A Simple Quantitative Test for Screening Cystinuria

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The specific techniques for determining urinary cystine levels include ion-exchange chromatography, high-voltage electrophoresis, and high-performance liquid chromatography.

The widely used colorimetric Brand's test requires a potentially dangerous substance - cyanide - and no qualitative paper methods are commercially available.

As a screening test, we used a colorimetric reaction that does not require cyanide. In this reaction, the cysteine sulphydryl groups reduce phosphotungstic acid to tungstate, which was then measured spectrophotometrically.

In order to diagnose cystinuria, urinary cystine levels must be determined; and the most specific techniques for doing this are ion-exchange chromatography, 1 high-voltage electrophoresis, 2 and high-performance liquid chromatography. 3 These methods require the use of complex and costly equipment only available in a small number of laboratories. No qualitative screening paper tests exist and the widely used colorimetric Brand’s technique, 4 and experiences derived from it, 5-8 involve the use of cyanide, a potentially dangerous substance. Consequently, many clinical chemistry laboratories do not test for cystinuria. Therefore, we tested the method of Shinohara and Padis modified 9 for clinical reliability and applicability. This method is a colorimetric reaction, which due to its simplicity, low cost, and use of routine equipment, could be used as a screening test for a large population.

Materials and Methods

Patients and Controls

Using the modified Shinohara and Padis method, 9 we determined the urinary cystine value for the 24-hour urine of 2,970 stone-forming patients (ages 14 to 80 years old), clinically distributed as follows: 2,936 calcium or uric acid stone-formers and 34 cystine stone-formers. Additionally, urinary cystine was measured in 238 normal subjects (ages 18 to 69 years old).

Given the greater solubility of cystine at a very low pH, we collected 24-hour urine in a container with 10 mL of 19% HCl.

Cystine was determined in urine samples by both the colorimetric technique and ion-exchange chromatography. Eighty samples were choosen based on the values of colorimetric cystine obtained: 32 with values ≤188 mg/24 hours (ie, mean normal value + 2SD); 10 with values between 189 and 242 mg/24 hours (ie, between the second and third SD from the normal value); and 38 with values exceeding 242 mg/24 hours, (ie, considered to be definitely pathological). Clinically, these 80 samples were distributed as follows: 46 calcium or uric acid stone-formers and 34 cystine stone-formers.

Colorimetric Determination of Cystine:

a) Principle

The reaction measures the quantity of urinary sulphydryl groups. The cysteine is reduced by the sodium sulfite to cysteine and cysteinesulfonic salt. The sulphydryl group (SH) of cysteine, and any other urinary compound containing this group, reduces phosphotungstic acid to tungstate, a blue color that is spectrophotometrically measured at 600 nm. The reduction of phosphotungstic acid can also occur as a result of the action of other substances such as ascorbic and uric acids. In order to eliminate the interference of these substances, 2 test tubes per urine sample are prepared. Mercuric chloride is added to 1 of the tube, which binds the SH groups. This binding makes the SH group non-reactive with phosphotungstic acid and does not interfere with other compounds ability to reduce phosphotungstic acid. The SH groups are represented by the difference in the intensity of the coloring with and without mercuric chloride.

b) Reagents

Acetate Buffer: add 2 parts of acetic acid (2 M) to 10 parts of sodium acetate (2 M); stable in refrigerator for several days.

Sodium Sulfite: add 12.6 g Na2SO3 and 0.2 g NaOH (or 5 mL NaOH 1 N) to 100 mL water; stable for approximately 1 month in refrigerator.

Mercuric Chloride: dissolve 2.7 g HgCl2 in 100 mL water; stable at room temperature.

Phosphotungstic Acid: dissolve 40 g molybdhenium-free sodium tungstate in about 300 mL water, add 32 mL 85% orthophosphoric acid, reflux gently for 2 hours, cool to room temperature and make up to 1 liter with water, mix, dissolve 32 g Li2SO4. Water in reagent is stable indefinitely in refrigerator.

Cystine standards: add cystine in 0.1 N HCl solution with concentrations ranging from 25 to 600 mg/L; stable in refrigerator.

c) Procedure

1. Test tubes: reagent blank (1 mL of water), standard (1 mL of standard), urine blank (1 mL of urine), urine (1 mL of urine).

2. 1 mL of acetate buffer and 0.3 mL of sodium sulfite were added to each tube and mixed.

Cystine was determined in urine samples by both the colorimetric technique and ion-exchange chromatography. Eighty samples were choosen based on the values of colorimetric cystine obtained: 32 with values ≤188 mg/24 hours (ie, mean normal value + 2SD); 10 with values between 189 and 242 mg/24 hours (ie, between the second and third SD from the normal value); and 38 with values exceeding 242 mg/24 hours, (ie, considered to be definitely pathological). Clinically, these 80 samples were distributed as follows: 46 calcium or uric acid stone-formers and 34 cystine stone-formers.
3. 1.5 mL of water and 0.2 mL of mercuric chloride were added to the reagent blank and urine blank. This was mixed and, after 2 minutes, 1 mL of phosphotungstic acid was added.

4. 1.7 mL of water and 1 mL of phosphotungstic acid were added to the standard and urine.

5. A reading at 600 nm of standard and urine against their respective blanks was made after 15 minutes, using a spectrophotometer (Shimadzu UV-160A). Mean time for procedure was approximately 30 minutes.

**Chromatographic Determination of Cystine**

Determination of cystine by ion-exchange chromatography was carried out at the Stone Center of the Mauriziano Hospital in Turin. A Biochrom 20 instrument (Pharmacia Biotech M, Buckinghamshire, England) was used, equipped with an autosampler column for the analysis of physiological liquids (20 cm, High Resolution Lithium Column), post-column reactor, and spectrophotometric detector.

**Statistical Analysis**

The data are expressed as mean ± SD. Student’s “t” test was used for comparing mean values. The relationship between values was tested by linear regression; $P<0.05$ was considered significant.

**Results**

**Colorimetric Determination of Cystine**

F1 shows the absorption curve of the reagent blank and the standard (300 mg/L) at various wavelengths. The absorption present at wavelengths below 450 nm is due to the phosphotungstic acid that absorbs in that range. We judged the most suitable reading to be at 600 nm.

In order to assess the linearity between absorption at 600 nm and cystine concentration, standards with concentrations ranging from 25 to 600 mg/L were prepared. F2 shows the reading of the standards at 600 nm at 5, 10, and 15 minutes following the addition of phosphotungstic acid. All 3 readings presented a good linear relation between absorption and cystine concentration ($r$-values between 0.923 and 0.998). The best stability was at 15 minutes.

To assess the accuracy of the method, we added 100, 200, 300, 400 mg/L of cystine (A, B, C, D of F3, respectively) to 5 urine samples, after which the samples were analysed and compared with the corresponding standard. The best recovery rate was at 15 minutes and was greater than 90%.

The precision of the technique was assessed by measuring urine cystine 10 times. The coefficient of variation was ±10.6%.

With colorimetric determination, the mean value ± SD of the urinary cystine in 238 normal subjects was 80 ± 54 mg/24 hours. In 2,936 calcium or uric acid stone patients, the results were 84 ± 52 mg/24 hours. In 34 cystine stone patients, the results were 599 ± 296 mg/24 hours.
Comparison of Colorimetric and Chromatographic Techniques

As mentioned, 80 urine samples were analysed in the Turin laboratory, also using the chromatographic method. The laboratory staff had no knowledge of the colorimetrically determined values. The upper reference limit of urinary cystine in that particular laboratory had been assessed at 28 mg/24 hours.\(^{10}\) \(\text{F4}\) shows a comparison of the 2 techniques.

The comparison shows that the 38 urine samples whose cystine excretion was found to be superior to 242 mg (mean urine value + 3SD) with the colorimetric method, all had cystine excretion greater than 28 mg/24 hours with the chromatographic method. Of these 38 samples, 34

\[\text{F3}\] Variation of the absorption at 600 nm in the colorimetric determination of the cystine 5, 10, 15, 30 mins of incubation: standards and urine. Quantities of 100 mg/L (A), 200 mg/L (B), 300 mg/L (C), and 400 mg/L (D) of cystine were added to the urine. The urinary absorption value shown here was obtained by subtracting the absorption value of the base urine from the urine to which the cystine was added, so that the urinary absorption curves should, theoretically, have coincided with those of the standards. For each curve the various points represent the mean values (the SDs are also shown), calculated on the 5 samples analysed on the same day or on different days.

\[\text{F4}\] Urinary cystine levels: comparison between colorimetric and chromatographic techniques in cystine stone formers and in calcium or uric acid stone-formers. Correlation for all values \(r=0.89, P<0.001\).
came from cystine stone patients, while 4 came from calcium stone patients (probably type III heterozygous cystinuria).

Additionally, the 32 urine samples with colorimetric cystine ≤188 mg/24 hours (mean normal value + 2SD), belonging to calcium or uric acid stone patients, all had a chromatographic cystine value less than 28 mg/24 hours. Therefore, the techniques gave overlapping results for these 2 groups.

On the other hand, of the 10 samples with colorimetric cystine between 188 and 242 mg/24 hours (ie, values between the 2nd and 3rd SD from the normal mean value), 3 had approximate cystinuria values with the chromatographic method (probably type III heterozygotes).

The correlation between the 2 techniques in the 80 samples analysed was 0.888 (P<0.001).

**Discussion**

This study suggests that the colorimetric technique used (which does not involve cyanide) is a simple and economical technique for cystinuria screening in large populations. However, it is important to take its specificity limits into account. This method does not measure cystine directly because it measures all SH groups. This explains the great difference found in normal subjects and in calcium or uric acid stone patients compared to the chromatographic technique.

Given the high levels of cystine excretion typical of cystinuric subjects, the method was able to discern 100% of cystinuric homozygotes.

In conclusion, when this technique produces cystine values greater than 242 mg/24 hours, a definitive diagnosis of cystinuria is possible. On the other hand, if values less than 188 mg/24 hours are produced, a negative diagnosis of cystinuria is possible. Diagnostic problems arise when values between 188 and 242 mg/24 hours are obtained (ie, values between the 2nd and 3rd SD from the normal mean value). In this range, a further check using a more specific technique would be required.


