T2034

Development of a Mass Spectrometry Based Pharmacokinetic (PK) Assay for the Measurement of RLIP76 Protein and Proteoliposome in Human Serum

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PURPOSE

Terapio is developing the RLIP76 protein for recovery for both traumatic brain injury and stroke. Oxidative stress produces toxic alkenals in cells. One of the primary detoxification mechanisms employs the transport protein RLIP76 to shuttle 4-HNE-glutathione conjugates (4-hydroxynonenal-GS) out of the cell. Mass spectrometry provides a solution for the absolute quantitation of certain proteins that may be difficult to accomplish by standard immunoassays. Thus, a quantitative liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS) based pharmacokinetic (PK) assay for the measurement of free and proteoliposome encapsulated RLIP76 in human serum was investigated.

Crude sample processing and preliminary MRM assay conditions using QTRAP® 5500/6500 (AB SCIEX) instruments were established to obtain an early indication of the sensitivity of the assay. In addition, a forced degradation experiment was performed to understand the degradation of RLIP76 peptides in serum. Subsequently preliminary optimization of MRM and sample processing conditions and preliminary precision & accuracy and matrix effect analyses were performed.

1. MRM Assay Development

Recombinant RLIP76 was digested with trypsin (10:1, Promega) and injected onto a QTRAP® instrument (AB SCIEX). The optimal 2 transitions (combination of peptide precursor and fragment ion mass-to-charge ratio that are monitored by the mass spectrometer) per peptide were determined using selected reaction monitoring (SRM)-triggered MS/MS. Collision energy (CE, the energy required to fragment peptide ions) optimization was then performed on each selected transition. Based on the optimization, 30 peptides (60 transitions) were successfully acquired and included in the MRM method.

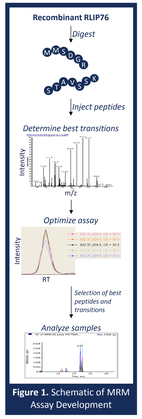


Figure 1. Schematic of MRM Assay Development

Preliminary MRM Assay Conditions and Sample Processing Methods for the Measurement of Recombinant RLIP76

2. No Enrichment Sample Preparation Method

RLIP76 was spiked in human serum and buffer (duplicate). Tween 80 is a necessary component of the parenteral buffer of RLIP76. Matrices with Tween 80 cause mass spectrometry ion suppression. Therefore, a chloroform/methanol extraction method to remove the Tween and disrupt the liposome was implemented. The precipitated proteins (pellet) were digested with trypsin overnight (37°C) and the peptides were reduced by adding TCEP. Samples were then injected onto the mass spectrometer.

Results: Twenty RLIP76 peptides were detected in serum. Calibration curves run on multiple days were linear for all peptides (median R-value = 0.970). Two peptides were detected in serum at 100 ng/mL.

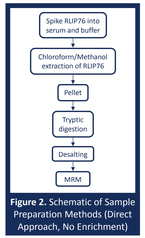


Figure 2. Schematic of Sample Preparation Methods (Direct Approach, No Enrichment)

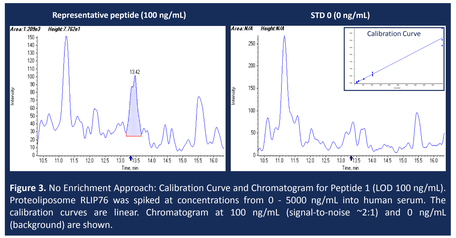


Figure 3. No Enrichment Approach: Calibration Curve and Chromatogram for Peptide 1 (LOD 100 ng/mL). Proteoliposome RLIP76 was spiked at concentrations from 0 - 5000 ng/mL into human serum. The calibration curves are linear. Chromatogram at 100 ng/mL (signal-to-noise ~2:1) and 0 ng/mL (background) are shown.

3. Forced Degradation

Serum was spiked with RLIP76 at 1 and 35 µg/mL (duplicate) and incubated at 37°C for 0, 3 and 18 hours. RLIP76 buffer positive control (1 µg) and serum negative controls (no RLIP76 spiked) were also included. Samples were snap-frozen and stored at -80°C after each incubation time. Samples were then thawed and proteins were extracted by chloroform/methanol and the pellet was resolublized with 10% SDS. One SDS-PAGE gel per spiking condition was performed. Five bands per sample were excised, destained and tryptic digested. MRM analysis was then performed.

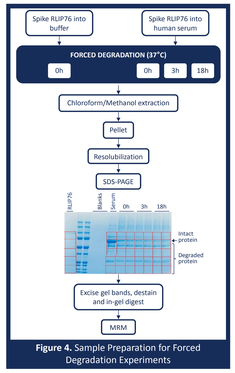


Figure 4. Sample Preparation for Forced Degradation Experiments

Results: Overall, there was a median loss of signal of ~30% in band 1 after 18 hours at 37°C in the monitored RLIP76 peptides. There was no loss at 3 hours. Two N-terminus peptides showed a loss in signal in band 1 by 18 hours and an increase in band 5, suggesting degradation in that region of RLIP76. These peptides were excluded from the assay. Peak areas were reproducible between the 2 replicates.

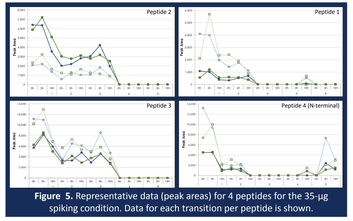


Figure 5. Representative data (peak areas) for 4 peptides for the 35-µg spiking condition. Data for each transition per peptide is shown.

4. Immunoprecipitation (IP) Sample Preparation Method

Streptavidin beads were coupled to biotinylated antibody. The mouse anti-RLIP76 (H10) (Santa Cruz) and mouse RALBP1 (2A1) antibody (Novus Biologicals) were tested. RLIP76 was spiked in human serum and buffer (duplicate). Disruption of the proteoliposome was achieved by adding 0.1% NP-40 and sonication. Streptavidin beads-biotinylated antibody was added to the serum and incubated overnight at 4°C with rotation and then washed with TRIS buffer. Samples were digested overnight with trypsin (37°C) and MRM analysis was performed.

Results: Eighteen RLIP76 peptides were detected in serum. Calibration curves run on multiple days were linear for all peptides (median R-value = 0.978). Five peptides were detected in serum at 5 ng/mL. **Figure 7** shows the results of a representative peptide with the mouse RALBP1 (2A1) antibody, which resulted in more efficient pull down of RLIP76.

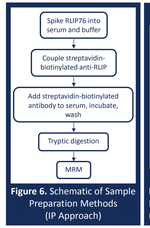


Figure 6. Schematic of Sample Preparation Methods (IP Approach)

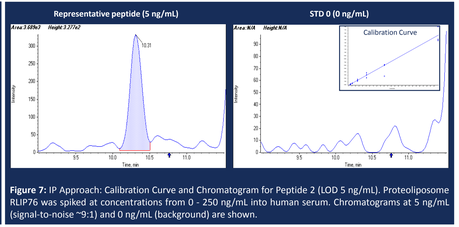


Figure 7: IP Approach: Calibration Curve and Chromatogram for Peptide 2 (LOD 5 ng/mL). Proteoliposome RLIP76 was spiked at concentrations from 0 - 250 ng/mL into human serum. Chromatograms at 5 ng/mL (signal-to-noise ~9:1) and 0 ng/mL (background) are shown.

Preliminary Assay Optimization for Measurement of Recombinant RLIP76

5. MRM Assay Optimization

Heavy labeled RLIP76 was provided by Terapio Corporation and was characterized for use as an internal standard (IS) for absolute quantification of RLIP76. The incorporation rate of the IS was determined to be 96% for heavy-Arg peptides and 84% for heavy-Lys peptides. For accurate quantification, an incorporation ≥95% is required, thus peptides containing Lysine residues were excluded from the MRM assay.

The assay was reduced to 8 peptides and the MRM method was optimized by reducing the column size, increasing the flow rate and increasing the slope of the gradient on the QTRAP® 6500. **Figure 8** compares the preliminary MRM gradient and the optimized MRM gradient.

Preliminary MRM gradient

Time (min)	% A	% B
Initial	92.5	7.5
0.2	92.5	7.5
23.00	75	25
23.60	40	60
24.60	40	60
24.61	92.5	7.5

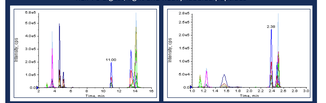
Run time: 30 min
Flow Rate: 10 µL/mL

Optimized MRM gradient

Time (min)	% A	% B
Initial	92.5	7.5
0.5	92.5	7.5
3.00	40	60
3.25	40	60
3.26	92.5	7.5

Run time: 4.5 min
Flow Rate: 30 µL/mL

RLIP76 digest, light and heavy-labeled peptides



Calibration curves, RLIP76 spiked in human serum, IP

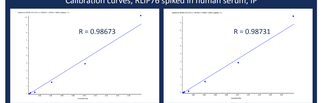


Figure 8. MRM Assay Parameters

Preliminary Assay Optimization for Measurement of Recombinant RLIP76

6. Sample Preparation Optimization (IP Method)

A number of sample preparation parameters were optimized as summarized below:

- Evaluation of magnetic beads: 4 different magnetic beads were evaluated. Dynabeads M-280 Streptavidin beads (Invitrogen) had highest binding capacity for the IP of RLIP76.
- Testing on bead: antibody ratio: Combinations of 1 – 40 µL beads and 0.5 – 4 µg antibody were evaluated. 10 µL (100 µg) beads and 1 µg RALBP1 (2A1) antibody were selected as the best condition.

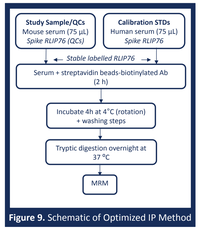


Figure 9. Schematic of Optimized IP Method

- Bead: antibody coupling time: 1, 2 and 4 hours were tested. There was no significant difference between the coupling times.
- IP incubation time: 2 and 4 hours and overnight incubation, at 4°C were tested. There was no significant difference between the different incubation times.
- Trypsin digestion time: 2, 4 and 6 hours and overnight incubation at 37°C were tested. The overnight digestion condition gave the best result.
- IP dilution and preparation of spiking solutions: Various serum dilutions with standard buffer (50mM Tris, 150mM NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4) resulted in variability between replicates, pointing to a solubility issue with spiking solutions or the spiked serum. Dilution of spiking solutions and serum with formulation buffer (including Tween80) gave reproducible results.

Optimized IP conditions include 2 hours coupling time, 4 hours IP at 4°C and overnight trypsin digestion at 37°C. 75 µL of serum is processed and diluted 1:7 in formulation buffer. Spiking solutions are all diluted in formulation buffer. Mouse and human QCs are back-calculated onto human calibration curves.

Table 1 shows representative results using the optimized method. The IS response is very reproducible for the calibration curves and QCs in both mouse and human serum. The calibration curves are linear (R-value = 0.998) and the IS successfully normalizes the data for any processing variability. Excellent accuracy is observed for all calibration standards and both mouse and human QCs.

Table 1: RLIP76 and IS Response and Accuracy in Calibration Standards (Human Serum) and QC Samples (Human and Mouse Serum).

Proteoliposome RLIP76 was used for spiking. IS was spiked at 250 µg/mL.

Calibration Standards	Area	IS Area	Area Ratio	Accuracy (%)
STD1 (50ng/mL) R1	1.48E+05	1.58E+05	0.9	102.9
STD1 (50ng/mL) R2	1.28E+05	1.49E+05	0.9	93.5
STD2 (250ng/mL) R1	4.93E+05	1.31E+05	3.8	90.5
STD2 (250ng/mL) R2	6.29E+05	1.34E+05	4.7	113.0
STD3 (1000ng/mL) R1	2.27E+06	1.34E+05	17.0	104.1
STD3 (1000ng/mL) R2	2.85E+06	1.68E+05	17.0	104.3
STD4 (2500ng/mL) R1	6.07E+06	1.58E+05	38.5	94.9
STD4 (2500ng/mL) R2	5.16E+06	1.38E+05	37.4	92.2
STD5 (5000ng/mL) R1	1.39E+07	1.74E+05	80.2	98.9
STD5 (5000ng/mL) R2	1.39E+07	1.63E+05	85.7	105.7

	IS Peak Area Standards	IS Peak Area Human QCs	IS Peak Area Mouse QCs
Average	1.50E+05	1.20E+05	1.50E+05
% CV	9.6	12.0	8.5

QCs	Area	IS Area	Area Ratio	Accuracy (%)
QC1-human (50ng/mL) R1	1.03E+05	1.18E+05	0.9	95.7
QC1-human (50ng/mL) R2	1.02E+05	1.10E+05	0.9	102.0
QC1-human (50ng/mL) R3	9.25E+04	1.01E+05	0.9	100.6
QC4-human (3500ng/mL) R1	4.74E+06	1.04E+05	45.6	80.3
QC4-human (3500ng/mL) R2	7.48E+06	1.23E+05	60.8	107.1
QC4-human (3500ng/mL) R3	8.26E+06	1.42E+05	58.2	102.5
QC1-mouse (50ng/mL) R1	1.07E+05	1.39E+05	0.8	82.2
QC1-mouse (50ng/mL) R2	1.26E+05	1.40E+05	0.9	97.9
QC1-mouse (50ng/mL) R3	1.37E+05	1.64E+05	0.8	90.6
QC4-mouse (3500ng/mL) R1	9.58E+06	1.48E+05	64.6	113.9
QC4-mouse (3500ng/mL) R2	8.59E+06	1.33E+05	64.7	114.0
QC4-mouse (3500ng/mL) R3	1.02E+07	1.61E+05	63.6	112.0

7. Preliminary Precision and Accuracy

Preliminary inter-assay precision and accuracy results are shown in **Table 2.** Overall, the results are very good for preliminary optimization since the accuracy is within 80-120% for the LLOQ and all other levels.

Table 2. Preliminary Precision and Accuracy Results for Calibration Standards (Human Serum) and Mouse QCs

Test #	Calibration Standards (ng/mL) Calculated concentration							
	50	150	250	750	1000	1500	2500	3750	5000
Test A	47.44	-	282.10	-	1039.00	-	2295.00	-	5260.00
	52.16	-	226.20		1037.00		2363.00		4924.00
Test B	52.59	123.50	165.30	629.40	-	1733.00	2809.00	3747.00	5243.00
	56.08	121.20	197.20	569.70	-	1799.00	2484.00	4625.00	5270.00
Mean	52.07	122.35	217.70	599.55	1038.00	1766.00	2487.75	4186.00	5174.25
%CV	6.82	1.33	22.79	7.04	0.14	2.64	9.16	14.83	3.23
%bias	104.14	81.57	87.08	79.94	103.80	117.73	99.51	111.63	103.49

Regression type: $1/x^2$ linear

Test #	Mouse QC samples (ng/mL) Calculated concentration			
	50	200	1000	3500
Test A	46.00	-	-	3901.00
	49.67	-	-	3970.00
Test B	41.82	-	-	3967.00
	57.52	170.20	854.80	3568.00
	55.58	155.60	829.00	4023.00
Mean	50.12	162.90	841.90	3885.80
SD	6.53	10.32	18.24	182.85
%CV	13.03	6.34	2.17	4.71
%bias	100.24	81.45	84.19	111.02

8. Matrix Effect

Matrix effect was tested by spiking 2 concentrations of RLIP76 into serum from 6 individual mice. The IP and MRM analysis was then performed. The results, shown in **Table 3**, indicate that there is no matrix effect.

Table 3. Matrix Effect Results

Sample ID	Matrix Effect Concentrations (ng/mL)	
	50 ng/mL	3500 ng/mL
QC1 mouse-1 + IS, R1	64.89	3546.00
QC1 mouse-1 + IS, R2	60.84	3671.00
mean	62.62	3608.50
SD	3.22	88.39
%CV	5.14	2.45
%bias	125.23	103.10
QC1 mouse-2 + IS , R1	53.27	2633.00
QC1 mouse-2 + IS , R2	51.39	3189.00
mean	52.33	2911.00
SD	1.33	393.15
%CV	2.54	13.50
%bias	104.66	83.17
QC1 mouse-3 + IS, R1	49.17	2706.00
QC1 mouse-3 + IS, R2	45.04	3407.00
mean	47.11	3056.50
SD	2.92	495.68
%CV	6.20	16.22
%bias	94.21	87.33
QC1 mouse-4 + IS , R1	48.74	3176.00
QC1 mouse-4 + IS, R2	52.88	3100.00
mean	50.81	3138.00
SD	2.93	53.74
%CV	5.76	1.71
%bias	101.62	89.66
QC1 mouse-5 + IS , R1	50.63	2565.00
QC1 mouse-5 + IS, R2	50.29	2503.00
mean	50.46	2534.00
SD	0.24	43.84
%CV	0.48	1.73
%bias	100.92	72.40
QC1 mouse-6 + IS , R1	54.48	3004.00
QC1 mouse-6 + IS, R2	60.65	3071.00
mean	57.57	3037.50
SD	4.36	47.38
%CV	7.58	1.56
%bias	115.13	86.79

CONCLUSIONS

A sensitive MRM assay was developed for the measurement of free and proteoliposome RLIP76 in human and mouse serum. The IP method showed increased sensitivity compared to no enrichment.

Preliminary MRM assay and sample processing conditions for the measurement of RLIP76 were developed:

- Preliminary MRM assay for 30 RLIP76 peptides
- LOD = 100 ng/mL for the no enrichment approach
- LOD = 5 ng/mL for the IP approach
- A unique method for determining whether RLIP76 peptides are affected by degradation indicated:
  - o Slight loss of signal in the main RLIP76 band after 18 hours at 37°C for all detected RLIP76 peptides
  - o N-terminus peptides appear to undergo degradation

Based on the above results, the IP method was chosen for further optimization

- MRM assay for 8 RLIP76 peptides
- Incorporation of heavy labeled RLIP76 as an IS for absolute quantification corrected for processing variability
- LOQ = 50 ng/mL
- Mouse QCs back-calculate on human serum calibration standards
- Preliminary Inter-day precision and accuracy is within 80-120% for the LLOQ and all other levels
- No matrix effect is observed

The next steps are to perform final assay optimization and proceed to method validation. The assay will be validated for PK studies in mice, non-human primate and human in parallel.

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