

HPLC/MS/MS with In-Source Collision-Induced Dissociation for Direct Measurement of PEGylated Compounds in Biological Matrices



Mark Dreyer¹, Linda Chen¹, Thomas Tarnowski¹, Oanh Dang², Dale Schoener², Patrick Lin², Mike Buonarati²

¹Elan Pharmaceuticals, South San Francisco, CA

²Alta-Intertek, El Dorado Hills, CA

Introduction

Extraction and quantification of PEGylated compounds from biological matrices represents a major challenge. We report the validation of a bioanalytical method for the determination of small molecules conjugated to polyethylene glycols (PEG's) in plasma by liquid chromatography-atmospheric pressure ionization/tandem mass spectrometry (LC-API/MS/MS) detection. A PEGylated small molecule analog was used as the internal standard. The lower limit of quantitation (LLOQ) for the analyte was 10.0 ng/mL. The calibration curves were acceptable over a range of 10.0 - 1000 ng/mL.

The analyte and internal standard were extracted from 100 μ L of plasma by protein precipitation by addition of acetonitrile containing 0.1% formic acid (FA). After evaporation to dryness and reconstitution, the extracts were analyzed by LC-API/MS/MS. Run times were approximately 4.5 minutes. Because of the nature of this compound (PEGylated), relaxed acceptance criteria were set for the validation ($\pm 20\%$).

Method

100 μ L aliquots of sample (diluted if necessary) or control plasma are pipetted into polypropylene tubes. 100 μ L of 2% Formic Acid in Acetonitrile (FA/ACN) is added and the tubes are vortexed for 60 seconds. An additional 1000 μ L of 0.1% FA/ACN is added, and the tubes are vortexed for at least 1 minute. The tubes are then centrifuged for 10 minutes at 3000 RPM, and the supernatants are decanted and evaporated to dryness.

Samples are reconstituted with 250 μ L of reconstitution solvent (30:70 ACN:0.1% FA), vortexed for 60 seconds, incubated at room temperature for at least 15 minutes, and then centrifuged for 5 minutes at 2500 RPM.

An aliquot of the sample is injected into an HPLC-MS/MS system, trapped on a 2x4 mm C18 guard column, and introduced into the mass spectrometer via a Turbo IonSpray atmospheric pressure ionization inlet for detection and quantification. High purity nitrogen gas is used for the nebulizer, TurboIonSpray and curtain gases.

Detection is in the multiple reaction monitoring (MRM) mode. The collision gas used to produce fragment ions is high purity nitrogen.

The PEGylated analyte of interest enters the TurboIonSpray source, is fragmented between the orifice and skimmer, and then a fragment of the small molecule moves through Q0 and is mass selected in Q1. Following fragmentation with nitrogen in the collision cell (Q2), the product ion is mass selected in Q3 and is detected.

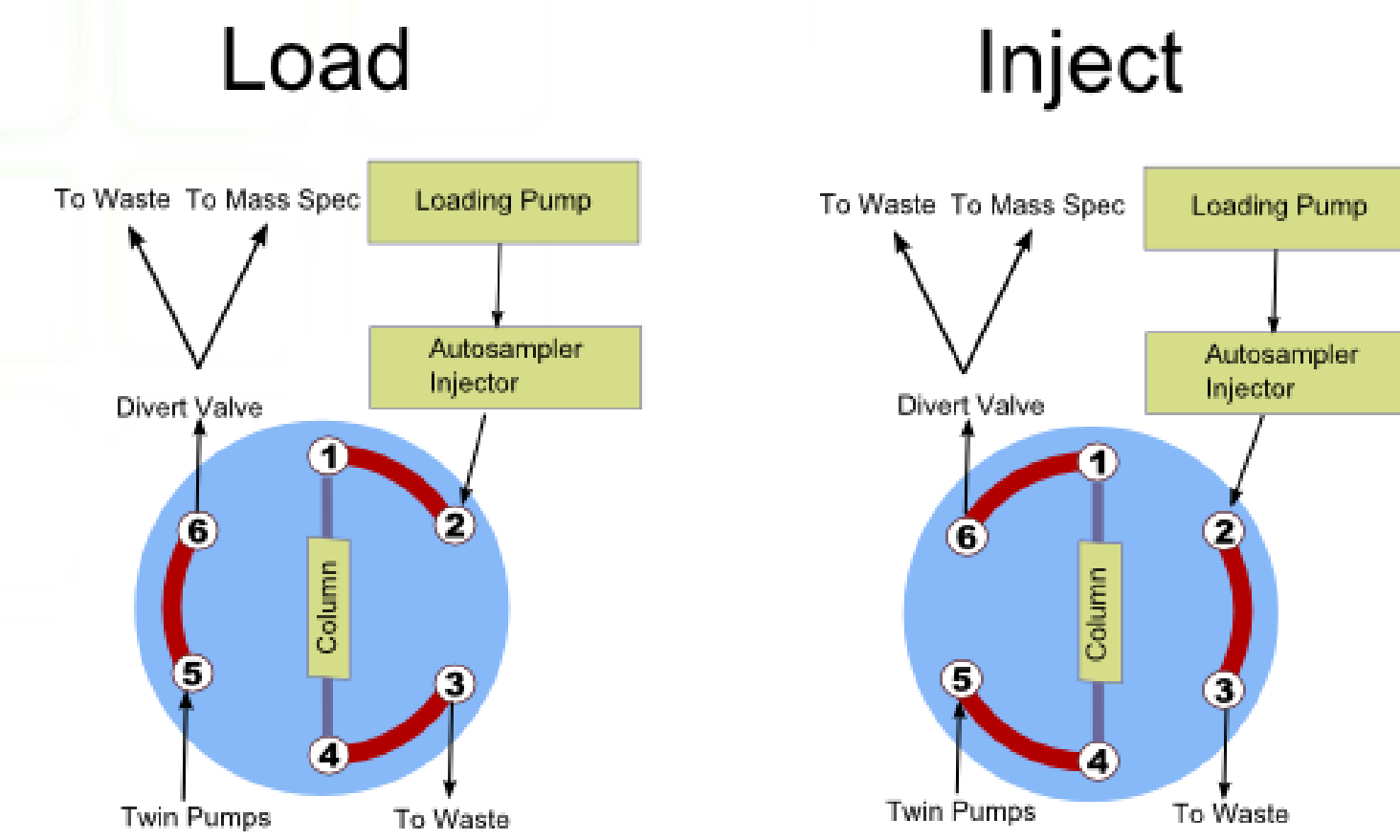


Figure 1 – Valve Configuration

- At 0.0 minutes the valve is in the load (left) position. Flow from the twin pumps (A/B) bypasses the column and goes toward the mass spectrometer (diverted to waste). Flow from the loading pump (C) goes through the column and then to waste.
- At 0.50 minutes, the valve switches to inject (right). Flow from pumps A/B backflushes the column and goes toward the mass spectrometer (diverted to waste). Flow from pump C goes to waste.
- At 0.90 minutes, the divert valve switches from waste to the mass spectrometer, at 4.20 minutes the divert valve switches back to waste.
- At 3.95 minutes the Load/Inject valve switches back to the Load position.

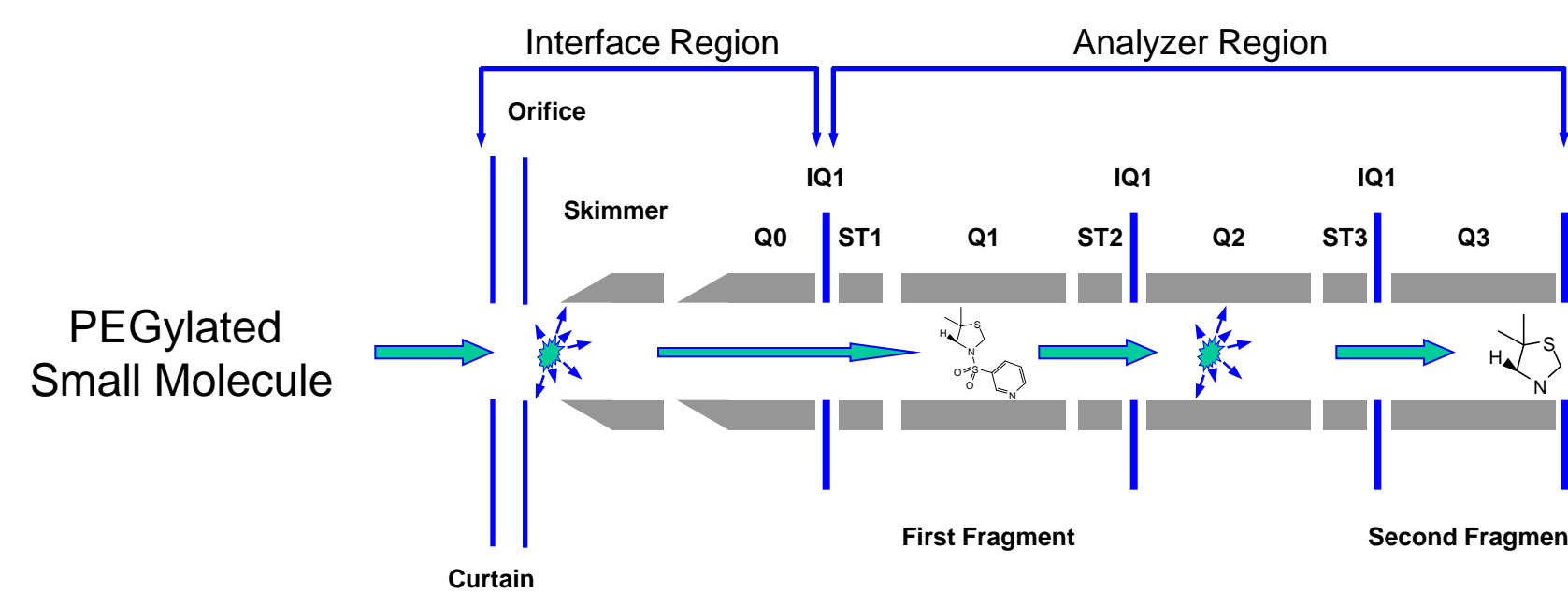


Figure 2 – Triple Quadrupole Mass Spectrometer

The instrument used for the method is an ABI4000 Triple quadrupole mass spectrometer.

- Column: Gemini C18 Guard, 4x2 mm (Phenomenex, Torrance, California)
- Column Temp: 50 °C
- Injection volume: 20 μ L
- Flow Rate: Pump A&B: 0.2 mL/min then 0.6 mL/min, Pump C: 0.6 mL/min
- Mobile Phase A: 0.1% FA
- Mobile Phase B: ACN with 0.1% FA
- Mobile Phase C: 40:60 ACN:0.01% FA
- Mobile Phase Composition: (v/v) Mobile Phase A: Mobile Phase B

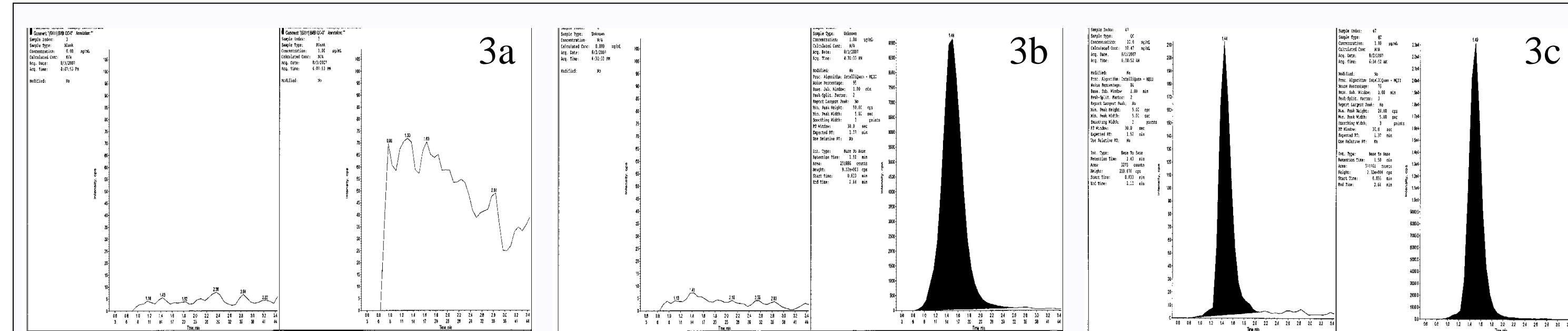


Figure 3 – Representative Chromatograms

- Figure 3a – Shows a representative chromatogram of control plasma extract without internal standard (Blank).
- Figure 3b shows a representative blank chromatogram of the analyte at the LLOQ without internal standard.
- Figure 3c shows a representative chromatogram of the analyte with the internal standard.

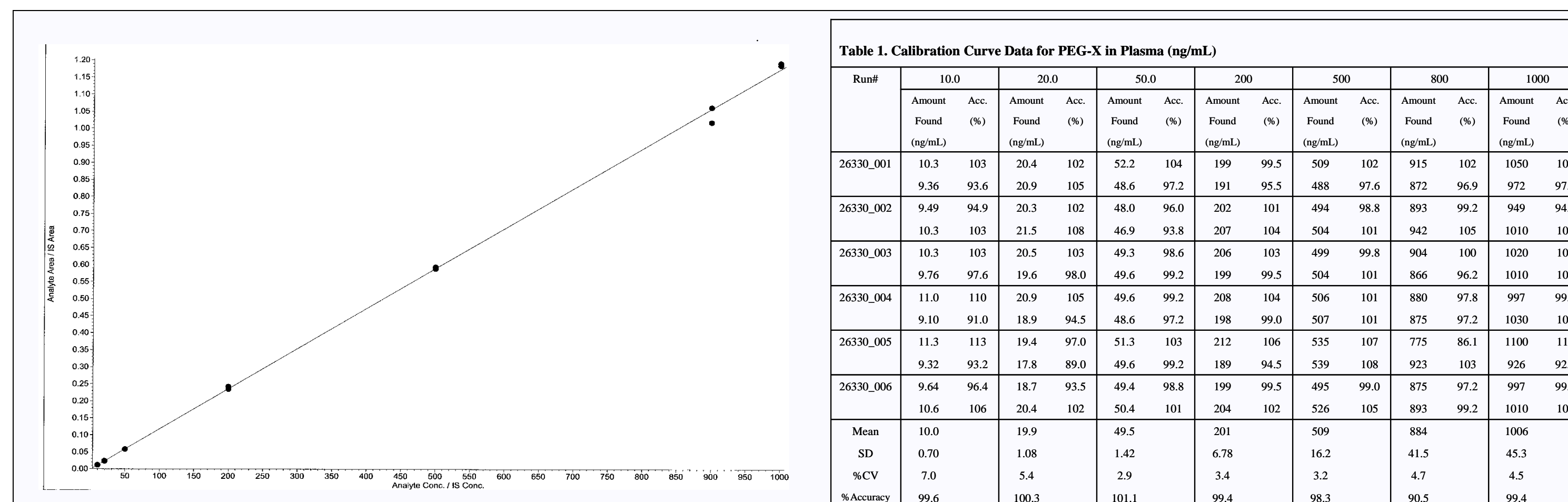


Figure 4 – Representative Standard Curve of Compound PEG-X

Run#	10.0	20.0	50.0	200	500	800	1000
26330_001	10.3	20.4	52.2	199	509	815	1050
26330_002	9.99	20.3	48.0	202	494	893	949
26330_003	10.3	20.5	49.3	206	499	904	1030
26330_004	11.0	20.9	49.6	208	506	101	1030
26330_005	11.3	19.4	51.3	212	535	107	1100
26330_006	9.64	18.7	49.4	199	495	875	997
Mean	10.0	20.4	50.4	204	504	893	1010
SD	0.70	1.08	1.42	4.78	16.2	41.5	43.1
%CV	7.0	5.4	2.9	2.4	3.2	4.7	4.5
%Accuracy	99.6	100.3	101.1	99.4	98.3	99.5	99.4

Table 1 – Calibration Curve Data for PEG-X in Plasma

Run Number	30.0		300		800	
	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)
26330_001	30.2	101	282	94.0	747	93.4
	29.3	97.7	297	99.0	771	96.4
	29.7	99.0	294	98.0	765	95.6
	29.7	99.0	298	99.3	763	95.4
	29.0	96.7	298	99.3	776	97.0
	29.7	99.0	300	100	778	97.3
Mean	29.6	295	767			
SD	0.410	6.59	11.3			
%CV	1.38	2.23	1.47			
%Accuracy	98.7	98.3	95.8			

Run Number	30.0		300		800	
	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)
26330_001	30.2	101	282	94.0	747	93.4
	29.3	97.7	297	99.0	771	96.4
	29.7	99.0	294	98.0	765	95.6
	29.7	99.0	298	99.3	763	95.4
	29.0	96.7	298	99.3	776	97.0
	29.7	99.0	300	100	778	97.3
26330_002	30.9	103	307	102	781	97.6
	30.4	101	306	102	772	96.5
	29.4	98.0	294	98.0	733	91.6
	26.4	88.0	285	95.0	745	93.1
	28.2	94.0	307	102	753	94.1
26330_003	29.1	97.0	279	93.0	733	91.6
	27.5	91.7	277	92.3	734	91.8
	28.1	93.7	281	93.7	704	88.0
	29.0	96.7	281	93.7	703	87.9
	27.3	91.0	275	91.7	721	90.1
Mean	29.0	291	749			
SD	1.21	11.2	25.5			
%CV	4.19	3.84	3.41			
%Acc.	96.6	97.1	93.6			

Run Number	30.0		300		800	
	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)
26330_002 (26 hours)	31.1	104	322	107	812	102
	30.2	101	304	101	786	98.3
	29.0	96.7	302	101	767	95.9
Mean	30.1	309	788			
SD	1.05	11.0	22.6			
%CV	3.50	3.56	2.87			
%Acc.	100	103	98.5			

Run Number	30.0		300		800	
	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)	Amount Found (ng/mL)	Acc. (%)
26330_003 Cycle-2	28.4	94.7	298	99.3	739	92.4
	28.6	95.3	297	99.0	735	91.9
	26.6	88.7	282	94.0	731	91.4
Mean	27.9	292	735			
SD	1.10	8.96	4.00			
%CV	3.95	3.07	0.544			
%Acc.	92.9	97.4	91.9			
26330_003 Cycle-3	28.2	94.0	274	91.3	729	91.1
	27.8	92.7	277	92.3	745	93.1
	27.8	92.7	280	93.3	737	92.1
Mean	27.9	277	737			
SD	0.231	3.00	8.00			
%CV	0.827	1.08	1.09			
%Acc.	93.1	92.3	92.1			

Run Number	Amount Found (ng/mL)	Acc. (%)	Run Number	Amount Found (ng/mL)	Acc. (%)
26330_001 ^a	10.5	105	26330_001 ^b	10.3	103
	11.0	110		9.75	97.5
	10.9	109		10.4	104
	10.1	101		11.2	112
	11.2	112		10.1	101
	11.4	114		10.6	106
Mean	10.9	104		10.4	
SD	0.476	0.490		0.490	
%CV	4.39	4.72		4.72	
%Acc.	109	104		104	

Run Number	30.0		300		800	
	Peak Area (cps) Processed	Peak Area (cps) Unprocessed	Peak Area (cps) Processed	Peak Area (cps) Unprocessed	Peak Area (cps) Processed	Peak Area (cps) Unprocessed
26330_003	12806	15541	135927	175312	325838	450142
	12553	16009	132788	171134	314705	465149
	13023	16488	135683	169965	305285	474782
	13858	17317	131875	177098	320745	465386
	12891	16832	133337	175122	322928	459759
Mean	13026	16437	133922	173726	317900	463044
SD	496	693	1799	3029	8151	9013
%CV	3.80	4.21	1.34	1.74	2.56	1.95
%Recovery	79.2	77.1	68.7		98.5	

Results

- Calibration range is 10 to 1000 ng/mL when 100 μ L of plasma is used for analysis.
- No significant matrix interference ($>20\%$ of the analyte peak response at the LLOQ) was observed in the assay.
- Injector carryover for the analyte and internal standard was not significant in extracted blank matrix samples injected immediately following the high calibration standards ($\leq 20\%$).
- The mean accuracy for LLOQ samples prepared in control plasma was within $\pm 20\%$ of nominal concentration. Precision at the LLOQ was $\leq 20\%$.
- Quality control (QC) samples (three concentrations) prepared in control plasma were: analyzed to determine intra- and inter-assay accuracy and precision of the method and were: also used to assess analyte stability. Intra- and inter-assay accuracy values were within 15% of the nominal concentrations. Intra- and inter-assay precision values (%CV) were $\leq 15\%$.
- For samples above the quantitation limit (AQL), QC samples (diluted by factors of 10, 20, and 50), the mean accuracy values were within 15% of nominal concentrations and precision (%CV) was $\leq 15\%$ for all dilutions, indicating that samples with concentrations above the calibration curve range could be successfully diluted and analyzed.
- Extraction efficiency (%Recovery) for the analyte in fortified QC samples was determined at the low, mid, and high QC concentrations. Mean %Recoveries of analyte in the low, mid, and high QC samples were 79.2%, 77.1%, and 68.7%, respectively.
- Mean %Recovery of the internal standard at all QC concentrations was 74.1%.

Discussion and Conclusions

Extraction of PEGylated compounds from biological matrices represents a major challenge. Traditionally PEGylated compounds are analyzed by first hydrolyzing the polymer and then extracting the small molecule for LC/MS/MS quantitation. This is an indirect approach, and errors may be introduced due to, for example, incomplete hydrolysis. The current method applies a two-step mass spectrometric fragmentation strategy after an HPLC column loading step and backflush that has resulted in improved selectivity. This not only avoids a hydrolysis step, thereby improving efficiency, but also improves assay accuracy.

The average analysis time is 9 minutes, and a large number of plasma samples pharmacokinetic studies have been analyzed by this method.

By the method reported here, the bioanalytical challenges of PEGylated compounds have been addressed by a method that is simple straightforward, accurate, with minimal sample preparation and is amenable to automation.