INTRINSIX™—A NOVEL CALIBRATION APPROACH FOR PERFORMING QUANTITATIVE LC-MS/MS ANALYSIS OF SERUM METHOTREXATE FOR CLINICAL RESEARCH

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INTRODUCTION

The IntrinsiX™ workflow enables analysis of serum methotrexate using ^{13}C-labeled analogs as internal calibrators. Results are generated from a single injection, eliminating batch analysis of calibrators and samples. Comparison studies with a conventional LC-MS/MS method using the same LC-MS/MS system provide confidence in the method. High dose methotrexate in combination with glucarpidase therapy requires selective quantification which immunoassay metabolite cross-reactivity can overestimate.

METHODS

Materials

- A methotrexate reference material and four ^{13}C-labeled analogs were supplied by Cerilliant (Round Rock, TX, USA).
- Analogs were used to prepare a 4-point IntrinsiX calibration curve over the range 0.025-10 µmol/L.
- NEQAS (Nottingham, UK) and WEQAS (Cardiff, UK) provided EQA samples in serum for accuracy testing.

Methods

- Samples (50 µL) supplemented with internal calibrators were pre-treated with methanol.
- Following mixing and centrifugation, an aliquot of supernatant was diluted in water in a 96-well 2mL plate.
- Using a Waters ACQUITY I-Class FTN UPLC® system, samples were injected onto a 2.1 x 30 mm Waters ACQUITY UPLC HSS C_{18} SB column using a water/methanol/ammonium acetate separation and analyzed with a Waters Xevo® TQD tandem quadrupole mass spectrometer.
- The analysis time per sample was approximately 5.7 minutes injection-to-injection.

Internal Calibration

- Four internal calibrators were simultaneously analyzed with methotrexate (Figure 1).

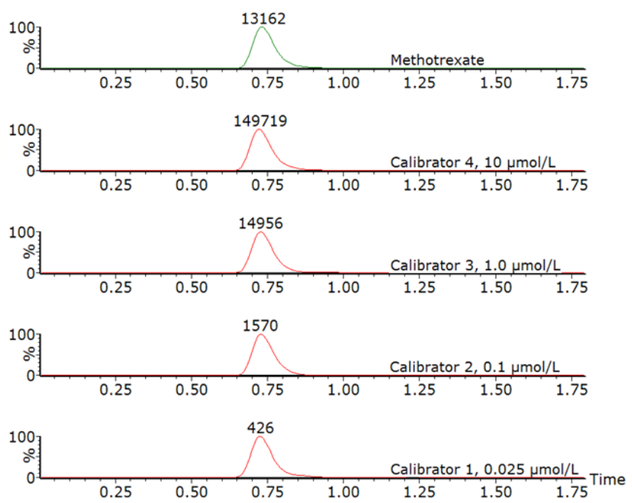

Figure 1. Example IntrinsiX chromatograms

- The acquired data was used to generate a sample associated calibration curve and determine the methotrexate concentration in the sample (Figure 2).

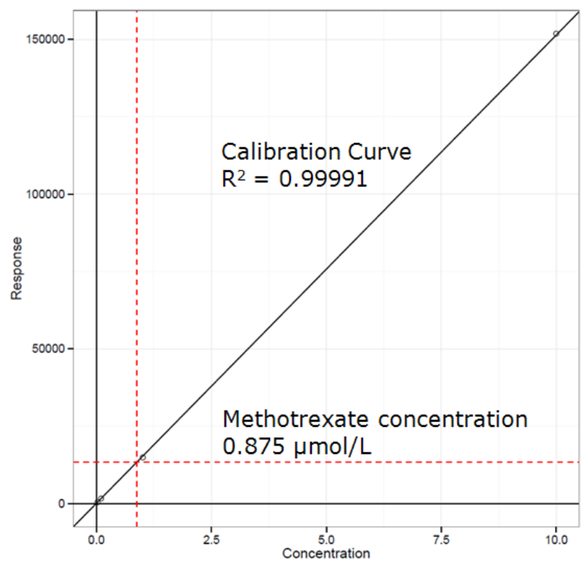

Figure 2. Calibration curve used to determine methotrexate concentration by IntrinsiX.

RESULTS

Linearity and analytical sensitivity

- Following CLSI-EP6-A, the method was shown to have a linear fit over the range of 0.0175—13.0 µmol/L.
- Analytical sensitivity investigations indicate the method would allow precise quantification (≤ 20%) at 0.015 µmol/L.

Carryover

- No significant carryover was observed following analysis of serum samples with methotrexate levels up to 100 µmol/L.

Precision

- Five replicates at each QC level in plasma were prepared daily over five days (n=25; Table 1).

Nominal Concentration (µmol/L)	Total Precision (% RSD)	Repeatability (% RSD)
0.1	6.8	6.3
1.0	3.3	1.8
2.5	2.8	1.3
7.5	2.2	0.9

Table 1. Total precision and repeatability for the IntrinsiX analysis of methotrexate.

Accuracy

- Correlation between the IntrinsiX approach and the all laboratory trimmed mean (ALTM) of EQA samples was described by the Deming equation y=0.94x+0.03 (n=14, range 0.030-2.14 µmol/L). A small but statistically significant constant bias was detected (p≤0.05), but no significant proportional bias.
- Agreement between the IntrinsiX approach and a conventional LC-MS/MS method using the same LC-MS/MS system for analysis of EQA samples was described by the Deming equation y=0.99x-0.02 (n=23, range 0.025—2.18 µmol/L). A small but statistically significant constant bias was detected (p≤0.05), but no significant proportional bias (Figure 3).

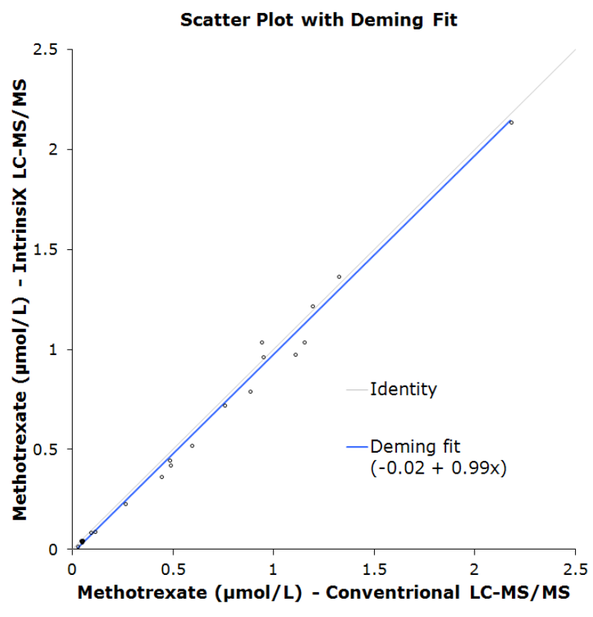

Figure 3. Deming fit of serum samples analysed by immunoassay and by the new IntrinsiX LC-MS/MS method.

Interference Testing

- Following CLSI EP7-A2, recovery of samples containing 0.1 or 1.0 µmol/L methotrexate (n=3) was unaffected (mean 101.0%, range 95.5-106.3%) when co-spiked with high concentrations of endogenous compounds (albumin, bilirubin, cholesterol, triglycerides or uric acid) or Intralipid®.
- Similarly, recovery was unaffected (mean 101.1%, range 98.2-104.9%) when methotrexate pools were supplemented with 5 or 50 µmol/L 7-hydroxymethotrexate or 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA; n=3), showing absence of interference from these metabolites.

CONCLUSION

- **We have successfully implemented a novel calibration approach for performing quantitative LC-MS/MS analysis of serum methotrexate for clinical research.**
- **Incorporation of calibrators into test samples allows improved throughput, shorter time to first result and allows the possibility of non-batched analysis.**
- **Samples are perfectly matrix-matched, as demonstrated by the excellent results of the interference testing (mean bias 101%).**

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