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Determination of sulfadimethoxine and ^{4}*N*-acetylsulfadimethoxine in bovine plasma, urine, oral fluid, and kidney and liver biopsy samples obtained surgically from standing animals by LC/MS/MS

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ABSTRACT

A quantitative method was developed and validated to measure the concentration of sulfadimethoxine (SDM) and its major metabolite, ^{4}*N*-acetylsulfadimethoxine (AcSDM), in bovine tissues and body fluids. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) gave quantitative results for these two analytes in extracts from bovine plasma, urine, oral fluid, kidney, and liver, using SDM-d_4 as internal standard (I.S.). The lower limit of quantitation (LLOQ) for both analytes in these matrices was validated at 2, 100, and 5 ng/mL in plasma, urine, and oral fluid respectively, and 10 ng/g in both kidney (cortex) and liver. The overall accuracy (average of 4 levels) is, for plasma, 104% (SDM) and 95% (AcSDM), with standard deviation of 9% (SDM) and 15% (AcSDM); for urine, 100% (SDM) and 106% (AcSDM), with standard deviation of 5% (SDM) and 6% (AcSDM); for oral fluid, 103% (SDM) and 103% (AcSDM), with standard deviation of 4% (SDM) and 4% (AcSDM); for kidney, 101% (SDM) and 111% (AcSDM), with standard deviation of 7% (SDM) and 6% (AcSDM); and for liver, 99% (SDM) and 115% (AcSDM), with standard deviation of 11% (SDM) and 9% (AcSDM). C18 SPE cartridges were used to clean-up these matrices, except for urine which was diluted directly with buffer before analysis by LC/MS/MS.

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1. Introduction

The use of sulfadimethoxine (SDM) in meat and milk-producing cattle has been approved by U.S. Food and Drug Administration (FDA) for treatment of shipping fever complex, bacterial pneumonia, calf diphtheria, and foot rot. This synthetic drug is effective against bacterial and coccidial infections, and can be administered orally [1] or intravenously [2]. Currently in U.S. the residue tolerance of SDM in edible tissue of cattle is 0.1 parts per million (ppm; 0.01 ppm in milk) [3].

Monitoring the proper use of animal drugs to ensure food safety is one important role of U.S. FDA. If SDM is not administered correctly to cattle or used off-label, elevated drug residue concentration may occur in edible tissues. The emergence of drug-resistant microbes to SDM is another public health concern [4–7]. Currently, the U.S. Department of Agriculture (USDA) uses the Fast Antibiotic Screen Test (FAST), a microbiology-based assay, for in-plant screening to determine the presence of antibiotics including SDM [8,9]. Bovine kidney is the primary monitoring site for the presence of antibiotics, which is only available after the animal is dead. The slaughter of animals containing residues can result in unsafe food entering the Nations food supply or the loss of a carcass. This can easily be avoided by extending the period the treated animal is held before slaughter. Even though the drug depletion time has been established, SDM residues are still a significant cause for carcass condemnation due to off label use. A simple testing method using easily obtainable animal fluid (serum, urine, saliva, etc.) to evaluate drug residue level is desirable. An earlier study has demonstrated that a tissue–fluid correlation exists for sulfamethazine residues in swine [10]. However, information is not available to develop a correlation of SDM residues in bovine tissues. The FDA Center for Veterinary Medicine (CVM) is conducting research to develop tissue–fluid correlations for a variety of veterinary drugs, such as penicillin G [11], gentamicin [12], and SDM in cattle.

Analytical methods were needed to determine the residual level of SDM and its major metabolite in bovine species, ^{4}*N*-acetylsulfadimethoxine (AcSDM) [13], to support the tissue–fluid correlation research. The target tissues and fluids from Holstein

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steers included kidney, liver, plasma, urine, and oral fluid. SDM is a chemically stable compound with a depletion half-life around 8–12.5 h in bovine species, if given intravenously [14,15]. Procedures are available from both published literature and government agencies on extracting and analyzing SDM in animal tissues and fluids from a variety of bovine matrices. Organic solvents such as ethyl acetate, methanol, and acetonitrile have been used for extraction, followed by either a solid phase extraction (SPE) or a liquid–liquid extraction (LLE) step for clean-up, and combined with the use of LC-UV, LC-fluorescence, or LC/MS/MS for detection and quantification [16–19]. Alternative methodologies like supercritical fluid extraction (SFE) and online SPE were also reported [20,21]. The collection of published methods provided valuable references for developing a "fit-to-purpose" method to achieve the goal of this study, which was to quantify both SDM and AcSDM over a wide concentration range in a variety of tissues and fluids. As part of experimental design, some of the tissue samples were acquired by the use of a laparoscopic procedure [22], to greatly reduce the number of animals in a depletion study. The design allowed multiple organ biopsy samples from an individual steer at designated time intervals to be taken before the animal was euthanized. However, the biopsy samples obtained as such would be of limited quantity and rather variable in weight (ca. 50–200 mg), which called for a method with high sensitivity and low susceptibility to matrix effect. The sample extraction and analysis throughput needed to be reasonably high to assay the large number of samples from multiple tissues and fluids.

Based on the experience at CVM in quantifying penicillin G and gentamicin in small size (ca. 100 mg) bovine kidney and liver biopsy samples [23,24], an LC/MS/MS method for quantifying SDM and AcSDM, using SPE for tissue and plasma clean-up and direct buffer dilution for urine sample preparation, has been developed and validated. The method contains sample extraction/clean-up procedures customized to each matrix, while it employs the same analytical equipment and instrumental conditions for all extracts. This method has been demonstrated to be sensitive, precise, and practical, to provide data for the continuing tissue–fluid correlation study.

2. Experimental

2.1. Apparatus

The LC/MS/MS system consists of a Waters Quattro Micro API bench-top triple quadrupole mass spectrometer (Milford, MA), and an Agilent 1100 LC system, including a binary pump, an autosampler with refrigerated sample tray, and a column compartment with temperature control (Santa Clara, CA). A Thermo BDS Hypersil C18 LC column, 150 mm × 2.1 mm ID, particle size 5 μm silica, fitted with Thermo C18 guard column, was used throughout the study (Thermo Fisher, Waltham, MA). A Sorvall 3C centrifuge (floor model) was used for centrifugation (Dupont Company, Wilmington, DE). The pH of buffer solutions was measured with a Fisher Accumet Research AR15 pH meter (Fisher Scientific, Pittsburgh, PA) equipped with an Accumet Ag/AgCl single junction probe. Liquid samples or extracts were accurately measured and transferred with calibrated variable micropipettes of proper capacity (Eppendorf North America, Westbury, NY). Organic solvents were measured with disposable graduated glass pipettes (Fisher Scientific). An Omni TH tissue homogenizer (Omni International, Marietta, GA) fitted with disposable plastic probes (7 mm × 110 mm) was used for blending kidney or liver lumps of approximately 100 mg. Biopsy samples were collected in pre-weighed round-bottom Falcon 14-mL centrifuge tubes (Becton Dickinson and Company, Franklin Lakes, NJ). PVDF filters with 0.22 μm pore size and 13 mm diameter (MilliPore, Billerica, MA) were used to filter the extract into 1.5-mL, amber glass autosampler vials. Varian Bond Elut C18 SPE cartridges (500 mg absorbent, 3 mL capacity, Varian Inc., Palo Alto, CA) were used to clean-up the raw sample extract.

2.2. Reagents and standards

Sulfadimethoxine was obtained from Sigma–Aldrich (St. Louis, MO) with 99% purity. Sulfadimethoxine-d_4 was purchased from Toronto Research Chemicals (Toronto, Canada) with overall purity of 98% (isotopic purity 99%). AcSDM was synthesized in-house from SDM according to a published procedure [25]. This compound was characterized with ^{1}H NMR and high resolution MS for identity, and its purity was evaluated with NMR and LC-UV (265 nm). No detectable impurities were found. Ammonium formate, ammonium acetate, and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich with analytical or higher purity. Other reagents were ACS grade formic acid (88%) from Mallinckrodt (Phillipsburg, NJ), glacial acetic acid and ammonia (~30%) from Fisher Scientific, and HPLC Grade acetonitrile and methanol from Burdick and Jackson (Morristown, NJ). Deionized (D.I.) water was produced in-house with the Milli-Q system (MilliPore, Billerica, MA) with a resistivity ≥18 MΩ cm.

2.3. Solutions

Stock solutions for SDM, AcSDM, and SDM-d_4 were made at approximately 1 mg/mL (weighed about 10 mg and recorded exactly to 0.01 mg) in 10 mL methanol in a volumetric flask. A 200 μg/mL working stock solution for each of SDM and AcSDM, and a 5 μg/mL solution for SDM-d_4, was then prepared by diluting a calculated amount of the 1 mg/mL stock solution with methanol. A series of mixed stock solutions containing an equal amount of SDM and AcSDM were prepared by mixing and diluting proper volumes of the 200 μg/mL stock solutions, or from a mixed stock solution of a greater concentration. These standard solutions were store at <−10 °C, and the analytes were found to be stable over a period of 6 months. The long time exposure to light was avoided while these stock solutions were in use.

A 0.05 g/mL TCA solution was prepared by dissolving 50.0 g TCA in D.I. water to make a 1 L solution. A 50 mM ammonium formate buffer solution (AFB) was prepared by dissolving 6.50 g ammonium formate (97%) in D.I. water to make a 2 L solution. The pH was then adjusted to 3.9 ± 0.1 with formic acid. A 50 mM ammonium acetate buffer (AAcB) was prepared by dissolving 15.9 g ammonium acetate (97%) in D.I. water to make a 4 L solution, and the pH was adjusted to 6.0 ± 0.1 with glacial acetic acid. LC mobile phase A consisted of 5% acetonitrile with 0.1% formic acid in D.I. water (v/v), prepared by mixing 200 mL acetonitrile, 3.8 L Milli-Q water, and 4.55 mL 88% formic acid. This solution was filtered through 0.2 μm Nylon disk before use.

2.4. Biological samples storage, and stability of analytes in matrices and extracts

The test system consisted of the following bovine tissues and body fluids: kidney (cortex), liver, plasma, urine, and oral fluid. Samples of these tissues and fluids were collected from 9 Holstein steers. The details of dosing and sample collection will be published separately [22]. Blank control kidney and liver were obtained either from stored samples generated previously known to be free of SDM, or from groceries and a farmer's market, which were screened to be free of detectable drug residues before use. Blank control urine, plasma, and oral fluid were obtained from each individual animal before dosing. All raw samples and extracts were stored at <−70 °C. The majority of the incurred samples were extracted and analyzed within a few days to a few weeks after collection.

The stability of both analytes in various matrices was studied for (1) prolonged storage under <−70 °C (long-term stability); (2) standing at room temperature for 6–8 h (short-term stability); and (3) subject to 3 freeze/thaw cycles before analysis. A set of incurred samples for each matrix with residues at 2–4 different levels were sub-divided and stored in freezer at <−70 °C at one time, and these sub-sets were analyzed at different time points over up to 6.5 months.

2.5. Sample extraction and clean-up/general

Prior to processing, frozen samples were maintained at room temperature until completely thawed. An "extraction batch" typically included a group of unknown samples of the same matrix, plus controls for "double blank" (not spiked), "zero blank" (spiked with I.S. only), and fortified controls (fortified at one of the low, middle, or high levels with both SDM and AcSDM, plus fixed amount of I.S.). All samples were processed in one session and were typically analyzed on LC/MS/MS continuously within one sequence (an "analysis batch").

2.6. Plasma extraction

Bovine plasma was vortexed and distributed in aliquots of 500 μL into 15-mL polypropylene (PP) centrifuge tubes with addition of 20 μL SDM-d_4 (I.S., 5 μg/mL). An aliquot of AFB (0.6 mL; 50 mM; pH 3.9) and 0.3 mL 1:1 methanol/H_2O was added to each tube, which was vortexed at high speed for 5 min. The sample had a moderate amount of precipitate. A 0.6 mL aliquot of AAcB (50 mM; pH 6.0) was added and the suspension was mixed briefly. All samples were centrifuged immediately for 10 min at 3900 RPM (∼4400 rcf) at 0–4 °C. The raw extract was then loaded onto an SPE cartridge (pre-conditioned with 2.5 mL methanol and 2.5 mL H_2O), and rinsed with 2.5 mL of AAcB (50 mM; pH 6) and 2.5 mL water. Analytes were then eluted with 2 portions of 2 mL 3:1 methanol/H_2O (v/v), which were collected and combined into a 15-mL centrifuge tube. After 0.2 mL AFB (50 mM; pH 3.9) was added to each tube, the extract was concentrated to about 0.9 mL by evaporation under nitrogen flow at 45 °C. The final volume was brought to the 1 mL mark on the 15-mL centrifuge tube with acetonitrile. The reconstituted extract was filtered through a 0.22 μm PVDF filter into an amber glass autosampler vial. This procedure also applied to pre-diluted plasma samples when the concentration of one or both analytes was above 2000 ng/mL in extract (4000 ng/mL in plasma), the upper limit of quantitation (ULOQ) of the analytical method. Under these conditions, 10 mM AAcB (pH ∼ 6.0; prepared by proper dilution of 50 mM AAcB with D.I. water) was used for pre-dilution.

2.7. Oral fluid extraction

The oral fluid from cattle is usually sticky, viscous, and contains solid particles. Centrifugation for 10 min at 4400 rpm (∼5000 rcf) at 0–4 °C yielded a clear liquid. Two hundred microliters (200 μL) of the clear liquid was dispensed with a micropipette into 3-mL PP tubes at a slow drawing speed. Twenty microliters (20 μL) SDM-d_4 (I.S., 5 μg/mL), followed by 0.4 mL AAcB (pH 6.0; 50 mM), 0.4 mL 1:1 methanol/H_2O, and 1.0 mL D.I. water were added to each sample, and the solution was vortexed. The SPE clean-up procedures were the same as used in the plasma treatment.

2.8. Kidney and liver extraction

The 50–200 mg kidney or liver tissue samples obtained by the laparoscopic surgery were place in pre-weighed tubes as a whole piece. To all samples, after addition of 20 μL SDM-d_4 (I.S., 5 μg/mL), aliquots of 0.5 mL 5% TCA solution and 0.5 mL methanol were added. The tissue was homogenized for 1 min, using a dedicated plastic Omni homogenizer probe tip for each sample. The probe tips were rinsed (after grinding) with minimal amount of methanol to recover drugs from the tissue residue, and the rinse was saved for the second extraction. The homogenates were centrifuged for 10 min at 3000 rpm (∼2600 rcf) at 0–4 °C. The supernatant was decanted into a separate set of graduated 15-mL PP tubes. One hundred microliters (100 μL) of 30% NH_3 (aq.) was added to each raw extract and the tubes were vortexed briefly. A second extraction was performed with 1 mL 1:1 H_2O/methanol, using the same homogenizer probes. The homogenization time for kidney and liver was 1 and 0.5 min respectively for the second extraction. The homogenate was then centrifuged for 10 min at 3900 rpm (∼4400 rcf) at 0–4 °C. The supernatant of the second extraction was combined with the first extract portion. After the solvent was evaporated to 0.5–0.8 mL under nitrogen flow at 45 °C, AAcB (50 mM; pH 6.0) was added to each tube to the 1-mL mark. The SPE clean-up procedure was the same as used for plasma.

2.9. Urine sample preparation

An aliquot of 500 μL urine sample was pipetted into a 50-mL volumetric flask. After the addition of 1.00 mL SDM-d_4 (I.S., 5 μg/mL), the flask was filled to volume with 10 mM AAcB in methanol/H_2O (pH ∼ 6; prepared by diluting the 50 mM AAcB with 3 volumes of D.I. water and 1 volume of methanol). The solution was mixed thoroughly. About 1 mL of the diluted urine sample was transferred directly to an autosampler vial through a 0.22 μm PVDF filter. If analysis indicated that the concentration of the 100-fold diluted urine exceeded ULOQ of this method (2000 ng/mL in extract or 200 μg/mL in urine), the diluted urine sample was to be further diluted, with proper addition of I.S. Furthermore, a separate SPE method for urine was developed to screen the blank control samples. In this case, 500 μL urine was first buffered with 0.5 mL AAcB (50 mM; pH 6) and spiked with 20 μL SDM-d_4 (I.S., 5 μg/mL), then underwent the same SPE procedure for the plasma clean-up.

2.10. Liquid chromatography/tandem mass spectrometry

The liquid chromatographic conditions were set as follows: flow rate 0.20 mL/min; column temperature 30.0 ± 0.1 °C; isocratic elution with 70% mobile phase A and 30% acetonitrile as mobile phase B; total run time 10 min. The injection volume was 40 μL. Under these conditions the retention times for SDM (SDM-d_4) and AcSDM were around 5.5–5.8 and 5.0–5.3 min respectively.

Parameters for the MS electrospray ionization (ESI) source were set as follows: desolvation gas (N_2) flow 300 L/h; cone gas (N_2) flow 50 L/h; desolvation temperature 200 °C; source temperature 100 °C; capillary voltage 3000 V. The cone voltage, extractor voltage, and RF lens voltage were optimized periodically over the MS/MS transition of SDM-d_4 (see below). Parameters for the MS analyzer were set at the following values: HM resolution 1, 13; ion energy 1, 0.2; LM resolution 1, 13; entrance, 5; collision, 15; exit, 1; HM resolution 2, 14; ion energy 2, 2.0; LM resolution 2, 14. Multiplier gain value was 650. The Collision Cell Pirani (Q2) was set at 3.0×10^{-3} Torr (Argon, purity >99.995%, from Roberts Oxygen Company Inc., Rockville, MD), and the actual read-back value was within $2.7–3.3 \times 10^{-3}$ Torr. The three transitions to monitor SDM, AcSDM, and SDM-d_4 were 311 → 156, 353 → 156, and 315 → 156 m/z respectively under MRM mode. See Fig. 1 for the structure of analytes and proposed MS fragmentation patterns. For all these transitions, the collision energy was 22 V, and the dwell time was 0.2 s. The other acquisition-related parameters were inter-channel delay, 0.02 s; inter-scan delay, 0.1 s; repeats, 1; and span, 0. In each analysis batch, the calibration standard mixture solutions were injected both before and after the unknown samples.

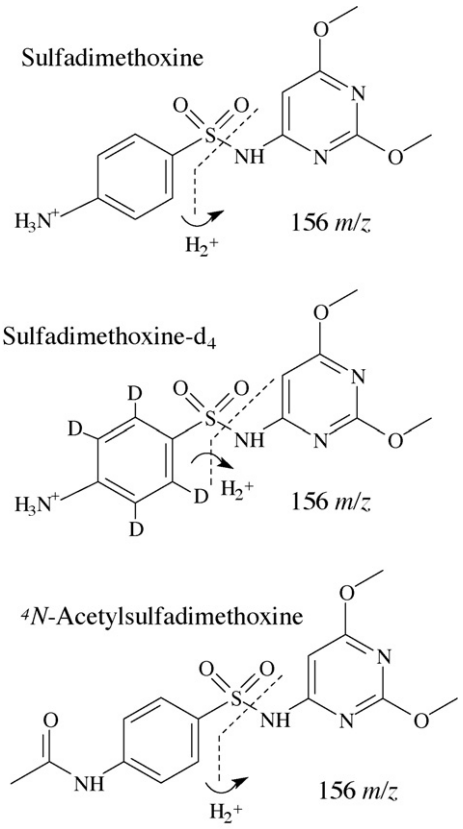

Fig. 1. Structure and MS^2 fragmentation for SDM, SDM-d_4, and AcSDM.

Occasionally there was a need to confirm the identity of residues, especially at low levels near the LLOQ. Therefore, an *ad hoc* confirmatory method for SDM and AcSDM was developed. The criteria stated in FDA's Guidance [26] were followed, but a full-scale validation was not performed. The 3 transitions for SDM confirmation were 311 → 156 (base peak), 108, and 218 *m*/*z*, and were 353 → 156 (base peak), 198, and 287 *m*/*z* for AcSDM. In each analysis batch, standards at appropriate levels were injected to establish the ion-ratio and retention time reference points. In each batch one blank control and one spiked extract (in same matrix as unknown samples) were used for negative and positive QC, respectively. Confirmation of selected incurred samples was performed as needed.

2.11. Data acquisition and processing

MassLynx 4.0 (Service Pack 3) was the controlling software to acquire raw data. Integration of peaks was conducted with TargetLynx with pre-set parameters, and the integrated area was manually adjusted if necessary. Calibration curve for quantitation was plotted with the peak area ratio of the analytes to I.S. against analytes' nominal concentration. For the calibration range of 0.5–2000 ng/mL, quadratic regression with weighting factor of 1/*X* and with the origin excluded was applied. Measured concentration of SDM and AcSDM was calculated with peak area ratio by the software. If the nominal concentration of the drugs was known, accuracy was then derived as the quotient (in percentage) of observed value to the nominal one.

2.12. System suitability and quality control

Basic equipment qualification tests were performed separately for the HPLC and MS systems, based on manufacturers' specifications and our established laboratory procedures. Additional quality control measures were taken to ensure the validity of the data obtained from each analysis batch. Integrated peaks for quantification purpose contained at least 15 scans, and all peaks' S/N value should be higher than 10. Accuracy and precision were evaluated for each set of measurement (e.g., SDM in plasma fortified at QC1 level) according to FDA's guidance on bioanalytical method development and validation [27]. The coefficient of determination (R^2) for calibration curves must be greater than 0.99. At least 4 out of 6 of the back-calculated values of the analytes in calibration solutions should be within ±15% of their nominal concentration, except ±20% at the LLOQ level. The LLOQ for the method was assigned to be the lowest calibration standard (0.5 ng/mL in buffer). For a particular analysis batch, the lowest acceptable quantitation level might be higher than LLOQ, should interference (S/N > 3; at or near the retention time of either SDM or AcSDM) appear in buffer or blank matrices, and the corresponding peak area was greater than 20% that of the LLOQ standard. If so, the next lowest calibration standard would be tested for the lowest acceptable quantitation level. In addition, three QC extracts at low, medium, and high levels were analyzed in the same sequence with the unknowns from the same extraction batch, and at least two of them had to meet the "within 15% (20% for the low-level QC) to nominal value" criterion. The peak area of SDM-d_4 in all standard solutions (100 ng/mL in matrix-free buffer), plus single blank in solvent, should constitute a no larger than 15% intra-day RSD. Peak area of I.S. from all extracted samples (unknowns and QCs) was checked against intra-day average and that of the standards'. If large deviation (>±30%) occurred, the corresponding sample(s) were either re-extracted or re-analyzed. Finally, the retention time for all analyte peaks (if present) had to be within ±0.1 min of the average intra-day value.

3. Results and discussion

Representative ion chromatograms for the three compounds in the five matrices are shown in Fig. 2. As SDM was the main analyte of interest, its deuterium-labeled analog, SDM-d_4, was used for internal standard. This compound was a viable I.S. for AcSDM as well, though differences existed in both absolute recovery and the extent of matrix effects. While the use of isotope-labeled AcSDM (not commercially available) as I.S. for AcSDM would likely give improved accuracy, its use was not essential to achieve the goals of the study. No cross-talk was found among these reference standards on LC/MS/MS.

3.1. Solid phase extraction and clean-up

In this study, the absolute recovery of the two analytes was evaluated by comparing the peak area of a set of "fortified" samples (standard solution added to samples BEFORE extraction) to that of "spiked" samples at same nominal level (standard solution added to extract AFTER extraction and clean-up). The results shown in Table 1 indicate that the absolute recovery is in a desirable range

Table 1
Completeness of the extraction and clean-up procedures for the matrices. The absolute recovery calculated by the "slope ratio method" is in brackets.

Matrix	Absolute recovery of drugs from 1st extraction, ±1 × standard deviation		
	SDM	AcSDM	SDM-d_4
Kidney	85 ± 1% (84%)	92 ± 5% (88%)	87 ± 5%
Liver	90 ± 3% (85%)	93 ± 2% (92%)	89 ± 4%
Plasma	96 ± 1% (97%)	70 ± 4% (70%)	97 ± 3%
Urine	100%[a]	100%[a]	100%[a]
Oral fluid	99 ± 2% (99%)	99 ± 2% (99%)	99 ± 8%

[a] Assumed value, as there should be no loss of drug substance by simple dilution.

for both analytes in all five matrices. No I.S. correction was applied as the co-spiked SDM-d_4 would compensate for the loss of residue in extraction. While in this way the measurement error resulting from the visual adjustment of the final volume of extracts to 1 mL could not be offset by I.S. either, the extent of such error was deemed insignificant. An alternative way to calculate the absolute recovery was to divide the slope from linear regression of fortified samples, using peak area at 3 levels, by the slope of the linear regression of the spiked ones. The calculated absolute recoveries from both algorithms were similar.

To further evaluate the completeness of residue extraction, a set of incurred samples for each of plasma, kidney, and liver was extracted two times consecutively. By calculation based on corresponding chromatogram peak area without I.S. correction, the percentage of the second extraction as of the sum of both rounds, for SDM and AcSDM respectively, were estimated to be both 9% for kidney, 16% and 12% for liver, and 2% and 1% for plasma. Most of these figures are in reasonable agreement with the absolute recoveries calculated from the first round of extraction.

The extent of matrix effect (matrix factor) in LC/MS/MS analysis was estimated by comparing the regression slope of spiked samples at 3 levels to that of the standard curve using peak area of SDM and AcSDM in each of the 5 matrices. For SDM-d_4, as the assumed concentration in both spiked extracts and matrix-free standard mix was always 100 ng/mL, the factor was approximated by dividing the average I.S. peak area from spiked samples by that from the standards in buffer. Overall, the matrix factors for all matrices are close to unity, indicative of a relatively clean environment for the analytes with minimal matrix effect (Table 2).

Table 2
Matrix factors for SDM, AcSDM, and SDM-d_4 in the 5 matrices.

Matrix	SDM	SDM-d_4	AcSDM
Kidney	0.98	1.04	1.01
Liver			
Lump	0.79	0.86	0.95
Homogenate	0.94	1.03	1.03
Plasma	0.98	1.06	0.98
Urine	0.99	1.11	1.01
Oral fluid	0.97	0.97	0.97

3.2. Selection of standardization model

A set of experiments were carried out to evaluate the best standardization model for quantification. Calibration curves for SDM and AcSDM were established for each of the options below (all with I.S. correction; weighting factor $1/X$; the origin excluded):

A. linear regression on standards spiked in matrix-free buffer;
B. quadratic regression on standards spiked in matrix-free buffer as above;
C. linear regression on standards fortified in each matrix;
D. quadratic regression on standards fortified in each matrix.

Extraction batches with fortified samples (fortified at 8 levels plus 3 QCs) for all 5 tissues or fluids were carried out as described above. Three batches were repeated for kidney, two for liver and plasma, and one for other matrices. In addition, the evaluation was also conducted using fortified samples in 20× pre-diluted plasma, and in undiluted urine which went through the same SPE proce-

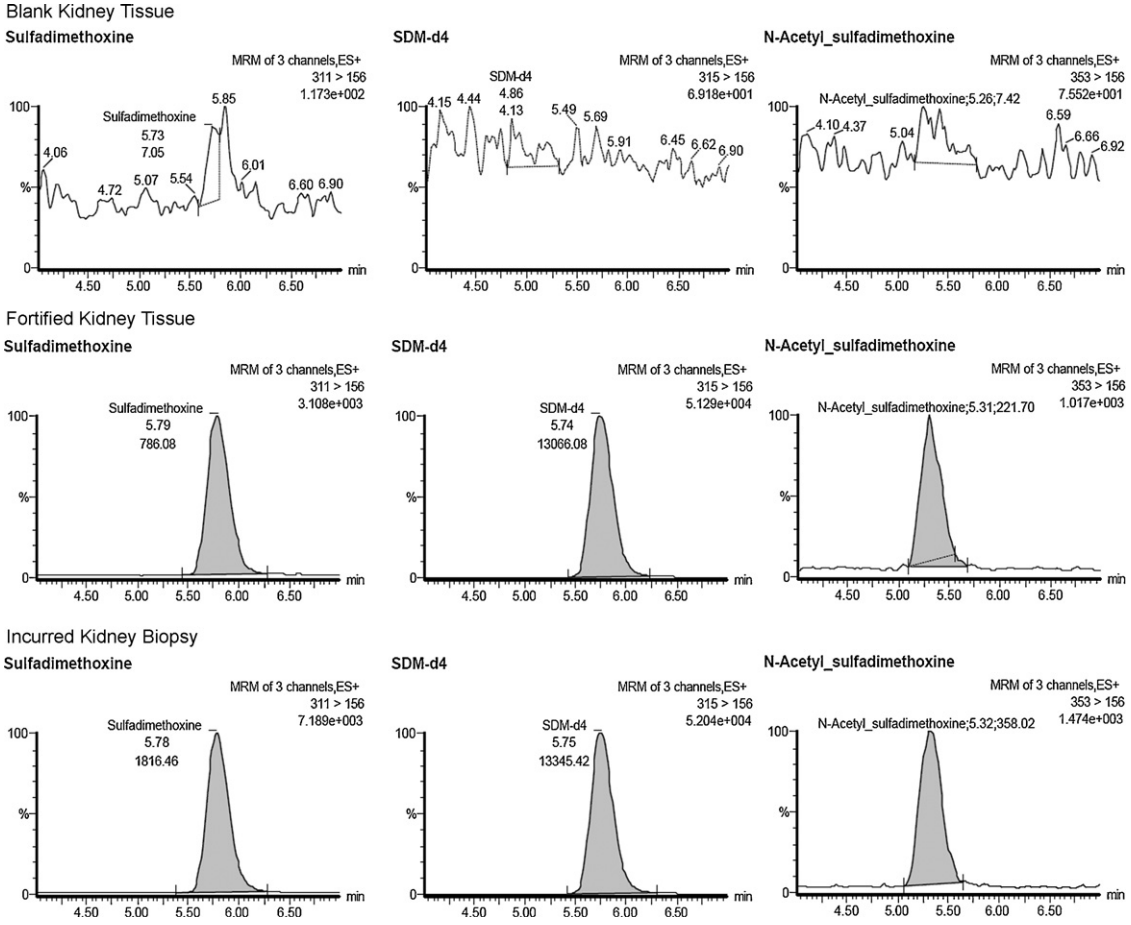

Fig. 2. Ion chromatograms of SDM, SDM-d_4, and AcSDM in kidney (a), liver (b), plasma (c), urine (d), and oral fluid (e): blank control (first row), fortified at 3 ng/mL (equivalent in-extract concentration; second row), and incurred (third row).

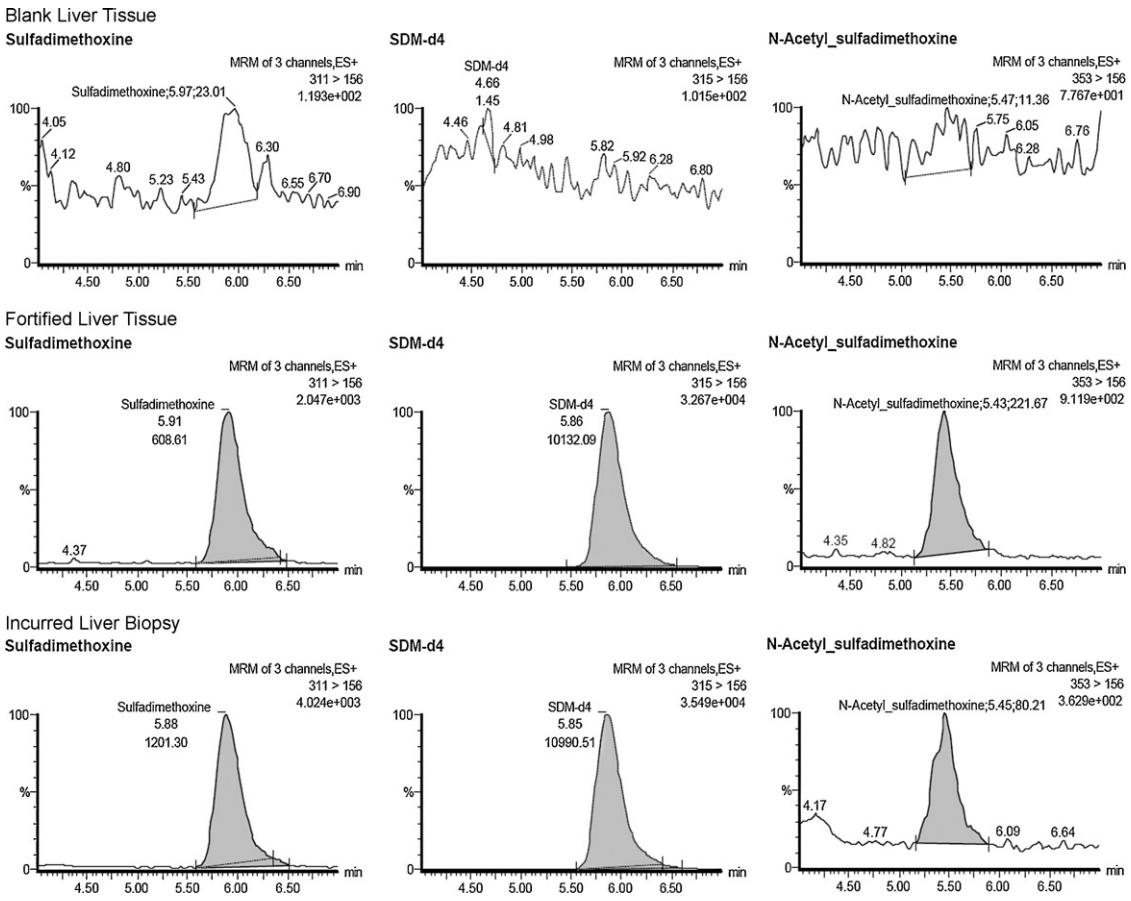

Fig. 2. (*Continued*)

dure as other matrices. The data showed that, for kidney and liver, B (quadratic regression) was deemed to be best among the 4 options, based on overall evaluation of coefficient of determination (R^2; quadratic regression) or correlation coefficient (r^2; linear regression), LLOQ, QC accuracy, and number of outlier points in calibration curves, though R^2 and r^2 values in all models were >0.992.

With quadratic regression, better results were obtained at the lower end of the calibration curve when back-calculating the values for fortified and QC samples. If linear regression was applied, there was a non-random, "U"-shaped pattern in the residual plot. The regression in oral fluid also showed better results with option B. For urine and plasma, there was little difference among the 4 models. For all 5 matrices, the matrix effects in extracted samples for SDM and AcSDM were minimal (see Table 2), and the effect was further reduced by I.S. correction. As the results substantiated the use of option B for all 5 matrices, and for consistency in data processing, all calibration curves were thus established based on spiked standards in matrix-free buffer, with quadratic regression.

3.3. Method performance

Accuracy and precision of the method were evaluated using the collective data from all QC samples at four different fortification levels from all acceptable batches. The inter-day accuracy of the method was between 95% and 115% for the 5 kinds of tissues and fluids in this work. The inter-day precision was within 15% based on the RSD at all spiking levels, and the intra-day precision was no greater than 6% for an average of 3 or 5 repeats. The accuracy and precision met the established criteria [27], except for AcSDM in the middle level QC spikes in liver extract (Table 3). It was noted that there was a moderate positive bias in the all-level accuracy value for AcSDM in all these matrices except plasma, substantiated by Student's *t*-test. The most significant effect was observed for AcSDM in liver, which might be qualitatively explained with the "process efficiency (PE)" concept conferred by Matuszewski et al. [28], which is the product of the absolute recovery (RE) and matrix effect (ME). Fitting in with our data (Tables 1 and 2) into the formula, RE and ME were both higher for AcSDM in liver extract (93% and 0.95) than for SDM-d_4 (89% and 0.86). The calculated ratio of PE values for AcSDM over SDM-d_4 was 1.15, corroborating well with the accuracy figure (AcSDM in liver, 115% for all levels combined; Table 3). In any event, considering the relatively small extent of the deviation, and the research objectives, the data for AcSDM in these tissues were deemed acceptable.

Specificity of the method was evaluated in two ways. First, interferences in the blank extracts were evaluated. Several blank controls (plasma and oral fluid samples) from a few steers (collected before first dosing) showed the presence of SDM as interference, some of which were confirmed by confirmatory LC/MS/MS analysis. The highest concentration measured in a steer was about 4 ng/mL SDM in plasma. This observation was consistent for the 3 double blank controls and 1 zero blank extract, prepared in separate batches. AcSDM was also confirmed in one of these extracts, though the estimated concentration is below 0.5 ng/mL in extract. Nevertheless, the lowest acceptable quantitation level in the affected analysis batches was well below the incurred plasma levels and did not compromise the results. Therefore, the cause of this observation was not investigated further. Secondly, potential interference from analogous compounds was checked. A set of samples of each matrix were spiked with 9 other sulfonamide drugs (sulfamerazine, sulfamonomethoxine, sulfacetamide, sulfaquinoxaline, sulfanilamide, sulfachloropyridazine, sulfamethoxazole, sulfathia-

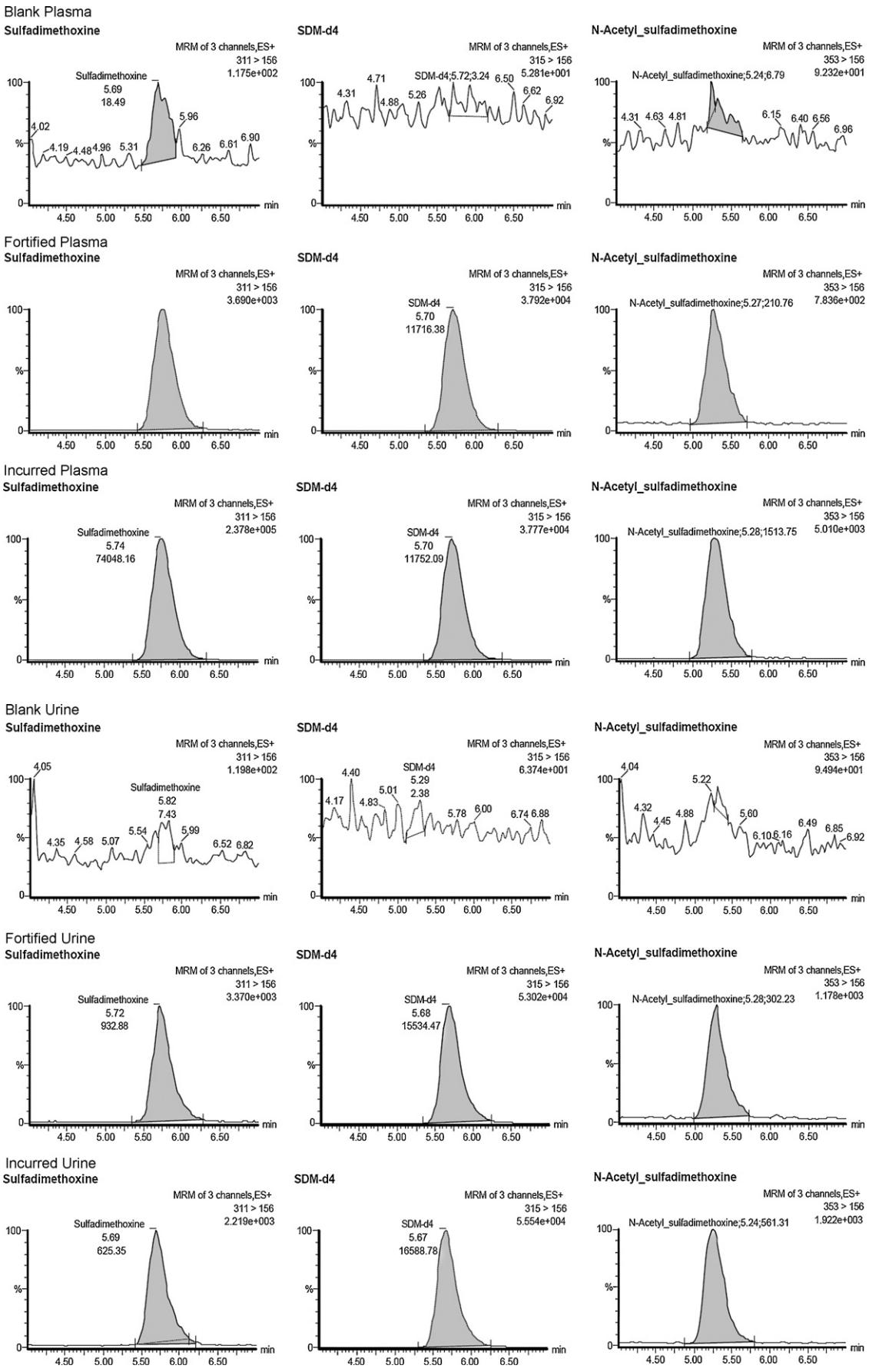

Fig. 2. (*Continued*)

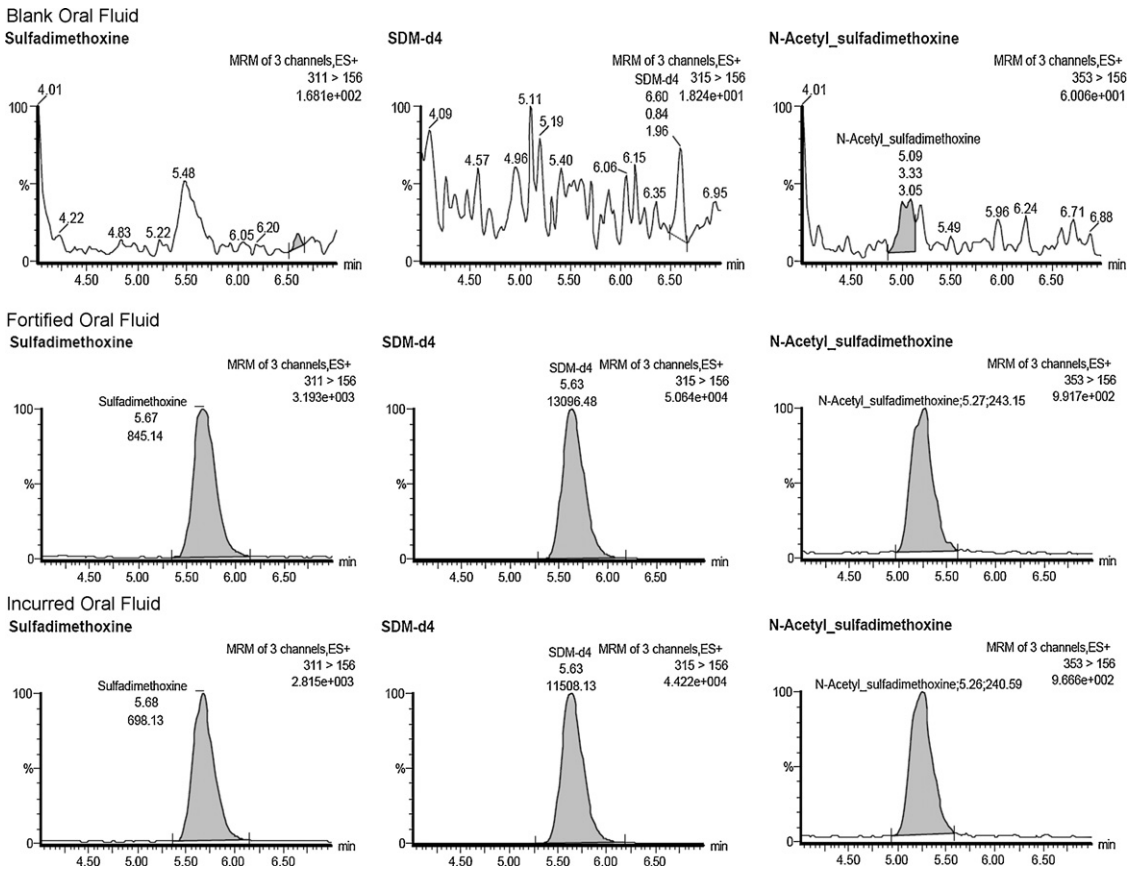

Fig. 2. (*Continued*).

zole, and sulfaguanidine) and were extracted and analyzed with the method. All samples were found negative for SDM or AcSDM.

3.3.1. Dynamic range and lower limit of quantitation for incurred sample analysis

For both SDM and AcSDM in all 5 matrices, the full quantification range was set as 0.5–2000 ng/mL (in extract). Virtually all calibration curves from more than a hundred batches passed the study criteria. However, when batches for incurred samples were analyzed, many of the "low QCs" were spiked at 3 ng/mL instead of 1.5 ng/mL (3 times LLOQ), to avoid unanticipated interference that would necessitate re-analyzing the whole batch. Therefore, the LLOQ for incurred sample batches was conservatively adjusted upwards, which were (after correction by dilution factors) 10 ng/g for kidney and liver, 2 ng/mL for plasma, 100 ng/mL for urine, and 5 ng/mL for oral fluid. The ULOQs were 2000 times the corresponding LLOQ.

3.4. Method robustness and validation

There were several factors that could potentially affect the performance of these methods, such as size (weight) variation of the tissue lumps, biopsy location on the kidney or liver, and potentially critical steps in sample preparation. Therefore, experiments were carried out to address these concerns.

The weight of the kidney or liver biopsy samples taken surgically from standing animals was usually in the range of 50–200 mg. As part of robustness testing, the effect of sample size on the method's accuracy and precision was tested with both fortified and incurred tissues. First, a group of fortified kidney and liver samples of varied weight from roughly 25–180 mg and 80–500 mg respectively, were extracted and analyzed. As the volume of all extracts was the same (1 mL), the concentration of spiked standards should be equal (Fig. 3a and b, *Y*-axis; each sample was fortified with 10 ng SDM and AcSDM). However, the amount of extracted matrix in these solu-

Table 3
Accuracy and precision data for QCs (CV and repeat numbers are in brackets).

Matrix	Analyte	Accuracy% at 3 ng/mL	Accuracy% at 5 ng/mL	Accuracy% at 100 ng/mL	Accuracy% at 1600 ng/mL	Accuracy% at all levels
Kidney	SDM	100 (11%, 22)	102 (6%, 12)	100 (5%, 25)	103 (4%, 24)	101 (7%, 83)
	AcSDM	108 (8%, 20)	113 (5%, 11)	114 (7%, 23)	110 (3%, 22)	111 (6%, 76)
Liver	SDM	101 (17%, 20)	95 (7%, 5)	98 (6%, 19)	99 (5%, 19)	99 (11%, 63)
	AcSDM	112 (11%, 13)	113 (9%, 5)	120 (10%, 12)	113 (4%, 12)	115 (9%, 42)
Plasma	SDM	108 (13%, 15)	110 (6%, 9)	103 (6%, 22)	100 (7%, 21)	104 (9%, 67)
	AcSDM	92 (17%, 19)	108 (6%, 9)	95 (15%, 22)	93 (13%, 21)	95 (15%, 71)
Urine	SDM	98 (4%, 21)	104 (6%, 10)	101 (6%, 22)	98 (5%, 22)	100 (5%, 75)
	AcSDM	107 (7%, 21)	110 (3%, 10)	108 (5%, 22)	101 (5%, 22)	106 (6%, 75)
Oral fluid	SDM	105 (5%, 8)	101 (4%, 4)	101 (5%, 12)	103 (4%, 12)	103 (4%, 36)
	AcSDM	102 (4%, 9)	105 (6%, 4)	103 (4%, 12)	103 (4%, 12)	103 (4%, 37)

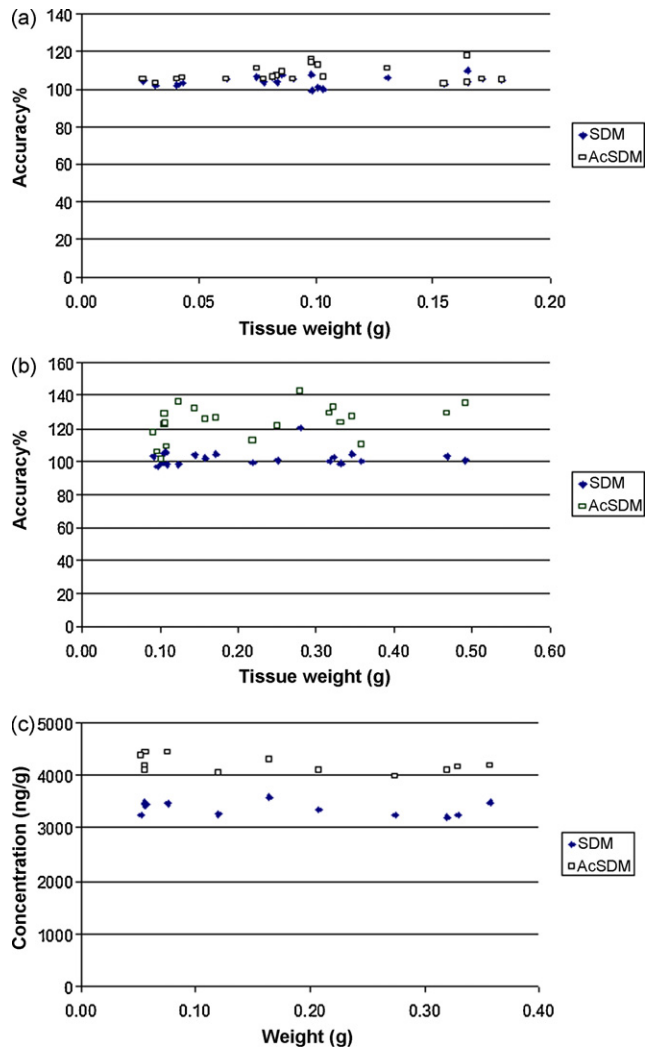

Fig. 3. Effect of tissue weight on measurements of analytes in fortified bovine kidney (a), fortified bovine liver (b), and incurred kidney (c).

tions would vary by several folds. In a second experiment, a set of incurred kidney samples of varying weight (assuming equal tissue concentration of drugs) was analyzed as well. In this case, the tissue residue concentration was presumably the same (Fig. 3c, *Y*-axis; measured tissue concentration was 3.37 ± 0.13 μg/g for SDM and 4.20 ± 0.15 μg/g for AcSDM) as was the analyte-to-matrix ratio in extract, but the in-extract concentration level would differ by a factor of the tissue weight ratio. In fact, the RSD values calculated thereof ranged from 2% to 8% for the two analytes in the three experiments. Combined with the apparently randomly distributed error over the examined weight ranges (plot not shown but can be easily derived from these figures), it is clear that the amount of matrix extract has no effect on accuracy (fortified) or measured value (incurred).

The variability of drug residue concentration over the surface of the incurred organs was also examined, using samples taken from various spots on post-mortem kidney (cortex) and liver. The RSD of both SDM and AcSDM among the two groups of kidney samples (left and right kidneys) was higher than that from the incurred sample group presented in Fig. 3c, which were obtained from a close proximity on a different kidney. Although an analysis of variance experiment was not run to determine the degree of variability deriving from locality relative to that from the analytical method, the RSD for SDM and AcSDM from two kidney sides combined, 28% and 31% respectively, is significantly larger than that in Fig. 3c (4% for both analytes). The reason for this seemingly large location-related variability is beyond the scope of this study. Nevertheless, in practice the location used to obtain kidney biopsy samples was not as spread-out as was deliberately performed in this experiment. In contrast, the RSD for both SDM and AcSDM among incurred tissues from various locations on the 3 lobes of bovine liver was only 6% and 9% respectively.

Variations in selected steps in extraction/clean-up procedures for kidney, liver, and plasma, including the lag time between spiking and sample homogenization, pre-dilution factor (plasma), tissue-grinding time (kidney and liver), the remaining volume after sample evaporation, neutralizing reagent volume, SPE cartridge lot, and SPE loading/washing/elution volumes, were examined using an approach based on the fractional factorial experimental design. Results indicated that in all cases there was no statistical difference (95% confidence level) between the two variation values (high/low) relative to the values specified in the method.

Overall the methods gave sufficiently clean extracts with 70–100% recovery for both analytes in 5 matrices. With intermittent column regeneration efforts, one LC column had been used over a thousand analysis cycles with matrix and still maintained satisfactory chromatography performance. There are a few additional items that deserve further elaboration. The SPE step of the oral fluid method proceeded less smoothly than those on other matrices, as the passing of solvent through the cartridge was slow and sometimes required positive pressure to push the diluted oral fluid through SPE cartridges. The extract also caused an increase in back-pressure on LC column requiring additional column cleaning with a high aqueous buffer. Using acetonitrile to precipitate protein might alleviate the problem but would increase the complexity and time required for sample preparation. The conditions used to precipitate protein from plasma with weakly acidic AFB yielded high recoveries for SDM but lowered recoveries for AcSDM. As both SDM and AcSDM are known to stick to serum proteins like BSA [29], precipitation with stronger denaturing solution might give a better recovery for AcSDM.

3.5. Stability of analytes in extracts and matrices

The stability of all 3 analytes in each matrix (after extraction) at 4 °C was evaluated first. During the normal analysis conditions and time span (8–16 h), both SDM and AcSDM in extracts from all 5 matrices remained stable, while concentration of the SDM-d_4 in kidney, liver, and plasma extract decreased by no more than 15%. Also, both analytes were found stable under the short-term standing and the 3-freeze/thaw-cycle conditions, except that the available data was inconclusive for AcSDM in kidney cortex due to a low recovery from sample preparation. To the best of our knowledge, there was no comprehensive data available on the stability of SDM and/or AcSDM in incurred bovine tissue/fluid upon long-term storage, although such data have been published for pig and chicken tissues [25,30]. In this study, it was found that there was little decrease of SDM concentration in all 5 matrices, over a period of 6.5 months in kidney and liver, 6 months in plasma, 5 months in urine, or 3.5 months in oral fluid. However, the concentration of AcSDM decreased moderately in incurred kidney, liver, and plasma samples, by approximately 20–35%, over a period of 6 months, under <−70 °C storage condition. In contrast there was no appreciable change of AcSDM level in urine and oral fluid.

4. Conclusions

An LC/MS/MS method has been developed and validated for quantifying SDM and AcSDM in a variety of matrices from bovine source, in concentration ranges relevant to the drug depletion process and residue tolerance level. The tissue–fluid correlation study

on SDM depletion in Holstein steers had been supplied with incursion data generated via this method (to be reported elsewhere). The combination of proper sample preparation procedure and liquid chromatography condition afforded these analytes with high recoveries and an ionization environment comparable to that in a neat buffer, which in turn allowed establishing calibration curves based on directly spiked standards in a matrix-free solution. Thorough ruggedness tests were conducted to ensure the stability of this method's performance, which is important for analytical works that deal with a wide variety of matrices and large number of unknown samples.

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