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Usefulness of cyanide-nitroprusside test in detecting incomplete recessive heterozygotes for cystinuria: a standardized dilution procedure

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Abstract We present the results of a cyanide-nitroprusside test (CNT) after a standardized dilution procedure of urine samples and report the efficiency of this method in detecting heterozygotes for cystinuria when applied on an open pediatric population. In the preliminary study we assayed by quantitative determination of amino acids 162 urine samples from a hospital population identifying 24 type III heterozygotes and 2 type II heterozygotes for cystinuria. The classic CNT gave 38 false positive results and 5 false negative results showing a sensitivity and specificity of 0.808 and 0.721, respectively. When progressively diluted, all samples of heterozygotes remained CNT positive up to a creatinine concentration of 90 mg/dl. At this level of dilution 31 out of 38 false positive turned to negative, thus obtaining a specificity of 0.922 without a lowering of the sensitivity in detecting heterozygotes. The standardized dilution at 90 mg/dl of creatinine concentration was applied to 74.7% of a population of 1024 schoolchildren. In this way 163 out of 210 positive results were eliminated and thus the specificity of CNT rose from 0.789 to 0.953. On the basis of these results, the method proposed can be regarded as reliable and useful for a screening program in detecting heterozygotes for cystinuria.

Key words Cystinuria · Cyanide-nitroprusside test · Heritable disorders · heterozygotes screening

Introduction

Cystinuria, the most common of the specific aminoacidurias, is a heritable disorder of intestinal and renal amino acid transport in which a large amount of cystine, arginine, ornithine, and lysine are excreted in urine. Three types of classic cystinuria have been described: type I is inherited in an autosomal recessive mode, heterozygotes showing normal aminoaciduria; in types II and III, inheritance is incompletely recessive and heterozygotes show cystine-lysinuria. The intestinal and renal defects and urinary amino acid excretion are lower in the type III phenotype [9]. Compound heterozygotes (I/III, I/II and II/III) can be revealed by the type of aminoaciduria in blood relatives [8].

A careful identification of the cystinuria phenotype may be useful in anticipating the relative risk of nephrolithiasis in incomplete recessive heterozygous children [4]. An identification of type II and III heterozygotes was obtained in previous studies using a cyanide-nitroprusside test (CNT) as a screening method [3, 7, 10], but further studies have shown that CNT does not ensure an accurate detection of these heterozygotes [1, 5].

It is well known that errors in the measurement procedure as well as some interfering substances can be source of potential errors of the CNT results. Urinary creatinine and large amounts of ketones produce a deep orange color that could be interpreted as a positive result. Sulfur-containing molecules (e.g., homocystine) deriving from endogenous metabolism or foods, react like cystine with cyanide-nitroprusside. Acified urine samples or an elevated concentration of ascorbic acid could produce a false negative reaction interfering with the development of the colored products. Among the factors affecting the sensitivity and specificity of CNT, the dilution of the urine sample, as indicated by the creatinine content, plays an important role. In the past, only the samples showing a marked purple color to CNT were scored as positive to minimize the false positive results that frequently occur in concentrated urines. This decreased the sensitivity of the test for detection of incomplete recessive heterozygotes. On the other hand, the excessive dilution of urine samples, which mainly occurs in infants and young children because of the inability of the renal concentrating system [11], has always repre-

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sented an obstacle in identifying heterozygotes by colorimetric tests. To date, no studies have evaluated the efficacy of CNT in detecting type II and III heterozygotes using an optimized dilution of urine samples on the basis of the creatinine content. For this reason, we developed a standardized dilution procedure and applied it to an open pediatric population.

Subjects and methods

Preliminary study

Subjects

In the preliminary study we examined 164 urine samples sent to our laboratory over a period of 2 years. The samples came from children aged 3 to 13 years, (mean 7.4 years, SD 2.9 years) who had been patients at the Department of Pediatrics, in which routine chemical assay, quantitative amino acid analysis and colorimetric tests (including CNT) were performed on suspicion of metabolic, urologic or neurologic disorders. Samples from 37 patients were sent to us with a presumptive diagnosis of cystinuria.

Dilution procedure

All the urine samples positive to CNT were firstly diluted on the basis of the originary creatinine concentration by adding distilled water to obtain a final concentration of 120 mg/dl and then progressively diluted up to 40 mg/dl in steps of 10 mg/dl. CNT was repeated at each step of dilution. The samples were tested with blinding in respect to the level of dilution and heterozygotic status.

Schoolchildren population

To evaluate the procedure described we applied the standardized dilution at 90 mg/dl creatinine concentration to the urine samples from an open pediatric population. A population of 1500 elementary schoolchildren was enroled from five schools located in the city of Rome. Superintendents and principals were contacted for permission to use their schools as study sites. The parents of study subjects received information about the objectives and methods of the study and were asked to give consent, which was obtained in all cases. The subjects had not been taking any drugs for at least 1 week before entering the study and were invited to avoid drinking 10 hours before urine collection. A morning sample of fresh urine was obtained from 1024 schoolchildren (compliance 68.3%), collected in vessels containing antibiotic and promptly sent to our laboratory. At the time of sampling a detailed chart reporting historical information was compiled. All subjects, aged 8 to 12 years (mean age 10.4 years, SD 1.3 years), were without any history of signs or symptoms related to urolithiasis and without any detectable active disease. The test was performed before and after dilution to obtain a final creatinine concentration of 90 mg/dl. The samples that proved CNT positive (before dilution) were assayed for quantitative amino acid analysis.

Laboratory investigations

Cyanide-nitroprusside test

The CNT was carried out as follows: mix 1 ml of urine with 0.2 ml of a 67 g/l acqueous sodium cyanide solution, let stand for 10 min at ambient temperature, then add dropwise up to 0.2 ml of a 50 g/l sodium nitroprusside solution. The test was considered positive when the sample turned pink to purple. Sodium cyanide and sodium nitroprusside were obtained from Carlo Erba (Milano, Italy) and BDH Chemicals (Poole UK) respectively. The within-observer variation in the interpretation of CNT was evaluated employing a pool of 20 urine samples from normal subjects (cystine = 23 μmol/l; creatinine = 89 mg/dl) in which cystine was added in order to obtain concentrations ranging from 30 μmol/l to 150 μmol/l, in steps of 10 μmol/l. Each specimen was tested 20-fold with blinding to cystine concentration. The results are reported in Fig. 1.

Urinary amino acids

Immediately after samples were collected 5-ml aliquots of urine were passed through C-18 Sep-Pack cartridges (Waters, Milford, Mass.) previously activated with water. Aliquots (100 μl) of eluate were mixed 1:1 with a 0.5 mM solution of norleucine in 0.1 M HCL as an internal standard. Aliquots (20 μl) were desiccated under vacuum and derivatized with 20 μl of methanol-water-triethylamine-phenylisothiocyanate solution (7:1:1:1) according to the Pico-Tag method for physiological amino acids [2]. The phenylthiocarbamyl derivatives of amino acids were stored at −70°C and assayed by reverse-phase high-performance liquid chromatography (HPLC) within one week after derivatization. The chromatographic system consisted of two 510 HPLC pumps for the high-pressure gradient, a rheodyne injector, a 484 absorbance detector (254 nm) and a baseline 810 chromatography data workstation, all obtained from Waters. Water for solutions and eluents was obtained from a Milli-Q purification system (Millipore, Bedford, Mass.). Phenylisothiocyanate was obtained from Waters; trietylamine, norleucine and amino acids standards were obtained from Sigma-Aldrich (Milan, Italy). Elution times of arginine, cystine, ornithine and lysine were 23.2, 49.1, 56.5, and 61.8 min respectively. The lower limit of cystine excretion found in our laboratory for the low-excretion heterozygotes (type III) was 135 μmol/g creatinine (Table 1).

Other laboratory data

Urine creatinine content was assayed colorimetrically by the alkaline picrate method.

Subjects' classification

Subjects of both groups (hospital and schoolchildren populations) were classified in their phenotypic category by calculating the sum of urinary cystine plus each of the other dibasic amino acids

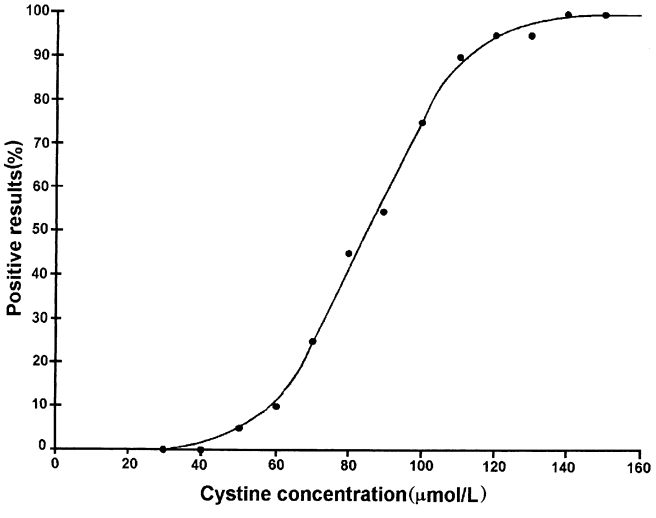

Fig. 1 Positive results rate of CNT as a function of sample's cystine concentration

Table 1 Urinary amino acid excretion by normal and heterozygous children in the hospital population[a]

Subjects	Cystine (μmol/g creatinine)	Ornithine (μmol/g creatinine)	Arginine (μmol/g creatinine)	Lysine (μmol/g creatinine)	Sum[b] (μmol/g creatinine)
Normal (*n* 136)	63.8 ± 18	17.6 ± 7.9	12.7 ± 2.9	126 ± 105	219 ± 111
Range	(24–112)	(5–40)	(7–21)	(11–512)	(61–588)
Type II (*n* 2)	673 ± 116	189 ± 17	146 ± 76	3204 ± 242	4214 ± 32
Range	(591–756)	(177–201)	(93–200)	(3033–3376)	(4191–4237)
Type III (*n* 24)	193 ± 66	62.2 ± 43	61.5 ± 48	1081 ± 469	1398 ± 534
Range	(135–342)	(20–186)	(17–202)	(590–2138)	(784–2664)

[a] Values are mean ± SD
[b] Sum of urinary cystine plus dibasic amino acids (ornithine, arginine, and lysine)

(arginine, ornithine, and lysine) [6]. The cut-off value of urinary cystine plus dibasic amino acids for the diagnosis of heterozygous cystinuria is 700 μmol/g creatinine. This approach allows good discrimination among the groups except that II/normal heterozygotes overlap completely with I/III double heterozygotes. Therefore, in subjects with uncertain genotype, the determination of urine amino acids excretion was extended to the relatives and probands were classified according to the parental phenotype.

Results

Preliminary study

Subjects

The quantitative determination of urinary amino acids in the 164 samples of the hospital population showed a normal excretion of cystine plus dibasic amino acids (sum <700 μmol/g creatinine) in 136 samples (82.9%) (Table 1) and an abnormal excretion (sum >700 μmol/g creatinine) in the remaining 28 samples (17.1%). In the latter group, 24 probands showing a cystine plus dibasic amino acid excretion from 784 to 2664 mmol/g creatinine were assigned to the III/normal genotype (type III). In two probands the sum of cystine plus dibasic amino acids excretion was over 4000 μmol/g creatinine. Both had one parent with an elevated excretion (>4000 μmol/g creatinine) whereas the other showed a normal excretion. Therefore they were assigned to the II/normal genotype (type II) (Table 1). Two subjects showing generalized aminoaciduria were eliminated from the study group. In the remaining 162 samples, CNT showed positive results in 59 samples (36.4%) and negative results in the remaining 103 samples (63.6%) (Table 2). Among the 59 CNT positive, 21 subjects were heterozygotes (19 type III and 2 type II) and 38 were false positive (Table 2). None of 38 false positive samples showed presence of ketone bodies, methionine, sulfocysteine, homocystine or mixed disulfide. Five out of 103 CNT negative were heterozygotes (all type III). In these samples pH ranged from 5 to 6 and ascorbic acid was below 10 mg/dl. The sensitivity and specificity of CNT were 0.808 and 0.721, respectively.

Plotting the values of cystine excretion as a function of urinary creatinine concentration, both false positive and false negative results appeared highly dependent on urinary creatinine concentration. As shown in Fig. 2, the

Table 2 Results of CNT in normal and heterozygous children in the hospital population

Subjects	CNT results		Total
	Positive	Negative	
Normal			
N/N or I/N (*n* 136)	38	98	136
Type II			
II/N (*n* 2)	2	0	2
Type III			
III/N (*n* 24)	19	5	24
Total	59	103	162

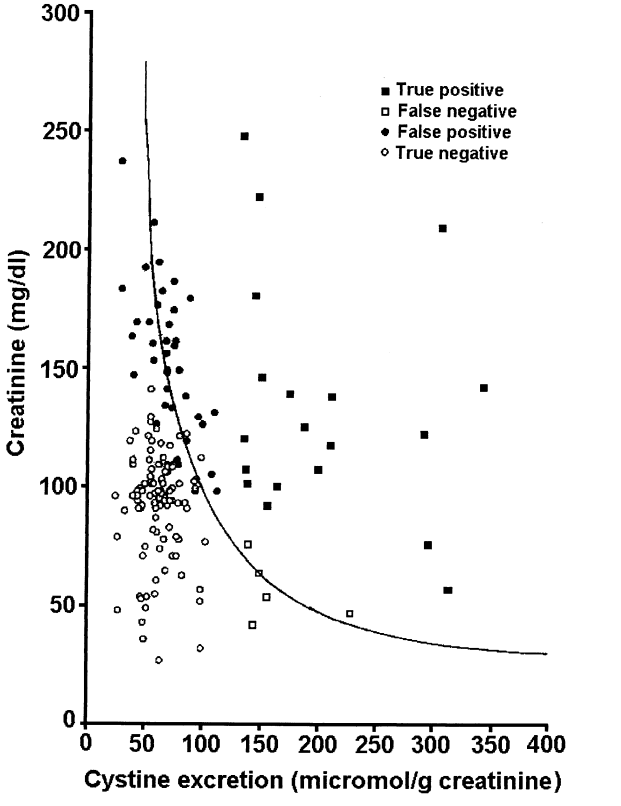

Fig. 2 Urinary cystine excretion from normal and heterozygous (type III) children as a function of the sample's creatinine concentration. Solid line shows the theoretical curve for a cystine concentration of 100 μmol/l computed by using the equation: [creatinine, mg/dl] = 10^4/ [cystine excretion, μmol/g]

false positive results occurred in samples with a creatinine level >98 mg/dl having a mean creatinine concentration significantly higher in respect to the total population (153 ± 31 vs 111 ± 31; $P < 0.01$), the false negative results occurred at a creatinine level <76 mg/dl with a mean creatinine concentration significantly lower than that of the total population (56.6 ± 13 vs 111 ± 31; $P < 0.001$) (*P*-value calculated by one-way analysis of variance).

Dilution procedure

The dilution procedure was applied on urine samples from 21 heterozygotes and 38 normal subjects who had been CNT positive at the initial determination. In the group of 21 heterozygotes (true positive) the excretion of cystine ranged from 135 to 756 μmol/g creatinine, thus including also the low-excretion type III heterozygotes. In the group of 38 normal subjects (false positive) the excretion of cystine was similar in respect to the CNT negative normal subjects (data not shown). Table 3 shows the performance of CNT at different levels of sample dilution. At a level of 90 mg/dl creatinine concentration, 113 samples were screened including 19 heterozygotes. The samples from heterozygotes remained CNT positive at this level of sample dilution, whereas 31 of 38 false positive changed to negative thus obtaining a specificity of 0.926 in respect to the proportion of population screened (69.8%). In order to evaluate whether persistent false positive results reflected the presence of interfering substances in unreacted urines or reagents, we measured the absorbance from unreacted urines, reagent blanc against water and completely reacted urines, at the peak absorbance of cystine-nitroprusside (521 nm). In each case the absorbance values of unreacted urines or reagent blanc were less than 5% of completely reacted urines. False positive results were eliminated at the creatinine level of 40 mg/dl but, at this level of sample dilution, the sensitivity in detecting heterozygotes decreased to 0.154.

Schoolchildren population

Classic CNT performed on 1024 schoolchildren gave positive results in 239 urine samples (23.3%). Among the 239 CNT positive, 29 heterozygotes (28 type III and 1 type II) were identified by quantitative amino acid analysis. The estimated frequency of type II and type III heterozygotes in the schoolchildren population was 0.97% and 2.7%, respectively.

The standardized dilution at a level of 90 mg/dl creatinine concentration involved 765 out of 1024 urine samples from schoolchildren (74.7%) showing an original creatinine concentration ≥90 mg/dl. The CNT resulted positive in 73 urine samples including all of the 29 samples from heterozygous probands. Three false positive samples showing an original creatinine concentration <90 mg/dl could not be diluted. Therefore, the standardized dilution was able to eliminate 163 of the 210 false positive results and the specificity of CNT in detecting heterozygotes rose from 0.79 to 0.94 or 0.953 whether calculated on the 765 dilute samples or on the total population (1024 samples), respectively (Table 4).

Discussion

The sensitivity of classic CNT in detecting type II and III heterozygotes for cystinuria showed variable values in previous studies, probably depending on the individual evaluation of the colored products scored as

Table 3 Results of dilution procedure on urine samples of the hospital population

Dilution[a]	Subjects[b] *n* (%)	Sensitivity[c]	Specificity[c]
Undiluted	162 (100%)	0.808	0.721
90	113 (69.8%)	1	0.926
80	133 (82.1%)	0.947	0.939
70	145 (89.5%)	0.810	0.960
60	151 (93.2%)	0.591	0.969
50	156 (96.3%)	0.250	0.985
40	159 (98.1%)	0.154	1

[a] Level of sample dilution expressed as final creatinine concentration (mg/dl)
[b] Population screened at the level of sample dilution reported in column 1
[c] Values calculated taking into account the population screened at the level of sample dilution reported in column 1

Table 4 Results of CNT before and after standardized dilution (90 mg/dl creatinine concentration) on urine samples of the school-children population

Samples	Subjects *n* (%)	CNT results			Specificity
		False positive	True positive	Negative	
Undiluted	1024 (100%)	210	29	785	0.79
Diluted	765 (74.7%)	44	29	692	0.94

positive. In fact Byrd et al. [1] reported in the same population a sensitivity that rose from 0.516 to 0.838 when borderline positive results were included. This latter approach may explain the low specificity found in our study, confirming that classic CNT is not efficient in detecting incomplete recessive heterozygotes for cystinuria. However, the data plotted in Fig. 2 show that false negative and false positive results of CNT are highly dependent on the urinary creatinine concentration. The dilution procedure performed in our preliminary study shows that it is possible to improve the specificity of this test without a lowering of its sensitivity. In fact, at the dilution level of 90 mg/dl creatinine concentration, almost all false positive results can be eliminated whereas the true positive results remain unchanged. At this level of urine dilution the CNT can identify correctly and with good reproducibility (>95%) the samples with a cystine excretion >122 μmol/g creatinine and <44 μmol/g creatinine having a cystine concentration >110 μmol/l and <40 μmol/l respectively (Fig. 1). Since the lower limit of cystine excretion in type III heterozygotes was reported of 120 μmol/g creatinine [6], the dilution at 90 mg/dl of creatinine concentration seems to ensure a good detection of the low-excretion heterozygotes. Below this level of sample dilution, the CNT shows a lower sensitivity in detecting heterozygotes for cystinuria, and thus of no use for screening purposes. Therefore, to minimize the impact of this limitation on a screening program, efforts should be made to obtain concentrated urine specimens.

On the basis of the preliminary study, we applied the standardized dilution on the 74.7% of urine samples of the schoolchildren population, eliminating 163 false positive results and thus obtaining a high specificity (0.953 in our series). The ambiguity of CNT results in the upper limits of cystine concentration of normal urine samples could explain the persistence of false positive results after standardized dilution. (Fig. 1). Otherwise, we have not found the presence of known interfering substances in the false positive samples.

Since observer variability always represents a bias in the evaluation of dichotomous tests, we provided a curve showing the within-observer variation found in our laboratory (Fig. 1), by which a comparison is possible and, if necessary, a correction of the optimized dilution according to the individual test performance.

In conclusion, our study outlines some of the main points for a more correct utilization of CNT and proposes a simple dilution procedure, available for a high percentage of the study population, which increases the efficiency of this widely used test in discriminating clinical cystinuric phenotypes. The method proposed, for its low cost and simplicity, can be regarded as reliable and useful for a screening program of heterozygous cystinuria.

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