



## High Sensitivity Methotrexate Quantitation in Human Serum with LC-MS/MS

### Quick Facts:

- LLOQ achieved is 0.03 ng/mL with 5 $\mu$ L injection
- Liquid Chromatography: Shimadzu Prominence UFLC
- Mass Spectrometry: IONICS 3Q Molecular Analyzer (triple quad)
- ESI Source
- Calibration Curve  $R^2 = 0.99999$

### 1. Introduction

Methotrexate is one of the oldest chemotherapy drugs and has been in use since the late 1950's. It was found effective in treating various neoplastic diseases such as acute leukemia, non-Hodgkin lymphoma and breast carcinoma. In low doses it has also been found effective in the treatment of certain autoimmune diseases. This research method presents a rapid LC-MS/MS approach with high selectivity and sensitivity. In this application note, a fast LC-MS/MS (research) method with IONICS 3Q Molecular Analyzer was tested and verified for quantitation of methotrexate in human serum.

### 2. Method

#### 2.1 Sample Preparation

Methotrexate was purchased from Toronto Research Chemicals (Toronto, ON). The stock solution 25 mg/mL of Methotrexate in methanol was prepared and stored at -15°C. Human serum was purchased from Sigma (Milwaukee, WI) and cleaned up by protein precipitation by mixing one volume of human serum, one volume of 0.1% formic acid and two volumes of acetonitrile. The mixture was vortexed for 1 min followed by centrifugation for 15 min. The supernatant was transferred to clean container and used as a matrix for methotrexate quantitation. A series of methotrexate standards from 0.03 to 125ng/mL were prepared by spiking the stock solution

into the clean protein precipitated human serum. All the solvents used in this application are HPLC grade.

#### 2.2 Mass Spectrometry Conditions

IONICS 3Q Molecular Analyzer equipped with an ESI source was used to perform the analysis. Instrument conditions were:

<b>Entrance Voltage (Volts)</b>	20
<b>HSID Temp. (°C)</b>	300
<b>Nebulizer Gas Setting</b>	400
<b>Drying Gas Setting</b>	120
<b>Heating Gas Setting</b>	350
<b>Source Temp. (°C)</b>	250
<b>Dwell Time (ms)</b>	100
<b>Pause Time (ms)</b>	5

**Table 1** Selected MRM operating conditions for methotrexate

MRM	CE	CCL2	CCL4	EV
455.1/308.0	28	-100	-90	30
455.1/134.0	48	-100	-90	30

#### 2.3 LC Conditions

The separation was performed on a Shimadzu Prominence UFLC system which includes binary pumps, autosampler, degasser, column oven. A sample volume of 5  $\mu$ L is injected into a Intakt C8 column (2.0 x 75 mm, 3  $\mu$ m) at room temperature. The LC time program is illustrated as **Table 2**. The total LC cycle time is 3 min and liquid flow rate is 0.4 mL/min.

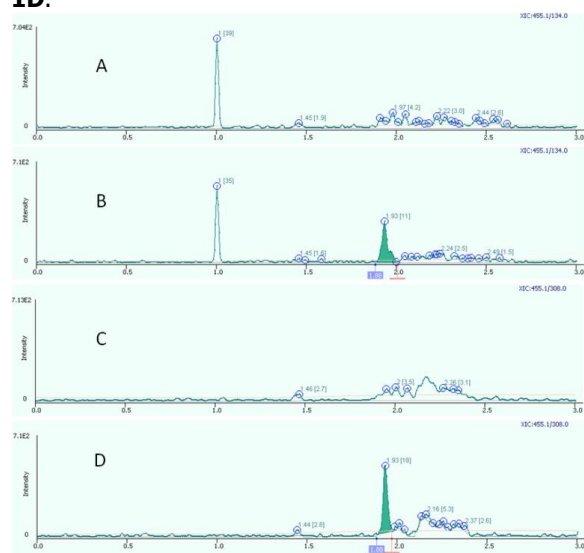
**Table 2** LC Gradient Conditions; Solvent A is 0.1% formic acid, Solvent B is methanol with 0.1% formic acid

Time (min)	Solvent B composition (%)
0	5
0.1	5
0.6	95
1	95
1.1	5
3	5

### 3. Results

#### 3.1 Extracted Ion Chromatogram (EIC)

The EIC for 455.1/134.0 and 455.1/308.0 for blank serum are shown in **Figure 1A and 1B** and the EIC for 455.1/134.0 and 455.1/308.0 for LOQ (0.03 ng/mL in human serum) are shown in **Figure 1C and 1D**.



**Figure 1.** (A) EIC of 455.1/134.0 in serum blank; (B) EIC of 455.1/134.0 for 0.03 ng/ml in human serum; (C) EIC of 455.1/308.0 in serum blank, and (D) EIC of 455.1/308.0 for 0.03 ng/ml in human serum

#### 3.2 Linearity

The calibration curve generated for both MRM transitions using 1/x weighting are good and the results for 455.1/134.0 transition (**Figure 2**) and 455.1/308.0 transition (**Figure 3**). Good linearity ( $R^2 = 0.9999$ ) was obtained for a concentration range of 0.03 to 125 ng/mL.

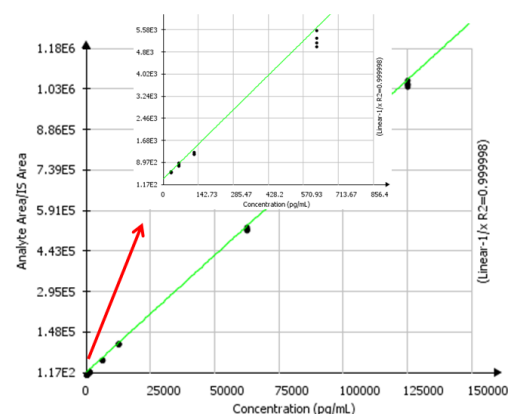
**Table 1** Intra-day variability (%RSD)

MRM	QC1(low)	QC2( med)	QC3(high)
455.1/134.0	5.1	1.2	1.0
455.1/308.0	6.9	1.8	0.5

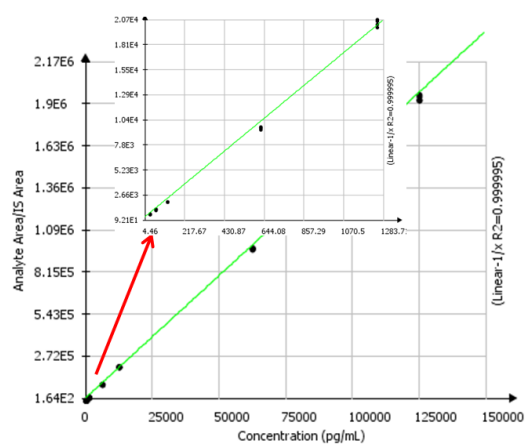
**Table 4** Inter-day variability (%RSD)

MRM	QC1(low)	QC2( med)	QC3(high)
455.1/134.0	2.6	3.0	3.5
455.1/308.0	7.0	3.6	3.8

**Figure 2** Calibration Curve for 455.1/134.0



**Figure 3** Calibration curve for 455.1/308.0



#### 3.3 Quantitation Results

The inter and intra-day variability for this experiment were excellent as shown in **Table 3 and 4**. The intra-day variability is determined by processing 4 replicates of each QC sample and the inter-day variability is determined with 4 replicates in 3 batches.

#### 4. Conclusion

A fast, sensitive, and accurate LC-MS/MS method was developed for methotrexate in serum using IONICS 3Q Mass Analyzer. An LLOQ of 0.03 ng/mL (5  $\mu$ L injection) was obtained. An excellent calibration curve for methotrexate was also obtained.

#### 5. Contact Information

To learn more about IONICS Mass Spectrometry, our products or services please visit our website or contact us directly.