Preanalytical variability of plasma samples, and mitigating strategies.

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Abstract

As the starting materials for proteomics experiments, complex biological samples pose very difficult challenges. Well-known issues about protein dynamic range, abundant proteins, signal suppression, and a host of other topics are routine considerations in virtually every experiment. Human plasma samples represent one of the most challenging samples, but wide and easy availability of sample and the potential value for biomarker discovery and future diagnostics exceeds the known challenges, making plasma a widely used sample. Virtually all plasma-based studies are designed to identify variations between samples, usually with samples from healthy subjects being compared to samples from patients with well-characterized disease states. The basic goal is to extract value from the variations between the samples being analyzed. Our projects focus on the flip-side of this situation, with specific attention paid to so-called preanalytical variability, or variations that arise *ex-vivo* either due to sample acquisition, sample (mis)handling and processing, and/or intrinsic biochemical processes. There are dozens of seemingly benign aspects to acquiring and preparing "standard" blood samples[1], few of which have been studied for their potential impact upon the protein content of blood samples. To date we have studied time (sample aging) effects on whole blood and serum/plasma, the effects of cell separators in the primary blood tube, and the nature of various common serum and plasma blood derivatives. This poster will provide an overview of our observations, and present some of our suggestions for optimal preparation and handling. In particular, we have examined the extent to which proteolytic activity intrinsic to blood samples can damage proteins. Such activity, if unchecked, adds further ex-vivo complications to an already intrinsically complicated sample, and, at worst, could be destroying protein biomarkers before they can even be discovered. Data suggest that much of the preanalytical variation in protein and peptide content is due to underlying, uncontrolled proteolytic activity intrinsic to the blood sample. The use of protease inhibitors to block or minimize preanalytical variables will be shown. The presence of protease inhibitors can offset the effects of many of the preanalytical variables, providing improved protein stability for biomarker discovery, validation and future diagnostic testing.

Overview

- **Drawn blood is a living tissue – and must be handled as such.**
- **There are many preanalytical variables associated with blood sample collection and processing.**
- **Reducing these variables by controlling or eliminating them will be a key aspect of enabling biomarker discovery and/or validation when using blood as the input sample.**

Objectives

- **Study certain preanalytical variables:**
  - **choice of blood samples**
  - **importance and effects of time as a handling variable.**
- **Demonstrate the value of protease inhibitors in stabilizing protein in plasma samples**
- **Exploit peptide analysis in several ways:**
  - ***In-vivo* peptide content should be maintained, as these have potential diagnostic value**
  - ***Ex-vivo* generation of peptides is a sensitive means of detecting protein degradation**

This poster presents a compilation of our observations to date, and summarizes strategies for mitigating preanalytical variability.

Experimental Designs

- Compare peptide spectra of same blood samples collected into different tubes: serum, EDTA, heparin, citrate and BD™ P100*.
- Monitor peptide content by time-course incubation of samples, followed by MALDI-TOF MS (ultraflex II, Bruker-Datonics) analysis.
- Sequence analysis of peptides by TOF/TOF MS (ultraflex II) and LC Q-TOF MS (Waters).

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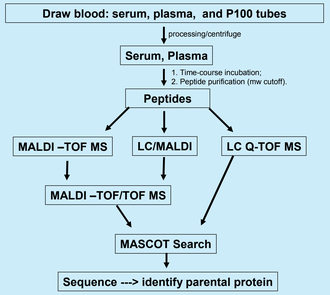
= Patented.

P100 tube contains:

- A proprietary cocktail of protease inhibitors resulting immediate inhibition during blood collection.
- Coated EDTA as anti-coagulant.
- A mechanical separator*, which provides a robust barrier between plasma and cells.

Procedures for peptide analysis



Results

Preanalytical variables associated with blood sample acquisition and handling

Patient Information
- Gender
- Age
- Diet
- Genetics
- Medical Background
- Health Background
- Special conditions (pregnancy, pre/post-menopausal, medications)
- Social history (alcohol intake, smoking status)

Collection Device
- Tube or bag?
- Glass or plastic?
- Gel separator?
- Non-gel separator?
- Other tube additives?

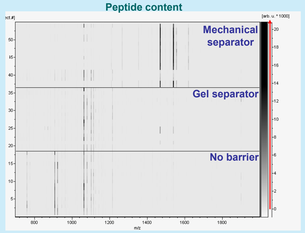
Venipuncture
- Needle gauge
- Blood collection set (details of)
- Phlebotomy
- Tourniquet technique?
- Patient position: seated/standing/lying
- First tube vs. last tube (or track tube order)
- Blood source: venipuncture or from existing line?

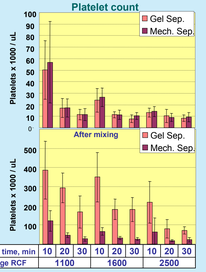
Blood Derivative and Processing
- Plasma? What anticoagulant?
- Serum? Clotting mechanism or activator?
- Details of processing:
  - Separate blood from cells?
  - Centrifugation? Speed and duration?
  - Aliquot before analysis? How were aliquots handled / stored?
  - Length of time before analysis
- Storage:
  - Frozen before analysis? What conditions?
  - Elapsed time prior to freezing?
  - Short vs. long-term storage
  - Storage temperature
  - Expiration dating
  - Storage materials: glass vs. plastic
  - Type of plastic
  - Number of freeze / thaw cycles`

- There are many variables associated with how blood samples are acquired and processed. Any or all of these could conceivably alter the protein content of a blood sample.
- We have analyzed only several of these many possibilities. To date, all variables that we have analyzed can be shown to affect one or more proteins or peptides. "No detail is trivial" with regard to proteomics.
- To improve the reproducibility of proteomics studies, as much information as possible relating to sample collecting and handling should be tracked, and variations in protocols should be minimized.

Adapted from (1)

Cell separation method affects plasma proteome content





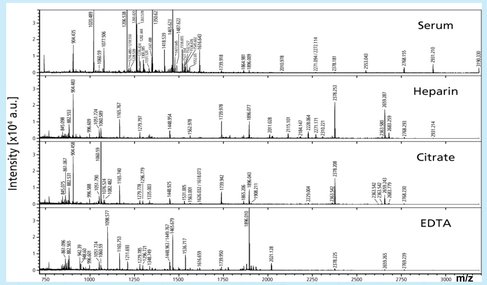
Centrifuge time, min	10	20	30	10	20	30	10	20	30
Centrifuge RCF	1100			1600			2500		

- Samples from 9 subjects were drawn, in duplicate, into EDTA plasma tubes with alternative cell separation devices. Peptides were prepared and analyzed with MALDI MS as described. The 18 MS spectra for each tube type are shown in "gel view".
- The intrinsic peptide content is different for each alternative cell separation method, despite the samples being otherwise chemically identical.
- Mechanical separator tubes show the fewest number of small peptides, which presumably come from protein degradation which is more prevalent in the other tubes.

- Samples from 12 subjects were drawn into EDTA plasma tubes having either a gel cell barrier (BD Vacutainer® PPT™ Plasma Preparation Tube) or a mechanical separator. A unique tube was drawn for each centrifugation time and speed condition cited. Cell counts were performed for each condition, both from the newly prepared samples (upper panel), as well as samples mixed after centrifugation (to release cells precipitated on top of the separator mechanism, lower panel.) Counts for the 12 subjects were averaged.
- Increased spin speed and time results in lower platelet counts.
- After mixing, mechanical separator tubes have fewer residual platelets.

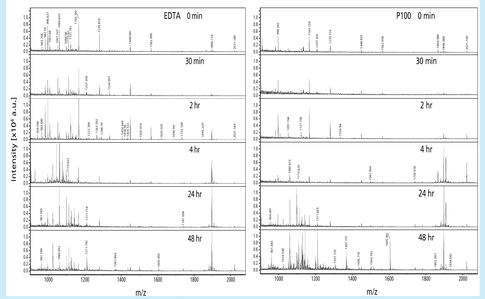
- The nature of the collection device itself can alter the sample protein content.
- This data represents another example of the need to carefully plan and track all aspects of blood sample acquisition for proteomics studies.

Variable peptide content in typical blood samples



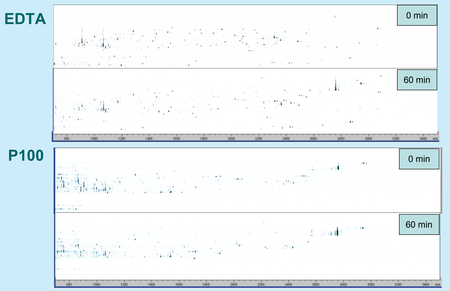
- Serum and plasma were drawn from the same individual. Data shown for fresh "time zero" samples.
- Serum contains significantly different peptides from plasma:
  - More peptides from 2000 to 3000 m/z.
  - Highly abundant peptides from 1000 to 2000 m/z.
  - The extra peptides are generated due to the *ex vivo* proteolysis during the clotting process[2,3].
- Differences between the three plasma samples are also observed.
- Since the nature of the blood sample imparts easily detected differences in the resulting peptide content, the choice of sample type is non-trivial, should be kept consistent throughout studies, and careful record-keeping of the nature of samples will be essential as part of later data analysis.

Protease inhibitors in plasma samples improve stability over time



- Variation in peptide content as a function of room-temperature incubation of EDTA plasma is evident. Similar extents of change are evident in citrate and heparin plasma and in serum[3], suggesting a general instability of human plasma due to intrinsic proteolysis.
- The time-dependent variations of peptide content are reduced in P100 samples.
- P100 sample is reasonably stable for > 2 hours after collection, suggesting improved handling conditions including:
  - Use of protease-inhibited plasma, such as provided by BD P100.
  - Analysis of the sample within a 2-hour timeframe, or
  - Freezing the collected sample within 2 hours.

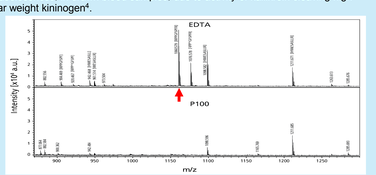
Stabilization by Protease Inhibitors: a "deeper look" with LC/MALDI



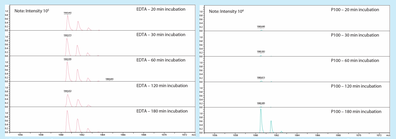
- LC/MALDI methods yield a virtual 2D plot (HPLC retention time vs. m/z), which shows significantly more peptides than direct MALDI shown in Figure 4. Data shown for one individual, with blood drawn into EDTA and P100 tubes. Peptides were prepared from "time zero" plasma, or from aliquots allowed to incubate at room temperature for one hour.
- Consistent with direct MALDI analyses, the EDTA samples have more peptides at "time zero" than P100. In addition, the P100 sample is noticeably more stable over time than the EDTA sample.
- Protease inhibitors provide stability over time to plasma peptide content.

Example with a specific peptide: bradykinin

- Bradykinin is a 9 residue peptide, shown to be a marker of cardiac trauma. Presumably at low levels in healthy blood samples, it has been described as being artifactually high, to the point of uselessness, in drawn blood samples, due to activity of kallikrein cleaving high molecular weight kininogen[4].



- EDTA and P100 samples from one healthy subject. Peptides from "time zero" plasma.
- Magnified view of MALDI spectra show that there are fewer low mass peptides in P100 than in EDTA sample, suggesting that P100 inhibits the generation of these peptides.
- Of particular note is bradykinin (red arrow), which is virtually absent in P100 samples, but easily detected in EDTA plasma.



- EDTA and P100 samples from a healthy subject, peptides isolated after room temperature plasma incubation times indicated. MALDI spectra at the same reverse-phase retention time are shown, providing a very clear view of bradykinin.
- LC/MALDI spectra as a function of time demonstrate that bradykinin concentration remains nearly undetectable in P100 plasma for at least 120 minutes.
- By comparison, EDTA samples maintain approximately 10x higher bradykinin levels from the earliest time points.
- These data suggest that P100 maintains a low, *in vitro* level for at least 2 hours, possibly enabling better utility for bradykinin in drawn blood samples.

Conclusions

- **Data demonstrate that many preanalytical variables (how blood samples are collected and handled) result in measurable changes in subsequent proteomics analyses.**
- **Reproducibility of data relies on reducing preanalytical variables, and many details of blood sample collection are important, often more so than researchers recognize.**
- **Every potential source of variation should be tracked, and minimized when possible.**

- **Choice of specific blood sample type directly influences observed proteins and peptides, both in "fresh" samples and also over time.**
- **Variations in residual cellular contamination appear to alter detectable protein content, with the least amount of degradation apparently associated with lowest cell counts.**
- **Inclusion of protease inhibitors in the plasma sample provide increased stability, with demonstrable improvement both at "time zero" and over at least 2 hours of room temperature incubation.**

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References

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(3) Yi, J. et al. *J. Proteome Res.* (2007), in press.
(4) Campbell, D. J. *Braz. J. Med. Biol. Res.* (2000) 33:665-677