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Absolute Quantitation of Five Antibody Drug Products in Dosed Monkey Serum, CSF and Brain Lysate for Lead Candidate Identification Using LC-MRM/MS

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PURPOSE

To identify the lead candidate drug in CSF, serum and brain tissue matrices obtained from monkey dosed with five different 5 antibodies. The five candidate drugs were bi-specific IgG-like antibodies based on the Azymetric™ platform. A single mass spectrometry-based MRM (Multiple Reaction Monitoring) assay was developed by targeting proteotypic peptides common to the five antibodies. Assay qualification was performed for the intended use of the assay, for absolute quantitation of the drug candidates in dosed monkey CSF, serum and brain tissue. The overall strategy for the study is shown in Figure 1.

FIGURE 1. Strategy overview.

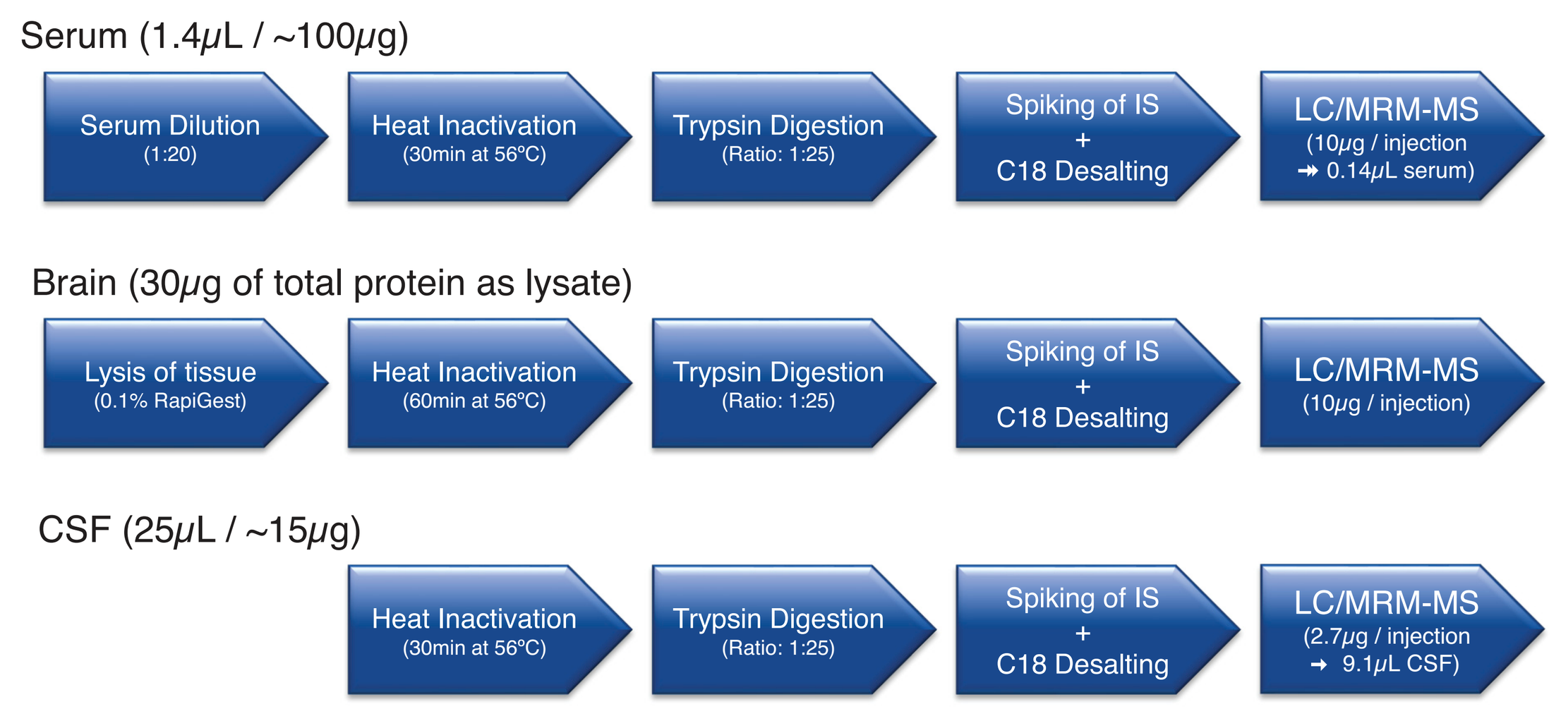
METHODS

MRM Assay Development - A bioinformatics analysis was performed to identify proteotypic peptides common to all five antibodies. The five antibodies were digested with trypsin, pooled and diluted to a final concentration of 200 pmol/mL total antibody (40 pmol/mL per antibody). Mass spectrometry analysis was performed using a nanoAcquity UPLC pump (Waters) coupled to a QTRAP 6500 mass spectrometer (AB Sciex). The total antibody digest was injected on the mass spectrometer to develop the MRM assay conditions against the common proteotypic peptides identified through bioinformatics analysis. Selected reaction monitoring (SRM)-triggered MS/MS was used to determine the optimal four transitions per peptide. The SRM-MS/MS method was developed by calculating the precursor mass of the doubly and triply charged peptide ions and the first and second y- fragment ion with an m/z greater than [m/z (precursor) + 20 Da], for each peptide. If the calculated transitions were observed during the MRM scan, the instrument switched automatically to MS/MS mode and acquired a full MS/MS spectrum of the precursor peptide ion. The four most intense b- or y- fragment ions in the MS/MS spectrum and the corresponding elution time were determined for each acquired peptide. After, the collision energy (CE) was optimized for each of the chosen transitions.

Preliminary Assessment of MRM Assay Performance - The six most intense peptides were selected for assay characterization in matrix, and the corresponding heavy labelled peptides were obtained for use as internal standards (IS). For a preliminary assessment of sensitivity, precision and accuracy, calibration curves were prepared in surrogate matrix due to the limited availability of the same matrix as study samples, and the exploratory nature of the analysis.

Calibration curves were thus generated by spiking one of the five antibodies into BSA-fortified buffer at different ranges based on the expected levels in the corresponding study sample matrix. For serum samples, a 100 to 100,000 ng/mL curve was used. For CSF the curve ranged from 2 to 2,000 ng/mL, and for brain tissue lysate the curve ranged from 2 to 2,000 pg/µg. QC samples were prepared in the same matrix as study samples at five levels (LOQ1, LOQ2, LOW, MID, HIGH). In addition, blank buffer (no IS, no antibody drug) for carryover assessment and double blank in matrix (no IS, no antibody drug) for assessment of interferences were prepared. All prepared samples were processed as shown in Figure 2 and analyzed using the developed MRM assay. Data were analyzed using MultiQuant software (AB Sciex).

FIGURE 2: Processing workflow for monkey serum, brain tissue lysate and CSF.



Assay Qualification - One intra-assay run was performed for each drug product. Three inter-assay runs were performed for one drug product since the five drug products shared the same peptides. Due to the limited availability of the same matrix as study samples, and the exploratory nature of the analysis the calibration curves were again prepared in surrogate matrix, using BSA-fortified buffer, and concentrations ranges for each matrix were adjusted based on the results from the preliminary assessment. For serum samples, the achievable LOQ was estimated to be in the medium ng/mL range but was set to 4 µg/mL based on independent ELISA data, and the ULOQ was set to 400 µg/mL. For CSF the curve ranged from 10 to 2,500 ng/mL, and for total brain lysate the curve range was 8 to 2,000 pg/µg. QC samples were also prepared in each of the matrices at four levels (LOQ, LOW, MID, HIGH). For carryover assessment and assessment of interferences, blank buffer (no IS, no antibody drug) for carryover assessment and double blank in matrix (no IS, no antibody drug) were also prepared. All samples were processed as shown in Figure 2 and analyzed using the MRM assay, followed by data analysis using MultiQuant software.

Sample Analysis – Exploratory sample analysis was performed with calibration curves and QC samples as per the assay qualification.

RESULTS

MRM Assay Development - Bioinformatic analysis identified 26 proteotypic peptides that were common across the five antibodies. MRM assay conditions were successfully developed for 24 of the 26 peptides.

Preliminary Assessment of MRM Assay Performance - The 6 peptides with the highest measured peak areas from the assay development step were selected for the preliminary assessment of assay performance. The retention time, carryover percentage, presence of interference at the light/heavy transition in buffer/matrix, the LOD, linear calibration curve range, r-score and how many of the QC samples were within an accuracy within a +/- 30% bias, were determined for each transition for each of the matrices. Complete results for CSF are shown in Figure 3A as an example (corresponding data for serum and brain lysate are not shown).

Assay sensitivity was found to vary significantly between the three matrices. This is shown in Figure 3B using data for peptide GEVQLVESGGGLVQPGGSLR. The three best performing peptides across the 3 different matrices were selected for the subsequent assay qualification:
FTISADTSK, GEVQLVESGGGLVQPGGSLR, and TTPPVLDSDGSFALVSK.
The other three peptides showed either poor sensitivity, excessive carryover, or interferences at the heavy/light transitions, and were not used.

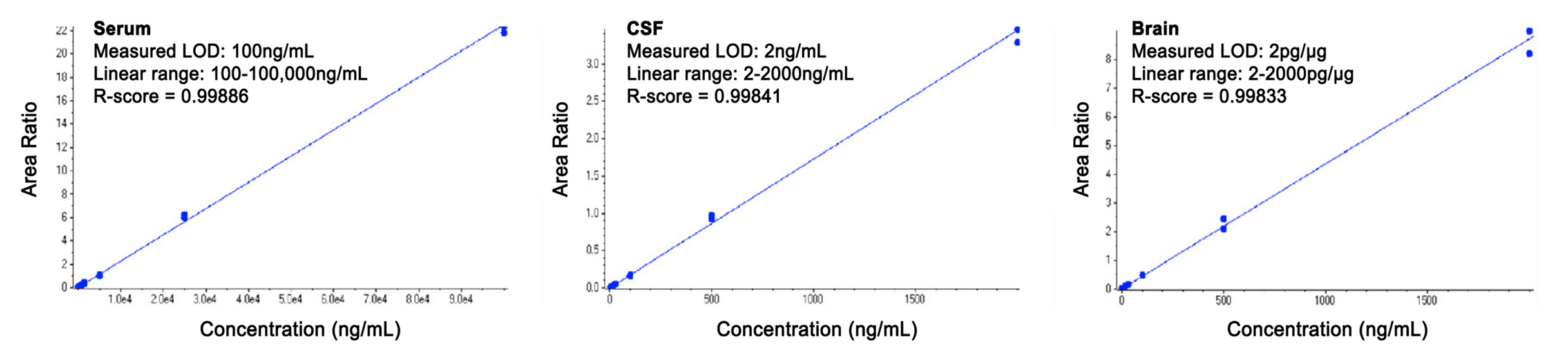
FIGURE 3. **[A]** Preliminary assay assessment results for CSF. Good results were obtained for peptides FTISADTSK, GEVQLVESGGGLVQPGGSLR and IYPTNGYTR.
[B] Calibration curves for GEVQLVESGGGLVQPGGSLR in serum, CSF and brain lysate. All curves were linear (r-score >0.998). The LOD was 100 ng/mL for serum, 2 ng/mL for CSF and 2 pg/µg for brain lysate.

[A]

Transition	RT	Peak Shape	Carry Over	Interferences (light B/M; heavy B/M)	LOD (ng/ml)	Linear calibration curve range	r-score	Accuracy of QC1 in matrix (2ng/mL)	Accuracy of QC2 in matrix (5 ng/mL)	Accuracy of QC3 in matrix (10 ng/mL)	Accuracy of QC4 in matrix (40 ng/mL)
FTISADTSK_485.25_721.4	7.27	Good	None	None	4	4 - 2000	0.99281	0/2	0/2	0/2	1/2
FTISADTSK_485.25_608.3	7.27	Good	None	Heavy M	30	30 - 2000	0.99260	0/2	0/2	0/2	0/2
FTISADTSK_485.25_822.4	7.27	Good	None	None	30	30 - 2000	0.98902	0/2	0/2	0/2	2/2
FTISADTSK_485.25_521.3	7.27	Good	None	None	100	100 - 2000	0.97499	0/2	0/2	0/2	0/2
GEVQLVESGGGLVQPGGSLR_970.02_586.3	17.46	Good	Yes (0.3%)	None	2	2 - 2000	0.99775	1/2	2/2	1/2	2/2
GEVQLVESGGGLVQPGGSLR_970.02_714.4	17.46	Good	Yes (0.3%)	None	4	4 - 2000	0.99841	0/2	2/2	1/2	2/2
GEVQLVESGGGLVQPGGSLR_970.02_626.4	17.46	Good	Yes (0.3%)	None	16	16 - 2000	0.99688	0/2	0/2	0/2	2/2
GEVQLVESGGGLVQPGGSLR_970.02_813.5	17.46	Good	Yes (0.3%)	Heavy M	16	16 - 2000	0.98758	0/2	0/2	0/2	1/2
IYPTNGYTR_542.78_808.4	5.9	Good	None	None	4	4 - 2000	0.99177	0/2	0/2	0/2	1/2
IYPTNGYTR_542.78_277.2	5.9	Good	None	None	16	16 - 2000	0.99220	0/2	0/2	0/2	1/2
IYPTNGYTR_542.78_610.3	5.9	Good	None	None	16	16 - 2000	0.99720	0/2	0/2	0/2	0/2
IYPTNGYTR_542.78_496.3	5.9	Good	None	None	500	500 - 2000	0.99661	0/2	0/2	0/2	0/2
TTPPVLDSDGSFALVSK_867.45_808.5	18.6	Good	None	Light M	8	8 - 2000	0.99841	0/2	0/2	0/2	0/2
TTPPVLDSDGSFALVSK_867.45_724.4	18.6	Good	None	Light M	16	16 - 2000	0.99760	0/2	0/2	0/2	0/2
TTPPVLDSDGSFALVSK_867.45_609.4	18.6	Good	None	None	16	16 - 2000	0.99721	0/2	0/2	0/2	1/2
TTPPVLDSDGSFALVSK_867.45_446.3	18.6	Good	None	None	16	16 - 2000	0.99855	0/2	0/2	0/2	0/2
LEESGGGLVQAGGSLR_765.40_688.4	11.4	Good	Yes (1.5%)	Light B	4	4 - 2000	0.99661	0/2	0/2	0/2	2/2
LEESGGGLVQAGGSLR_765.40_787.4	11.4	Good	Yes (1.5%)	Light B	2	2 - 2000	0.99254	0/2	0/2	1/2	2/2
LEESGGGLVQAGGSLR_765.40_560.3	11.4	Good	Yes (1.5%)	None	16	16 - 2000	0.99595	0/2	0/2	0/2	1/2
LEESGGGLVQAGGSLR_765.40_489.3	11.4	Good	Yes (1.5%)	None	16	16 - 2000	0.99775	0/2	0/2	0/2	1/2
LLIYSASFLYSGVPSR_886.98_359.2	25.1	Good	Yes (0.6%)	None	16	16 - 2000	0.99534	0/2	0/2	2/2	0/2
LLIYSASFLYSGVPSR_886.98_765.4	25.1	Good	Yes (0.6%)	None	4	4 - 2000	0.99723	0/2	0/2	0/2	0/2
LLIYSASFLYSGVPSR_886.98_602.3	25.1	Good	Yes (0.6%)	None	30	30 - 2000	0.99691	0/2	0/2	0/2	0/2
LLIYSASFLYSGVPSR_886.98_515.3	25.1	Good	Yes (0.6%)	None	100	100 - 2000	0.99386	0/2	0/2	0/2	0/2

Notes:
Accuracy : If both transitions pass (OK), if only 1 pass (1/2), of none pass (0/2); OK < 30%

[B]



MRM Assay Qualification - For assay qualification, three peptides and two transitions per peptide were monitored. Of the two transitions, the best transition was used for quantification and the second transition was used as a qualifier. Since the same peptides are used for the quantification of the five drug products, one intra-assay run was performed for each of the 5 drug products, and 3 inter-assay runs were performed on one of the five drug products in each matrix. Sensitivity was found to be identical across all five antibodies, and the intra-assay precision and accuracy was comparable (data not shown). The inter-assay results for all three matrices are shown in Figure 4.

FIGURE 4. Inter-assay precision and accuracy for the three matrices. Precision and accuracy was ≤16.4% for **[A]** serum, ≤17.7% for **[B]** CSF, and ≤21.9% for **[C]** brain lysate.

[A] Serum

Curve Code (drug; peptide_transition; run)	Standard concentrations (µg/mL serum)								QC samples (µg/mL)			
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	LOQ	Low	Med	High
	4	10	20	50	100	200	350	400	4	12	75	300
Drug #1; FTISADTSK_485.25_721.4; run 1	4.3	9.3	19.1	44.3	102.2	201.9	356.2	340.2	3.6	11.4	70.2	291.6
	3.9	9.6	20.6	51.2	108.4	215.4	394.8	386.4	3.6	11.7	71.6	285.3
Drug #1; FTISADTSK_485.25_721.4; run 2	3.9	9.5	22.2	53.5	108.0	205.4	365.9	387.3	3.5	9.8	58.2	239.6
	3.9	9.3	18.0	48.9	106.0	193.4	343.2	389.8	3.1	10.2	58.8	233.5
Drug #1; FTISADTSK_485.25_721.4; run 3	4.8	9.6	20.2	45.6	96.8	218.8	352.8	431.6	4.1	N/A	86.9	286.8
	4.4	9.8	19.0	46.8	92.9	193.5	336.4	385.3	4.3	N/A	82.0	294.4
n	6	6	6	6	6	6	6	6	6	4	6	6
Mean	4.2	9.5	19.9	48.4	102.4	204.7	358.2	386.8	3.7	10.8	71.3	271.9
SD	0.4	0.2	1.5	3.5	6.4	10.7	20.7	28.9	0.4	0.9	11.7	27.6
Precision (%CV)	8.8	2.0	7.4	7.2	6.2	5.2	5.8	7.5	11.7	8.5	16.4	10.2
% bias	5.0	-4.8	-0.7	-3.2	2.4	2.4	2.3	-3.3	-7.5	-10.2	-5.0	-9.4

[B] CSF

Curve Code (drug; peptide_transition; run)	Standard concentrations (ng/mL CSF)								QC samples (ng/mL)			
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	LOQ	Low	Med	High
	10	20	50	100	500	1000	2000	2500	10	30	600	1800
Drug #1; FTISADTSK_485.25_721.4; run 1	12.2	20.9	51.8	86.2	440.7	942.8	2091.2	2500.9	10.9	27.6	502.8	1636.6
	11.6	20.4	51.1	76.7	E346.5	938.9	2102.9	2511.7	10.0	27.8	500.7	1522.9
Drug #1; FTISADTSK_485.25_721.4; run 2	12.6	20.7	53.6	102.2	510.0	923.2	2164.0	2616.4	12.8	34.3	672.9	2041.2
	11.6	19.7	41.7	91.7	399.9	819.5	2026.8	2546.5	13.3	36.1	666.2	2210.3
Drug #1; FTISADTSK_485.25_721.4; run 3	10.8	19.9	50.9	95.0	468.8	886.1	2201.8	2601.8	11.4	33.2	609.7	2052.2
	11.8	20.5	52.3	98.9	441.6	830.8	2135.2	2433.8	12.0	31.4	588.4	1914.3
n	6	6	6	6	5	6	6	6	6	6	6	6
Mean	11.8	20.4	50.2	91.8	452.2	890.2	2120.3	2535.2	11.7	31.7	590.1	1896.3
SD	0.6	0.5	4.3	9.3	40.6	54.4	61.1	68.1	1.2	3.5	75.7	265.0
Precision (%CV)	5.2	2.3	8.5	10.1	9.0	6.1	2.9	2.7	10.4	11.0	12.8	14.0
Accuracy (% bias)	17.7	1.7	0.5	-8.2	-9.6	-11.0	6.0	1.4	17.3	5.8	-1.6	5.3

[C] Brain lysate

Curve Code (drug; peptide_transition; run)	Standard concentrations (pg/µg Brain lysates)								QC samples (pg/µg)			
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	LLOQ	Low	Med	High
	8	16	50	100	400	1000	1600	2000	8	24	500	1500
Drug #1; FTISADTSK_485.25_721.4; run 1	8.3	15.1	45.9	94.2	388.7	1017.6	1638.0	1939.0	7.7	21.2	446.4	1396.4
	8.5	16.9	46.5	93.4	402.5	1034.5	1550.1	2048.7	7.0	21.7	449.8	1342.6
Drug #1; FTISADTSK_485.25_721.4; run 2	8.7	18.3	56.8	112.3	482.5	972.8	1635.2	2022.7	6.0	19.1	432.7	1389.6
	7.6	17.7	47.3	98.9	386.1	1040.2	1595.8	1846.6	4.5	17.0	405.8	1182.3
Drug #1; FTISADTSK_485.25_721.4; run 3	9.0	17.8	56.5	110.0	474.4	1069.8	1534.3	1970.5	7.6	19.4	452.5	1389.4
	7.5	17.8	53.0	107.2	434.4	936.4	1639.2	1912.2	4.9	20.4	473.0	1372.3
n	6	6	6	6	6	6	6	6	6	6	6	6
Mean	8.3	17.3	51.0	102.7	428.1	1011.9	1598.8	1956.6	6.3	19.8	443.4	1345.4
SD	0.6	1.2	5.1	8.2	42.7	48.8	47.0	74.1	1.4	1.7	22.5	82.2
C.V.	7.3	6.7	9.9	8.0	10.0	4.8	2.9	3.8	21.9	8.6	5.1	6.1
% bias	3.3	7.9	2.0	2.7	7.0	1.2	-0.1	-2.2	-21.5	-17.5	-11.3	-10.3

The qualification indicated an inter-assay precision (%CV) and accuracy (%bias) <25% and +/- 25% for all matrices. The monkey CSF and serum samples results revealed excellent agreement between MRM and ELISA (independent laboratory). Analysis of eight blinded CSF QC samples resulted in very good accuracy. Overall, the results indicated that the MRM assays have very good sensitivity without the use of hybrid immunoaffinity-MRM and are well suited for an exploratory analysis of monkey samples.

Sample Analysis

The MRM assay was used for the analysis of three dosed CSF study samples and eight blinded CSF samples spiked with antibody drug. The results from this analysis are shown in Figure 5.

FIGURE 5. **[A]** Measured antibody drug concentrations in the three study samples using MRM and ELISA. The MRM measurements from this study correlate well with the independent ELISA data. **[B]** Measured antibody drug concentrations in the eight blinded spiked CSF samples. A negative bias was observed for the blinded samples, but the overall accuracy was consistent at <20% bias.

[A]

Sample ID	Measured concentration by MRM (ng/mL)	Measured concentration by ELISA (ng/mL)
B6535	48.2	29.6
C4062	70.5	40.0
B6248	69.4	69.8

[B]

Sample ID	Measured concentration (ng/mL)	Nominal concentration of spiked sample (ng/mL)	% difference
Blinded Sample #1	0	0	0
Blinded Sample #2	0	0	0
Blinded Sample #3	29.3	35	-16.4
Blinded Sample #4	101.1	120	-15.8
Blinded Sample #5	801.9	1000	-19.8
Blinded Sample #6	99.4	120	-17.1
Blinded Sample #7	30.5	35	-12.8
Blinded Sample #8	810.9	1000	-18.9

CONCLUSIONS

- A single mass spectrometry-based MRM assay was successfully developed for the measurement of five distinct but structurally related Azymetric™ antibody drugs, by targeting proteotypic peptides common to the five antibodies.
- Data presented shows that the assay is sensitive, precise and accurate for the intended use, for the absolute quantitation of five antibody drug candidates in dosed monkey serum, CSF and brain lysate, towards identification of the lead candidate drug.
- Inter-assay precision and accuracy (expressed as % bias) was ≤16.4% for serum, ≤17.7% for CSF and ≤21.9% for brain lysate.
- The three CSF study samples analyzed showed very good agreement between MRM and ELISA (independent laboratory).
- The eight blinded spiked CSF samples were quantified with good accuracy, with % bias <20% for all samples.

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