
Special Issue

Matrix Normalization Techniques for Definitive Urine Drug Testing

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Abstract

Analytical performance of stable isotope-labeled internal standardization (SIL-IS) and threshold accurate calibration (TAC) methods of matrix normalization are compared for quantitation of 51 drugs and metabolites (analytes) in urine with analysis by ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS-MS). Two SIL-IS methods of analysis were performed, one method using analyte-specific internal standardization (ASIL-IS) and another method using a shared stable isotope from another analyte for internal standardization (SSIL-IS). Variance in inter-specimen matrix effect, without the use of a matrix normalization method, was studied by UPLC-MS-MS analysis of 338 urine donor samples and showed >200% variation in ion response for some analytes. Matrix normalization methods were evaluated for precision, accuracy, calibration, multi-matrix recovery and positive casework quantitation. Acceptable calibration and quality control criteria were achieved for all methods when calibrators and controls were prepared from the same urine matrix pool. Quantitative accuracy, determined by the addition of analytes to multi-donor urine pools at two concentration levels, resulted in acceptable percent relative standard deviation (%RSD) and bias for TAC and ASIL-IS methods. SSIL-IS method quantitations in analyte-supplemented donor pools revealed a %RSD ranging from 20% to 60% for >30% of the analytes and a method bias that ranged up to 87%, with a differential matrix effect on analyte and shared internal standard accounting for the imprecision and bias. Analyte quantitation in 162 authentic case samples showed close agreement for TAC and ASIL-IS methods, with greater variance in the SSIL-IS method. The study demonstrates effective matrix normalization by ASIL-IS and TAC methods and a matrix-caused bias in the SSIL-IS method.

Introduction

Accurate quantification of drugs and their metabolites (analytes) is an analytical foundation of forensic and clinical toxicology, and for more than two decades, liquid chromatography interfaced with tandem mass spectrometry (LC-MS-MS) has been a key analytical technology used in quantitative toxicology casework. As continuing instrumental developments lead to faster MS data acquisition capabilities, targeted analysis of an ever-expanding panel of analytes is enabled. These large multi-analyte drug panels improve laboratory efficiency by combining what had been traditionally individual

drug assays or drug class-specific methods into a single analytical procedure.

It is well known, however, that LC-MS-MS analysis with electrospray ionization is susceptible to matrix effects caused by co-eluting endogenous and exogenous compounds that compete with the target analytes for ion charge in the MS ion source or that affect droplet formation/extraction into the MS or that cause ion pairing (1–4). If not addressed, differences in the MS response in the presence and absence of biological matrix can affect the accuracy and precision of an assay, leading to compromises in the robustness of the analytical method. While matrix effects cannot

be completely eliminated, with attention, they can be compensated for by normalization of the inter-sample variability in MS response.

Normalization of matrix effect is most often achieved by an isotope dilution technique. Stable isotopes have been used in isotope dilution studies since initial applications were reported in 1939 and 1940 (5, 6), and advances in isotope enrichment and molecular labeling have made stable isotope dilution the primary technique for quantitative analysis in the field of analytical toxicology, as well as many other areas of biological science (7). The use of analyte specific stable isotope-labelled internal standards (ASIL-IS) is considered to be the best strategy for stable isotope-labeled internal standardization (SIL-IS) because the co-eluting analyte and matched internal standard should, theoretically, experience the same competing matrix ions in the MS ion source and should interact similarly with co-eluting matrix ions because of shared molecular structures (8, 9). Likewise, they should share chromatographic behavior. Due to cost and availability of analyte-specific internal standards for the large multi-analyte panels quantified in the field of toxicology, the shared use of a stable isotope internal standard from another analyte (SSIL-IS) has increased in routine practice. While efforts are made to utilize shared internal standards from within a drug class or within a region of chromatographic retention, the practice can result in differential matrix exposure of the analyte and its shared internal standard. The effect of this practice on the accuracy of quantitation in forensic and clinical casework should be evaluated carefully during method validation as it may not be revealed in routine testing if calibrator and quality control material are prepared from the same urine matrix source.

An alternate approach to matrix normalization, called threshold accurate calibration or TAC, was reported in 2016 (4) by two of the authors of the current investigation. In the TAC method, a standard addition or fortification of analyte is prepared for a second analysis to establish a normalized ratio between the response of an initial neat-analyte analysis and the response of a second fortified-analyte analysis. The dual samples are prepared without extraction and directly analyzed after appropriate reagent additions, hydrolysis incubation and dilution. Like the use of an internal standard in the ASIL-IS method, the fortified analyte elutes from the analytical column and enters the MS source simultaneously with the neat analyte. Equivalent effects of matrix on neat and fortified analyte therefore normalize the inter-sample variability in ion suppression or enhancement, thus allowing accurate quantitation in each case sample. The TAC method of matrix normalization was initially reported for use with a single calibrator at a threshold concentration and was further validated and reported in a multi-calibration protocol (10). An adaptation of the TAC method was also reported for use in high-resolution MS analysis (11). Based on results of analyte identification and quantitation in 5,000 cases, the efficacy of the TAC method coupled with UPLC-MS-MS analysis has been established (12).

The objective of this study is to co-evaluate the analytical performance of ASIL-IS, SSIL-IS and TAC methods for urine drug testing and to compare quantitative results obtained by the three methods in case samples.

Materials and Methods

Materials

Chemicals included formic acid, phosphoric acid and ammonium hydroxide (Fisher Scientific, Fair Lawn, NJ). Glucuronide hydrol-

ysis was performed with genetically modified beta-glucuronidase (IMCSzyme catalog number 04-E1F-005, Irmo, SC; specific activity >50kU/mL). Solvent use included Optima LC-MS grade acetonitrile (Fisher) and LC-MS grade methanol (Burdick and Jackson, Muskegon, MI). Reference analytes, internal standards, gabapentin reference standard and hydrolysis control (morphine-3-glucuronide) were obtained from Cerilliant, Sigma-Aldrich (Round Rock, TX). Methapyrilene was from Sigma-Aldrich (St. Louis, MO). Analyte-negative urine was collected to produce urine pools from differing donor sources, and the analyte-negative status of each pool was verified by the TAC method of analysis, using limit of detection (LOD) criteria. A single analyte-negative pool was used for the preparation of all calibrators and controls for the methods, and the additional pools were used in the recovery studies.

Reagents, calibrators and controls

Calibrator and quality control material preparation was the same for TAC, ASIL-IS and SSIL-IS methods. Separately prepared stock multi-analyte reference solutions were used in the preparation of working calibrators, quality controls and fortification reagent. Multi-analyte calibrator and quality control reference solutions were prepared in methanol and stored at -10°C for use in the preparation of working calibrator and control material as well as in the preparation of a working fortification reference reagent for the TAC method. The multi-analyte stock solutions were prepared at analyte concentrations that were 10 times the upper limit of quantitation (ULOQ) concentrations as listed in Table I. A stock internal standard mixture for ASIL-IS and SSIL-IS testing was prepared at the ULOQ concentration in methanol and was stored at -10°C. Methapyrilene, which was used as an injection recovery control in the TAC method, was prepared in methanol with a stock concentration of 10 µg/mL and was stored at -10°C. For hydrolysis control in each of the methods, stock morphine-3-glucuronide was prepared at a concentration of 10,000 ng/mL and was also stored at -10°C. Working reagents were prepared on the day of analysis. Working calibrators and controls were prepared the same for ASIL-IS, TAC and SSIL-IS analysis, using analyte-negative urine pool supplemented at analyte concentrations listed in Table I. For the working hydrolysis control, a dilution of the stock hydrolysis control in analyte-negative urine was used to achieve free-morphine concentration equivalent to 400 ng/mL. The TAC fortification reagent was prepared at an analyte concentration of 60% ULOQ, and a working injection recovery reagent containing 400 ng/mL of methapyrilene was prepared in water with buffered hydrolysis reagent containing purified beta-glucuronidase for TAC analysis. The working internal standard for the ASIL-IS and SSIL-IS methods employed a 4-fold dilution of the stock internal standard solution in a buffered hydrolysis enzyme solution containing purified beta-glucuronidase. For the recovery studies, seven analyte-negative donor urine pools were supplemented with multi-analyte reference standard at two concentrations to produce two sets of seven recovery pools, one set with an analyte target concentration of 25% ULOQ and the second set with an analyte target concentration of 50% ULOQ.

TAC analysis method

The TAC method of matrix normalization and quantitation with UPLC-MS-MS analysis was performed as previously reported (10), with a modified application of a liquid handler for automated sample preparation, which was performed using a Hamilton MicroLab STARlet liquid handler (Hamilton Company, Reno, NV).

Table I. Analyte Limits of Detection and Quantification along with Calibrator and Working Quality Control Pool Concentrations

Analytes	Limits (ng/mL)				Quality control (ng/mL)			Calibration		Internal standards	
	LOD	LLQ	ULQ	Q75	Q125	Q750	LOD	Range (ng/mL)	TAC	ASIL-IS	SSIL-IS
Alprazolam	20	50	1000	75	125	750	20	40-1,000	Alprazolam	Oxazepam-d ₅	Oxazepam-d ₅
Alprazolam, alpha-OH	20	50	1000	75	125	750	20	40-1,000	Alprazolam, alpha-OH	Alprazolam, alpha-OH-d ₅	Methamphetamine-d ₅
Amphetamine	20	50	1000	75	125	750	20	40-1,000	Amphetamine	Amphetamine-d ₆	Cocaine-d ₃
Benzoyleggonine	10	25	500	37.5	62.5	375	10	20-500	Benzoyleggonine	Benzoyleggonine-d ₈	Methadone-d ₉
Buprenorphine	2	5	100	7.5	12.5	75	2	4-100	Buprenorphine	Buprenorphine-d ₄	Methadone-d ₉
Buprenorphine, nor	2	5	100	7.5	12.5	75	2	4-100	Buprenorphine, nor	Buprenorphine, nor-d ₃	Benzoyleggonine-d ₈
Carisoprodol	20	50	1000	75	125	750	20	40-1,000	Carisoprodol	Carisoprodol-13C ₃	Oxazepam-d ₅
Clonazepam	20	50	1000	75	125	750	20	40-1,000	Clonazepam	Clonazepam-d ₄	Oxazepam-d ₅
Clonazepam, 7-amino	20	50	1000	75	125	750	20	40-1,000	Clonazepam, 7-amino	Clonazepam, 7-amino-d ₄	Benzoyleggonine-d ₈
Cocaine	10	25	500	37.5	62.5	375	10	20-500	Cocaine	Cocaine-d ₃	Oxazepam-d ₅
Diazepam	20	50	1000	75	125	750	20	40-1,000	Diazepam	Diazepam-d ₅	Diazepam, nor-d ₃
Diazepam, nor	20	50	1000	75	125	750	20	40-1,000	Diazepam, nor	Dihydrocodeine-d ₆	Dihydrocodeine-d ₆
Dihydrocodeine	20	50	1000	75	125	750	20	40-1,000	Dihydrocodeine	EDDP-d ₃	Methadone-d ₉
EDDP	20	50	1000	75	125	750	20	40-1,000	EDDP	Fentanyl-d ₅	Methadone-d ₉
Fentanyl	1	2.5	50	3.75	6.25	37.5	1	2-50	Fentanyl	Fentanyl, nor-d ₃	Oxazepam-d ₅
Fentanyl, nor	1	2.5	50	3.75	6.25	37.5	1	2-50	Fentanyl, nor	Flurazepam, 2-OH ethyl-d ₄	Dihydrocodeine-d ₆
Flurazepam, 2-OH ethyl	20	50	1000	75	125	750	20	40-1,000	Flurazepam, 2-OH ethyl	Hydrocodone-d ₆	Dihydrocodeine-d ₆
Hydrocodone	20	50	1000	75	125	750	20	40-1,000	Hydrocodone	Hydrocodone, nor-d ₃	Dihydrocodeine-d ₆
Hydrocodone, nor	20	50	1000	75	125	750	20	40-1,000	Hydrocodone, nor	Hydromorphone-d ₃	Dihydrocodeine-d ₆
Hydromorphone	20	50	1000	75	125	750	20	40-1,000	Hydromorphone	Ketamine-d ₄	Benzoyleggonine-d ₈
Ketamine	2	5	100	7.5	12.5	75	2	4-100	Ketamine	Ketamine, nor-d ₄	Benzoyleggonine-d ₈
Ketamine, nor	2	5	100	7.5	12.5	75	2	4-100	Ketamine, nor	Lorazepam-d ₄	Oxazepam-d ₅
Lorazepam	20	50	1000	75	125	750	20	40-1,000	Lorazepam	MDA-d ₃	Methamphetamine-d ₈
MDA	20	50	1000	75	125	750	20	40-1,000	MDA	MDEA-d ₅	Methamphetamine-d ₈
MDEA	20	50	1000	75	125	750	20	40-1,000	MDEA	MDMA-d ₅	Methamphetamine-d ₈
MDMA	20	50	1000	75	125	750	20	40-1,000	MDMA	Meperidine-d ₄	Methadone-d ₉
Meperidine	20	50	1000	75	125	750	20	40-1,000	Meperidine	Meperidine, nor-d ₄	Oxazepam-d ₅
Meperidine, nor	20	50	1000	75	125	750	20	40-1,000	Meperidine, nor	Methadone-d ₉	EDDP-d ₃
Methadone	20	50	1000	75	125	750	20	40-1,000	Methadone	Methamphetamine	Amphetamine-d ₆
Methamphetamine	20	50	1000	75	125	750	20	40-1,000	Methamphetamine	Methphenidate-d ₉	Methamphetamine-d ₈
Methylphenidate	20	50	1000	75	125	750	20	40-1,000	Methylphenidate	Midazolam-d ₄	Oxazepam-d ₅
Midazolam	20	50	1000	75	125	750	20	40-1,000	Midazolam	Morphine-d ₆	Dihydrocodeine-d ₆
Morphine	20	50	1000	75	12.5	75	2	4-100	Morphine	Morphine, 6-acetyl-d ₆	Dihydrocodeine-d ₆
Morphine, 6-acetyl	2	5	100	7.5	12.5	75	2	4-100	Morphine, 6-acetyl	Naloxone-d ₅	Dihydrocodeine-d ₆
Naloxone	20	50	1000	37.5	62.5	750	20	20-500	Naloxone		

The internal standards used in the TAC, ASIL-IS and SSIL-IS methods are also listed.

(Continued)

Table I. Listing of Analyte and Internal Standard Transition Ion Acquisition Masses along Optimized Cone Voltage and Collision Energy Conditions for TAC, ASIL-IS and SSIL-IS Methods

Analytes	Limits (ng/mL)				Quality control (ng/mL)				Calibration		Internal standards
	LOD	LLOQ	ULOQ	Q75	Q125	Q750	LOD	Range (ng/mL)	TAC	ASIL-IS	
Oxazepam	20	50	1000	75	125	750	20	40-1,000	Oxazepam	Oxazepam-d ₅	
Oxycodone	20	50	1000	75	125	750	20	40-1,000	Oxycodone	Oxycodone-d ₆	Dihydrocodeine-d ₆
Oxycodone, nor	20	50	1000	75	125	750	20	40-1,000	Oxycodone, nor	Oxycodone, nor-d ₃	Dihydrocodeine-d ₆
Oxymorphone	20	50	1000	75	125	750	20	40-1,000	Oxymorphone	Oxymorphone-d ₃	Dihydrocodeine-d ₆
Phencyclidine	2	5	100	7.5	12.5	75	2	4-100	Phencyclidine	Benzoyllegomine-d ₈	Benzoyllegomine-d ₈
Phentermine	20	50	1000	75	125	750	20	40-1,000	Phentermine	PVP, alpha-d ₈	Benzoyllegomine-d ₈
PVP, alpha	2	5	100	7.5	12.5	75	2	4-100	PVP, alpha	Ritalinic acid-d ₁₀	Methamphetamine-d ₈
Ritalinic acid	20	50	1000	75	125	750	20	40-1,000	Ritalinic acid	Tapentadol	Methadone-d ₉
Tapentadol	20	50	1000	75	125	750	20	40-1,000	Tapentadol	Temazepam	Oxazepam-d ₅
Temazepam	20	50	1000	75	125	750	20	40-1,000	Temazepam	Tramadol-13 C-d ₃	Methadone-d ₉
Tramadol	20	50	1000	75	125	750	20	40-1,000	Tramadol	Tramadol, N-desmethyl	Methadone-d ₉
Tramadol, N-desmethyl	20	50	1000	75	125	750	20	40-1,000	Tramadol	Trazadone-d ₆	Oxazepam-d ₅
Trazadone	20	50	1000	75	125	750	20	40-1,000	Trazadone	Triazolam-d ₄	Oxazepam-d ₅
Triazolam	20	50	1000	75	125	750	20	40-1,000	Triazolam, alpha-OH	Triazolam, alpha-OH-d ₄	Oxazepam-d ₅
Triazolam, alpha-OH	20	50	1000	75	125	750	20	40-1,000	Zolpidem	Zolpidem-d ₆	Benzoyllegomine-d ₈

The internal standards used in the TAC, ASIL-IS and SSIL-IS methods are also listed.

Robotically, two 20- μ L aliquots of calibrators, quality controls, a hydrolysis control and case samples were added to paired analysis wells in a 96-well plate for neat and fortified analysis. Quantitation and detection limits along with calibrator and control concentrations for the methods are shown in Table I. A fortification reagent (20 μ L) was added to the fortification wells and 20 μ L of water to the neat wells, followed by 20- μ L additions to all wells of buffered-hydrolysis reagent (2 μ L beta-glucuronidase stock per well) containing 400 ng/mL of methapyrilene. After rotational mixing, the prepared plates were incubated for 1 h at 55°C on the liquid handler platform. Post-hydrolysis, the neat and fortified samples were diluted with 200 μ L of 2% (v/v) methanol in water containing 0.1% (v/v) formic acid for a final 13-fold dilution of the original urine specimen. A fortification blank without urine matrix was robotically prepared with each plate to contain a water sample along with hydrolysis reagent, fortification reagent and diluent. UPLC-MS-MS analysis was performed using a Waters ACQUITY UPLC I-Class (FTN) system in combination with a Xevo® TQD tandem mass spectrometer (Waters Corporation, Milford, MA) using conditions previously reported (10). Essentials of the analysis include use of a BEH Phenyl column (1.7 μ m, 2.1 \times 50 mm, Waters) maintained at 45°C. The flow rate was 0.6 mL/min with chromatographic separation in a gradient elution program as follows: 2% B (0–0.5 min), 2–70% B (0.5–2.2 min), 70–90% B (2.2–2.7 min) and 90–2% B (2.7–3.0 min), with a final 0.3-min hold. Mobile phase solvent A contained 2 mM ammonium formate in water with 0.1% formic acid and solvent B contained 2 mM ammonium formate in methanol with 0.1% formic acid. The mass spectrometer was operated in electrospray-positive ionization mode using the following MS-MS conditions: capillary voltage (0.55 kV), RF 2.5 V, source temperature (150°C), desolvation gas (nitrogen) delivered at a flow rate of 800 L/h and at a temperature of 550°C, collision gas (argon) maintained at a pressure of 0.5 Pa. MS acquisition parameters for analytes and transition ions were optimized for cone voltage and collision energy, respectively, as shown in Table II. Ion acquisition data were processed using TargetLynx™ with MassLynx control of instrument conditions and data acquisition (Waters). Additional data handling for TAC analysis was achieved by exporting the TargetLynx data into Microsoft Excel for calculation of a matrix normalized TAC response ratio [neat peak ion area/(fortified ion area – neat ion area)] and for evaluation of calibration, quality control, hydrolysis control and case sample criteria including acceptable transition ion ratio and quality control quantitation, along with retention time within 0.2 min of average reference standard retention time and injection recovery for neat and fortified analysis within 20%.

SIL-IS methods

The ASIL-IS method of matrix normalization with UPLC-MS-MS analysis was performed as reported by Danaceau and coworkers (13), with modified acquisition conditions and application of the Hamilton Starlet liquid handler for automated sample preparation. Robotically, 100 μ L of calibrators, controls and hydrolysis control and case samples were added to individual wells of an Oasis MCX μ Elution plate (Waters), followed by multi-aspiration addition of 100 μ L of buffered hydrolysis reagent (10 μ L glucuronidase stock per well) containing the internal standards listed for the ASIL-IS method in Table I with internal standard at 25% ULOQ concentration listed for the analyte. After addition, the plate was mixed using a flatbed microplate mixer prior to incubation for 1 h at 55°C in an oven. After incubation, 200 μ L of 4% phosphoric

acid was added to all wells and mixed by aspiration. The mixture was drawn through the column sorbent bed by vacuum and then the wells were washed with 200 μ L of 20% (v/v) methanol in water. After vacuum drying of the sorbent, analytes were eluted with two 25- μ L aliquots of acetonitrile:methanol (1:1) containing 5% of a strong ammonia solution (28–30%). The combined eluent was diluted with 150 μ L of 2% acetonitrile in water containing 1% formic acid. UPLC-MS-MS analysis was then performed using a Waters ACQUITY UPLC I-Class (FTN) system in combination with a Xevo® TQD tandem mass spectrometer (Waters) with the chromatographic conditions previously reported (13). Essentials of the UPLC-MS-MS analysis include use of an ACQUITY UPLC BEH C₁₈ column (1.7 μ m, 2.1 \times 100 mm, Waters) maintained at 40°C. The flow rate was 0.6 mL/min, with chromatographic separation in a gradient elution program as follows: 2% B (0–0.6 min), 2–67% B (0.6–3.33 min), 67–90% B (3.33–3.5 min) and 90–2% B (3.5–3.6 min), with a final 0.4-min hold. Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. The mass spectrometer was operated in electrospray-positive ionization mode using multiple reaction monitoring for the detection of analytes and internal standards as listed in Table II. Ion acquisition data were integrated with calibration and concentration determined by use of Target Lynx™ software with MassLynx control of instrument conditions and acquisitions (Waters). Retention time limit range and transition ion ratio criteria were the same as used in the TAC method.

The SSIL-IS method was performed by reprocessing the Target-Lynx data for the ASIL-IS methods runs using shared, rather than analyte-specific, internal standards as shown in Table II. The calibration and quality control criteria were the same as used in the ASIL-IS and TAC methods.

Matrix effect study

The TAC method of testing involves preparation of a 51-analyte fortification well (60% ULOQ concentration) for each case sample analyzed. Matrix effect can be calculated in routine casework by comparing fortification analysis in case samples without neat drug response to that of a fortification blank using a water sample in place of urine. This allows an expanded look at the effect of matrix across many authentic case samples. Thus, in the present study, any case samples found initially to have no analyte response can be reserved for an expanded look at the variability on matrix effect by comparing the response for the fortified urine sample (A) with that of a fortified water sample (B), which is included on each analytical run, using the formula [(A/B) – 1] \times 100%. In this study, matrix effect was statistically evaluated in 338 urine samples from routine casework to determine the variability in matrix effect between case samples.

Comparison of TAC, ASIL-IS and SSIL-IS methods

Calibration performance was assessed by regression re-analysis of calibrators (\pm 20% criteria) and analysis of the regression line fit using coefficient of determination (r^2) with a >0.99 acceptance criteria. Inter-assay precision and bias were assessed over 10 analytical runs with a <15% acceptance criteria for precision and \pm 15% for bias. Bias and precision in the recovery studies with multi-donor urine pools was determined with acceptance criteria of \pm 20%. Additional assessments included carryover following high calibrator (criteria: <50% of LOD concentration, hydrolysis control (criteria: \pm 20% of target concentration), LOD control criteria for all methods, negative control (criteria:< 50% of LOD concentration) and

Table II. Listing of Analyte and Internal Standard Transition Ion Acquisition Masses along Optimized Cone Voltage and Collision Energy Conditions for TAC, ASIL-IS and SSIL-IS Methods

Analyte	Molecular ion <i>m/z</i>	Cone voltage, V	Target ion <i>m/z</i>	Target ion collision energy, eV	Qualifier ion <i>m/z</i>	Qualifier ion collision energy, eV
Alprazolam	309	50	205	40	281	26
Alprazolam-d ₅	314	50	210	40		
Alprazolam, alpha-OH	325	51	297	23	243	30
Alprazolam, alpha-OH-d ₅	330	51	302	25		
Amphetamine	136	5	91	8	119	8
Amphetamine-d ₆	142	5	93	28		
Benzoyllecgonine	290	39	168	19	105	30
Benzoyllecgonine-d ₈	298	39	171	19		
Buprenorphine	468	60	55	70	84	45
Buprenorphine-d ₄	472	60	400	40		
Buprenorphine, nor	414	70	101	40	83	50
Buprenorphine, nor-d ₃	417	70	101			
Carisoprodol	261	18	176	9	158	8
Carisoprodol-13C3	264	18	179	9		
Clonazepam	316	50	270	25	241	35
Clonazepam-d ₄	320	50	274	25		
Clonazepam, 7-amino	286	32	121	28	222	22
Clonazepam, 7-amino-d ₄	290	32	221	28		
Cocaine	304	40	182	14	82	29
Cocaine-d ₃	307	40	185	14		
Diazepam	285	55	154	29	193	29
Diazepam-d ₅	290	55	154	29		
Diazepam, nor	271	55	140	29	165	27
Diazepam, nor-d ₅	276	50	140	29		
Dihydrocodeine	302	50	199	32	128	60
Dihydrocodeine-d ₆	308	25	128	60		
EDDP	278	20	249	23	234	30
EDDP-d ₃	281	20	234	30		
Fentanyl	337	18	188	22	105	38
Fentanyl-d ₅	342	25	188	22		
Fentanyl, nor	233	38	84	15	177	15
Fentanyl, nor-d ₅	238	38	84	15		
Flurazepam, 2-OH ethyl	333	45	109	28	211	30
Flurazepam, 2-OH ethyl-d ₄	337	45	113	28		
Hydrocodone	300	60	199	35	171	36
Hydrocodone-d ₆	306	60	202	35		
Hydrocodone, nor	286	25	199	25	128	50
Hydrocodone, nor-d ₃	289	25	220	25		
Hydromorphone	286	60	185	30	157	40
Hydromorphone-d ₃	289	60	185	30		
Ketamine	238	35	125	25	179	15
Ketamine-d ₄	242	35	129	25		
Ketamine, nor	224	25	125	25	179	15
Ketamine, nor-d ₄	228	25	129	25		
Lorazepam	323	50	277	41	229	30
Lorazepam-d ₄	327	50	281	20		
MDA	180	20	163	15	105	20
MDA-d ₅	185	20	168	15		
MDEA	208	30	163	13	105	30
MDEA-d ₅	213	30	163	13		
MDMA	194	27	105	25	163	13
MDMA-d ₅	199	27	165	13		
Meperidine	248	15	174	18	220	22
Meperidine-d ₄	525	15	178	18		
Meperidine, nor	234	45	160	15	131	28
Meperidine, nor-d ₄	238	45	164	15		
Methadone	310	10	265	12	105	18
Methadone-d ₉	319	15	286	15		
Methamphetamine	150	27	119	11	91	17
Methamphetamine-d ₈	158	27	93	17		

Internal standard acquisitions were not performed in the TAC method.

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(Continued)

Table II. Listing of Analyte and Internal Standard Transition Ion Acquisition Masses along Optimized Cone Voltage and Collision Energy Conditions for TAC, ASIL-IS and SSIL-IS Methods

Analyte	Molecular ion <i>m/z</i>	Cone voltage, V	Target ion <i>m/z</i>	Target ion collision energy, eV	Qualifier ion <i>m/z</i>	Qualifier ion collision energy, eV
Methylphenidate	234	30	84	40	91	40
Methylphenidate-d ₉	243	30	93	18		
Midazolam	326	55	291	26	223	36
Midazolam-d ₄	330	55	295	26		
Morphine	286	50	201	25	165	40
Morphine-d ₆	292	25	153	40		
Morphine, 6-acetyl	328	45	165	35	211	30
Morphine, 6-acetyl-d ₆	334	45	165	45		
Naloxone	328	48	253	25	212	38
Naloxone-d ₅	333	48	258	25		
Oxazepam	289	50	243	20	104	30
Oxazepam-d ₅	292	50	246	20		
Oxycodone	316	40	241	20	256	25
Oxycodone-d ₆	322	40	304	15		
Oxycodone, nor	302	40	227	28	187	25
Oxycodone, nor-d ₃	305	40	190	25		
Oxymorphone	302	43	227	31	242	25
Oxymorphone-d ₃	305	43	230	31		
PCP	244	19	86	11	159	12
PCP-d ₅	249	19	86	11		
Phentermine	150	18	133	10	91	18
Phentermine-d ₅	155	18	138	10		
PVP, alpha	232	45	91	20	126	25
PVP, alpha-d ₈	240	45	91	20		
Ritalinic acid	220	25	84	30	56	38
Ritalinic acid-d ₁₀	230	28	93	20		
Tapentadol	222	40	121	20	107	25
Tapentadol-d ₃	225	40	221	20		
Temazepam	301	30	255	22	177	40
Temazepam-d ₅	306	50	260	22		
Tramadol	264	10	58	21		
Tramadol-13C-d ₃	268	10	58	21		
Tramadol, N-desmethyl	250	30	44	15	232	7
Tramadol, N-desmethyl-d ₃	253	30	47	15		
Trazadone	372	28	176	25	148	35
Trazadone-d ₆	378	28	150	35		
Triazolam	343	56	308	26	239	44
Triazolam-d ₄	347	56	243	44	312	26
Triazolam, alpha-OH	359	50	176	27	141	38
Triazolam, alpha-OH-d ₄	363	50	176	27		
Zolpidem	308	20	235	35	92	48
Zolpidem-d ₆	314	20	235	20		

Internal standard acquisitions were not performed in the TAC method.

absence of negative urine matrix interference with internal standards. In addition, comparison of quantitation was performed with 67 de-identified case samples in an exempt study protocol reviewed by the Institutional Review Board of the Albany Medical College.

Results and Discussion

Urine matrix effect without normalization

The variation in percent matrix effect, determined by direct analysis of 338 donor urine samples diluted 13-fold according to the TAC fortification procedure, is displayed in Figure 1. The mean percent matrix effect along with 95% confidence limits ($\pm 2SD$) are shown in the open bar graph along with the 2SD range (dashed error bars), while the gray bar graph shows the median matrix effect with the

solid error bars representing the full data range. The analytes on the x-axis are aligned from left to right in increasing order of retention time during chromatographic separation to evaluate trends in matrix effect associated with chromatographic separation. Early-eluting analytes up to phentermine with a retention time of 1.4 min have mean and median ion suppression in the range of 50%. Later-eluting analytes, with a few exceptions, demonstrate less ion suppression based on mean and median data, with a few late-eluting analytes showing mean and median ion enhancement. Matrix effects in this study are substantial and are consistent with the findings of prior TAC method evaluations (4, 10).

Matrix effects have also been reported for the ASIL-IS method (13) that employs a solid-phase sample clean-up, which is a common practice intended to reduce matrix effect. Solid-phase extraction may

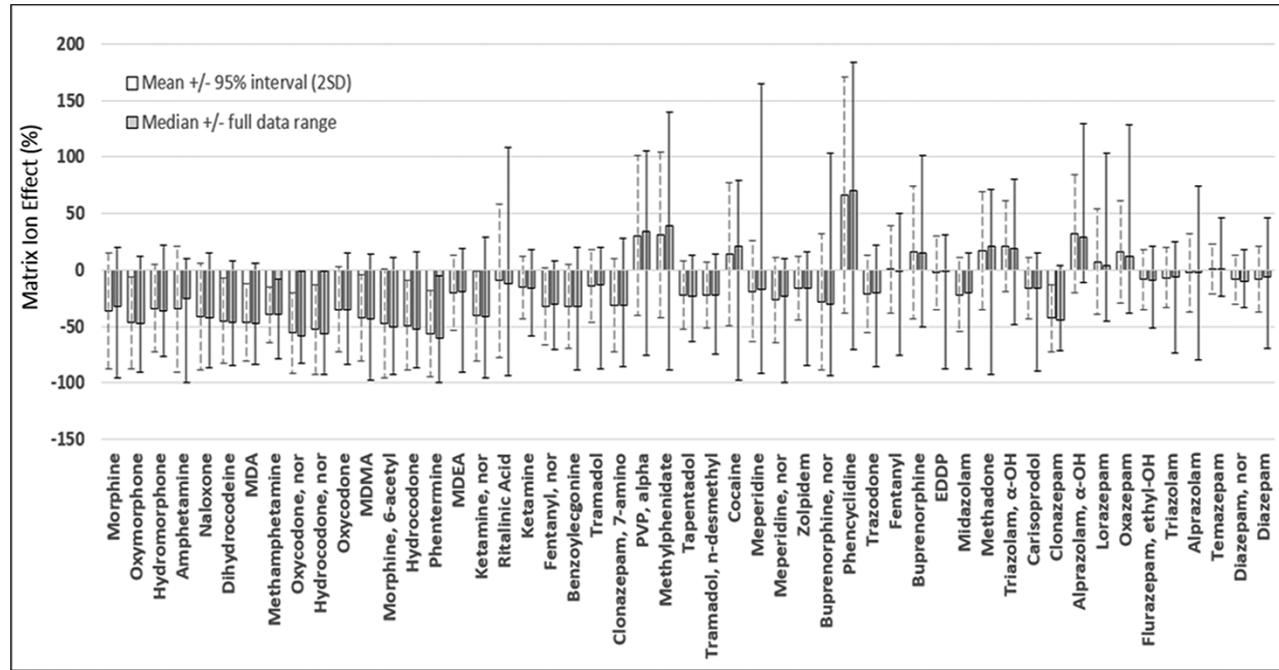


Figure 1. Percent matrix effect determined in 338 donor urine specimens. The clear bars represent mean effect with 95% (2SD) confidence limits (dashed line), and gray bars present median percent matrix effect with full range error bars (solid line). Positive percent matrix effects indicate ion enhancement and negative percent represents ion suppression relative to the analysis of a blank sample without urine matrix.

not, however, be fully effective in removing the co-eluting matrix because the technique typically removes components with differing, rather than similar, polarity and hydrophobicity properties; thus, the analyte along with interfering compounds that have similar functional groups may be more concentrated after the extraction. Ion suppression has been reported to occur even with extensive clean-up strategies (14, 15), and protocols incorporating a sample concentration step have demonstrated a magnified matrix effect (16). This may explain why significant matrix effect occurs with both, direct analysis (TAC approach) which involves a 13-fold dilution of the urine, as well as in the solid-phase extraction methods (ASIL-IS and SSIL-IS) which employ a 2-fold dilution of urine.

The full range of matrix effects as shown in Figure 1 may not be fully revealed in standard method validation protocols, which typically evaluate biological matrices from 10 or less donors and often do not take into consideration significant differences in urine dilution due to the variable hydration status of donors. In this study, we tested 338 donor urine specimens and provide a statistical summary of matrix effect for >17,000 analyte measurements. Assessment of variance by Gaussian or full-range analysis confirms ion suppression in a subset of donor specimens for all analytes as well as significant ion enhancement in a subset of the later-eluting analytes. The intra-analyte difference in matrix effect between donor specimens averaged 90% and 156%, using the 95% confidence limits and full ranges limits, respectively. Differences of over 200% in the ion response between urine samples were found for several of the analytes (ritalinic acid, phencyclidine and alpha-hydroxytriazolam), and the findings suggest caution in the use non-normalized ion response data for either quantitation or threshold-calibrated drug identification.

Calibrator and quality control performance for matrix normalization methods

Initial evaluation of the matrix normalization methods focused on the performance of calibrators and controls which were prepared in a single urine matrix, consistent with routine practice in many laboratories. A comparison of inter-assay precision for SIL-IS, TAC and SSIL-IS methods has been performed (see data in Supplemental Figure S1). The low concentration controls were within criteria for all three methods, with the lowest concentration control showing %RSD and range for ASIL-IS (5.5% mean; 2.7–11.8% range), TAC (7.1%; 4.4–12.6%) and SSIL-IS (5.5%; 1.2–12.2%). All methods had %RSDs <13% in the low control ranges. The high concentration control also showed within-criteria %RSD and ranges for ASIL-IS (4.7% mean; 2.2–12.6% range), TAC (8.4%; 5.0–12.2%) and SSIL-IS (4.3%; 2.0–10.2%) methods. Overall, precision was therefore acceptable across the methods.

Results for inter-assay control bias are shown in Supplemental Figure S2. Low controls were within criteria, with the lowest concentration control showing a percent bias mean and range for ASIL-IS (4.6% mean; 0.1 to 8.8% range), TAC (-2.6%; -6.8 to 3.8%) and SSIL-IS (3.3%; -1.8 to 13.9%) and with bias for all three methods within the $\pm 15\%$ bias criteria. The 12.5% LOQ control performed similarly except for n-desmethyl tramadol where the bias exceeds criteria (17.7%) in the SSIL-IS method. High control bias for ASIL-IS (2.9% mean; -0.9 to 10.1% range), TAC (-1.2%; -8.1 to 1.6%) and SSIL-IS (1.4%; -6.8 to 6.8%) was within criteria for all methods.

Additional quality control performance criteria were also met for all methods, including carryover, glucuronide hydrolysis, LOD and negative control limits. Analysis of the calibration data also showed

acceptable performance for the three methods, based on the coefficient of determination limits ($r^2 > 0.99$) over 10 analytical runs as shown in Supplemental Figure S3. Calibrator concentration based on the regression equation also met the $\pm 20\%$ of target criteria for all methods.

Quantitation accuracy of matrix normalization methods

The evaluation of quality control and calibration performance in a single matrix as reported above would indicate acceptable routine assay performance for each of the matrix normalization methods.

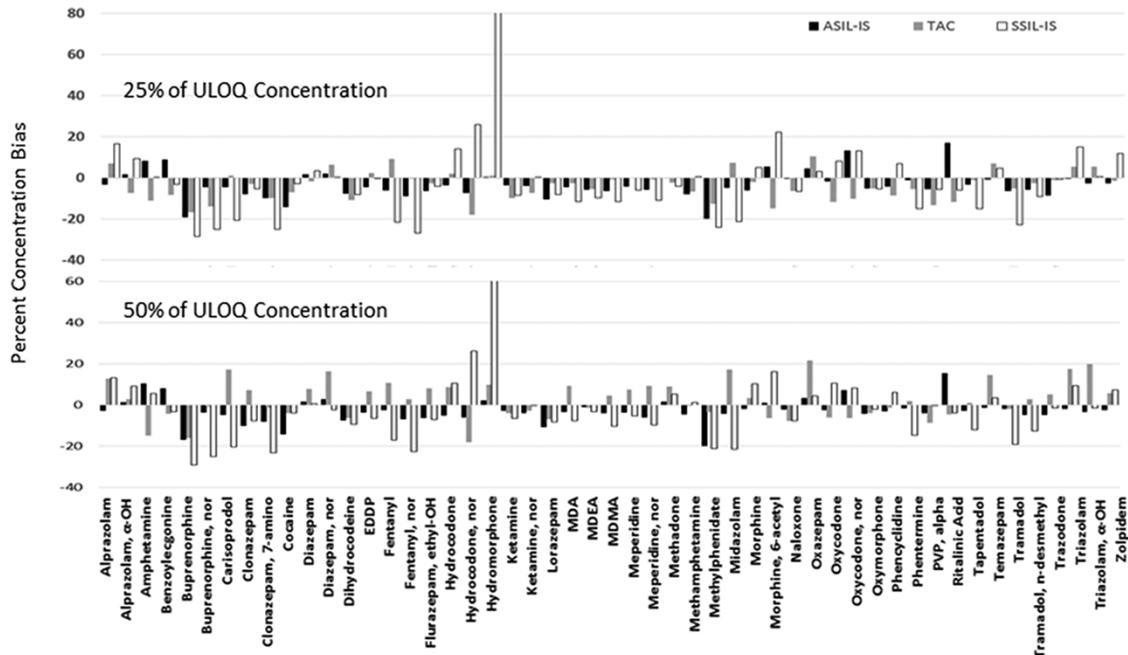


Figure 2. Evaluation of analyte concentration bias in seven urine pools containing analyte at 25% (upper panel) and 50% (lower panel) of ULOQ concentration determined by ASIL-IS, TAC and SSIL-IS methods.

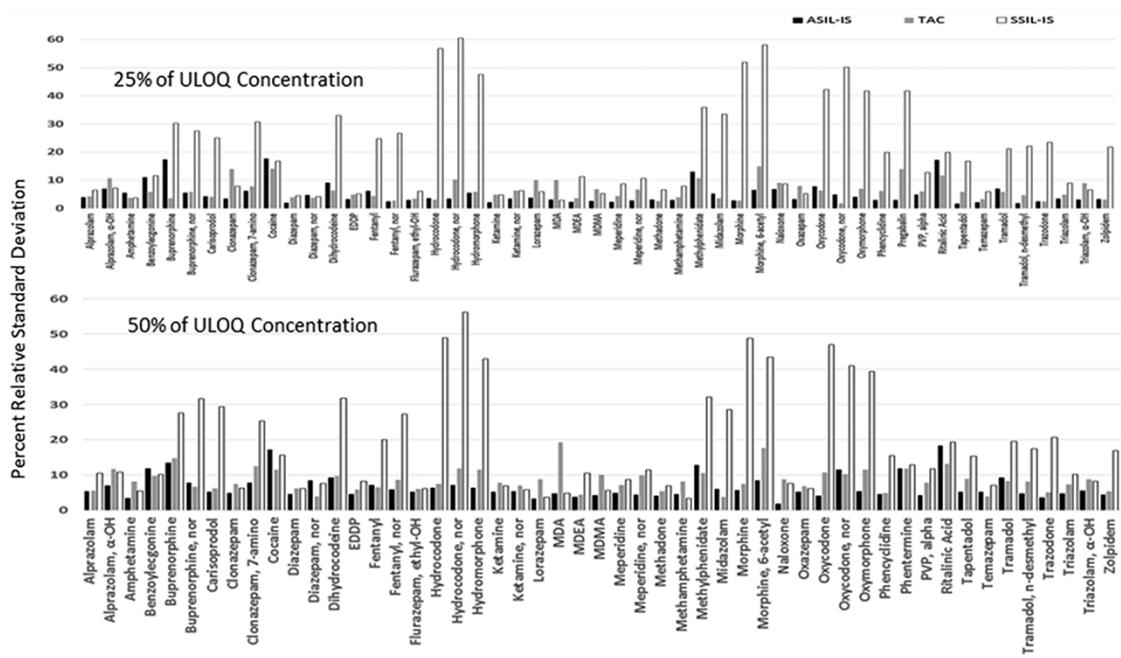


Figure 3. Percent relative standard deviation (%RSD) for analyte concentrations in seven urine pools containing analytes at 25% (upper panel) and 50% (lower panel) of ULOQ concentration as determined by ASIL-IS, TAC and SSIL-IS methods.

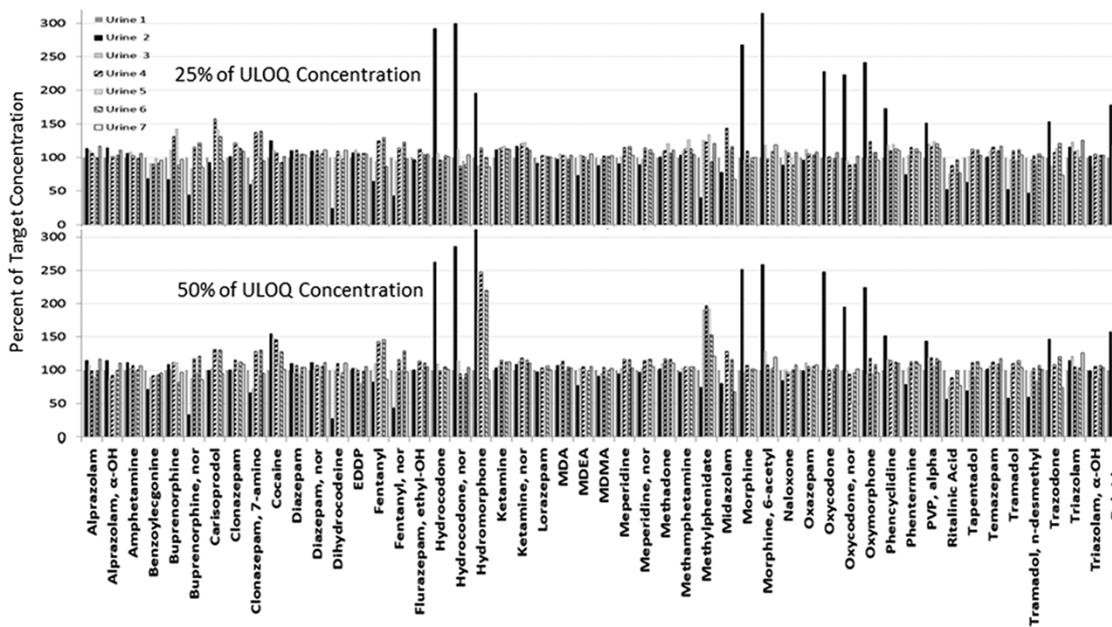


Figure 4. Percent of target concentration for analytes in seven donor urine pools containing reference analytes at 25% (upper panel) and 50% (lower panel) of ULOQ concentration. Determination was performed by the SSIL-IS method using a shared internal standard for each analyte.

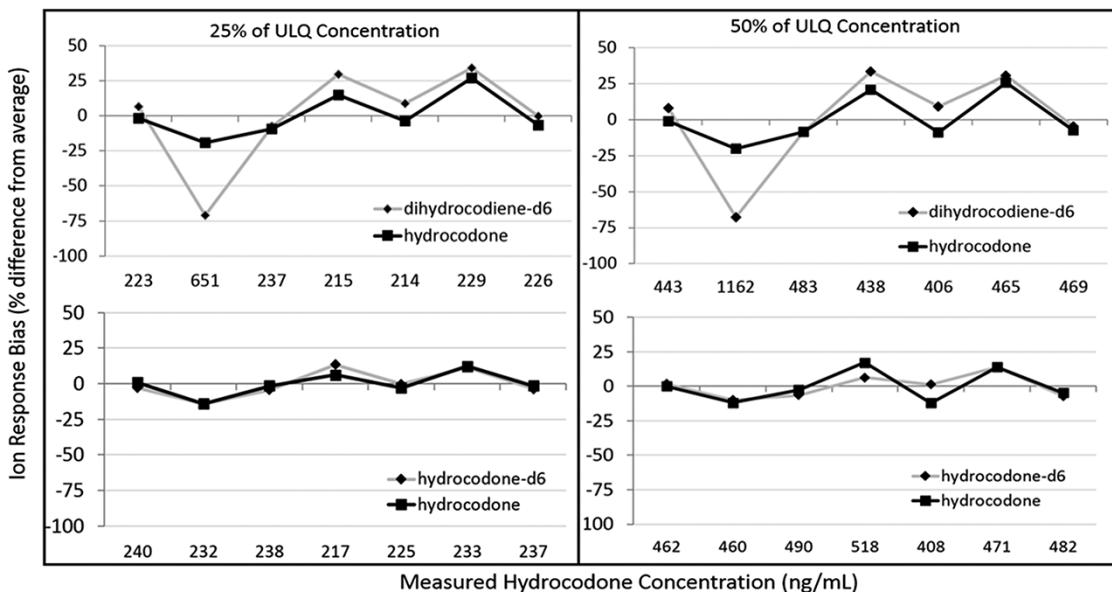


Figure 5. Ion response bias (percent difference from the average ion response) in seven recovery pools plotted for the analyte and its internal standard with the x-axis shows the concentration of analyte measured in the analysis. Upper panels show results for analyzed by SSIL-IS and lower panels by ASIL-IS. The study was performed at fortification concentrations of 250 ng/mL (left panels) and 500 ng/mL (right panels).

These routine assay criteria, however, do not provide an assessment of inter-sample matrix effects that may influence quantitative performance in actual casework analysis. Quantitative accuracy in urine from multiple donors ($n=7$) with analyte-negative urine by supplementing each donor urine with low (25% of ULOQ) and high (50% of ULOQ) analyte concentrations prior to analysis by the methods. A graphic comparison of percent concentration bias at the two concentration levels is shown in Figure 2 for the three methods of matrix normalization. Analyte concentration bias averaged 6.8% and 8.7% for analysis by the ASIL-IS

and TAC methods, respectively, with bias less than $\pm 20\%$ for both methods across all analytes at low and high concentrations. The SSIL-IS method study revealed a significantly higher range of bias. SSIL-IS concentration bias at 25% of ULOQ concentration ranged from -28% to 84% and at 50% of ULOQ ranged from -29% to 87% .

The variance observed in these multi-pool accuracy studies was also evaluated. As shown in Figure 3, the %RSD of analyte concentration in the analytical results for the seven donor pools was within the $\pm 20\%$ criteria for both the ASIL-IS and TAC methods,

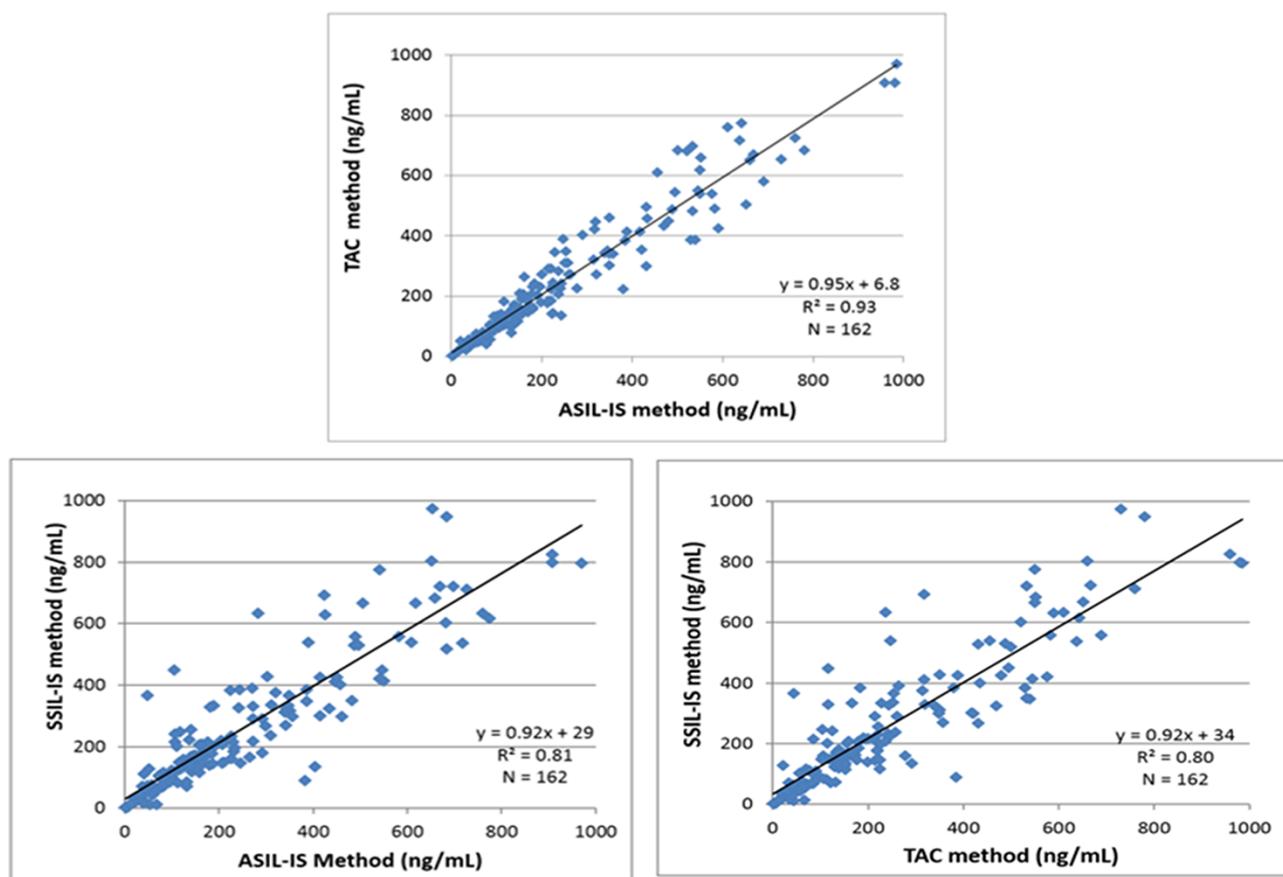


Figure 6. Linear regression analysis of quantitative findings for 67 de-identified urine samples obtained by ASIL-IS, TAC and SSIL-IS methods. The data for 162 comparative results are plotted. The upper graph compared TAC to ASIL-IS results, lower-left graph compares ASIL-IS to SSIL-IS results and lower-right graph compares SSIL-IS to TAC results.

but a pattern of increased variance was observed in SSIL-IS analysis. Many of the opiates (hydrocodone, norhydrocodone, hydromorphone, morphine, 6-acetylmorphine, oxycodone, noroxycodone and oxymorphone) showed %RSD of 40–60%, and the pattern of imprecision was similar in both the low and high concentration pools. Other analytes also had %RSD >20% and showed similar patterns of recovery imprecision at low and high pool concentrations.

Inter-matrix quantitation discrepancies in SSIL-IS analysis are shown more clearly for the individual donor pools in Figure 4. Donor 2 urine demonstrated a high concentration bias across the opiate analytes and was a major contributor to the imprecision and quantitative inaccuracy for these analytes. Inaccurately high results were found consistently at low and high analyte fortification concentrations. Additionally, a pattern of low concentration bias was found for other analytes, including dihydrocodeine, also in the analysis of Donor 2 urine.

Inaccurate quantitation of a series of opiates in the Donor 2 pool can be explained by differential ion suppression of the analytes and their shared internal standard, dihydrocodeine-d₆. The upper panels of Figure 5 show the relative ion response of hydrocodone and its shared internal standard, dihydrocodeine-d₆ in the seven recovery pools, with SSIL-IS measurements at low (left) and high (right) concentrations. The upper panels show a discordant matrix effect between the analyte and internal standard in Donor 2 urine analysis, with a resultant inaccuracy in quantitation as displayed on

the x-axis. Ion suppression effects observed for the shared internal standard, dihydrocodeine-d₆ with a chromatographic retention time of 1.10 min, are not observed for the analyte, hydrocodone, with a retention time of 1.23 min. An inaccurately high hydrocodone concentration was measurable for Donor 2 urine at both low and high analyte fortification levels. Use of an analyte-matched internal standard in the ASIL-IS (lower-left and lower-right panels) shows a normalized response, resulting in an accurate quantitation of hydrocodone concentration for all donor urine pools. Similar SSIL-IS method inaccuracy in Donor 2 urine at the low and high recovery fortifications was found for norhydrocodone, hydromorphone, morphine, 6-acetylmorphine, oxycodone, noroxycodone and oxymorphone, which all shared dihydrocodeine-d₆ as internal standard. Use of analyte-specific internal standard in the ASIL-IS method averted this bias for each of these analytes.

Inaccurately low dihydrocodeine concentrations in Donor 2 urine, as shown in Figure 4, are also logically explained when using hydrocodone-d₆ as a shared internal standard. Dihydrocodeine is ion suppressed in the matrix of Donor 2 urine while hydrocodone-d₆ is not, thus causing discordance and the resultant quantitative inaccuracy for dihydrocodeine. Further testing of Donor 2 urine by other laboratory methods with an expanded analyte panel that included the gabapentinoids identified gabapentin at a concentration exceeding 100 $\mu\text{g/mL}$ in Donor 2 urine. The retention time of gabapentin in the SSIL-IS and ASIL-IS methods was found to

be 1.14 min, compared with dihydrocodeine and dihydrocodeine-d₆ retention times of 1.10 min. It is likely that competition for ionization in the ion source between co-eluting gabapentin and either dihydrocodeine or its stable isotope dihydrocodeine-d₆ resulted in the observed increase in ion suppression of this analyte and internal standard. Quantitation inconsistencies were not observed by the ASIL-IS method, where co-elution of the analytes and their analyte-specific internal standard allow similar ion matrix effects on both the analyte and analyte-specific internal standard. In Donor 2 urine, the high concentration of gabapentin resulted in a similar ion suppression of coeluting dihydrocodeine and dihydrocodeine-d₆, therefore allowing accurate quantitation by the ASIL-IS method in the presence of significant matrix suppression.

Quantitation comparison of matrix normalization methods in case samples

The performance of the ASIL-IS, TAC and SSIL-IS methods was also evaluated in routine casework specimens by co-analysis of 67 de-identified urine samples. A total of 162 analytes were co-identified by the three methods with 100% qualitative concordance. The quantitative comparisons are graphically displayed along with linear regression data in Figure 6. The three methods showed a significant correlation. ASIL-IS versus TAC methods (upper graph) showed the lowest variance in quantitative results with a coefficient of determination of 0.93 and analysis of variance analysis resulting in a slope and intercept indistinguishable from 1.0 and 0, respectively, with 95% confidence. SSIL-IS results, compared with either ASIL-IS (lower-left graph) or TAC (lower-right graph) results, showed a statistically significant positive intercept bias and lower coefficients of determination than determined in the ASIL-IS versus TAC method comparison. Regression analysis of ASIL-IS versus SSIL-IS concentrations revealed a coefficient of determination of 0.8, a slope <1.0 (95% confidence) and a positive intercept of 29 ng/mL ($P=0.014$). A similar analysis of TAC versus SSIL-IS concentrations resulted in a coefficient of determination of 0.80, a slope of <1.0 (95% confidence) and a positive intercept bias of 34 ng/mL ($P=0.006$).

Conclusion

The study shows that, without matrix normalization techniques, the effect of urine matrix compounds on the ion response of co-eluting drugs and metabolites varies significantly both among analytes and among donor samples tested for the same analyte, highlighting the importance of a thorough evaluation of matrix effects and the requirement for mitigation. The validity of matrix normalization by the classical ASIL-IS method was verified and compared closely with analytical results for normalization by the newer TAC method. Multi-matrix accuracy studies found quantitation bias when using a shared non-coeluting internal standard for matrix normalization, and the bias was not revealed by calibration and quality control monitoring in routine analytical testing. The findings of the study support the use of ASIL-IS or TAC as matrix normalization methods for accurate quantitation of urine drugs and metabolites in the routine practice of forensic and clinical toxicology.

Supplementary data

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

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