
**CONFIRMATION OF METHOTREXATE IN EQUINE URINE BY HIGH
PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS
SPECTROMETRY**

**DEVELOPED FOR THE
TESTING INTEGRITY PROGRAM
BY**

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Confirmation of Methotrexate in Equine Urine by Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)

INTRODUCTION

Methotrexate (MTX, 4-amino-N10-methylpteroyl glutamic acid, Amethopterin) is a folic acid antagonist, and acts as an inhibitor of dihydrofolate reductase, leading to DNA damage and cell death (8). Intravenous administration of Methotrexate at high dose is widely used as chemotherapy in humans for various neoplastic diseases such as acute leukemia, osteogenic sarcoma (7, 11), non-Hodgkin lymphoma and breast carcinoma (6). Recently, however, lower doses of MTX have been used in humans to treat rheumatoid arthritis (2). MTX usage in racehorses has been reported to the Pennsylvania Racing Commissions, most likely due to the reported effectiveness of treating rheumatoid arthritis in humans. Most equine lameness conditions are not due to rheumatoid arthritis, thus with the existence of regulatory limits on select non-steroidal anti-inflammatory medications (Phenylbutazone; Flunixin), the use of MTX to manage lameness in race horses is without merit.

Methotrexate (MTX) is practically insoluble in all water immiscible solvents; therefore solid phase extraction (SPE) is a more efficient choice for MTX enrichment from aqueous biofluids (5). While polyglutamates and 7-hydroxy Methotrexate have been reported (1, 4, 5, 7, 8) as detectable metabolites of MTX, this method focuses on the parent Methotrexate in equine urine as the target analyte. Several SPE methods are reported in the literature (3, 8, 9), but this SOP utilizes a modification of the SPE method reported by Rule and Henion (3). This reference cites the use of C-18 impregnated glass fibers in a 384-well format coupled with the use of a valve-switching trap-and-analysis dual column configuration. This particular SPE substrate is also available in conventional SPE 3 mL format (OroChem[®] Part #SYC18203CC). After investigating several SPE methods reported in the literature, the OroChem SPE column was chosen for providing the most useful results in the enrichment of MTX from urine.

SCOPE

Initial screening for Methotrexate suspect samples is performed using enzyme-linked immunosorbent assay (ELISA, Neogen[®]). This method describes the solid phase extraction of Methotrexate from equine urine, and its subsequent analysis and confirmation by positive ion electrospray liquid chromatography-tandem mass spectrometry (LC/MS/MS). The mass spectrometer operating conditions described within this method are specific to the Micromass Q-ToF (Series 1) instrument. This method does not include instrumental screening for MTX, although it may be easily adapted for this purpose.

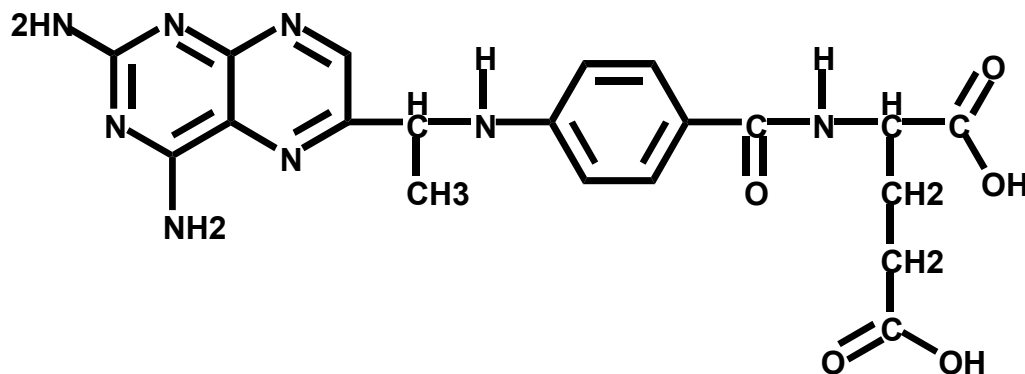
LIMITATIONS

Methotrexate is a very polar, poly-functional compound, closely related to Folic Acid. This polar nature precludes extraction by conventional liquid-liquid extraction procedures. By this chemical characteristic, ionization efficiency is also decreased, resulting in instrument sensitivities much higher than normally observed with mono functional basic compounds. The dicarboxylic acid functionalities tend to suggest negative ionization as the preferred mode of ionization; however, comparison of negative ion and positive ion electrospray results demonstrated better sensitivity levels using positive ionization mode.

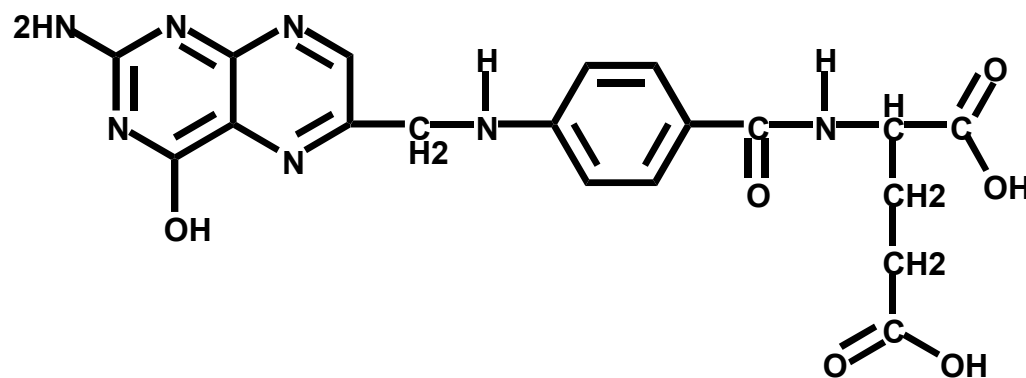
PRINCIPLE OF METHOD

One hundred microliters of sample (urine) is first acidified with aqueous formic acid (0.5%), applied to an OroChem C18 SuPERScreen solid phase cartridge (3 mL C18 Fine #SYC18203CC, OroChem, Chicago), eluted with 200 μ L 0.1% Formic Acid (in 9:1 Methanol:Water), then evaporated to dryness. The residue from the dried organic solvent is reconstituted with injection solvent (50:50:1 Acetonitrile: Water: Acetic Acid). The analyte is identified by liquid chromatography-mass spectrometry operated under positive ion electrospray ionization conditions. The mass spectrometer is operated in full scan MSMS product ion mode for the pseudomolecular ion of Methotrexate at 455.3 amu. Methotrexate concentration is estimated by external standard calibration using the principle product ions of Methotrexate (308.1+175.1). The limit of quantitation and confirmation is 0.5 μ g/mL for urine.

STRUCTURES OF METHOTREXATE AND FOLIC ACID



Methotrexate (Amethopterin) ; MW 454



Folic Acid ; MW 441

WORKING DRUG STANDARD SOLUTION

Methotrexate reference stock solution is prepared at 0.5 mg/mL by weighing 1-5 mg of traceable primary reference standard (Sigma cat # A-9898) and transferring to a 16x125 mm screw cap culture tube. A volume (accuracy to 10 μ L - ie x.xx mL) of water is added to the tube to produce a 0.5-mg/mL solution (2*(x.xx) mL equal to x.xx mg).

Working drug standards are prepared from the traceable primary reference stock solution of Methotrexate (0.5 mg/mL in HPLC grade water) at 10 ng/ μ L and 1 ng/ μ L.

Obtain primary reference materials from the pharmacy. Record accession of these materials on the pharmacy log sheet.

Complete Balance Use Log and QA Primary Reference Standard Log for this process.

Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# 597 and Primary Reference Powder Designation (i.e. R-Amet-1).

Using the 0.5 mg/mL Methotrexate reference stock:

- A) Add 100 μ L of Methotrexate primary reference stock to 9.9 mL Acetonitrile to produce a 10 μ g/mL secondary stock solution. Label according to the format mmddy $\underline{\text{MTX}}$ 10.
- B) Add 1 mL A to 9 mL 50:50:1 Acetonitrile: Water: Acetic Acid to produce a 1 μ g/mL column test solution. Label this Column Test Solution mmddy $\underline{\text{MTX}}$ coltest1.

Store the Reference stock and working stock solutions A and B at 2-8 $^{\circ}$ C.

Record the preparation and labeling of these secondary stock solutions in the secondary preparation logbook in the appropriate Unit of the Laboratory.

CONTROL SAMPLES

The Negative Control Sample is equine urine demonstrated by ELISA analysis and this SOP to contain no detectable Methotrexate.

The Positive Control Sample is equine urine supplemented with Methotrexate at 50 μ g/mL. Calibrators, Negative and Positive Control samples are prepared according to Table 1, logged, labeled, and then stored at -70° C.

CONTROL AND CALIBRATOR PREPARATION TABLE

Calibrators and Controls are prepared from negative control urine, and reference stock Solution (0.5 mg/mL) of Methotrexate. The mmddy $\underline{\text{MTX}}$ 0 calibrator also serves as Negative Control, and the mmddy $\underline{\text{MTX}}$ 50 calibrator serves as the positive control.

Table 1.

Batch preparation for a total of 15 mL per item

LABEL (format MMDDYYMTX μ g/mL)

Print using AVERY Template 5267 in MS Word) 14 of each

LABEL	μ g/mL	Total μ g required	0.5 mg/mL stock (μ L needed)	Negative Control (mL used)
MMDDYY <u>MTX</u> 0	0	0	0	15
MMDDYY <u>MTX</u> 1	1	15	30	14.97
MMDDYY <u>MTX</u> 5	5	75	150	14.85
MMDDYY <u>MTX</u> 10	10	150	300	14.7
MMDDYY <u>MTX</u> 25	25	375	750	14.25
MMDDYY <u>MTX</u> 50	50	750	1500	13.5

- A. Prepare 15 mL of batch calibrators in 50 mL labeled bottles as described in Table 1.
- B. Label 84 screw cap culture tubes (16x125 mm) in 6 x 14 format, and aliquot 1 mL of the appropriate calibrator from step A into each respective tube. Cap and store at -70°C .
- C. The zero calibrator doubles as the negative control, and the 50 μ g/mL calibrator doubles as the positive control.

METHOTREXATE SOLID PHASE EXTRACTION PROCEDURE

Reagents Needed

- 1) 0.1 % Formic Acid
- 2) 0.5 % Formic Acid
- 3) 0.1 % Formic Acid in 9:1 MeOH: H₂O
- 4) 50:50:1 Acetonitrile: Water: Acetic Acid
- 5) 0.05 M pH 5 Ammonium Acetate = 100 mL 0.5 M Ammonium Acetate (pH 5) plus 900mL HPLC grade water.

Sample Prep: Add 100 μ L Sample, Calibrator, and control urine and 2.5 mL of 0.5% Formic Acid (in water) to labeled 16x125 mm culture tubes and mix by vortexing for 10 seconds.

SPE Column: Orochem C18 SuPEerScreen 3 ml C18 Fine #SYC18203CC

(Phone: 630-887-0616 : 888-404-8401, www.orochem.com)
SPE Manifold: Varian VacElute SPS 24
Vacuum Pump: VacuuBrand Model MZ 2C oil-less pump

Adjust the vacuum pressure to produce a slow dropwise elution from the cartridge (~ 1 mL per minute) for all steps listed.

Condition Column using: 3 mL Methanol
3 mL 0.1% Formic Acid (in H₂O)

Add Sample(s) to the SPE cartridges and slowly elute (~ 1 mL per minute)

Wash Column using: 3 mL 0.1 % Formic Acid (in H₂O)

Dry Column by: Adjusting vacuum pressure to maximum for 5 minutes and drawing air through the SPE cartridge

Elute SPE Column with: 200 µL 0.1% Formic Acid (in 9:1 MeOH: H₂O)

Evaporate to dryness at ~ 75°C with air or nitrogen as drying gas.

Add 100 µL 50:50:1 Acetonitrile: Water: Acetic Acid and vortex for 10 seconds.

Transfer the above solution into labeled auto sampler vials fitted with limited volume inserts, then cap. All the samples are ready for LC/MS/MS analysis.

CHROMATOGRAPHY

Column: None Used
Guard: Agilent Zorbax XDB-C18 4.6x12.5 mm 5 u (Part # 820950-925)
Mobile Phase: 20:80 MeCN/ 0.001 M Ammonium Acetate (pH 5)
Flow 0.065 – 0.1 mL/min

Flow conditions may vary. Flow is optimized for each use to achieve optimum sensitivity of 1 µg/ml column test solution of Methotrexate in 50:50:1 Acetonitrile: Water: Acetic Acid, with retention time of 6 minutes or less.

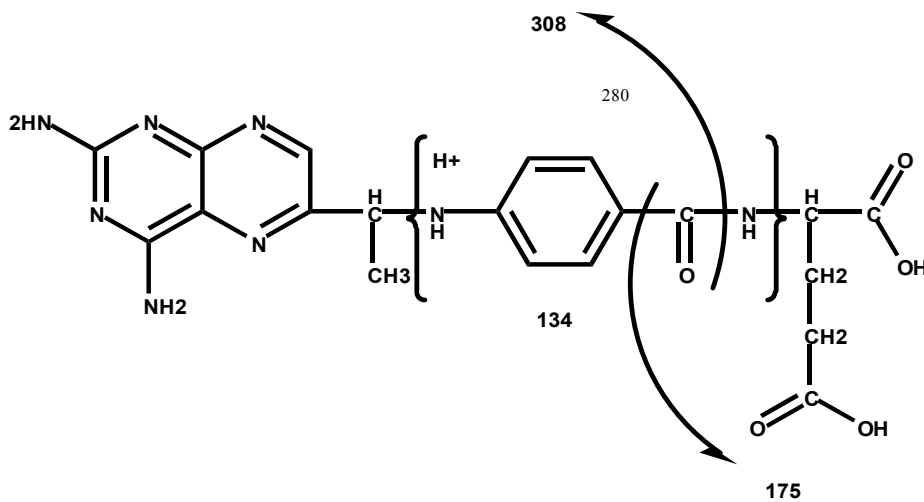
LCMSMS CONDITIONS (Micromass Q-Tof)

Method is based on immunoassay (ELISA) screening. Limit of Confirmation for this method is 0.5 µg/ml.

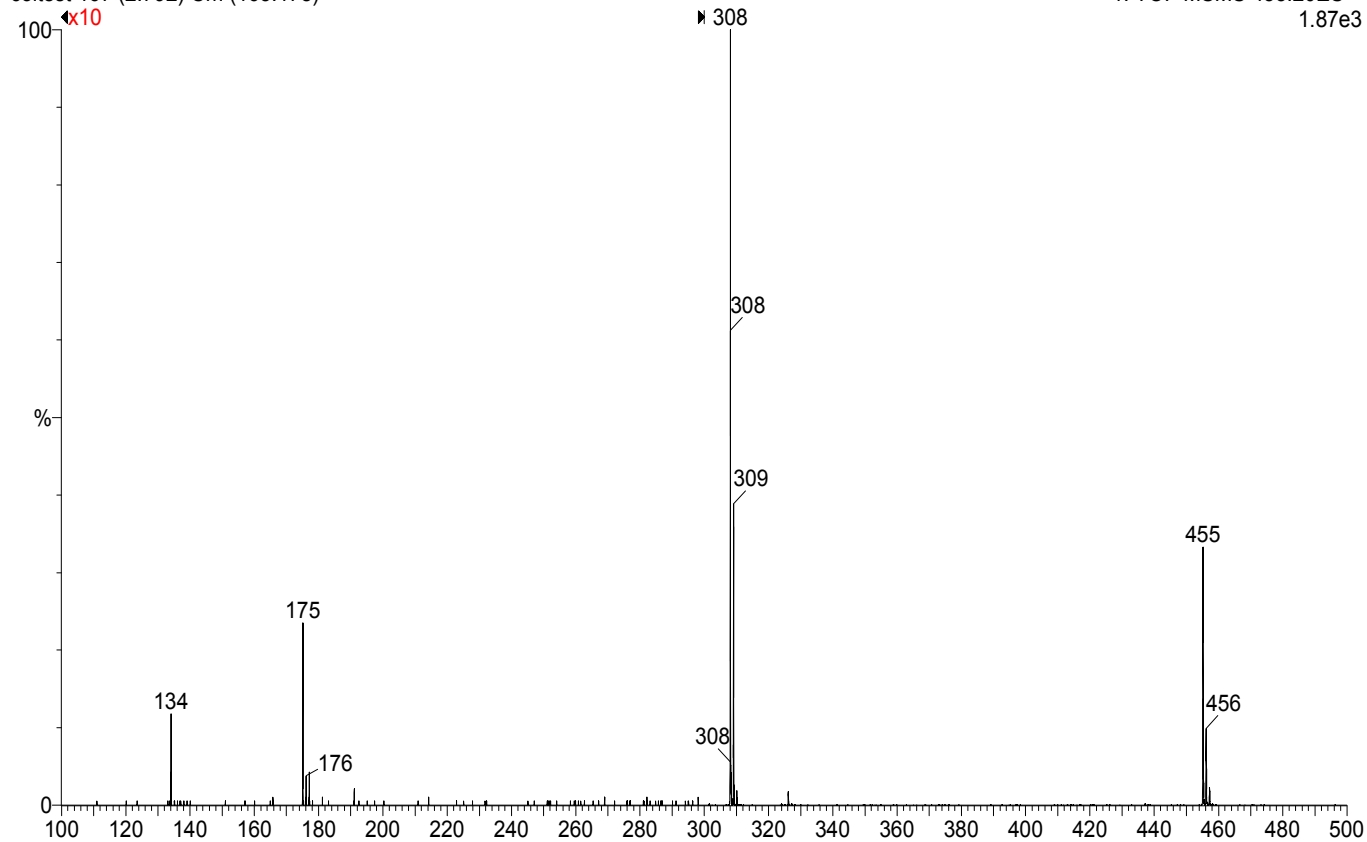
Confirmation is by Positive Ion Electrospray mode (ESI+), with external standard calibration.

Scan Range 100-500 amu
Scan Time 0.3
Inter-scan 0.03
MS1 ion 455.3
Cone Voltage 30
Collision energy 17
Quantification Ions 308.1, 175.1

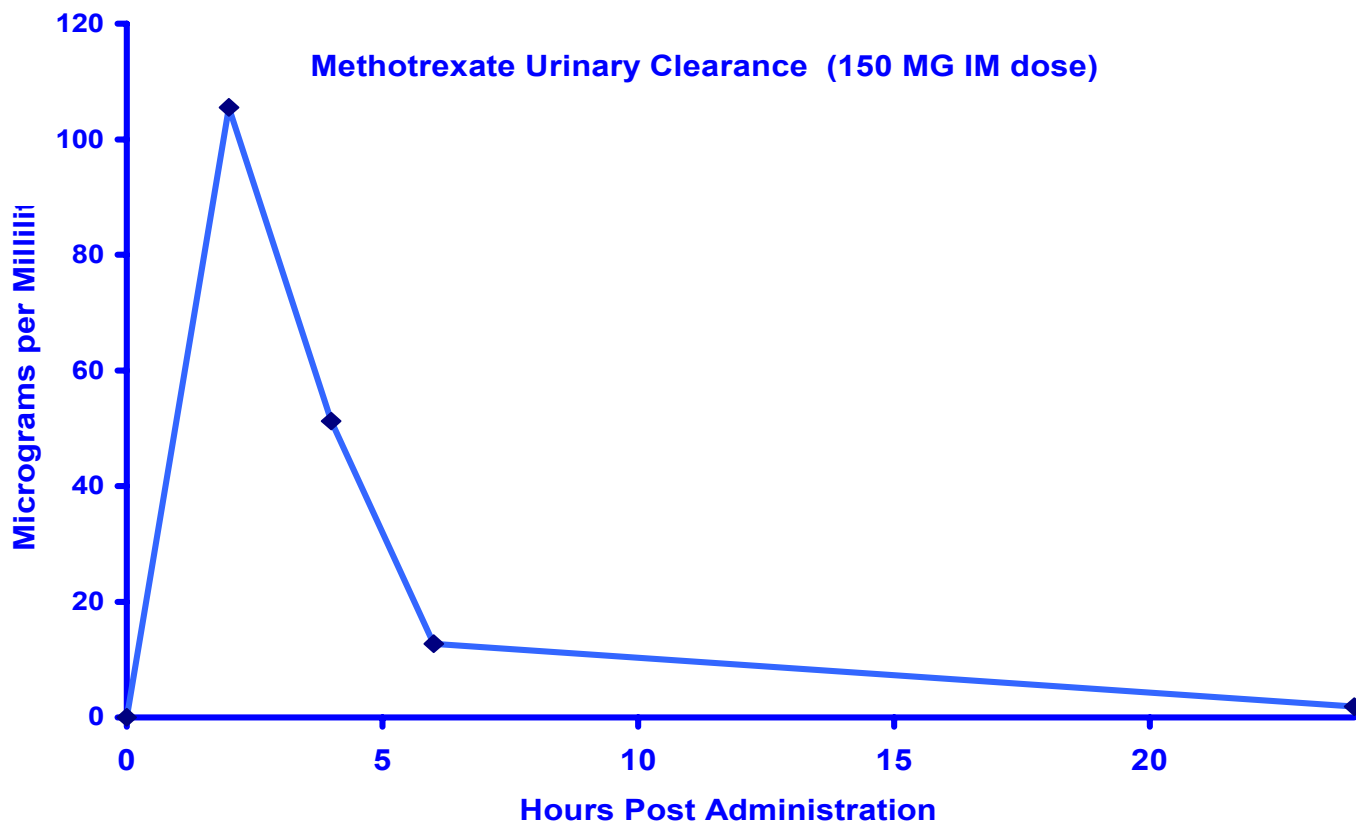
Methotrexate (Amethopterin) MH+ 455.3



coltest 167 (2.792) Cm (165:178)



**Chemical structure and proposed fragmentation (top)
and MSMS spectrum of Methotrexate (bottom)**



Urinary clearance of 150 mg IM dose of Methotrexate. (Less than 1 µg/mL at 24 hours.)

SAMPLE ANALYSIS SEQUENCE FOR ANALYSIS OF METHOTREXATE

The following sequence is followed for the analysis of Methotrexate.

1. Column Test
2. Calibrators (0 to 150 ng/ml)
3. Solvent Blank (acetonitrile)
4. Negative Control (0 calibrator)
5. Solvent Blank (acetonitrile)
6. Sample 1

Repeat steps 5 and 6 for each additional sample

7. Solvent Blank (acetonitrile)
8. Positive Control

CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR METHOTREXATE

Report a test sample as positive per this standard operating procedure for Methotrexate if ALL of the following criteria are met:

The LC retention times for the extracted ion chromatogram of the quantifying ions (308.1 + 175.1 m/z) for Methotrexate in the sample, 10 $\mu\text{g/mL}$ calibrator, and the positive control are within +/- 0.15 minutes. This is determined by inspection of the extracted ion chromatogram comparisons that are included in the analysis data packet. These chromatograms may be subtracted and/or smoothed.

The signal to noise ratio of the quantifying ions (308.1 + 175.1) for Methotrexate is greater than 10. This is determined by inspection of the extracted ion chromatogram comparisons, which are included in the analysis data packet. These chromatograms may be subtracted and/or smoothed.

The MSMS full scan spectrum for the suspect sample contains no mass spectral peaks greater than 25% of the 308.1 quantifying ion for Methotrexate. This is determined by inspection of the mass spectrum comparison of the sample, standard, and control. The presence of such peaks indicates the possibility that the values determined for the integration of the quantifying ion chromatograms may be skewed due to the presence of unknown co-eluting substances; and, that the ion abundance ratios are representative of compounds in addition to MTX.

The relative abundances in the full scan MSMS spectrum of the quantifying ions (308.1, 175.1) and precursor ion (455.1) must be within +/- 35% of those of the standard. This is determined by inspection of the full scan MSMS spectrum comparison of the sample, standard, and control.

All Blanks and Negative controls demonstrate the absence of MTX (by "absence" in the use of this method, it is meant no quantifiable Methotrexate concentration greater than 0.1 $\mu\text{g/mL}$).

Liquid Chromatography

A. General Considerations

1. The polar nature of MTX causes poor peak shape on conventional LC and LCMS analytical columns. Some success has been reported using trapping columns (4, 10), which are then back flushed to the analytical column. In the absence of this configuration, success has been achieved using analytical guard columns. Equilibration of the column to the mobile phase and running the column test mix is necessary to characterize the condition of the column prior to analysis.
2. Recording of the column serial number is recommended to ensure the use of the same column from the last known set of conditions and performance.

B. Performance Criteria

1. The minimum calibrator (1 $\mu\text{g/mL}$) should be detectable and have a chromatographic signal to noise greater than 10:1 (using summed 175.1+308.1 m/z extracted ion chromatogram, subtracted and smoothed).

C. Corrective Actions

1. If the test solution peak shape is split, the chromatographic system, column and connections must be inspected
 - a. Verify mobile phase settings in method, and at the local instrument. Verify the correct solutions are applied to the appropriate channels and that the bottle assignments and percentages correspond to the method settings.
 - b. The PEEK connections should be examined to determine that the PEEK tubing is flush to all mating surfaces (zero dead volume)
 - c. The PEEK tubing may have become crimped at the ferrule. Slide the PEEK nut and ferrule back and trim the PEEK tubing using the PEEK Tubing-cutting tool to remove the crimped section.
 - d. The column may not be equilibrated for the mobile phase being used. Re-equilibrate at analysis flow rate for at least 15 minutes in both directions.
 - e. The column may need to be replaced (Agilent XDB-C18 4.6x12.5 mm, Agilent Technologies, Part No. 820950-925).

2. If the test solution peak response (TIC) is diminished, the test mix, and mass spectrometer system must be examined. (These steps assume that the mass axis calibrator produced normal response and that chromatographic retention and peak width are acceptable.)
 - a. Verify the reagent gas is on, both at the tank, and in the software settings on the tune page.
 - b. Verify the desolvation gas is on, both at the tank, and in the software settings on the tune page.
 - c. Verify the source temperature and desolvation temperature are on by inspecting the tune page read backs.
 - d. Verify the acquisition method is correct, particularly parent ion, scan time, and scan range. Additional parent ion functions, smaller scan times, or increased scan windows will produce a proportionate decrease in the observed signal.
 - e. Verify the injection volume is correctly set
 - f. Fresh column test mix may be needed. (1 $\mu\text{g}/\text{mL}$ Methotrexate –50:50:1 Acetonitrile: Water: Formic Acid)

If all else fails, the flow injection response to the mass axis calibrator should be re-examined to determine if the instrument response is still the same.

Mass Spectrometry

It was previously described in the SCOPE section that this procedure is based upon the MicroMass Q-ToF (series 1) instrument. Some of the following considerations can be generalized to other LCMSMS instrumentation while some may be unique to this particular instrument.

Front-end Dissociation

While overall sensitivity can be increased by sample preparation strategies (increased sample volume, multi dimensional SPE, or switched trapping column enrichment), the following steps are critical to ensure optimal sensitivity, regardless of the target concentration range of the analysis.

The precursor ion (455 m/z) must be optimized in MS mode to eliminate dissociation or adduct formation in the source. On the MicroMass Q-Tof, this is done by adjusting the entrance lens and capillary voltages, as well as the probe and source temperatures. This process would be similar on other instruments, although the names of the elements could be different for various instruments.

Collision Energy Optimization

The MicroMass Q-Tof usually yields best sensitivity in full scan MSMS mode. Simulation of selected reaction monitoring (SRM) experiments does not result in appreciable improvement of the limits of detection. Therefore, the collision energy should be optimized to produce the highest total ion chromatogram (TIC) signal. Such optimization usually occurs when a normally distributed MSMS spectrum is produced, which includes at least 10% (or greater) precursor ion.

Interferences

Folic acid shows no interference with this method. Screening of several hundred randomly selected race samples indicates other unidentified materials can be detected coming through the 455 amu precursor channel. These have MSMS spectra that are clearly distinguishable from Methotrexate. Therefore, quantification estimations must also be supported by full scan MSMS spectrum comparison for a finding of fact.

REAGENTS

0.5% Formic Acid

Reagent for Solid Phase Extraction (SPE) of Methotrexate using OroChem C-18 cartridges

Procedure for 1000 mL:

Mix 5 mL Formic Acid with 995 mL HPLC grade water

0.1% Formic Acid

Reagent for Solid Phase Extraction (SPE) of Methotrexate using OroChem C-18 cartridges

Procedure for 1000 mL:

Mix 1 mL Formic Acid with 999 mL HPLC grade water

0.1% Formic Acid in 9:1 methanol: water

Reagent for Solid Phase Extraction (SPE) of Methotrexate using OroChem C-18 cartridges

Procedure for 1000 mL:

Mix 1 mL Formic Acid with 900 mL HPLC grade methanol and 99 mL HPLC grade water

50:50:1 FIA Reagent

LCMS Reagent for Flow Injection Analysis (FIA) and Liquid Chromatograph (LC) injection solvent

Procedure for 101 mL:

1. Combine 50 mL HPLC grade (or better) Acetonitrile with
2. 50 mL HPLC grade (or better) water, and
3. Add 1 mL Formic Acid (high purity if available)

500 mM Ammonium Acetate pH 5

Stock solution for LCMS mobile phase dilutions

Procedure for 1000 mL:

1. Dissolve 29 mL Glacial Acetic Acid in 800 mL HPLC grade (or better) water
2. Adjust to pH 5.0 with Ammonium Hydroxide
3. Bring to volume (1000 mL) with HPLC grade (or better) water

Dilute 1:10 to 1:100 with HPLC grade water for 0.05 to 0.005 M solutions

References

1. **Trace Analysis of Methotrexate and 7-Hydroxy Methotrexate in Human Plasma and Urine by a Novel High Performance Liquid Chromatographic Method**, Ther. Drug Monit., 13(6):528-532 (1991),. Beck, O., et al.,
2. **Rapid Quantitation of Methotrexate and its Metabolites in Human Serum, Urine and Bile, using Solid-Phase Extraction and High-Performance Liquid Chromatography** J. Chromatogr., Biomed. Applic., 487:476-482 (1989),. Nuernberg, B., Kohlbrenner, M., Faulkner, R., Furst, D.E.,
3. **A 384-Well Solid-Phase Extraction for LC/MS/MS Determination of Methotrexate and Its 7-Hydroxy Metabolite in Human Urine and Plasma** Anal. Chem. 73(3), 439-443 (2001) Rule,G., Chapple, M., Henion, J.
4. **Liquid-Liquid Extraction in the 96-Well Plate Format with SRM LC/MS Quantitative Determination of Methotrexate and Its Major metabolite in Human Plasma** Anal. Chem 71, 2340-2345 (1999) Steinborner, S., Henion, J.
5. **Determination of methotrexate in human urine at trace levels by solid phase extraction and high performance liquid chromatography/tandem mass spectrometry** Rapid Commun. Mass Spectrom. 14, 173-179 (2000) Turci, R., Fiorentino, M.L., Stanni, C., Minoia, C.
6. **Determination of methotrexate in environmental samples by solid phase extraction and high performance liquid chromatography: ultraviolet or tandem mass spectrometry detection?** Rapid Commun. Mass Spectrom. 14, 685-691 (2000) Turci, R., Micoli, G., Minoia, C.
7. **Evaluation of clinical assays for measuring high-dose methotrexate in plasma** Clinical Chemistry 42:1, 39-44 (1966) Albertioni, F., Rask, C., et al.
8. **Simultaneous quantitation of methotrexate and its two main metabolites in biological fluids by a novel solid-phase extraction procedure using high-performance liquid chromatography** J. Chrom. B, 665 163-170 (1995) Albertioni,A., Pettersson, B., Beck, O., et al.
9. **Determination of methotrexate in serum by high-performance liquid chromatography** J. Chrom B, 681, 317-322 (1996) Aboleneen, H., Simpson, J., Backes, D.
10. **Ion-pair chromatography of methotrexate in a column-switching system using an alkyl-diol silica precolumn for direct injection of plasma** J. Chrom A, 742, 113-120 (1996) Yu, Z., Westerlund, D.
11. **Analysis of methotrexate and 7-hydroxymethotrexate by high-pressure liquid chromatography** Cancer Treatment Reports, 62:4, 529-532, (1978) Wisnicki, J.L., Tong, W.P., Ludlum, D.B.