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Use of a commercially available reagent for the selective detection of homocysteine in plasma

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Abstract

A procedure for the detection of homocysteine (Hcy) in blood plasma is described. A commercially available chromogen is added to the plasma sample. The plasma solution turns from yellow to blue upon heating for 4 min when a detectable threshold level of Hcy is present. Chromatographic separations and immunogenic materials are not needed. The protocol takes ~ 30 min.

INTRODUCTION

Biological thiols are challenging analytes. They posses similar structures, exhibit complex redox chemistry and are transparent in the visible spectral region. Over 50 thiol-reactive reagents are currently sold[1]. These typically contain universal electrophilic alkylating groups and are therefore nonselective for specific thiols. Many of these reagents must be handled with caution to prevent unwanted degradation and side-product formation. For instance, monobromobimane is unstable in H_2O (ref. 2), iodoacetamides are cross-reactive with histidine, tyrosine and methionine[1], *o*-phtha-laldehyde adducts exhibit high photoinstability[3] and maleimide labeling produces unwanted rearrangements of conjugates[4]. Due to the difficulties involved in thiol detection, determinations using colorimetric or fluorimetric reagents must be carried out in conjunction with separations[5]. There are numerous other procedures, which include chromatographic separations, immunoassays and enzymatic assays, and electrochemical, mass spectrometric and flow-injection technologies[6,7].

Elevated plasma levels (>12–15 μM) of the thiol amino-acid homocysteine (Hcy) have been associated with increased risk of myocardial infarction, stroke and venous thromboembolism. Hcy has also been linked to increased risk of Alzheimer's disease, neural tube defects, complications during pregnancy, inflammatory bowel disease and osteoporosis[7–10]. In addition to analytical HPLC with a derivatization step (*vide supra*), gas chromatography/mass spectrometry (GC-MS) and commercial immunoassays (e.g., the fluorescence polarization immunoassay run on Abbott's Imx and AxSYM platforms[11,12]) are often used to monitor Hcy. GC-MS requires instrumentation that is not typically applied at point-of-care. The immunoassays require enzymes, antibodies and biomolecules that are expensive and intrinsically unstable; therefore, careful attention to storage conditions is required.

The protocol described here is based on the kinetically favored formation of the alpha-amino carbon-centered radical of Hcy, which allows for the selective reduction of methyl viologen dication to its corresponding radical cation[13–15] (Fig. 1). This characteristic reduction mechanism has proven useful in promoting the selective development of chromogen solution color by Hcy. Other commonly occurring thiols (such as cysteine and glutathione) as well as

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other amino acids do not produce interference[14,15]. The method should be suitable for those who require a highly selective and simple off-to-on colorimetric test for detecting threshold levels of Hcy in its reduced (i.e., thiol rather than disulfide) form. Protection from air is not necessary during the assay; we believe that this is because dissolved O_2, which would quench the radical reactions involved (Fig. 1), is eliminated upon heating. All reagents are commercially available and relatively inexpensive. The protocol is illustrated using commercial reconstituted human blood plasma as a matrix. This method should also be suitable for monitoring reduced Hcy in other biological matrices, such as urine. Changes in reagent and/or buffer concentration allow for adjustment to higher Hcy threshold detection limits[15] than those shown here.

MATERIALS

REAGENTS

- Certified buffer solution, pH 7, color code yellow (Fisher, cat. no. SB107)
- Certified buffer solution, pH 10, color code blue (Fisher, cat. no. SB116)
- L-cysteine (Sigma-Aldrich, cat. no. C-7352)
- Methyl viologen dichloride hydrate (Sigma-Aldrich, cat. no. 856177)
- DL-Hcy ≥95% (Sigma-Aldrich, cat. no. 193143)
- Human plasma (Sigma-Aldrich, cat. no. P9523)
- Hydrochloric acid (HCl) 37–38% (EMD, cat. no. HX0607-2)
- Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, cat. no. T87602)

EQUIPMENT

- Hot plate, type 2300 (Barnstead/Thermolyne, cat. no. HP2305B)
- Volumetric flask, 100 ml (Pyrex, cat. no. 5580)
- pH meter (Orion, cat. no. 410A)
- Glass vials, 15 × 45 mm, screw thread with rubber-lined cap (Gerresheimer, cat. no. 60910L-1)
- Teflon-coated stir bar (VWR, cat. no. 58948-091)
- Magnetic stirrer (Barnstead/Thermolyne, cat. no. S130815)
- Plastic transfer pipettes (Samco, cat. no. 202)
- Glass microsyringe, 10 μl (Hamilton, cat. no. 701)

EQUIPMENT SETUP

pH meter calibration—Turn on the pH meter. Select calibration mode from 7 to 10. Immerse the electrode into the certified buffer solution (pH 7). Wait until the reading stops blinking and a beeping sound is heard. Remove the buffer solution. Rinse the electrode with deionized H_2O. Remove any excess H_2O from the electrode with a paper tissue. Immerse the electrode into the certified buffer solution (pH 10). Wait until the reading stops blinking and a beep sound is heard. Remove the buffer solution. Rinse the electrode with deionized H_2O. Remove the excess water with a paper tissue. Timing is 10 min.

REAGENT SETUP

Tris 0.5 M pH 7—Weigh 6.06 g Tris(hydroxymethyl)aminomethane into a 150-ml beaker. Add 90 ml deionized H_2O and a Teflon-coated stir bar. Stir until all the solid is dissolved using a magnetic stirrer. Dip the pH meter electrode in the solution and keep stirring. Add concentrated HCl dropwise using a plastic transfer pipette until pH 7 is reached. Timing is 10 min. **! CAUTION** HCl is corrosive. Wear gloves, apron and safety glasses, and avoid direct contact with vapors.

Plasma—Allow the bottle of lyophilized plasma to warm up to room temperature. Remove the metallic seal from the bottle and insert a needle through the rubber septum to relieve the inner pressure. Reconstitute the plasma by adding the appropriate amount of deionized water. Swirl the liquid gently until a suspension is obtained. Timing is 5 min (15 min with sample warm up).

Hcy stock solution—Weigh out 10.5 mg DL-Hcy (95%). Transfer to a 100-ml volumetric flask. Add 90 ml deionized H_2O. Dissolve the solid by gently swirling the flask. Add deionized H_2O until reaching the mark in the flask. Swirl the flask gently. Timing is 5 min. ▲ **CRITICAL** Hcy solutions should be used fresh.

Cysteine stock solution—Weigh out 272.6 mg L-cysteine. Transfer to a 100-ml volumetric flask. Add 90 ml deionized H_2O. Dissolve the solid by gently swirling the flask. Add deionized H_2O until reaching the mark in the flask. Swirl the flask gently. Timing is 5 min. ▲ **CRITICAL** Cysteine solutions should be used fresh.

PROCEDURE

1. Turn on the hot plate (select setting number 7) in a fume hood.
2. Weigh out 8 mg methyl viologen dichloride hydrate in a glass vial.

   ! CAUTION Methyl viologen dichloride hydrate is toxic. Wear gloves and safety glasses.
3. Add 0.1 ml Tris(hydroxymethyl)aminomethane buffer 0.5 M, pH 7 with a 1-ml plastic syringe.
4. Add 0.5 ml reconstituted plasma with a 1-ml plastic syringe.
5. Add 3.5 μl Hcy standard solution using a 10 μl glass microsyringe (the final concentration of Hcy should be 5 μM).
6. Close the vial tightly (using the appropriate screw cap).
7. Place the vial on the hot plate.
8. Shake the vial twice during the first minute of heating.

   ! CAUTION Handle with care as burns are possible. Wear gloves and safety glasses.
9. A faint blue color will develop in the refluxing solution after 4 min (the air stream entering the fume hood will moderately cool the walls of the vial being heated prompting solvent condensation).

   ▲ **CRITICAL** Run a replicate; be sure that heating is uniform if running samples concurrently.

10. Repeat Steps 1–9 for additions of 7, 10.5 and 21 μ Hcy standard solution (the final concentrations of Hcy should be 10, 15 and 30 μM, respectively). Different visual color intensities will be obtained (Fig. 1).
11. Run a control sample with no Hcy added.
12. Run a sample with the addition of 10 μl cysteine standard solution (the final concentration of cysteine should be 450 μM) to rule out interferences.

• **TIMING** 30 min for each sample if not run concurrently

• **? TROUBLESHOOTING**

• **TIMING**

Steps 1–11: 30 min

Step 12: 30 min for each sample if not run concurrently

• **? TROUBLESHOOTING**

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

Figure 2 shows that solutions containing Hcy can be visually distinguished from the blank plasma sample and the cysteine-containing control solutions. As the concentration of Hcy is increased, the solution color intensifies.

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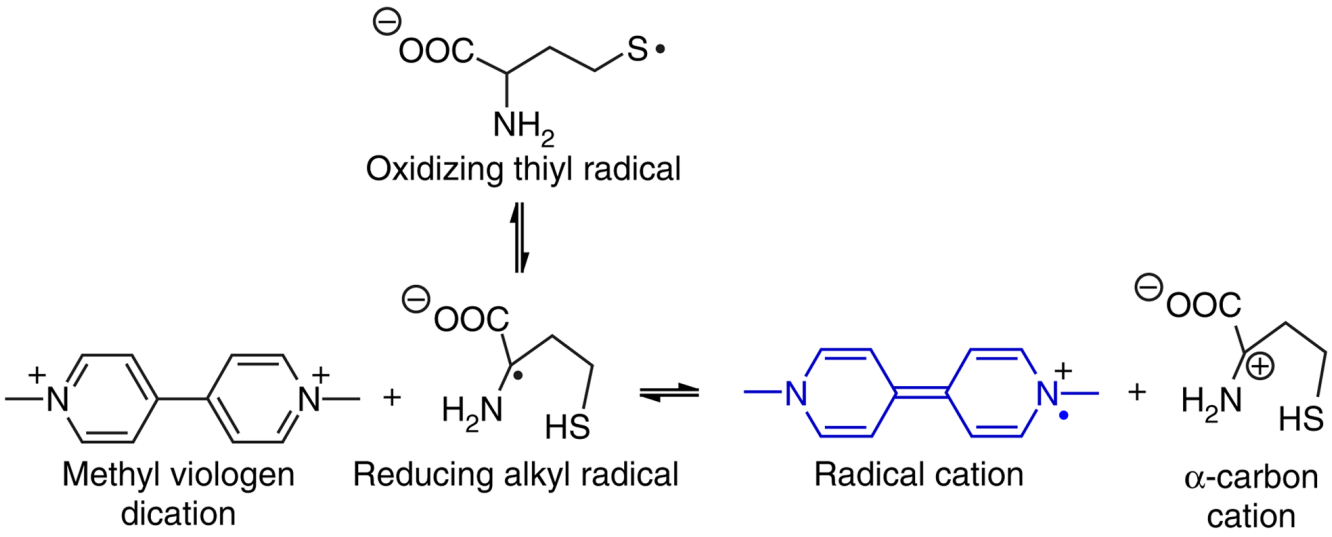

Figure 1.

Hcy undergoes a thiyl radical to carbon radical rearrangement more readily than other biothiols. This is attributed to the favored formation of a five-membered ring (H-atom transfer) transition state, as opposed to four-membered and nine-membered ring transition states as in the cases of cysteine and glutathione, respectively. Captodative stabilization of the alpha-amino carbon-centered radical renders the alpha C–H bond weaker than the S–H bond by ~ 4 kcal/mol. Alpha-amino-acid carbon radicals are well-known strong reductants. Favored formation of the reducing alpha-amino radical in the case of Hcy allows for the selective reduction of weakly oxidizing chromogens, such as methyl viologen dication at neutral pH.

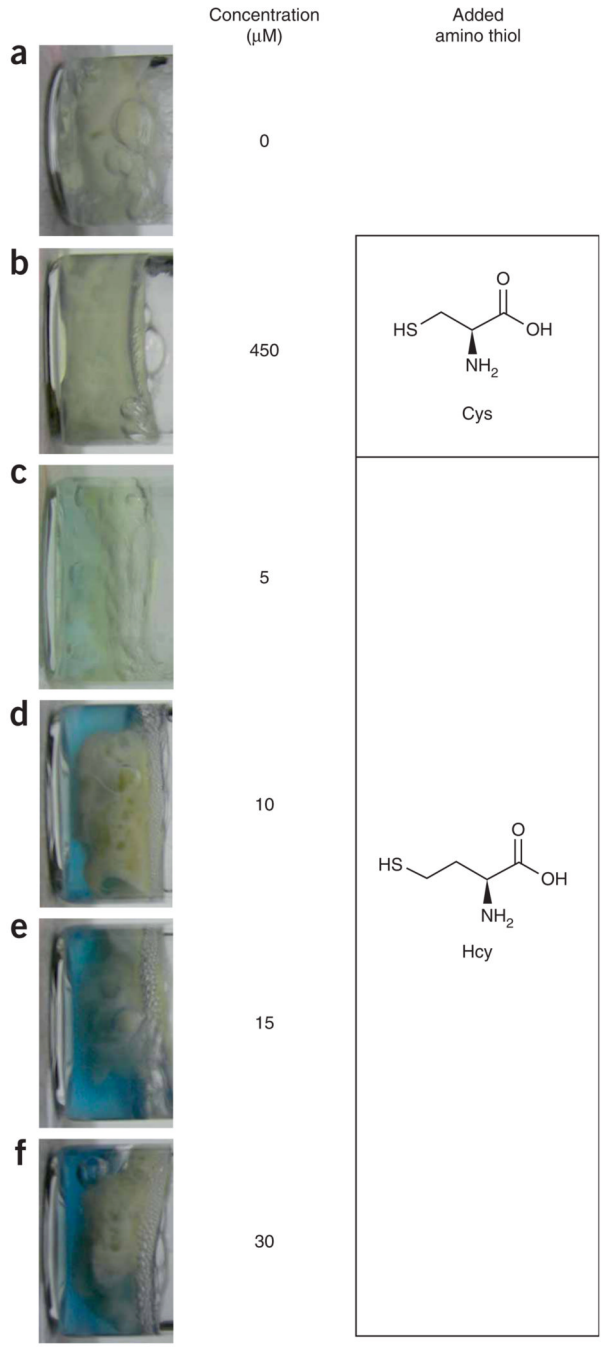

Figure 2.

Photographs of vials containing human blood plasma and methyl viologen dication in buffer solution containing (from upper to lower) (**a**) no added reduced Hcy, (**b**) 450 μM cysteine, (**c**) 5 μM Hcy, (**d**) 10 μM Hcy, (**e**) 15 μM Hcy and (**f**) 30 μM Hcy. The results demonstrate that the color formation and intensity are due to the Hcy levels. Threshold values denoting dangerous Hcy concentrations in human blood plasma are ≥12–15 μM. Normal urinary values might vary but are generally <9.5 μM The shades of the solutions shown are intended as a general guide, along with the protocol, to allow the user to adjust the sensitivity and detection limit. The solid material in the vials apparently comprises denatured proteins and related materials that congeal upon heating.

TABLE 1

Troubleshooting table.

Problem	Possible reasons	Solution
Color formation from Hcy is not observed at desired detection limit	Hcy solution is not fresh	Use sample containing reduced Hcy immediately
	Low Hcy levels	Run replicates; concentrations of methyl viologen dichloride hydrate and/or buffer might need adjustment (e.g., see ref. [15]); time and/or temperature might need adjustment
	Wrong buffer	Buffer must be Tris; no substitutes are acceptable
Solution color fades rapidly	Methyl viologen dichloride hydrate radical cation is quenched	Sample should not be allowed to cool before visual inspection; do not move sample
Solution color formation is nonselective; observed with cysteine and/or other thiols	pH is too high, resulting in nonselective formation of reducing disulfide radical cation	Check buffer solution and check that the pH in the plasma sample is neutral using pH paper
	Metal catalysis	Avoid contact with metals; use deionized water
Solution turns dark greenish after 4 min	Temperature is too high or heating period is too long	Use milder heating
Foaming observed in vial	Leak	Make sure vials are tightly closed before heating