Reproducibility and Accuracy of Measurements of Free and Total Prostate-Specific Antigen in Serum vs Plasma after Long-Term Storage at −20 °C

David Ulmert,1* Charlotte Becker,1 Jan-Åke Nilsson,5 Timo Piironen,2 Thomas Björk,3 Jonas Hugosson,4 Göran Berglund,5 and Hans Lilja1,6

Background: Long-term frozen storage may alter the results of prostate-specific antigen (PSA) measurements, mainly because of degradation of free PSA (fPSA) in vitro. We compared the effects of long-term storage on fPSA, total PSA (tPSA), and complexed PSA (cPSA) in serum vs EDTA-plasma samples.

Methods: We measured fPSA and tPSA concentrations in matched pairs of archival serum and EDTA-plasma samples (stored frozen at −20 °C for 20 years) from a large population-based cohort in Malmö, Sweden. We also compared concentrations in age-matched men with those in samples not subjected to long-term storage, obtained from participants in a population-based study of prostate cancer screening in Göteborg, Sweden. These contemporary samples were handled according to standardized preanalytical and analytical protocols aimed at minimizing in vitro degradation. tPSA and fPSA measurements were performed with a commercial assay (Prostatus Dual Assay; Perkin-Elmer Life Sciences).

Results: Concentrations of tPSA and fPSA and calculated cPSA (tPSA − fPSA) in archival plasma were not significantly different from those in contemporary serum from age-matched men. In archival serum, however, random variability of fPSA was higher vs plasma than in contemporary samples, whereas systematic error of fPSA analyses was similarly small in archival and contemporary serum and plasma.

Conclusions: Concentrations of tPSA and calculated cPSA were highly stable in plasma and serum samples subjected to long-term storage at −20 °C. Greater random variability, rather than a systematic decrease, may explain differences in fPSA analyses observed in archival serum.

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Prostate-specific antigen (PSA)7 is widely used as a serum marker for prostate cancer (PCa) (1). The secretory epithelium of the human prostate produces and releases high concentrations of PSA into seminal fluid (2). In healthy men younger than 50 years, small but measurable PSA concentrations (≤1 µg/L) occur in the circulation. Diseases of the prostate that affect tissue architecture and/or gland size (e.g., PCa and benign hyperplasia of the prostate) lead to increased concentrations of all forms of PSA in serum (3). Concentrations may increase as much as 10 000-fold in advanced stages of PCa or more moderately in early PCa and benign hyperplasia of the prostate. PSA in blood [total PSA (tPSA)] occurs as free PSA (fPSA) and complexed PSA (cPSA), which consists mainly of PSA bound to α1-antichymotrypsin (ACT) (4,5). In patients with PCa, the ratio of fPSA to tPSA (i.e., percent fPSA) is larger than in patients with benign prostate enlargements but no evidence of PCa (4,6). Thus, measurement of percent fPSA can be used to differentiate benign from advanced prostate cancer.
malignant prostatic disease, particularly in men with a moderately increased tPSA (e.g., 2–10 µg/L) (6–8).

One of the problems in studying the utility of PSA measurements is that PCa often progresses slowly over many years before detection (9). Because prospective screening studies take many years to finish, archival serum or plasma samples are often evaluated retrospectively as a complement. Retrospective analyses of PSA in archival samples are feasible, however, only if the different forms of PSA are sufficiently stable during long-term storage.

Stability studies performed in vitro have demonstrated that the immunoreactivities of purified fPSA and tPSA are stable for 8 months at storage temperatures of 4 °C or −20 °C (10). The stability in serum and plasma, however, may differ from that of purified protein. In one study of samples stored for 25 years at −20 °C, 38% of tPSA had deteriorated in serum thawed once before PSA was measured, and there was a preferential loss of PSA-ACT in archival samples (8), results that may be consistent with later findings of a decrease of cPSA and an increase of fPSA after thawing of serum samples stored for 2 years at −20 °C (11). On the other hand, a comparison of the stabilities of the different molecular forms of PSA in different sample media, i.e., serum, heparin-plasma, and EDTA-plasma (12), suggested that rates of decrease of fPSA are higher in serum than in anticoagulated plasma and that fPSA decay may be accelerated by higher temperatures (12), results largely consistent with data reported by Woodrum et al. (13).

The finding that different forms of PSA have different stability properties in vitro clearly has implications for the interpretation of data obtained from archival samples. We therefore compared the stabilities of the molecular forms of PSA in archival EDTA-plasma and serum with the stabilities in contemporary samples.

**Materials and Methods**

**SAMPLES**

Archival samples used in this study were from a population study, the Malmö Preventive Medicine Study, performed in Malmö, Sweden from 1974 to 1986. Both serum and EDTA-plasma samples were obtained from 22,439 men, and the median storage time was 20 years. Blood cells were separated immediately, and the samples were frozen at −20 °C and never thawed. By use of the Swedish National Cancer Registry, we found that up to December 31, 1999, a total of 586 men in the sample pool were diagnosed with incipient PCa. For our current study, we randomly selected 2 subsets of samples from the large cohort of men who were not registered with a diagnosis of PCa until that date: 125 matched pairs of plasma and serum samples for comparison of long-term storage effects and 524 plasma samples from participants (mean age, 55 years at the time of venipuncture) age-matched with controls from Göteborg from whom contemporary serum samples were obtained for PSA measurements.

Before analysis, all archival samples were thawed and then centrifuged for 5 min to prevent clotting.

The contemporary samples subjected to PSA measurements were obtained from the first round of the European Randomized Screening Study for Prostate Cancer (ERSPC), performed in Göteborg, Sweden in 1995 and 1996. Of 9972 randomly selected men, 5855 men 50 to 66 years of age underwent PSA measurement, and those with a tPSA concentration ≥3 µg/L were offered a transrectal ultrasound and sextant biopsy of the prostate. Serum was separated from blood cells, frozen at −20 °C within 3 h, and then stored at −20 °C for <2 weeks before analysis, which was performed <3 h after the samples were thawed. This procedure was followed rigorously to minimize the effects of preanalytical handling on the PSA forms (12). After samples were matched for age with the Malmö Preventive Study group (n = 524), PSA concentrations in sera from 1056 men (mean age, 55 years at the time of venipuncture) in the Göteborg screening study were available for comparison. To compare data from contemporary PSA measurements in matching pairs of serum vs plasma samples, we studied a subset of participants (n = 279) in the Göteborg screening study (with tPSA ≥3 µg/L but negative biopsies) who had both EDTA-anticoagulated plasma and serum drawn at the same time.

Men registered with a diagnosis of PCa were excluded from all evaluations.

**TIME-RESOLVED IMMUNOFLUOROMETRIC ASSAYS**

We measured fPSA and tPSA with a dual-label immunofluorometric assay (DELFIA Prostast™ PSA Free/Total PSA; Perkin-Elmer Life Sciences). This assay measures fPSA and cPSA in an equimolar fashion (14), and the cross-reaction for PSA-ACT in the PSA assay is <0.2% (10). The lower limits of detection are 0.05 µg/L for tPSA (CV = 5.0% at 2.32 µg/L) and 0.04 µg/L for fPSA (CV = 5.9% at 0.25 µg/L). For detection, the 1235 automatic immunoassay system from Perkin-Elmer Life Sciences was used. cPSA concentrations were calculated by subtracting fPSA from tPSA. The correlation coefficient (r) of the calculated cPSA and measured cPSA (ACS:180; Bayer Diagnostics) (15) was 0.99 when evaluated in archival plasma samples from 984 men.

**STATISTICAL ANALYSIS**

For both contemporary and archival samples, we used paired Student t-tests to evaluate PSA concentrations obtained from serum and plasma. We used unpaired Student t-tests to compare PSA concentrations in archival plasma samples and contemporary serum samples. Logarithmic transformation was used to obtain a gaussian distribution. Box plots were used to visualize distributions. Calculations of random error (i.e., random fluctuations in these measurements, which closely correspond to the mean of the intraindividual fluctuations as an absolute number), systematic error (i.e., constantly reproduced...
inaccuracy, which corresponds to one half of the mean difference between plasma and serum), and CV (i.e., random error/overall mean) were performed as recommended by Bland and Altman (16).

**Results**

**COMPARISON OF MEASURED PSA CONCENTRATIONS IN MATCHED PAIRS OF CONTEMPORARY SERUM VS PLASMA SAMPLES**

We used matched pairs of samples from 279 men in the Göteborg screening study to compare PSA concentrations in serum and EDTA-plasma. The random error, systematic error, and CV for fPSA were 4.3%, −0.8%, and 3.9%, respectively. The corresponding data for tPSA were 3.8%, −3.4%, and 5.7%. Thus, the random and systematic errors in plasma compared with serum were negligible, and CVs were small and in the same range as the assay imprecision. In addition, according to paired Student t-tests, the concentrations in contemporary serum samples were not significantly different from those in plasma [mean tPSA, 6.59 μg/L in serum vs 6.66 μg/L in plasma (P = 0.58); mean fPSA, 1.08 μg/L in serum vs 1.09 μg/L in plasma (P = 0.75)].

**COMPARISON OF PSA CONCENTRATIONS IN MATCHED PAIRS OF ARCHIVAL SERUM VS PLASMA SAMPLES**

We also compared PSA concentrations measured in matched pairs of archival serum and EDTA-plasma samples from the Malmö Preventive Medicine Study (n = 125). The random error, systematic error, and CV were 7.6%, −0.6%, and 27% for fPSA and 12%, −2.2%, and 12% for tPSA. Because of the measured range, the systematic error was again small but not negligible, whereas random error and CV were higher than expected from assay imprecision alone. As indicated by the box plots shown in Fig. 1A, in archival samples there was higher random variability in fPSA in serum than in plasma. According to paired Student t-tests, in the matched pairs of archival samples fPSA was significantly lower (P = 0.009) in serum (mean, 0.20 μg/L) than in plasma (mean, 0.22 μg/L) after a median storage time of 20 years at −20 °C (Fig. 1A). There was, however, no significant difference between serum and plasma for either tPSA or calculated cPSA (Fig. 1, B and C).

**COMPARISON OF PSA CONCENTRATIONS IN ARCHIVAL VS CONTEMPORARY SAMPLES**

We compared the PSA concentrations measured in 524 archival EDTA-plasma samples (stored for 20 years at −20 °C) with those obtained for 1056 contemporary age-matched serum samples from Göteborg (stored ≤2 weeks at −20 °C before analysis). Geometric means with 95% confidence intervals as well as the unpaired Student t-test (Table 1) demonstrated no statistically significant difference between archival EDTA-plasma vs contemporary serum for either tPSA (P = 0.88) or fPSA (P = 0.54). In addition, there was no significant difference for calculated cPSA (P = 0.47).

**Discussion**

Measurement of PSA in blood has well-established roles in diagnosis and monitoring of PCa (1). Analysis of tPSA is not, however, an ideal means of differentiating between early PCa and benign prostatic disease. Analysis of the different molecular forms of PSA (fPSA, tPSA, and cPSA) may improve this differentiation. Many studies on the utility of PSA forms have been performed on archival serum samples stored frozen for different time intervals. In vitro studies indicate that proper handling of blood is...
samples that had undergone long-term storage at
plasma for measurement of fPSA and tPSA in archival
temperature and, less rapidly, at 4 °C

cPSA
tPSA 0.95 (0.91–0.98) 0.95 (0.90–1.01)
cPSA\(^b\) 0.62 (0.58–0.66) 0.65 (0.60–0.68)

\(^a\) Geometric means were calculated individually for each PSA subform, which may explain why the sum of fPSA and cPSA is not entirely identical (although closely similar) to tPSA.

\(^b\) Calculated (tPSA – fPSA).

Table 1. Free, total, and complexed PSA measured in contemporary serum samples and archival plasma samples.

<table>
<thead>
<tr>
<th>PSA Type</th>
<th>Contemporary Serum (n = 1056)</th>
<th>Archival Plasma (n = 524)</th>
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<tbody>
<tr>
<td>fPSA</td>
<td>0.28 (0.27–0.29)</td>
<td>0.29 (0.27–0.30)</td>
</tr>
<tr>
<td>tPSA</td>
<td>0.95 (0.91–0.98)</td>
<td>0.95 (0.90–1.01)</td>
</tr>
<tr>
<td>cPSA(^b)</td>
<td>0.62 (0.58–0.66)</td>
<td>0.65 (0.60–0.68)</td>
</tr>
</tbody>
</table>

important to minimize degeneration of the different molecular forms. fPSA concentrations in freshly drawn sera have been shown to decrease during storage at room temperature and, less rapidly, at 4 °C (11). It has also been demonstrated that cPSA concentrations decrease and fPSA concentrations may increase in serum stored for 2 years at −20 °C (11).

We addressed the issue of whether serum is equivalent to plasma for measurement of fPSA and tPSA in archival samples that had undergone long-term storage at −20 °C. By comparing the data from archival samples with measurements of contemporary plasma vs serum samples, we found that serum and plasma were equivalent for assessment of fPSA and tPSA if samples had been handled according to a strict preanalytical protocol.

We also investigated whether long-term storage affects concentrations of tPSA and fPSA in serum and plasma by comparing PSA concentrations in 125 matched pairs of archival serum and plasma samples. In these archival samples, fPSA concentrations, but not tPSA or cPSA concentrations, were lower (P = 0.009) in serum than in plasma. According to the Bland–Altman evaluation results, however, fPSA had a CV >25%, which suggests much higher variability of fPSA measurements in the archival compared with contemporary samples (CV = 4%). The analyte concentration influences the CV, and a higher CV is expected at lower concentrations. This explanation does not account for the entire difference, however, because the fPSA assay has a CV no greater than 5% at the concentrations representative of the archival samples, whereas the random variability of fPSA was <5% in the contemporary samples. These data indicate that the variability (random error) in fPSA measurements was much lower in the contemporary than in the archival samples. These data also suggest, however, that the magnitude of differences observed in matched pairs of archival serum vs plasma may be smaller than would be expected from the previously reported data (8, 10–13). Distribution of fPSA over a wider range of concentrations in serum than in plasma in the archival samples (Fig. 1A) may illustrate the random fluctuation of fPSA in these samples. The magnitude of systematic error in the archival samples was small (−0.6%), but it did indeed correspond to the difference in median concentrations in plasma vs serum (0.22 vs 0.20 µg/L). The systematic error was quite similar in magnitude in the matched pairs of contemporary plasma and serum samples, but because fPSA was ~5-fold higher (~1 µg/L) in this biopsied subset of a screening study cohort, the systematic error was much too small to give a significant difference in the paired t-test. These findings correspond to other reports that fPSA is less stable than PSA-ACT (12), but they differ from the report by Stenman et al. (8) of a lack of tPSA and PSA-ACT stability. We thus conclude that for measurements of tPSA only, serum or plasma may be used, but when fPSA measurements are required, such as when percent fPSA or cPSA are to be calculated, plasma should be used. The cause of the decrease in or instability of fPSA in serum is not known and is likely of a complex character. Contributing variables could include the release of proteolytic enzymes during blood clotting and the formation of complexes between fPSA and α-2-macroglobulin (17). Because of the large proportional difference in the amounts of cPSA vs fPSA, these changes are likely to have only a minimal effect on the concentrations or clinical interpretation of fPSA results, provided that long-term storage of serum has been performed under conditions similar to those reported here.

We performed all analyses with the same assay and internal controls for each assay run. During the entire study period, the total CV was regularly verified to closely adhere to previously cited values. However, subtle changes attributable to calibration shifts cannot be entirely excluded because the Malmö Preventive Medicine Study samples were analyzed during 1 month with a single batch of PSA reagents, whereas the ERSPC screening study measurements were performed 5 years earlier with other batches of the commercial PSA reagents. Our conclusions are based on the findings that in the contemporary samples concentrations of all PSA forms in plasma were identical to those measured in the matched serum pairs. Furthermore, our comparison of PSA concentrations measured in archival plasma vs contemporary serum from age-matched men showed no differences for fPSA or tPSA. However, in archival samples, comparison of fPSA concentrations measured in matched pairs of serum vs plasma indicated that fPSA concentrations were lower in the archival serum samples. Because fPSA constitutes a small percentage of tPSA, the significant, but small, loss of fPSA did not impact tPSA concentrations.

Our conclusions are based on comparisons of 3 different sets of matched samples from population-based cohorts of similar ethnicity: (a) matched pairs of contemporary plasma vs serum; (b) matched pairs of archival plasma vs serum; and (c) archival plasma vs contemporary serum from age-matched samples. Our current data do not provide any ultimate guidelines, but in combination with previously reported data, they confirm that in archival samples subjected to long-term storage, fPSA mea-
measurements in serum are (somewhat) less reliable than in plasma. The comparison between archival serum vs plasma does not account for a significant delay in measuring serum or plasma (several hours) after thawing. After thawing, loss of fPSA in archival serum occurs at approximately twice the rate in archival plasma (data not shown), which is highly compatible with the previously reported findings (11, 12).

We performed the comparison of the preventive medicine cohort in Malmö vs the PCs screening study cohort in Göteborg with the utmost care to minimize putative differences. Furthermore, to select study cohorts that were comparable and not confounded by men with PCs, men from both study cohorts with a diagnosis of PCs were not eligible to be included in any subsets of samples selected for this investigation. Study data suggest, however, that ≤10% of cancer cases may be considered clinically insignificant (18); therefore, some men with small but aggressive undetected PCs may have been included in our comparison.

We found that both tPSA and fPSA in plasma subjected to long-term storage were comparable to contemporary analyzed samples despite the possible slight difference in exclusion of men with PCs. We therefore conclude that collection of anticoagulated plasma may be sufficient to enable valid evaluation of all major forms of PSA in samples stored for a median of 20 years at −20 °C and that plasma samples from our material stored for a long term in the Malmö Preventive Medicine Study are therefore suitable for use in future studies on PCs.

In conclusion, these studies demonstrate that it is possible to perform reliable analyses on archival plasma samples stored for more than 2 decades at −20 °C. Because of a statistically significant loss and above all a higher variability of fPSA in serum after long-term storage, plasma is recommended for prospective studies and is optimal for retrospective studies.

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References