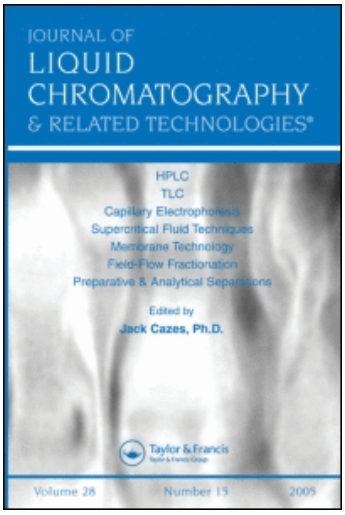

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of the Isoforms of a Monoclonal Antibody by Gel Isoelectric Focusing, High Performance Liquid Chromatography and Capillary Isoelectric Focusing

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To cite this Article Silverman, C. , Komar, M. , Shields, K. , Diegnan, G. and Adamovics, J.(1992) 'Separation of the Isoforms of a Monoclonal Antibody by Gel Isoelectric Focusing, High Performance Liquid Chromatography and Capillary Isoelectric Focusing', Journal of Liquid Chromatography & Related Technologies, 15: 2, 207 — 219
To link to this Article: DOI: 10.1080/10826079208017165
URL: http://dx.doi.org/10.1080/10826079208017165

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(2), 207-219 (1992)

SEPARATION OF THE ISOFORMS OF A MONOCLONAL ANTIBODY BY GEL ISOELECTRIC FOCUSING, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ISOELECTRIC FOCUSING

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ABSTRACT

The isoforms of a monoclonal antibody have been resolved by three different techniques: polyacrylamide gel isoelectric focusing, cation exchange HPLC, and capillary isoelectric focusing. The three techniques gave similar results; five major and one minor fraction were observed, corresponding to pIs in the pH range 6.0 to 6.5. The methods are compared and the advantages as well difficulties of each are discussed.

INTRODUCTION

Recent advances in recombinant DNA and hybridoma technologies have made the commercial production of proteins for use as biopharmaceuticals feasible. Twelve biotechnologically derived drugs have been approved in the US and more than 100 genetically

engineered proteins and antibodies are currently in clinical trials or under review by the FDA.

Due to the structural complexity of biopharmaceuticals, the techniques used to analyze them continue to evolve. In this report, the advantages and disadvantages of determining the heterogeneity of a monoclonal antibody by isolectric focusing on gels and in capillaries and by cation exchange high performance liquid chromatography are evaluated.

Isoelectic focusing (IEF) techniques are recognized as having high-resolving power for proteins that differ in their net charge (1). The net charge of any protein is a sum of all the positive and negative charges of the ionizable acidic and basic side chains of its constituent amino acids and carbohydrates. Consequently, there is a specific pH for every protein at which the net charge is zero. When a protein is placed in a matrix with varying pH, subject to an electric field, it will move toward the electrode with the opposite charge. The protein will eventually migrate to a point in the pH gradient where its net charge is zero, which is known as the isoelectric pH value, termed pI.

Horizontal gel slabs made of polyacrylamide are the most common configuration of IEF. These gels are formed with large pores which allow for relatively unimpeded motion of proteins. Visualization of the proteins as discrete bands is achieved by staining, usually with silver salts or by Commassie Brilliant Blue. Quantitation of the bands is usually determined by gel densitometry.

Capillary electrophoresis IEF of proteins has only been recently demonstrated (2-4). The procedure requires two steps. The first is the establishment of the stable pH gradient, which is carried out by the migration of ampholytes in the capillary upon application of an electrical field. A protein will migrate along the capillary pH gradient until it reaches a point of zero net

charge, at which time migration ceases. In order to detect the protein, sodium hydroxide and sodium chloride are placed in the cathode reservoir and voltage is again applied. Proteins are mobilized through the capillary and pass the detector in order of decreasing pI. An essential feature of this procedure is that the silica capillary is internally coated so as to minimize protein adsorption and electroendosmosis. Electroendosmosis is the bulk flow of liquid toward the negative electrode resulting from the effect of the electric field on the positive counterions in the double-layer adjacent to the non-coated capillary wall. The elimination of electroendosmotic flow is critical for isoelectric focusing to be performed (2-8).

In general, HPLC has been found not as useful a technique as electrophoresis in analyzing proteins, particularly those of high molecular weight such as monoclonal antibodies (150,000 daltons). Ion-exchange chromatography, particularly the anion exchangers such as DEAE have been used in the analysis and preparative isolation of antibodies (9). Separations of proteins on ion-exchangers are usually best affected with an ionic strength gradient. The pH is usually selected to be near the isoelectric point to maximize retention. The only optimization commonly exploited is the adjustment of the ionic strength gradient slope.

METHODS AND MATERIALS

Monoclonal Antibodies

The antibody studied is referred to as B72.3 (10) which is a murine IgG1 and is reactive with a wide range of carcinomas while demonstrating little or no reactivity towards normal human tissue. A DTPA conjugate of B72.3 was recently approved in Europe for *in vivo* imaging for colorectal cancer using indium-111 as the radioisotope. B72.3 was harvested from tissue culture and purified by Protein A and DEAE chromatography. The samples were prepared by Celltech Limited, Berkshire, UK.

TABLE 1
CAMAG Scanner Parameters

A.	Dialog Mode	1
B.	Scanlength	44.0 mm
C.	Trackspace	1.0 mm
D.	Scanning Speed	0.2 mm/s
E.	Wavelength	557 nm
F.	Zero All Tracks	Yes
G.	Scanning Sensitivity	Yes
H.	Sensitivity	200 N
I.	Span	10
J.	Offset	10
K.	Lamp Used	Mercury or Tungsten

Polyacrylamide Gel Isoelectric Focusing and Gel Densitometry

Slab gel isoelectric focusing was performed using the PhastSystem and PhastGel IEF 5-8 (Pharmacia LKB Biotechnology, Piscataway, NJ). The bands were fixed with 20% trichloroacetic acid and stained with 1.725 g/L Coomassie Brilliant Blue R250, 30% methanol, and 10% acetic acid. The sample concentrations were adjusted to within 1 to 2.5 mg/mL protein and 0.5 uL volumes were applied to the gel.

Densitometer scans of the IEF gels were obtained using a Camag TLC Scanner II (Camag, Switzerland) and Waters 745 Data Module (Millipore Corporation, Milford, MA). Transmission scans were determined at 557 nm using a mercury or tungsten source. Scanning parameters were adjusted to ensure proper intensities and resolution of the bands as listed in Table 1.

High Performance Capillary Isoelectric Focusing

Capillary isoelectric focusing was performed using a Bio-Rad HPE-100 system (Bio-Rad Laboratories, Richmond, CA). The instrument was

equipped with a UV/VIS detector and high voltage power supply. The capillary (20 cm length and 25 micron diameter) was enclosed in a cartridge which was inserted between the inlet (anode) and outlet (cathode) reservoirs. The silica capillary was internally coated with a linear polymer which prevents protein adsorption and electroendosmosis (Bio-Rad HPE Cartridge, 148-3002).

Prior to capillary experiments, the samples were dialyzed against 2 mM sodium phosphate buffer, pH 7.0. Final concentration of antibody was approximately 5 mg/mL. The dialyzed sample was mixed (1:1) with a 2% solution of pH 5-8 range ampholytes (Bio-Lyte 5/8) and pressure loaded into the capillary. 10 mM phosphoric acid (Bio-Rad IEF Electrolyte, 148-5019) was added to the inlet (anodic) reservoir and 20 mM sodium hydroxide solution (Bio-Rad IEF Electrolyte, 148-5018) was added to the outlet (cathodic) reservoir. An applied potential caused the ampholytes to migrate, establishing a pH gradient along the capillary. Focusing was achieved by migration of the sample along the pH gradient. The focusing step was performed at constant voltage and monitored by following the current. As focusing proceeded, the current decreased to a minimum value. In all experiments, the focusing was stopped when the current registered 0.3 microamperes. Typically, the focusing step lasted about 3 minutes.

The focused zones were mobilized electrophoretically by exchanging the NaOH catholyte solution for a solution containing 20 mM sodium hydroxide and 80 mM sodium chloride (Bio-Rad IEF Mobilizer, 148-5020). The migration of chloride anions into the capillary continously decreased the pH from the cathodic end of the tube (4) causing the proteins to move towards the outlet (3, 4). Detection of the zones as discrete peaks was achieved by UV absorption determination at 280 nm.

High Performance Cation-Exchange Liquid Chromatography

The HPLC column was a Bakerbond wide-pore CBX column, 7.75 x 100 mm (J.T. Baker Incorporated, Phillipsburg, NJ). The gradient

was linear from 100% solvent A (0.01 M 2-[N-Morpholino] ethanesulfonic acid (MES) buffer, pH 5.0) to 100% solvent B (0.01 M MES buffer, pH 8.5) over 60 minutes. The flow rate was 1.0 mL/minute with detection at 280 nm. Under these conditions, the MAb fractions eluted after the gradient, between 65 and 95 minutes. For all HPLC experiments performed, a Varian Vista 5500 LC and DS data station were used.

RESULTS AND DISCUSSION

Polyacrylamide Gel Isoelectric Focusing and Gel Densitometry

The conventional means of performing isoelectric focusing is by gel electrophoresis using polyacrylamide as the anticonvective medium. Figure 1 shows the IEF gel of MAb B72.3 and Figure 2 represents a densitometer scan of a comparable gel of the same lot of material. Five major peaks are observed corresponding to pIs in the pH range 6.0 to 6.5. A low intensity peak is also observed on the acidic side of the profile corresponding to a pI of about 5.9. Densitometer scanning provided a means to quantitate the individual isoforms and the reproducibility of this method was examined for nine samples run as three lanes on three gels. The tabulated area counts for the six peaks are presented in Table 2. Relative standard deviations (standard deviation/average area) were calculated for the nine replicates of each fraction and these values range from approximately 10 to 20%. The factors contributing to these rather large variations include minor imperfections which often appear in gels and the staining reaction which is only linear over a narrow concentration range. Gel electrophoresis and gel scanning is therefore labor intensive and capable of providing only limited quantitative information.

Capillary Isoelectric Focusing

The capillary IEF electropherogram of MAb B72.3 is presented in Figure 3. Like the gel scan, the capillary IEF profile shows five major

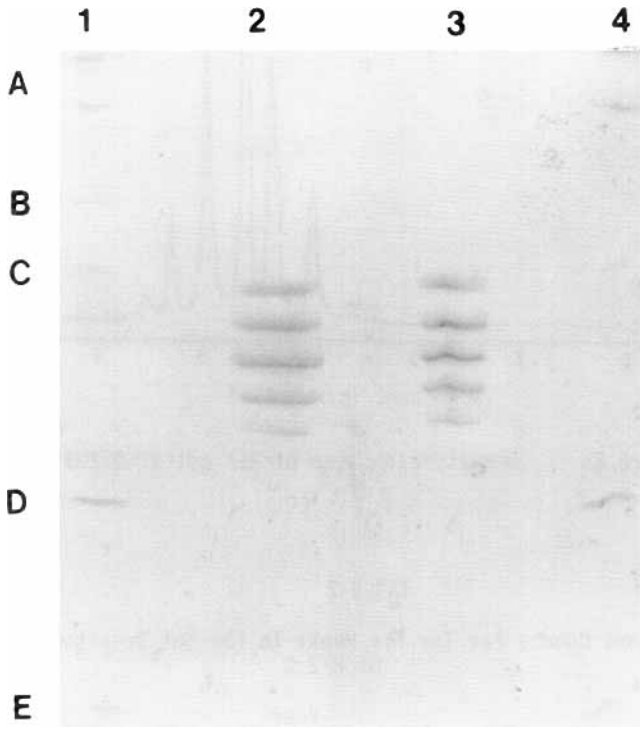

Figure 1. IEF gel of B72.3. High pI calibration markers were run on tracks 1 and 4. The B72.3 replicates were run on tracks 2 and 3. Bands (A) through (E) correspond to the following: (A) pI marker proteins near cathode wick, pIs 8.0-8.5; (B) myoglobin-basic band, pI 7.35; (C) human carbonic anhydrase B, pI 6.55; (D) bovine carbonic anhydrase B, pI 5.85; (E) Beta-lactoglobulin-A, pI 5.20.

and one minor component which eluted approximately 10 minutes after mobilization. Resolution of six components by capillary IEF for B72.3 was achieved using the pH 5-8 range ampholytes. Use of the pH 3-10 range ampholytes resulted in a single broad peak which could not be resolved by varying the run conditions of the experiment. The critical parameter affecting the resolution when using the pH 5-8 range ampholytes appeared

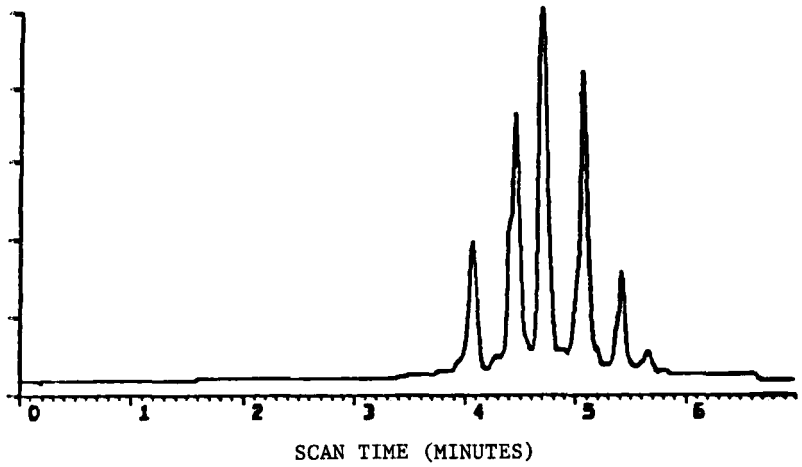

Figure 2. Densitometer scan of IEF gel of B72.3

TABLE 2

Tabulated Area Counts For The Six Peaks In The Gel Densitometer Scan Of B72.3

	Peak					
Replicate	1	2	3	4	5	6
1	238261	493981	667527	713537	417450	114450
2	282681	613118	817267	772043	458828	120902
3	244606	532015	635652	635652	368959	121876
4	293980	622796	765919	669483	384510	128657
5	221120	488071	619343	563005	317742	103402
6	228881	520964	610768	505673	259491	78969
7	351438	610699	745914	672586	436156	155760
8	275810	554546	669019	646751	405683	145458
9	311545	510013	654279	589544	402756	156708
Average Area	272036	549610	687299	640919	383508	125131
Standard Dev.	40583	50239	67878	75534	58097	23820
RSD	9.1%	9.9%	11.8%	15.1%	19.0%	19.0%

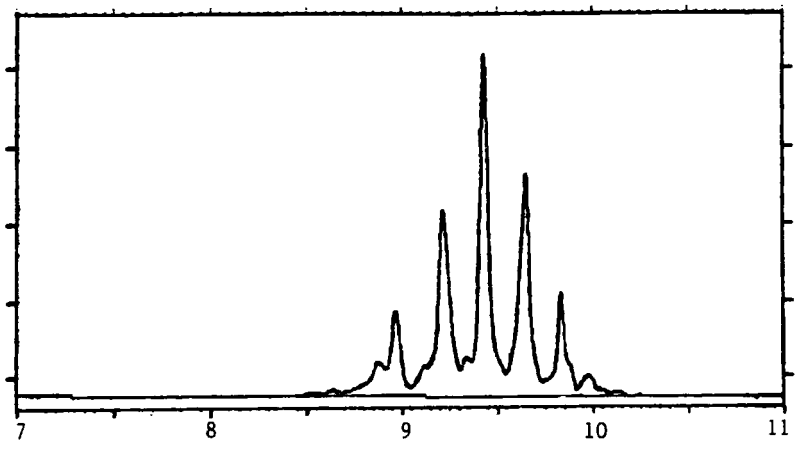

Figure 3. Capillary IEF electropherogram of B72.3.

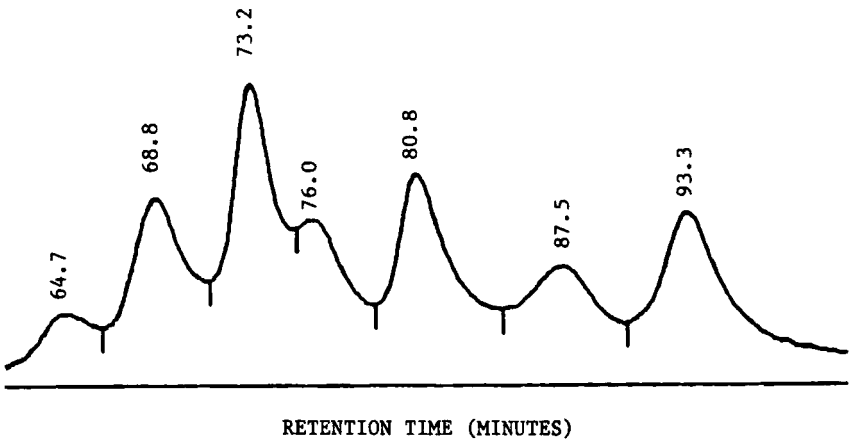

Figure 4. Cation-exchange HPLC chromatogram of B72.3.

to be the focusing time. Longer focusing times often resulted in better separation of the fractions. The focusing step was stopped, however, when the current registered 0.3 microamperes since at lower currents protein precipitation in the capillary may occur.

The major advantage of capillary isoelectric focusing over gel isoelectric focusing was the relative speed of the technique. The focusing step usually required approximately 3 minutes and mobilization took 8 to 10 minutes. However, the elution times by capillary IEF for B72.3 were observed to vary by as much as 33% between runs (i.e., 15 $\pm$ 5 minutes). This problem may be minimized by the use of one or more internal pI standards, with elution times of the sample measured relative to the standards. Although a systematic determination of standard deviations for the capillary results was not attempted, the reproducibility of the relative percent areas was better than that found for the gel scans (data not included).

The major drawback concerning the capillary method is the instability of the capillary coating. Typically, when using the narrow range pH 5-8 ampholytes, the capillaries were only useable for 3-5 runs after which there was observed a dramatic loss of resolution. By about the fifth run, the method failed and no signals were obtained. It has also been observed that the capillary does not fail as quickly when the broad range pH 3-10 ampholytes are used (11). The capillary instability may be due to an "edge effect" such that the steeper gradient, from pH 8 to 12 at the edge of the capillary contributes more to coating instabilities than the pH 10 to 12 gradient when the broad range ampholytes are used (11). This problem may not be a trivial one, as different types of capillary coatings as well as different catholyte solutions may have to be investigated in order to develop a stable system useful for routine testing.

Cation Exchange High Performance Liquid Chromatography

The HPLC chromatogram of B72.3 is shown in Figure 4. The method employed a linear gradient from pH 5.0 to pH 8.5 over 60 minutes. At pH

5.0, all of the protein components, also referred to as isoforms of B72.3, were positively charged and thus retained by the column. As the pH was increased, the isoforms eluted as resolved peaks in order of increasing pI. Seven peaks were observed at retention times between 64 and 94 minutes. The identities of the peaks were investigated by collecting fractions with retention times of approximately: 65, 69, 73, 76, 81, 88, and 93 minutes. The samples were then concentrated using a Savant Speed Vac Concentrator, and focused by gel electrophoresis versus a B72.3 sample. The first six peaks in the HPLC chromatogram which eluted between 65 and 88 minutes were each observed to be single pI fractions, corresponding to the five major bands of B72.3 as follows: 65 minutes, pI = 6.0; 69 minutes, pI = 6.1; 73 and 76 minutes, pI = 6.2; 81 minutes, pI = 6.3; and 88 minutes, pI = 6.4. The minor fraction with a pI of about 5.9 which is observed as a faint band by gel isoelectric focusing, and as a low intensity band by capillary electrophoresis, was not evident in the HPLC chromatogram of B72.3. Also, the two overlapping peaks at 73 and 76 minutes in the chromatogram were observed to focus at a pI of approximately 6.2. The results confirmed that the peaks eluted in order of increasing pI, with the exception of the peak with a retention time of 94 minutes which focused by gel IEF electrophoresis as all five isoforms.

The major disadvantage of the HPLC method for the analysis of B72.3 was the long run time. The lack of baseline resolution is another disadvantage as indicated by the presence of one intense and one or two faint bands sometimes appearing on the IEF gels of the HPLC fractions. Multiple HPLC purifications of fractions, however, could be used for the isolation of single isoforms for further characterization by, for example, carbohydrate analysis or mass spectrometry. In studying MAbs with pIs which differ from B72.3, additional method development is required. This method is not universally applicable to all antibodies. On the other hand, HPLC provided a means to collect fractions of purified material, which was not as readily feasible by the other two techniques.

CONCLUSIONS

Polyacrylamide gel isoelectric focusing results in highly resolved separations of proteins yielding semiquantitative information by gel

densitometry. This method is more labor intensive and time consuming relative to either capillary isoelectric focusing or HPLC. Capillary IEF is fast, requires minimum sample consumption and affords quantitative information by relative peak area integration. Unfortunately, difficulties still remain which prevent the routine use of the technique due to instabilities of the capillary coating. Several groups have suggested solutions to these problems including Mazzeo and Krull (8) who investigated the use of methyl celluose and other additives for performing capillary IEF in uncoated fused silica capillaries. The advantage of their method is the continuous renewal of the "coating effect" such that capillary degradation was not a problem. In addition to the use of polyacrylamide-coated capillaries, another group has reported the use of PEG-coated capillaries (J & W Scientific, Folsom, CA) for capillary IEF (12). The long-term stability of these coatings has not been reported.

Finally, the HPLC method provides a means to purify and collect fractions which is useful for further sample analysis by other methods. Disadvantages include the lack of baseline resolution as well as the long run time which prohibits this technique from being used routinely for the quality assurance of B72.3

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