Development and Application of Mass Spectroscopy Assays for N^ε-(1-Carboxymethyl)-L-Lysine and Pentosidine in Renal Failure and Diabetes

Katherine L. O'Grady, Sundeep Khosla*, Joshua N. Farr, Olga P. Bondar, Elizabeth J. Atkinson, Sara J. Achenbach, Brittany A. Eckhardt, Brianne S. Thicke, Amanda J. Tweed, Tammie L. Volkman, Matthew T. Drake, Jolaine M. Hines, and Ravinder J. Singh*

Background: Advanced glycation end products (AGEs) are formed via the nonenzymatic glycation of sugars with amino acids. Two AGEs, N^ε-(1-carboxymethyl)-L-Lysine (CML) and pentosidine, have been observed to be elevated in subjects suffering from a multitude of chronic disease states, and accumulation of these compounds may be related to the pathophysiology of disease progression and aging.

Methods: We describe here the development and validation of a specific and reproducible LC-MS/MS method to quantify CML and pentosidine in human serum with lower limits of quantitation of 75 ng/mL and 5 ng/mL, respectively. The analyte calibration curve exhibited excellent linearity at a range of 0–10 900 ng/mL for CML and 0–800 ng/mL for pentosidine. High-low linearity of 5 serum pairs was assessed, with a mean recovery of 103% (range 94–116%) for CML, and 104% (range 97–116%) for pentosidine.

Results: Serum concentrations of CML and pentosidine were quantified in 30 control and 30 subjects with chronic renal insufficiency. A significant increase in both analytes was observed in renal failure compared to control subjects (2.1-fold and 8.4-fold, respectively; $P < 0.001$ for both). In a separate cohort of 49 control versus 95 subjects with type 2 diabetes mellitus (T2DM), serum CML but not serum pentosidine, was significantly elevated in the T2DM patients, and CML was also correlated with glycemic control, as assessed by hemoglobin A1c ($r = 0.34$, $P < 0.001$).

Conclusions: These mass spectroscopy-based assays for serum CML and pentosidine should be useful in accurately evaluating circulating levels of these key AGEs in various disease states.

Mayo Clinic College of Medicine, Rochester, MN.

*Address correspondence to: S.K. at College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Fax: 507-293-3853; e-mail khosla.sundeep@mayo.edu. R.J.S. at College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Fax 507-293-3853; e-mail singh.ravinder@mayo.edu

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IMPACT STATEMENT

We describe the development of a specific and reproducible mass spectroscopy-based assay for serum advanced glycation endproducts (CML and pentosidine). A significant increase in circulating levels of both analytes was observed in renal failure compared to control subjects; serum CML, but not pentosidine, was elevated in patients with type 2 diabetes mellitus. Given the growing research and clinical interest in the role of AGEs in the pathogenesis of a number of diseases, including the complications of diabetes mellitus and renal failure, these novel assays should be an important tool to advance research and clinical practice in this area.

INTRODUCTION

Advanced glycation endproducts (AGEs) accumulate in various tissues with aging and may contribute to the pathology of diabetes mellitus, atherosclerosis, and induction of oxidative stress and inflammation (1, 2). AGEs are a heterogeneous group of compounds (3, 4) formed via a complex pathway known as the Maillard Reaction (5). One condition known to stimulate the production of these compounds is prolonged hyperglycemia, whereby nonenzymatic glycation of reducing sugars with amino acid groups, lipids, or nucleic acids generate AGEs (6). In addition to diabetes, AGEs have also been shown to accumulate in patients with renal failure (7, 8). AGEs are thought to have a cascading effect that further increases oxidative stress via free radical damage, which subsequently promotes additional formation of AGEs as well as chronic inflammation.

Although AGEs accumulate in tissues, circulating levels of AGEs may also be useful in monitoring potential tissue complications, exemplified best by the measurement of glycated hemoglobin (HbA1c) and its known relationship to diabetes complications (9). However, in addition to HbA1c, circulating levels of pentosidine and CML have also been measured in patients with diabetes, most commonly using enzyme-linked

immunosorbent assays (ELISAs). The use of ELISAs for AGEs has been associated with several problems, however, including: i) the affinity of antibodies can vary for different protein residues; ii) characterization of antibody specificity may be lacking; and iii) differences may exist between the antigens present in biological samples and the highly modified standard antigens used for antibody production (10).

We sought to develop a quantitative method for two AGEs: CML, the most abundant AGE, and pentosidine, without the use of antibodies. In this report we describe the development and clinical validation of a novel assay that is highly specific, sensitive, and reproducible using liquid chromatography combined with tandem mass spectroscopy (LC-MS/MS) for the detection of circulating levels of CML and pentosidine in human serum.

METHODS

Study Subjects

The protocol was approved by the Mayo Clinic Institutional Review Board.

Controls versus renal insufficiency/failure patients. Sixty serum samples were collected in control and renal insufficiency/failure groups, each containing 30 subjects (15 male/15 female)

and renal insufficiency/failure was defined as serum creatinine concentration above 1.2 mg/dL (the cutoff reference level).

Control versus type 2 diabetes (T2DM) patients.

We recruited postmenopausal women and men ≥ 50 years old. T2DM subjects were defined as having a HbA1c level $\geq 6.5\%$, and we studied subjects with T2DM for at least 5 years. Exclusion criteria included: *a*) significant abnormality in any of the screening laboratory studies [complete blood count, serum calcium (>10.5 mg/dL), eGFR based on creatinine (<30 mL/min/1.73 m²), TSH (<0.1 mIU/L), and 25-hydroxyvitamin D (<15 ng/mL)]; *b*) presence of clinically noted chronic liver disease, unstable cardiovascular disease, malignancy, chronic gastrointestinal disease, autoimmune rheumatologic conditions, hypo- or hyperparathyroidism, Cushing's syndrome, severe chronic obstructive pulmonary disease, alcoholism, or type 1 diabetes; *c*) undergoing treatment with any of the following drugs: corticosteroids (>3 months at any time or >10 days within the previous year); treatment within the past year with any of the following: anti-convulsants, pharmacological doses of thyroid hormone, adrenal or anabolic steroids, aromatase inhibitors, calcitonin, estrogen, selective estrogen receptor modulators, parathyroid hormone, denosumab, or thiazolidinediones. We also excluded subjects who have been exposed to a bisphosphonate within the past 5 years.

Materials and methods. N^ε-(1-carboxymethyl)-L-lysine (CML), N^ε-(1-carboxymethyl)-L-lysine-d4 (CML-d4), and pentosidine-d3 trifluoroacetic acid salt were purchased from Toronto Research Chemicals. Pentosidine was obtained from Cayman Chemicals. Hydrochloric acid (36.5–38.0%), perfluoropentanoic acid (97%), and ammonium hydroxide (28–30%) were purchased from Sigma Aldrich; acetonitrile, methanol, and acetone were obtained from Fisher Scientific.

Stock solutions of both CML and pentosidine were prepared, separately, in water at 1 mg/mL and 100 μ g/mL concentrations. Calibration curves were constructed from a dilution of an intermediate stock containing 15 μ g/mL CML and 1 μ g/mL pentosidine. This intermediate was diluted in water to prepare standards with the following CML/pentosidine concentration ratios (ng/mL): 0/0, 75/5, 150/10, 300/20, 750/50, 1500/100, 3000/200, 5250/350, 7500/500, and 10900/800.

Stock solutions of deuterated internal standards, CML-d4 and pentosidine-d3, were prepared, separately, in concentrations of 1 mg/mL. Working solutions were made at 10 μ g/mL for CML-d4 and 1 μ g/mL for pentosidine-d3.

Quality controls of low, medium, and high concentrations were prepared by diluting pooled human serum 1:2 with nanopure water (low), left neat (medium), or spiked with +1250 ng/mL CML and +500 ng/mL pentosidine (high). All three quality controls were run at the beginning and end of each sample batch.

Sample preparation. Standards, controls, and samples were stored frozen prior to use. For hydrolysis, 50 μ L of control or sample was pipetted into 13 $\times$ 100 mm borosilicate glass culture tubes with 475 μ L of 6 M HCl prepared in water, 25 μ L of 10 μ g/mL CML-d4, and 25 μ L of 1 μ g/mL pentosidine-d3. Samples were capped with Teflon lined screw caps, vortexed for 1 min at ~ 1500 rpm, and hydrolyzed for 20 h at 110 °C in a protected heat block. After hydrolysis, samples were cooled to room temperature and centrifuged at 3000 rpm for 10 min to remove particulates. To extract analytes, 0.6 mL volume of activated AG-50W-X8 resin (Bio-Rad Laboratories) was added to filter tubes in a Cerex-48 SPE ware holder and then washed five times with 1 mL nanopure water. Hydrolysates were transferred onto the resin using disposable transfer pipets. Each filter tube was washed five times with 2 mL nanopure water, then

CML and pentosidine were eluted into 13 × 100 mm glass tubes with 3 consecutive additions of 1 mL 4 M NH₄OH. Extracts were dried down under nitrogen at 50°C and 15–20 psi for 2–3 h in a Zymark Turbo Vap water bath. Once dry, extracts were reconstituted with 90:10 water: acetonitrile with 5 mM perfluoropentanoic acid (NPPA), vortexed for 2 min, filtered into 96-well collection plates, and stored refrigerated until analysis.

LC-MS/MS. A triple-quadrupole mass spectrometer, Sciex API 5000 (Applied Biosystems) utilizing an electrospray ionization source (ESI) in positive ion mode was used in combination with a Cohesive TLX4 liquid chromatography system. Ion-pairing chromatography was performed on a Phenomenex Synergi 4 μ , Max RP 80 A, 2.0 × 150 mm analytical column with a C₁₂, 4 × 2 mm guard cartridge (Phenomenex). The injection volume was 75 μ L. Mobile phases were prepared using NPPA as the ion-pairing agent. Mobile Phase A contained 5 mM NPPA in 90:10 water: acetonitrile, while Mobile Phase B consisted of 5 mM NPPA in 10:90 water: acetonitrile. Mobile phases were degassed for 5 min prior to use. The liquid chromatography method is detailed in [Supplemental Table 1](#). The total method length was 19.25 min, with analyte retention times of ~7.3 min for CML, and ~8.8 min for pentosidine. Flow was diverted from the mass spectrometer for the first 5 min of chromatography to prevent contamination.

Ionization was performed in positive mode with multiple reaction monitoring (MRM) transitions. Mass transitions and compound parameters used for CML and pentosidine and the associated internal standards are included in [Supplemental Table 2](#) while source and gas settings are detailed in [Supplemental Table 3](#).

Total Advanced Glycation Endproducts (AGEs)

Prior to addition of samples, the background of an empty, black microtiter plate was acquired

and wells with greater than 100 AU (arbitrary units) were excluded for use in analysis. For Total AGEs analysis using fluorescence detection, samples were diluted 5 times with PBS (pH 7.4) and 300 μ L of the diluted sample was added to the plate. The samples were read at excitation wavelength of 370 nm and emission wavelength of 440 nm using a Spectra Max Gemini XS (Molecular Devices). The fluorescence was normalized to CML, used as a surrogate for total AGEs.

Biochemical assays and estimated glomerular filtration rate (GFR). HbA1c and creatinine were measured in the Mayo Clinical Laboratory by ion-exchange high-performance liquid chromatography and an enzymatic colorimetric assay, respectively. The CKD-Epi equation was used to estimate GFR based on age, sex, race, and serum creatinine levels ([11](#)).

Skin AGEs measurements. Skin AGEs were measured using the AGE Reader[®] (Diagnoptics), which measures an index of tissue accumulation of AGEs by means of skin autofluorescence ([12](#)). The AGE reader measures skin autofluorescence with an excitation light source with wavelength between 300 and 420 nm. Previous studies have shown an error rate of ~5% when repeated skin autofluorescence measurements are made in control and T2DM patients ([12](#)).

Statistical analyses. Clinical characteristics of the various cohorts were summarized using medians and interquartile ranges. Chi-square and Kruskal-Wallis tests were used to assess group differences for categorical and continuous variables. Linear regression models were used to test for group differences in CML, pentosidine, and total AGEs values adjusting for eGFR values. Spearman correlations were assessed to compare the various lab measurements.

RESULTS

Quantification of CML and pentosidine

Standards spiked with CML-d4 and pentosidine-d3 internal standards were analyzed via LC-MS/MS. Using linear regression analysis, calibration curves were found to be extremely reproducible over 6 days (Fig. 1) with results for CML and pentosidine presenting slopes of 1.0014 and 1.0061, and r^2 values of 0.997 and 0.9999, respectively.

Chromatographic separation of CML and pentosidine was achieved using an ion-pairing reagent combined with reversed-phase chromatography. This facilitated optimization of peak shapes for both analytes and their internal standards (Fig. 2).

To verify precision, both intra- and inter-assay imprecision was performed. Intra-assay imprecision was determined by running 3 levels of quality control 20 times within the same assay. Inter-assay imprecision was determined by running 3 levels of quality control material over the course of 8 days. Values of the controls spanned the analytical measurable range and results are listed in Supplemental Table 4.

Spiked recovery and admixture experiments were performed to ensure accuracy. CML and pentosidine were spiked into human serum in varied amounts. CML was spiked at 300, 750, and 1500 ng/mL concentrations with mean recovery ranging from 96–102%. Pentosidine was spiked at 20, 50, and 100 ng/mL concentrations with mean recovery ranging from 92–104%. High: low admixture studies were performed in ratios of 90:10, 75:25, 50:50, 25:75, and 10:90 for 5 pairs of serum samples. Recoveries ranged from 94–116% (mean 104%) for CML (645–7080 ng/mL) and 97–116% (mean 104%) for pentosidine (5.4–389 ng/mL). Dilutional linearity experiments were performed on 6 different samples diluted with water x2, x5, and x10. The series showed excellent linearity with measured/expected values ranging from 81–

110% (mean 93% for values 230–16 800 ng/mL) for CML and 87–114% (mean 99% for values 5.8–534 ng/mL) for pentosidine.

Control versus renal failure patients

We next evaluated CML, pentosidine, and total AGEs in a cohort of control versus renal failure patients. Supplemental Table 5 shows the clinical characteristics of the control and renal failure patients. Fig. 3, A–C shows the values for CML, pentosidine, and total AGEs in the control versus renal failure patients. As is evident, all 3 AGEs parameters were markedly higher in the renal failure as compared to the controls. Table 1 shows the correlations between CML, pentosidine, and serum AGEs in the renal failure and control subjects. CML and pentosidine were strongly correlated in both groups. Total serum AGEs were correlated with CML and pentosidine in the renal failure but not the control group.

Control versus T2DM patients

We also evaluated CML, pentosidine, and total AGEs in a second cohort consisting of control versus T2DM patients. [Supplemental Table 6](#) shows the clinical characteristics of the control and T2DM patients used in these analyses. The median duration of T2DM was 14 years (range, 5–30 years). The median HbA1c over the preceding 5 years in the T2DM patients was 7.4%, with a fairly wide range (6.5–11.4%). Skin AGEs were significantly higher in the T2DM patients. Serum creatinine and eGFR did not differ between the control and T2DM subjects.

[Fig. 4](#) shows the CML, pentosidine, and total AGEs values in the control versus T2DM patients. CML was significantly higher in the T2DM as compared to the control subjects, whereas serum total AGEs or pentosidine were not different

between the 2 groups. Since a previous analysis had suggested that CML was elevated only in T2DM patients with renal insufficiency ([13](#)), we repeated the comparisons in [Fig. 4](#) following adjustment for eGFR, with very similar results (data not shown). We further examined the group of T2DM patients with the worst glycemic control (median 5 year HbA1c > 8%; $N=30$), with very similar results: CML (median [IQR]) was higher in the T2DM (2110 [1700–2370] ng/mL) versus the control subjects [1540 (1350–1720) ng/mL, $P<0.001$], whereas pentosidine (T2DM, 17.1 [14.1–19.2] versus control, 15.6 [12.9–20.9] ng/mL, $P=0.68$) and serum AGEs (T2DM, 0.93 [0.86–1.01] versus control, 0.96 [0.89–1.05] μM , $P=0.42$) did not differ between groups.

[Table 2](#) shows the correlations between serum CML, pentosidine, and AGEs, as well as the other

Table 1. Spearman correlation coefficients adjusted for age and sex between CML, pentosidine, and serum AGEs in the renal failure and control subjects.

	Renal failure		Control	
	CML	Pentosidine	CML	Pentosidine
CML	1.0	0.87***	1.0	0.54**
Serum AGEs	0.69***	0.48**	0.13	0.20

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

variables (median HbA1c over 5 years, and skin AGEs). CML was correlated with pentosidine and HbA1c in both the control and T2DM subjects. CML did not correlate with serum AGEs or skin AGEs in either group. Pentosidine was correlated with CML in both groups and with serum AGEs in the T2DM subjects. Serum AGEs correlated with pentosidine and skin AGEs in the T2DM but not the control subjects.

Comparison of changes relative to controls in the disease cohorts

Supplemental Table 7 compares the fold increases in serum CML, pentosidine, and total

AGEs in the renal failure and T2DM cohorts. As shown, although the 1.2-fold increase in serum CML in the T2DM subjects was statistically significant (Fig. 4), it was much smaller than the fold increase in CML (2.1) in the renal failure patients, who also had a marked increase (8.4-fold) in serum pentosidine; the latter did not change in the T2DM subjects. Total AGEs were also increased 1.9-fold in the renal failure patients, but did not change in the T2DM patients.

DISCUSSION

Utilizing LC-MS/MS, we developed a novel, reproducible assay to detect and quantitate two select AGEs, CML and pentosidine in serum. Although previous studies have described mass spectroscopy-based assays for CML and/or pentosidine (14–16) and discussed issues related to mass spectrometric detection of these compounds in clinical samples (17, 18), our study provides extensive validation of our assay that we can now offer to the scientific community as an orderable test in a College of American Pathologists (CAP)- and Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. In

Fig. 4. Serum a) CML, b) pentosidine, and c) total AGEs concentrations in control versus type 2 diabetes mellitus patients. Bars indicate median values.

Table 2. Spearman correlation coefficients adjusted for age, sex, and BMI in the control and T2DM patients.

Control	CML	Pentosidine	Serum AGEs	HbA1c	Skin AGEs
CML	1.0	0.70***	0.05	0.30*	0.11
Pentosidine	0.70***	1.0	0.04	0.13	0.12
Serum AGEs	0.05	0.04	1.0	0.01	-0.19
T2DM					
CML	1.0	0.47***	0.19	0.34***	-0.11
Pentosidine	0.47***	1.0	0.41***	0.02	0.16
Serum AGEs	0.19	0.41***	1.0	-0.10	0.37***

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

addition, our simultaneous evaluation of CML, pentosidine, and total AGEs in control versus renal failure and T2DM patients provides additional validation to our assay and shows the relationships between these AGEs and other parameters, including HbA1c and skin AGEs, in the diabetic cohort.

Using LC-MS/MS for the quantification of AGEs is more sensitive and specific than fluorescence or ELISAs, due to instrumentation specificity and capacity to avoid quantitating miscellaneous interference signals. Preliminary fluorescence studies on renal failure subject hydrolysate did not

correlate linearly with LC-MS/MS values, and we postulate that untargeted, fluorescing compounds in the matrix are likely to blame. Hydrolysis, extraction, and chromatography were optimized for both analytes to successfully eliminate interference peaks and reduce ion suppression. Inter-assay ($n = 15$) and intra-assay ($n = 20$) imprecision are acceptable for both analytes. Spiked recovery, dilutional linearity, and admixture studies performed all met our standardized criteria ($< 20\%$ CV).

In serum, pentosidine is primarily protein-bound; thus, only extremely low concentrations of

free pentosidine are present prior to acid hydrolysis (19). In order to quantitate CML and pentosidine accurately using an LC-MS/MS method, protein conjugated analytes must be cleaved off before detectable levels are achieved; therefore, hydrolysis is a crucial step in sample preparation prior to LC-MS/MS analysis. Hydrochloric acid hydrolysis is one of the most commonly reported methods used to free CML and pentosidine, due to the acid stability of these compounds (14). Kinetics studies that determined analyte concentrations present in serum matrix at a hydrolysis time spanning from zero to 24 h were undertaken to refine our hydrolysis method, and it was concluded that a 20 h acid hydrolysis was optimal for both analytes, as both CML and pentosidine plateaued in their response (Supplemental Fig. 1). Additional experiments were conducted to determine the most efficient hydrolysis procedure with regard to the volumetric ratio of serum to acid, and differences between varying molarities of hydrochloric acid. The final method is a representation of the conclusions reached from the above preliminary experiments.

With regard to assay performance, it is important to note that each batch requires substantial preparation prior to injection (30–32 h), which is tedious and may interfere with standard laboratory workflow. A significant amount of time is required to prepare daily/weekly reagents and to maintain proper functioning of equipment. Diligent efforts to replace guard cartridges and monitor changes in chromatography are needed to ensure proper sample processing. Further research must be conducted to determine whether a build-up from particulates in the sample matrix, or degradation to columns and cartridges due to the acidity of mobile phases are behind the previously stated nuances. In the case this assay is used in a clinical setting, subsequent efforts to reduce method duration may prove to be beneficial.

AGEs have been found to be significantly elevated in the serum, tissue, and bones of subjects

suffering from a variety of chronic disease states. Pentosidine and CML have been well characterized as biomarkers for the formation and accumulation of AGEs (20), and are known to play an important role in diabetes and vascular complications (21, 22). In our study, serum CML was significantly elevated in the diabetic subjects, and was found to correlate with glycemic control measured by HbA1c. However, no significant difference in pentosidine concentration was observed between the T2DM cohort and controls. In contrast with our study, Yoshida et al. found that serum pentosidine concentrations were significantly higher in patients with diabetes than in age-matched control subjects (23). These differing findings may reflect the fact that Yoshida et al. (23) used an immunoassay, rather than mass spectrometry, to measure serum pentosidine and thus their assay may not have been entirely specific for pentosidine. Since both serum pentosidine (24) and CML (13) may be altered by renal function, we also performed analyses adjusting for eGFR. After adjustment for eGFR, there remained no significant difference in pentosidine between diabetic and healthy subjects. Conversely, CML concentrations were significantly higher in the diabetic cohort relative to nondiabetic control subjects, both with and without adjustment for eGFR, and in both the T2DM and control cohorts, CML concentrations correlated with HbA1c.

Analyte concentrations were also determined in a separate cohort of 30 normal subjects and 30 subjects with renal failure. A significant increase in both analytes was observed in the renal failure cohort compared to controls. Prior studies have documented a dramatic accumulation of pentosidine in the plasma of subjects with end-stage renal disease (25), likely due, at least in part, to decreased renal elimination (26). A study utilizing stable isotope mass spectrometry found both CML and carboxyethyl-lysine (CEL) to be significantly increased in dialysis patients compared to controls (14). The accumulation of CML and

pentosidine in chronic kidney disease is likely due to a lack of excretion from the kidneys and increased production of AGEs from chronic inflammation and oxidative stress. Mounting evidence indicates that under chronic hyperglycemic or renal insufficiency conditions, the accumulation of AGEs, which are consequences of nonenzymatic reactions between sugar and free amino acid groups of proteins, lipids, and nucleic acids, promote the generation of reactive oxygen species (ROS), leading to oxidative stress and additional AGE production (27). One possible consequence of elevated synthesis of AGEs may paradoxically be irreversible damage to the kidney tissues over time via overproduction of ROS (27).

In this study we observed that skin AGEs measured by autofluorescence were markedly higher in the renal failure cohort compared to controls. Skin AGEs were also found to be significantly higher in the T2DM cohort compared to controls (28). In our study, serum creatinine and eGFR did not differ between the control and T2DM group, which demonstrates that T2DM serves as a separate risk factor for AGE accumulation in the absence of impaired renal function. Prior studies have shown that patients with diabetes currently on hemodialysis have even higher skin autofluorescence values than diabetes patients without

impaired renal function, which supports the conclusion that both T2DM and end stage renal disease are independent risk factors for AGE accumulation (28).

In comparing the fold increases in serum CML, pentosidine, and total AGEs in the renal failure versus T2DM patients, we found that the magnitude of the increases were much greater and included all 3 analytes in the renal failure patients, whereas only serum CML was significantly elevated in the T2DM patients. Whether this is due to a greater production or decreased clearance (or both) of AGEs in renal failure versus T2DM requires further evaluation.

In summary, we have developed and clinically validated a specific and reproducible LC-MS/MS method to quantify pentosidine and CML in human serum that should facilitate future studies examining circulating levels of these analytes in various disease states, including diabetes mellitus and renal insufficiency/failure.

SUPPLEMENTAL MATERIAL

[Supplemental material](#) is available at *The Journal of Applied Laboratory Medicine* online.

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

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