ELSEVIER

Journal of Chromatography B, 775 (2002) 63–70

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of poorly separated monoclonal serum proteins by capillary zone electrophoresis

Thierry Le Bricon[a,*], Erika Launay[a], Pascal Houze[a], Djaouida Bengoufa[b], Bernard Bousquet[a], Bernard Gourmel[a]

[a]Laboratoire de Biochimie A, Hôpital St.-Louis AP-HP, 1 Avenue Claude Vellefaux, 75475 Paris Cedex 10, France
[b]Laboratoire d'Immunologie et d'Histocompatibilité, Hôpital St.-Louis AP-HP, 1 Avenue Claude Vellefaux, 75475 Paris Cedex 10, France

Received 14 February 2002; received in revised form 24 April 2002; accepted 24 April 2002

Abstract

A capillary zone electrophoresis (CZE) technique was developed for the determination of poorly separated monoclonal serum proteins by agarose gel electrophoresis (AGE). A P/ACE 5500 capillary instrument (Beckman) was used under the following conditions: 57 cm×50 μm I.D. fused-silica capillary, pH 9.6 borate buffer, and 214 nm on-line detection. Sixty patients (61±13 years) with a well isolated (n=24, group A) or poorly separated monoclonal band(s) by AGE (n=36, group B) were included in this study. Within- and between-run precision for CZE was below 4% for albumin and 7% for γ-globulin. A 100% (group A) or 61% agreement (group B, more bands detected by CZE in 10 cases) was obtained between CZE and AGE for the number of monoclonal bands. In group B, quantification was possible in 92% of samples by CZE vs. 64% by AGE (P<0.05, chi-square). The proposed CZE method appears as an additional helpful technique for the determination of poorly separated monoclonal serum proteins by AGE. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Monoclonal proteins

1. Introduction

Agarose gel electrophoresis (AGE) of serum proteins is routinely used in clinical laboratories for the diagnosis and monitoring of human B cell malignancies [1]. When produced by a single clone of cells, immunoglobulins are detected in serum as a narrow band most frequently in the γ-region of the gel; the amount of monoclonal component measured by densitometric scanning reflects tumor mass [2]. Detection and quantification of β-migrating monoclonal immunoglobulins (when <1 g/l) remain problematic because of their comigration with other β-globulins: C_3 fraction of the complement, transferrin and β-lipoprotein [3]. In our experience, they are encountered in ~10% of serum containing a monoclonal component, although this might be underestimated [3]. Oligoclonal profiles, large or weak suspect γ bands are observed in another ~20% of these samples and their poor separation does not frequently allow a reliable quantification and/or interpretation. An alternative electrophoresis technique would be helpful for the analysis of these

*Corresponding author. Tel.: +33-1-4249-9931; fax: +33-1-4249-9247.
E-mail address: thierry.le-bricon@sls.ap-hop-paris.fr (T. Le Bricon).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00284-2

complex serum profiles, especially in clinical laboratories performing a large number of serum electrophoresis.

Capillary zone electrophoresis (CZE) represents a significant advance in current electrophoresis technology [4–7]. Using a fused-silica capillary and high-pH buffer, CZE efficiently separates serum proteins according to their charge-to-mass ratio into five major regions (γ, β, α-2, α-1, and albumin) with a particular ability to separate immunoglobulin A (IgA) from other β-globulins [3,8–11]. CZE requires small amount of sample and reagents with no time consuming staining-destaining and densitometric scanning of gel. Fully automated CZE instruments are now available on the market, but they are specifically designed for serum analysis (with multiple sample analysis) and therefore cost-effective only in large laboratories. In addition, the reliability of serum monoclonal band quantification by UV detection is still uncertain with significant positive bias vs. AGE reported [9–11]. Here we developed a CZE technique on a semi-automated P/ACE 5500 instrument (Beckman) for the determination of poorly separated β-migrating immunoglobulins and γ-oligoclonal/large band profiles by conventional AGE.

2. Materials and methods

2.1. Patients and samples

Sixty hospitalised patients (61±13 years, M/F: 30/30) with at least one monoclonal fraction by AGE in their serum were separated into two groups based on their AGE profile, group A: a single well-defined monoclonal band in γ (n=24: IgG 83%, IgM 13%, IgA 4%) and group B: monoclonal band(s) poorly separated by AGE (n=36), either β-migrating (n=19: IgA 74%, IgG 26%) or oligoclonal/large bands profiles (n=17: IgG 59%, IgM 24%, IgA 18%, free light chains 12%). Twenty patients from group B were re-examined after 3 months in the course of their therapeutic follow-up with the following interpretation: >10% decrease in serum monoclonal protein concentration=treatment efficacy, >10% increase=disease progression.

Blood samples were collected in 7-ml thrombin tubes (Beckton Dickinson) and centrifuged for 15 min at 1500 *g* (at 20 °C). Total protein determination and AGE were performed on fresh serum samples, before storage at −20 °C for CZE analysis.

2.2. Materials

2.2.1. Capillary zone electrophoresis

CZE was performed using a P/ACE 5500 semi-automated system (Beckman) with a 57 cm (50 cm effective length)×50 μm I.D. fused-silica capillary at 25 °C. Serum samples were diluted 50-fold in demineralized water (Fresenius) as described elsewhere [12], before hydrodynamically injected under pressure (15 s). The running buffer was a pH 8.6 0.3 *M* borate buffer (Protein separation buffer A, Sigma) adjusted to pH 9.6 with 1 *M* NaOH. Proteins were separated at a constant voltage of 17 kV for 20 min and the electrophoretic profile was obtained by direct absorbence of the peptide bonds at 214 nm at the cathodic end. Between runs, the capillary was sequentially washed and reconditioned for 1 min with demineralized water, 2 min with 0.1 *M* NaOH, 2 min with demineralized water and 1 min with running buffer.

Two serum samples were selected for estimation of within- and between-run precision (RSDs, n=20): a normal control (65 g/l total protein) and a pathological one (78 g/l total protein) with a well-defined monoclonal band (19.9 g/l) in the γ-region of the agarose gel. The sensitivity of CZE for monoclonal detection was evaluated by serial dilution of a serum containing a γ-migrating monoclonal IgG (10 g/l) in a normal serum (polyclonal γ: 10 g/l).

2.2.2. Agarose gel electrophoresis

AGE was performed on a semi-automated gel electrophoresis apparatus (Hydrasys, Sébia) using the Hydragel Kit (Sébia) [13]. Briefly, 10 μl of undiluted serum was applied to each well (15/30 wells/gel), and allowed to diffuse for 5 min before separation at 272 V at 20 °C during 7 min in a Tris–barbital, pH 9.0 buffer. Gels were dried at 65 °C for 10 min, stained with 4 g/l amidoschwartz in a 100 ml/l acetic acid solution, and destained with 0.5 g/l citric acid before final drying at 75 °C for 8 min. The electrophoretic profile was obtained by densitometric scanning of the gel at 570 nm on a Hyrys densitome-

ter (Sébia). Total analysis time was 45 min. In our hands, between-run precision (monthly controls, $n=30$) is comprised between 5% (for albumin) and 13% (for α-1-globulins).

2.2.3. Other biochemical analysis

Total serum protein was determined on a Hitachi 747 analyser (Boehringer) by the Biuret reaction (Boehringer). Quantitative results for each fraction of the CZE or AGE electrophoregram are expressed in g/l by multiplying the % of total area under the curve (AUC) with total protein concentration. Serum immunofixation was performed using the Paragon IFE kit (Beckman).

2.3. Statistical analysis

Population distribution was Gaussian and results are expressed as mean and standard deviation. Correlation between techniques was evaluated by linear regression and analysis of variance (ANOVA), and differences between techniques by the chi-square test. Significance level was set at $P<0.05$ for all tests.

3. Results

3.1. Analytical performances

Precision (within/between-run RSDs) for albumin and γ-globulin quantification was below 4 and 7% for both controls, respectively (Table 1). For other globulins, RSDs ranged from 6% (within run) for β-globulins to 25.1% (between run) for α-2-globulins. For retention times, RSDs below 5% were obtained for the fastest proteins (γ-globulins), and in the 5–10% range for the slowest protein (albumin). Sensitivity of the technique for monoclonal component detection was 0.5 g/l. A CZE electrophoregram of the normal control is presented in Fig. 1.

Table 1
Analytical performance of the CZE technique

Fraction	Normal control		Pathological control	
	g/l	RSD* (%)	g/l	RSD* (%)
Albumin	43.5	1.6/3.6	41.3	1.4/2.3
α-1-Globulins	1.5	18.0/23.3	2.0	10.4/15.6
α-2-Globulins	5.8	18.7/14.7	4.8	17.6/25.1
β-Globulins	5.1	7.3/13.9	6.6	6.0/9.8
γ-Globulins	8.1	3.8/6.8	23.5	1.6/4.1

Total protein: normal control 65 g/l, pathological control 78 g/l. *RSD: Within/between run precision ($n=20$).

3.2. Electrophoretic separation

As for AGE, all patients from group A presented a single sharp spike by CZE migrating in the γ-region (mean concentration: 26.6 g/l, 1.0 to 57.8 g/l). Results obtained in group B are summarised in the form of a flowchart (Fig. 2). The number of suspects bands detected by CZE was identical to AGE in 61% of cases. In all other cases, except one ($n=10$), the number of bands detected by CZE was superior to AGE (Fig. 2, Table 2). The exact number of fractions could not be determined in three poorly separated profiles by CZE ($n=1$) or AGE ($n=2$). Selected examples of CZE electrophoregrams from poorly separated monoclonal proteins by AGE are presented in Fig. 3.

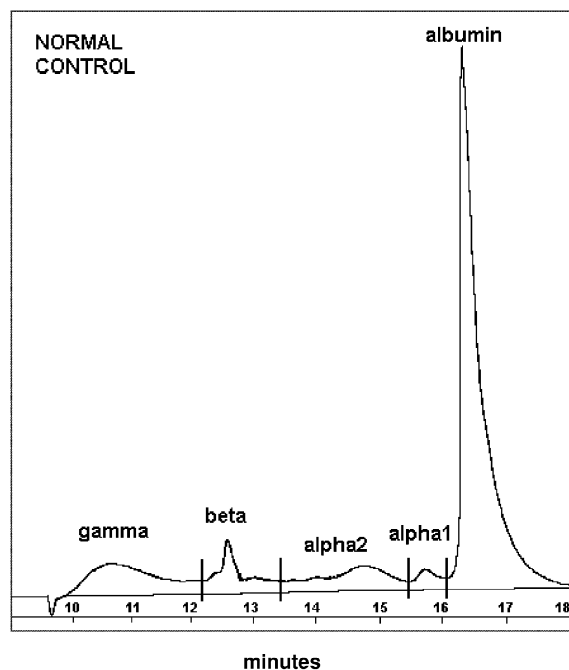

Fig. 1. CZE electrophoregram of the normal control. Normal control (65 g/l total protein, smooth broad γ-zone: 8.1 g/l). Electrophoretic separation was obtained in 20 min.

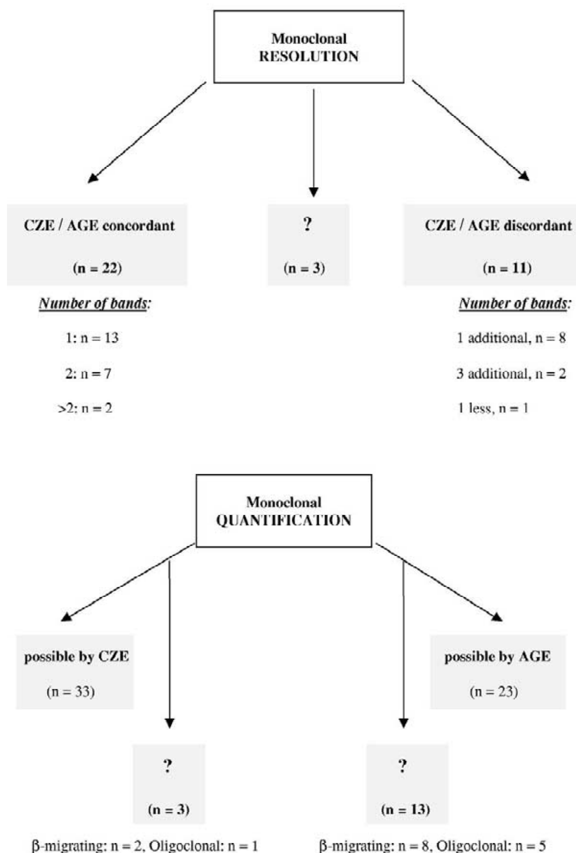

Fig. 2. Separation and quantification of poorly separated monoclonal bands. Performance of CZE and AGE was compared in patients from group B having in their serum either a monoclonal immunoglobulin comigrating with β-globulins (n=19) or an oligoclonal or large band profile (n=17).

3.3. Monoclonal band quantification

In group A, the absolute difference with AGE was 0.9±1.8 g/l. Results of CZE and AGE significantly correlated (r=0.992, P<0.0001): CZE (g/l)=1.02× AGE (g/l)+1.37 (Fig. 4). In group B, quantification of monoclonal bands was possible in 92% of samples by CZE vs. 64% by AGE (P<0.05), including 10 of 13 (19.8±9.9 g/l) not measurable proteins by AGE (six β-migrating, four oligoclonal profiles). In samples quantified by AGE, results of CZE and AGE correlated (r=0.987, P<0.0001): CZE (g/l)=1.03× AGE (g/l)+3.33 (Fig. 4), with a 4.2±3.5 g/l absolute difference between CZE and AGE.

3.4. Therapeutic follow-up

Fourteen out of 20 monoclonal bands were quantified by AGE and patients were classified as displaying therapeutic efficacy (n=5), relapse (n=3) or stable disease (n=6). A 100% concordance was obtained with CZE (Fig. 5). Among those, two patients with a β-migrating monoclonal immunoglobulin had apparent total remission by AGE (no peak detected); two bands were still detected by CZE (2.7 and 1.1 g/l).

Five oligoclonal profiles and one β-migrating monoclonal band could not be quantified by AGE. They were classified as apparent stable disease (n= 5) or treatment efficacy (n=1). The β-migrating band measured by CZE was 42.3 g/l before and 9.6 g/l after treatment confirming treatment efficacy. All

Table 2
Electrophoregram resolution: discordant samples between CZE and AGE

Subject (sex/age)	Age (No. bands)	CZE (No. bands, quantification)	IFE (typing)
1 (F/89 years)	1	2 (56.7, 0.4 g/l)	2 IgG Kappa
2 (M/50 years)	1	4 (16.4 g/l poorly separated.)	1 IgG Kappa
3 (M/68 years)	1	2 (26.4, 15.8 g/l)	1 or 2 IgA Lambda
4 (F/81 years)	1	2 (21.3, 2.3 g/l)	1 IgA Lambda
5 (F/77 years)	1	4 (48.1, 28.9, 22.6, 0.3 g/l)	3 IgG kappa
6 (M/38 years)	1	2 (13.9, 1.4 g/l)	2 or 3 IgA Kappa
7 (M/82 years)	2	3 (5.4, 5.4, 4.4 g/l)	3 BJ Lambda
8 (F/48 years)	2	3 (6.6, 2.2, 1.2 g/l)	4 IgG: 2 Kappa+2 Lambda
9 (M/66 years)	2	3 (19.4, 14.0, 5.7 g/l)	2 or 3 IgA Kappa
10 (M/52 years)	2	3 (27.5, 16.1, 12.3 g/l)	3 IgA Lambda
11 (M/36 years)	4	3 (5.4, 4.8, 4.5 g/l)	1 IgG+2 IgM Lambda

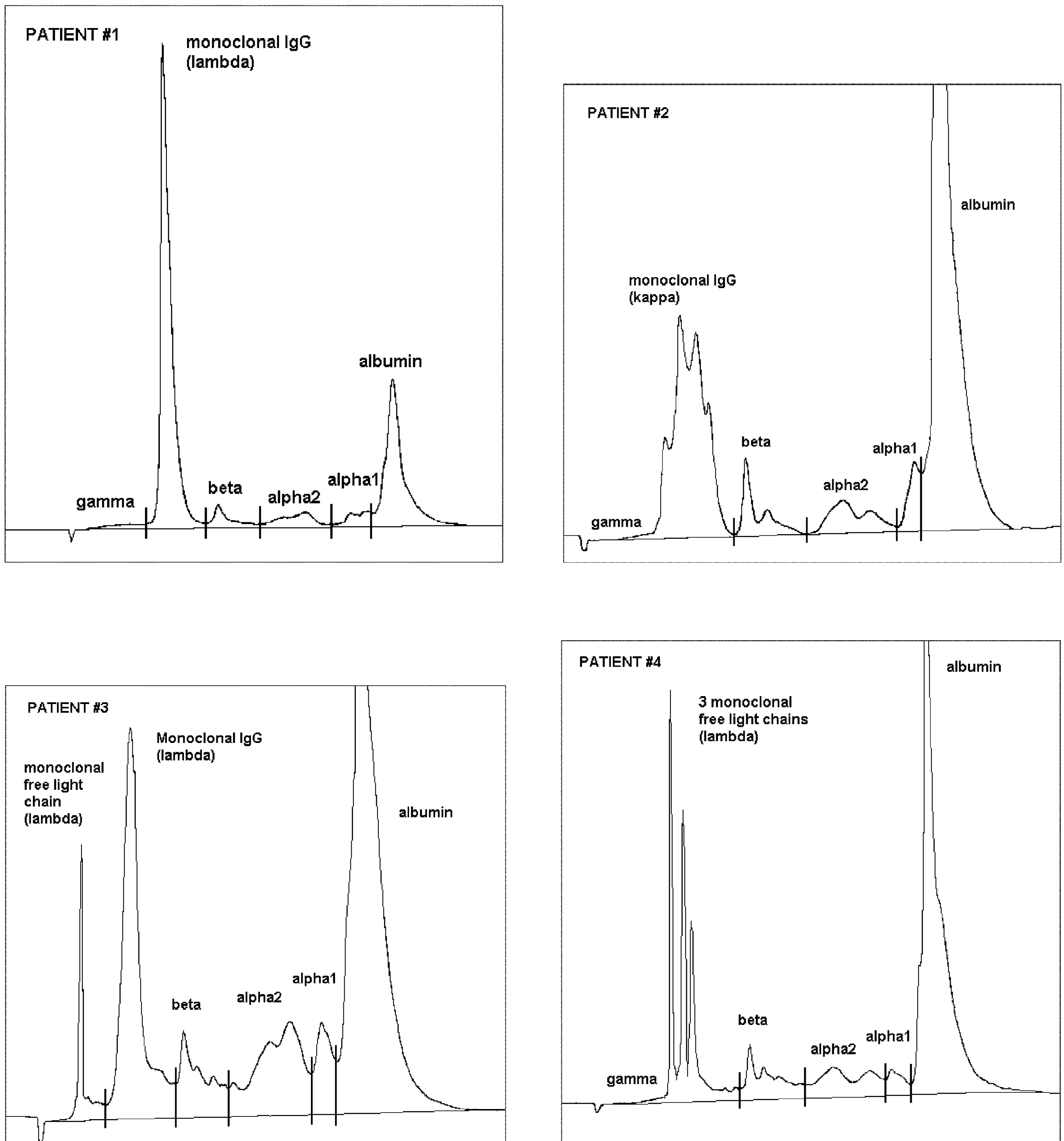

Fig. 3. Selected CZE electrophoregrams from poorly separated monoclonal bands by AGE patient 1 (total protein: 107 g/l), by AGE: one band co-migrating with β-globulins, by CZE: a well separated band in γ; patient 2 (total protein: 64 g/l), by AGE: a large band in the γ region of the gel, by CZE: heterogeneity of a large monoclonal band (four peaks?); patient 3 (total protein: 61 g/l), by AGE: one band in the γ region of the gel, by CZE: two bands (intact immunoglobulin and free light chain in the form of a sharp peak); patient 4 (total protein: 64 g/l), by AGE: two bands in the γ region of the gel, by CZE: three sharp peaks of free light chain.

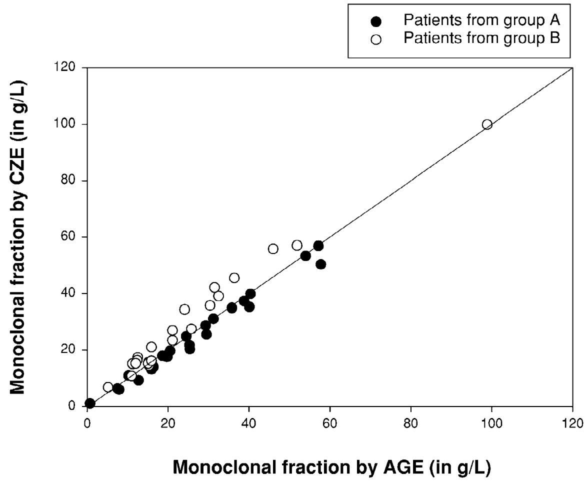

Fig. 4. Correlation between CZE and AGE for monoclonal band quantification. Correlation between the two techniques was examined in patients from group A ($n=24$) and those with poorly separated monoclonal band(s) by AGE (group B), comigrating in the β-region ($n=19$) or oligoclonal profiles ($n=17$).

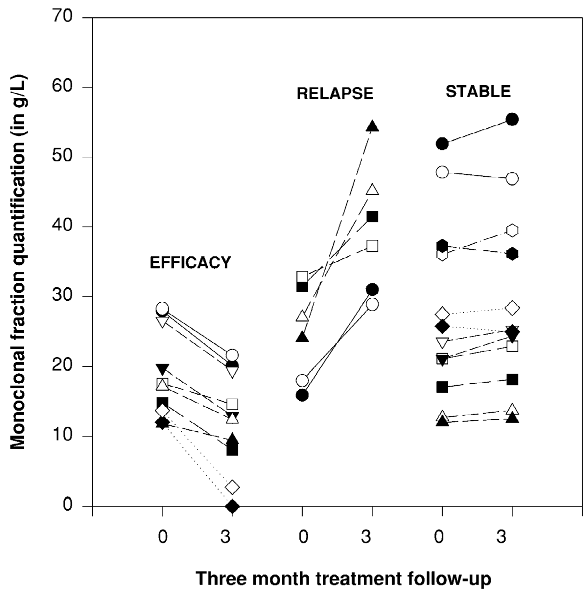

Fig. 5. Therapeutic follow-up of myeloma patients by CZE and AGE. Twenty patients from group B were reexamined 3 months later in the course of their treatment. Therapeutic efficacy was defined as a >10% decrease in monoclonal fraction concentration and disease progression as a >10% increase. Each symbol represents a patient (open symbols: AGE, closed symbols: CZE).

oligoclonal profiles (except one) were quantified by CZE confirming the diagnosis suggested by AGE.

4. Discussion

In the last 5 years, capillary zone electrophoresis has begun to have an impact in some clinical laboratories for analysis of proteins in serum [8–15], and in a lesser extend in urine [16] and in cerebrospinal fluid (CSF) [17]. Advantages of CZE over conventional AGE are related to full automation [13–15] and/or improved electrophoretic separation [10,11,17,18]. Recent results suggest, however, that quantification of polyclonal [15] and/or monoclonal [9] γ-globulins by CZE might be subjected to positive bias vs. AGE [11].

Current CZE methodologies for serum protein analysis (adapted on fully automated systems) are based on multiple sample analysis with 20 cm×25–40 μm diameter untreated fused-silica capillaries ($n=7$ for the Paragon CZE 2000) [9,13–15]. High voltage (~10 kV) with a buffer having a pH substantially higher than isoelectric points of the sample proteins separates serum proteins in ~5 min [13–15]. Here we increased the length of the capillary to 57 cm to improve protein separation (especially in the β-γ zone), an approach already described for serum (100 cm total length) [12] and CSF analysis (80 cm) [17]. We also selected a high-ionic-strength 0.3 *M* borate buffer since ~1% of monoclonal proteins (usually slow IgG or IgM γ-migrating by AGE) are not detected by CZE when a low-ionic-strength buffer is used (50 m*M* borate) [9,19,20]. The pH of the Protein separation buffer A was further raised from 8.6 to 9.6, as isoelectric points as high as 9.0 have been described for some monoclonal immunoglobulins [20]. The use of a pH lower than the isoelectric point of a monoclonal immunoglobulin will result in no peak detection at the cathodic end. The use of a high ionic strength buffer in CZE, however, resulted in high current so that a maximum of 17 kV could be applied for protein separation with a total analysis time of 20 min/sample.

Quantification of each protein fraction by electrophoresis techniques always relies on two measurements: total protein by the Biuret technique (usually automated on a clinical chemistry analyser) and %

peak AUC by densitometric scanning (AGE) or direct UV absorbence (CZE). The Biuret is the most widely accepted method to quantify total protein in human serum, because of specificity, low cost, and analytical performances (in our hands, between-day variation: RSD=0.8% for a control at 65 g/l, $n=27$). Our CZE method was as precise as other CZE semi-automated techniques [9–11] or our routine AGE technique for separation of albumin (RSD~5%) and γ-globulins (RSD~10%). The poor precision for α-1- and α-2-globulins (RSDs~10–25%) mostly reflects the protein heterogeneity of these zones, leading to irreproducible automated integration [9]. While less precise than the high-volume throughput Paragon CZE 2000 (RSDs: 2–10%) [13–15], our method fulfils the analytical requirements of clinical laboratory electrophoresis, especially for albumin and γ-globulins. The 0.5 g/l sensitivity for monoclonal detection is also comparable to that of conventional AGE and automated CZE. Obviously, the main limitation of the system is the single-run capability of the P/ACE instrument. Our method cannot compete with semi-automated AGE (~30 samples/1 h) [13] or automated CZE with simultaneous multiple-sample analysis (up to 60 samples/1 h) [13–15].

Improved CZE resolution resulted in the detection of additional bands in γ and/or β vs. AGE. Most frequently, a single additional clone was evidenced and confirmed by IFE as IgA ($n=4$), IgG ($n=3$) and free light chain ($n=1$). The exact nature of additional bands detected by CZE was not always established by IFE and might represent true oligoclonal gammopathies, structural heterogeneity of monoclonal immunoglobulins (different glycosylation or polymerization levels), CZE artefacts or another unknown phenomenon. The oligosaccharide pattern of IgG is, for example, constant in every healthy individual, but variations may be encountered under pathological conditions, including multiple myeloma, due to differences in glycosyltransferase activity [21]. Interference studies performed on the CZE Paragon 2000 provided no evidence for a significant alteration of the CZE pattern by bilirubin, lipids, or fibrinogen; hemolysis resulted in a peak in γ–β region [14,15], but none of our samples was hemolyzed. However, it has been showed that radiocontrast agents absorbing at 214 nm can simulate a monoclonal component [22]. In our study, CZE allowed the detection of one case of bisalbuminemia (out of 60 patients, not detected by AGE) as recently observed by others [23]. Another interesting feature of our CZE technique is that monoclonal free light chain (present in three patients, lambda type) produces a distinctive sharp peak in the γ-region of the electrophoregram (Fig. 3).

More than the determination of the exact number of clones, the presence of free light chains vs. intact immunoglobulins or the detection of a bisalbuminemia, our CZE technique allows a reliable quantification of previously not measurable monoclonal protein(s) by AGE. Of the complex protein profiles we analysed, 92% could be quantified by CZE, a significant improvement over AGE (64%). In a recent study, β-migrating immunoglobulins (by AGE) were efficiently separated from transferrin by the Paragon CZE 2000 system, thus allowing their quantification in 70% of cases (45 out of 65) [3]. We here obtained an excellent correlation with minimum bias ($r>0.90$, +2.8% in group A) between CZE and AGE for monoclonal protein quantification. There was no influence of the monoclonal protein concentration. A 4.2±3.5 g/l difference was, however, observed in group B. Because they are shifted toward γ-globulins in their mobility, β-migrating immunoglobulins should be more accurately quantified by CZE than by AGE. In CZE, the γ-fraction does not overlap with the β-fraction [10–12] thus leading to 10–15% lower values for β-globulins and 10–15% higher values for γ-globulins compared to AGE [15]. Importantly, our technique allowed the therapeutic follow-up (over a 3-month period) of 20 patients with poorly separated monoclonal bands by AGE.

Until now, the reliability of monoclonal band quantification by previously published manual or automated CZE methods remained uncertain [9–11]. Excellent results were first reported in large groups of patients ($r>0.96$ with AGE, slope ~1.0) [10,11]. In ~10% of samples, however, the absolute difference between CZE and AGE for monoclonal protein quantification was important (up to +20 g/l) [11]. In a more recent study [9], CZE quantification of monoclonal proteins yielded to ~40% higher mean values than AGE. This phenomenon was not dependent on the class of monoclonal protein: IgG

(+49%), IgM (+47%) and IgA (+37%). The direct measurement at 214 nm might be strongly affected by the UV-active side chains which absorb in the 240–280 nm range and are found in aromatic amino acids and histidine. The specific absorption coefficient of most abundant serum proteins varies from 13.32 for IgG to 17.96 $l\ g^{-1}\ cm^{-1}$ for α1-antitrypsin [15]. This might be also the case for individual monoclonal proteins, thus leading to significant over- [9] and/or underestimation [11] in their measurement and a converse error in other fractions, since CZE results are obtained in % of total AUC.

In conclusion, our CZE method designed for the analysis of poorly separated monoclonal proteins by conventional AGE is efficient, sensitive and precise at low cost. Caution should be, however, paid to monoclonal protein detection and quantification by CZE. The buffer conditions should be carefully optimised for the detection of all monoclonal immunoglobulins. Using a pH 9.6 buffer with high ionic strength, 100% of monoclonal proteins were detected and a large majority of them successfully quantified.

References

[1] P. Riches, in: H.C. Gooi, H. Chapel (Eds.), Clinical Immunology. A Practical Approach, IRL Press, Oxford, 1990, p. 111.
[2] R.A. Kyle, Clin. Chem. 40 (1994) 2154.
[3] J.C. Meunier, Ann. Biol. Clin. 57 (1999) 605.
[4] J.P. Landers, Clin. Chem. 41 (1995) 495.
[5] R.L. St. Claire, Anal. Chem. 68 (1996) 569R.
[6] R. Lehmann, W. Voelter, H.M. Liebich, J. Chromatogr. B 697 (1997) 3.
[7] M.V. Novotny, J. Chromatogr. B 689 (1997) 55.
[8] M.A. Jenkins, M.D. Guerin, J. Chromatogr. B 699 (1997) 257.
[9] Y. Henskens, J. De Winter, M. Pekelharing, G. Pnjee, Clin. Chem. 44 (1998) 1184.
[10] M.A. Jenkins, M.D. Guerin, Ann. Clin. Biochem. 32 (1995) 493.
[11] M.A. Jenkins, E. Kulinskaya, H.D. Martin, M.D. Guerin, J. Chromatogr. B 672 (1995) 241.
[12] J.W. Kim, J.H. Park, J.W. Park, H.J. Doh, G.S. Heo, K.J. Lee, Clin. Chem. 39 (1993) 689.
[13] F. Godey, M. Ropert, A. Bouasria, C. Lucas-Clerc, L. Guenet, N. Savoure, A. Le Treut, Ann. Biol. Clin. 57 (1999) 337.
[14] J. Bienvenu, M.S. Graziani, F. Arpin, H. Bernon, C. Blessum, C. Marchetti, G. Righetti, M. Somenzini, V. Gioacchino, F. Aguzzi, Clin. Chem. 44 (1998) 599.
[15] X. Bossuyt, G. Schiettekatte, A. Bogaerts, N. Blanckaert, Clin. Chem. 44 (1998) 749.
[16] M.A. Jenkins, T.D. O'Leary, M.D. Guerin, J. Chromatogr. B 662 (1994) 108.
[17] E. Sanders, J.A. Katzmann, R. Clark, R.P. Oda, Z. Shihabi, J.P. Landers, Clin. Chem. Lab. Med. 37 (1998) 37.
[18] F.T.A. Chen, Clin. Chem. 38 (1992) 1651.
[19] M.A. Jenkins, M.D. Guerin, Clin. Chem. 42 (1996) 1886.
[20] T. Murray, W. Long, R.G. Narins, New Engl. J. Med. 292 (1975) 574.
[21] S. Fujii, T. Nishiura, A. Nishikawa, Y. Tomiyama, M. Iida, T. Karasuno, H. Nakao, T. Yonezawa, N. Taniguchi, S. Tarui, Cancer Res. 50 (1990) 5345.
[22] X. Bossuyt, A. Mewis, N. Blanckaert, Clin. Chem. 45 (1999) 129.
[23] S.E. Jaeggi-Groisman, C. Byland, H. Gerber, Clin. Chem. 46 (2000) 882.