Spectrophotometric Screening Method for Acetaminophen in Serum and Plasma

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We describe a simple, economical procedure for rapidly detecting acetaminophen in serum or plasma. The method is based upon the reduction by the drug of ferric 2,4,6-tris(2-pyridyl)-S-triazine, an acidic pH, to ferrous 2,4,6-tris(2-pyridyl)-S-triazine complex, which absorbs maximally at 593 nm. Absorbance and acetaminophen concentration are linearly related from 25 to 400 mg/L, and so therapeutic and toxic concentrations can be measured. The method is accurate; day-to-day CV’s for two pooled control specimens (103 and 227 mg/L) were 4.4 and 6.6%. Correlation studies, with an established nitrination method and with the free-radical diphenylpicrylhydrazyl dye method, showed correlation coefficients of 0.985 and 0.915, respectively. Of 25 commonly used drugs tested, only levodopa, oxyphenylbutazone, and phenylephrine interfere significantly. Interference from salicylate, salicylamide, and phenylbutazone was insignificant.

Additional Keyphrases: emergency drug assay • toxicology • acid ferric reduction of phenolic hydroxyl group • extraction with ethyl acetate

During the last few years the misuse of acetaminophen (N-acetyl-p-aminophenol) has been associated with severe and sometimes fatal hepatotoxicity (1, 2) and nephrotoxicity (3).

The diagnosis and treatment of acetaminophen overdose are based largely on information concerning the acetaminophen concentration in plasma and the time of drug ingestion. Overdosed patients must be given an antidote within 10 h of drug ingestion; after 10 to 12 h the antidote not only is ineffective but also potentially hazardous to the patient. The clinical laboratory therefore plays a critical role in the diagnosis and treatment of acetaminophen overdoses and must be able quickly to provide the clinicians with accurate data on plasma acetaminophen. Several methods for this drug have good accuracy and specificity (4–8), but they are time consuming, technically demanding, and require the use of costly, highly specialized instruments.

We describe here a simple and economical procedure for rapidly detecting acetaminophen in serum or plasma, for which only a standard spectrophotometer is required and which can be completed in 20 min.

Materials and Methods

Reagents

**Acetate buffer, 0.3 mol/L, pH 3.6.** Dissolve 3.1 g of sodium acetate trihydrate in 800 mL of distilled water, and add 16.0 mL of glacial acetic acid. Adjust the pH to 3.6 and dilute to a final volume of 1.0 L with water.

**2,4,6-**

**Tris(2-pyridyl)-S-triazine, 0.8 mmol/L.** Dissolve 624 mg of this compound (Sigma Chemical Co., St. Louis, MO 63176) in 250 mL of HCl (36 mmol/L).

**Ferric chloride solution.** Dissolve 540 mg of FeCl₃·6H₂O (Sigma Chemical Co.) in 100 mL of HCl (20 mmol/L).

**Stock acetaminophen standard, 1000 mg/L.** Dissolve 100 mg of acetaminophen (Sigma Chemical Co.) in 100 mL of distilled water.

**Working standards.** Prepare working standards (100 and 200 mg/L) by making a 10- and fivefold dilution of the stock standard with drug-free pooled serum, respectively. Store aliquots of working standards in the freezer when not in use.

**Procedure**

Place exactly 0.5 mL of unknown serum or plasma in a 10 × 75 mm glass tube containing 1.0 mL of ethyl acetate and about 0.1 g of sodium chloride. Prepare standards and controls in the same manner. Vortex-mix the contents of each tube vigorously for 30 s.

At 30-s intervals, add 0.1 mL of the organic (upper) layer of the standards, controls, and unknowns to appropriately marked tubes containing 2.5 mL of the acetate buffer, 0.3 mL of 2,4,5-tris(2-pyridyl)-S-triazine solution, and 0.1 mL of FeCl₃·6H₂O solution, gently mixing the contents of every tube after each addition. Also prepare a reagent blank.

Set the spectrophotometer at 593 nm and zero it on the reagent blank. At the same timed intervals, read the absorbance of each tube after it has stood for exactly 15 min at room temperature. Determine the acetaminophen concentration of unknown and control specimens from a standard curve, or calculate it on the basis of the absorbance of the standards.

Results

**Absorption Spectrum**

The absorption spectrum of the product of direct acid reduction of ferric 2,4,6-tris(2-pyridyl)-S-triazine by acetaminophen (Figure 1) was determined with a scanning spectrophotometer for two different acetaminophen concentrations, 40 and 100 mg/L, 15 min after starting the reaction. Both absorption spectra exhibit a maximum at 593 nm.

**Analytical Variables**

**Color stability.** Color stability was determined (Figure 2). The change in absorbance per minute was constant up to about 20 min for both concentrations. An optimum reaction time of 15 min was chosen. The graph indicates that the reaction time may be shortened to 10 min in case of an emergency determination.

**Linearity.** Absorbance at 593 nm is linearly related to acetaminophen concentrations from 25 to 400 mg/L (Figure 3). Thus the method is suitable for measuring toxic as well as therapeutic acetaminophen concentrations.

**Precision.** The reproducibility of the method, either day-to-day or within-run (Table 1), was very good. The relative variability about the mean of both controls was within acceptable limits.

**Analytical recovery.** To 40 mL of acetaminophen-free pooled serum, 10 mL of aqueous acetaminophen standard (500 mg/L) was added. The mixture was equally divided into 20 parts. To each portion, acetaminophen was added to give

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Fig. 1. Absorption spectrum for the direct acid reduction of ferric 2,4,6-tris(2-pyridyl)-S-triazine by acetaminophen
A, 100 mg/L; B, 40 mg/L; and C, blank.

Fig. 2. Color stability of the reaction product
Acetaminophen concentration: A, 50 mg/L; B, 100 mg/L.

Fig. 3. Relation between acetaminophen concentration and absorbance at 593 nm
Each point represents an average value of four determinations.

various concentrations; each portion was then assayed and the measured value compared with the expected value. The mean percent recovery for 20 specimens was 100.5% (SD, 6.5%).

Correlation Studies

We compared results by the proposed method with those by two well-established colorimetric methods: the free-radical diphenylpicrylhydrazyl dye method of Routh et al. (9) (Figure 4A), for which the regression line was $y = 1.01x + 5.24$, and the nitration method of Glynn and Kendal (10) (Figure 4B), for which the regression line was $y = 0.99x + 4.06$. Comparison of data obtained by our proposed method against those by a high-performance liquid-chromatographic method (8) for 17 samples yielded a correlation coefficient of 0.95 with $y = 0.92x + 2.02$.

Drug Interferences

Twenty-five drugs often used alone or in combination with acetaminophen were added to drug-free serum and tested with our procedure. Only drugs with a phenolic hydroxyl group, such as levodopa, phenylephrine, and oxyphenylbutazone, caused interference. Although salicylate and salicylamide possess a phenolic hydroxyl group, the interference of either of them in a concentration of 1000 mg/L corresponds to an apparent 15 mg of acetaminophen per liter. Drugs not interfering with the procedure included: phenylbutazone, phenacetin, acetylsalicylate, diazepam, chlordiazepoxide, doriden, methaqualone, phenobarbital, pentobarbital, secobarbital, amobarbital, butobarbital, propoxyphene, primidone, phenytoin, theophylline, codeine, caffeine, ascorbic acid, and meprobamate.

Discussion

Clinical diagnosis and management of acetaminophen overdose would be greatly enhanced if a rapid, reliable screening-method for this drug were available. We believe that

Table 1. Reproducibility of the Analysis for Acetaminophen

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<thead>
<tr>
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<th>Control I</th>
<th>Control II</th>
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<tbody>
<tr>
<td></td>
<td>Within-run ($n = 5$)</td>
<td>Day-to-day ($n = 60$)</td>
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<tr>
<td>Mean, mg/L</td>
<td>103.0</td>
<td>225.0</td>
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<tr>
<td>SD, mg/L</td>
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<tr>
<td>CV, %</td>
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<td>3.3</td>
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<td></td>
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<td></td>
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<tr>
<td>Mean, mg/L</td>
<td>103.0</td>
<td>227.0</td>
</tr>
<tr>
<td>SD, mg/L</td>
<td>6.8</td>
<td>10.0</td>
</tr>
<tr>
<td>CV, %</td>
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the present method meets this need. It is simple, quick, and economical. The reaction time we used is 15 min, but if necessary could validly be shortened to 10 min. Only a standard spectrophotometer is required.

Recently, Meola (11) introduced a simple colorimetric procedure for emergency determination of acetaminophen. However, the relation between concentration and color intensity is only linear to 120 mg/L. This can result in having to repeat assays in the most serious overdose cases. Furthermore, salicylate and salicylamide interfere more in Meola’s procedure.

The procedure has been shown to be specific to compounds that have a phenolic hydroxyl group located either meta or para to the functional group on the benzene ring. Thus, potential interference of a drug can be predicted from its chemical structure, which may be important in the treatment of multiple-drug overdoses involving acetaminophen. Both salicylate and salicylamide have a hydroxyl group located ortho to the carboxylic acid group; a hydrogen bond with the carboxyl may account for the lesser interference from these two compounds.

Results by our procedure correlate excellently with those by the established nitration method of Glynn and Kendall (10), which requires more manipulative steps and includes a deproteinization procedure that is considerably longer than our 30-s extraction.

Direct sunlight will reduce ferric 2,4,6-tris(2-pyridyl)-S-triazine by photoreduction, but indirect sunlight or normal fluorescent room lighting have no such effect, for all practical purposes.

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References