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Rapid capillary electrophoretic analysis of human serum proteins: qualitative comparison with high-throughput agarose gel electrophoresis

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Abstract

This study details the qualitative results from a comparative evaluation of agarose gel electrophoresis (AGE) and (CZE) as a screening procedure for monoclonal proteins in serum. Three hundred and five serum samples were analyzed by the two techniques; samples suspected of containing monoclonal proteins based on abnormalities observed with AGE or CZE were confirmed by immunoelectrophoresis and/or immunofixation. CZE separation conditions were simple (requiring only a bare silica capillary and 150 m*M* borate buffer, pH 10.0) and separation was complete in under 120 s. The results obtained by CZE were comparable or better than those obtained with AGE. Samples displaying "point-of-application" artifacts on AGE were not problematic by CZE analysis; an abnormal profile, due to the presence of a monoclonal protein, or a normal profile were clearly observable. The rapid analysis, excellent reproducibility, automation and relatively high throughput (≈90 samples per 8 h on a single instrument) sets the stage for CZE analysis of serum proteins to be used routinely in a clinical setting.

Keywords: Proteins

1. Introduction

Human serum is extremely heterogeneous with respect to its protein content: more than 300 different proteins have been identified, most of which are below the detection limit of the standard electrophoretic techniques. At the present time, clinical diagnostic information is obtained from serum through the non-denaturing electrophoresis of the proteins on cellulose acetate (CAE) or, more commonly, agarose gels (AGE). Both of these provide separation of the proteins into five or more regions or zones, of which the major are albumin, alpha-1 (α_1), alpha-2 (α_2), beta (β) and gamma (γ) globulin. A densitometric scan of the Ponceau S-stained agarose gel provides a profile from which clinical diagnostic information can be obtained. Qualitative information is obtained from the profile itself as well as the peak shapes and symmetry, while quantitative information is extracted from peak areas which allow for the relative concentrations of the proteins in the five major zones to be determined. Variation in the quantity of protein in any of the zones outside of a "normal range" is often clinically significant; quali-

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tative information is obtained based on subtle differences in the profile itself. This can range from changes as subtle as a slight asymmetry in the gamma zone to more obvious abnormalities, like the presence of an additional protein peak(s) in any given zone. Of particular importance is the use of this information in identifying monoclonal proteins in patients with a variety of disorders [1].

One of the clinical applications identified when capillary electrophoresis (CE) was still a relatively unknown technique was the analysis of proteins in human serum. Jorgenson and Lukacs [2] were the first to show that serum proteins could be successfully separated by CZE. While their separations were prohibitively long (up to 40 min), the same major zones found with agarose electrophoresis were observed. Almost a decade later, Chen and co-workers [3,4] demonstrated CZE as a viable alternative to AGE. In 1993, Kim et al. [5] carried out electrophoresis with the serum from patients with cirrhosis, nephrotic syndrome and polyclonal gammopathy. Also in 1993, Klein and Jolliff [6] carried out a comparative evaluation of serum protein analysis by CZE and AGE using 100 serum samples from normal adults (both sexes). These studies found that, in general, capillary electropherograms provided comparable or better detail than the densitometric scans of agarose gels. In addition, analysis by CZE appeared to provide a peak area response that was linear with concentration for each of the five major zones and correlated well with results from AGE. More recently, studies by Jenkins and Guerin [7], Dolnik [8] and Lehmann et al. [9] echoed similar results. Unfortunately, most of these studies are associated with lengthy analysis times (8–24 min) that do not provide the necessary throughput for CZE on a single capillary instrument to compete favorably with agarose or acetate systems. It is in this respect that the report by Chen [4] is so significant. In that study, they demonstrated that CZE analysis of serum proteins could be extremely rapid (separation in less than 90 s), highlighting for the first time the potential of CZE for high sample volume throughput.

The use of CZE for serum protein analysis could revolutionize monoclonal protein detection as a result of a design which incorporates the instrumental control typical of micro-HPLC while offering resolution superior to standard electrophoresis. Of importance to the clinical laboratory, these translate into a rapid turn-around time, small sample requirements, and the potential for automation of the serum protein electrophoresis procedure. It is important to note that, while the initial expense of equipment may be higher than that for agarose gel electrophoresis, the operating cost of reagents should be small in comparison.

This study presents the initial qualitative results from the analysis of over 300 patient serum samples for monoclonal proteins. The diagnostic results from capillary electrophoretic separation in less than 120 s are compared with those obtained from AGE. In order to obtain a meaningful comparison, AGE was carried out on a commercial clinical instrument designed specifically for high throughput (not high resolution) AGE of serum samples. This study shows that CZE not only provides comparable information to that from a high throughput clinical AGE instrument but, in fact, allows for the identification of a number of abnormalities which were not detected by AGE.

2. Materials and methods

2.1. Patient samples

Forty fresh normal serum samples and 73 frozen normal serum samples were obtained from a population of normal donors. These donors were 50% male and 50% female and ranged in age from 23 to 75 years. Aliquots of patient sera ($n=192$) were obtained from the Clinical Protein Laboratory. These samples were analyzed by AGE and CZE for the presence of a monoclonal protein.

2.2. Materials

Sodium borate and boric acid were obtained from EM Science (Gibbstown, NJ, USA), cesium chloride was obtained from Sigma (St. Louis, MO, USA) and benzy alcohol and 3-chloro benzoic acid were obtained from Aldrich (Milwaukee, WI, USA). AGE reagents were obtained from Helena Labs. (Beaumont, TX, USA) and used according to the manufacturers' instructions. Fused-silica capillaries were

obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.3. Buffer and sample preparation

Borate buffer (150 m*M*, pH 10.0) was prepared with Milli-Q (Millipore) purified water by mixing of 150 m*M* sodium borate solution and boric acid until pH 8.5 was obtained. This solution was adjusted to a pH of 10.0 with a concentrated solution of cesium hydroxide (avoiding the addition of Na^+ with NaOH). Phosphate-buffered saline (PBS; 20 m*M* monosodium phosphate, 150 m*M* NaCl, pH 7.4) was prepared using 2.6 g NaH_2PO_4, 8.69 g Na_2HPO_4 and 35.1 g NaCl dissolved in 4 l of distilled water and filtered with a 0.22 μ Nalgene filter. Standards for serum protein analysis by CZE were prepared by dissolving benzyl alcohol and 3-chlorobenzoic acid in PBS at a concentration of 10 μg/ml.

2.4. Agarose gel electrophoresis

This analysis was performed according to the instructions provided by the supplier (Helena Labs.), and involved electrophoresis with prepoured REP SPE–30 agarose gels on a Helena REP system. The separated protein was visualized with Ponceau S stain and peak areas were calculated using Helena Software following scanning densitometry using the Helena EDC laser scanner.

2.5. Capillary electrophoresis instrumentation

CZE separation was carried out on a Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA, USA), interfaced with an IBM Value Point 486 computer utilizing System Gold software (V. 8.1, Beckman Instruments) for control and data collection. Peak migration times, area and height were obtained through the System Gold software. An Excel spreadsheet was used to store and collate data including conversion of peak area to g/dl protein and statistical analysis.

2.6. Capillary electrophoresis separation conditions

New uncoated fused-silica (27 cm×20 μm) capillaries were prepared by extensively rinsing with 0.1 *M* NaOH, water and separation buffer. The following method was used for the routine analysis of serum proteins. The analysis was initiated with a two column volume rinse with separation buffer; sample (serum diluted 1:10 in PBS) was injected for 2 s by pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) followed by a 2 s injection by pressure (0.5 p.s.i.) of standards (benzyl alcohol and 3-chlorobenzoic acid) in PBS; separation was carried out at 20 kV with the inlet as the anode and the outlet as the cathode; following separation, a two-column volume rinse with 1.0 *M* NaOH was followed by a one-column volume rinse with water and a three-column volume rinse with unelectrophoresed separation buffer. Additional washing with 1.0 *M* NaOH was sometimes necessary to ensure that the migration time for albumin was maintained at 1.55±0.01 min. The external capillary temperature was maintained at 25°C and detection was by absorbance at 214 nm.

3. Results and discussion

3.1. Routine analysis of human serum

Routine serum electrophoresis, whether on acetate or agarose gels, is the first stage screening tool used for the detection of monoclonal proteins. Any serum protein electrophoretic patterns that look suspicious are subjected to immunoelectrophoresis (IEP) or immunofixation (IF) for identification and typing of monoclonal proteins. It is important to have a primary screening technique that is both sensitive and specific for the detection of monoclonal proteins because immunofixation is time-consuming and, subsequently, expensive.

As can be seen by the normal serum profiles represented in Fig. 1, there are a number of differences between the normal serum pattern obtained with AGE-scanning densitometry and CZE. The first obvious difference between the two is that the electropherogram presents a profile that has a reversed peak order in comparison with that of the densitometer scan. This results from the fact that AGE is carried out with the serum loaded onto the gel at the cathode, with the proteins drawn electrophoretically to the anode. Under these conditions,

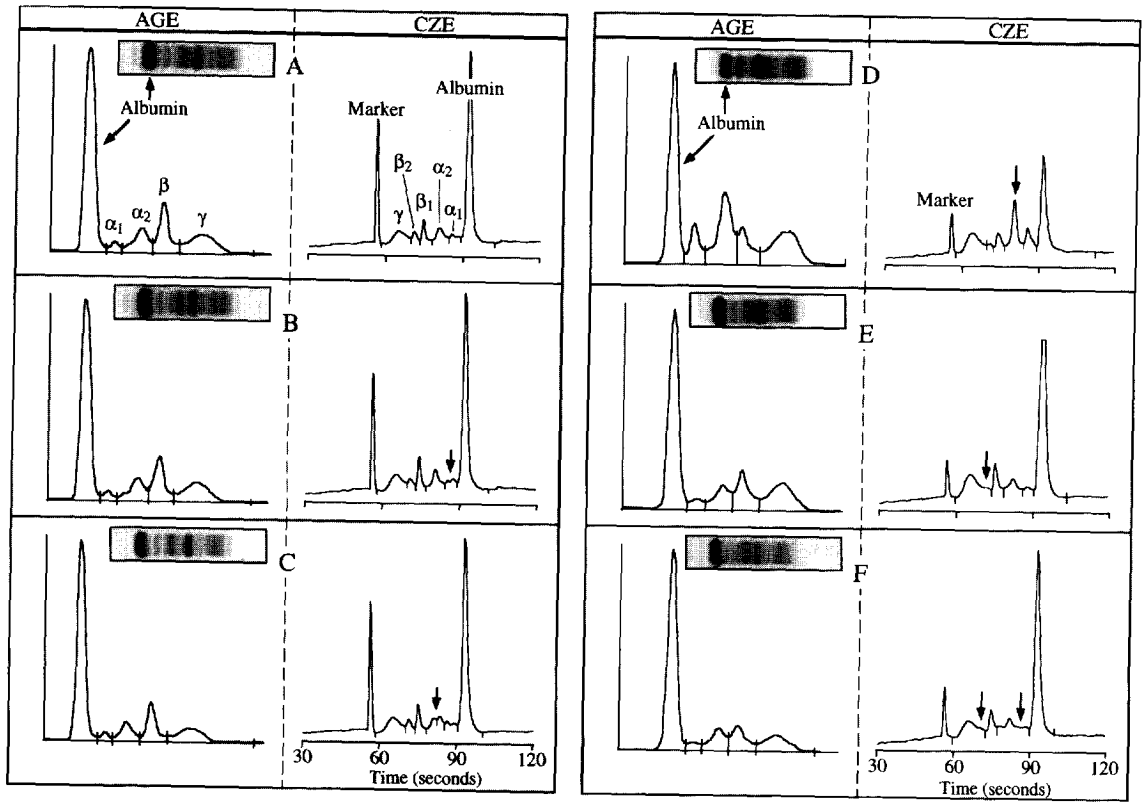

Fig. 1. Profile variations associated with AGE and CZE of normal human serum. The profiles in panels A–F result from the electrophoretic analyses of six normal sera. Panel A represents the pattern encountered most frequently with both AGE and CE. Panels B, C, and D represent normal variations observed in the alpha region. Panel B shows a split alpha-1, C, an extra alpha peak, and D, a prominent alpha-2 peak. Panel E represents a normal profile with very little beta-2 peak detected by CE while F illustrates a norm serum profile with low quantities of both alpha-1 and beta-2. Separation conditions for both methods were as described in Section 2. The agarose gel is inset in the left panels. The arrow in the right panels designates the variation.

albumin migrates the fastest and the immunoglobulins (the gamma zone) the slowest. Scanning the gel from the bottom to the top (left to right as shown in the figures) results in the profile shown in Fig. 1A. With capillary electrophoresis, however, the serum is injected into the capillary at the anode (inlet) and, while the proteins are still electrophoretically drawn to the anode (the site of loading), a strong electroosmotic flow (EOF) towards the cathode draws all molecules to the outlet and detector. As a result of this EOF, albumin, which migrates the fastest and will travel the furthest against the EOF, reaches the detector last. The gamma zone, which migrates the slowest and will travel minimally against the EOF, reaches the detector first. The net result is that the peak order is reversed. The second major difference is the resolution of a second beta peak. This is most likely the result of the enhanced resolution associated with CE in comparison with the more conventional electrophoretic analyses.

3.2. Information from AGE and CZE analysis of normal serum

As would be expected with the analysis of a significant number of "normal" serum samples, there is a variation in the observed CE profiles (Fig. 1A–F). It is not uncommon to observe serum that possesses additional peaks in the alpha region other than the two peaks most frequently found at 1.45 and 1.39 min (e.g., alpha-2, Fig. 1C). Additionally, some samples display the presence of very large peaks in

the alpha region that may be considered "normal" or, at least, are not associated with the presence of a monoclonal protein (Fig. 1D). It is also not uncommon to observe the absence of a beta-2 peak in normal serum (Fig. 1E), an occurrence that appears to be associated more commonly with stored serum samples than with fresh specimens. The correlation between the disappearance of these peaks and the length of storage is not clear at this time. Fig. 1F represents another variation of a normal profile in which both the alpha-1 and the beta-2 regions are markedly depleted of protein. These summarize the variability that can be expected with CZE analysis of normal serum under these conditions. It is noteworthy that these normal specimens were negative for the presence of monoclonal proteins as determined by IF.

3.3. Qualitative information from AGE and CZE analysis of abnormal serum

3.3.1. Obvious gammopathies

A number of the serum samples had clear abnormalities, whether deduced from the AGE densitometry profile or the capillary electropherogram (Fig. 2). Fig. 2A shows a large (3.3 g/dl) spike in the gamma region which was determined to be an IgGκ by IF. Fig. 2B shows yet another distinct example of a clear-cut abnormality which is manifested as a large spike in the beta-gamma region by AGE and as a beta-2 spike on CZE and was determined to be a IgAκ monoclonal protein (1.3 g/dl). Although less obvious, Fig. 2C illustrates a small (0.55 g/dl) spike in the fast gamma region. This abnormality is obvious from both AGE and CZE despite its accounting for a small percentage of the total gamma region; IF showed the protein to be an IgGκ monoclonal protein. Fig. 2D illustrates the increased sensitivity associated with CZE. This patient serum contains a small (0.46 g/dl) IgMκ protein that was not obvious on either the AGE gel or the densitometry scan but is suspicious as an abnormality in the gamma region on the CE profile.

3.3.2. "Point-of-application" artifacts

There is a small subset of serum samples which are inherently difficult to analyze by gel electrophoresis and for which CZE provides a clear advantage. These are serum samples which contain monoclonal proteins that remain at the point of sample application on agarose gels (and cellulose acetate as well). These precipitates may be euglobulin or cryoprecipitates and may or may not contain a monoclonal protein. It is impossible to determine without IEP or IF of the serum sample whether or not these are monoclonal proteins. Fig. 3 clearly illustrates that the "questionable" nature of the interpretation with this group of samples is eliminated with CZE for two reasons. First, because electrophoretic mobility with AGE is bi-directional from the point of application, the point of application

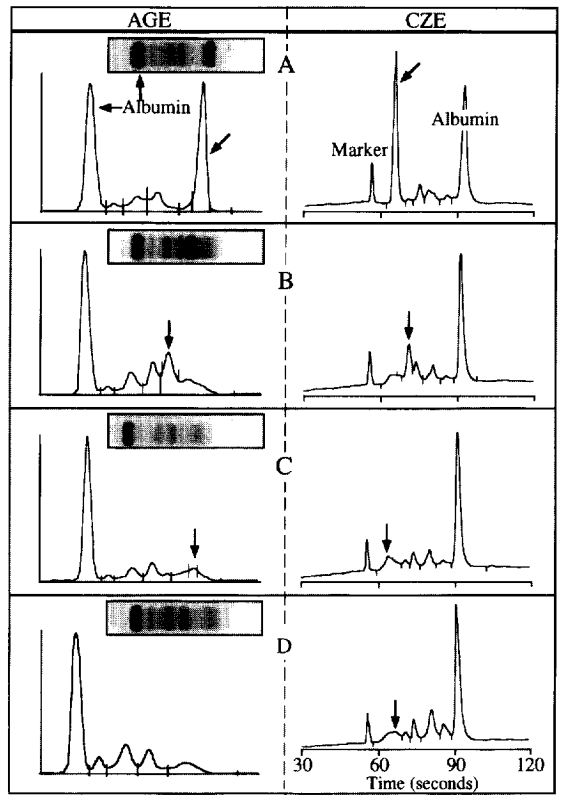

Fig. 2. AGE and CZE profiles of sera containing monoclonal proteins. Panels A and B represent monoclonal proteins obvious on both the AGE and CE profiles. In panel A, the monoclonal protein is detected in the gamma region while in panel B, it is found to migrate in the beta region. Panels C and D represent more subtle abnormalities, both of which lie in the gamma region. Separation conditions for both methods were as described in Section 2. The agarose gel is inset in the left panels. The arrow in the right panels designates the abnormality.

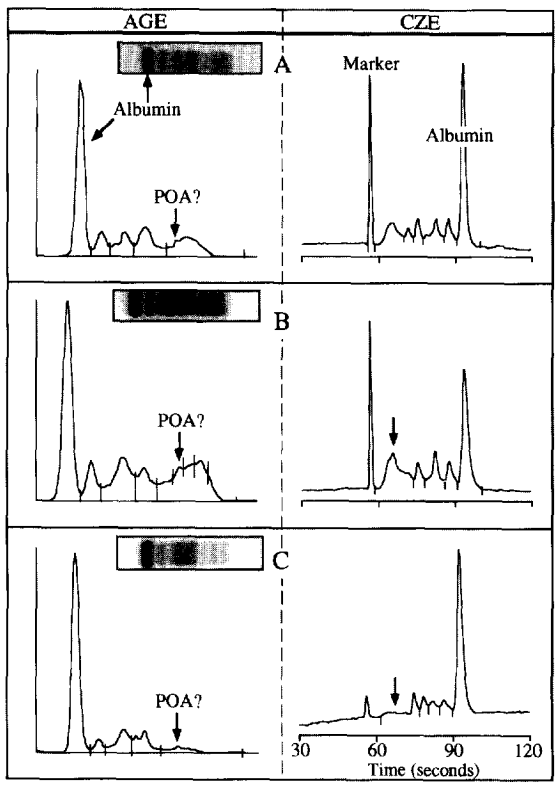

Fig. 3. Advantages of CZE for analysis of serum samples prone to generating "point-of-application" artifacts on AGE. Panels A, B and C typify a subset of patient serum samples that present point-of-application artifacts on AGE. In panel A, observing a normal profile by CE confirms the AGE artifact. Panels B and C each show a suspicious spike by CE which could be indicative of a monoclonal protein. Separation conditions for both methods were as described in Section 2. The agarose gel is inset in the left panels. The arrow in the right panels designates the abnormality.

remains part of the scanned area of interest. With CZE, net mobility (electrophoretic+EOF) is unidirectional (towards the detector) and the point of application is remote from the detector. Secondly, unlike AGE where precipitates cannot exit the loading well and enter the gel, there is no gel matrix in CZE to impede electrophoretic migration since analysis occurs in free solution.

The AGE densitometry profiles and capillary electropherograms shown in Fig. 3 result from the analysis of serum samples that were categorized as point-of-application artifacts. This categorization indicates that some component in the sample did not enter the gel, whether it be a monoclonal protein precipitate or some form of other precipitate, perhaps due to storage. As shown by the CE profiles in Fig. 3, some of these samples were clearly normal. For example, Fig. 3A shows that interpretation of the AGE densitometry profile can only classify this sample as a point-of-application artifact or a monoclonal spike. However, analysis by CZE clearly defined this as an artifact of AGE. In some cases (Fig. 3B), what was suspected as a combined point-of-application artifact/monoclonal spike is confirmed by CZE by the clear presence of a monoclonal protein. The advantages of CZE are most clearly seen with samples exhibiting the presence of a monoclonal protein that might be mistakenly dismissed as a point-of-application artifact. Fig. 3C shows an example of a sample where the nature of the peak in the AGE gamma region is suspected to be a point-of-application artifact, but is interpretable as a monoclonal protein spike with CZE. This patient was diagnosed with a cryoglobulin disorder that was typed as an IgGκ with polyclonal IgG (mixed cryoglobulinemia-type II).

3.3.3. Other sample groups where CZE provides a qualitative advantage over AGE

There are several other categories of abnormalities in which capillary electrophoresis seems to demonstrate a definite advantage over agarose gels. Fig. 4 illustrates a number of different examples of subtle abnormalities that went undetected by AGE (i.e. the AGE profiles appeared normal) but were detected by CE. Small shoulders are apparent (the left side of the gamma region) in the electropherograms shown in panels A and B. The sample in panel A was found to contain a λ light chain, which migrated at the point of application even on IF, while that in panel B was found to be a IgMκ monoclonal protein. Panel C shows the presence of an abnormal rise in the center of the gamma region which was eventually identified as an IgMκ type I cryoglobulin. The AGE densitometry scans shown in panels D and E show polyclonal increases in the gamma region; however, the capillary electropherograms also show a small shoulder on the right side of the gamma peak. These patients were found to have, respectively, a type II cryoglobulin with an IgMκ with a polyclonal IgG increase and a free λ light chain migrating at the point of application. Panel F represents the separated

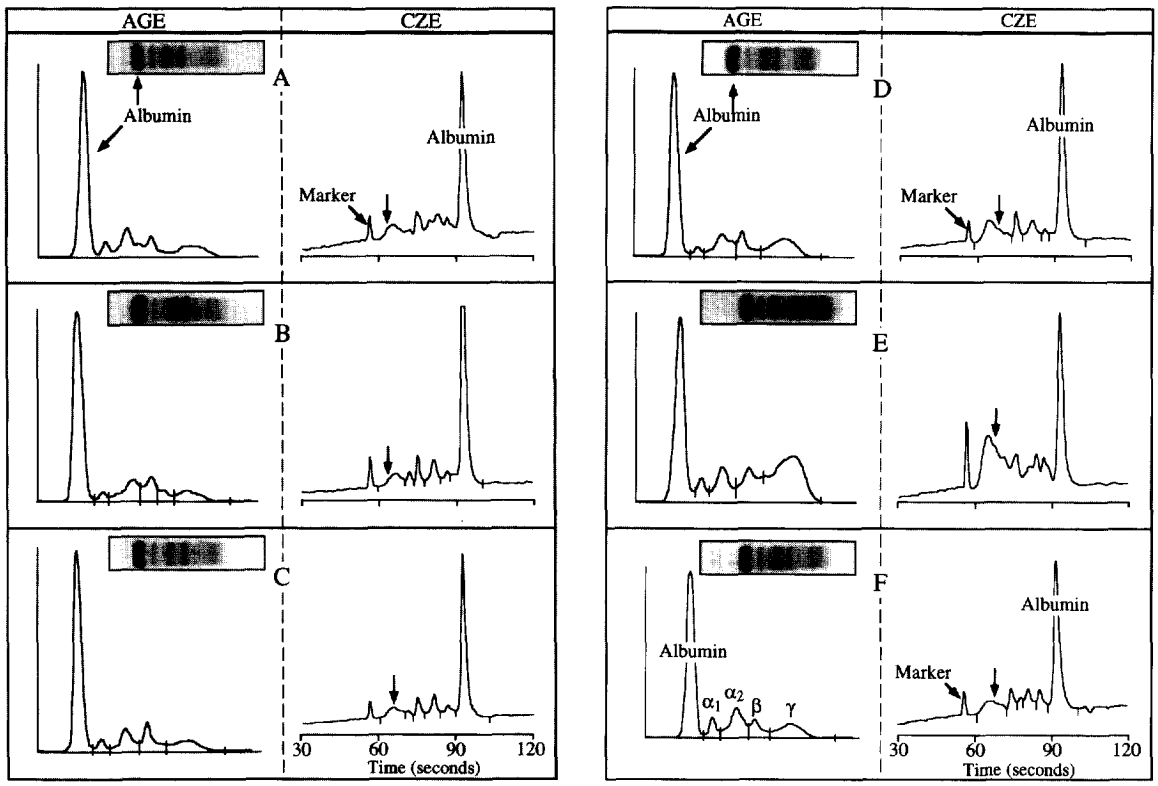

Fig. 4. Agaraose gel and capillary zone electrophoresis of patient sera with subtle abnormalities. The capillary electropherograms in panels A–F show subtle changes in the shape of the gamma region that were not detected by AGE. These abnormalities were confirmed as monoclonal proteins by subsequent IF. Separation conditions for both methods were as described in Section 2. The agarose gel is inset in the left panels. The arrow in the right panels designates the abnormality.

serum from a patient who was diagnosed with amyloidosis with a λ monoclonal protein. This is seen in the CE profile as a small abnormality on the right side of the gamma zone. All of the aforementioned represent monoclonal proteins confirmed by immunofixation that might have been inadvertently missed by examining the information obtained from AGE densitometry profiles or visual inspection of the gels.

3.4. Migration time reproducibility

Migration time reproducibility with CZE was evaluated through the analysis of the same serum sample at the beginning and end of each day for 25 days. The conditions associated with this study were developed such that the migration time of the albumin peak was 1.55±0.01 min. These conditions included a rinse with 1.0 *M* NaOH each day. The serum sample used for this study produced a normal CE profile but had markedly depleted α1 and β2 regions. Therefore, coefficient of variance (C.V) values were only calculated for the albumin, alpha-2, beta-1 and gamma peaks (Table 1). Consistent with other reports describing the reproducibility associated with CZE separations of proteins, the reproducibility for these four regions was acceptable,

Table 1
Reproducibility associated with migration for multiple analysis of a single sample on different days (n=51).

	Albumin	Alpha-2	Beta-1	Gamma
Mean migration time (min)	1.55	1.36	1.25	1.09
S.D.	0.008	0.007	0.009	0.007
C.V. (%)	0.49	0.50	0.75	0.61

ranging from 0.49% (albumin) to 0.75% (beta-1) over 51 analyses. Achieving this level of reproducibility is important in light of the fact that a slight shift in the mobility of a given peak could have diagnostic significance.

3.5. Why CE instead of AGE?

The results of this study show that CZE not only provides comparable information to that from high-throughput, standard AGE but also allows for the identification of a number of abnormalities not detected by AGE. In addition, the general synopsis from the existing literature, comparing the performance of CE with conventional electrophoresis for analysis of serum proteins, is that CE provides peak area responses that are linear with concentration for each of the five major zones and that correlate well with results from AGE. Any discrepancies in the relative values were thought to be attributable to either slight variations in the distribution of specific proteins (as a function of the two techniques) or differences due to the direct absorbance at 214 nm versus the measurement of stained protein. In this respect, it is important to distinguish one of the major differences between CE and conventional electrophoresis, that CE involves "on-line detection". It is also important to remember that the densitometric scans of serum proteins resolved on acetate and agarose gels are obtained from stained proteins and, hence, are subject to errors in quantitation due to two independent factors. The first involves sample-to-sample differences in post-translational modification of some proteins in serum, which can lead to subtle but significant differences in the amount of stain that is bound. The second involves methodology-based parameters such as staining time and temperature, stain concentration and age, destaining time and temperature, etc., all of which can influence dye binding and, hence, quantitation. In contrast, the CE profile represents the direct on-line measurement of protein via the peptide bonds (λ_{max}=214 nm). This approach eliminates concerns about varying quantitation due to the methodological factors delineated above and should, therefore, be less susceptible to changes in post-translational modification. For these reasons, the CE profile should be more reliable for quantifying relative protein concentrations.

4. Conclusions

There appear to be a number of serum sample subcategories for which CE more easily and accurately is able to detect abnormalities. Of particular note, point-of-application artifacts that are frequently observed with conventional gel techniques are not observed with CE. This eliminates the effort and cost associated with having to confirm the presence or absence of monoclonal proteins with immunofixation. Furthermore, it appears that subtle abnormalities in the pattern and smaller quantities of monoclonal proteins can be detected from the CE profiles. It is clear that a larger number of samples from within these patient subcategories must be evaluated to provide concrete numbers conveying the comparative sensitivity and specificity of the two techniques. However, preliminary quantitative comparisons look very promising [10]. It is important to note that there were *no* cases where the monoclonal proteins detected by AGE were not seen on the CE profile. However, we were able to cite several cases where abnormalities were detected by CE and not by AGE.

Based on the qualitative results from this study, capillary electrophoresis appears to be a viable alternative to conventional electrophoretic techniques for evaluating serum protein profiles. Separation times are rapid (<120 s) without compromising the quality of the diagnostic information obtained. The volume of sample required is quite reasonable (<30 μl total serum volume) and the reagent costs low or negligible. An added advantage is the real-time on-line detection as opposed to the staining and scanning associated with gel techniques. In short, CE promises to be rapid, inexpensive and a more sensitive method than AGE for the evaluation of serum proteins.

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References

[1] R.A. Kyle and J.A. Lust, in D. Wiernik et al. (Editors), Neoplastic Diseases of the Blood, Churchill Livingstone, New York, 3rd ed., 1996.
[2] J.W. Jorgenson and K.D. Lukacs, Science, 222 (1983) 266.
[3] F-T.A. Chen, C-M. Liu, Y-Z. Hsieh, and J.C. Sternberg, Clin. Chem., 37 (1991) 14.
[4] F-T.A. Chen, J. Chromatogr., 559 (1991) 445.
[5] J.W. Kim, J.H. Park, J.W. Park, H.J. Doh, G.S. Heo and K-J. Lee, Clin. Chem., 39 (1993) 689.
[6] G. Klein and K. Jolliff in J.P. Landers (Editor), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1993, p. 419.
[7] M.A. Jenkins and M.D. Guerin, Ann. Clin. Biochem., 32 (1995) 493.
[8] V. Dolnik, J. Chromatogr. A, 709 (1995) 99.
[9] R. Lehmann, H. Liebich, G. Grubler, and W. Voetler, Electrophoresis, 16 (1995) 998.
[10] J.A. Katzmann, R.J. Clark, C. Namyst-Goldberg, E. Sanders, R.A. Kyle, and J.P. Landers, Clin. Chem., (submitted for publication).