

Ključne reči: slobodan PSA, imuno-test, anti-PSA antitela, standardizacija

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