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Separation and quantitation of monoclonal antibodies in cell growth medium using capillary zone electrophoresis

H.J. Dai, G. Li, I.S. Krull *

Department of Chemistry, Northeastern University, 102 Hurtig Building, 360 North Avenue, Boston, MA 02115, USA

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Abstract

IgG1 is separated from its impurities in cell growth medium under simple CZE conditions without specific sample pretreatment. Linearity, limit of quantitation, limit of detection, precision and accuracy for the method are demonstrated. The quantitation for IgG1 in the cell growth medium is obtained by generating a calibration curve and by using standard additions. This CE method can offer a good alternative to conventional HPLC methods. Attempts are also made to separate the heterogeneous species in monoclonal antibodies using both CZE and MECC.

Keywords: Monoclonal antibodies; Capillary zone electrophoresis; Cell growth medium

1. Introduction

Monoclonal antibodies (Abs) have become of commercial interest in recent years. They have found utility in several therapeutic applications [1]. For example, they can be used to focus inflammatory responses on target cells, as carriers to deliver small molecules to a specific site in vivo, to exert regulatory functions on hormones, as vaccines to generate active immune responses, and they can also be used to alter pharmacokinetics, and biodistributions. Monoclonal Abs have also found applications in immunoassay and immunochemistry studies.

The production and purification of Abs present some challenges. Abs are quite heterogeneous, even for monoclonal Abs. The microheterogeneity in/for monoclonal Abs is found in the Fc (crystalline portion) or the carbohydrate regions. Different numbers and types of carbohydrates can be found in the same type of monoclonal Abs. It is extremely difficult to separate the microheterogeneous species in the same monoclonal antibodies. Since the differences are small, the different species often migrate very closely together under most flat-bed electrophoresis, modern high performance liquid chromatography (HPLC), and high performance capillary electrophoresis (HPCE (CE)) conditions. Even in mass spectrometry

* Corresponding author. Tel.: +1 617 437 4818; fax: +1 617 437 8795.

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(MS), Abs appear as broad, diffuse, and wide peaks with an average molecular weight (MW) of about 150 000 Da.

Analyses of Abs using CE have been reported in numerous papers [2–34]. However, most of these were for the separation of Abs from antibody–antigen conjugates. Such papers rarely dealt with real samples. Also, a large number of these reports involved CIEF [2–15,31,33]. In this study, we explored different conditions in order to separate the heterogeneous species in the IgG1 monoclonal Abs. We also derived a simple approach for the separation and quantitation of a monoclonal Ab in cell growth medium.

2. Experimental

2.1. Chemicals

Sodium tetraborate was purchased from Aldrich (Milwaukee, WI). Sodium phosphate monobasic and sodium acetate were purchased from J.T. Baker (Phillipsburg, NJ). Tricine (*N*-tris(hydroxymethyl)methylglycine), SDS, citric acid and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO). FC surfactants were obtained from J&W Scientific (Folsom, CA). Methanol (MeOH) was obtained from EM Science (Gibbstown, NJ).

Both standard IgG1 and cell growth medium samples were supplied by a pharmaceutical company. Standard IgG1 was obtained in solution with a 5.7 mg/ml concentration.

2.2. Capillary electrophoresis instrumentation

The experiments were performed on a Waters Quanta 4000 CE system. Data were collected with a Macintosh Classic (Apple Computer, Cupertino, CA) via SMADchrom 3.0 interface software (Marc S. Nathanson, Sharon, MA). The data were integrated with the same software. Uncoated capillaries used were either 50 or 75 μm i.d. × 60 cm total length (52 cm effective length), except in quantitation, in which the capillary was 70 cm total length (62.5 cm effective length) (Polymicro Technology, Bloomfield, NJ). FC-coated capillaries were 50 μm i.d. × 60 cm total length (52 cm effective length) (J&W Scientific). The eCAP neutral coated capillary was 50 μm i.d. × 37 cm (30 cm to detector) (Beckman Instruments, Palo Alto, CA). Injections were either hydrostatic or electromigration. The detection was set at 214 nm and the output range was selected at 0.005 AUFS. For the separation of IgG1 in cell growth medium and the quantitation of IgG1, the injections were 10 s at 12.5 kV. The run voltage was 17.5 kV and the current was ~16 μA.

2.3. Reagents

The running buffers consisted of 20 mM borate (pH 9.3), 20 mM borate + various concentrations of SDS (pH 9.4), 10 mM tricine + 50 mM SDS (pH 7.6), and 20 mM tricine + 10% MeOH (pH 8.0) (used in uncoated capillaries), 15 mM borate + 0.005% FC-WA:FC-N (1:2) (pH 9.0), 15 mM borate + 0.005% FC-WA:FC-N (1:3) (pH 9.0), 15 mM borate + 0.005% FC-N (pH 9.0), 15 mM borate + 0.01% FC-pN, 10 mM phosphate + 0.005% FC-WA:FC-N (15:85) (pH 8.9), 10 mM phosphate + 0.005% FC-N (pH 8.9) and 40 mM acetate + 0.02% FC-N (pH 4.6) (for FC-coated capillaries), 20 mM citrate (prepared from citric acid and pH adjusted to 3.0 with sodium hydroxide) (for eCAP neutral coated capillary). All reagents were analytical reagent grade.

2.4. Operating conditions

For uncoated capillaries, the capillary was rinsed with 1 N NaOH at the beginning of each day. Prior to each injection, the capillary was first rinsed with 100 mM NaOH for 2 min, then 3 min with deionized water, and finally 4 min with running buffer, prior to each injection. The temperature was maintained at 27°C for all runs.

2.5. Sample preparation

2.5.1. Separation of heterogeneous species in standard IgG1

The denatured antibody samples were first diluted with equal volumes of 20 mM borate + 100

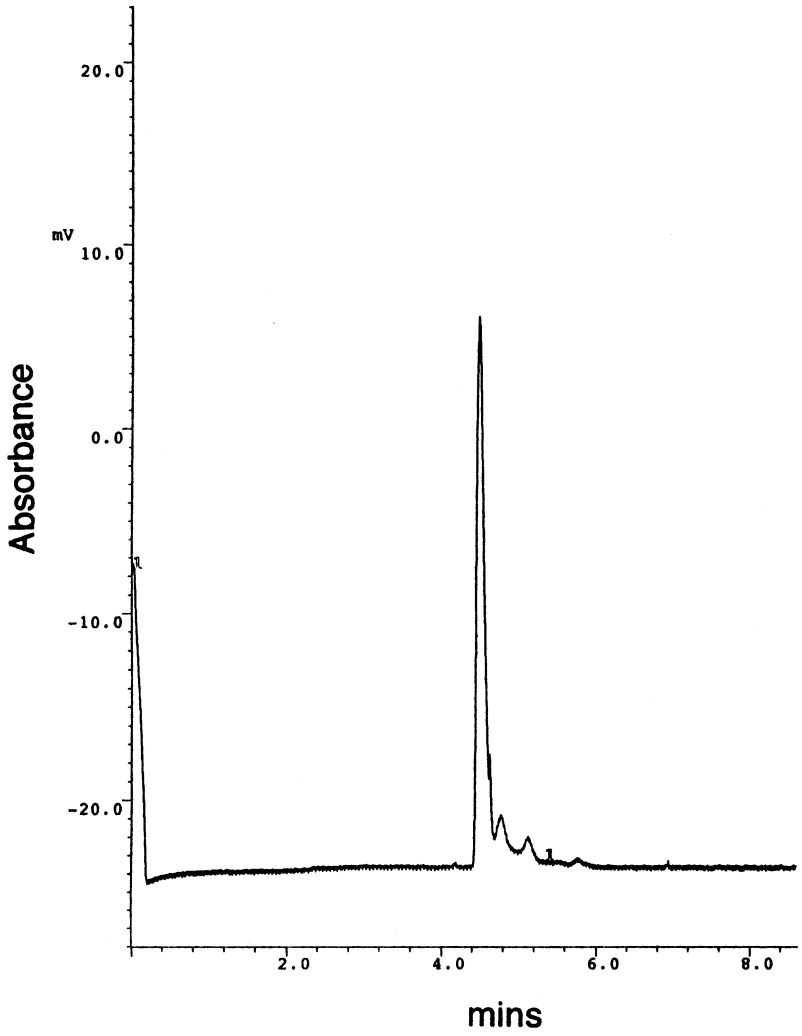

Fig. 1. Electropherogram of standard IgG1. Capillary, uncoated, 75 μm × 60 cm (52 cm effective length); running buffer, 20 mM borate + 0.005% SDS, pH 9.4; injection, 5 s hydrostatic; run voltage, 20 kV; detection, 214 nm.

mM SDS (pH 9.4) and incubated at room temperature for 3 h, or were first diluted 5-fold with the 20 mM borate + 100 mM SDS solution and incubated at 50°C for 2 h prior to injection. The non-denatured samples were all diluted with the running buffer for that separation capillary, prior to injection.

2.5.2. Calibration of standard IgG1

The standard IgG1 was diluted with running buffer (20 mM borate at pH 9.3) 10-fold. It was then further diluted to 20, 40, 60, 80, 100, and 200 × from the original concentration, by diluting the 10-fold diluted sample with running buffer.

2.5.3. Quantitation of antibodies in cell growth medium

Unknown nos. 1 and 2 were 20-fold diluted from the original sample with 20 mM borate running buffer. Unknown 3 was 30-fold diluted with the same buffer. Five samples were prepared

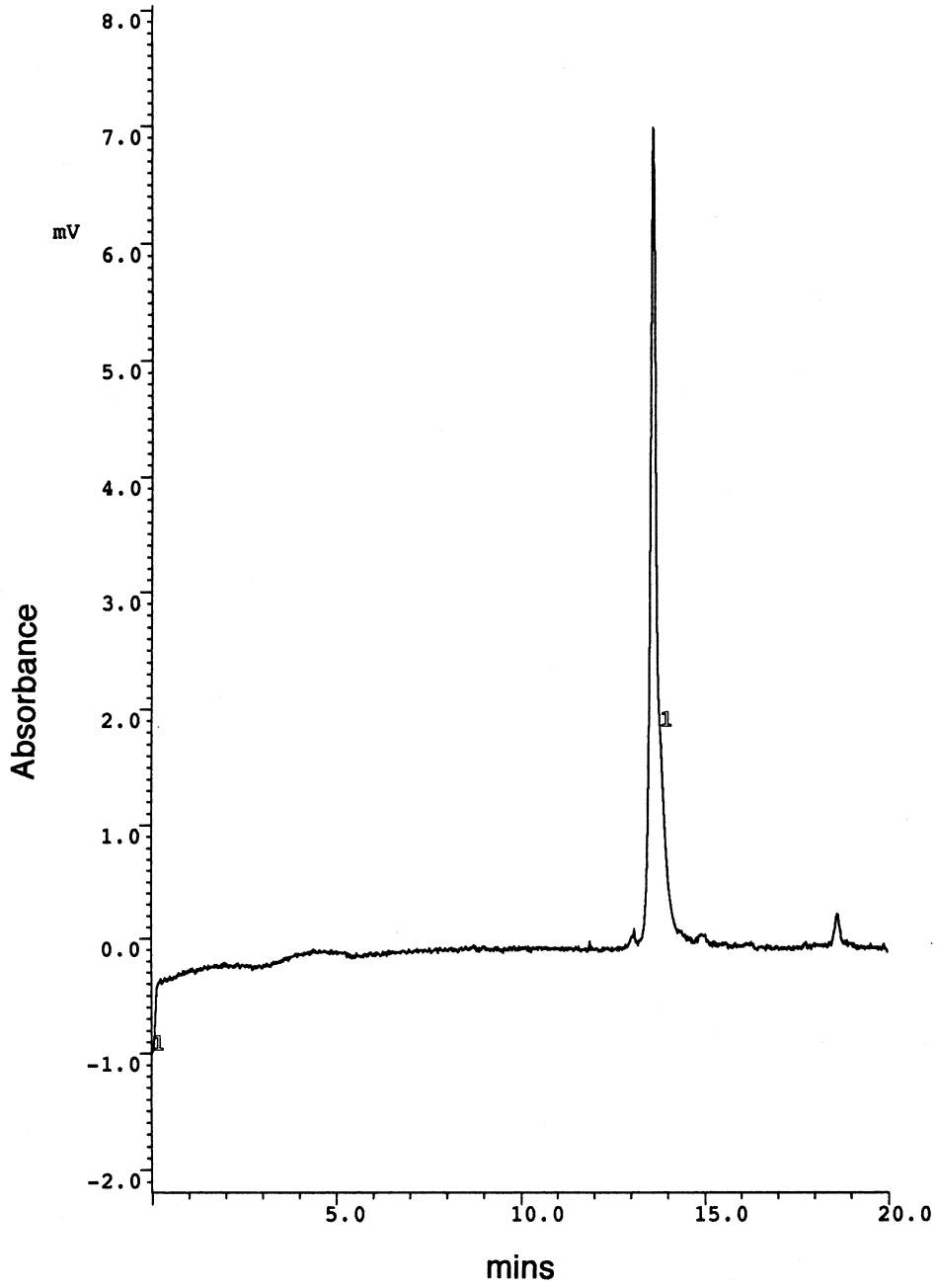

Fig. 2. Electropherogram of 20 × diluted denatured IgG1. 1.5 h after heating at 50°C. Capillary, uncoated, 75 μm × 60 cm (52 cm effective length); running buffer, 20 mM borate + 50 mM SDS, pH 9.4; injection, 10 s, hydrostatic; run voltage, 15 kV; detection, 214 nm.

for each unknown: the first one with no standard IgG1 spiking; the second one spiked with 57 μg ml^{-1} of standard IgG1; the third one spiked with 114 μg ml^{-1} of standard IgG1; the fourth one spiked with 171 μg ml^{-1} standard IgG1; and the fifth one spiked with 228 μg ml^{-1} IgG1.

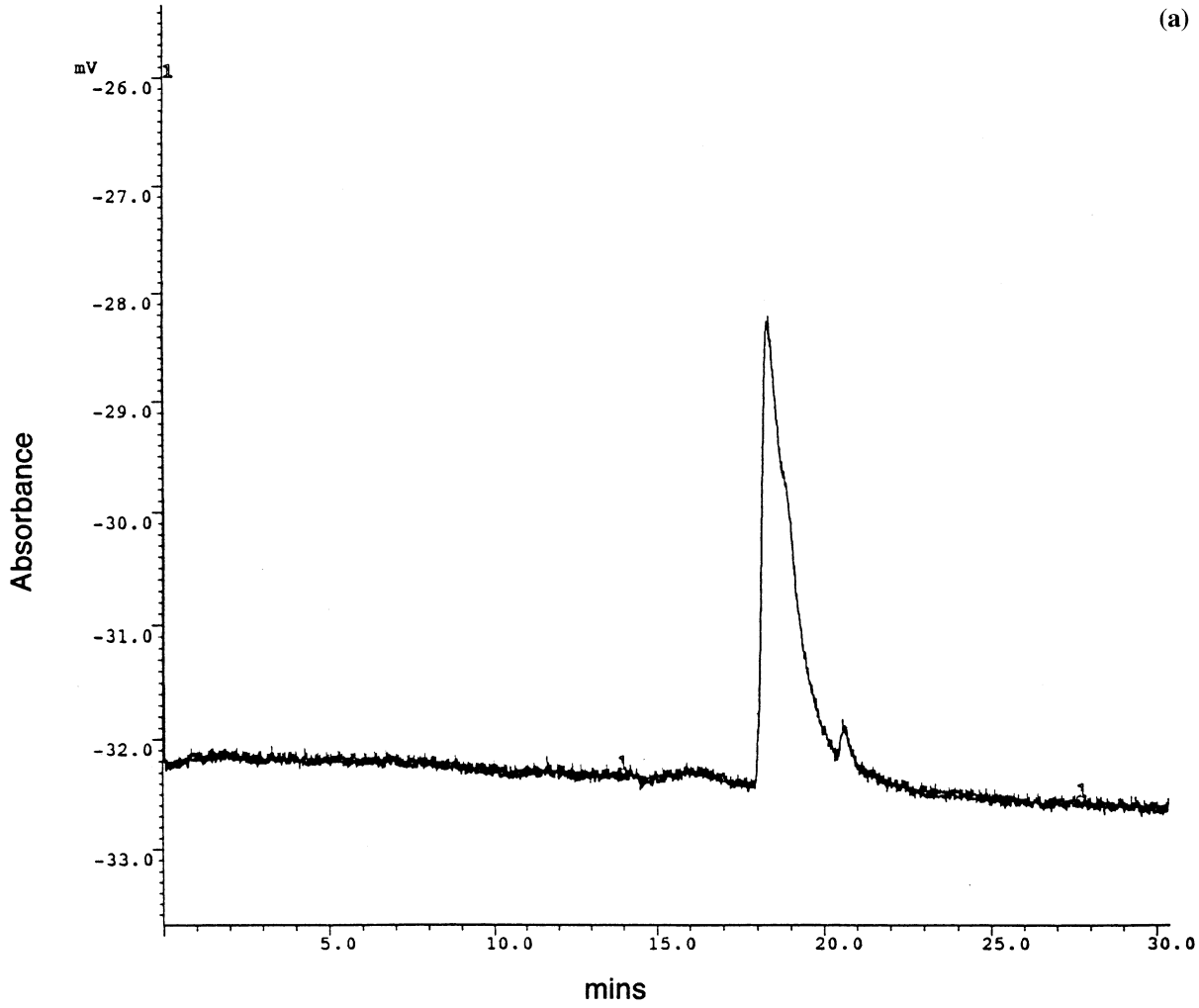

Fig. 3. CE separation of IgG1. (a) Capillary, FC-coated, 50 μm × 60 cm (52 cm effective length); running buffer, 15 mM borate + 0.005% FC-WA/FC-N (1:2), pH 9.0; injection, 5 s, hydrostatic; run voltage, 20 kV; detection, 214 nm. (b) Capillary, FC-coated, 50 μm × 60 cm (52 cm effective length); running buffer, 15 mM borate + 0.005% FC-WA/FC-N (1:3), pH 9.0; injection, 5 s, hydrostatic; run voltage, 20 kV; detection, 214 nm. (c) Capillary, FC-coated, 50 μm × 60 cm (52 cm effective length); running buffer, 15 mM borate + 0.005% FC-N, pH 9.0; injection, 5 s, hydrostatic; run voltage, 20 kV; detection, 214 nm.

3. Results and discussion

3.1. Separation of different species in standard IgG1

Initial efforts involved the development of CE methods that would separate some or all of the different species present in the sample of standard IgG1. This material represented a purified state of the recombinant (cell growth) Ab, IgG1. In the numerous attempts to develop such methods, we evaluated both coated and uncoated capillary columns, all commercially available.

3.1.1. Uncoated capillaries

Borate and tricine were the first buffers tried in this study because they were the most common buffers found in the literature used for Ab analysis with uncoated capillaries. A single peak was observed when using 20 mM borate (pH 9.3) as

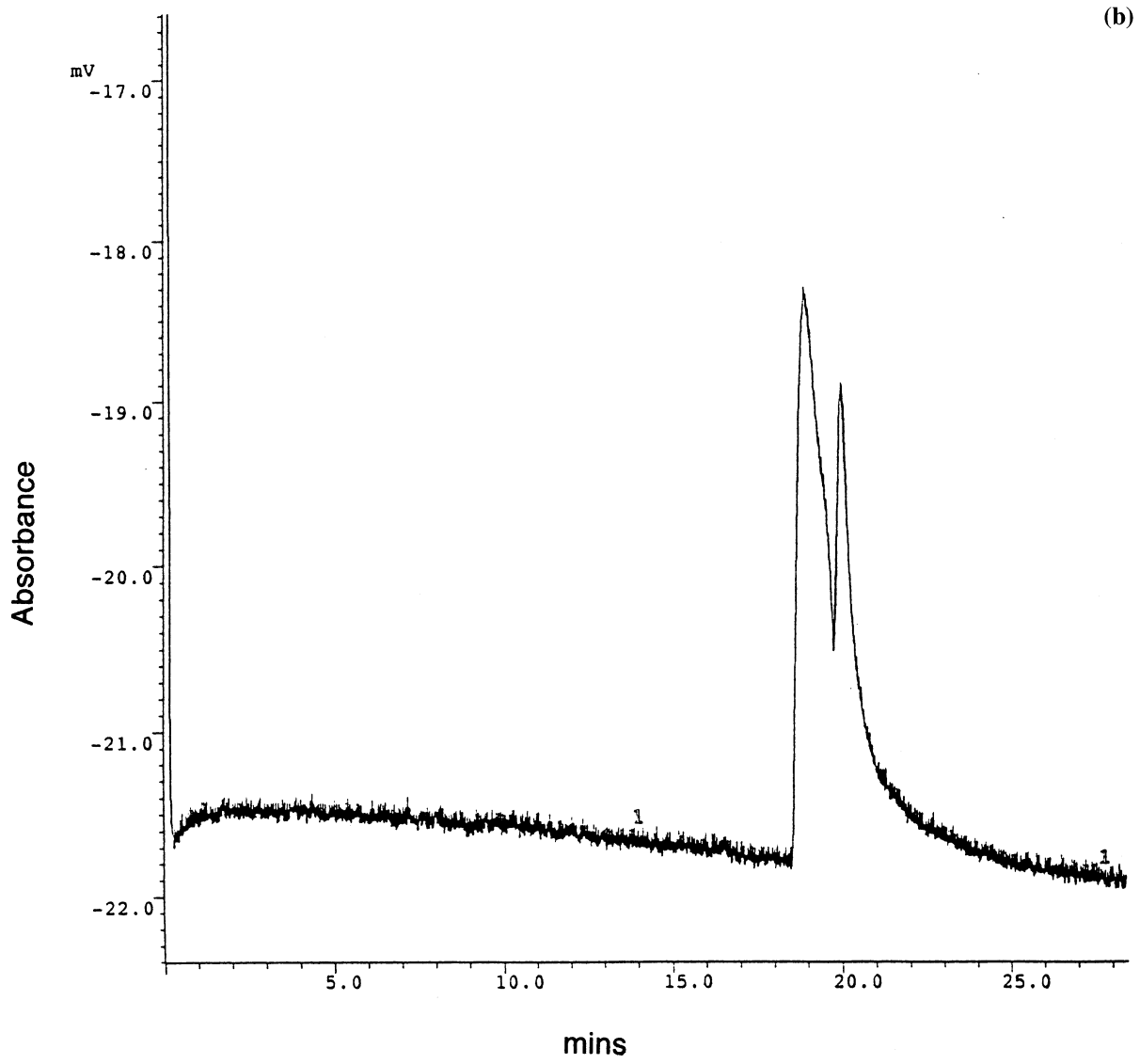

Fig. 3. (*Continued*)

the running buffer. The separation was then attempted with 20 mM borate + SDS at various concentrations. Usually, a single peak was observed under these conditions, even with SDS at 25 mM concentration. However, with the particular IgG1 used in this study, the antibodies were starting to show signs of denaturation, even at a SDS concentration as low as 0.005% (Fig. 1). However, when the SDS concentration was increased to 50 mM, and the standard IgG1 was mixed with the running buffer 3 h before injection, two peaks were observed. Unfortunately, further studies showed that the two peaks were unstable. Eventually, one peak was observed after complete denaturation of the Abs sample (Fig. 2) (several peaks can be seen). The peak shape for the Abs obtained with the tricine buffers was poor. These results all suggest that under these running buffer conditions, the original, active Abs slowly degrade and probably became denatured as

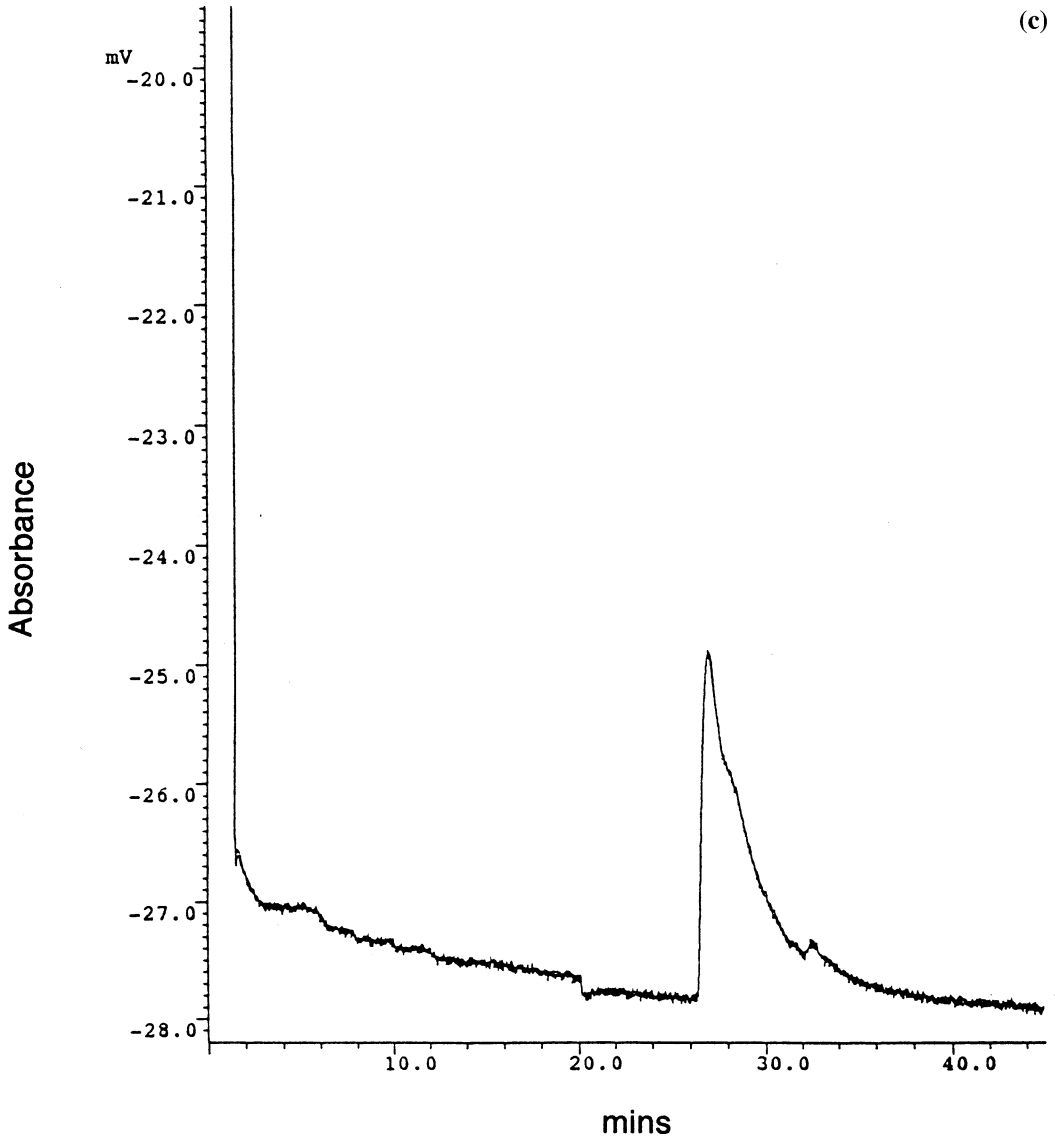

Fig. 3. (*Continued*)

a function of SDS concentration, pH, and time in storage in the running buffer. It also seemed likely that the Abs needed to be fully denatured prior to introduction into the CE capillary, in order to realize acceptable reproducibility of the final electropherograms. This most likely altered the amount of active Ab species present in the final sample being introduced into the instrument , and may have changed the ratio of aggregates to monomer Ab, if these were present at the start. (We know nothing about Ab activity at this stage.)

3.1.2. FC-coated capillaries

Two unresolved peaks were observed for IgG1 when using the 15 mM borate + 0.005% FC-WA:FC-N (1:2) buffer (Fig. 3). However, the peaks were not as sharp as the ones obtained with

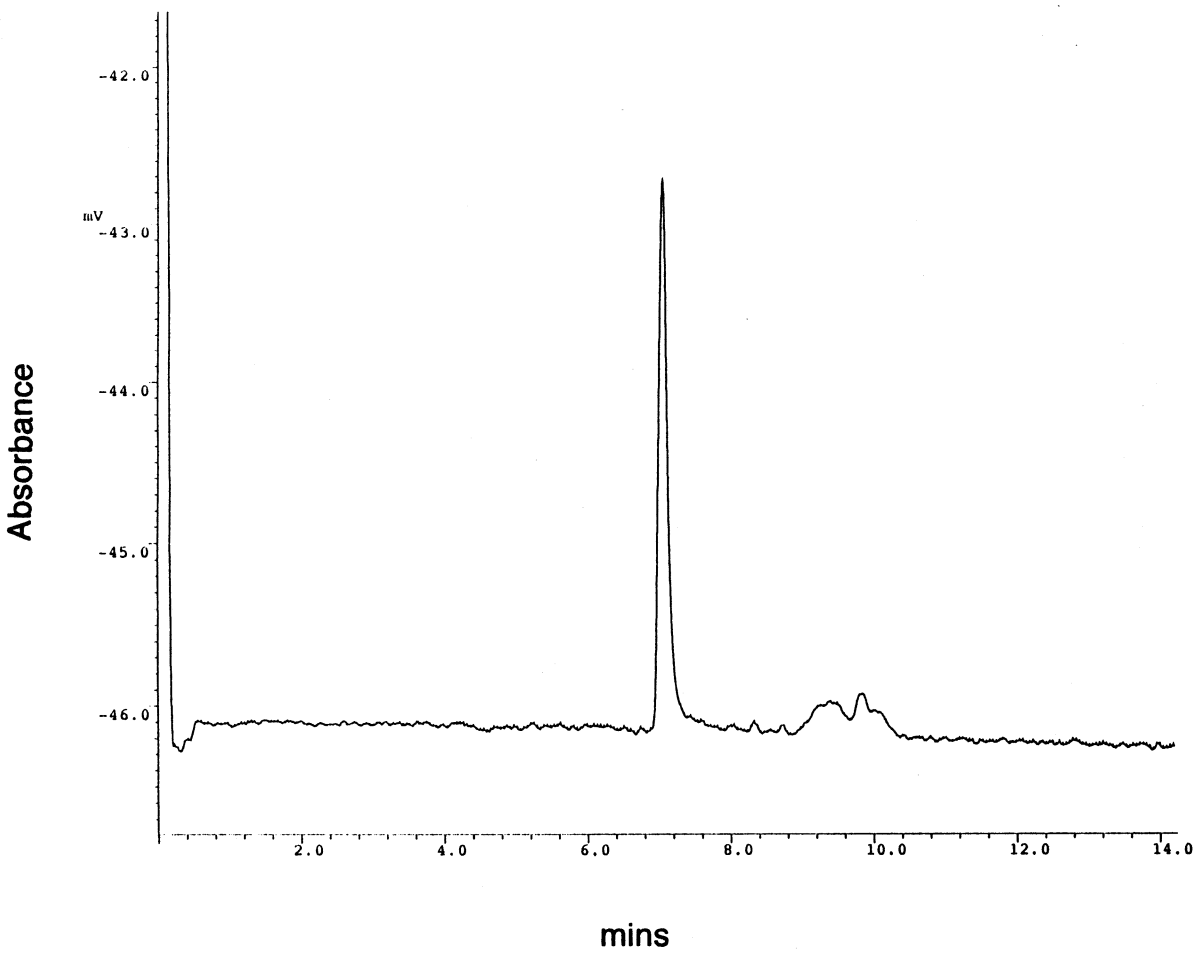

Fig. 4. Typical electropherogram of IgG1 separation in cell growth medium. Capillary, uncoated, 50 μm × 70 cm (62.5 cm effective length); running buffer, 20 mM borate, pH 9.3; injection, 10 s at 12.5kV; run voltage, 17.5 kV; current, 16 μA; detection, 214 nm.

uncoated capillaries. When the surfactant concentration ratio was increased to 1:3, the peaks were further separated, but still no baseline separations were achieved (Fig. 3b). Changing the surfactant to 0.005% FC-N did not help the separations (Fig. 3c). When the surfactant was changed to 0.01% FC-pN, only spikes were observed. Only one peak was observed when the buffer was changed to 10 mM phosphate + 0.005% FC-WA:FC-N (15:85), 10 mM phosphate + 0.005% FC-N, or to 40 mM acetate + 0.02% FC-N. The peaks observed were sharper than those observed when using borate + FC-N as running buffer.

3.1.3. eCAP neutral coated capillary

Only one sharp peak was observed when using 20 mM citrate at pH 3.0 with the neutral coated capillary (figure not shown).

3.2. Separation of IgG1 in cell growth medium

Using all the above conditions, it was not possible to separate the different species present in the standard Abs. The separation and quantitation of the Abs were thus done under simple CZE conditions where they appeared as a single, sharp peak. An uncoated capillary was chosen over coated ones because of its tolerance to-

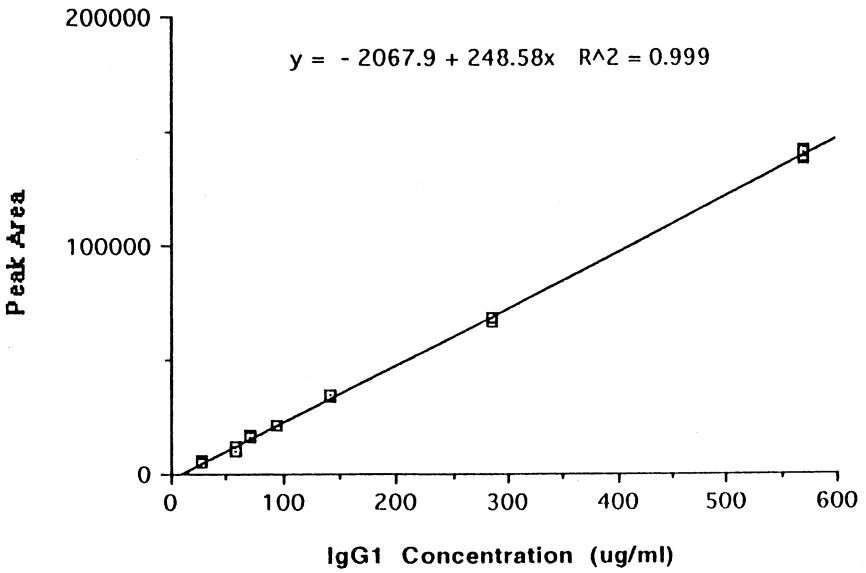

Fig. 5. Calibration curve for the standard monoclonal Ab.

wards various pH buffers and low cost. Different concentrations of IgG1 were present in different cell growth medium samples used in this study. IgG1 was successfully separated from the unknown impurities by using 20 mM borate (pH 9.3) in an uncoated capillary without sample pretreatment (Fig. 4). An electropherogram of the cell growth medium blank showed no Ab present (data not shown).

3.3. Quantitation of IgG1.

3.3.1. Calibration curve

The calibration curve was found to be linear for a range from 28.5 to 570 μg ml^{-1} under CZE conditions (Fig. 5). The R^2 was 0.999. The percent RSD was from 0.78–6.13 (Table 1). The limit of quantitation and the limit of detection were the same because the percent RSD for the concentration of 28.5 μg ml^{-1} was less than 10%, and it would be hard to detect Abs at lower concentrations under these conditions.

Table 1
Data used for IgG1 external standard calibration curve

IgG1 concentration (μg ml^{-1})	Average peak area	RSD (%)
28.50	5272	6.1
57.00	10 856	5.8
71.25	16 819	3.2
95.00	21 334	0.8
142.50	34 481	2.1
285.00	67 141	1.8
570.00	140 160	1.2

$n = 5$.

3.3.2. Quantitation of IgG1 in cell growth medium

The final quantitaion of Abs in cell growth medium was done by comparing the results obtained for an external calibration curve and a standard additions method (Tables 2–5). For the

Table 2
Line equations of standard additions calibration plots for quantitation of IgG1 in unknowns nos. 1–3, demonstration of linearity obtained for such plots

Unknown No.	Linear regression	R^2
1	$y = 3.1905e+4+258.18x$	0.992
2	$y = 1.4459e+4+247.16x$	0.998
3	$y = 1.7850e+4+237.87x$	0.993

Table 3
Data for standard additions of unknown no. 1

Conc. of IgG1 spiked (μg ml^{-1})	Average peak area	RSD (%)
0	32273	1.7
57	46437	1.3
114	59485	2.1
171	78842	1.9
228	89652	1.0

$n = 5$.

Table 5
Data for standard additions for unknown no. 3

Conc. of IgG1 spiked (μg ml^{-1})	Average peak area	RSD (%)
0	17330	3.0
57	32259	4.2
114	44415	4.7
171	59157	3.8
228	76174	2.0

$n = 5$.

standard additions method, the correlation coefficient (R^2) value for the first unknown was 0.992, that for the second unknown was 0.993, and that for the third unknown was 0.993 (Table 2). In general, the overall results obtained using the standard additions method were somewhat higher than those obtained with the external standard, calibration plot. This may have been due to the additional errors involved in preparing the spiked standard additions samples. However, even with these significant differences (Table 6), the overall results were in good ageement with each other.

3.3.3. Validation for quantitation of IgG1 in cell growth medium

Different concentrations of IgG1 were spiked into a blank cell growth medium, and the results were compared with the data obtained using the external standard calibration curve. This was a zero blind or open proficiency validation approach. The percent accuracy for the method was, at worst, ± 1.3 (Table 7). The percent RSD value ranged from 1.5 to 3.8, in the direction expected as the concentration being determined approached lower and lower (limit of quantitation) values. In general, the final assay method has demonstrated acceptable accuracy and precision for these first samples of cell growth medium utilized for quantitation purposes.

Table 4
Data for standard additions for unknown no. 2

Conc. of IgG1 spiked (μg ml^{-1})	Average peak area	RSD (%)
0	14480	1.5
57	28463	2.2
114	42746	1.0
171	45519	1.6
228	70814	2.4

$n = 5$.

4. Conclusion

The data obtained have shown that the IgG1 from SB could be easily separated from other components in the cell growth medium under simple CZE conditions. The overall method was fast, simple and there was no need for sample pretreatment. All data showed that the method was accurate and precise with suitable validation data obtained for single blind spiked studies. Under the conditions evaluated here, individual protein species contained within the Abs mixture were not resolved from each other. Other CE conditions might be more suitable to resolve such microheterogeneous species [16]. However, for the absolute quantitation of total (active/inactive) Abs present in a typical cell growth medium sample, the optimized conditions described above appear quite suitable and acceptable.

Acknowledgements

We would like to thank J&W Scientific for the generous donation of all the FC-coated capillaries and FC-surfactants used in this study. We would also like to thank Dr. David Mao from J&W Scientific for this valuable advice on antibody separation using FC-coated capillaries.

Table 6
Comparison of quantitation results obtained for three samples (unknowns) using both an external standard calibration curve and standard additions methods

Unknown No.	Calculated IgG1 conc. in original sample[a] (mg ml^{-1})	Calculated IgG1 conc. in original sample[b] (mg ml^{-1})	Error
1	2.47	2.76	±5.57
2	1.17	1.33	±6.45
3	2.25	2.34	±1.20

[a] Using the calibration curve.
[b] Using the standard additions.

Table 7
Validation data for quantitation of IgG1 in cell growth medium

Concentration of IgG1 spiked into blank (μg ml^{-1})	Calculated IgG1 concentration in blank (μg ml^{-1})	RSD (%)	Accuracy (%)
71.25	72.18	3.8	+1.3
95.00	93.80	2.7	−1.3
142.50	141.64	1.5	−0.6

$n = 5$.

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