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# Analysis of Rituximab, A Therapeutic Monoclonal Antibody by Capillary Zone Electrophoresis

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## Abstract

This paper focuses on the applicability of capillary zone electrophoresis (CZE) using uncoated fused silica capillaries for the determination of heterogeneity of rituximab (MabThera, Roche) and for the study of its solution and thermal stability. The best resolution for the charge variants of the main component was obtained with a buffer electrolyte containing 800 mM 6-amino caproic acid, 2 mM triethylene tetramine and 0.05% hydroxypropyl methylcellulose at pH = 5.2. It was found that the pH and the components of the buffer used for the electrophoretic separation and for the dilution of the sample prior to the analysis are important to the stability of the rituximab. We demonstrated the rituximab is stable in the pharmaceutical product MabThera due to the stabilizing additives, but the dilution of the MabThera caused a slow formation of acidic variants, while the amount of the basic variants did not change. After incubation of the diluted rituximab at higher temperature several charge variants could be determined by CZE.

**Keywords:** Monoclonal antibody; Charge heterogeneity; Capillary zone electrophoresis

## Introduction

The biotechnologically engineered monoclonal antibody (mAb) contains four polypeptides linked via disulfide bonds: two light and two heavy chains. It is produced in mammalian cell culture. The mAbs can undergo post-translational modification (deamidation, oxidation, glycosylation, lysine truncation, aggregation) resulting in molecular heterogeneity, charge and size variants. Since these varieties of the mAb have effects on its pharmaceutical properties (antigen binding) and stability, it is important to investigate its molecular heterogeneity. Therefore numerous works were published about the analysis of mAb and the characterization of the molecular heterogeneity [1]. The rituximab (Rtx) is a monoclonal antibody used to treat cancers of blood system such as B cells leukemia (non Hodgkin's lymphoma) and some autoimmune diseases (rheumatoid arthritis) [2].

While the size heterogeneity of the monoclonal antibodies was studied by size-exclusion chromatography [3,4], peptide mapping [4], LC-MS [5] or gel electrophoresis [6-8], the charge heterogeneity was mostly studied by cation-exchange chromatography [3,4]. The different methods of capillary electrophoresis (CZE, CGE, CIEF) are useful in the complex analysis of the mAbs. The capillary zone electrophoresis (CZE) can reveal the charge variants of the mAbs. High separation efficiency could be achieved when the interaction between protein and the inner wall of the capillary was eliminated. In order to overcome this problem several strategies, such as appropriate choice of the pH and different additives of the background electrolyte and use of capillary coatings were applied [9]. The simplest way to reduce these interactions is if very low or high pH values are used. In this case the protein and the inner surface have the same charge and thus the proteins do not tend to adsorb onto the surface. However, the large difference between the pH of the buffer and the pI of the protein can cause structural changes in the protein resulting in low recovery [10]. This can often suggest modifying the inner wall of the capillary for preventing protein adsorption. Using bare fused silica capillaries different additives like 6-amino caproic acid (EACA) [11], triethylene tetramine (TETA) [12] or hydroxypropyl methylcellulose (HPMC) [11] are given into the background electrolyte. Shi et al. evaluated the separation of charge variants of mAb in fused silica capillary using a CZE running buffer containing polyethylene oxide (PEO) and TETA [13]. Five different commercially marketed mAb products were investigated by CZE in eCAP coated neutral capillary using EACA and polysorbate 20 in the running buffer [14]. Several mAbs, among them the rituximab, were analyzed in coated capillary with polyacrylamide-based hydrophilic surface [15]. Gassner et al. tested various statically coating (polyvinylalcohol, polybrene, dextran sulphate, polyethylenimine) for the analysis of rituximab by CZE [16].

This paper focuses on the applicability of CZE using uncoated fused silica capillaries for the determination of heterogeneity of rituximab (MabThera, Roche) and for the study of its solution and thermal stability.

## Material and Methods

### Instrumentation

The capillary electrophoresis instrument was a 7100 model (Agilent, Waldbronn, Germany). In the case of CZE measurements hydrodynamic sample introduction (30 mbar, 2 s or 30 mbar, 5 s) was used for injecting samples. The sample solutions were introduced at the capillary end closer to detection and the anodic end of the capillary as well. Separations were performed using a fused-silica capillary of 64.5 cm × 50 μm i.d. (Polymicro, Phoenix, USA). The applied voltage was +10-15 kV. The detection was carried out by on-column diode array photometric measurement at 200 nm. The electropherograms were recorded and processed by ChemStation computer program of B.04.02 version (Agilent).

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## Chemicals

Reagents of analytical grade were obtained from various distributors. Disodium hydrogen phosphate, HCl, NaOH, EACA, HPMC and TETA for preparing buffer electrolytes were purchased from Sigma-Aldrich (St. Louis, USA). The 1 mg/mL Rtx sample solutions were prepared shortly before CE separation by dissolving the rituximab (10 mg/mL MabThera, Roche) in water. The CGE separations in sieving matrix gel buffer (Beckman Coulter SDS-MW, USA) were performed. In this case the 1 mg/ml sample sample solution is made by diluting the rituximab with SDS-MW sample buffer solution (Beckman Coulter). The post conditioning was carried out with 1 M NaOH or 1 M HCl (in case of using of EACA-TETA-HPMC) for 5 min and with background electrolyte for 6 min.

## Results and Discussion

### CZE separation of rituximab in uncoated capillary

The most simple approach to analyze Rtx with CZE if uncoated fused silica capillary is used. A phosphate buffer of pH= 9.3 (a higher pH than the pI value of the Rtx (pI=7.3 - 9.1 [13,16]) was selected to minimize interactions of Rtx with the deprotonated silanol groups of the surface. At pH 9.3 the surface became negatively charged and therefore the predominantly negatively charged Rtx was repelled by the surface (the estimated negative charge of the Rtx at pH= 9.3 is 40 according to the Protein Calculator version 3.4). In this condition a sharp peak was obtained for the Rtx (less than 0.08 min peak width at 16.6 min migration times), which indicates that only minimal adsorption occurred (Figure 1a). However, the electrophoresis of Rtx

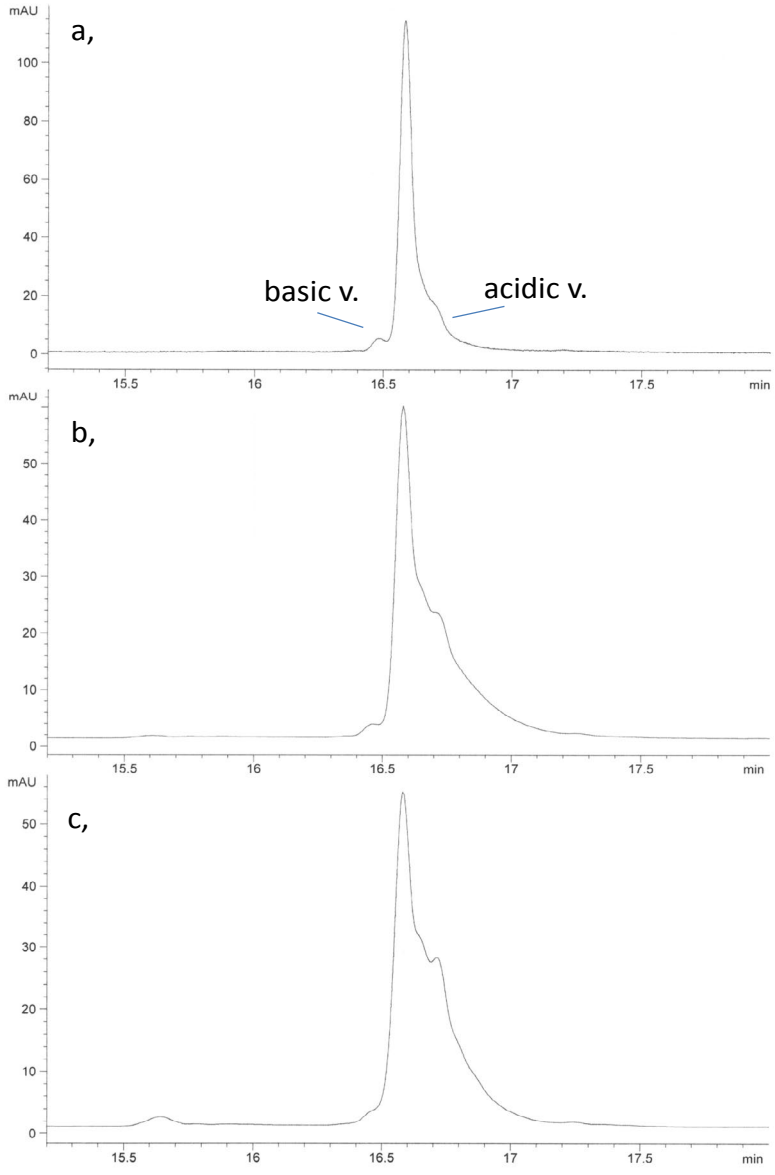



**Figure 1:** CZE separations of rituximab. The 10 mg/ml pharmaceutical product MabThera was tenfold diluted with water and the samples were injected within 5 min (a,), in 3 days (b,) and 7 days (c,) after the dilution.
Conditions: 100 mM phosphate buffer pH 9.3, capillary: 64.5 cm x 50 µm i.d., $l_{eff}$: 56.5 cm, 60 mbar s, +10 kV, λ= 200 nm.

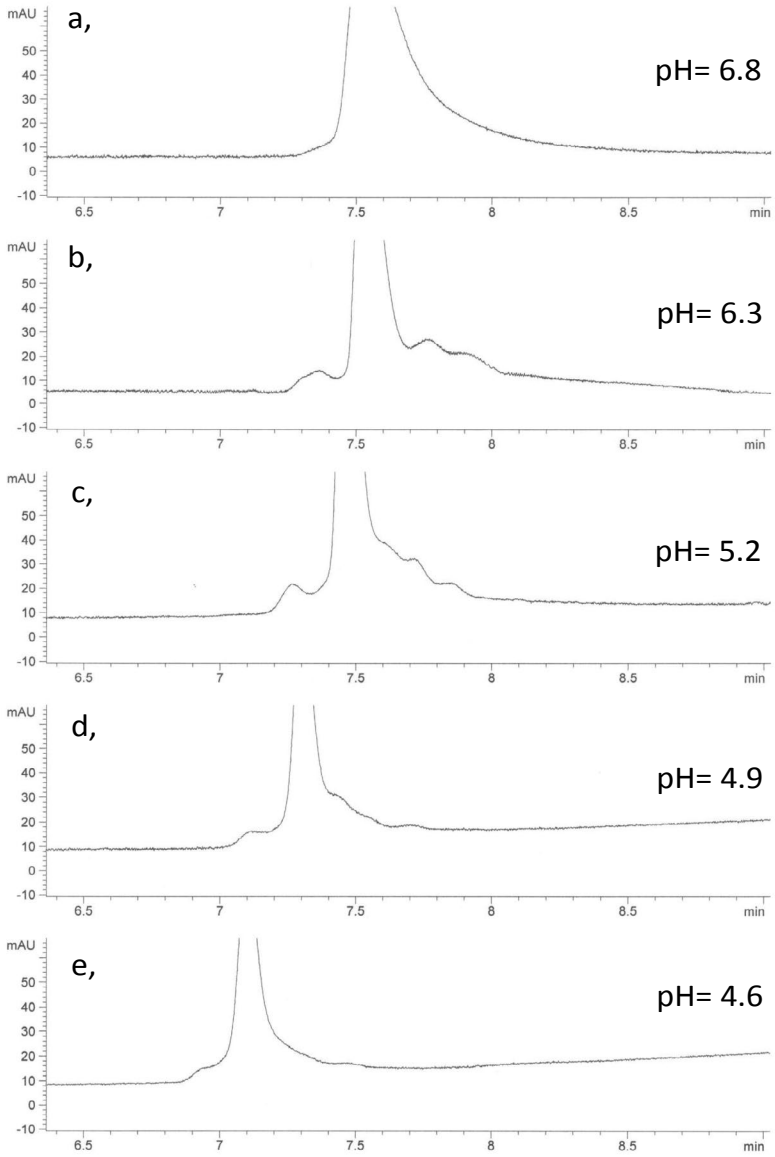



**Figure 2:** CZE separations of rituximab using running buffer of different pH. Conditions: 400 mM EACA, 2 mM TETA, 0.05% HPMC, capillary: 64.5 cm x 50 µm i.d., $l_{eff}$: 8.5 cm, samples were injected by 150 mbars at the anodic end of the capillary, closer to the detection window ("short end injection"), -15 kV, λ= 200 nm. The 10 mg/ml pharmaceutical product MabThera was tenfold diluted with water.

at pH lower than 8 resulted in a very broad signal if any. Although the CZE separations of proteins are generally carried out in coated (PVA, µSIL, PB, PEI, etc. [9,10]) capillaries, we did not get considerably better peak shape for Rtx using µSIL capillary. The CZE separation above pH= 10 could not be carried out because the Rtx degradated.

The 10 mg/ml pharmaceutical product was tenfold diluted with water and the samples were injected immediately, in 3 days and in 7 days after the dilution. In the obtained electropherograms of (Figure 1b) the basic and acidic variants can be observed at the sides of the main component, Rtx. In general, the acidic variants are formed by glycosylation with sialic acid or uronic acid, or by deamidation of asparagine or glutamine, or by pyroglutamate formation from glutamine and glutamate, whereas the basic variants are formed by formation of Fc-1 lysine or Fc-2 lysine, or by the noncyclization of N-terminal glutamine, or by succinimide formation from aspartic acid or by C-terminal proline amidation [14]. In the electropherograms the basic variant is probably 1-lysine variant (~2.5 %), which is relatively well separated from the main component (0-lysine). The shoulder of the main component contains one or more acidic variants. The dilution of the pharmaceutical product, which contains stabilizing components (neutral detergent) caused a slow formation of acidic variants, while the amount of the basic variants did not change. Mainly the acidic variants in the drug product could have impact on biological activity, so the fast and reliable determination of the amounts of these variants is required [17]. The electrophoretic patterns presented in (Figure 1 c) are in good agreement with the electropherogram obtained in neutral coated capillary [16].

Since, we did not reach proper resolution for acidic variants using simple phosphate buffer in uncoated silica capillary, the application of different dynamic coatings was tested. Several components (detergents,

polyamins, polymers) added to the running electrolyte have been investigated as dynamic coatings on the surface of the capillary [11-16]. Often a mixture of different additives with complex mechanism are applied [11-16]. Below pH= 8 the Rtx can be analyzed by CZE only in a (dynamically or statically) coated capillary. For the CZE of mAbs the mixture of a nonionic, hydrophilic polymer (HPMC), a compound forming ion pairs with peptides (EACA) and a polyamine (TETA) was suggested by Shi et al. in order to dynamically coat the capillary [13]. In our work this suggested mixture was used for the separation of Rtx. The nonionic linear polymer HPMC tends to adsorb to the neutral surfaces suppressing protein-wall interactions. Prior to conditioning the capillary with the running electrolyte short rinsing with 1 M HCl was used to protonate the silanol groups (and to remove the adsorbed proteins, as well). Using a higher polymer concentration a sieving effect can also be in play. The EACA on one side can interact with the residual silanol groups of the capillary surface that are not covered by HPMC, and on the other hand it can form ion pairs on the protein reducing its net charge. The addition of TETA to the buffer can also form a dynamic coating on the inner wall of the capillary reducing the adsorption of proteins. Additionally, it competes with the protein to interact with the silanol groups. However, the excess of TETA can modify the charge heterogeneity of mAb caused by the interaction of amines of TETA with the carboxyl groups of the mAb. The CZE separations of Rtx shown in Figure 2 were performed using running buffer containing 400 mM EACA, 2 mM TETA and 0.05% HPMC (suggested concentrations from [12] for several mAbs but not Rtx) of different pH values. At pH= 6.8 (close to the pI of Rtx) the adsorption of the Rtx to the inner wall is still considerable, which resulted in peak tailing (Figure 2a,b). However, in the pH range of 4.9-6.3 the peaks at the bottom of the main peak Rtx were resolved to two-three acidic variants and one basic variant. At pH below 4.6 the separation of the variants from the main component became worse. The best resolution for the acidic variants were obtained at pH= 5.2 (Figure 2c,d,e). Similar patterns of peaks of basic/acidic

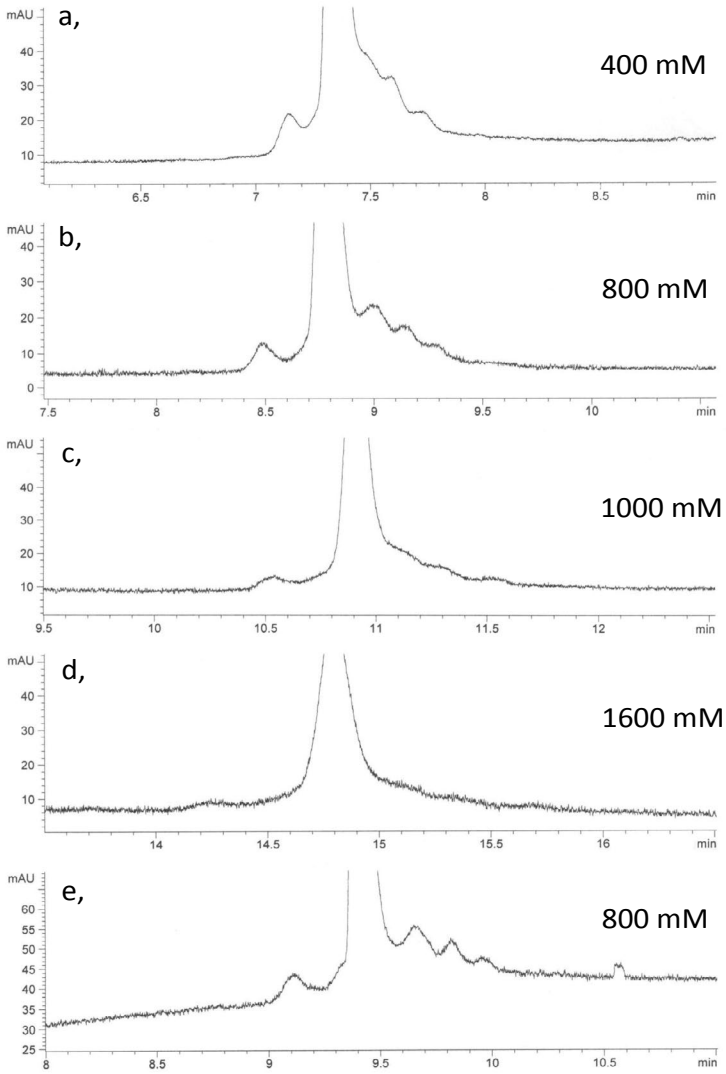



**Figure 3:** CZE separations of rituximab using running buffer of pH=5.2 with different concentration of EACA.
Conditions: 400-1600 mM EACA ((a,): 400 mM, (b,): 800 mM, (c,): 1000 mM, (d,): 1600 mM, (e,): 800 mM), 2 mM TETA, 0.05% HPMC, capillary: 64.5 cm x 50 µm i.d., $l_{eff}$: 8.5 cm, sample was injected by 150 mbars at the anodic end of the capillary, closer to the detection window ("short end injection"), -15 kV, λ= 200 nm. The 10 mg/ml pharmaceutical product MabThera was tenfold diluted with water. in case of (e,): capillary: 30 cm x 50 µm i.d., $l_{eff}$: 21.5 cm, +15 kV, sample was injected further to the detection window (normal mode).

variants were reported by He et al. for other mAbs [12,17], using a bit higher optimal pH (pH= 5.7).

The electropherograms of (Figure 3a,b,c,d) illustrate the CZE separations of Rtx using running buffer of pH= 5.2 including the three additives (EACA, TETA and HPMC) with 400-1600 mM concentration of EACA. When the concentration of EACA was doubled from 400 mM to 800 mM the improvement in the separation of acidic variants was quite significant, but the further increase in the concentration of the EACA led to a gradual disappearance of the variants. The considerable increase of the EACA in the buffer accompanied a slower migration of the components due to the suppressed of (increased ionic strength, smaller surface charge of the capillary). The phenomenon of the gradual disappearance of the variants above 1000 mM EACA could not be explained. A little better resolution was obtained for a slightly larger electric field and longer separation length (Figure 3e), but the further increasement of the length of the capillary deteriorated the efficiency of the separation. Interestingly, the increase of the EACA concentration in the running buffer improved the resolution only for the basic variants for other mAbs [12].

## Study of solution and thermal stability of rituximab

The stability of the mAb is critical in terms of its applicability and pharmacological properties. The characterization of the stability of the therapeutic monoclonal antibodies is important since they can go through several chemical and physical degradation/transformation processes, thus their proteic natures can alter. During the biotechnological preparation and the storage the mAbs are exposed to a lot of stress factors. The chemical stability relates to the integrity

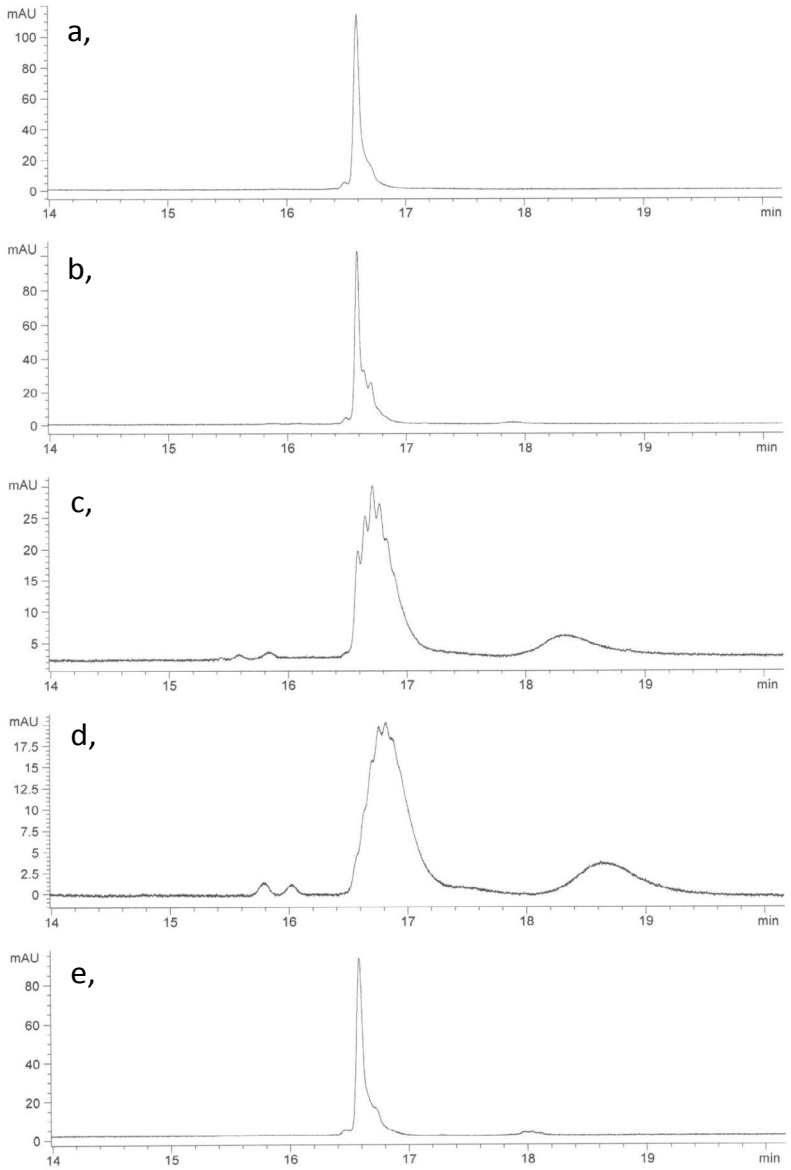



**Figure 4:** Thermal stability (55°C for 1.5 h (b,), 15 h (c, and e,) and 40 h (d,)) studies of rituximab.
(b,) - (d,): the samples (MabThera) were diluted prior the thermal treatment,
(a,) and (e,): the samples were diluted just before their injections. (a): the sample was kept at room temperature.
Conditions: 100 mM phosphate buffer pH 9.3, capillary: 64.5 cm x 50 µm i.d., 60 mbars, +10 kV, λ= 200 nm, the samples are diluted with water.

of covalent linkages, while the secondary bonds (hydrogen bond, electrostatic interaction) are responsible for the physical stability. The Rtx is formulated for parenteral administration in sodium citrate dihydrate buffer (pH 6.5) containing nonionic surfactant (Tween 80) to stabilize the aqueous formulation [4].

As it was stated in the previous section the acidic variants are slowly formed by diluting the pharmaceutical product by water, while the amount of the basic variants did not change. At higher temperature the acidic variants are formed with a much higher rate than at room temperature. Incubating the mAb samples at 55°C for 1.5 hours the degradation of the main component was around 10% and two peaks (acidic variants) appeared in the shoulder of the main peak (Figure 4a,b). The acidic variants are generally formed due to deamidations or pyroglutamate formation. In accelerated stability testing the deamidation

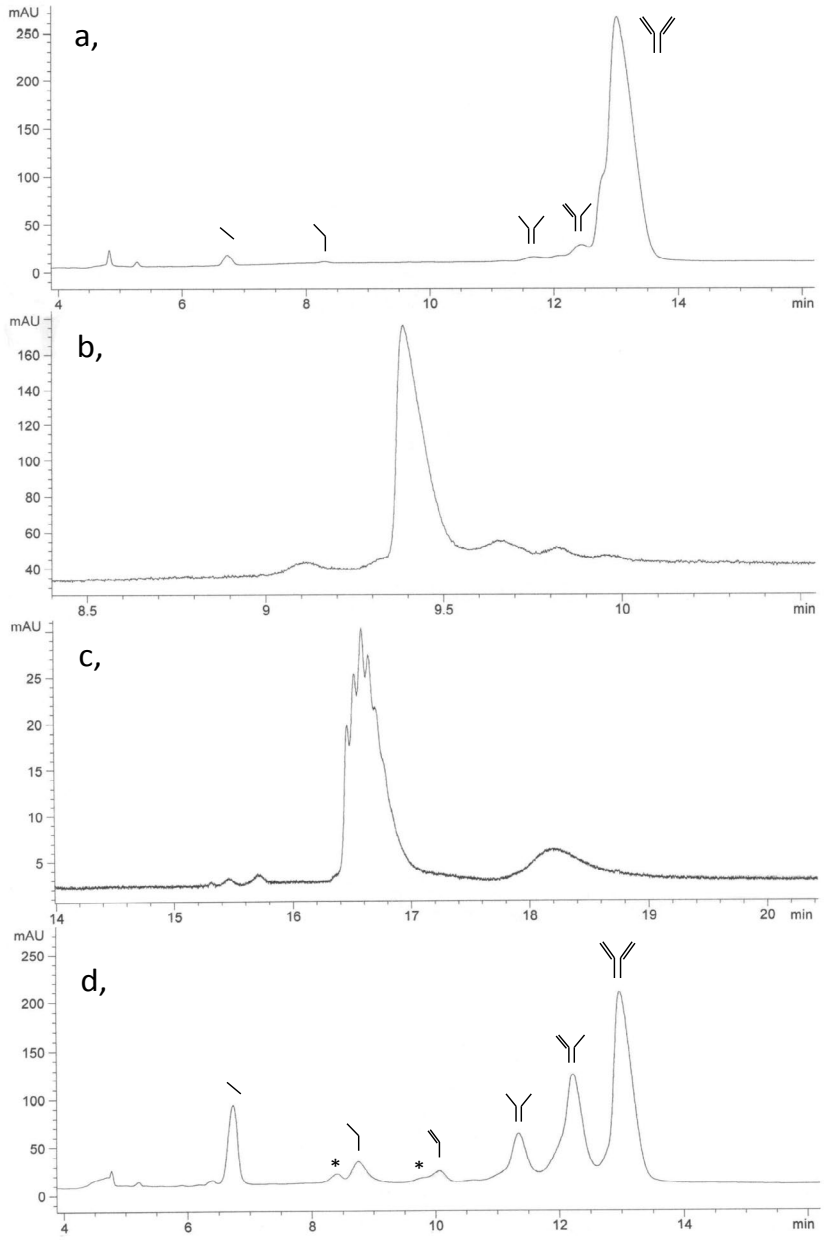



**Figure 5:** Comparison of electropherograms of rituximab obtained by CGE using sieving matrix (a, d), CZE using EACA-TETA-HPMC additives in the buffer (b,) and CZE using phosphate buffer (c,), (b,).
Conditions:
(a, d): CGE separations, Beckman Coulter SDS-MW gel buffer sieving matrix, electrokinetic (short-end) injection: +7.5 kV for 50 sec, capillary: 30 cm x 50 µm i.d., U= +15 kV, λ= 200 nm.
(b,): CZE separation, 800 mM EACA, 2 mM TETA, 0.05% HPMC, pH= 5.2, capillary: 30 cm x 50 µm i.d., 150 mbar s, +15 kV, λ= 200 nm.
(c,): CZE separation. The sample was stressed by 55°C for 15 h, 100 mM phosphate buffer pH 9.3, capillary: 64.5 cm x 50 µm i.d., 60 mbar s, +10 kV, λ= 200 nm
(d,): CGE separation. The sample was stressed by 55°C for 15 h, 100 mM phosphate buffer pH 9.
1 mg/ml pharmaceutical product MabThera samples were prepared by dilution with Beckman Coulter SDS-MW sample buffer (a, d,), water (b,) and with the buffer solution (c).

and the cyclization of N-terminal glutamate to pyroglutamate were verified by MALDI-TOF MS [5]. Incubating the mAb samples for 15 and 40 hours 5-6 intensive but overlapped peaks of acidic variants could be observed (Figure 4c,d). These results correspond with the statements of others, that is, at high temperature and at high pH the deamidation (the most common covalent modifications for protein pharmaceuticals) becomes faster [3]. A similar increase in acidic variants after stress (0,5 M phosphate, pH 8.0 at 40°C for 8 day) was obtained by Han et al. using conventional and microchip CZE [14]. When the mAb samples were incubated at 55°C for 15 and 40 hours (Figure 4c,d) two peaks could be observed between 15.5-16.5 min. According to the suggestion of ref. [18] these peaks can be assigned to the components formed by the oxidation of methionine.

The Rtx in the formulated product MabThera without dilution shows very good stability. (The dilution of the formulated product MabThera reduces the stabilizer effect of the nonionic surfactant and citrate buffer additives. While the citrate moderates the deamidation by changing the conformation of the protein, the phosphate ions may catalyze the deamidation [3].) Incubating the undiluted MabThera at 55°C for 15 hours it practically did not undergo any transformation (Figure 4e). Based on these obtained and reported results it can be stated that the roles of the pH and the buffer species are important in the stability of Rtx, the formulated product mAbs should not dilute with buffer (phosphate, pH=9.3), or if it is necessary it should be done just prior to the sample injection in order not to accelerate the degradation of the Rtx.

### Comparison of CZE and CGE using SDS-sieving matrix for heterogeneity of rituximab

The SDS-protein complexes can be separated by CGE in the sieving matrix according to their molecular weight and shape. Earlier the CGE was also applied to separate the size variants (fragments, aggregates) of mAb [6-8]. In our experiments the Rtx was diluted with the sample matrix buffer just before the injection. Little peaks of four low molecular weight (LMW) fragments (light chain, heavy chain, two heavy chains and two heavy chains with one light chain) can be seen before the main component (monomer) (Figure 5a). These fragments observed on the CGE electropherogram are in a good agreement with the electrophoretic pattern obtained by Hunt [6]. Interestingly, in the CZE electropherogram of the same sample (Figure 5b) also four components could be observed next to the main component, however, the four components separated by CZE and CGE are probably not identical. The charge variants of the main component separated by CZE (Figure 5b) appeared as one peak (monomer with a given molecular size) in CGE electropherogram (Figure 5a). In this sample stored at room temperature mainly the peak of the light chain could be found.

The CZE separation of the components of the thermally degradated sample revealed a broad peak after the main component (monomer) (Figure 5c). Although this broad peak at 18.2 min seems to be a possible dimer (the increase of the temperature may alter the secondary, tertiary and quaternary structures of the protein and led to aggregation), the CGE measurement clearly shows the lack of aggregates of mAbs (Figure 5d). It is more likely that the effect of heating caused the rupture of the Y-shape in the hinge region resulting in formation of heavy chains and half antibodies (heavy chain + light chain) (Figure 5d). These two components could not be observed at room temperature. The amounts of the LMW components were increased by thermal stress (Figure 5d). Since the release of a light chain is easier than that of the heavy chains (which are held together by two disulfide bonds in hinge region), the light chain is predominant LMW fragment in the sample. The shoulders of the peaks of the heavy chain and half antibody (marked with *) are probably their non-glycosylated forms (as suggested by [8]) or the results of incomplete SDS binding [6] and/or unfolding of the mAb [6]. With the CGE the LMW components could be well separated from each other and from the monomer. For the monomer the precision of migration time and peak area were 0.27% and 6.16%, respectively. The limit of detection (LOD, 6s) was 0.69 μg/ml using UV detection at 200 nm.

According to the obtained results it can be stated that the CZE analysis could reveal the charge variants of Rtx, while the CGE with sieving matrix gave information about the size heterogeneity. There is no chance to determine either the charge or the size variants by the same separation method.

## Conclusions

In this work the applicability of CZE for the determination of the heterogeneity of rituximab was studied. A buffer of pH=5.2 with several additives was used to suppress the interactions between the Rtx and the capillary, thus even an uncoated fused silica capillary was applicable for the efficient separations of several charge variants and the main component. In order to support some conclusions obtained by CZE, CGE in sieving matrix separation was used. For instance, the charge variants of the main component that can be separated by CZE provide the same molecular size.

It was found that the dilution of the pharmaceutical product, which contains stabilizing components (neutral detergent) caused a slow formation of acidic variants. Incubating the Rtx at higher temperature or basic condition, charge variants and fragments of the Rtx were formed by deamidation and fragmentation. The CZE analysis could reveal the charge variants of Rtx, while the CGE with sieving matrix gave information about the size heterogeneity.

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