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Preparation of prostate specific antigen standards for immunoradiometric assay

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Background: Immunoradiometric assay is one of the most common and precise methods for determination of prostate specific antigen (PSA) in clinical laboratories. Usual use of human serum in routine assays has many disadvantages; such as easy contamination and precipitation, instability and unavailability. Thus in order to avoid these problems the artificial matrix was used which acts similar to human serum. ***Materials and Methods:*** In order to design immunoradiometric assay for prostate specific antigen, series of standards in different concentrations were needed for special artificial matrix preparation. The influence of artificial matrix in standards was studied to determine prostate specific antigen in comparison with human serum. Some different factors, such as the amount of non-specific bonding (NSB), precision, and accuracy, conditions of storage and stability of these standards prepared by artificial matrix were investigated. ***Results:*** The most appropriate artificial matrix (Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) +Triton X-100 (0.5 ml/L) + HSA (1.2 g/L) + Urea (0.5 mol/L)) for preparing the standards was selected in comparison with human serum (HSA) and a commercial kit standards. HSA and Urea concentration have more critical influences on the properties of the standards. The amount of NSB of the selected matrix was the lowest one, so the selected matrix was the most suitable for preparing the standards. The results show the optimum condition of storage duration of our standards for one year was in refrigerator (2-8°C). It was observed that preparation of standards with selected matrix had acceptable accuracy and precision. ***Conclusion:*** According to the results, standards which were prepared with this matrix had suitable and appropriate properties and it could be utilized to prepare PSA standards in immunoradiometric assay. **Iran. J. Radiat. Res., 2008; 6 (1): 51-58**

Keywords: *Prostate specific antigen (PSA), immunoradiometric assay (IRMA), immunoradiometric kit, artificial matrix, human serum.*

INTRODUCTION

Prostate cancer is the sixth most common cancer in the world, the third most common cancer in men. In 2000, the number of new cases of prostate cancer was estimated at 513000 worldwide and this disease accounts for 9.7% of all cancers in men [1, 2]. Thus early detection and local treatment have been advocated in an effort to influence the significant morbidity and mortality associated with the disease.

Prostate specific antigen (PSA) is a glycoprotein of 30 KDa found mainly in prostatic tissue and specific for prostatic tissue normal or malignant. It has been identified to be present in normal, benign hyperplastic and malignant prostatic tissue, in metastatic prostate carcinoma, and also in prostatic fluid and seminal plasma. Pathologically increased production of PSA is a characteristic for malignant prostatic tissues. Considerably increased PSA levels in patients with bone metastases demonstrate their prostatic origin.

The determination of PSA is based on the biochemistry and immunoassay methods, such as: radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [5, 6]. Today, one of the most common methods for analysis of PSA is immunoradiometric assay (IRMA) which is one of the RIA methods. In this method two monoclonal antibodies against two different epitopes of PSA molecule are used.

An IRMA kit used in clinical laboratories is consisted of four main components including,

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anti-PSA monoclonal antibody – coated tubes; monoclonal I-125 – labeled anti-PSA antibody; series standards for concentration range from 0 to 100 ng/ml and lyophilized control samples

It should be kept in mind that in preparation of these kits having a stable series standard is of prime importance. The standards are prepared by adding known amounts of purified PSA obtained from human seminal fluid to a suitable matrix.

An ideal standard should have certain characteristics such as stability during storage; not contain substances which could interfere in the assay; chemical and physical similarities to real matrix (human serum); presence of low non-specific bonding (NSB) in the assay [7, 8].

In order to design immunoradiometric assay kit for prostate specific antigen in our laboratory, series of ideal standards were needed in different concentrations, so special matrix was studied as a replacement for human serum. A matrix ought to be obtained which was selected to simulate the effects of the real one. First, women serum was used as free serum (without PSA) for the preparation of the matrix, but problems such as high NSB, instability, easy contamination and precipitation showed up, therefore, the preparation of an artificial matrix was studied as a replacement for free serum.

MATERIALS AND METHODS

All reagents were analytical grade and purchased from Merck (Germany), Aldrich (Germany) and Fluka (United Kingdom). PSA/IRMA KIT were obtained from Immunotech Company (Czech Republic).

Preparation of women serum (free serum) and men serum

Women and men blood in tubes containing no additive was collected separately. Serum from cells was separated by centrifugation, and then stored at 2-8°C, if the assay was to be performed within 24 hours. For longer storage, they were kept frozen at -20°C.

Preparation of purified PSA from seminal fluid

Seminal fluid was collected from healthy donors in Day hospital (Tehran, Iran) and then transferred to the laboratories in a well-kept cold ice container. In the first step seminal fluid dialysis was done in buffer solution (Citrate buffer 0.1 M, pH=6.8) which contained 0.1 mmol/L of phenyl-methyl-sulfonyl fluoride; in second step, dialysised sample was passed through an Affigel-Blue column and then through Sephacryl S-200 HR column, FPLC (model: Pharmacia/ Uppsah/Sweden). The purified sample was collected and stored at 4°C [9]. Also, the concentration of purified PSA was estimated by Luary method [10] and PSA/IRMA kit (Immunotech).

Preparation of artificial matrix

Free serums as well as different artificial matrices consisting of the following materials were prepared; Free serum (without PSA); Tris-glycine (25.0 mmol/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + HSA (1.2 g/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + Urea (0.5 mol/L); Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + HSA (1.2 g/L) + Urea (0.5 mol/L); HSA (1.25 g/L) + PBS (KCl (0.2 g/L) + NaCl (8.0 g/L) + KH2PO4 (0.2 g/L) + NaH2PO4 (1.1 g/L)); HSA (1.2 g/L) + Hank's solution (MgSO4.7H2O (0.2 g/L) + KCl (0.4 g/L) + NaCl (8.0 g/L) + NaN3 (1.0%) + Na2PO4 (0.2 g/L) + KH2PO4 (6.0 g/L) + CaCl2.H2O (0.1 g/L)).

A solution of NaN_3 (1%) also was added to all materials.

In addition, the concentration of HSA and Urea were varied between 0-2.5 g/L and 0-3 mol/L. The effects of these materials in matrices with different compositions were

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examined and compared with free serum as well. Then, the best matrix was selected and standardized using the prepared matrix.

Preparation of PSA standard

At first, master standards with purified PSA were prepared in free serum and artificial matrices. Then in accordance with estimated protein concentration, PSA sample was diluted to several concentration (0-100 ng/ml range) with free serum and artificial matrices. Their PSA concentrations were determined by PSA/IRMA KIT (Immunotech,) three times to obtain the mean value used as a standard.

Determination of PSA by IRMA method

Determination of PSA was performed by a commercial kit (Immunotech). The samples or standards were determined as the instruction of Immunotech Company. The samples or standards were determined as the instruction of Immunotech Company. The bound radioactivity was measured in a gamma counter (model Oakfield instrument LTD, England). The calibration curve was constructed as ratio of bound to total radioactivity versus the concentration of the standards. PSA concentration of the unknown samples was read off from the calibration curve.

The concentration of real sample (men serum) was analyzed by Immunotech kit with different standards so that the best artificial matrix was selected which was acting similar to free serum.

Finally, the concentration of real samples (men serum consisted PSA), kit control samples and diluted control samples of Immunotech kit were compared by IRMA kit with using kit standards and the standards applied in the present research.

Determination of non-specific bonding (NSB)

Non-specific bonding means the effective interaction of other compounds included in standards which are non-specific. So, all matrices without PSA were analyzed by PSA/IRMA kit (Immunotech) to measure their NSB amounts.

Stability and storage conditions

For the comparison of the stability of Immonutech kit standards, and the obtained standards, both series of standards were evaluated for the duration of a year. According to the instruction of Immunotech Company, PSA/IRMA kit standards should be kept in 2-8°C for a year. In order to obtain the best condition of storage, the prepared standards were stored in different conditions for the same period, such as room temperature (25°C), refrigerator (2-8°C), freezer (-20°C) and as lyophilized samples which were kept in freezer (-20°C). During that period, real men sample was determined by applying kit standards and selected standards. In addition, the concentration of standards prepared with free serum and selected artificial matrix was analyzed in different time period.

Accuracy and precision

To study accuracy and reproducibility of standards, each of the standards (0-100 ng/ml) was prepared from purified PSA and selected matrix triplet. The concentrations of standards were measured by Immunotech kit, and then the concentration of purified PSA was estimated. Finally, reproducibility of the standards was obtained.

The precision was evaluated by comparison of the concentrations of control samples of Immunotech kit, real samples and diluted samples with artificial matrix analyzed by two different kinds of standards (the present study's standards and Immunotech kit standards).

RESULTS

Comparison of artificial matrices and free serum

As shown in figure 1, the initial concentration of purified PSA in different matrices and free serum separately by IRMA method was estimated. The concentration of PSA in free serum at the 95% confidence was 33.60 ± 0.30 mg/L. Only in the matrix including (Tris-glycine (25.0 mmol/L) + NaCl

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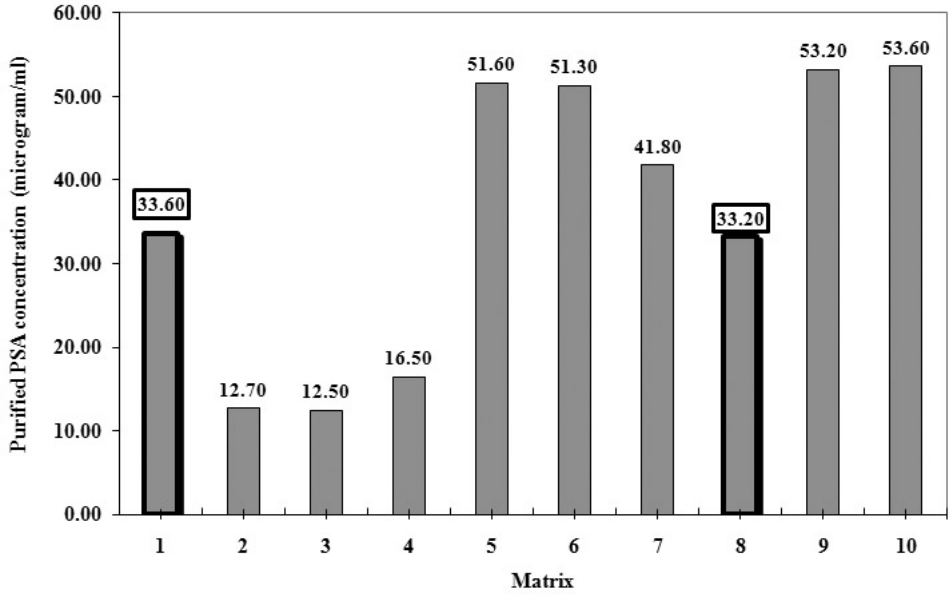

Figure 1. The determination of purified PSA concentration by using standards with different matrices.

1- Free serum (without PSA)
2- Tris-glycine (25.0 mmol/L)
3- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L)
4- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L)
5- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L)
6- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + HSA (1.2 g/L)
7- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + Urea (0.5 mol/L)
8- Tris-glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) +Triton X-100 (0.5 ml/L) + HSA (1.2 g/L) + Urea (0.5 mol/L)
9- HSA (1.25 g/L) + PBS (KCl (0.2 g/L) + NaCl (8.0 g/L) + KH2PO4 (0.2 g/L) + NaH2PO4 (1.1 g/L))
10- HSA (1.2 g/L) + Hank's solution (MgSO4.7H2O (0.2 g/L) + KCl (0.4 g/L) +NaCl (8.0 g/L) + NaN3 (1.0%) +Na2PO4 (0.2 g/L) + KH2PO4 (6.0 g/L) + CaCl2.H2O (0.1 g/L))

A solution of NaN3 (1%) also was added to all matrices.

(75.0 mmol/L) + Tris (12.5 mmol/L) + Triton X-100 (0.5 ml/L) + HSA (1.2 g/L) + Urea (0.5 mol/L)), the concentration of PSA (33.20 + 0.38 mg/L) was similar to the concentration of PSA in free serum.

Figure 2 shows the standard curve which was obtained by the standards prepared with selected matrix and immnunotech kit. Using this calibration curve, PSA concentration of the unknown samples could be read off appropriately. Also, table 1 shows the amounts of NSB in all matrices and free serum. As the results showed, the amount of NSB of free serum was the highest amount among all matrices and the amount of NSB of the selected matrix was the lowest one. Therefore, the selected matrix did not manifest an important specific interaction with PSA.

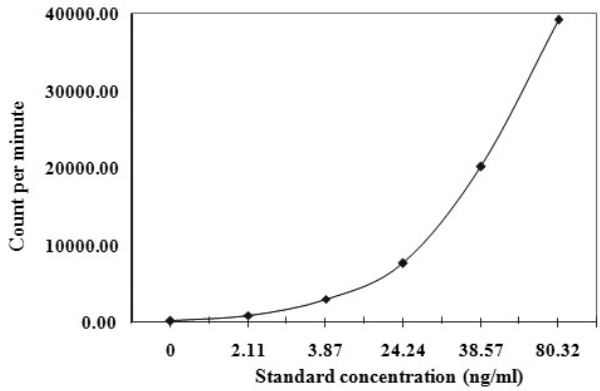

Figure 2. Calibration curve for artificial standards.

The effects of HSA and Urea concentration

Figures 3 and 4 show the effect of HSA and Urea concentration respectively. For the results shown in figure 3 the concentrations of other chemicals were the similar (Tris-

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Table 1. The comparison of the NSB amounts of matrices and free serum

Matrix	Count per minute	% NSB*
1	574±10	0.40±0.01
2	413±12	0.28±0.03
3	411±14	0.28±0.06
4	382±12	0.25±0.04
5	370±13	0.25±0.05
6	235±13	0.14±0.04
7	240±12	0.15±0.05
8	212±11	0.12±0.02
9	402±15	0.27±0.06
10	502±14	0.37±0.05

Total count (count per minute) = 12978
Background count (count per minute) = 50

$$\%NSB = \frac{\text{Standard zero count} - \text{background count}}{\text{Total count} - \text{background count}} \times 100$$

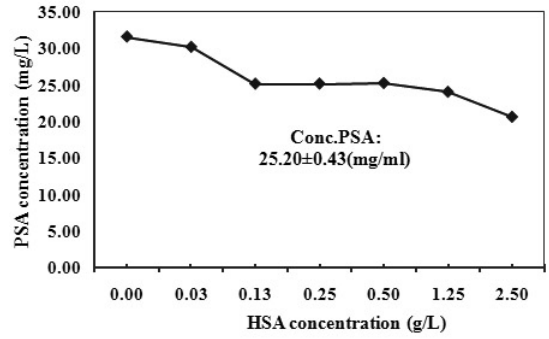


Figure 3. The effect of HSA concentration on the measurement of PSA concentration.
(The concentrations of other chemicals are the same and Urea concentration equals to 0.5mol/L)

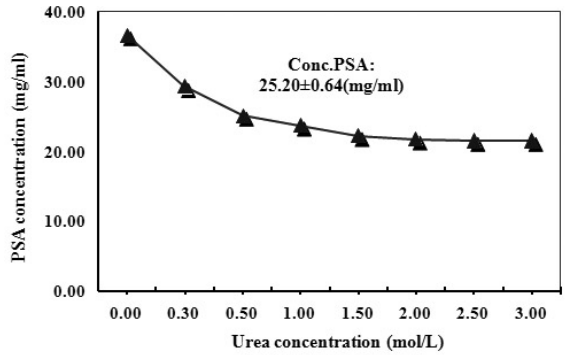


Figure 4. The effect of Urea concentration on the estimation of PSA concentration.
(The concentrations of other chemicals are same and HSA concentration equals to 1.25g/L)

glycine (25.0 mmol/L) + NaCl (75.0 mmol/L) + Tris (12.5 mmol/L) +Triton X-100 (0.5 ml/L) + Urea (0.5 mol/L)) and the concentration of HSA had changed (0-2.5g/L). In addition in figure 4 the concentration of chemicals were the same and only the concentration of urea was changed (0-3 mol/L).

The best concentration for HSA and urea were determined, as 1.2 (g/L) and 0.5 (mol/L), respectively. As shown in figures 3 and 4, increasing the concentration of HSA and urea had caused interference in the reaction between antigen and antibody, leading to negative error when each concentration had increased. As shown in figure 5 the bovine serum albumin (BSA) and HSA produced similar effects meaning that BSA could be replaced by HSA.

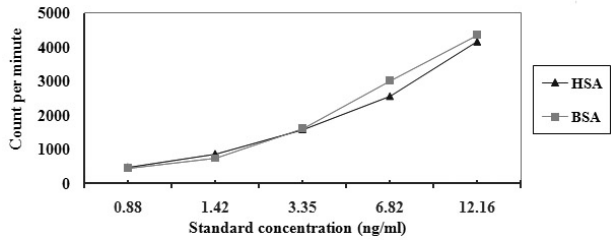
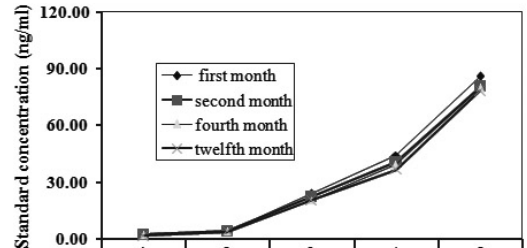


Figure 5. The comparison of HSA and BSA concentration effects in preparation of standard matrix. (Initial PSA concentration was 0.61 mg/L)

Stability and storage conditions

The stability of Immunotech standards and the obtained standards of this research with selected matrix and free serum were measured for a period of 12 months. Figures 6 and 7 show the results of the prepared standards with artificial matrix and free serum. The decrease of primary concentration of antigen in Immunotech, artificial standards with matrix and free serum were compared (table 2). The stability



	1	2	3	4	5
first month	2.21	3.92	23.93	44.14	85.81
second month	2.01	3.72	22.22	40.34	80.51
fourth month	1.72	3.65	20.42	38.83	79.75
twelfth month	1.47	3.63	20.31	36.56	78.23

Figure 6. The stability of artificial standards in different time period.

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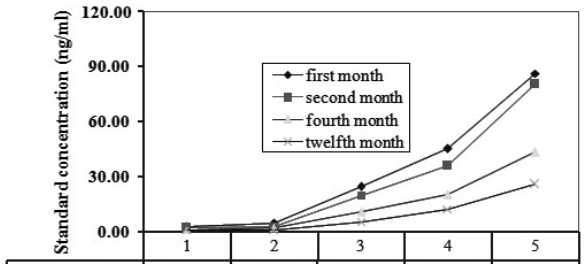

	1	2	3	4	5
first month	2.33	4.32	24.54	45.21	86.21
second month	1.79	2.87	19.76	35.56	80.18
fourth month	0.83	1.68	10.66	20.12	43.45
twelfth month	0.41	0.89	5.32	12.12	25.66

Figure 7. The stability of free serum standards in different time period.

Table 2. The comparison of the stability of Immunotech kit standards and our standards for period of one year.

Standard	Decrease initial concentration %
Immunotech	0.34±16.34
F	0.46± 76.75
A1	0.56± 13.69
A2	0.32 ±14.27
A3	0.29 ± 13.15
A4	0.87±77.85

Immunotech standards in refrigerator
F: standard with free serum in refrigerator
A1: standard with selected matrix in refrigerator
Standard with selected matrix In freezer A2
A3: standard with selected matrix as lyophilized in -4°C
A4: standard with selected matrix in 25°C

of prepared standards with free serum diminished considerably.

The selected standards were stored in different conditions: 25°C, 2-8°C, -20°C and as a lyophilized sample. After six months the concentrations of PSA in standards were measured and the results were compared (table 3). As shown, the optimum condition belonged to those samples which were kept in refrigerator (2-8°C).

Determination of accuracy and precision

Each standard was prepared from purified PSA triplet, then the primary concentration of PSA was calculated: 33.43 + 0.26 mg/L (CV= 0.31% and 95% confidence interval). The result proved that the preparation of standards with selected matrix had an acceptable accuracy.

Table 3 shows the precision for determined PSA concentration in control kit, diluted

Table 3. The comparison of the results obtained by kit standards and standards prepared in the present study.

Sample	Concentration range with Immunotech kit (ng/L)	Concentration of prepared standards (ng/L)
C1	3.62-7.82	4.09 ± 0.11
C2	13.45-20.34	0.22± 15.35
C1/2	1.81-3.91	1.97 ± 0.34
C2/2	6.70-10.17	7.38 ± 0.51
S1	0.52 ± 0.01	0.49 ± 0.02
S2	1.22 ± 0.12	1.46 ± 0.21
S3	3.51± 0.41	3.60 ± 0.36
S4	5.33 ± 0.23	5.01 ± 0.42
S5	10.77± 0.56	10.06 ± 0.47

C1, C2 = kit control samples as lyophilized
C1/2, C2/2 = diluted kit control samples with prepared matrix
S1-S5 = real samples

control samples and real samples. It was found that there was a good agreement between the measured concentrations with using the obtained standards and kit standards.

DISCUSSION

The aim of the present work was to prepare the artificial matrix for prostate specific antigen standards of immunoradiometric assay. Different matrices and free serum were studied in using the mentioned standards and the results were compared with commercial kit (Immunotech) standards. An important finding of the study was the good agreement between the prepared standards with Immunotech kit standards (table 3).

A suitable artificial standards properties must have the special properties; such as, chemical and physical similarities to free serum, high stability, suitable accuracy and precision, low NSB, easy storage condition, low price and availability [6]. Every component in the matrix has an important role in achieving suitable and ideal properties for the standard (figure 1). For example, sodium chloride for providing a suitable electrolytic medium similar to free serum, Tris-glycine and Tris as an adjusting buffers (pH=7.4), Triton X-100 as surfactant

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agent and NaN3 as an antimicrobial were used. Albumin serum acted as a carrier protein and could prevent for specific-bonding of materials in the matrix. Also it caused stabilization of antibody [9]. It appeared that urea had an important role for maintaining suitable osmosis pressure in reaction between antibody and antigen [8]. It was found that HSA and urea concentration had more critical influences on the properties of the standards [8] (figures 3 and 4). According to the results, it could be concluded that substitution of a selected matrix for a real free serum did not have any interference in the reaction between antigen and antibody. It could act chemically and physically similar to free serum.

One of the advantages of the prepared standards was its stability for one year in refrigerator (2-8°C). It appears that PSA is very sensitive, and in fact it decays in free serum easily and rapidly (figure 7). But no significant changes were observed in calibration curves with standards which were made with artificial matrix even more than one year period (figure 6, table 2).

Finally, taking into account, the obtained artificial matrix can serve as a suitable matrix for production of PSA/IRMA kit standards. The results of the present research concerning the preparation IRMA standards indicated that the application of the standards for the production of PSA/IRMA kit was feasible. Therefore, the IRMA kit can be considered as a useful diagnostic tool for PSA measurement.

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