Indophenol Method for Acetaminophen in Serum Examined

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We show that an indophenol-based colorimetric method for acetaminophen (Clin Toxicol 15: 67–73, 1979) has less than maximal sensitivity because of (a) incomplete hydrolysis, related to suboptimal acid concentration; (b) suboptimal hydrolysis time; and (c) possible deterioration of the ammonium hydroxide reagent after 2.5 months. We have modified the method by optimizing duration of hydrolysis and color development. Results by the method correlate linearly with acetaminophen concentrations to 250 mg/L (1.66 mmol/L). Moreover, the modified method is free from interference by compounds in serum from uremic patients.

Additional Keyphrases: drug assay • hydrolysis • variation, source of

Use of acetaminophen (paracetamol, N-acetyl-p-aminophenol), a well-known antipyrctic and analgesic substitute for salicylate, has increased in recent years (1, 2). However, the potential for hepatotoxicity and renal failure accompanying chronic excessive use or suicidal overdose of the drug (3–5) necessitates a reliable, interference-free procedure for determination of acetaminophen in serum.

The colorimetric method of Frings and Saloom (6) is based on obtaining a protein-free serum filtrate, with subsequent hydrolysis of acetaminophen to p-aminophenol. The resulting p-aminophenol reacts with phenol in the presence of ammonium hydroxide to form a blue indophenol product having maximal absorbance at 620 nm.

During a study of this method, we encountered incongruities involving linearity and analytical recovery of the acetaminophen standards, which led to a complete investigation of this method. Here we report the results of this investigation and present a modified optimized method for acetaminophen determination. In light of Bailey’s (7) findings of false-positive and erroneously high concentrations of acetaminophen in uremic patients, as determined by the method of Glynn and Kendall (8), we also investigated the effect of uremic serum on the recovery of acetaminophen measured by the modified indophenol-based colorimetric method.

Materials and Methods

Apparatus

We measured absorbances with a Gilford 300-N spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH) equipped with a flow-through cuvet.

Reagents

Concentrated HCl, reagent grade.

Phenol, 20 g/L. Dilute 23 mL of liquid phenol to 1 L with de-ionized water. Stored in a brown glass bottle at 4–7 °C, the reagent is stable for four months.

NH4OH, 4 mol/L. Add 142 mL of concentrated NH4OH to a 500-mL volumetric flask and dilute to volume with de-ionized water. Stored in a brown glass bottle at room temperature, the reagent is stable for two months.

Trichloroacetic acid, 200 g/L. Stored in a brown glass bottle at 4–7 °C, the reagent is stable for six months.

Color reagent: phenol reagent/NH4OH reagent, 7/3 by vol. Prepare just before use, mixing well.

Acetaminophen stock standard, 1.000 g/L (6.62 mmol/L). Dissolve 100.00 mg of acetaminophen in about 50 mL of de-ionized water and dilute to 100 mL. Stored in a brown glass bottle at 4–7 °C, the standard is stable for six months.

Blank serum. Pool samples and store at 4–7 °C for at least 16 h, then filter through glass wool. Using the proposed method, check that the acetaminophen concentration in the filtered serum pool is less than 2 mg/L (10 µmol/L). Store in frozen 1-mL aliquots. Similarly, prepare pooled serum from patients with above-normal concentrations of urea.

Acetaminophen serum standard, 100 mg/L (0.66 mmol/L). Dilute 1.00 mL of the acetaminophen stock standard to 10 mL with the blank serum. Store frozen in 1-mL aliquots.

Acetaminophen working serum standards. Dilute 0.20, 0.80, 1.20, 1.60, 1.80, 2.20, and 2.50 mL of acetaminophen stock standard to 10 mL each with the blank serum; dilute another series of standards with the pooled uremic serum. Final concentrations will range from 20 to 250 mg/L (0.13–1.66 mmol/L). Store frozen in 1-mL aliquots.

Procedure

Method 1: Colorimetric procedure of Frings and Saloom (6). In brief, to 1.0 mL of sample in labeled 16 × 100 mm tubes, add 2.0 mL of the trichloroacetic acid reagent and vortex-mix for 5 s. Centrifuge until a clear supernate is obtained. Transfer 1.0 mL of the clear supernate to a labeled 16 × 150 mm tube, add 0.20 mL of concentrated HCl (12 mol/L), and place in a boiling water bath for 10 min. Remove the tubes from the water bath, add 5.0 mL of the freshly prepared color reagent to each tube, and vortex-mix for 5 s. After 30 min, but before 1 h, measure the absorbance of the standards and unknowns against a reagent blank at 620 nm in a 1-cm pathlength cuvet.

Method 2: Modified procedure. After mixing the sample with the trichloroacetic acid reagent, centrifuge the tubes at 1500 × g for 5 min. Acidify the 1.0 mL of clear supernate with 0.40 mL of less concentrated HCl (7 mol/L) and leave this in the boiling water bath for 35 min. After adding the color reagent, vortex-mix for 5 s. After 15 min, but before 45 min, measure the absorbance of each tube at 620 nm, having adjusted the absorbance of a serum blank to zero.

Results

Optimization of the Indophenol-Based Colorimetric Procedure

Effect of HCl concentration on final reaction pH. Using Method 1 and 80 mg/L (0.53 mmol/L) serum standards of acetaminophen, we evaluated the effect of HCl concentration, during hydrolysis, on the pH of the final reaction mixture. Reaction conditions were identical to Method 1 except that 0.40 mL of HCl solution (ranging in concentration from 0.75 to 12.0 mol/L) was added before the hydroly-
sis. As Figure 1 indicates, absorbance was maximum at the HCl concentration of 6.75 mol/L; the final pH of that test solution (after the color development) was 9.0.

Effect of hydrolysis time on color development. With 80 mg/L (0.53 mmol/L) serum standards and 0.40 mL of 6.0, 6.75, and 7.5 mol/L HCl solutions, hydrolysis was essentially complete after 30 min (Figure 2). Use of the more concentrated HCl solutions resulted in greater absorbances than the 6 mol/L acid at all hydrolysis times [0.20 mL of 12 mol/L HCl (6) is equivalent to 0.40 mL of 6.0 mol/L HCl], but the difference in absorbances between 6.75 and 7.5 mol/L HCl was less than 2%. The final reaction mixtures corresponding to the samples treated with 6.0, 6.75, and 7.5 mol/L HCl had respective pH values of 9.45, 9.35, and 9.29 after 35 min of hydrolysis.

Color stability. To evaluate the stability of the color formed, we used an 80 mg/L acetaminophen serum standard, 7.5 mol/L HCl, and 35 min of hydrolysis. Figure 3 shows that the development of color after the addition of the color reagent, vortex-mixing, and measurement of the absorbance at 620 nm vs de-ionized water was complete and stable after 15 min.

Absorbance characteristics of the reaction product. In the modified method, as in the method of Frings and Saloom (6), the absorbance of the reaction product (vs de-ionized water) was maximum at 620 nm.

Stability of NH₄OH. Not only did the absorbances of standards decrease as the NH₄OH aged, but also the rate of color development was slower for reagents older than about two months.

Linearity. Absorbance at 620 nm correlated linearly with acetaminophen concentration from 0 to 250 mg/L (0–1.66 mmol/L) (Figure 4).
Effect of Uremia on the Modified Method

We determined concentrations of urea nitrogen (560 ± 40 mg/L, mean ± SD; n = 8) and creatinine (46 ± 4 mg/L; n = 8) in the pooled uremic serum by using an Astra 8 (Beckman Instruments, Inc., Brea, CA 92621); urea was measured by a urease-based method and rate of changes in conductivity of the reaction medium; creatinine was measured by the rate of the absorbance change 25.6 s after mixing of the samples with sodium picrate solution. The content of the acetaminophen standards affected the urea nitrogen and creatinine concentrations only to the extent expected by dilution. Analytical recovery of acetaminophen from the uremic serum pool ranged from 97 to 109% (average 103%). Urea nitrogen and creatinine in specimens from six uremic patients ranged from 100 to 1360 and from 8 to 63 mg/L, respectively. We also measured electrolytes for these samples, to allow us to calculate anion gap values. To each plasma sample we added 1.00 g (0.66 mmol) of acetaminophen per liter to yield a final acetaminophen concentration of 910 mg/L (0.60 mmol/L). The analytical recovery data (average 103%) indicated that neither urea nitrogen plus creatinine nor unmeasured anions interfered with acetaminophen determination by the modified colorimetric procedure.

Discussion

In the chemical reaction of this colorimetric method (6), both chromogen and chromophore production appear to be pH dependent. Optimal acid concentration and time of hydrolysis are important in the conversion of acetaminophen to p-aminophenol. The rate of chromophore generation is greatest when the pH of the reaction mixture is between 9.2 and 9.5.

Under the conditions of the modified procedure, hydrolysis is complete, so that the indophenol color reaction takes less time.

Given the pH dependence of this reaction, the stability of the NH₂OH reagent is important. As the reagent ages, it loses ammonia by volatilization. Although this effect is mainly a function of the number of times the reagent bottle is opened, we recommend a limit of two months as the useful lifetime of the reagent.

Fringes and Saloom (6) showed the indophenol method to be free from interference by the 70 drugs tested. We found that the modified indophenol color reaction is also free from interference from uremic serum samples. Advantages of our procedure over the procedure presented by Bailey (7) are the elimination of ether and hexane extractions and a reduction in the number of procedural steps.

Stewart et al. (9) have raised the issue of measuring unconjugated acetaminophen vs total acetaminophen, including sulfate and glucuronide conjugates. Bailey (7) has included an ether extraction to eliminate interferences by substances in uremic serum. Because unconjugated acetaminophen may represent 14 to 75% of total acetaminophen (8), the use of methods that measure unconjugated acetaminophen poses the problem of underestimating the true risk of hepatic toxicity to the patient. Although the active drug is the unconjugated acetaminophen, the nomogram most widely used in emergency treatment of acetaminophen overdose (10) in the United States is based on the data from measurements of total acetaminophen (8). Currently, appropriate guidelines for estimating the risk of hepatic damage from concentrations of unconjugated acetaminophen have not been established. Until such guidelines have been promulgated, we believe that total acetaminophen should be measured when toxic overdose is assessed.

The indophenol-based colorimetric method presented here provides results that are congruent with the data used to establish guidelines for therapeutic intervention with sulfhydryl compounds.

References