

# LC-MS-MS Quantitative Analysis of Folic Acid, Its Metabolites and Derivatives in Serum for Research Use

## ABSTRACT

**Introduction:** Liquid chromatography triple quadrupole mass spectrometry is suited for rapid analysis of multiple analytes of similar and different structures and physicochemical properties. Folic acid and its derivatives are a diverse group of biologically active compounds that physiologically impact the bodies' functions and include- folic acid, dihydrofolate, tetrahydrofolate, 5-methyl-tetrahydrofolate, 5,10-methylene-tetrahydrofolate, folinic acid, N- formyl-tetrahydrofolate, and the oxidation product of 5-methyl-tetrahydrofolate known as MeFox. A sensitive and specific LC-MS/MS analytical research method was developed and optimized for the quantitation of folates in serum. Various sample preparation techniques were evaluated. A one dimensional chromatographic configuration achieved the required sensitivity, accuracy and robustness to measure the compounds over their relevant dynamic range.

**Method:** A Thermo Scientific™ TSQ Quantiva™ tandem mass spectrometer in positive Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 200 µl of serum was used for the analysis of the folates. A Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 2.6 µm column with a water:methanol :mixture containing 0.5% acetic acid was used. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive mode due to the compounds different chemistries and structures. The accuracy of these methods were verified using standardized reference materials and from donor individuals.

**Results:** Good linearity and reproducibility were obtained with the concentration range from 25 pg/ml to 1000 ng/ml for the folates with a coefficient of determination  $R^2 > 0.98$  or better for all compounds in the various matrices. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to the pg/ml levels of the folates in biological samples. Excellent reproducibility was observed for all compounds (CV < 10%) for the techniques and configurations used.

**Conclusion:** A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry research method was developed and verified for the simultaneous measurement of folates, derivatives and metabolites in serum. The sample preparation techniques are easily applied for high throughput analysis.

**Novel Aspect:** A sensitive method for the analysis of folates and metabolites that achieves the desired levels of detection and quantitation.

## INTRODUCTION

Folates act as endogenous one-carbon donors and in the form of tetrahydrofolate is involved in purine and pyrimidine nucleotide synthesis during normal cell division and is particularly active in pregnancy and infancy. Folates also aid in the production of red blood cells. Tetrahydrofolate and its metabolites are involved in the synthesis of amino acids and nucleic acids while dihydrofolate regulates this synthesis.

In this research study, we evaluated various columns and solvent combinations as well as sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of folates, their metabolites and derivatives that include Folic acid (FA), Dihydrofolate (DHF), Tetrahydrofolate (THF), 5,10-Methylenetetrahydrofolate (5,10-CH<sub>2</sub>-THF), 5,10-Methenyltetrahydrofolate (5,10-CH=THF), 5-Formyltetrahydrofolate (5-CHO-THF), 10-Formyltetrahydrofolate (10-CHO-THF), 5-Methyltetrahydrofolate (5-M-THF) and a Pyrazino-s-triazine oxidation product of Tetrahydrofolate (MeFox). The sample preparation choices were kept simple and included protein crash and a simple solid phase extraction. The methodologies were developed on a TSQ Quantiva tandem mass spectrometer in positive Electrospray ionization modes with a Vanquish HPLC system for a 6 minute analytical gradient.

## MATERIALS AND METHODS

### Standards

The following analytical reference standards and Internal standards were obtained from-

Merck & Cie, Schaffhausen, Switzerland

Folic Acid (FA):	100 mg
Folic Acid-13C5:	20 mg
Dihydrofolate (DHF):	100 mg
Tetrahydrofolate (THF):	100 mg
Tetrahydrofolate-13C5:	20 mg
5,10-Methylene-THF (5,10-CH <sub>2</sub> -THF):	100 mg
5,10-Methenyl-THF (5,10-CH=THF):	100 mg
5,10-Methenyl-THF-13C5:	20 mg
5-Formyl-THF (5-CHO-THF):	100 mg
5-Formyl-THF-13C5:	20 mg
10-Formyl-THF (10-CHO-THF):	100 mg
5-Methyl-THF (5-M-THF):	100 mg
5-Methyl-THF-13C5:	20 mg

## Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Acetic Acid
Methanol	Ascorbic Acid
Acetonitrile	Ammonium Formate

The standards and internal standards were made up in 1 g/L of Ascorbic acid in water to prevent the degradation of the compounds and were stored in amber vials wrapped in aluminum foil at -25°C to further prevent degradation as the folates are very sensitive to light.

## Sample Preparation- Solid Phase Extraction

- 200 µL of vitamin depleted serum/water mixture calibrators, controls and serum sample were added to 1.5 ml eppendorf tubes and 20 µL of folate ISTD mixture at 1000 ng/mL were added to each tube and vortexed briefly
- 400 µL of SPE sample buffer (10 g/L Ammonium Formate and 1 g/L Ascorbic Acid in water at pH 3.2) was added to each tube followed by 200 µL of water and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The solid phase extraction plate- 10 mg/2mL SOLA 96 well SPE plate, was conditioned with 2 mL of Acetonitrile followed by 2 mL of Methanol and then 2 mL of SPE sample buffer
- The supernatant was then added to the wells of SPE plate and allowed to drip through under gravity at room temperature
- The SPE plate was then washed with 3 mL of the SPE wash buffer (0.5 g/L Ammonium Formate and 0.05 g/L Ascorbic Acid at pH 3.4
- The folates were then eluted using 400 mL/L Acetonitrile:100 mL/L Methanol:10 mL/L Acetic Acid:1 g/L Ascorbic Acid solution under positive pressure
- The elute was dried down under nitrogen at room temperature
- The extract was reconstituted in 200 µL of 9:1 water and methanol
- The supernatant was transferred to an MS vial and capped
- All in-house calibrators were prepared in vitamin depleted serum (Golden West Biological, Inc, Temecula, CA)

Figure 1- SOLA SPE Plate



The calibration curves ranged from 0.01 ng/mL to 1000 ng/mL and various pooled donor samples were used as control material.

## Data Analysis

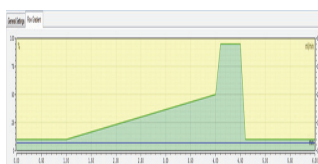
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Quantiva Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

## METHOD

### HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 100 x 2.1 mm, 2.6 µm
Column Temperature:	30 °C
Injection Volume:	10 µL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.5% Acetic Acid
Mobile Phase B:	80%:20%-Methanol:Acetonitrile
Flow Rate:	0.35 ml/min
Gradient:	0.0 min- 90%A:10%B 1.0 min- 90%A:10%B 4.0 min- 50%A:50%B 4.1 min- 5%A:95%B 4.5 min- 5%A:95%B 4.6 min- 90%A:10%B 6 min
Run time:	



## MS and Ion Source Conditions-

TSQ Quantiva triple quadrupole mass spectrometer	
Ion mode:	Positive Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 °C
Ion Transfer Tube Temperature:	300 °C
Sheath Gas:	50
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):1350 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
Tetrahydrofolate	0.94	446.23	299.1/166.1	19.6/41.3	69
Tetrahydrofolate-13C5	0.94	451.5	299.1	16.9	77
10-Formyl-THF	1.04	474.23	456.2/412.2	20.3/32.9	88
5-Methyl-THF	1.18	460.2	313.1/180.1	19.1/38.4	70
5-Methyl-THF-13C5	1.18	465.2	313.1	19.4	76
5,10-Methenyl-THF	1.48	456.1	412.2/327.1	29.1/31.3	77
5,10-Methenyl-THF-13C5	1.48	461.2	416.2	30.1	83
MeFox	1.62	474.1	327.1/284.1	23.1/36.6	79
MeFox-13C5	1.62	479.1	284.1	37.1	72
5-Formyl-THF	2.76	474.1	327.1/299.1	19.6/31.2	71
5-Formyl-THF-13C5	2.76	479.1	299.1	31.3	72
5,10-Methylene-THF	2.95	458.28	311.1/166.1	19.2/45.8	78
Dihydrofolate	3.00	444.2	297.1/178.1	16.9/12.9	60
Folic Acid	3.03	442.2	295.04/176.1	17.0/38.2	68

## RESULTS

### Linearity/Sensitivity

The assay was linear over the calibration curve for the Foliates in the vitamin depleted serum/water mixture as shown in the table with their mean of coefficient of determinations ( $R^2$ ) for positive mode using solid phase extraction. The linearity of the extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination ( $R^2$ ) > 98 for compound and the %CV for each calibration point were all <10% in order to be accepted.

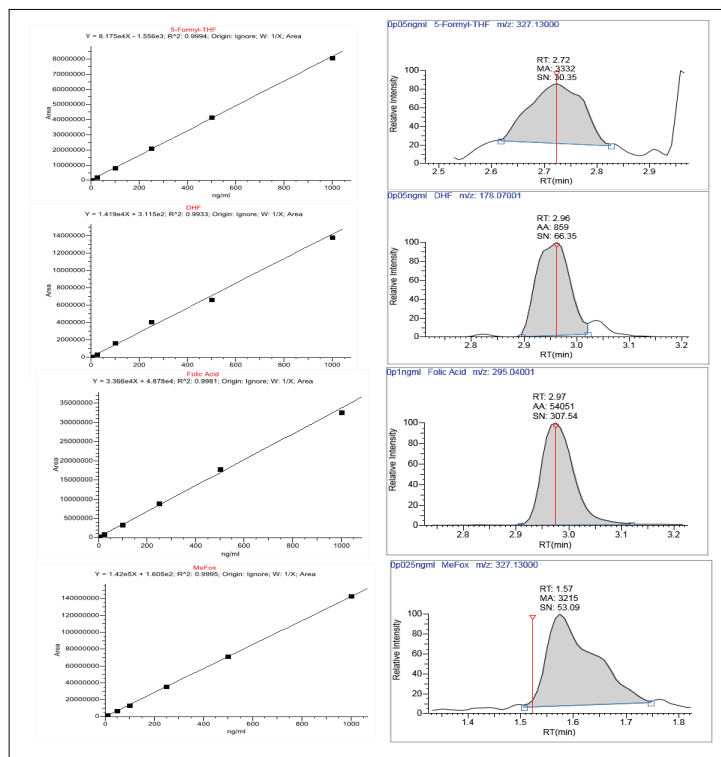
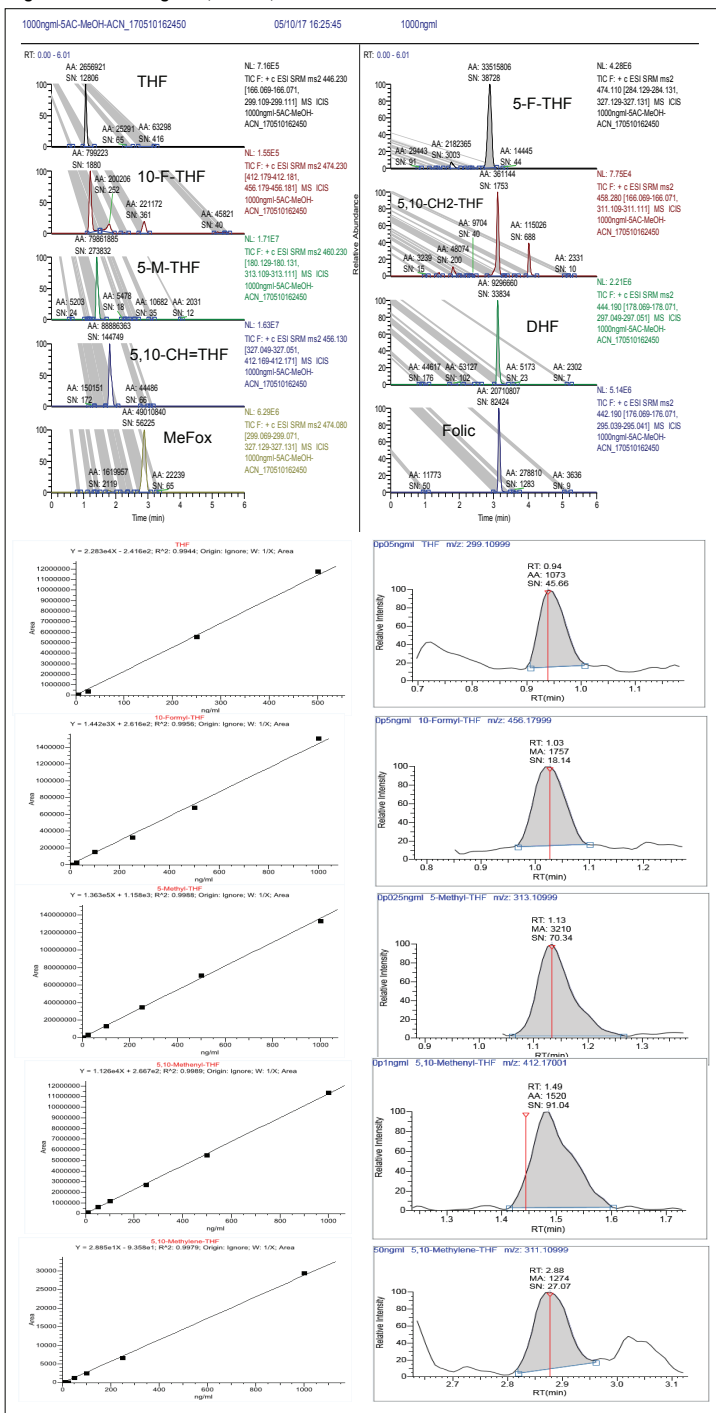
Table 2- Linearity and Sensitivity

Compound	Linearity (ng/ml)	LOQ (ng/ml)	$R^2$	S/N
Tetrahydrofolate	0.05 - 1000	0.05	0.9944	45.66
10-Formyl-THF	0.5 - 1000	0.5	0.9954	18.14
5-Methyl-THF	0.025 - 1000	0.025	0.9988	70.34
5,10-Methenyl-THF	0.1 - 1000	0.1	0.9989	91.04
MeFox	0.025 - 1000	0.025	0.9995	53.09
5-Formyl-THF	0.05 - 1000	0.05	0.9994	30.35
5,10-Methylene-THF	50 - 1000	50	0.9979	27.07
Dihydrofolate	0.05 - 1000	0.05	0.9933	66.35
Folic Acid	0.1 - 1000	0.1	0.9981	307.54

### Accuracy

The accuracy was determined by the analysis of pooled sample control material as the percentage deviation from the targeted mean and the results were <10% for all levels. The serum pooled control material concentrations were 25 ng/ml and 250 ng/ml.

Figure 2 - Chromatograms, Curves, LOQ.



## CONCLUSIONS

- Baseline separation of the folates, derivatives and metabolites in 6 minutes with good LOQ in positive mode and a folate clean serum matrix is required to achieve the desired calibration curve and LOQ as the folate can be found in serum to significant  $\mu\text{M}$  levels
- Very good linearity of calibration curves with very good accuracy, precision and reproducibility but the compounds are very susceptible to light degradation and this can be seen particularly with 5,10-Methylene-THF in this case
- Further evaluate other more simple sample preparation techniques other than solid phase extraction. Protein crash with 5% Trichloroacetic acid demonstrated to be a potential option but not for all the folates described in this case.

## REFERENCES

- Determination of Folate Vitamins in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay  
Clinical Chemistry 50:2- 423-432 (2004)
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