Evaluation of Renal Biomarkers, Including Symmetric Dimethylarginine, following Gentamicin-Induced Proximal Tubular Injury in the Rat

Diane M. Hamlin,1 A. Eric Schultze,1 Michael J. Coyne,2 Donald J. McCrann III,2 Rebekah Mack,2 Corie Drake,2 Rachel E. Murphy,2 Julie Cross,2 Marilyn Strong-Townsend,2 Maha Yerramilli,2 and Mary K. Leissinger2

Key Points
- This study demonstrates the utility of serum symmetric dimethylarginine (SDMA) as an excretory renal function biomarker in a rat model of gentamicin-induced proximal tubular injury.
- Analytical and biologic validation of an SDMA immunoassay allows for future studies in rats using a high-throughput method.

Abstract
Symmetric dimethylarginine (SDMA) is an excretory renal function biomarker shown to correlate well with glomerular filtration rate in dogs, cats, humans, and rats. The objectives of this study were to determine utility of serum SDMA as a renal biomarker in a rat model of gentamicin-induced renal injury and to provide validation of a commercially available SDMA immunoassay for rat serum. Rats were randomly assigned to one of three dose levels of gentamicin (20, 50, or 100 mg/kg) or a vehicle control group and dosed once daily by subcutaneous injection for either four or ten days. Serum and urine renal biomarker evaluation, including serum SDMA, hematologic and serum biochemical analysis, urinalysis, and histologic examination of kidney, were performed. Before biologic validation, analytic validation of the SDMA immunoassay for rat serum was performed, including assessment of assay accuracy, precision, analytical sensitivity, linearity, analyte stability, and interference testing. Among markers of excretory renal function, SDMA and serum creatinine increased earliest and at the lowest gentamicin concentrations and were significantly increased in both the 50- and 100-mg/kg dose levels in the four- and ten-dose treatment groups compared with controls. Time- and dose-dependent increases were noted for all urinary biomarkers investigated in this study, with microalbumin being most responsive and osteopontin least responsive for detection of gentamicin-induced injury across dose levels and schedules investigated. The SDMA immunoassay met all set quality requirements assessed in analytical validation. This study is the first to investigate performance of serum SDMA compared with other excretory renal function markers in a rat gentamicin acute toxicity model. In this study, serum SDMA was an earlier biomarker for detection of gentamicin-induced toxicity than serum cystatin C, BUN, and creatinine clearance. The SDMA immunoassay provides a reliable commercially available assay for future renal investigations in rat models.

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Introduction
Toxicity is the main cause of candidate attrition at all stages of drug development, with hepatic, cardiac, and nephrotoxicity representing the most frequently cited organ-specific toxicities encountered (1). In the preclinical space, test article–related nephrotoxicity most commonly manifests as renal tubular lesions, although glomerular lesions have been noted more frequently in recent years with the increased investigation of biotherapeutics (2). Standard components of preclinical toxicity studies, including renal histopathologic assessment and the serum biomarkers creatinine (sCr) and BUN, are often effective in identifying nephrotoxicity, although emerging renal biomarkers may be used in concert to provide additional mechanistic and time-course information to define toxicity further. Urinary biomarkers of renal injury were the first to be qualified under the US Food and Drug Administration’s (FDA) biomarker qualification program; these and other biomarkers have since been utilized extensively in preclinical and clinical studies to enhance understanding of kidney injury and function in experimental models and natural disease states (3–7).

Symmetric dimethylarginine (SDMA) is an emerging biomarker of renal excretory function. SDMA has been shown to outperform creatinine and creatinine-based equations for estimating GFR and has demonstrated promise as a more sensitive biomarker for
assessing renal dysfunction in animals and people compared with other serum biomarkers of renal function (8–16). Likely biologically inert, SDMA is produced in a stable manner from degradation of methylated proteins in nucleated cells, released to the blood after proteolysis, and is subsequently cleared from the plasma primarily by renal excretion with little to no renal metabolism reported (10,17). SDMA has shown promise as a more sensitive biomarker for assessing renal dysfunction in animals and people and is routinely used in diagnostic veterinary medicine (10,17). SDMA has shown promise as a more sensitive biochemical marker because gentamicin-induced nephrotoxicity is well-described on each testing day. Temperatures were chosen to mean SDMA concentration was determined for each sample was then compared with the mean SDMA concentration obtained by LC-MS/MS. Forty individual serum samples were used in the method comparison, and a subset of samples contained spiked exogenous SDMA. Exogenous SDMA was used to ensure sample concentrations spanned the assay analytical range. For spiked samples, volume of exogenous SDMA added was <4% of original specimen volume to minimize dilution effects on sample matrix.

A spike recovery study was also performed to assess for proportional systematic bias attributable to matrix effect. Three pooled rat serum samples were analyzed in triplicate before and after spiking with 10, 40, or 80 µg/dl SDMA and mean SDMA values obtained. The volume of exogenous SDMA added was <6% of original specimen volume to minimize dilution effects on sample matrix. Percent recovery for each sample was determined as follows: ![Percent recovery formula](MathJax_JavaScript.png). Mean recovery percentage was required to be within 100% ± a total error allowable (TEa) of 20%.

Interassay (within laboratory) precision was determined by evaluating pooled rat serum samples at three concentrations (“normal,” ≤9 µg/dl; “cutoff,” 10–20 µg/dl; and “elevated,” 21–100 µg/dl), with two replicates twice a day for 15 days. The normal sample contained endogenous SDMA. In addition, the cutoff and elevated samples contained spiked exogenous SDMA. Percent coefficient of variation (% CV) and SD were determined using all replicate runs on the basis of Clinical and Laboratory Standards Institute (EP5-A2) guidelines. For SDMA, the precision criteria for validation were: ≤20% CV from limit of quantification (LOQ) to 9 µg/dl, ≤15% CV between 10 and 20 µg/dl, and ≤8% CV between 21 and 100 µg/dl.

Analytical sensitivity of the assay was determined by measuring the limit of blank (LOB), limit of detection (LOD), and LOQ. The LOD was determined as meanblank+1.645(SDblank) by assaying four replicates of 0.9% NaCl solution per day for 15 days. The LOD was determined as LOB+1.645 (SDlow concentration sample) by assaying six samples containing SDMA ranging from 2 to 9 µg/dl, four replicates per day for 15 days. The mean value of different reagent lots was used to determine the LOB and LOD. LOQ was determined to be the SDMA concentration at or above the LOD with precision of ≤30% CV. To define the analytical measurement range further, linearity was assessed over the SDMA concentration range of LOQ to 100 µg/dl using mean sample concentrations within this range as documented in the comparison of methods study. The stability of SDMA in serum samples from ten rats was evaluated. Samples were stored at −20°C, 4°C, and 25°C and assayed in triplicate at 0, 2, and 7 days of storage. A mean SDMA concentration was determined for each sample on each testing day. Temperatures were chosen to reflect temperatures commonly encountered in shipment or laboratory storage or processing.

For interference testing, assay interference by lipids, bilirubin, and hemoglobin at physiologically relevant concentrations was evaluated. Pooled rat serum samples with normal (approximately 8 µg/dl) or increased

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### Materials and Methods

#### SDMA Immunoassay Validation

All analytical validation testing used methods that were based on the best practices of IDEXX Laboratories, Inc., and on the Clinical and Laboratory Standards Institute guidelines (22–24). A high-throughput immunoassay to measure SDMA (IDEXX SDMA Test; IDEXX Laboratories, Inc., Westbrook, ME) was evaluated in this study. Accuracy was evaluated using a comparison of methods study, and liquid chromatography tandem mass spectrometry (LC-MS/MS) was considered the reference method (13,19,25). The concentration of SDMA was measured in triplicate for each individual rat sample using LC-MS/MS following previously described methods. The samples were also evaluated in duplicate using the immunoassay on a Beckman Coulter AU680 (Brea, CA). Mean SDMA concentration by immunoassay for each sample was then compared with the mean SDMA concentration obtained by LC-MS/MS.
analyses was also evaluated. Arginine (cat. no. A5006; Sigma–Aldrich) at physiologically relevant concentrations was used to create a spiked sample with 25 mg/dl arginine. ADMA (cat. no. D4269; Sigma–Aldrich) was used to create a spiked sample with 50 mg/dl ADMA. L-MMA (cat. no. 475886; Millipore, Burlington, MA) was used to create a spiked sample with 50 mg/dl L-MMA.

To assess interference by lipids, 20% intralipid emulsion (cat. no. 201102; Calbiochem) was used to create a sample spiked with 25 mg/dl bilirubin. For hemoglobin, EDTA anticoagulated canine whole blood was used to create spiked samples at hemoglobin concentrations of 100, 250, and 500 mg/dl. Interference from related compounds (arginine, asymmetric dimethylarginine [ADMA], and Nω-L monomethyl arginine [L-MMA]) at physiologically relevant concentrations was also evaluated. Arginine (cat. no. A5006; Sigma–Aldrich) was used to create a spiked sample with 25 mg/dl arginine. ADMA (cat. no. D4269; Sigma–Aldrich) was used to create a spiked sample with 50 µg/dl ADMA. L-MMA (cat. no. 475886; Millipore, Burlington, MA) was used to create a spiked sample with 50-µg/dl L-MMA.

Animals

A total of 80 male Sprague Dawley CD IGS rats (Charles River Laboratories, Raleigh, NC), approximately 7–9 weeks old and weighing 150–350 g, were used in the study. The study was conducted at Covance Laboratories, Inc. (Greenfield, IN). Rats were group housed in a limited access animal facility. Temperature and relative humidity were maintained between 20°C and 26°C, a relative humidity of 30%–70%, and a 12-hour/12-hour light/dark cycle. Rats were acclimatized for a minimum of 3 days before onset of treatment. Greenfield city water and a certified rat diet (#2014C; Envigo RMS, Inc., Madison, WI) were supplied ad libitum. Rats were given various cage-enrichment devices and dietary enrichment on full feeding days.

Facilities and Animal Use Statement

The Institutional Animal Care and Use Committee at Covance Laboratories reviewed and approved the study (protocol 8373371, approved November 2017). This study was conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and the animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals. For SDMA immunoassay analytical validation, samples were obtained from a bank maintained at the IDEXX BioAnalytics facility (Columbia, MO) containing residual aliquots of serum originally collected for serologic testing of rats to determine health status. Serum samples from male and female rats of various inbred strains and outbred stocks ranging in age from 1.5 to 18 months were used for components of assay validation. Residual serum aliquots were from blood samples originally obtained after rats were euthanized for postmortem examinations. Serum was collected into tubes without additive and stored at −80°C for a period not exceeding 1 year before analysis. The IDEXX BioAnalytics-Columbia MO facility is part of an AAALAC-accredited research animal care and use program at the University of Missouri. Studies involving animals were reviewed and approved by the University of Missouri’s Animal Care and Use Committee.

Test Material and Treatment Protocol

Three preparations of a gentamicin sulfate solution, with concentrations of 10, 25, and 50 mg/ml, were used in this study. A vehicle solution containing 0.5% hydroxypropyl methylcellulose (w/v), 0.2% polysorbate 80 (w/v), in purified water was used as vehicle control.

The study design is detailed in Table 1. Rats were randomly assigned to treatment group using a computerized procedure designed to achieve body weight balance with respect to group assignment. Rats were dosed at a volume of 2 ml/kg to provide dose levels of 0 (vehicle), 20, 50, or 100 mg of gentamicin per kilogram. Doses were based on the most recently recorded scheduled body weight. The subcutaneous dose was administered via syringe and needle in the mid-scapular region (alternating left and right sides). The dose site was clipped free of hair before dosing. Rats were dosed once daily for four or ten consecutive days depending on treatment group. Experimental approach, including dosing schedule, route of administration, and volume and concentration of gentamicin, were similar to other published gentamicin-induced acute nephrotoxicity models in rats (20,21).

| Treatment Group | Test Article | Dose, mg/kg | Route | Number of Rats per Group (N=80) |
|----------------|--------------|-------------|-------|---------------------------------|
|                |              |             |       | 4 Doses | 10 Doses |
| 1              | Vehicle      | 0           | sc    | 10       | 10       |
| 2              | Gentamicin   | 20          | sc    | 10       | 10       |
| 3              | Gentamicin   | 50          | sc    | 10       | 10       |
| 4              | Gentamicin   | 100         | sc    | 10       | 10       |

sc, subcutaneous injection.
Body Weight, Clinical Observations, and Mortality
Each rat was weighed at the time of allocation to treatment group, and on days 1, 4, 7, and 10. Rats were observed twice daily to evaluate food and water intake and for signs of illness. Any change in food and water intake or signs of clinical illness were recorded.

Urine and Blood Collection
The night before necropsy, rats were fasted and placed in individual metabolic cages. During this period, urine was collected chilled for 12–16 hours and then submitted to the clinical pathology laboratory for urine creatinine (uCr) and urine biomarker measurement. On the day of necropsy, rats were anesthetized with isoflurane by inhalation. Blood for hematologic analysis and clinical chemistry determinations was obtained from the orbital plexus. Blood for complete blood counts was collected into tubes containing K2EDTA. Blood for clinical chemistry parameter measurements was collected in tubes without anticoagulant. Blood intended for biomarker analysis was obtained from the abdominal aorta and collected in tubes without anticoagulant.

Euthanasia and Necropsy
After collection of blood for hematology, clinical chemistry, and creatinine clearance, rats were euthanized using isoflurane anesthesia and exsanguination, and a complete necropsy was performed.

Clinical Pathology and Kidney Biomarkers
Hematologic analysis consisted of complete blood counts and included: erythrocyte, reticulocyte, total and differential leukocyte, and platelet counts; hematocrit; hemoglobin concentration; mean corpuscular hemoglobin (MCH); MCH concentration (MCHC); and mean corpuscular volume determined using an ADVIA 120 Hematology System with Multispecies Software (v3.1; Siemens Medical Solutions, Norwood, MA) and Siemens reagents. Differential leukocyte counts and blood cell morphology were reviewed using Wright-Giemsa stained (ADVIA S60 Auto Slide Stainer; Siemens Medical Solutions) blood smears.

Clinical chemistry parameters measured in serum included concentrations of albumin, calcium, chloride, cholesterol, creatinine, glucose, inorganic phosphorus, potassium, urea, sodium, total bilirubin, total protein, triglycerides, and activities of alanine aminotransferase, alkaline phosphatase, \( \gamma \)-glutamyltransferase, aspartate aminotransferase, and creatine kinase using a Modular P Analyzer (Roche Diagnostics, Nutley, NJ) and Roche reagents. The concentration of globulin and the albumin/globulin ratio were calculated.

uCr was measured using the same methodology as sCr, and these values were used to determine creatinine clearance via standard methodology (26). Creatinine clearance was adjusted for body weight and reported as milliliters per minute per kilogram. Complete urinalysis (urine volume, color, clarity, specific gravity, pH, protein, blood, ketones, glucose, bilirubin, urobilinogen, and sediment examination) was performed using standard methods.
| Doses | Treatment Group | N  | Creatinine Clearance, ml/min per kg | Creatinine, mg/dl | BUN, mg/dl | Cystatin C, ng/ml | SDMA, µg/dl | µALB, µg/mg | Clusterin, ng/mg | Cystatin C, ng/mg | KIM-1, pg/mg | NGAL, ng/mg | Osteopontin, ng/mg |
|-------|-----------------|----|------------------------------------|-------------------|--------|-----------------|----------|--------|---------------|----------------|-------------|-----------|-------------------|
| 4     | Vehicle         | 10 | 3.80±0.71                          | 0.52±0.04         | 7.4±1.7 | 1704±409.5      | 8.9±1.1  | 1.7±0.1 | 3.1±1.1       | 77.5±29.9       | 1263.8±188.9 | 900.5±373.6 | 368.9±92          |
|       | 20 mg/kg gentamicin  | 10 | 3.37±0.72                          | 0.54±0.05         | 7.6±2.2 | 1567.2±194.5    | 7.9±1.4  | 1.3±0.4 | 135.4±414.3  | 61.7±32.7       | 1762.2±444.9 | 451.2±295  | 434.4±118.6       |
|       | 50 mg/kg gentamicin | 10 | 3.26±0.79                          | 0.58±0.04         | 7.8±1.6 | 1578.9±488.1    | 10.6±2.1 | 1.5±0.3 | 524.6±865.5  | 103.6±30.3      | 1940.5±467.6  | 249.2±299.2 | 504±134.3         |
|       | 100 mg/kg gentamicin | 10 | 3.02±0.85                          | 0.57±0.05         | 9.6±2.2 | 1547.6±254.6    | 11.7±1.9 | 1.7±0.4 | 2871.6±762.5 | 312.4±95.4      | 2615.0±777.9 | 988.4±597.5 | 730.5±301.9       |
| 10    | Vehicle         | 10 | 3.60±0.99                          | 0.55±0.05         | 7.5±1.4 | 1513.3±430.1    | 9.8±1.5  | 1.7±0.2 | 170.5±528.1  | 63.8±21.7       | 1501.7±425.9 | 798±320.2  | 498.5±204.9        |
|       | 20 mg/kg gentamicin | 10 | 4.15±0.52                          | 0.55±0.05         | 8.8±1.1 | 1897.4±499.3    | 9.7±1.1  | 1.2±0.5 | 1291.7±1413.1 | 135.2±26.2      | 1515.4±207.6 | 1270.4±430.9 | 488±144           |
|       | 50 mg/kg gentamicin | 10 | 3.37±0.33                          | 0.67±0.05         | 10.9±2.5 | 1797.7±410.7    | 14±2.2   | 2±1.2   | 7381.7±6549.1 | 653.9±308.1     | 3526.6±1020.4 | 13514.8±4963.7 | 1259±399.8       |
|       | 100 mg/kg gentamicin | 10 | 1.22±1.01                          | 3.43±2.62         | 69.8±28.3 | 5560.8±2369.4   | 60.3±40.5 | 31756.9| 13079.4| 2238.4±920.9 | 16024±6830.6    | 37059.7±23964.8 | 6379.3±6618.2 | 286.7±157.2       |

Data are presented as the mean±SD. SDMA, symmetric dimethylarginine; µALB, microalbumin; KIM-1, kidney injury molecule 1; NGAL, neutrophil gelatinase-associated lipocalin. Values normalized to urine creatinine. Gentamicin-treated group is significantly different (P<0.02) from vehicle control group at the same number of doses using a pairwise one-sided Wilcoxon rank sum test after a significant Kruskal–Wallis test.
Concentration of the urine biomarkers μALB, CLU, cystatin C, KIM-1, NGAL, and OPN was measured. Urine biomarker results were normalized to uCr concentration. Urine cystatin C, μALB, and NGAL were measured using the Luminex (Fl-labeled beads) platform. Urine CLU was performed on a Biovendor ELISA (RD391034200CS; Asheville, NC). Testing of serum cystatin C was conducted using a Biovendor ELISA (RD391009200R). Urine KIM-1 and urine OPN were performed using R&D Systems ELISAs (RKM100 for KIM1 and MOST00 for OPN; Minneapolis, MN). Serum SDMA was measured using the IDEXX SDMA Test on a Beckman Coulter AU series chemistry analyzer.

Anatomic Pathology

Both kidneys were excised and examined grossly. The right kidney was fixed in 10% neutral-buffered formalin. Kidney sections cut at 5 μm were stained with hematoxylin and eosin and periodic acid–Schiff stains. Histopathologic evaluation was performed on the right kidney on all rats in all groups by a board-certified veterinary pathologist, and results were peer-reviewed by an additional board-certified veterinary pathologist. The histologic evaluation of the kidney was made by a subjective description of microscopic changes. Similar to other renal microscopic grading schemes used in assessment of gentamicin-induced injury, scoring focused predominately on changes observed within the renal tubules (21). The five-point severity grading score outlined by the Critical Path Institute’s Predictive Safety Testing Consortium Nephrotoxicity Working Group was utilized to assign histopathologic scores ranging from no or equivocal lesions or alterations, minimal, slight, moderate, marked, or severe changes (27,28).

Laboratories

Clinical chemistry, hematology, urinalysis, and uCr concentration were measured following standard methods at Covance Laboratories, Inc. Anatomic pathology (gross and microscopic examination) was evaluated at Covance Laboratories, Inc. Urine biomarkers for KIM-1, NGAL, and urine cystatin C and μALB were tested at Charles River Laboratories (Mattawan, MI). Urine biomarker levels for CLU and OPN were measured at Eli Lilly and Company (Indianapolis, IN). Serum Cystatin C was measured at Covance Laboratories, Inc. SDMA was measured at IDEXX Laboratories, Inc.

Statistical Analyses

Correlations between markers were computed using Kendall’s τb, a nonparametric correlation coefficient on the basis of the ranks of result values. Kendall’s τb was chosen over the more familiar Pearson’s r for its ability to assess correlations of both ordinal markers and markers with nonlinear associations. Resulting correlations were categorized as weak (τb<0.30), moderate (0.30≤τb<0.70), or strong (0.70≤τb<1.00). For serum and urine kidney biomarkers including creatinine clearance, a Kruskal–Wallis test was used to determine whether analyte values differed between treatment levels for a given number of doses. When the Kruskal–Wallis test was significant (P<0.05) for a biomarker and dose count, pairwise one-sided Wilcoxon rank sum tests were used to determine which, if any, treatment level(s) differed from the control. One-sided tests were chosen because treatment was expected to increase biomarker values over controls for all markers except creatinine clearance, which was expected to decrease with respect to the control group. For complete blood count and clinical biochemistry data.

Figure 2. | Creatinine clearance (ml/min per kilogram) by group and dose in control and gentamicin-treated rats. Outlier boxplot: Horizontal line within the box represents the median sample value, box represents IQR, whiskers extend to 1.5×IQR. *Treatment group is significantly different (P≤0.02) from vehicle control group at the same number of doses using a one-sided Wilcoxon rank sum test after a significant Kruskal–Wallis test. IQR, interquartile range.

Creatinine Clearance (ml/min/kg)

| Vehicle | 20 mg/kg | 50 mg/kg | 100 mg/kg |
|---------|---------|---------|-----------|
| 4 Doses |         |         |           |
| 10 Doses|         |         |           |

* Treatment group is significantly different (P<0.02) from vehicle control group at the same number of doses using a one-sided Wilcoxon rank sum test after a significant Kruskal–Wallis test.
presented in the Supplemental Material, pairwise two-sided Wilcoxon rank sum tests were used after a significant (P<0.05) Kruskal–Wallis test because the direction of change was not hypothesized in advance. For kidney biomarkers, complete blood count, and clinical biochemistry data, a Bonferroni correction (α<0.05, m=3) was applied to all Wilcoxon rank sum tests to offset the false detection rate associated with multiple comparisons. Weight data were modeled using a mixed effects linear model with fixed effects of day and treatment and a random intercept for each animal. R v4.0.0 (The R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analysis. For complete blood count, clinical chemistry, and body weight data, statistically significant differences reported in the results were further defined by percent change from vehicle control at the same number of doses using a one-sided Wilcoxon rank sum test after a significant Kruskal–Wallis test. IQR, interquartile range.

Figure 3. | Urine renal biomarkers by group and dose in control and gentamicin-treated rats. (A) Microalbumin (μg/mg); (B) clusterin (ng/mg); (C) cystatin C (ng/mg); (D) kidney injury molecule 1 (pg/mg); (E) neutrophil gelatinase-associated lipocalin (ng/mg); (F) osteopontin (ng/mg). Biomarker data are expressed as urine biomarker concentration/urine creatinine (mg/dl). Horizontal line within the box represents the median sample value, box represents IQR, whiskers extend to 1.5×IQR. *Treatment group is significantly different (P<0.02) from vehicle control group at the same number of doses using a one-sided Wilcoxon rank sum test after a significant Kruskal–Wallis test. IQR, interquartile range.

accuracy of the immunoassay compared with the reference standard in the comparison of methods study. TEa was used as a quality requirement for immunoassay method performance, with a value of 20% set for TEa at predetermined decision limits. Decision limits for total error were chosen at a cutoff SDMA concentration (e.g., 10–20 μg/dl) to reflect SDMA values spanning the range of expected and elevated, and at an elevated SDMA concentration (e.g., 21–100 μg/dl). For method acceptance, the total error observed (TEobs) was required to be less than the predetermined TEa of 20% at each of the decision limits evaluated. TEobs was calculated by the formula 2×CV (%) + Bias (%). Coefficient of variation was the relevant CV for the decision limit evaluated, as determined in the interassay precision study. Bias at each decision limit was determined as follows: absolute value [(SDMAnew method–SDMAreference method)/SDMAreference method]×100. SDMAnew method represented the mean immunoassay value of SDMA at each decision limit, and the corresponding SDMAreference method as...
determined by LC-MS/MS in the comparison of methods study. The effects of six interfering substances were evaluated using a multiple linear regression model. The six interferents evaluated were lipids, bilirubin, hemoglobin, arginine, ADMA, and MMA. The interferent effects were evaluated using two SDMA pooled samples at different target concentrations: approximately 8 \text{ mg/dl} (“normal”) and approximately 55 \text{ mg/dl} (“increased”). Acceptable bias was defined as ±2.91 \text{ mg/dl} for the normal sample pool and ±3.63 \text{ mg/dl} for the increased sample pool. The two one-sided test procedure was used to test whether SDMA concentration in samples containing interferents was equivalent to those without, i.e., whether there was evidence that the estimated bias was contained within the above defined range of acceptable bias. Sample stability was assessed using samples from ten individual patients. These samples were run in triplicate immediately to establish a baseline/control concentration and then aliquoted into four tubes for future testing. Then, one of each of the four tubes for each patient were stored at −20°C, 4°C, 25°C, and 45°C. Each aliquot was then run in triplicate after two days’ storage and seven days’ storage at the assigned temperature. A linear mixed effect model was used to estimate time and temperature interaction fixed effects with sample ID random effect. Contrasts of the resulting parameter estimates were used to determine whether the difference in SDMA concentration was statistically significant.

### Results

#### Body Weight, Clinical Observations, and Mortality

No mortality or signs of clinical illness were observed in any of the rats during gentamicin treatment, irrespective of dose administered. All control and treatment groups demonstrated weight gain over the course of the study with the exception of the 100-mg/kg ten-dose group (Supplemental Table 1). A minimal (9%) decrease in body weight at dosing day 10 was observed in the 100 mg/kg group compared with the vehicle control (Supplemental Table 1).

#### Kidney Biomarkers

Data for the serum excretory renal function biomarkers (sCr, BUN, SDMA, and cystatin C) in gentamicin-treated and control rats are shown in Figure 1 and Table 2. For the four-dose treatment groups, serum cystatin C and BUN levels were not significantly different between gentamicin-treated and control rats at any of the dose levels, whereas sCr and SDMA levels were significantly increased (P≤0.02) at both the 50- and 100-mg/kg dose levels when compared with controls. For the ten-dose treatment groups, sCr, SDMA, and BUN were significantly increased (P≤0.02) at the 50- and 100-mg/kg dose levels, whereas cystatin C was significantly increased (P≤0.02) at the 100 mg/kg dose level only when compared with controls. For creatinine clearance there was no significant difference between any of the treatment groups and the vehicle control group with the four-dose regimen (Figure 2, Table 2). With the ten-dose regimen, creatinine clearance was significantly decreased (P≤0.02) at the 100-mg/kg dose level compared with the control group (Figure 2, Table 2).

Data for the six urine renal biomarkers (μALB, CLU, cystatin C, KIM-1, NGAL, and OPN) in gentamicin-treated and control rats are shown in Figure 3 and Table 2. In the four-dose groups, gentamicin-treated rats had a significant increase (P≤0.02) in urine μALB and cystatin C at all dose levels, for NGAL at the 50- and 100-mg/kg dose levels, and for CLU at the 100-mg/kg dose level only compared with controls. Urinary KIM-1 and OPN did not increase significantly in gentamicin-treated rats compared with controls (Figure 3, Table 2). In the ten-dose groups, gentamicin-treated rats had significantly increased (P≤0.02) urine μALB, KIM-1, and CLU at all dose levels, for NGAL and cystatin C at the 50- and 100-mg/kg dose levels, and for OPN at the 100-mg/kg dose level only compared with controls (Figure 3, Table 2).

#### Clinical Pathology

On complete blood count, statistically significant differences in parameters were observed mostly in the 100 mg/kg ten-dose gentamicin-treated group compared

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**Table 3. Kidney microscopic alterations after treatment with gentamicin in rats**

| Number of male rats | 10 | 10 | 10 | 10 | 10 | 10 |
|---------------------|----|----|----|----|----|----|
| Number of doses     | 4 doses | 10 doses | 4 doses | 10 doses | 4 doses | 10 doses |
| Microscopic alteration | 4 MI; 9 MI; 1 SL | 7 MI | 4 MI; 5 MO; 1 MA | 8 MI; 1 SL | 8 MA; 2 SE |
| Dose, mg/kg         | 20 | 50 | 100 | 20 | 50 | 100 |
| Number of rats affected, severity score | Basophilic tubules | 4 MI; 9 MI; 1 SL | 7 MI | 4 MI; 5 MO; 1 MA | 8 MI; 1 SL | 8 MA; 2 SE |
| Degeneration, tubes | – | – | – | 7 MI; 2 SL; 1 MO | – | 1 MI; 2 MO; 4 MA; 3 SE |
| Necrosis, tubules   | 1 MI; 3 MI; 3 MI | 7 MI; 6 SL | 5 MI | 3 MI; 3 MO; 4 MA |
| Cast, granular      | – | 9 MI; 1 SL | – | 3 MI; 3 MO; 4 MA |
| Cast, proteinaceous | – | – | 4 MI | – | 1 MI; 6 SL; 3 MA |
| Hyaline droplets    | – | – | – | 5 MI; 3 MO; 2 MA |
| Infiltrate, mixed cells | 8 MI; 3 MI | 1 MI; 6 SL; 3 MO | 9 MI | 2 MI; 3 MO; 5 MA |
| Mineralization      | – | – | – | 1 MI; 2 MO |

Severity grading scale: –, equivocal change or finding not observed; MI, minimal; SL, slight; MO, moderate; MA, marked; SE, severe.
### Table 4. Kendall’s $\tau_b$ correlation matrix

|                       | Creatine clearance, ml/min/kg | sCr, mg/dl | BUN, mg/dl | SDMA, $\mu$g/dl | Serum cystatin C, ng/ml | Clusterin, ng/mg | Osteopontin, ng/mg | KIM-1, pg/mg | NGAL, ng/mg | $\mu$ALB, ng/mg | mALB, mg/mg | Basophilic tubule | Cast, granular | Cast, proteinaceous | Degeneration, tubule | Dilatation, tubule | Hyaline droplet, tubule | Cell Infiltrate | Necrosis |
|-----------------------|-------------------------------|------------|------------|----------------|------------------------|-----------------|-------------------|---------------|-------------|-------------------|---------------|------------------|---------------|------------------|------------------|----------------|--------------------|-------------|---------|
| Creatinine clearance  | 1                             | 0.54       | 0.47       | 0.55          | 0.44                   | 0.37            | 0.34              | 0.47          | 0.39        | 0.41              | 0.32          | 0.27             | 0.63           | 0.53             | 0.54             | 0.59             | 0.28             | 0.39             | 0.41             | 0.42             | 0.52             | 0.54             |
| sCr, mg/dl            |                               | -0.54      | 1          |               |                        |                 |                   |               |             |                   |               |                  |               |                  |                  |                  |                   |                  |                 |                 |                 |                 |
| BUN, mg/dl            |                               | 0.47       | 1          |               |                        |                 |                   |               |             |                   |               |                  |               |                  |                  |                  |                   |                  |                 |                 |                 |                 |
| SDMA, $\mu$g/dl       |                               | 0.55       | 0.44       | 1             |                        |                 |                   |               |             |                   |               |                  |               |                  |                  |                  |                   |                  |                 |                 |                 |                 |
| Serum cystatin C, ng/ml |                             | 0.44       | 0.37       | 0.34          | 0.24                   | 1               |                   |               |             |                   |               |                  |               |                  |                  |                  |                   |                  |                 |                 |                 |                 |
| Clusterin, ng/mg      |                               | 0.37       | 0.34       | 0.24          | 0.17*                  | 0.47            | 1                 |               |             |                   |               |                  |               |                  |                  |                  |                   |                  |                 |                 |                 |                 |
| Osteopontin, ng/mg    |                               | 0.34       | 0.29       | 0.48          | 0.36                   | 0.55            | 0.48              | 0.48          | 0.39        | 0.58              | 0.48          | 0.36             | 0.63           | 0.53             | 0.55             | 0.48             | 0.48             | 0.48             | 0.48             | 0.54             | 0.54             |
| KIM-1, pg/mg          |                               | 0.47       | 0.49       | 0.42          | 0.55                   | 0.41            | 0.59              | 0.41          | 0.59        | 0.41              | 0.59          | 0.41             | 0.59           | 0.59             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             |
| NGAL, ng/mg           |                               | 0.54       | 0.25       | 0.32          | 0.10*                  | 0.45            | 0.49              | 0.49          | 0.49        | 0.45              | 0.52          | 0.45             | 0.45           | 0.46             | 0.53             | 0.56             | 0.56             | 0.56             | 0.56             | 0.56             |
| $\mu$ALB, ng/mg       |                               | 0.48       | 0.28       | 0.35          | 0.35                   | 0.44            | 0.62              | 0.44          | 0.59        | 0.41              | 0.59          | 0.41             | 0.59           | 0.59             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             |
| mALB, mg/mg           |                               | 0.56       | 0.61       | 0.61          | 0.55                   | 0.53            | 0.48              | 0.53          | 0.53        | 0.48              | 0.54          | 0.54             | 0.54           | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             |
| Basophilic tubule     |                               | 0.48       | 0.52       | 0.61          | 0.61                   | 0.46            | 0.46              | 0.52          | 0.52        | 0.52              | 0.52          | 0.52             | 0.52           | 0.52             | 0.55             | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             |
| Cast, granular        |                               | 0.54       | 0.46       | 0.45          | 0.45                   | 0.58            | 0.54              | 0.58          | 0.53        | 0.45              | 0.45          | 0.45             | 0.45           | 0.46             | 0.56             | 0.56             | 0.56             | 0.56             | 0.56             | 0.56             |
| Cast, proteinaceous   |                               | 0.54       | 0.52       | 0.55          | 0.55                   | 0.53            | 0.54              | 0.53          | 0.53        | 0.54              | 0.54          | 0.54             | 0.54           | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             | 0.54             |
| Degeneration, tubule  |                               | 0.56       | 0.61       | 0.61          | 0.61                   | 0.46            | 0.46              | 0.46          | 0.46        | 0.46              | 0.46          | 0.46             | 0.46           | 0.46             | 0.46             | 0.46             | 0.46             | 0.46             | 0.46             | 0.46             |
| Dilatation, tubule    |                               | 0.54       | 0.61       | 0.61          | 0.61                   | 0.45            | 0.45              | 0.58          | 0.58        | 0.58              | 0.58          | 0.58             | 0.58           | 0.58             | 0.58             | 0.58             | 0.58             | 0.58             | 0.58             | 0.58             |
| Hyaline droplet, tubule|                               | 0.48       | 0.62       | 0.62          | 0.62                   | 0.54            | 0.54              | 0.47          | 0.47        | 0.47              | 0.47          | 0.47             | 0.47           | 0.47             | 0.55             | 0.55             | 0.55             | 0.55             | 0.55             | 0.55             |
| Cell Infiltrate       |                               | 0.54       | 0.62       | 0.62          | 0.62                   | 0.62            | 0.62              | 0.62          | 0.62        | 0.62              | 0.62          | 0.62             | 0.62           | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             |
| Necrosis              |                               | 0.54       | 0.62       | 0.62          | 0.62                   | 0.62            | 0.62              | 0.62          | 0.62        | 0.62              | 0.62          | 0.62             | 0.62           | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             | 0.62             |

sCr, serum creatinine; SDMA, symmetric dimethylarginine; KIM-1, kidney injury molecule 1; NGAL, neutrophil gelatinase-associated lipocalin; $\mu$ALB, microalbumin.

*Not significant as estimation of confidence interval included zero.
with vehicle control (Supplemental Table 2). These changes consisted of a marked (77%) decrease in reticulocyte counts, moderate (109%) increase in absolute neutrophil counts, moderate (166%) increase in absolute monocyte counts, slight (20%) increase in platelet counts, minimal (7%) decrease in mean corpuscular volume, and a minimal (4%) increase in MCHC in the 100-mg/kg ten-dose treatment group. In the 50-mg/kg ten-dose treatment group, a slight (21%) increase in platelet counts and a slight (24%) decrease in reticulocyte counts were observed compared with vehicle control (Supplemental Table 2). In rats treated with four daily doses of gentamicin, the only statistical differences noted between gentamicin-treated and control rats occurred in the 100 mg/kg treatment group and consisted of a minimal (12%) decrease in reticulocyte counts and a moderate (33%) decrease in absolute neutrophil count compared with controls. On clinical chemistry evaluation, statistically significant differences in biochemistry analytes were confined to the 100-mg/kg ten-dose gentamicin-treated group (Supplemental Table 3). Changes consisted of a minimal (80%) increase in aspartate aminotransferase activity, minimal (14%) decrease in chloride concentration, minimal (6%) decrease in sodium concentration, and a minimal (6%) decrease in potassium concentration in the 100-mg/kg ten-dose gentamicin-treated group compared with vehicle control (Supplemental Table 3).

On urinalysis, salient findings included the presence and magnitude of proteinuria and ketonuria, which were greatest in the 100-mg/kg ten-dose gentamicin-treated group, although statistics were not applied to urinalysis results (Supplemental Table 4). Rats treated with four daily doses of gentamicin had no salient differences noted in urinalysis values compared with vehicle controls.

**Anatomic Pathology**

The changes in kidney microscopic appearance in gentamicin-treated rats are listed in Table 3. Microscopic changes in the kidney were dose and time dependent. Minimal infiltration of mixed population of inflammatory cells, minimal degeneration and necrosis of renal tubules, and minimal to slight basophilic tubules occurred in the four-dose treatment group at the ≥20-mg/kg dose level and at 20 mg/kg in the ten-dose treatment group. Microscopic changes in the ten-dose treatment group at the ≥50-mg/kg dose level consisted of minimal to severe degeneration and necrosis of renal tubules, slight to severe basophilic tubules (regeneration), minimal to severe dilation of renal tubules, minimal to marked granular casts, minimal to moderate proteinaceous casts, and minimal to marked infiltration of mixed population of inflammatory cells (mononuclear and neutrophils). Degeneration and necrosis of proximal tubular epithelial cells were mostly concentrated in the cortex, frequently extended into medulla, and occasionally affected papilla of the kidney. Degeneration and necrosis were characterized by hypereosinophilia, vacuolation, or fragmentation of cytoplasm, pyknotic nuclei, or sloughed epithelial cells into the lumens, and occasional mineralization. The dilated tubules were lined by flattened epithelial cells and filled with degenerated eosinophilic granular cell debris or

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**Figure 4.** Accuracy of the SDMA immunoassay compared with liquid chromatography tandem mass spectrometry reference method for determination of SDMA in rat serum using Passing and Bablok regression. Slope=0.99 (95% confidence interval, 0.97 to 1.01), N=40 samples. SDMA, symmetric dimethylarginine.

| Sample | Immunoassay Mean, µg/dl | Symmetric Dimethylarginine Spiked, µg/dl | Spiked Recovery, µg/dl | Total Spike Recovery % | Total Mean Recovery % |
|--------|-------------------------|----------------------------------------|------------------------|------------------------|-----------------------|
| 1      | 7.9                     | N/A                                    | N/A                    | N/A                    | 104.9                 |
|        | 17.7                    | 10                                     | 9.8                    | 98                     |
|        | 52.8                    | 40                                     | 44.8                   | 112.1                  |
|        | 91.7                    | 80                                     | 83.8                   | 104.7                  |
| 2      | 8.4                     | N/A                                    | N/A                    | N/A                    | 106.9                 |
|        | 18.6                    | 10                                     | 10.2                   | 102                    |
|        | 52.8                    | 40                                     | 44.3                   | 110.8                  |
|        | 94.7                    | 80                                     | 86.3                   | 107.9                  |
| 3      | 8                       | N/A                                    | N/A                    | N/A                    | 116.6                 |
|        | 20.6                    | 10                                     | 12.5                   | 125.3                  |
|        | 54.3                    | 40                                     | 46.3                   | 115.7                  |
|        | 95.2                    | 80                                     | 87.1                   | 108.9                  |

Neat and SDMA spiked aliquots from each sample were analyzed in triplicate to obtain immunoassay mean values. N/A, not applicable. SDMA, symmetric dimethylarginine.
Correlations between Kidney Biomarkers and Pathology Scores

Kendall’s $\tau_b$ correlations for the results of creatinine clearance, serum and urine biomarkers, and microscopic pathology scores are presented in a correlation matrix (Table 4). Significant ($P<0.01$) moderate to strong positive correlations were found between sCr and all urinary biomarkers and the microscopic pathology scores with the exception of a weak correlation between sCr and OPN. Significant ($P<0.01$) moderate positive correlations were found between BUN and all urinary biomarkers and microscopic pathology scores. Significant ($P<0.01$) moderate positive correlations were found between SDMA and all urinary biomarkers and microscopic pathology scores. Significant ($P<0.01$) but weak to moderate positive correlations were found between serum cystatin C and the urinary biomarkers and microscopic pathology scores with the exception of NGAL, for which no significant correlation was noted with serum cystatin C. Significant ($P<0.01$) but weak to moderate negative correlation was found between creatinine clearance and all of the biomarkers except for serum cystatin C, for which no significant correlation with creatinine clearance was identified.

SDMA Immunoassay Analytical Validation

Accuracy of the SDMA immunoassay was assessed against LC-MS/MS reference method. A Passing and Bablok linear regression analysis of a scatter plot, representing mean SDMA concentrations from individual rat samples measured by LC-MS/MS compared with immunoassay, had a slope of 0.99 (95% confidence interval [CI], 0.97 to 1.01), an intercept of −0.53 (95% CI, −1.22 to 0.15), and a $R^2$ of 0.1 (Figure 4). Additionally, spiked recoveries were between 105% and 117% for each individual sample tested (Table 5) and within 100±TEa (80%–120%). Precision of the assay was found to be <1.2 SD from LOQ to 100 $\mu$g/dl (Table 6). At predetermined decision limits for SDMA concentrations, the assay % CV was 17% at 5.7 $\mu$g/dl (“normal”), 6% at 15.3 $\mu$g/dl (“cutoff”), and 2% at 57.6 $\mu$g/dl (“elevated”). TEobs was within TEa (<20%) at the cutoff and elevated decision limits evaluated, with values of 16% and 4%, respectively. The sensitivity testing of the assay showed that the mean LOB for three different reagent lots was 1.1 $\mu$g/dl and that the LOD mean for three different reagent lots was 1.5 $\mu$g/dl. The low end of the analytical measurement range was defined as 3.4 $\mu$g/dl (Table 7). The analytical measurement range for rats was determined to be 3–100 $\mu$g/dl. SDMA concentrations from ten individual serum samples were found to be stable after 2 and 7 days of storage at −20°C, 4°C, and 25°C (Figure 5). No interference from bilirubin was observed at any level. The measurable SDMA concentration was significantly decreased compared with control in moderate and high hemoglobin or lipid spiked samples (250 and 500 mg/dl; $P<0.05$; Figure 6). For the related compounds such as arginine, L-MMA, and ADMA, only the high spiked ADMA sample (50 $\mu$g/dl) resulted in a significantly decreased measurement in SDMA concentration, although results were still within the range of predetermined acceptable bias to be considered equivalent to control (Figure 7).

| Sample, µg/dl | Target, µg/dl | Mean, µg/dl | Total SD | Total % Coefficient of Variation | Total % Coefficient of Variation Criteria |
|---------------|--------------|-------------|----------|---------------------------------|------------------------------------------|
| LOQ-9         | 6            | 5.7         | 0.97     | 17%                             | ≤20%                                     |
| 10–20         | 14           | 15.3        | 0.89     | 6%                              | ≤15%                                     |
| 20–100        | 50           | 57.6        | 1.21     | 2%                              | ≥8%                                      |

$N=60$ measurements from each sample pool. LOQ, limit of quantitation.

Discussion

The SDMA immunoassay demonstrated acceptable performance for measurement of SDMA in rat serum as determined by validation criteria and excellent correlation to the LC-MS/MS reference method. Choice of reference method was based upon peer-reviewed literature, which has established LC-MS/MS as a sensitive, accurate, and rugged method for SDMA determination in serum or plasma matrix as previously investigated in species including rats, mice, dogs, and humans (13,19,29,30). The validated SDMA immunoassay provides a readily accessible high-throughput alternative for measurement of SDMA in the rat. This study provides biologic validation on the performance of serum SDMA as a renal excretory function biomarker in the context of acute renal toxicity in rats. A well-described gentamicin model was used initially to characterize the relationship of serum SDMA response to other biomarkers, including soluble urine and serum biomarkers and histopathologic examination of kidneys, in a preclinical study design. In this study, SDMA was compared with both other serum excretory renal function

Table 6. Precision of the symmetric dimethylarginine immunoassay

| Sample Range | % CV, % Coefficient of Variation Criteria |
|--------------|------------------------------------------|
| LOQ-9        | 17% ≤20%                                 |
| 10–20        | 6% ≤15%                                  |
| 20–100       | 2% ≥8%                                   |

Table 7. Analytical sensitivity of the symmetric dimethylarginine immunoassay

| Analysis               | Average Symmetric Dimethylarginine, µg/dl |
|------------------------|------------------------------------------|
| Limit of blank (LOB)   | 1.1 (n=60)                               |
| Limit of detection (LOD)| 1.5 (n=60)                               |
| Limit of quantitation (LOQ)| 3.4 (at 30% CV)                      |

% CV, percent coefficient of variation.
biomarkers and urinary renal injury biomarkers. Excretory renal function biomarkers such as SDMA, serum cystatin C, BUN, and creatinine increase, whereas creatinine clearance decreases as significant functional renal mass is lost and renal ability to filter and clear these biomarkers from blood is impaired. Newer urinary renal injury biomarkers have been evaluated to compliment renal function biomarkers with biomarkers that are more sensitive or specific to various types of renal injury. Urine cystatin C and μALB are functional biomarkers that can increase in urine with dysfunction or damage of the glomerulus and/or proximal tubules. CLU, KIM-1, NGAL, and OPN are biomarkers that increase in urine in response to renal tubular injury (31). Serum SDMA in this study of gentamicin-induced renal injury was more responsive than other renal excretory function biomarkers, including serum cystatin C, BUN, and creatinine and complimented sCr in detection of gentamicin-induced renal toxicity. There is interest in alternative biomarkers for excretory renal function because serum BUN and creatinine have been found to lack sensitivity, increasing when approximately 50%–60% of renal function has been compromised (32). Whereas in this study serum SDMA and creatinine were both more responsive than the tubular injury markers KIM-1 and CLU for identification of gentamicin-induced renal toxicity in the four-dose treatment groups. The tubular injury biomarkers KIM-1 and CLU have been shown to provide earlier detection of drug-induced renal injury compared with the traditional biomarkers of renal function, sCr and BUN (31,34). Yet, as in our study, some authors have failed to demonstrate enhanced responsiveness of KIM-1 and CLU compared with creatinine in certain gentamicin-induced injury models (35). It is possible these discrepancies reflect variance in experimental design elements, including dose level and schedule, methods of measurement, and biologic variation.

The urinary biomarkers measured in this study were qualified by the FDA for assessment of renal toxicity in rats, and the physiology and relative performance of these biomarkers have been recently reviewed (31,36); information on urinary biomarker variation and expected values in health for Sprague Dawley rats has also been recently published (37). Whereas all serum and urine biomarkers demonstrated weak to moderate correlation with histopathologic changes in kidneys, soluble biomarkers were of variable responsiveness for detection of gentamicin-induced injury or dysfunction across dose levels and between the four- and ten-day dose schedules. Urinary μALB was the most responsive soluble biomarker in this study across both the four- and ten-dose groups and was increased significantly at all dose levels compared with controls. Like μALB, urinary cystatin C was significantly increased in gentamicin-treated animals.

**Figure 5.** SDMA immunoassay sample stability. (A) SDMA sample stability at −20°C. (B) SDMA sample stability at 4°C. (C) SDMA sample stability at 25°C. Storage of rat serum at the temperatures and time periods investigated did not significantly affect measurement by SDMA immunoassay. All testing was done with rat serum samples run in triplicate. Error bars represent 1 SD around the mean. SDMA, symmetric dimethylarginine.
Figure 6. | SDMA immunoassay analyte-independent interference testing—lipids, bilirubin, and hemoglobin. Pooled rat serum samples at “normal” (approximately 8 μg/dl) and “increased” (approximately 50 μg/dl) SDMA concentrations were evaluated. Increased SDMA sample differed significantly from control when spiked with 250- and 500-mg/dl hemoglobin or 500-mg/dl lipids, and sample containing normal SDMA differed significantly from control when spiked with 500-mg/dl hemoglobin. (A) Assessment of interference by lipids. (B) Assessment of interference by conjugated bilirubin. (C) Assessment of interference by hemoglobin. All testing was done with rat serum samples run in triplicate. Error bars represent 1 SD around the mean. SDMA, symmetric dimethylarginine.

Figure 7. | SDMA immunoassay analyte-dependent interference testing—related compounds. Pooled rat serum samples at “normal” (approximately 8 μg/dl) and “increased” (approximately 50 μg/dl) SDMA concentrations were evaluated. Concentration of SDMA in the increased sample spiked with asymmetric dimethylarginine (ADMA) was significantly decreased compared with control. (A) Assessment of interference by arginine. (B) Assessment of interference by ADMA at 50 μg/dl. (C) Assessment of interference by N⁶-L mono methylarginine (L-MMA). All testing was done with rat serum samples run in triplicate. Error bars represent 1 SD around the mean. SDMA, symmetric dimethylarginine.
compared with controls at all dose levels in the four-dose group but was only significantly increased at the 50- and 100-mg/kg dose level in the ten-dose group. Cystatin C and µALB are both reabsorbed from the glomerular filtrate by the renal proximal tubular epithelium. Compromised absorption capacity in the proximal tubular epithelium from gentamicin-induced dysfunction or damage is a plausible explanation for the increased responsiveness of these urinary biomarkers in the four-dose group. These results agree with a previous study in which urinary cystatin C was found to be a more responsive indicator of gentamicin-induced nephrotoxicity compared with sCr and urine biomarkers, including NGAL, KIM-1, and CLU, although in this study, urinary µALB was not evaluated (35).

NGAL, KIM-1, and CLU are all tubular injury biomarkers, and dose- and time-dependent increases in these urinary biomarkers have been shown previously with gentamicin-induced injury. Our NGAL, KIM-1, and CLU results are similar to previous reports, although responsiveness among the three urinary injury markers has been somewhat variable across studies (20,21,35,38). The least responsive soluble biomarkers in this study included the renal excretory function biomarkers serum cystatin C and creatinine clearance and the tubular injury marker OPN, which were not significantly increased in gentamicin-treated animals compared with controls except for in the ten-dose group at the 100 mg/kg dose level. Creatinine clearance is commonly used as a surrogate GFR marker in preclinical and clinical studies; yet, tubular secretion of creatinine, reported to be as high as 30%-60% in mice, can negatively affect the sensitivity of creatinine clearance as an excretory function marker, resulting in overestimation of GFR (39). Direct measures of GFR, including inulin clearance or newer transcutaneous methods, may provide a more sensitive and reliable estimate of GFR but were not components of this study (40). The observed relative unresponsiveness of OPN is similar to a previous study that found that in response to a 7-day course of gentamicin at dose levels of 60 or 120 mg/kg, urinary markers, including cystatin C, KIM-1, NGAL, and CLU, increased in a time- and dose-dependent manner, whereas urinary OPN was not significantly increased in gentamicin-treated animals compared with controls (35). Gentamicin-induced histopathologic changes agreed with those previously described in similar study designs (21,41). Clinical pathology findings in the ten-dose 100 mg/kg treatment group, including marked increase in BUN and sCr concentrations and presence of proteinuria, were likely attributable to the gentamicin-induced renal injury, with changes on the complete blood count supportive of systemic inflammation (increases in neutrophil and monocyte counts, decreases in reticulocyte counts). Ketonuria in the ten-dose 100 mg/kg treatment group was consistent with negative energy balance as supported by the weight loss observed in this group. Use of only male rats in this study is a noted limitation that did not allow for evaluation of sex as a biologic variable affecting biomarker data.

In summary, this study is among the first to evaluate the serum renal function biomarker SDMA in an acute toxicity model in the rat and found SDMA compliments sCr and outperforms other renal function biomarkers, including serum cystatin C, creatinine clearance and BUN, increasing both earlier and at a lower dose level in response to gentamicin-induced renal toxicity. Among urinary biomarkers in this study µALB was the most responsive across both the four- and ten-day dose schedules, and performance was matched by urinary cystatin C in the four-dose group and by KIM-1 and CLU in the ten-dose group. Creatinine clearance, serum cystatin C, and OPN were the least responsive biomarkers for gentamicin-induced toxicity in this study. This study also provided analytical validation of a commercially available SDMA immunoassay for rat serum, opening further opportunities to study utility of SDMA in rodents and to investigate translational relevance of this biomarker in preclinical studies.

Disclosures
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Author Contributions
M.J. Coyne, J. Cross, D.M. Hamlin, R.M. Mack, R.E. Murphy, and A.E. Schultze conceptualized the study; M. Strong-Townsend and M. Yerramilli were responsible for data acquisition; M.J. Coyne, C. Drake, and D.J. McCrann III performed data analysis; M.J. Coyne, D.M. Hamlin, M.K. Leissinger, and A.E. Schultze drafted the original work; and all authors participated in editing and final approval of the manuscript.

Supplemental Material
This article contains supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0006420201/-/DCSupplemental.

Supplemental Table 1. Body weight data by group and dose in vehicle control and gentamicin-treated rats.
Supplemental Table 2. Complete blood count data by group and dose in vehicle control and gentamicin-treated rats.
Supplemental Table 3. Clinical chemistry data by group and dose in vehicle control and gentamicin-treated rats.
Supplemental Table 4. Urinalysis data by group and dose in vehicle control and gentamicin-treated rats.

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