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Thermal stability studies of immunoglobulins using capillary isoelectric focusing and capillary zone electrophoretic methods

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Abstract

The affinity of an antibody towards its antigen is highly specific to its conformation, in order to have optimal antibody–antigen interaction. The increase of temperature might cause changes in antibody conformations. The change of structure conformations may be reflected in the isoelectric points (p*I* values), peak shape and absorbance of the antibody. In this study, a monoclonal antibody was heated over a period of time. Capillary isoelectric focusing (cIEF) and capillary zone electrophoresis (CZE) were used to monitor the change in the antibody. The longer the heating period, the lower the p*I* values were under cIEF conditions. CZE also showed changes in peak shapes and decreases in absorbance of the antibody with heating. © 1998 Published by Elsevier Science B.V.

Keywords: Thermal stability; Stability studies; Temperature effects; Isoelectric focusing; Immunoglobulins; Antibodies; Proteins

1. Introduction

The antibody–antigen interaction is a biomolecular interaction involving various noncovalent interactions between the variable-region domain of the antibody molecule, particularly the hypervariable regions, or complementarity-determining regions (CDRs) and the antigenic determinant, or the epitope of the antigen. These noncovalent interactions are hydrogen bonds, ionic bonds, hydrophobic interactions and Van der Waals interactions [1,2]. These forces are responsible for the stability of the antibody–antigen complexes. Because the strength of these interactions are relatively weak compared to covalent bonds, a strong antibody–antigen interaction requires a large number of such interactions. The strength of the antibody–antigen complexes is also determined by the distance between the two proteins. The closer the two proteins, the stronger the interactions. Therefore, a small change in the structure of the variable region of the antibody (Ab) could lead to a decrease or even loss of affinity for the antigen.

Changes in factors such as temperature, ionic strength and solution pH can alter the structural integrity and flexibility of the immunoglobulins [3,4]. With all the papers published on the analysis of antibodies by CE, only a very limited number of them have been devoted to the structural change of the antibody with a change of temperature and pH [4,5]. If there is any change in the structure or conformation of the antibodies, it is possible that the isoelectric points (p*I* values) and absorbances of the isoforms of the antibodies can also be affected. Size-exclusion chromatography (SEC) was used to analyze a sample of Abs which was incubated at

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40°C at pH 10.3 for 2 days. SEC showed that 92% of the Ab was still in monomer form, 4% in dimers, and 4% in fragments. The monomers for this sample had the same elution time as the standard Ab. If antibodies were sensitive to a rise of temperature, there should be more changes to them than what the SEC data found. Capillary electrophoresis (CE) can be used to supply more information on the effect of temperature on the antibodies.

Several papers published using capillary isoelectric focusing (cIEF) to separate antibodies had demonstrated the high-resolution power of the method [6–11]. cIEF is a high-resolution technique that can be used to separate proteins, peptides and antibodies based on their p*I* values. cIEF is run in a gradient where the pH is acidic in the anode (usually phosphoric acid) and basic in the cathode (usually sodium hydroxide). The sample is mixed with carrier ampholytes. When a high voltage is applied, the ampholyte mixture separates in the capillary with negatively charged ampholytes moving towards the anode and the positively charged ampholytes moving towards the cathode. When the ampholyte reaches its p*I* and is no longer charged, it will stop migrating. The protein can be detected by salt or electroosmotic flow (EOF) driven mobilization [12–14]. The detection is usually set at 280 nm because the ampholytes absorb strongly in the low UV range.

Both J&W Scientific and Chiron Diagnostics have used fluorocarbon (FC)-coated capillaries for the separation of antibodies [15–18]. They showed that using the FC-coated capillary with various ratios of surfactants and various pH values of the buffer, different isoforms in antibodies can be separated.

These two forms of CE can be used complementary to each other in looking at the change of antibody conformations and isoforms affected by a rise of temperature.

2. Experimental

2.1. Chemicals

Sodium acetate, sodium hydroxide and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ). Sodium phosphate dibasic, HPMC (hydroxylpropylmethylcellulose), all standard proteins and ampholytes were purchased from Sigma (St. Louis, MO, USA). *N*,*N*,*N*′*N*′-Tetramethylethylenediamine (TEMED) was from Bio-Rad Labs. (Hercules, CA, USA). FC surfactants were obtained from J&W Scientific (Folsom, CA, USA). The standard IgG1 was supplied by a pharmaceutical company at a concentration of 5.7 mg/ml.

2.2. Instrumentation

Initial methods development in capillary zone electrophoresis (CZE) was performed on a Waters Quanta 4000 CE system (Milford, MA, USA), and data were collected with a Macintosh Classic (Apple Computer, Cupertino, CA, USA) via a SMADchrom 3.0 interface software (Marc S. Nathanson, Sharon, MA, USA). Peaks were integrated with the same software. The final CZE experiments were performed on a Waters Quanta 4000E CE system, and data were collected with a Macintosh Classic via a Dynamax system from Rainin (Woburn, MA, USA). For cIEF, all experiments were performed with an Isco model 3850 CE (Isco, Lincoln, NE, USA) in the constant voltage mode. Uncoated capillaries used were 60 cm (40 cm from injection side to detection window)×50 μm I.D. (Polymicro Technology, Bloomfield, NJ, USA). FC-coated capillaries were 50 cm (42.5 cm effective length)×50 μm I.D., or 40 cm (32.5 cm effective length)×50 μm I.D. (J&W Scientific). Injections were hydrostatic for the Waters CE system, the detection was set at 214 nm and the output range was selected at 0.005 AUFS. For cIEF, a syringe flush port was used to rinse capillaries and load samples for analysis.

2.3. CZE operating conditions

For the FC-coated capillaries, they were rinsed with deionized water for 3 min and with running buffer for 5 min prior to injection. The temperature was maintained at 27°C for all runs.

2.4. Preparation of run buffer

For cIEF, the cathodic buffer was 20 m*M* NaOH, and anodic buffer was 20 m*M* phosphoric acid. For CZE, the buffer was phosphate and/or acetate +0.005% FC surfactants at various pH values.

2.5. Sample preparation for thermally stressed IgG1

Two different conditions were used in the preparation of the thermally stressed antibodies. For the first one, 60 μl of 1 *M* NaOH was added to 5 ml of purified IgG1 (original concentration at 5.7 mg/ml), bringing the pH from 7.0 to 10.3. The sample was incubated at 40°C for 2 days. The pH of the sample was then brought back down to 7.0. The second method followed the work from Bristol-Myers Squibb [5]. 8.4 μl of 5 *M* NaCl was added to 300 μl of purified IgG1. 50 μl of this sample was taken out as a control, and the rest was incubated at 60°C. A sample was taken out after 2 days, another one after 4 days and the rest was heated for 7 days.

2.6. Sample preparation for CZE

Standard IgG1 was diluted ten times with 20 m*M* phosphate at pH 7.0 before injection. The sample incubated at 40°C for 2 days was diluted 8 times with 20 m*M* phosphate at pH 7.0 before injection. All samples incubated at 60°C were diluted 7 times with 20 m*M* phosphate at pH 7.0 prior to analysis.

2.7. Sample preparation for cIEF

A 10% ampholyte with 0.2% HPMC, 2.8% TEMED was first prepared. The sample was then prepared by mixing 1 part of IgG1, 1 part of cytochrome *c* (original concentration at 4 mg/ml), 1 part of myoglobin (original concentration at 4 mg/ml), 1 part of deionized water, and 4 parts of the 10% ampholyte. The final concentration of the sample would be 5% ampholyte with 0.1% HPMC, 1.4% TEMED, 0.5 mg/ml of cytochrome *c* and myoglobin. The antibodies would be 8 times diluted from the original concentration.

3. Results and discussion

3.1. Results for cIEF

Methods development in cIEF was started by using a wide range ampholyte (pH 3–10) to find the approximate p*I* values of the standard IgG1 and the thermally stressed samples. A 3-protein standard (cytochrome *c* with pI at 9.3, α-chymotrypsinogen A with p*I* at 8.7, and myoglobin with p*I* values at 7.4 and 7.0) was first run for a calibration of the capillary and ampholyte performance. Analysis of the standard IgG and one of the thermally stressed samples showed that their p*I* values were in the range 7–9.

Cytochrome *c* (p*I* 9.3) and myoglobin (p*I* values 7.4, 7.0) were added to all the antibody samples for the calibration of the antibody p*I* values via cIEF. α-Chymotrypsinogen A was not used in this case because there were a lot of impurities in this protein and they would interfere with the antibody p*I* values.

cIEF of the standard IgG1 showed five peaks (Fig. 1). Their p*I* values ranged from 8.24 to 7.77 and the R.S.D. for the p*I* values were all less than 0.4% (Table 1).

The IgG1 incubated at 40°C for 2 days showed three major peaks under the same cIEF conditions, and the peaks migrated slower than the standard IgG1 (Fig. 2). This suggested that the IgG was starting to change its conformation or even breaking down into fragments, or forming aggregates. The p*I* values for this sample ranged from 7.99 to 7.78 and the R.S.D. values were under 0.2%.

An IgG1 sample which had been incubated at 60°C for 2 days also showed the same pattern and similar p*I* values as the one incubated at 40°C for 2 days (Fig. 3 and Table 1).

An IgG sample incubated at 60°C for 4 days also showed three major peaks, but two more peaks started to form at the tail of the third peak (Fig. 4). However, those two peaks were too close to each other for integration. The p*I* values for the three major peaks were slightly lower than the ones incubated for only 2 days (Table 1). The tails of the peaks also started to fuse with the myoglobin standard, which had one of its p*I* values at pH 7.4.

An IgG1 sample which was incubated at 60°C for 7 days showed substantial differences from the other samples. At least five peaks were observed (Fig. 5). The p*I* values for the five peaks integrated ranged from 7.90 to 7.53 with R.S.D. values less than 0.3% (Table 1). Some species in this sample should have p*I* values lower than 7.5, but they were interfered by the myoglobin standard which had one p*I* at 7.4. There were other choices for standard proteins. However, those proteins had enzymatic properties

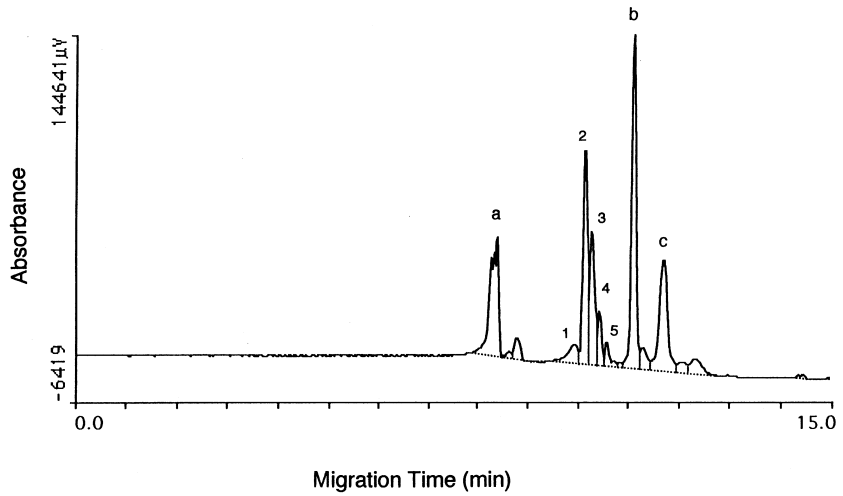


Fig. 1. cIEF electropherogram of standard IgG with protein standards. Capillary: uncoated, 60 cm (40 cm from injection end to detection window)×50 μm; catholyte: 20 m*M* NaOH; anolyte: 20 m*M* phosphoric acid; run voltage −20 kV; initial current: 39 μA; temperature: 25°C; standard proteins and sample were dissolved in 5% pH 3–10 ampholyte, 0.1% HPMC, 1.4% TEMED. Peaks: a=cytochrome *c*, p*I* 9.3; b and c=myoglobin, p*I* values 7.4 and 7.0; 1–5=IgG.

Table 1
Summary of p*I* values and %R.S.D. of standard IgG1 and thermally stressed samples (n=3)

Peak no.	Standard IgG1	40°C, 2 days	60°C, 2 days	60°C, 4 days	60°C, 7 days	R.S.D. (%) for p*I* values
1	8.24	8.00	7.99	7.94	7.90	±0.39
2	8.07	7.91	7.90	7.89	7.80	±0.27
3	7.99	7.80	7.78	7.82	7.72	±0.32
4	7.87				7.62	±0.22
5	7.77				7.53	±0.15

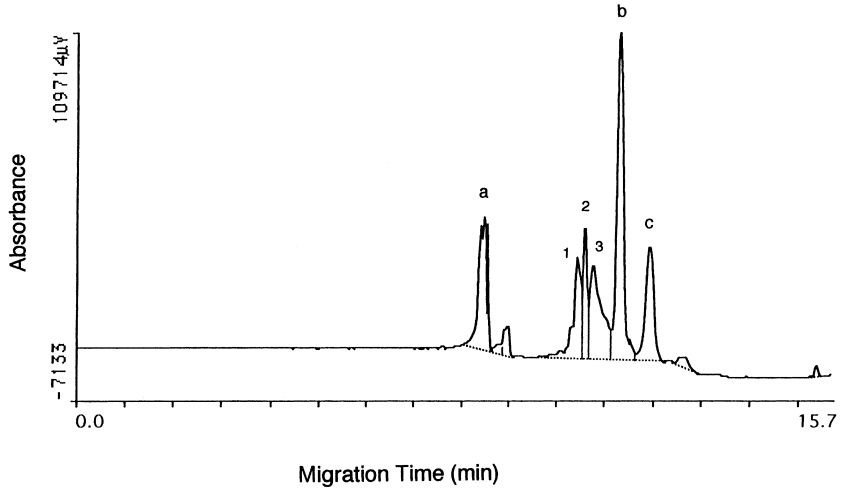


Fig. 2. cIEF electropherogram of thermally stressed IgG at 40°C for 2 days with protein standards. All conditions were the same as Fig. 1.

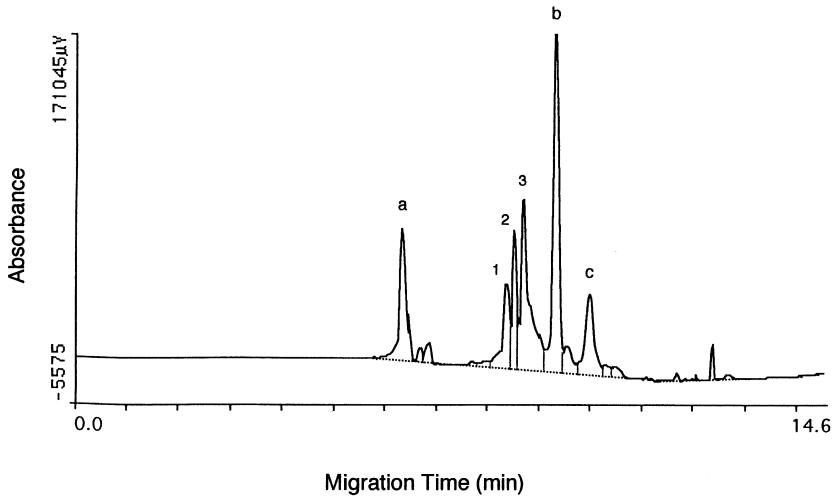


Fig. 3. cIEF electropherogram of thermally stressed IgG at 60°C for 2 days with protein standards. All conditions are the same as Fig. 1.

which might have added unnecessary interferents to the samples.

The cIEF data provided substantial evidence that there were changes in the antibodies with a rise of temperature. Although the p*I* values for some of the isoforms of the antibodies could be separated using the wide range ampholyte, there were still species in the antibodies that could not be accounted for because the resolving power of the wide range ampholyte was insufficient. The resolving power could be improved by using a longer capillary. However, with a longer capillary, a higher voltage would need to be applied to achieve the same analysis time. The higher voltage could lead to higher Joule heating which could split protein peaks. Using standards with p*I* values lower than myoglobin would also avoid the interference of a standard from the lower p*I* isoforms of the antibodies. With the narrow range ampholyte, the peak width might be decreased by using a more dilute sample.

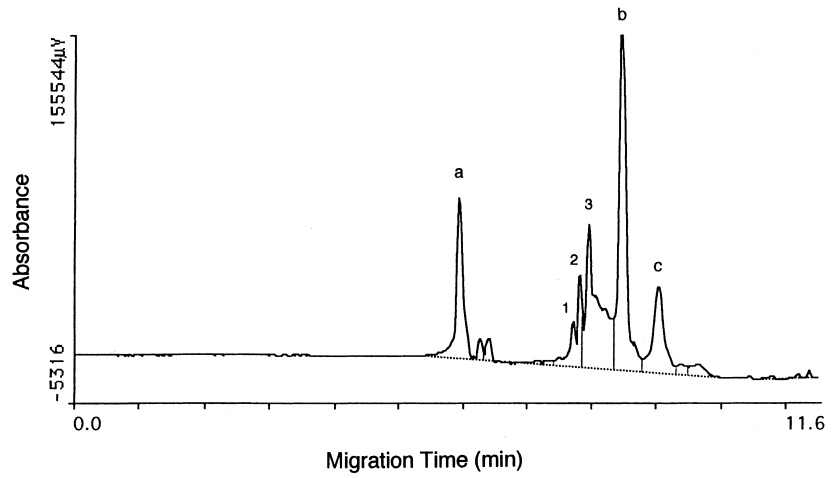


Fig. 4. cIEF electropherogram of thermally stressed IgG at 60°C for 4 days with protein standards. All conditions are the same as Fig. 1.

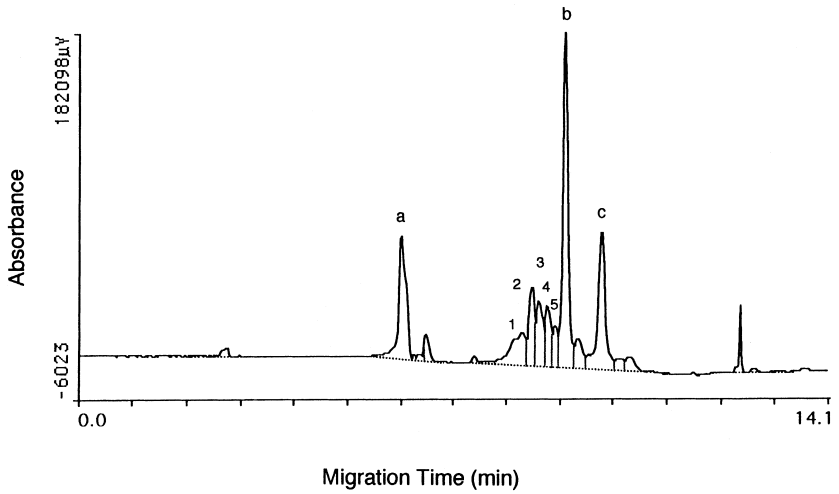


Fig. 5. cIEF electropherogram of thermally stressed IgG at 60°C for 7 days with protein standards. All conditions are the same as Fig. 1.

3.2. Results for CZE

The separation of isoforms of the antibody was also done with a FC-coated capillary. The recommended buffer to be used with the FC-coated capillary was 20 m*M* phosphate buffer +0.005% FC surfactants [17]. A first attempt was made with 20 m*M* phosphate +0.005% FC-WA–FC-N (1:1) mixture at pH 7.2, which was close to the pH of the antibody solution. Four peaks were observed under these conditions. When the pH of the solution was changed to 7.9, six peaks were obtained, and the resolution was slightly improved. With the pH increasing to 8.8, the peaks collapsed. It appeared that pH 7.9 was optimal.

The next step was to optimize the surfactant ratios. FC-N was a neutral surfactant which was added to decrease the EOF and prevent the adsorption of protein onto the capillary wall. With an increase of the FC-N surfactant, the migration time of the antibody increased, but the resolution decreased. The optimal surfactant ratio for 20 m*M* phosphate at pH 7.9 was 0.005% FC-WA–FC-N (1:1).

Once the optimal pH and surfactant ratio were obtained, the next step was to test the reproducibility of the method. The FC-coated capillary was proven by Mao et al. as a stable capillary for protein and antibody analyses [15,17]. However, different samples would show very different behavior under the same separation conditions. With the antibodies used in this experiment, the migration time was different by minutes from run to run under the optimized conditions. Since the surfactants also acted as a dynamic coating in the FC-coated capillary, the changes in migration time from run to run might be due to inconsistency of the surfactant coating on the capillary after each run. The migration time for the antibodies was stabilized by the addition of sodium acetate. The reason why sodium acetate stabilized the migration time remains unknown. The final optimized buffer conditions were 12.5 m*M* phosphate+12.5 m*M* acetate+0.005% FC-WA–FC-N (1:2) at pH 7.5. One major and three small peaks were observed (Fig. 6).

CZE of the standard IgG1 showed four major peaks which were at 17–19 min (Fig. 6). The sample, which was incubated at 60°C for 2 days, started to show changes in the peak shapes (Fig. 7). The first peak was at the same migration time as the standard IgG but the absorbance had decreased and the other peaks became more noticeable.

The sample, which was incubated at 60°C for 4 days, showed a more similar pattern to the one that was incubated at 60°C for 2 days, but the peak was more broad and the migration time of the first peak increased by 1 min (Fig. 8).

When the sample which was incubated at 60°C for 7 days was run, the peak almost disappeared (Fig. 9). The broad peak appeared from approximately 19 min to almost 28 min. One possible explanation was that

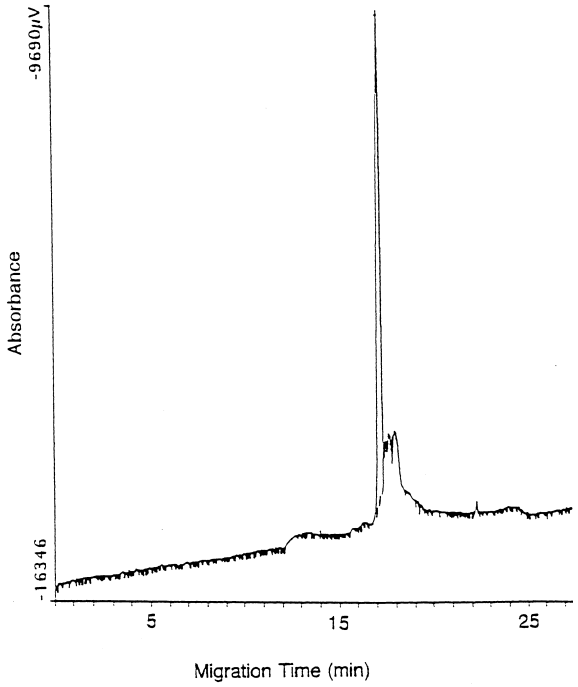


Fig. 6. CZE electropherogram of standard IgG. Capillary: FC-coated, 40 cm (32.5 cm effective length)×50 μm; buffer: 12.5 m*M* phosphate+12.5 m*M* acetate+0.005% FC-WA–FC-N (1:2), pH 7.5; injection: hydrostatic, 9.8 cm injection head, 10 s; run voltage: 15 kV; current: 14.5 μA; temperature: 25°C; detection: 214 nm.

the antibody was breaking down or changing into other species upon heating.

The sample incubated at 40°C for 2 days only showed one broad peak at ~17–24 min under the same CZE conditions (Fig. 10). This sample showed more subtle changes in peak shapes than the one incubated at 60°C for 2 days, possibly due to the harsher incubation conditions in which NaOH was added.

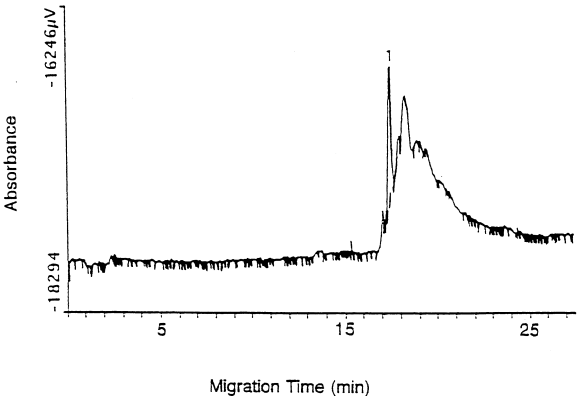


Fig. 7. CZE electropherogram of thermally stressed sample at 60°C for 2 days. All conditions are the same as Fig. 6.

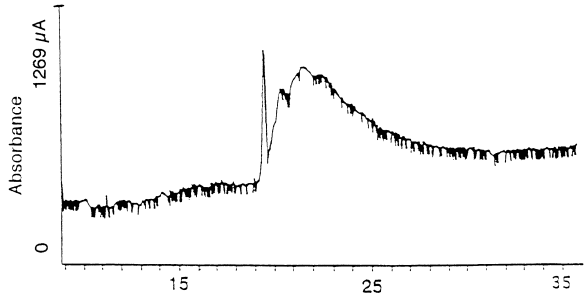


Fig. 8. CZE electropherogram of thermally stressed sample at 60°C for 4 days. All conditions are the same as Fig. 6.

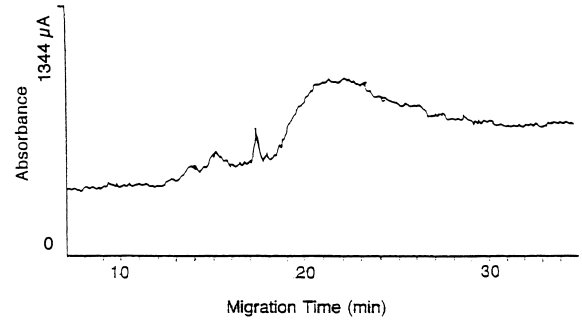


Fig. 9. CZE electropherogram of thermally stressed sample at 60°C for 7 days. All conditions are the same as Fig. 6.

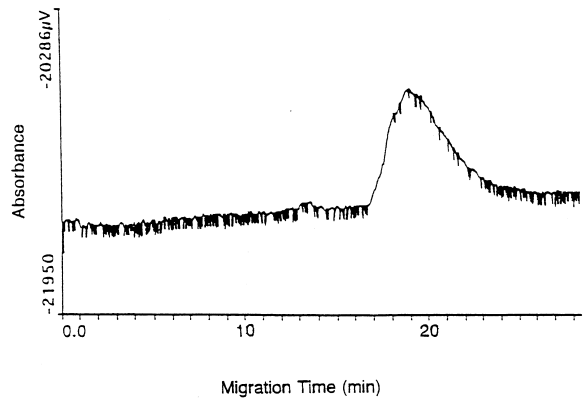


Fig. 10. CZE electropherogram of thermally stressed sample at 40°C for 2 days. All conditions are the same as Fig. 6.

These CZE results showed that there were changes in the antibodies when heated over a period of time. This supported the results by cIEF. The resolution of the peaks could be improved by using a longer capillary.

4. Conclusion

Data from both the cIEF and CZE methods support the idea that a rise in temperature changed the conformation of the antibodies. The rise of temperature definitely had more of an effect on the antibodies than the SEC data showed. However, the data only provided evidence there were changes of p*I* values, peak shape and absorbance of the isoforms. One would not be able to determine if the temperature change was causing the antibody to unfold, breaking down into fragments, or if it was actually changing the antibodies into other species. Interfacing CE with MS will help to determine if the increase of temperature is breaking the antibody into fragments. Circular dichroism (CD) can be helpful in determining any change in the protein functional groups or secondary structure for the antibody due to a period of heating.

These studies have proven that both cIEF and CZE can be effective tools in monitoring the change in antibodies. They can show changes in antibodies which HPLC might not be able to detect. The drawback of the cIEF method in the analyses of antibodies was that the isoforms would give different p*I* values and lead to complicated cIEF patterns. At the same time, with the more information that cIEF provides, any changes in the antibodies could be easily detected.

Since the antibody activity towards its antigen is highly specified by its protein conformation, any change in the conformation could decrease its activity towards the antigen. To determine the effect of rising temperature on the activity of the antibodies, one would have to apply affinity CE.

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