Impact of Assay Parameters on the Accuracy of Free PSA Test: Source and Stability of Calibrator, Calibration Curve Fitting, and Level of Total PSA in the Serum

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The measurement of PSA is recommended for men over 50 years of age for screening of prostate cancer. However, proper differentiation of prostate cancer from benign prostate hyperplasia (BPH) relies on an accurate measurement of free PSA (fPSA) and a correct calculation of percent fPSA. Because of the extremely low concentration of fPSA in the serum, any slight deviation from its true value may produce large errors in percent fPSA calculated. Therefore, we undertook a study examining carefully those parameters of the fPSA assay which might affect the fPSA determination. We found that the integrity of the calibrator, the computer curve-fitting program selected, the source of the calibrator, and the total PSA or fPSA + PSA complexes (tPSA) concentration of the specimen all had an impact on the accuracy of the fPSA

value assayed. We found that an examination of the slope of the calibration curve was important to reveal whether the calibrator had or had not been denatured during storage. We also found that the 4-parameter curve fitting program was best suited for plotting the fPSA calibration curve. The calibrator we isolated from LNCaP cells was acceptable for our assay because it had an affinity for the assay antibody very similar to that of serum fPSA. We also determined the effect of tPSA concentration on the fPSA determinations and found that within the concentration range of 4–10 ng/mL the impact on the percent fPSA calculated was not significant. We believe that our assay produces accurate fPSA values when all these assay parameters are well controlled. *J. Clin. Lab. Anal.* 12:304–309, 1998. © 1998 Wiley-Liss, Inc.

Key words: prostate cancer; screening; 4 parameter; benign prostate hyperplasia

INTRODUCTION

Two major PSA isoforms are immunologically detectable in the human serum, namely free PSA (fPSA) and the PSA- α 1-antichymotrypsin (PSA-ACT) complex (1–5). The amount of fPSA found in patients with prostate cancer is usually small, between approximately 0 and 5% of the total PSA, whereas a much higher percentage of fPSA is present in patients with benign prostate hyperplasia (BPH). Because of its relative tissue specificity, PSA was the first tumor marker recommended, in combination with digital rectal examination, for the screening of prostate cancer (6).

However, the PSA test is not cancer specific. Serum PSA values from prostate cancer overlap with that from patients with BPH, especially when the tumor is still organ-confined. Determination of serum fPSA and the calculation of %fPSA ($\text{fPSA/tPSA} \times 100$ where tPSA is equal to fPSA plus PSA complexes) has been found to help differentiate BPH from prostate cancer and to avoid many unnecessary biopsies (7). In other words, determination of the %fPSA cutoff improves the specificity of prostate cancer screening in men with se-

rum PSA levels between 4 and 10 ng/mL. The result of our own and other studies suggested that when the %fPSA is above a certain cutoff (e.g., in this case 23%) it is most likely that patients have BPH, whereas below a certain cutoff (such as 6%) they probably have cancer (8–11). Because the concentration of serum fPSA is usually much lower than that of total serum PSA, any slight deviation of fPSA from its true value will conceivably impact the %fPSA calculation. Therefore, an accurate determination of fPSA is critical in order to produce an accurate %fPSA value.

Here we developed a simple enzyme immunoassay for fPSA on microplate (Fig. 1). Polyclonal anti-PSA antibodies from Dako were coated on a microplate which would capture all free and complexed PSA molecules in the sample. The amount of fPSA bound was quantified by further reacting it with the monoclonal, anti-fPSA specific antibody from CanAg and

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Assay Specific for free PSA

Fig. 1. Test format for our microplate in-house fPSA assay.

visualized by a second, horseradish peroxidase conjugated polyclonal, rabbit antimouse antibody (12). However, in order to produce accurate fPSA values for %fPSA calculation, several assay parameters needed to be carefully examined including the integrity of the stored calibrator, the computer program selected for plotting the calibration curve, the source of calibrator isolated, and the effect of tPSA concentration in the sample. We found that making certain that the calibrator was not denatured during storage and the selection of a 4-parameter logistic standard curve-fitting program were most important to ensure the accuracy of our fPSA assay.

MATERIALS AND METHODS

BSA (Bovine serum albumin, fraction V), Tween 20, casein were all purchased from Sigma (St Louis, MO). Microtiter Strips (immulon 4) were from Dynatech Laboratories (Chantilly, VA). Polyclonal anti-PSA antibodies were from Dako Corporation (Carpinteria, CA). The antifree PSA specific antibody was from CanAg (Gothenburg, Sweden). Sheep antirabbit IgG HRP-conjugate solution was obtained from Zymed Laboratories (South San Francisco, CA). TMB substrate kit was from Pierce (Rockford, IL).

fPSA Assay

The procedure for the fPSA assay was established in house and was described in detail in our earlier publication (12). The assay requires only one addition and one incubation before quantification. Essentially 50 μ L serum and 50 μ L monoclonal antifree PSA antibody (diluted 1:5,000 in PBS containing 1% BSA) were added at the same time to the Dako polyclonal anti-PSA coated micro-well (total 100 μ L). After mixing, the microplate was incubated for 2 hr at rt. The wells were washed and HRP-conjugated antimouse IgG antibody

(100 μ L, 1:5,000 dilution) was added for quantification. Pierce TMB Substrate solution was used for color development following the kit instruction. Our in-house fPSA assay can detect as low as 0.06 ng/mL and the crossreactivity with PSA-ACT complex is only 0.27%. Our calibrator was isolated from LNCaP cells and the calibration curve covers the concentration range from 0.06 to 4 ng/mL.

RESULTS

Effect of Denaturation

Free PSA at nanogram concentrations in the absence of other proteins is extremely unstable. As shown in Figure 2, significant denaturation occurred even after 2 hr at either room temperature or at 4°C. It only took 15 hr at room temperature or 24 hr at 4°C to abolish all fPSA immunoreactivity. On the other hand, 1% BSA effectively protected the denaturation of fPSA at identical fPSA concentrations. This is demonstrated in the control in Figure 2 and again in Figure 3. In the presence of 1% BSA, nanogram concentrations of fPSA were stable for seven days at 37°C. The fPSA did not show any denaturation until we raised the temperature to 50°C. Even at elevated temperatures, such as 50°C, the fPSA was stable in the presence of 1% BSA; fPSA did not show any denaturation after 7hr incubation. Since fPSA in separate tubes was subjected to different incubation temperature the time-dependent slope change shown in Figure 2 could not be the result of absorption of fPSA on the tube. The denaturation shown in Figure 3 in the presence of overwhelming amount of BSA at 50°C also could not be attributed to fPSA absorption on the tube wall. All denaturations showed a decrease in slope. Therefore our results clearly suggest that the calibrator for this fPSA assay needs to be maintained at high protein concentrations. Any dilution should be performed with buffer containing 1% BSA. When in doubt of the integrity of the calibrator or of the calibrator after long term storage, the slope of the calibration curve should be compared with that of the early curve to ensure that the slope of the curve has not changed. It should also be noted that such slope change only occurs in immunoassays, in which a binding between the antigen (analyte) and the assay antibody follows a saturable fashion. Denaturation of either the antigen or the antibody will usually decrease the affinity between them and result in a curve with a diminished slope.

Curve Fitting

During an earlier stage of assay development we designed the calibrator curve to cover the fPSA concentration from 0 to 40 ng/mL. A study was carried out to determine which computer curve-fitting program available on our microplate reader (Molecular Device) should be used to construct the calibration curve (13–15). We took a set of calibrators with known fPSA values and subjected them to the assay. The ab-

Effect of the Stability of fPSA on the Slope

Fig. 2. Stability of fPSA at 4°C and at room temperature in the absence of other proteins. Two aliquots of LNCaP free PSA, approximately 10 µg/mL each, diluted 500-fold with PBS and incubated at two different temperatures. At various time intervals, aliquot (75 µL) was withdrawn and trans-

ferred into plastic tubes containing 75 µL 1% BSA and kept at 4°C. At the end of the incubation time all samples were analyzed by the in-house fPSA assay. The control was stored with PBS containing 1% BSA at 4°C throughout the incubation period.

Fig. 3. Stability of fPSA in the presence of 1% BSA. The only difference in the stability studies between Figure 2 and Figure 3 was that in Figure 3, all the PBS buffer used for fPSA dilution contained 1% BSA.

sorbencies at 450 nm obtained from the assay were read against the four calibration curves plotted using four different curve-fitting programs. The fPSA values obtained from reading these four different calibration curves are listed in Table 1. Conceivably, the ideal calibration curve should produce fPSA values exactly the same as those known values listed in the first column of the table. *Italic figures designate estimated fPSA concentrations deviating more than 10% from the known values.* Consequently, the more italic values produced, the worse the curve-fitting program. Therefore, it is clear from Table 1 that the “linear fitting” was the worst program while “4-parameter fitting” was the best. The reason that there was such a difference between linear fitting and 4-Parameter fitting was due to the very narrow linear range of our calibration curve. As illustrated in Figure 4, the linear por-

TABLE 1. Effect of Different Curve-Fitting Programs on the Estimated fPSA Value

Known fPSA value (ng/mL)	4 Parameter (ng/mL)	Quadratic (ng/mL)	Linear (ng/mL)	Log-log (ng/mL)
0	0.18	<i>0.6</i>	<i>0.92</i>	<i>1.25</i>
2.5	2.25	<i>1.98</i>	<i>2.02</i>	2.41
5.0	5.21	4.75	4.78	5.03
10.0	10.05	9.90	<i>11.34</i>	<i>10.33</i>
20.0	19.86	20.41	22.98	<i>21.24</i>
40.0	40.2	39.85	38.29	<i>37.57</i>

Italic figures are values deviated more than 10% of the corresponding known fPSA value.

Fig. 4. Dose-response curve for fPSA derived from different sources.

tion of the calibration curve is limited to about 2 ng/mL. If one draws a linear line to accommodate all points up to 5 ng/mL, it will produce falsely high fPSA values at the low concentration end of the curve, and both falsely high and low fPSA values for the upper concentration curve. Since we are usually dealing with specimens containing fPSA below 4 ng/mL during screening, our calibration curve only needs to cover fPSA concentrations up to 4 ng/mL (Figure 4).

Selection of fPSA From Different Sources as Calibrator

Free PSA was usually isolated from seminal plasma, a rich source of fPSA, which contains approximately 0.6 mg fPSA/mL. However, fPSA can also be isolated from LNCaP cell cultures. Recently we described a procedure to grow LNCaP cells in a Hollow fiber bioreactor in the presence of artificial androgen, R 1881, which allowed us to collect milligram concentrations of relatively pure fPSA (16). Since the LNCaP cell line was derived from a human prostate carcinoma, the fPSA it produces should be similar to fPSA found in patients with prostate cancer and could be different from the fPSA derived from a normal source, such as seminal plasma. Because it is not practical to isolate fPSA from patients' sera (the concentration is too low), we need to choose between fPSA from seminal plasma or from LNCaP cell culture for the preparation of calibrator. However, any calibrator selected should also have an affinity for the assay antibody identical to that of the analyte found in the serum sample, otherwise the assay results may not be accurate. A simple method for determining whether or not analytes from different sources have the same affinity for the assay antibody is to run a dose-response curve. Compare the slopes of various dose-response curves obtained by plotting the signal (in this case, plot the $A_{450\text{ nm}}$ against the concentration of the analytes; the values

were assigned based on dilution). If the selected calibrator and the sample analyte exhibit a similar slope in their dose-response curves, this suggests that both the calibrator and sample analyte have the same affinity for the assay antibody. As shown in Figure 4, fPSA from either seminal plasma, LNCaP cells, or patients' sera all exhibit practically the same slope in the linear portion of their dose-response curves. In other words, they all appear to have the same affinity for the assay antibody even though they are from different sources. Conceivably, fPSA from either seminal plasma or LNCaP cell cultures can be used as calibrator for the fPSA assay. In Figure 5 we demonstrated why the 4-parameter logistic curve-fitting program is the best program. We took a set of our calibrators with known fPSA values and subjected them to our in-house fPSA assay. Different sets of fPSA values were obtained from the same absorbency reading against calibration curves constructed based on different curve-fitting programs. We then plotted the known fPSA values on the dotted horizontal line in different columns and set them as 0%. The rest of fPSA values were then plotted as deviations from the known fPSA values. Apparently, the set of fPSA values obtained from the 4-parameter-calibration curve had values tied most closely to the known fPSA values associated with the smallest deviation.

Effect of tPSA or PSA-ACT Concentration

Our fPSA kit uses microplate coated with polyclonal anti-PSA antibodies for the assay. It is likely that all PSA isoforms

Fig. 5. Selections from four different computer curve-fitting programs for the calibration curve. A set of calibrators with known fPSA values (0) was assayed by our in-house fPSA assay. The final absorbencies obtained from the assay were read against calibration curves whose construction was based on these different curve-fitting programs. Differences between the set of fPSA values from the known fPSA values were plotted in terms of percent of deviation.

in the serum sample including fPSA, PSA-ACT, and other minor PSA complexes will be captured by the polyclonal anti-PSA antibodies during sample incubation. As a consequence, there will be competition between fPSA and PSA-ACT for the limited amount of anti-PSA antibodies coated on the microwell. Since the concentration of PSA-ACT complex is usually much higher than that of fPSA in most specimens, determination of fPSA will be impacted by the level of PSA-ACT complex in the same sample. In other words, fPSA value will most likely be underestimated if there is high concentration of PSA-ACT present in the serum. Calculated values of %fPSA undoubtedly will be affected under these situations. As shown in Table 2, the %fPSA was only 2.1 at tPSA of 34.1 and increased to 2.9 when sample was further diluted (less PSA-ACT present in the sample to compete with fPSA). Fortunately %fPSA is most useful in the range of tPSA between 2.5 and 15 ng/mL in which %fPSA determination is used to differentiate BPH from cancer. In this concentration range of tPSA very little difference in %fPSA was found, regardless of whether the calculation was based on tPSA ($\%fPSA = fPSA / tPSA \times 100$) or PSA-ACT complex ($\%fPSA = fPSA / PSA-ACT \times 100$) concentrations.

DISCUSSION

Stability of the analyte is a major concern in any type of assay. In an immunoassay it is well known that denaturation of the analyte is usually associated with a decrease of affinity for the assay antibody and results in falsely low values as shown in Figure 6A. However, very few of us question the stability of the calibrator. Perhaps it is because the calibrators are usually maintained at very stable conditions, such as storage at -70°C or are lyophilized. But it also may be due to the fact that very few people know how to detect the denaturation of a calibrator. While the stability of the analytes can be measured against the calibrator, for calibrators however, there is nothing for the calibrator to be compared with. Unfortu-

TABLE 2. Effect of tPSA Concentration on the Value of fPSA and %fPSA^a

Dilution factor	fPSA	tPSA/ %fPSA ^b	PSA-ACT/ %fPSA ^c
1	2.12	>100/	>20/
2.5	1.42	60.4/	>20/
5.0	0.87	34.1/2.1	>20/
10	0.49	16.9/2.9	13.85/3.5
20	0.26	7.86/3.3	7.46/3.4
40	0.13	3.89/3.4	3.81/3.5

^aSerum used in this study was from a patient with prostate carcinoma and had a total PSA value of approximately 170 ng/mL. The diagnosis was confirmed by biopsy. fPSA, tPSA and PSA-ACT all are in ng/mL. tPSA was determined by Hybritech Tandem E assay, and fPSA and PSA-ACT were by in-house assays.

^bCalculated based on tPSA.

^cCalculated based on the concentration of PSA-ACT.

Fig. 6. Effect of the denaturation of the sample-analyte and calibrator on the assay value. (A) Denaturation of sample analyte will result in a lower signal and falsely low assay values; (B) Denaturation of calibrator will result in a less steep slope and falsely high assay values for the sample.

nately the impact of calibrator denaturation is far more damaging than the denaturation of individual analytes. When a denatured calibrator is used in an assay all the assay values will be falsely elevated. As shown in Figure 6B the reason for the false elevation of assay values is due to the change of the slope of the calibration curve when the calibrator becomes even slightly denatured. Gradual denaturation can occur in analytes stored at -70°C and in lyophilized analytes if the moisture stored was not completely removed during lyophilization. In this investigation we found that monitoring the slope of the calibrator is the best way of detecting whether or not the calibrator was denatured during storage. In our experimental design we made sure that the decrease in slope of heat-denatured fPSA was not due to absorption of fPSA by the tube. We recommend that when comparing the slope of the stored calibrator with that of the early slope of a freshly prepared calibrator it is important to make certain that scales of the X and Y axes in both figures are exactly the same.

It is not generally realized that the entire calibration curve of an immunoassay is not always linear. The signal produced by the immunoassay is similar to the enzymatic activity displayed by an enzyme in which only the initial portion of the curve is linear; the entire curve is actually hyperbolic in shape,

which is related to the affinity (equilibrium dissociation constant) between the analyte and the assay antibody. When the attempt is made to draw a straight line through all points generated by calibrators of varying concentrations, falsely lower values at low concentrations and falsely higher values at higher concentrations often result. Curve-fitting computer programs are now available to assist the construction of a proper calibration curve in order to produce accurate assay values. By assaying the calibrators with known values we were able to assess which of the different curve-fitting programs was most suitable. The plot shown in Figure 5 clearly indicates that the 4-parameter fitting program generates fPSA values with the least deviation and linear fitting produces the largest deviation from those known values. Both Log-Log and Quadratic are acceptable for most situations but showed a large deviation at 0.125 fPSA concentration. Since the fPSA concentration in many serum specimens is close to this concentration, their application for the fPSA assay is discouraged.

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