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Calibrating from Within: Multipoint Internal Calibration of a Quantitative Mass Spectrometric Assay of Serum Methotrexate

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BACKGROUND: Clinical LC-MS/MS assays traditionally require that samples be run in batches with calibration curves in each batch. This approach is inefficient and presents a barrier to random access analysis. We developed an alternative approach called multipoint internal calibration (MPIC) that eliminated the need for batch-mode analysis.

METHODS: The new approach used 4 variants of ^{13}C-labeled methotrexate (0.026–10.3 μM) as an internal calibration curve within each sample. One site carried out a comprehensive validation, which included an evaluation of interferences and matrix effects, lower limit of quantification (LLOQ), and 20-day precision. Three sites evaluated assay precision and linearity. MPIC was also compared with traditional LC-MS/MS and an immunoassay.

RESULTS: Recovery of spiked analyte was 93%–102%. The LLOQ was validated to be 0.017 μM. Total variability, determined in a 20-day experiment, was 11.5%CV. In a 5-day variability study performed at each site, total imprecision was 3.4 to 16.8%CV. Linearity was validated throughout the calibrator range ($r^2 > 0.995$, slopes = 0.996–1.01). In comparing 40 samples run in each laboratory, the median interlaboratory imprecision was 6.55%CV. MPIC quantification was comparable to both traditional LC-MS/MS and immunoassay (r^2 = 0.96–0.98, slopes = 1.04–1.06). Bland-Altman analysis of all comparisons showed biases rarely exceeding 20% when MTX concentrations were >0.4 μM.

CONCLUSION: The MPIC method for serum methotrexate quantification was validated in a multisite proof-of-concept study and represents a big step toward random-access LC-MS/MS analysis, which could change the paradigm of mass spectrometry in the clinical laboratory.

Introduction

The conventional workflow for LC-MS/MS clinical assays includes a calibration curve with each batch of samples, leading to a substantial increase in the cost per batch *(1)*. Performing batch-mode analyses also increases turnaround time (TAT) and makes STAT sample analysis challenging. The utility of including a full calibration curve in each batch has been questioned *(2)*; however, most clinical laboratories continue to operate in this fashion. Batch testing is also less than ideal in personalized medicine strategies, including therapeutic drug monitoring, where rapid reporting of serum concentrations is essential for optimizing dosage. To improve laboratory efficiency and to make therapeutic drug-monitoring analysis by LC-MS/MS more accessible, alternative calibration strategies have been used, including reduction in the number of calibrators and internal calibration *(1, 3, 4)*. Others have explored response factor–based calibration schemes or single-point calibration to eliminate the need to run a calibration curve with every sample batch *(5, 6)*. Our goal was to develop a new calibration strategy using multipoint internal calibration (MPIC) in a modified isotope-dilution approach in which every sample contained its own matrix-matched calibration curve. The proof-of-concept was demonstrated using the quantification of methotrexate (MTX) in serum.

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We selected MTX as the model because high-dose MTX therapy requires therapeutic monitoring to mitigate nephrotoxicity by administering leucovorin or glucarpidase when supratherapeutic MTX serum concentrations are observed *(7)*. MTX's narrow therapeutic window and highly variable intrapatient drug absorption and metabolism also necessitate frequent monitoring until plasma concentrations below 0.05 to 0.1 μM are achieved *(7, 8)*.

Immunoassays for MTX are subject to interference with different compounds and metabolites (e.g., deoxyaminopteroic acid) depending on which assay is used *(8–11)*. Coadministration of MTX with glucarpidase rapidly converts MTX to deoxyaminopteroic acid *(12)*, which can lead to assay interference for days following treatment *(13)*. LC-MS/MS has been used by some clinical laboratories to overcome interference issues with MTX immunoassays *(9, 14, 15)*. Although LC-MS/MS is a powerful tool resulting in analytically sensitive and specific measurements, reliance on external calibration curves and sample batching makes it difficult for clinical laboratories to provide the rapid TATs that are required for optimal rescue therapy.

The MPIC method described includes multiple isotope-labeled MTX internal standards, each at a different concentration, as calibrators in each sample; this approach eliminates the need to batch samples and reduces the costs of external calibrator preparation and analysis. We describe the multisite assay validation of MPIC analysis of MTX in serum.

Materials and Methods

CHEMICALS AND REAGENTS

MTX-certified reference material and powdered pure MTX were purchased from Cerilliant (M-136) and EMD Millipore, respectively. Defibrinated plasma (UTAK) was used for spiking experiments. MPIC internal calibrators were custom synthesized by Cerilliant to specifications developed by Waters Corp (see Supplemental Fig. 1 in the online Data Supplement). Detailed summaries of calibrator purity assessment and value assignment are described in the Supplemental Methods section of the online Data Supplement (see Supplemental Figs. 2 and 3 and Supplemental Tables 1 and 2). MPIC microcentrifuge tubes containing four ^{13}C-labeled MTX analogs (isotopologues; 0.026–10.3 μM) were prepared centrally and sent to each of the 3 sites on dry ice. They were stored locally at −20°C until used. LC-MS/MS–grade solvents, ammonium acetate, and formic acid were purchased from Fisher Scientific, and water was either LC-MS/MS grade or 18.2 mΩ purified water (Purelab Ultra).

PATIENT SERUM SAMPLES

All samples used for these studies were leftover, deidentified, and uncoded samples from the clinical laboratories (exempt from institutional review board approval). Site 1 prepared high- and low-spiked samples for precision and linearity experiments by dissolving pure MTX into defibrinated plasma. At Site 2, high- and low-concentration serum pools were prepared using excess sera from patients treated with MTX that had been analyzed by immunoassay (ARK^{TM}; ARK Diagnostics). These samples had been refrigerated for up to 5 days and were then frozen at −20°C until analysis. To make a high pool, patient samples with high concentrations (i.e., >10 μM) were combined and then diluted with MTX-negative serum (verified by immunoassay) to approximately 4 μM. Samples with 0.05 μM MTX were combined to form the low-pool sample. These 2 pools were used for the precision and linearity experiments.

Samples for the method comparison study were prepared at Site 1. Forty samples were distributed to Sites 2 and 3; of those, 17 were from single individuals. The other 23 samples were blends made using 10 samples with high amounts of MTX (>10 μM) mixed with 23 different leftover deidentified clinical samples that had undetectable concentrations of MTX to cover the clinically relevant range.

MPIC SAMPLE PREPARATION

Samples were prepared at room temperature by adding 50 μL of serum directly to the MPIC vials containing the dried MTX internal calibrators, then vortexed for 30 s on a multitube vortex mixer. Next, proteins were precipitated with 250 μL methanol and then vortexed on a multitube vortex mixer for another 30 s. Samples were centrifuged at 16 100*g* for 2 min. The supernatant was transferred to a 96-well plate or Eppendorf tube and either analyzed directly (Site 1, 20 μL injection volume) or diluted (Sites 2 and 3, 50 μL supernatant plus 950 μL water for a final concentration of 5% vol:vol methanol, 10 μL injection volume).

LC-MS/MS

Three different LC-MS/MS platforms were used: ACQUITY-Xevo TQD, ACQUITY-Xevo TQ-S micro, and Alliance 2795-Quattro micro. All HPLCs used 2 mM ammonium acetate and 0.1% formic acid (v/v) in water as mobile phase A and methanol with the same modifiers as mobile phase B (MPB). At Site 1, separation was achieved using an Ascentis Express C18 column (2.1 mm i.d. × 20 mm, 2.7-μm particles) and a 0.6-min gradient (10%–100% MPB). Sites 2 and 3 used an ACQUITY UPLC HSS C18 SB column (2.1 mm i.d. × 30 mm, 1.8-μm particles; Waters Corp)

with isocratic elution (23% MPB) followed by a 0.5-min regeneration gradient (23%–95% MPB). The multiple-reaction monitoring parameters used for MTX analysis, including transitions and dwell times, are shown in Supplemental Table 3. Longer dwell times were used at lower concentrations to improve signal-to-noise. The cone voltage was 30 and 36 V, and the collision energy was 20 and 18 eV at Site 1 and Sites 2 and 3, respectively. Example chromatograms from the different chromatographic methods are provided (Fig. 1, A, and Supplemental Fig. 4).

RECOVERY AND INTERFERENCES

Certified reference MTX was spiked into defibrinated plasma at a concentration of 11.92 μM. This sample was then gravimetrically added to 3 samples. One sample was defibrinated plasma with no detectable MTX; the other 2 were patient samples that contained MTX. The samples were each analyzed as 3 technical replicates. Recovery was calculated based on the expected Δ for each sample.

To evaluate the impact of interferences on MPIC quantification, samples with high concentrations of hemoglobin (>0.9 g/dL, n = 2), high triglycerides (>600 mg/dL, n = 2), and high total protein (>9 g/dL, n = 3) were spiked as described, and MTX was measured.

MATRIX EFFECTS

Extraction efficiency and ion suppression were evaluated based on the recovery of multipoint internal calibrators *(16)*. The internal calibrators in MPIC tubes were reconstituted in 3 different ways: (*a*) 300 μL solvent was added and incubated at room temperature and vortexted for 30 s; (*b*) leftover clinical samples were extracted in a separate microcentrifuge tube as normal, and the supernatant was transferred to an MPIC tube; and (*c*) the same leftover clinical samples were extracted in an MPIC tube as normal. The slope of the calibration curve in each sample was determined, and extraction efficiency was calculated as the ratio of slope *C* to slope *B*, expressed as a percentage. Ion suppression was then calculated as 1 minus the ratio of slope *C* to slope *A*, expressed as a percentage, for each sample (n = 16). Before being extracted (reconstitution approach B or C), the samples were volumetrically spiked to a final concentration of 2.07 μM MTX, and the slopes of the internal calibration curves were compared with the peak area of the spiked unlabeled MTX to evaluate the relative matrix effects on MTX vs the internal calibrators.

PRECISION

The high- and low-pooled samples sourced at each site were used for the evaluation of precision measurement, based on CLSI EP05-A3 guidelines. The respective high and low concentrations for each site were as follows: 0.404 and 4.3 μM at Site 1, 0.032 and 10.7 μM for Site 2, and 0.052 and 8.2 μM for Site 3. To estimate long-term assay variability, Site 1 analyzed the same pooled samples in duplicate on each of 20 days. All sites conducted a 5-day, multisite precision study with 5 replicates per patient pool for each of the 5 days. Intra- and interday variability for both precision studies was calculated using ANOVA. Total variability was estimated by the sum of squares method using Eq. 1:

$$\text{Total Assay Variability (\%)} = \sqrt{(\text{CV}_{\text{within day}})^2 + (\text{CV}_{\text{between day}})^2} \quad (1)$$

LOWER LIMIT OF QUANTIFICATION

The standard solution of MTX was serially diluted into defibrinated plasma to generate samples with the following target concentrations: 0.008, 0.016, 0.024, 0.032, and 0.040 μM. Each dilution was measured in 5 replicates on a single day. Intraday variability was determined as the percent CV of the 5 replicates, and the precision and deviation from target concentrations of these measurements were used to determine the lower limit of quantification (LLOQ; <20%CV, within 20% of target).

LINEARITY

The linear range of the assay was evaluated by performing a 2-sample mixing experiment that spanned the analytical measurable range (AMR), in accordance with CLSI EP06-A2 guidelines. High- and low-pooled samples, which were sourced by each of the 3 locations, as described, were mixed in 10% increments to generate a total of 11 concentrations. Each concentration was prepared and analyzed in triplicate.

METHOD COMPARISON

Forty patient samples or sample pools with MTX concentrations across the AMR were sent to all 3 sites for a method comparison study, based on CLSI EP09-A3 guidelines. Site 1 compared MPIC with its in-house, previously validated LC-MS/MS MTX clinical assay *(14)*, whereas Sites 2 and 3 compared the new method with a commercial, 510(K)-cleared immunoassay (ARK).

Modified Bland-Altman plots *(17)*, or difference (in percentage) plots, were used to assess agreement between 2 methods in the method comparison study. In each case, the differences were calculated using Eq. 2:

$$\text{Difference (\%)} = \frac{\text{MPIC} - \text{Method A}}{\text{Method A}} \times 100\% \quad (2)$$

Method A was either LC-MS/MS or ARK immunoassay, as appropriate. Results collected from these

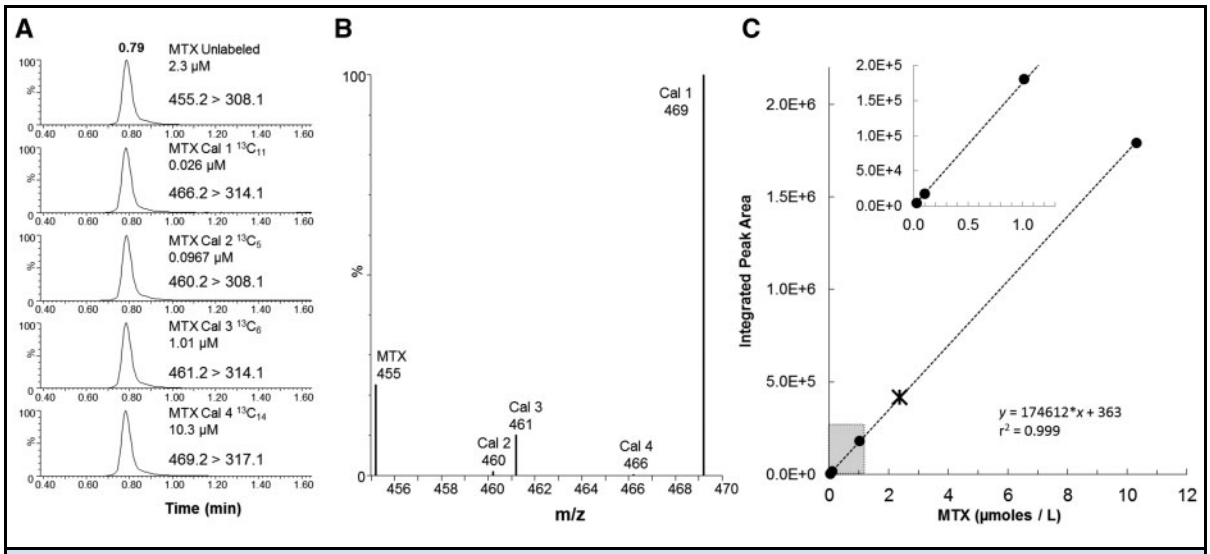

Fig. 1. Representative data from Site 2. (A) MTX and calibrators elute at 0.79 min. Peak areas are normalized to 100%. (B) The mass spectrum shows the unique parent *m/z* of the 4 internal calibrator transitions and MTX. (C) The calibration curve generated from the integrated peak areas is used to quantify MTX. Of note, the origin is not included in the regression, and the unlabeled transition is not included in the calculation of r^2. The inset plot is zoomed in to the shaded region near the origin of the plot. Cal, calibrator.

samples were also used to evaluate reproducibility of MPIC MTX quantification across 3 sites. To assess interlaboratory variability, the percent CV of 1 measurement per sample at each site was calculated. MTX serum concentrations measured by Site 1's LC-MS/MS method were compared with the mean MPIC MTX concentration from all 3 sites and the mean immunoassay MTX concentration at Sites 2 and 3.

DATA ANALYSIS

Results were processed using TargetLynx (Waters Corp) and a Microsoft Excel macro. The MPIC macro determined the concentration of unlabeled MTX in each sample using peak areas from the coeluting isotopically labeled MTX ions and linear regression with $1/x$ weighting and did not include the origin. For this analysis, the regression line r^2 was always >0.995 (not including the unlabeled transition). Calibrators with residual bias exceeding 20% were either removed or the sample was reextracted. Other data analysis and figure generation were performed in Excel and with the *ggplot2 (18)* and other *tidyverse (19)* packages in R Studio *(20)*.

Results

MPIC OF MTX

The stable isotope-labeled internal calibrators are chemically identical to the unlabeled MTX but with between 5 and 14 [^{13}C] atoms incorporated (see Supplemental Fig. 1). ^{13}C was selected instead of deuterium to avoid retention time shifts. Calibrators were carefully designed to mitigate any isotopic contribution to or from any other calibrator or unlabeled MTX. The target concentrations for the internal calibrators were 0.025, 0.1, 1.0, and 10.0 µM, and a certified reference material was used to determine the exact concentrations for data analysis: 0.026, 0.0967, 1.01, and 10.3 µM (Fig. 1). Calibrator value assignment is described in detail in the online Data Supplement. Each internal calibrator transition consists of a unique parent and product ion pair. Unlabeled MTX and all calibrators coelute at 0.79 min (Fig. 1, A), with unique parent *m/z* corresponding to each internal calibrator transition observed (Fig. 1, B). The coefficient of determination for the calibration lines in all samples analyzed exceeded 0.999 (Fig. 1, C).

MULTISITE VALIDATION OF MPIC

Validation experiments performed by each site are summarized in online Supplemental Table 4. Using MPIC, recovery of MTX gravimetrically spiked into normal matrix was 93.3%–102.0% (see Supplemental Table 5). The impact of common interferences was evaluated in samples with high concentrations of hemoglobin, triglycerides, and total protein. Quantification of MTX spiked into all common interference samples (n = 7) was within 93.4%–117.0% of expected values (see

Supplemental Table 6). Matrix effects were evaluated, and mean ± SD ion suppression of 42.3 ± 11.1% and mean ± SD extraction efficiency of 78.3 ± 22.1% were observed (see Supplemental Table 7). The slopes of the calibration curves in 16 different samples were strongly correlated to the observed peak area of the spiked MTX, suggesting that matrix effects affect the internal calibrators and the unlabeled MTX similarly (see Supplemental Fig. 5). Site 1 also determined the LLOQ by evaluating intraday imprecision of 5 low-concentration samples ranging from 0.008 to 0.04 μM and applying 20%CV and ±20% bias cutoffs (see Supplemental Fig. 6). The LLOQ for quantifying MTX in serum with MPIC was validated at 0.017 μM.

The full 20-day precision study demonstrated mean inter- and intraday imprecision of 8.9%CV and 7.3%CV (see Supplemental Tables 8–10). Total assay variability, determined by ANOVA, was 11.9%CV and 11.1%CV at low and high concentrations, respectively. The shortened, multisite, 5-day precision study estimated assay variability similar to the 20-day, single-site study and is summarized in Table 1. Total imprecision (calculated using Eq. 1) was 10.8%, 5.95%, and 16.8% at low concentrations and 14.3%, 3.36%, and 8.98% at high concentrations at Sites 1, 2, and 3, respectively.

All 3 sites validated linearity across the calibrator range (0.026–10.3 μM) with all $r^2 > 0.995$ and slopes between 0.996 and 1.01 (Fig. 2). The observed biases for each concentration at all sites (n = 33) ranged from −6.3% to 8.2%.

INTERLABORATORY REPRODUCIBILITY

Forty samples with MTX concentrations spanning the linear range of the assay were quantified by 2 methods at each of the 3 sites. Results from MPIC analysis at each of the 3 sites were used to assess inter-laboratory reproducibility. Across all sites and samples, MPIC quantification resulted in median imprecision of 6.55%CV (interquartile rage: 3.9–13.2%CV, Fig. 3). Three of the 6 samples with variability exceeding 20% had MTX concentrations < 0.04 μM, which is below most clinically used cutoffs (Supplemental Table 11). One of the 3 sites had values that differed substantially from the other 2 for these 6 samples.

Table 1. Imprecision of the MPIC method at 3 sites.

	Site 1		Site 2		Site 3	
CV (%)	Low	High	Low	High	Low	High
Interday	7.79	12.6	3.79	2.27	14.1	7.85
Intraday	7.45	6.81	4.59	2.47	9.15	4.35
Total[a]	10.8	14.3	5.95	3.36	16.8	8.98

Imprecision was evaluated by analyzing 5 replicates of a low and high QC material on each of 5 days at each of the 3 participating laboratories.
[a]The total variability was calculated using Eq. 1.

COMPARISON OF MPIC AND TRADITIONAL LC-MS/MS

MPIC compared favorably with traditional LC-MS/MS *(14)*, with $r^2 = 0.98$ and slope = 1.04 (see Supplemental Figs. 7 and 8, A). The differences plot shows a mean bias of −5.3% when comparing the mean MPIC quantification across the 3 sites to the LC-MS/MS comparator method (Fig. 4, A). All but 2 values were within ±20%, and those were both <0.04 μM.

COMPARISON OF IMMUNOASSAY AND TRADITIONAL LC-MS/MS

The mean immunoassay MTX concentrations from Sites 2 and 3 were compared with the LC-MS/MS comparator method (Site 1). A mean difference of 5.7% was achieved between the 2 methods (Fig. 4, B), and all values except 1 exceeding 20% bias were <0.1 μM.

COMPARISON OF MPIC AND IMMUNOASSAY

MPIC also compared favorably with the ARK immunoassay with r^2 values of 0.96–0.98 and slopes of 1.04–1.06 (see Supplemental Fig. 8, B and C). Differences between the immunoassay and MPIC rarely exceeded ±20% for samples with MTX concentrations >1 μM (Fig. 5). Greater biases were observed at low MTX concentrations, as expected *(21, 22)*.

Discussion

In this article, we describe a new calibration approach suitable for mass spectrometry, MPIC, which demonstrated excellent analytical performance in a multisite study on 3 different LC-MS instruments following CLSI guidelines. This approach was applied to MTX analysis in serum as proof-of-concept. MPIC quantification of MTX in serum showed good recovery in spiking experiments; had no interference from high protein, lipid, and hemoglobin concentrations; and was linear throughout the calibrator range. The 5-day, multisite and full 20-day, single-site precision studies both demonstrated within- and between-day imprecision of 2.3–14.1%CV and total variability of <17%CV. MTX quantification in serum by MPIC showed low between-laboratory variability and had strong correlations with a previously validated LC-MS/MS method *(14)* and a commercially available immunoassay.

MPIC offers the advantage of matrix-matched calibration with every sample. This reduces the need for extensive matrix-effects studies *(16)* because external calibrators are not spiked into a separate matrix. In

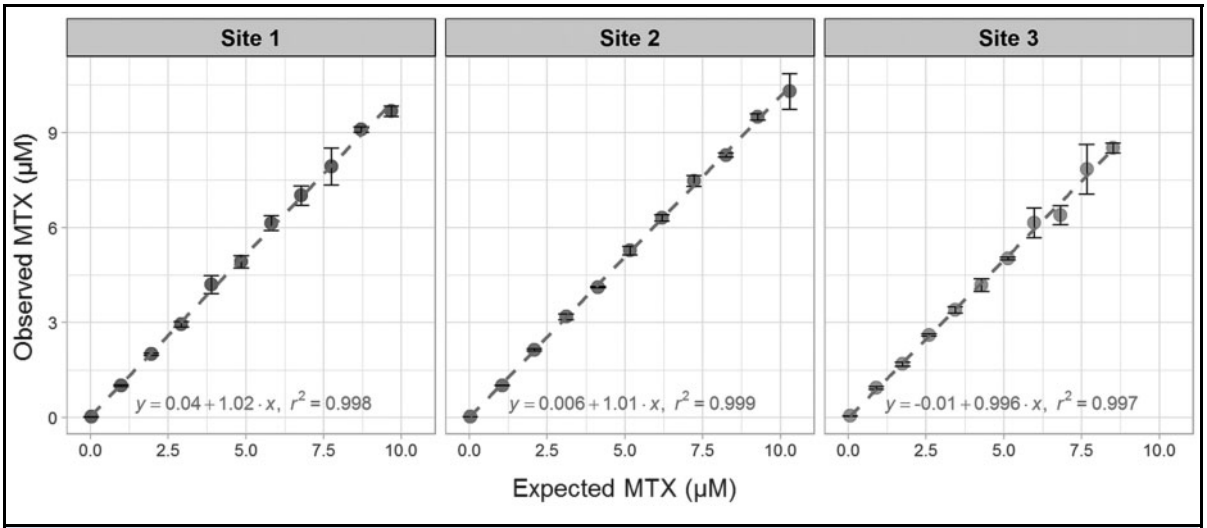

Fig. 2. MPIC linearity. Two pooled samples with either a high or low concentration of MTX were mixed in 10% increments, generating 11-point curves. Each point represents the mean ± SD (n = 3). The dashed line represents the linear regression line; the corresponding equation and r^2 are at the bottom of each panel.

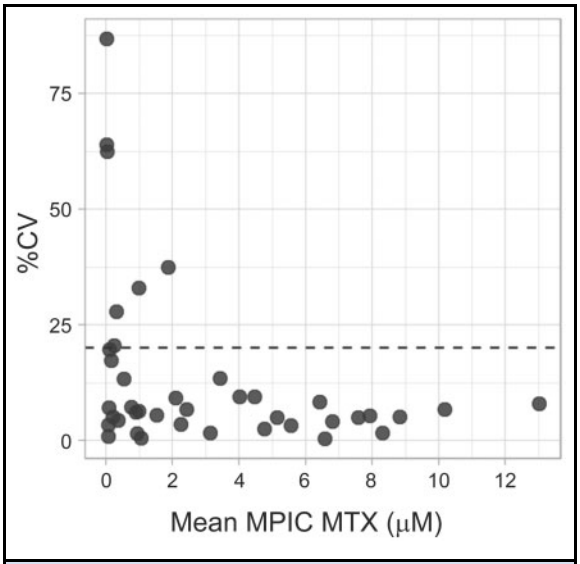

Fig. 3. Interlaboratory reproducibility. Serum samples (n = 40) were split and analyzed once at each site. The mean MTX concentration is plotted against percent CV. The dashed line is at 20%.

addition, the calibrators were designed with ^{13}C to avoid retention time shifts so that the internal calibrators are subjected to the same ion suppression or enhancement effects as the analyte *(23)*.

Increased biases and variability were observed at the low end of MTX concentrations (<0.1 μM). Multiple reasons are possible. First, the contradictory biases observed between MPIC and the immunoassay run at Sites 2 and 3 (mean bias, −11.4% and +8.3%, respectively) are most likely the result of variable calibration across the 2 sites, which was particularly problematic at low concentrations and consistent with previous studies *(24)*. Second, there could be variability in pipetting in the MPIC assay, which would be improved with automated liquid handling. Third, the number of transitions monitored at the same time point in the chromatographic program is higher with internal calibration and limits the number of points across the chromatographic peak, which could lead to higher imprecision. Finally, there could be matrix effects that affect the unlabeled MTX differently than the internal calibrators; although more extensive evaluation is warranted, our data seem to suggest otherwise (see Supplemental Fig. 5).

Importantly, 6 specimens were highly variable between laboratories by the MPIC approach. In a post hoc analysis, we examined the variability of the slopes of the internal calibration curves and noticed that a cutoff of 30% below the mean slope for the day would have led to 3 of the 6 samples being repeated at Site 3. This QC approach would complement the stringent criteria for r^2 (>0.995) and for the residuals of calibrators from the regression line (<20%) that were used in this proof-of-concept study. Although the residual of the calibrators may provide an alternative to qualifier ion ratios for the internal calibrators, it seems that including qualifier ion ratios for the endogenous MTX would be prudent in future configurations of this assay. Additional quality

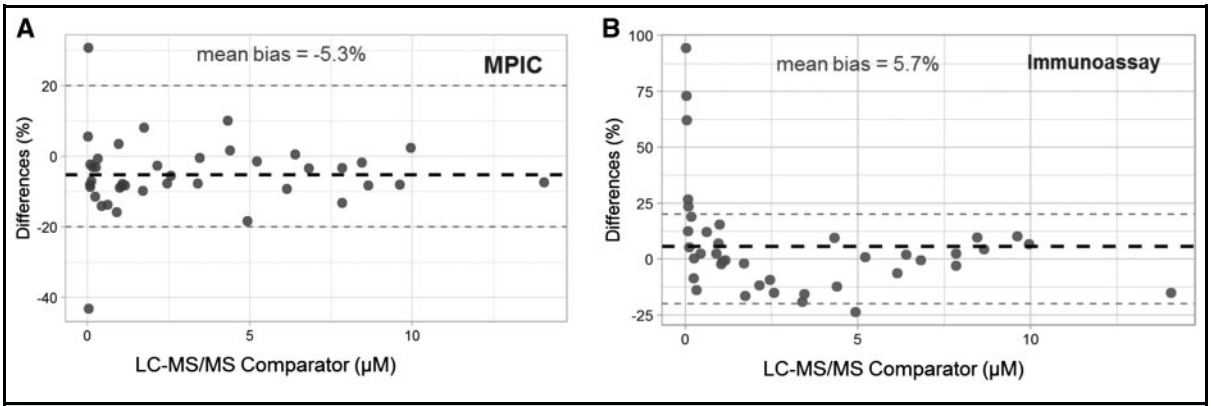

Fig. 4. Method comparison to traditional LC-MS/MS. Serum samples were split and analyzed by MPIC at all 3 sites. (A) Differences plot of mean MPIC value (all sites) vs the LC-MS/MS comparator method. (B) Differences plot of mean immunoassay value (Sites 2 and 3) vs LC-MS/MS comparator method. The thin dashed lines are at ±20%, and the thick dashed lines are at the mean biases.

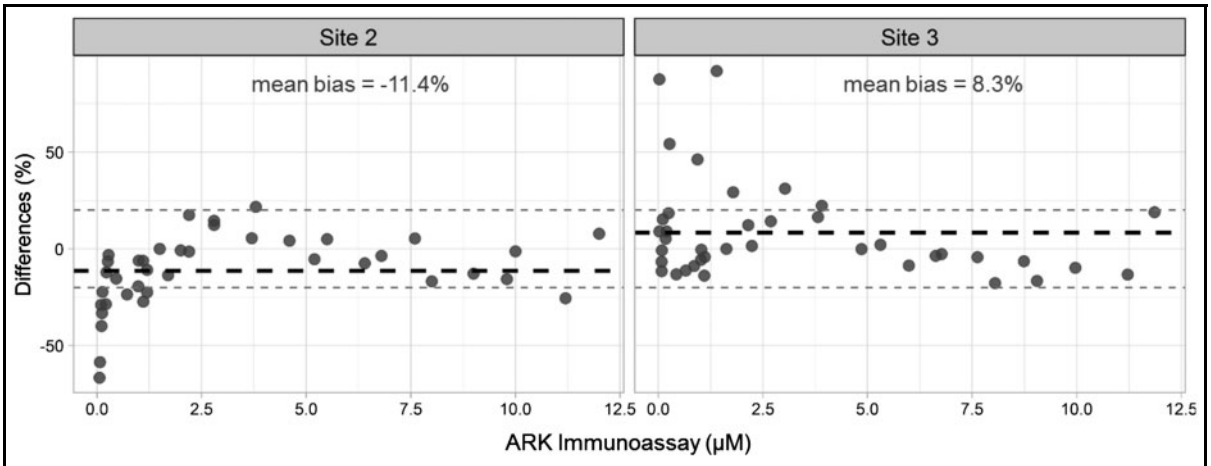

Fig. 5. Comparison to an immunoassay. Serum samples were split and MTX analyzed by both immunoassay and MPIC at Sites 2 and 3 (same samples as in Fig. 4). The differences between methods were calculated using Eq. 2. Dashed lines are at ± 20%. The *y*-axis is shared between the 2 plots.

assurance would include an MTX-free sample to evaluate carryover daily.

The biases observed need to be examined from the perspective of patient management. The plasma concentration of MTX is commonly used in 2 ways. First, the observed half-life is used to identify individuals for whom pharmacological rescue will be required. Second, the absolute concentration is used to clear patients to be discharged from the hospital. In the first instance, concentrations above the upper limit of quantification of the immunoassay are common, and although time-consuming dilutions are required for patient care, there does not appear to be significant bias. In the second instance, accuracy around the medical decision limit (varies by center from 0.01 to 0.05 μM) is required, and thus adequate QC is required for a novel MPIC assay. Fortunately, more data elements are included in a mass spectrometric result (in this case, including an entire calibration curve) than in an immunoassay, and this will help ensure accurate results.

We have shown >2-fold improvement using MPIC on the LLOQ compared with immunoassay (0.017 vs 0.040 μM), based on the limit of quantification defined in the ARK MTX assay package insert

(25). In addition, the immunoassay has an upper limit of quantification of 1.2 μM, which is an order of magnitude lower than the highest calibrator used for MPIC (10.3 μM). As mentioned briefly, in routine clinical analysis, samples exceeding the upper limit of quantification are serially diluted until the result is within the AMR. Dilution is not automatable on many platforms; therefore, it takes additional time and labor to repeatedly analyze samples until they are within range. More than half of the patient samples required 1 dilution and several required multiple dilutions to obtain immunoassay results in the reportable range in this study. MPIC could help improve TAT by reducing the need for dilutions in the vast majority of samples.

Currently, batch-mode testing performed by LC-MS/MS in the clinical laboratory results in 12-h or longer TATs for most assays. Increased TATs are a major limitation of LC-MS/MS (26). Longer TATs lead to delayed patient treatment to prevent MTX toxicity, resulting in worse clinical outcomes and prolong hospital length of stay. MPIC quantification is capable of reducing TAT by allowing samples to be analyzed as they arrive, enabling random-access clinical LC-MS/MS. With the synthesis of the relevant isotopologues, MPIC quantification should be applicable to other compounds that require quick TATs (e.g., immunosuppressants). In addition, laboratory efficiency is improved by reducing resources required to prepare and analyze calibrators with every batch of samples (27).

Conclusion

In a multisite study, we developed and validated an alternative calibration strategy for clinical LC-MS/MS assays that uses multiple internal calibrators. For proof-of-concept, the chemotherapeutic drug MTX was used. MPIC analysis correlated well with both immunoassay and LC-MS/MS using a traditional calibration scheme. Excellent reproducibility and linearity across 3 sites, each with a different LC-MS/MS system, demonstrates assay robustness. Elimination of external calibration runs requiring sample batching can significantly reduce TATs and improve the economic feasibility of translating therapeutic drug-monitoring assays from immunoassay to an LC-MS/MS platform.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard abbreviations: TAT, turnaround time; MPIC, multipoint internal calibration; MTX, methotrexate; MPB, mobile phase B; LLOQ, lower limit of quantification; AMR, analytical measurement range.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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