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SDS Non-Acrylamide Polymeric Gel-Filled Capillary Electrophoresis for Molecular Size Separation of Protein

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"SDS Non-Acrylamide Polymeric Gel-Filled Capillary Electrophoresis for Molecular Size Separation of Protein"

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ABSTRACT

Sodium dodecyl sulfide (SDS) non-acrylamide gel-filled capillary columns are a new technology being used for analysis and separation of biotechnology-derived proteins. This research was to compare this new technology to the current methods of SDS polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance size-exclusion chromatography (HPSEC). The molecular mass of four different recombinant proteins were determined by two commercialized SDS non-acrylamide gel-filled capillary columns, SDS-PAGE, and HPSEC. The data obtained showed that the SDS non-acrylamide gel-filled capillary columns were compatible with the SDS-PAGE technique for molecular mass determination. HPSEC was shown to be unreliable for molecular weight determination. SDS non-acrylamide gel-filled capillary columns have the advantages of being fast reliable, automated, and has improved reproducibilty and quantification.

INTRODUCTION

Pharmaceutical industries are shifting their research interests from small organic molecules to biotechnology-derived proteins (1). Protein separation, purification, and characterization are becoming an important aspect of biotechnology. High-performance size-exclusion chromatography (HPSEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are currently being applied to determine the composition and molecular weight of these recombinant proteins. Even though these methods are being used, some problems with them have been noticed (2).

HPSEC is effective in determining protein composition, but the separation efficiencies (2) and peak resolution capability of HPSEC is not very ideal (3). It is, however, capable of being able to analyze greater amounts of sample (4).

An advantage that SDS-PAGE has over HPSEC is its mode of separation. SDS-PAGE consists of cross-linked polyacrylamide gel that with the presence of SDS is able to separate proteins by molecule mass (3,5,6). Even though this is an indispensable method for the separation of proteins, it is slow, difficult to quantitate (1), and labor intensive (1,3).

With the commercialization of the high-performance capillary electrophoresis (HPCE) instrumentation, there has been advances with SDS cross-linked gel-filled columns (2) and SDS polymer gel-filled capillary column (3). The commercialization of the latter of these two methods has given pharmaceutical companies a major separation method for their biotechnology-derived proteins. SDS polymer gel-filled capillary columns offer the possibility of transferring routine electrophoretic techniques to the automated capillary format, since it is very similar to SDS-PAGE (1). The SDS polymer gel-filled capillary columns have the same molecular sieving action of SDS-PAGE, which is

accomplished by a gel matrix formed with the presence of a linear or cross-linked polyacrylamide or non-acrylamide polymers and SDS molecules (7).

High-performance gel-filled capillary electrophoresis has many advantages over HPSEC and SDS-PAGE. The main advantages consist of rapid separation at the nanogram level, improved reproducibility and qualification (2,3,9), and high-speed and high-performance separations for analysis and molecular weight determination (1-4,7,9). Most of these advantages are due to on-line detection by UV absorbance at an appropriate wavelength (8), no need to spray, blot, or stain in order to determine solutes and molecular weights (7,9), on-column injection (9), and that it is currently automated and low in energy (2,3,7-9).

The polymer gel-filled capillary columns present an advantage over the cross-linked gel-filled capillary columns in that filling a capillary with linear or cross-linked solutions containing polyacrylamide or non-acrylamide polymers and SDS molecules that will form a gel matrix for each run, than to prepare a polymerized gel within the capillary. A capillary column can be filled approximately 40 times, with rinsing in between each run, before peak resolution declines (3), while a cross-linked gel-filled columns lose resolution after more than one run (5). Therefore, the polymer gel-filled capillary columns offers a less time consuming and longer lasting technique than the cross-linked gel-filled capillary columns. Now with the commercialized SDS non-acrylamide, polymer gel-filled capillary column, it is possible to obtain this advantageous method for separation and analysis of proteins. In this paper, we examine the feasibility of this new HPCE technology with an SDS non-acrylamide, polymer gel-filled capillary column as a rapid means of characterization and quantitation of proteins of pharmaceutical interest. The results will be compared to those of SDS-PAGE and HPSEC.

MATERIALS AND METHODS

Instrumentation

The instrument that was used was Beckman P/ACE system 2100 high-performance capillary electrophoresis (HPCE) instrument (Beckman instruments, Inc., Fullerton, CA).

Non-acrylamide based gel-filled capillary system from Beckman: Each analytical run consists of rinsing a coated capillary column (parts #241521, Beckman; 100 um i.d. x 375 um o.d.; effective length, 40 cm) with 1.0 N HCl for two minutes and the column was filled with an SDS non-acrylamide gel solution (parts #241522, Beckman) for four minutes. Use of a coated capillary column is required to eliminate electroendosmosis.

The column temperature was maintained at 20^{o}C by a circulating coolant to minimize band diffusion for effective size separation. An electrophoretic run was conducted at -14.1 KV (-300 V/cm, 24 uA) using the SDS non-acrylamide based gel solution (Beckman) as reservoirs at both anode and cathode terminals. About 1 mg of protein per ml solution was injected for sixty seconds under nitrogen pressure (total injection volume, ca. 60 nl) onto the SDS gel-filled capillary column.

Non-acrylamide based gel-filled capillary system from ABI.

A bare fused-silica capillary column (50 um i.d. x 375 um o.d.; effective length, 20 cm, Polymicro Technologies Inc., Phoenix, AZ) was activated by rinsing with a 0.1 N NaOH solution for fifteen minutes. Analytical run starts by filling the capillary with the SDS non-acrylamide based gel solution (parts #401482, Applied Biosystems, Inc. (ABI), Foster City, CA) for sixty minutes. Since the gel system from ABI contains a chemical to minimize eletroendosmotic flow, use of a coated capillary column is not required.

The column temperature was maintained at 30°C by a circulating coolant. Each electrophoretic run was conducted at -8.1 KV (-300 V/cm, 20 uA) using the SDS non-acrylamide based gel solution (ABI) as reservoirs at both anode and cathode terminals. About 1 mg of protein per ml of solution was injected for sixty seconds under nitrogen pressure (total injection volume, ca. 6 nl) onto the SDS gel-filled capillary column.

Peaks migrating in the capillary columns were monitored on-column by UV at 214 nm. The area under the peak was integrated by means of an in-house GC/LC program residing on a VAX mainframe computer and with an electronic integrator (model 3392A, Hewlett Packard, Palo Alto, CA).

HPSEC: A series II 1090 Liquid Chromatograph with a low volume autosampler/injector (Hewlett Packard) was used for the high-performance size-exclusion chromatographic (HPSEC) assay. Zorbax GF-250 column (9.4 mm i.d. x 25 cm; MAC-MOD Analytical Inc. Chadds For, PA) was used for the analysis of rbSt and Bio-Sil TSK-250 column (7.5 mm i.d. x 60 cm, catalog no. 125-006, Bio-Rad Laboratories, Richmond, CA) for ATGAM. ATGAM and rbSt peaks eluding from the column were monitored at 280 nm.

SDS-PAGE: A BioRad Mini-PROTEAN II Electrophoresis Cell (part #165-2940) was used as the electrophoresis apparatus. A separating and stacking gel were prepared with 10 wells for each gel. The percentage of the Acrylamide:Bis was varied to change the amount of cross-linkage to allow the separation of both large and small protein molecules. The percentage of the Acrylamide:Bis for each of the proteins used was 15% for rbSt, 12% for ATGAM, 17% for hemoglobin, and 15% for Hemopure 2.

The proteins were then separated by applying 10 mAmp of electric current at room temperature. The current was applied until the proteins had reached about 0.5 cm from the bottom of the gel. Each gel was then silver stained using

the Daichi Silver Staining Kit (Daichi Pure Chemicals Co. LTD; Tokyo, Japan). About 1 mg of protein per ml of solution was loaded into a well on the gel. For molecular weight determination, each gel was photographed and also scanned by a densitometer.

Molecular Dynamics densitometer: A laser densitometer (parts #300b; Hewlett Packard) was used to scan the SDS-PAGE gels to be able to use the Image Quant software (Molecular Dynamics; Sunny Vale, CA) to calculate the molecular weights of the samples that were separated.

Reagents

The molecular mass protein standard solution containing hen egg white lysozyme (molecular mass: 14,400 daltons), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase (31,000), hen egg white ovalbumin (45,000), bovine serum albumin (BSA) (66,200), rabbit muscle phosphorylase b (97,400) was obtained from Bio-Rad Laboratories (#161-0304, SDS-PAGE Low Range Molecular Weight Standard).

The sample buffer solution, containing 1% SDS (Sigma Chemical Company, ST. Louis, MO) in 0.12 M Tris/HCl, pH 6.6 (part #241525, Beckman), was used to dilute the protein sample. The 2-mercaptoethanol (Sigma) was used to reduce the protein sample.

For the HPSEC assay, a thoroughly de-gassed mobile phase composed of 150 mM NaCl (Mallinckrodt Inc., Paris, KY), 25 mM NaH_2PO_4 (J. T. Baker Chemical Co., Phillipsburg, NJ) at pH 8.0 and 0.1% SDS was pumped at a flow rate of ca. 1.0 ml/min.

For the SDS-PAGE assay, procedures described in the Current Protocols in Molecular Biology (10) were followed. The separation gel was prepared using

Acrylamide:Bis (BioRad), water, 1.5 M Tris (Sigma) at pH 8.8, 10% Sodium Dodecyl Sulfate (SDS) (Sigma), 10% Ammonium Persulfate (BioRad), and 100% N,N,N',N' Tetramethylene-diamine (TEMED) (BioRad). The stacking gel was prepared with the same reagents as the separating gel except 0.5 M Tris at pH 6.8 was used in place of the 1.5 M Tris at pH 8.8. The sample solubilization buffer contained 0.5 M Tris at pH 6.8, glycerol (Sigma), 10% SDS (Sigma), and 0.5% bromophenol blue (BioRad).

Preparation of Protein Samples

Molecular mass protein standard: 5 ul quantity of molecular mass protein standard solution was diluted in 40 ul of the sample buffer solution. After thorough mixing, 2 ul of 2-mercaptoethanol was added and the mixture was heated at 80^{o}C for five minutes.

Recombinant proteins: Samples of proteins, rbSt (recombinant bovine somatotropin, molecular mass: 21,812), ATGAM (polyclonal anti-thymocyte equine immune globulin, molecular mass: ca. 150,000), used in this study were all manufactured by The Upjohn Company (Kalamazoo, MI, USA), and hemoglobin (molecular mass: alpha chain = 15,053 daltons, and beta chain = 15,854 daltons) and Hemopure 2 (blood substitute synthesized from bovine hemoglobin) were manufactured by Biopure (Boston, MA).

Protein samples were diluted in the sample buffer solution to approximately 1 mg/ml concentration. To try and separate the alpha and beta chains in the hemoglobin samples a range of different concentrations (0.1 - 1.0 mg/ml) were used. To reduce and denature the protein, 2 ul of 2-mercaptoethanol was pipetted into a solution of ca. 1 mg protein concentration per ml. After thorough mixing, the sample was heated at 80^{o}C for five minutes.

For SDS-PAGE analysis, the protein sample was prepared in the sample buffer solution then diluted 4 parts sample with 1 part sample solubilization buffer 4X to get a 1 mg of protein per ml of solution. If the sample was required to be reduced then 2 M Dithiothreitol (DTT) (BioRad) was used.

For the HPSEC analysis, approximately 10 mg of protein per ml in 0.1% SDS were prepared. About 100 ul of the sample solution was injected onto the size-exclusion column.

RESULTS AND DISCUSSION

Molecular Mass Determination of SDS-PAGE

There were three methods of molecular mass determination available for SDS-PAGE. These consisted of manual measuring of distances and then plotting the log molecular weight of a protein and its log of the "relative" mobility, which was found by dividing the migration distance for each protein by the migration distance of the dye front, on a graph to determine the molecular weight of the recombinant proteins or by using the linfit program available in Upjohn's VAX mainframe. Also, the molecular densitometer was used with Image Quant software to obtain a mechanical measuring of the molecular weight.

Several lots or concentrations were used to obtain an average of the different components of each recombinant protein. Five different lots of rbSt were used. Figure 1 shows a picture of a SDS-PAGE slab gel for rbSt. Table 1 contains the results of the three methods for determining the molecular weight of the compounds. All three methods gave relatively close molecular weight data. The method using the Molecular Dynamics scanner does show the best correlation with the theoretical molecular weights (Table 11). Figure 2 shows the scan of the same rbSt SDS-PAGE slab gel shown in figure 1. The scan and the picture are relatively the same. Figure 3 represents a line graph obtained from the Image Quant software that was used to calculate the molecular weight of the enriched dimer sample of rbSt.

To look at the reducibility of the Molecular Dynamics scanner with the Image Quant software for calculating the molecular weight, duplicate SDS-PAGE gels were done on different rbSt samples. Both gels were then scanned six times a piece. The molecular weights were calculated for each of the scans and then averaged. The results are shown in Table 2. The average standard deviation and average

relative deviation show that this method is valid to use to obtain molecular weights of recombinant proteins when they have been separated by SDS-PAGE.

The molecular weights for ATGAM, hemoglobin, and Hemopure 2 were relatively close with all three methods of determination. These results are shown in Tables 3, 4, 5. They were also relatively close to the theoretical molecular weights for each of the recombinant proteins (Table 11). The scans of the SDS-PAGE for these recombinant proteins are shown in figure 4 for ATGAM, figure 5 for hemoglobin, and figure 6 for Hemopure 2. The method of molecular weight using the Image Quant software and densitometer for SDS-PAGE was able to completely resolve the alpha and beta chains of hemoglobin with all of the concentrations used, which is hard to accomplish in most analytical methods (Figure 7).

Molecular mass determination of the Beckman and ABI SDS non-acrylamide based gel-filled Capillary Column

A protein standard (Bio Rad) and the recombinant proteins were used to look at the type of separation that is possible. The two systems were able to separate the six proteins in the standard (ABI Figure 8, and Beckman Figure 9), except that the Beckman system had difficulty achieving a base-line separation between BSA and phosphorylase b proteins. The time to fully separate the proteins was twenty minutes for the Beckman system and six minutes for the ABI system.

The electropherograms of the recombinant proteins are shown in Figures 10-14 for the ABI system. For rbSt a typical lot will show the monomer and dimer of the protein (Figure 10), but with the enriched dimer sample the trimer and tetramer are able to be seen (Figure 11). When ATGAM wasn't reduced, a single, not very sharp peak, was seen (Figure 12). This is probably due to the

microheterogeneity of the polyclonal antibody of IgG (11). When it was reduced the L- and H- chains were able to be resolved (Figure 12). Both the ABI and Beckman systems were unable to separate the alpha and beta chains of hemoglobin totally, since the two subunits only differ by 800 daltons (Figure 13). The reason for this is because 1,500 daltons has been estimated as the minimum peak resolution of the SDS gel-filled capillary electrophoresis (11). The electropherogram of Hemopure 2 is shown in figure 14.

The molecular weights for the proteins are shown in Tables 6-9 for the Beckman system and Table 10 for the ABI system. These values are relatively close to the theoretical values for each of the recombinant proteins.

Tsuji speculated on the differences between these two SDS non-acrylamide based gel-filled capillary electrophoresis systems in his paper "Factors Affecting Performance of SDS Non-acrylamide Gel-Filled Capillary Electrophoresis and its Application for Molecular Mass Determination of Proteins" (11). The two SDS gel-filled capillary systems separate the recombinant proteins similarly by the means of molecular sieving action, although the chemical components of their gel system are different (11).

Molecular mass determination by HPSEC

The data on the molecular mass determination by HPSEC were done by H. E. Simmonds, J. S. Bourdage, and L. C. Eaton of the Upjohn Company. Table 11 compares the molecular weights for the four recombinant proteins for SDS Gel-filled capillary systems from ABI and Beckman, SDS-PAGE, HPSEC, and the theoretical values. The data from the HPSEC was the least reliable of the methods. The difficulties can be attributed to the pH and ionic strength of the mobile phase, which can affect the hydrodynamic radius of a protein and can cause ionic

interaction or hydrophobic binding of the protein to the bonded phase of the HPSEC column (11,12).

CONCLUSION

This research has shown that the SDS non-acrylamide gel-filled capillary electrophoresis systems are compatible with the methods being used today by pharmaceutical companies to analysis and separate their biotechnology-derived proteins. It has the advantage of being fast, automated, non-labor intensive, and has improved quantification and reproducibilty compared to the SDS-PAGE technique. It has superiority over the HPSEC method since the HPSEC is unreliable in molecular weight determination. Currently there is more research being done on this new technology by Kiyoshi Tsuji and other research laboratories.

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Table 1.
Comparison of methods (scanner, manual, and linfit) for the determination of molecular weight of rbSt from SDS-PAGE.

rbSt Samples	Molecular Weight (daltons) Scanner	Manual	Linfit
Control Reference Standard			
Fragment	-	-	-
Monomer	20,600	20,000	20,700
?	26,700	24,000	24,000
?	31,800	27,600	28,100
Dimer	46,000	36,800	37,300
Trimer	64,700	58,200	58,000
#55,503 Sublot #2			
Fragment	-	-	-
Monomer	20,400	20,200	20,900
?	26,500		
?	31,800		
Dimer	46,200	36,000	36,700
Trimer	64,700	60,300	59,700
#55,503 Sublot #3			
Fragment	15,800	-	-
Monomer	21,200	20,000	20,700
?	26,800	24,000	24,000
?	31,500	29,000	30,000
Dimer	46,400	36,000	36,700
Trimer	65,600	60,300	59,700
Enriched Dimer Sample			
Fragment	15,800	-	-
Monomer	21,200	20,000	20,600
?	26,400	24,000	24,000
?	31,700	29,800	30,600
Dimer	43,100	38,000	39,200
Trimer	64,500	58,200	56,400

Table 2.
Results from the SDS-PAGE technique for different samples of rbSt. These results were obtained from two SDS-PAGE gels, which were scanned six times each.

	Average Molecular Weight	Average Standard Deviation	Average Relative Deviation
Fragment	15,800	689.0	4.36%
Monomer	20,800	449.0	2.16%
?	26,600	527.0	1.98%
?	31,700	976.0	3.08%
Dimer	46,200	1140.0	2.52%
Trimer	64,700	1660.0	2.55%

Table 3.
Comparison methods (scanner, manual, and linfit) for the determination of molecular weight of ATGAM from SDS-PAGE.

ATGAM Samples	Molecular Mass (daltons) Scanner	Manual	Linfit
#18494-DAS-135c			
L-chain	24,000	23,000	23,000
F_c Fragment	46,000	46,000	45,000
H-chain	57,000	55,000	57,000
$F_{(ab')2}$	99,000		
IgG	≥ 120,000	≥ 120,000	≥ 120,000
#918BK			
L-chain	25,000	25,000	24,000
F_c Fragment	46,000	40,000	38,000
H-chain	58,000	51,000	53,000
$F_{(ab')2}$	100,000		
IgG	≥ 110,000	≥ 120,000	≥ 120.000
#17,910 Fraction #1			
L-chain	24,000	25,000	24,000
F_c Fragment	47,000	40,000	39,000
H-chain	61,000	53,000	53,000
$F_{(ab')2}$	100,000		
IgG	≥ 110,000	≥ 120,000	≥ 120,000
Fraction #3			
L-chain	25,000	24,000	23,000
F_c Fragment	47,000	39,000	39,000
H-chain	56,000	55,000	56,000
$F_{(ab')2}$	99,000		
IgG	≥ 110,000	≥ 120,000	≥ 120,000
Fraction #9			
L-chain	23,000	24,000	23,000
F_c Fragment	46,000	39,000	39,000
H-chain	60,000	55,000	55,000
$F_{(ab')2}$	98,000		
IgG	≥ 110,000	≥ 120,000	≥ 120,000
Fraction #7a			
L-chain	24,000	24,000	23,000
F_c Fragment	47,000	45,000	44,000
H-chain	60,000	57,000	47,000
$F_{(ab')2}$	98,000		
IgG	≥ 110,000	≥ 120,000	≥ 120,000

Table 4.
Comparison of the methods (scanner, manual, and linfit) for the determination of molecular weight of hemoglobin from SDS-PAGE. This is the average of four different concentrations of hemoglobin.

Molecular Weight (daltons)		
Scanner	Manual	Linfit
36,000	28,200	28,300
35,000	26,900	26,800
14,000	16,200	17,000
13,000	15,800	16,400

Table 5.
Comparison of methods (scanner, manual, and linfit) for the determination of molecular weight of Hemopure 2 from SDS-PAGE.

Hemopure 2 Samples	Molecular Weight (daltons) Scanner	Manual	linfit
#H2C026			
	15,000	18,000	17,000
		28,000	28,000
	34,000	32,000	32,000
	42,000	40,000	40,000
	54,000	49,000	50,000
	68,000		
	72,000		
	83,000		
	≥91,000	≥90,000	≥96,000
#H2C028			
	15,000	18,000	17,000
	26,000	28,000	28,000
	34,000	33,000	33,000
	42,000	41,000	41,000
	54,000	49,000	50,000
	68,000		
	71,000		
	83,000		
	≥90,000	≥90,000	≥96,000
#H2C031			
	15,000	17,000	17,000
		28,000	28,000
	34,000	32,000	32,000
	41,000	41,000	41,000
	54,000	49,000	50,000
	68,000		
	72,000		
	83,000		
	≥91,000	≥90,000	≥96,000

Table 6.
Molecular weight determination of rbSt by the Beckman SDS non-acrylamide based gel-filled capillary column (100um id x 40 cm).

rbSt Samples	Molecular Weight (daltons)
Control Reference	20,500
Standard	31,300
#55,503	20,400
Sublot #1	31,400
#55,503	20,100
Sublot #2	31,800
Enriched Dimer	17,800
	34,400
	45,700
	58,400

Table #7.
Molecular weight determination of ATGAM by the Beckman SDS non-acrylamide based gel-filled capillary column (100 i.d. x 40 cm).

ATGAM Samples	Molecular Weight (daltons)
#918BK	
reduced	38,000
	63,200
non-reduced	246,000
#18494-DAS-135C	
reduced	37,000
	63,000
non-reduced	247,100
#17,910	
Fragment 1	
reduced	67,800
	≥150,000
Fragment 3	
reduced	46,800
	51,000
	68,300
	114,900
	≥150,000
Fragment 9	
reduced	28,100
	41,200
	55,200
	≥150,000
Fragment 7A	
reduced	27,400
	40,600
	46,000
	≥150,000
non-reduced	23,200
	35,100
	56,900
	≥150,000

Table #8.
Moleculare weight determination of hemoglobin by the Beckman SDS non-acrylamide based gel-filled capillary column (100 um id x 40 cm).

Concentration	Molecular Weight (daltons)
0.1 mg/ml	14,400
0.2 mg/ml	14,700 23,800
0.5 mg/ml	14,600 23,200
0.75 mg/ml	14,600 22,800
1.0 mg/ml	15,500 23,300

Table #9.
Molecular weight determination of Hemopure 2 by the Beckman SDS non-acrylamide based gel-filled capillary column (100 i.d. z 40 cm).

Hemopure 2 Samples	Molecular Weight (daltons)
H2C026	13,900
	24,600
	36,000
	46,500
	57,600
	69.600
	90,500
	≥100,000
H2C028	13,700
	24,000
	35,200
	45,500
	55,800
	68,400
	88,100
	≥100,000
H2C031	12,400
	21,800
	31,500
	41,800
	53,300
	81,800
	≥100,000

Table #10.
Molecular weight determination for the different recombinant proteins by the ABI SDS non-acrylamide based gel-filled capillary system (50 um i.d. x 20 cm).

Recombinant Protein	Molecular Weight (daltons)
rbSt	18,900
	31,000
ATG	
non-reduced	103,300
	138,300
	320.900
reduced	31,300
	56,700
Hemoglobin	16,400
	25,600
Hemopure 2	17,600
	25,600
	40,400
	51,600
	66,300
	81,400
	109,500
	$\geq$130,000

Table 11.
Comparison of Analytical Methods for the Determination of Molecular Mass of Recombinant Proteins. SDS Gel-Filled Capillary Columns from ABI (50 µm id x 20 cm) and Beckman (100 µm id x 40 cm) were used at 30° C. and 20° C., respectively.

Recombinant Proteins	Molecular Mass (daltons)					
	SDS Gel-Filled Capillary		SDS-PAGE	HPSEC	Theory	
	ABI	Beckman				
rbSt	13,500	17,800	15,800	11,900		
	18,900	21,300	20,800	19,200, 36,900	monomer:	21,812
	42,200	45,700	46,200	43,500, 80,300		
	80,800	> 58,400	64,700	68,000, 113,000		
	131,000		> 126,000			
ATGAM	31,300	27,700	24,000		L-chain:	23,000
	56,700	60,500	58,000		H-chain:	50,000
	43,000	40,700	46,000		Fc fragment:	46,000
	95,300		99,000		$F_{(ab')2}$:	100,000
	142,000	152,000	> 100,000	207,000	IgG:	150,000
Hemoglobin	16,400	14,400	15,800	-	α-chain:	15,053
	25,600	23,200	16,200		β-chain:	15,854
Hemopure	17,600	13,300	15,000	-	monomer: α- and β-chain	
	25,600	23,500	28,000	17,600	dimer	
	40,400	34,200	34,000			
	51,600	44,600	42,000	31,700	tetramer	
	66,300	69,000	68,000			
	81,400	86,800	83,000			
	109,000		> 91,000	100,000		
	> 150,000	> 178,000				

Figure 1. Picture of SDS-PAGE gel for rbSt. The gel percentage (Acrylamide:Bis) was 15%. Lane assignments, 2 and 7: BioRad low molecular weight standard; 3: Control Reference Standard; 4: lot #55.503 sublot #2; 5: lot #55,503 sublot #3; and 6: Enriched dimer sample.

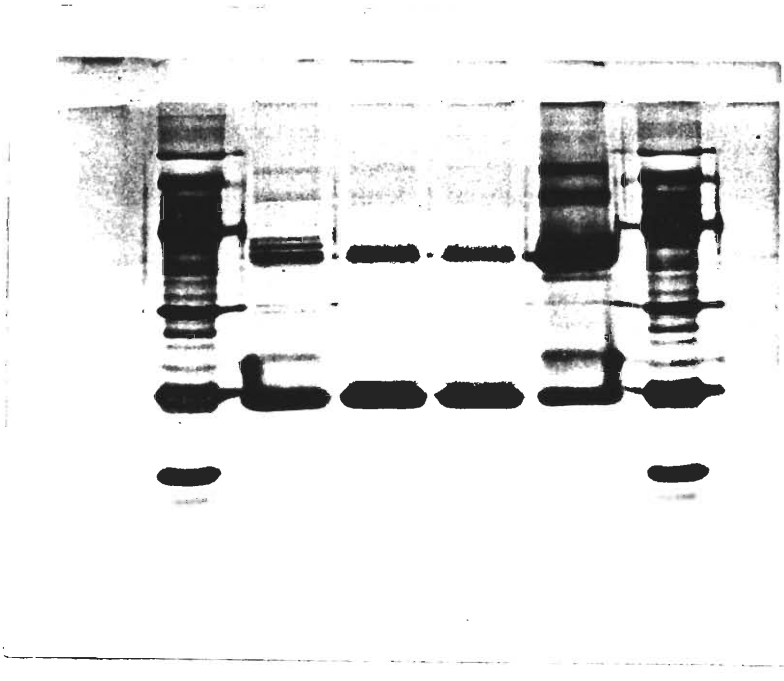

Figure 2. The scan of the SDS-PAGE gel for rbst. The gel percentage (Acrylamide:Bis) was 15%. Lane assignments, 2 and 7: BioRad low molecular weight standard; 3: Control Reference Standard; 4: lot #55.503 sublot #2; 5: lot #55,503 sublot #3; and 6: Enriched dimer sample.

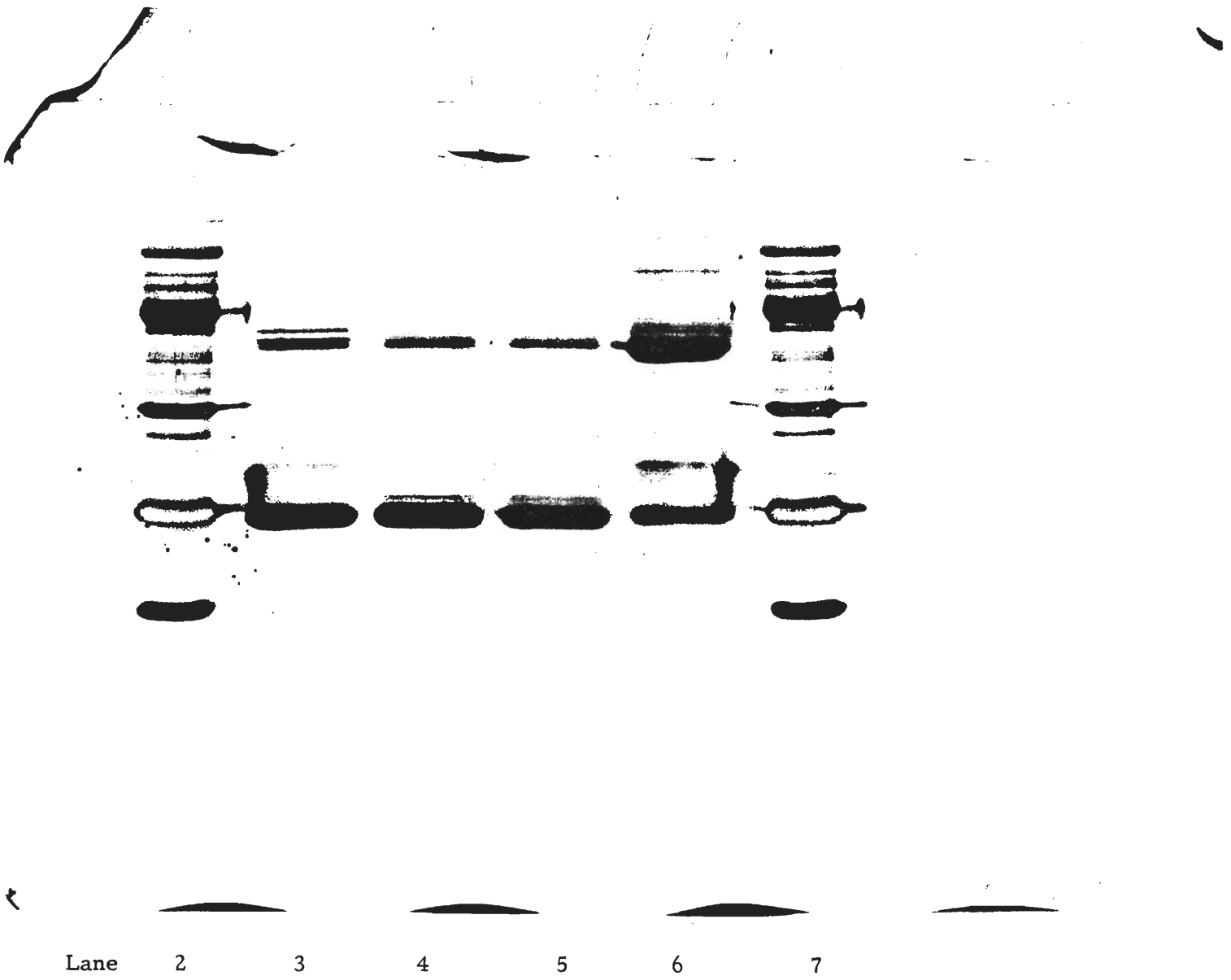

Figure 3. Linegraph obtained from the Image Quant software for the enriched dimer sample on the SDS-PAGE gel of rbSt. The peaks of rbSt are #6 for the trimer, #8 for the dimer, and #13 for the monomer. The first peak under #13 was the peak used to determine the molecular weight.

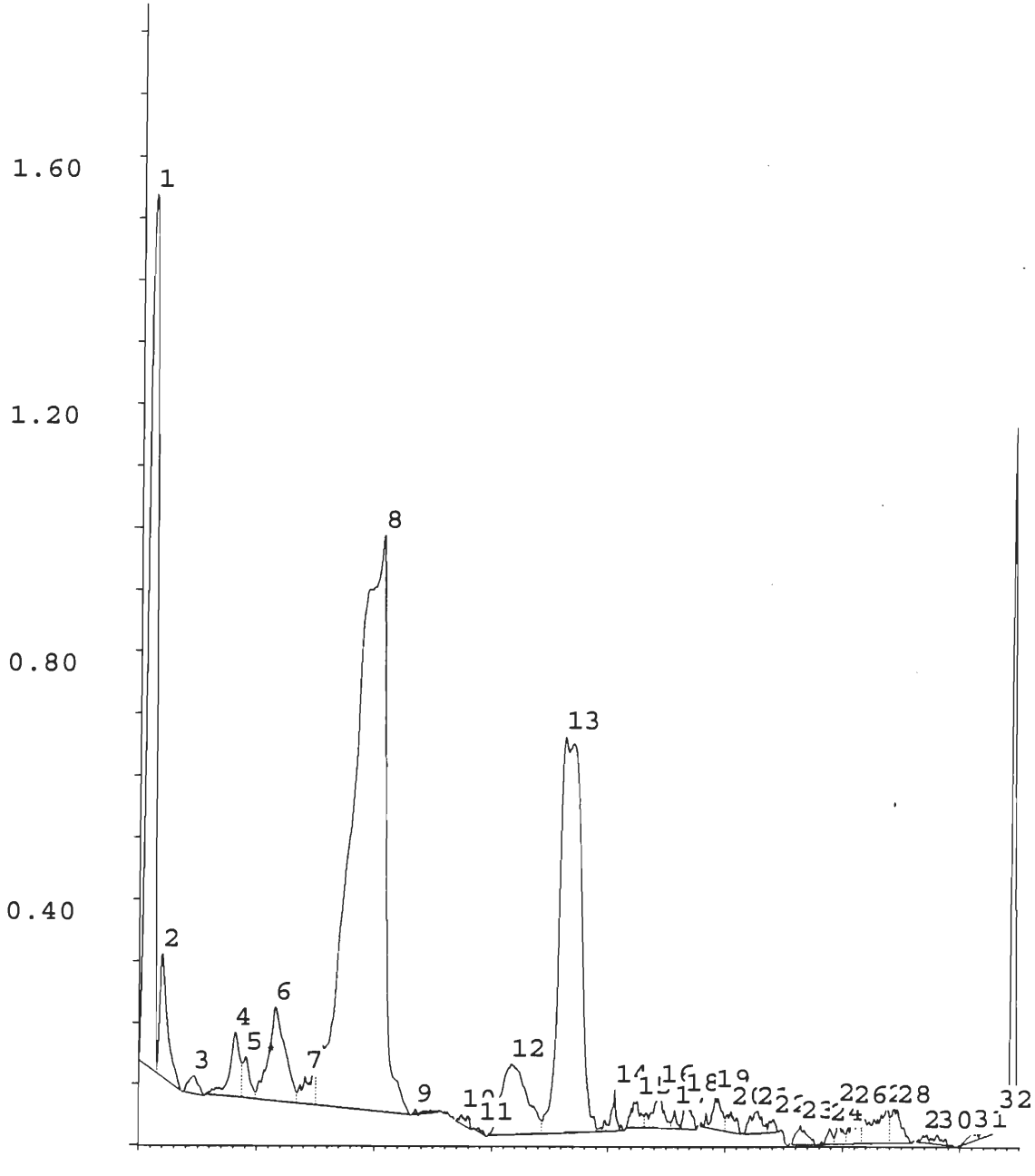

Figure 4. The scan of the SDS-PAGE gel for ATGAM. The gel percentage (Acrylamide:Bis percentage) was 12%. Lane assignments, 2 and 9: BioRad low molecular weight standard; 3: lot #17,910 Fraction #1; 4: lot #17,910 Fraction #3; 5: lot #17,910 Fraction 9; 6: lot #17,910 Fraction #7; 7: lot #18494-DAS-135c; and 8: lot #918BK.

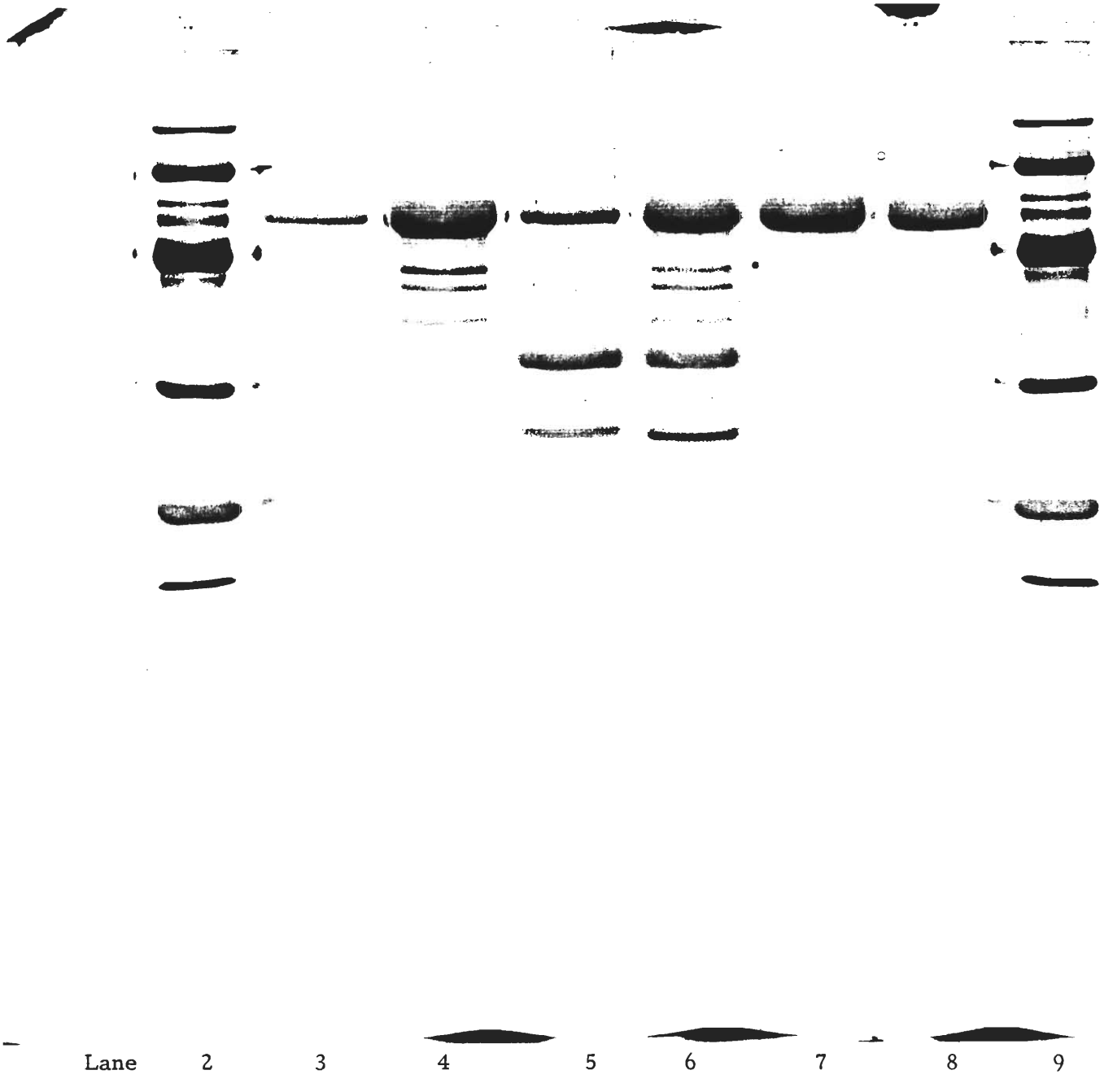

Figure 5. The scan of the SDS-PAGE gel for hemoglobin. The gel percentage (Acrylamide:Bis percentage) was 17%. Lane assignments, 2, 7, and 8: BioRad low molecular weight standard; 3: 0.7 mg/ml conc.; 4: 0.5 mg/ml conc.; 5: 0.2 mg/ml conc.; and 6: 0.1 mg/ml conc.

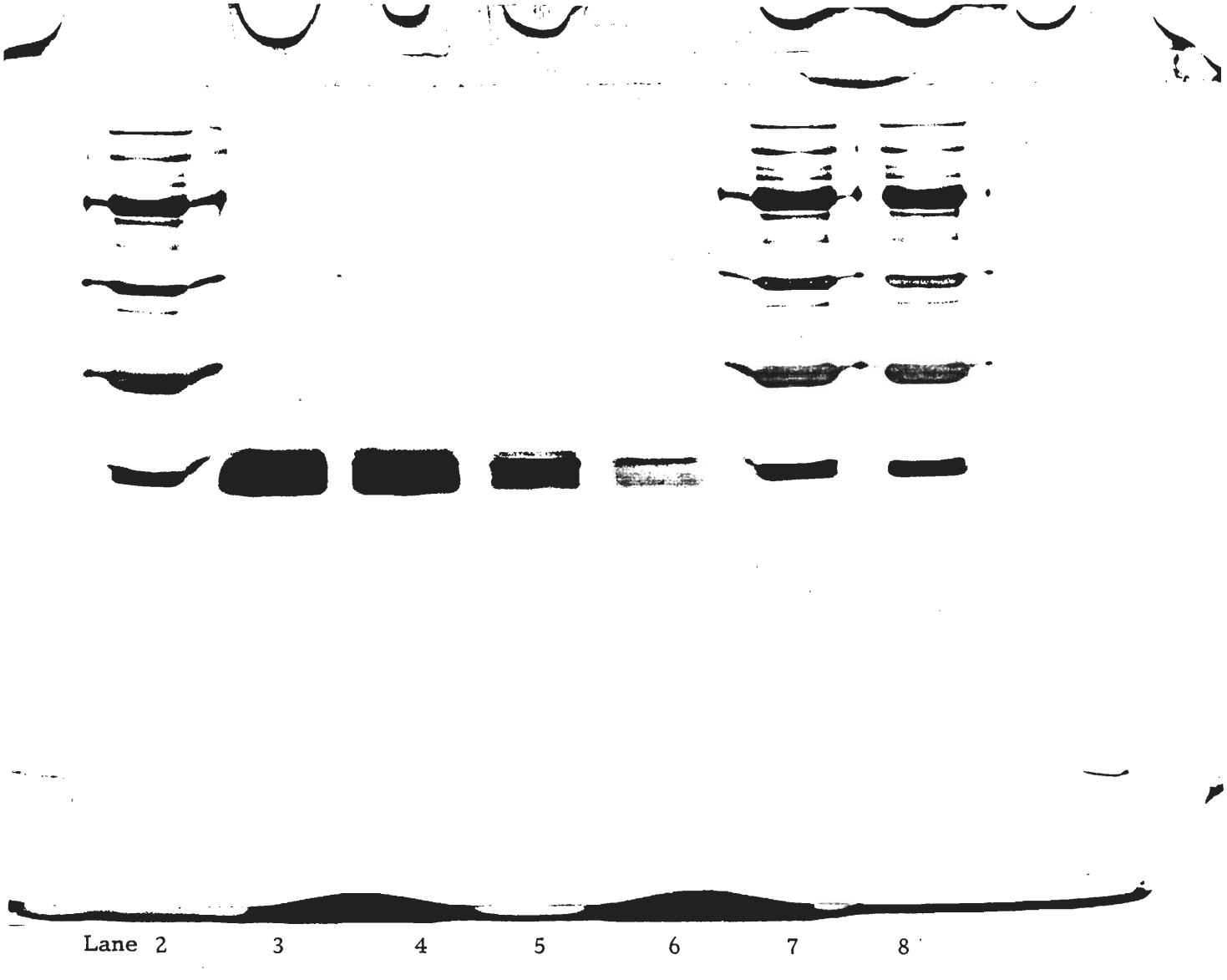

Figure 6. The scan of the SDS-PAGE gel for Hemopure 2. The gel percentage (Acrylamide:Bis percentage) was 15%. Lane assignments, 2 and 9: BioRad low molecular weight Standard; 3 and 4: lot #H2C026; 5 and 6: lot #H2C028; and 7 and 8: lot #H2C031.

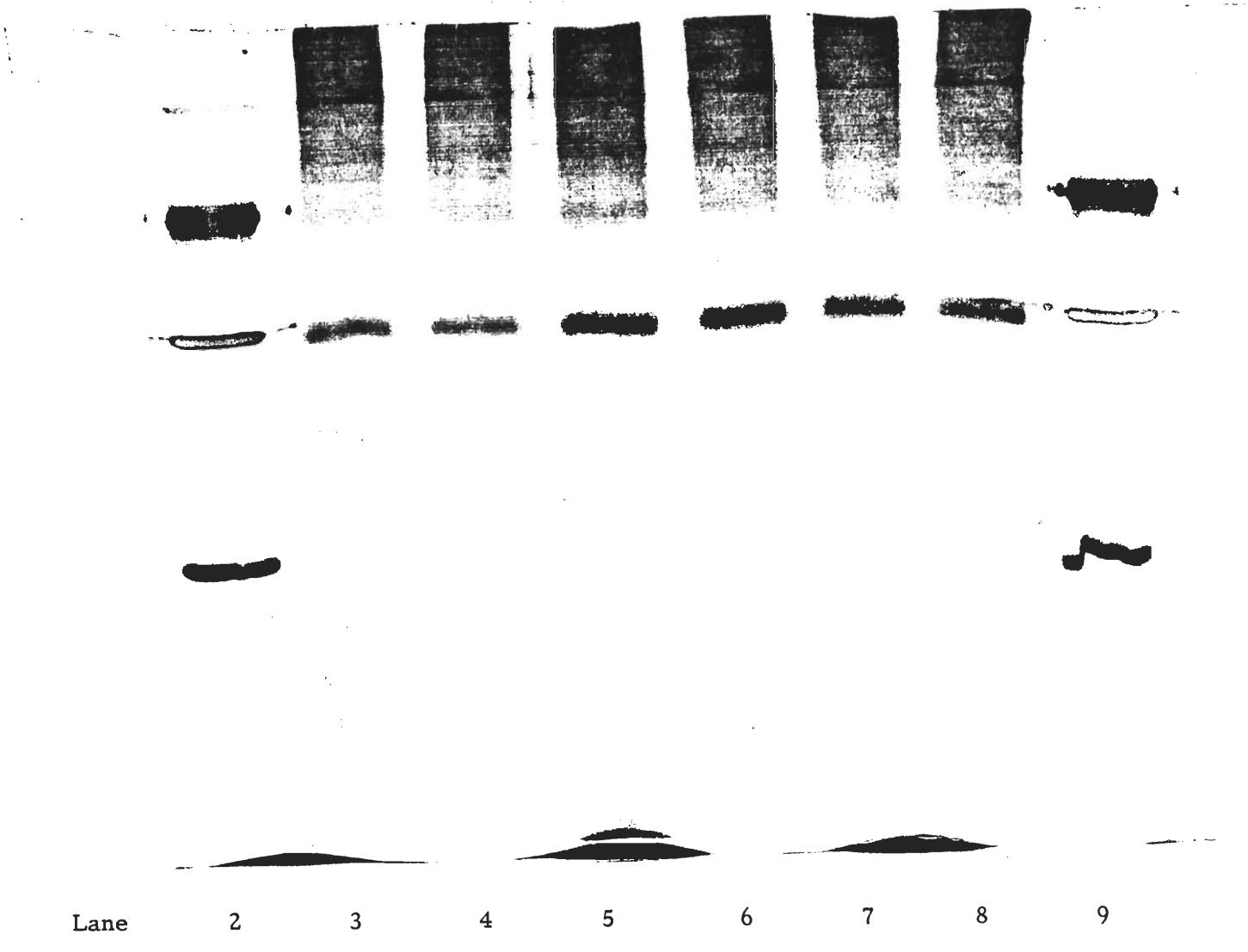

Figure 7. Line graph of 0.1 mg/ml conc. of hemoglobin. Peak #6 is the beta chain and peak #7 is the alpha chain.

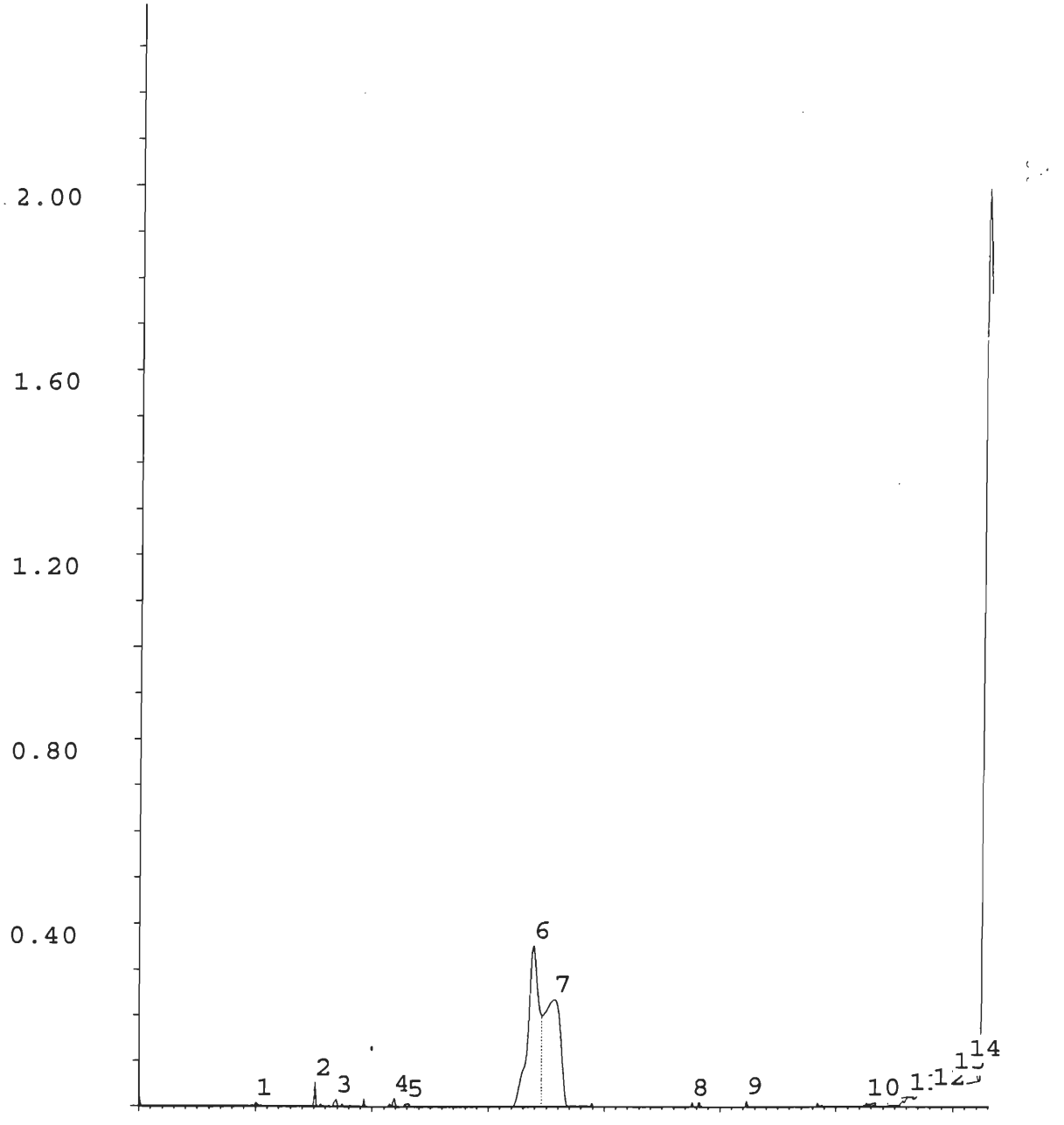

Figure 8. SDS gel-filled capillary electrophoetic separation of the molecul protein reference standard as monitored at 214 nm using the ABI SDS ge capillary system. Peak migration order, A: lysozyme (14,400 daltons); B: inhibitor (21,500); C: carbonic anhydrase (31,000); D: ovalbumin (45,000); (66,400); and F: phosphorylase b (97,400). Conditions, -300 V/cm (20 uA); temperature: 30°C; effective peak migration distance: 20 cm; bare fused si capillary: 50 um i.d.; running buffer: SDS non-acrylamide based based gel from ABI.

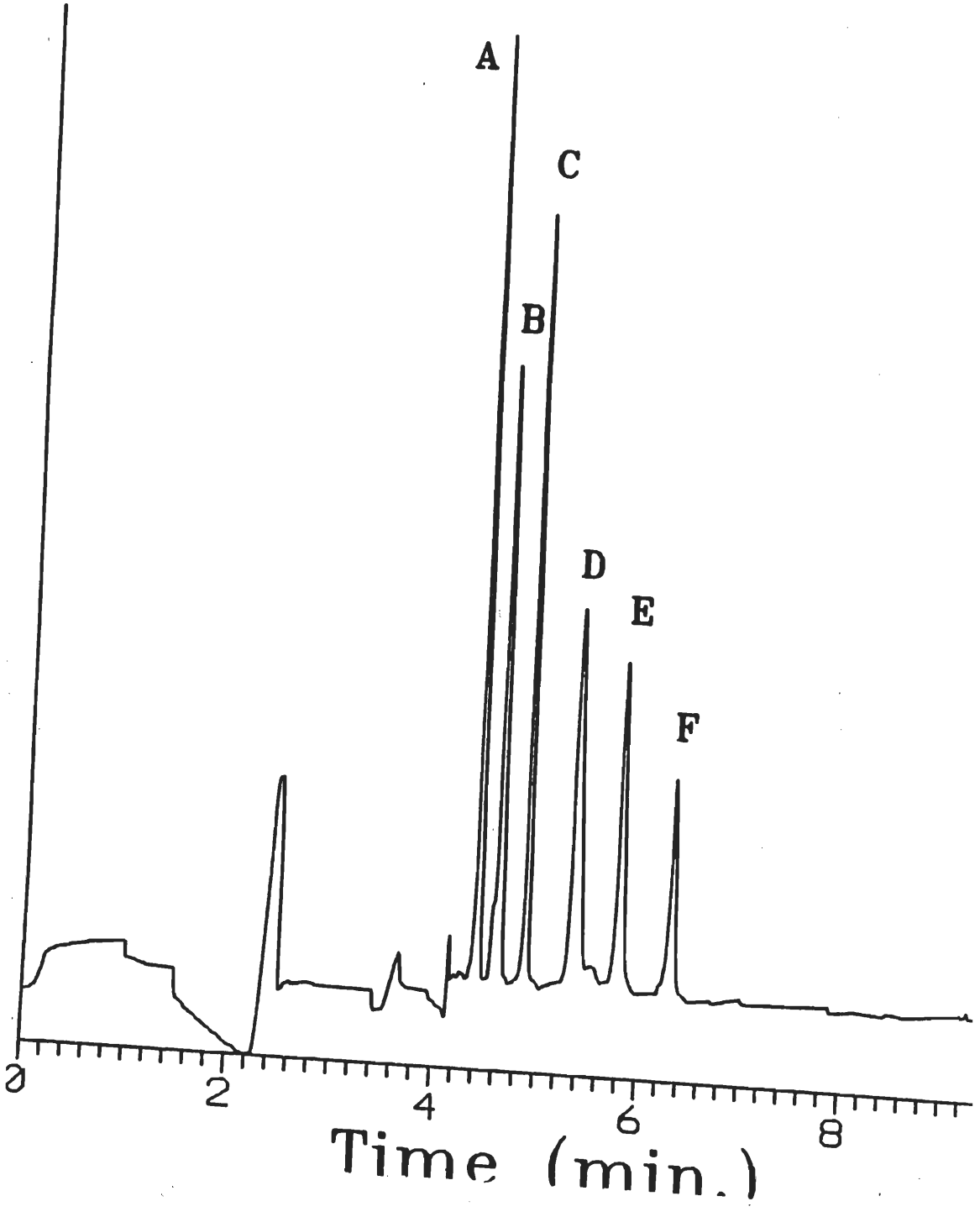

Figure 9. SDS gel-filled capillary electrophoretic separation of the molecular mass protein reference standards as monitored at 214 nm for the Beckman gel-filled capillary system. Peak migration order, A: lysozyme (14,400 daltons); B: trypsin inhibitor (21,500); C: carbonic anhydrase (31,000); D: ovalbumin (45,000); E: BSA (66,400); and F: phosphorylase b (97,400). Conditions, -300 V/cm (20 uA); column temperature: 20°C; effective peak migration distance: 40 cm; bare fused silica capillary: 100 um i.d.; running buffer: SDS non-acrylamide based based gel solution from Beckman.

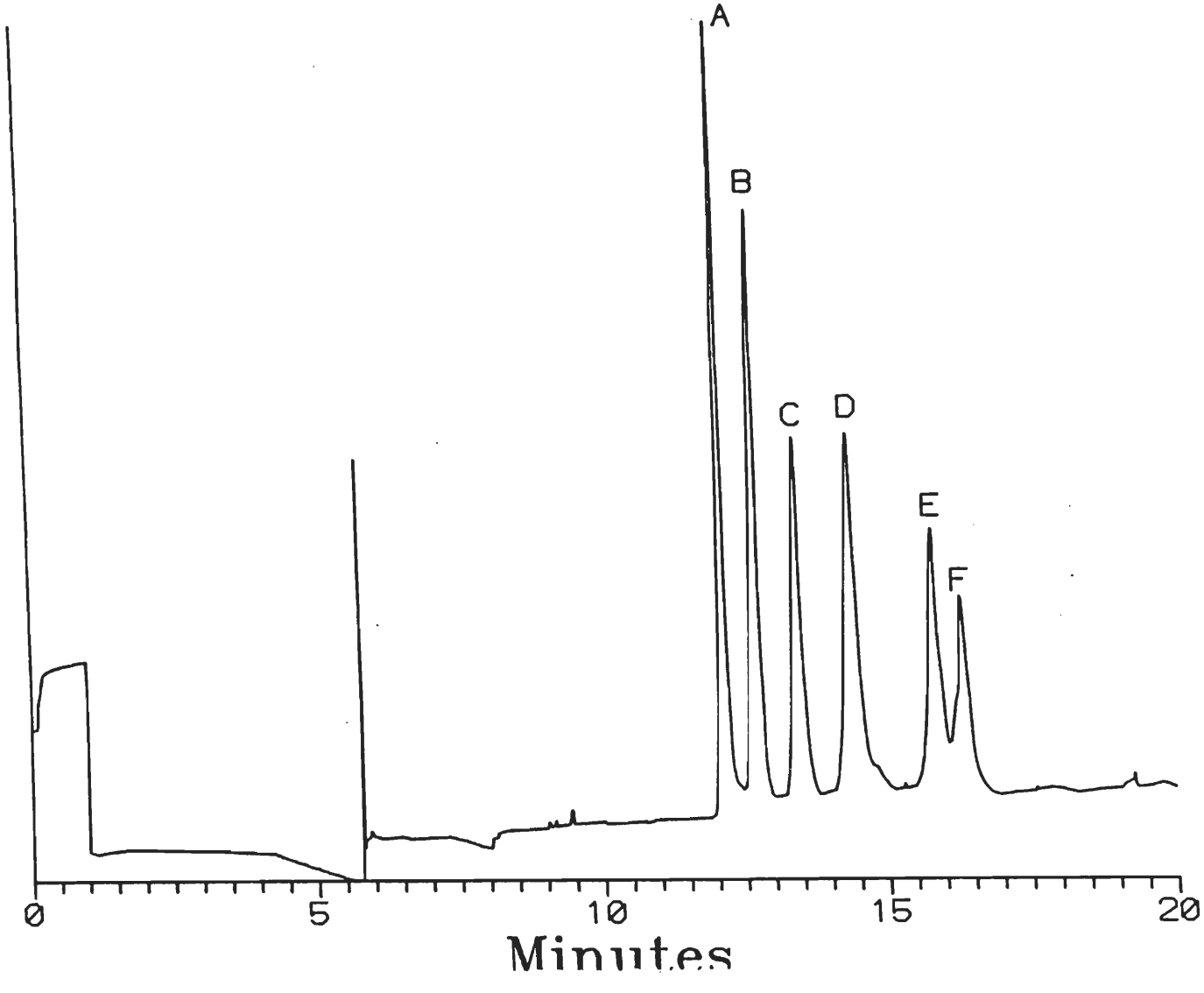

Figure 10. SDS non-acrylamide gel-filled capillary electropherogram of an rbSt sample as monitored at 214 nm indicating base-line resolution of monomer and dimer peaks. Conditions, -300 V/cm (20 uA); column temperature: 30°C; effective peak migration distance: 20 cm; bare fused silica capillary: 50 um i.d.; running buffer: SDS non-acrylamide based based gel solution from ABI. Peak identification, A: monomer; B: dimer.

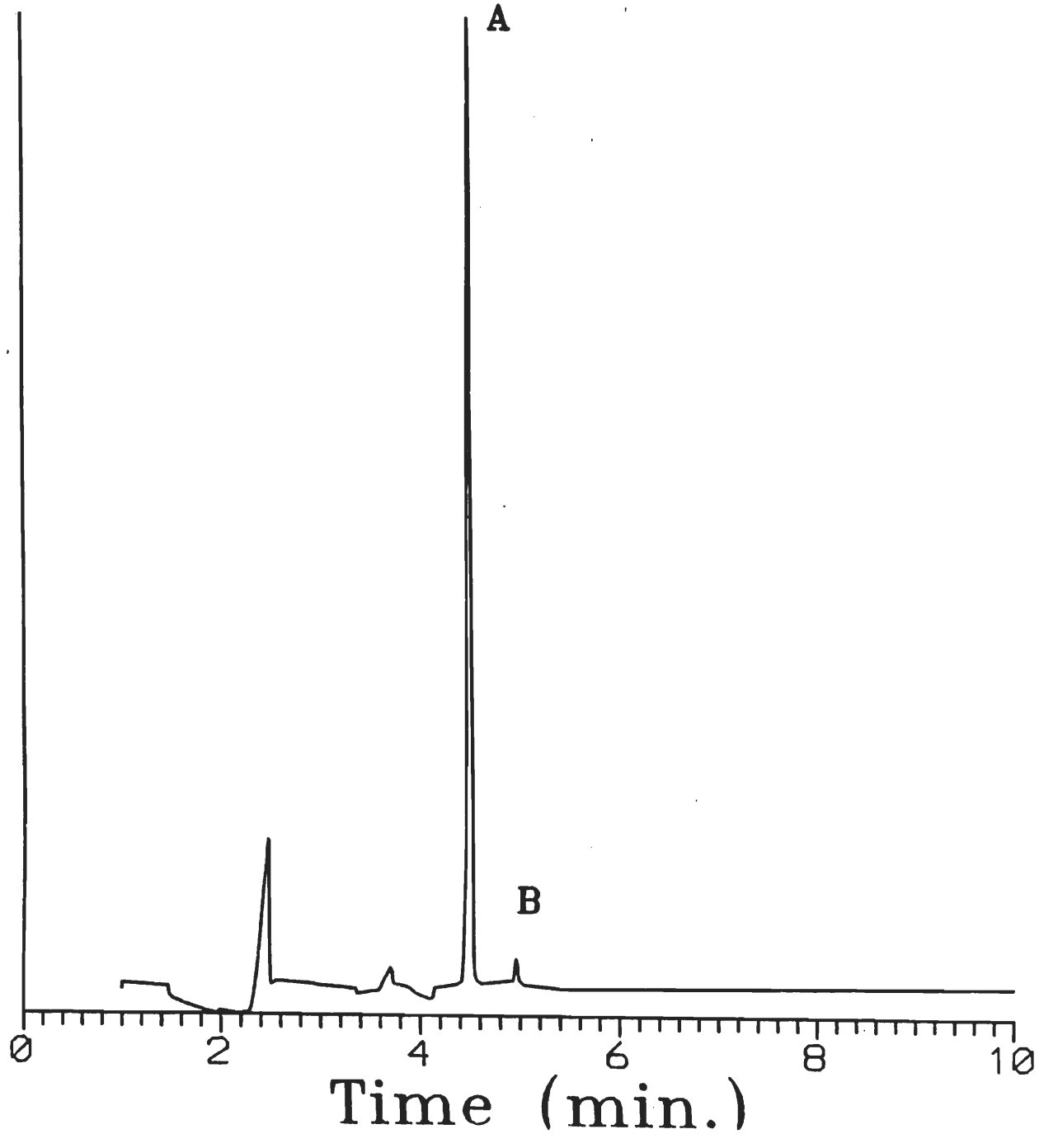

Figure 11. SDS gel-filled capillary electropherogram of the dimer enriched rbSt sample indicating a base-line resolution of monomer, dimer, trime, and tetramer peaks. Conditions, -300 V/cm (20 uA); column temperature: 20°C; effective peak migration distance: 40 cm; bare fused silica capillary: 100 um i.d.; running buffer: SDS non-acrylamide based based gel solution from Beckman.
Peak identification, A: monomer; B: dimer; C: trimer; D: tetramer.

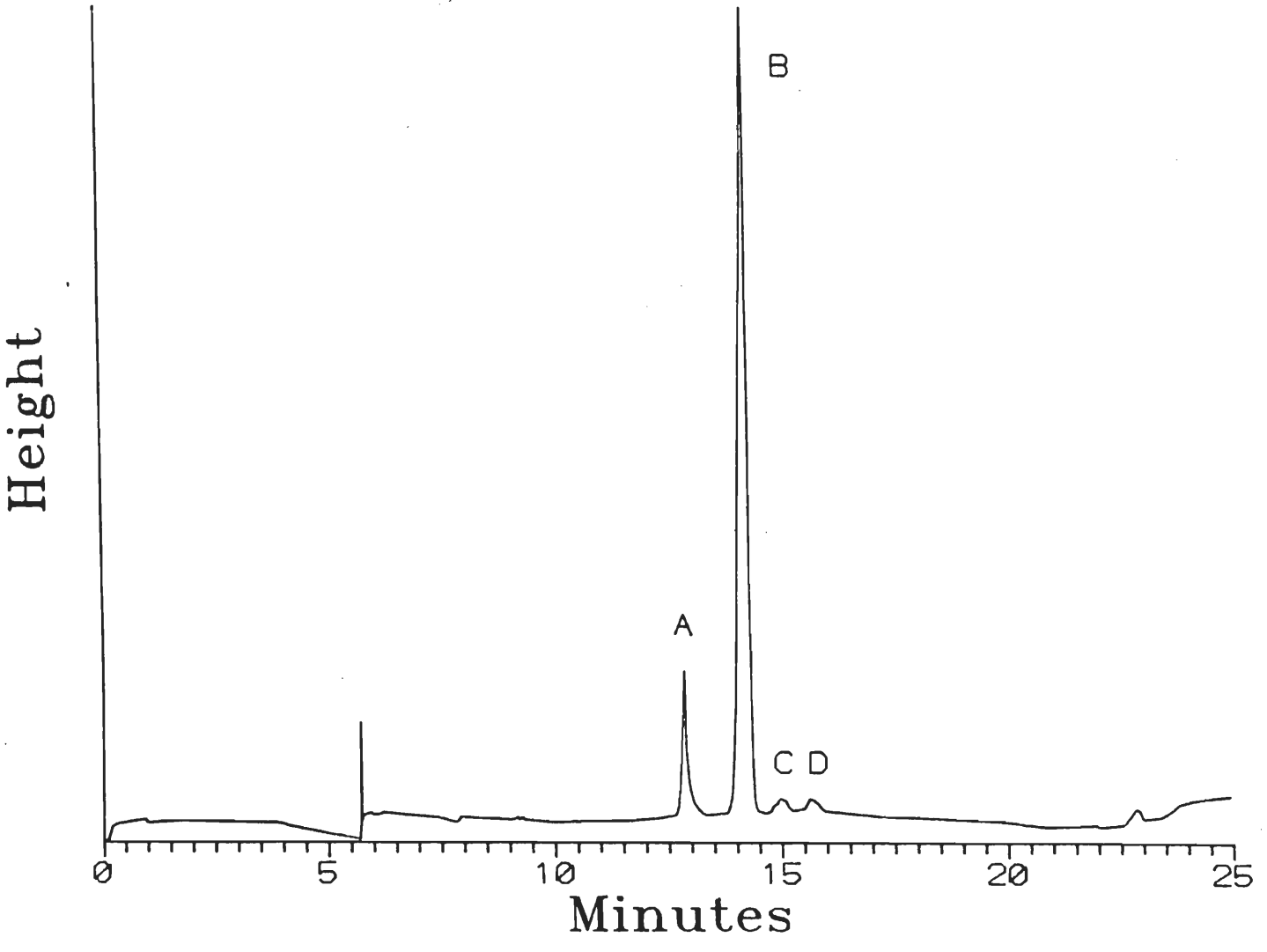

Figure 12. SDS non-acrylamide gel-filled capillary electropherogram of a reduced and non-reduced samples of polyclonal IgG as monitored at 214 nm indicating resolution of L- and H-chaims and the intact IgG molecule. Conditions, -300 V/cm (20 uA); column temperature: 30°C; effective peak migration distance: 20 cm; bare fused silica capillary: 50 um i.d.; running buffer: SDS non-acrylamide based based gel solution from ABI.

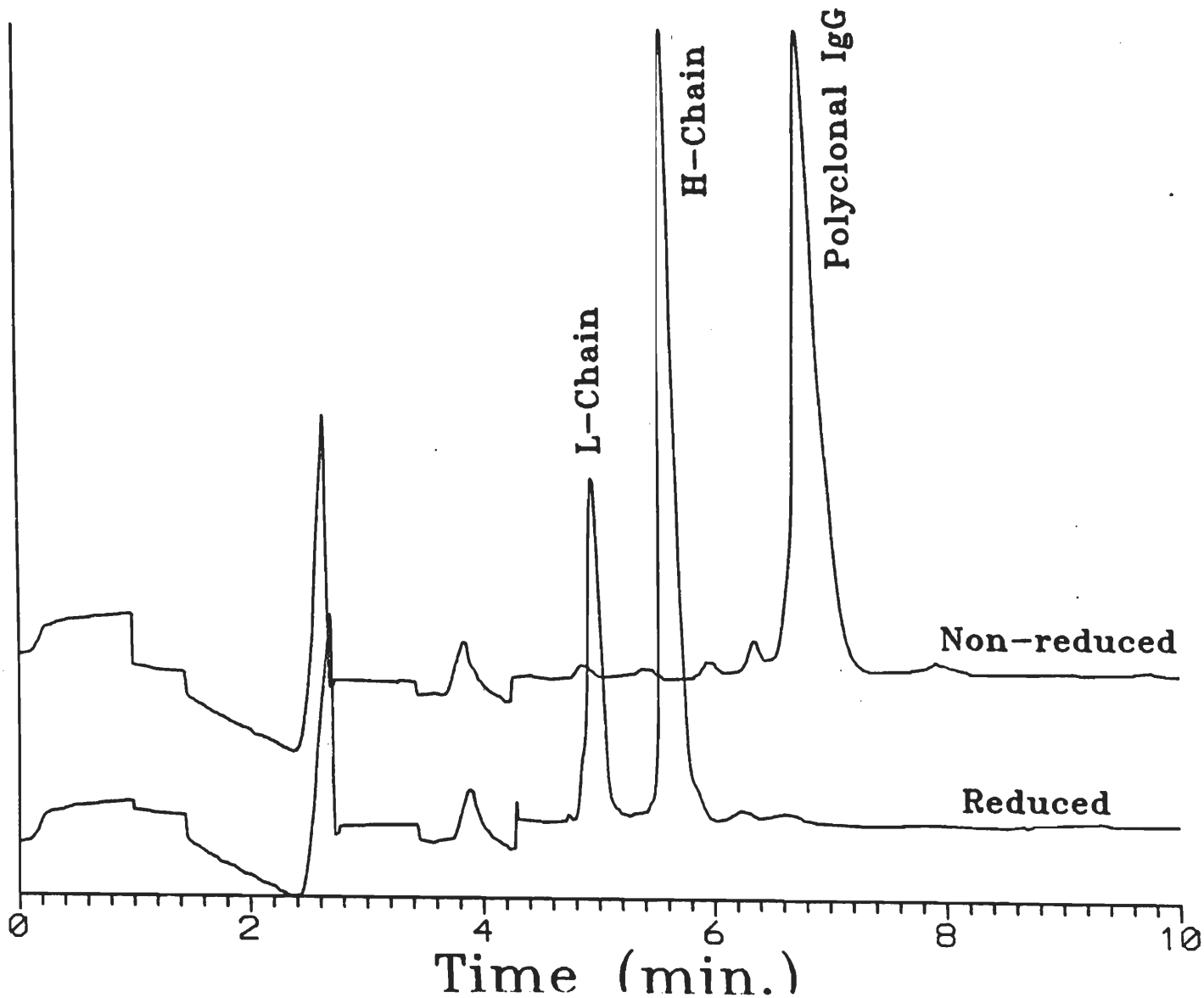

Figure 13. SDS non-acrylamide gel-filled capillary electropherogram of hemoglobin wih a faint indication fro the presence of the alph- and beta-subunit peaks (15,053 and 15,954 daltons). Conditions, -300 V/cm (20 uA); column temperature: 30°C; effective peak migration distance: 20 cm; bare fused silica capillary: 50 um i.d.; running buffer: SDS non-acrylamide based based gel solution from ABI.

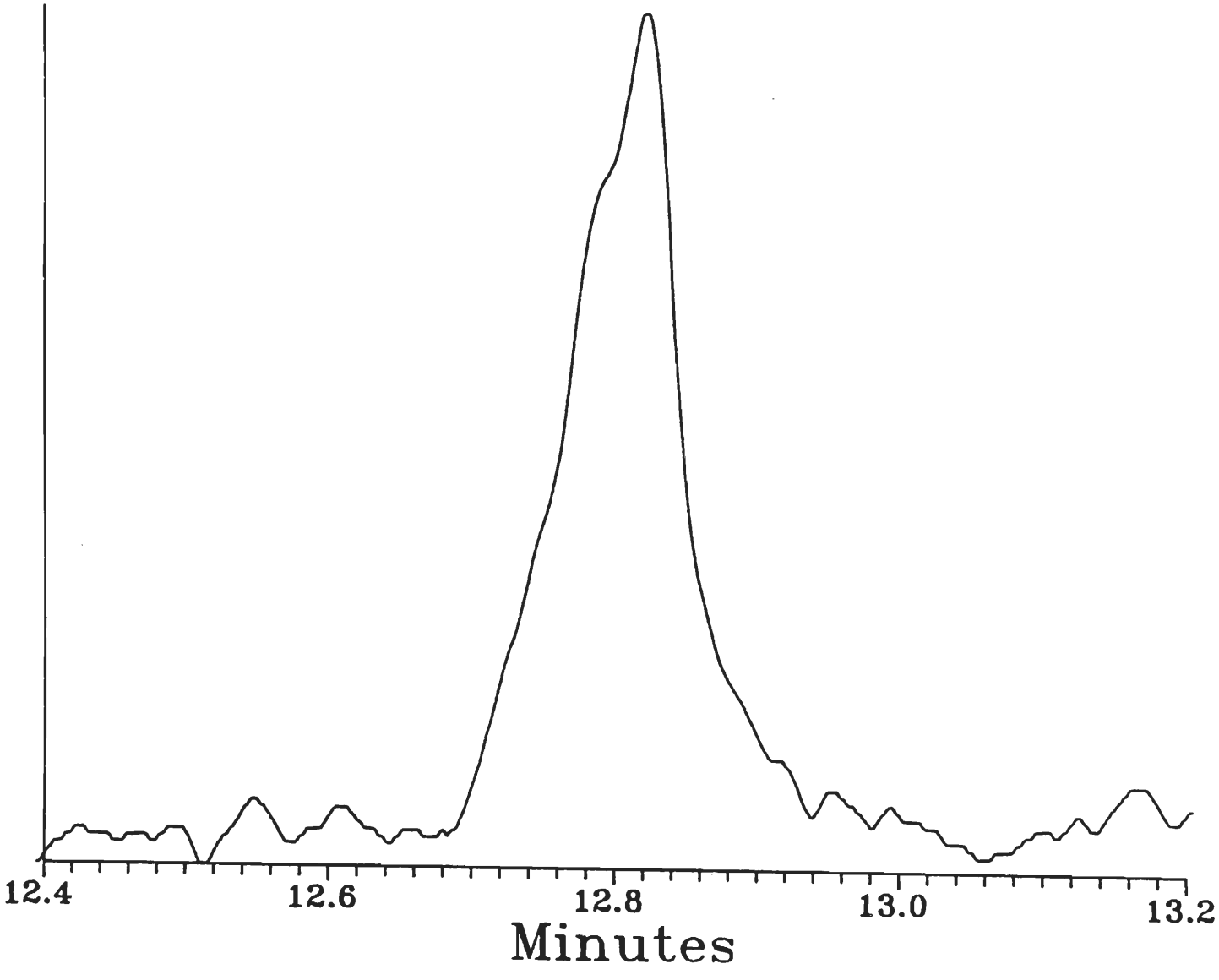

Figure 14. SDS non-acrylamide gel-filled capillary electropherogram of Hemopure 2. Conditions, -300 V/cm (20 uA); column temperature: 30°C; effective peak migration distance: 20 cm; bare fused silica capillary: 50 um i.d.; running buffer: SDS non-acrylamide based based gel solution from ABI.