Evaluation of Protease Inhibitors Containing Tubes for MS-Based Plasma Peptide Profiling Studies

Vanessa Pérez,^{1,2*} Javier Juega-Mariño,¹ Anna Bonjoch,³ Eugenia Negrodo,³ Bonaventura Clotet,³ Ramón Romero,^{1,2} and Josep Bonet^{1,2}

¹Department of Nephrology, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

²Laboratory of Experimental Nephrology, Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

³HIV Unit & Fundació Lluïta contra la SIDA, Hospital Universitari Germans Trias i Pujol, Badalona, Spain

Background: Peptide profiling of biological fluids is a promising tool for biomarker discovery. Blood is an ideal entity for proteomic studies but it is subjected to a proteolytic activity that sets up just at the moment of phlebotomy. Intending to prevent this proteolytic activity, tubes containing protease inhibitors (PI) have been developed. In this study, we evaluated the effect on plasma peptide profile of using tubes containing PI and the evolution of this effect over time. **Methods:** Blood samples from ten subjects were drawn into conventional tubes containing ethylenediaminetetraacetic acid (EDTA) and tubes containing PI. Samples were processed at time “zero” and after 1, 2, 4, and 8 hr. Plasma peptide profiles were analyzed by magnetic bead based technology coupled to matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry readout. **Results:** When comparing plasma peptide profile of blood samples collected into tubes containing PI with samples collected into conventional EDTA tubes, differences in the area of 13 peaks were detected at time “zero”; after 8 hr these differences tended to disappear. Moreover, bradykinin and C3- and C4-derived peptides were produced promptly after blood extraction when samples were collected into conventional EDTA tubes, and the use of PI prevented their generation. **Conclusion:** Considering that time taken to process blood samples affects their peptide profile and a decrease in PI's effect occurs over time, it may be concluded that the use of tubes containing PI for blood collection may be advantageous in the context of research, but may have some limitations regarding clinical practice. *J. Clin. Lab. Anal.* 28:364–367, 2014. © 2014 Wiley Periodicals, Inc.

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Advances in the field of proteomics have promoted a growing number of clinical studies with the exciting challenge of discovering new biomarkers for diagnosis and prognosis of human diseases (1). A magnetic bead based fractionation procedure coupled to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) readout has been successfully used to profile the low molecular weight proteome of different body fluids in a rapid, reproducible, and highly automatable way (2–11). The fact that almost every cell of the organism sheds products into blood, leaving a record of its physiological state, makes blood an attractive source for biomarker discovery (12). Moreover, blood collection is minimally invasive, of low risk, and cheap (13). The Human Proteome Organization (HUPO)

in the collaborative Human Plasma Proteome Project recommended the use of plasma instead of serum for

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Author responsible for reprints: Josep Bonet, Department of Nephrology, Hospital Universitari Germans Trias i Pujol, Carretera del Canyet, 08916 Badalona, Spain. E-mail: jbonet.germanstrias@gencat.cat
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*Correspondence to: Vanessa Pérez, Laboratory of Experimental Nephrology, Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Carretera de Can Ruti, Camí de les Escoles, 08916 Badalona, Spain. E-mail: vperez@igtp.cat

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peptidomic studies; explicitly, EDTA plasma was proposed as the preferred specimen from human blood (14,15).

EDTA inhibits blood coagulation but does not prevent the activation of the first enzymatic steps of the cascade, with the consequent *ex vivo* generation of peptides. To avoid protease activity during and after blood collection, tubes containing protease inhibitors (PI) have been designed, which stabilize the blood proteome at time “zero,” that is, 15 min after blood extraction (14). This report aims (a) to evaluate the intrinsic protease activity in plasma samples by monitoring changes in peptide profile over time and (b) test the effect of using blood collection tubes containing PI and the evolution of this effect over time.

This prospective study included a training group of ten male subjects (47 ± 10 years) and two validation groups, group A with five subjects (38 ± 6 years, 5 men) and group B with 27 subjects (52 ± 9 years, 23 men). All subjects were older than 18 years, with normal renal function and without proteinuria. Local Ethical Committee approved the study protocol and all subjects gave their written informed consent to participate.

Two blood samples were collected from all subjects, one into a conventional EDTA tube (Becton Dickinson, Franklin Lakes, NJ) and the other into a BD P100 tube, that is, an EDTA tube containing PI (Becton Dickinson). Samples obtained from subjects of the training group were centrifuged immediately after phlebotomy at $2,500 \times g$ for 20 min at room temperature; plasma was aliquoted at time “zero” and after 1, 2, 4, and 8 hr. Blood samples from subjects of the validation groups A and B were centrifuged at time “zero” and 4 hr after phlebotomy, respectively. Aliquots were frozen at -80°C until use.

Samples were prefractionated, in duplicate, with IMAC-Cu magnetic beads (Bruker Daltonics, Bremen, Germany) according to the manufacturer’s protocol (7). Eluates were diluted 1:5 with liquid chromatography (LC) grade water and mixed 1:2 with matrix solution (1.84 mg/ml 2,6-dihydroxyacetophenone, 20% acetonitrile, 40 mmol/l ammonium citrate dibasic). One microliter of the mixture was spotted, in duplicate, onto an AnchorChip MALDI target plate (Bruker Daltonics). A liquid-handling platform (Freedom Evo, Tecan, Männedorf, Switzerland) was used to ensure assay reproducibility.

Spots were analyzed with an ultrafleXtreme MALDI-TOF/TOF instrument (Bruker Daltonics) operated in positive linear mode. Ionization was achieved with 337 nm nitrogen laser and repetition rate of 1,000 Hz. Spectra were acquired with 1,000 laser shots, acceleration voltage of 25 kV, pulsed ion extraction time 300 ns, matrix suppression up to 800 Da, and laser energy 70%. The instrument was externally calibrated using peptide calibrants (Bruker Daltonics), reaching a mass accuracy

<10 ppm. Peaks with signal-to-noise ratio >3 in the m/z range 1–10 kDa were recorded with the Flex Control software (version 2.0, Bruker Daltonics).

Four spectra were obtained from each sample, and a detailed analysis was performed with DataAnalysis software (version 3.4, Bruker Daltonics), seeking to select the spectra with the highest number of peaks and the highest intensity. ClinProTools software (version 2.2, Bruker Daltonics) was used to calculate peak areas, and spectral data were analyzed with SPSS software (version 15.0, SPSS Inc., Chicago, IL).

For the identification of peptides, samples were prefractionated using magnetic beads and then trapped and desalted using ZipTip C18 pipette tips (Millipore, Bedford, MA). Peptides were mixed 1:1 with matrix α -cyano-4-hydroxy cinnamic acid and 1 μl was spotted onto a MALDI target plate (Bruker Daltonics). Spectra were obtained with an ultrafleXtreme MALDI-TOF/TOF instrument (Bruker Daltonics). The MS/MS data were searched using the MASCOT program (Matrix Science, London, UK). Proteins identified with a score confidence interval $\geq 95\%$ were accepted.

The nonparametric Wilcoxon test was performed to compare the peptide profile of samples frozen at different time points with the peptide profile of the same samples frozen at time “zero.” Results are expressed as median (interquartile ranges). $P < 0.05$ was considered statistically significant.

Our results showed that in the training group, the peptide profile of blood samples collected into EDTA tubes remained unchanged during the first hour (Supplementary Table S1). After 2-hr substantial changes were observed; peaks m/z 1,098.67, 1,505.06, 1,606.81, and 2,660.35 showed a decreased area regarding the same samples frozen at time “zero,” while the area of peaks m/z 1,740.92, 1,882.25, and 1,897.00 increased. After 4 hr, the area of peak m/z 2,428.30 decreased. After 8 hr, the area of peaks m/z 1,973.13, 1,987.70, 2,007.94, and 2,084.25 increased. When blood was collected into P100 tubes, the area of peaks with m/z 1,740.92, 1,882.25, and 2,428.30 increased or decreased in the same way and at the same hour after phlebotomy than when using EDTA tubes; other peaks showed the same change but at a different moment. Some peaks increased when using P100 tubes but remained stable at EDTA tubes (m/z 1,060.73, 1,076.74, 1,847.10, 2,485.32, and 2,862.57); the area of these peaks was higher at time “zero” in samples collected into EDTA tubes, and this change was observed also after 4 hr, both in training and validation groups. Eight peaks remained stable when blood was collected into P100 tubes.

Peak m/z 1,060.73, identified as bradykinin, showed higher area at time “zero,” and after 1, 2, and 4 hr when samples were collected into EDTA tubes, compared with P100 tubes. These results match with those obtained in

Fig. 1. Average peak area, in vertical axis (arbitrary units, AU), of peptide signals m/z 1,060, 1,076, 1,098, 1,505, 1,367, 1,606, 1,847, 1,897, 1,973, 1,987, 2,007, 2,084, 2,485, 2,660, and 2,862, when blood from subjects of the training group was collected into EDTA (continuous lines) or P100 tubes (dotted lines) and when samples were processed at time “zero” or 8 hr after phlebotomy. The nonparametric Wilcoxon test was performed to determine differences between blood samples collected into EDTA and P100 tubes. * P -value <0.05.

validation groups (Supplementary Table S2). After 8 hr, no differences were observed in the area of this peak depending on the collection tube (Fig. 1).

Peaks m/z 1,098.67 and 1,505.06, identified as complement C3f fragment, showed higher area when using EDTA tubes (Fig. 1). These differences were observed over time and were also detected in both validation groups.

At time “zero,” peak m/z 1,897.00, identified as fragments of complement C4-A, showed higher area when using EDTA tubes. The area of peaks m/z 1,973.13, 1,987.70, 2,007.94, and 2,084.25 at time “zero” was higher in samples collected into P100 tubes.

In proteomic studies focusing on the low molecular weight proteome of biological fluids, preanalytical variables must be considered because they can significantly modify the results of the analysis and lead to imprecise conclusions; researchers should be conscious of the instability of plasma due to the proteolytic activity that sets up at the moment of phlebotomy, just when blood contacts the needle or the walls of the collection tube. Intending to prevent this proteolytic activity, tubes containing PI have been designed, although their benefits have not been clearly recognized when samples are not rapidly processed (14, 16, 17).

At time “zero” and when blood was collected into P100 tubes, we observed lower peak areas in peptides identified as bradykinin and C3- and C4-derived peptides, which are generated from endogenous peptidase activity; these peptides were produced promptly after blood extraction and PI impeded their generation. Moreover, at time “zero”

some peaks showed higher area when using PI, maybe due to a diminished protease activity.

As more hours passed, greater differences were observed in the peptide profile of samples collected into EDTA tubes. Moreover, the effect of PI decreased over time; when comparing the peptide profile of samples collected into P100 with samples collected into EDTA tubes at time “zero,” we detected differences in the area of 13 peaks. These differences tended to disappear over time, and after 8 hr only the area of four peaks was different. Because each pair of samples compared in this study were obtained from the same subject and processed identically, differences observed in their peptide profile may be attributed to the presence or absence of PI in the collection tube and the time taken to analyze samples.

Clinical staff responsible for blood extraction is not usually the same that performs the analysis at the laboratory. Thus, processing samples at time “zero” is almost impossible and may elapse a considerable time until samples are analyzed. During this time, proteolytic activity takes place, critically changing the content of plasma proteins.

Researchers contemplating the search for biomarkers in plasma samples should consider whether the beneficial effects of PI counterbalance the cost of specialized collection tubes, and the requirement of dedicated staff to minimize the time from sampling to processing. In conclusion, blood collection is a crucial step for successful peptide biomarker discovery in plasma samples and further studies about its standardization are required.

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CONFLICT OF INTEREST

The authors confirm that they have no financial or personal relationships that cause a conflict of interest regarding the work in the manuscript.

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