percentile was substantially different (four- to fivefold higher) than the analytical sensitivity, allowing for acceptable clinical specificity. Furthermore, there was excellent clinical concordance between the Vitros ECi and Dade RxL cTnI assays. The cTnI values obtained with the ECi were approximately one-half of the those obtained with the RxL cTnI, probably because of the lack of antigen and antibody standardization between assays (4). Thus, the Vitros ECi Troponin I immunodiagnostic assay appears to be an acceptable alternative for monitoring plasma or serum cTnI for ruling in and ruling out AMI. As for risk stratification in acute coronary syndrome patients (6), additional clinical evaluation studies will be necessary to validate it applicability in clinical practice.

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References

Semiautomated Method for Determination of Cystine Concentration in Polymorphonuclear Leukocytes, Anna Pastore,1 Anna Lo Russo,1 Marcella Greco,2 Gianfranco Rizzoni,2 and Giorgio Federici1 1Laboratory of Clinical Biochemistry and 2Division of Nephrology and Dialysis, Children’s Hospital and Research Institute “Bambino Gesù”, Piazza S. Onofrio 4, 00165 Rome, Italy; * author for correspondence: fax 39-06-68592210, e-mail Federici@obg-irccs.rm.it)

Cystinosis is an autosomal recessive disease caused by impaired transport of cystine across lysosomal membranes. The subsequent lysosomal storage of the poorly soluble cystine produces crystal formation and cellular damage in many tissues. The earliest involvement occurs in the renal tubules and causes Fanconi syndrome, with polyuria, dehydration, acidosis, rickets, and failure to thrive. In untreated cystinosis, the progression of renal glomerular dysfunction leads to uremia and death by 9–10 years of age unless dialysis or renal transplantation is initiated (1). The therapy of nephropathic cystinosis involves treatment with the cystine-depleting agent cysteamine (2).

The most direct diagnostic method for cystinosis is measurement of leukocyte cystine content by an Escherichia coli cystine-binding protein assay. This assay is time-consuming and involves competition between non-radioactive cystine and [14C]cystine for a cystine-binding protein. The protein-bound radioactivity is then trapped on nitrocellulose filters and is inversely correlated to nonradioactive cystine (3).

Here we present a method for measuring the cystine content of polymorphonuclear (PMN) leukocytes that is based on HPLC with fluorescence detection (FD) (4, 5); this method is reproducible, sensitive, and requires no radioactive reagents.

Blood (4.5 mL) was drawn by venipuncture and was collected into a heparin-containing tube. PMN leukocyte separations were carried out as described by Smolin et al. (6). After sonication of the PMN leukocytes three times for 2 s (each) in 0.1 mL of 0.1 mol/L phosphate buffer, pH 7.2, containing 5 mmol/L N-ethylmaleimide (NEM), 50 µL of 120 g/L sulfosalicylic acid was added, and the cystine content in the acid-soluble fraction was determined. The protein pellet was then dissolved in 150 µL of 0.1 mol/L NaOH, and the protein concentration was determined with the BCA protein assay (Pierce).

The derivatization and chromatography procedures were performed, with little modification, as reported previously (4). Briefly, the autosampler collected 3 µL of 4 mol/L NaBH4, 2 µL of 2 mmol/L EDTA-dithiothreitol, 1 µL of 1-octanol, and 2 µL of 1.8 mol/L HCl and placed the mixture in the derivatization vial containing 10 µL of sample. After the mixture was incubated for 3 min, 10 µL of 1.5 mol/L N-ethylmorpholine buffer, pH 8.0, 40 µL of distilled water, and 2 µL of 25 mmol/L bromobimane were added. After an additional 3-min incubation, 4 µL of acetic acid was added, and 40 µL of this mixture was injected into the column.

Known concentrations of cystine were added to samples. The concentrations in samples with added cystine were determined in five replicates, and analytical recoveries were calculated. The intraassay precision was obtained by analyzing 10 replicates of the biological samples on the same day. The interassay precision was determined by analyzing the same biological samples on 10 different days over 1 month.

Calibration curves for cysteine (0.3–10 µmol/L) were prepared in duplicate by diluting the stock solutions with 0.1 mol/L HCl containing 100 µmol/L dithiothreitol. The linearity of the assays was also studied in the following range: 0–10 µmol/L cysteine. The limit of detection, defined as the concentration that produces a signal-to-noise ratio >3, was ~0.4 pmol in the assay.

In a typical HPLC-FD chromatogram of a sample from a healthy subject (Fig. 1A), the retention time for cysteine-S-bimane was 3.7 min. The cysteine peak at 3.7 min in Fig. 1A was given by a sample concentration of 0.08 nmol half-cysteine/mg protein. In a typical chromatogram from an individual with cystinosis (Fig. 1B), the cysteine-
S-bimane peak gave a sample concentration of 3.5 nmol half-cysteine/mg protein.

A linear relationship was obtained between the peak area and cysteine concentration in the ranges studied in an aqueous matrix (data not shown). The correlation coefficient was >0.99. The equation for the regression line \((n = 8)\) was: \(y = 0.03x + 0.16\), where \(y\) is the peak area, and \(x\) is the concentration of the analyte. The lowest concentrations in the linearity studies were above the limit of detection of 0.4 pmol in the assay. No significant matrix effect was observed when linearity studies were performed in samples diluted up to 1:32 with water containing 100 \(\mu\)mol/L dithiothreitol (data not shown).

The intraassay CV for the cysteine measurements was 2%; the interassay CV, determined by assaysing on 10 different days over 1 month, was 4.7%. The mean recovery was 99.6%.

A discontinuous gradient of Ficoll-Hypaque (Sigma) was used to separate PMN leukocytes from blood. The PMN cells were 78–96% pure as determined by cell counting with a hemocytometer. The residual leukocyte material contained <4% PMN cells.

The PMN leukocyte cystine concentrations in 20 healthy children (Fig. 1C) were 0.01–0.19 nmol half-cysteine/mg protein (mean ± SD, 0.08 ± 0.06), consistent with other studies (1). As reported previously (5), the PMN leukocyte cystine concentrations in subjects before treatment with cysteamine were 3.5–3.8 nmol half-cysteine/mg protein \((n = 3; \text{age range, 6 months to 1 year})\);

the cysteine-depleting effect of cysteamine was shown during treatment, with PMN leukocyte cystine concentrations ranging from 0.7 to 1.23 nmol half-cysteine/mg protein \((n = 16; \text{age range, 1–20 years})\).

In obligate heterozygotes, PMN leukocyte cystine concentrations were intermediate between those in homozygotes and healthy individuals \((n = 24; \text{mean ± SD, 0.9 ± 0.6 nmol half-cysteine/mg protein}); \) this finding is consistent with other studies (1, 6).

Early diagnosis of cystinosis is essential for timely treatment, and follow-up of a patient in treatment is essential for a correct therapy. Moreover, determination of PMN leukocyte cysteine concentrations in individuals with a family history of nephropathic cystinosis is important for identifying heterozygotes for this heritable disease.

The method presented here is based on the measurement of cystine with a simple, fully automated HPLC-FD assay after isolation of PMN leukocytes and sonication of cells. We measured the cystine concentrations in PMN leukocytes fraction because Smolin et al. (6) demonstrated that the use of this fraction provides an accurate method for detecting heterozygotes. Our results in obligate heterozygotes agree with the report of Smolin et al. (6) in that >90% of individuals who are heterozygotes will have values that fall above the reference interval; in the present study, none of the values for heterozygotes were within 2 SD of the mean for the healthy subjects (Fig. 1C).

In all experimental systems where both cysteine and

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Fig. 1. HPLC-FD chromatograms obtained for PMN leukocytes of a healthy subject (A) and a cystinotic patient before treatment with cysteamine (B), and cystine content of leukocytes (C).

(A and B), cysteine eluted at 3.7 min. Peaks that eluted after 3.7 min are unidentified compounds with bromobimane fluorescence. (C), cystine content of PMN leukocytes from healthy subjects (a), affected homozygotes (b), and obligate heterozygotes (c). Cystine concentrations in 20 healthy donors were 0.01–0.19 nmol half-cysteine/mg protein (mean ± SD, 0.08 ± 0.06 nmol half-cysteine/mg protein); cystine concentrations in affected subjects before treatment with cysteamine were 3.5–3.8 nmol half-cysteine/mg protein \((n = 3)\). Obligate heterozygotes show cystine concentrations intermediate between those in affected homozygotes and healthy subjects \((n = 24; \text{mean ± SD, 0.9 ± 0.6 nmol half-cysteine/mg protein})\).
cystine are present, care must be taken to prevent the spontaneous oxidation of cysteine to cystine, which would spuriously increase the results for cystine. This is accomplished using NEM, which forms an irreversible adduct with free thiols such as cysteine.

The current method measures cysteine after reduction of cystine with NaBH₄; before this reduction, free cysteine and other free thiols are blocked by the use of 5 mmol/L NEM in the sonication mixture to prevent oxidation of free cysteine and the disulfide exchange reaction of cystine. The use of high NEM concentrations is justified by the presence in the cell of high concentrations (1–2 mmol/L) of reduced glutathione, which also react with NEM (7). It was demonstrated that excess NEM has essentially no effect on the determination of oxidized thiols with this method (8). Moreover, samples are at acid pH before and during the first step of derivatization (reduction), and NEM is almost inactive at acid pH (9).

In conclusion, this HPLC assay is sensitive and precise and provides high sample throughput; moreover, the cost of the procedure is lower than the cystine-binding protein assay. This assay thus enables reliable determination of intracellular cystine in both homozygous and heterozygous subjects, which allows simple and rapid diagnosis of cystinosis in newborns as well as identification of heterozygous individuals.

References