2013 © Curr. Issues Pharm. Med. Sci. Vol. 26, No. 3, Pages 299–304

Current Issues in Pharmacy and Medical Sciences

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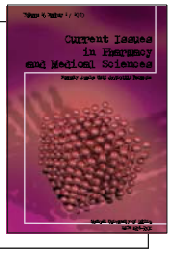

Comparison of agarose gel and capillary electrophoresis for the characterization of serum monoclonal paraproteins

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ABSTRACT

Serum protein electrophoresis is an especially useful method to detect and semi-quantify monoclonal proteins in patients with multiple myeloma and other plasmocyte dyscrasias. The presence of monoclonal protein (M protein) in electrophoretic separation is indicated by a sharp spike in gamma-globulin fraction that is sometimes located in alpha-2-globulins and beta-globulins. Semi-quantification of M protein is a basic method to monitor therapy of patients with multiple myeloma (MM) and monoclonal gammopathies of undetermined significance (MGUS). The purpose of the study was to compare concentrations of M protein obtained by agarose gel (AGE) and capillary electrophoresis (CE) and to evaluate diagnostic usefulness of both electrophoretic techniques for the identification of M protein. The investigations were carried out in the group of 90 patients with monoclonal gammopathies, 42 females and 48 males aged 65±9 years. Patients with monoclonal gammopathies had lower concentrations of monoclonal proteins determined by AGE in comparison to CE. High positive correlation between the results of monoclonal protein concentrations obtained by AGE and CE was observed. Both AGE and CE seem to be equally useful diagnostically in the detection of paraproteins.

Keywords: monoclonal proteins (M protein), agarose gel electrophoresis (AGE), capillary electrophoresis (CE), monoclonal gammopathy

INTRODUCTION

The detection of monoclonal protein (M protein) is of key importance for the diagnosis of monoclonal gammopathies [14,17]. In the course of the disease, the plasmocytes and/or B lymphocytes that have been changed by neoplastic disease produce monoclonal immunoglobulins [1,29]. When monoclonal gammopathy is suspected it is necessary to test patient's blood/urine samples to detect and identify the type of paraprotein using serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE). Both agarose gel electrophoresis (AGE) and capillary electrophoresis (CE) are recommended techniques for electrophoretic separations [12,13,28].

In electrophoretic separation M protein often generates additional spike in the gamma-globulin fraction. However, M protein is sometimes detected in the fractions of alpha-2-globulins and beta-globulins. A characteristic spike is an indication for IFE to confirm whether a homogenous protein has been found [5]. Next, semi-quantification of monoclonal immunoglobulin concentration is recommended. To calculate the concentration of monoclonal protein, the area under the spike is measured and expressed as absolute value after total protein concentration has been measured in the examined sample [3,24].

Semi-quantitative evaluation of M protein content is a basic method used to monitor therapy in patients with monoclonal gammopathies, i.e. multiple myeloma (MM), Waldenström macroglobulinemia, monoclonal gammopathy of undetermined significance (MGUS) and other plasmocyte dyscrasias [13,31].

M protein concentration <30 g/l is one of the main criteria to differentiate MGUS from MM and is useful to diagnose MGUS and smoldering multiple myeloma (SMM) [13,19]. Increased concentration of M protein in patients with MGUS is often associated with the disease progress [13,20].

The purpose of the study was to compare concentrations of M protein obtained by gel and capillary electrophoresis and to evaluate diagnostic usefulness of both electrophoretic techniques for the identification of M protein.

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DOI: 10.12923/j.2084-980X/26.3/a.13

MATERIAL AND METHODS

The investigations were carried out in the group of 90 patients with plasmocyte myeloma and other plasmocyte dyscrasias. The examined group consisted of 42 females and 48 males aged 65±9 years, treated in The Clinic of Hematology and Bone Marrow Transplantation, SPSK-1, Lublin.

Blood serum samples were taken from the patients fasting from the cubital vein to the test tubes without anticoagulants. The test tubes were centrifuged for 10 min at 2,000 rpm. Thus obtained serum underwent testing by gel and capillary electrophoresis and immunofixation to detect M protein and determine total protein concentration. The testing was carried out in The Unit of Laboratory Diagnostics, SPSK-1 in Lublin.

Serum protein electrophoresis was performed using the semiautomated Sebia HYDRASYS system with the Hydragel protein 15/30 reagent set (Sebia, France) according to the manufacturer's instructions. Proteins were stained with amido black and densitometric scanning was performed at 570 nm.

Capillary electrophoresis was done on Minicap with Minicap Protein 6 kit (Sebia, France) according to the manufacturer's instructions. The kit is designed for the separation of human serum proteins into six regions with alkaline buffer (pH=9.9). It is equipped with two parallel capillaries, internal diameter <100 μm, which allows two analyzing processes to be performed simultaneously. Buffered samples are inserted into the capillary by aspiration at its anode end. Direct reading of the protein separation output is read at the cathode end at 200 nm wave length.

Immunofixation was performed using Sebia HYDRASYS system according to the manufacturer's instructions using fixative or monospecific antisera for IgG, IgA, IgM kappa or lambda and was considered the "golden standard" as to whether there was a band and to determine the identity of bands.

Quantitative results for monoclonal protein of the AGE or CE electrophoretogram were expressed in g/dl by multiplying the percentage of total area under the spike by total serum protein concentration which was measured on the biochemical autoanalyser Cobas Integra 400 (Roche, Basel, Switzerland).

STATISTICAL ANALYSIS

The results were analyzed statistically by STATISTICA 10.0 (StatSoft) and MedCalc 7.0. The values of M proteins evaluated semi-quantitatively were read from AGE and CE electrophoretograms and expressed descriptively as arithmetic mean (X), standard deviation (SD), median (Me) and percentile range 25-75%.

Distribution of the results was analyzed by Shapiro-Wilk test and differences between variables were evaluated by Wilcoxon signed rank test; correlations between semi-quantified values of M protein depending on the method were determined by Spearman's rank correlation test. To present the character of differences between the results graphic analysis by Bland-Altman plot and Mountain plot was used. Statistical significance was assumed at $p<0.05$.

To evaluate diagnostic usefulness of an electrophoretic method to identify abnormal monoclonal components on electrophoretic separation, sensitivity, specificity and diagnostic compatibility of the results was calculated with relation to immunofixation results according to standard formulas. Diagnostic sensitivity is the proportion of true positive results to the sum of true positive and false negative results. Diagnostic specificity is expressed as the proportion of true negative results to the sum of true negative and false positive results.

RESULTS

Immunofixation was done in all patients of whom 79 tested positively. M protein (IgG or IgA) was identified in 73 patients and 6 patients had monoclonal free light chains (FLC) detected: kappa (2 patients) and lambda (4 patients). IFE results were negative in 11 patients. In one patient, the electrophoretograms obtained by both methods were false positive.

In the group of 62 patients it was possible to determine semi-quantitative concentrations of M protein from the electrophoretograms obtained by either gel or capillary electrophoresis. Table 1 presents the results.

Table 1. Mean protein concentrations [g/dl] of monoclonal components (n=62) on AGE and CE

Type of monoclonal protein	Agarose Gel Electrophoresis (AGE)				Capillary Electrophoresis (CE)			
	n	X±SD	Me	25-75%	n	X±SD	Me	25-75%
IgG	53	0.97±1.45*	0.56	0.22-0.86	53	1.09±1.35	0.68	0.29-1.1
IgA	9	0.46±0.73*	0.06	0.05-0.44	9	0.66±0.9	0.14	0.12-0.81
Total	62	0.90±1.38*	0.42	0.16-0.86	62	1.03±1.3	0.65	0.23-1.1

N – number of examined

*$p<0.01$

Mean concentration of M protein determined by AGE was 0.9±1.38 g/dl and was statistically significantly decreased ($p<0.01$) in comparison to its concentrations obtained by CE (1.03±1.3 g/dl).

In 7 patients AGE and CE separations produced divergent values of M protein. Table 2 presents the results of the patients in whom semi-quantification on AGE or CE was not possible.

In the group of 10 patients who tested positively on immunofixation, the electrophoretograms did not show spikes that could indicate M protein component. It was not possible to detect and quantify M protein in the following cases: 2 IgG, 5 FLC and 3 IgA.

Table 2. Specification of paraproteins missed by detection on AGE or CE, or that could not be quantified by AGE or CE

N	Detection		Quantification				Identification	
	AGE	CE	AGE	Protein, (g/dl)	CE	Protein, (g/dl)	IFE	Type
1	+	-	+	0.05	NA	NA	+	IgG K
2	-	+	NA	NA	+	0.05	+	IgG L
3	+	-	+	0.04	NA	NA	+	IgG L
4	-	+	NA	NA	+	0.04	+	FLC L
5	-	+	NA	NA	+	0.36	+	IgA K
6	-	+	NA	NA	+	0.19	+	IgA K
7	-	+	NA	NA	+	0.09	+	IgA L

N – number of examined, NA – not applicable, K – kappa, L – lambda, AGE – agarose gel electrophoresis, IF – immunofixation electrophoresis, CE – capillary electrophoresis

Moreover, the correlations between the values of M protein concentrations obtained by AGE and CE were analyzed (Fig. 1).

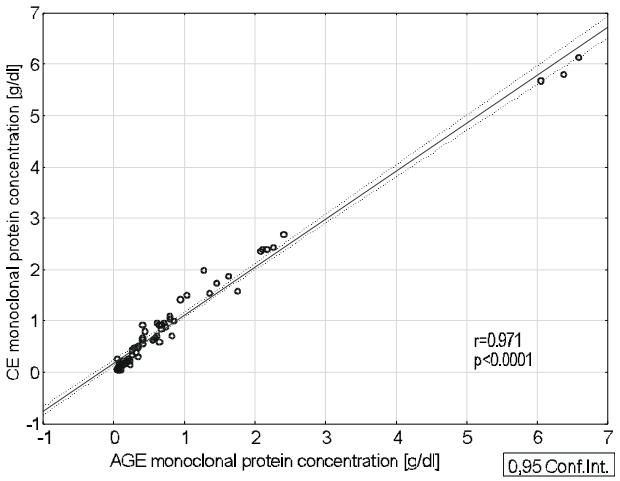

Fig. 1. Correlations between M protein concentrations determined by AGE and CE (n=62)

The concentrations of M protein determined by AGE and CE were significantly highly correlated (r=0.971, p<0.0001).

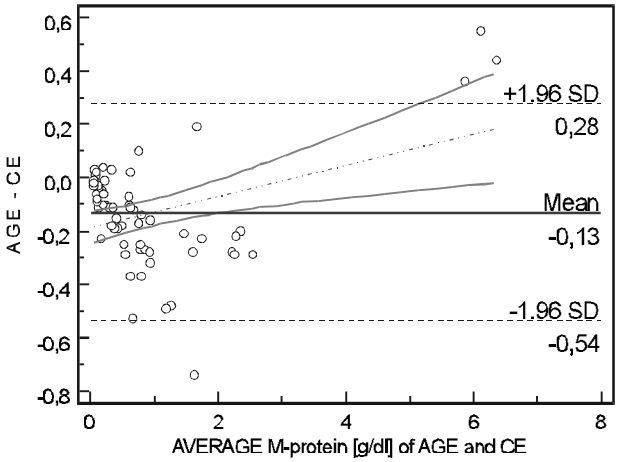

Fig. 2. Bland-Altman plot. Correlations between the differences of M protein concentrations determined by two methods and its mean concentrations. Center line represents the mean difference, and top and bottom lines represent the mean difference ± 2 SD

The statistical results were evaluated graphically by Bland-Altman and Mountain plots (Fig.2 and Fig. 3).

Graphic analysis by Bland-Altman plot (Fig. 2) shows significant differences between M protein values with reference to the range of determined concentrations, gradient coefficient was 0.06 and was significantly different from zero (p<0.01) (Fig. 2). The most differences were observed for M protein concentration >5 g/dl.

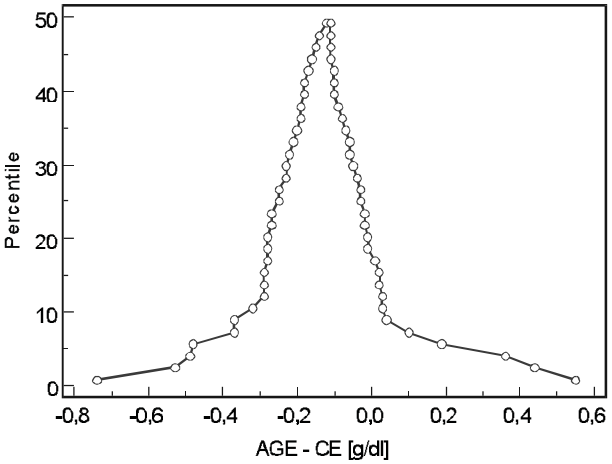

Fig. 3. Mountain plot. Accumulated distribution of differences between the results of M protein concentrations

The range of differences on Mountain plot was symmetrical and ranged from -0.74 to 0.55 g/dl, Me=-0.11 g/dl (Fig. 3).

Finally, diagnostic sensitivity, specificity and compatibility of the results obtained by AGE and CE with reference to IFE results was evaluated (Table 3 and Table 4).

Table 3. AGE vs. IFE. Classification of „Band" or „No band" in specimens tested for monoclonal proteins*

AGE	IFE		
	Band	No band	Total
Band	64	1	65
No band	15	10	25
Total	79 (81%)	11 (91%)	90 (82.2 %)

AGE – agarose gel electrophoresis, IF – immunofixation electrophoresis
* Totals are given as number (percentage) and percentage indicates the number of specimens correctly identified based on IFE as the golden standard

Table 4. CE vs. IFE. Classification of „Band" or „No band" in specimens tested for monoclonal proteins*

CE	IFE		
	Band	No band	Total
Band	67	1	68
No band	12	10	22
Total	79 (84,8 %)	11 (91 %)	90 (85,5%)

CE – capillary electrophoresis, IF – immunofixation electrophoresis
* Totals are given as number (percentage) and percentage indicate the number of specimens correctly identified based on IFE as the golden standard

Both AGE and CE were 91% specific, the sensitivity of AGE and CE was 81% and 84.8%, respectively. The compatibility of the results obtained by AGE and CE with reference to IFE was close, 82.2% and 85.5%, respectively.

DISCUSSION

Gel and capillary electrophoresis are two diagnostic techniques currently used for the separation of proteins to detect and quantify monoclonal component. The purpose of our study was to evaluate their diagnostic sensitivity in the detection of monoclonal immunoglobulins and to analyze possible differences in M protein concentrations measured by those techniques.

Our results demonstrated statistically significantly higher ($p<0.0001$) values of paraproteins determined semi-quantitatively by CE than AGE. Henskens et al. [16] and Gay Bellile et al. [15] observed similar results. However, the investigations by Mussap et al. [24] did not find significant differences between the concentrations of M protein obtained by CE and AGE. They found that densytometric readings from agarose gels were higher at M protein <20 g/l and that CE results were higher compared to AGE when M protein was >20 g/l.

That discrepancy of the results was possibly due to differences in direct protein measurement by UV absorption used in CE and staining used in AGE, the fact was noted by numerous authors, too [6,8,9]. Moreover, it has to be emphasized that quantification of M protein by AGE is non-linear at concentrations >20 g/l. It results from the saturation of narrow dense monoclonal bands with a dye in the gel, which in turn produces imprecise and inaccurate reading. Non-linear distribution means that changed plasmatic cell populations cannot precisely reflect the changes in M protein concentrations [24]. However, such dependences are not observed when UV absorption is used at 214 nm wavelength. Also Mussap et al. [24] found that different concentrations of M protein did not depend on the type of immunoglobulin, which complies with our observation.

Graphic analysis according to Bland-Altman plot revealed significant correlations between the values of differences in the results obtained by both methods and the range of monoclonal protein measured. Similar results were demonstrated by Gay Bellile et al. [15] and Henskens et al. [16]. The results by McCudden et al. [23] were different, though. In the group of 42 patients with M protein detected (>3g/l) they did not find significant differences with reference to the range of paraprotein concentrations determined.

Our results confirmed high correlation ($r=0.971$; $p<0.0001$) for M protein concentrations determined by AGE and CE. Many other authors found similar correlation [15,16,23]. Barlow et al. [4] showed good correlation between M protein ($r=0.96$), and they graphically demonstrated good compatibility of the result differences. There were three exceptions but none of them produced difference >5g/l. They found only one significant inconsistency, i.e. AGE and CE results were 85 g/l and 68 g/l, respectively. They concluded that such single difference was due to the differences between direct protein measurement, i.e. UV absorption and staining.

In addition to that, the study evaluated diagnostic usefulness of two electrophoretic methods to detect suspicious spikes on electrophoretograms that could indicate M protein component. The results were referred to immunofixation considered as "golden standard".

The data from accessible literature indicate high sensitivity and specificity of capillary technique in the detection of M protein [9]. The sensitivity of CE was higher (93-100%) compared to cellulose gel electrophoresis (74%) and agarose gel electrophoresis (86-95%) [7]. The authors [7,16,22] reported on the cases when it was difficult to detect monoclonal immunoglobulin either by CE or AGE. In most cases non-detection of M protein resulted from its low concentration or its migration to beta-globulin fraction where it was masked by other proteins like tranferrin and C3 complement component [7].

In our study, a group of 10 patients tested positively on immunofixation. However, the electrophoretograms obtained by both methods did not reveal suspicious spikes that could indicate paraprotein content. The majority of those cases were associated with FLCs (5 patients) or IgA monoclonal protein (3 patients). Bossuyt and Mariën [7] analyzed 481 patients and found that CE did not detect M protein in 24 (5%) patients. Generally, false negative results were noted in the group with FLCs and low concentrations of IgA or IgM. That complies with our observations, too. Chartier et al. [11] presented similar results. They analyzed the results obtained by gel electrophoresis and two capillary systems Capillarys 2 and V8. They found that false negative results might have been due to conditions related to hypogammaglobulinemia (45%) and low paraprotein concentration (50%).

In our study, there were cases when paraprotein was not detected by either electrophoretic method which could have been due to trace amounts of those proteins, which was detected by immunofixation though. Immunofixation is more sensitive than electrophoresis [7,11]. Another factor that made identification more difficult was location of monoclonal proteins (IgA) in other fractions than gamma-globulins, which was shown on densytometric graph; most frequently they were located in alpha-2--globulin and beta-globulin or in beta-globulin fraction alone. Such location lowers the possibility to detect monoclonal immunoglobulin on electrophoretograms. Other authors confirm that finding, too [2,21,30]. Ludwig et al. [21] could not detect and quantify IgA in 46% patients and in 4% patients with IgG. Similarly, Wang et al. [30] and Avet-Loiseau et al. [2] could not determine IgA on the electrophoretograms in 57% and 33% patients, respectively.

Moreover, in our study CE did not detect 2 cases of monoclonal protein present (2 IgG); AGE failed in 5 cases

(1 IgG, 1 FLC, 3 IgA) [Tab. 2]. The findings seem to confirm observations by other authors that IgA band is a bit more distinctly separated from beta- globulin fraction and better detected by CE, which results in higher sensitivity of the method [6, 8-10, 16, 27].

It is worth mentioning that the evaluation of therapeutic process loses objectiveness when monoclonal band cannot be distinctly separated from alpha- or beta-globulin fraction. Then, total immunoglobulin profile is recommended to estimate the amount of M protein. However, caution is required as nephelometry and turbidimetry can either heighten or lower estimated amounts of M protein [12, 26].

The evaluation of diagnostic usefulness revealed higher sensitivity of Minicap system of CE compared to Hydrasys apparatus for AGE; the respective values for sensitivity were 85% and 81%. Diagnostic specificity for both methods was 91%. Our results evaluating sensitivity were lower compared to those reported by Bossut and Mariën [7] and Katzmann et al. [18]. The difference may be due to different apparatuses used for CE and a bigger investigated group (>1500 patients).

However, Yang et al. [31] obtained slightly different results: diagnostic sensitivity of capillary and gel electrophoresis was 81% and 90% respectively. Slightly lower sensitivity of capillary electrophoresis could have resulted from the fact that not all cases of IgM and FLCs were detected. Both methods were 100% specific.

In our study, lower specificity was due to one false positive result in both electrophoretic methods. The result was not confirmed by immunofixation. A little spike on the electrophoretograms could have been caused by high concentration of CRP or fibrinogen in the sample. Fibrinogen can migrate to gamma-globulin fraction thus stimulating paraprotein and makes interpretation of the results more difficult [7,8].

In comparison to our results McCudden et al. [23] found a bit higher sensitivity of capillary and gel electrophoresis (92% and 91% respectively). They concluded that higher sensitivity of CE is associated with lower specificity of this technique (74%) compared to AGE (81%). They also investigated immunotyping (IT) for its diagnostic sensitivity and specificity. It is the newest method used in apparatuses for capillary electrophoresis to identify the type of monoclonal protein and provides an alternative to traditional immunofixation. IT was found to be less sensitive compared to IFE, which is confirmed by other studies [9,23,31]. Detecting FLCs and low concentrations of monoclonal immunoglobulins was more difficult on IT and those are often observed in patients with MGUS [25,31].

Finally, the interpretation of separations obtained by AGE and CE in patients with gammopathies should consider slight differences in the detection and quantification of monoclonal proteins between those two electrophoretic systems.

CONCLUSIONS

1. Patients with monoclonal gammopathies seem to have lower concentrations of monoclonal proteins determined by gel electrophoresis in comparison to capillary electrophoresis.
2. The results of monoclonal protein concentrations obtained by gel and capillary electrophoresis were positively correlated.
3. Both gel and capillary electrophoresis are equally useful diagnostically to detect paraproteins.

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