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Three methods of capillary electrophoresis compared with high-resolution agarose gel electrophoresis for serum protein electrophoresis

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Abstract

We assessed the BioFocus 2000 capillary electrophoresis instrument for use in a routine clinical laboratory. We examined 210 serum samples received for serum protein electrophoresis by four methods: (1) The Bio-Rad HR015EC high-resolution serum protein kit on the BioFocus; (2) the Jenkins–Guerin (JG) method on the Applied Biosystems 270A HT Capillary Electrophoresis System (JG-ABI); (3) the Jenkins–Guerin method using the BioFocus (JG-BF); and (4) the quantitation of monoclonal bands found in 76 of the 210 samples was assayed by Helena Titan Hi-Res agarose gel electrophoresis (HRAGE). The correlation coefficient between the three sets of capillary electrophoresis monoclonal band results and the Helena quantitation was 0.92 or better. Although the quantitative comparison of monoclonal bands by HR015EC was very good, the lack of sharpness of monoclonal bands using the HR015EC kit meant our preference was to use the JG method on either the ABI or on the Biofocus.

Keywords: Proteins

1. Introduction

Capillary electrophoresis (CE) is an exciting technique which is slowly gaining acceptance within the clinical environment. Since 1991 many scientific papers have been published using CE for serum protein electrophoresis [1–7], urine protein electrophoresis [8,9], and haemoglobin variant analysis [10–13]. Since 1995 papers describing techniques for cryoglobulin assessment [14], lipoprotein separation [15,16], oxalate–citrate separations [17], drug analysis, cerebrospinal fluid protein electrophoresis [18], HbA_{1c} analysis [19,20] and steroid analysis [21] have also been published.

To date the routine use of CE in the clinical laboratory appears to have been mainly for serum protein electrophoresis, where large numbers of samples are handled by teaching hospitals and private pathology. The cost effectiveness of using the technique of CE has been demonstrated by a number of authors [13,22]. Since 1993 several commercial companies have recognised the advantages of serum protein electrophoresis (SPE) by CE, and have introduced instruments designed for the clinical laboratory [23–25].

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Whilst our laboratory has routinely performed serum protein electrophoresis by CE for the past 5 years on an Applied Biosystems instrument (ABI), we have recently acquired an instrument which has been designed for the clinical laboratory, namely the Biofocus 2000 (BF).

In this study we have compared 210 samples using a recently released Bio-Rad commercial high resolution CE kit (HR015EC) with our previously published optimized method on the Applied Biosystems instrument (JG-ABI) [26]. We utilised software specifically written by Bio-Rad to allow the BioFocus to be used as an open instrument, i.e. not only for methods confined to the manufacturer's reagents. This meant that we were also able to study the samples using our routine method on the BioFocus (JG-BF). Finally, the quantitation of monoclonal bands obtained by the various CE methods was compared to the quantitation obtained by running the samples on commercial high-resolution agarose gel electrophoresis (HRAGE).

2. Experimental

2.1. Method 1: HR015EC

The Bio-Rad HR015EC kit was used according to the manufacturer's instructions. The CE separation was carried out with a 24 cm×25 μm I.D. fused silica capillary (19.4 cm length to the detector) contained in a cassette. Measurement of protein absorbance was made at 204 nm. A reference or normal sample supplied with the kit was run at the beginning of each run. This could then be used to verify system performance as well as to overlay patient samples for comparison purposes. Two control samples with specified values for all nine protein components were also included in the kit. The purpose of these samples was as quality control for the HR015EC kit.

The total protein in either g/dl or g/l of each patient was entered into the computer before each run. The A/G ratio was adjusted prior to calculating other results due to the UV absorbance of albumin and globulins being not exactly equal. This calculation was done based on a factor developed with the international IFCC/CAP protein calibrator. The total area (100%) was divided up amongst the various proteins. The shape of the gamma area was reviewed by a special software function which identified possible paraproteins with a 'Check Gamma' alert.

After each run the electropherogram printed showed axes labelled normalized mobility and normalized absorbance. The normalized mobility was calculated for each sample using an internal marker (hippuric acid) which was assigned a mobility of −1.00. The relative mobility was determined by the location of each protein relative to the marker and the electroosmotic front, which was assigned a mobility of 0.00. The width of the peaks were corrected according to the migration time using classical formulae for mobility. This meant that the γ area was expanded by approximately 25% (manufacturer's personal communication).

For the normalized absorbance the width of the peaks were converted from the time domain to the mobility domain, as described above. Then the internal marker was assigned an area of exactly 1000. The *y*-axis was scaled so that the height of the internal marker would produce the desired area (1000). When comparing different electropherograms, the internal markers will have the same area and essentially the same height; the serum proteins in the patient sample will appear larger or smaller depending on the total protein content of the patient sample.

2.2. Method 2: JG-ABI

The Jenkins–Guerin optimised method uses 75 m*M* boric acid buffer, pH 10.3, containing 0.2 m*M* calcium lactate (Sigma L-2000, St. Louis, MO, USA). CE was performed using an Applied Biosystems Model 270A-HT CE system (Foster City, CA, USA) with a standard 72 cm×50 μm I.D. fused-silica capillary (Scientific Glass Engineering, Ringwood, Australia). The effective length of this capillary for separation of samples was 50 cm. Electrophoresis was performed for 12 min at 20 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

Turbochrom 1V software package supplied with the instrument.

Calibration of the instrument was performed by measurement of the area under the curve of albumin of three normal serums with concentrations ranging from 28 to 40 g/l. The capillary was calibrated on installation in the instrument, and once weekly thereafter. Quality control was ensured by running a normal serum once a week, and an IgG monoclonal band of 20 g/l daily. The allowable limits of variation for the control band was ±2 g/l.

2.3. Method 3: JG-BF

The same optimized method was also performed on the BioFocus 2000 CE System (Hercules, CA, USA) using a 54.6 cm×50 μm I.D. fused-silica capillary (Scientific Glass Engineering) contained in a cassette. The effective length of the capillary for separation of samples was 50 cm. Electrophoresis was performed for 12 min at 12 kV constant voltage, the sample being injected for 13.5 kPa·s. Detection was at the cathodic end by on-column measurement of absorbance at 200 nm. A voltage of 12 kV in the BioFocus produced an equivalent near baseline separation to the 20 kV applied in the ABI. Quantitation of proteins by the instrument utilised Bio-Rad CDM2.0A software which had been specifically written to allow the BioFocus to be used as an open instrument. Calibration standards and quality control specimens were identical to those used on the ABI instrument.

2.4. Method 4: HRAGE

For comparison purposes, all the monoclonal bands were run on high-resolution agarose gel electrophoresis (HRAGE) using commercially prepared gels (Helena Titan High-Res Cat. 3040; Beaumont, TX, USA). The gels were used according to the manufacturer's instructions. The band intensity was scanned using a Helena Cliniscan 2 at 610 nm.

3. Results

The HR015EC kit measured the absorbance of the proteins and the internal marker in the buffer system at 204 nm. These analytes showed as a running electropherogram on the computer monitor of the CE instrument (see Fig. 1a). On completion of the run, the computer calculated the normalized mobility and normalized absorbance for each sample (see Section 2.1). The same sample in the form reported by the instrument is shown in Fig. 1b.

The HR015EC kit detected normal variations in routine electropherograms such as increase in α1 and 2 (acute phase reactants), split α1 (unusual A1AT phenotypes), polyclonal increase in gamma globulins, decrease in γ-globulins and β–γ bridging. The HR015EC kit also detected the presence of pathological conditions such as the presence of any monoclonal bands (see Fig. 2).

Of the 210 clinical samples assayed, 76 samples contained one or more monoclonal protein bands, the levels of which varied between 1 and 79 g/l as determined by HRAGE. The 210 samples included eight samples which contained more than one monoclonal band, as well as two triple monoclonal IgG(κ) samples. Table 1 shows the distribution of types of monoclonal bands found in the 210 samples.

Quantitative comparison of 76 monoclonal bands by HR015EC versus densitometric measurement of bands by HRAGE gave the equation $y=1.07x+1.06$ (method of Bablock et al. [27]). The correlation coefficient was 0.85. These figures included one specimen with a gelling cryoglobulin which gave results greater than three standard deviations from the line of best fit. Excluding this gelling cryoglobulin specimen from the correlation the equation became $y=1.07x+0.96$. However, the correlation coefficient improved from 0.85 to 0.95.

The HR015EC kit failed to detect two of the monoclonal bands that were detected by the other three methods. In both cases the residual γ-globulins were not reduced. In the first case we failed to detect a 1–2-g/l IgG(λ) band since there was no 'Check Gamma' alert (see Fig. 3). In the second case we failed to find a 5-g/l IgA(λ) band. This failure may have related to lack of experience with the kit. The 'Check Gamma' alert does not extend to bands in the β region.

Quantitative comparison of the monoclonal bands in 76 samples by method 2 (JG-ABI) versus densito-

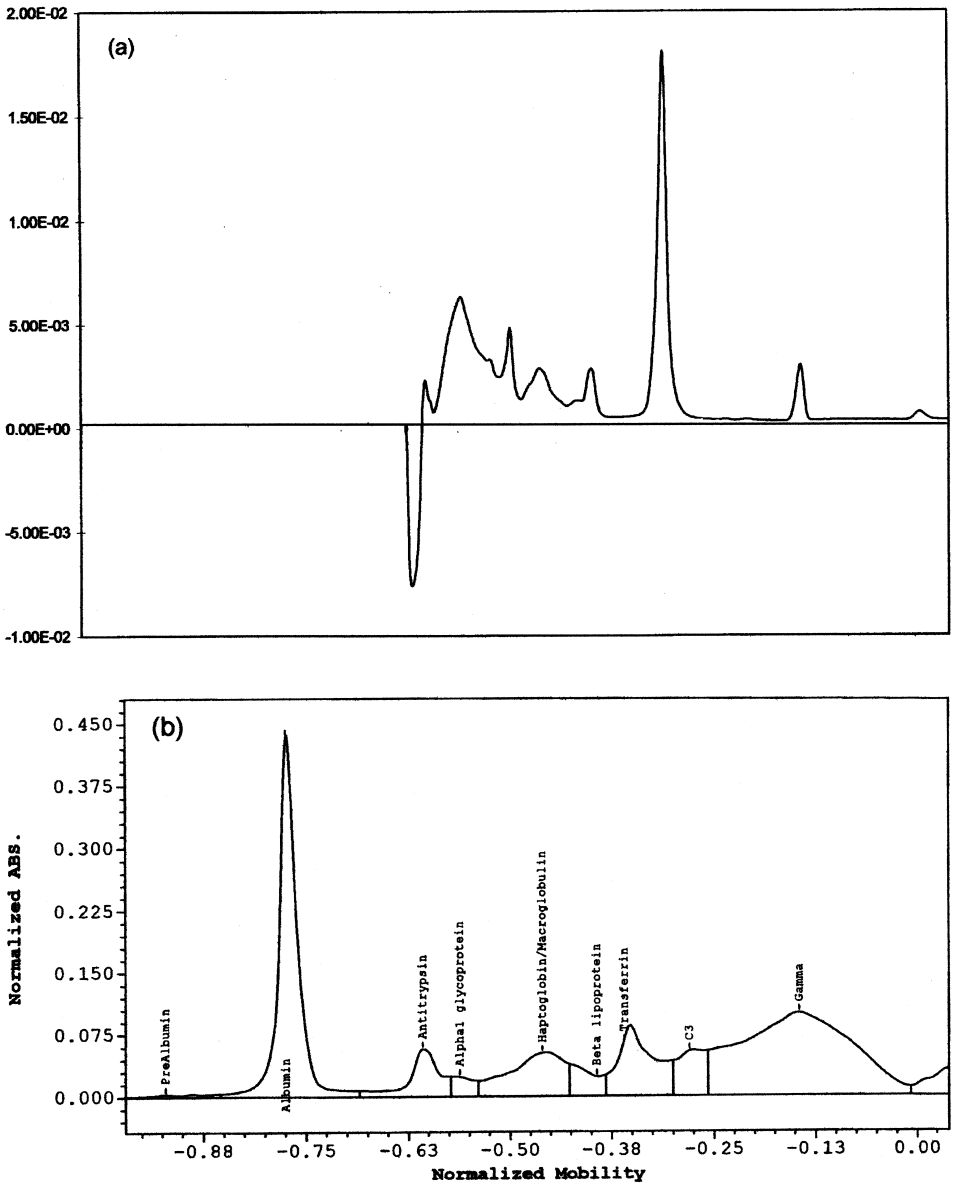

Fig 1. Using the HR015EC kit. (a) An electropherogram as seen on the computer screen of a sample from a patient with a polyclonal increase in γ-globulins. (b) The printed electropherogram of the same sample.

metric measurement of bands by HRAGE gave the equation $y=x+1.0$, the correlation coefficient being 0.971. This was slightly better than the original correlation made on the same instrument [4].

With the BioFocus, the correlation of the monoclonal bands in 76 samples by Method 3 (JG-BF) versus densitometric measurement of bands by HRAGE gave the equation $y=x+1.0$, the correlation coefficient being 0.923. Examples of this method on the Biofocus are shown in Fig. 4.

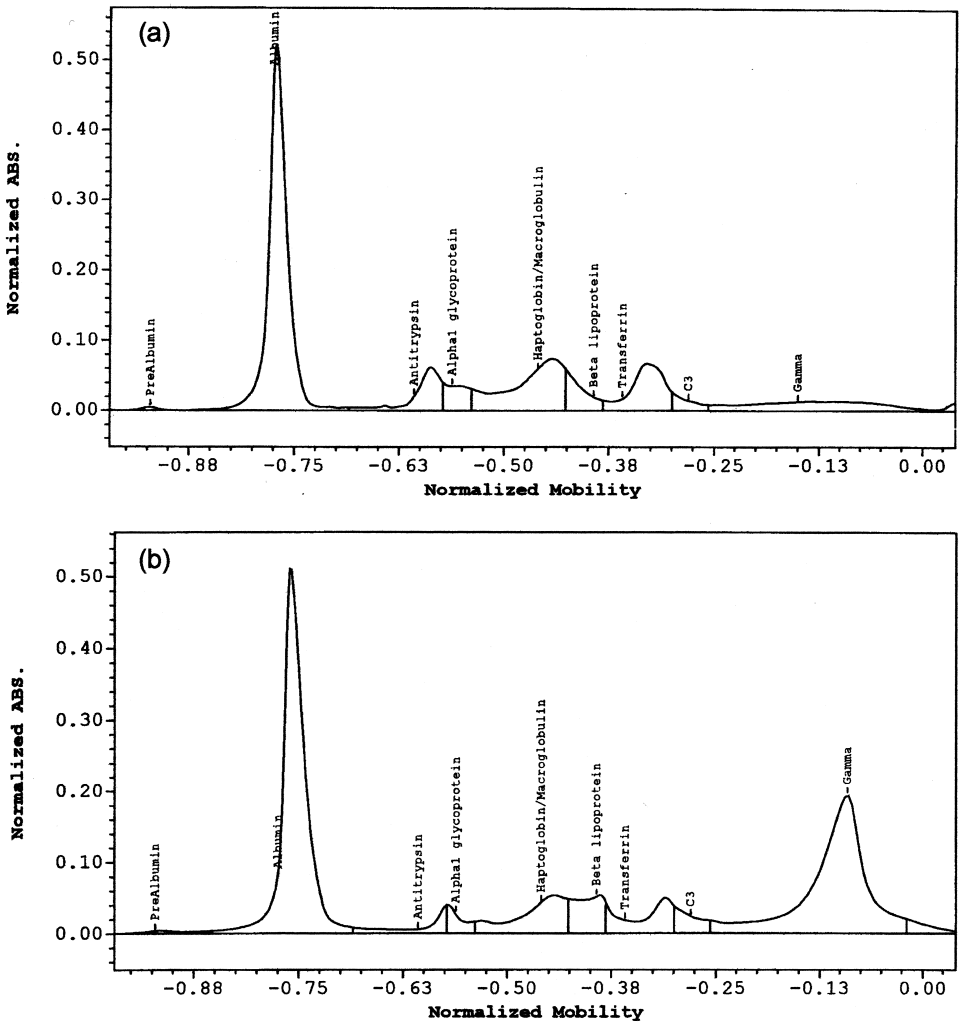

Fig. 2. Capillary electropherograms, using the HR015EC kit, of (a) patient with decreased γ- globulins and (b) a patient with 18 g/l monoclonal IgG(κ) band.

4. Discussion

It is important that Bio-Rad have included software that makes the Biofocus an open instrument. Hence alternative methods for serum protein electrophoresis by CE can be run on the instrument, as well as other methods such as drug analysis.

Two major factors influence how tight or broad the bands are by capillary electrophoresis. The first of these is the length of capillary, the second the buffer used. Many other operating parameters also affect resolution and efficiency of CE separations. We noted that the field strength in Method 2 was 277 V/cm, whereas the field strength in Method 3 was 219 V/cm. The voltage calibration on one instrument may be incorrect. Using the Poiseuille equation to calculate injection volumes, Method 1 has 3 nl injected per sample, Method 2 has 8 nl and Method 3 has 3 nl.

The HR015EC kit uses a 24 cm×25 μm I.D.

Table 1
Distribution of numbers and types of monoclonal bands detected in 210 samples by three capillary electrophoresis methods and high resolution agarose gel electrophoresis. Samples with two monoclonal bands of differing heavy or light chain type are counted as two bands

Type of monoclonal band	HR015EC	JG-ABI, JG-BF, HRAGE
IgG(κ)	23	24
IgG(λ)	13	15
IgA(κ)	7	7
IgA(λ)	6	7
IgM(κ)	15	16
IgM(λ)	6	7
Free κ light chain band	1	1
Free λ light chain band	3	3
IgD(λ)	1	1
IgG heavy chain disease	1	1

capillary, which has an effective length of 19.4 cm to give analysis of one sample in 4.5 min, whereas the JG-ABI method uses a capillary with an effective length of 50 cm to give a separation in 12 min. Obviously, if a large number of samples are to be handled each day, the shorter the separation, the quicker the results will be available. The buffer used in the HR015EC kit is a proprietary preparation, the pH being measured at pH 9.6.

The quantitation approach for the two CE methods is quite different. The HR015EC kit uses areas that have been corrected by time for conversion into the mobility domain. The user enters the total protein (in either g/dl or g/l) before the run is commenced. Because the HR015EC kit acknowledges that the UV absorbance of albumin and globulins is not exactly equal, the A/G ratio is adjusted prior to calculating other results. The calculation is done, based on a factor developed with the international IFCC/CAP protein calibrator. The total area (100%) is divided up amongst the various proteins.

Improved reproducibility in capillary electrophoresis through the use of mobility and migration time ratios has been discussed by several authors [28,29]. If necessary, a monoclonal band may be manually cut to give better quantitation. With the HR015EC kit, the use of the computer program gave results which were visually broader for the monoclonal bands, than those for the JG-ABI or JG-BR methods.

In contrast, the JG method uses calibration of the capillary with albumin standards, and assumes that 1 g of albumin is equivalent to 1 g of monoclonal protein. Previous assessment of 362 monoclonal bands have supported this hypothesis [4]. Comparison of Figs. 2 and 4 demonstrate both the trade off of resolution for speed, and the different buffer compositions have on the resulting electropherograms for the two CE methods. The resolution obtained with a 12-min separation as in the JG-ABI or JG-BF methods appears superior to the resolution obtained by the HR015EC kit.

The drawbacks of the HR015EC kit included two monoclonal bands that we failed to detect. The 'Check Gamma' feature of the HR015EC kit alerted the operator to monoclonal bands in the γ region. However, it was not geared to inspect the β region, nor to comment on any monoclonal bands in the β region. Reviewing the monoclonal bands, eight of the 76 samples contained more than one monoclonal band. These included samples with multi-banded IgAs. Other samples contained monoclonal bands with varying heavy chain types and similar light chains, or alternatively, monoclonal bands with the same heavy chain and varying light chain types (see Table 2). Whilst Methods 2, 3, and 4 in all eight cases showed multiple monoclonal bands, the HR015EC kit did not resolve the monoclonal bands into multiple bands in four out of eight cases. We considered this lack of resolution a drawback of the HR015EC kit.

Gelling cryoglobulins are a problem by CE depending on the amount of cryoglobulin in the specimen. This is particularly apparent when there is 4 g/l of cryoglobulin (reference range cryoglobulin, <0.1 g/l). From experience, quantitation of paraprotein is affected even when the amount of cryoglobulin is considerably less. Using the HR015EC kit, the quantitation of the paraprotein where a cryo-

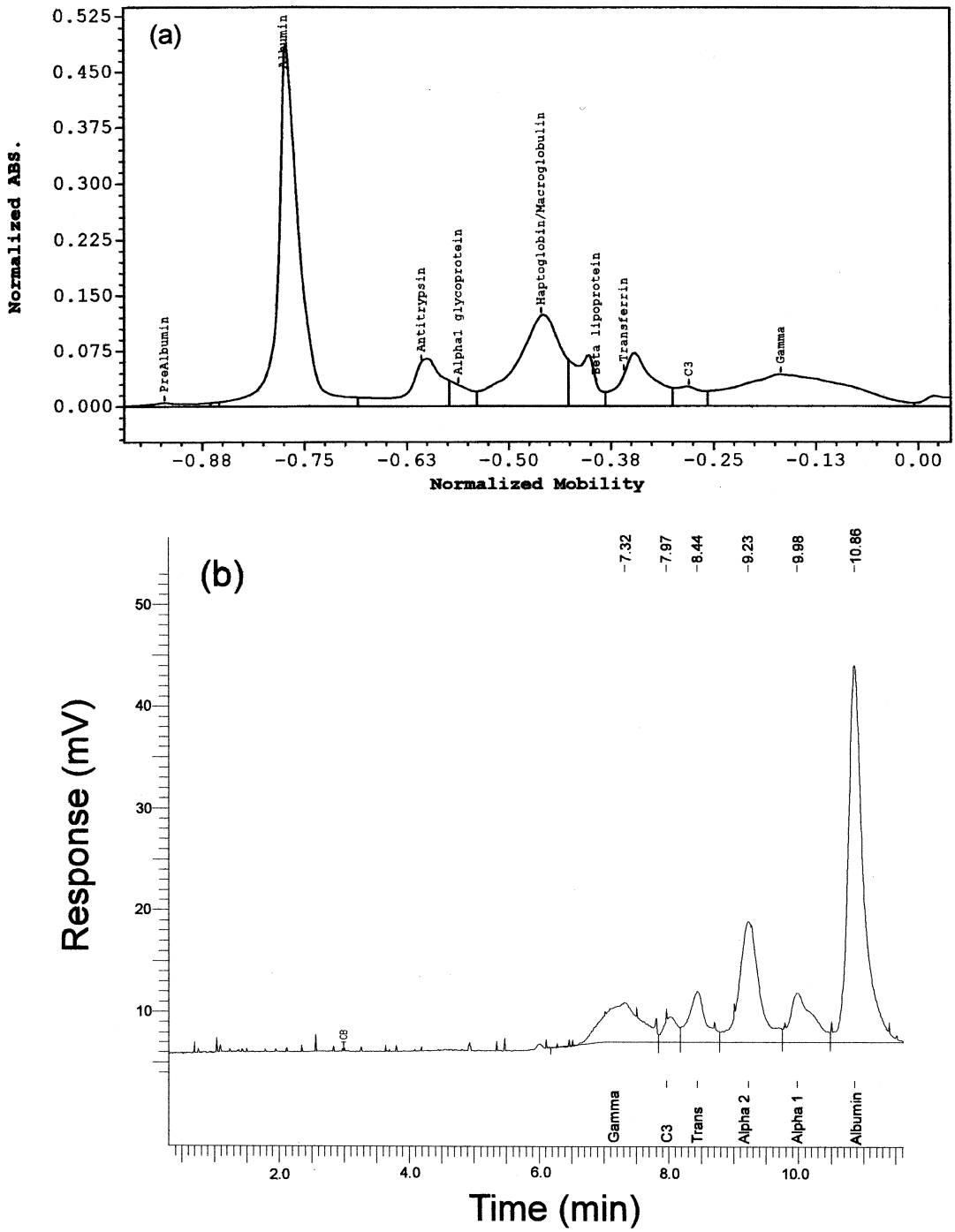

Fig. 3. Capillary electropherograms: (a) using the HR015EC kit on a patient with a 1–2-g/l IgG(λ) band which did not give the 'Check Gamma' alert using the HR015EC kit; (b) same specimen by the JG method on the ABI instrument.

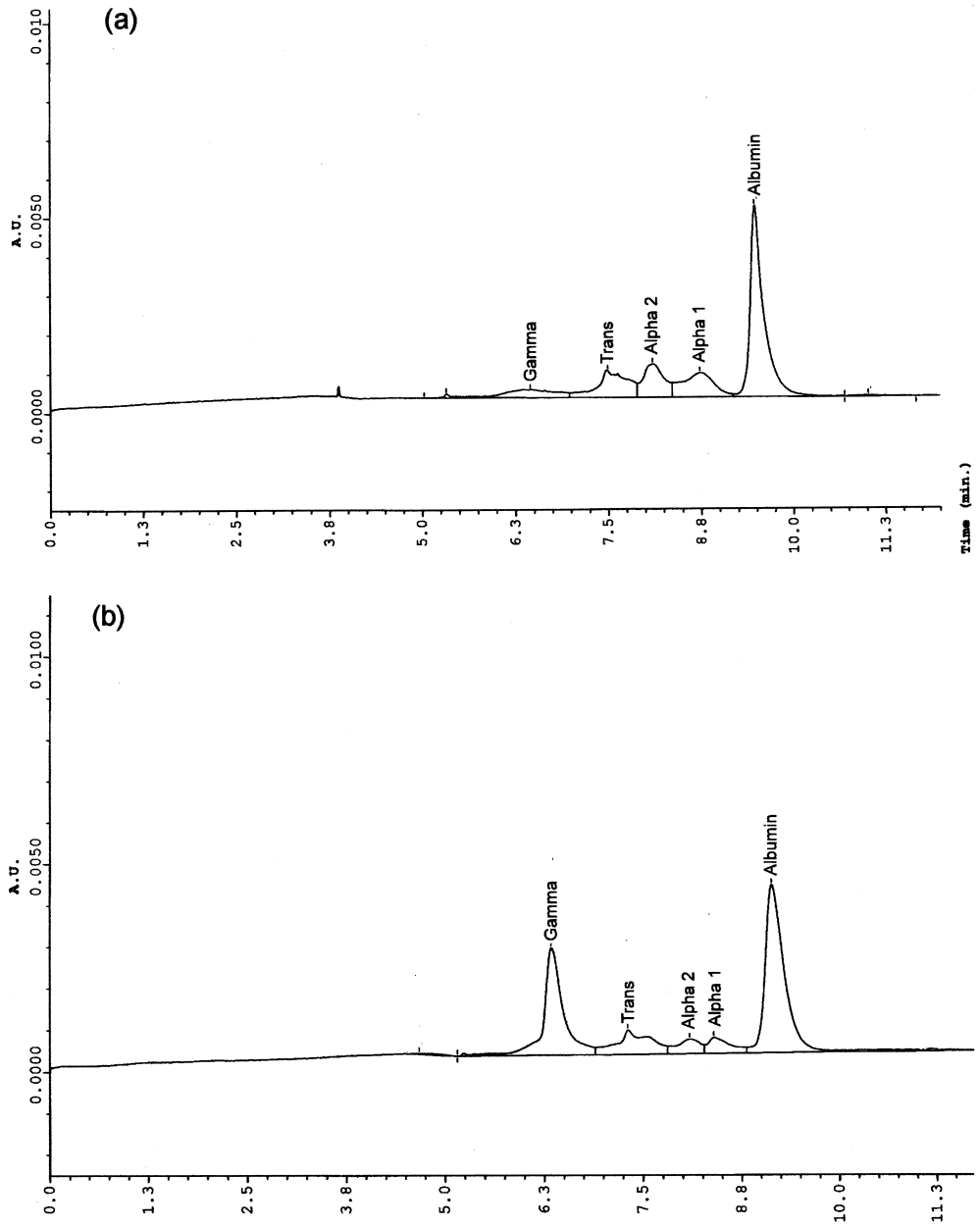

Fig 4. Capillary electropherograms using the JG method on the BioFocus of the same specimens as in Fig 2. (a) Patient with decreased γ-globulins; (b) patient with 18 g/l monoclonal IgG(κ) band.

globulin was involved, was grossly underestimated. The HR015EC kit distributed the protein over the various fractions from the total protein result. In the case of the cryoglobulin specimen, it could be easily seen that there was a problem as the albumin was >80 g/l.

Table 2
Comparison of quantitation of monoclonal bands contained in eight specimens which showed two monoclonal bands by high-resolution agarose gel electrophoresis

Band type	HR015EC band size (g/l)	JG-ABI band size (g/l)	JG-BF band size (g/l)	HRAGE band size (g/l)
2×IgA(κ)	5+12	4+14	3+10	5+11
IgA(λ)+IgG(λ)	16+1	18+1	10+3	17+1
IgM(κ)+IgG(κ)	14	14+8	16+6	16+12
2×IgA(λ)+IgG(λ)	5+5	14+1	9+5+1	9+7
IgM(κ)+IgG(κ)	13	9+14	9+14	11+10
IgM(κ)+IgM(λ)	5	5+5	5+5	4+3
IgM(κ)+IgG(κ)	8+2	9+2	9+2	8+4
2×IgA(κ)	13	17+4	17+4	18+6

Whilst the JG-ABI and the JG-BF quantitation of the gelling cryoglobulin were both 10 times that of the HR015EC kit, they were still affected by the nature of that sample. Since the HR015EC separation was run at 37°C, whereas the JG-ABI separation was run at 30°C, the temperature that the test was run at does not explain the poor result on the HR015EC. The difference in the cryoglobulin quantitation is probably related to the composition of the HR015EC proprietary buffer which is different to the boric acid/calcium lactate used in the JG-ABI method.

In conclusion, the quantitative comparison of monoclonal bands by HR015EC for paraproteins was very good. However, due to the broadness of monoclonal bands using the HR015EC kit, our preference for serum protein electrophoresis by CE would be to use the JG method on the ABI. The drawback to this situation is that the ABI is a discontinued instrument. From the data presented the JG-BF was shown to detect all protein abnormalities studied, and to have a correlation for monoclonal bands of 0.92. Hence our conclusion is that the JG method on the Biofocus is a satisfactory method for routine serum protein electrophoresis.

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