expansions observed in fragile X syndrome and myotonic dystrophy, which are often mitotically unstable and result in diffuse smears on Southern transfers, HD alleles exhibit little if any mitotic instability. Thus, the expanded alleles in HD always appear sharp and just as intense as the normal alleles.

The direct measurement of the CAG repeat in the HD gene is a sensitive and highly accurate molecular diagnostic test. Although linkage testing has been available for HD families for several years, the linkage testing is more expensive and less accurate, especially when key family members are unavailable. The described Southern protocol has provided us with an additional confirmation of the PCR results. We have found excellent agreement between the results of the two types of mutational analyses. The Southern test has been particularly useful when dealing with affected patients with highly expanded alleles (>70 repeats), which often fail to amplify well, and nonaffected patients who are homozygous for normal-size alleles. Use of the Southern test also represents an excellent quality-assurance step, which is particularly important in the presymptomatic cases. Given the seriousness of the diagnosis and the clinical variability of HD, all efforts should be made to reduce the possibility of a misdiagnosis. All results in our laboratory have been generated by two independent types of DNA tests, thus reducing the chance of error.

References


Shortcomings of an Automated Assay for Total Antioxidant Status in Biological Fluids, David Schofield* and Joan M. Braganza (Pancreato-Biliary Serv., Manchester Royal Infirrn., Oxford Rd., Manchester M3 9WL, UK; *author for correspondence: fax Int+44-161-276-4168, e-mail mommfcl@stud.mann.ac.uk)

Oxidative stress, the potential for tissue damage from an excess of free radical production over available antioxidant defenses, [1] is now incriminated in >100 disease states [2]. Although individual antioxidant moieties play specific roles in combating oxidative stress, the physiological concentrations and effects of each antioxidant should not be interpreted in isolation [3]. In recent years, several methodologies designed to measure the total antioxidant status of body fluids with a view to diagnostic and (or) prognostic use in humans have been described [4]. The most widely used of these methods, the total (peroxyl) radical-trapping antioxidant potential (TRAP) assay [5], is unfortunately too lengthy to permit the analysis of large batches of samples and is also technically difficult, requiring specialized equipment. A more recent technique [6] measures the ability of antioxidants within physiological fluids to quench the absorbance of the radical cation formed by the reaction of 2,2'-azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with a peroxidase and hydrogen peroxide. This method is suitable for automation with a centrifugal analyzer and thus permits rapid throughput of samples. The assay is now available as a "Total Antioxidant Status" kit (Randox Labs., Crumlin, UK). The kit has recently been used to monitor possible changes in serum total antioxidant status after consumption of red wine [7].

An integral part of our ongoing studies in patients with pancreaticitis [8] is to monitor changes in concentrations of blood antioxidants, particularly micronutrients, and markers of oxidative stress. The performance characteristics of this kit were, therefore, assessed in the current study, with a Roche Cobas Bio centrifugal analyzer (Roche Analytical Instruments, Nutley, NJ). This centrifugal analyzer permits simultaneous monitoring of absorbance, in the ultraviolet (UV)/visible range, of several samples at time intervals as short as 10 s and is, therefore, ideally suited for following the kinetics of ABTS+ generation. Chromogen, containing ABTS and metmyoglobin, which acts as a peroxidase in this assay, was prepared in phosphate-buffered saline (5 mmol/L, pH 7.4) as instructed. Generation of the blue-green radical cation ABTS+ was initiated by addition of hydrogen peroxide. Quenching of the absorbance of this species at 600 nm, by plasma or individual antioxidants, was compared with that from a Trolox® (6-hydroxy-2,5,7,8-tetramethyl
chroman-2-carboxylic acid (Hoffmann-LaRoche); included as part of the "Total Antioxidant Status" kit (Randox Labs.) calibrator.

Initial studies were promising, showing a "lag time" for ABTS\(^{+}\) production for the Trolox calibrator (2.5 mmol/L) of almost 2 min; a linear absorbance change with Trolox concentration (0–2.5 mmol/L) at time 3 min \((r > 0.999)\); and an acceptable within-batch CV \(<2\%\) for total antioxidant capacity of control plasma, when measured at 3 min.

However, a number of problems with the methodology soon became apparent:

1) The measured Trolox equivalent antioxidant capacity (TEAC) of human plasma increased markedly with run time. For example, the TEAC value for plasma from one healthy individual was 1.06 mmol/L when measured at 120 s, 1.21 mmol/L at 140 s, 1.37 mmol/L at 160 s, and 1.53 mmol/L at 180 s, thus showing a rise of 44\% when measured at 3 min as opposed to 2 min, and continuing to increase thereafter. Indeed, at longer run times, the absorbance spectrum of the assay mixture containing plasma was markedly different from that containing Trolox, to the extent that the reaction mixture was pale purple rather than blue-green as characteristic of ABTS\(^{+}\). Thus, plasma appears to become a more efficient antioxidant as the assay incubation time increases.

2) Twofold dilution of the same plasma sample resulted in an increase in plasma TEAC at 3 min, from 1.51 mmol/L to 1.82 mmol/L, consistent with the increase in values on dilution noted in the instruction sheet for the kit.

3) Attempts to supplement plasma samples with known quantities of Trolox gave low recoveries. Dilution of aliquots of this control plasma with equal volumes of water and 2.40 mmol/L Trolox gave TEAC values for the diluted samples of 0.91 and 1.85 mmol/L respectively, a recovery of only 78\% for Trolox.

Taken together, these results raised grave doubts as to the accuracy of any quantitative measure of total antioxidant capacity using this assay. Consequently, further studies using individual antioxidants were undertaken to attempt to pinpoint the source of the problems.

It is known that albumin, urate, and ascorbate make the major contributions to the total antioxidant capacity of human plasma \([6]\), largely because of their high concentrations relative to those of other blood antioxidants such as bilirubin, \(\alpha\)-tocopherol, and \(\beta\)-carotene. Albumin fraction V (bovine; Sigma, Poole, UK), uric acid (Sigma), and ascorbic acid (Merck, Lutterworth, UK) were therefore prepared as calibrators, in the concentration range 0–2 mmol/L for comparison with Trolox.

Figure 1 summarizes the time course of ABTS\(^{+}\) production, as monitored by absorbance at 600 nm, in the presence of each of these substances, together with the profiles for plasma and a "blank" reaction, for comparison. Ascorbate behaves in an analogous manner to Trolox, showing a lag phase during which no color is generated, followed by a rapid production of ABTS\(^{+}\). The TEAC value for ascorbate derived from these data (0.95) is independent of run time, and suggests that ascorbate, like Trolox, acts as a sacrificial antioxidant in that no ABTS\(^{+}\) is observed until the antioxidant is consumed.

However, the kinetics for albumin are entirely different. No lag phase is observed, but the rate of color development is lower than that for the blank reaction, Trolox, or ascorbate. These data suggest that albumin reacts slowly to quench ABTS\(^{+}\) after its formation and (or) inhibits the peroxidase-catalyzed reaction generating the chromophore. Albumin therefore mimics plasma in that it apparently becomes a more efficient antioxidant as incubation time increases, the TEAC value rising from 0.24 at 2 min to 0.47 at 3 min. Furthermore, the reaction mixture for cuvettes containing albumin ultimately turns pale purple, as for plasma.

urate displays a reaction profile showing mixed characteristics; a lag phase during which absorbance increases little, followed by a "slow" generation of the radical cation. The TEAC therefore again rises with increasing run time.

In summary, these three key antioxidants of plasma apparently influence ABTS\(^{+}\) production by differing mechanisms, because the kinetics of chromophore generation vary markedly. It is a key requirement of any technique purporting to measure "total antioxidant capacity" that all antioxidants contributing to this capacity behave in an analogous manner. This requirement is clearly not met for this "Total Antioxidant Status" kit; thus, any results obtained must be regarded as, at best, semiquantitative because they are critically dependent on the analyzer parameters used. In particular, attempts to correlate the contributions of individual antioxidants with the measured total antioxidant capacity are doomed to failure. We recommend that in validating any novel methodology for total antioxidant status, close attention be paid to confirming that the kinetics of reaction are equivalent for all the key physiological antioxidants, thereby ensuring that the measured antioxidant capacity is essentially independent of the precise assay conditions selected.

Furthermore, our interest in a clinical setting is to monitor changes in blood antioxidant status after supplementation with micronutrient antioxidants. As noted by others \([5, 6, 9]\), the major contributions to total antioxidant status are provided by albumin and urate, rather than micronutrients such as ascorbate and \(\alpha\)-tocopherol. Total antioxidant capacity is raised in the serum of patients predialysis, substantially because of high serum urate, despite low ascorbate \([9]\). The fall after dialysis is
largely due to reduced urate concentrations, although protein thiols rise [9]. Thus, despite an apparently normal total antioxidant capacity in a patient, the monitoring of individual micro-nutrient antioxidants, each of which may play key roles, remains essential.

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References

HPLC Screening Method for Cystinuria, John F. Livesey, James G. Donnelly, Daylily S. Ool
Cystinuria is a disorder of amino acid transport with an autosomal recessive inheritance pattern. It is one of the most common genetic disorders, with an overall prevalence of 1 in 7000 worldwide [1]. Several mutations of the SLCA31 gene on chromosome 2 have been identified [1–3]. The mutated gene produces a defective protein whose normal function facilitates reabsorption of cystine (CYSS) and dibasic amino acids in the proximal renal tubules. Excessive elimination of CYSS increases the risk of crystal and stone formation. Maximum solubility of CYSS in urine is 1.2–1.6 mmol/L in the physiological pH range of 5.0 to 7.0 [4]. Stone formation may be prevented by increasing fluid intake, alkalizing the urine, changing the diet, or treating with 2-mercaptopropionylglycine (2-MPG) or penicillamine, which forms soluable mixed disulfides with cysteine (CYSH).

In the absence of stones for analysis, cystinuria can be diagnosed by the presence of crystals or by the chemical analysis of urine. Microscopic examination of urine for CYSS crystals is not dependable because at least 75% of patients do not present with crystalluria [4]. The colorimetric assay involving cyanide-nitroprusside is not specific for CYSS, and a previous adaptation of this method for detection of cystinuria and homocystinuria [5] does not account for the presence of other endogenous thiols or thiol-containing drugs. Other reported methods include thin-layer chromatography [6], which is semiquantitative, or quantification by gas chromatography with flame photometric detection [7], capillary electrophoresis [8], or HPLC-mass spectrophotometry [9], each of which requires equipment and expertise not commonly found in a routine clinical laboratory. The usual method for diagnosing cystinuria in the absence of stone analysis is amino acid analysis. However, automated analyzers are not specific for thiols; rather, they generate derivatives of all amino compounds in the sample. The result is a complex chromatogram with long run times. This is undesirable for a screening program, considering that even among patients with renal calculi, <2% contain CYSS [4, 10].

The recent identification of the genetic basis of cystinuria by using molecular biological techniques [3] raises interesting possibilities for future detection methods, but for initial detection of affected individuals, genetic analysis is neither practical nor necessary.

Previous reports of HPLC methods for urine thiols involve long columns and have long analysis times [11], and involve methanol [11] or acetonitrile [12] mobile phases, which are undesirable in terms of hazardous waste disposal.

We report here a rapid, thiol-specific HPLC method that we use to identify cystinuric patients. Using a short column permits injection of samples every 10 min, and using ethanol in the mobile phase reduces laboratory costs and exposure to toxic chemicals.

We adapted the derivatization method of Jacobsen et al. [11] for use with urine samples. Into 1.5 mL microcentrifuge tubes we added 10 μL of n-amyl alcohol, 25 μL of sample (or standard or quality control), and 25 μL of 2.0 mol/L NaBH4 in 0.1 mol/L NaOH. After gentle vortex-mixing, we added 25 μL of 1.0 mol/L HCl and mixed gently. Derivatization of reduced thiols was accomplished by adding 40 μL of 10.0 mmol/L monobromobimane (mBB) (in 220 mL/L acetonitrile with 4 mmol/L EDTA, pH 7.0). The samples were mixed well and incubated at 42 °C for 15 min. The reaction was quenched with 50 μL of 3.0 mol/L HCl. Samples were further diluted to yield suitable peak heights by adding 400 μL of 1.0 mol/L NaH2PO4, pH 4.6.

Separation of thiol–bimanes was performed by injecting 5 μL onto a 125 × 4 mm Superspher®60 3-μm RP-SelectB cartridge preceded by a 5-μm LiChrospher® 60 RP-SelectB 4 × 4 mm guard cartridge (Merck, BDH, Toronto, Canada).

Mobile phase buffer A was 50 mmol/L NaH2PO4 adjusted to pH 3.6 with glacial acetic acid; buffer B was 1:1 acetone:buffer A; solvent C was 600 mL/L ethanol. Linear gradients were run at a flow rate of 1.2 mL/min from 90:5:5 (by vol) A:B:C, to 5:5:90 (by vol) in 2 min, followed by a linear return in 3 min to initial conditions. Equilibration was complete within 5 min, and injections were made every 10 min. Fluorescence was measured at λEX = 392 nm and λEM = 482 nm with 15 × 10 nm bandwidths.

Aqueous standards were prepared by dissolving CYSS (NIST SRM #143c, US Dept. of Commerce, Gaithersburg, MD) in HPLC-grade water. This was stored at 4 °C, and diluted daily as required. Aqueous standards of other thiols that might be expected to appear in urine were also analyzed. There was no chromatographic interference from cysteinylglycine, penicillamine, mercaptopethylamine, N-acetyl-CYSH, or 2-MPG. Chro-