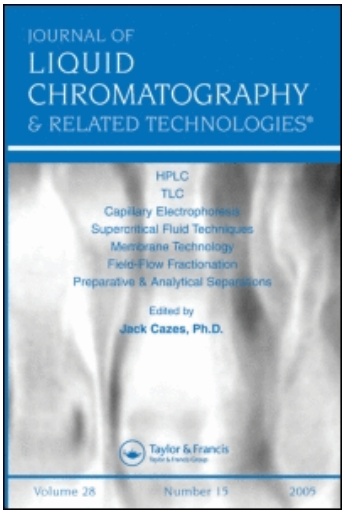

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CHARACTERIZATION OF HUMANIZED ANTI-TAC MONOCLONAL ANTIBODY BY TRADITIONAL SEPARATION TECHNIQUES AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

Humanized anti-Tac monoclonal antibody has been characterized by both SDS-PAGE and isoelectric focusing using traditional polyacrylamide methods. In addition to these techniques, capillary electrophoresis has been adapted to isoelectric focusing in a liquid phase. The humanized antibody has a pI in the range of 8.2-9.0, a region in which precast polyacrylamide or agarose gels are not stable in the 7-10 pH range. Experiments have been conducted to generate gradients in the 3-10 pH range and these gradients have successfully separated differently charged subspecies of humanized anti-TAC. Because of the simplicity in generating a variety of gradients, the power of resolution in different modes, its high efficiency, and the minimum sample volume requirements, capillary electrophoresis may become the method of choice for the routine characterization of therapeutic monoclonal antibodies and their stability profiles.

INTRODUCTION

Therapeutic monoclonal antibodies are gaining great importance in the new generation of pharmaceutical drugs targeted for human and animal consumption (1). Therefore, in the manufacture of monoclonal antibodies the purity of the final product is of primary importance. Protein impurities have the potential to effect the biochemical quality of the drug and may result in detrimental effects to the patient. In order to facilitate research and development, process monitoring, and quality control of monoclonal antibodies, a battery of analytical methods for protein characterization is required. To date, a variety of methods are routinely used for purity testing and stability of monoclonal antibodies, including various chromatographic procedures, electrophoretic methods and bioassays (2-5). Although these methods have received wide acceptance in the pharmaceutical industry over many years, several limitations still need to be overcome.

The utility of capillary electrophoresis (CE)[1] for the analysis of immunoglobulins has been previously demonstrated (6-13). In this work, we examined CE as an alternative analytical method to separate and quantitate monoclonal antibodies, using free-zone and isoelectric focusing modes. As a model system we have used a humanized antibody, termed anti-TAC. Anti-TAC is an IgG1 class genetically-engineered hybrid antibody, containing approximately 90% human sequence and 10% murine sequence (14-16). The antibody is directed against, and is specifically recognized by, the human receptor for interleukin-2, which is a well characterized lymphokine involved in the complex network of cellular communications (17-22). Another characteristic of humanized anti-TAC is that it contains isoforms differing only slightly in their migratory pattern in traditional isoelectric focusing gels. Changes in the pattern due to deamidation or deglycosylation are readily discernible in the slab isoelectric focusing gels. Our objective is to eventually quantitate the degree of change using a gradient in the 7-10 pH range to permit more effective measurement of a shift in pI.

[1]Abbreviations used here: CE, capillary electrophoresis; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; tris, tris(hydroxymethyl)aminomethane; TEMED, N, N, N', N'-tetramethylenediamine; bis, N,N'-methylene-bisacrylamide; pI, isoelectric point; kV, kilovolts; mA, milliamps; W, watts.

EXPERIMENTAL

Reagents and samples

All chemicals were obtained at the highest purity level available from the manufacturer, and were used without additional purification. N-Decane and sulfosalicylic acid were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Sodium hydroxide, borax ($Na_2B_4O_7 \cdot 10H_2O$), γ-methacryloxypropyltrimethoxysilane (Bind-Silane) and lithium chloride were obtained from Sigma Chemical Company (St. Louis, MO). Acetone (HPLC grade), trichloroacetic acid, and hydrochloric acid solution (12 M) were purchased from Fisher Scientific (Fair Lawn, NJ). Broad range pI standards 3-10, LKB ampholine PAG (polyacrylamide gel) plates (pH 3.5-10.0) were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Acrylamide, ammonium persulfate, TEMED, tris, bis, glycine, bromophenol blue, Coomassie brilliant blue R-250, sodium dodecyl sulfate, 2-mercaptoethanol, Bio-Lyte 3/10 (2% in water), phosphoric acid (20 mM), sodium hydroxide solution (20 mM), and sodium hydroxide (20 mM)-sodium chloride (80 mM) solutions were purchased from Bio-Rad Laboratories (Richmond, CA).

Humanized monoclonal antibodies were supplied by Hoffmann-La Roche Inc. (Nutley, NJ). Reagent solutions and buffers were prepared using triply distilled and deionized water, and routinely degassed and sonicated under vacuum after filtration.

Millex disposable filter units (0.22 μm) were purchased from Millipore Corporation (Bedford, MA), and fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX), and Polymicro Technologies (Phoenix, AZ).

Instrumentation

A commercially available CE instrument (P/ACE System 2000, Beckman Instruments, Palo Alto, CA) was used for this work. Samples were stored in a microapplication vessel assembly, consisting of a 150 μl conical microvial inserted into a standard 4 ml-glass reservoir and held in position for injection by an adjustable spring. In order to minimize evaporation of the sample volume (100 μl), about 1-2 ml of cool water was added to the microapplication vessel housing the microvial. The external water serves as a cooling bath for the sample in the microvial, and as a source of humidity to prevent sample evaporation and concentration. After insertion of the microvial, the microapplication vessel assembly was covered with a rubber injection septum and placed into the sample compartment of the CE instrument. Samples were injected into the capillary column by pressure. Peak visualization and data acquisition were

performed using the UV detection system of the CE-instrument and the System Gold Chromatography Software Package (Beckman Instruments, San Ramon, CA). Data integration was also carried out with a model D-2500 Chromato-Integrator (Hitachi Instruments, Inc., Danbury, CT).

Procedure

Free-zone capillary electrophoresis

Humanized anti-TAC samples for analysis in microapplication vessels were placed into the sample holder of the analyzer. The analysis program was initiated and the first sample automatically injected into the capillary by positive nitrogen pressure (0.5 psi for 4 seconds). At the completion of each run, the capillary column was sequentially washed by injection of 2.0 N sodium hydroxide solution, 0.1 N sodium hydroxide solution, distilled-deionized water, and then regenerated with running buffer.

The CE separations reported were performed using 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl. The CE instrument was equipped with a 57 cm (50 cm to the detector) x 75 μm I.D. capillary column. The CE separation was performed at 18 kV. Capillary temperature was maintained at 25°C during the run. Under these conditions, approximately 24 nl (6 nl/sec) of a bulk solution of humanized anti-TAC monoclonal antibody (6 mg/ml) was injected into the capillary column (23). Monitoring of the analytes was performed at a wavelength of 214 nm.

Polyacrylamide slab gel electrophoresis

Humanized anti-TAC was analyzed by SDS-PAGE, under reduced and nonreduced conditions, according to the method of King and Laemmli (24). Samples at a concentration of 1 mg/ml were combined 1:1 (v/v) with the appropriate denaturing sample buffer and heated in boiling water for three minutes. Nonreduced samples were electrophoresed in a 8.7% resolving, 4% stacking gel. Reduced samples, containing a 5% (v/v) final concentration of 2-mercaptoethanol, were electrophoresed on a 12.5% resolving, 4% stacking gel. The concentrations of protein used (for reduced and nonreduced conditions) were 10, 15, and 25 μg/well. The protein bands were visualized by staining with Coomassie brilliant blue R, and subsequently destained with acetic acid:methanol:water (7.5%:15%:77.5%) (v/v). The relative percentage represented by each band was quantitated using a Model 300A Laser Computer Densitometer (Molecular Dynamics, Sunnyvale, CA).

Isoelectric focusing in slab gels

Samples of humanized anti-TAC were analyzed by isoelectric focusing in slab gels according to procedures supplied with Pharmacia-LKB PAG plates, pH 3-10 using an LKB flatbed Multiphor II electrophoresis apparatus. Samples at a concentration of 15 to 20 μg were applied approximately 1.5 inches from the cathodic buffer strip. The protein was placed on the gels using rubber wells rather than on paper strips to avoid adsorption to the paper applicator. Focusing took place over a period of 90 minutes at settings of 1.5 kV, 50 mA and 30 W. The pI range of humanized anti-TAC was derived from a linear curve generated by standards focused in the same gel (Pharmacia-LKB broad range pI standards, 3-10).

Isoelectric focusing in capillary electrophoresis

Humanized anti-TAC monoclonal antibody samples were analysed by capillary electrophoresis, in the isoelectric focusing mode, according to a modification of the method of Kilár and Hjertén (25) as described by Zhu ***et al.*** (11). In this method, a pH gradient was created by means of carrier ampholytes when voltage was applied, and the proteins were then separated according to their isoelectric points. However, in order to eliminate electroendosmotic flow, the interior surface of the capillary was coated with non-crosslinked acrylamide by the method of Kilár and Hjertén (25). Several applications (9-11,26,27) and some modifications of the method have been described (28).

The capillary column (37 cm x 30 cm x 75 μm I.D.) containing a preformed window was placed into a cassette-cartridge (Beckman). The capillary was aligned and fixed, under a microscope, before subjected to chemical coating of the internal surface. The coating of the capillary was carried out as described elsewhere (25) and as depicted in Figure 1. After intensive rinsing of the capillary with deionized water, a 0.5% solution of γ-methacryloxypropyltrimethoxysilane (Bind-Silane) in 50% acetone was introduced into the column by using controlled aspiration generated by a peristaltic pump. After 1 hr the solution was removed and replaced by freshly-prepared non-crosslinked acrylamide reagent. The acrylamide reagent consisted of 4% (w/v) acrylamide solution containing 0.4 μl TEMED and 0.5 mg ammonium persulfate per ml solution. After 10 min (or before any polymerization occurs) the capillary column was extensively rinsed with water (to remove excess of non-attached polyacrylamide reagent) and dried by aspiration.

When the process of coating was finished, the cassette-cartridge device containing the coated capillary was placed into the CE P/ACE instrument. The monoclonal antibody sample, placed into a

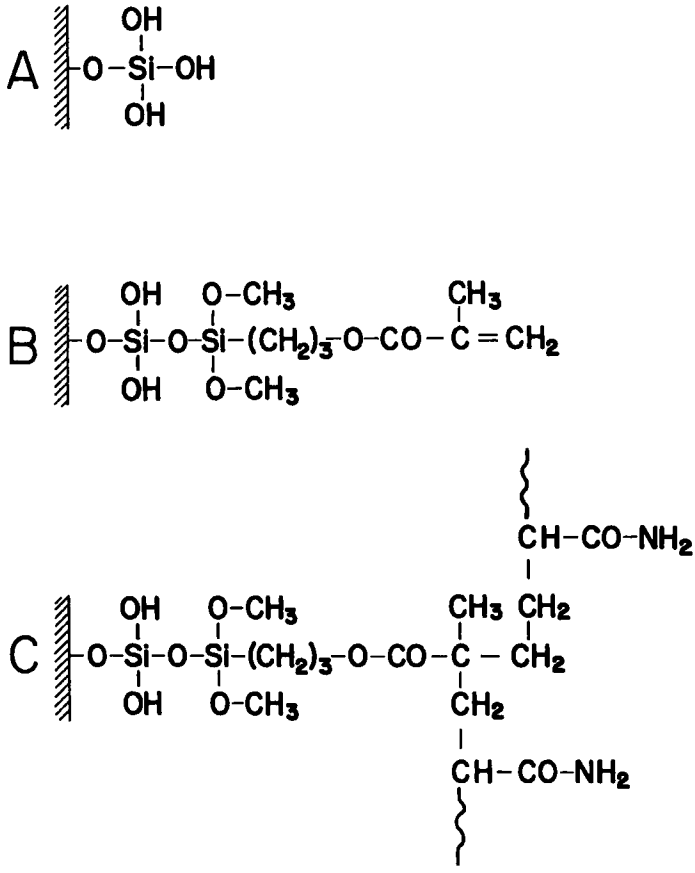

FIGURE 1. A schematic representation of the various chemical reactions necessary for the covalent crosslink of polyacrylamide to the surface of the fused-silica capillary column.

microapplication vessel, was then applied into the column. Approximately 50 µl of 6 mg/ml of humanized anti-TAC were mixed with 150 µl of the carrier ampholytes (Bio-Lyte 3/10). An aliquot of this antibody-ampholytes mixture was injected into the capillary column by pressure for 10 min to ensure complete filling of the

capillary[2] (the rest of the solution seems to be stable and can be reused after a few hours). At this time, the process of isoelectric focusing was performed in two stages (see Figure 2). The first stage consisted of a prefocusing step, and was carried out by placing a solution of 20 mM of H_3PO_4 in the anolyte (positive end), and a solution of 20 mM NaOH in the catholyte (negative end). The monoclonal antibody, which was dispersed along the column, was prefocused at 6 kV for approximately 10 min. The current decreased during this step. The second stage of the isoelectric focusing consisted of a mobilization step of the focused protein zones. This step was started by replacing the 20 mM NaOH solution in the catholyte with a solution of 20 mM NaOH containing 80 mM NaCl (cathodic mobilization). When cathodic mobilization is initiated by addition of chloride ions into the capillary, a reduction in the hydroxyl ion concentration at the cathodic end of the capillary occurs. As the pH drops, focused proteins and carrier ampholytes acquire charge and thus are mobilized towards the cathode (29). This step was performed at 8 kV for approximately 40 min. A small increase in current occurs during mobilization. The migration of the protein zones was monitored at 280 nm. Temperature was maintained at 25°C during the entire process of isoelectric focusing. To assure reproducibility, cleaning of the capillary was carried out after every injection with 0.02 M sodium phosphate buffer, pH 2.3, followed by a water rinse.

RESULTS

The electrophoresis profile (SDS-PAGE) of anti-TAC, analyzed with and without reducing conditions, are shown in Figure 3. Three different concentrations of protein were tested (10, 15, and 25 μg/well). At the lowest concentration of protein (Figure 3, lane 1) a minor band was observed in addition to the main band of protein. The minor band also increased with increasing concentration of protein (Figure 3, lanes 2 and 3). At present, the nature of the separation achieved and the identity of the second band are currently being investigated. Under reducing conditions a minor band is also observed in addition to the two major protein bands, heavy and light chains (Figure 3, lanes 4-6).

[2]The capillary column (37 cm x 75 μm I.D.) has a total internal volume of approximately 1.63 μl. Since 6 nl/sec are injected under these experimental conditions, it takes about 4.5 min to completely fill the capillary.

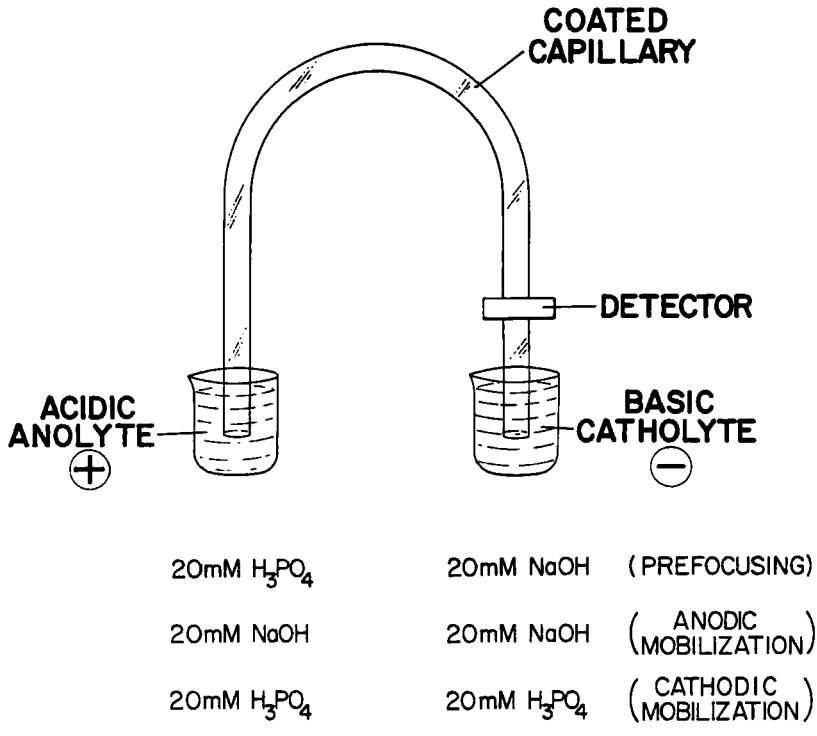

FIGURE 2. A schematic representation of the principle of capillary electrophoresis in the isoelectric focusing mode as proposed by Kilár and Hjertén (25). However, in this report the cathodic mobilization was performed as a modification of the method described by Zhu ***et al.*** (11). A solution of 20 mM of H_3PO_4 was used as anolyte and 20 mM NaOH containing 80 mM NaCl was used as catholyte.

As shown in Figure 4, capillary electrophoresis profile produced a single sharp peak under the concentration of sample tested (6 mg/ml). However, at higher concentrations of protein it was possible to observe an additional band in minute quantities (data not shown). By determination of peak area, both electrophoretic profiles (SDS-PAGE under nonreducing conditions, and capillary electrophoresis) produced more than 98% purity (data not shown). However, when the native antibody protein was subjected to flat gel isoelectric focusing, five distinct bands were observed (Figure 5,

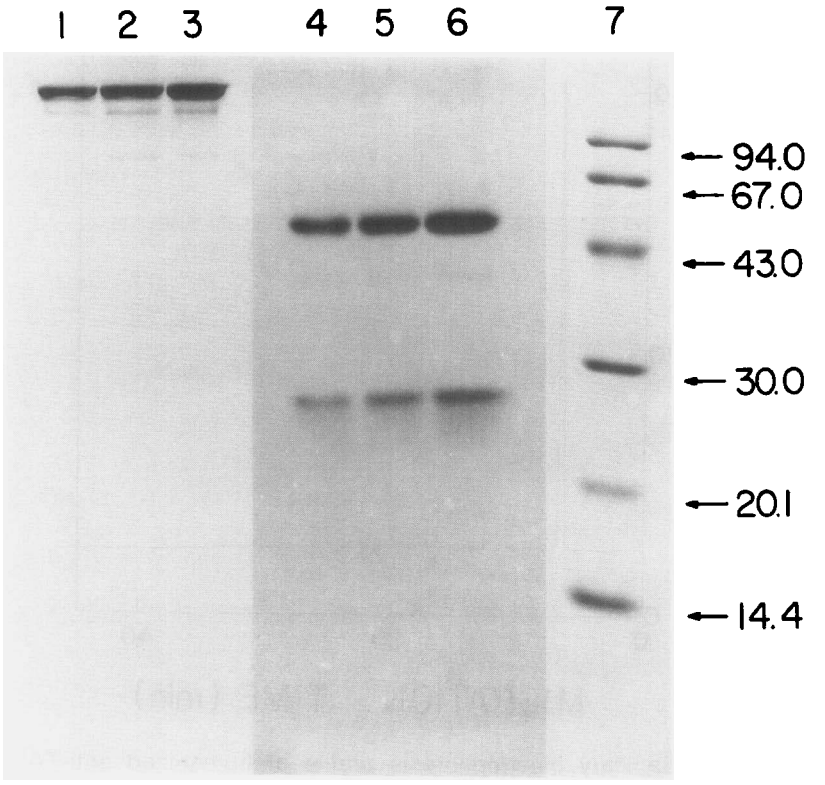

FIGURE 3. Polyacrylamide slab gel electrophoresis in SDS of humanized anti-TAC monoclonal antibody. **Lanes 1, 2, and 3:** 10, 20 and 40 μg of antibody electrophoresed under nonreducing conditions. **Lanes 4, 5, and 6:** the same quantities of antibody electrophoresed under reducing conditions. **Lane 7:** molecular weight standards, expressed as kd.

lane 1). These separated protein bands migrated in a region corresponding to a pI range of 8.2-9.0. It was possible to obtain quantitative data expressed as peak areas, using a laser computing densitometer, of which bands 2, 3, and 4 have the highest peak areas (Figure 6, and Table I). Similarly, capillary electrophoresis in the isoelectric focusing mode yields a qualitative profile of 5 peaks (Figure 7), somewhat different to the quantitative profile obtained in Figure 6 (see Table I).

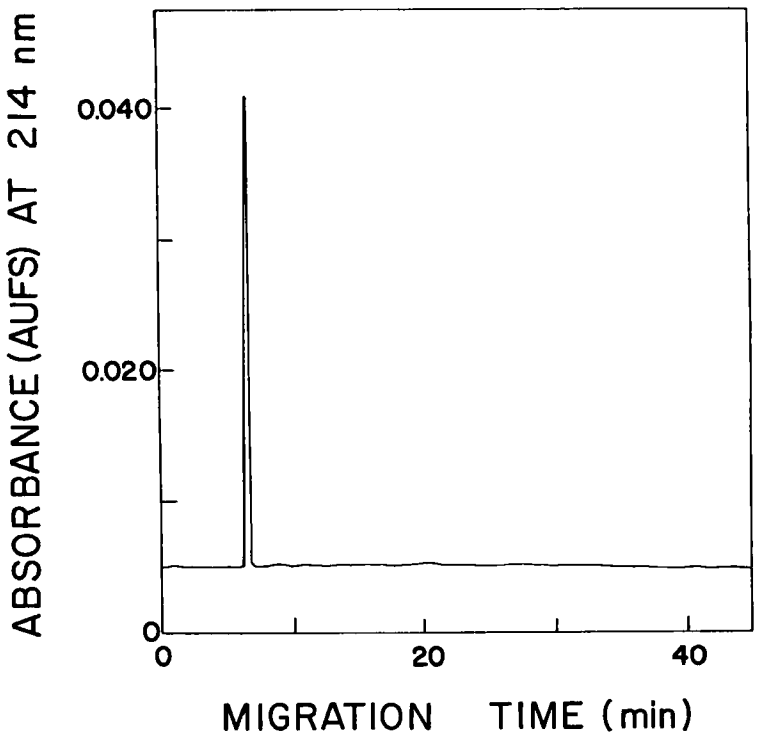

FIGURE 4. Capillary electrophoresis profile of humanized anti-TAC monoclonal antibody.

DISCUSSION

Monoclonal antibodies play several roles in the pharmaceutical industry. For example, they serve as purification (affinity matrices), diagnostic and therapeutic agents. As therapeutic agents, monoclonal antibodies offer an exciting potential for the treatment of a variety of life-threatening diseases (1). These protein drugs will most often be administered intravenously. Thus, safety considerations arise that must be addressed by the development of new technology.

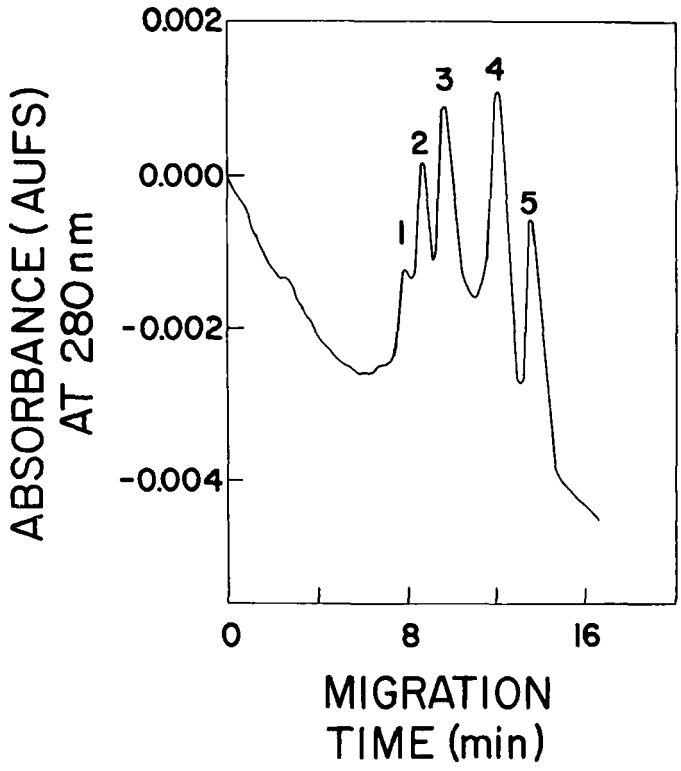

FIGURE 5. Isoelectric focusing profile of humanized anti-TAC monoclonal antibody in slab gels. **Lane 1:** monoclonal antibody. **Lane 2:** isoelectric focusing markers, expressed as pI.

Therapeutic monoclonal antibodies are routinely produced in mammalian hybridoma cells or recombinantly-modified mammalian cells and then secreted into the growth medium. The growth medium generally contains protein supplements and the cell itself produces proteins for secretion, in addition to the desired monoclonal antibody. Throughout the synthesis and secretion process, the cell may also modify the protein molecule (i.e., via glycosylation, phosphorylation, etc.), or some artifacts or variants (degradant) of the recombinant product may be formed (i.e., translation errors, improper folding, premature termination, incomplete or incorrect post-

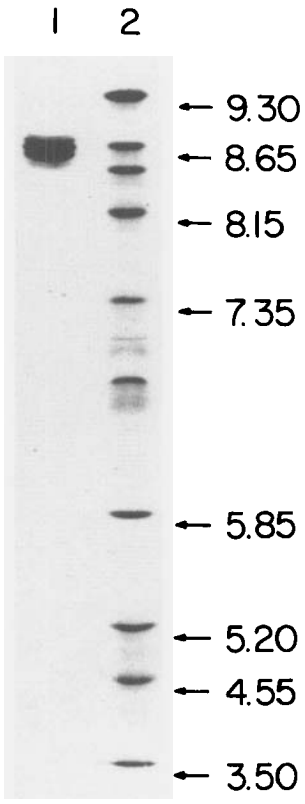

FIGURE 6. Staining intensity peak areas profile of isoelectric focusing electrophoresis in slab gels of humanized anti-TAC monoclonal antibody. Linear densitometry was obtained from the Coomassie brilliant blue stained bands (insert) by using a computing densitometer. Number 1 represents the more basic protein zone and number 5 the more acidic. Numerical values are presented in Table I.

translational modification, chemical or proteolytic modification, and protein aggregates). All these modifications may contribute to the production of polypeptide species with structure similar to the desired native antibody. Additionally, these modifications by themselves or in association with extraneous molecules from the growth medium or cell, or pharmaceutical formulation excipients, may provoke an antigenic response in the drug recipient. Therefore, a high-

TABLE I. COMPARATIVE PEAK AREA VALUES BETWEEN TRADITIONAL SLAB GEL ISOELECTRIC FOCUSING AND CAPILLARY ELECTROPHORESIS ISOELECTRIC FOCUSING

PEAK NUMBER	PEAK AREA (percentage of total)	
	Slab Gel	Capillary Electrophoresis
1	6.5	5.5
2	26.6	15.3
3	45.4	29.5
4	17.3	33.1
5	4.2	16.6

Peak areas for slab gel isoelectric focusing were obtained from the profile described in Figure 6 (Laser Computer Densitometer). Peak areas for capillary electrophoresis isoelectric focusing were obtained from the profile described in Figure 7 (System Gold Chromatography Software Package).

resolution method such as capillary electrophoresis would be helpful for monitoring biosynthetic fidelity and assessing high levels of protein purity (detecting minute structural changes) during the production of therapeutic monoclonal antibodies.

The analytical methods should be adaptable over a wide range of conditions. The protein molecule of interest must be examined throughout the production process, from low purity in the harvested growth medium to greater than 95% purity in formulated products. Examination of the consistency and stability of the protein produced from lot to lot is required to ensure the safety and efficacy of the protein drug.

In this communication, we demonstrate the importance of electrophoretic analytical techniques as a routine tool for the evaluation of the quality of a protein which exhibits microheterogeneity, as in the case of humanized anti-TAC monoclonal antibody.

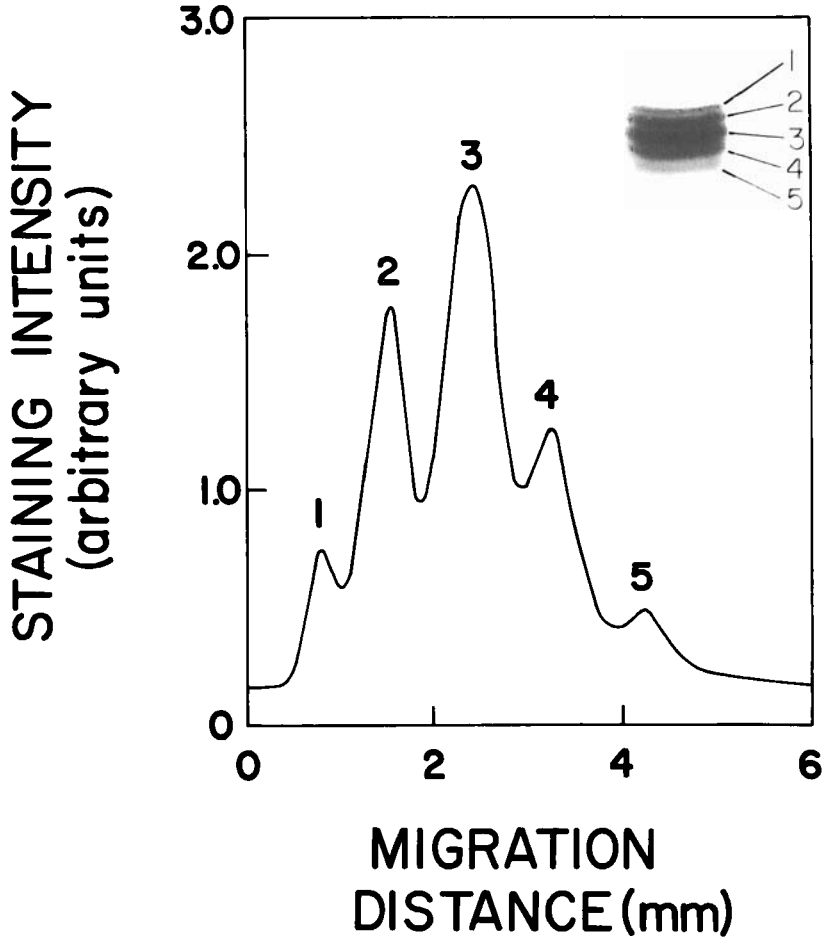

FIGURE 7. Peak areas profile of isoelectric focusing of humanized anti-TAC monoclonal antibody in capillary electrophoresis. Densitometry was obtained directly from the electropherographic profile by using the System Gold Chromatography Sofware Package. Number 1 represents the more basic protein zone and number 5 the more acidic. Numerical values are presented in Table I.

Traditional slab gel electrophoresis and isoelectric focusing, in a polyacrylamide gel matrix, have provided significant information about the purity and charge heterogeneity of monoclonal antibodies (30,31). In particular, isoelectric focusing suggests that several isoforms of a particular antibody exist due to different degrees of modification and/or differential folding patterns. However, the quantification of these individual protein bands remains difficult especially when using a densitometric scanner, which is limited to yielding semi-quantitative information.

The utility of two powerful analytical techniques have been simultaneously assessed in this report for the analysis of humanized monoclonal anti-TAC. Capillary electrophoresis isoelectric focusing (CE-IEF) and slab gel isoelectric focusing (SG-IEF) patterns each contain at least five partially resolved bands. Slight differences are observed in the resulting data when comparing the two techniques, which may be due to the measurement of a relative concentration of the protein antibody at a wavelength of 280 nm (CE-IEF) versus staining with an organic dye and densitometric scanning (SG-IEF), or some other technical aspects currently being investigated. In fact, it is well known that the exact quantification of the components present in complex biological material is one of the most difficult tasks in protein chemistry, especially when staining the proteins with organic dyes (32). Although it is quite easy to measure stained protein bands with a densitometer, it is more difficult to evaluate the measured absorbance and to obtain reliable quantitative data reflecting the amount of the protein actually present. Capillary electrophoresis may be the alternative method to quantitate heterogeneous proteins, such as monoclonal antibodies.

Recently, a similar electrophoresis profile was obtained for a monoclonal antibody used as an imaging agent for the diagnosis of certain types of cancer (33). The separation of the antibody protein bands, however, were achieved in the pH range of 6.0-6.5. Interestingly, a preparation of the antibody which has been stored over time contained an additional protein band when compared to a more recent lot of antibody. Although the authors did not speculate about the nature of the additional protein band, it is possible that a process of deamidation or other chemical changes generated a form of the antibody during storage. Capillary electrophoresis in the isoelectric focusing mode may become a routine procedure for the determination of stability of a protein in quality control laboratories as suggested by Shields and Silverman (33).

In conclusion, we have succeeded in obtaining the capillary electrophoresis profile of a humanized monoclonal antibody. Although experiments are underway to improve the technical aspects

of this CE mode, we have demonstrated that capillary electrophoresis is a useful analytical tool for the characterization of antibodies. In addition, capillary electrophoresis may have some potential to generate the actual quantification of the various heterogeneous components of a monoclonal antibody.

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