Journal of Chromatography B, 877 (2009) 1281–1291

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Influence of clotting time on the protein composition of serum samples based on LC–MS data☆

Natalia I. Govorukhina[a], Marcel de Vries[b], Theo H. Reijmers[c], Péter Horvatovich[a], Ate G.J. van der Zee[d], Rainer Bischoff[a,*]

[a] Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[b] Mass Spectrometry Core Facility, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[c] Division of Analytical Biosciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[d] Department of Gynecology, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands

ARTICLE INFO

*Article history:*
Received 24 April 2008
Accepted 16 October 2008
Available online 1 November 2008

*Keywords:*
Biomarker
Clotting time
LC–MS
Proteomics
Serum

ABSTRACT

Many large, disease-related biobanks of serum samples have been established prior to the widespread use of proteomics in biomarker research. These biobanks may contain relevant information about the disease process, response to therapy or patient classifications especially with respect to long-term follow-up that is otherwise very difficult to obtain based on newly initiated studies, particularly in the case of slowly developing diseases. An important parameter that may influence the composition of serum but that is often not exactly known is clotting time. We therefore investigated the influence of clotting time on the protein and peptide composition of serum by label-free and stable-isotope labeling techniques. The label-free analysis of trypsin-digested serum showed that the overall pattern of LC–MS data is not affected by clotting times varying from 2 to 8 h. However, univariate and multivariate statistical analyses revealed that proteins that are directly involved in blood clot formation, such as the clotting-derived fibrinopeptides, change significantly. This is most easily detected in the supernatant of acid-precipitated, immunodepleted serum. Stable-isotope labeling techniques show that truncated or phosphorylated forms of fibrinopeptides A and B increase or decrease depending on clotting time. These patterns can be easily recognized and should be taken into consideration when analyzing LC–MS data using serum sample collections of which the clotting time is not known. Next to the fibrinopeptides, leucine-rich alpha-2-glycoprotein (P02750) was shown to be consistently decreased in samples with clotting times of more than 1 h. For prospective studies, we recommend to let blood clot for at least 2 h at room temperature using glass tubes with a separation gel and micronized silica to accelerate blood clotting.



## 1. Introduction

The discovery and validation of biomarkers for early diagnosis of disease at a stage where successful therapy is still possible is an important goal of modern biomedical research. To achieve this goal, high-resolution analytical techniques are applied to complex clinical samples, mostly body fluids. Serum is a body fluid that is representative of the composition of soluble proteins and peptides in blood and is thus a suitable starting material for biomarker discovery studies. Moreover, many existing large sample collections at major hospitals consist of serum that is stored frozen at −80 °C. Since these collections may well contain important information about the health status of the corresponding patients and controls, especially when followed over long periods of time, it is critical to evaluate under which conditions it is possible to compare samples from these collections with modern proteomics approaches.

The generation of serum requires that blood be coagulated and that the cellular components as well as the blood clot be removed by centrifugation or filtration. It has notably been argued that the time and conditions under which blood is allowed to clot (clotting time) are important parameters that must be controlled and kept constant in order to compare protein and peptide profiles [1–5]. However, most existing sample collections have not been obtained with subsequent proteomics analyses in mind and clotting time and conditions have often not been rigorously controlled. Many of the studies evaluating the influence of pre-analytical parameters on serum protein composition have been performed by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [4], a method that suffers from rather poor concentration sensitivity and that may be prone to

☆ This paper is part of the special issue "Quantitative Analysis of Biomarkers by LC–MS/MS", J. Cummings, R.D. Unwin and T. Veenstra (Guest Editors).

* Corresponding author. Tel.: +31 50 363 3338; fax: +31 50 363 7582.
*E-mail address:* r.p.h.bischoff@rug.nl (R. Bischoff).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.029

mass spectrometric artifacts [6]. More sensitive approaches using enrichment of proteins and peptides on magnetic bead separators or by liquid chromatography (LC) followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) have also indicated that conditions of sample handling and preparation are critical [2,3,5,7,8]. Our previous studies and those of others have shown that the combination of LC with electrospray-ionization mass spectrometry (ESI-MS), abbreviated LC–MS, is suitable to analyze body fluids such as serum or urine [9,10]. The increasing number of applications of LC–MS and LC–MS-MS for the profiling of body fluids or the targeted detection of individual proteins underscores furthermore that this method is capable of achieving concentration sensitivities in the ng–pg/mL range [11–18]. In return, LC–MS provides highly complex data sets when used in the profiling mode (measurement of all detectable compounds in a sample) and it is thus not easy to assess the effect of a given pre-analytical parameter on the overall result.

We describe here an approach to assess the effect of clotting time on LC–MS profiles of serum obtained from a healthy volunteer by univariate and multivariate statistical analysis after data processing. In order to render serum samples suitable to high-performance LC–MS analysis, proteins were digested with trypsin. Additionally, we investigated the supernatant of acid-precipitated serum samples which are highly enriched in low-molecular weight proteins and peptides (the so-called peptidome) [3,19–24]. For comparison of samples we used label-free as well as stable-isotope labeling (iTRAQ™) [25] approaches.

## 2. Materials and methods

### 2.1. Description of samples

Serum samples were prepared at the Department of Gynecological Oncology (University Medical Center Groningen, Groningen, The Netherlands) and stored at −80 °C in aliquots until analysis. All intermediate fractions that were obtained during sample preparation were stored at −20 °C. Glass tubes (Becton Dickinson, #367953), with a separation gel and micronized silica to accelerate clotting, were used for blood collection. Serum was obtained from a single healthy female volunteer, who consented to this study, after different clotting times. Serum was prepared by letting the freshly collected blood coagulate at room temperature for 1, 2, 4, 6 or 8 h followed by centrifugation at room temperature for 10 min at 3000 rpm.

### 2.2. Preparation of serum samples

20 μL of serum were mixed with 80 μL of buffer A (Agilent, Santa Clara, California, USA) of which 80 μL were injected on a Multiple Affinity Removal column (Agilent, 4.6 × 50 mm, Part #5185-5984) after filtration through a 0.22 μm spin filter (Part #5185-5990) at 13,000 × *g* and 4 °C for 10 min to remove particulates. The multiple affinity removal system designed for human serum samples allows to remove of albumin, IgG, alpha-1-antitrypsin, IgA, transferrin and haptoglobin in a single step by immobilized antibodies (www.agilent.com/chem). Removal of abundant proteins was performed on a LaChrom HPLC System (Merck Hitachi, www.merck.com) with detection at 280 nm using the following timetable: 0–9 min, 100% buffer A (0.25 mL/min); 9.0–9.1 min, linear gradient 0–100 B % (1 mL/min), 9.1–12.5 min, 100% buffer B (1 mL/min); 12.5–12.6 min, linear gradient 100–0% buffer B (1 mL/min); 12.6–20 min, 100% buffer A (1 mL/min). The flow-through fraction (depleted serum collected between 2 and 6 min) of a total volume of approx. 1 mL was collected [9]. Each serum sample obtained after different clotting times (1, 2, 4, 6, 8 h) was depleted in duplicate.

Protein concentrations were determined with the Micro BCA™ Protein assay reagent kit (www.piercenet.com) and calculated for an average protein molecular weight of 50 kDa. BSA was used as the calibration standard. Depleted serum samples were digested with trypsin (sequencing grade modified trypsin, Promega, cat. #V5111, Madison, Wisconsin, USA) at an enzyme-to-substrate ratio of 1:20 overnight at 37 °C with shaking at 400 rpm (Eppendorf Thermomixer) and 4% of the digest were subjected to capillary LC–MS analysis.

### 2.3. Standard addition of horse heart cytochrome c

Serum samples were spiked with 21–50 pmol of horse heart cytochrome *c* (Sigma, www.sigmaaldrich.com, cat. #9007-43-6) prior to immunodepletion (21 pmol (+) or 50 pmol (++)). Ten percent of each spiked serum sample was subjected to LC–MS analysis after trypsin digestion. To evaluate the repeatability of the LC–MS part of the procedure alone, cytochrome *c* was digested with trypsin and added at the same amounts to depleted and trypsin-digested serum (1 h clotting time sample) directly prior to LC–MS (21 pmol addition denoted as "n+"; 50 pmol addition denoted as "n++").

### 2.4. Cap-LC–MS

All LC–MS analyses were performed on an Agilent 1100 capillary HPLC system coupled on-line to an SL ion-trap mass spectrometer (www.home.agilent.com; cat. #G2445A) equipped with an Atlantis™ dC 18 (1.0 × 150 mm, 3 μm) column that was protected by an Atlantis™ dC 18 in-line trap column (3 μm, 2.1 mm × 20 mm guard column). 40 μL of the pretreated (depleted and digested) fractions corresponding to ∼8 μg or 160 pmol of total protein digest (calculated based on a 50 kDa protein) were injected. The autosampler (cat. #G1367A) was equipped with a 100 μL injection loop and a temperature-controlled cooler (cat. #G1330A) maintaining the samples at 4 °C. The HPLC system had the following additional components: capillary pump (cat. #G1376A), solvent degasser (cat. #G1379A), UV detector (cat. #G1314A) and column holder (cat. #G1316A). The sample was injected and washed in the back-flush mode for 30 min (0.1% aq. formic acid (FA) and 3% acetonitrile (AcN) at a flow rate of 50 μL/min. Peptides were eluted in a linear gradient from 0 to 70% (0.5%/min) AcN containing 0.1% FA at a flow-rate of 20 μL/min. After each injection, the in-line trap and the analytical column were equilibrated with eluent A ($H_2O$/AcN/FA; 950:50:1) for 20 min prior to the next injection.

The following settings were used for mass spectrometry during LC–MS. Nebulizer gas: 16.0 psi $N_2$, drying gas: 6.0 L/min $N_2$, skimmer: 40.0 V, ionization voltage: 3500 V, cap. exit: 158.5 V, Oct. 1: 12.0 V, Oct. 2: 2.48 V, Oct. RF: 150 $V_{pp}$ (voltage, peak power point), lens 1: −5.0 V, lens 2: −60.0 V, trap drive: 53.3, *T*: 325 °C, scan resolution: enhanced (5500 *m*/*z* per second scan speed). Target mass: 600. Scan range: 100–1500 *m*/*z*. Spectra were saved in centroid mode. LC–MS chromatographic data were analyzed with Bruker Data Analysis software, version 3.4 (Build 181).

### 2.5. TCA precipitation of serum samples and MALDI-TOF-MS analysis

TCA, dissolved in 40 μL ice-cold water, was added to 20 μL of the original serum samples to reach a final concentration of 5%. After 30 min on ice, samples were filtered through 0.22 μm spin filters (Part #5185-5990, Agilent) at 13,000 × *g* at 4 °C for 10 min to remove particulates. Filtrates were used for further analysis.