Serum Protein Profiles by "High-Performance" Liquid Chromatography with Detection at Multiple Wavelengths

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We have separated serum proteins in only 15 min by high-performance liquid chromatography. The identification of several peaks in the chromatographic profile was greatly aided by the use of multiple-wavelength detection. We have found a good correlation between retention times and electrophoretic mobilities. Serum samples with increased γ- or β-globulins, as determined by electrophoresis, resulted in chromatographic profiles with strongly increased peaks in the appropriate regions. This chromatographic method revealed both relative and absolute differences in individual protein concentrations among several serum samples.

The separation of serum proteins by "high-performance" liquid chromatography (HPLC) was first reported several years ago (1, 2). Serum proteins were separated with an anion-exchange column in 30 min or less by gradient elution chromatography. The profile of serum proteins detected by HPLC closely resembled the profiles obtained with gradient elution chromatography on anion-exchange gels (3), except that the HPLC separations were much faster. In a more recent separation of serum proteins by HPLC, the γ- and β-globulin fractions were identified in addition to albumin (4).

The general detection of proteins in a chromatographic separation is readily accomplished by monitoring the absorbance of the column effluent at 280 nm. Typically, chromatographic peaks are identified by determining the retention times of purified standards. However, because of the lack of purified standards and the plethora of proteins in serum, the identity of many of the peaks in a HPLC profile has remained a mystery.

Peak identification can be greatly improved with the use of specific detectors. The identification of serum proteins in electrophoretic profiles was greatly facilitated by the development of immunoelectrophoresis (5). Unfortunately, such a method requires many hours and is not readily compatible with HPLC separations.

Although it lacks the specificity of immunological methods, monitoring the chromatographic separation at multiple wavelengths can provide additional information about peak identity. This method was first used some years ago by Anderson (6) to identify nucleotides in a complex profile by means of their distinct ratios between absorbances at 280 and 260 nm.

Proteins also contain distinctive chromophores, which are associated with the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The spectral bands observed in these aromatic amino acids were also observed in the spectra of proteins by difference spectroscopy (7). Thus, the absorption spectra of proteins should reflect the relative abundance of residues of aromatic amino acids. If these spectral differences are to be recorded at only a few wavelengths, then such wavelengths must accentuate spectral differences, to obtain the maximum amount of qualitative information.

We have obtained purified serum proteins to determine their retention times and relative absorbances at 254, 280, and 305 nm in our chromatographic system, hoping to use this information to identify specific proteins in serum protein profiles obtained by HPLC. We have also obtained clinical samples to determine whether our method can detect both qualitative and quantitative differences in protein profiles between normal and abnormal sera (determined immunoelectrophoretically).

Materials and Methods

Apparatus

Serum proteins were separated with a 4.2 × 250 mm Synchropak AX300 column (Syn Chrom Inc., Linden, IN 47955) and a Varian Model 5020 liquid chromatograph (Varian Instrument Group, Palo Alto, CA 94303). Three variable wavelength detectors (Varian Model UV-50) were connected in series with 150 × 0.23 mm, i.d., stainless steel tubing. These detectors monitored the column effluent at 254, 280, and 305 nm. Absorbance tracings at 254 and 280 nm were displayed with a dual pen recorder; the 280 nm detector output was also connected to a Varian Vista 401 Data System for the determination of retention times and peak areas. The features of the data system are such that peaks appearing to be off-scale in the recorder tracings were fully recovered by the data system.

Chromatography

Gradient elution was begun immediately after a 10-μL injection of raw sera or protein solution. The 15-min linear gradient began at 20 mmol/L tris(hydroxymethyl)aminomethane (Tris), pH 8.0, and ended with 300 mmol/L sodium acetate in Tris buffer, at a flow rate of 1.5 mL/min. Between injections the column was re-equilibrated for 10 min with the starting buffer.

Reagents

L-Tyrosine, DL-tryptophan, L-tryptophylglycine, L-tryptophyl-L-phenylalanine, L-tyrosylglycine, L-tyrosyl-L-phenylalanine, L-phenylalaninylglycine, human serum albumin, transferrin, human immunoglobulin G, and human γ-globulins at the highest available purity were obtained from Sigma Chemical Co., St. Louis, MO 63178. Highly purified α1-acid glycoprotein was from Calbiochem-Behring, LaJolla, CA 92037.

Saturated solutions of amino acids and dipeptides were prepared in starting (Tris) buffer. The solutions were filtered with 0.22-μm av. pore size Millex filters (Millipore Co., Bedford, MA 01730) and diluted until they produced an appropriate detector response at 254 nm. Serum protein solutions, 20 g/L, were also prepared in starting buffer, except α1-glycoprotein, which was only 10 g/L in the same buffer.

Samples

Frozen serum samples were obtained from the Clinical Laboratory at the University of California Medical Center in San Francisco. Normal serum controls were obtained from
Prealbumin, Transferrin ratios, resulting for IgG peptides. 

Table 1. Absorbance Ratios for Some Amino Acids and Dipeptides a

<table>
<thead>
<tr>
<th>Compound</th>
<th>(A_{280}/A_{254})</th>
<th>(A_{305}/A_{280})</th>
</tr>
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<tbody>
<tr>
<td>Tryptophan</td>
<td>1.94 ± 0.02</td>
<td>0.10 ± 0.002</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.06 ± 0.02</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>Phenylalanlyglycine</td>
<td>0.06 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>Tyrosylglycine</td>
<td>3.04 ± 0.05</td>
<td>0.03 ± 0.004</td>
</tr>
<tr>
<td>Tryptylglycine</td>
<td>2.01 ± 0.04</td>
<td>0.13 ± 0.008</td>
</tr>
<tr>
<td>Tyrosylphenylalanine</td>
<td>2.08 ± 0.02</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>Tryptylphenylalanine</td>
<td>1.97 ± 0.02</td>
<td>0.14 ± 0.002</td>
</tr>
</tbody>
</table>

a Six replicate determinations were made by direct injection of a 10-μL solution into detectors. Values shown are mean ± SD.

Helena Labs., Beaumont, TX 77704, and reconstituted according to the manufacturer's instructions.

Procedures
Normal serum, as determined by electrophoresis, was supplemented with purified serum proteins by adding 50 μL of the appropriate protein solution (or buffer, for the control) to 100 μL of serum. The absorbance detectors were calibrated so that the responses at 280 nm were equivalent to within 1%.

Results
Peptide Absorbance Ratios
Peak absorbances at 254, 280, and 305 nm were determined for some peptides and amino acids by direct injection. The resulting absorbance ratios are listed in Table 1. Tyrosine and tyrosylglycine have the highest 280 to 254 nm absorbance ratios, as would be expected from examination of the ultraviolet spectrum of tyrosine (8). Conversely, phenylalanlyglycine exhibits the lowest 280 to 254 nm ratio, which is consistent with the phenylalanine absorption spectrum (8). Predictably, the combination of phenylalanine with tyrosine in a dipeptide results in a lower 280 to 254 nm ratio than that for tyrosine alone. This effect is much more diminished in tryptophylphenylalanine because of the much stronger absorbance of tryptophan. The only aromatic amino acid to absorb appreciably at 305 nm is tryptophan, hence the higher 305 to 280 nm absorbance ratios for tryptophan and related dipeptides. This ratio should be a particularly good index for tryptophan-rich proteins.

Absorbance Ratios for Serum Proteins
Absorbance ratios for a few serum proteins are listed in Table 2. Purified serum proteins were individually chromatographed as previously described. The retention times for each protein and the reported electrophoretic mobilities of these proteins are also listed in Table 2. Compared with the dipeptides in Table 1, the range of absorbance ratios is more limited for the serum proteins. Undoubtedly, primary protein structure is not the sole determinant of absorbance values, and secondary and tertiary structure must also have a role.

The predominance of phenylalanine over the other aromatic residues in albumin (9) is the most likely cause of the lower 280 to 254 nm ratio for albumin in Table 2; this same effect was observed when phenylalanine was combined with tyrosine in the dipeptide listed in Table 1. The other remarkable ratio in this table is the high 305 to 280 nm ratio for immunoglobulin (IgG). This ratio reflects, most probably, the large number of tryptophan residues in IgG (10).

There is substantial similarity between the trends in retention with this method and electrophoretic mobility. The correlation coefficient for these two sets of numbers was 0.99, indicating that electrophoretic mobilities could be used to predict the protein elution order. This relationship was first observed many years ago by Peterson and Chiazze, who found good agreement between the retention times of serum proteins on diethylaminoethylcellulose and the electrophoretic mobilities of the collected peaks (3).

Serum Protein Profile of a Control
The profile of serum proteins in a control material is shown in Figure 1. When the 280 and the 254 nm profiles are compared, the IgG peak changes much more in intensity than the albumin peak does. The 280 to 254 nm peak height ratios for IgG and albumin were 2.51 and 1.84, respectively, and the retention times were 4.5 and 10.9 min, respectively. These data are in reasonable agreement with the corresponding ratios and retention times in Table 2.

The identification of the IgG and serum albumin (SA) peaks in Figure 1 was quite straightforward. These peaks were readily identified by comparing their retention times and peak
Serum Protein Profiles in Human Sera

Normal serum. We chromatographed a serum sample with a normal electrophoretic protein profile. The resulting chromatographic profiles at 280 and 254 nm are shown in Figure 2. The transferrin peak was identified on the basis of its retention time and of the profile resulting from supplementing this serum with purified transferrin.

The albumin peak in Figure 2 is substantially larger than that in the control material. Using the latter albumin peak area as an external standard with a concentration of 39 g/L, we calculated the approximate albumin concentration in the profile shown in Figure 2 to be 53 g/L.

The chromatographic profile recorded at 305 nm revealed a somewhat different profile than those shown in Figure 2. At 305 nm IgG and prealbumin were the highest peaks. The 305 to 280 nm ratio for IgG was 0.19, which agrees reasonably well with that listed in Table 2. But the 305 to 280 nm ratio for prealbumin was 0.71, which cannot be explained by the six tryptophan residues in prealbumin (13). Most likely, prealbumin is binding a ligand with a strong chromophore in the near ultraviolet region of the spectrum. Consequently, the area of the prealbumin peak does not reflect the actual prealbumin concentration.

Abnormal sera. The chromatographic profiles shown in Figure 2 were from a serum sample that exhibited a sharp peak in the gamma region, as determined by electrophoresis. The strong peaks observed near the origin in Figure 3 likely contain IgM. When a gamma-globulin fraction was chromatographed, we observed a diffuse peak near the origin (little or no retention by the column) and a sharp peak at 4.5 min (IgG). The diffuse peak eluting before IgG was probably IgM and polymeric IgA, both of which are too large to penetrate the column.

height ratios with those in Table 2, and confirmed by chromatography of the serum supplemented with albumin or IgG. Also, these peaks are characterized by their large peak areas.

The identification of the peak appearing after albumin was more difficult because purified prealbumin (PA) was not available. On the basis of the relationship between retention time and electrophoretic mobility, prealbumin was a likely choice, being the most prominent serum protein with an electrophoretic mobility greater than albumin (11). Schlabach et al. (12) recently observed a similar peak, appearing just after albumin in the chromatographic profile, that was detectable at both 280 and 340 nm and by fluorescence with excitation around 360 nm and emission above 450 nm; this peak could be selectively removed by immunoprecipitation with anti-human prealbumin.

The within-run precision of the chromatographic analysis was determined by detection at 280 nm. Six replicate injections of the serum control resulted in retention times of 4.67 (SD 0.06) min, 11.37 (SD 0.06) min, and 12.90 (SD 0.09) min, respectively, for IgG, albumin, and prealbumin. The corresponding relative standard deviations ranged from 0.95 to 1.3%. The percentages of total peak area were 53.07 (SD 1.47)%, and 16.11 (SD 0.36)%, respectively, for albumin and IgG. The relative standard deviations in the corresponding area determinations ranged from 0.95 to 4.95%.

The relative amount of albumin in the control was reported by the manufacturer to range between 53.7 and 61.7%. This agrees fairly well with the percentage of albumin found in our chromatographic analysis. From the information supplied by the manufacturer, the mean albumin concentration in the control was 39 g/L.
material and would be expected to elute early. The albumin in this profile was about 37 g/L, as calculated from the previously described external standard.

Although prealbumin appears to be a substantial peak in Figure 3, it was not prominent in the electrophoretic profile. Evidently some strong chromophore enhanced the intensity of the prealbumin peak, particularly at 305 nm. Both thyroxine and retinol-binding protein bind specifically with prealbumin (14, 15). The complex between prealbumin and retinol-binding protein has been reported to have a fairly strong absorption band beyond 300 nm, but prealbumin alone does not (16).

The chromatographic protein profiles in another serum sample are shown in Figure 4. This serum sample had a diffuse increase in the gamma region of the electrophoretic profile. Again absorbance in the region near the origin was increased, but not nearly as much as in Figure 3. The peak at 2.27 min is likely an immunoglobulin, IgM or perhaps IgA; the 280 to 254 nm ratio is about 2.3, which is similar to IgG and suggests a similar tyrosine and tryptophan composition. The increase in IgG absorbance was more than twice that in the normal serum profile. Using the same method as before, we calculated the approximate albumin concentration in this serum sample to be 33 g/L.

A serum sample with increased β-globulins was chromatographed, and the resulting profiles are shown in Figure 5. There is indeed a dramatic increase in the size of the peaks occurring between IgG and albumin. The identity of those peaks is not known, but β-lipoproteins would be good candidates. The approximate albumin concentration in this profile was 35 g/L.

Another serum sample with an increase in the beta region...
of the electrophoretic profile produced the chromatographic profiles shown in Figure 6. These profiles exhibited the least IgG of all. There is an apparent increase in the transferrin peak, and an increase in the region between transferrin and albumin. The large peak immediately after albumin has a different 280 to 254 nm ratio (about 2.2), which suggests that the peak occurring at 12.0 min is neither albumin nor an albumin dimer. This peak could possibly be α-lipoprotein.

Assessment of Protein Profiles in Human Sera

The mean retention times (and SD) for IgG, transferrin, albumin, and prealbumin in the profiles shown in Figures 2–6 were 4.50 ± 0.05 min, 7.25 (0.02) min, 10.85 (0.26) min, and 12.76 (0.09) min, respectively; the range in relative standard deviation was from 0.3 to 2.4%. We estimated that the approximate albumin concentrations in these serum samples varied from 33 to 53 g/L, using the albumin peak in Figure 1 as an external standard.

Discussion

Serum samples found by electrophoresis to have increased concentrations of components in the gamma or beta regions, produced higher peaks in the appropriate regions of the chromatographic profile, as predicted from the relationship between retention time and electrophoretic mobility. Increases in the gamma or beta regions were found to yield several higher peaks in the chromatographic profile. Changes in serum IgG concentration could be readily identified because IgG produces a distinct peak in the chromatographic profiles. Monitoring the chromatographic separation at multiple wavelengths provided qualitative information that could be used to confirm or eliminate possible identifications. When necessary, additional qualitative information can be obtained by collecting peaks and performing further, qualitative tests.

Prealbumin and retinol-binding protein are known to complex, and the retinol content of this complex may have produced the unusually large prealbumin peaks in our profiles. This complex, which was also found to be precipitable with anti-prealbumin (17), fluoresces with excitation around 330 nm and emission above 450 nm owing to the presence of retinol (17). Our conditions are very similar to those reported earlier (12), in which a strongly fluorescent, prealbumin peak, appearing just after albumin in a comparable serum protein profile, was identified by immunoprecipitation (12). Thus, the peak appearing after albumin is probably prealbumin complex with retinol-binding protein.

The chromatographic method we have described seems well suited for serum-protein profiling. Both relative and absolute changes in the protein concentrations can be identified. The speed of analysis, the ability to automate, the good qualitative and quantitative results are all factors that make such a method ideal for establishing normal serum protein profiles, identifying specific abnormalities in consecutive samples, and following specific changes in sequential samples.

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References