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Stability of [−2]Pro-PSA in Whole Blood and Serum: Analysis for Optimal Measurement Conditions

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Background: The clinical usefulness of [−2]pro-PSA (where PSA is prostate-specific antigen) in prostate cancer diagnosis has been emphasized in recent studies. To determine proper blood sample handling conditions for [−2]pro-PSA evaluation, we analyzed the preanalytical stability of [−2]pro-PSA. *Methods:* Blood samples from 22 Japanese males were stored under various conditions before total PSA (tPSA), free PSA, and [−2]pro-PSA concentrations were measured, and the preanalytical stability of [−2]pro-PSA and the changes in the Prostate Health Index (*phi*) were assessed. *Results:* [−2]Pro-PSA was stable in serum for at least 24 hr at both room temperature (RT) and at 4°C. However, [−2]pro-PSA levels in whole blood increased rapidly over time, particularly at RT. Mean recovery (%) of [−2]pro-PSA in whole blood at RT was >110% at 1 hr after drawing of blood. The *phi* tended to increase over time in a pattern similar to the change in[−2]pro-PSA. *Conclusions:* Preanalytical stability was lower for [−2]pro-PSA than for free PSA or tPSA. Whole-blood [−2]pro-PSA increased in a time-dependent manner, particularly at RT. Thus, whole blood samples collected at RT should be centrifuged within 1 hr after drawing. The [−2]pro-PSA in serum is stable for at least 24 hr at both RT and at 4°C. J. Clin. Lab. Anal. 28:315–319, 2014.

Key words: [−2]pro-PSA; prostate cancer; free PSA; Prostate Health Index; preanalytical stability

INTRODUCTION

Prostate-specific antigen (PSA) is widely used as a serum marker for prostate cancer (PCa; (1, 2)). However, its use is limited and it lacks cancer specificity, particularly when PSA < 10 ng/ml. Therefore, new markers that can accurately detect PCa are required. Recent studies have focused on the "fraction of free PSA" (fPSA), also known as "pro-PSA," for this purpose (3–5). Native pro-PSA (also known as [−7]pro-PSA) exists in a truncated form and contains a seven-amino acid *N*-terminal proleader peptide (6). [−7]Pro-PSA is partially cleaved by human kallikrein family or other proteases, resulting in the generation of three truncated forms of pro-PSA: [−5]pro-PSA, [−4]pro-PSA, and [−2]pro-PSA. [−5]Pro-PSA and [−4]pro-PSA are further cleaved to the active form of PSA (7). However, [−2]pro-PSA cannot be cleaved further. In addition, pro-PSA is more often associated with peripheral zone cancer than transition zone hyperplasia (8). Thus, [−2]pro-PSA may be a more cancer-specific isoform of pro-PSA. Several studies on the clinical utility of [−2]pro-PSA for early PCa detection have been reported (3, 5, 9). For example, Catalona et al. analyzed serum specimens obtained from 1,091 men who underwent prostate biopsy. They concluded that percent pro-PSA was superior to total PSA (tPSA) or percent free PSA within a PSA range of 2–10 ng/ml for predicting PCa (5). Moreover, percent [−2]pro-PSA (defined as [−2]pro-PSA/free PSA) showed the highest cancer specificity among all other variables in a PSA range of 2–4 ng/ml. Ito et al. reported that laboratory-based indices containing [−2]pro-PSA, particularly the Prostate Health Index

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(*phi*), in serum samples from Japanese males have greater specificity for PCa in comparison to percent free PSA (9).

As described above, [−2]pro-PSA has high clinical potential to improve the quality of PCa screening, but it is important to determine the measurement accuracy and reproducibility of this strategy. Thus, it is important to evaluate preanalytical stability under various conditions. For example, free PSA is less stable than tPSA under storage conditions in both a time- and temperature-dependent manner (10). Only one study from Europe investigated [−2]pro-PSA stability; the results indicated that mean [−2]pro-PSA increased with time in whole blood at room temperature (RT; (11)). As the epidemiology of PCa and basic PSA behaviors show ethnic variations, evaluation of [−2]pro-PSA stability in the Asian population is an interesting area of investigation. Therefore, the present study was performed to evaluate the short-term [−2]pro-PSA preanalytical stability in serum and whole blood from Japanese males to determine the parameters for optimal specimen handling.

TABLE 1. Patient Characteristics

Age (years)	
Median	63.5
Range	52–75
tPSA (ng/ml)	
Median	5.40
Range	3.62–43.30
fPSA (ng/ml) at baseline	
Median	0.92
Range	0.23–5.89
[−2]Pro-PSA (pg/ml) at baseline	
Median	12.06
Range	3.55–71.04
phi at baseline	
Median	26.13
Range	16.53–89.47
Prostate volume (cc)	
Median	34.3
Range	16.9–67.8
Prostate biopsy	
Yes (cancer positive/negative)	21 (7/14)
No	1

phi, Prostate Health Index; PSA, prostate-specific antigen.

MATERIALS AND METHODS

Specimen Collection and PSA Measurement

Twenty-two Japanese patients who were referred to Nagasaki University Hospital from May to September 2011 due to elevation of tPSA were enrolled in this study. Blood samples were collected from each patient under an Internal Review Board-approved protocol after receiving informed consent and in accordance with the practices and ethical standards of the Declaration of Helsinki and Nagasaki University. The basic characteristics of the 22 patients are shown in Table 1. Blood was drawn in the morning, and samples were divided into two groups. In group 1, after allowing for clotting for about 10 min, blood sample was then centrifuged, and part of the collected serum was frozen at −70°C within 15 min after centrifugation; this was used as a reference sample, because two freeze-thaw cycles do not affect [−2]pro-PSA stability (11). The remaining serum was aliquoted and stored at 21°C (RT) or 4°C for 1, 3, 8, or 24 hr before being frozen at −70°C. In group 2, blood samples were stored as whole blood at RT or at 4°C for 1, 3, 8, or 24 hr. The serum was then isolated and frozen at –70°C until PSA measurement. The tPSA, fPSA, and [−2]pro-PSA measurements were performed simultaneously, including reference samples for baseline values, using an Access Immunoassay Analyzer (Beckman Coulter, Brea, CA). Each measurement result represents the mean of duplicate determinations. To evaluate preanalytical stability, data from each of the 22 patient samples at the different time points were transformed to percentage of recovery from the respective baseline value. The *phi* was defined as ([−2]pro-PSA/fPSA)×(square root of tPSA; (7,9)).

Statistical Analysis

Data for the percentage recovery of [−2]pro-PSA, fPSA, and *phi* are expressed as means ± SD. Comparisons between baseline and the values at each time point were conducted using ANOVA for repeated measurements. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The time course of [−2]pro-PSA percentage recovery is summarized in Table 2. The [−2]pro-PSA preanalytical stability in serum was favorable at time points within 24 hr regardless of temperature (RT or 4°C). The actual recovery percentage increased slightly over time (Fig. 1A). In contrast, the percentage recovery of [−2]pro-PSA in whole blood increased significantly, particularly at RT. As shown in Figure 1B, the mean recovery percentage at 1 hr was already >110% and increased continuously with time thereafter. The changes in percentage recovery of [−2]pro-PSA in whole blood at 4°C showed a trend similar to that at RT; however, the increases in percentage recovery were much smaller than those at RT. We also evaluated the stabilities of tPSA and fPSA under the same specimen handling conditions. Changes in mean recovery percent of fPSA are shown in Table 2. Both tPSA (data not shown) and fPSA were almost stable for at least 24 hr, and

TABLE 2. Percentage Recovery of [−2]Pro-PSA, Free PSA, and the Prostate Health Index (*phi*) at Various Time Points and Temperatures

Hours	In serum at RT	In serum at 4°C	In whole blood at RT	In whole blood at 4°C
		Mean ± SD [−2]pro-PSA recovery %		
Baseline (0 hr)	100	100	100	100
1 hr	101.7 ± 6.2	104.3 ± 5.6	111.0 ± 9.3[a]	103.6 ± 9.1
3 hr	104.7 ± 8.9	104.9 ± 9.1	123.1 ± 14.1[a]	106.5 ± 7.4
8 hr	104.8 ± 7.0	103.9 ± 8.7	142.3 ± 24.9[a]	114.1 ± 11.0[a]
24 hr	104.5 ± 8.9	103.9 ± 6.7	244.7 ± 135.3[a]	116.3 ± 20.9[a]
		Mean ± SD free PSA recovery %		
Baseline (0 hr)	100	100	100	100
1 hr	96.9 ± 5.0	97.3 ± 5.9	97.8 ± 5.3	98.1 ± 5.5
3 hr	94.9 ± 4.5	97.1 ± 4.6	96.5 ± 3.8	99.1 ± 5.0
8 hr	93.2 ± 4.3[a]	95.7 ± 3.9	93.5 ± 4.5[a]	98.4 ± 4.3
24 hr	91.9 ± 6.5[a]	92.5 ± 5.3[a]	91.9 ± 4.8[a]	93.9 ± 5.5[a]
		Mean ± SD *phi* recovery %		
Baseline (0 hr)	100	100	100	100
1 hr	105.1 ± 6.9	107.0 ± 6.7	113.3 ± 8.3[a]	104.0 ± 9.0
3 hr	110.2 ± 8.5[a]	108.4 ± 7.4	127.2 ± 16.8[a]	107.9 ± 8.7
8 hr	111.7 ± 8.7[a]	107.3 ± 8.1	151.9 ± 27.9[a]	114.0 ± 10.8[a]
24 hr	113.3 ± 8.6[a]	112.2 ± 8.0[a]	265.3 ± 144.5[a]	120.2 ± 19.0[a]

PSA, prostate-specific antigen; RT, room temperature.
[a] $P < 0.05$ compared with baseline.

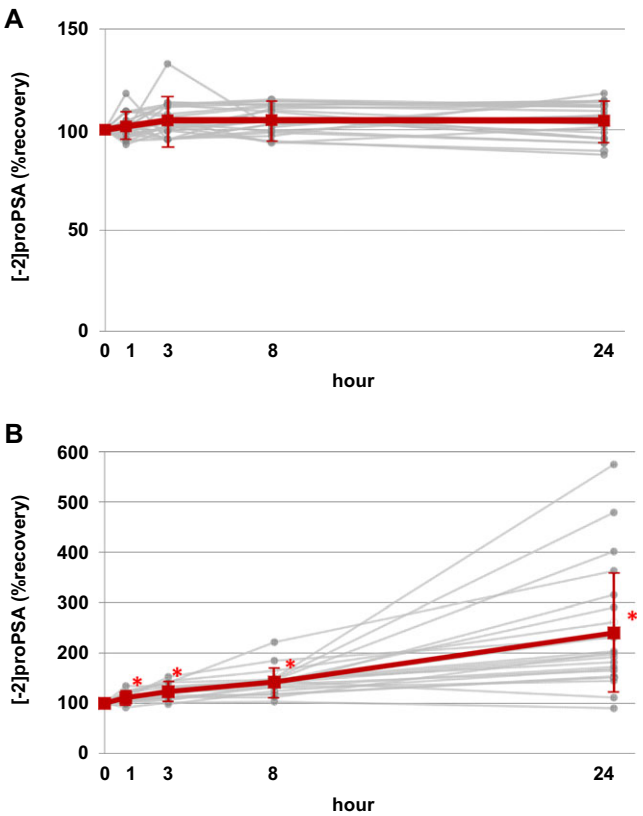

Fig. 1. Time course of percentage recovery of [−2]pro-PSA (where PSA is prostate-specific antigen) in serum (A) and whole blood (B) at room temperature. Gray lines indicate individual values, and the red line indicates mean ± SD. *$P < 0.05$ compared with baseline value.

the percentage recovery of fPSA decreased gradually with time. This tendency of fPSA to decrease was consistent with patterns described in previous reports (12, 13).

The *phi* value, which is used in an early PCa detection mathematical algorithm in Europe, was also evaluated. As shown in Table 2 and Figure 2, the changes in mean *phi* were similar to those in [−2]pro-PSA under each handling condition. The percentage change in *phi* in whole blood at RT increased rapidly over time. In contrast, the percentage change of *phi* in serum was relatively stable for 24 hr after drawing blood. The increase in the *phi* value in serum reached almost +10% by 3 hr after drawing of blood but was stable until 24 hr.

DISCUSSION

Preanalytical and analytical stability of a tumor marker is essential for accurate cancer diagnosis (14). PSA is a reliable and useful serum marker for PCa, but its clinical utility is limited by its moderate cancer specificity. Free PSA is usually used as a supplemental serum marker, which may improve the specificity of tPSA during the PCa detection process (15–17). Among the different forms of free PSA, [−2]pro-PSA is a cancer-specific pro-PSA that can serve as a new screening marker, particularly in the PSA range of 2–10 ng/ml. In this study, the [−2]pro-PSA preanalytical stability in serum was acceptable for at least 24 hr after drawing of blood in Japanese males, regardless of sample temperature (4°C or RT). Moreover, unlike fPSA, changes in percentage recovery of [−2]pro-PSA in

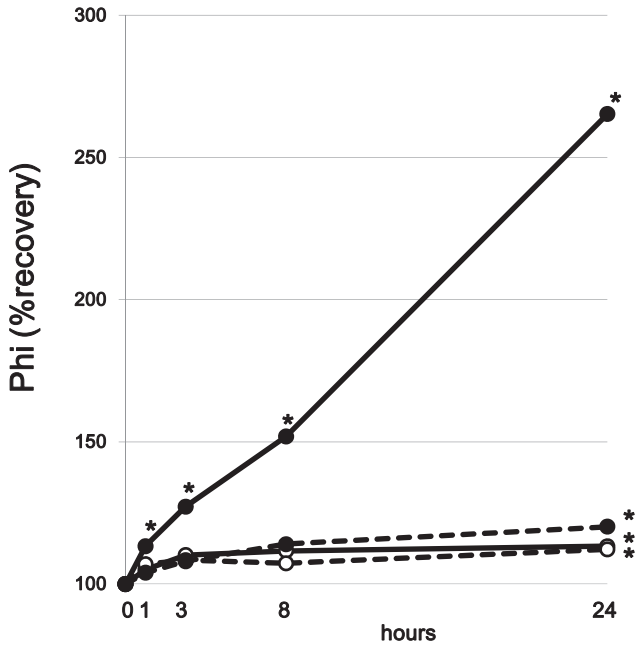

Fig. 2. Changes in Prostate Health Index (*phi*) over time in serum at 4°C (---○---) and at room temperature (—○—), and in whole blood at 4°C (---●---) and at room temperature (—●—). *$P < 0.05$ compared with baseline value.

whole blood increased significantly with time and temperature. This same trend in [−2]pro-PSA preanalytical stability was reported by Semjonow et al. in Caucasian males (11). Thus, this indicates that ethnic differences do not affect the preanalytical stability of [−2]pro-PSA. To our knowledge, this is the first report regarding [−2]pro-PSA preanalytical stability in Asian males.

Regarding [−2]pro-PSA increase in whole blood at RT, Semjonow suggested that the proteolytic activity of the blood, possibly mediated by human kallikrein-related or other endopeptidases, resulted in an increase in the [−2]pro-PSA value. Human kallikrein-related peptidases (hKs) consist of a single family of 15 highly conserved trypsin- or chymotrypsin-like serine proteases (18, 19). hK3 or PSA is the most widely known hK due to its clinical application. PSA and other hKs are synthesized as inactive pre–pro forms that are proteolytically processed to secreted inactive pro-forms. Then, pro-hKs are subsequently activated to mature forms by autocatalytic activity or by another hK or other endopeptidase. It has been demonstrated that hK2, hK4, hK5, and hK15 can cleave pro-PSA to the active form of PSA (19–21). Following activation, mature hK enzymes are inactivated by endogenous inhibitors, such as α2-macrogloblin, antichymotrypsin, or kallistatin. Interestingly, after internal cleavage and activation of pro-PSA by hK5, hK5 also deactivates this cleavage process upon prolonged incubation (22). These findings highlight the complexity of hK regulatory mechanisms, particularly with regard to pro-PSA in the human prostate or human serum. Specific enzymes serially cleave [−7]pro-PSA to the active form of PSA or to other truncated forms of pro-PSA, such as [−5]pro-PSA, [−4]pro-PSA, or [−2]pro-PSA. As hK2 and trypsin are unable to activate [−2]pro-PSA further, [−2]pro-PSA is the most stable form of pro-PSA in prostate tissue and serum (19). Thus, the [−2]pro-PSA molecule accumulates, and levels of [−2]pro-PSA eventually increase in whole blood. However, the difference in proteolytic activity (i.e., the hKs/endopeptidase content) between serum and whole blood is unknown. To ensure optimal clinical application of [−2]pro-PSA, the precise mechanism of [−2]pro-PSA alteration in blood specimens must be elucidated. The present study did not assess preanalytical stability at time points beyond 24 hr because clinical blood samples are nearly always processed within 24 hr after being drawn.

We also characterized changes in *phi*. It has been suggested that higher *phi* values are associated with an increased probability of PCa detection (7, 9). In fact, *phi* and percent [−2]pro-PSA were the strongest predictors of a positive prostate biopsy when compared with tPSA, fPSA, and percent free PSA. In the present study, changes in *phi* were similar to those of [−2]pro-PSA. Based on the results of [−2]pro-PSA or fPSA preanalytical stability, and on the *phi* calculation formula, the calculated *phi* results were most affected by the [−2]pro-PSA or fPSA values. As [−2]pro-PSA increased and fPSA decreased over time in the sample after the drawing of blood, the tendency of *phi* to increase increases with time. Thus, the *phi* was affected by blood sample handling conditions over the short term after drawing of blood. As the increase of *phi* in the serum specimen was almost constant, around +10%, until 24 hr after drawing blood, this change in *phi* in the serum specimen may be acceptable for clinical use. However, it should be noted that the results of *phi* in whole blood, particularly at RT, increased markedly with time. This may considerably affect clinical decision-making for PCa diagnosis. Blood sample handling conditions should be taken into consideration when assessing [−2]pro-PSA or *phi*. This may be the first report of changes of *phi* in clinical blood specimens under various handling conditions.

CONCLUSIONS

In summary, [−2]pro-PSA is relatively unstable in comparison to tPSA or fPSA. Whole-blood samples collected at RT should be centrifuged and separated into serum at least within 1 hr after drawing of blood to obtain accurate [−2]pro-PSA and *phi* results. Whole-blood samples can be kept at 4°C for up to 3 hr until serum separation. Once the serum is separated, the [−2]pro-PSA value is stable for at

least 24 hr. Moreover, as *phi* values tend to increase over time after drawing of blood, sample handling conditions should be taken into consideration when evaluating *phi* for PCa diagnosis.

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