ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 605–612

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Development of an equilibrium immunoassay using electrochemiluminescent detection for a novel recombinant protein product and its application to pre-clinical product development [1]

Charles Grimshaw [a], Carol Gleason [b], Eric Chojnicki [c,*], John Young [c]

[a] *IGEN, 1620 Industrial Drive, Gaithersburg, MD 20877, USA*
[b] *J.M. White Associates, P.O. Box 335 Jamesville, NY 13087, USA*
[c] *Amgen Inc, Department of Pharmacokinetics and Drug Metabolism, 1840 DeHavilland Drive, Thousand Oaks, CA 91320-1789, USA*

Received 6 January 1997; received in revised form 23 September 1997

Abstract

In order for a biotechnology derived protein product to be considered 'well characterized', a thorough understanding of the pharmacokinetic and metabolic fate of the product is essential. Specifications for such products need to be based on historical data obtained in the pre-clinical, clinical and manufacturing experience through the use of specific, accurate, precise and validated assays. Typically, assays such as ELISA or RIA that have the disadvantages of limited dynamic range, matrix interactions and hazardous waste generation are used to gather this data. We present data in this abstract that demonstrates the utility of an equilibrium immunoassay that uses electrochemiluminescent detection in the assessment of the pharmacokinetic and metabolic fate of a biotechnology derived product based on either the human (AMG1h) or murine (AMG1m) protein sequence. The assay uses biotinylated antibody in a sandwich format with antibody labeled with the *N*-hydroxysuccinimide ester of a ruthenium (II) tris–bipyridine chelate for detection in the Origen™ System. Streptavidin-coated paramagnetic beads are used to capture the antibody–antigen–antibody sandwich complex and facilitate electrochemiluminescent detection. The assay standard curve for AMG1h ranges from a lower limit of quantitation (LOQ) of 2.5 ng ml^{-1} to an upper limit of quantitation (ULQ) of 2000 ng ml^{-1} with accuracy and precision of not greater then 15% CV and deviation from nominal over the range. The corresponding LOQ (0.5 ng ml^{-1}) and ULQ (200 ng ml^{-1}) values determined for AMG1m displayed similar accuracy and precision. In addition, we demonstrate that the assay as performed is insensitive to matrix effect up to addition of 7% of the total reaction volume. General guidelines for developing similar electrochemiluminescent based assays and their applications, advantages and limitations will be discussed. © 1997 Elsevier Science B.V.

Keywords: Electrochemiluminescence; Sandwich assay; Streptavidin; ECL

* Corresponding author. Fax: +1 805 4996913; e-mail: eric.chojnicki@amgen.com

[1] Presented at the Well Characterized Biotechnology Pharmaceuticals Meeting in San Francisco, 6–8 January 1997.

PII S0731-7085(97)00218-5

1. Introduction

The ORIGEN® electrochemiluminescence (ECL) detection system has replaced numerous radioimmunoassays and ELISAs for a wide variety of analytes [1,2]. A variety of assay formats may be used with this detection system, but all are based on the magnetic capture of ORIGEN TAG-labeled immune complexes to an electrode surface via a magnetic particle [3]. The ORIGEN Analyzer 1.5 (IGEN, Gaithersburg, MD) generates and measures the ECL signal of the TAG (a ruthenium tris–bipyridine chelate) after automated capture and washing of the magnetic particles. In the assay described here, an antibody sandwich is formed between the analyte and two antibodies. One antibody is biotinylated for capture by the streptavidin-coated magnetic particle and the other antibody is TAG-labeled for the ECL signal generation. The ECL signal is directly proportional to the analyte concentration.

The particular analyte assayed here is a biotechnology product derived by genetic engineering methods and prepared in both a murine- and human-derived form. Both the murine and human products, hereafter referred to as AMG1m and AMG1h, respectively, contain in addition a human IgG Fc fragment which forms the basis for the specificity of the biotinylated antibody serving as the capture agent. The antibody used for TAG-labeling and ECL signal generation, derived from polyclonal rabbit antiserum elicited in response to the murine antigen, displays 10-fold lower reactivity against the human antigen and forms the basis for the second half of the sandwich assay. ECL assays developed for AMG1m and AMG1h using this optimized antibody pair display a nearly 3-log detection range with excellent precision and accuracy. For AMG1m, a range of 0.5–200 ng ml^{-1} was achieved, while for the human counterpart, AMG1h, the range was 2.5–2000 ng ml^{-1}.

Because of the unique design of the ORIGEN 1.5 Analyzer, the need for extensive washes and lengthy incubations is eliminated. Separation of bound from free analyte is achieved through the capture of the antibody sandwich complex onto streptavidin-coated paramagnetic beads, and the single wash is conducted automatically within the electrochemical cell after the beads have been captured onto the surface of the electrode. In addition, because light is generated in an electrochemical reaction and not via an enzyme-catalyzed reaction, there is little or no matrix effect. Thus, variable sample volumes can be employed to effectively extend the range of the calibration curve at least 10-fold and eliminate the need for extensive dilution of biological samples prior to analysis. The general flexibility of the ECL assay

Table 1
Validation of AMG1h assay: summary of accuracy and precision for standards

	Standard (ng ml^{-1})						
	2.00 ($n=18$)	6.32 ($n=18$)	20.0 ($n=18$)	63.2 ($n=18$)	200 ($n=18$)	632 ($n=18$)	2000 ($n=18$)
Accuracy							
Mean	1.73	6.70	21.5	64.8	186	677	1900
Deviation (%)	−13.5	6.0	7.6	2.4	−7.1	7.1	−5.0
Precision							
Between-day (%CV)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Between-assay (%CV)	0.0	1.2	2.7	0.0	0.0	0.0	0.0
Within-assay (%CV)	23.7	8.9	5.8	7.9	10.6	8.4	9.1

Deviation (%): percent deviation from nominal level.
Within-assay precision: estimate of average variation of triplicate predicted concentrations, averaged over the three assays on 2 days.
Between-assay precision: estimate of average variation among the three assays, averaged over the 2 assay days.
Between-day precision: estimate of variation between 2 days, averaged over assays.
An estimate of '0%CV' indicated that variation within assays was greater than that observed between days and/or between assays.

Table 2
Validation of AMG1h assay: summary of accuracy and precision for all QC samples

	Standard (ng ml^{-1})				
	2.50 ($n=17$)	5.00 ($n=18$)	35.0 ($n=18$)	250 ($n=18$)	1250 ($n=18$)
Accuracy					
Mean	2.36	5.31	37.8	255	1068
Deviation (%)	−5.5	6.1	8.0	2.0	−14.6
Precision					
Between-day (%CV)	0.0	0.0	0.0	0.0	0.0
Between-assay (%CV)	1.8	9.9	0.0	0.0	4.8
Within-assay (%CV)	11.1	7.1	15.7	8.7	6.9

See footnotes to Table 1.

format without the need for extensive dilution of biological samples makes this an ideal method for characterization of biotechnology-derived products and an excellent candidate for automation.

2. Experimental

2.1. Materials

Biotin-SP-conjugated affinity purified rabbit polyclonal anti-human IgG Fc$_\gamma$ fragment specific antibody (Bio–Ab) obtained from Jackson Immunoresearch Labs (West Grove, PA) was reconstituted to 1.0 mg ml^{-1} protein concentration according to the manufacturers instructions and stored frozen at −80°C until use. Affinity purified rabbit polyclonal anti-AMG1m antibody prepared at AMGEN (Thousand Oaks, CA) was labeled with TAG–NHS Ester™ (IGEN) using a TAG:protein challenge ratio of 7:1 and separated from unreacted TAG according to the manufacturers instructions resulting in a final conjugation ratio of 2.35:1 (TAG:Ab). The TAG–Ab at a final protein concentration of 0.284 mg ml^{-1} was stored in amber microtubes at 4°C until use.

2.2. Preparation of standards

Stock solutions of AMG1m (2.17 mg ml^{-1}) and AMG1h (0.64 mg ml^{-1}) in PBS buffer were used to prepare standard calibrators (STD). Standards for AMG1m were prepared from an initial 200 ng ml^{-1} working solution by serial half-log dilution (i.e. $10^{0.5}=3.162$-fold dilution) into 100% rat plasma covering the range 0.2–200 ng ml^{-1}. Similarly, standards for AMG1h were prepared from an initial 2000 ng ml^{-1} working solution by serial half-log dilution into 100% rat plasma covering the range 2–2000 ng ml^{-1}. Standard calibrators prepared in bulk and aliquots stored in polypropylene microtubes at −80°C until use.

2.3. Quality control samples

Pooled quality control (QC) samples were prepared from an independent spike of the AMG1m and AMG1h stock solutions into 100% rat plasma. QC samples for AMG1m (0.25, 0.5, 35, 25 and 125 ng ml^{-1}) and AMG1h (2.5, 5, 35, 250 and 1250 ng ml^{-1}) prepared in bulk and aliquots stored in polypropylene microtubes at −80°C until use.

2.4. Assay format

The assay premix was prepared by diluting the Bio–Ab and TAG–Ab to final concentrations of 67 ng ml^{-1} and 38 ng ml^{-1}, respectively, into assay diluent which consists of a mixture of 1 part by volume of ORIGEN PBS-1 buffer (150 mM Na-phosphate, 150 mM NaCl, pH 7.8) containing 1% (w/v) Tween 20 (BioRad, Richmond, CA) plus 5% (v/v) fetal bovine serum (Sigma, St. Louis, MO) with 2 parts by volume of ORIGEN Assay Buffer (phosphate buffer containing tripropylamine). The Dynabeads® M-280 streptavidin-coated paramagnetic beads (IGEN) were diluted to 1 mg ml^{-1} in assay diluent.

Table 3
Validation of AMG1m assay: summary of accuracy and precision for standards

	Standard (ng ml^{-1})						
	0.200 ($n=18$)	0.632 ($n=18$)	2.00 ($n=18$)	6.32 ($n=18$)	20.0 ($n=18$)	63.2 ($n=18$)	200 ($n=18$)
Accuracy							
Mean	0.2	0.63	1.99	6.35	20.0	63.3	201
Deviation (%)	−0.2	−0.6	−0.3	0.5	−0.1	0.1	0.5
Precision							
Between-day (%CV)	0.0	0.0	0.0	0.5	0.3	0.0	0.0
Between-assay (%CV)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Within-assay (%CV)	52.6	14.4	6.7	6.8	8.0	7.3	10.9

See footnotes to Table 1.

In the standard assay format, 10 μl of STD, QC or unknown sample was pipetted into 12 × 75 mm polypropylene tubes, followed by addition of 300 μl of the assay premix containing both the Bio–Ab and TAG–Ab. Following gentle vortexing, the tubes were covered with foil and incubated at 37°C for 3 h, at which time 25 μl of the 1 mg ml^{-1} Dynabeads working solution was added. The tubes were then incubated with vigorous shaking for 2 h at room temperature, at which time the ECL was read using the ORIGEN 1.5 Analyzer.

2.5. Validation

To confirm the stability of the ECL signal at equilibrium and establish that sequential analysis of several carousels would yield reproducible results without having to stagger the incubation times, three identical carousels each containing duplicate standard curves were prepared and analyzed in sequence for both AMG1m and AMG1h. Thus, incubation with Bio–Ab + TAG–Ab for all three carousels was conducted for 3 h at 37°C in parallel, with the first carousel subjected to a 2 h incubation with Dynabeads, 3 h incubation for the second carousel, and 4 h for the third.

Triplicate calibration curves for AMG1m (0.20, 0.63, 2.0, 6.3, 20, 63, and 200 ng ml^{-1}) and AMG1h (2.0, 6.3, 20, 63, 200, 630, and 2000 ng ml^{-1}) were analyzed on each of three separate ORIGEN 1.5 Analyzers on each of 2 days for a total of six assays. Each assay consisted of two 50-tube carousels run sequentially using a single analyzer. Standard curves were positioned at the beginning, middle and end of the assay, such that the middle standard curve spanned the last four tubes of the first carousel and the first four tubes of the second carousel. Triplicate sets of QC samples were similarly positioned across each 2-carousel assay. The calibration curves were obtained by weighted (1/concentration) nonlinear least squares regression analysis of the ECL counts versus the concentration of AMG1h or AMG1m using the 4PL model:

$$Y = [(\min - \max)/1 + (\text{conc}/\text{ED}_{50})^b] + \max$$

To compare and contrast precision and accuracy obtained using variable sample volumes with that obtained using the standard fixed sample volume format, a sequential 2-carousel assay was conducted consisting of triplicate AMG1m standard curves prepared using a fixed 10 μl STD sample volume and duplicate standard curves prepared using 2, 5, 10 or 20 μl STD sample volumes. The ECL results for the variable volume STD samples were then backfitted against the ECL versus concentration standard curve calculated using the fixed 10 μl volume STD samples to determine the effect of volume on %CV and %recovery.

Table 4
Validation of AMG1m assay: summary of accuracy and precision for all QC samples

	Standard (ng ml^{-1})				
	0.25 ($n=18$)	0.50 ($n=18$)	3.5 ($n=18$)	25 ($n=18$)	125 ($n=18$)
Accuracy					
Mean	0.21	0.45	3.29	24.3	116
Deviation (%)	−14.1	−9.4	−6.0	−2.9	−7.3
Precision					
Between-day (%CV)	0.0	6.1	0.0	0.0	0.0
Between-assay (%CV)	24.4	6.2	0.0	2.0	6.1
Within-assay (%CV)	15.9	13.5	5.8	6.0	9.6

See footnotes to Table 1.

3. Results and discussion

3.1. Validation of the AMG1h Assay

Precision and accuracy of the AMG1h standards are contained in Table 1. The standards >2 ng ml^{-1} show low values in deviation ($<7.6\%$) and all of the respective %CV values are $<11\%$. Calibration curves for AMG1h in 100% rat plasma when fitted to the 4PL model showed correlation coefficients >0.994 for all curves. Similar results were obtained using either raw ECL counts or using corrected ECL counts with the background value subtracted (mean ECL counts for blank 100% rat plasma). The absolute ECL counts for blank 100% rat plasma (1500–2400 counts depending on the particular analyzer used) represented 3–4% of the ECL counts (50 000–65 000) observed at the midpoint (63.2 ng ml^{-1}) of the standard curve.

Data from the spiked quality control samples are shown in Table 2. The accuracy of the method, as measured by the %deviation from the mean was $<14.6\%$ at the five control concentrations in rat plasma. The overall precision as measured by the between-day and between assay %CV value, was $<10\%$ for all the quality control standards. Similarly, the within-assay %CV was $<15.7\%$ for all the quality control samples. The lower limit of quantitation (LOQ) was thus set at 2.5 ng ml^{-1} of AMG1h in rat plasma.

3.2. Validation of the AMG1m assay

Precision and accuracy of the AMG1m standards are contained in Table 3. The standards above 0.2 ng ml^{-1} show low values in deviation ($<0.6\%$) and the respective %CV values are $<14.4\%$. Calibration curves for AMG1m in 100% rat plasma when fitted to the 4PL model showed correlation coefficients >0.995 for all curves. Similar results were obtained using either raw ECL counts or using corrected ECL counts with the background value subtracted (mean ECL counts for blank 100% rat plasma). The absolute ECL counts for blank 100% rat plasma (1500–2400 counts depending on the particular analyzer used) represented 5–7% of the ECL counts (30 000–35 000) observed at the midpoint (6.32 ng ml^{-1}) of the standard curve.

Data from the spiked quality control samples are shown in Table 4. The accuracy of the method, as measured by the %deviation from the mean was $<14.1\%$ at the five control concentrations in rat plasma. The overall precision as measured by the between-day and between assay %CV value, was $<6.2\%$ for all but the lowest (0.25 ng ml^{-1}) quality control sample. Similarly, the within-assay %CV was $<13.5\%$ for all but the lowest quality control sample. The lower limit of quantitation (LOQ) was thus set at 0.50 ng ml^{-1} of AMG1m in rat plasma.

Table 5
Verification of equilibrium conditions for AMG1h and AMG1m assay: summary of precision and drift for standards

	AMG1h Standard (ng ml^{-1})						
	2.00 ($n=6$)	6.32 ($n=6$)	20.0 ($n=6$)	63.2 ($n=6$)	200 ($n=6$)	632 ($n=6$)	2000 ($n=6$)
%CV in ECL counts	2.6	3.1	5.0	3.1	6.8	2.8	3.1
%Drift car 1→car 2	−2.1	0.2	6.3	−1.3	10.6	0.3	−1.3
%Drift car 1→car 3	1.6	0.2	5.9	−1.3	13.6	1.3	0.0
	AMG1m Standard (ng ml^{-1})						
	0.200 ($n=6$)	0.632 ($n=6$)	2.00 ($n=6$)	6.32 ($n=6$)	20.0 ($n=6$)	63.2 ($n=6$)	200 ($n=6$)
%CV in ECL counts	8.5	7.8	7.6	6.4	4.9	7.9	6.0
%Drift car 1→car 2	11.8	9.1	1.2	5.6	0.7	10.3	4.1
%Drift car 1→car 3	17.8	17.2	9.6	11.4	6.1	15.9	11.8

Table 6
Comparison of constant volume vs. variable volume AMG1m standards

	AMG1m Standard (ng ml^{-1})						
	0.200	0.632	2.00	6.32	20.0	63.2	200
Constant volume ($n=3$) %CV: (10 μl sample)	9.0	10.5	2.9	4.1	2.2	3.7	13.2
%Recovery	100.0	96.5	104.0	99.1	99.3	99.8	102.8
Variable volume ($n=8$) %CV: (2, 5, 10, and 20 μl sample)	15.8	19.6	7.7	5.8	2.7	12.3	7.4
%Recovery	95.0	83.6	87.5	93.5	90.3	86.7	97.0

3.3. Verification of stable equilibrium conditions

Comparison of the raw ECL counts determined for three consecutive carousels as shown in Table 5 confirms that the antibody sandwich formation has reached equilibrium by the end of the initial 2 h incubation period with the Dynabeads, and that the signal remains stable over the course of an additional 2 h period. The overall %CV value for the raw counts is $<6.8\%$ across the three carousels for AMG1h and $<8.5\%$ for AMG1m. Slightly more drift (increasing counts) was observed for AMG1m ($<17.8\%$ change from carousel one to carousel three) than for AMG1h ($<13.6\%$ carousel one to carousel three). However, for both analytes the data support a simplified protocol in which two or three carousels are prepared and incubated with Bio–Ab and TAG–Ab simultaneously, and then the ECL counts read on the ORIGEN 1.5 Analyzer in sequence, one carousel after the other.

3.4. Assay performance with variable sample volume

Precision and accuracy for analysis of AMG1m standards was retained when variable sample volumes (2, 5, 10, and 20 μl) were compared with an independent set of standards assayed using the standard fixed 10 μl sample volume format. As shown in Table 6, the %CV values for the mean averaged over the four different sample volumes was in all cases $<20\%$. In addition, the percent recovery calculated again as the mean was between 83.6 and 97%, as compared to a range of 96.5–104.0% for the constant volume standards. Thus, there is no evidence for any significant matrix effect when up to 20 μl of rat plasma sample (7% of the total assay volume) is assayed, and accurate results are obtained over the 2–20 μl range.

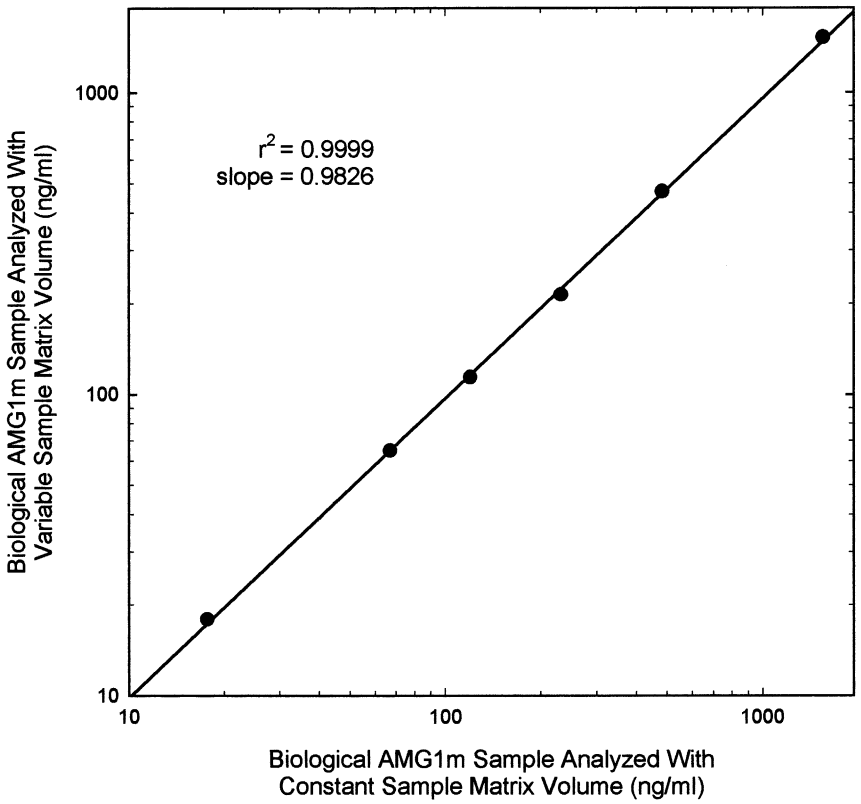

Fig. 1. Orthogonal comparison of biological samples from a dose-ranging pharmacokinetic study of AMG1m assayed using varied volumes (2–20 μl) of sample matrix vs. a constant volume (10 μl) of sample matrix.

3.5. Application to biological samples

The ruggedness of the assay was demonstrated by comparing biological samples from a dose-ranging pharmacokinetic study of AMG1m in rats using both a variable dilution (1:10-1:1000) with constant sample volume (10 μl) assay format and a single (or no) dilution with variable sample volume (2–20 μl) format. As shown in Fig. 1, the correlation was excellent ($r^2 = 0.9999$) for AMG1m concentrations determined using the two different assay formats.

3.6. Advantages of the assay format

The data presented here demonstrates the utility of measuring either AMG1h or AMG1m concentrations in 100% rat plasma using an electrochemiluminescent sandwich assay format. The assay format is quite flexible, allowing for accurate and precise quantitation of either analyte over nearly a 3-log concentration range. In addition, the demonstrated ability to accurately measure the analyte concentration using different volumes of sample (2, 5, 10 or 20 μl) results in effective assay range of nearly 4-logs. Thus, it should be possible using this method to precisely determine the concentration of AMG1h in rat plasma samples at concentrations from 1.25–10 000 ng ml^{-1} without the need for any dilution of the experimental sample, by simply testing 2–20 μl of the unknown sample and using the present standard curve range (2.5–2000 ng ml^{-1}). Similarly, for AMG1m rat plasma sample at concentrations from 0.25–1000 ng ml^{-1} should be readily measured using the presented method.

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