Albumin-Bound Fluorescence in Serum of Patients with Chronic Renal Failure

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A band that is strongly fluorescent and migrates electrophoretically with serum albumin is commonly found in electrophoretograms of sera from patients with chronic renal failure. We sought to determine whether the fluorescence originates from binding of certain still-unidentified metabolites or drugs, from an abnormal albumin species, or from some other protein entity. Molecular-exclusion column chromatography, polyacrylamide gel isoelectric focusing, and cellulose acetate electrophoresis, along with results of charcoal treatment and alcohol extraction, provided evidence that the fluorescence comes from fluorescent ligands tightly bound to albumin. The fluorescent intensity of the albumin fraction, isolated by molecular-exclusion chromatography, coincides with the albumin-associated fluorescence determined electrophoretically and with the intensities of the fluorescence emission spectrum for serum. A fluorescent species with an emission maximum of 415 ± 5 nm, separated by thin-layer chromatography, appears to account for the increased serum fluorescence.

Additional Keyphrases: chronic renal disease · chromatography, molecular-exclusion and thin-layer · electrophoresis, cellulose acetate · isoelectric focusing · creatine kinase

On electrophoresis of sera from patients with chronic renal failure a distinct fluorescent band is seen situated close to the creatine kinase BB isoenzyme or between the MB and the BB isoenzymes (1-7). Studies in which iodoacetate (2) and anti-BB antibodies (3, 7) were used to inhibit the enzyme, as well as studies in which the enzyme substrates were omitted (2-6) or where the changes in fluorescence were monitored kinetically (4), provide evidence that the fluorescence in the serum of such patients is not ascribable to enzyme activity. The fluorescent species has not yet been identified, nor is it known whether it is naturally occurring or arises from exogenous dietary or drug sources.

Some studies have shown that the fluorescence is present in end-stage renal failure (4, 8); others indicate that it occurs more widely in chronic renal failure (1, 2, 6) but not in severe, acute renal failure (6, 8). The fluorescence ordinarily seen in serum of patients with chronic renal failure appears to decrease with improvement of the disease and after successful renal transplantation (1, 4, 6, 8).

Few studies have been reported on the biochemical properties of the fluorescent substance. Binding to albumin of the material responsible for the fluorescence is suggested by the fact that it migrates electrophoretically with albumin (1-7), but there is some disagreement as to whether it is actually associated with albumin. For example, the fluorescent substance may not be a protein, because it remains in the supernatant phase after serum proteins are precipitated with trichloroacetic acid (4). Other studies with ultrafiltration suggest that the fluorescence is bound to a substance with a relative molecular mass (Mr) in excess of 100 000 (6). In one study, the material responsible for the fluorescence was precipitated with anti-albumin (9), but in another it was not (6). The substance appears to be non-dialyzable and it is not believed to be a contaminant of the hemodialysis equipment, because it also is present in sera from patients on peritoneal dialysis (6).

Because the fluorescent material in the serum of patients with chronic renal failure could be useful in differentiating between acute and chronic renal failure, we attempted to characterize it further and to provide some quantitative information on its relative concentrations in the serum of patients with chronic renal failure. We used molecular-exclusion column chromatography and isoelectric focusing to show that the fluorescence is indeed associated with albumin. Treatment of samples with charcoal and ethanol provided evidence that the fluorescence comes from ligands bound to albumin. We also describe a preliminary method for isolating the fluorescent material from serum, and discuss its chromatographic properties.

Materials and Methods

Subjects. We studied 12 renal patients with end-stage renal disease and on maintenance hemodialysis. Characteristic laboratory findings for the patients included above-normal values for creatinine and urea and, on occasion, phosphate. Most had low or subnormal erythrocyte counts, hemoglobin concentration, and hematocrit.

Fluorescence emission spectroscopy. To quantitate the fluorescence spectrum, we diluted a 100-µL aliquot of serum to 3 mL with de-ionized water. We recorded the fluorescence emission spectrum from 300 to 580 nm at an excitation wavelength of 345 nm (Hitachi/Perkin-Elmer MPF-214 fluorescence spectrophotometer; Perkin-Elmer Corp., Norwalk, CT 06856). Excitation and emission slit widths were 5 and 16 nm, respectively.

Cellulose acetate electrophoresis. For electrophoresis of the serum samples, we used Titan III Iso-Flur membranes, electrophoretic chambers, buffers, and applicators (all from Helena Laboratories, Beaumont, TX 77701). We made three applications per sample (0.8-1.0 µL/application) to the membranes, followed by electrophoresis at 300 V for 15 min. After electrophoresis, we dried the membranes and scanned them with a scanning fluorometer. The scanning fluorometer had an excitation peak emission of 366 nm and a 410-nm emission ultraviolet cut-off filter. We quantitated peak areas by triangulation. Results are expressed in terms of albumin concentration determined after precipitation with sodium sulfite, ether extraction of globulins, and biuret reaction.

Molecular-exclusion chromatography. To separate albumin and globulin fractions of serum from normal subjects and renal patients on the basis of relative molecular mass, we used a glass column (85 X 1 cm i.d.) packed with Bio-Gel P-200, 100-200 mesh (Bio-Rad Laboratories, Richmond, CA 94894). The column was standardized with a mixture of Blue Dextran (Mr, 2 x 10⁶; Sigma Chemical Co., St. Louis, MO 63178) and purified human serum albumin (cat. no. A9511; Sigma). For

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each analysis, we layered 10 μL of test serum on the top of the packed column and eluted with phosphate buffer (0.1 mol/L, pH 7.2) prepared by mixing appropriate amounts of 0.1 mol/L Na₂HPO₄ and KH₂PO₄. A flow rate of about 80 μL/min was maintained throughout the experiment. The column effluent was monitored for fluorescence and absorbance (280 nm) with a "Fluorichrom" fluorescence detector (Varian Associates, Inc., Palo Alto, CA 94303) and "Fracto-Scan" ultraviolet photometer (Buchler Instruments Division, Fort Lee, NJ 07024), respectively. Filters selected for fluorescence monitoring provided an excitation wavelength of 360 nm and an emission bandpass above 420 nm.

The column eluate passed through the flow-through cell of the ultraviolet monitor, then through the flow-through cell of the fluorescence monitor. Each monitor was connected to a strip-chart recorder (Model 056; Perkin-Elmer). Peak areas were quantitated by cutting out the peaks and weighing them on an analytical balance. The values are expressed as the ratio of albumin fluorescence to ultraviolet absorbance.

Isoelectric focusing. For analytical-scale disc isoelectric focusing of serum samples we used a Bio-Rad Model 150-A electrophoresis cell. Each 20-μL serum sample was mixed with 1.3 mL of freshly prepared gel solution immediately before casting in a 75 × 5 mm (i.d.) gel tube. The gel solution contained, per liter, 75 g of acrylamide, 2.5 g of N,N'-methylene-bisacrylamide, 18.8 mL of 3/10 and 31.3 mL of 4/6 carrier ampholytes, and 9 μmol of riboflavin ("Biolyte") ampholytes were supplied in 400 g/L solutions; Bio-Rad). After casting, each gel was photo-polimerized for 1 h and then focused at 4 °C for 2 h at 200 V. The cathode and anode solutions were 2.5 g/L NaOH and 30 mmol/L H₂SO₄ respectively. After focusing was completed, the polyacrylamide gels were removed from the tubes, viewed under long-wavelength ultraviolet light, then stained with 40 g/L bromphenol blue in ethanol/water/acetic acid (10/9/1 by vol) to locate the point of migration of the fluorescent substance and of albumin. Purified human serum albumin was used as a standard.

Fluorescence removal with charcoal. We removed the fluorescent substance from serum with charcoal by adjusting 200 μL of each sample to pH 3.0 with 0.2 mol/L HCl, diluting with water to a total volume of 0.8 mL, adding 9 mg of Norit A charcoal, and mixing with a magnetic stirrer for 1.5 h in a cold room at 4 °C. We removed the charcoal by centrifugation (20 000 × g, 20 min). We removed the supernate, diluted to 3 mL, and verified the removal of fluorescence by fluorometric analysis as previously described.

Fluorescence removal with alcohol. We added 4 mL of absolute ethanol or methanol to 400 μL of serum, shook the mixture for 15 min on an Eberbach shaker, then centrifuged to precipitate proteins.

Results

Molecular-exclusion chromatography and cellulose acetate electrophoresis. We examined sera from renal-disease patients and controls by molecular-exclusion chromatography and cellulose acetate electrophoresis, to determine whether the fluorescence seen by fluorometric emission spectroscopy is associated with serum albumin (Table 1). The fluorescence values for the P-200 method are expressed as the ratio of albumin fluorescence to ultraviolet absorbance of the column effluents. Values by the electrophoretic method are expressed in terms of albumin concentration. For patients with renal disease, the mean P-200 albumin fluorescence ratio was 0.35 (SD 0.10), with a range of 0.23 to 0.50. For apparently normal subjects, the mean ratio was 0.10 (SD 0.02), with a range of 0.07 to 0.12. The albumin fluorescence ratio determined after electrophoresis on cellulose acetate was 1.63 (1.1) for patients with chronic renal disease and 0.47 (0.20) for normal subjects. The standard deviations indicate that each method gives comparable separations between values for renal-disease patients and those for the normal group.

The intensities of emission fluorescence for serum from both patients and normal individuals are related to the P-200 albumin fluorescence ratios (r < 0.01, r = 0.67, n = 17), additional evidence that the 440-nm fluorescence in serum is associated with albumin. The albumin fluorescence measured after electrophoresis on cellulose acetate is also correlated with that derived from the P-200 method (r < 0.05, r = 0.63).

Because fluorescence increases and albumin generally decreases in sera of patients with chronic renal failure, values for both the P-200 and the electrophoretic method are expressed in terms of albumin. The albumin-bound fluorescence separated by gel-filtration was 2.24-fold that of the normal individuals; however, when expressed on an albumin basis, the difference between renal patients and normals is 3.5-fold (Table 1). As a result, the means—and presumably diagnostic sensitivity—between disease and non-disease are more distinct. With the electrophoretic method, the mean fluorescence intensities of albumin from renal-disease patients were 3.04-fold those for normals; when calculated on an albumin basis, they were 3.47-fold greater.

Isoelectric focusing. Observations of the polyacrylamide gel patterns after isoelectric focusing further support the contention that the fluorescence is associated with albumin. The two fluorescent bands observed immediately after isoelectric focusing corresponded in position to the two albumin bands seen after staining. The albumin bands reportedly have isoelectric points of about 4.8 and 5.0 (12). Each serum sample

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<th>Table 1. Relative Intensities of Serum- and Albumin-Bound Fluorescence in Sera of Patients with and without Chronic Renal Failure</th>
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<td>Mean (SD)</td>
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* Average creatinine values for persons with chronic renal failure and for normal individuals were 98 (SD 35) and 8.4 (SD 0.05) mg/L, respectively. These values showed no correlation with fluorescence intensity.

** Serum fluorescence (arbitrary units) was determined from the fluorescence emission spectra of a 30-fold dilution of serum.

* Ratio of albumin-bound fluorescence to ultraviolet absorbance as determined after molecular exclusion chromatography.

* Ratio of albumin-bound fluorescence to albumin concentration as determined after electrophoresis on cellulose acetate.
from a renal-disease patient displayed fluorescence associated with both albumin bands. The relative fluorescence intensities of the albumin-associated bands were not quantitated by scanning fluorometry, but the patterns from 10 patients with renal disease were correctly distinguished from the patterns for five normal persons by an individual who was uninformed as to the patient status.

Removal of fluorescence with charcoal and alcohol. Results of the three methods provide evidence that the fluorescence seen in serum is associated only with albumin, because it is unlikely that a fluorescent species distinct from albumin would migrate with albumin on electrophoresis, display the same two isoelectric points as albumin, and yet have the same molecular-exclusion chromatographic properties. Even though it appeared likely that the fluorescence was due to a ligand bound to albumin, structural alterations in the albumin molecule could be the cause of the increased fluorescence. Albumin does fluoresce naturally (10) and, in uremic patients, there are changes in the amino acid composition of albumin (12), in the ratios of the two albumin bands found after isoelectric focusing (12, 13), in the alpha-helix content (14), and in the drug-binding properties (11–13). We therefore performed experiments with charcoal and with ethanol to investigate which of the two possibilities is responsible for the fluorescence.

We first examined the effect of pH on serum fluorescence stability and found that it did not change between pH 3 and 12, but declined rapidly at values <3 or >12. The loss in fluorescence is irreversible: the fluorescence is not restored by readjusting the pH of the samples to neutral. With charcoal present, a lowering of pH from 7.5 to 3.0 increased the removal of fluorescence from serum, from 25.7% to 50.7%. This decrease in fluorescence was not the result of pH quenching, because a corresponding sample treated identically, except without charcoal, was used to establish total fluorescence. Also, the decreased fluorescence was not caused by adsorption of protein on charcoal, as determined by protein analysis of the supernatant phase.

Our attempts to desorb the fluorescent material from charcoal with methanol were not successful, but we could remove a constant proportion of the fluorescence from serum with a simple alcohol precipitation of protein, 4 mL of ethanol or methanol being added to 400 μL of serum. The samples were mixed for 15 min, centrifuged, and the fluorescence spectra were determined on the resulting alcohol-aqueous phase. Emission maxima for the samples from renal patients were at 415 (range 5 nm) and the fluorescence intensities of the ethanol extracts correlated with that of serum (γ = 0.31x + 4.9, r = 0.60, n = 18, P < 0.01). The presence of native fluorescence in serum makes an accurate calculation of recovery difficult, but, based on total serum fluorescence, our recoveries were approximately 34% with ethanol and 50% with methanol.

Thin-layer chromatographic properties of the fluorescent compounds. To obtain basic information as to the number of fluoroescing compounds and their chemical properties, we used thin-layer chromatography. The ethanol-aqueous phases were evaporated and the residue was reconstituted in methanol before being applied to a reversed-phase thin-layer chromatographic plate (Analtech, Inc., Newark, DE 19711). The plates were developed with methanol/water (40/60 vol) and different areas of the plate were removed and eluted with methanol. Based on the characteristic emission spectrum at 415 nm, a fluorescent species with a Rf value of 0.76 appears to account for the increased fluorescence in the ethanol extracts and in serum.

A single spot (Rf 0.90–0.70) was also found when the samples were chromatographed on silica gel plates (Analtech, Inc.) with methanol as a developing solvent. Methods with greater resolution may show that more than one fluorescent compound is present.

Fluorescence spectra of metabolites. We checked the fluorescence spectra of several vitamins and a number of metabolites that are known to increase with renal disease: uric acid, urea, creatinine, β-phenylpyruvic acid, methyl guanidine, guanidinosuccinic acid, hippuric acid, p-hydroxyphenylacetic acid, indoleacetic acid, indolepropionic acid, pyridoxine, and pyridoxal-5-phosphate. These compounds, added to serum, either did not exhibit fluorescence or the emission spectrum differed clearly from that of the material from renal patients.

Discussion

Thus far, our characterization of the fluorescent material in serum of patients with chronic renal disease has established that the fluorescence is not attributable to albumin itself, but to a fluorescent species that is bound to albumin, which can be removed with charcoal treatment under acidic conditions or with ethanol or methanol. As determined by reversed-phase and silica gel thin-layer chromatography, at least one fluorescent species with an emission maximum at 415 nm accounts for the increased serum fluorescence. The compound is soluble in water, methanol, ethanol, and butanol, but not in less polar immiscible solvents. Its fluorescence is greatly diminished at pH values of <3 or >12. In disagreement with a previous report (4), we found no fluorescence in the supernatant phase after serum proteins were precipitated with trichloroacetic acid (200 g/L). If any of the fluorescent material were transferred from albumin to the supernatant phase under these acidic conditions, our studies indicate that its fluorescence would be quenched.

We have presented some quantitative data on the amount of albumin-bound fluorescence in patients with end-stage renal disease. Differences between albumin-bound fluorescence in patients with renal disease and those without renal disease are greater if the fluorescence is expressed in terms of albumin. Extraction of the fluorescent material with alcohol and subsequent analysis of the alcohol phase, directly or by some other technique such as liquid-chromatography, should result in even greater separation as well as in increased specificity.

References

Stability of Standard Curves Prepared for EMIT® Homogeneous Enzyme Immunoassay Kits Stored at Room Temperature after Reconstitution

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We examined the stability of standard curves obtained with use of homogeneous enzyme immunoassay reagents (EMIT®, Syva Corp., Palo Alto, CA) for assay of lidocaine, procainamide, N-acetylprocainamide, gentamicin, and theophylline, when stored at room temperature (23–24 °C) after reconstitution of the lyophilized materials. Standards were run and curves obtained for as long as 16 days after reconstitution. All standard curves were acceptable, according to the criteria specified in Syva product literature. Absorbance changes for all calibrators, including the zero calibrator, increased gradually with time. Increases of non-zero calibrators were largely offset by a parallel increase in the zero calibrator, such that the quantity ΔA – ΔA₀ was very nearly constant with time. Standard curves based on the quantity ΔA – ΔA₀ can be used for longer than curves based only on ΔA.

Additional Keyphrases: economics of laboratory operation, variation, source of, quality control, drug assay, enzyme-multipled immunoassay

The homogeneous enzyme immunoassay technique (EMIT®, Syva Corp., Palo Alto, CA 94304) is widely used to measure and monitor drug concentrations in serum (1). The EMIT system provides rapid determinations, requires only small sample volumes and no specialized or expensive apparatus, and obviates use of radioactive material. Results correlate well with those by other techniques (2–4). Calibration curves for EMIT assays are said to be usable for one working day (1), if not longer, and the reagents, reconstituted according to the manufacturer’s instructions and stored at 2–8 °C when not in use, are said to be usable for 12 weeks (Syva package insert).

We desired to use the EMIT system for urgent ("stat") assays on a 24-h basis. Reconstituted reagents had to be stored at room temperature, rather than at 2–8 °C as recommended by Syva, to avoid the delay of warming the reagents before use. Two questions immediately arose. How long is a standard curve valid under these conditions? And how quickly do the reconstituted reagents deteriorate if stored at room temperature (23–24 °C) rather than at 2–8 °C as the manufacturer recommends? We undertook the following experiments to answer these questions.

Materials and Methods

EMIT kits (all lot no. J02) for assay of lidocaine, procainamide, N-acetylprocainamide (NAPA), gentamicin, and theophylline were provided by Syva. These kits consist of two reagents and six calibrators, all lyophilized, and a buffer in concentrated liquid form.

For all assays we used a Stasar III spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074) coupled to a Syva Model CP-5000 clinical processor. For sample and reagent pipetting we used a Syva Model 1500 pipettodilutor.

Kits were reconstituted with de-ionized water as prescribed in the instructions provided with the kit. For the 50-test kits (lidocaine, procainamide, and NAPA), two kits were reconstituted at the same time and combined. [Because reagents A and B are matched to each other within each lot (Syva package insert), only kits from the same lot may be combined.] We combined buffer from all kits, because the same one is used in all.

Assays were performed as described in the kit instructions. We used the "two-point" mode of the CP-5000, in which the calculator reads the absorbance of the reaction mixture after 15 and 45 s and prints out the change in absorption (ΔA). Once the mean ΔA value for duplicate zero calibrators is determined (ΔA₀), the calculator subtracts this number from the ΔA values of all subsequent standards and unknowns. The resulting "ΔA – ΔA₀" values, five points corresponding to the five non-zero calibrators in each kit, are then plotted vs drug concentration to give the calibration curve.

All kits were reconstituted on Day 0 of the experiment, and the lidocaine, procainamide, and NAPA kits were combined as described above. All kits were then stored overnight at 4 °C.

1 These values are not standard absorbance values as defined by Beer's law. According to the instructions provided with each EMIT kit, the spectrophotometer is run in concentration mode with a concentration factor of 2.667. Although the readings are referred to by the manufacturer as absorbance, they are actually 2.667 times the absorbance. All commercial EMIT assays use this nonstandard terminology, so we have chosen to avoid confusion by using it throughout this paper.