Cyanide-Nitroprusside Colorimetric Assay: A Rapid Colorimetric Screen for Urinary Cystine

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Background: Cystinuria is an autosomal recessive disorder resulting in poor proximal tubule reabsorption of cystine in the nephron, increasing the risk of cystine stone formation. A fast, inexpensive assay to screen for urinary cystine is needed because cystine stones are difficult to noninvasively differentiate from more common calcium-containing ones. Tandem mass spectrometry (MS/MS) is sensitive and specific but is labor-intensive and costly. Alternatively, a colorimetric assay is fast and cost-effective; however, creatinine interference is an issue.

Methods: A published cyanide-nitroprusside colorimetric assay was modified for a high-throughput microplate format. Creatinine interference was reduced using 0.1 mol/L PBS and a standard reaction time of 60 s and was further corrected using a formula derived from the slope of multiple creatinine standard curves.

Results: The limit of blank was determined to be 2.6 mg/L, the limit of detection 11.9 mg/L, and the limit of quantitation 15.3 mg/L. The analytic measurement range was established as 15.3–100 mg/L cystine. Intraassay and interassay CV was calculated to be 9.6% and 8.0%, respectively, for a high-level cystine concentration (83.6 mg/L). Low-level cystine (36.4 mg/L) intraassay and interassay CV was determined to be 18.1% and 17.6%, respectively. Passing–Bablok regression analysis of colorimetric vs LC-MS/MS results revealed a slope of 1.10 and *y* intercept of −7.14 mg/L, with an overall bias of 2% by Bland–Altman plot analysis.

Conclusions: We analytically validated a rapid colorimetric assay suitable to quantify urinary cystine. The effect of thiol drugs on this assay remains to be determined.

IMPACT STATEMENT

Kidney stones are a common occurrence and require specific treatment depending on the type of stone. While cystine is a less common stone type, they are difficult to detect noninvasively. Furthermore, cystinuria patients face a tremendous stone burden over the course of their lifetime, necessitating frequent monitoring. The current technology used to detect urinary cystine is expensive and untimely. In this study, we present a modified version of a colorimetric assay that corrects for creatinine interference and is suitable as a high-throughput, screening assay for urinary cystine that can be used in a clinical setting.

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Cystine is a dibasic amino acid formed by 2 cysteine monomers joined by a disulfide bond. In the kidney, cystine is freely filtered by the glomerulus and reabsorbed in the proximal tubule (*1*). Among individuals unaffected by cystinuria, only 0.4% of filtered cystine is lost in the urine (*2*). A lack of proximal tubular reabsorption of cystine results in urine cystine concentrations that can reach and exceed saturation by the end of the collecting duct, putting patients at risk for cystine stone formation (*3*). This condition, termed "cystinuria," is caused by autosomal recessive inheritance of mutations in 1 of 2 genes [solute carrier family 3 member 1 (*SLC3A1*)[4] and solute carrier family 7 member 9 (*SLC7A9*)], for which protein products form the heterodimer transporter on the apical membrane of the proximal tubule that facilitates reabsorption of dibasic amino acids (cystine, ornithine, lysine, and arginine) (*1*, *4*, *5*). Cystine kidney stones are the only known phenotype of affected patients, presumably because cystine is less soluble than the other 3 amino acids. Cystinuria is responsible for 6% of pediatric and 1% of adult renal calculi (*6*). At physiological urine pH (between 5 and 7), cystine stones are most likely to form at concentrations exceeding 240–300 mg/L (*7*). Current therapies are directed at keeping cystine concentrations below this level of saturation through dietary adjustments (most importantly high fluid and low sodium intake) and/or increasing the solubility of cystine by increasing urinary pH (*3*, *8*–*10*). Thiol drugs [i.e., tiopronin (thiola) and D-penicillamine] are also effective to reduce cystine into soluble cysteine monomers and thus reduce the concentration of cystine and the risk of stones (*11*–*13*).

The high recurrence rate (up to 60%) of cystine stone formation and risk of chronic kidney disease further highlights the need to detect cystinuria and initiate appropriate treatment (*8*). Because treatment strategies depend on stone composition, it is important to differentiate between stone types (*14*). Although composition is easily differentiated by infrared spectroscopy of stone material in vitro, stones are not always available and/or submitted for analysis. Further, cystine stones cannot be distinguished from calcium stones by current imaging techniques (*15*–*17*). Microscopic urine examination may reveal hexagonal crystals unique to cystine; however, only 20%–25% of patients will manifest these definitive crystals in their urine (*8*).

Several assays have been developed to detect cystine in urine. Quantitative cystine measurement by tandem mass spectrometry (MS/MS),[5] either alone or in combination with liquid or gas chromatography (LC-MS/MS or GC-MS/MS), is very sensitive and specific but is relatively labor-intensive and costly (*18*). A colorimetric assay is relatively fast and inexpensive; however, publications suggest possible interference with creatinine and/or thiol compounds (*12*, *19*, *20*). A solid-phase assay has been proposed that physically measures urinary cystine capacity (*21*). However, it is entirely manual and involves a 48-h incubation period of urine with cystine crystals and thus is not practical for most clinical laboratories.

A colorimetric assay that corrects for creatinine interference could provide a high-throughput cystinuria screening process for use in a clinical environment (*22*). The objective of this study was to (*a*) modify a cyanide-nitroprusside colorimetric assay using automated microplate settings and adjusting for creatinine interference, (*b*) analytically validate this assay as a high-throughput screening method for urinary cystine, and (*c*) compare urinary cystine measurements by this colorimetric assay and the LC-MS/MS method.

DOI: 10.1373/jalm.2016.022582

[4] **Human genes:** *SLC3A1*, solute carrier family 3 member 1; *SLC7A9*, solute carrier family 7 member 9.
[5] **Nonstandard abbreviations:** MS/MS, tandem mass spectrometry; CLRW, clinical laboratory reagent water; AMR, analytic measurement range; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantitation.

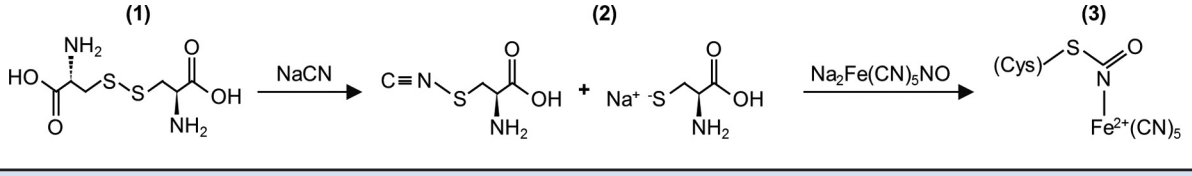

Fig. 1. Cystine and cyanide-nitroprusside reaction scheme.
Cystine (1) is reduced to cysteine monomers (2) using sodium cyanide. Addition of sodium nitroprusside results in the formation of a colored complex (3) with max absorbance at λ = 521 nm.

MATERIALS AND METHODS

Colorimetric assay

Materials. Chemicals used were the highest grade available: ammonium bicarbonate (≥99.0%), creatinine (≥98%, anhydrous), L-cystine (≥99.7%), sodium cyanide (97%), sodium nitroprusside dihydrate (≥99%), and PBS tablets (pH 7.4). All reactions were performed in Corning Costar microplates (96-well, clear, flat-bottom, polystyrene). Plates and all reagents were purchased from Sigma-Aldrich. Clinical laboratory reagent water (CLRW) (18 Ωm) used in all reagent preparation was obtained from a Barnstead NANOpure Diamond system (Thermo Fisher Scientific).

Preparation of samples and standards. The Mayo Clinic Institutional Board approved this study and consent was waived. Waste urine samples from patients without known cystinuria were prospectively obtained in the Mayo Clinic Renal Testing laboratory. Urine samples were alkalized with 10-N sodium hydroxide (aq) to pH >8, measured using a pH electrode (Broadley James) in conjunction with a meter calibrated with reference solutions of pH 2, 4, 7, and 10 (Thermo Fisher Scientific). A 2-point calibration curve was generated with each run. CLRW was used as the low concentration (0 mg/L) standard, and a 100-mg/L cystine solution was prepared gravimetrically in 10 mmol/L ammonium bicarbonate as the high standard, pH 8.1; standards, controls, and samples were adjusted to pH >8.0 to maximize cystine solubility. Sodium hydroxide (10 N) was used to adjust sample pH to avoid unnecessarily large changes in sample volume, and ammonium bicarbonate was used in preparation of the high standard due to its stability. Absorbances of the low and high standards were monitored as quality controls.

Undiluted samples that were initially measured above the analytic measurement range (AMR) were diluted with CLRW to concentrations predicted to lie within the standard curve from the initial reading. Creatinine concentrations were determined using a Cobas 6000 c501 automated chemistry analyzer and an enzymatic creatinase assay (Roche Diagnostics).

Assay design. The colorimetric reaction (Fig. 1) and initial conditions of our assay were based on those previously reported by Nakagawa and Coe (*20*). Half of the microplate was used per assay (48 wells). Cystine standards were run in quadruplicate (8 wells), and samples were run in duplicate (40 wells), allowing for a maximum of 20 patient samples per run. An ELISA STARlet liquid handler (Hamilton Robotics) was used to aliquot the samples/reagents into the microplate as follows: 50 µL sample, 90 µL 0.1 mol/L PBS, 30 µL 10% (w/v) sodium cyanide, and 20 µL 10% (w/v) sodium nitroprusside. The sodium cyanide solution was prepared under a fume hood. Addition of sodium cyanide was followed by a 20-m incubation period at room temperature, during which the plate was gently agitated on the STARlet's integrated microplate shaker. Addition of sodium nitroprusside

was followed by a 60-s standing incubation period at room temperature. Absorbance of the cysteine-nitroprusside complex was measured at 521 nm immediately after the 60-s incubation period using a BioTek Epoch plate spectrophotometer controlled with Gen5 software. Results were imported into Microsoft Excel and corrected for creatinine interference.

The effect of PBS concentration on cystine and creatinine signals was determined by measuring the absorbance at 521 nm of 4 cystine and creatinine dilution series, each ran in 4 different PBS concentrations: 0.01, 0.05, 0.1, and 0.2 mol/L. Linear regression analysis was used to compute the slope of each dilution series. The cystine dilutions were made from a 100-mg/L stock solution prepared in 10 mmol/L ammonium bicarbonate, pH 8.1; and the creatinine dilutions were made from a 538-mg/dL stock solution prepared in CLRW. Creatinine concentrations in the stock and dilutions were confirmed using an enzymatic creatinine assay performed on a COBAS c501 analyzer.

Creatinine interference was quantitated using an equation derived from 12 separate creatinine dilution series. Dilutions were made from a 401-mg/dL creatinine solution prepared in CLRW, and the concentrations were confirmed using the enzymatic creatinine assay. Slope (*m*) and *y* intercept (*b*) of the linear regression curve resulting from the average measured signal at each concentration of creatinine was used to estimate creatinine interference. Because the interference was directly linear to the amount of creatinine (mg/dL) measured in each sample, the following equation was derived to estimate the total amount of cystine signal:

$$\text{Corrected signal} = \text{Measured signal} - (m \times [\text{creatinine}] + b)$$

LC-MS/MS assay

Method description. Samples were analyzed by LC-MS/MS in the Mayo Clinic Biochemical Genetics Laboratory using a validated assay as previously described (*18*). Briefly, quantitative analysis of the amino acids cystine, ornithine, lysine, and arginine was performed by LC-MS/MS by labeling amino acids present in urine with aTRAQ Reagent 121 (AB Sciex). Samples were dried and reconstituted with aTRAQ Reagent 113-labeled Standard Mix (AB Sciex). Amino acids were separated using a C18 (150 × 4.6 mm) column (Higgins Analytical) and detected by an API 3200 tandem mass spectrometer (AB Sciex). The concentrations of amino acids were established by comparison of their ion intensity (121-labeled amino acids) to that of their respective internal standards (113-labeled amino acids). Results of the LC-MS/MS assay were not known when the colorimetric assay was run.

Validation

Linearity was established using normal urine samples gravimetrically spiked to pathologically high cystine concentrations and diluted with CLRW (n = 3). Linearity of cystine (n = 3) and creatinine (n = 3) standards in CLRW was similarly established. Linear regression analysis for each experiment achieved our acceptance criterion of $R^2 > 0.95$.

The limit of blank (LOB) was defined as the concentration mean of zero-standard replicates (n = 40) plus 2 SDs in accordance with the CLSI EP17-A2 guidelines (*23*). The limit of detection (LOD) was determined using both the measured LOB and replicates (n = 40) of a urine sample spiked gravimetrically to a low-level (mean 8 mg/L) cystine concentration. We thus defined the LOD as the LOB plus 2 SDs of the low analyte concentration sample. The limit of quantification (LOQ) was established as the lowest analyte concentration at which the intraassay CV was below 20% for multiple sample replicates (n = 39). The lower bound of the AMR was defined as the LOQ and the high bound was defined as the high standard concentration.

Precision was assessed using high and low cystine standards in accordance with CLSI EP15-A3 guidelines (*24*). These samples were prepared by

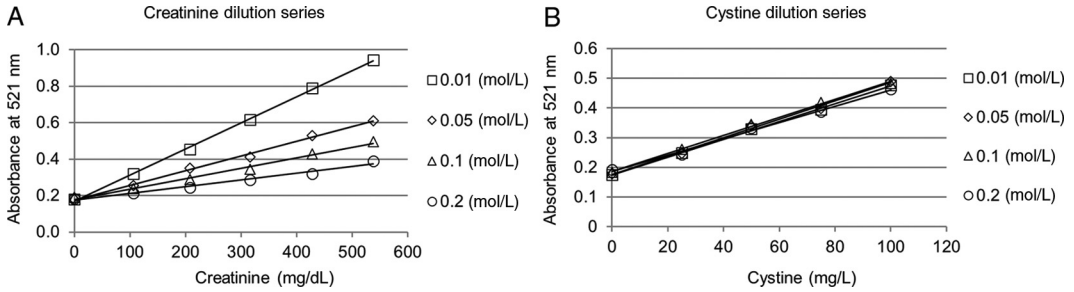

Fig. 2. Effect of PBS on creatinine and cystine reaction with cyanide-nitroprusside.
Increase in PBS concentration from 0.01 to 0.2 mol/L progressively decreased the creatinine signal (A) with little effect on that of cystine (B).

spiking normal patient urine samples to an artificially high and low cystine concentration within the AMR and were measured in 5 replicates, over 5 days, in 5 independent assays. Intraassay SD (within-run precision) and interassay SD (between-run precision) were calculated, from which the CV for both components were determined, with a CV acceptance criteria of <20%.

To assess cystine stability in solution, random urine samples (n = 7), and standard samples prepared in 10 mmol/L ammonium bicarbonate (n = 3), were spiked gravimetrically to varying cystine concentrations. Stability was assessed over 7 days, with measurements taken on days 1, 3, and 7. Stability was assessed at ambient (20 °C), refrigerated (4 °C), and frozen (−80 °C) temperatures and for 3 freeze-thaw cycles. Percent difference calculations were in reference to day 0 values. Stability was deemed acceptable at the last time point that the mean percentage difference [(observed − expected)/expected × 100] was within ±20%, and no more than n = 3/10 samples displayed a greater than ±20% result change.

Comparison between the colorimetric and LC-MS/MS assays was assessed by measuring the cystine concentration of random urine samples (n = 47), spiked gravimetrically to normal and disease range cystine concentrations, and quantitated using both methods, with a mean bias acceptance criteria of within ±20%.

Statistical analysis

Analyse-it® software for Microsoft Excel was used for statistical analyses. SDs were calculated under the assumption of data being a sample of the entire population. LOB and LOD calculations were based on the assumption of a standard normal Gaussian distribution. Linear and Passing–Bablok regression analyses were used to compare colorimetric and LC-MS/MS results. The slope, *y* intercept, correlation coefficient (R^2), SD, and SD of residuals were calculated. Bland–Altman plots were used to graphically display bias between methods.

RESULTS

Assay design

The volumes of samples and reagents were proportionately reduced for microplate settings. Increasing PBS concentration proportionally decreased the interfering creatinine signal while having no effect on the measured cystine signal (Fig. 2); therefore, 0.1 mol/L PBS was used as the reaction buffer.

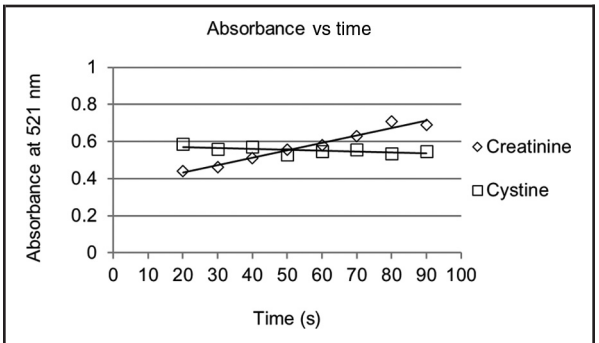

Fig. 3. Change in creatinine and cystine signal over time.
Over a period of 90 s, absorbance due to cystine (100 mg/L) fell while that of creatinine (400 mg/dL) increased.

Spectral analysis across wavelengths (300–700 nm, 10-nm intervals) of a 100-mg/L cystine solution confirmed previous reports of 2 absorbance maxima, at 400 and 521 nm, with the 521-nm peak representing the cysteine-nitroprusside complex. A linear decrease in the signal of a 100-mg/L cystine stock solution was observed over a time period of 90 s. Conversely, a linear increase in the absorbance of a 400-mg/dL creatinine stock solution was observed (Fig. 3).

The incubation time during each run was optimized to account for the time sensitivity of the nitroprusside reaction. A total of 17 s elapsed between the addition of nitroprusside to the first and last columns of the microplate. Subsequent spectrophotometer measurements took place over a time span of 60 s. Across-plate analyses of both cystine (100 mg/L) and creatinine (400 mg/dL) solutions revealed that the %CV decreased between 0 and 120 s for both solutions, as summarized in Table 1. Note "incubation time" refers to the time between addition of nitroprusside to the last column of the microplate and the beginning of spectrophotometer measurements. Based on these results, an incubation time of 60 s was chosen to maintain intraassay precision <20% and limit the amount of creatinine interference.

Table 1. Across-plate %CV for cystine and creatinine measurements by incubation time.

	Time, s		
	0	**60**	**120**
Cystine	9%	7%	6%
Creatinine	30%	11%	8%

Acceptable linearity (R^2 > 95%) was obtained at 4-, 8-, 16-, and 32-fold dilutions once the cystine concentration was well below the upper limit of solubility in urine (approximately 250 mg/L) (Fig. 4). Thus, the observed concentrations of the neat samples were lower than expected (back calculated from the 4-fold dilution). Acceptable linearity was also observed for cystine (100 mg/L) and creatinine (400 mg/dL) standard solutions at 1-, 2-, and 4-fold dilutions.

The LOB was determined to be 2.6 mg/L, the LOD 11.9 mg/L, and the LOQ 15.3 mg/L. The AMR was established as 15.3–100 mg/L cystine. Intraassay and interassay CVs were calculated to be 9.6% and 8.0%, respectively, for a high-level cystine concentration (mean 83.6 mg/L). Low-level cystine (mean 36.4 mg/L) intraassay and interassay CVs were determined to be 18.1% and 17.6%, respectively.

The average percentage difference between cystine measurements on day 0 and day 7 at 20 °C was 13.4% (1 sample with >20% difference); at 4 °C, it was 13.8% (3 samples with >20% difference), and at −80 °C, it was 22.6% (3 samples with >20% difference); after 3 freeze-thaw cycles, it was 18.9% (1 sample with >20% difference). Stability was better for cystine dissolved in a buffered solution. The average percentage difference between samples with cystine prepared in 10 mmol ammonium bicarbonate measured on day 0 and day 7 at 20 °C was 4.8% (0 samples with >20% difference), at 4 °C it was 10.2% (0 samples with >20% difference), at −80 °C it was 6.6% (0 samples with >20% difference), and after 3 freeze-thaw cycles it was 12.8% (0 samples with >20% difference).

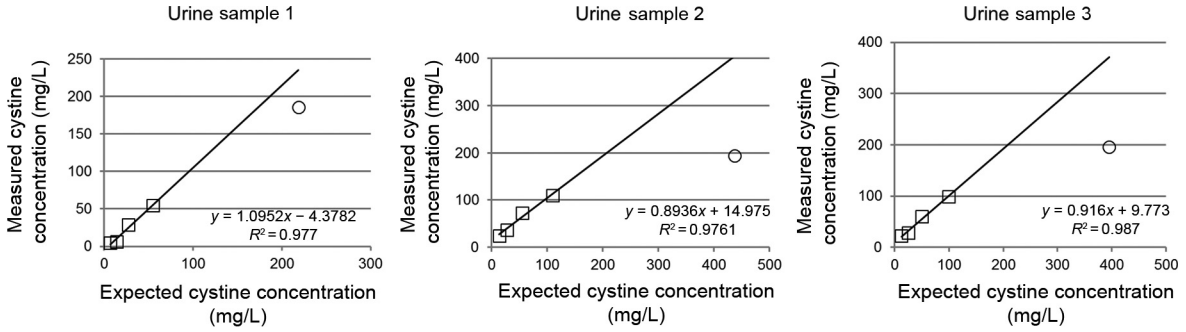

Fig. 4. Linearity of cystine measurement in spiked urine.
Linearity for 3 representative urine samples is shown and was acceptable once the concentration was below the solubility limit in urine (~250 mg/L). Circle markers represent samples with cystine concentrations above the solubility limit of cystine and were not included in R^2 or linear regression calculations.

Linear regression analysis of colorimetric and LC-MS/MS results revealed a slope of 1.06, a *y* intercept of −5.31 mg/L, and an R^2 value of 0.81. The mean and SD of the absolute difference (colorimetric LC-MS/MS) were 10.2 and 118.1 mg/L, respectively. The residual SD was 119.8 mg/L. Passing–Bablok regression analysis revealed a slope of 1.10 and a *y* intercept of −7.14 mg/L (Fig. 5A). Bland–Altman plot analysis of the percentage difference between assays revealed a 2% bias (colorimetric MS/MS), with 95% limits of agreement of −79% to 83% (Fig. 5B). Overall assay parameters and characteristics are summarized in Table 2.

DISCUSSION

Determining stone composition is clinically important, since medical prophylaxis differs by stone type. While thiazides are useful for calcium stones, they have no known benefit for cystine stones, and thiol drugs would be an unacceptable choice for calcium stones. Stones of unknown composition

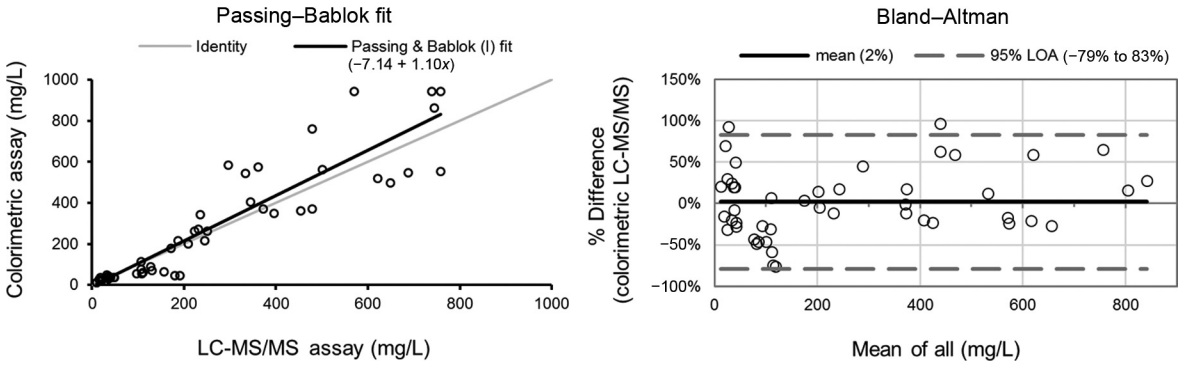

Fig. 5. Comparison (n = 47) of cystine measurement by colorimetric and LC-MS/MS assays.
Both assays were comparable across the measurement range as shown by Passing–Bablock (A) and Bland–Altman (B) plots. Notably, the Bland–Altman analysis revealed a mean 2% bias between the assays.

Table 2. Summary of cystine assay performance characteristics.

Assay parameter	Assay characteristics
Assay format	Spectrophotometry
Analyte, unit	Cystine, mg/L
Assay time	30 min
Standards	High: cystine 100 mg/L (prepared in 10 mmol ammonium bicarbonate)
	Low: blank (CLRW)
Sample type	Urine, pH >8
Amount of sample required	50 µL
Total assay volume	190 µL
Number of samples per assay	20
LOB = $mean_{blank}$ + 2 SD	2.7 mg/L, n = 40
LOD = LOB + 2 $SD_{low\ [analyte]}$	11.9 mg/L, n = 40
LOQ: lowest [analyte] with CV <20%	15.3 mg/L, n = 39, SD 2.2 mg/L, CV 14.6%
AMR: LOQ and high standard	15.3 mg/L and 100 mg/L
Intraassay variation (2 levels, 5 replicates each day for 5 days)	High: mean 83.6 mg/L, SD 8.0 mg/L, CV 9.6%
	Low: mean 36.4 mg/L, SD 6.6 mg/L, CV 18.1%
Interassay variation (2 levels, 5 replicates each day for 5 days)	High: mean 83.6 mg/L, SD 6.7 mg/L, CV 8.0%
	Low: mean 36.4 mg/L, SD 6.4 mg/L, CV 17.6%
Stability (n = 7) (7-day acceptance criteria: <3 samples with >20% difference, mean % difference <20%)	Ambient (20 °C), 1 sample >20%, mean 13.4%

are not easily distinguished by common imaging procedures (radiographs, computed tomography scans, or ultrasound) (*16*, *17*). Although a stone analysis by infrared spectroscopy would easily detect composition, stones are not always captured and/or sent for testing. Cystine crystals are pathognomonic, but are only visible in the urine of affected individuals in a minority of samples analyzed. Previous studies have suggested a colorimetric assay could be used as a screening tool for cystinuria, although creatinine interference can be an issue. To correct for creatinine interference, previous assays have measured creatinine in each urine sample and then ran a concurrent blank at the creatinine concentration; however, this is inefficient and not applicable for clinical testing laboratories. Assays were also manual, introducing further error.

To address these gaps, in the current study, a rapid microplate assay for urinary cystine was developed and validated. A 0.1-mol/L PBS buffer (10× concentrated) reduced the signal of the creatinine-nitroprusside complex but had no effect on the cysteine-nitroprusside complex. Furthermore, creatinine interference is linear and thus was accounted for with a calibration curve. Using an equation derived from creatinine dilution curves, the creatinine signal was estimated and subtracted. Therefore, creatinine interference could be accurately and efficiently accounted for in each individual urine sample.

Comparison between the colorimetric and LC-MS/MS methods revealed a 2% bias between assays, well within our acceptance criteria of ±20%. However, linear regression analysis revealed a correlation coefficient of 0.81, indicating that there are large variations observed in some individual samples. This result may in part be due to the difference in analyte selectivity of the LC-MS/MS assay and the colorimetric assay. The LC-MS/MS

assay only measures intact cystine, whereas the colorimetric assay measures all free cysteine in solution, since nitroprusside reacts with the thiol group of cysteine monomers. Thus, if any cystine is reduced, the LC-MS/MS assay will not detect the cysteine monomers, whereas the colorimetric assay will, perhaps explaining the high positive percentage bias (colorimetric assay higher) observed in some high concentration samples.

Furthermore, the intraassay CV was found to be larger than the interassay CV. We believe this to be due to the time sensitivity of the assay, resulting in variable incubation times between individual wells. However, by only using half of the microplate per assay, automating the liquid handling, and strictly adhering to a 60-s incubation time after addition of nitroprusside to the last column of the microplate, we achieved an acceptable across-plate %CV for cystine and creatinine stock of <20%.

Because of this variation, we advocate the colorimetric assay as a clinical screening tool rather than a definitive quantitative measure. Pathogenic concentrations of cystine (240–300 mg/L) are much greater than cystine concentrations in normal patients (<50 mg/L), and the colorimetric assay has proven suitable to distinguish pathogenic concentrations from normal concentrations of urinary cystine. Patients testing positive for cystinuria on the colorimetric assay can then be followed up with more quantitative mass spectrometry assays. Further work needs to be completed to discern clinical cutoff values for the assay.

This study has certain limitations. Previous studies have suggested that thiol drugs also interfere with the sodium nitroprusside assay (*20*, *21*). However, Nakagawa et al. (*20*) examined the effect of 3 thiol drugs (captopril, thiola, and penicillamine) as well as other possible interferents, including albumin, uric acid, ammonia, ascorbic acid, and iron (III) chloride. They reported linear color development with captopril upon increasing concentration, with thiola and penicillamine not developing color with increasing concentration. This result suggests that sodium cyanide does not reliably reduce thiola-cysteine disulfide and thiola disulfide, or penicillamine complexes. Furthermore, albumin, uric acid, and ammonia did not interfere with the reaction; however, they reported significant interference from ascorbic acid and iron (III) chloride, and suggest eliminating these daily supplements before analysis (*20*). We did not test for interference from all of these or possibly other compounds (such as homocystine) in the current study; thus, we cannot exclude the possibility of other factors contributing to the positive bias discussed earlier. Nevertheless, we believe this assay has a role as a rapid screen that is more cost-effective and potentially widely available compared to MS/MS technology. It is also important to note its intended use for patients with known stone disease of uncertain type, among whom these potential confounders (like homocystine) are less likely.

In conclusion, the modified colorimetric assay presented here is suitable as a rapid high-throughput and cost-effective screen to identify and potentially monitor patients with cystinuria. Future studies are needed to clarify the effect thiol drugs have on the colorimetric and MS/MS assays.

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.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form.* **Employment or Leadership:** None declared. **Consultant or Advisory Role:** J.C. Lieske, Alleena, Alnylzm, Dicerna, Oxthera, Retrophin. **Stock Ownership:** None declared. **Honoraria:** None declared. **Research Funding:** J.C. Lieske, Retrophin. **Expert Testimony:** None declared. **Patents:** None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: This study was supported by (*i*) the Rare Kidney Stone Consortium (U54KD083908), a part of the Rare Diseases Clinical Research Network, an initiative of the Office of Rare Diseases Research, National Center for Advancing Translational Sciences (NCATS); this consortium was funded through a collaboration between NCATS and the National Institute of Diabetes and Digestive and Kidney Diseases; (*ii*) Mayo Clinic O'Brien Urology Research Center, DK100227; and (*iii*) an investigator-initiated research grant from Retrophin.

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