



# Investigation of Lot-to-Lot Matrix Variation and Carryover for a Small Peptide in Human Serum

## Authors

*Yifei Liu, Jessica Schofield*

## Introduction

A new derivative peptide, being developed as a potential drug, was assayed in human serum by LC/MS/MS. Despite the usage of isotope-labeled internal standard, it was observed that the instrument response of compound vs. internal standard varied between different lots of matrices. It was suspected that the recovery between lots of matrix was not consistent. An investigation was conducted to eliminate the lot-to-lot variation and the carryover in assay.

## Method

### ANALYTE AND INTERNAL STANDARD:

	MW	MRM monitored
Molecular (A)	~ 1600 g/mol	814.6 → 144.1
Internal standard	A-d8 (labeled by 8 deuterium)	818.6 → 144.1

### EXTRACTION:

200 µL human serum samples were added with 50.0 µL of IS working solution (100 ng/mL) and 750 µL of 1% formic acid in acetonitrile. Vortex and centrifuge at 3000 rpm at ambient temperature for 10 minutes. Supernatant were transferred to 96-well plate and evaporated at 50°C to dryness and reconstituted with 100 µL of acetonitrile: water: formic acid (20:80:0.1, v:v:v).

## Method

### LC/MS CONDITION:

The assay was conducted on API-5000 in order to reach the maximum sensitivity to achieve the LLOQ (50.0 pg/mL). Mobile phase contains 0.1% formic acid as aqueous (A) and 0.1% formic acid in acetonitrile as organic phase (B). Genesis C8 2.1 x 50 mm, 4  $\mu$  was used as HPLC column. The original LC method applied regular gradient from 5% B to 90% B in 3 minutes.

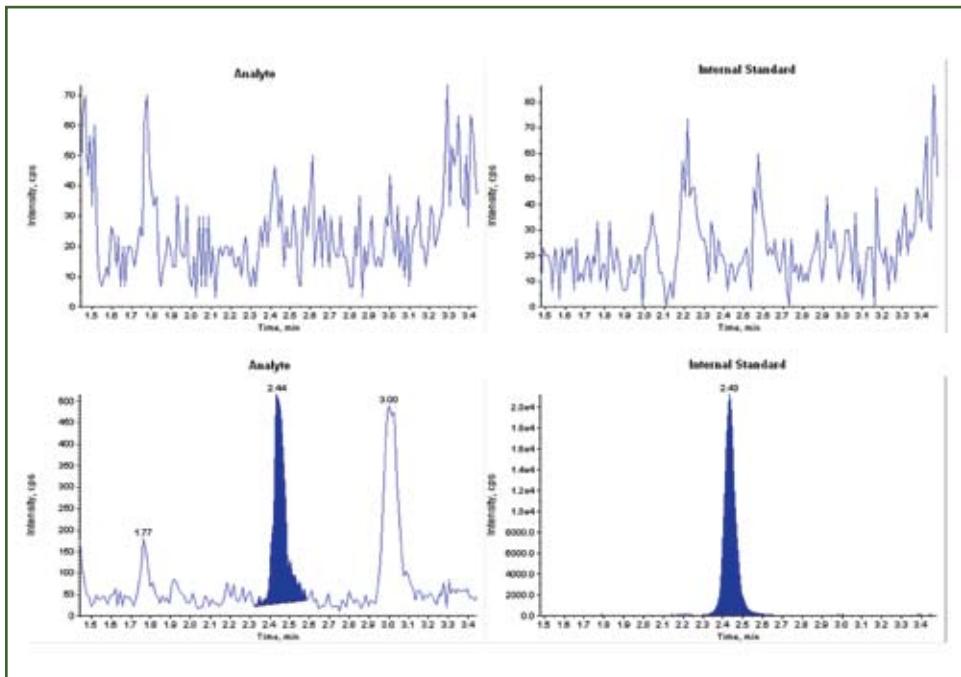
## Experimental

The test accuracy and precision run used the same lot of pool serum to prepare the standard curve and QC samples. The result is as following:

TABLE 1: Accuracy and precision test with same lot of matrix for curve and QCs					
	LLOQ QC	QC1	QC2	QC3	QC4
Theor. Conc. (ng/mL)	0.05	0.1	0.3	8	40
Found Conc. (ng/mL)					
Replicate #1	0.0479	0.11	0.294	7.95	41.1
Replicate #2	0.0442	0.102	0.288	8.29	41.6
Replicate #3	0.0493	0.102	0.303	8.44	43.2
Replicate #4	0.0428	0.101	0.311	8.51	42
Replicate #5	0.0428	0.108	0.328	8.44	42.6
Replicate #6	0.0429	0.111	0.301	8.57	41.5
Mean	0.045	0.106	0.304	8.37	42
S.D.	0.00289	0.0045	0.0141	0.225	0.777
%CV	6.4	4.2	4.6	2.7	1.9
%Theoretical	90	106	101.3	104.6	105
n	6	6	6	6	6

## Experimental continued

FIGURE 1: Extracted Blank and LLOQ (50.0 pg/mL)



In selectivity test, up to 10 different lots of human serum spiked at the LLOQ level (50.0 pg/mL) and/or low QC level (150 pg/mL) was extracted vs. regular standard curves and QCs to evaluate the extraction efficiency and chromatography. It was found that individual lots of human serum sample vary dramatically in MS response at same nominal concentration. Each specificity sample (LLOQ SPEC or QC1 SPEC) was extracted as duplicated. The result confirms that reliability of the extraction compare to the duplicated sample values. All lots of blank serum plasma were also screened to confirm no interference peak at the retention time of the analyte and internal standard.

## Experimental continued

TABLE 2: Accuracy and Precision between 10 lots of human serum at 50.0 and 100 pg/mL				
	LLOQ SPEC Set 1	LLOQ SPEC Set 2	QC1 SPEC Set 1	QC1 SPEC Set 2
Theor. Conc. (ng/mL)	0.05	0.05	0.1	0.1
Found Conc. (ng/mL)				
Lot #1	0.425	0.389	0.395	0.535
Lot #2	0.162	0.158	0.213	0.211
Lot #3	0.0974	0.0770	0.168	0.144
Lot #4	0.0839	0.0901	0.132	0.141
Lot #5	0.0935	0.0660	0.136	0.108
Lot #6	0.640	0.531	0.637	0.583
Lot #7	0.0611	0.0505	0.0968	0.108
Lot #8	0.0819	0.0826	0.125	0.133
Lot #9	0.0435	0.0546	0.102	0.102
Lot #10	0.0830	0.0871	0.145	0.140
Mean	0.177	0.159	0.215	0.221
S.D.	0.196	0.165	0.172	0.181
%CV	110.7	103.8	80	81.9
%Theoretical	354	318	215	221
n	10	10	10	10
Unacceptable sample values				

It was suspected that different recovery occurs in protein crash during extraction. A test was conducted by spiking the same level of analyte and IS into 10 lots of extracted blank human serum. The results were similar as above. Therefore it is concluded that it is not a recovery issue that causes the different response between lots.

To solve the selectivity issue, different mobile phase and LC conditions were tried as well as modification of extraction. The final method was applying the same extraction procedure while the LC conditions were modified by extending the gradient time and reducing the gradient slope. LLOQ was raised to 100 pg/mL to ensure the sufficient sensitivity and less variation of accuracy and precision at low level QCs.

Table 3 shows that the final method solves the selectivity issue and eliminates the response variation between different human serum lots.

**Experimental** continued

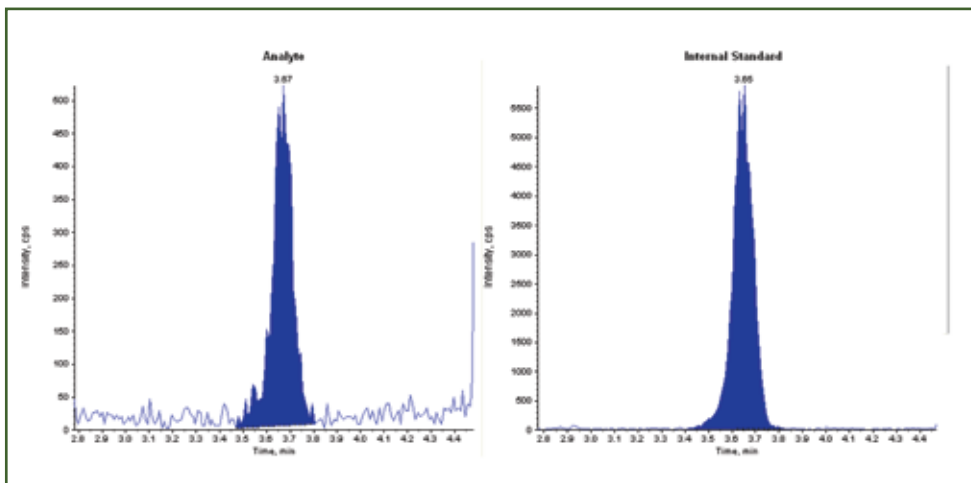
**TABLE 3:**  
Accuracy and Precision between 10 lots of human serum at 50.0 and 100 pg/mL

	SPEC Set 1	SPEC Set 2
Theor. Conc. (ng/mL)	0.100	0.100
Found Conc. (ng/mL)		
Lot #1	0.0939	0.0912
Lot #2	0.101	0.0877
Lot #3	0.101	0.0952
Lot #4	0.0893	0.0909
Lot #5	0.0845	0.0912
Lot #6	0.0939	0.0877
Lot #7	0.0842	0.0903
Lot #8	0.0949	0.0955
Lot #9	0.0872	0.0854
Lot #10	0.105	0.0914
Mean	0.0935	0.0907
S.D.	0.00724	0.00317
%CV	7.7	3.5
%Theoretical	93.5	90.7
n	10	10

**TABLE 4: Final LC-condition and gradient:**  
Initial flow 0.300 mL/min., 20% Mobile Phase B

Time (Min.)	Module	Events	Parameter
0.100	Pumps	Pump B Conc.	20
4.00	Pumps	Pump B Conc.	30
4.01	Pumps	Total Flow	0.3
4.02	Pumps	Total Flow	0.0
4.03	Pumps	Pump C Flow	0.0
4.05	Pumps	Pump C Flow	0.3
4.50	Pumps	Pump C Flow	0.7
5.50	Pumps	Pump C Flow	0.7
6.00	Pumps	Pump C Flow	0.0
6.01	Pumps	Total Flow	0.0
6.02	Pumps	Total Flow	0.3
6.05	Pumps	Pump B Conc.	20
7.00	System Controller	Stop	

FIGURE 2: Extracted LLOQ (revised) with internal standard (100 pg/mL)





---

## Discussion

The investigation showed that the extraction method did not contribute to the lot-to-lot variations. Differences in the recoveries between lots were not significant; however, the LC conditions had a dramatic impact on the instrument response. Utilizing a fine-tuned mobile phase composition and gradient successfully eliminated the lot-to-lot matrix variation. Applying the optimized LC condition also reduced the carryover between samples.

---

## Conclusion

Unlike the small molecules, the ionization consistency of the peptide has a major contribution to lot-to-lot response variation because multi-charged MRMs were usually selected for MS detection. It is very important to establish and optimize the proper LC conditions for small peptide type compounds to ensure the reliability of the sample results in GLP studies.