Colorimetry of Serum Acetaminophen (Paracetamol) in Uremia

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A popular colorimetric analysis for serum acetaminophen, based on ring-nitration of the drug, is demonstrated to yield both "false positives" and erroneously high results for serum from uremic patients. The interference appears to be anionic at physiological pH, and correlates significantly with serum creatinine concentration and with the magnitude of the "anion gap." A modification of the analysis involving extraction of the acetaminophen with ether eliminates the interference. As little as 10 mg of drug per liter can be accurately measured in serum by the proposed procedure. Analytical recovery was 97% at a concentration of 1 g/L. Coefficients of variation for the analysis at respective concentrations of 100 and 500 mg/L were: within-run, 2.7% and 2.0%; between-run, 3.1% and 4.2%. I encountered no serious interferences from other drugs. The proposed method, rapid and reliable, is recommended for routine use in the clinical laboratory.

Acetaminophen (paracetamol) is widely used as a non-prescription analgesic and antipyretic agent. It is available both alone (e.g., Tylenol; McNeil) and in more than 200 formulations in the United States (1). Because it is widely available, cases of overdose are relatively common. Acute acetaminophen intoxication and even chronic excessive ingestion of the drug have been characterized by hepatotoxicity (including fatal hepatic necrosis) (1−4). Although less well documented, nephrotoxicity has also been associated with acetaminophen overdose (2, 5).

The diagnosis and treatment of acetaminophen poisoning are based largely on information concerning the concentration of the drug in serum rather than on the dose of drug ingested (1, 3). Treatment consists of oral administration of massive doses of sulfhydryl-containing compounds (e.g., cysteine) over several days, which requires hospitalization of the patient and is not without major side effects (9). Consequently, it is usually utilized only when serum acetaminophen concentrations suggest the likelihood of hepatotoxicity (i.e., when the concentration exceeds 300 mg/L at 4 h or more after ingestion, or 50 mg/L at 12 h or more after ingestion, or when the drug's biological half-life in serum exceeds 4 h) (3). Treatment is best initiated as quickly as possible. The implications of these factors for the clinical laboratory are obvious: a rapid, reliable method for acetaminophen analysis should be available at all times.

Acetaminophen has been measured in serum by various techniques: ultraviolet (6) and visible (7−12) spectrophotometry, gas−liquid chromatography (13), and "high-pressure" liquid chromatography (14−16). Despite the advent of chromatographic assays for acetaminophen, spectrophotometric techniques have remained popular in many laboratories owing to their relative simplicity, economy, and quick turnaround time, one of the most widely used assays for acetaminophen being the colorimetric method of Chafetz et al. (7), as modified for use in serum by Glynn and Kendal (8) and Walberg (9). This method has been recommended by the American Academy of Pediatrics Committees on Drugs (17) and is even available in a commercial kit (Acetaminophen Rapid Stat Diagnostic Kit; Lancer, Division of Sherwood Medical, St. Louis, MO 63103). The analysis involves mild ring-nitration of acetaminophen with nitrous acid to yield 2-nitro-4-acetamidophenol, which, at alkaline pH, forms the yellow phenolate-anion chromophore with maximum absorbance at 430 nm (Figure 1).

I find this method to be generally quite suitable for use in the rapid diagnosis of acetaminophen intoxication, but I have encountered serious endogenous interferences (both "false positives" and erroneously increased acetaminophen concentrations) when the procedure is applied to the serum of uremic patients. This is of particular concern when the analysis is requested to explain the etiology of renal failure.

In the present study this interference is characterized, and a rapid, reliable modification of the procedure suitable for use in uremia is presented.

Materials and Methods

Apparatus

Absorbances were measured with a Model 25 double-beam ultraviolet−visible spectrophotometer (Beckman Instruments, Inc., Fullerton, CA 92634).

Reagents and Standards

All reagents were of analytical (AR) grade.

Ammonium sulfate, 1.3 mol/L in water.

Ammonium hydroxide.

Diethyl ether.

HCl, 6.0 mol/L.

NaNO2, 1.4 mol/L in water. Store at 4 °C in an amber-colored glass bottle. Prepare freshly at least once a week.

NaOH, 12.5 mol/L in water.

Na2SO4, anhydrous.

Trichloroacetic acid, 0.6 mol/L in water. Store at 4−8 °C in a brown bottle.

Acetaminophen stock standard, 100 g/L, in methanol. Dissolve 200 mg of acetaminophen in 2 mL of methanol. Store at −20 °C. Prepare fresh at least once a month. The standard was from Applied Science Laboratories, Inc., State College, PA 16801.

Acetaminophen working standards. To prepare the 1000 mg/L acetaminophen standard, evaporate 0.5 mL (50 mg) of

\[
\text{HNCCH}_3\text{OH} + \text{HNO}_2 \rightarrow \text{HNCCH}_3\text{HNO}_2 \rightarrow \text{HNCCH}_3\text{HO}^-\text{NO}_2
\]

Fig. 1. Structures of (A) acetaminophen (p-acetamidophenol); (B) 2-nitro-4-acetamidophenol, formed by nitration with nitrous acid; and (C) 2-nitro-4-acetamidophenolate anion, a yellow chromophore with maximum absorbance at 430 nm

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the stock standard and dissolve the residue in 50 mL of water (for method 1 below) or drug-free serum (for method 2). For working standards, appropriately dilute the 1000 mg/L standard with water or drug-free serum to produce the desired concentrations (usually 20–300 mg/L). Prepare at the time of analysis.

Procedure

Method 1 (conventional colorimetric procedure) (7–9). Pipet 1 mL of sample (patient’s serum, serum working standards, and a suitable commercial lyophilized control) into 15-mL glass test tubes. Add 2 mL of 0.6 mol/L trichloroacetic acid to precipitate the proteins and mix well. Centrifuge (3000 ×g, 10 min) and carefully transfer exactly 2 mL of each clear, colorless supernate to a second series of test tubes. Pipet 0.5 mL of 6.0 mol/L HCl and mix. Generate nitrous acid by adding 0.5 mL of 1.4 mol/L NaNO₂, mix well, and let stand for 2 min. Add 0.5 mL of a 1.3 mol/L ammonium sulfamate solution dropwise to neutralize excess nitrous acid. Mix well and immediately add 0.5 mL of 12.5 mol/L NaOH. Vortex-mix for about 1 min.

Measure the absorbance of each sample at 430 nm, with water as the reference. Calculate the concentration of acetaminophen in the patients’ sera and control by comparing their absorbances with those of the working standards.

Method 2 (proposed modification for use in uremia). Pipet 1 mL of sample (patient’s serum, serum working standards, and a suitable commercial lyophilized control) into 20-mL glass culture tubes equipped with Teflon-lined screw caps. With a calibrated spatula, add about 500 mg of solid ammonium sulfate to each tube. Pipet 10 mL of diethyl ether into each tube, cover the opening of the tubes with plastic sheeting (Saran Wrap), and apply the screw cap firmly. Extract by shaking vigorously for 5 min, and centrifuge (3000 ×g, 5 min). Transfer the ether (upper) extracts to 15-mL glass test tubes and add about 500 mg of anhydrous Na₂SO₄ with a calibrated spatula. Pipet 8 mL of each dehydrated extract into a second series of test tubes, and evaporate in a gentle current of air.

Reconstitute each extract residue with 1 mL of water, vortex-mix, add 2 mL of 0.6 mol/L trichloroacetic acid, and again vortex-mix. Pipet 2 mL of hexane into each tube and vortex-mix for about 30 s. Centrifuge, aspirate, and discard the hexane (upper) layer. Transfer 2 mL of the remaining aqueous phase into a third series of test tubes. Add 0.5 mL of 6.0 mol/L HCl and mix. Generate nitrous acid by pipetting 0.5 mL of 1.4 mol/L NaNO₂ into each tube, mix well, and let stand for 2 min. Add 0.5 mL of the 1.3 mol/L ammonium sulfamate solution dropwise to neutralize excess nitrous acid. Mix well, immediately add 0.5 mL of 12.5 mol/L NaOH, and vortex-mix for about 1 min.

Measure the absorbance of each sample at 430 nm, with water as the reference. Calculate the concentration of acetaminophen in the patients’ sera and control by comparing their absorbances with those of the working standards.

Serum Samples from Uremic Patients

Sera having a creatinine concentration >30 mg/L and (or) urea nitrogen concentration >500 mg/L were gleaned as residual samples from 58 different patients known not to have ingested acetaminophen. The samples were analyzed by the conventional colorimetric procedure (method 1). Additional sera from 20 of these patients were analyzed by both the conventional colorimetric procedure (method 1) and the proposed modification of the procedure (method 2).

Results

The urea nitrogen concentrations in the sera of the 58 different uremic patients ranged from 80 to 1420 mg/L (x̅, 700; SD, 260), and the creatinine concentrations from 9 to 239 mg/L (x̅, 88; SD, 53). The patients with normal urea nitrogen concentrations had abnormal creatinine concentrations, and vice versa. The apparent "acetaminophen" concentrations in these sera ranged from 3 to 58 mg/L (x̅, 28; SD, 13) when the samples were analyzed by method 1. In contrast, "background" concentrations in the sera of 10 drug-free volunteers with normal renal function ranged from 5 to 16 mg/L (x̅, 8; SD, 4).

When additional sera from 20 of the 58 uremic patients were analyzed by both the conventional colorimetric procedure (method 1) and the proposed extraction procedure (method 2), significant differences were found (Table 1). "Acetaminophen" concentrations obtained by method 1 ranged from 17 to 50 mg/L (x̅, 33; SD, 10), while concentrations obtained by method 2 ranged from 0 to 6 mg/L (x̅, 3; SD, 2). This represents a reduction of "background" by 89 ± 8% (x̅ ± SD) (range 68–100%).

For the proposed procedure a standard curve was prepared by supplementing drug-free serum with acetaminophen. A plot of concentration (x) vs absorbance (y) was linear (r = 0.9998) for seven concentrations in the 0–1000 mg/L range studied ("least-squares" regression equation: y = 0.0021x + 0.0188). Although as little as 5 mg of acetaminophen per liter could be detected by the procedure, the "background" determined in 20 drug-free sera (range 0–6 mg/L) limited the working analytical sensitivity to 10 mg/L.

Analytical recoveries for the proposed method were de-

| Table 1. Comparison of "Acetaminophen" Concentrations in 20 Uremic Patients by a Conventional Colorimetric Method vs the Proposed Method |
|---------------------------------|---------------------------------|---------------------------------|
| Creatinine, mg/L | "Acetaminophen," mg/L | | |
| x̅ | 13.775 | p < 0.01 |
| SD | 51 | 2 |

1 One such control (concentration, 20 mg/L) is supplied by Utak Laboratories, Saugus, CA 91350.

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determined by comparison of absorbances obtained in acetaminophen-supplemented sera with those obtained in nonextracted aqueous standards of the compound. The recoveries at various concentrations ranged from 88 to 97%: 25 mg/L (89%), 50 (88%), 100 (91%), 200 (90%), 500 (94%), and 1000 (97%). Recoveries from uremic serum supplemented with acetaminophen were 94% at a concentration of 100 mg/L, 88% at 500 mg/L.

The precision of the proposed method was evaluated by making 12 measurements on each of two sera supplemented with acetaminophen to concentrations of 100 and 500 mg/L, respectively: within-run CVs were 2.7% and 2.0%; between-run, 3.1% and 4.2%. Acetaminophen was stable in serum for at least nine days at −20 °C. The phenolate chromophore was stable for at least an hour at room temperature.

Amitriptyline, amphetamine, caffeine, chloridiazepoxide, chloromezalone, chlorpromazine, chlorproamide, cocaine, diazepam, diphenhydramine, dihydrocodeine, imipramine, indomethacin, lorcazepam, memprobamate, methadone, methaqualone, morphine, nitrazepam, oxypertine, pentazocine, pentobarbital, phenacetin, phenytoin, promethazine, strychnine, theophylline, and tolbutamide reportedly do not interfere with the method for acetaminophen (7, 8). Strong acids such as salicylate and its congeners are not extracted under the conditions of the proposed assay.

After correction for aliquotting losses with the proposed method, a plot of absorbances obtained by the conventional colorimetric method (x) vs the proposed extraction method (y) was linear (r = 0.9993) for seven concentrations over the 0–1000 mg/L range studied in non-uremic serum ("least-squares" regression equation: y = 0.9866x − 0.0185).

The mean apparent "acetaminophen" concentration (28 mg/L) by method 1 in the 58 uremic patients differed significantly from that in the 10 normal volunteers (9 mg/L) when Student's t-test was applied (t = 8.842, p < 0.01). The "acetaminophen" concentration showed a statistically significant correlation with the serum creatinine by "least-squares" regression analysis (r = 0.698, p < 0.01) but no significant correlation with urea nitrogen. Of the uremic patients, 11 had normal "background" when their sera were analyzed by method 1. However, these 11 had significantly lower creatinine concentrations (3, 35 mg/L; SD, 12) than the remaining 47 (102 mg/L; SD, 51) (t = 8.376, p < 0.01). For the entire series, whenever the creatinine was >86 mg/L (as was the case for 36 sera), the "background" concentration exceeded that for normal serum by method 1.

For 49 uremic patients the "anion gap" was determinable and was estimated by the difference (Na⁺ + K⁺) − (Cl⁻ + HCO₃⁻). The apparent acetaminophen concentration by method 1 in these 49 sera correlated significantly with the anion gap (r = 0.548, p < 0.01).

In my initial attempts to resolve the uremic "interference," I used adsorption techniques: Amberlite XAD-2 nonionic polymeric adsorbent (Malinckrodt Inc., St. Louis, MO 63160), silica gel (Matheson Coleman & Bell, East Rutherford, NJ 07070), and AG50W-X2, AG50W-X4, and AG50W-X8 cation-exchange resins (Bio-Rad Laboratories, Richmond, CA 94804); all failed to eliminate the interference. When AG1-X10 anion-exchange resin (Bio-Rad Labs.) was used, however, the interference was eliminated, but so was 58% of acetaminophen added to the serum.

Discussion

Removal of the interference by the anion-exchange resin and the statistically significant correlation of the degree of interference with the magnitude of the "anion gap" suggest that the interference is anionic in nature. It is conceivable, if not probable, that the interference may be due to ring nitration of the phenolic acids encountered in uremic serum. Creatinine and urea themselves are not responsible for the interference, because an aqueous creatinine standard of concentration 10 g/L and an aqueous urea standard of concentration 100 g/L failed to react when method 1 was used.

Use of the proposed extraction method decreases interferences encountered in uremia to the "background" concentrations noted in normal sera. The anionic interferences are not recovered under the conditions of physiological pH at which the ether extraction is performed. However, acetaminophen is almost completely recovered. Hexane is utilized to remove turbidity due to lipids that are co-extracted with acetaminophen.

The conventional colorimetric assay (7–9) should be considered unsuitable for use in uremia. In the present study, use of this assay gave "false positive" acetaminophen concentrations ranging up to 88 mg/L. Although this concentration may not appear to be extraordinarily high, it can be consistent with acetaminophen overdose if more than 12 h have elapsed since drug ingestion (3). Furthermore, such a finding may suggest a false etiology for the patient's compromised renal status if it is not already explained. Finally, if acetaminophen is truly present in uremic serum, the use of method 1 would yield inappropriately high results, perhaps suggesting overdose in an otherwise therapeutically dosed patient.

If no other reason, analysis for acetaminophen should be included in every routine toxicology panel because use of the "antidote" (e.g., acetylcysteine) is based upon information on the drug concentration in serum. The proposed method can be performed rapidly, easily, and economically. In addition, samples can be conveniently batched and assayed simultaneously. The immediate development of color in the assay permits rapid identification of "positives" before spectrophotometric quantitation. Current chromatographic techniques may not offer all of these advantages.

The proposed assay can be used either as the laboratory's primary method for acetaminophen analysis or as an alternative method whenever the conventional procedure yields a "positive" result and the renal function of the patient is unknown. I consider the proposed method to be reliable, accurate, and specific, and recommend it for routine use in the clinical laboratory.

References

Glucose Determinations in Plasma and Serum: Potential Error Related to Increased Hematocrit

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This study reports the effect of increased hematocrit (packed cell volume) on the rate of decrease in plasma glucose during 6 h in blood specimens from seven newborns (cord blood) and heparin-treated blood specimens from eight adults. Mean hematocrits ranged from 0.43 to 0.75 for the adults and from 0.51 to 0.81 for the infants. The decrease in glucose was related to time and to hematocrit. For example, in adults with a mean hematocrit of 0.43, glucose decreased from 890 ± 40 mg/dL to 570 ± 30 mg/dL (mean ± SEM) at 6 h, whereas in adults with a mean hematocrit of 0.75, glucose decreased from 890 ± 40 to 200 ± 30 mg/dL. In infants with a mean hematocrit of 0.51, glucose decreased from 1010 ± 20 to 480 ± 70 mg/dL at 6 h; infants with a mean hematocrit of 0.81 had blood glucose decrease from 1010 ± 20 to 50 ± 30 mg/dL. These results indicate that the incidence of hypoglycemia in infants with polycythemia may be overestimated, and emphasize the need for prompt handling of blood samples collected from newborns for glucose determinations.

Additional Keyphrases: pediatric chemistry • newborns • sample handling • glycolysis in blood drawn for glucose determinations • variation, source of

The accuracy of plasma glucose determinations is related to the method of sample collection (1–4); addition of glycolytic inhibitors such as NaF (2–4); separation of serum or plasma from cells; and sample treatment—i.e., cooling specimens on ice or refrigerating them immediately (1) and, particularly, the interval between sampling and assay (1–5). These problems are particularly relevant to glucose determinations in newborn infants. Several investigators (6–8) have demonstrated that glucose consumption is greater in erythrocytes from either premature or full-term infants than in erythrocytes from adults. Because newborns commonly have hematocrits >0.60 and not uncommonly >0.70, we evaluated the effect of hematocrit on results for glucose in blood specimens from newborns and adults as another potential source of laboratory error in glucose determinations.

Methods

Whole blood was obtained from eight healthy adult volunteers and from the umbilical cord of seven normal, full-term infants of appropriate weight for gestational age. Without delay, 4-mL aliquots were treated with sodium heparin (5 int. units/mL of blood), placed in 10 test tubes, and centrifuged (5000 rpm, 5 min). The 10 tubes per specimen were then allocated to five groups. Group 1 was the control and had no further manipulation; the remaining groups had various volumes of plasma removed, resulting in the hematocrit values listed in Table 1. Samples with hematocrits exceeding 0.85 were eliminated from further consideration. Hematocrits were determined in duplicate. In addition, non-heparinized blood from each adult and infant was placed into a tube containing 10 mg of potassium oxalate and 12.5 mg of NaF, and into a tube containing no anticoagulant (clot tube). We determined plasma glucose in duplicate 20-μL heparin-treated samples at 0, 1, 2, 4, and 6 h with a glucose analyzer (Model 23A; Yellow Springs Instrument Co., Yellow Springs, OH 45387). Duplicate glucose determinations were made at 1, 2, 4, and 6 h for the NaF-treated samples and at 2, 4, and 6 h for the clotted specimens. The serum and clot were not separated or agitated during the study. The glucose value at time 0 for the heparin-treated samples for both the infant and adult specimens was used as the reference (time-zero) glucose value for NaF-treated and clotted specimens from infants and adults. All samples were left at room temperature throughout the study (25–27 °C). Results were evaluated by the Student’s t- and paired t-tests.

The study was approved by the Committee of Associates on Human Investigation at the University of South Florida.

Results

Table 1 shows the glucose changes with time for each group. The higher baseline glucose value in infants than in adults partly obscures the significantly more rapid changes in glucose concentrations in infants, both with increasing time and increasing hematocrits.

Decreases in glucose in samples from infants were signifi-