High-Resolution Densitometry: Analysis of Stained Albumin Bands as a Model for Electrophoresis of Serum Proteins

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In discussing the principles of quantitative analysis in thin-layer media, we show that requirements for quantitative analysis are not satisfied when stained protein electrophoretic bands are scanned with a conventional rectangular-slit densitometer. We investigated a high-resolution densitometer based on a linear photodiode array as an alternative analytical tool, using stained electrophoretic bands of radio-labeled human serum albumin as a simplified model for results of serum protein electrophoresis. Identical protein bands scanned with both the high-resolution densitometer and a conventional densitometer were quantified with improved accuracy and precision by the new instrument. We also used the high-resolution densitometer to develop a computer model for performance characteristics of a rectangular-slit densitometer.

Additional Keyphrases: rectangular-slit densitometer compared · radioassay · computer model · "thin-layer" analysis

Quantitative analysis of thin-layer separation methods requires fulfillment of several basic criteria:

- The analyte or analytes of interest must be retained quantitatively in the thin-layer support during the separation process. This requirement is generally fulfilled in clinical electrophoretic and TLC methods.

- After the separation step, a chemical reaction generates a chromophore or fluorophore in the support medium wherever the analyte is present. The amount of chromophore generated by this staining reaction should be proportional to the mass of analyte present (7).

- The chromophore must be quantitatively measured in situ in the thin-layer support.

This last requirement defines the scope of densitometry, and the lack of adequate densitometric measurements has been a major obstacle to implementation of accurate and precise quantitative methods with thin-layer media (8).

The theoretical and technical limitations of traditional densitometric methods have been discussed by several authors (7, 8), who generally agree that the rectangular-slit design of conventional densitometers limits the potential accuracy and precision of these instruments. We recently described a new instrument in which a photodiode array and computer reduction of data achieve quantitative analysis of chromophores in thin-layer media (3). Using this instrument as a high-resolution densitometer, one can map the light intensity at every point in the plane of a thin-layer support medium. The digitized image represents a complete quantitative description of the photometric data in the thin-layer plane. Appropriate mathematical algorithms may then be devised to calculate chromophore concentration, the shape and location of chromophore zones, and other quantities of interest.

In the study we report here, we used stained electrophoretic bands of human serum albumin as a simplified model for protein electrophoresis. After scanning these bands with the high-resolution densitometer, we used mathematical algorithms to simulate the operation of a traditional densitometer. This analysis provides new insight into the limitations of fixed-slit densitometers, and suggests methods whereby accuracy and precision in quantitative analysis of thin-layer techniques can be improved.

Materials and Methods

Instrumentation. For high-resolution densitometry of stained electrophoresis bands we used an experimental apparatus based on a linear photodiode array. Details of the instrument design and performance characteristics have been previously published (3). In brief, we used a linear photodiode array to subdivide stained protein bands in a grid-like arrangement and measure the transmittance in each small surface element. The photodetector consists of 512 discrete photodiodes (pixels) arranged in a 1.28-cm linear array. We positioned a stained protein band over the array and measured the light intensity at each small surface element above the array. A stepping motor and gear drive moved the cellulose acetate sheet over the detector in small increments. The inherent spatial resolution of the photodiode array is 25 × 25 μm, but we measured individual surface elements of about 150 × 150 μm. Analog values of light intensity were converted to binary values with a high-speed analog-to-digital converter. The digitized data were transmitted to the computer, and the digitized image was stored in memory or on magnetic tape.

In the experiments with a conventional rectangular-slit densitometer, we used a commercial instrument (AutoScanner Flur-Vis; Helena Laboratories, Beaumont, TX 77704), following the manufacturer’s operating instructions. In all experiments, we used the slit template that generated a rectangular beam of about 4 × 0.5 mm. We used the integrator feature of the instrument to determine the areas under the individual densitometer peaks.

Human serum albumin. We labeled serum albumin with 125I by using a commercial preparation of immobilized glucose oxidase/lactoperoxidase (Enzymo-Beads; Bio-Rad Laboratories, Richmond, CA 94804), and crystalline human serum albumin (Sigma Chemical Co., St. Louis, MO 63178), 1 g/L, dissolved in sodium phosphate buffer (0.2 mol/L, pH
7.2), according to the procedure recommended by Bio-Rad. After incubating the reaction mixture for 40 min at room temperature, we poured it onto a 0.5 × 15 cm column of Sephadex G-25 M (Pharmacia Fine Chemicals, Piscataway, NJ 08854) and eluted the labeled albumin with more of the same sodium phosphate buffer. The labeled albumin was eluted in the void volume. We used it without further purification. The specific activity of the $^{125}$I-labeled albumin was about 5 Ci/g. We then added 0.1 mL of the radiolabeled albumin to 50 mL of 40 g/L human albumin in phosphate-buffered saline and serially diluted to obtain labeled albumin solutions of 40, 20, 10, 5, and 2.5 g/L.

Protein electrophoresis. We used the "Zip Zone" electrophoresis system (Helena Laboratories) to prepare stained albumin bands. Known concentrations of labeled human serum albumin were applied to presoaked cellulose acetate sheets with the manufacturer's applicator. After electrophoretic migration, the albumin samples were fixed and stained with Ponceau S, according to the Helena protocol for serum protein electrophoresis (9). The cellulose acetate sheets to be scanned with the densitometer were cleared according to the manufacturer's directions. In other experiments we eluted Ponceau S dye from uncleared sheets by cutting out the bands of interest and soaking them in 2.0 mL of 0.1 mol/L NaOH for 1 h with intermittent vortex-mixing (10). The eluted dye solution was cleared by centrifuging (15 min, 2000 rpm). We quantified the concentration of Ponceau S dye in the eluate by measuring absorbance at 550 nm.

Data analysis. For all data analysis we used a desk-top computer (Model 9845B; Hewlett-Packard, Fort Collins, CO 80525). Computer programs were written in Hewlett-Packard BASIC. Selected densitometer response curves were fit to the function $y = Ax - Bx^{2}$ where $y$ was the densitometer integral and $x$ was the actual albumin mass in an electrophoretic band. We used a computer algorithm in which a value for the coefficient $A$ was selected, after which an iterative procedure determined the value of $B$ that gave the lowest variance. By repeating this process with small increments in the value of $A$, we determined the values for $A$ and $B$ that gave the minimum combined variance. Plots of computer-generated curves were prepared on an $x$-$y$ plotter.

Results and Discussion

Theory of the fixed-slit densitometer. Figure 1A shows an idealized model of a stained albumin electrophoretic band being scanned with a rectangular slit of light. The slit travels along the $y$ axis, which is the direction of electrophoretic migration. Note that slit width is defined as the dimension of the slit in the $y$ direction, and slit length is the dimension of the slit in the $x$ direction. Absorbance at each point in the $x$-$y$ plane is represented by elevation above the plane. The diagram emphasizes several prerequisites for accuracy of this method: (a) Slit length in the $x$ direction is shorter than the chromophore band and the slit is accurately centered so that the edges of the band are not included in the slit; (b) slit width in the $y$ direction is small with respect to the $y$ dimension of the chromophore band; and (c) at any instant in its traversal of the band, the chromophore under the slit is uniformly distributed. The last point can be expressed mathematically by saying that within the part of the chromophore band examined by the slit, the gradient of light intensity is uniformly 0 (df/d$x$ = 0 and df/d$y$ = 0). An example of uniform chromophore distribution is shown in Figure 1B, which represents a cross section of Figure 1A at the $y$-coordinate indicated with an arrow.

The reason for these criteria is that a single photodetector behind the rectangular slit measures the average of transmitted light in the slit, whereas the quantity of interest is

![Idealized Protein Band](image1)

Fig. 1. (A) Three-dimensional plot of an idealized stained protein band being scanned with a rectangular slit of light moving in the $y$ direction; (B) cross section of A at the $y$-coordinate indicated by the arrow.
over the full range of albumin concentrations indicated that protein elution was also quantitative, and that dye binding was linear with respect to albumin mass.

Quantification of protein in noneluted protein bands. Because clearing cellulose acetate sheets before densitometry precludes eluting the protein separately, we needed to quantify the protein in cut-out albumin bands without elution. Figure 4 shows the results of an experiment in which, after electrophoretic migration and staining, we cut out each stained zone from the surrounding cellulose acetate. The stained protein band was not cleared. After the radioactivity of intact protein band was counted, we eluted the protein and measured the radioactivity in the eluate. Precise linearity of this plot of these two values for each standard establishes that counting an intact protein zone yields a precise determination of protein mass, and that individual protein bands can be accurately quantified without elution. In subsequent experiments, we measured protein mass by counting the $^{125}$I radioactivity in intact, cleared, and stained protein zones.

Photometric accuracy of the high-resolution densitometer. Before comparing the performance of the high-resolution densitometer with that of a conventional scanned slit densitometer, we wanted to ascertain that both instruments were photometrically accurate; that is, we wanted to demonstrate that differences between measurements made by the two densitometers derived from different handling of an inhomogeneous chromophore distribution and not from an error in optical or electronic design. Consequently, we determined the absorbance of a graduated series of neutral density filters on both instruments. The high-resolution densitometer and the commercial densitometer were both accurate for analysis of homogeneous specimens; with neutral density filters, the absorbance measurements from the two instruments were in excellent agreement up to an absorbance of 1.6.

Advantages of the high-resolution densitometer. As a model for actual electrophoretic specimens, we migrated, stained, and cleared 20 radiolabeled albumin bands. We scanned each band with the fixed-slit densitometer, using standard procedures to align and center the chromophore zones under the slit. The quantity of protein in each band was determined as the integral of the densitometer peak for that band. The slit length was 4 mm, compared with an average length of the albumin bands of 7 mm. We then scanned the same specimens with the high-resolution densitometer. Centering the specimens over the detector was not important because the photodiode array extended well beyond the edges of the albumin band. The absorbance was calculated for each pixel in the digitized image, and all absorbances were summed to yield the integrated absorbance over the entire albumin band.

The most conspicuous difference between these plots (Figure 5) is the increased curvature in the line of best fit for the conventional densitometer. The high-resolution response curve has significantly less curvature, and underestimation of high-protein concentrations is markedly lessened. This improvement is expected because chromophore gradients are larger in high-concentration samples, and averag-
ing over a large slit will introduce greater relative errors in this setting. Also, the high-concentration protein bands are longer, so that a fixed-length slit will subdue a proportionally smaller fraction of the total band.

Another difference between the curves in Figure 5 is the scatter of individual points around the line of best fit. In the response curve for the high-resolution densitometer, all points are very close to the line of best fit ($r = 0.9992$). Scatter of individual points around the line of best fit is conspicuous in the conventional densitometer data ($r = 0.9907$) and can be attributed to variation of chromophore distribution in each band and random errors in positioning and centering the specimens in the densitometer. These sources of error are minimized with the high-resolution densitometer.

**Deficiency of the staining reaction.** Although the response curve from the high-resolution densitometer represents a definite improvement in accuracy and precision, the integrated absorbance of protein bands is still not a linear function of protein mass. The residual inaccuracy at high concentrations of protein is explained by the fact that the absorbance of Ponceau S dye in cleared cellulose acetate does not follow Beer’s law at higher concentrations. Microscopic examination of a stained and cleared protein band reveals that the dye is not in solution, but rather is a suspension of stained particles in the cleared cellulose acetate. The failure to follow Beer’s law is thus not surprising. The availability of an accurate, precise photometric technique highlights this failure of the staining method, which had largely been obscured by the superimposed inaccuracy of conventional densitometry. Several remedial approaches can rectify this deviation from Beer’s law—use a stain that follows Beer’s law or, alternatively, simply abandon the traditional dependence upon Beer’s law. When analyte concentration is computed with digital processing instead of analog electronics, the only requirement for quantitative analysis is that light intensity and analyte mass be related by a smooth and reproducible function. Rather than transforming light intensity to absorbance, one could store an empirical function in computer memory to transform light intensity measurements to analyte concentration. We have used this approach extensively in the quantitative analysis of reflectance from multilayer films (6, 11, 12).

**Computer model for a rectangular-slit densitometer.** The digitized photometric image of an albumin band generated by the high-resolution densitometer represents a complete optical description of the specimen. Thus, mathematical algorithms can be devised to simulate the performance of any densitometer design that measures light transmittance of albumin bands. Figure 6 demonstrates the results of an experiment in which a computer program modeled the performance of a fixed-slit densitometer. In the top tracing the slit length was about one-half of the length of the albumin band and slit width was about 1/10 of the width of the albumin band. By the computer algorithm, this slit was moved over the center of the digitized image and the average light transmittance for all pixels within the model slit was calculated at each point in the scan. Average transmittance within the slit was converted to absorbance, and absorbance was integrated over the entire scan. Note that the upper curve of Figure 6 is very similar to the curve produced by a commercial fixed-slit densitometer (lower plot, Figure 5); imprecision and curvature of the two plots are similar. In the lower curve of Figure 6, the width of the model slit was extended to 2.4 mm (more than one-half of the albumin band width). The excessively wide slit results in severe attenuation of the bands for high albumin concentra-

In summary, although Ponceau S dye uptake by human serum albumin is a linear function of albumin mass, quantification of stained albumin bands with a conventional fixed-slit densitometer is both inaccurate and imprecise. The inaccuracy of such measurements is attributable to two factors: (a) A single photodetector determines the average transmittance of light within the slit, from which absorbance is calculated; nonuniform distribution of chromophore within the large slit will cause systematic underestimation of albumin concentration. (b) Transmittance of light by dye in the cleared cellulose acetate sheet does not obey Beer’s law, so that the logarithmic transformation of transmittance measurements to absorbance is not appropriate. By using a high-resolution densitometer based on a photodiode array, we can solve the first problem by sampling light intensity at each point in a highly resolved grid. Replacing the logarithmic transformation with a computer-derived calibration curve could render unimportant the non-Beer’s law behavior of stained protein aggregates in the cleared cellulose acetate.

**References**


