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Absolute Myoglobin Quantitation in Serum by Combining Two-Dimensional Liquid Chromatography−Electrospray Ionization Mass Spectrometry and Novel Data Analysis Algorithms

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To measure myoglobin, a marker for myocardial infarction, directly in human serum, two-dimensional liquid chromatography in combination with electrospray ionization mass spectrometry was applied as an analytical method. High-abundant serum proteins were depleted by strong anion-exchange chromatography. The myoglobin fraction was digested and injected onto a 60 mm × 0.2 mm i.d. monolithic capillary column for quantitation of selected peptides upon mass spectrometric detection. The addition of known amounts of myoglobin to the serum sample was utilized for calibration, and horse myoglobin was added as an internal standard to improve reproducibility. Calibration graphs were linear and facilitated the reproducible and accurate determination of the myoglobin amount present in serum. Manual data evaluation using integrated peak areas and an automated multistage algorithm fitting two-dimensional models of peptide elution profiles and isotope patterns to the mass spectrometric raw data were compared. When the automated method was applied, a myoglobin concentration of 460 pg/μL serum was determined with a maximum relative deviation from the theoretical value of 10.1% and a maximum relative standard deviation of 13.4%.

Keywords: absolute quantitation • serum • myoglobin • high-performance liquid chromatography • electrospray ionization mass spectrometry • two-dimensional HPLC • standard addition • monoliths • computational proteomics • algorithms

Introduction

In recent years, there has been growing interest in applying proteomics to clinical diagnostics and predictive medicine. Protein biomarkers can assist in the diagnosis of diseases in order to reduce the time and cost of clinical and medical treatment.[1] Myoglobin, a low molecular mass heme protein present in the cytosol of cardiac and skeletal muscle, represents the earliest known biochemical marker for myocardial necrosis associated with myocardial infarction.[2] Its serum level increases within 2 h after infarction from normal concentrations of less than 90 pg/mL to values which may be as high as 5500 pg/mL, reaching peak levels within 6−9 h.[3−5] Serum concentrations of myoglobin after myocardial infarction can be measured by immunoassays which are commercially available from different suppliers.[6] However, results from different analytical procedures for myoglobin determination showed differences of up to 36% (frozen human serum[7]) and even 40% (fresh human plasma[8]). Since this variability among commercial myoglobin immunoassays exists, standardization of myoglobin determination is mandatory according to existing regulations and standards, such as Directive 98/79/EC and ISO/CEN 17511. This requires both reference materials as well as development and implementation of a reference measurement procedure to reduce the bias observed between commercially available myoglobin assays and to provide traceable results.

In this respect, a need exists for accurate and precise technologies that permit traceable, absolute quantitation of myoglobin in serum or plasma. Mass spectrometry has evolved as a powerful tool for the selective and sensitive detection of proteins and peptides. In due consequence, it should be highly

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appropriate as an analytical method for the quantitative determination of myoglobin in complex biological matrices such as serum or plasma.

Relative quantitation strategies have been developed based on two-dimensional gel electrophoresis or multidimensional chromatography−mass spectrometry in combination with stable isotope labeling.[9−11] In these experiments, two protein samples are compared in which one is labeled with heavy isotopes either by growing the cells in isotope-enriched media[12−16] or by labeling of the proteins after isolation in the different stages of sample preparation.[10,17−21] Moreover, matrix proteins in the sample, which are assumed to be present in a constant level, can serve as internal standards to monitor changes in the relative concentration of proteins of interest.[21,22]

In addition to the relative quantitation strategies described above, mass spectrometry can also be utilized to derive information about absolute protein concentration. This can be accomplished by mass spectrometric measurement of the signal intensity of the intact protein in relation to an isotope-labeled protein which is added in known amount to the sample and serves as internal standard.[23] In another method, synthetic isotope-labeled peptides, with sequences derived from proteolytic peptides of the protein under investigation, have been utilized for calibration and quantitative determination of biomarkers at the subfemtomole level.[24−30]

In two publications, Chelius et al. successfully demonstrated the applicability of liquid chromatography−mass spectrometry to the relative quantitation of myoglobin in biological samples.[31,32] It was shown that 250 fmol myoglobin is detectable in a background of human serum; however, no absolute quantitation was attempted. Gerber et al. utilized synthetic isotope-labeled peptides of horse myoglobin for its detection in a yeast background.[25] Operating an ion-trap mass spectrometer in the selected reaction monitoring mode, they were able to detect and determine as little as 300 amol myoglobin. However, this method is not directly transferable to human serum samples because of the interference of the high-abundant proteins that have to be depleted before reliable quantitation.

In the present work, we have significantly extended the ideas discussed above toward absolute quantitation of myoglobin in human serum in two ways. First, we have developed and optimized a two-dimensional chromatographic separation system to reduce the background of proteins, which are abundant in the sample matrix. This sample preparation is based on intact protein separation by anion-exchange chromatography in the first dimension and peptide separation after tryptic digestion in the second dimension. The addition of known amounts of human myoglobin was chosen to calibrate the method, and horse myoglobin served as internal standard to account for variation in the whole analytical process, including sample preparation, tryptic digestion, and peptide measurement. Second, we have implemented novel automated algorithms for rapid data evaluation. Using improved feature finding and automatic peak integration routines, we could show that both quantitation accuracy and precision can be considerably improved compared to peak integration performed by the instrument software following manual data evaluation. The resulting methods represent a significant step toward establishing a measurement protocol and reference measurement procedure for the quantitative determination of myoglobin in human serum.

Table 1. Concentrations of Added Human Myoglobin Calibrant in the Aliquots of Myoglobin-Spiked Human Serum

standard no.	standard concentration [ng/μL]	myoglobin standard added [μL]	water added [μL]	final myoglobin standard concentration[a] [ng/μL]
0	5.2	0	10	0.000
1	5.2	5	5	0.236
2	5.2	10	0	0.473
3	52.0	2	8	0.945
4	52.0	3	7	1.418
5	52.0	4	6	1.891
6	52.0	5	5	2.364
7	52.0	6	4	2.836
8	52.0	7	3	3.309

[a] Standard solutions were added to 100 μL aliquots of myoglobin-spiked serum, final volume, 110 μL.

Experimental Section

Chemicals and Materials. Acetonitrile (HPLC gradient-grade) was obtained from Riedel-deHaën (Seelze, Germany). Water was purified by a Purelab Ultra System from Elga (Siershahn, Germany). Trifluoroacetic acid (TFA, for protein sequence analysis), Trizma Base (analytical reagent grade), and ammonium bicarbonate (analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). Reagent-grade sodium chloride was from Grüssing GmbH (Filsum, Germany). Horse myoglobin was obtained from Sigma (St. Louis, MO). Myoglobin-depleted human blank reference serum and a stock solution of human myoglobin (520 ng/μL) were provided by the Institute for Reference Materials and Measurements (Joint Research Center of the European Commission, Geel, Belgium). Trypsin (sequencing grade) was purchased from Promega (Madison, WI) and RapiGest SF reagent from Waters (Milford, MA). Serum samples were filtrated using Vivaclear centrifugal filters from Vivascience (Hannover, Germany).

Samples and Standards. To determine the accuracy and reproducibility of quantitative myoglobin determinations, myoglobin-depleted blank reference serum was spiked with 456−463 pg/μL human myoglobin. This concentration represented the target value to be quantitated. To 1000 μL serum containing human myoglobin, 10 μL of a 500 ng/μL solution of horse myoglobin was added as internal standard. For calibration, known amounts of human myoglobin standard were added to the serum sample. Nine aliquots of 100 μL of the prepared serum were pipetted into vials, and different volumes of deionized water and human myoglobin standard solution were added to a final volume of 110 μL. The volumes of myoglobin standard solution and the final concentrations of human myoglobin in the aliquots are collected in Table 1.

Depletion of High-Abundant Serum Proteins. High-abundant serum proteins were removed form the myoglobin-spiked serum sample by strong anion-exchange (SAX) HPLC using a ProPac SAX-10 column (250 mm × 4.0 mm i.d.) and guard column (50 mm × 4.0 mm i.d.) from Dionex (Idstein, Germany). The instrument employed for SAX experiments included a low-pressure gradient HPLC pump (model 480 G, Gynkotek, Germering, Germany) equipped with a variable wavelength UV detector (model Lambda 1000, Bischoff Analysentechnik und -geräte, Leonberg, Germany), and a six-port injection valve with a 20 μL external sample loop (model 8125, Rheodyne, Rohnert Park, CA). The gradient applied to separate the myoglobin fraction from the rest of the high-abundant

human serum proteins was 0−500 mmol/L sodium chloride in 10 mmol/L Tris-HCl, pH 8.0, in 10 min for the experiments shown in Figure 2, or otherwise 0−35 mmol/L sodium chloride in 10 mmol/L Tris-HCl, pH 8.5, in 1.0 min, 4.0 min isocratic hold at 35 mmol/L sodium chloride, then a washing step from 35 to 500 mmol/L sodium chloride in 2.0 min followed by 500 mmol/L sodium chloride for 2 min. To keep fraction volumes as small as possible, the proteins were eluted at a flow rate of 1.0 mL/min. The reequilibration time was 10 min to ensure reproducible elution of myoglobin. Usually, the myoglobin peak was not discernible in the chromatogram so that the elution window, which was typically between 4.25 and 4.85 min, was determined in advance from the injection of 10 μL of a 52.0 ng/μL solution of plain human myoglobin. Subsequently, 20 μL myoglobin-spiked serum was injected, and the myoglobin fraction was collected in preweighted 1.5 mL Eppendorf vials. The volume of the fraction was reduced in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany) to approximately 80 μL, and then adjusted to a defined mass of 100.0 mg with water using an analytical balance.

Digestion of the Myoglobin Fraction. RapiGest denaturant (10.0 μL, 1.0% w/v) was added to the fractions, and the solution was equilibrated at 37 °C for 15 min. Trypsin solution (4.0 μL, 0.50 μg/μL) was added, and the digestion was allowed to proceed for 2 h at 37 °C. Digestion was terminated by the addition of 10.0 μL of 500 mmol/L hydrochloric acid. After hydrolysis and precipitation of the denaturing agent for 1 h at 37 °C, the vials were centrifuged at 13 000 rpm for 10 min. Subsequently, the supernatant was transferred to glass vials and put in the autosampler for reversed-phase HPLC−ESI−MS analysis.

For the experiments presented in Figure 3a,b, a sample of horse myoglobin was digested as described above and another was digested according to the following protocol. Myoglobin (100 μg) was dissolved in 50 μL of a solution containing 8.0 mol/L urea and 0.50 mol/L ammonium bicarbonate and kept at 37 °C for 45 min. Trypsin (3.3 μg) was activated in 50 mmol/L acetic acid for 13 min at 30 °C and added to the protein solution in a ratio of trypsin/protein of 1:30. Digestion was allowed to proceed for 24 h at 37 °C. Digestion was terminated by the addition of TFA to a final concentration of 0.1% (v/v).

Reversed-Phase HPLC−Electrospray Ionization Mass Spectrometry. Reversed-phase HPLC was performed using monolithic capillary columns (60 mm × 0.20 mm i.d.), which were prepared according to the published protocol.[33] Monolithic capillary columns (50 mm × 0.20 mm i.d.) are commercially available (Monoliths from LCPackings-A Dionex Co., Amsterdam, The Netherlands). The system used for HPLC−ESI−MS experiments consisted of a fully automated capillary HPLC system (Ultimate with Famos, LCPackings). ESI−MS was performed on a quadrupole ion-trap mass spectrometer (esquire HCT, Bruker Daltonics, Bremen, Germany). Total ion current chromatograms and mass spectra were digitally recorded using esquireControl and DataAnalysis software version 3.1 (Bruker Daltonics).

The monolithic capillary column was connected to the spray capillary (fused silica, 90 μm o.d., 20 μm i.d., Polymicro Technologies, Phoenix, AZ) by means of a microtight union (Fritz Gyger AG Swiss, Gwatt-Thun, Switzerland). For analysis with pneumatically assisted ESI, an electrospray voltage of 3.5 kV and a nitrogen sheath gas flow of 15 psi were employed. Mass calibration and tuning were performed according to the manufacturer's recommendations. Fine-tuning for ESI−MS of peptides[34] was accomplished by infusion of a 0.40 pmol/μL solution of cytochrome C in 0.050% aqueous TFA solution containing 20% acetonitrile (v/v) at a flow rate of 3.0 μL/min.

The elution conditions for the analysis of the digested myoglobin fractions incorporated a 6.0-min isocratic hold of 0.050% aqueous TFA, followed by a 15.0-min gradient of 0−40% acetonitrile in 0.050% aqueous TFA at a flow rate of 2.0 μL/min. The injection volume was 5.0 μL, and each fraction was analyzed four times. The initial 6-min isocratic elution step at 0% acetonitrile was applied to completely remove the salt from the SAX fraction, which was otherwise a source of sodium adducts in the mass spectra of the peptides. Identification of the myoglobin peptides in the serum sample was based on the measured molecular masses of the peptides and comparison of their elution times with those obtained from the analysis of a tryptic digest of pure human or horse myoglobin under identical chromatographic conditions.

Manual data evaluation was performed with the esquireData software Package (Bruker). The peak areas of the human and horse myoglobin peptides were obtained from extracted ion chromatograms with isolation widths of ±0.5 m/z units after applying smoothing based upon a Gaussian function. Manual data evaluation and calculation of the results were accomplished with Microsoft Excel 2000.

Automated Data Analysis. The instrument software was configured to store the measurement data in its most unprocessed form available. We refer to this type of data as *raw data*. One RP−HPLC−MS analysis consisted of about 1830 scans. The time intervals of scans were roughly evenly spaced over retention time with an average of 0.9 scans/s. The sampling accuracy was 0.2 in m/z dimension. The raw data was exported from the Bruker instrument software using Bruker's CDAL library, resulting in raw data files of ca. 300 MB per experiment. To speed up the analysis, all HPLC−ESI−MS raw data files were truncated to a retention time range from 900 to 1600 s and an m/z from 600 to 1000. These ranges were used to ensure all relevant myoglobin peaks were included, with ample margins. Moreover, very weak signals (ion count $<$ 100) were filtered out. These ranges and cutoff values were chosen after some initial runs of the complete analysis pipeline. This data preprocessing was not essential for performing the analysis, but it saved disk space and processing time.

Fully automated detection and quantitation of myoglobin peptides was accomplished with the feature finder algorithm of the OpenMS proteomics framework (http://www.openms.de). As algorithmic details have been described elsewhere,[35] we will only give a brief overview of the main algorithmic ideas. The key concept is an accurate identification of a *feature*, which is defined as the part of the mass spectrometric data caused by a single charge variant of an individual peptide. The volume of a feature thus corresponds to the total ion current of its peptide and is proportional to its concentration in the sample. The algorithm identifies features automatically by fitting two-dimensional models of theoretical feature shape. These models are based on Gaussian elution profiles along the retention time dimension and averagine-derived isotope patterns along the m/z dimension. The model accounts for differing charge states and instrument resolution as well. A numerical fit of raw data regions to a two-dimensional feature model identifies raw data points belonging to a specific feature. Summing over the intensities of these raw data points yields the feature volume. The feature-finder application reads the

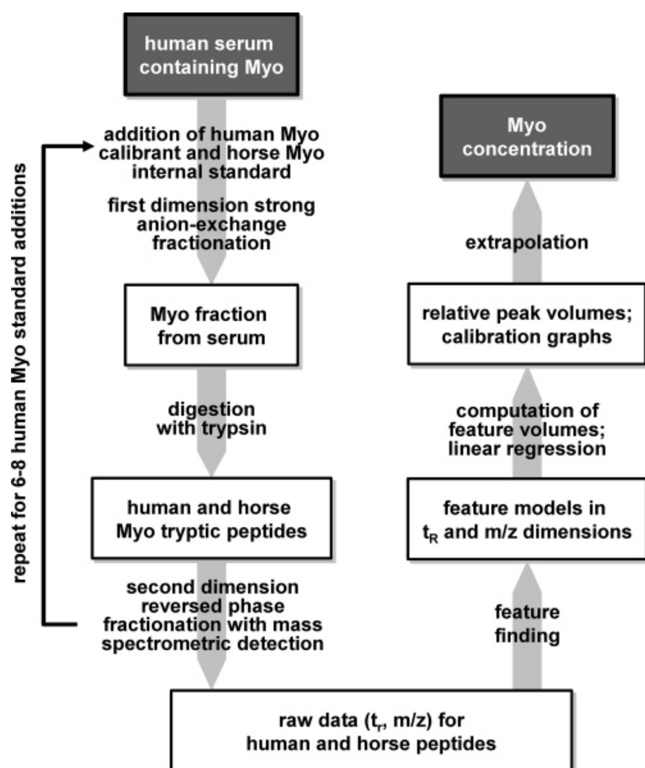

Figure 1. Generic scheme for the absolute quantitation of myoglobin in human serum. For details, see text.

raw data sets as described above and automatically extracts a list of features. These feature lists are then cut down to the relevant myoglobin peptides based on known rough estimates of retention time and m/z ratio of these peptides. A subsequent linear regression over measurements then yields the statistical data required to determine the peptide concentration. Statistical analyses were performed using code from the OpenMS system and GNUplot.

Results and Discussion

Concept for Absolute Quantitation of Myoglobin in Serum. Because of the extremely high number and the broad dynamic concentration range of components present, human serum constitutes one of the most difficult matrices for absolute quantitative measurements of one or more specific proteins. Depletion of high-abundant proteins is generally mandatory to be able to detect confidently medium- to low-abundant proteins in human serum. We decided to apply the method of standard addition for calibration, because calibration standards and internal standard can be added at the very beginning of the analytical protocol (Figure 1). In this way, calibration comprehensively accounts for variability in overall serum composition, sample preparation, and measurement of the analyte. Moreover, calibration standards can be added as authentic protein so that there is no need for isotope-labeling of myoglobin, which could be achieved for a human protein only by applying very expensive and elaborate recombinant protein expression technology. The approach for using isotope-labeled peptides as internal standards is not applicable in this case because they would be lost in the first stage of sample preparation. As we will show later, the addition of an internal standard proved useful for improving the reproducibility of the results for measured peak intensities.

The generic scheme of data generation and evaluation for the quantitation of myoglobin in serum is outlined in Figure 1. Human myoglobin calibrants of known concentration as well as horse myoglobin, which served as internal standard, were added to a filtered human serum sample containing the myoglobin to be quantified. Total myoglobin was separated from high-abundant serum proteins by strong anion-exchange chromatography (SAX). Following isolation, the myoglobin fraction was digested with trypsin without reduction and alkylation, because myoglobin does not contain any disulfide bridges. The tryptic peptides were desalted, separated, and detected by reversed-phase HPLC−ESI−MS. Peptides generally eluted from the monolithic capillary columns as very sharp peaks of only 5−10 s peak width at base. Therefore, full-scan mass spectra were recorded in the fast-scanning, ultrascan-mode of the ion-trap mass spectrometer in order to obtain sufficient data points for a description of well-defined chromatographic peaks. All myoglobin peptides could be unambiguously identified on the basis of their molecular masses and retention times, which had been previously determined from the analysis of a tryptic digest of plain myoglobin. The complete procedure of myoglobin fractionation, digestion, and peptide analysis was repeated for six to eight different additions of human myoglobin calibrant to the serum sample. Manual or automated data evaluation and computation of the myoglobin concentration were performed by calculating the peak area or volume ratios of the human and horse peptides, subsequently plotting the relative peak areas or volumes versus the concentration of added human myoglobin, linear regression analysis, and finally extrapolation of the regression line to $y = 0$, which yielded the myoglobin concentration directly as the negative x-intercept of the regression line.

Depletion of High-Abundant Serum Proteins Using Strong Anion-Exchange Chromatography. Almost 75% of the protein content of serum comprise only three proteins, namely, human serum albumin (HSA), transferrin (TRA), and immunoglobulins G (IgG). In due consequence, the first step of sample preparation aimed at depletion of these high-abundant serum proteins. Multiaffinity columns are commercially available that retain the six most abundant serum proteins. However, such columns are very expensive and have only limited lifetime. Given an isoelectric point of 7.3 for myoglobin and comparatively low isoelectric points of most of the high-abundant serum proteins (4.9 for HSA, 5.9 for TRA, 5.5−9.5 for IgGs), we anticipated a good chance of being able to achieve a separation at pH 8.5 based on SAX. Figure 2a shows the fractionation of 10 μL of serum spiked with human myoglobin. It can be seen that myoglobin eluted as a well-defined peak at a retention time t_R around 2.6 min in front of the IgG, TRA, and HSA peaks and that most of the high-abundant proteins could be successfully removed. Only a small part of the IgG fraction was coeluting with myoglobin. The myoglobin fraction was collected, digested, and reanalyzed by reversed-phase HPLC−ESI−MS/MS. In fact, database search using the MASCOT software package yielded significant protein hits only for myoglobin and immunoglobulins, proving that purification was successful.

The loading capacity of the 250 mm × 4 mm i.d. SAX column was evaluated by injecting increasing amounts of human serum. With 15-μL injections, the myoglobin peak was still eluting in the IgG front, but a second peak was observed at 1.7 min shortly after the injection peak, which was shown to contain myoglobin upon reanalysis of the corresponding

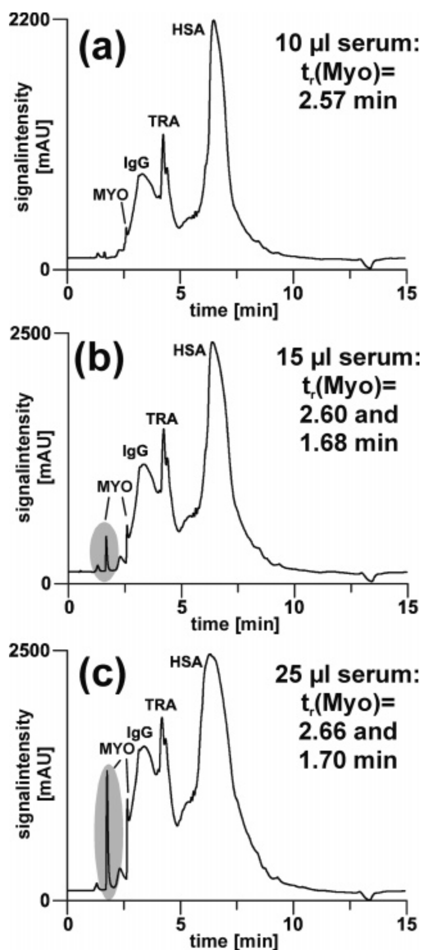

Figure 2. UV-chromatograms of 10−25 μL of myoglobin-spiked blank reference serum. Column, ProPac, SAX-10, 250 mm × 4.0 mm i.d.; gradient, 0−500 mmol/L sodium chloride in 10 mmol/L aqueous Tris-HCl, pH 8.5, in 10.0 min; flow rate, 1.0 mL/min; room temperature; sample, 10−25 μL blank reference serum spiked with 1.53 pmol/μL myoglobin.

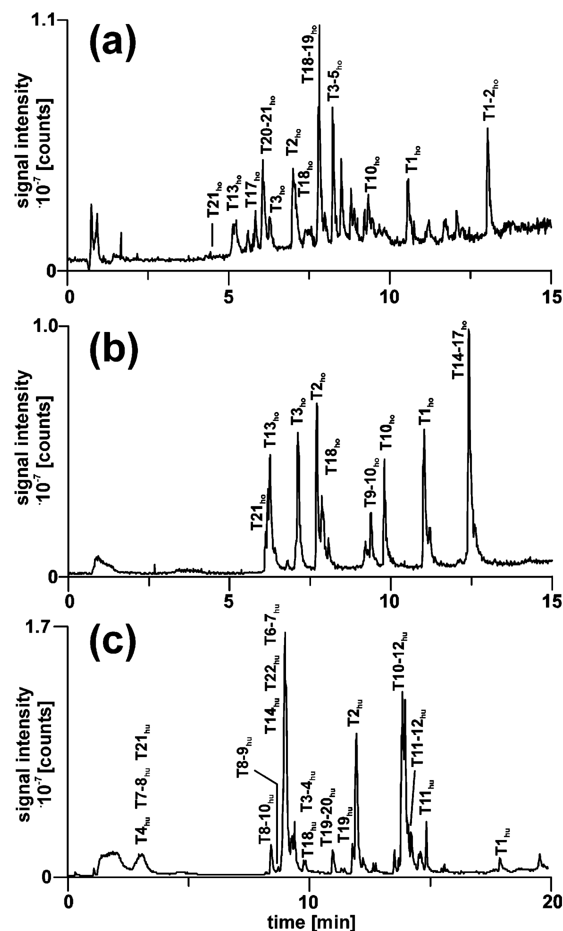

Figure 3. Reversed-phase HPLC−ESI−MS analysis of tryptic digests of horse and human myoglobin. Column, poly(styrene-divinylbenzene) monolith, 60 mm × 0.20 mm i.d.; gradient, (a and b) 0−50% (c) 0−35% acetonitrile in 0.050% TFA in 15.0 min, 2.0 μL/min; temperature, 55 °C; detection, full-scan ESI−MS; scan range, m/z 500−1500; electrospray voltage, −3.5 kV; spray gas, nitrogen, 15 psi; dry gas, nitrogen, 4 L/min; sample, 0.25 pmol myoglobin digested with (a) urea and (b and c) RapiGest as denaturant.

fraction. Injections of 20 μL finally resulted in a significant increase in the signal intensity of the additional peak, which is probably a consequence of peak deformation and peak splitting due to overloading and nonlinear elution effects.[36] We concluded that the maximum loading capacity of the column lies between 10 and 15 μL serum. Upon connection of an additional 50 mm × 4 mm i.d. precolumn and application of a shallower gradient including an isocratic step during elution of myoglobin, 20 μL of serum could be readily loaded and fractionated without any untoward displacement effect.

Quality and Reproducibility of Myoglobin Digestion. Since myoglobin lacks any disulfide bridges, the laborious step of reduction and alkylation, as well as the time-consuming removal of excess reagents by overnight dialysis, can be omitted during sample preparation. Nevertheless, such sample preparation steps can be readily included into the experimental protocol, making the procedure generally applicable to other proteins having a p*I* higher than 7.5. Moreover, an acid-labile denaturing agent is available (RapiGest), which can be very easily removed by acid hydrolysis and centrifugation of the insoluble reaction products. Two digests were prepared from horse myoglobin, one applying the classical protocol with 8 mol/L urea as denaturant and an alternative protocol with 0.1% RapiGest, in order to investigate their influence on digest quality. Although the signal intensities were quite similar, Figure 3a,b clearly shows that significantly improved signal-to-noise ratios were achieved in the chromatogram of the digest performed with RapiGest as denaturant. Moreover, it becomes clear that more peptides with one or more missed cleavages were present in the classical digest (four versus two), despite a 6-fold longer reaction time. The total sequence coverages with tryptic peptides were 80 and 75% using RapiGest and urea, respectively. Based on these results, we decided to continue method development with the RapiGest protocol.

Figure 3c illustrates the results of tryptic digestion of human myoglobin in the presence of RapiGest. The quality of the chromatogram was comparable to that obtained with horse myoglobin, and 80% of the sequence was covered with tryptic peptides. The reproducibility of digestion as well as of the analysis of the digests was evaluated by the independent preparation of three digests of human myoglobin and quadruplicate analysis of the generated peptides. For manual data evaluation, ion chromatograms were extracted in a window of m/z ±0.5 corresponding to the singly or doubly charged peptide ions. Subsequently, the peaks at the expected retention times were integrated using the esquire DataAnalysis software,

Table 2. Comparison of Reproducibilities of Peak Areas of Selected Tryptic Peptides from Human Myoglobin in Three Different Digests Determined by Manual and Bioinformatic Data Evaluation

			RSD of peak areas [%] (manual//automated)			
peptide	sequence	average retention time [min]	digest 1 $N = 4$	digest 2 $N = 4$	digest 3 $N = 4$	all $N = 12$
$T22_{hu}$	ELGFQG	7.9	1.1//1.7	3.8//4.0	4.4//2.4	5.1//5.6
$T14_{hu}$	GHHEAEIKPLAQSHATK	7.9	9.8//4.5	9.4//4.7	8.2//4.0	9.1//6.7
$T18_{hu}$	HPGDFGADAQGAMNK	8.5	2.6//3.7	4.0//3.9	3.7//4.1	8.4//8.9
$T19_{hu}$	ALELFR	10.4	4.4//5.1	5.0//2.8	4.2//4.6	6.6//7.6
$T2_{hu}$	VEADIPGHGQEVLIR	10.5	3.3//3.8	6.6//5.2	2.2//3.8	5.8//6.4
$T11_{hu}$	HGATVLTALGGILK	13.3	7.9//5.3	3.6//2.8	2.3//3.1	8.3//6.5
	Average	-	6.0//4.0	5.8//3.9	4.4//3.7	8.1//7.0

and statistical data were obtained using Microsoft Excel. Alternatively, the raw data generated by the ion-trap mass spectrometer were treated using the fully automated algorithm as described above. Solely completely digested peptides were considered for quantitation. The peptides $T1_{hu}$ and $T4_{hu}$ showed significantly elevated relative standard deviations between 13 and 36%, presumably because of broad peak profiles resulting in low signal-to-noise ratios in the extracted ion chromatogram.

Table 2 summarizes the relative standard deviations of the peak areas for the peptides $T22_{hu}$, $T14_{hu}$, $T18_{hu}$, $T19_{hu}$, $T2_{hu}$, and $T11_{hu}$ within the four replicate analyses of the digests as well as those calculated for all 12 analyses performed in this study. The relative standard deviations (RSDs) in peak areas among the four runs of a single digest varied between 1.1 and 9.4%, and the average RSDs ranged from 3.7 to 6.0%. Moreover, the RSDs values for all 12 analyses demonstrate that the intermediate precision for the described analytical procedure, comprising protein digestion and peptide analysis, is below 10%. The results of manual peak area and automated peak volume determination were equivalent, with a slight tendency of lower RSD values when using automated data evaluation. This congruence was not unexpected since the analyte mixture was evaluated in a nonserum matrix.

Evaluation of Myoglobin Quantitation. Utilizing the protocol outlined in detail in the Experimental Section and in Figure 1, we performed two independent studies for myoglobin quantitation in serum. To obtain data suitable for method evaluation, a known amount of myoglobin was spiked into myoglobin-depleted human blank reference serum. A number of different peptides, excluding those that were only partially digested, were evaluated for quantitation. Some of the peptides were readily detectable but not suitable for quantitation because of identical sequences of the human and horse peptides (e.g., $T19_{hu}$ and $T18_{ho}$, ALELFR, or $T22_{hu}$ and $T21_{ho}$, ELGFQG). Among the peptides unique for human myoglobin, $T11_{hu}$ and $T1_{hu}$ were found to yield the most reproducible and accurate results, and hence, these two peptides were utilized for evaluation of the quantitation procedure.

An example for the reconstructed total ion current chromatogram, obtained by reversed-phase HPLC−ESI−MS analysis of the digested myoglobin fraction, and selected ion chromatograms of the human peptides $T11_{hu}$ and $T1_{hu}$ and the horse peptides $T10_{ho}$ and $T2_{ho}$ are illustrated in Figure 4. The difference between the two peptides $T11_{hu}$and $T10_{ho}$ rests within the N-terminal sequences of the human and horse peptides, HGAT and HGTV, respectively. It is seen that both peptides were clearly and unambiguously distinguishable in the chromatograms based on their molecular masses. Furthermore, coelution of the two peptides facilitated optimal correction for signal suppression by utilizing relative peak areas of the human and horse peptides. The myoglobin concentration was obtained from the x-intercept upon linear regression analysis of a plot of the absolute peak areas or volumes of myoglobin peptides or the relative peak areas or volumes of myoglobin and horse peptides as a function of the amount of added human myoglobin standard.

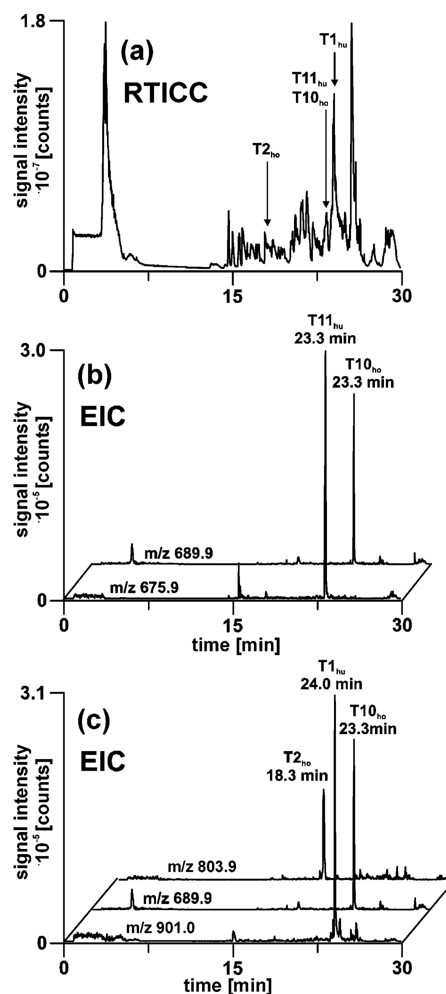

Figure 4. Reconstructed total ion current chromatogram (a) and extracted ion chromatograms of the peptides $T11_{hu}$ and $T10_{ho}$ (b), $T1_{hu}$, $T2_{ho}$, and $T10_{ho}$ (c) used for quantitation of myoglobin in serum. Gradient, 6.0 min isocratic elution with 0.050% aqueous TFA, followed by 0−40% acetonitrile in 0.050% TFA in 15.0 min; sample, 5.0 μL digested myoglobin fraction, containing 1.84 ng/μL human myoglobin standard and horse myoglobin as internal standard. Other conditions as in Figure 3.

Table 3. Results of Linear Regression Analysis Applying Manual and Automated Data Evaluation

human/horse peptides used for quantitation[a]	standards used for calibration[b]	regression parameters[c] a, b, R^2	measured concentration[d] [ng/μL]	relative error[e] [%]	RSD[f] (N) [%]
		Manual Data Evaluation			
Study 1					
$T11_{hu}$/without I. S.	1−6	5.84×10^5, 1.49×10^5, 0.991	0.256	−43.9	11.3 (24)
$T11_{hu}/T10_{ho}$[a]	1−7	0.635, 0.267, 0.971	0.420	−7.89	13.3 (28)
$T1_{hu}/(T2_{ho} + T10_{ho})$[a]	1−7	0.346, 0.132, 0.939	0.384	−16.0	21.0 (28)
Study 2					
$T11_{hu}$/without I. S.	1−7	3.12×10^5, 1.46×10^5, 0.991	0.470	1.51	7.74 (28)
$T11_{hu}/T10_{ho}$[a]	1−7	0.250, 0.096, 0.991	0.382	−17.5	8.94 (28)
Average of relative error and RDS of results obtained with I. S.				13.8	14.4
		Automated Data Evaluation			
Study 1					
$T11_{hu}$/without I. S.	1−7	3.33×10^6, 1.14×10^6, 0.992	0.342	−24.9	8.1 (28)
$T11_{hu}/T10_{ho}$[a]	1−7	0.599, 0.301, 0.967	0.502	10.1	12.5 (28)
$T11_{hu}/T10_{ho}$[a]	1−6	0.649, 0.275, 0.965	0.424	−6.9	13.4 (24)
$T1_{hu}/T1_{ho}$[a]	1−6	0.393, 0.191, 0.976	0.486	6.65	12.5 (24)
Study 2					
$T11_{hu}$/without I. S.	1−8	1.81×10^6, 1.04×10^6, 0.984	0.574	23.9	8.56 (32)
$T11_{hu}/T10_{ho}$[a]	1−8	0.221, 0.105, 0.992	0.474	2.46	7.0 (32)
Average of relative error and RDS of results obtained with I. S.				6.4	11.4

[a] The ratio indicates the peptides that were utilized to calculate relative peak areas. Sequences of the human peptides are given in Table 2; sequences of the horse peptides: $T1_{ho}$, GLSDGEWQQVLNVWGK; $T2_{ho}$, VEADIAGHGQEVLIR; $T10_{ho}$, HGTVVLTALGGILK; $T16_{ho}$, YLEFISDAIIHVLHSK. [b] Concentrations are given in Table 1. [c] (Relative) peak area or volume = $a \times$ standard concentration + b. [d] The measured concentration was calculated as the x-intercept of the regression line. [e] Relative error = $100 \cdot (x_{meas} - x_{true})/x_{true}$; $x_{true} = 0.456$ ng/μL in study 1, 0.463 ng/μL in study 2. [f]

$$\mathrm{RSD} = \frac{100}{x_{meas}} \times \frac{s_y}{a} \times \sqrt{\frac{1}{N} \times \frac{y_{average}}{a^2 \sum (x_i - x_{average})^2}}$$

where s_y, residual standard deviation of regression line; N, number of measurements; $y_{average}$, average of abscissa values, x_i, individual ordinate values, $x_{average}$, average of ordinate values in regression line.

Table 3 collects the quantitative results of manual or automated data evaluation. When the peptide $T11_{hu}$ was used for quantitation without internal standard, the results were spread over a considerably broad range with relative errors of −43.9% to 23.9% and RSDs of 8.1−11.3%. This distribution is most probably a consequence of matrix interferences of other components coeluting with myoglobin in the strong ion-exchange separation, resulting in signal suppression because of coelution of other tryptic peptides with $T11_{hu}$ in the reversed-phase separation. This situation is not uncommon in quantitative analysis of compounds in complex matrices and is usually corrected by relating analyte response to the signal response of an internal standard, present in the sample at constant concentration. Quantitation using the ratio of peak areas of selected human and horse peptides facilitated a significant improvement both in relative deviations form the true value and relative standard deviations. With manual data evaluation, the relative errors and RSDs were consistently below 21% (Table 3), which represent quite decent values for absolute protein quantitation in a complex matrix such as serum.

A significant further improvement in quantitative results was feasible by means of fully automated analysis of the data. In contrast to the manual method of quantitation utilizing the instrument software, this algorithm is able to distinguish between peaks belonging to the peptide and noise based on a sound statistical model. This results in a more precise determination of the regions summed up as mass tracks and thus in a more accurate quantitation. The average relative error (Table 3) in the calculations using relative peak volumes dropped by more than a factor of 2 from ±13.8% with manual data evaluation to ±6.4% with automated data evaluation. Moreover, the average RSD decreased from 14.4% to 11.4%. These quality criteria qualify the elaborated method as among the most accurate and precise so far reported for the absolute quantitation of a medium-abundant protein in serum.

Concluding Remarks

The reliable quantitation of proteins in serum or plasma as matrix still represents one of the most difficult analytical challenges. The difficulties arise from the presence of a few, but highly abundant, proteins in serum, which have to be removed before quantitation of medium- to low-abundant proteins in serum can be performed, and from the unavailability of isotope-labeled proteins, which serve to calibrate the method and to account for losses during sample preparation. This study showed that first-dimension separation at the intact protein level, followed by digestion and second-dimension separation at the tryptic peptide level represents a suitable generic approach to reduce sample complexity before quantitation of selected proteins in serum by liquid chromatography−mass spectrometry. Moreover, it is demonstrated that high-performance anion-exchange chromatography is suitable for the separation of serum proteins having pI values above about 7.5 from the high-abundant proteins albumin, transferrin, and immunoglobulins G in serum. Calibration is feasible upon addition of known amounts of authentic protein to the serum sample. This is essential for the analysis of human serum samples, for which isotope-labeled protein standards are usually not available. In the present form, the proposed method is applicable to proteins present in serum at concentrations of a few hundred picograms per microliter serum. In the future, we will aim at decreasing the detection limits of the method by increasing the loading capacity of first-dimension separation, by using affinity chromatography to

selectively enrich target proteins, and by using the newest generations of high-sensitivity mass spectrometers. The methodological approach can serve as a candidate reference measurement procedure for determination of myoglobin in a human serum matrix. In conjunction with suitable reference materials, it provides traceability of the currently used commercial assays based on antibody recognition of the analyte.

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