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Evaluation of serum protein separation by capillary electrophoresis: prospective analysis of 1000 specimens

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Abstract

To critically assess the method of capillary electrophoresis (CE) we examined 1000 prospective serum samples submitted for protein electrophoresis by both high-resolution agarose gel electrophoresis (HRAGE) and CE. CE was performed using a 72 cm (50 cm to detector) × 50 μm I.D. fused-silica capillary with detection of absorbance at 200 nm. The 1000 samples examined contained 362 monoclonal paraproteins with concentrations ranging from 1 to 71 g/l. We evaluated the individual paraprotein correlations, the overall correlation between the two methods being 0.96. We found that HRAGE gave slightly higher values for the monoclonal bands than CE and the difference was statistically significant. We conclude that CE is a viable alternative to HRAGE for the determination of protein dyscrasias in a routine clinical laboratory.

1. Introduction

Serum protein electrophoresis, first demonstrated by Tiselius in a wholly liquid medium in a glass tube in 1937 [1], is a technique which separates serum proteins in an electric field. The use of inert support media, such as paper and cellulose acetate [2], enhanced the initial resolution of protein separations. This was further improved by the use of agarose gel [3] which has been in common use for the past twenty years. High-resolution agarose gel electrophoresis (HRAGE) has provided a universally accepted method for the separation of serum proteins [4] in most clinical laboratories. Monoclonal proteins are currently quantitated densitometrically from HRAGE using the total globulin quantitation derived from the total protein and albumin analyses.

Similar to the original Tiselius method, capillary electrophoretic (CE) separation is performed in a capillary by the application of a very high voltage [5]. However, by reducing the diameter of the capillary [6] the resolution of CE has greatly improved. Other major advantages of CE relate to speed of analysis, automation of the technique and sensitivity of detection.

The use of CE to separate human serum

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proteins has already been reported in the clinical laboratory [7,8], with real-time data analysis enabling automated quantitation of particular protein peaks from electropherograms [9]. Kim et al. [10] compared 37 samples by agarose gel electrophoresis and CE with favourable outcome.

Our aim was to evaluate the use of CE in quantitation of serum protein electrophoresis, for clinical purposes, by a prospective comparison of 1000 clinical specimens with HRAGE.

2. Experimental

HRAGE was performed using commercially prepared gels (Helena Titan High-Res Cat. 3040; Beaumont, TX, USA). The gels were used according to the manufacturer's instructions with the exception that staining was carried out using 0.2% Amido black. The band intensity was scanned using a Helena Cliniscan 2 at 610 nm.

For CE an Applied Biosystems Model 270A-HT CE system (Foster City, CA, USA) with standard 72 cm × 50 μm I.D. fused-silica capillary was used. Electrophoresis was performed for 15 min at a 18-kV constant voltage with detection at the cathodic end by on-column measurement of absorbance at 200 nm. Quantitation of the various parameters was by calculation of the area under the curve utilising the Turbochrom III software package supplied with the instrument. On identification of a monoclonal band by either technique, the immunoglobulin type was established using isoelectric focusing with immunofixation of the patient's serum.

Calibration of the instrument was performed by measurement of area under the curve of appropriate dilutions of a serum albumin standard (Boehringer-Mannheim, Indianapolis, IN, USA). The calibration was verified by comparison to other commercially available albumin standards including purified human albumin (Baxter Healthcare, Dade Division, Miami, FL, USA).

Each capillary was calibrated on installation in the instrument, and once weekly thereafter. Initially, to ensure adequate quality control, at least two points on the calibration curve were re-run daily. Subsequently a normal serum with an albumin of 40 g/l and a serum containing a monoclonal band of 14 g/l were run each day for control purposes. The allowable limits of variation for each control were ± 2 g/l.

The capillary was conditioned prior to each separation by sequentially rinsing for 2 min with 100 m*M* sodium hydroxide, 2 min with distilled water and 3 min with assay buffer (50 m*M* boric acid, pH 9.7, containing 1 m*M* calcium lactate). Addition of calcium lactate to the boric acid buffer enhanced the CE separation, particularly the beta components transferrin and C3.

Sera were diluted 1:49 (v/v) in the assay buffer and 200-μl aliquots loaded into individual sample cups in the 50-space autosampler. Each sample was introduced to the capillary by a 2-s $1.27 \cdot 10^2$ mm vacuum injection.

3. Results

The electropherograms obtained by CE were a virtual mirror image of the densitometer-derived electropherograms of HRAGE. Various examples, including a normal serum, are shown in Fig. 1. Reproducibility studies for both methods are detailed in Table 1. A limited literature review was unable to establish acceptable confidence limits for HRAGE. A proposed selected method [3] reported that subjective evaluation of precision as determined by different observers appeared to be excellent but did not report limits of acceptability.

Of the 1000 clinical samples studied, 362 contained one or more monoclonal protein bands, the levels of which varied from 1 to 71 g/l (see Table 2) as determined by HRAGE.

The comparison of the quantitation of the monoclonal bands by CE and HRAGE is shown in Fig. 2. The correlation coefficient between the two methods was 0.96. The quantitative correlations of the monoclonal bands by paraprotein type are shown in Fig. 3. We have analysed the data according to the difference between the two methods in the standard way [11] (see Fig. 4).

We examined the data to see if there was a

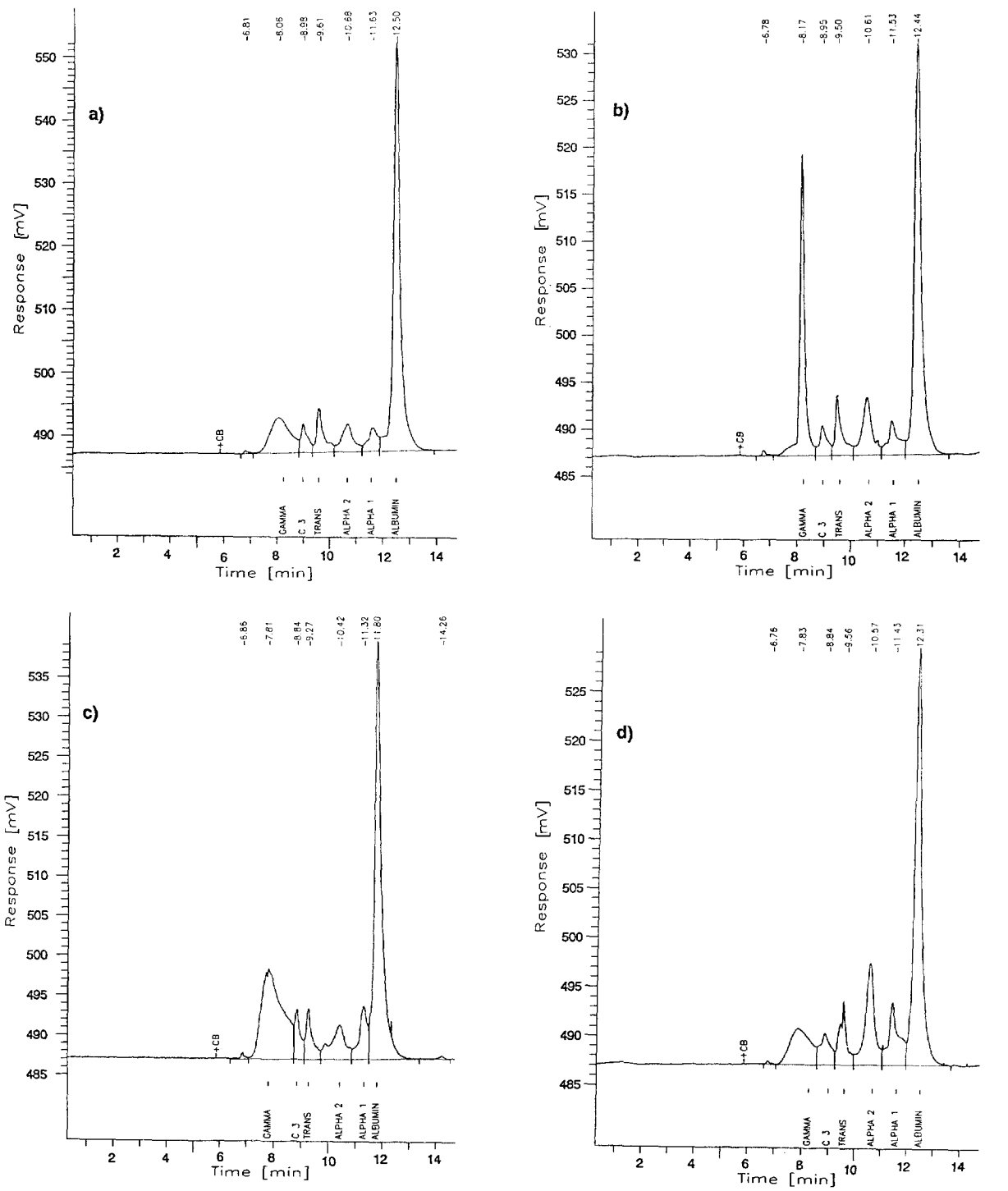

Fig. 1. Capillary electropherograms of (a) normal laboratory control, (b) IgG paraprotein of 15 g/l, (c) polyclonal increase in gamma-globulins and (d) increased acute phase reactants. Albumin, alpha 1, alpha 2, transferrin, C3 and gamma components are marked on each tracing.

Table 1
Coefficients of variation of paraprotein quantitation at two levels by capillary electrophoresis and three levels by high-resolution agarose gel electrophoresis.

Protein concentration (g/l)	n	Concentration found (g/l)		C.V. (%)
		Minimum	Maximum	
CE				
19.5	34	15.3	26.3	10.4
36	40	32.5	39.8	5.0
HRAGE				
16	22	14.0	17.9	5.2
33	22	26.2	34.2	8.1
53	22	43.0	54.5	6.8

n refers to number of runs in which sample was assayed.

statistical difference between the paraprotein values obtained by HRAGE and CE. The standardised skewness and kurtosis were very large indicating that the distribution of observations was significantly different from normal. Since the t-test was not appropriate, the sign test and Wilcoxon signed rank test were used. These statistical methods indicated that HRAGE gave a slightly greater result for the monoclonal bands than CE ($p < 0.05$) (see Table 3).

CE detected three immunoglobulin A (IgA) paraproteins which were not detected by HRAGE due to their being "disguised" in the transferrin or C3 bands. However, on CE, they showed either a distinct extra band or a bizarre-shaped C3 peak. Reanalysis by specific immuno-

Table 2
Distribution of monoclonal paraproteins (in g/l) found in 362 specimens of the 1000 samples studied (based on HRAGE data)

Monoclonal protein	0–10	11–20	21–30	31–40	41–50	50 +	Total
IgG	80	52	37	28	8	8	213
IgA	20	13	12	4	7	10	66
IgM	24	24	7	6	3	1	65
Free light chain	13						13
Biclonal proteins	1	4					5

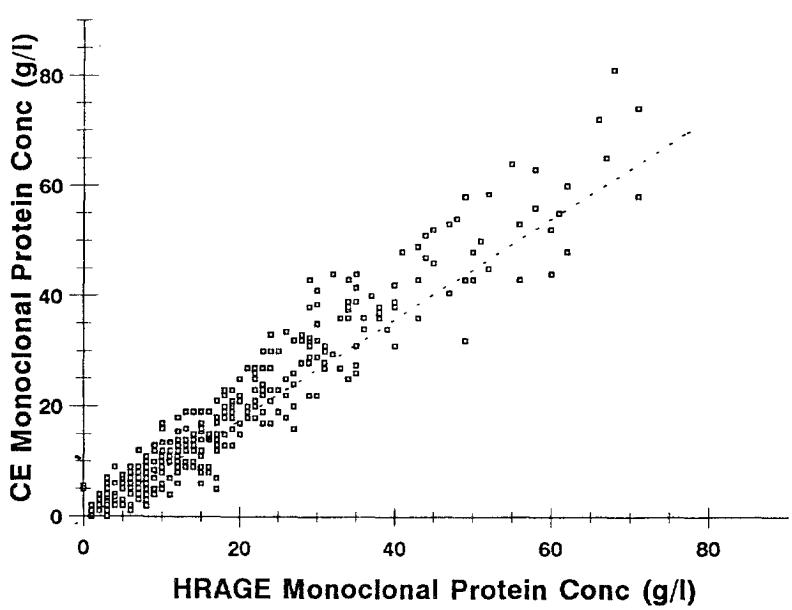

Fig. 2. Comparison of monoclonal protein quantitation by CE and HRAGE.

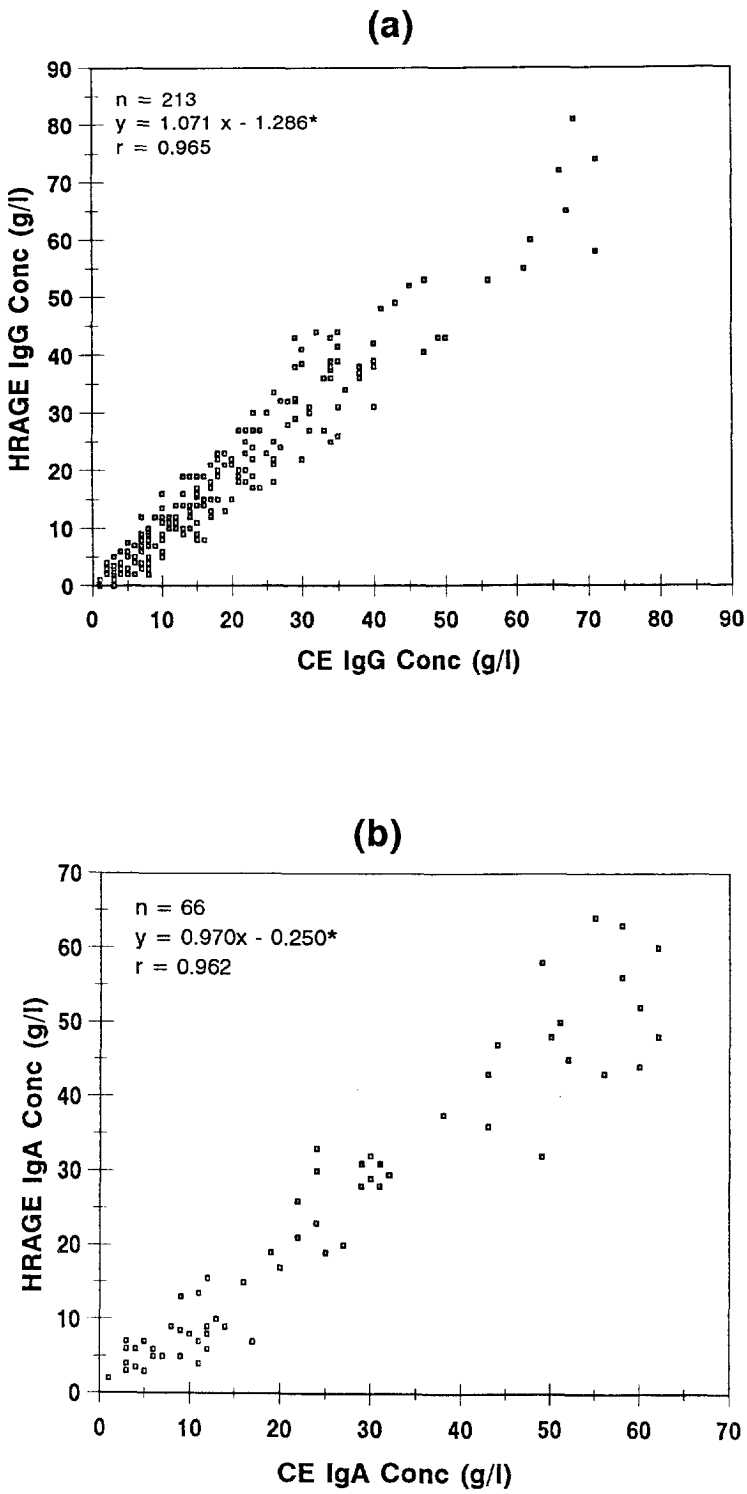

Fig. 3. (*continued on p. 246*)

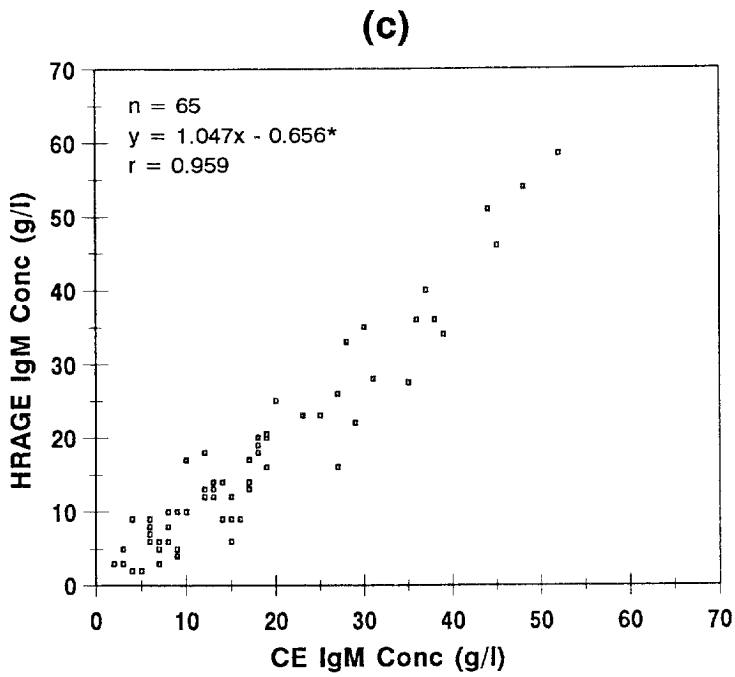

Fig. 3. (a) Comparison of quantitative monoclonal IgG by densitometric analysis of HRAGE versus quantitation by CE. The asterisk indicates the method of Passing et al. [17]. (b) Comparison of quantitative monoclonal IgA by densitometric analysis of HRAGE versus quantitation by CE. The asterisk indicates the method of Passing et al. [17]. (c) Comparison of quantitative monoclonal IgM by densitometric analysis of HRAGE versus quantitation by CE. The asterisk indicates the method of Passing et al. [17].

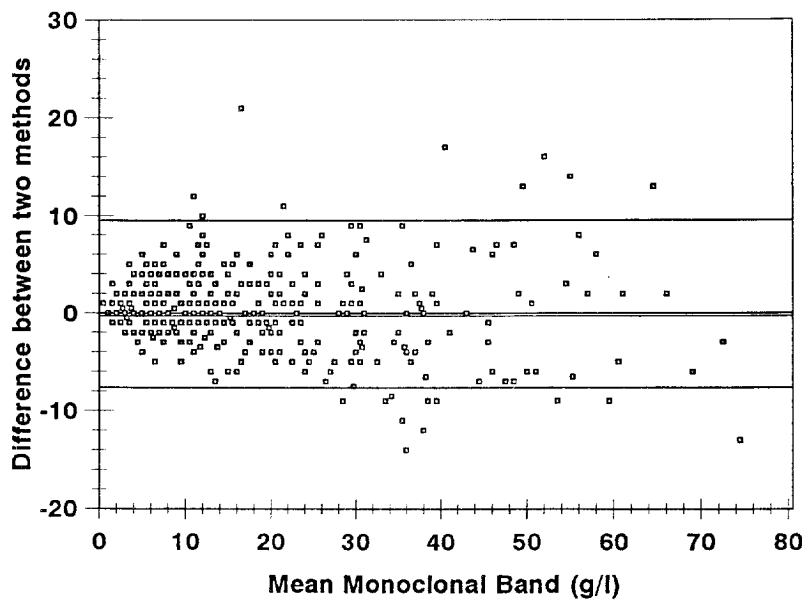

Fig. 4. Bland Altman analysis of data of 362 monoclonal bands. Average mean differences of bands −0.53, standard variance 4.435.

Table 3
Statistical analysis of data obtained from quantitation of monoclonal bands by HRAGE and CE

Variable	HRAGE	CE
Average	19.15	18.70
Median	15	14
Standard error	0.82	0.84
Minimum	1	0
Maximum	71	81
Skewness	1.15	1.14
Standardised skewness	8.95	8.86
Kurtosis	0.85	0.88
Standardised kurtosis	3.31	3.41

Sign test statistic $S = 177$ ($z = 2.32$ and $p = 0.02$), $W^+ = 27678$ ($z = 2.05$, $p = 0.04$).

fixation of HRAGE showed the presence of the IgA monoclonal bands.

One serum with an elevated level of IgA (7.9 g/l) showed a clonal spike by CE, but no monoclonal band by HRAGE.

One monoclonal IgG band was detected by CE but not by HRAGE. On re-examination of the immunofixation of isoelectric focusing of the serum of this patient, there was a diffuse banding pattern of two populations of IgG. This unusual pattern had not been classified as a monoclonal band on inspection of the HRAGE.

Two cases of monoclonal IgM paraproteinaemia were detected by HRAGE but were significantly distorted when detected by CE. The retention times of all proteins on the CE electropherograms in these two cases were markedly prolonged, the albumin retention times of these samples being over 60 s longer than usual. When the calcium lactate was removed from the boric acid buffer, the monoclonal bands became evident.

In two cases where HRAGE showed a 1 g/l light-chain band in the slow gamma, CE did not detect a corresponding band. In all other cases ($n = 11$) when light chains were detected by HRAGE, they were also detected by CE. Eight specimens with small monoclonal bands of less than 3 g/l migrating with C3 by HRAGE were not detected by CE due to concurrent migration with C3. The small monoclonal band was indistinguishable from C3 and showed no obvious distortion of the electropherogram.

Comparison of the two sets of data showed there were 35 occasions which the standardised residual calculation indicated outliers. These are further described in Table 4.

4. Discussion

Using HRAGE, an ideal protein stain should have consistent dye uptake for all protein types. In practice, this does not occur as dye binding

Table 4
Detailed analysis of the 35 monoclonal bands which the standardised residual calculation indicated were outliers

Monoclonal protein	Number in study	Number of outliers	Detailed differences	HRAGE > CE	CE > HRAGE	Comment
IgG	213	20	11	1	10	Total protein > 100 g/l. Possible incomplete staining by HRAGE.
			9	5	4	Total protein < 100 g/l
IgA	66	9	7	6	1	Multibanded IgA: probable over-estimation by HRAGE due to inclusion of β bands.
			2	0	2	Single banded IgA.
IgM	65	6	5	2	3	Total protein > 100 g/l.
			1	1	0	Diffuse IgM band

The table shows a breakdown of the paraprotein types shown to be outliers, which method gave the higher value, and the possible causes for the outlier values.

initially occurs by electrostatic attraction of anionic dye to protonated amino groups of proteins with secondary hydrogen bonding and hydrophilic binding between non-polar constituents also being involved [12]. For example, serum albumin has a different dye uptake to IgG protein components. It has also been noted that an excessively high protein concentration within a separated band may exhibit incomplete staining or metachromacy [13]. Hence, in cases where the total protein was greater than 100 g/l the CE probably gave the more accurate quantitation as it is difficult to get dye evenly into a very large band.

In the multi-banded IgAs, there was a probable over-estimation by HRAGE due to frequent inclusion of transferrin and complement components in the quantitation (as seen in Table 4). CE in many cases was able to separate the IgA band from the beta components.

The IgG and IgA monoclonal proteins showed a statistically significant difference between the two methods (multiple-comparison LSD procedure at overall significance level of 0.05). The Box and Whisker plots for the individual paraproteins (see Figs. 5–7) are a convenient graphical representation of the data. These plots reveal (in Fig. 5) that the majority of the IgG

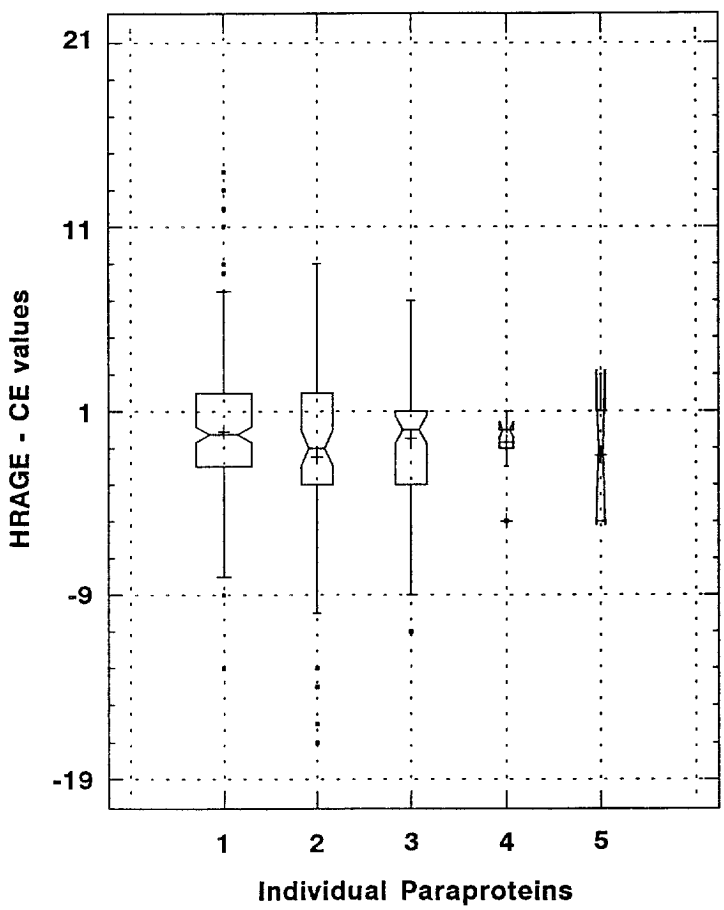

Fig. 5. Notched Box and Whisker plots showing differences between CE and HRAGE. The boxes shown extend from the 25th to the 75th percentile, the central line is the median, and the notches around them indicate the 95% confidence interval of the median. Individual outliers shown as dots. Identity of the paraproteins: 1 = IgG; 2 = IgA; 3 = IgM; 4 = free light-chain bands; 5 = biclonals.

outliers relate to higher CE values, whereas the IgA outliers are all due to higher HRAGE results. The boxed areas of the individual Box and Whisker plots show in grams the spread of the 25th–75th percentile for each paraprotein. The spread of this mid-50 percentile for IgA results is significantly greater than the spread of the mid-50 percentile for IgG or IgM results (see Figs. 6 and 7).

To determine the greatest differences between HRAGE and CE we applied two methods. The first was the regression of one method on another, which confirmed that 69% of the outliers as determined by examination of the standardised residuals from the regression have bands greater than 30 g/l by both HRAGE and CE. The second method, the Bland and Altman plot of the differences, showed 75% of the outliers to be bands greater than 30 g/l. Hence, there was good agreement between two statistical methods.

With HRAGE the technique is highly labour-dependent, the skill of the operator significantly contributing to the reproducibility of the tech-

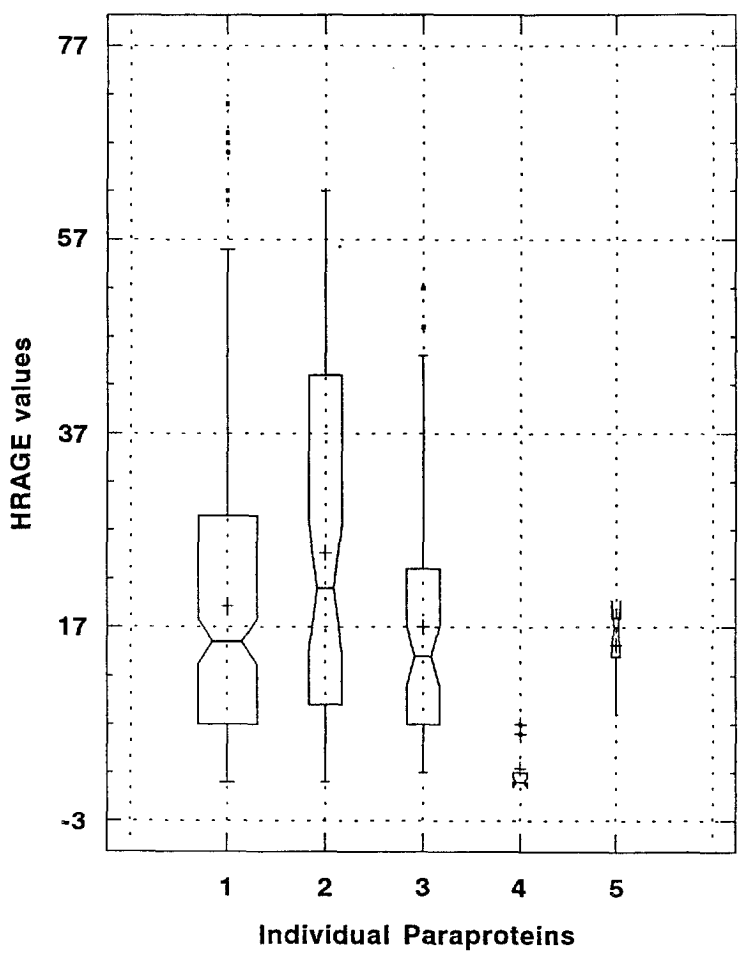

Fig. 6. Box and Whisker plot showing the spread of HRAGE data for individual paraproteins. The boxes shown extend from the 25th to the 75th percentile, the central line is the median, and the notches around them indicate the 95% confidence interval of the median. Individual outliers shown as dots. Identity of the paraproteins: 1 = IgG; 2 = IgA; 3 = IgM; 4 = free light chain bands; 5 = biclonals.

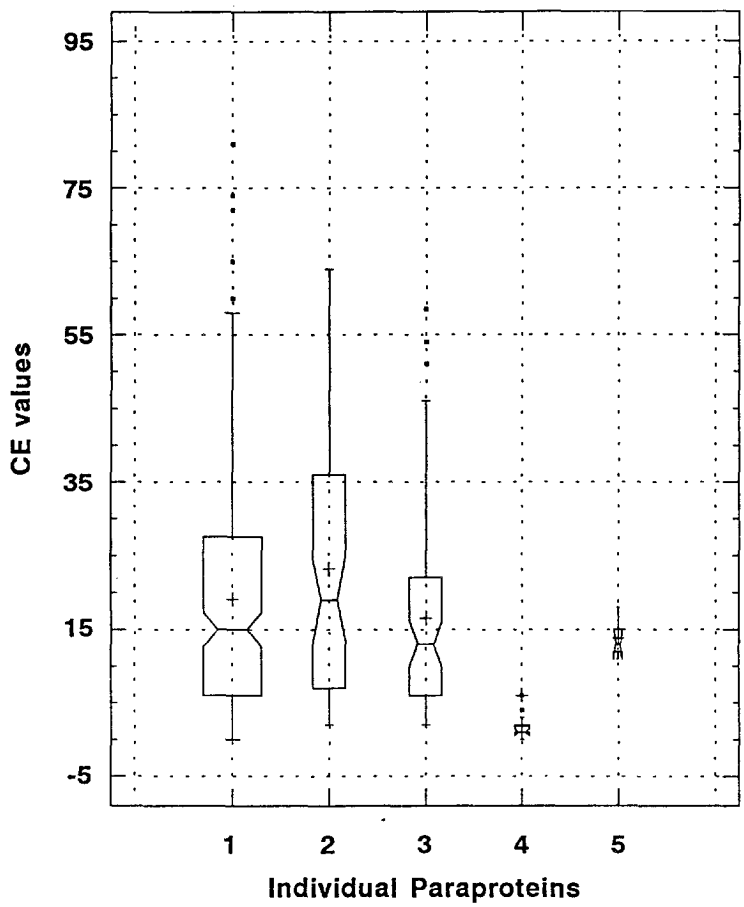

Fig. 7. Box and Whisker plot showing the spread of CE data for individual paraproteins. The boxes shown extend from the 25th to the 75th percentile, the central line is the median, and the notches around them indicate the 95% confidence interval of the median. Individual outliers shown as dots. Identity of the paraproteins: 1 = IgG; 2 = IgA; 3 = IgM; 4 = free light chain bands; 5 = biclonals.

nique. With the use of commercially produced gels, small variations in the amount of applied sample, run time, staining time, destaining technique and use of the densitometer can produce significant variation with the result.

With CE, however, the technical skills required are limited to the dilution of the sample and preparation of the buffer. Adhesion of proteins to the fused-silica capillary has been a problem with CE in the past. We have overcome this problem in two ways: first by choosing a buffer with an extreme pH at which adhesion is less likely [14–16] and second by strictly maintaining our wash protocol between runs which aids conditioning of the capillary surface and promotes reproducibility of results.

We conclude that CE is a viable alternative to HRAGE for the determination of protein electrophoresis in a routine clinical laboratory.

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