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Capillary isoelectric focusing and sodium dodecyl sulfate–capillary gel electrophoresis of recombinant humanized monoclonal antibody HER2

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Abstract

Capillary isoelectric focusing (cIEF) and IEF of recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) show five charged isoforms with estimated p*I* values ranging from 8.6–9.1. The cIEF assay demonstrated good precision with relative standard deviations (R.S.D.) 0.7–3.7% and 0.4–4.2% for intra and interassay analysis, respectively. The method was linear for the area of the main peak over the concentration range 2–250 μg/ml with a Pearson correlation coefficient >0.99. The limit of detection for the main peak was determined to be 2 ppm. With both sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE) and SDS–polyacrylamide gel electrophoresis, the nonreduced rhuMAbHER2 migrated as a single major peak with minor peaks in the aggregate and clip regions. After reduction, the electropherogram and the slab gel showed the expected heavy chain and light chain fragments with minor peaks in the aggregate and clip regions. The SDS–CGE assay showed good precision with R.S.D. values of 0.1–7.8% and 0.1–8.1% for intra and interassay analysis, respectively. The Pearson correlation coefficient for the area of the main peak was >0.99 demonstrating linearity for the concentration range 0.5–500 μg/ml. The limit of detection for intact rhuMAbHER2 was determined to be 0.5 ppm. The data presented demonstrates the feasibility of replacing the slab gel techniques with capillary electrophoresis in a quality control environment.

Keywords: Monoclonal antibodies

1. Introduction

Recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) is an antibody directed against the human epidermal growth factor-2 p185 gene product $p185^{HER2}$ [1]. This gene product is greatly over expressed in a subset of certain cancers (such as breast cancer) and is closely correlated with poor prognosis [1]. rhuMAbHER2 consists of two 214-residue light chains and two 449-residue or 450-residue heavy chains, with the 449-residue form predominating. Each light chain is attached to a heavy chain by disulfide bonds and interchain disulfide bonds attach the heavy chains to each other. One conserved Asn-linked glycosylation site is found within the constant region of each heavy chain. rhuMAbHER2 is a non-sialic acid containing glycoprotein subject to charged heterogeneity resulting from C-terminal clipping and deamidation [2,3].

Capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE) were evaluated for the quantitative analysis of rhuMAbHER2 and the relative merit of replacing the

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conventional slab gel techniques. IEF and SDS–polyacrylamide gel electrophoresis (PAGE) are important techniques for the characterization and quality control of proteins in biotechnology and are routinely used to evaluate identity, purity, lot to lot consistency, and stability of a protein product [4]. Traditionally, these techniques have been performed using slab gel technology with detection by means of silver or Coomassie Blue staining. While good separations can be obtained with these methods, they suffer from several limitations which have been well described [5]. Because of demonstrated correlation with the slab gel techniques [6–10,15], high-performance capillary electrophoresis with its automated and quantitative features demonstrates great potential for replacing its more lengthy and labor intensive slab gel counterparts. Previous work has shown the feasibility of using cIEF and SDS–CGE with monoclonal antibodies [10–15]. In this report we demonstrate that these methods are rugged and have adequate sensitivity, precision and linearity for use in a quality control environment.

2. Experimental

2.1. Materials

All chemicals used were of analytical reagent grade. Hydroxypropylmethylcellulose (HPMC), β-mercaptoethanol, Pharmalytes 8-10.5 and Ampholines 3.5-9.5 were purchased from Sigma (St. Louis, MO, USA). p*I* markers for IEF were purchased from Pharmacia (Piscataway, NJ, USA). Bio-Lyte 3-10 and 7-9 ampholytes, cathodic mobilizer, N,N,N′,N′-Tetramethylethylenediamine (TEMED), SDS-sample buffer, SDS-running buffer, and broad range molecular mass standards were purchased from Bio-Rad (Hercules, CA, USA). Synthetic p*I* markers for cIEF were a gift from Bio-Rad. rhuMAbHER2 was manufactured in-house.

2.2. cIEF

cIEF was performed using a Bio-Rad BioFocus 3000 capillary electrophoresis (CE) System with a BioCAP LPA capillary, 50 μm I.D., 24 cm total length and 19.5 cm to the detector (Bio-Rad). The ampholyte solution (2% w/v) consisted of a 8:1:1 ratio of Pharmalyte 8-10.5, Bio-Lyte 7-9, Bio-Lyte 3-10, respectively, containing 0.5% (v/v) TEMED and 0.2% (w/v) HPMC. Samples were prepared by adding 100 μl of rhuMAbHER2 (0.25 mg/ml) to 100 μl of the ampholyte solution, this solution was briefly mixed and centrifuged for 10 s at 5000 *g*. Capillaries were rinsed with purified water for 90 s prior to each injection. Sample plus ampholytes were injected by applying pressure (40 s at 100 p.s.i; 1 p.s.i.=6894.76 Pa). Focusing was performed at 625 V/cm, constant voltage, for 5 min using 20 m*M* phosphoric acid and 40 m*M* sodium hydroxide as the anolyte and catholyte, respectively. Chemical mobilization was carried out at 625 V/cm, constant voltage, for another 17 min. Capillary and sample temperature were maintained at 20°C.

2.3. IEF

IEF gels containing 4% (w/v) acrylamide, 0.5% (v/v) Triton X-100, 0.2% (w/v) pH 3.5–9.5 ampholines, were cast on a GelBond PAG backing (FMC, Rockland, MD, USA). The anode and cathode solutions were 1 *M* H_3PO_4 and 1 *M* NaOH, respectively. Samples and p*I* markers were applied to sample wicks approximately 2 cm from the anode. Focusing proceeded for 1 h with 10 W, 25 mA, and 1200 V limits at 4°C. The sample wicks were then removed and focusing continued for another hour. The focused gels were fixed, stained with Coomassie brilliant blue, destained and dried between two sheets of dialysis membrane soaked in 2.5% (v/v) glycerol.

2.4. SDS–CGE

SDS–CGE was performed on a Bio-Rad BioFocus 3000 CE system. The capillary was fused-silica with a 75 μm I.D., 24 cm total length and 19.5 cm to the detector (Polymicro, Phoenix, AZ, USA). rhuMAbHER2 was diluted to approximately 1 mg/ml and 15 m*M* ionic strength with purified water and analyzed in both the nonreduced and reduced form. The nonreduced sample contained equal volumes of rhuMAbHER2 and the CE-SDS sample buffer. The reduced sample contained 95 μl of rhuMAbHER2, 100 μl of CE-SDS sample buffer and 5 μl of

β-mercaptoethanol. The capillary was rinsed with 0.1 *M* NaOH, 0.1 *M* HCl and SDS-run buffer, for 90, 60, and 120 s respectively, prior to each injection. Samples were injected using electrophoretic injection (40 s at 10 kV). Separation was performed at 333 V/cm, constant voltage, for 25 min. Capillary and sample temperature were maintained at 20°C.

2.5. SDS–PAGE

SDS–PAGE was performed according to the procedure of Laemmli [16]. Aliquots containing 20 μg of rhuMAbHER2 or molecular mass standards were analyzed using a 4% acrylamide stacking gel and a 4–20% acrylamide gradient resolving gel. Electrophoresis proceeded at 20 mA for approximately 3 h. The protein bands were visualized with an Oakley silver stain [17].

3. Results

3.1. cIEF and IEF

In our labs, IEF is used primarily to ensure the identity, consistency and stability of a protein as demonstrated by the correct p*I*, a profile which is consistent with a reference material and the absence of new or more intense bands, respectively. The use of IEF stained with Coomassie Brilliant Blue and quantitated by densitometry, at best provides a somewhat linear, semi-quantitative approach to monitor the charged isoforms of rhuMAbHER2. cIEF, on the other hand, offers a fast means of online detection for monitoring the distribution (i.e. peak area percent) of charged isoforms and calculation of p*I*. A comparison of the results of IEF and cIEF analysis of rhuMAbHER2 is shown in Fig. 1. This figure demonstrates a correlation between the number and relative intensity of the IEF bands and the cIEF peaks, each peak or band representing a charged isoform of rhuMAbHER2. In addition, by interpolation of the rhuMAbHER2 migration times using synthetic standards, the isoelectric points of the major species agree well with the p*I* range determined for the same sample run on the slab gel (8.6–9.1). The cIEF analysis of rhuMAbHER2 at two storage temperatures, 5°C and 37°C, held for 27

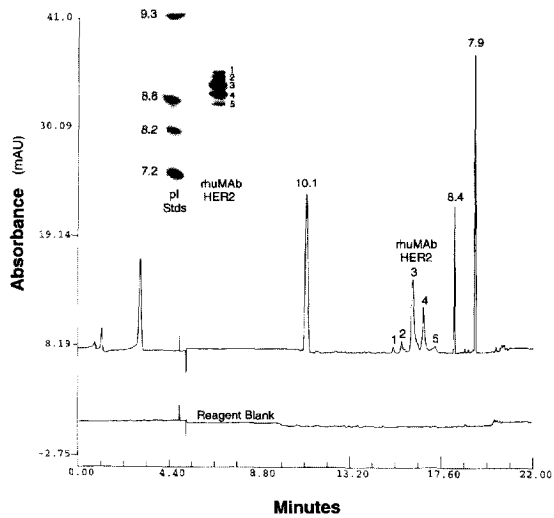

Fig. 1. IEF (insert) and cIEF of rhuMAbHER2. cIEF of rhuMAbHER2 is shown with p*I* markers (top) and reagent blank (bottom). Analysis conditions were as described in Section 2.

days is shown in Fig. 2A. There is a slight decrease in peak area percent for peaks 1–3, the more basic peaks, and a slight increase for peaks 4 and 5, the more acidic peaks. This indicates that some of the peaks may be affected by storage at elevated temperature and the assay may be stability indicating, as such a shift is consistent with protein deamidation. The limit of detection (LOD) for the cIEF method was determined to be 2 ppm (μg/ml), Fig. 2B. Sensitivity may be increased further by using a longer capillary which results in a larger sample load. The Pearson correlation coefficient for the area of the main peak (peak 3) was >0.99 demonstrating assay linearity for the concentration range 2–250 μg/ml. Intra-assay precision was demonstrated using five replicate injections of a single sample (Table 1). The relative standard deviations (R.S.D.) of migration time ranged from 0.7–0.9%, for peak area R.S.D. values ranged from 0.8–3.0%, and for peak area percent R.S.D. values ranged from 1.0–3.7%. The interassay precision was determined using five replicate injections of three different preparations of a sample run on three separate days (Table 1). The R.S.D. values for migration time ranged from 0.4–0.6%, for peak area R.S.D. values ranged from 1.2–3.2%, and for peak area percent R.S.D. values ranged from 1.1–4.2%. Fresh reagents were used

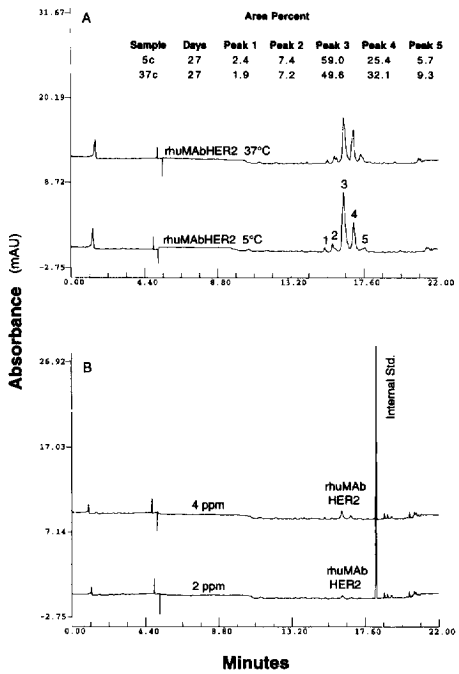

Fig. 2. (A) cIEF analysis of rhuMAbHER2 held at 5°C or 37°C, for 27 days, The table (insert) shows the changes in peak area percent due to storage at 37°C. (B) cIEF analysis of rhuMAbHER2 at concentrations of 4 and 2 μg/ml.

each day but the capillary remained the same. The above R.S.D. values are considered within acceptable limits for a quality control assay.

3.2. SDS–CGE and SDS–PAGE

SDS–PAGE visualized with silver stain is a highly sensitive method used to monitor the purity, consistency and stability of a protein as demonstrated by a profile which is consistent with a reference material and by the absence of new or more intense bands. The main disadvantage of this method is that it is generally nonquantifiable and variations have been observed in binding of silver from protein to protein and gel to gel [4]. SDS–CGE offers fast, direct on-line detection for monitoring the peak profile of a protein as it relates to fragmentation and aggregation. Changes in the peak profile are monitored and reported as peak area percent for the peaks of interest. An SDS–CGE method was compared to the traditional slab gel SDS–PAGE method for evaluation of the purity, consistency and percent distribution of rhuMAbHER2. Seven peaks were detected by SDS–CGE of a nonreduced sample. This correlated well with the seven bands observed for rhuMAbHER2 on SDS–PAGE (Fig. 3). In addition,

Table 1
Intra-assay and interassay precision for rhuMAbHER2 using cIEF

Analyses	Value	Peak				
		1	2	3	4	5
Migration Time[a] *(min)*						
Intra-assay	Mean	15.64	16.05	16.59	17.14	17.69
	R.S.D.	0.9	0.8	0.7	0.8	0.8
Interassay	Mean	15.58	15.99	16.54	17.09	17.64
	R.S.D.	0.5	0.5	0.6	0.5	0.4
Peak Area[a]						
Intra-assay	Mean	42 406	126 229	1 020 284	453 526	94 353
	R.S.D.	3.0	1.9	0.8	3.0	1.6
Interassay	Mean	2 769	7 880	61 181	25 967	5 428
	R.S.D.	1.2	1.3	1.3	3.2	0.8
Area %[a]						
Intra-assay	Mean	2.4	7.3	58.8	26.1	5.4
	R.S.D.	3.7	1.6	1.0	2.6	1.0
Interassay	Mean	2.5	7.3	58.8	25.8	5.6
	R.S.D.	4.2	2.9	1.1	3.4	4.2

[a] Values represent the mean of five replicate injections

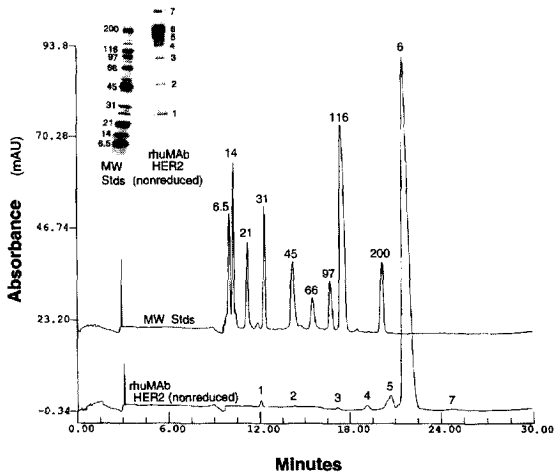

Fig. 3. SDS–PAGE (insert) and SDS–CGE of rhuMAbHER2 under nonreduced conditions (bottom) and molecular mass markers (top). Analysis conditions were as described in Section 2.

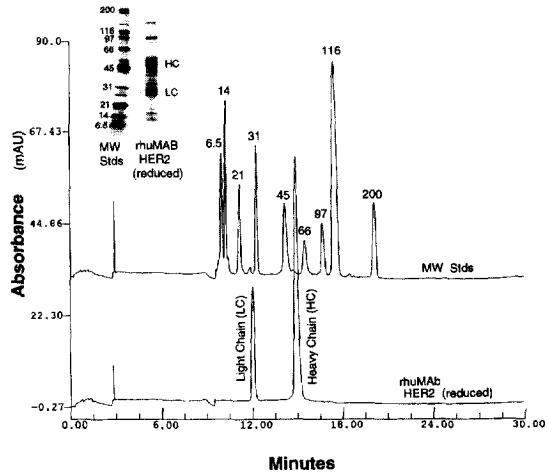

Fig. 4. SDS–PAGE (insert) and SDS–CGE of rhuMAbHER2 under reduced conditions (bottom) and molecular mass markers (top).

the peak area percent attributed to high-molecular-mass aggregates (peak 7) seen using SDS–CGE of nonreduced samples is consistent with that found with size-exclusion chromatography using a SDS containing mobile phase (data not shown). SDS–CGE provided excellent resolution of rhuMAbHER2 light and heavy chains. Silver stained SDS–PAGE was however more sensitive in detecting minor species than the SDS–CGE method for rhuMAbHER2 under reduced conditions (Fig. 4). The SDS–CGE analysis of rhuMAbHER2 at two storage temperatures, 5°C and 37°C, held for 27 days is shown in Fig. 5A. There is a slight increase in peak area percent for the lower-molecular-mass peaks (peaks 1–5) for the sample stored at 37°C. This indicates that the assay can detect changes in fragmentation which may be caused by storage at elevated temperature and hence, could be used for stability indicating purposes. The LOD (Fig. 5B) for peak 6 (intact rhuMAbHER2), under nonreduced conditions, was determined to be 0.5 ppm (μg/ml) or 0.1%. In general, impurities at levels below 0.5% are considered minor impurities and require identification but the toxicologic, pharmacologic, and immunologic profiles need not be obtained [18]. The Pearson correlation coefficient for the peak area of intact rhuMAbHER2 was >0.99 demonstrating linearity for the concentration range 0.5–500 μg/ml.

Intra-assay precision was demonstrated using three replicate injections of a single sample (Table 2). The R.S.D. values of migration time ranged from 0.8–

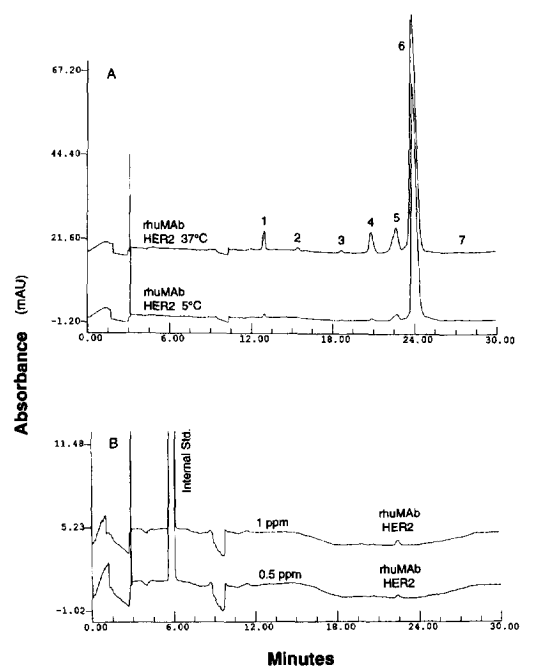

Fig. 5. (A) SDS–CGE of rhuMAbHER2 under nonreduced conditions. The samples have been held at 5°C or 37°C, for 27 days. (B) The limit of detection for intact rhuMAbHER2 (peak 6) was determined to be 0.5 μg/ml.

Table 2
Intra-assay and interassay precision for rhuMAbHER2 using SDS–CGE

Analyses	Value	Peak					
		1	3	4	5	6	7
Migration Time (min)							
Intra-assay[a]	Mean	12.36	17.67	19.67	21.34	22.29	25.91
	R.S.D.	1.1	0.9	0.9	0.8	0.8	1.7
Interassay[b]	Mean	12.32	17.6	19.61	21.28	22.23	25.57
	R.S.D.	0.5	0.5	0.5	0.4	0.3	1.3
Normalized Area[c]							
Intra-assay[a]	Mean	10 860	3118	10 815	39 997	1 118 783	4175
	R.S.D.	6.1	7.1	3.8	7.8	5.3	2.7
Interassay[b]	Mean	10 345	3239	10 330	37 545	1 070 701	4094
	R.S.D.	8.1	3.4	7.2	6.5	4.4	5.9
Normalized Area %[d]							
Intra-assay[a]	Mean	0.91	0.26	0.91	3.36	94.19	0.35
	R.S.D.	4.4	2.2	6.0	3.4	0.1	1.6
Interassay[b]	Mean	0.91	0.28	0.91	3.30	94.23	0.36
	R.S.D.	7.0	7.3	3.9	2.0	0.1	4.8

[a] Values represent the mean of three replicate injections
[b] Values represent the mean of three replicate injections on three separate days
[c] Area/migration time
[d] Area %/migration time

1.7%, for peak area normalized to migration time the R.S.D. values ranged from 2.7–7.8%, and for peak area percent normalized to migration time R.S.D. values ranged from 0.1–6.0%. The interassay precision was determined using three replicate injections of three different sample preparations run on three separate days (Table 2). The R.S.D. values for migration time ranged from 0.3–1.3%, for normalized peak area the R.S.D. values ranged from 3.4–8.1%, and for normalized peak area percent the R.S.D. values ranged from 0.1–7.3%. Fresh reagents were used each day but the capillary remained the same. These values are considered within acceptable levels for a quality control assay.

4. Discussion

One of the first demonstrations of cIEF of monoclonal antibodies was presented by Costello et al. [10]. They compared the antibody under investigation with the traditional slab gel methods and detected five peaks on cIEF which compared to five bands on slab gel IEF. Their advantage for using cIEF was that precast gels were not stable in the pH 7–10 range and the antibody had a p*I* in the range of 8.2–9.0. cIEF of another monoclonal antibody (anti-CEA) was also demonstrated by Huang et al. [19]. The p*I* of this antibody ranged from 6.31–6.83 and these authors also observed five isoforms. The cIEF assay reported here has shown good precision for all parameters (migration time, peak area, and area %) which would be routinely monitored in a quality control system. R.S.D. values for these parameters fell within the range of 0.4–4.2% for both intra and interassay analyses. These observed values for precision compare favorably with values reported in the literature [7,15]. Another parameter which is commonly measured by other laboratories is the isoelectric point of a protein. This value has been shown to be measured reproducibly by others using cIEF [12,19].

When using SDS–CGE for the analysis of antibody molecules, the method appears to be precise and compares favorably to analysis by Coomassie blue stained SDS–PAGE but was less sensitive than silver stain. We reported here intra and interassay precision of migration time that were 1.7% or less, and precision of normalized peak area for smaller peaks between 2.7 and 8.1%. These data compare

favorably with that published for bovine immunoglobulin G (IgG) (2.7% for migration time and 10.3 to 15.1% R.S.D. for normalized peak areas) [13]. The pattern seen for purified antibodies as shown by Kroon et al. [14], indicates that the same number of peaks (i.e. 7) can be detected; one that is larger than the monomer and may represent aggregate and five that are probably IgG fragments, H2L, H2, HL, H, and L. Kroon et al. [14] also showed that for quantitation of peak areas, using a reference peak to normalize area improved precision of area quantitation when using pressure injection. This resulted in 3.1% R.S.D. for a peak (half antibody molecule) that was 5.76% of total area. In the study reported here, electrophoretic injection was used demonstrating excellent precision of migration time and good precision of peak areas. It is noteworthy that the intact rhuMAbHER2 migrates at a position which results in a higher than expected molecular mass determination under nonreduced conditions for both SDS–PAGE and SDS–CGE (Fig. 4). Possible explanations for this anomalous migration have been offered by previous authors [20], who state that basic proteins and glycoproteins tend to have a decreased charge-to-mass ratio when complexed to SDS, resulting in a decreased migration rate and overestimations of molecular mass. Their proposed solution for a better estimation of molecular mass for this class of proteins is to use the conditions for a Ferguson plot. However, as our primary goal is to monitor purity and consistency using estimations of percent distribution (i.e. peak area percent) this discrepancy is not important.

5. Conclusions

The data presented here demonstrates the feasibility of using CE as a replacement for the conventional slab gel techniques used in a quality control environment. A good correlation between the number and relative intensity of bands on slab gels and peaks in CE has been demonstrated. In addition, both cIEF and SDS–CGE separations have been shown to be linear and precise.

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