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Original Article

Mass Determination of Major Plasma Proteins by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Glen L. Hortin[1,*] *and Alan T. Remaley*[1,2]

[1]Department of Laboratory Medicine, National Institutes of Health, Bethesda, MD; and [2]National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) serves as a rapid and accurate means to determine masses of proteins independent of their shapes or interactions with other molecules. It provides one of the most fundamental characterizations of major plasma proteins. Purified proteins in saline or serum specimens were prepared for analysis by dilution, mixing with a solution of sinapinic acid, and drying on a target plate. Specimens were analyzed in a linear TOF mode with external calibration. Analyses of 24 purified plasma proteins showed predominance of singly charged ions with lesser amounts of dimer and doubly charged monomer, and provided measured masses for these proteins. A number of proteins, including albumin, transferrin, apolipoproteins A-I, A-II, C-I, C-II, and C-III, and prealbumin, could be analyzed directly in serum with appropriate dilution. Measured values for masses of major plasma proteins will assist in analysis of serum and plasma. It is possible to analyze a number of components by MALDI-TOF/MS directly in diluted serum. Extremely simple sample preparation techniques may be useful in analyzing structural variation of several major plasma proteins, particularly those with masses <30 kDa, including a number of apolipoproteins and markers of nutritional status or acute phase responses.

*Author to whom all correspondence and reprint requests should be addressed:
Glen L. Hortin, Department of Laboratory Medicine, National Institutes of Health, Building 10, Room 2C-407, Bethesda, MD 20892.
E-mail: ghortin@mail.cc.nih.gov

Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), invented in the late 1980s (*1*,*2*), serves as a technique for analyzing peptides and proteins in relatively complex samples. It has even been employed for the direct analysis of tissue specimens (*3*). In this technique, specimens containing peptides and protein are dried on a target plate together with a light-absorbing matrix molecule. Vaporization of the mixture of protein and matrix by a laser releases ionized protein molecules, often with a single charge, which can be analyzed in a TOF mass spectrometer. It serves as a simple and rapid analytical technique for determining the molecular weights and heterogeneity of small amounts of protein. In general, intact molecular ions are formed from individual peptide chains, allowing accurate determination of polypeptide mass (*4–6*). However, there may be some technique-dependent variation in measured mass related to variable loss of labile groups, such as sialic acids or association of sodium or matrix with protein molecular ions (*4–10*).

Sample preparation is a key step in the analysis of complex specimens by MALDI-TOF/MS. Variables, such as protein concentration, salt concentration, or specimen fractionation, may strongly influence the observed spectra. Usual approaches for sample preparation for MALDI-TOF/MS involve desalting and cleanup of specimens by techniques such as dialysis, solid-phase extractions, or fractionation through absorption to a specialized target surface (in a variant of MALDI-TOF/MS that has been termed surface-enhanced laser desorption/ionization or SELDI) or to magnetic beads (*5–16*). These preparation steps involve significant time, expense, and potential losses of selected components. In the present study, we examined direct analysis of serum and purified serum components as a very simple approach that might be applicable for routine in the clinical laboratory and that would avoid modification or selective losses of components during preparation.

Materials and Methods

Peptides and proteins used as calibrators and sinapinic acid used as the specimen matrix were purchased from Bruker Daltonics (Billerica, MA). Masses of calibrators were: (bovine insulin)$^+$, 5734.61; (equine cytochrome c)$^+$, 12,361.20; (equine myoglobin)$^+$, 16,952.62; (bovine serum albumin)$^{2+}$, 33,216; (bovine serum albumin)$^+$, 66,431; (bovine serum albumin dimer)$^+$, 132,859. Purified proteins were obtained from the following sources: human holo-transferrin, prealbumin, immunoglobulin G (reagent grade), α_1-antitrypsin, and α_1-acid glycoprotein, from Sigma Chemical (St. Louis, MO); human haptoglobin (mixed types), α_2HS-glycoprotein, Gc globulin, α_2-macroglobulin, immunoglobulin A, neutrophil lysozyme, and C-reactive protein from Calbiochem (La Jolla, CA); β_2-glycoprotein I, Glu-plasminogen, and antithrombin III from Haematologic Technologies, Inc. (Essex Junction, VT). Lipoproteins including apolipoproteins A-I, A-II, C-I, C-II, C-III, and E were purified by standard published procedures (*17*). Purified proteins were generally in stock solutions from 1 to 10 g/L in concentration. Serum specimens were remnants of human specimens submitted to the clinical laboratory used in accordance with a protocol approved by an Institutional Review Board.

Calculated masses of proteins were based on amino acid sequences in well-validated databases, usually the Swiss-Prot database (www.ebi.ac.uk/swissprot). Calculations of polypeptide masses were performed with software offered by the National Institute of Diabetes and Digestive and Kidney Diseases mass spectrometry group (sx.102a.niddk.nih.gov/peptide). Calculated peptide

masses did not include the masses of oligosaccharide components.

Analyses were performed with an Ultraflex TOF mass spectrometer (Bruker Daltonics) in a linear positive ion mode similar to previously described analyses (*12*). Specimens were analyzed with two programs of separation parameters. To examine an m/z range of 5000 to 30,000, pulsed extraction had a delay of 300 ns. To examine m/z > 30,000, there was a delay of 550 ns. Specimens were prepared by dilution with 10 mmol/L ammonium acetate and then by mixing 1 vol of diluted specimen with 2 vol of matrix solution containing 10 g/L sinapinic acid in 40% acetonitrile/10% ethanol/50% water containing 0.1% trifluoroacetic acid. Specimens were applied twice to a 384-position sample plate as 1-μL aliquots and allowed to air-dry. Specimens were analyzed with manual laser positioning and adjustment of laser power. All figures shown represent data collected from 10 positions, summing a total of 300 laser pulses. Measurements of m/z were performed using external calibration. Masses of proteins were measured at the peak apex.

Results

Most serum proteins are globulins that require the presence of some salt to maintain solubility, but salts tend to suppress ionization of proteins in MALDI-TOF/MS. Therefore, there is a compromise between physiological salt conditions that maintain protein solubility and the efficiency of ionization. We examined the ability to analyze purified proteins in saline solutions by MALDI-TOF/MS, using simple dilution of the proteins with matrix solution. Generally, these analyses yielded a pattern in which the singly charged molecular ion predominated, but lesser amounts of dimer, trimer, or doubly charged monomer often were observed, as shown for the examples of α_1-acid glycoprotein, transferrin, and immunoglobulin G as shown in **Fig. 1** panels **A–C**, respectively, or for apolipoprotein A-I and transthyretin in **Fig. 2**. These analyses served as a relatively rapid method to determine the mass of specific components and to evaluate the tendency to form dimers and multiply charged ions.

Table 1 lists the measured masses of 23 plasma proteins that were available as purified components, plus serum amyloid A, which was observed in serum from a patient with an acute phase response as characterized in other studies (*18*). The broader peaks of high molecular weight proteins, probably reflecting both lower resolution and increased microheterogeneity from variable glycosylation, led to greater imprecision in the measurement of masses for these components. Comparison of measured values in **Table 1** vs older published values based on techniques such as compositional analysis, electrophoresis, and ultracentrifugation generally showed fairly good agreement for proteins lacking oligosaccharides. MALDI-TOF/MS provided lower estimates of masses for most glycoproteins than the classical textbook values. Measured values for several proteins with high carbohydrate content such as α_1-acid glycoprotein, antithrombin III, α_2-HS-glycoprotein, and α_1-antichymotrypsin were more than 10% lower than older published values. Measured values for glycoprotein I and plasminogen were about 10% higher than published values.

MALDI-TOF/MS should provide highly accurate of proteins without posttranslational modifications, and, in most cases, there is good agreement for these proteins with calculated masses based on amino acid sequence databases. For proteins bearing complex oligosaccharides and other substantial posttranslational modifications, the sequence databases are not very useful for estimating masses. Values measured by MALDI-TOF/MS should represent accurate estimates with the caveat that there may be some underestimation of mass as a result of loss of some groups

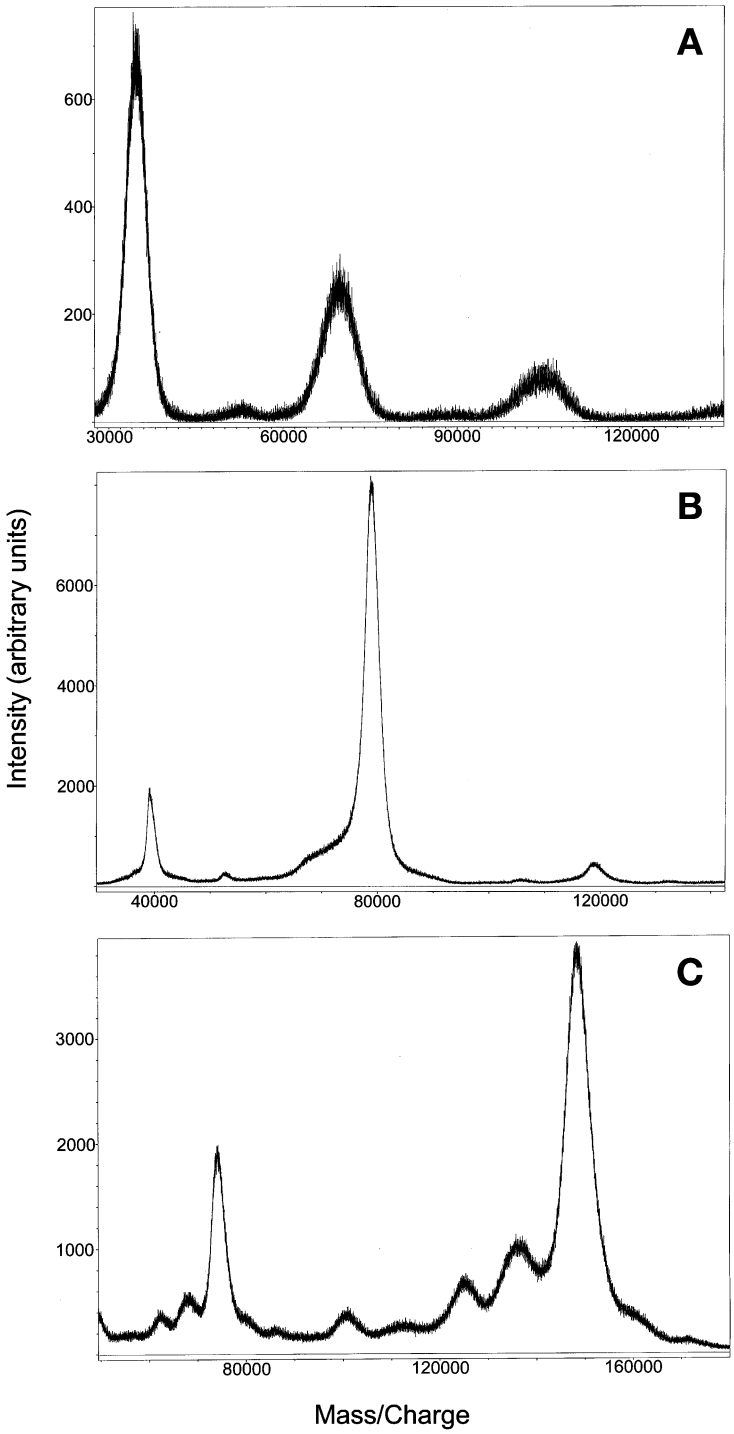

Fig. 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of α_1-acid glycoprotein **(A)**, transferrin **(B)**, and immunoglobulin G **(C)**.

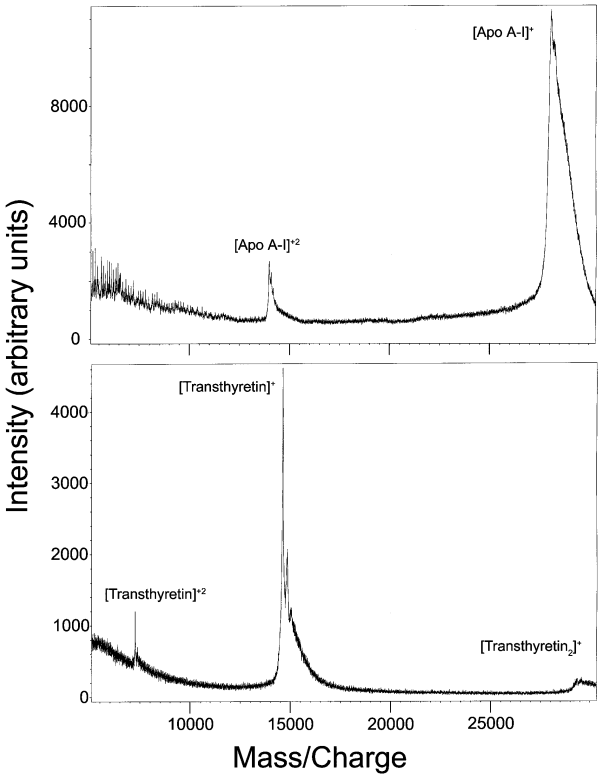

Fig. 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of apolipoprotein A-I **(Top)** and transthyretin also known as pre-albumin **(Bottom)**.

such as sialic acids during desorption and ionization *(6–10)*. Losses of sialic acid may vary depending on technique of analysis, leading to differences in measured mass; as an example, measurements of the mass of α_1-acid glycoprotein by MALDI-TOF/MS vary from about 32,000 to 36,000 in different studies *(5,9,21)*. In general, the ionization efficiency appeared to be poorer for high molecular weight and highly glycosylated components. Haptoglobin and α_2-macroglobulin were not included in **Table 1** as they yielded very weak and diffuse peaks at m/z of about 96,000 and 124,000 for haptoglobin (mixed types) and 178,000 for α_2-macroglobulin.

Serum required an initial dilution by a factor of about fivefold with aqueous diluent before further threefold dilution with matrix to obtain any detectable spectra. The high protein content of serum probably did not allow a high enough ratio of matrix to protein to provide for efficient ionization of undiluted specimens. Matrix usually needs to be in more than 1000-fold molar excess relative to protein to yield good spectra *(6)*. Several diluents were examined such as 10 m*M* ammonium acetate, 10 m*M* ammonium bicarbonate, normal saline, and other solutions with varying ionic strength. Diluents with an ionic strength above that of normal saline suppressed ionization. The solutions of 10 m*M* ammonium acetate and 10 m*M* ammonium bicarbonate were subjectively considered to yield similar results with greatest peak intensities (not shown). A solution of 10 m*M* ammonium acetate was used as diluent for subsequent experiments described here. Dilution of serum with 19 vol of 10 m*M* ammonium acetate plus 40 vol of matrix solution yielded spectra as shown in **Fig. 3**. In the top panel, the m/z range from 5000 to 30,000 is shown and, in the bottom panel, m/z from 30,000 to 100,000 is shown. For m/z > 30,000, singly and doubly charged albumin is the dominant component. A peak corresponding to expected position of transferrin generally was visible and a number of other minor unidentified peaks were observed. If the analysis was extended beyond m/z of 150,000, peaks were observed in the expected positions for albumin dimer and IgG. In the m/z range from 5000 to 30,000, there were a number of sharper peaks, corresponding to the expected positions of apolipoproteins A-I, A-II, C-I, C-II, C-III, and transthyretin. Although some of these proteins, such as apolipoprotein C-I, C-II, and C-III, are often considered relatively minor serum components, they are among the 20 most abundant components on a molar basis *(20)*. In general, ion intensities for individual proteins in MALDI-TOF/MS should relate to the molar concentration of a protein, and there is a strong bias for detection of low molecular weight proteins *(20,21)*.

Table 1
Comparison of Protein Masses Measured by MALDI-TOF/MS vs Reported Values for Traditional Analytical Techniques and Calculated Peptide Mass[a]

Protein	Measured (m/z)	SD	Reported mass (Ref)	Calculated peptide mass/length in amino acids	Peptide modifications. Percentage carbohydrate (CHO)	Database entry
Albumin	66,400	200	66,000 (*38*)	66,438/585 aa or 66,557/586 aa	None or cysteinylated, 0% CHO	P02768 6/15/02
IgG	148,420	180	160,000 (*38*)	Not calculated variable sequence	3% CHO	
Apolipoprotein A-I	28,077	8	28,016 (*39*)	28,079/243 aa	None (*see* text for possible)	P02647 6/15/02
Transferrin	79,110	134	77,000 (*38*)	75,144/679 aa	6% CHO	P02787 6/15/02
α_1-Acid glycoprotein	35,290	350	40,000 (*38*)	21,631/183 aa	45% CHO	P19652 6/15/02
α_1-Proteinase inhibitor	50,300	130	55,000 (*38*)	44,325/394 aa	12% CHO	P01009 6/15/02
Apolipoprotein A-II	8696 17,393	3 12	17,414 (*39*)	8690.9 (monomer)/77 aa 17,379.8 (dimer)/2 X 77 aa	N-terminal pyroGlu monomer or disulfide-linked dimer, 0% CHO	NP001634 10/31/00
Transthyretin (prealbumin) (four subunits)	13,826 13,862	6 4	54,400 4 X 13,600 (*38*)	13,761.5 (subunit)/127 aa	None or cysteinylated, 0% CHO	P02766 6/15/02
α_2HS-Glyco-protein	42,420	130	50,000 (*40*)	32,944/A-chain 282 aa + B-chain 27 aa	Phosphorylation X 2, 13% CHO	P02765 6/15/02
Apolipoprotein C-III	9132 9427	3 3	8800 (*39*)	8764.7 or 9130.0 or 9421.3 or 9712.6/each 79 aa	No CHO or GalNAcGal* or GalNAcGalSA or GalNAcGalSA$_2$	P02656 6/15/02
IgA	160,820	1340	170,000 (*38*)	Not calculated, variable sequence	8% CHO	
Apolipoprotein C-I	6630 6431	1 1	6630 (*39*)	6630.6 or 6432.4/57 or 55 aa	None or loss of N-terminal ThrPro, 0% CHO	P02654 6/15/02

Gc globulin	51,250	160	51,000 (*41*)	51,216/458 aa	4% CHO	P02774 6/15/02
α_1-Antichymo-trypsin	52,330	510	68,000 (*41*)	45,266/400 aa	27% CHO	P01011 6/15/02
β_2-Glyco-protein I	44,940	160	40,000 (*41*)	36,233/326 aa	19% CHO	P02749 6/15/02
Apolipoprotein C-II	8916 10,342	2 3	8900 (*39*)	8915.0/79 aa	None, 0% CHO	P02655 12/8/02
Antithrombin III	57,150	270	65,000 (*41*)	49,033/432 aa	15% CHO	P01008 6/15/02
Plasminogen	90,100	500	81,000 (*41*)	88,386/791 aa	Phosphorylation X 1, 3% CHO	P00747 6/15/02
Apolipoprotein E	34,420	170	34,145 (*39*)	34,237 (E3) or 34,186 (E2) or 34,288 (E4)/299 aa	Sequence polymorphism, 0% CHO	P02649 6/15/02
Serum amyloid A	11,683 11,528	1 1	11,685 (*37*)	11,682.8/104 aa or 11,526.6/103 aa	None or truncated chains, 0% CHO	P02735 6/15/02
C-reactive protein (X 5 subunits)	23,006	15	118,000 5 X 23,600 (*38*)	23,029 (subunit)/206 aa	N-terminal pyroGlu, 0% CHO	P02741 6/15/02
β_2-microglobulin	11,727	4	11,800 (*38*)	11,729.2/99 aa	None, 0% CHO	P01884 9/15/00
Lysozyme	14,690	7	14,500 (*38*)	14,692.7/130 aa	None, 0% CHO	NP00230 1/17/03

[a]The imprecision (SD) was determined by 10 measurements within a single run. Estimated carbohydrate contents were from the references cited for reported masses. Calculated peptide masses were based on indicated database entries.

GalNAc, Gal, and SA indicate N-acetylgalactosamine, galactose, and sialic acid.

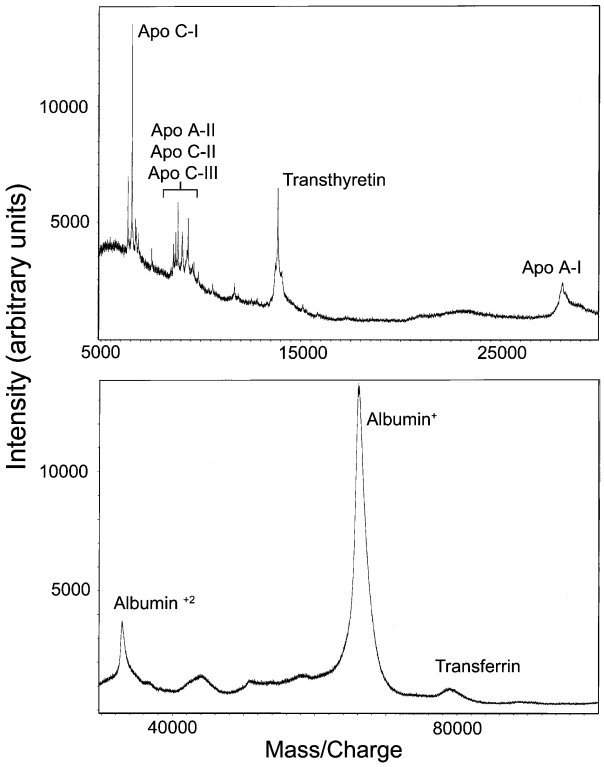

Fig. 3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of diluted serum. **(Top)** m/z range from 5000 to 30,000 with tentative identification of major peaks. **(Bottom)** m/z range from 30,000 to 100,000.

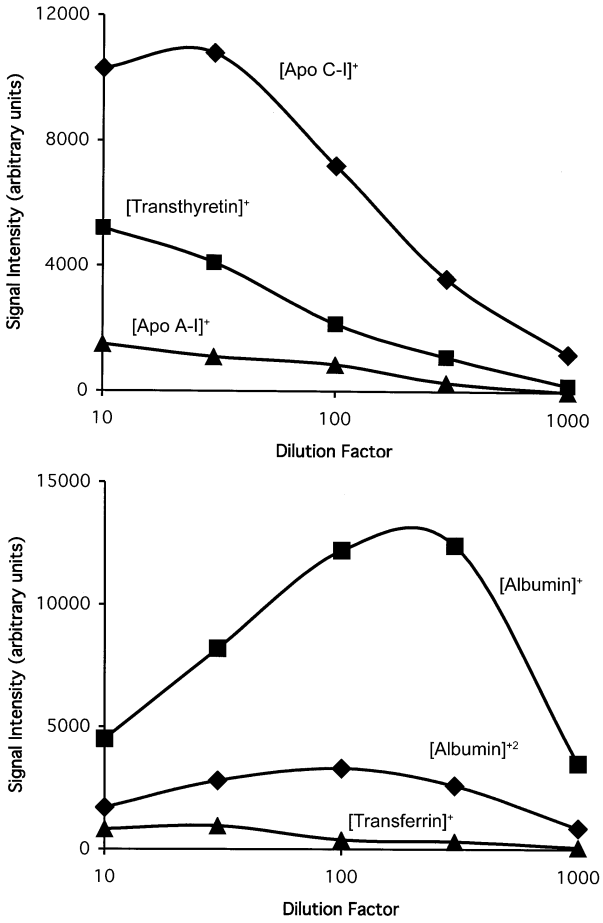

Fig. 4. Peak intensities (peak heights) with varying dilution of serum. Listed dilutions reflect dilution with 10 m*M* ammonium acetate prior to further threefold dilution with matrix solution.

A peak corresponding to the expected position (m/z = 6630) of apolipoprotein C-I was one of the strongest peaks, suggesting unusually efficient ionization of this protein, whereas the ionization of apolipoprotein A-II appeared relatively inefficient considering its higher abundance relative to apolipoproteins C-I, C-II, and C-III. Different peptides and proteins are recognized to have highly variable ionization in MALDI-TOF/MS with content of arginine residues a significant factor in ionization of peptides *(22)*. Although there are reported to be a large number of small peptide components in serum *(23–26)*, components with m/z < 5000 were not observed clearly under the conditions of analysis used here and this region of the spectrum is not shown. Detection of small peptides probably is suppressed owing to their much lower abundance relative to proteins.

Most peaks in the spectra appeared to decrease in signal intensity with increasing dilution with 10 m*M* ammonium acetate as shown in **Fig. 4** (prior to further threefold dilution with matrix solution). Most components had decreasing intensities for dilutions from about 10- to 300-fold; however, peaks related to albumin increased in intensity over this range of dilutions and began to decrease in intensity only at dilutions more than 100-fold. Apparently the concentrations of albumin were saturating at low dilutions. Absolute

Table 2
Major Peaks in MALDI-TOF Mass Spectrometric Analysis of Two Serum Specimens and Tentative Assignments[a]

Measured m/z	Assignment	Expected mass
High m/z region (>30,000)		
147,860 ± 490/147,170 ± 700	$[IgG + H]^+$	~148,000
133,320 ± 410/132,960 ± 270	$[Albumin_2 + H]^+$	132,855
79,180 ± 240/79,360 ± 310	$[Transferrin + H]^+$	79,110
66,540 ± 90/66,550 ± 140	$[Albumin + H]^+$	66,428
33,290 ± 130/33,240 ± 170	$[Albumin + 2H]^{+2}$	33,216
Low m/z region (5000–30,000)		
28,015 ± 15/28,038 ± 12	$[Apo A\text{-}I + H]^+$	28,078
13,876 ± 2/13,868 ± 1	[Prealbumin + Cys + H]	13,946
13,756 ± 2/13,747 ± 2	$[Prealbumin + H]^+$	13,827
9424 ± 1/9417 ± 3	$[Apo C\text{-}III_1 + H]^+$	9422.3
8914 ± 1/8911 ± 1	$[Apo C\text{-}II + H]^+$	8917
8809 ± 1/8806 ± 1	$[Apo A\text{-}II + Cys + H]^+$	8811
8686 ± 3/8680 ± 1	$[Apo A\text{-}II (monomer) + H]^+$	8692
6630 ± 1/6631 ± 1	$[Apo C\text{-}I + H]^+$	6631
6433 ± 1/6432 ± 1	$[Apo C\text{-}I (–ThrPro-) + H]^+$	6433

[a]Values for m/z are means ± SD of six measurements.

intensities of signals in MALDI-TOF/MS usually are subject to substantial variation from one laser shot to the next. The fairly dense application of matrix with serum in this study, however, yielded fairly consistent signals, and variation was reduced somewhat by summing signals from 300 laser shots for each analysis. Because peaks were compared within the same analysis, other peaks within an analysis served as controls for consistency of the results. Comparison of peak ratios between components might be considered a more valid approach for this analytical technique, but it leads to similar results. These dilution studies led to a conclusion that there was an optimal dilution for analysis of most components other than albumin that was about a 10- or 20-fold dilution with ammonium acetate prior to further dilution with matrix.

The measured m/z for a number of peaks in serum samples appeared to correspond to the expected values for several major serum components. Data for repeated analysis of two specimens are shown in **Table 2**. Most peaks corresponded to expected values within the precision of measurement. An exception was transthyretin, which is known to be polymorphic and to undergo a variety of modifications that may affect it mass (*27*,*28*). The mass measurements indicated that it was possible to obtain accurate mass measurements of several of the most abundant components in the complex mixture of serum proteins without extensive fractionation or removal of salts. For the smaller proteins, the pairing of sodium ions or matrix molecules with the protein would have been apparent in mass measurements.

Discussion

Significant information about molecular masses and heterogeneity of concentrated solutions of purified proteins or even the complex mixture represented by serum was obtained by MALDI-TOF/MS simply by

dilution of specimens together with matrix and drying on a target plate. Measured mass values in **Table 1** serve as a database for the expected positions of peaks for a number of serum components when analyzed by MALDI-TOF/MS. These data may be of value in helping to identify which proteins are forming peaks and in identifying peaks that may be useful for internal calibration of masses during an analyses. Of course, masses of some proteins may vary owing to sequence polymorphism, variable posttranslational modification, or degradation during processing or storage. Measured masses of highly sialylated proteins by MALDI-TOF/MS also may vary depending on several variables, as previously noted. Also, it must be considered that there is structural variation in glycosylation of proteins in different physiological states that may lead to changes in glycoprotein mass *(27,28)*.

Direct analysis of diluted serum specimens by MALDI-TOF/MS may offer a rapid and inexpensive approach to analyze several major serum components with minimal opportunities for modification or selective loss of components. For proteins lacking labile posttranslational modifications, the mass measurements determined by MALDI-TOF/MS should be accurate measures of protein mass. For several of the most abundant proteins with masses less than 30,000, mass measurements can be made with sufficient precision and accuracy to allow detection of most chemical modifications of proteins or sequence polymorphisms.

Two drawbacks of the analysis of a complex mixture, such as serum, without fractionation are the detection of only a few of the most abundant components and the general suppression of signals by the high protein and salt concentrations. Dominance of the albumin peaks for monomer, doubly charged monomer, and singly charged monomer interfere with the ability to detect other peaks with $m/z >$ 30,000. Selective immunodepletion of albumin or albumin plus immunoglobulin G may be helpful in examining other protein components in this region. This may be a practical procedure considering that less than 1 µL of serum is required per analysis. For ions with $m/z < 30{,}000$, only several of the most abundant components are observed. Specimen fractionation would be required to see lower abundance components. Consequences of the general suppression of ionization by other ions and proteins are relatively weak signals, detection only of high abundance compoents, and the need to spend more time with data acquisition to sum data from a large number of laser shots. Use of relatively high laser power may be necessary to obtain measurable peaks, and this may lower resolution and promote fragmentation of proteins. Low peak intensities and resolution also can be a limiting factor in obtaining precise and accurate mass measurements.

The method of sample preparation described here offers a simple approach to analyze a number of major serum components. In particular, it appears best suited to analyze components with a mass less than 30,000 where there is better sensitivity, resolution, and less interference from albumin. This technique would be useful for identifying polymorphisms or modifications of components, such as by cysteinylation in the example of transthyretin *(29,30)*. A significant portion of apolipoprotein A-I, appeared as a hump following the major expected peak, suggesting a modification increasing its mass by about 150 Da, which merits further investigation; this protein may undergo fatty acylation and is susceptible to nitration and chlorination *(31,32)*. Alternatively, this may represent adduct formation with a matrix molecule *(6)*. Variants of a number of other apolipoproteins have been described including a truncated form of apolipoprotein C-I, which has lost two amino acids from its N-terminus *(33,34)*. The latter truncated form of apolipoprotein C-I was observed consistently in our analyses.

In conclusion, considerable information about structural variation of small apoplipoproteins and prealbumin may be obtained by the simple technique described here. It would offer applications similar to the previously described characterization of hemoglobin variants by simple dilution of extracts from dried blood spots (*35*). As presented here, the analysis is primarily qualitative in nature. Any effort to apply this a quantitative technique for profiling the relative amounts of different proteins would require extensive investigation of appropriate dilutions, the linearity, and calibration of responses, and standardization of procedures. Qualitative analysis of the global pattern of most abundant proteins may have value for a number of purposes. It may assist in interpreting the degree of fractionation achieved by other techniques such as SELDI-TOF/MS. The major components observed here are likely to show up frequently as peaks during SELDI-TOF/MS analysis (*18*). During the acute phase response, other components, such as serum amyloid A and C-reactive protein, become substantially elevated so that they also may observed (*18*,*20*,*36*,*37*). The simple "dilute and shoot" approach for MALDI-TOF/MS analysis of serum or plasma may be of greatest value in identifying qualitative changes of proteins, such as provided by the previously listed examples. This approach may be of value for clinical applications owing to its simplicity. Some protein variants may be indicators of risk for amyloidosis, cardiovascular disease, or of inflammatory responses. Although there is great interest in trying to apply MALDI-TOF or SELDI-TOF/MS for the analysis of low abundance serum and plasma components, the most abundant components in serum or plasma tend to dominate observed spectra unless there is extensive effort at specimen fractionation (*20*). The present report considers that there is potentially valuable information to be gained from analysis of several of the most abundant components that tend to dominate analyses of unfractionated serum or plasma, just as important clinical information is provided by routine clinical laboratory quantitative or qualitative analysis of major components such as albumin, immunoglobulins, transferrin, and apolipoproteins.

Acknowledgments

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