MS-Based Monitoring of Proteolytic Decay of Synthetic Reporter Peptides for Quality Control of Plasma and Serum Specimens

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ABSTRACT

Objectives: To determine the preanalytical quality of serum and plasma by monitoring the time-dependent ex vivo decay of a synthetic reporter peptide (RP) with liquid chromatography/mass spectrometry (LC/MS).

Methods: Serum and plasma specimens were spiked with the RP and proteolytic fragments were monitored with LC/MS at different preanalytical time points ranging from 2 to 24 hours after blood withdrawal.

Results: The concentration of fragments changed in a time-dependent manner, and respective peptide profiles were used to classify specimens according to their preanalytical time span. Classification accuracy was high, with values always above 0.89 for areas under receiver operating characteristic curves.

Conclusions: This “proteomics degradation clock” can be used to estimate the preanalytical quality of serum and plasma and might have impact on quality control procedures of biobanking repositories.

The discovery and validation of biomarkers for diagnosis, therapeutic monitoring, and prognosis of many diseases is an important goal of modern biomedical research. However, in complex biological samples such as serum and plasma, the preanalytical variability in sample handling causes substantial in vitro changes of protein and peptide content or enzyme activities.1,2 These modifications not only affect in vitro diagnostic tests3 but also have a major impact on multiparametric “-omics” approaches and can override disease-related patterns4 or even abolish meaningful data interpretation.5,6

Unfortunately, the preanalytical quality of serum and plasma specimens that is related to sample handling can hardly be measured or quantified. For most possible analytes, quality control (QC) tools are not yet available and correspond to the “QC gap.” Up to now, only a few degradation markers—namely, fibrinopeptide A,2,7 protein S, MMP7, MMP9, and CD40L8—have been identified. However, the concentration of these decay markers is related to not only the preanalytical time span but also several disease states, making the monitoring of a time-dependent decay process more difficult. A proteomics approach to systematically identify and thoroughly validate further decay markers that could serve as a QC tool is still missing.

Here, we propose the addition of a synthetic reporter peptide (RP) to blood specimens and subsequent relative quantification of proteolytically derived fragments for monitoring of the preanalytical time span. Blood has inherent proteolytic activity that is related to various endoproteases (eg, from protease cascades of coagulation, fibrinolysis, and the complement activation)9 as well as diverse exoproteases.10 Consequently, proteins and peptides are continuously processed in a time-dependent manner, causing “ladder-like”
degradation patterns. We hypothesized that the mass spectrometry (MS)–based monitoring of the proteolytic processing of the spiked RP might function as a proteomic degradation clock to estimate the preanalytical quality of blood specimens.

The synthetic RP consists of an amino (N)–terminal octapeptide with sequence homology to the complement factor C4 (P0C0L4). The carboxyl (C)–terminal mass tag comprises aminohexanoic acid as a linker and d–amino acids that are protease resistant to ensure signal accumulation of the so-called anchor peptide (AP) during prolonged incubation.12 The RP was added to blood collection tubes, and proteolytic decay was initiated during blood withdrawal. The proteolytic cleavage of the RP resulted in continuous and time-dependent elimination of amino acids from the N-terminus of the reporter peptide. Serum and plasma specimens were incubated together with the RP at room temperature for different time points ranging from 2 to 27 hours after blood withdrawal. An intermediate fragment with a reduced length of 4 amino acids (RP-4) and the protease-resistant AP were quantified with liquid chromatography (LC)/MS. The quotient AP/RP-4 constantly increased with a prolonged preanalytical time span. However, the proteolytic activity of blood specimens is altered during the course of various diseases. Specifically, malignant disease can be associated with increased proteolytic activity of distinct proteases (for review, see Findeisen and Neumaier13). Ideally, the RP is processed by “housekeeping” proteases in a strictly time-dependent manner. However, various proteases are upregulated in the serum of patients with malignant disease. To analyze the impact of malignant disease on the processing of the RP, we analyzed the blood specimens from healthy controls (n = 15) and patients with late-stage gastrointestinal tumors (n = 15). Furthermore, the proteolytic activity of serum is generally higher when compared with plasma. To investigate the impact of an activated coagulation cascade on the processing of the RP, we analyzed the EDTA plasma in parallel. With this approach, a highly accurate classification of specimens according to the respective preanalytical time span was possible.

To avoid bias in proteomics biomarker discovery trials, it is crucial to monitor the preanalytical status of clinical specimens, and consequently, appropriate QC analyses should be introduced for future studies.

Materials and Methods

Materials and Chemicals

The reporter peptide LSQKPRL-Ahx-ateelkal Table II comprises an amino acid sequence that is derived from complement factor C4 (P0C0L4). The RP was synthesized in the functional genome analysis laboratory of the German Cancer Research Centre (Heidelberg, Germany). High-performance liquid chromatography (HPLC)–grade acetonitrile was purchased from Fisher Chemicals (Schwerte, Germany). Formic acid and trichloroacetic acid (TCA) were purchased from Sigma (Seelze, Germany). All reagents and chemicals were at least of analytical grade.

Serum and Plasma Samples

Whole-blood specimens were taken from patients with late-stage gastrointestinal tumors (n = 15) and healthy controls (n = 15) at the University Hospital Mannheim. Blood collection was performed after we obtained institutional review board approval from the Medical Faculty Mannheim of the University of Heidelberg and patients’ written informed consent. The RP was added to serum and EDTA tubes (Sarstedt, Nürnberg-Rommlersdorf, Germany) prior to blood collection (see below). The specimens were kept at room temperature for 30 minutes prior to centrifugation at 20 °C for 10 minutes at 3,000g. The specimens were further kept at room temperature, and 50-µL aliquots were taken 2, 5, 8, and 24 hours after blood withdrawal and stored at −80 °C until further use. Any handling and processing of serum specimens from tumor patients and controls was performed in a strictly randomized and blinded manner. We further elucidated the effects of freeze/thaw cycles on RP processing. After blood draw, specimens that contained 10 µmol/L RP were stored at room temperature for 1 hour prior to freezing. Frozen specimens were kept at −80 °C for 60 hours. Then, samples were thawed and stored at room temperature for another 1, 3, 7, and 26 hours. In parallel, serum specimens spiked with 10 µmol/L RP were incubated for 2, 4, 8, and 27 hours continuously at room temperature. After incubation, all specimens were stored at −80 °C prior to LC/MS analysis.

Sample Preparation and Peptide Extraction

The lyophilized RP was dissolved in dimethyl sulfoxide to a concentration of 8 mmol/L. This stock was diluted with phosphate-buffered saline to 500 µmol/L. Then, 25 µL of this
stock solution was added to 1.2-mL serum tubes (Sarstedt) and 54 µL to 2.6-mL EDTA-plasma tubes (Sarstedt). The complete filling of tubes with blood resulted in a final concentration of 10 µmol/L for the RP. To investigate the impact of varying tube filling, we additionally prepared serum specimens from 4 healthy individuals with a final RP concentration of 5, 10, and 20 µmol/L for a 2-, 4-, 8-, and 27-hour incubation. After blood withdrawal, the serum and plasma were processed as described above and stored at –80°C. For deproteinization, the samples were thawed and 50 µL of cold 10% (v/v) TCA was added. The resulting mixture was kept at 4°C for 30 minutes prior to centrifugation for 15 minutes at 4°C and 12,000 rpm in a microcentrifuge (Eppendorf, Wesseling-Berzdorf, Germany). The supernatant was again centrifuged for 5 minutes at 4°C and 12,000 rpm, and 1.5 µL of the supernatant was injected into the HPLC column.

LC/MS Analysis

LC/MS was performed using a nano-HPLC system (RSLCNano; Dionex, Idstein, Germany) coupled to a linear ion trap–Orbitrap hybrid mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher Scientific, Bremen, Germany) with a chip interface (TriVersa NanoMate; Advion, Ithaca, NY). Analytical chromatography was performed on a 75-µm ID C-18 column (Dionex) with a flow of 300 nL/min and a gradient from 3% to 60% of buffer B in 37 minutes. The composition of buffer A was water with 0.1% formic acid, and buffer B was 80% acetonitrile with 0.1% formic acid. Each LC run was preceded by a blank run, ensuring lack of carryover of the material from the previous run. MS analysis was performed in positive ion mode, with a mass range of 340 to 1,700 m/z. Tandem MS (MS/MS) analyses were performed data dependently for sequence confirmation. Qualitative (LC/MS/MS for verification of peptide sequence) and quantitative data sets (profile-type LC/MS for peak amplitude data) were collected in the same runs. Peak areas were extracted for the peptides of interest (AP and RP-4) from measurements of the individual specimens using the Xcalibur software (Thermo Fisher Scientific). The AP/RP-4 ratio is the quotient of the peak areas of these 2 peptides at a given time point.

Reproducibility of RP Spiking

To monitor the reproducibility of RP spiking, we spiked a QC sample with RP and incubated it for 2 hours and 24 hours. Aliquots were stored at –80°C until further use. Each QC specimen was processed 5 times and median, standard deviation, and coefficient of variation (CV) were calculated with Excel (Microsoft, Redmond, WA).

Statistics

The Wilcoxon rank sum test and area under the receiver operating characteristic (AUROC) calculations were performed with MedCalc (MedCalc Software, Ostend, Belgium). P values below .05 were assumed to be statistically significant.

Results

Relative Quantification

The proteolytic processing of the RP leads to a ladder-like degradation and formation of various fragments (Table 1). The peak areas of RP-4 and AP were determined for each time point, and the quotients of AP/RP-4 were calculated respectively. Figure 1 shows an exemplary LC/MS result with determination of the respective peak area on the basis of an extracted ion chromatogram. Even at a low concentration of 40 nmol/L, the extracted ion chromatogram of AP shows only 1 peak (Figure 1A), and this excellent signal-to-noise ratio makes quantitative LC/MS analyses highly specific. The quantification of RP-4 with LC/MS could also be performed unambiguously in Figure 2. In addition, the proteolytic fragments AP and RP-4 were confirmed by LC/MS/MS analyses in Figure 3.

The linearity of LC/MS quantification was determined with 5 different concentrations of AP (0.08, 0.4, 2, 10, and 50 µmol/L). Linear regression was calculated for medians of triplicate measurements, and the coefficient of determination (R²) was 0.98 (data not shown). The reproducibility of RP spiking was determined by independent processing of 6 serum aliquots from 1 healthy donor that were incubated for 2 and 24 hours. Peak areas of AP and RP-4 were extracted from LC/MS measurements and the respective quotients were calculated in Table 2. The 2 time points were chosen to investigate the reproducibility of AP and RP-4 at different concentrations (low/high). In no case did the CVs exceed values of 15%, indicating a good reproducibility of the method.

Furthermore, the impact of varying RP concentrations caused by varying tube filling with blood during withdrawal was analyzed. Specimens with various final concentrations of RP (5, 10, and 20 µmol/L) were analyzed as described in the Materials and Methods. The quotients of AP/RP-4 for RP concentrations of 5 and 20 µmol/L were not significantly different from those with 10 µmol/L, which was chosen as the standard concentration in Figure 4. This demonstrates that the quotient of AP/RP-4 is stable in a broad concentration range of RP, and accordingly, variations in draw volume do not substantially affect the final results.

Finally, we examined the impact of a freeze/thaw cycle on RP processing. Serum specimens were spiked with 10 µmol/L RP and incubated for 2, 4, 8, and 27 hours either without or with an additional freeze/thaw cycle as described in the Materials and Methods. The quotients of AP/RP-4 for specimens with an intermediate freeze/thaw cycle were not different from those specimens that were continuously kept...
at room temperature for 2, 4, 8, and 27 hours, as shown in Figure 4. This indicates that the proteolytic activity toward the RP is conserved after 1 freeze/thaw cycle and the “proteomics degradation clock” is proceeding.

Kinetics of RP Decay

The RP was added to blood specimens of 15 healthy control individuals, and time-dependent proteolytic processing was monitored with LC/MS. Peak areas of AP and RP-4 were extracted from the LC/MS data and quotients of AP/RP-4 were calculated accordingly. As shown in Figure 5, the concentration of AP is constantly increasing with prolonged incubation time, whereas the concentration of the intermediate fragment RP-4 is decreasing. Accordingly, the quotient of AP/RP-4 in serum is exponentially increasing with a prolonged incubation time. The quotients in serum had median values of 5.9, 21.7, 74.7, and 663.3 for respective time points of 2, 5, 8, and 24 hours. The quotients in EDTA plasma were lower, with values of only 1.1, 5.3, 20.9, and 240.5 for the respective incubation times Table 3. This indicates a decreased proteolytic activity in these specimens, and EDTA is a well-known inhibitor for various metalloproteases, including aminopeptidases.14

Impact of Disease Status on RP Decay

Assessing the preanalytical quality of blood specimens with an exogenous decay marker is unambiguously possible if the processing of the RP is strictly related to the time of incubation. However, the proteolytic activity in blood specimens might be altered during the course of various diseases, including cancer.15 To test for disease-related impact on the proteolytic processing of the decay marker, we compared the quotient of AP/RP-4 from healthy controls (n = 15) and tumor patients (n = 15) at different time points. The Wilcoxon rank sum test showed no statistically significant differences for 5 of 8 possible comparisons (Table 3). In contrast, 3 P values were below .05, indicating statistically significant differences.
Furthermore, the standard deviations of the quotient (AP/RP-4) in specimens from tumor patients are higher when compared with healthy controls, and this might also affect the classification accuracy. Despite this interference factor, the classification accuracy was high, with AUROC values always above 0.89 (see below). This indicates that disease-related bias in the processing of the RP is minor compared with the preanalytical impact of sample handling.

Classification

The quotients (AP/RP-4) were calculated from serum and plasma for any individual time point (2, 5, 8, and 24 hours). The box-and-whisker diagrams of Figure 6 comprise values from 30 specimens (15 from tumor patients and 15 from healthy controls) for each time point. As already shown in Figure 5, the medians of the quotients (AP/RP-4) are constantly increasing over the time. For serum and EDTA plasma, the boxes of any respective time point that comprise 50% of the data (2 quartiles) have no overlap (Figure 6). However, the whiskers of proximate time points have a distinct overlap that limits the classification accuracy. AUROC calculations were used to estimate the quality of prediction for respective time points. The lowest AUROC value of 0.89 was seen for the differentiation of EDTA plasma with preanalytical time spans of 2 and 5 hours, respectively. The classification of other proximate time points ranged between 0.90 and 0.99. The AUROC values for classification of distant time points were 1.00 Table 4 and Figure 7.

Discussion

Proteomics approaches are powerful tools for biomarker discovery and thereby can improve early detection, staging, therapeutic monitoring, and prognosis of various diseases, including cancer. Despite much progress in the field, the introduction of new biomarkers for routine diagnostic
applications is rare. In contrast, the number of possible biomarker candidates from a multitude of proteomic profiling studies is rather high. However, most of these biomarkers could not be validated independently and consequently had to be classified as false positives. Most important, the preanalytical bias in sample handling has been identified as one of the most serious threats to profiling experiments. Specifically, MS-based proteomic profiling approaches are prone to preanalytical interference factors that profoundly affect the peptide profiles of blood specimens. These preanalytical variabilities comprise blood collection, transport, and storage. A summary of the reproducibility of the anchor peptide and intermediate fragment over different incubation times is provided in Table 2.

**Table 2** Reproducibility

| Incubation Time, h | Peptide | Median (SD) | CV, % |
|-------------------|---------|-------------|-------|
| 2                 | AP      | 6.26E+07 (1.85E+06) | 3.0 |
| 24                | AP      | 2.02E+08 (2.27E+07) | 11.2 |
| 2                 | RP-4    | 4.68E+07 (4.06E+06) | 8.7 |
| 24                | RP-4    | 7.05E+05 (1.03E+05) | 14.6 |
| 2                 | Q       | 1.4 (0.1) | 7.5 |
| 24                | Q       | 291.2 (11.8) | 4.0 |

*The reporter peptide (RP) was spiked in 6 aliquots of 1 serum specimen and incubated for 2 and 24 hours. Peak areas of the anchor peptide (AP) and intermediate fragment (RP-4) were extracted from the liquid chromatography/mass spectrometry data, and SD and coefficients of variation (CV) were calculated. Q is the quotient of AP/RP-4.*

**Figure 3** Amino acid sequence confirmation. Print screen of the tandem mass spectrometry spectra decoding of (A) anchor peptide m/z 494.29 and (B) RP-4 m/z 741.46 that was performed with PEAKS studio software version 5.2 (Bioinformatics Solutions, Waterloo, Canada). The synthetic amino acid Ahx could not be handled by the software and instead is displayed as lysine (L), which is an isomer of Ahx and thus produces a fragment with the same mass.
centrifugation, and storage and can hardly be controlled and standardized under routine laboratory conditions. More impeding, there are only limited solutions for the assessment of a given sample regarding its quality that is related to preanalytical variabilities. Consequently, it is important to evaluate tools for the assessment of sample quality. Specifically, biobank repositories are facing this problem when specimens with comparable preanalytical “history” have to be identified that can be analyzed in comparative proteomics approaches without any bias.

Serum and plasma have high intrinsic proteolytic activity that is shaping the peptidic profiles of these specimens in a time-dependent manner. For few high-abundance plasma proteins such as fibrinopeptide A and inter-α-trypsin inhibitor, the respective preanalytical variabilities have been

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**Table 3**

### Disease-Related Processing of RP

| Individuals | Incubation Time, h | Q (AP/RP-4), Median (SD) | P Value
|-------------|------------------|--------------------------|-------|
| Serum       |                  |                          |       |
| HC          | 2                | 5.9 (1.8)                | .01b  |
| TU          | 5                | 21.7 (11.8)              | .71   |
| HC          | 8                | 17.5 (22.1)              | .77   |
| TU          | 24               | 44.8 (172.6)             |       |
| EDTA plasma |                  |                          |       |
| HC          | 2                | 1.1 (0.2)                | .63   |
| TU          | 5                | 5.3 (2.8)                | .04b  |
| HC          | 8                | 20.9 (19.1)              | .68   |
| TU          | 24               | 240.5 (81.1)             | .18   |
| AP, anchor peptide; HC, healthy controls (n = 15); Q, quotient calculated from peak areas of AP/RP-4; RP, reporter peptide; TU, tumor patients (n = 15).

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**Table 4**

### Sixteen-Field Matrix of AUROC Values

| Time, h | 2 h | 5 h | 8 h | 24 h |
|---------|-----|-----|-----|------|
| AUROC serum |     |     |     |      |
| 2       | —   | 0.98| 1.00| 1.00 |
| 5       | 0.98| —   | 0.90| 1.00 |
| 8       | 1.00| 0.90| —   | 0.99 |
| 24      | 1.00| 1.00| 0.99| —    |
| AUROC EDTA |   |     |     |      |
| 2       | —   | 0.89| 1.00| 1.00 |
| 5       | 0.89| —   | 0.92| 1.00 |
| 8       | 1.00| 0.92| —   | 0.98 |
| 24      | 1.00| 1.00| 0.98| —    |

AUROC, area under the receiver operating characteristic curve.

a Quotient (anchor peptide/reporter peptide 4) was used for classification of specimens with different preanalytical time spans.

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This approach requires 4 main prerequisites. (1) The peptidic fragments of the synthetic RP must be protected from complete proteolytic processing by genuine serum proteases to ensure signal accumulation. (2) An excess of synthetic RP is necessary to ensure signal accumulation over a prolonged incubation time. (3) The proteases that participate in trimming down the synthetic RP should have housekeeping qualities with minimal interindividual variance and independence from various disease states. (4) Serum and plasma are highly complex specimens, and sample preparation prior to LC/MS analyses should be simple to ensure good reproducibility. Here we present an approach that mainly fulfills all 4 of these requirements. (1) Multiple aminopeptidases are present in blood and are involved in the metabolism of proteins and various peptide hormones. The successive proteolytic processing of the RP and its consecutive intermediate fragments results in the accumulation of a protease-resistant C-terminus depicted as the AP. This AP consists of the linker amino-hexanoic acid and d-amino acids that are not proteolytically processed. (2) The RP is spiked into serum specimens in a final concentration of 10.5 μmol/L. This is comparable to the concentration range of fibrinopeptide A, which is one quantified. However, the concentration of plasma proteins that serve as substrates for proteases can markedly vary during the course of various diseases (eg, cancer and systemic inflammation). Furthermore, the polymorphism of serum proteins is an additional source of sample heterogeneity that makes standardization of endogenous protein decay difficult. Consequently, the addition of synthetic reporter peptides has been proposed to increase standardization of proteolytic reactions in serum specimens.

We hypothesized that the monitoring of the preanalytical time span can be realized by relative quantification of proteolytically derived fragments of a synthetic RP that are generated in serum and plasma specimens in a time-dependent manner.

Figure 6 Classification of serum and plasma according to the preanalytical time span. The distribution of the quotient (anchor peptide/intermediate fragment [RP-4]) in (A) serum and (B) EDTA plasma from 30 individuals (15 healthy controls and 15 tumor patients) is shown for respective incubation times. The line inside each box represents the median; the limits of each box represent the 25th and 75th percentiles. The whiskers represent the minimum and maximum values, excluding outside (open circles) and far-out (solid squares) values, which are displayed separately. The brackets indicate classification accuracy of proximate time points expressed as area under the receiver operating characteristic values (see Table 4).

Figure 7 Receiver operating characteristic (ROC) curves for comparison of serum specimens with different preanalytical time spans. The area under the ROC (AUROC) values are displayed in parentheses. Comparisons of proximate time points (2 vs 5 hours, 5 vs 8 hours, and 8 vs 24 hours) have AUROC values less than 1.00. Comparisons of distant time points (2 vs 8 hours, 2 vs 24 hours, and 5 vs 24 hours) have AUROC values of 1.00.
of the most abundant peptides in serum.\(^27\) With prolonged incubation, the velocity of product accumulation slows, and the kinetics of the protease reaction changes from zero order to first order. However, the quotient AP/RP-4 shows an exponential increase with prolonged incubation (see Figure 5). (3) The disease state has only a minor influence on the proteolytic processing of the RP when tumor patients and healthy controls are compared. Different aminopeptidases have a closely related enzymatic activity with broad overlap in substrate specificities, and natural as well as synthetic substrates can be hydrolyzed by different enzymes.\(^26\) The pleiotropic activity of aminopeptidases might be the reason for minor effects of the disease state on the time course–related processing of RP. Most interesting, RP is even cleaved in EDTA plasma but with clearly diminished turnover. Many aminopeptidases are zinc metalloenzymes, and their activity is inhibited by EDTA.\(^1\) However, Figure 5 shows highly parallel curves with nearly the same slope, indicating that the inhibition of metalloenzymes is compensated to a certain extent. (4) A sample cleanup with TCA has recently been described for effective recovery of peptides from complex matrices such as serum or plasma. Specifically, peptides with a molecular weight of less than 3,000 Da are concentrated in the supernatant, whereas abundant proteins with high molecular weight are precipitated. This leads to a dramatic reduction of the complexity of specimens prior to further LC/MS analyses.\(^28\) Accordingly, the detection of AP and RP-4 can be performed unambiguously in combination with high-accuracy mass spectrometry.

In conclusion, the preanalytical variability has been recognized as a major interference factor in laboratory testing,\(^29\) and there is increasing awareness that research studies for biomarker identification also are critically affected.\(^30,31\) Consequently, analytical methods are urgently needed to confirm or deny equivalent quality of biobanking specimens before their use in a given experiment.\(^21\) For this purpose, we suggest the addition of an exogenous RP for monitoring the preanalytical quality of serum and plasma specimens.

However, the results of our proof-of-concept experiment have to be validated prospectively by including other disease states and more patients. Furthermore, a broader pattern of preanalytical variability that includes different long-term storage conditions\(^32\) and freeze/thaw cycles\(^33\) should be investigated systematically. The monitoring of an external decay marker can be useful for assessing sample quality and therefore might be a valuable tool for biobanks to facilitate biomarker discovery.

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