Urinary Urobilinogen Determined by a Mercuric Chloride Procedure

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In this quantitative assay, urinary urobilinogen is oxidized to urobilin with iodate in an acid medium, the pH is adjusted to 6 with sodium acetate, and the mixture is reacted with alcoholic HgCl₂ solution, extracted with CHCl₃, and measured spectrophotometrically at 513 nm. The artificial standards of previous methods have been replaced with crystalline stercobilin IX (commercially available), a urobilin closely related to the urinary urobilins. The reproducibility of the method, as assessed from 10 replicates of a single urine specimen to which urobilinogen was added, gave a coefficient of variation of 3.9%. Analytical recovery of urobilinogen added to urines was 90.4% (SD 14.5%). Bilirubin, biliverdin, mesobilirubin, coproporphyrin I, uroporphyrin I, and porphobilinogen do not interfere.

Additional Keyphrases: spectrophotometry • oxidation of urobilinogen to urobilin • liver disease assessment • stercobilin or urobilin as standard • reference interval • molar absorptivities

The procedures most commonly used to estimate urobilinogen are those based upon the method of Watson et al. (1): the reaction of p-dimethylaminobenzaldehyde reagent (Ehrlich's reagent) with urobilinogen. In the simplest procedure, the reagent is added directly to urine, followed immediately by saturated sodium acetate to inhibit the color formation of non-urobilinogen components and to intensify the urobilinogen–aldehyde color.

These procedures have not been wholly satisfactory. Urobilinogen is unstable. It is easily oxidized to urobilin, which does not react with Ehrlich's reagent. Substances in urine other than urobilinogen produce interfering colors. Slight procedural variations and inadequate standards (1, 2) have all contributed to the unreliable results encountered in urobilinogen assay.

The need for a more reliable procedure prompted us to re-examine the various methods for the quantitative estimation of urobilinogen. Here we describe a quantitative procedure in which urobilinogen is oxidized to urobilin and estimated colorimetrically as a mercury–urobilin complex. Crystalline synthetic stercobilin or urobilin is used as a standard.

Materials and Methods

Reagents

All chemicals are reagent grade.

Methanol • HCl. Prepare freshly each day by adding 1 mL of concentrated hydrochloric acid to 14 mL of methanol.

Acetic acid, 2 mol/L. Dissolve 120 mL of glacial acetic acid in distilled water and dilute to 1 L.

Sodium acetate buffer, pH 6.0, 2 mol/L. Dissolve 155 g of anhydrous sodium acetate in about 700 mL of distilled water; adjust the pH to 6 with acetic acid, add 10 g of sodium chloride, and dilute to 1 L with distilled water.

Potassium iodate, stock, 0.2 mol/L. Prepare 100 mL by dissolving 4.5 g of KI₃ in distilled water and diluting to 100 mL with distilled water. This solution is stable indefinitely.

Potassium iodate reagent. Prepare freshly each day by diluting 1 mL of the potassium iodate stock to 20 mL with acetic acid, 2 mol/L.

Mercuric chloride solution. Dissolve 10 g in absolute ethanol and dilute to 100 mL with absolute ethanol.

Chloroform.

Sterobilin IX or urobilin IX stock, 1 g/L of 0.1 mol/L NaOH. Dissolve approximately 1 mg of crystalline material in 1 mL of the sodium hydroxide solution. Keep refrigerated and prepare freshly each week.

Sterobilin IX (cat. no. S 594-9) and urobilin IX (cat. no. U 590-9) in purities of about 95% can be obtained from Porphyrin Products, Logan, UT 84321. Because stock solutions deteriorate at the rate of about 1% per day, they must be assayed just before the working standards are prepared.

Procedures

Preparation of standard. Dilute 10–50 μL of the stock solution to 10 mL with the methanol-HCl reagent, mix and leave in the dark for 30–60 min, to convert the stercobilin to its hydrochloride. Then measure the absorbance in the spectrophotometer at 489 nm against a methanol-HCl blank. When Urobilin IX is used, make the readings at 492 nm. Calculate the concentration of stercobilin or urobilin in the stock as follows:

\[ \text{Concn, g/L} = \frac{A \times M_r \times \text{diln}}{c_{492 \text{nm}} \times 1 \text{cm}} \]

where \( A \) = absorbance, \( M_r = 594 \) diln = dilution factor, and \( c_{492 \text{nm}} = \) molar absorptivity = 93 000.

Prepare working standards containing 0 to 50 mg/L by appropriately diluting aliquots of the stock solution with distilled water.

Place 1-mL samples of urine, standard, or water for reagent blank in glass-stoppered test tubes having a volume of 12–15 mL. To each tube, add 0.25 mL of the potassium iodate reagent to convert the urobilinogen to urobilin, mix, and place the tubes in the dark.

After 20 min, add 1.0 mL of the acetate buffer and 0.5 mL of the mercuric chloride solution, mix, and leave on the bench for 10 min for the mercury–urobilin complex to form. Add 5 mL of chloroform. Securely stopper the tubes with glass stoppers and gently invert each tube 15 to 20 times to extract the mercury–urobilin complex from the aqueous layer. Set the tubes aside for 5 min to allow the two phases to separate. Aspirate and discard the aqueous phase and pass the chloroform phase through Whatman no. 1 filter paper (7 cm diameter) to remove traces of water. Measure the absorbance of the filtered solution vs a reagent blank, at 513 nm.

Results

Standard curve. Aqueous standards of stercobilin were prepared to cover the range 0 to 50 mg/L and taken through the above procedure. The curve shown (Figure 1) is typical of curves obtained over an eight-month period with different stock solutions of stercobilin. The Lambert–Beer law is sat-

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is factorially obeyed by this curve, although parabolic expression better fits the data for a more extended range.

Gray and Nicholson (3) reported stercobilin to be stable. For this reason, we selected it as a standard, although we later found that urobilin can serve equally well as a standard. Superimposed upon the stercobilin standard curve in Figure 1 is one obtained by using urobilin as a standard.

The chloroform extract, a colloidal suspension of the mercury–urobilin complex, is stabilized by adding sodium chloride to the buffer. Without the sodium chloride, some urines may cause the suspension to deposit as a red film on the walls of the tubes.

Spectra of stercobilin-HCl and urobilin-HCl. Figure 2 shows absorption curves for the hydrochlorides of synthetic stercobilin and of synthetic urobilin in methanol-HCl. Stercobilin-HCl has a single sharp peak at 489 nm and urobilin-HCl a similar peak at 492 nm. For solutions in chloroform, the stercobilin-HCl peak is at 496 nm and that for urobilin-HCl at 498 nm, in agreement with Jackson et al. (4), who reported that stercobilin-HCl has a maximum absorption peak in chloroform at 496 nm with $\varepsilon = 93,000$ and the peak for urobilin-HCl is at 499 nm with $\varepsilon = 93,000$. We found both stercobilin-HCl and urobilin-HCl to have molar absorbivities of 93,000 in either chloroform or methanol-HCl.

Spectrum of a urobilin-HCl from urine. A sample of a patient’s urine was oxidized with iodate, and aliquots of the urine before and after oxidation were added to the methanol-HCl reagents to form the urobilin hydrochloride. The resulting spectra (Figure 2) indicate that urobilin in urine responds as does stercobilin.

Spectra of the mercury complexes of stercobilin and urobilin. The spectra of the mercury complexes of stercobilin, urobilin, and the urobilin from urine in chloroform (Figure 3) are quite similar. Mercury–stercobilin has a peak at about 512 nm; that of mercury–urobilin is at 514 nm. The spectrum of the mercury–urobilin complex from urine appears to fall between that of the mercury–stercobilin complex and of the mercury–urobilin complex.

Molar absorptivity of mercury–stercobilin, mercury–urobilin complexes. Working standard solutions of stercobilin and urobilin containing 10, 15, 20, 25, 30, 40, and 50 mg/L were subjected to the mercuric chloride procedure, and the molar absorptivity was calculated from the absorbance of the fivefold diluted chloroform solution of each standard. For stercobilin it was 54120 (SD 1100) and for urobilin 54200 (SD 1700).

Response of related compounds. Individual aqueous solutions containing 20 mg/L prepared from alkaline stock solutions of bilirubin, biliverdin, mesobilirubin, coproporphyrin I, uroporphyrin I, and porphobilinogen were taken through the procedure as described. The chloroform extracts of the first three showed absorption peaks at 450, 385, and 431 nm, respectively. Coproporphyrin I extract gave two peaks: at 412 and 437 nm. Uroporphyrin I gave a green color in the aqueous phase, which was not extracted by chloroform. Porphobilinogen gave no color. None of these compounds showed any absorption at 513 nm.

Reproducibility. Between-assay variation was determined on working standards prepared on 11 different days to cover a concentration range from 5 to 50 mg/L. These solutions were subjected to the analytical procedure the same day that the standards were prepared. The overall CV was 4.3%.

Within-assay CV was determined by use of a single normal urine containing 0.32 mg of urobilinogen per liter. To this urine, 2.87 mg/L of urobilinogen [produced by the reduction of bilirubin according to Watson (5)] was added. Ten replicates were measured by our procedure. The average value

Fig. 1. Standard curves for the mercuric chloride assay for stercobilin and urobilin

Fig. 2. Spectra of the hydrochlorides of stercobilin, urobilin, and urinary urobilins

Fig. 3. Spectra of mercury complex of stercobilin, urobilin, and urinary urobilins
Discussion

It is well known that urinary urobilinogen is a sensitive indicator of liver damage. Nevertheless, the popularity of urobilinogen testing varies widely in different parts of the world. Sherlock (8), an English author, regards urinary urobilinogen as one of the more sensitive indicators for the early detection of hepatocellular dysfunction. Free and Free (9) state that urobilinogen testing is widely used in Japan in diagnosing liver disease. In the United States, urobilinogen measurement has not been widely accepted, perhaps because the tests commonly used in clinical laboratories produce unreliable results. Interfering substances, variation in testing procedures, and variation in reagents, especially in the dyes used as standards, have added to the confusion associated with urobilinogen testing.

Most of these problems are eliminated in our procedure. The results are reproducible. The procedure itself, although demanding good analytical technique, is easily performed and requires no special equipment. No compounds have been found in urine that interfere with the test. Closely related compounds such as bilirubin, biliverdin, and mesobilirubin may form colored chloroform extracts, but their absorption peaks differ from those of the urobilins, so they do not interfere.

Lack of adequate standards has been the major obstacle to quantitative urobilinogen testing. We propose the use of either stercobilin IX or urobilin IX as standards. These compounds are closely related to the urobilins found in urine, and they respond in a similar manner in the mercuric chloride procedure. They are stable and can be stored in crystalline form in the cold for several months. A high degree of purity, although desirable in these standards, is not essential because the actual amount of either compound can be determined by colorimetry of their hydrochloride forms, thus making it possible to establish the concentration and purity of the standard independent of the mercuric chloride procedure.

References