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Computerized Quantitative Analysis of Coomassie-Blue-Stained Serum Proteins Separated by Two-Dimensional Electrophoresis

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Two-dimensional electrophoresis in combination with Coomassie Blue staining was refined for use as a quantitative method. Microcomputer software was developed for use with the IBM AT and compatible computers for analyzing the gels. To test the refined method to determine its usefulness in simultaneous measurements of 28 human serum proteins, we measured each protein relative to a single standard (bovine serum albumin) polymerized at different concentrations in a calibration scale, rather than using 28 individual standards. All samples were analyzed in triplicate. We evaluated calibration, linearity of response, recoveries, units, within-run CV, and between-run CV. The five isoforms of apolipoprotein A-I were analyzed in samples from 16 healthy donors and the isoform ratios determined. The method as presented here should prove useful for diagnosis of nonurgent disease states and for analysis for protein isoforms in relation to disease; it should also be applicable to assays of proteins in other fluids and tissues.

Two-dimensional electrophoresis (2DE) (*1*) is a powerful technique for resolving complex protein mixtures, first separating proteins according to charge (isoelectric point), then according to molecular mass.[3] The sample is subjected to isoelectric focusing (IEF) in a tube gel, then this tube gel is anchored at the top of a polyacrylamide slab gel, and the sample is electrophoresed in the presence of SDS. Depending on the sample and the electrophoretic conditions used, more than a thousand proteins (e.g., see *2–4*) may be resolved on a single 2D gel.

However, this high-resolution method has been little used in clinical settings since its description 14 years ago. 2DE is labor-intensive, with a turnaround time of several days; its detection limits (nanograms of protein) are rather high; and most importantly, the results of 2D gel analyses are generally expressed photographically (by arrows pointing to spots), rather than numerically. The proteins separated by 2DE are generally quantified immunochemically (e.g., see *5–8*).

Nonetheless, despite the labor required and limited sensitivity of 2DE, the method may have potential clinical applications for which commonly used immunoassays cannot substitute. For example, it may be used to study proteins for which no antibody is yet available because of antigen impurity or low antigenicity. In addition, post-translational modifications may change the charge or molecular mass of proteins in complex mixtures; such variants are often not differentiated by antibodies but almost always are resolved by 2DE. Also, many proteins may be monitored simultaneously in a 2D gel, rather than requiring the use of many individual immunoassays for these different proteins. Although 2DE will never substitute for high-volume "stat" assays, the ability to express 2DE results numerically in a statistically meaningful way could lead to improved utility of this method in the clinical research setting.

To this end, we have optimized the 2DE method for use with Coomassie Blue stain and developed software for analysis of 2DE results on IBM AT or compatible personal computers. Specifically we have addressed calibration scales, gel staining protocol, scanning linearity and reproducibility, linearity of response for 28 proteins in human serum, recovery data, and within-run and between-run coefficients of variation. Although this report is concerned with human serum samples, the method is potentially applicable to other biological fluids such as cerebrospinal fluid, saliva, and urine as well as tissues and cultured cells.

Materials and Methods

Reagents and chemicals. Ampholines were purchased from Pharmacia LKB, Gaithersburg, MD; Coomassie Blue (Brilliant Blue R250) was from Aldrich, Milwaukee, WI; bovine serum albumin (BSA, cat. no. A 7030) was from Sigma Chemical Co., St. Louis, MO; and [^{14}C]formaldehyde used for labeling was from New England Nuclear, Boston, MA. All other chemicals were of "electrophoresis" grade.

Human serum. Normal human serum samples were purchased from a plasma-collection facility (Biological Products, Inc., Madison, WI). Two 1-mL aliquots were removed from each tube of serum, and the aliquots frozen at −80 °C. Reference serum [the U.S. National Reference Preparation for specific human serum proteins; purchased from the Centers for Disease Control (CDC), Atlanta, GA] was reconstituted and stored at −80 °C in separate 52-μL aliquots in 500-μL microcentrifuge tubes until needed. Each time the reference serum was run on 2DE, a new aliquot was used.

Sample preparation. Serum samples were thawed, then treated with an immunoaffinity matrix (ALB-AWAY; Kendrick Labs., Madison, WI) to remove most of the albumin, as described previously (*9*). Briefly, 12 μL of serum was mixed with a 300-μL suspension of ALB-AWAY in a 500-μL microcentrifuge tube. The mixture was shaken on an orbital tabletop shaker at 200 revolutions per minute for 1.5–2 h at room temperature (22–25 °C), then microcentrifuged for 10 s. The supernatant liquid (typically 166.4 μL of the total 312 μL volume) was removed, frozen at −80 °C, and lyophilized. This material, the equivalent of 6.4 μL of albumin-depleted serum, was resuspended in 25 μL of SDS-sample buffer, pH 6.8 (per liter, 100 g of glycerol, 50 mL of beta-mercaptoethanol, 50 g of SDS, and 62.5 mmol of Tris), and boiled for 5 min in preparation for 2DE.

For the linearity response study, a large-scale version of this assay was carried out. We used 240 μL of serum (from an apparently normal woman) and 6 mL of ALB-AWAY

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[3] Nonstandard abbreviations: 2D, two-dimensional; 2DE, 2D electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CDC, Centers for Disease Control; and IEF, isoelectric focusing.

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suspension for each immunodepletion. After boiling the sample in SDS-sample buffer, we loaded appropriate volumes onto the tube gels to represent 3, 5, 7, and 9 μL of albumin-depleted serum, in triplicate.

Electrophoresis. The 2DE method used was essentially as described by O'Farrell (*1*), with the following modifications: 150 × 2.3 mm (i.d.) tube gels were incubated in SDS equilibration buffer (same as SDS boiling buffer, but containing 50 mmol of dithiothreitol per liter in place of beta-mercaptoethanol) for 10 min instead of 2 h. Isoelectric focusing was carried out for 9600 V-h (overnight at 600–700 V); prefocusing was omitted. The ampholine mix was 1.5% pH 5–7, 1.5% pH 5–8, and 1.0% pH 3.5–10. The second-dimension slab gels were 12.5 × 15 cm (0.75 mm thick) with a 2.5 × 15 cm stacking gel. Slab gel electrophoresis was carried out at 12.5 mA/gel for about 4 h, until the bromphenol blue marker-dye front was at the bottom of the gel.

Staining/destaining. The slab gels and accompanying calibration scales were stained according to the technique of O'Farrell (*1*), with modifications. One gel and one calibration scale were placed for 16 h in a plastic box containing 250 mL of staining solution (per liter, 500 mL of ethanol, 100 mL of acetic acid, and 100 mg of Coomassie Blue). The staining solution was poured off and 250 mL of rehydration solution (per liter, 100 mL of acetic acid and 100 mg of Coomassie Blue) was added; rehydration was allowed to proceed with shaking for 2 h. The gels were destained for 30 h in 250 mL of destaining solution (100 mL/L acetic acid solution containing 27 mL of Dowex 1 × 8, 50-mesh ion-exchange resin, chloride form) with shaking. All acetic acid-containing solutions were neutralized (assessed with pH paper) with sodium hydroxide pellets before discarding. The destained gels and accompanying calibration scales were treated with an aqueous 80 mL/L glycerol solution for 30 min before transparency drying between cellophane sheets. Once dried, these gels appeared to remain unchanged for at least several months.

Preparation of calibration scales. Calibration scales were prepared by polymerization of serial dilutions of BSA in successive layers of acrylamide (0.75 mm × ~15 mm × 150 mm). Radiolabeled BSA was used so that the exact amount of protein (ng/mm^2) in each layer of the dried calibration scale could be determined. The stock solution of BSA (10 g/L), determined by absorbance at 280 nm, with an assumed absorptivity of 0.677 $L \cdot mol^{-1} \cdot cm^{-1}$, contained added [^{14}C]BSA.

BSA was radiolabeled by reductive alkylation with [^{14}C]formaldehyde according to the procedure of Jentoft and Dearborn (*10*). The reaction was allowed to proceed for 16 h at 4 °C and then for 2 h at room temperature with 2.25 μCi of [^{14}C]formaldehyde per milligram of protein. The [^{14}C]BSA stock solution was extensively dialyzed before use, then diluted fivefold with unlabeled BSA solution before polymerization in the scales. The calibration scales consisted of 10 layers of serially diluted BSA; the first layer contained about 85 mg of protein per liter before polymerization. An eleventh layer was usually poured that contained a small amount of India ink to make the polyacrylamide strips easily visible in the staining solution.

After the last layer of the scales had polymerized, we separated the plates and cut the layered gels vertically into 1-cm strips to make individual calibration scales with 10 BSA layers and 1 black layer. These narrow strips were placed in a plastic sealable box in a solution containing 100 mL/L acetic acid, 500 mL/L ethanol, shaken gently for at least 1 h, then stored at −20 °C. To determine nanograms of protein per square millimeter in each layer of the blue scale, four calibration scales were stained, rehydrated, and destained with gels, then transparency dried. A known area was cut from each layer and oxidized in a Packard sample oxidizer by Hazleton Labs, Madison, WI, to determine disintegrations per minute per square millimeter. The number of nanograms of protein per square millimeter was calculated by using ng/mm^2 = dpm/mm^2 ÷ dpm/ng.

Gel scanning device. An LGS-50 laser scanner (Digital Instruments, Inc., Kemblesville, PA) was used to digitize all transparency-dried gels for computer analysis. This instrument is capable of scanning a 20 × 25 cm^2 area of 1024 × 1024 resolution (200 μm) at the HeNe laser wavelength of 632.8 nm in less than 4 min. We found that the scanner response was linear with absorbance over the 0–3.0 absorbance range (data not shown); we routinely checked this, using factory-calibrated neutral-density filters (Melles Griot, Irvine, CA). Scanning the same gel five times followed by analysis of three polypeptides on each scanned image (apolipoprotein A-I, haptoglobin α2 chain, and prealbumin) gave CVs of 1.0%, 1.1%, and 2.4%, respectively.

The gel images were formatted by using the software's file utility so that pixel density values between 0 and 1.5 absorbance units were linearly converted to integers between 0 and 255, 1 byte per pixel. The formatted image file of a 2D gel with this system is just about 1.1 MByte, so it will fit conveniently on a 1.2 MByte (high density) floppy diskette.

Software and computers. We developed the QGEL and QBASE software programs for the analysis of 2D gels, using the IBM AT computer and compatibles. Demonstration diskettes for QGEL software are available upon request. The software requires an IBM AT or PS/2 computer (or compatible) with 640 kByte RAM, hard drive, EGA or VGA card and monitor, and mouse device. Commands commonly used for analysis of 2D gel images are Load image; Blowup (magnify a section); Erase background; Contrast the image; Calibrate the image (enter units, generate protein vs stain density standard curve); Outline protein spots; Set spot background; and Show spot table (list μg protein values per spot); Determine molecular weight; and Determine isoelectric point. Occasionally used ancillary commands are Show reference image; Make reference image, Cursor stats (check pixel values within a cursor box); Delete spots; and 3-D plot.

After a polypeptide spot(s) has been manually outlined on the computer monitor by use of the mouse device, the integrated density within the outline above a preset background is automatically determined. If a calibration curve has been entered, the integrated value is also expressed in nanograms of protein, relative to the standard curve. Pixels within the outline that have values at or below the preset background are excluded from the calculation.

QBASE software was developed as a simplified database manager for 2D spot data obtained by using QGEL software. It enables users to extract spot density information from QGEL image files, to automatically average data for many protein spots within a sample (up to 5 gels), and to average data from sets of samples. Once appropriate conversion factors are entered, spot data are converted automatically to clinical units (e.g., mg/dL) by the QBASE program.

We used any of three computers to carry out these

analyses: an IBM PS/2 system 60 (10 MHz 60 MByte hard drive); a Televideo Telecat-286 (8 MHz, 70 MByte hard drive); and a PC-COM AT (15 MHz, 70 MByte hard drive). All three have either EGA or VGA monitors and either Microsoft or Logitech mouse devices.

For this report, we ran each serum sample in triplicate (i.e., each is represented by three 2D gels). For each of these triplicates, we ran the depletion assays at the same time, the electrophoretic steps at the same time (not necessarily in the same electrophoresis tanks) and the stain and destain steps at the same time, using the same batches of the various solutions. We simultaneously processed one triplicate of the CDC Reference Serum with each batch of samples.

Corrections for system drift and protein recovery values. When carrying out quantitative 2DE, it is important to run a CDC reference serum each time samples are run. Analysis by 2DE is a multi-step process and is more susceptible to assay drift than are less complicated assays. For example, changes in the temperature of the laboratory owing to lowered nightly thermostat settings subtly yet detectably affect the overnight steps of staining and destaining. If the reference serum values change from run to run, the changes in the individual proteins may be calculated and a correction factor applied to samples run on the same day.

The protein recoveries in 2DE gels generally range from 50% to 60% (*11*), as we confirmed by tracking the 2DE recoveries of several ^{14}C-labeled serum proteins. To obtain accurate results, it is necessary to know the percent recovery for each protein in the gel. The most convenient way to determine these variables is from known reference values for proteins analyzed daily in the reference serum. In fact, QBASE requires the input of a recovery factor in the conversion to clinical units.

Proteins measured by 2DE with values expressed as BSA equivalents do not necessarily have the same normal reference intervals as proteins measured by RIA or nephelometry. By running a reference serum with predetermined protein concentrations, it is possible to include a stainability factor as part of the recovery number.

We currently run a reference serum sample with every set of unknowns; we use the U.S. National Reference Preparation for Specific Human Serum Protein from the CDC, Atlanta, GA. The CDC supplies known concentrations for 15 proteins in this serum. In cases where values are not supplied, we use the mean value from a published range. In cases where unknown proteins are measured, the nanogram amount of the unknown protein in the reference serum is considered to have undergone the same recovery as a known protein of the same molecular mass. The CDC reference serum must be analyzed first, so that recovery factors (including stainability) can be entered into QBASE for the samples.

Results

Measurements Relative to Bovine Serum Albumin

To quantify the many stained proteins resolved by 2DE, individual proteins must be compared with a single protein standard rather than with multiple individual standards. To do this we polymerize the standard protein at different concentrations in otherwise identical acrylamide layers, to make a calibration scale for generating a standard curve. A calibration scale then accompanies each 2D gel through the staining, destaining, and drying processes.

BSA, a commonly used standard for total protein determinations, was chosen as the calibration scale protein for this study. The molar absorptivity of BSA is known, so its concentration can be determined with high accuracy. We determined the nanograms of BSA per square millimeter in each layer of stained, dried calibration scales by oxidation and scintillation counting as described in *Materials and Methods*. Figure 1 shows a typical BSA calibration curve relating BSA concentration to stain intensity. Once the calibration density values and their corresponding concentration values are entered into QGEL to obtain this curve, we could then automatically calculate the relative amount (in nanograms) of protein in any polypeptide spot or any area on the image.

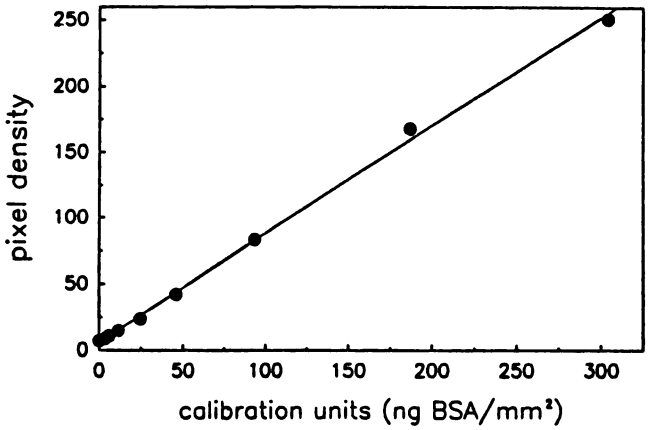

Fig. 1. Bovine serum albumin calibration curve showing average stain density vs protein concentration (ng/mm²)

Serial dilutions of BSA were polymerized in layers to make protein calibration scales as described in *Materials and Methods*. To obtain this typical curve, a calibration scale was stained, destained, dried, and scanned along with a 2D gel. With the QGEL software, the average stain density for each of the nine layers was determined and the curve plotted automatically. All polypeptide spot integrations described in this report were made relative to such a calibration curve. The linear regression line is shown (correlation coefficient = 0.999; slope = 0.82; *y*-intercept = 6.2, corresponding to the background of the image)

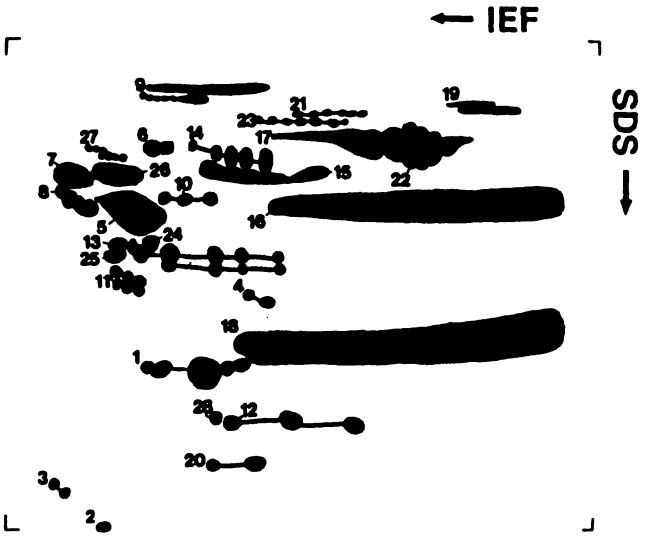

Fig. 2. 2DE gel positions of the serum proteins listed in Table 1

In many cases, individual proteins consisted of multiple spots included in the same protein outline. The acidic end of the pH gradient is positioned to the *left*, and low-molecular-mass proteins are at the *bottom* of the figure. Other conditions were as described in *Materials and Methods*

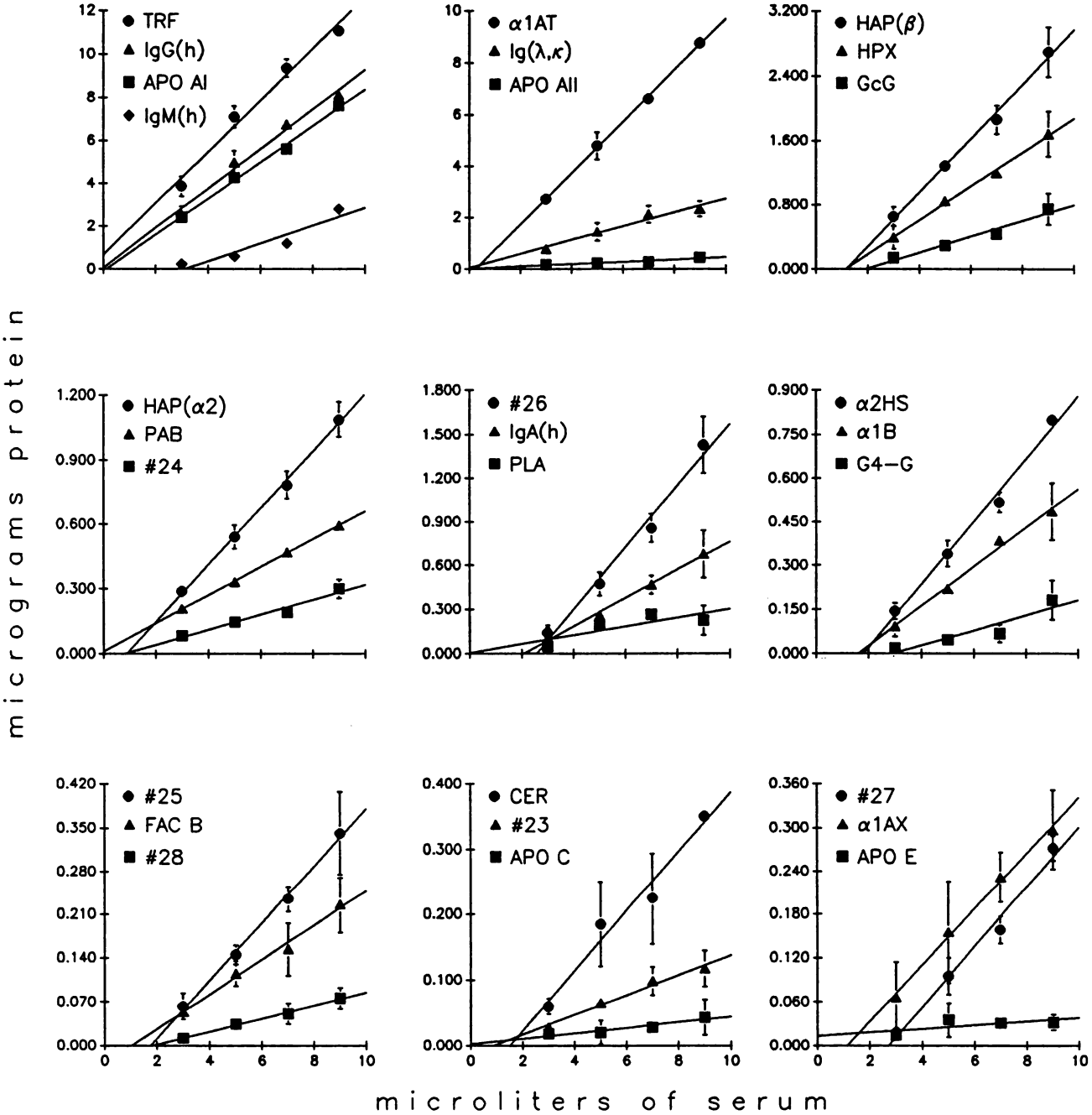

Fig. 3. Linearity plots: aliquots representing 3, 5, 7, and 9 μL of serum were removed from a large volume of albumin-depleted serum, subjected to 2DE, and resulting images were analyzed by QGEL and QBASE, except that no conversion was made to clinical units

Error bars show SD for triplicate gels (protein measured vs serum volume loaded), except for the ceruloplasmin 9 μL point (n = 2). The linearity of the plots (except for plasminogen and apolipoprotein E) indicates the validity of measuring these proteins relative to a BSA standard curve. TRF, transferrin; IgG(h), immunoglobulin G heavy chain; APO, apolipoprotein; IgM(h), immunoglobulin M heavy chain; α1AT, α_1-antitrypsin; Ig(λ,κ), immunoglobulin light (lambda, kappa) chains; HAP (β), haptoglobin β chain; HPX, hemopexin; GcG, Gc-globulin; HAP (α2), haptoglobin α2 chain; PAB, prealbumin; IgA(h), immunoglobulin A heavy chain; PLA, plasminogen; α2HS, α_2HS-glycoprotein; α1B, α_1B-glycoprotein; G4-G, G4-glycoprotein; FAC B, properdin factor B; CER, ceruloplasmin; α1AX, α_1-antichymotrypsin

Proteins Studied

Figure 2 shows a diagram of the 2D gel locations of the 28 polypeptides analyzed for this study: apolipoprotein A-I, apolipoprotein A-II, apolipoprotein C, apolipoprotein E, α_1-antitrypsin, α_1B-glycoprotein, α_1-antichymotrypsin, α_2HS-glycoprotein, ceruloplasmin, Gc-globulin, G4-glycoprotein, haptoglobin α2 chain, haptoglobin β chain, hemopexin, IgA heavy chains, IgG heavy chains, IgM heavy chains, Ig light chains, plasminogen, prealbumin, properdin factor B, transferrin, and six unknown proteins. We determined the locations of the known proteins on 2D gels by comparing them with other 2D maps (*12, 13*) and (or) by co-migration with purified standards. Usually in instances where proteins were composed of multiple spots, we outlined those spots simultaneously, so that the integrated density measured represented a sum of isoform values. The polypeptides range in concentration from about 30 to 3000 mg/L and vary in molecular mass from about 10 to 150 kDa.

Stain Uptake vs Protein Load

It is known that the amount of Coomassie Blue dye bound per unit of protein varies from protein to protein (*14*). Thus, it is essential to establish that both BSA and the proteins of interest are being measured in a linear range. If so, values for proteins in unknown samples expressed in BSA equivalents will be proportional to the amount of protein present, and the method will be valid.

To test whether the 2DE method gives stoichiometric results for individual proteins, we analyzed aliquots of albumin-depleted serum (representing 3, 5, 7, and 9 μL of serum) by 2DE as described in *Materials and Methods*. We analyzed the 28 proteins shown in Figure 2 on those images, using a microcomputer and QGEL/QBASE software, and raw data (averaged nanograms of protein ± 1 standard deviation) were plotted for each individual protein vs. the serum volume loaded. Figure 3 shows that a linear staining response relative to BSA was observed for nearly all of the 28 serum proteins under consideration, indicating the validity of the method for those proteins. The lowest correlation coefficients were for apolipoprotein E (r = 0.683), plasminogen (r = 0.819), G4-glycoprotein (r = 0.919), and IgM heavy chains (r = 0.945). Correlation coefficients for the remaining 24 proteins were ≥0.95.

Apolipoprotein E and G4-glycoprotein gave relatively low correlation coefficients, but these proteins, along with apolipoproteins A-II and C, were at the lower limit of detection for this Coomassie Blue staining method. To measure apolipoproteins A-II, E, and C and G4-glycoprotein accurately by 2DE, one would have to load larger serum volumes onto the gels, so as to increase the signal. However, this might cause overload problems for other proteins, such as transferrin.

The reasons for greater nonlinearity of plasminogen and IgM heavy chains are unclear. Possibly the dye binds to these proteins anomalously; saturation may occur at relatively low protein concentration. The IgM heavy chains tended to be poorly resolved from transferrin in this pH gradient; thus there is some operator variability in drawing the outlines. We consider 2DE measurements of plasminogen and IgM to be qualitative rather than quantitative.

Within-Run and Between-Run Variation

Table 1 shows the average values (n = 23 apparently healthy adult donors; four women, 19 men) for 15 proteins along with within-run and between-run CVs for simultaneous 2D analyses of up to 28 serum proteins; CV calcula-

Table 1. Reference Intervals, Within-Run CVs, and Between-Run CVs for Serum Proteins by 2DE

		[Protein](mg/L)		CV,%		
				Within run		
Spot	Protein	Measured[a]	Reference interval[b]	Run 1[c]	Run 2[d]	Between run[e]
1	Apolipoprotein A-I	1530 ± 320	960–2030	1.3	2.8	4.8
2	Apolipoprotein A-II	nd	nd	nd	np	70
3	Apolipoprotein C	nd	nd	nd	np	56
4	Apolipoprotein E	nd	nd	nd	16	67
5	α_1-Antitrypsin	2160 ± 180	930–2240	3.4	5.0	4.5
6	α_1B-Glycoprotein	240 ± 60	150–300	4.9	7.1	11
7	α_1-Antichymotrypsin	310 ± 110	300–600	22	16	21
8	α_2HS-Glycoprotein	570 ± 150	400–850	nd	23	10
9	Ceruloplasmin	430 ± 400	150–600	11	16	39
10	Gc-Globulin	200 ± 50	200–550	9.7	17	17
11	G4-Glycoprotein	nd	nd	nd	15	32
12	Haptoglobin (α2-chain)	200 ± 180	nd	8.3	5.2	7.6
13	Haptoglobin (β-chain)	990 ± 430	nd	5.6	5.0	8.5
14	Hemopexin	nd	nd	nd	7.1	7.8
15	IgA (heavy chain)	nd	nd	nd	13	13
16	IgG (heavy chain)	5270 ± 1110	nd	9.4	7.0	14
17	IgM (heavy chain)	nd	nd	nd	8.6	31
18	Ig (light chain)	1640 ± 830	nd	14	5.0	13
19	Plasminogen	100 ± 90	60–250	20	21	32
20	Prealbumin	290 ± 50	100–400	5.7	1.4	6.6
21	Properdin Factor B	170 ± 60	100–450	14	10	52
22	Transferrin	2860 ± 500	2520–4290	5.2	5.1	7.7
23	Unknown 23	nd	nd	24	12	86
24	Unknown 24[f]	nd	nd	nd	7.3	12
25	Unknown 25[g]	nd	nd	nd	9.6	18
26	Unknown 26	nd	nd	nd	11	11
27	Unknown 27	nd	nd	nd	nd	10
28	Unknown 28	nd	nd	nd	16	12

nd = not determined. np = not present (not detected on these gels). [a] Mean ± SD for 23 healthy adult donors; four women, 19 men. [b] The sources for reference intervals were spot nos. 1, 5, 22 (reference *15*); spot nos. 6–10, 19, 20 (reference *16*); spot no. 21 (reference *17*). [c] Pooled normal human serum; "within run" does not mean that gels were necessarily electrophoresed in the same tank, only that they were processed at the same time, with use of the same reagents and conditions. [d] Normal female donor. [e] CDC reference serum. [f] Probably apolipoprotein A-IV (*12, 13, 18*). [g] Probably Zn-α_2-glycoprotein (*13*).

tions were done with different serum samples. For both sets of within-run CV determinations we performed five analyses of a single serum on the same day; each analysis was run in triplicate. Not all the proteins were analyzed during the first test and not all the proteins were present on the gels during the second test. Notably, apolipoproteins A-II and C, usually in low abundance, were not detectable at all in the serum from the woman that we used for the second within-run CV study. The average within-run CV for run 1 was 10.6% (n = 15 proteins) and the average for run 2 was 10.8% (n = 25 proteins).

To obtain the between-run CVs we ran aliquots of the same serum sample in triplicate on eight different days during about one month. The average CVs shown for eight measurements are those of the CDC Reference Serum and are uncorrected for assay drift. The assay drift correction (which takes into account the recoveries for each day's run) decreases the between-run variability in the case of unknowns.

Isoform Analysis

A single protein on 2DE may be comprised of many spots occupying different positions on the slab gel. This phenomenon may be due to secondary structure (different peptide chains comprising the protein), to post-translational modification (e.g., proteolytic cleavage, alkylation, glycosylation, sialylation), or to genetic polymorphism.

Serum apolipoprotein A-I, for instance, is optimally separated into five distinct spots on 2D gels (Figure 4A); the forms are designated apo A-I_{-2}, apo A-I_{-1}, apo A-I_0, apo A-I_{+1}, and apo A-I_{+2}, based on their lateral position relative to the most abundant apo A-I_0 form (*18*). The four more acidic isoforms (increasingly acidic pIs towards the left, increasingly basic pIs towards the right) are all derived from the basic pro-apo A-I (apo A-I_{+2}) form. A post-translational cleavage of the six N-terminal amino acids of apo A-I_{+2} yields apo A-I_0 (*19*), and deamidations of these two forms yield apo A-I_{+1} and apo A-I_{-1}, respectively. All five forms must be summed to obtain a concentration value for this important indicator of coronary vascular disease risk (*20, 21*). We can derive ratios of these isoforms easily from 2D gels, even without absolute quantification. Altered apolipoprotein A-I isoform ratios have been documented for rare dyslipoproteinemias such as fish-eye disease (*22*) and Tangier disease (*18, 23, 24*).

We evaluated isoforms of apolipoprotein A-I, using QGEL and QBASE. We used sera from 16 apparently normal donors (three women, 13 men) in this study; the results are presented in Figure 4. The relative abundance of each form, expressed as a percentage of the total, was: apo A-I_0, 76.3 ± 3.5; apo A-I_{-1}, 13.7 ± 2.2; apo A-I_{-2}, 4.5 ± 1.9; apo A-I_{+1}, 3.5 ± 0.7; and apo A-I_{+2}, 2.1 ± 0.8. These percentages agree fairly well with earlier estimates (*19, 23*) of the two most abundant forms. Data were ranked based on relative abundance of apo A-I_0 to illustrate potential biochemical relationships between apo A-I_0 and other isoforms. Regression analyses (not shown) show correlation coefficients of 0.69–0.70 for forms 0 and −2, 0 and −1, and 0 and +2.

Discussion

Known disease states, for which the *simultaneous* measurement of several serum proteins provides more-accurate diagnostic information, include diseases of depleted nutritional status (*25*), and inflammation, which cause changes in the acute-phase reactants (*26*). Endocrine status may also affect multiple serum protein concentrations (*26*). A major advantage of 2DE over multiple immunoassays is that new or unusual polypeptides may be observed during 2DE analyses. For example, we have observed monoclonal gammopathies, presumably benign, in serum from apparently otherwise healthy donors. 2DE might be expected to be especially useful for idiopathic disease states.

Coomassie Blue stain uptake by proteins can vary depending on staining or destaining conditions, gel thickness, and temperature. Under some conditions (e.g., protein saturation), it may be a nonquantitative stain (*27*). However, the conditions for Coomassie Blue staining described here allow good quantification of proteins resolved by 2DE over the range 30–3000 mg/L. The key requirement is that a BSA calibration strip be included during the staining and subsequent treatment of each 2D gel. Our results indicate that it is necessary to run 2DE analyses in triplicate to obtain quantitative results. This is in agreement with Miller et al. (*28*), who quantitatively analyzed 2D autoradiograms.

Although several groups have described computerized analytical methods for 2D gels, using mini-computer systems such as the PDP-11/60 and VAX 11/750 (e.g., *29, 30*), to our knowledge this is the first work describing quantitative analysis of serum proteins on 2D gels with use of software for the popular and more affordable IBM AT compatible computers. The manual, microcomputer analysis described here is relatively time-consuming and is best suited for nonurgent samples or research samples. For example, once the gels have been run and scanned, a practiced operator using an 8 MHz personal computer will require about 1 h per sample (triplicate gels) to obtain a printout showing concentration values for the 28 polypeptide spots shown in Figure 2.

The approach taken here utilized manual outlining of protein spots with a mouse device and the computer-generated image. We felt it was important to first verify the method and to work out problems with human serum samples before trying to develop a semi-automated or automated analysis. In light of the steadily increasing speeds and storage capabilities of personal computers, automatic spot outlining and spot matching between and among many images will eventually be possible for inexpensive lab computers. While the fast laser scanner described in *Materials and Methods* performed well, any densitometer—a CCD camera, for example—that gives linear readings over the 0–1.5 absorbance range would be suitable.

The largest CVs for the proteins listed in Table 1 tend to be those present in lower concentrations in the serum: apolipoproteins A-II, C, and E, as well as properdin factor B and unknown no. 23. The error for these faintly staining proteins would probably be decreased by increasing the serum load, but this probably would cause overloading of other serum proteins. Thus, there are difficulties in keeping all the serum proteins simultaneously within a measurable range for 2DE. In those cases where 2DE would be useful for quantitative analysis of a single protein (or for isoform ratios of a single protein), the method may be simplified. By clipping appropriate sections from three IEF tube gels containing the protein of interest, it is possible to run all three (for the triplicate measurement) simultaneously on a single slab gel.

For the method described here, it was essential that all users circle the same proteins in the same manner. To help operators handle image complexity and maintain reproducibility, we incorporated a set of four reference-image com-

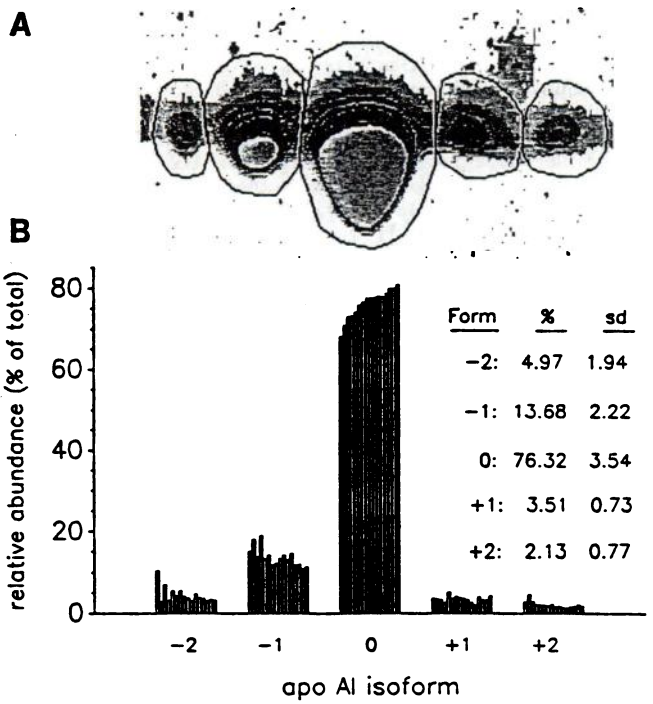

Fig. 4. Isoform analysis: (*A*) computer printout of the area of a 2DE gel, showing the five individually circled apolipoprotein A-I isoforms; (*B*) the corresponding histograms, showing the relative abundance of each isoform

The five isoforms in sera from 16 normal donors (three women, 13 men) were analyzed with QGEL and QBASE, then ranked (low to high) according to abundance of the major form, apo A-I_0. The *inset* on the histogram plot shows the mean abundance and standard deviations for each isoform

mands into QGEL that enable a user to visually "page through" a series of previously-saved images or "protein profiles" during the outlining process, demonstrating to the *current* user precisely how a protein spot or series of spots was circled by a previous user, and thereby revealing how the current image should be handled. The reference images appear in a window at the upper left of the monitor. Similarly, the operator may save a portion of a screen showing an unusual form of a protein by adding it to a reference image file. Several operators may then take advantage of reference images on one computer or copy these files for use on any computer configured to run QGEL.

There would seem to be a stringent biological control on the apo A-I isoform ratios, given the unanticipated low standard deviations. Further studies with larger populations and various disease states (such as coronary vessel diseases) are necessary to establish the potential utility of this type of analysis. Preliminary evidence suggests, for instance, that in sera from AIDS patients (kindly provided by Dr. D. Maki, University of Wisconsin Clinics), the mean value for the apo A-I_0 abundance may be lower than normal, the apo A-I_{+2} abundance higher than normal, and the SDs about these means higher than normal.

The landmark 2DE techniques of O'Farrell have been in use now for several years. To date, most 2DE use has been geared toward the visualization of all-or-none changes between two gels. We believe that the availability of microcomputer software such as the QGEL/QBASE system will make quantitative analysis of 2D gel images possible on a routine basis, especially by research laboratories that are equipped with or have access to microcomputers and appropriate gel-scanning hardware.

In terms of clinical assays, 2DE will never replace stat assays, but it will play an increasingly important role among the protein separation and characterization techniques available at the research level. Not only will 2DE be useful for the discovery of new proteins associated with disease states, its utility will be extended to include subtle changes in known proteins and their isoforms brought on by disease.

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