High Sensitivity Methotrexate Quantitation in Human Serum utilizing LC-MS/MS

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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's lymphoma and breast carcinoma. In low doses it has also been found effective in the treatment of certain autoimmune diseases. However, when taken in overdose, methotrexate may cause a variety of health issues such as renal or gastrointestinal damage. In order to avoid these potentially serious adverse effects, an accurate quantification method is needed.

This research method presents a rapid LC-MS/MS approach utilizing IONICS' 3Q 210 Molecular Analyzer. The method provides high selectivity and sensitivity, which was tested and verified for the quantitation of methotrexate in human serum.

Method

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 using 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 a 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.

Mass Spectrometry Conditions: An IONICS 3Q 210 equipped with an ESI source was used to perform the analysis. Instrument conditions were: Electrospray Voltage: 5000V, HSID Temperature: 300°C, Nebulizer Gas Setting: 400, Drying Gas Setting: 120, Heating Gas Setting: 350, Source Temperature: 250°C, Dwell Time: 100 ms, Pause Time: 5 ms.

This method measured MRM transitions 455.1/308.0 and 455.1/134.0, **Table 2** provides a brief overview of the settings utilized while monitoring these transitions

Table 2: Monitored MRM Transitions and Associated Instrument Settings

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

LC Conditions: The separation was performed on a Shimadzu Prominence UFLC system which includes binary pumps, an autosampler, degasser, and a column oven. A sample volume of 5 µL was injected into a Imtakt C8 column (2.0 x 75 mm, 3µ) at room temperature. The LC time program is illustrated in **Table 3.** The total LC cycle time was 3 min with a liquid flow rate of 0.4 mL/min.

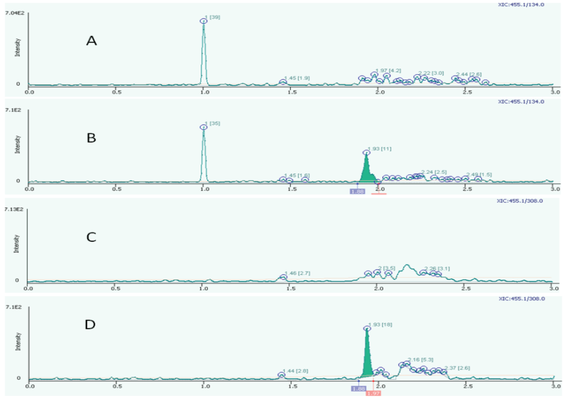
Table 3: LC Gradient Conditions; Solvent A is H20 with 0.1% formic acid, Solvent B is methanol with 0.1% formic acid

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

Results

Extracted Ion Chromatograms

Figure (1A): The EIC for 455.1/134.0 Serum Blank **(1B)** EIC of 455.1/134.0 for 0.03 ng/mL in Human Serum. **(1C)** EIC of 455.1/308.0 Serum Blank, **(1D)** EIC of 455.1/308.0 for 0.03 ng/mL in Human Serum



Linearity

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

Table 4 and Table 5 show the intra and inter-day variability for the results. 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.

Table 4: 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 5: 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 2a: Calibration Curve for 455.1/134.0

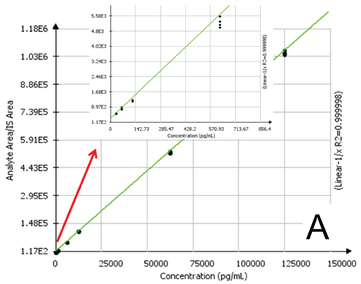
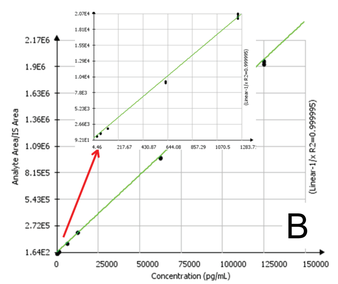


Figure 2b: Calibration curve for 455.1/308.0



Conclusion

A fast, sensitive, and accurate LC-MS/MS method was developed for methotrexate in serum using IONICS 3Q 210. An LLOQ of 0.03 ng/mL (5 µL injection) was obtained. An excellent calibration curve with R^2=0.9999 for methotrexate was also obtained. The sample preparation for this LC-MS/MS method is simple and well suited for routine clinical analysis of methotrexate.