The Development and Validation of Methods for the Quantitation of Polyethylene Glycol in Serum via ELISA with Methoxy Terminal (mPEG) Detection Following the Evaluation of Commercially Available Kits

Sarah K. Peters, Richard L. Sukovaty, Curtis E. Sheldon
Celerion, Lincoln, NE, USA

PURPOSE

Polyenthylene glycol (PEG) can be attached to proteins for therapeutic use to slow the proteolytic degradation and the clearance of the carried protein from the circulatory system. A method for the accurate and selective quantitation of mPEG in both human and cynomolgus monkey serum was required as part of a program focusing on the development of a PEGylated molecule.

METHODS

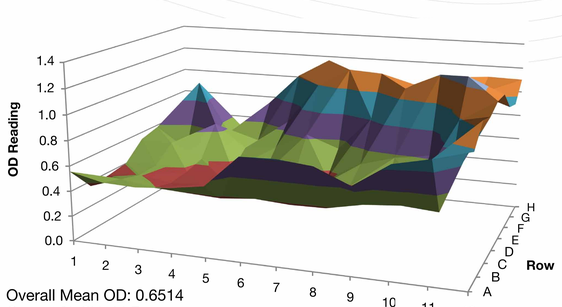
Three commercially available kits (direct competitive ELISA) were purchased and evaluated using a proprietary PEGylated molecule as the calibrator. Kit A used an anti-mPEG antibody coated plate and an HRP-PEG molecule as the competitor, kit B used a plate coated with BSA-mPEG and an AP-anti-PEG backbone antibody detector, and kit C used a plate coated with BSA-mPEG and an HRP-anti-PEG backbone antibody detector.

In addition, a *de novo* assay was developed in which samples were added to microtiter plates coated with an anti-PEG (terminal methoxy specific) capture antibody. The wells were washed to remove the unbound sample material and an anti-PEG (backbone specific) detection antibody added. Unbound labeled antibody was removed and an HRP-strepdavidin conjugate added. The unbound conjugate was removed and a chromogenic substrate added to the HRP/ antibody complex bound to the plate wells. The development of the colored reaction product was directly proportional to the amount of mPEG present in the sample and was detected using a colorimetric plate reader.

RESULTS

An approximate 5-fold analytical range from 450 – 3000 ng/mL was achieved using Kit A (Chart 1), however, this was not acceptable for the analysis of clinical samples due to the nature of some of the dosing schedules, and the fact that many samples would require dilution to obtain concentrations within the analytical curve range. The 5-fold range could potentially have made it challenging to determine the appropriate dilution factor for some of the samples. Kit B had a more appealing analytical range of 50.0 – 750 ng/mL (15-fold), but the samples would have again required significant dilutions. Additionally, the manufacturer discontinued its production during the qualification of the method in favor of a higher sensitivity kit (Kit C). Kit C was evaluated and a broader range of 100 – 2000 ng/mL seemed possible (Chart 2), until variability attributed to the coated plates supplied proved too great to overcome (Chart 3).

Chart 3: OD Response vs. Well Position of 500 ng/mL Solution (Kit C)



Overall Mean OD: 0.6514
Overall % CV: 44.7
High Value: 1.2666 (E11)
Low Value: 0.3257 (E5)

The *de novo* methods (direct ELISA) were validated in human and cynomolgus monkey serum over the analytical range of 300 to 10,000 ng/mL (4PL curve fit) (Chart 4). Inter-batch precision (C.V.) of quality control samples at 600, 3000, and 7500 ng/mL was 9.5%, 8.3%, and 9.8%, respectively for human samples and 7.4%, 9.2%, and 11.2%, respectively for cynomolgus monkey samples. Inter-batch accuracy (% Bias) of the same quality control samples was +16.3, +13.3, and +2.4, respectively for human samples and +13.3, +12.0, and -5.9, respectively for cynomolgus monkey samples (Table 1).

Table 1: Validation Summaries

	Human Serum Assay	Cynomolgus Monkey Serum Assay
Limit of Quantitation	300 ng/mL	300 ng/mL
QC Concentrations	600, 3000, and 7500 ng/mL	600, 3000, and 7500 ng/mL
QC Inter-Batch Precision Range (% CV)	8.3 to 9.8%	7.4 to 11.2%
QC Inter-Batch Accuracy Range (% Bias)	2.4 to 16.3%	-5.9 to 13.3%
Short-Term Stability (Hrs)	24 hours at ambient temperature under white light	24 hours at ambient temperature under white light
Freeze-Thaw Stability (Cycles)	9 freeze (-80°C)-thaw (ambient temperature) cycles	6 freeze (-80°C)-thaw (ambient temperature) cycles
Long-Term Storage Stability	307 days in polypropylene tubes at -80°C (nominal)	76 days in polypropylene tubes at -80°C (nominal)
Dilution Integrity	Samples diluted up to 40-fold can be quantified	Samples diluted up to 250-fold can be quantified
Stability of Analyte During Sample Collections and Handling	Up to 120 minutes in human whole blood at ambient temperature under white light	Up to 120 minutes in cynomolgus monkey whole blood at ambient temperature under white light

Short-term stability in serum was established for 24 hours at ambient temperature under white light for both species. Freeze and thaw stability in serum was established for nine and six freeze (-80°C) and thaw (ambient temperature) cycles in human and monkey serum, respectively. Sample collection and handling stability was established in whole blood for 2 hours at ambient temperature under white light for both species (Tables 2 and 3).

Table 2: Stability of PEG During Sample Collection and Handling from Human Whole Blood at Ambient Temperature Under White Light Conditions

	Low QC Sample		DF = 10 Dilution QC Sample	
	0 minutes	120 minutes	0 minutes	120 minutes
mPEG (ng/mL)	302 294 292 293 269 254	300 268 275 232 238 222	23100 23800 22100 21400 23800 21900	23800 19800 20800 21900 20100 19000
Mean	284	256	22700	20900
% CV	6.5	11.7	4.5	8.3
% of Control		90.1		92.1
n	6	6	6	6

Table 3: Whole Blood Stability from Cynomolgus Monkey Whole Blood at Ambient Temperature Under White Light Conditions

	Low QC Sample		DF = 10 Dilution QC Sample	
	0 minutes	120 minutes	0 minutes	120 minutes
mPEG (ng/mL)	320 316 357 286 271 292	331 303 344 318 286 360	36200 38800 36600 33900 37200 34600	41600 38900 40500 39300 37900 38500
Mean	307	324	36200	39500
% CV	10.0	8.4	4.9	3.5
% of Control		105.5		109.1
n	6	6	6	6

Chart 1: OD Response vs. Concentration (Kit A)

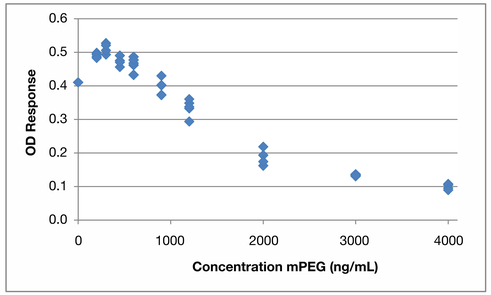


Chart 2: OD Response vs. Concentration (Kit C)

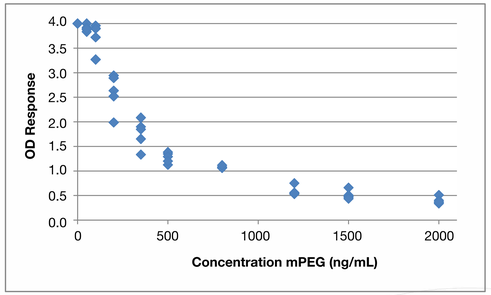
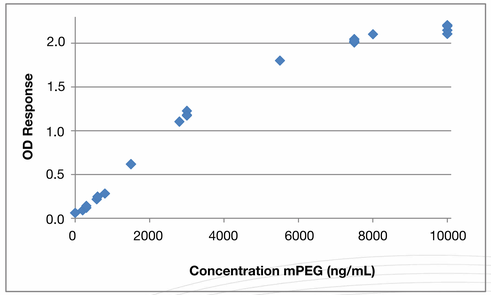


Chart 4: OD Response vs. Concentration (*de novo* assay)



CONCLUSIONS

The validated double antibody sandwich method was found to be more appropriate than single antibody competitive ELISA methods and allowed for rapid, selective, accurate and reproducible quantitation of mPEG in human and cynomolgus monkey serum samples. Additionally, it offered an appropriate compromise between the sensitivity required for the analysis of clinical samples, a reasonable broad assay range required for the analysis of non-clinical samples, and a robust assay required for method validation and sample analysis. These assays have been utilized in the evaluation of over 2000 human samples and over 2000 monkey samples with a greater than 90% batch acceptance rate.