Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 703–708
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A one-step, competitive electrochemiluminescence-based immunoassay method for the quantification of a fully human anti-TNFα antibody in human serum

D. Horninger, E. Eirikis, C. Pendley, J. Giles-Komar, H.M. Davis, B.E. Miller*

Department of Clinical Pharmacology and Experimental Medicine, Centocor, Inc., 145 King of Prussia Road, Radnor, PA 19087, USA

Accepted 26 January 2005
Available online 11 March 2005

Abstract

A quantitative, one-step, competitive electrochemiluminescence (ECL)-based immunoassay for the determination of a fully human, anti-TNFα monoclonal antibody in human serum has been developed. A biotinylated, mouse anti-variable region-specific antibody and a ruthenium-labeled anti-TNFα antibody were the only specific reagents needed to develop the assay. A single incubation step of 2 h followed by ECL detection was used. The assay was capable of measuring the analyte in neat serum over approximately a 1600-fold range with higher concentrations measured following a single dilution. Assay accuracy, precision, and reproducibility were suitable to support pharmacokinetic studies of the analyte. This competitive assay format offers an alternative approach to the development of immunoassays for the measurement of macromolecules in complex matrices to support preclinical and clinical studies.

Keywords: Electrochemiluminescence; Competitive immunoassay; Anti-TNFα; Monoclonal antibody

1. Introduction

Conventional analytical methods such as HPLC and mass spectrometry are typically not suitable for the quantification of macromolecule therapeutics in serum or plasma samples from preclinical or clinical studies. The quantification of macromolecules with sufficient fidelity to support pharmacokinetic studies usually requires the development and validation of a suitable immunoassay [1]. Frequently, sandwich-type ELISA methods are employed to achieve the desired sensitivity and selectivity. These assays require the development of antibody reagents that bind specifically to the therapeutic macromolecule. In the case of fully human therapeutic monoclonal antibodies, it can be difficult to generate a pair of suitable antibody reagents because of the lack of sufficient unique amino acid sequences that do not overlap with endogenous immunoglobulin sequences. Besides the limitation of antibody reagents, conventional ELISA methods generally involve multiple incubation and wash steps making them both time-consuming and labor intensive. In addition, ELISA methods often have a limited dynamic range, necessitating multiple sample dilutions to measure the full range of concentrations necessary to determine the pharmacokinetic profile of a macromolecule.

Electrochemiluminescence (ECL) provides an alternative to conventional colorimetric methods [2,3]. ECL is a technique that allows for high sensitivity, good reproducibility, and generally little interference from components in complex matrices such as serum or plasma [3,4]. Several multi-step, non-competitive sandwich ECL methods suitable for use as assays to support pharmacokinetic studies have been reported [5,6]. The high sensitivity possible with ECL should also allow ECL detection to be used to develop competitive immunoassays that require only a single analyte-specific antibody.

This paper describes the development of a one-step, competitive electrochemiluminescence-based immunoassay

* Corresponding author. Tel.: +1 610 651 7336.
E-mail address: bmiller4@cntus.jnj.com (B.E. Miller).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.01.036

method for the quantification of a novel fully human anti-TNFα monoclonal antibody. In this method, the antibody in the test sample competes with Ru-labeled antibody for binding to an immobilized anti-variable region monoclonal antibody. The method requires a single incubation of 2 h, is highly sensitive, and has the required accuracy and precision to support pharmacokinetic studies. The method has a dynamic range of greater than 3 logs and the range of sample concentrations expected in clinical study samples can be quantified in neat serum or after a single dilution.

2. Experimental

2.1. Reagents

Phosphate-buffered saline, Ca^{++}- and Mg^{++}-free (pH 7.8) was obtained from Cellgro (Herndon, VA). Bovine serum albumin (fraction V), complete and incomplete Freund's adjuvants, and glycine were from Sigma Chemical Co. (St. Louis, MO). Tween-20 was from Mallinckrodt (Paris, KY). Dimethyl sulfoxide was from EM Science (Gibbstown, NJ). Bovine IgG standard was from Pierce Chemical Co. (Rockford, IL). Biotin-LC-Sulfo-NHS Ester, ORI-TAG®-NHS ester (ruthenium (II) tris-bipyridine, *N*-hydroxysuccinimide), Dynabeads® M-280 streptavidin-coated magnetic beads, ORIGLO plus solution, and ORICLEAN plus solution were obtained from IGEN International, Inc. (Gaithersburg, MD). Normal human serum was obtained, under informed consent, from volunteer donors in the Centocor, Inc. blood donor database. Serum from rheumatoid arthritis patients was obtained from Bioreclamation, Inc. (Hicksville, NY).

2.2. Preparation of biotinylated mouse anti-variable region monoclonal antibody

To generate anti-variable region antibodies, female Balb/c mice (Charles River Laboratories, USA) were immunized by intraperitoneal injection with 25 μg of the anti-TNFα antibody in Freund's complete adjuvant. After two subsequent intraperitoneal injections with the anti-TNFα antibody in incomplete Freund's adjuvant and a final intravenous boost, individual spleens were collected and cell fusions performed with the mouse myeloma cell line FO (American Type Culture Collection, MD, USA) according to the method of Groth and Scheidegger [7]. Hybridomas specific for the anti-TNFα variable region were determined by antigen capture ELISA and subsequently subcloned by limiting dilution. One anti-variable region monoclonal antibody was selected for assay development, and the IgG was purified from culture supernatant by Protein A chromatography.

The anti-variable region antibody was conjugated with biotin using Biotin-LC-Sulfo-NHS Ester with a commercial kit according to the manufacturer's instructions (IGEN International, Inc.). Briefly, anti-variable region IgG (1 mg) was dissolved in PBS (1 mL). The biotin was prepared at 2 mg/mL in sterile, distilled water. A 20:1 challenge ratio of biotin:IgG was used. The solution was incubated for 60 min at room temperature and quenched with glycine (20 μL, 2 M). The biotinylated antibody was purified and the buffer exchanged with PBS using a PD-10 size exclusion column (Amersham Biosciences, Uppsala, Sweden). Protein concentration was determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL) using bovine IgG as the standard. The biotin conjugate was stored in PBS at 4 °C.

2.3. Preparation of ruthenium-labeled human anti-TNFα

The anti-TNFα antibody was labeled with ruthenium using ORI-TAG®-NHS ester. The anti-TNFα antibody was prepared in PBS (1 mg/mL). DMSO (50 μL) was added to a tube containing 75 μg of ORI-TAG®-NHS ester. To achieve an 8:1 ORG-TAG®-NHS ester:IgG challenge ratio, 37.6 μL of the ORI-TAG-NHS ester solution was added to the 1 mL of IgG. This solution was incubated at room temperature for 60 min followed by the addition of glycine (20 μL, 2 M). Following the addition of glycine, the solution was incubated at room temperature an additional 10 min. The labeled antibody was purified and the buffer exchanged with PBS using a PD-10 size exclusion column. Protein concentration was determined with the BCA protein assay kit using bovine IgG as the standard. The ruthenium-conjugated anti-TNFα was stored in PBS at 4 °C.

2.4. Competitive ECL immunoassay

The anti-TNFα antibody standard stock solution was prepared in PBS (20 mg/mL). A working solution was prepared in PBS at a concentration of 1 mg/mL. The standard curve concentrations were prepared from this working solution by serial dilution with pooled, normal human serum and covered the range 10–300,000 ng/mL. Pooled, normal human serum was used as the 0 standard. The biotinylated anti-variable region monoclonal antibody, streptavidin-coated magnetic beads, and the Ru-labeled anti-TNFα antibody were titrated to provide an approximately linear relationship over the range of quantification. An assay master mix consisting of Ru-labeled anti-TNFα antibody (62.5 ng/mL), biotinylated anti-idiotypic antibody (250 ng/mL), and Dynabeads® M-280 streptavidin-coated magnetic beads (50 μg/mL) was prepared. The assay was performed in 96-well round bottom polypropylene plates (Costar, NY, USA). Standard or serum sample (25 μL) and the master mix (200 μL) were added to the appropriate wells. All standards and samples were analyzed in duplicate unless indicated otherwise. Plates were incubated for 2 h at room temperature on a plate shaker at 400 ± 100 rpm (Titer Plate Shaker Model 4625, Labline, Melrose Park, IL). Following the incubation period, ECL was read on an ORIGEN analyzer (ORIGEN M-Series 384 Analyzer, IGEN International, Inc., MD, USA). The standard curve was established using a four-parameter logistic equation (Soft-

maxPro Software, v 3.1.2, Molecular Devices Corp., CA, USA). The concentration of the anti-TNFα antibody in test samples was determined by interpolation from the standard curve.

3. Results

3.1. Standard curve and range of quantification

Representative standard curves from five separate assays performed over a 3-week period are shown in Fig. 1 . The standard curve ranged from 10 to 300,000 ng/mL. The standard curve was prepared with neat, pooled, normal human serum. Preliminary experiments had determined that in order to achieve acceptable accuracy and precision with test samples, a serum matrix was required for the standard curve (data not shown). Performance parameters for the standard curve are shown in Table 1. Examination of the back-calculated values for the standard curve concentrations indicated that acceptable accuracy (defined as within ±25% of the nominal concentration) was observed from 300 to 50,000 ng/mL and this was established as the range of quantification for the assay. Concentrations outside of the range of quantification were used to improve the fit to the 4-parameter equation.

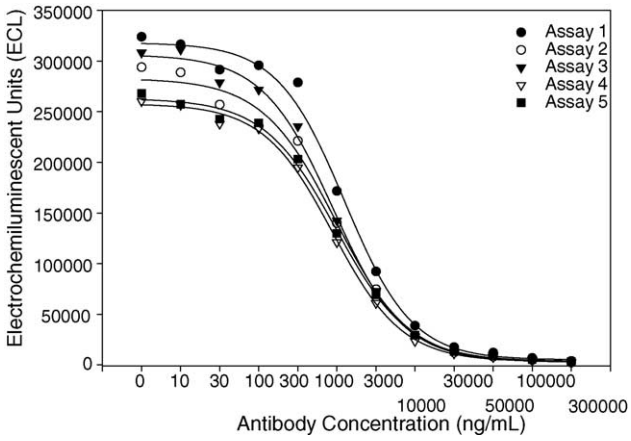

Fig. 1. Representative standard curves.

3.2. Lower limit of quantification (LLOQ)

The lower limit of quantification was established by determining the recovery of the anti-TNFα antibody added to pooled, normal human serum. For the LLOQ determination, concentrations ranging from 1000 to 100 ng/mL were tested. Four independent experiments were performed. The results are shown in Table 2. In each of the individual assays, recovery within 25% of the nominal concentration was

Table 1
Standard curve performance data

	Assay 1		Assay 2		Assay 3		Assay 4		Assay 5	
Standard concentration (ng/mL)	Back-calculated value (ng/mL)	%CV	Back-calculated value (ng/mL)	%CV	Back-calculated value (ng/mL)	%CV	Back-calculated value (ng/mL)	%CV	Back-Calculated Value (ng/mL)	%CV
300000	NR	N/A	123249.32	0.90	801486.13	16.20	NR	NA	1862592.11	41.10
	NR		121634.75		1009368.78		NR		3386609.21	
100000	NR	N/A	74204.81	1.70	104875.73	5.20	123362.81	1.70	120401.90	3.10
	NR		72472.62		112818.30		120504.61		115209.64	
50000	41691.52	14.90	49058.72	4.30	44831.89	9.40	43580.51	1.40	47859.64	1.60
	51511.70		46167.67		39271.43		44435.82		46767.36	
30000	22554.89	9.40	31857.49	2.10	26861.73	3.20	27314.10	9.30	26488.01	2.30
	25759.92		30922.09		28120.23		23947.87		27358.87	
10000	7722.04	14.20	12220.79	2.00	10085.56	0.90	10064.33	1.10	9528.34	0.70
	9449.01		11880.22		10207.74		10215.72		9622.26	
3000	2826.84	5.90	3225.07	0.90	2944.30	3.50	2965.47	2.10	2970.20	3.10
	3071.50		3182.96		3095.54		3053.82		3104.69	
1000	1049.25	8.60	978.23	1.20	1014.71	4.10	1004.99	2.60	1019.15	2.60
	1185.63		995.03		1075.37		1042.79		1056.96	
300	183.06	19.00	189.59	19.20	259.19	1.10	256.83	14.60	296.58	9.80
	239.78		249.01		263.13		316.07		258.02	
100	116.24	3.70	78.02	72.20	126.79	25.90	115.54	40.50	33.27	89.70
	110.28		240.61		87.48		64.11		148.49	
30	118.83	19.30	90.20	36.40	78.38	5.20	79.88	19.60	76.29	11.90
	156.34		53.31		84.33		60.43		64.42	
10	NR	NA	2.89	0.00	NR	NA	11.01	0.00	27.61	133.80
	30.61		NR		NR		NR		0.77	

Table 2
Limit of quantification determination

Nominal concentration (ng/mL)	Assay 1		Assay 2		Assay 3		Assay 4		Mean recovery (%)
	Observed concentration (ng/mL)	Recovery (%)	Observed concentration (ng/mL)	Recovery (%)	Observed concentration (ng/mL)	Recovery (%)	Observed concentration (ng/mL)	Recovery (%)	
1000	1037.83	103.78	1137.54	113.75	1004.96	100.50	1126.57	112.66	107.67
750	766.00	102.13	ND	ND	ND	ND	ND	ND	102.13
500	495.06	99.01	520.22	104.04	556.95	111.39	671.42	134.28	112.18
400	388.96	97.24	393.61	98.40	448.06	112.02	485.27	121.32	107.24
300	309.67	103.22	214.90	71.63	291.14	97.05	325.51	108.50	95.10
250	231.21	92.48	ND	ND	ND	ND	ND	ND	92.48
200	205.03	102.52	131.52	65.76	213.67	106.83	228.60	114.30	97.35
150	162.91	108.61	ND	ND	ND	ND	ND	ND	108.61
100	117.48	117.48	41.38	41.38	24.62	24.62	70.76	70.76	63.56

ND: not done; recovery (%): (observed concentration/nominal concentration) × 100.

most consistently observed at concentrations of 300 ng/mL and higher. The average percent recovery at 300 ng/mL was 95.10%. The LLOQ for the assay was established as 300 ng/mL.

3.3. Accuracy and precision in normal serum and serum from rheumatoid arthritis patients

To determine the performance of the assay over the range of serum concentrations expected in clinical study samples, the accuracy and precision of the assay were determined by adding the anti-TNFα antibody to both pooled, normal human serum and serum from rheumatoid arthritis patients. Rheumatoid arthritis is one of the intended indications for the antibody and it was important to determine if any interference with the assay would occur with disease-specific serum. Antibody concentrations ranging from 500 up to 500,000 ng/mL were tested. Based on the standard curve performance, concentrations from 500 to 50,000 ng/ml were evaluated using neat serum and concentrations greater than 50,000 ng/mL were tested following a 1:10 dilution with pooled, normal human serum in order to bring the concentration in the diluted sample into the range of quantification for the assay. The results are shown in Table 3. Across the concentration range tested, recovery was within ±25% of the nominal concentration. As shown in Table 3, there was no difference in accuracy or precision in the serum from rheumatoid arthritis patients compared with normal serum, demonstrating that disease-

Table 3
Accuracy and precision in normal serum and serum from RA patients

Nominal concentration (ng/mL)	Dilution	Observed concentration (ng/mL)	Recovery (%)	%CV
(A) Normal serum				
0	Neat	<LOQ	NA	NA
500	Neat	391.94	78.39	0.40
750	Neat	582.49	77.67	3.30
1000	Neat	904.51	90.45	5.10
2000	Neat	2000.97	100.05	1.20
5000	Neat	4759.13	95.18	0.40
10000	Neat	9816.23	98.16	1.30
20000	Neat	18815.74	94.08	2.20
50000	Neat	44038.65	88.08	0.90
100000	10	98925.39	98.93	0.70
500000	10	479281.01	95.86	4.60
(B) Serum from rheumatoid arthritis patients				
0	Neat	<LOQ	NA	NA
500	Neat	579.22	115.84	1.40
750	Neat	793.89	105.85	10.10
1000	Neat	978.26	97.83	0.30
2000	Neat	2195.33	109.77	2.20
5000	Neat	6401.31	128.03	1.90
10000	Neat	12806.98	128.07	2.70
20000	Neat	23437.73	117.19	0.30
50000	Neat	54776.14	109.55	1.60
100000	10	104409.68	104.41	1.90
500000	10	467886.49	93.58	3.70

Table 4
Intra-assay precision

Replicate	Nominal concentration (ng/mL)							
	500000	100000	50000	10000	5000	2000	1000	500
1	599577.43	104395.84	63017.08	9180.61	4921.40	2010.31	1037.35	412.67
2	583801.00	99805.81	56155.03	10256.95	5004.05	2324.54	1129.37	539.94
3	579075.16	100847.53	52318.76	9731.53	5167.91	2247.94	1168.97	568.04
4	533304.37	93663.62	49764.05	9147.01	4887.95	2111.52	1098.45	491.82
5	550306.28	101441.46	49720.10	9573.60	5099.11	2208.62	1143.77	564.53
6	507481.49	98000.25	55057.30	9291.41	5077.67	2175.77	1091.78	563.85
7	552540.54	101282.15	53493.24	9854.00	5320.12	2213.22	1174.56	629.49
8	511604.38	97450.71	52419.44	9469.42	5197.24	2256.82	1223.55	606.76
Mean	552211.33	99610.92	53993.12	9563.06	5084.43	2193.59	1133.48	547.14
S.D.	33751.22	3234.58	4291.38	377.31	144.99	96.63	57.80	68.19
Mean recovery (%)	110.44	99.61	107.99	95.63	101.69	109.68	113.35	109.13
%CV	6.11	3.25	7.95	3.95	2.85	4.40	5.10	12.46

specific serum does not interfere with the quantification of the anti-TNFα antibody.

3.4. Intra-assay precision

Intra-assay precision (%CV) was determined by measuring the anti-TNFα antibody in pooled, normal human serum. Concentrations ranging from 500 to 500,000 ng/mL were used. Eight replicates at each concentration were tested. The results are shown in Table 4. At all concentrations, the intra-assay precision was <20%, ranging from 3.95 to 12.46%.

3.5. Inter-assay precision and analyte stability in serum

Inter-assay precision and the stability of the anti-TNFα antibody in serum were determined by measuring the con-

Table 5
Inter-assay precision and analyte stability

Sample	Concentration (ng/mL)					Mean	S.D.	Precision, %CV
	Day 1	Day 4	Day 7	Day 14	Day 21			
1	10694.10	9408.93	8384.13	9903.31	8609.63	9400.02	946.67	10.07
2	4167.23	3667.48	3349.80	3748.93	3768.41	3740.37	291.95	7.81
3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
4	20775.39	22707.96	24509.76	29670.19	19555.12	23443.68	3958.78	16.89
5	27726.04	24271.18	25198.23	29244.94	18573.16	25002.71	4102.61	16.41
6	22646.79	21488.35	21165.73	26501.15	15723.85	21505.18	3866.60	17.98
7	5996.52	6230.69	6269.71	7862.05	5091.32	6290.06	999.78	15.89
8	2290.03	2539.59	2530.08	3157.92	2375.28	2578.58	340.62	13.21
9	698.07	943.50	941.26	1084.68	1015.70	936.64	145.90	15.58
10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
11	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
13	239914.95	220928.27	238306.83	268589.92	184850.41	230518.07	30724.30	13.33
14	209920.33	185473.17	198044.96	219015.23	157944.91	194079.72	23812.94	12.27
15	89893.46	89612.81	91659.56	113328.69	88884.46	94675.80	10477.02	11.07
16	190900.85	172583.15	162767.55	192922.36	154240.08	174682.80	17029.34	9.75
17	182875.63	166122.22	172678.57	190715.61	164861.00	175450.61	11124.23	6.34
18	86622.50	69166.55	69997.77	77555.64	47380.14	70144.52	14539.97	20.73
19	148861.66	132935.52	136081.33	164832.92	145949.37	145732.16	12565.55	8.62
20	17843.12	16319.49	15120.61	17873.25	12334.79	15898.25	2300.19	14.47
21	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
22	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
23	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA
24	37449.54	38876.59	41017.63	46874.08	37439.50	40331.47	3938.98	9.77
25	27788.98	20197.67	20306.22	23607.30	22602.34	22900.50	3103.28	13.55
26	98106.73	70052.97	95751.43	90679.91	74186.47	85755.50	12817.31	14.95
27	141179.85	141156.13	132023.37	151310.66	133993.69	139932.74	7588.47	5.42
28	125782.31	123591.08	115214.14	142337.22	135014.79	128387.90	10510.06	8.19
29	93397.36	102682.27	162678.35	139191.31	122380.19	124065.89	27930.09	22.51
30	21225.02	26332.41	26948.13	34504.84	31648.49	28131.77	5131.40	18.24

centration of the anti-TNFα antibody in serum samples from rheumatoid arthritis patients that received the anti-TNFα antibody by intravenous infusion. Serum samples were stored at 4 °C and then analyzed in five separate assays over 21 days. Samples were selected to cover a range of concentrations from <LLOQ up to approximately 200,000 ng/mL. The results are shown in Table 5. Across all of the samples tested, the mean inter-assay precision was 13.05 ± 4.55% for samples with results above the LLOQ. Of the 23 study samples with results above the assay LLOQ, the inter-assay precision was <20% for all but two of the samples. The results demonstrate excellent inter-assay precision over the 3-week period and also demonstrate that the analyte is stable in serum when stored for up to 3 weeks at 4 °C. The results of the pharmacokinetic analysis for the study will be reported separately (manuscript in preparation).

3.6. Specificity

The specificity of the assay for the anti-TNFα antibody was determined by analyzing serum samples containing several other chimeric, humanized, or fully human therapeutic antibodies. These included an anti-CD3 antibody, an anti-IL-12 antibody, an anti-tissue factor antibody, and the anti-TNFα antibody, infliximab. When added to normal human serum at a concentration of 10,000 ng/mL the samples gave results that were all below the lower prior to limit of quantification of the assay (data not shown).

4. Discussion

The pharmacokinetic assessment of macromolecules in clinical studies is important in understanding the concentration–response relationship and in assessing safety and efficacy. For quantification of macromolecules to support pharmacokinetic assessments, immunoassays are commonly employed. A critical element to the successful development of an immunoassay is the specific binding between the analyte (the macromolecule) and the specific antibody reagent(s) used. In the vast majority of cases, suitable immunoassay reagents are not commercially available and high affinity and selective antibodies must be specifically generated. For the typical non-competitive sandwich-type immunoassay, the generation of two suitable antibodies is not always possible, especially for immunoassays that measure fully human monoclonal antibodies. The production of these specific reagents is time-consuming, expensive, and success is difficult to predict.

A competitive immunoassay format is an alternative to sandwich-type ELISA methods that require the availability of one specific reagent and a labeled analyte that can be used to compete with the unlabeled analyte in the sample. To achieve the needed sensitivity, competitive immunoassays frequently employ radioactive labels, which are undesirable due to the issues relating to the handling and disposal of radioactive materials. It was hypothesized that the technique of ECL could be used to develop a competitive immunoassay with sufficient sensitivity and other performance characteristics necessary to allow pharmacokinetic assessments in support of drug development studies. In the present study, only a single, high-quality anti-variable antibody was available. Using ECL detection, a single step, competitive immunoassay was developed to quantify the concentration of a fully human anti-TNFα antibody in human serum. The assay showed the accuracy, precision, and reproducibility necessary to be used to support pharmacokinetic studies [8]. In neat serum, an approximately 1600-fold range in serum concentrations could be measured directly. Higher concentrations could be accurately determined after a single 1:10 dilution. In addition, the assay required a single incubation of the serum samples with the assay reagents, avoiding the multiple incubation and wash steps typical of conventional ELISA methods. As antibody and other macromolecule therapies have become increasingly more common, the need for better immunoassays will likely continue to increase. The ECL-based competitive immunoassay approach described here should be broadly applicable for developing immunoassays for the quantification of macromolecules.

References

[1] J.W.A. Findlay, W.C. Smith, J.W. Lee, et al., J. Pharm. Biomed. Anal. 21 (2000) 1249–1273.
[2] D.R. Deaver, Nature (London) 377 (1995) 758–760.
[3] G.F. Blackburn, H.P. Shah, J.H. Kenten, et al., Clin. Chem. 37 (1991) 1534–1539.
[4] S.J. Swanson, S.J. Jacobs, D. Mytych, et al., Dev. Biol. Stand. 97 (1999) 135–147.
[5] C. Grimshaw, C. Gleason, E. Chojnicki, J. Young, J. Pharm. Biomed. Anal. 16 (1997) 605–612.
[6] A.S. Yuan, M.L. Morris, K.C. Yin, Y.-K. Hseih, B.K. Matuszewski, J. Pharm. Biomed. Anal. 33 (2003) 719–724.
[7] F.S. De St. Groth, D. Scheidegger, J. Immunol. Meth. 35 (1980) 1–21.
[8] United States Food Drug Administration, Fed. Reg. 66 (2001) 28526–28527.