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Method development and qualification of capillary zone electrophoresis for investigation of therapeutic monoclonal antibody quality

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ABSTRACT

Capillary electrophoresis techniques are widely used in the analytical biotechnology. Different electrophoretic techniques are very adequate tools to monitor size—and charge heterogenities of protein drugs.

Method descriptions and development studies of capillary zone electrophoresis (CZE) have been described in literature. Most of them are performed based on the classical *one-factor-at-time* (OFAT) approach. In this study a very simple method development approach is described for capillary zone electrophoresis: a "**two-phase-four-step**" approach is introduced which allows a rapid, iterative method development process and can be a good platform for CZE method. In every step the current analytical target profile and an appropriate control strategy were established to monitor the current stage of development. A very good platform was established to investigate intact and digested protein samples.

Commercially available monoclonal antibody was chosen as model protein for the method development study. The CZE method was qualificated after the development process and the results were presented. The analytical system stability was represented by the calculated RSD% value of area percentage and migration time of the selected peaks (<0.8% and <5%) during the intermediate precision investigation.

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1. Introduction

Monoclonal antibodies are the most emerging classes of therapeutic biosimilar protein drugs. Their overall characterization is a critical in the point of the patients' safety. General papers were published recently focusing on the comprehensive comparability of biosimilar candidates to the originator [1,2].

Monoclonal antibodies are very heterogeneous molecules in both size and charge. Antibody characterization and homogenity assessment require a huge analytical arsenal of separation techniques [3,4]. For primer sequence and microheterogenity determination the mass spectrometry coupled liquid chromatography or capillary electrophoresis has the major importance [5,6]. Secondary and tertiary structure determination is performed by different spectroscopic methods such as fluorescence or circular dichroism as far as infrared spectroscopy-based methodology [7–9].

Charge variant analysis has big impact among the routine analytical methods since certain type of chemical or biological modifications have influence on the biological activity of the antibody. Such modifications like deamidation of asparagines may have influence on biological activity of the molecule [10,11].

Many basic laboratory techniques are used in research laboratories to determine charge variant distribution [12–14]. Techniques like gel-based isoelectric focusing provide very good resolution but time- and buffer-consuming and very hard to use in regulated environment. Capillary isoelectric focusing also provide very good resolution and give adequate information about the isoelectric points of each charge variants [15,16], but the analysis is relatively slow (takes about 30–50 min for each runs) and very sensitive for the way of sample preparation because its complexity.

In routine analytical applications the different separation techniques are the most commonly used. Mainly strong cation exchange chromatograpic techniques play the most important role in quality control processes of therapeutic MABs regarding the charge heterogeneity assessment [17].

Among them the capillary zone electrophoresis is one of the most popular tools for protein characterisation. This is a very good

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methodology for a rapid, cost-effective analysis of therapeutic proteins [18,19,22]. Different background electrolyte systems were described in previous studies. Shi et al. developed and validated method based on 20 mM acetate–acetic acid (at pH 6.0) with the addition of 0.3% polyethylene oxide (PEO) and 2 mM triethylenetetramine (TETA) [19]. He et. al developed a CZE method for charge variant analysis based on 600 mM ε-aminocaproic acid (EACA) buffer at pH 5.4 and 0.1% HPMC as polymeric additive [18].

Most recently an intercompany study of capillary zone electrophoresis method for charge hetereogenity analysis of monoclonal antibodies was published by Moritz et. al [20]. This paper enhances the advantages of EACA system in an international precision study, where repeatability was <1% within all of the companies.

In this study a novel and very simple method development strategy for capillary zone electrophoresis methods is introduced to investigate a commercially available monoclonal antibody drug product as a model protein. EACA buffer system was chosen as background electrolyte. Our goal was to develop an analytical method which can be a good platform both for "top-town" and "middle-up" analysis of monoclonal antibodies. I order to establish a fast and simple method development strategy the "two-phase-one-step" approach was introduced, which could be easily adopted into any biopharmaceutical laboratory.

Our aim was also to adjust a proper method which is capable of resolving the main isoforms of a commercially available antibody chosen for this study as model protein. Method qualification was also performed to prove that the developed method is serves its purpose. Linearity, level-of-detection (LOD), level-of-quantification (LOQ), intermediate precision and specificity were tested. Effects of oxidation and deamidation on the charge profile of the investigated monoclonal antibody were also studied during specificity testings.

2. Materials and methods

2.1. Materials

Hydroxi-propyl methyl cellulose (HPMC), triethylenetetramine (TETA), ε-aminocaproic acid (EACA), hydrogen peroxide, phosphoric acid, papain enzyme, Carboxypeptidase-B (CPB) enzyme, Tris(hydroxymethyl)aminomethane (TRIS), hydrogen peroxide, methionine, ammonium hydrogen carbonate and phosphoric acid were purchased form Sigma (St Louis, USA, Missouri). Commercially available monoclonal antibody was purchased from Janssen Biotechnology Inc. (Horsham, USA, Pennsylvania). Amicon Ultra 3 K Centrifugal Filter system was purchased from Merck Millipore (Darmstadt, Germany)

2.2. Instrumentation

All of the measurements were performed on Beckman Coulter(Hercules, USA Califonia) PA800 and PA800 Plus capillary electrophoresis systems. 50 μm inner-diameter fluorocarbon coated capillaries (μSil-FC) were purchased from Agilent Technologies (Santa Clara,USA California) The polyimide coating onthe both ends of the capillaries were removed by Microsolv CE Window Maker apparatus (Microsolv, USA Monmouth). Hettich Mikro 200 benchtop centrifuge (Andreas Hettich GmbH & Co.KG, Germany Tuttlingen)

2.3. Buffers and solutions

100 mM phosphoric acid was used as cleaning solution during capillary cleaning. 25 mM TRIS ph 7.0 was used for papain enzymatic reaction. 25 mM, 50 mM and 100 mM EACA buffers was made by diluting stock EACA solution with purified water.

2.4. Sample solutions

Papain digested sample solution contained monoclonal antibody in 1 mg/ml concentration diluted with 20 mM TRIS pH 7.0 solution the digestion was performed during 2.5 h at 37 °C. 200 μl solution was containing 4 μl papain enzyme.

Intact antibody solution was used in 10 mg/ml concentration, where the original formulation buffer was removed and the protein was replaced into CZE running buffer by Amicon Ultra 10 K filtration unit after spinning 30 min at 14000 g speed.

2.5. Deamidated samples

10 mg/ml intact antibody solution was incubated for 30 h at room temperature in 0.1 M ammonium hydrogen carbonate solution. The buffer of the solution was removed and the protein was replaced into CZE running buffer by Amicon Ultra 10 K filtration unit after spinning 30 min at 14000 g speed.

2.6. Oxidized samples

10 mg/ml intact antibody solution was incubated for 90 min at room temperature with 1 v/v% 1 M H2O2 solution. The oxidation procedure was stopped by adding methionine to the reaction mixture. The buffer of the solution was removed and the protein was replaced into CZE running buffer by Amicon Ultra 10 K filtration unit after spinning 30 min at 14000 g speed.

2.7. General zone electrophoresis setup

The basic setup were not modified during method development were the following: injection was performed during 10 s with 0.5 psi hydrodinamical pressure. 30 kV separation voltage was applied during all of the measurements in 50 cm long (40 cm effective length) coated capillary. Before daily use the capillary was flushed by purified water for 5 min at 50 psi, for 5 min with 100 mM phosphoric acid at 50 psi, followed by a 5 min rinse with purified water at 50 psi. In every single run a 5 min-long water flushing was used at 50 psi in the beginning and at the end of the method.

3. Results and discussions

3.1. Method development strategy (MDS)

In our study a simple '*two-phase and four-step*' approach was introduced (Table 1) which can speed up the time of method developments of CZE systems. Two phase means a fast *screening phase*, where the pH and the ionic strength are being adjusted to receive the optimal migration time stability and the most optimal resolving capacity of the method, and a *fine tuning phase* where the amount of organic additives and viscosity enhancers are adjusted to be optimal to reach the maximum in peak resolving with the maximum migration time and area percentage stability. Some physical parameters (separation voltage, injection time and pressure, capillary length) were not investigated since the choice of buffer system (EACA, TETA, HPMC) was based on the literature available. To minimize electroosmotic flow and minimize protein precipitation a fluorocarbon coated capillary was chosen. This choice was based on costs and availability on the market.

Table 1
(a) The migration time (minutes) results of pH screening in Phase I.–step 1. (b) RSD% reults of the migration times (minutes) of the six peaks in Phase I–Step 2. (c) Mean value and RSD% reults of the migration times (minutes) and area percentage of the six peaks. (d) RSD% results of the six peaks of Phase I–Step 4 optimizations. HPMC in different amounts were used to enhance viscosity.

Phase I.–Screening							
Migration times (min)							
	pH adjustment	Peak 1	peak 2	peak 3	peak 4	peak 5	peak 6
(a) Step 1–pH screening	pH 3.0	14.342	14.704	–	–	–	–
	pH 3.5	14.242	14.171	15.321	–	–	–
	pH 4.0	15.333	16.071	17.529	17.825	18.475	18.871
	pH 4.5	15.046	15.996	18.896	19.688	20.354	21.021
	pH 5.0	13.813	14.554	19.346	20.825	21.346	–
	pH 3.6	14.229	14.738	15.096	–	15.637	–
	pH 3.8	14.825	15.417	16.233	16.317	16.933	17.371
	pH 4.2	15.213	16.017	17.829	18.229	18.892	19.321
	pH 4.1	14.979	15.675	17.167	17.483	18.113	18.462
Deviations of migration times (RSD%)							
	Ionic strength	peak 1	peak 2	peak 3	peak 4	peak 5	peak 6
(b) Step 2–Adjustment of ionic strength	25 mM	1.2	0.9	0.5	0.5	0.5	1.4
	50 mM	0.5	0.1	0.1	0.1	0.1	0.8
	100 mM	0.5	0.6	0.6	0.6	0.5	1.3

Phase II.–"Fine tuning"													
	Organic modifier	Peak1		Peak2		Peak3		Peak4		Peak5		Peak6	
	(TETA)	time	area%	time	area%	time	area%	time	area%	time	area%	time	area%
							1 mM						
(c) Step 3–Adjusting optimal level of organic additive	mean	15.04	2.04	15.67	59.67	17.15	7.30	17.46	1.93	18.09	14.06	18.76	1.20
	RSD (%)	0.08	1.61	0.00	0.22	0.04	0.49	0.04	3.00	0.03	0.26	0.02	4.75
							5 mM						
	mean	15.01	2.39	15.68	58.98	17.17	7.36	17.48	1.90	18.11	14.57	18.46	1.15
	RSD (%)	0.37	8.83	0.08	0.95	0.07	2.16	0.05	1.00	0.08	3.61	0.01	8.47
							10 mM						
	mean	14.99	2.47	15.68	58.69	17.44	7.01	17.48	1.79	18.11	14.14	18.42	1.04
	RSD (%)	0.00	2.00	0.02	0.79	2.22	0.26	0.04	2.17	0.04	1.35	0.27	11.14
	Visvosity enhancer	Peak1		Peak2		Peak3		Peak4		Peak5		Peak6	
	(HPMC)	time	area%	time	area%	time	area%	time	area%	time	area%	time	area%
							0,05 V/V%						
(d) Step 3–Enhance viscosity by using polymer additive	mean	15.14	2.06	15.77	50.76	17.26	1.74	17.42	8.25	17.77	0.64	18.41	15.18
	RSD (%)	0.04	2.89	0.04	1.60	0.03	5.15	0.05	2.13	0.01	1.73	0.05	0.90
							0,1 V/V%						
	mean	15.18	1.94	15.80	51.803	17.31	1.52	17.46	8.65	17.80	0.56	18.45	15.84
	RSD (%)	0.12	15.13	0.13	0.64	0.13	12.72	0.12	1.77	0.11	21.81	0.13	1.46
							0,2 V/V%						
	mean	15.21	2.13	15.84	51.06	17.36	1.38	17.49	8.43	17.83	0.63	18.49	15.77
	RSD (%)	0.08	0.63	0.08	0.12	0.13	18.08	0.8	4.88	0.06	0.33	0.10	0.50

3.2. Method goal determination—analytical target profile (ATP)

Main goal was to obtain a method which is efficient enough to be able to resolve the main charge variants (eg. C-terminal lysine heterogenity) and their deamidated forms in MABs. Further aim was to obtain a method which can be a good platform both for intact and for digested sample investigations.

To reveal and to demonstrate the maximal peak resolving capability and maximal resolution stability of the developed buffer system papain digested Mab samples were used for method developments to calculate the standard deviations during development phase (migration time and time corrected area% of the selected peaks). Six different peaks were selected to monitor system stability during the different method development phases.

3.3. Method development

3.3.1. Phase I–screening pH and ionic strength

According to the literature available EACA as primary buffer system was chosen [19]. Many other buffer systems were used for CZE method optimizations, but EACA was mostly used for therapeutic protein separations [19–21].

3.3.1.1. Step 1: pH screen—a short iterative approach. To control the development process and obtain information about the stability of the method during screening, the migration times of the six main peaks were chosen as outputs for monitoring parameters. One main goal was to obtain as short method as possible with all of the six peaks resolved, consequently the migration time of the lastly migrating peak was the most informative.

3.3.1.2. Establishing control strategy for pH screening. For basic screening we adjusted five pH points between 3.0 and 5.0 (3.0, 3.5, 4.0, 4.5 and 5.0) of the 100 mM EACA solution. After the five-point screening the pH 4.0–4.5 range was chosen for further investigations based on the results of migration behavioral of the main peaks listed in Table 1a. Under pH 4.0 and art pH 5.0 not all of the six indicated peaks were properly resolved. At pH 4.5 the migration time of the lastly migrating peak was too high (over 20 min).

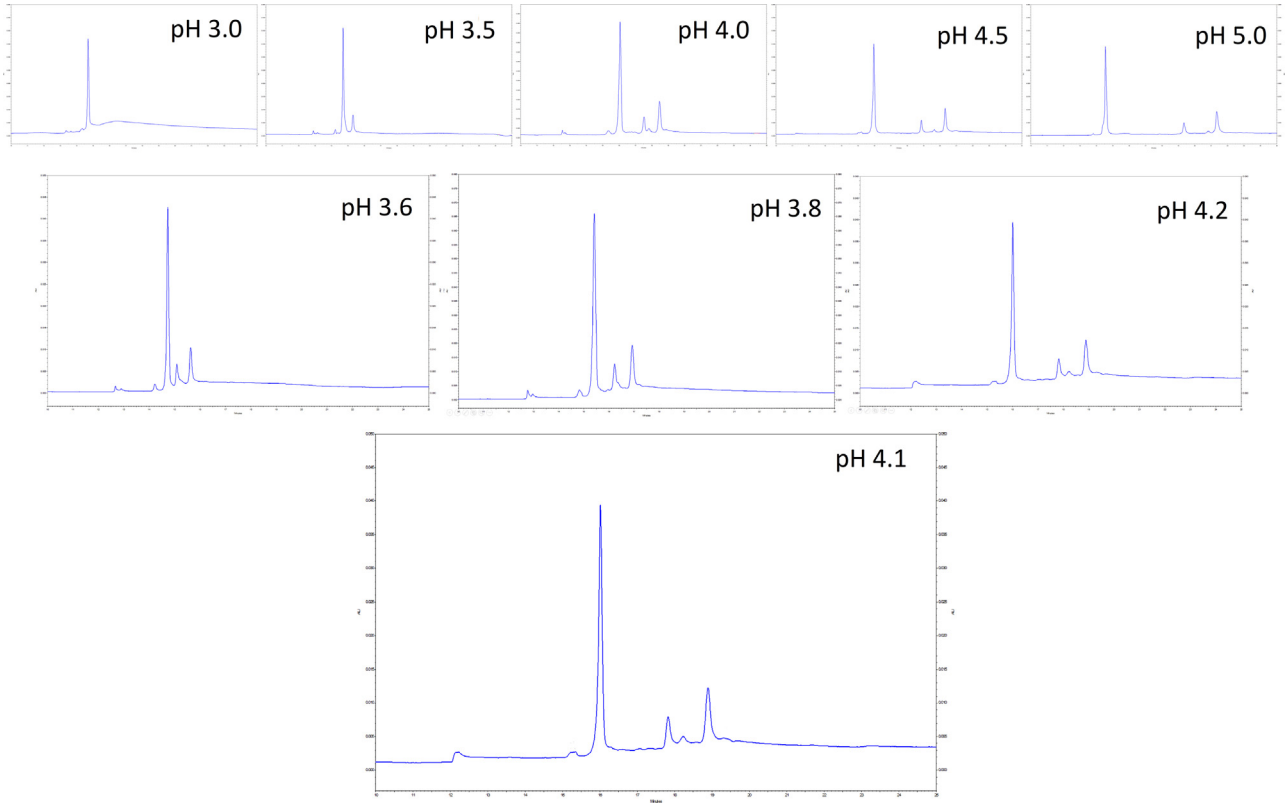


Fig. 1. Electrophoretic profile of papain digested MAB sample. Separation voltage: 30 kV, 214 nm, 100 mM EACA buffer, pH 3.0, 3.5, 4.0, 4.5, 5.0, 3.6, 3.8, 4.2 and 4.1.

Finally pH 4.1 was proven to be the optimal point for the next step of development Fig. 1.

3.3.1.3. Step 2: adjusting ionic strength. Aim of Step 2 was to find the optimal ionic strength for the buffer system by adjusting three different level of EACA buffer (25 mM, 50 mM and 100 mM). For each level the pH was adjusted to 4.1 according to Step 1.

With the 25 mM and the 100 mM buffer the RSD%s for the migration times of the peaks were much more higher (RSD% ≤ 1.3 and 1.4) than in the case of 50 mM ionic strength buffer (RSD% ≤ 0.8).

According to the data of Table 1b, 50 mM EACA buffer was found to be optimal for further optimization Fig. 2a.

3.3.1.4. Establishing control strategy for finding the optimal ionic strength. As method optimization control the relative standard deviations of migration times of the six main peaks were calculated and used for primary indicator for the migration stability of the system. RSD% for each peaks were calculated from six–six independent runs for each ionic strength levels.

3.3.2. Phase II. "Fine tuning"

3.3.2.1. Step 3–adjusting the optimal level of TETA as organic modifier. Organic modifiers are used as selectivity enhancers in CZE systems [19]. Effect of TETA on the separation system was investigated on three different levels: 1 mM, 5 mM and 10 mM. Each data were calculated by six–six runs on each TETA levels.

As Table 1c shows minimal amount of TETA was enough to significantly enhance migration time stability. In the case of 1 mM TETA level, the greatest relative standard deviation percent was <5% concerning the area percent variability from run to run since at higher TETA level (5 mM and 10 mM) the area percentage stability was lower (RSD% for Peak 6 was over 8 and 11%). By adding organic modifier to the sample buffer, the migration time stability enhanced significantly and on the other hand the baseline stability improved dramatically (Fig. 2b).

3.3.2.2. Establishing control strategy for optimizing amount of TETA in the separation buffer. RSD% values of migration times and area percent of the selected peaks were chosen as output parameters. We intended to accept RSD% values for migration times <0.1 percent and <5% for area%.

3.3.2.3. Step 4–investigation of viscosity enhancer (HPMC). In our study HPMC was used to enhance viscosity of our CZE system. Effect of HPMC on the separation system was investigated on three different levels: 0.05 v/v%, 0.1 v/v% and 0.2 v/v% of the separation buffers were containing cellulose. Each data were calculated by six–six runs on each HPMC levels.

On higher viscosity level the separation efficacy of the CZE system increased significantly. In the region between Peak 2 and Peak 3 the resolution of the peaks increased dramatically and a peak indicated as Peak3* is migrating before Peak 3 (Fig. 2c). This new peak was chosen also as an indicator of the system stability during the application of the viscosity modificator HPMC. Table 1d shows the results obtained with using different levels of HPMC in the separation buffer. In the case of 0.05% HPMC added level, the greatest relative standard deviation percent was <5.2% concerning the area percent variability of Peak3* from run to run since at higher HPMC levels (0.1 v/v% and 0.2 v/v%) the area percentage stability was significantly lower (RSD% for Peak 3* was over 12 and 18%).

3.3.2.4. Establishing control strategy for HPMC adjustment. RSD% values of migration times and area percent of the selected peaks were chosen also as output parameters. We intended to accept RSD% values for migration times <0.1 percent and <5% for area%.

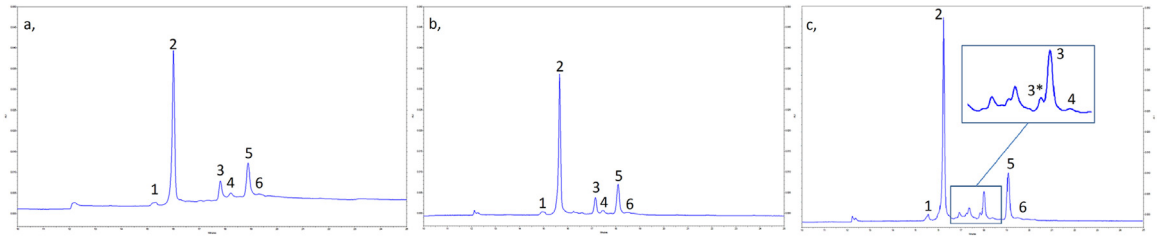


Fig. 2. (a) Electrophoretic profile of papain digested MAB sample. Separation voltage: 30 kV, 214 nm, 50 mM EACA buffer, pH 4.1. 1–6: indication of peaks used as output for control strategy. (b) Electrophoretic profile of papain digested MAB sample. Separation voltage: 30 kV, 214 nm, 50 mM EACA buffer, pH 4.1 and 1 mM TETA as organic additive. 1–6: indication of peaks used as output for control strategy. (c) Electrophoretic profile of papain digested MAB sample obtained with the finally adjusted method setup. Separation voltage: 30 kV, 214 nm, 50 mM EACA buffer, pH 4.1, 1 mM TETA and 0.05 v/v% HPMC as viscosity enhancer. 1–6 including 3*: indication of peaks used as output for control strategy. Zoomed area shows the improved resolution of the electropherogram region between Peak 2 & 3.

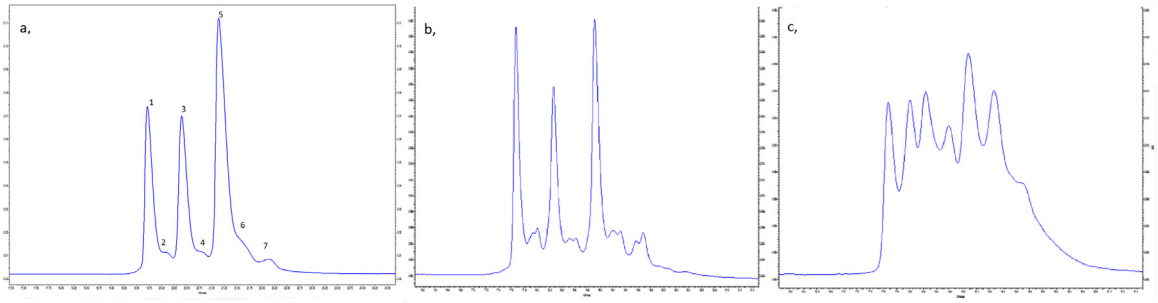


Fig. 3. (a) Electrophoretic profile of intact MAB obtain with the developed method. Separation voltage: 30 kV, 214 nm, 50 mM EACA buffer, pH 4.1, 1 mM TETA and 0.05 v/v% HPMC. 1–7: indication of peaks used for evaluation in the method qualification process. (b and c) Deamidated and oxidized MAB sample used for specificity testing. Separation voltage: 30 kV, 214 nm, 50 mM EACA buffer, pH 4.1, 1 mM TETA and 0.05 v/v% HPMC.

Table 2
Intermediate precision (18 runs) results for intact antibody.

	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5		Peak 6		Peak 7	
	Area%	Time (min)	Area%	Time (min)	Area%	Time (min)	Area%	Time (min)	Area%	Time (min)	Area%	Time (min)	Area%	Time (min)
Mean value	20.22	18.32	3.21	18.46	21.19	18.91	3.57	19.08	41.86	19.61	7.05	19.82	2.96	20.32
SD	0.11	0.13	0.11	0.06	0.22	0.07	0.15	0.06	0.36	0.07	0.32	0.07	0.09	0.07
RSD%	0.52	0.71	3.28	0.35	1.03	0.36	4.13	0.32	0.85	0.37	4.51	0.35	3.17	0.33

3.4. Justification of the final method applicability for intact protein

After development procedure performed by using papain digested MAB sample in 1 mg/ml solution the intact MAB in 10 mg/ml solution was investigated. Fig. 3a. shows the electrophoretic profile of the untreated protein. 7 main peaks could be separated with the developed method and optimal settings: 30 kV separation voltage, 50 mM EACA buffer, 1 mM TETA and 0.05 v/v% HPMC.

3.5. Method qualification

Qualification procedure of the developed method with the optimal parameters was performed. The untreated, intact MAB was used as analyte for the validation process.

Qualification of the method was performed by testing the main performance characteristics. LOD, LOQ, scpecificity, precision and linearity of the method was tested.

3.5.1. Level-of detection, level-of quantification, linearity

LOD, LOQ determinations were performed according to the recent ICH Q2(R1) guideline and linearity investigation were performed by the different dilutions of the original intact 10 mg/ml Mab solution by separation buffer between 0.05% and 100%. 0.05% of the original solution was proven to be LOD and 0.01% as for LOQ. Linearity of the method was found to be also adequate since the R^2 value of the main peak area – sample concentration function was $\geq$0.99.

3.5.2. Specificity

During specificity testing the deamidation and oxidation were used as chemical stress (Fig. 3b and c). The C-terminal lysine heterogeneity was justified by Carboxipeptidase B enzymatic reaction (data not shown). Peak 1 & 3 and 5 were belonging to the Lysine variants on C-terminal of the Fc region. Peak 2 & 4 & 6 and 7 are containing the deamidated forms of Peak 1 & 3 and 5. The resolution and the quality of the information was lower in the case of oxidative stress treatment probably due to the harsh chemical treatment with H_2O_2.

3.5.3. Intermediate precision

Intermediate precision tests were performed to investigate the effects of random errors of the analytical method. This precision study was carried out on three different days, on two different instruments and with two different operators. Six independent sample preparations with single injections were applied on each days. All 18 runs were evaluated and the mean values, the standard deviations and the relative standard deviations of the individual peak area percentage and migration times were calculated Table 2.

Very good results were obtained during evaluation. RSD <0.8% was obtained concerning migration times, and RSD <5% was calculated for area% values.

4. Conclusions

This study was aimed to establish a simple method development model for capillary zone electrophoresis systems. We introduced an iterative *'two-phase and four-step'* approach to accelerate electrophoretic method developments for monoclonal antibody investigations could be easily introduced into any biopharmaceutical laboratory.

Papain digested Mab samples were used for method development. Six different peaks of the digested samples were chosen to monitor system stability during the different method development phases.

For preliminary screening a 100 mM EACA for buffer system was chosen. Since pH is one of the main factors which affect the separation, the parameter screening was started with adjusting the pH. The optimal pH value was found to be 4.1, the optimal ionic strength was found to be 50 mM and the optimal amount of polymeric solution to be 0.05 v/v%. In every step the output parameters were declared (migration time, area percent of the individual peaks) and a control strategy was established to have verification over each steps in order to reach the optimum.

During optimization steps the migration time stability and the relative standard deviations for the area percentage values of the individual peaks increased significantly: from 1.2% to 0.04%, from 0.9% to 0.0%, from 0.6% to 0.04%, from 0.6% to 0.04%, from 0.5% to 0.03% and from 1.4% to 0.02% for Peak 1,2,3,4,5 and 6 respectively.

In the last step the viscosity was enhanced by the addition of polymeric solution (HPMC) to the buffer system and the resolving capacity increased dramatically as a consequence (Fig. 2c) and a new pre-peak (Peak 3*) could be separated from Peak 3. New peaks appeared since the values for output parameters (RSD% of the area% and migration time of the "indicator" peaks) remained within the limits appointed in the control strategy. The RSD% values for area percentages remained around 5%.

To reveal the maximal peak resolving capability of the developed buffer system papain digested MAB samples were used for method development.

Intact antibody was used for the qualification procedure, where LOD, LOQ, linearity, specificity and intermediate precision was investigated according to ICH Q2(R1). The developed method proved to be linear between 0.1 and 100%. The C-terminal lysine variants could be separated from the peaks containing deamidated forms. Very good results were obtained during intermediate precision where 18 runs on 3 different days were investigated. RSD < 0.8% was obtained for migration times and RSD < 5% was calculated for area% values of the individual peaks.

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