T1230

Determination of Modified Thymidine Phosphorylase Concentration and its Activity in Mouse Serum by LC-MS/MS

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PURPOSE

Thymidine phosphorylase (TP) catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogues to their respective nucleobases and 2-deoxy-α- d -ribose-1-phosphate[1]. Defect of TP activity related to some diseases, such as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) [2, 3]. Ectogenic enzyme is one of the important treatments for such diseases. Then monitoring the concentration of the ectogenic enzyme is necessary to help understanding the metabolism and efficiency of the drug *in-vivo*. For measuring the concentration of enzyme, ligand binding assay is a default choice, but the feasibility and accuracy of such kind of assays are excessively relied on the generation of specific antibody reagents, and the generation of the reagent is time consuming and quality uncertainty.

dThd and Pi binding are favorable processes to monitor the activity of TP[4]. In this study, a sensitive and specific liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method was established to measure the concentration and activity of modified thymidine phosphorylase in mouse serum by quantifying of thymine, the product generated by TP catalyzes phosphorolysis of thymidine.

To our best knowledge, this is the first time an LC-MS/MS method is reported to measure the concentration of an enzyme via enzyme catalyze reaction.

METHOD(S)

An enzyme reaction system was established based on the study of a kinetic mechanism of HumanThymidine Phosphorylase[5]. Final concentrations of components in the reaction mixture: 10 mM Pi and 10 mM thymidine, pH of reaction system is 7.4, and incubation time is 1 hour at 37℃, then the reaction was terminated by adding methanol, and the thymine was extracted by methanol for LC-MS/MS analysis.

Table 1 Enzyme Reaction System Preparation

Solution	Negative Enzyme-Control Sample (μL)	Reaction Sample (μL)	Final Concentration
10 x PBS	20	20	1 x PBS
Water	60	60	NA
10.0 mM thymidine	20	20	1.00 mM
Enzyme samples (Last step)	100 PBS	100 serum samples	STD sample: 0.0250 to 3.00 nM
Final volume	200	200	NA

For thymine qualntification:

- Column: CAPCELL PAK ADME 2 μm 2.1×100 mm
- Mobile phase A: 0.1% formic acid and 1mM Ammonium formate in H_2O
- Mobile phase B: 0.1% formic acid in acetonitrile

Mass Spectrometer： AB Sciex 6500+

- Ionization Mode-ESI Positive Ion Detection
- Ion transitions:

  Analyte: Thymine 127/110; Internal standard: Thymine-d7 134/116

RESULT(S)

Enzyme activity was calculated from concentration change of thymine in the reaction system, and a linearity relationship was established for enzyme concentration and thymine LC-MS/MS response to determine the concentration of the modified TP enzyme. Figure 1 showed that there is good linearity between thymine response and enzyme concentration when the thymidine phosphorylase ranged from 0.0250-3.00 nM. Table 2 showed that the accuracy and precision results is also good.

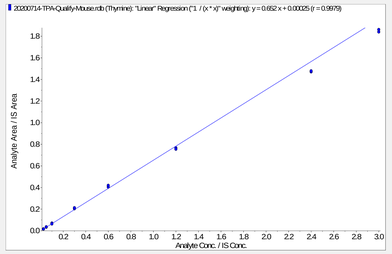

Figure 1 Calibration curve of modified TP concentration against thymine response when concentration of modified TP is ranged from 0.0250-3.00 nM in mouse serum

Table 2 Accuracy and Precision results of modified TP concentration against thymine response when concentration of modified TP is ranged from 0.0250-3.00 nM in mouse serum

Matrix	Mouse serum				
Sample ID	LLOQ QC	LQC	GMQC	MQC	HQC
Conc. (nM)	0.0250	0.0750	0.250	1.50	2.25
%DEV	106	95.3	100	106	111
	103	102	102	103	110
	109	98.9	94.2	102	107
	106	101	100	103	111
	96.8	97.7	102	102	108
	109	103	96.1	100	109
Mean %DEV	105	99.6	99.1	103	109
%CV	4.3	2.8	3.3	1.9	1.5

The LC-MS/MS method was applied in a mouse PK study dosed with the modified TP. Figure 2 and figure 3 demonstrated that opposite trend of TP concentration and thymidine and 2-deoxyuridine concentration when dosing modified TP enzyme at different level, which showed that the activity and concentration of the modified TP in mouse serum can be effectively monitored by this method.

Reproducibility of the method was also evaluated, difference of all 24 selected samples were within 20.0% between the results got from different batches. It demonstrated that the assay the reliable and reproducible.

Figure 5 showed that the serum concentration data and PK profile of the modified TP in mouse obtained by this LC-MS/MS method was comparable with traditional ligand binding assay for quantifying the enzyme, not only in the trend of PK profile, but also in concentration levels.

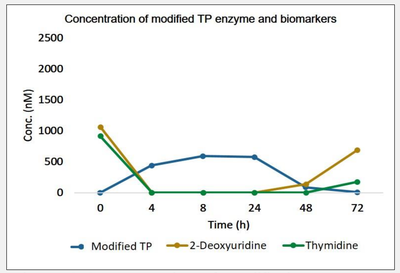

Figure 2 Concentration of modified TP enzyme, 2-deoxyuridine and thymidine at lower dose of modified TP enzyme

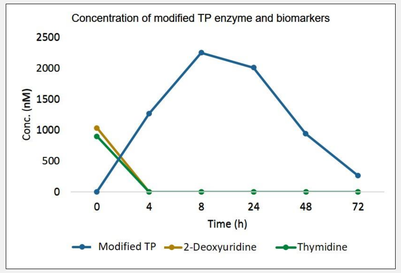

Figure 3 Concentration of modified TP enzyme, 2-deoxyuridine and thymidine at higher dose of modified TP enzyme

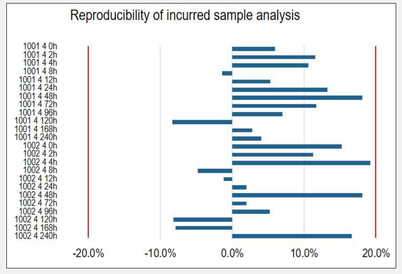

Figure 4 Enzyme conc. of PK samples tested in different batches of assay

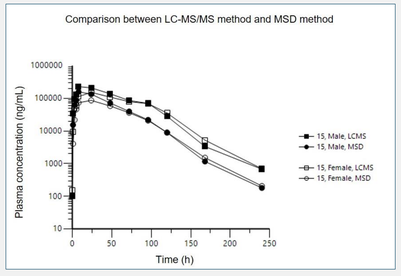

Figure 5 Comparison of serum concentration and PK profile obtained by LC-MS/MS method and Ligand binding assay (MSD) method

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CONCLUSION

A sensitive and specific LC-MS/MS method was established to measure the concentration and activity of a modified thymidine phosphorylase in mouse serum. The performance and application results of the assay illustrated that LC-MS/MS is specific and accurate for measuring enzyme concentration by means of monitoring enzyme activity reaction. It can be considered as an alternative approach for measuring concentration of enzyme, and it possesses great advantage when there is difficulty to generate regent for ligand binding assay.

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