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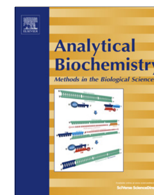

The highly abundant urinary metabolite urobilin interferes with the bicinchoninic acid assay

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ABSTRACT

Estimation of total protein concentration is an essential step in any protein- or peptide-centric analysis pipeline. This study demonstrates that urobilin, a breakdown product of heme and a major constituent of urine, interferes considerably with the bicinchoninic acid (BCA) assay. This interference is probably due to the propensity of urobilin to reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}), thus mimicking the reduction of copper by proteins, which the assay was designed to do. In addition, it is demonstrated that the Bradford assay is more resistant to the influence of urobilin and other small molecules. As such, urobilin has a strong confounding effect on the estimate of total protein concentrations obtained by BCA assay and thus this assay should not be used for urinary protein quantification. It is recommended that the Bradford assay be used instead.

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The determination of total protein concentration in a sample prior to liquid chromatography, immunochemical, electrophoretic, or mass spectrometry analysis is fundamental to achieve accurate and reproducible experimental results. Typically, there are two groups of spectrophotometric protein concentration assays: (1) dye binding assays, e.g., the Bradford, and (2) biochemical assays such as bicinchoninic acid (BCA) and Lowry assays. Each of these approaches has advantages and disadvantages in the determination of total protein concentration with the caveat that different chemicals within the sample matrix can interfere with the final result [1–8]. Thus it is crucial to select an appropriate protein quantification method on a sample-type by sample-type basis.

The BCA assay is a two-step process largely based on the biuret reaction. Initially peptide bonds and tyrosine, tryptophan, cystine, and cysteine residues [9] in a protein/peptide reduce copper ions from cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}), a reaction that occurs in an alkaline solution [9,10]. The reduced copper is then chelated by two bicinchoninic acid residues, which form a metal–acid complex. This metal–acid complex produces the characteristic deep purple color that absorbs at 562 nm and can be quantitatively measured in a spectrophotometer [10] (Fig. 1). Since the production of Cu^{1+} is theoretically a function of the number of peptide bonds, with cysteine, tyrosine, and tryptophan residues reducing Cu^{2+} more favorably, in a protein preparation, the total protein concentration can then be estimated by comparison to a known protein standard such as bovine serum albumin.

We recently noticed that, while attempting to quantify the concentrated protein in the retentate from ultrafiltered urine samples, the reaction time of the BCA assay seemed to correspond to the intensity of pigment present in the sample. Since a number of molecules are known to interfere with the BCA assay, we wanted to know if one of the major pigments in urine interfered with the BCA assay.

There are many molecules contributing to the yellow pigments attributed to urine. Some well-known molecules include the riboflavins [11], in particular vitamin B2, which has a pronounced orange color (see database entry HMBD00244 for details [12]). However, we were particularly interested in the various derivatives of heme because of their metal-chelating and electron transfer characteristics [13], as this is the foundation of the BCA assay. The cyclic tetrapyrrole (porphyrin), iron-chelating pigment hemoglobin is responsible for the sequestration and then transport of molecular oxygen in the blood. Heme and hemoglobin are synthesized by developing erythrocytes in the bone marrow; however, the life span of erythrocytes extends for only about 120 days, following which the old cells are removed from the circulation by Kupffer cells and macrophages within the liver and spleen. Oxidative catabolism of the heme molecule results in the release of the coordinate iron, which is recycled for use in de novo hemoglobin

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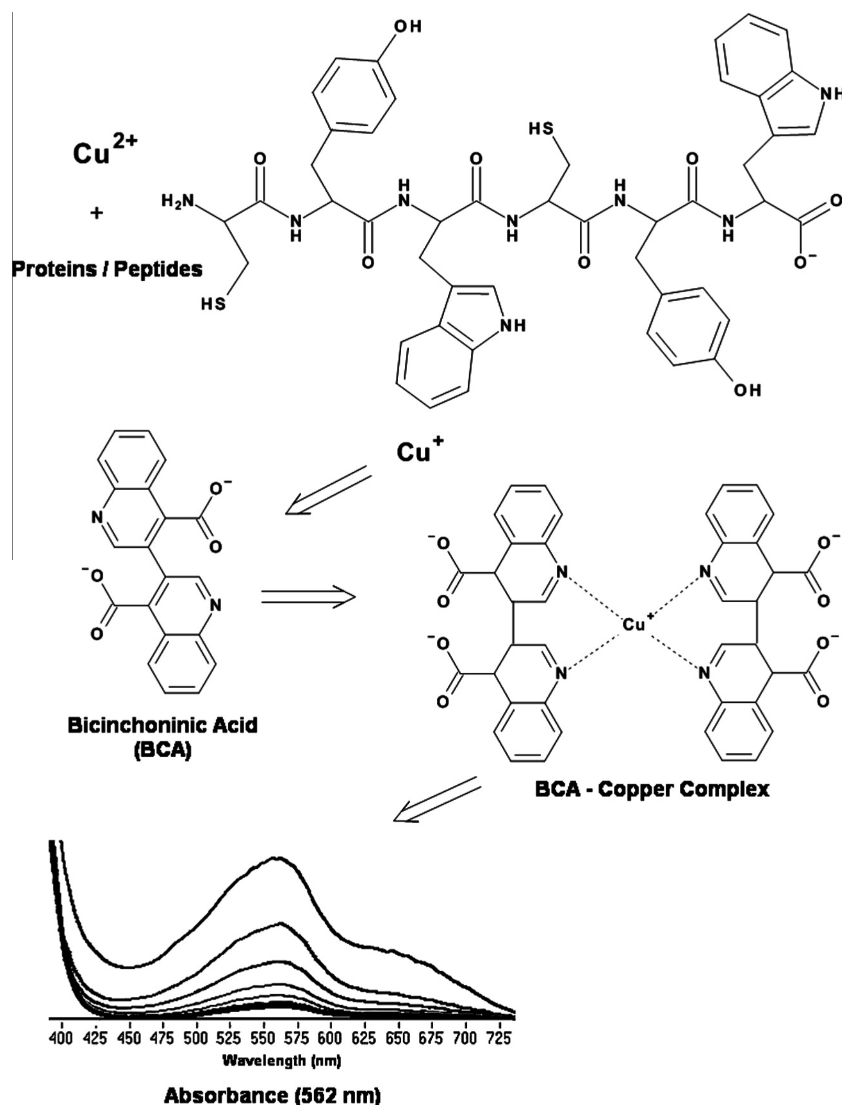

Fig.1. The BCA reaction. All reagents are combined with the analyte (protein). The protein's cysteine, cystine, tyrosine, and tryptophan residues [9] and the peptide bonds reduce the Cu^{2+} to Cu^{1+}, which is then chelated by two bicinchoninic acid residues, which form the purple metal–acid complex that absorbs at 562 nm.

synthesis (reviewed by [14]), while the insoluble derivative biliverdin undergoes reduction to bilirubin (Fig. 2A), which is released into the circulation where it binds within a deep pocket on serum albumin [15]. The liver takes up bilirubin from the circulation and then excretes it as a digluconide conjugated form into the bile [16], where it is metabolized to urobilinogen (Fig. 2B) by intestinal flora. Some urobilinogen is reabsorbed into the circulation and then excreted via the kidneys into the urine [17], where it is further oxidized to form urobilin [18] (Fig. 2C), while the remainder is metabolized to stercobilin and excreted with the feces (Fig. 2D). Here we demonstrate that the end product found in urine, urobilin, interferes with the BCA assay during protein quantification. This was tested using physiological levels of urobilin in normal subjects (~3.9 mg/L) [19] and using higher concentrations of urobilin, to mimic the increased amount observed when it is bound to carrier proteins, such as albumin [20].

Materials and methods

The BCA assay, Bradford reagent kits, bovine serum albumin (BSA), and Nunclon Delta surface flat-bottom, polystyrene, 96-well plates were purchased from Thermo Scientific (Rockford, IL, USA). Urobilin hydrochloride (>95% purity) was sourced from Frontier Scientific, Inc. (Logan, UT, USA). NuPAGE 4–12% Bis–Tris gradient gels and NuPAGE electrophoresis reagents were purchased from Life Technologies (Carlsbad, CA, USA). Methanol (MeOH) and sodium hydroxide (NaOH) were purchased from Chem-Supply (Gillman, SA, Australia). Amicon Ultra-15, 3-kDa nominal molecular weight limit (NMWL) centrifugal ultrafiltration devices were purchased from Millipore (Billerica, MA, USA), phosphoric acid (H_3PO_4) and potassium sodium tartrate tetrahydrate ($C_4H_4KNaO_6 \bullet 4H_2O$) were from Merck (Darmstadt, Germany), Brilliant Blue-G was from Sigma–Aldrich (St. Louis, MO, USA), and ammonium sulfate was from Ajax Finechem (Seven Hills, NSW, Australia). All solutions were prepared with Milli-Q water (Millipore) unless otherwise described.

Preparation of BSA standards and urobilin solutions

BSA standards were prepared by performing 1:2 serial dilutions from a 1 mg/ml stock solution with 20 mM $C_4H_4KNaO_6 \bullet 4H_2O$ in 75 mM NaOH (final concentrations as indicated). In addition, a single 1 mg/ml stock solution of urobilin was also prepared in 20 mM $C_4H_4KNaO_6 \bullet 4H_2O$/75 mM NaOH. To evaluate the effect of urobilin

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