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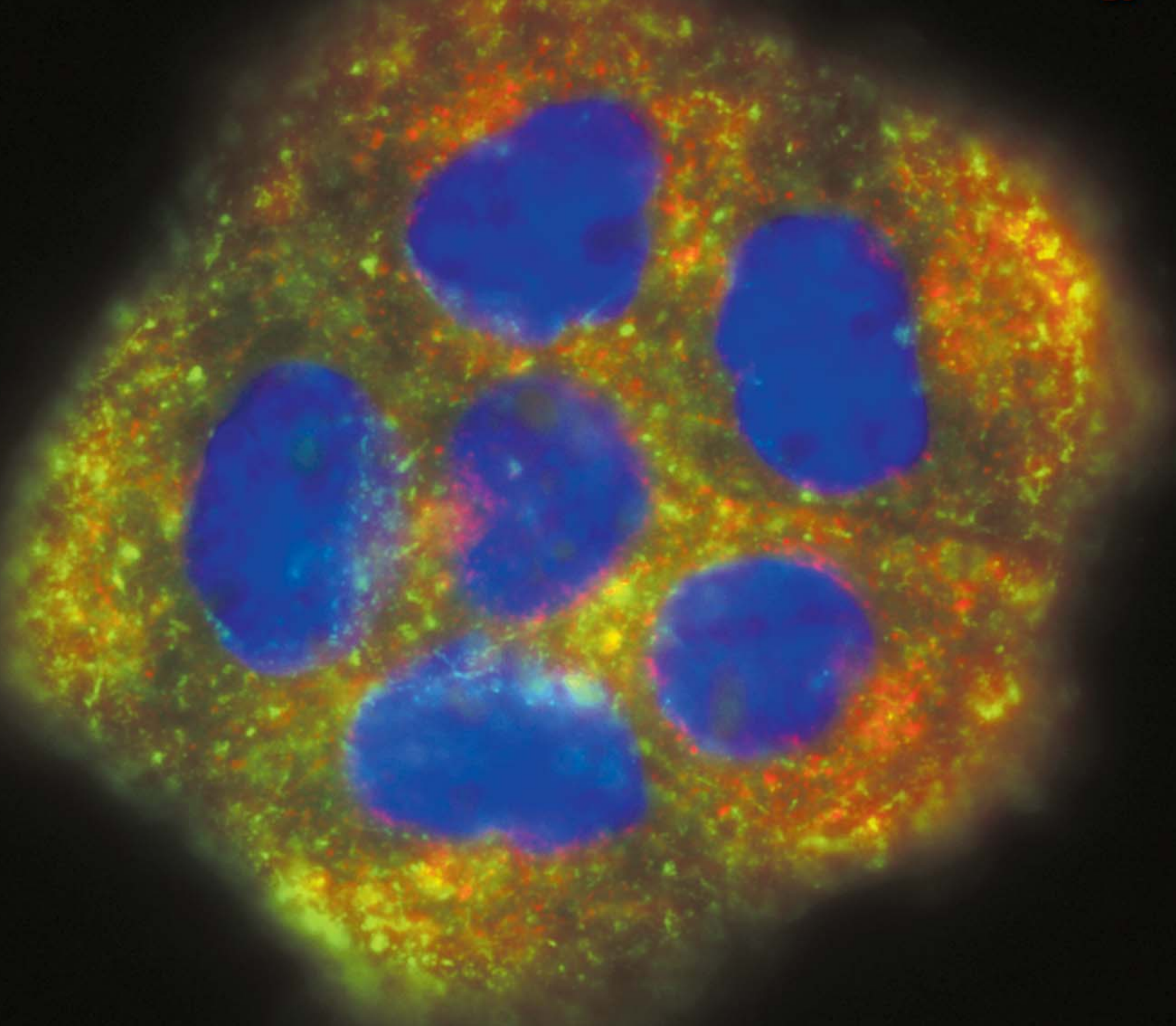

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Polysorbate-80 Determination in BG00002 MAb Using Cobalt Complexation

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Table 1. Summary of cobalt complexation methodology.

1. Prepare polysorbate-80 standards containing 20 mg/ml carrier protein.
2. Pipette standard, control, or sample.
3. Add cold (–20° C) reagent alcohol to standards, controls, and samples.
4. Incubate for four hours at –20° C.
5. Remove from freezer and centrifuge.
6. Remove supernatant.
7. Dry overnight by speed vacuum concentration.
8. Reconstitute with HPLC-grade water.
9. Transfer contents to glass centrifuge tubes.
10. Add cobalt thiocyanate (prepared from cobalt II nitrate hexahydrate and ammonium thiocyanate). Incubate (ambient conditions) for 15 minutes.
11. Extract with dichloromethane.
12. Remove organic phase.
13. Measure on spectrophotometer, $\lambda = 620$ nm.

Polysorbate-80, or polysorbitan mono-oleate, a non-ionic surfactant commercially referred to as Tween-80, is commonly employed as an excipient in the biopharmaceutical industry due to its low toxicity as well as its ability to solubilize hydrophobic molecules. Thus, polysorbate-80 is employed as a stabilizer in order to control the formation of protein aggregates and larger particulates in biopharmaceutical drug substance and drug product formulations.

Guidelines published by The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the United States Pharmacopeia (USP) classify excipients such as polysorbate-80 as major components in biopharmaceutical formulations and specify that these types of compounds be quantified using a validated analytical method.[1–3]

On a practical level, polysorbate-80 is highly viscous, making it a challenge to work with during both preparation and addition to biopharmaceutical formulations. Thus, accurate and precise measurement of this surfactant is critical to ensure that the concentrations of polysorbate-80 are well within established specifications for the respective biopharmaceutical drug substance or drug product.

Various methods have been used to measure non-ionic surfactants. Methods include high performance liquid chromatography in line with evaporative light scattering detection (HPLC-ELSD), analysis by fluorescent micelle measurement, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), among other methods. Additionally, measurement of non-ionic surfactants using cobalt complexation has been widely used during product development applications at Biogen Idec because of reliable performance data that has been demonstrated with

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the procedure. Monoclonal antibody (MAb) BG00002, a biopharmaceutical molecule in late phase development at Biogen Idec, is formulated with 0.02% polysorbate-80. Because of the relatively late phase status of this particular molecule in the development pipeline, it became paramount to validate the method in a current good manufacturing practice (cGMP) laboratory for routine implementation, and to transfer the method to a second qualified quality control testing laboratory. The method for measurement of polysorbate-80 in MAb BG00002 using cobalt complexation was further optimized and fully validated consistent with ICH Q2A and Q2B guidelines. The method was also successfully transferred to the Biogen Idec Quality Control, Biochemistry Department in Oceanside, CA for routine implementation.[4–6]

Table 2. Assay optimization. Evaluation of different protein matrices for preparation of standards.

Protein Matrix and Standard Curve	Absorbance of Blank at 620 nm	Polysorbate-80 Concentration of BG00002 MAb Reference Material (%)
Deformulated MAb, 4 Data Points	0.0007	0.013
Deformulated MAb, 6 Data Points		0.013
Preformulated MAb, 4 Data Points	0.0007	0.014
Preformulated MAb, 5 Data Points		0.013
Bovine IgG, 4 Data Points	0.0061	0.013
Bovine IgG, 6 Data Points		0.013
No Protein, 4 Data Points	0.0004	0.014
No Protein, 6 Data Points		0.014

Deformulated MAb is BG00002 MAb that has polysorbate-80 removed as described by protein-A chromatography. Preformulated MAb did not contain any polysorbate-80 in its formulation.

Table 3. Accuracy of polysorbate-80 method by cobalt complexation.

Percent Polysorbate-80, Theoretical Concentration	Measured Concentration in Assay (Percent)	Mean Measured Concentration (Percent)	Average Recovery (Percent)
0.010	0.0100	0.0101	101.0
	0.0101		
	0.0102		
0.020	0.0209	0.0208	103.8
	0.0207		
	0.0207		
0.030	0.0291	0.0295	98.3
	0.0302		
	0.0292		
0.000	0.0010	0.0010	NA
	0.0010		
	0.0010		

Measurement of polysorbate-80 by cobalt complexation is a relatively simple method based on the procedure published by Crabb and Persinger. It is based on the characteristic absorbance of the cobalt-polyoxyethylene (PNS) complex at a wavelength of 620 nm. The complex has a proposed structure consisting of a cobaltous ion surrounded by at least six moles of ethylene oxide. Five of the coordinate positions are occupied by oxygens of the polyether chain while the sixth position is occupied by a terminal hydroxide or one of the oxygens of the ether moieties.[7]

The assay procedure consists of five major steps:

1. Precipitation of the MAb using cold reagent alcohol
2. Concentration of the supernatant containing polysorbate-80 to a residue using speed vacuum concentration
3. Reaction of the reconstituted residue with cobalt thiocyanate (prepared by mixing cobalt nitrate hexahydrate with ammonium thiocyanate)
4. Extraction of cobalt-PNS complexes with dichloromethane
5. Spectrophotometric measurement of the complexes (visible absorbance, 620 nm)

The description of the method is summarized in Table 1.

One advantage of implementing the cobalt complexation methodology in a cGMP environment is that the instrumentation and ancillary equipment requirements of the assay are consistent with qualified instrumentation, typically available in most biopharmaceutical quality control laboratories. For instance, the major instrumentation requirements are a qualified and calibrated spectrophotometer and a speed vacuum concentration apparatus.

Table 4. Polysorbate-80 determination by cobalt complexation, assay specificity, effect of protein aggregation in sample.

Sample	% Polysorbate-80 Determined in Assay	Sample	% Polysorbate-80 Determined in Assay
Aggregate at 2.6%		**Aggregate at 0.5%**	
A	0.0175	A	0.0178
B	0.0175	B	0.0181
C	0.0173	C	0.0180
D	0.0173	D	0.0176
E	0.0180	E	0.0178
F	0.0179	F	0.0176
Mean	**0.0176**	**Mean**	**0.0178**
SD	**3.0×10^{-4}**	**SD**	**2.0×10^{-4}**
RSD (%)	**1.70**	**RSD (%)**	**1.15**
		p-value F-test of data	**0.2101**
		p-value Two-tailed t-test, equal variances	**0.1458**

An F-test was performed on the data in order to determine if the variances of the sample sets were significantly different. The F-test value of 0.2101 indicated that the variances were not significantly different at a 95% confidence level. Thus, the two-tailed t-test that was performed to measure the statistical significance of the difference of the means from the two sample sets used equal variances.

Table 5. Assay linearity.

Polysorbate-80 Measurement by Cobalt Complexation
ANOVA Statistics from Least Squares Linear Regression Analysis

Statistical Parameter	Value Obtained from Regression Analysis	Specification	Pass/Fail
Coefficient of Determination (R^2)	0.9989	≥ 0.98	Pass
Slope	10.624	N/A	N/A
Y-Intercept	-0.0013	N/A	N/A
Residual Sum of Squares	9.03×10^{-5}	N/A	N/A
F Ratio	4374.2	> 6.61 ($F_{critical}$)	Pass

Optimization of the Cobalt Complexation Method for Quality Control Applications

Quality control chemistry staff, trained to perform the assay procedure, further optimized the method by evaluating different protein matrices used to prepare the standards utilized in the assay. Different spectrometer cells were also evaluated as part of the assay optimization. Finally, the method (which had previously only used three assay standards) was modified to add a fourth standard at the low end of the curve to accommodate the measurement of samples containing low concentrations of polysorbate-80. Thus, assay standards contained 0.005, 0.01, 0.02, and 0.03% polysorbate-80.

Early developmental data had shown that the addition of protein in the matrix of the polysorbate-80 assay standards was essential because non-protein standards correlated with the accuracy shortcoming of the method. During assay optimization, various proteins were tested as suitable matrix candidates. Three different protein matrices were evaluated as suitable media for preparation of the assay standards: bovine serum albumin, preformulated MAb, and de-formulated MAb. Deformulated MAb is BG00002 protein that had originally been formulated with polysorbate-80 at 0.02%. The polysorbate-80 was removed by adsorption of the MAb onto protein A. The MAb was then subsequently eluted and ultrafiltered/diafiltered back into the original formulation (10 mM sodium phosphate, 140 mM sodium chloride, pH 6.1) not containing any polysorbate-80.

Reference materials containing polysorbate-80 were measured by using calibration curves generated by using standards prepared in the different test matrices. Measurements using these calibration curves resulted in comparable data, as summarized in Table 2. Deformulated BG00002 MAb was chosen as the matrix for preparing standards during the assay validation studies. This material was chosen because it was readily available in sufficient quantities to perform a complete method validation.

Spectrophotometer cells (Hellma USA, Plainview, NY) containing an 8.5 mm center quartz were chosen for use because they could accommodate suitable volumes needed for the assay and were compatible with the spectrophotometers used during the method validation. Additionally, it took much less time to generate stable measurements with these particular Hellma cells. Other cuvettes that were tested during assay optimization were not practical because it took inordinate amounts of time (minutes) before stable measurements could be obtained.

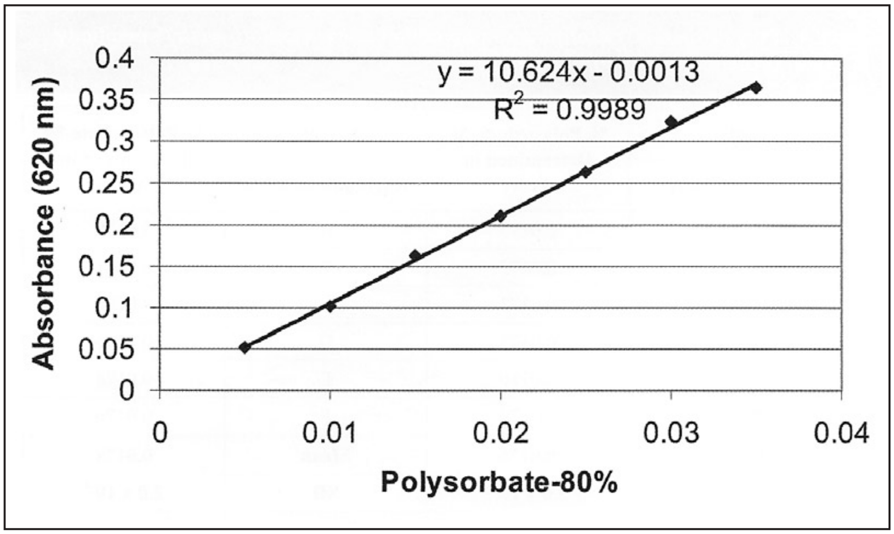


Figure 1. Determination of polysorbate-80 by cobalt complexation. Linearity plot by least squares linear regression analysis.

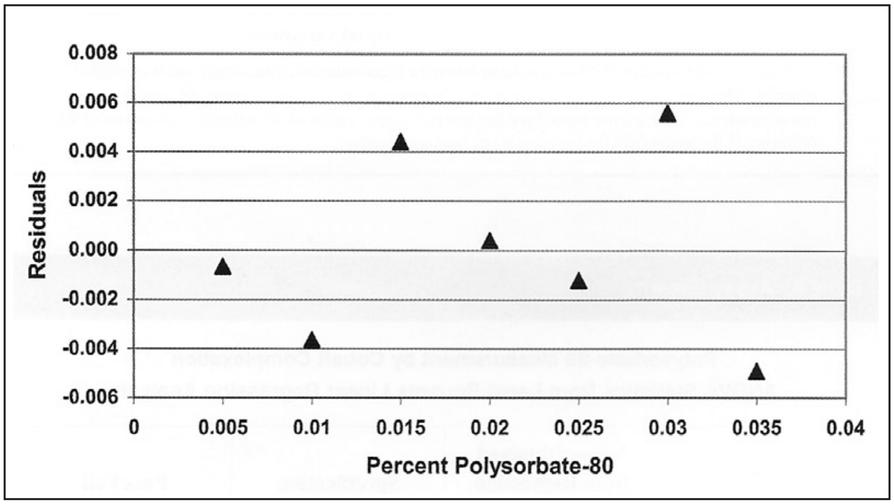


Figure 2. Determination of polysorbate-80 by cobalt complexation. Analysis of residuals from linearity plot.

Table 6. Polysorbate-80 measurement by cobalt complexation.

Lower Limit of Quantitation (LLOQ) and Limit of Detection (LOD)[1]

N	7
Residual Sum of Squares (from ANOVA)	9.03×10^{-5}
Residual Variance (Residual SS)/(N-2)	1.81×10^{-5}
Residual Standard Deviation (Residual Variance)$^{1/2}$	0.0042
Assay Noise (Residual SD/Slope)	0.0004
LOD (assay noise x 3.3)	0.0013 % Polysorbate-80
LOQ (assay noise x 10)	0.0040 % Polysorbate-80

[1] Statistics were determined according to Reference 8.

Validation of the Cobalt Complexation Method for the Measurement of Polysorbate-80 in BG00002 MAb

The method validation followed ICH harmonized tripartite guidelines Q2A and Q2B. Because polysorbate-80 is considered a major component under "quantitative tests of the active moiety or other selected components," the assay parameters that were evaluated according to the guidelines included accuracy, specificity, precision, and linearity/range. In addition, limit of quantitation, limit of detection (LOD), robustness, and assay drift were also evaluated during the validation study. [1,2]

The accuracy of the method was assessed by performing a spiking-recovery experiment. Polysorbate-80 was added to MAb formulations at three concentrations at the low, middle, and high range of the assay. Each concentration was tested in independent replicates of three, for a total of nine data points. The data summarized in Table 3 clearly demonstrate the accuracy of the method as all measurements throughout the range resulted in approximated 100% recovery.

The specificity of the assay was evaluated by performing two independent experiments. The first experiment tested assay interference by comparing the measurement of polysorbate-80 in a BG00002 MAb preparation containing high aggregate (~2.6% high molecular weight aggregate) to a preparation of MAb containing low aggregate (0.5% high molecular weight aggregate). Aggregation was measured using a validated size-exclusion chromatographic method for MAb BG00002. Six replicates were independently prepared for both sample populations. Data were compared using a two-tailed student's t-test with equal variances. Data are summarized in Table 4. As demonstrated by the data analysis, the two sample populations (low aggregate 0.5% vs. high aggregate 2.6%) are not statistically different ($\alpha = 0.05$).

Specificity was also evaluated by testing the drug substance/drug product formulation without the addition of polysorbate-80. Data generated from different studies performed during the assay validation showed that the polysorbate-80-free formulation resulted in measurements that were ≤ the LOD of the method.

The linearity/range of the method was evaluated by generating a calibration curve containing seven concentrations of polysorbate-80 (0.005% – 0.035%), each independently prepared in replicates of three. Linearity of the method was shown using a seven-point calibration curve. The curve analyzed by least squares linear regression had a coefficient of determination (R^2) > 0.98, residuals that were small and randomly distributed and an F ratio consistent with an acceptable curve fit. Data are summarized in Table 5 and Figures 1 and 2.

The lower limit of quantitation (LLOQ) and LOD of the method were determined by additional analysis of the linearity data. The analysis of variance (ANOVA) statistics generated by the least squares linear regression analysis were used to provide estimates of the LLOQ and LOD of the assay. The assay noise was measured by determining the residual standard of the least squares linear regression line. The residual standard deviation of the regression line, divided by the respective slope, provided an estimate of the assay noise. Consistent with the ICH Guidelines, the LLOQ was determined by taking the assay noise and multiplying by 10. LOD was determined by multiplying the assay noise by 3.3. Data are shown in Table 6. The LLOQ of the assay is estimated to be 0.004% polysorbate-80 while the LOD is estimated to be 0.001% polysorbate-80.

The precision of the method was evaluated in a single assay (repeatability) and at intermediate levels (days, analysts, and instruments). The repeatability study evaluated a reference material, BG00002 MAb containing polysorbate-80, analyzed in independently prepared replicates of six. Data are summarized in Table 7. Intermediate precision of the method was evaluated between two trained analysts, over three days, and between two different spectrophotometers. Data for day-to-day and analyst-to-analyst precision are summarized in Table 8. The method is precise as demonstrated by low determinations of percent relative standard deviation (% RSD) at all precision levels tested.

Table 7. Polysorbate-80 measurement by cobalt complexation.

Repeatability

Sample	Absorbance (620 nm)	Percent Polysorbate-80 Determined in Assay
A	0.1839	0.0187
B	0.1831	0.0187
C	0.1900	0.0193
D	0.1817	0.0185
E	0.1883	0.0192
F	0.1839	0.0187
	Mean	**0.0189**
	SD	**0.0003**
	RSD (%)	**1.70**
	Mean - 95% Confidence Interval	**0.0186**
	Mean + 95% Confidence Interval	**0.0191**

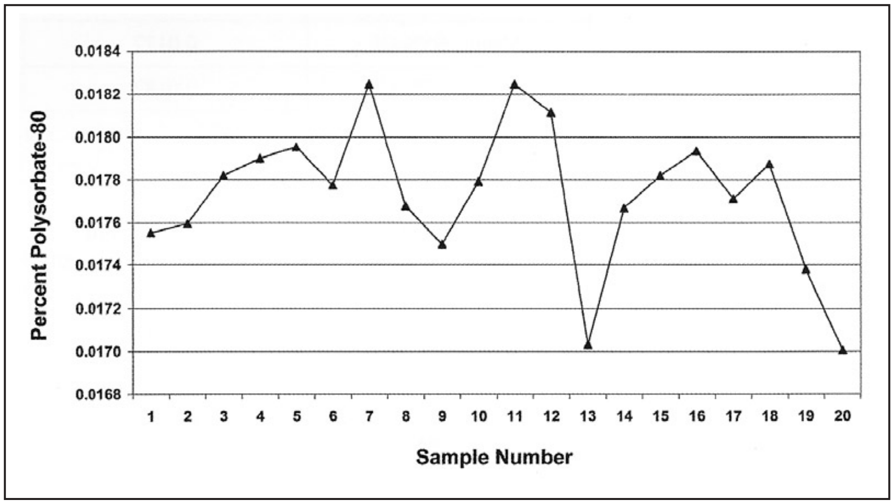


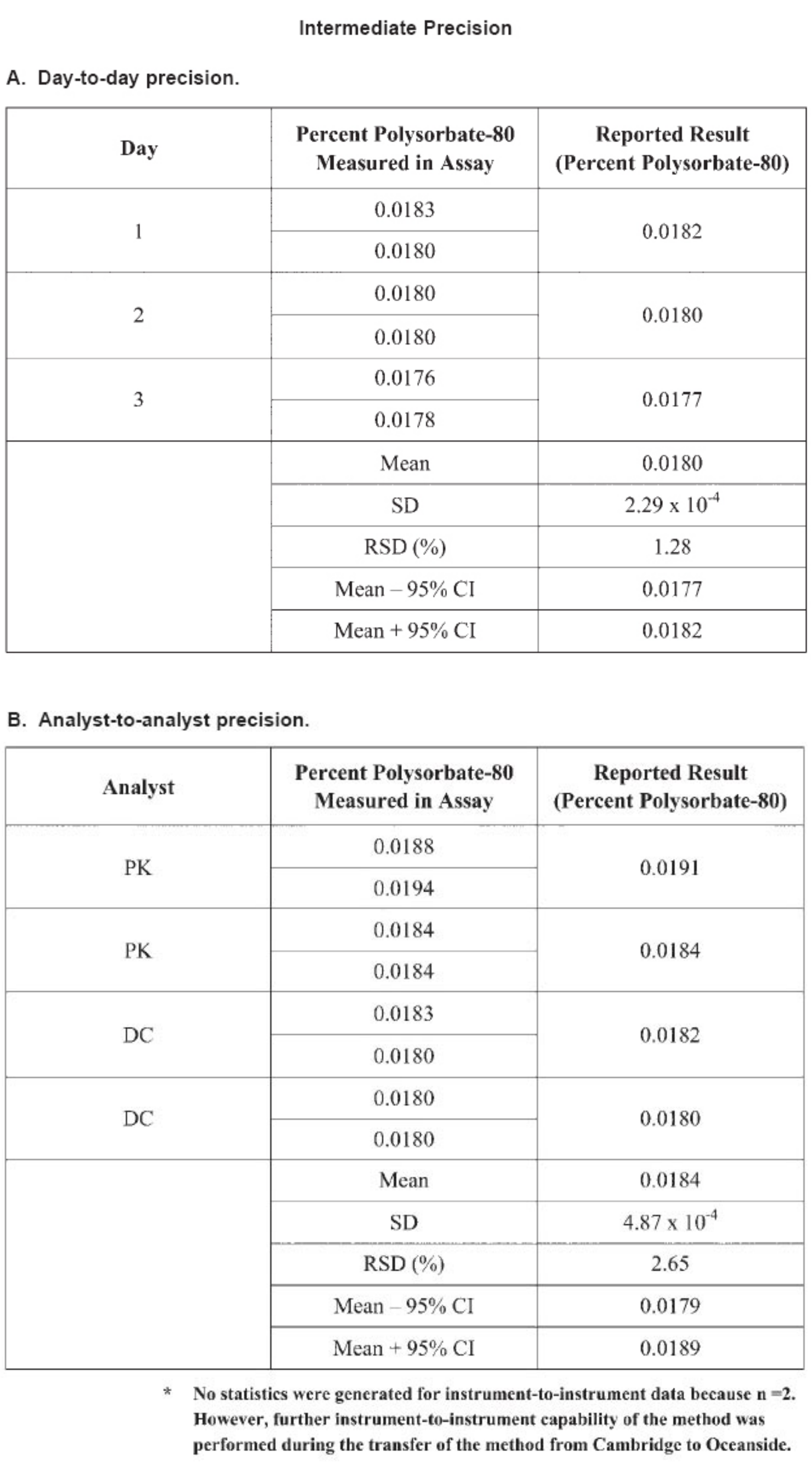
Figure 3. Determination of polysorbate-80 by cobalt complexation. Analysis of assay drift.

Assay Robustness: The robustness of the method was evaluated by measuring respective assay responses generated by deliberately perturbing selected variables. Protein precipitation time, volume of cobalt thiocyanate, and time of extraction with dichloromethane were independently tested. The method was shown to be robust to the deliberate perturbations that were selected. All data were indicative of a robust method from analysis of precision data. RSDs of the control conditions and test conditions were all less than 3%.

Assay Drift: Although not specified by the ICH guidelines for assay validation, assay drift was evaluated in order to establish a maximum sample limit during routine testing applications. Twenty replicates of a sample were independently prepared and analyzed in a single assay. The percent polysorbate-80 determined from each replicate was plotted against the replicate number as shown in Figure 3.

Data from the drift study demonstrate that the assay can accommodate the analysis of ten samples, each in replicates of two.

Table 8. Polysorbate-80 determination by cobalt complexation.



Intermediate Precision

A. Day-to-day precision.

Day	Percent Polysorbate-80 Measured in Assay	Reported Result (Percent Polysorbate-80)
1	0.0183	0.0182
	0.0180	
2	0.0180	0.0180
	0.0180	
3	0.0176	0.0177
	0.0178	
	Mean	0.0180
	SD	2.29×10^{-4}
	RSD (%)	1.28
	Mean – 95% CI	0.0177
	Mean + 95% CI	0.0182

B. Analyst-to-analyst precision.

Analyst	Percent Polysorbate-80 Measured in Assay	Reported Result (Percent Polysorbate-80)
PK	0.0188	0.0191
	0.0194	
PK	0.0184	0.0184
	0.0184	
DC	0.0183	0.0182
	0.0180	
DC	0.0180	0.0180
	0.0180	
	Mean	0.0184
	SD	4.87×10^{-4}
	RSD (%)	2.65
	Mean – 95% CI	0.0179
	Mean + 95% CI	0.0189

* No statistics were generated for instrument-to-instrument data because n =2. However, further instrument-to-instrument capability of the method was performed during the transfer of the method from Cambridge to Oceanside.

Transfer of the Method To a Second cGMP Laboratory

The method was transferred from Biogen Idec's quality control laboratories in Cambridge, MA to its quality control laboratories in Oceanside, CA. Analysts from the Oceanside laboratories were trained to perform the assay by Cambridge staff experienced with the method. In order to show that the method was suitable for implementation in the Oceanside laboratories, a study was designed to demonstrate that the method was precise and accurate at the Oceanside facility. A comparability study was also performed between the Cambridge and Oceanside laboratories.

The Oceanside quality control laboratory analyzed six samples in a single assay (repeatability) over two days and between two analysts. Additionally, the samples were run at both the Oceanside and Cambridge facilities for a laboratory-to-laboratory reproducibility comparison.

As shown by the data in Table 9, the method demonstrates acceptable precision at the Oceanside laboratory. Repeatability, day-to-day, and analyst-to-analyst studies resulted in comparable data as shown by analysis of the data using the student's t-test ($\alpha = 0.01$). A similar study comparing the data from the Cambridge and Oceanside laboratories also demonstrated that the data were comparable. A α value of 0.01 (or 99% confidence) was used in these studies rather than the traditional α value (0.05, 95% confidence) because of the extremely tight precision shown by the method during the validation.

Summary

Measurement of the surfactant polysorbate-80 in the biopharmaceutical MAb BG00002 using cobalt complexation has been successfully validated and transferred to a second cGMP laboratory for routine implementation. The method is suitable for routine measurement of polysorbate-80 as shown by data demonstrating acceptable accuracy, specificity, linearity, and precision. The assay procedure also has an LLOQ of 0.004% polysorbate-80 and an LOD of 0.001%

polysorbate-80. The method is capable of analyzing up to ten samples, in replicates of two in a single assay. Finally, the method was shown to be comparable at both the Cambridge and Oceanside quality control laboratories. The method is adaptable to most quality control laboratories in the biopharmaceutical industry.

Table 9. Transfer of method from Cambridge Quality Control Laboratory to Oceanside Quality Control Laboratory.

Performance Parameter	Criterion (Student's t-value)	Results
Repeatability — Oceanside Laboratory	≤ 2.8188	t-value = 0.3040
Inter-Analyst Variability – Oceanside Laboratory	≤ 2.8188	t-value = 1.8121
Laboratory-to-Laboratory Variability, Cambridge to Oceanside	≤ 2.9208	t-value = 1.8775

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