Conversion of a Qualitative Screening Test to a Quantitative Measurement of Urinary Cystine and Homocystine

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ABSTRACT

Qualitative urinary screening procedures were converted to quantitative methods for urinary cystine and homocystine based on the reactions between these amino acids and cyanide-nitroprusside reagents. Cystine and homocystine are quantified by the measurement of absorbances at 521 and 524 nm, respectively. Cyanide-nitroprusside reacts with both cystine and homocystine. However, in the presence of silver nitrate, only homocystine reacts to produce a magenta color. Following the cyanide-nitroprusside reaction, absorbance must be read within three minutes for cystine and immediately for homocystine. The stability of the absorption spectra has no apparent effect on these quantitative assays. Amino acid concentrations are expressed as ratios to creatinine, which tends to eliminate false negative results in dilute urine specimens. The normal urine value for cystine and homocystine combined is 66.8 ± 52 (n = 50) mg per g creatinine. The normal value for homocystine alone is 29.9 ± 16.8 (n = 24) mg per g creatinine. The simplicity of these procedures allows these quantitative methods to be used as screening tests for cystinuria and homocystinuria.

Introduction

One of the chemical tests performed on urines during routine screening for inborn errors of metabolism is a cyanide-nitroprusside reaction designed to test for the presence of excessive concentrations of cystine or homocystine. After incubation with cyanide, a magenta color develops immediately upon addition of nitroprusside solution to urine containing an increased concentration of either cystine or homocystine. Because the color product is unstable, the test is generally regarded as unsuitable for the quantitative measurement of urinary cystine. The test has sufficient sensitivity to detect homozygotes of cystinuria, but it may fail to detect heterozygotes because of their lower urinary cystine concentrations. In the past, either an automated amino acid analyzer or a high performance liquid chromatograph (HPLC) was needed for the quantification of uri-
Because nitroprusside reacts with both penicillamine and cystine indiscriminatively, amino acid analyzer or HPLC were also relied upon for separating cystine from penicillamine for patients on penicillamine therapy. The cyanide-nitroprusside reaction does not distinguish cystine from homocystine. Since homocystinuria and cystinuria are associated with two different disease entities, it is important to differentiate between these two biochemical abnormalities. It has been demonstrated that cystinuria is related to defective renal tubular reabsorption of the dibasic amino acids cystine, arginine, ornithine and lysine whereas homocystinuria is associated with deficiency of cystathionine β-synthase. When silver nitrate is included in the reaction mixture, nitroprusside reacts only with homocystine and allows differentiation between homocystine and cystine. Because the color products of the nitroprusside reactions are unstable, nitroprusside reactions have been used only as screening tests.

In this investigation, the absorption spectra of the reaction products produced by the cyanide-nitroprusside reagent with both amino acids have been studied, and quantitative procedures for both cystine and homocystine were developed. The sensitivities of these modified procedures are comparable to either that of an automatic amino acid analyzer or high performance liquid chromatograph (HPLC), but here they are sufficiently simple to be used as screening tests for cystinuria and homocystinuria.

**Materials and Methods**

L-cystine (>99 percent in purity), L-homocystine (>98 percent in purity), and silver nitrate of American Chemical Society grade were all purchased.

Sodium nitroprusside (or sodium nitroferricyanide, dihydrate, 99.7 percent) was also obtained.†

**Quantitative Measurement of Urinary Cystine**

1. Precisely 0.3 mL of urine, 1.2 mL of phosphate buffered saline (PBS), and 0.3 mL of 100 g per L NaCN solution (10 percent) are mixed in a test tube. (Blank: 0.3 mL of urine is mixed with 1.5 mL of PBS and thereafter the procedure is the same as the sample).
2. The solution is allowed to stand at room temperature for five min.
3. The sample absorbance is read against the blank at 521 mm within three min following the addition of 0.1 mL of 200 g per L, nitroprusside (20 percent).

(This procedure does not distinguish homocystine from cystine. Both amino acids react similarly. When the test is positive, a separate aliquot of urine should be used to determine whether the elevation is due to cystine or homocystine by the following procedure).

**Quantitative Measurement of Urinary Homocystine**

(Cystine does not react under these conditions)

1. Urine specimens are saturated with NaCl (about 0.35 g NaCl per ml of urine).
2. Test and blank: 0.5 mL salt-saturated urine is mixed with 1 mL of saturated NaCl solution
3. Test: 0.15 mL of 3 percent NH4OH containing 1 percent AgNO3 is added to the tube. Blank: 0.15 mL of 3 percent NH4OH containing no AgNO3 is added to the tube.

* Sigma Chemical Co., St. Louis, MO.
† VWR Scientific, San Francisco, CA.
4. The mixture is allowed to stand at room temperature for one min.
5. Precisely 0.15 mL of 10 g per L nitroprusside (1 percent) and 0.15 mL of 7 g per L NaCN solutions (0.7 percent) are added to both “Test” and “Blank”.
6. The absorbance of the “Test” solution against the blank is read immediately at 524 nm.

Results

Absorption Spectrum Derived from Cystine

The absorption spectrum for the reaction product of cystine with nitroprusside was scanned to determine the absorbance peak related to the concentration of cystine. As shown in figure 1, two peaks were identified. The peak at 394 nm is derived mainly from the blank (figures 1A and 1B) or the nitroprusside solution; only the absorbance peak at 521 nm is related to the reaction product produced from the cystine and nitroprusside reaction.

The absorption spectrum is not stable; it is shown in figure 1B how rapidly both peaks change with time. At 394 nm, the peak first rises and then declines, whereas the peak at 521 nm remains stable for a few minutes and then begins to decrease with time. However, the stability of the absorbance at 521 nm during the first few minutes allows enough time to read the absorbance. Apparently the absorption of nitroprusside (blank) will contribute to the absorbance at 521 nm (figure 1A, dotted line) if the sample is not read against a blank. The change that took place at peak 521 nm in the initial few minutes is most likely due to a combined effect of the increase of the peak at 394 nm and the decline of the peak at 521 nm during the early stages of the reaction.

Absorption Spectrum Derived from Homocystine

When urine tests positive with cyanide-nitroprusside, the next step is to determine which of these amino acids, cystine or homocystine, is responsible for the positive result. Therefore, the screening test designed to react only with homocystine was also converted to a quantitative procedure. The absorption spectrum of the nitroprusside-homocystine reaction in the presence of silver nitrate is shown in figure 2. There are only minor differences between the absorption spectrum of nitroprusside-homocystine and that of nitroprusside-cystine. For example, because relatively less nitroprusside is used in the homocystine reaction, the absorbance of the blank at 394 nm is relatively smaller; the absorption peak related to homocystine concentration is at 524 nm instead of 521 nm identified for cystine.

The absorption spectrum of nitroprusside-homocystine is also unstable and changes rapidly within minutes (figure 2B). Presumably owing to the presence of more unreacted nitroprusside reagent, the absorbance at peak 394 nm was highest in the lowest concentrations of homocystine (figure 2C). Peak 394 nm also increases first at an early stage of the reaction and then declines with time (figure 2B). However, none of these changes affect the quantitative measurement of cystine and homocystine.

Effect of Amino Acid Concentration

To determine how quickly the absorbance should be read following the nitroprusside reaction, the change of the absorbances related to the reaction products was monitored at both 521 and 524 nm. Apparently absorbance at 521 nm is
slightly more stable than that at 524 nm (figure 3). While the absorbance at 521 nm for cystine remains essentially the same in the first five minutes, the absorbance for homocystine starts to decline as soon as recording begins. There appeared to be no lag phase at the beginning of the reaction (figure 3B). The stability of the absorbance at
Absorption spectra of the homocystine-nitroprusside reaction and their stability. Absorption spectra in figure 2A was produced by 0.5 mL NaCl-saturated urine containing 0.5 mg per mL homocystine after nitroprusside reaction in the presence of silver nitrate. The dotted line is the absorption spectrum of a blank in which urine was replaced by PBS. In figure 2B is shown the change of absorption spectrum with time. 1, 15 sec; 2, 2 min.; 3, 5 min.; 4, 10 min. In figure 2C shown the effect of homocystine concentrations (mg per ml). The reaction mixture contained 1 mL of urine and 0.5 mL of saturated NaCl solution. Other ingredients are as same as in figure 2A.
either 521 or 524 nm is also related to the amino acid concentration (figure 3). It appears that the higher the amino acid concentrations the more rapid the absorbances decrease. Since the cystine concentration of the majority of the positive urines are below 0.5 mg per milliliter, the reaction rate will be sufficiently slow to allow an accurate reading of the absorbance before significant change occurs at 521 nm (figure 3A). However, in the case of homocystine, the rapid decrease of 524 nm (figure 3B) suggests that the absorbance at 524 nm should be read immediately after the addition of nitroprusside. An effort should also be made to read the absorbance for standards, controls, and samples at the same time interval. Conceivably, an automated instrument capable of standardizing the time for pipetting, mixing, and reading will further improve the assay precision.

**Titration of Cystine with Nitroprusside**

Apparently the concentration of nitroprusside used in the reaction is also critical. On one hand, it has to be maintained in excess in respect to cystine; on the other hand, it should be kept low to avoid
high background. In figure 4, a fixed amount of cystine was titrated with increasing amounts of nitroprusside to determine the proper concentration of nitroprusside to be used for the quantitative determination of cystine. Since the amount of cystine (0.5 mg per mL) used in the titration experiment (figure 4) is greater than that found in most urines from patients with cystinuria, the addition of 40 μL of 20 percent (200 g per L) nitroprusside to the reaction mixture (final volume, 0.65 mL) should be sufficient to maintain nitroprusside in excess concentration.

**STANDARD CURVES**

Nitroprusside will react with either free sulphydryl (SH) groups, or disulfide bonds after reduction. As demonstrated in figure 5A, both cystine and cysteine react with nitroprusside under our assay condition and produce linear curves with increasing amino acid concentration. Since cystine weighs about twice as much as cysteine but yields only one sulphydryl group upon reduction by cyanide, it produces only one-half the absorbance that cysteine produces on the same weight basis. No color was generated for methionine.

The standard curves for both cystine and homocystine, used in our newly established procedures, are presented in figure 5. As shown in figure 5B, the absorbance at 521 nm produced by cystine is linear up to 0.5 mg per mL. Cystine concentrations higher than 0.5 mg per mL were not explored. Reactions using different amounts of nitroprusside solution (100 or 60 μL) resulted in almost the same standard curve. For homocystine, the absorbance is linear up to 0.2 mg per mL. Although there is a slight deviation from linearity at 0.5 mg per mL of homocystine (figure 5C), the curve is still usable to provide acceptable result for the diagnosis of homocystinuria.

**EXPRESSION OF CYSTINE PER CREATININE**

In the quantitative procedure, the concentration of cystine is expressed in
Absorbance produced by reaction of nitroprusside with various concentrations of several amino acids. In figure 5A it is shown that the absorbance at 521 nm for cysteine is twice that of cystine on the same weight basis. In figure 5B are presented the standard curves of cystine. The numbers 60 and 100 represent aliquots (in mL) of nitroprusside (0.2 g per mL) used. In figure 5C is shown the standard curve for homocystine.
terms of creatinine to minimize the effect of fluctuation of urine volume. Expressing cystine concentration in terms of creatinine also narrows down the reference range, provides better differentiation of abnormal from normal levels, and helps to eliminate false negative results. One such example is illustrated in table I. Two urine specimens were received from twin baby girls for urine metabolic screening. When the urines were tested by the screening nitroprusside procedure, one urine was positive and the other was negative, as judged by the color that was produced. However, both urines were positive when they were tested again by the newly established procedure. The discrepancy was due to the fact that one of the urine samples had a very low creatinine concentration (0.11 g per L), and the color produced with nitroprusside was similar to that of normal urines. When the cystine concentration was expressed in terms of creatinine according to the new procedure, the result then became positive.

**Stability of Nitroprusside Solution**

The nitroprusside solution was found to be stable for at least one month if stored at 4°C and protected from light. The stability of the nitroprusside solution was tested based on the change of its absorbance over time (figure 6A, -cystine) and also on the absorbance produced by the reaction of nitroprusside with cystine (figure 6A, + cystine). As shown in figure 6A, both absorbances for the nitroprusside at 521 nm, in the absence and presence of cystine, remain unchanged after one month of storage. No difference was found between the fresh solution and the one month-old solution in terms of time-dependent changes in absorption spectra (figure 6B). These results indicate that the nitroprusside solution can be used for at least one month.

**Normal Ranges and Precisions**

Because nitroprusside reacts with both cystine and homocystine in the urine in the absence of silver nitrate, the normal concentration range established by the first procedure actually includes both urinary cystine and homocystine (table II). On the other hand, the normal range established in the presence of silver nitrate relates only to homocystine. The within day, and day to day precisions were established for both cystine and homocystine. The CV of 4.5 and 4 percent for both amino acids indicates excellent precision with these manual tests. A even greater accuracy is expected when an automated instrument, such as a microplate reader is used.

**Discussion**

In the past, an automated amino acid analyzer was required to quantify urinary cystine for monitoring patients on penicillamine therapy. Since an automated amino acid analyzer is not commonly available in the clinical laboratory, there is a demand for a simpler and less expen-
sive quantitative assay for urinary cystine. Therefore, it was decided to reexamine the screening procedure for cystinuria and homocystinuria, and an attempt was made to convert them to quantitative assays. One of the major reasons that the nitroprusside procedure was thought not suitable for the quantitative measurement of cystine is that the color derived from the nitroprusside-cystine product is not stable. After carefully studying the stability of the absorption spectra, it appeared to us that the labile nature of the color product actually is not a problem if absorbance is recorded immediately after the addition of the nitroprusside reagent. This is supported by both the linearity of the standard curves and the precision of both procedures presented in the present study. Conceivably the precision of these reactions can be further improved if these
modified procedures are automated to allow precise timing for pipetting, mixing, and absorbance recording. In fact, a microplate reader coupled with any automatic pipetting station is exactly what is needed for this purpose.

The original cyanide-nitroprusside screening procedure has no problem detecting homozygotes, whose urinary cystine concentrations are usually above 250 mg per g creatinine or greater than 0.1 mg per mL urine. However, cystine in urines from heterozygous patients may only be slightly increased, and the color produced by the cyanide-nitroprusside reaction is not as distinct. Technologists frequently have difficulty interpreting the screening test when there is turbidity or other colored substances present in the urine specimen. Under these conditions, the new quantitative procedure will be very helpful. With the new procedure, cystine may be detected accurately at concentrations of 20 µg per mL, which will allow the identification of any urine containing only a slightly elevated urinary cystine. In fact, the sensitivity of this assay can be increased easily by simply using a larger aliquot of urine for the assay. Our procedure starts with a mixture of 0.3 mL urine and 1.2 mL of phosphate-buffered saline (PBS). All of the PBS can be replaced by urine if higher sensitivity is needed. Therefore, a 5-fold increase in sensitivity can be achieved if 1.5 instead of 0.3 mL of urine is used for the assay.

It should be noted that no attempt was made to determine accurately the normal concentration of urinary cystine because urine contains both cystine and homocystine, and nitroprusside will react with both amino acids in the absence of silver nitrate. The normal range determined in the absence of silver nitrate only allows the identification of abnormal urines. The abnormal urine may contain elevated urinary concentration of cystine or homocystine (table II). To determine the urinary cystine concentration, it will be necessary to perform both procedures and subtract the concentration of homocystine from the total concentration determined in the absence of silver nitrate.

These new quantitative methods can replace the screening procedures because of their simplicity. False negative results may be avoided as was demonstrated with the case of twin baby girls. The sensitivity of these modified procedures will also allow the detection of heterozygotes that normally might be missed by the screening methods.

References


