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Mitigating Matrix Effects in LC–ESI–MS/MS Analysis of a Urinary Biomarker of Xylenes Exposure

Brett A. Bowman[1,2], **Elizabeth A. Ejzak**[1,2], **Christopher M. Reese**[1], **Benjamin C. Blount**[1], **Deepak Bhandari**[1,*]

[1]Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Hwy NE, Atlanta, GA 30341, USA

[2]Life Sciences Research, Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201, USA

Abstract

Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) with stable isotope-labeled internal standards (SIL-ISs) is the gold standard for quantitative analysis of drugs and metabolites in complex biological samples. Significant isotopic effects associated with deuterium labeling often cause the deuterated IS to elute at a different retention time from the target analyte, diminishing its capability to compensate for matrix effects. In this study, we systematically compared the analytical performance of deuterated (^{2}H) SIL-IS to non-deuterated (^{13}C and ^{15}N) SIL-ISs for quantifying urinary 2-methylhippuric acid (2MHA) and 4-methylhippuric acid (4MHA), biomarkers of xylenes exposure, with an LC–ESI–MS/MS assay. Analytical method comparison between ISs demonstrated a quantitative bias for urinary 2MHA results, with concentrations generated with 2MHA-[$^{2}H_7$] on average 59.2% lower than concentrations generated with 2MHA-[$^{13}C_6$]. Spike accuracy, measured by quantifying the analyte-spiked urine matrix and comparing the result to the known spike concentration, determined that 2MHA-[$^{2}H_7$] generated negatively biased urinary results of −38.4%, whereas no significant bias was observed for 2MHA-[$^{13}C_6$]. Post-column infusion demonstrated that ion suppression experienced by 2MHA and 2MHA-[$^{13}C_6$] was not equally experienced by 2MHA-[$^{2}H_7$], explaining the negatively biased 2MHA results. The quantitation of urinary 4MHA results between ISs exhibited no significant quantitative bias. These results underscore the importance of the careful selection of ISs for targeted quantitative analysis in complex biological samples.

*Author to whom correspondence should be addressed. dbhandari@cdc.gov.

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Supplementary Data

Supplementary data are available at *Journal of Analytical Toxicology* online.

Introduction

Xylenes are volatile aromatic hydrocarbons that have widespread industrial applications due to their superior solvent properties (1). They are one of the top 30 most produced chemicals in the USA by volume and are frequently used in commercial products such as petrochemicals, paints and plastics (2, 3). Major environmental sources of exposure to xylenes include petroleum refinery emissions, vehicle exhaust fumes, landfill gases and tobacco smoke (4, 5). Acute exposure above the permissible exposure limit set by the Occupational Safety and Health Administration has resulted in reduced muscular strength and coordination, depressed respiration, coma and death; chronic exposure to low concentrations of xylenes can cause nausea, gastrointestinal discomfort, vomiting, increased anxiety and difficulties concentrating (1, 4, 6). Due to these public health concerns, it is important to accurately quantify biomarkers of exposure to xylenes. In this study, we investigated the effect of various stable isotope-labeled internal standards (SIL-ISs) on the accuracy of measurement of urinary xylene metabolites using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

A known challenge to the quantitative capability of LC–MS/MS is the matrix effects that can occur at the electrospray ionization (ESI) interface between the LC and the MS (7, 8). Complex sample matrices contain thousands of chemicals that can interfere with the ionization efficiency of the analyte through various mechanisms, including competition for ion charge and changes in the electrospray droplet formation (7–9). The co-eluting species can increase or decrease the ionization potential of the targeted analyte, referred to as ion enhancement or ion suppression, respectively. Consequently, an artificially low or high analyte signal will be detected in the MS, leading to erroneous resulting concentrations if the matrix effect is not accounted for. The large variation of matrix components between samples leads to a wide variation in matrix effects that can dramatically impact the accuracy of LC–ESI–MS/MS analytical results.

The selection of IS to account for matrix effects is a critical factor in analytical method development to ensure the expected accuracy of LC–MS/MS (10, 11). The predominant technique to normalize analyte signal from matrix effects is isotope dilution (12, 13). For isotope dilution, the SIL form of the analyte is used as an IS that closely matches the physicochemical properties of the analyte, mimicking the behavior of the analyte through sample preparation, chromatographic separation, and ionization and mass fragmentation (10, 14). Common isotopic labels include ^{2}H, ^{13}C, ^{15}N and ^{18}O, with ^{2}H being the most frequently used due to availability and affordability (13, 15, 16). Although ^{2}H-labeled ISs are often used in isotope dilution LC–MS/MS, slightly earlier elution than the unlabeled target analyte occurs in reverse-phased columns, particularly with an increased number of ^{2}H atoms (15, 17, 18). In contrast, ^{13}C- and ^{15}N-labeled ISs typically coelute with target analytes (14, 18, 19).

In this study, we aim to systematically examine and compare the analytical figures of merit of an LC–ESI–MS/MS assay for measuring two urinary metabolites of exposure to xylenes, 2-methylhippuric acid (2MHA) and 4-methylhippuric acid (4MHA), by using deuterated (^{2}H) and non-deuterated (^{13}C and ^{15}N) SIL-ISs. The analytical performance was

compared between 2MHA-[$^{2}H_{7}$] and 2MHA[$^{13}C_{6}$] for 2MHA and between 4MHA-[$^{2}H_{7}$] and 4MHA-[$^{13}C_{2}$-^{15}N] for 4MHA.

Experimental

Materials

OPTIMA LC–MS-grade acetonitrile, methanol and water were purchased from Fisher Scientific (USA). LiChropur LC–MS-grade ammonium acetate was purchased from Sigma Aldrich (USA). The unlabeled analytes 2MHA and 4MHA were purchased from Toronto Research Chemicals (Toronto, Canada). The SIL-ISs 2MHA-[$^{2}H_{7}$], 2MHA-[$^{13}C_{6}$] and 4MHA-[$^{2}H_{7}$] were purchased from Toronto Research Chemicals (Toronto, Canada), and 4MHA-[$^{13}C_{2}$-^{15}N] was purchased from Cambridge Isotopes Laboratory (Tewksbury, MA). Anonymized spot urine samples were purchased from Tennessee Blood Services (Memphis, TN) after collection from 12 self-identified nonsmokers and 25 self-identified smokers.

LC–ESI–MS/MS analytical method

The analytical method in this study has been previously reported and used here with minor modifications (20). Briefly, the sample preparation and instrumentation are described. The calibration material, quality controls and urine specimens were prepared by diluting 10 times with working IS and buffer (50μL calibration material or quality control or urine specimen+25μL working IS+425μL 15mM ammonium acetate pH 6.8). This sample preparation is commonly described as dilute-and-shoot. The prepared solutions were then analyzed using LC–ESI–MS/MS. Chromatographic separation was performed using an Acquity I-Class LC system (Waters Corporation, Milford, MA) equipped with Acquity LC HSS T3 1.8μm×2.1mm×150mm column (Waters Corporation, Milford, MA). The LC system was coupled to a 5500 triple quadrupole mass spectrometer equipped with an ESI source (Sciex, Framingham, MA). Analyte separation was performed using a gradient elution with 15mM ammonium acetate pH 6.8 in water as mobile phase *A* and acetonitrile as mobile phase *B*. The gradient elution and chromatographic parameters are found in the original publication. The mass spectrometer was operated in negative ion mode with ESI in scheduled multiple reaction monitoring modes. Optimized source parameters were IonSpray voltage −4,500V, curtain gas flow 45 (arbitrary units), GS1 gas flow 55 (arbitrary units), GS2 gas flow 65 (arbitrary units), collision gas flow 7 (arbitrary units) and probe temperature 650°C. Compounddependent parameters are displayed in Table I.

Calibration

A set of eight calibrants was analyzed with each set of unknown samples. A weighted $1/x$ (where x is the standard concentration) least-square model was fit to the calibration. Calibration curves were linear, with r^2 >0.99. All calibrants were prepared in 15mM ammonium acetate solution.

Analytical method comparison

The described LC–ESI–MS/MS analytical method was used to quantify urinary concentrations of 2MHA and 4MHA in 37 urine specimens using both a deuterated and carbon-labeled IS. Urine specimens from smokers (N=25) and nonsmokers (N=12) were

assayed to ensure analytical results that spanned the entire calibration range and to capture any differences arising with smoking status. Analytical results were excluded from the comparison if a result from either IS was below the limit of detection or above the upper limit of quantitation.

Urinary results produced by ^{2}H-labeled and ^{13}C- and ^{15}N-labeled ISs were treated as results from separate analytical methods. Thus, the conventional analytical method comparison tools of correlation-regression analysis and Bland–Altman (BA) analysis were performed to assess and visualize the agreement in results between the SIL-ISs. In the correlation-regression analysis, the coefficients of determination were calculated to assess the correlation between method results, and the linear regression was calculated and compared to the line of equality to assess the quantitative agreement between methods. The BA analysis was performed to further assess the quantitative agreement between methods. The BA analysis involved plotting the percent differences of each paired result (*y*-axis) against the average of each paired result from the two methods (*x*-axis). The overall mean percentage differences and the 95th percentile limits of agreement were also calculated and included in the plots. Percent difference was used instead of absolute difference to meet the BA assumption that the differences are from an approximately normal distribution (21, 22). The absolute difference data rejected the null hypothesis in the Shapiro–Wilk test that the data came from a normally distributed population, whereas the percent difference data did not reject the null hypothesis.

Accuracy through spiking

The accuracy of the urinary concentration of the targeted analytes measured using each SIL with the LC–ESI–MS/MS was assessed in the urine matrix by completing a spike accuracy experiment. Unlabeled 2MHA and 4MHA were spiked into urine at three levels, and analyte concentrations were measured for the non-spiked and spiked urine using both deuterated and carbon-labeled ISs. The endogenous 2MHA and 4MHA concentrations measured in the non-spiked matrix were subtracted from urine-spiked levels to determine the resulting spike concentration. The relative error was then calculated by comparing this resulting spike concentration with the known spike concentration using the formula:

$$\text{Relative eror (\%)} = \frac{\text{Resulting spike concentration} - \text{known spike concentration}}{\text{known spike concentration}} \times 100$$

The relative error was measured in 14 human urine samples, sampling nonsmoker (N =8) and smoker (N=6) samples to capture the matrix effects of potentially exposed and unexposed individuals. Spike levels were chosen to span the calibration range for each analyte to examine if there were concentration-dependent biases.

Post-column infusion

The urinary matrix effects associated with the analytical method were examined using post-column tee infusion, similar to procedures reported in the literature (23, 24). The LC was operated following the chromatographic conditions described in this experimental section. The eluate from the LC was infused with an aqueous solution containing the unlabeled

2MHA or 4MHA and their corresponding ^{2}H-labeled and ^{13}C- and ^{15}N-labeled ISs via a tee connector on the flow path to the MS. The mass spectrometer-integrated syringe pump was used to infuse the aqueous solution of the pure analyte at 10μL/min. Separate post-column infusions were performed for 2MHA and 4MHA to avoid overlapping mass spectra.

Solid-phase extraction sample preparation

The original analytical method utilized a dilute-and-shoot sample preparation prior to LC–MS/MS analysis. A solid-phase extraction (SPE) method was developed for the isolation of 2MHA and 4MHA to reduce or eliminate matrix effects. The SPE was performed by diluting 250μL of urine to 1,800μL with aqueous ammonium acetate buffer (pH 9.0). Evolute AX Express (60mg) mixed-mode strong anion exchange 96-well plates from Biotage (Charlotte, NC) were used for sample matrix cleanup. SPE steps involved plate conditioning with 1mL of methanol followed by equilibration using 1mL of ammonium acetate buffer (pH 9.0). The diluted urine samples were then loaded and washed with 1mL of LC–MS grade water followed by 1mL of methanol. Analytes were eluted in 500μL of 10% formic acid in acetonitrile:methanol (3:2) followed by 500μL of 10% formic acid in acetonitrile. SPE was performed using a Biotage Extrahera sample preparation system (Charlotte, NC). Following SPE, eluents were evaporated to dryness in an SPE Dry evaporation system from Biotage (Charlotte, NC) under nitrogen gas at 60°C. Prior to LC–MS/MS analysis, dried samples were reconstituted in 500μL 15mM aqueous ammonium acetate (pH 6.8) and homogenized for 10min.

Data analysis

All LC–MS/MS data were generated in Analyst 1.7 (Sciex, Framingham, MA) and processed in MultiQuant 3.0.3 (Sciex, Framingham, MA). Graphing and statistical analysis were performed with GraphPad Prism 9.0.0 (San Diego, CA).

Results and discussion

Analytical method comparison

In this study, we characterized methodological accuracy when using different ISs (i.e., ^{2}H-labeled vs. ^{13}C- and ^{15}N-labeled) and found that significant bias can result from using non-coeluting ISs such as ^{2}H-labeled 2MHA. Specifically, the analyte concentrations of 2MHA and 4MHA were measured in 37 urine samples using the described LC–ESI–MS/MS analytical method with ^{2}H-labeled and ^{13}C- and ^{15}N-labeled ISs. Although arising from the same analytical procedure, the results produced by the differently labeled ISs were considered to arise from separate analytical methods, and thus typical statistical tools of correlation plot-regression and BA analyses were performed to assess the agreement in results. The quantitative comparisons are graphically displayed in Figure 1, and the results are detailed in Table II. Individual urinary results can be found in Supplementary Table SI.

The regression analysis for urinary 4MHA concentration using a ^{13}C- and ^{15}N-labeled IS (4MHA-[$^{13}C_2$-^{15}N]) versus a ^{2}H-labeled IS (4MHA-[$^{2}H_7$]) demonstrated a slope and intercept statistically indistinguishable from 1.0 and 0, respectively, with 95% confidence interval (CI), and a strong correlation with a coefficient of determination of 0.998. This

indicates that both SIL-ISs produce equivalent analytical results for urinary 4MHA. In addition, the BA analysis showed a statistically insignificant bias of −0.642%, further demonstrating that both SIL-ISs will produce the same urinary concentration for the dilute-and-shoot LC–ESI–MS/MS method. The regression analysis for urinary 2MHA concentrations using a ^{13}C-labeled IS (2MHA-[$^{13}C_6$]) versus a ^{2}H-labeled IS (2MHA-[$^{2}H_7$]) demonstrated an intercept indistinguishable from 0 and a slope of 2.23, significantly different from 1.0 with 95% CI. The BA analysis for the 2MHA concentration comparison demonstrated a statically significant bias of −59.2%. Here, the conventional method comparison approach demonstrated that urinary 2MHA concentrations resulting from the ^{2}H-labeled IS were negatively biased by ~2-fold on average when compared to the ^{13}C-labeled IS.

Although the linear regression analysis for 2MHA concentrations demonstrated a strong correlation between results, the percent difference in individual urinary concentrations between SIL-ISs ranged widely from −97.9% to −15.9% with a percent coefficient of variation of −32.4%. In contrast, the difference in urinary 4MHA concentrations between SIL-ISs was minimal, ranging from −6.67% to 8.85% with an average difference of 0.642%. The wide variation in the 2MHA concentrations between ^{2}H-labeled and ^{13}C-labeled ISs indicated major matrix-to-matrix variations between urine samples. Therefore, the disagreement revealed by the analytical method comparison required further exploration to understand the most effective SIL-IS for the accurate measurement of 2MHA in urine matrix.

Accuracy through spiking

The analytical method comparison demonstrated that the selection of a ^{2}H-labeled IS versus a ^{13}C-labeled IS was a critical factor for the quantitation of urinary 2MHA concentrations but did not assess which IS generated the most accurate results. The accuracy of each SIL-IS paired with the dilute-and-shoot LC–MS/MS method was measured by spiking known amounts of 2MHA or 4MHA into 14 urine samples and comparing the resulting concentration to the known spiked amount. The average spike results and the relative error across all urine samples are summarized in Table III. Individual spike accuracy results are summarized in Supplementary Tables SII and SIII.

For 4MHA, the spike accuracy experiment revealed an average relative error of −2.3% and −0.9% for ^{13}C-/^{15}N-labeled and ^{2}H-labeled ISs, respectively. For 2MHA, the average relative error was −3.5% and −38.4% for ^{13}C-labeled and ^{2}H-labeled ISs, respectively. It is generally regarded that accuracy should be within ±15% for quantitative LC–MS/MS methods (25, 26). Both ^{13}C-/^{15}N-labeled and ^{2}H-labeled ISs for 4MHA as well as the ^{13}C-labeled IS for 2MHA readily meet this criterion with average relative errors all <5% across 14 urine samples and therefore demonstrate that each can be used to generate accurate LC–MS/MS data in urine matrix. The ^{2}H-labeled IS 2MHA-[$^{2}H_7$] exhibited an average relative error of −38.4%, experiencing a significant negative bias that misses the accuracy criterion for a quantitative bioanalytical method. On an individual urine matrix basis, the ^{2}H-labeled IS for 2MHA exhibited relative errors ranging from −12.6% to −59.2%.

The spike accuracy experiment demonstrated that both 4MHA ISs produced accurate results and justified the statistically equivalent urinary 4MHA concentrations discussed earlier (Figure 2). For 2MHA, the negative bias observed in the spike accuracy experiment with the ^{2}H-labeled IS follows the linear regression line exhibiting a positive slope of 2.23 for the ^{13}C-labeled IS versus the ^{2}H-labeled IS and the BA analysis with the average bias of −59.2%. The ^{2}H-labeled IS underestimated the 2MHA spike concentration in the spike accuracy experiment and similarly produced lower urinary concentrations when compared to the ^{13}C-labeled IS in the method comparison analysis. Furthermore, the large variation in individual spike biases for the ^{2}H-labeled IS reflects the variation of percent differences in the analytical method comparison for 2MHA. These wide-ranging differences seen between urine matrices indicate that there are matrix effects not accounted for by 2MHA-[$^{2}H_7$] that are the source of inaccurate urinary concentrations.

Common guidance provided for analytical method development recommends that calibration standards be prepared in the same biological matrix of unknown specimens (25). This guidance is expected to improve the accuracy of an analytical method by accounting for matrix effects experienced by the analyte in unknown specimens. Although the calibration standards used in this study were prepared in non-matrix aqueous 15mM ammonium acetate, the wide variation in individual spike error for 2MHA results generated with the 2MHA-[$^{2}H_7$] IS indicates that matrix-matching the calibration standards would be an insufficient solution across population-based samples. In this study, the selection of an averagely biased (−38.4%) urine matrix for calibration standards would result in an error of 25.8% and 20.8% for the lowest and highest biased specimens, respectively. Both accuracies are still outside of the ±15% guidelines for analytical methods, and this matrix-matching procedure requires the arduous and time-consuming selection of an averagely biased urine matrix. Furthermore, the selection of an authentic matrix with negligible analyte, particularly in multi-analyte panels that examine exposure to environmental chemicals or of endogenous nature, can be challenging or impossible (27). The use of an IS that accounts for matrix effects is the simplest procedure for accurate quantitation of urinary concentrations.

Post-column infusion: explaining method comparison and accuracy

Post-column infusion experiments were employed to better understand the fundamental reason behind the poor accuracy of urinary 2MHA concentrations with the ^{2}H-labeled IS and its lack of agreement with the ^{13}C-labeled IS. Post-column infusion of an aqueous solution of analyte and its labeled ISs was mixed in-line with the LC eluent as described in the experimental section.

The ion current plot from post-column infusion was paired with the chromatograms to explore the capability of each SIL-IS to account for matrix effects experienced by the unlabeled analyte. The paired plots are displayed in Figure 2 with the ion current resulting from a post-column tee infusion and the chromatogram resulting from injected standards and ISs. Examining the chromatograms, the ^{2}H-labeled ISs 2MHA-[$^{2}H_7$] and 4MHA-[$^{2}H_7$] both elute ~1.8s earlier than the corresponding unlabeled forms, whereas the ^{13}C-/^{15}N-labeled ISs 2MHA-[$^{13}C_6$] and 4MHA-[$^{13}C_2$-^{15}N] elute at virtually the same retention time of the corresponding unlabeled forms. The earlier retention time of the deuterated ISs reflects

relatively significant differences in physicochemical properties of deuterium compared to hydrogen (isotope effects) that is well known to cause earlier retention in reversed-phase LC (15, 28). ^{13}C-/^{15}N-labeled ISs do not experience this relative phenomenon to the same extent and therefore match the retention time of the unlabeled analyte as seen with 2MHA-[$^{13}C_6$] and 4MHA-[$^{13}C_2$-^{15}N] (14).

Notably, over the retention time difference window for 2MHA and 2MHA-[$^{2}H_7$], the ion current dropped ~50% (see Figure 2, left) with unlabeled 2MHA experiencing significant ion suppression from coelution with urine matrix components. In contrast, 2MHA-[$^{13}C_6$] eluted off the LC simultaneously with 2MHA, and both experienced equivalent ion suppression. Since quantitation with isotope dilution LC–MS is achieved by ratioing the analyte to a spiked IS, unequally suppressed or enhanced analyte signal compared to the IS signal will lead to biased results. The greater ion suppression experienced by 2MHA and 2MHA-[$^{13}C_6$] compared to 2MHA-[$^{2}H_7$] explains the lower urinary results produced by 2MHA-[$^{2}H_7$] in the method comparison and the negative bias for 2MHA-[$^{2}H_7$] in the urine spike accuracy.

In contrast, regardless of the difference in retention times, the 4MHA-[$^{2}H_7$] experienced identical matrix effects to 4MHA and 4MHA-[$^{13}C_2$-^{15}N], reflecting stable matrix effects over this chromatographic period (Figure 2, right). This observation aligns well with the previously discussed analytical method comparison results, demonstrating statistically equivalent urinary 4MHA concentrations and the spike accuracy results passing for both SIL-ISs.

Addressing matrix effects with SPE

Sample preparation such as SPE is often employed to extract and preconcentrate analytes from matrix components to reduce or eliminate matrix effects. In the original analytical method, dilute-and-shoot sample preparation was completed prior to LC–ESI–MS/MS analysis. In order to overcome the matrix effect observed for urinary 2MHA measurement, we performed several anion-exchanged SPE experiments as discussed in the experimental section. Experiments were performed to improve the wash step of the SPE to selectively remove matrix interference as well as change the elution strength of the elution solvent by altering its pH and organic solvent composition. However, there was no notable reduction in ion suppression for the samples prepared by the SPE method compared to the dilute-and-shoot method. Therefore, in this study, SPE was not a suitable approach to reduce matrix effects for the measurement of urinary 2MHA.

Conclusion

Selection and validation of a SIL-IS is a critical step in the development of an effective LC–MS/MS assay for biomonitoring applications. In this study, a systematic approach for examining matrix effects was employed to assess the quantitative performances of ^{2}H-labeled and ^{13}C-/^{15}N-labeled ISs in a dilute-and-shoot LC–ESI–MS/MS method that measures urinary biomarkers of xylene exposure. The approach revealed that the ^{13}C-/^{15}N-labeled IS was superior to the ^{2}H-labeled IS by accounting for matrix effects experienced by the unlabeled analyte. The minor difference in elution time between ^{2}H-labeled ISs and

their unlabeled analog can lead them to experience significantly different matrix effects that negatively impact method accuracy. ^{13}C-/^{15}N-labeled IS more closely matches its unlabeled counterpart and elutes at virtually the same retention time, experiencing equivalent matrix effects. Due to greater availability and significant cost benefits, ^{2}H-labeled IS remains the most commonly used SIL-IS. The development of LC–MS/MS methods with non-coeluting IS, such as ^{2}H-labeled IS, should be completed with more rigorous method validation to ensure that matrix effects experienced by the analyte are still compensated by the IS. This systematic study aimed for mitigating the matrix effect for quantifying urinary 2MHA using isotope dilution LC–ESI–MS/MS can be universally applied for various other LC–MS applications including pharmaceuticals, drug therapeutics and clinical diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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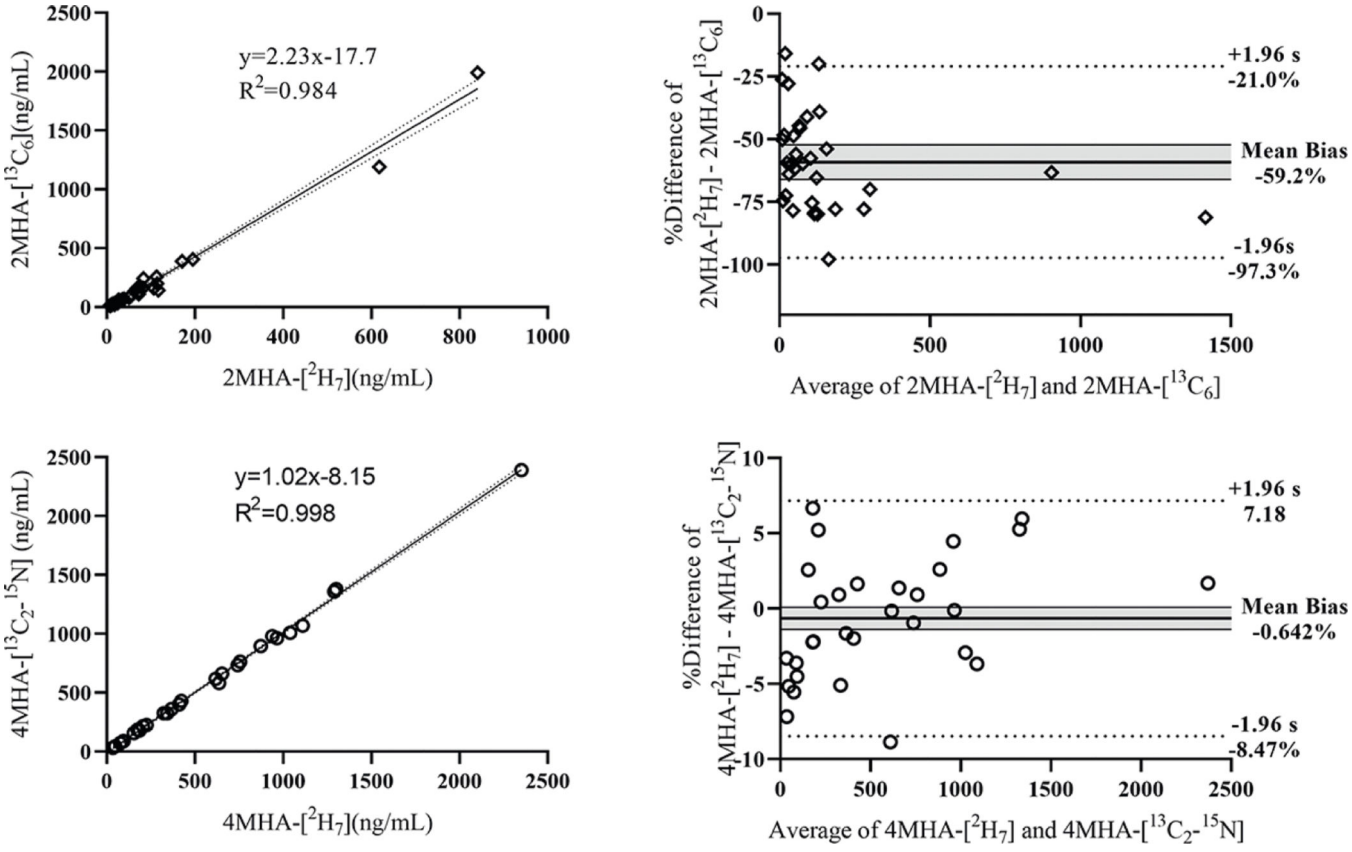

Figure 1.

The linear regression-correlation (left) and BA plots (right) for the analytical method comparison. Urinary 4MHA concentrations (bottom) are equivalent between ^{2}H-labeled IS and ^{13}C-/^{15}N-labeled IS, whereas for 2MHA (top), there is a marked bias between ^{2}H-labeled and ^{13}C-labeled IS. The shaded gray area in the BA plot indicates a 95% CI of BA bias.

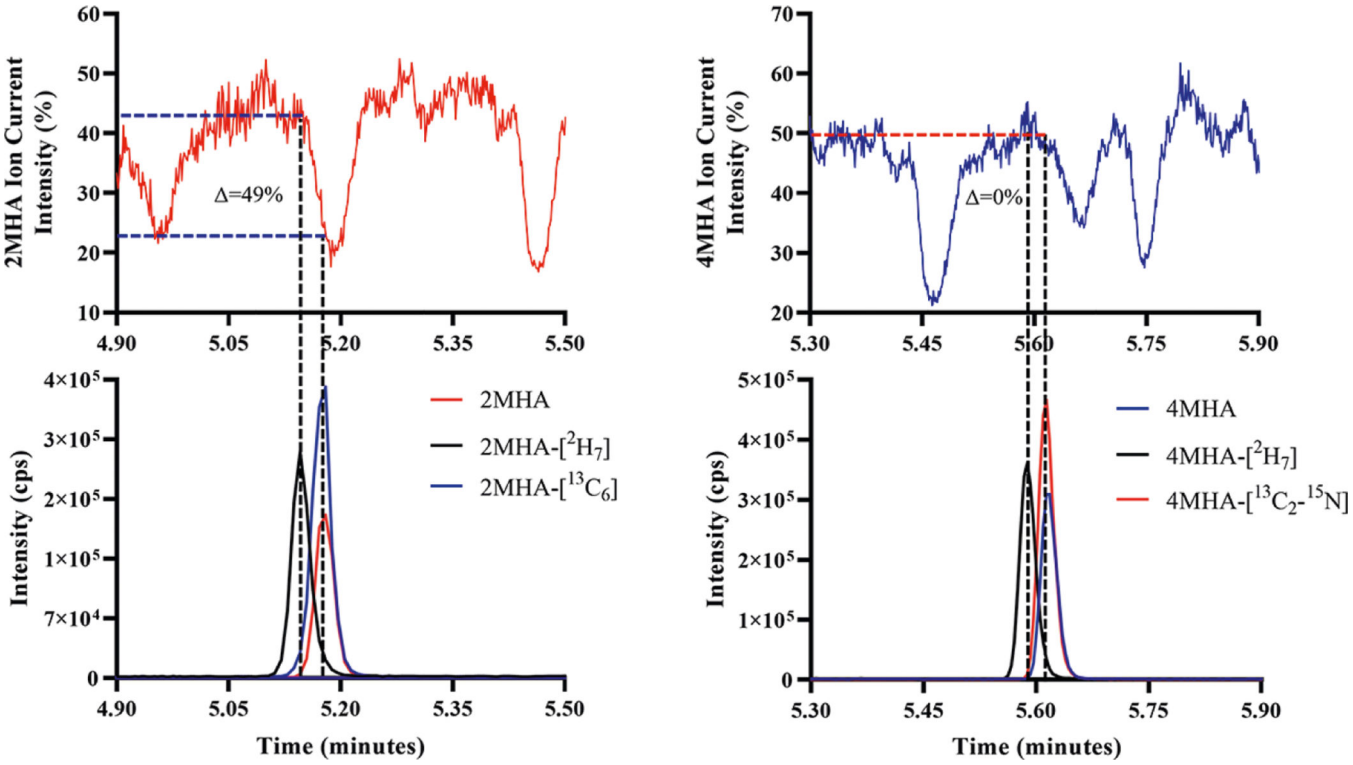

Figure 2.

Ion current from post-column infusion paired with chromatograms demonstrates the varied matrix effects experienced by IS and analyte. 2MHA and 2MHA-[$^{13}C_6$] experience ion suppression that is not equally experienced by 2MHA-[$^{2}H_7$]. Although eluting at different times, 4MHA and 4MHA-[$^{13}C_2$-^{15}N] experience similar matrix effects to 4MHA-[$^{2}H_7$]. The results shown are with a representative nonsmoker urine sample with low endogenous 2MHA and 4MHA concentrations.

Table I.

Compound-Specific Mass Spectrometric Parameters.

Analyte	Retention time (min)	Ion transition (m/z)		DP	EP	CE	CXP
		Quantitation	Confirmation				
		192.1➔148.3		−40	−10	−16	−19
2MHA	5.18		192.1 ➔91.2	−40	−10	−22	−7
2MHA-[$^{2}H_{7}$]	5.15	199.2 ➔155.3		−35	−10	−16	−11
2MHA-[$^{13}C_{6}$]	5.18	198.0 ➔154.1		−40	−10	−17	−11
		192.2 ➔148.2		−40	−10	−16	−17
4MHA	5.62		192.2 ➔91.2	−40	−10	−21	−11
4MHA-[$^{2}H_{7}$]	5.59	199.2 ➔155.3		−45	−10	−17.5	−5
4MHA-[$^{13}C_{2}$-^{15}N]	5.62	195.0 ➔150.0		−40	−10	−17	−8

DP=declustering potential, EP=entrance potential, CE=collision energy, CXP=cell exit potential. All units are in V.

Table II.

Analytical Method Comparison Results Comparing ^{2}H-Labeled IS and ^{13}C-/^{15}N-Labeled IS

Method pairs (X vs. Y)	Slope (95% CI)	Intercept (95% CI)	Correlation coefficient (r^2)	Bland–Altman bias (95% CI) (%)
2MHA-[$^{2}H_7$] versus 2MHA-[$^{13}C_6$]	2.23 (2.12 to 2.33)	−17.7 (−37.9 to 2.61)	0.984	−59.2 (−66.0 to −52.3)
4MHA-[$^{2}H_7$] versus 4MHA-[$^{13}C_2$–^{15}N]	1.02 (1.00 to 1.04)	−8.15 (−21.9 to 5.64)	0.998	−0.642 (−1.37 to 0.087)

The BA bias between ISs is significant for 2MHA but not for 4MHA.

Table III.

Spiked Concentrations, Mean Resulting Concentrations and Mean Relative Error for Each IS at Each Spike Level, Averaged over 14 Spiked Urine Specimens

IS	Spike level	Spike concentration (ng/mL)	Mean resulting concentration (ng/mL)	Relative error (%)	Average relative error (%)
2MHA-[$^{2}H_{7}$]	1	47.0	29.4	−37.4	−38.4
	2	312	184	−41.0	
	3	2,740	1,730	−36.9	
2MHA-[$^{13}C_{6}$]	1	47.0	46.4	−1.3	−3.5
	2	312	288	−7.7	
	3	2,740	2,700	−1.5	
4MHA-[$^{2}H_{7}$]	1	45.0	46.6	3.6	−0.9
	2	295	283	−4.1	
	3	2,760	2,700	−2.2	
4MHA-[$^{13}C_{2}$-^{15}N]	1	45.0	45.6	1.3	−2.3
	2	295	275	−6.8	
	3	2,760	2,720	−1.5	