Liquid-Chromatographic Determination of Acetaminophen in Serum

Carol G. Fletterick,1,2 Thomas H. Grove,1,3 and David C. Hohnadel1,4

We describe a sensitive and precise “high-pressure” liquid-chromatographic method for determining acetaminophen in serum. The 2-acetaminophenol and 3-acetaminophenol structural isomers of acetaminophen are used as internal standards. The method, which involves solvent extraction and adsorption chromatography on silica, provides excellent sensitivity, accuracy, and selectivity. The standard curve is linear over the range of acetaminophen concentrations of 0.5 to 300 mg/L, which makes the method useful for both pharmacokinetic studies and overdose monitoring. Analytical recovery is 97% for acetaminophen concentrations ranging from 5 to 300 mg/L. Many commonly used drugs were tested and found not to interfere. The procedure has been successfully adapted as a microscale method requiring only 50 µL of sample. The microscale method is particularly useful for pediatric and neonatal patients for whom sample size is a major concern.

Additional Keyphrases: Kinetics of drug metabolism • monitoring therapy • pediatric and obstetric chemistry • microscale adaptation • drug assay • “high-pressure” liquid chromatography

Acetaminophen (e.g., Tylenol, McNeil; and Datril, Bristol-Myers) is generally considered to be a safe analgesic and antipyretic substitute for aspirin. Acute acetaminophen overdose is associated with hepatic necrosis and, in severe cases, fulminant hepatic failure (1–4). Because hepatic injury only becomes biochemically apparent 24 to 48 h after overdose ingestion (5, 6), the potential for developing severe hepatic damage can be much more quickly assessed by quantitating the serum concentration of acetaminophen. Certain concentrations in serum at 4 and 12 h after overdose are associated with probable hepatic damage (5). However, because there is often difficult in determining the time of ingestion, the serum acetaminophen biological half-life (t1/2) obtained from data on two or more sequential samples is considered the most reliable indicator of impending hepatic necrosis (5).

“High-performance” liquid chromatography is currently the method of choice for measuring serum acetaminophen concentration and biological half-life. It excels ultraviolet spectrophotometric (7, 8), colorimetric (9–11), and gas–liquid chromatographic (12–16) methods in rapidity, specificity, sensitivity, and simplicity of sample preparation. High-performance liquid chromatographic methods involving ion-exchange (17, 18), reversed-phase (19–21), and silica adsorption (22) have been described. The last-mentioned method is simple and rapid but includes no internal standard and potential interferences were not evaluated.

A high-performance liquid chromatographic method for determining acetaminophen in serum is described here in which a silica adsorption column is used, and two structural isomers of acetaminophen are internal standards.

Materials and Methods

Apparatus

All analyses were performed with a du Pont Model 830 high-pressure liquid chromatograph with a 254-nm ultraviolet detector (du Pont Instruments, Wilmington, DE 19889). The chromatograph is equipped with a 2.1 mm (i.d.) × 25 cm column containing “ZORBAX SIL” silica (du Pont Instruments) pre-packed with 5- to 6-μm spherical particles. With a 10-μL syringe, injections are made directly into the column.

Reagents and Standards

Acetaminophen (4-acetaminophenol), 3-acetaminophenol, and 2-acetaminophenol were purchased from Aldrich Chemical Co., Milwaukee, WI 53233. A stock solution (1.0 g of each compound per liter) is prepared by dissolving 10 mg of each in 10 mL of absolute ethanol. Working standards are prepared by appropriate dilution of stock standards with drug-free serum.

Chloroform, ethyl acetate, and heptane (“Photrex” grade) were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Absolute ethanol was purchased from IMC Chemical Group, Inc., Sobin Park, MA. Chloroform for use in column conditioning and in the mobile phase was water-saturated before use by adding 3.0 mL of distilled water to each liter, stirring (magnetic stirrer) for 3 h, and aspirating any undissolved water. Other chemicals were reagent grade.

Extraction and Macro Procedure

Place serum or working standards (1.0 mL) in 16 × 125 mm borosilicate glass tubes, each containing 1.0 mL of sodium acetate buffer (1.0 mol/L, pH 4.8). To determine acetaminophen in the low concentration range (0.5 to 40 mg/L), add 10 µL of the 1 g/L 2-acetaminophenol and 3-acetaminophenol
Fig. 1. Chromatograms of a serum blank (A) and of serum fortified with 100 mg each of acetaminophen and internal standards per liter (B)

stock solution to the tubes, resulting in a serum concentration of each internal standard of 10 mg/L. To determine acetaminophen in the high concentration range (50 to 300 mg/L), or for an unknown overdose, add 100 μL of the internal standard stock solution to the tubes, giving a final concentration in serum of each internal standard of 100 mg/L. The samples are vortex-mixed. Then add 10 mL of ethyl acetate to each tube and cap the tubes.

Mix the contents of the tubes for 5 min at 40 rpm on an angled turntable, then centrifuge for 5 min at 900 × g. Transfer the organic phase to a clean tube and evaporate at 65 °C under a slow stream of dry nitrogen. Dissolve the residue in 100 μL of absolute ethanol and chromatograph 5 μL of this. Elute the column with a mixture of water-saturated chloroform/heptane/ethanol/glacial acetic acid (540/360/100/0.72 by vol). Adjust the liquid-inlet pressure to 6.0 MPa (1000 psi), resulting in a flow rate of 0.4 mL/min.

Quantify sample concentrations by measuring the ratio of the peak area of acetaminophen to that of one of the internal standards. The 3-acetaminophen internal standard is the one routinely used in calculations.

Microprocedure

For determination of acetaminophen in pediatric or neonatal samples where sample volumes are limited, a 20-fold scaledown of the macro procedure is used. Place 50 μL of serum or working standards in 13 × 100 mm borosilicate glass tubes containing 50 μL of 1 mol/L sodium acetate buffer (pH 4.8), 5 μL of 1 g/L internal standard, and 0.5 mL of ethyl acetate. Then follow the macroscale protocol except dissolve the extracted residue in a final volume of 50 μL of ethanol and then inject 10 μL into the column.

For comparative purposes, we also determined acetaminophen concentrations by the reference colorimetric method of Glynn and Kendall (11) as modified by Dechtiaruk et al. (16). Values for the colorimetric procedure were read in absorbance units at 430 nm.

Results and Discussion

Analytical Variables

Figure 1 shows typical chromatograms for sera. Retention times were 3.4 min for 2-acetaminophenol, 5.4 min for 3-acetaminophenol, and 7.7 min for acetaminophen. As the column ages, retention times decrease. Increasing the percentage of ethanol in the mobile phase will also decrease the retention times, as will increasing the operating pressure.

Linearity. Figure 2 shows the standard curves, with 2-acetaminophenol and 3-acetaminophenol as internal standards. The concentration of acetaminophen ranges from 5 to 100 mg/L. Acetaminophen concentrations are quantitated by calculating the ratio of the peak area of the drug to the peak area of the internal standard. The peak height can also be used for quantitation with no differences in linearity. Experiments using an internal-standard concentration of 100 mg/L readily allow quantitation of acetaminophen from 10 to 300 mg/L. We determined the linearity of extraction, chromatography, and detector response for low acetaminophen concentrations from 0.5 to 30 mg/L, using an internal standard concentration of 10 mg/L. No differences in slope related to acetaminophen concentration were observed for the standard curves of either internal standard from 0.5 to 300 mg/L when adjustments were made to a common scale. Figure 2 shows part of these data. The range of linearity in the microscale method (with use of the 100 mg/L internal standard) extends from 10 to 300 mg/L.

Reproducibility. Both the macro and micro-methods were evaluated for within-day and between-day variation by analyzing thawed aliquots of a frozen serum pool that had been fortified with acetaminophen. The between-day precision data were obtained during several weeks by different analysts. The within-day studies were done by a single analyst. Table 1 gives the results for several concentrations of acetaminophen.

<table>
<thead>
<tr>
<th>Table 1. Reproducibility Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample vol</strong></td>
</tr>
<tr>
<td>50 μL</td>
</tr>
<tr>
<td>1 mL</td>
</tr>
<tr>
<td>1 mL</td>
</tr>
<tr>
<td><strong>Day-to-day</strong></td>
</tr>
<tr>
<td>50 μL</td>
</tr>
<tr>
<td>1 mL</td>
</tr>
<tr>
<td>1 mL</td>
</tr>
</tbody>
</table>

Fig. 2. Standard curves for acetaminophen, with 2-acetaminophenol (●—●) and 3-acetaminophenol (O—O) as internal standards.
Fig. 3. Regression plot comparing results of a colorimetric method and the present method

**Accuracy.** Known amounts of acetaminophen were added to drug-free serum and processed as described. Standards were added directly to the ethyl acetate for comparison and then further processed. Analytical recoveries for the macro method with duplicate serum samples for the concentration range of 5 to 30 mg/L, with a 10 mg/L internal standard, were 94 to 100%. Recoveries of similar duplicate samples in the concentration range 50 to 300 mg/L and with a 100 mg/L internal standard were 91 to 100%. The mean recovery for all samples was 97%. For acetaminophen concentrations ranging from 10 to 100 mg/L, the average recovery for 16 samples assayed by the micro-method was 100% (range 98–105%).

The acetaminophen concentration in 21 sera was measured by the macro liquid chromatographic and colorimetric methods (Figure 3). The acetaminophen concentrations ranged from 0.5 to 300 mg/L, and a correlation coefficient of 0.990 was found for these data. The colorimetric method appears to be accurate for acetaminophen concentrations between 50 and 250 mg/L. Within this range, recoveries from the high-performance liquid chromatographic and colorimetric methods agree well. Sera containing concentrations of less than 50 mg/L cannot be distinguished from drug-free serum by the colorimetric method, but there is no difficulty in measuring concentrations of acetaminophen by the high-performance liquid chromatographic method at 5 mg/L or even less if the 10 mg/L internal standard is used.

**Interference.** Potential interference by other drugs was studied by chromatographing extracted sera containing other medications (Table 2). Of the 28 drugs studied, none co-eluted with acetaminophen to produce falsely elevated peaks and make quantitation impossible. Acetanilide and caffeine co-elute with the 2-acetaminophen internal standard, making 3-acetaminophenol the preferred internal standard. However, acetanilide would not be expected in most serum specimens, because it is rarely used, and the interference of caffeine at normal physiological concentrations (0.1 the concentration in this study) will only depress the acetaminophen by less than 20% if the 2-acetaminophen internal standard was inadvertently used. Sulfamethoxazole and trimethoprim (found in Septra™ and Bactrim™) co-elute at the position of 3-acetaminophenol. Physiological concentrations of these drugs in serum may surpass the concentrations studied here and necessitate the use of the 2-acetaminophen internal standard to measure acetaminophen. Interference from these compounds is always obvious, because a much larger apparent 3-acetaminophenol peak is observed. In practice, it is also possible to quantitate the Septra concentration in serum by using 2-acetaminophenol as the internal standard. The macro method as described gives linear standard curves for the Septra concentrations in the range of 10–200 mg/L. Theophylline elutes as only a very small shoulder on the 3-acetaminophenol peak and does not alter the quantitation of acetaminophen when using either internal standard. Routinely, 3-acetaminophenol is used as the internal standard for all calculations, because Septra is the only drug interfering under these circumstances. When a patient's sample is observed to have a much larger 3-acetaminophenol peak, Septra is presumed to be present and 2-acetaminophenol is used for quantitation.

**Volunteer Study**

To further demonstrate the in vivo validity of our method in the low concentration (therapeutic) range, five healthy, nonfasting volunteers, three men and two women, each ingested 1200 mg of acetaminophen (Tylenol, McNeil). Blood was sampled from antecubital fossa before this ingestion and 30 min after. The sera obtained were analyzed in duplicate for acetaminophen by our method. All samples collected before ingestion were negative (i.e., $\bar{x} = 0.0$, SD = 0.1 mg/L). The average concentration found after 30 min was 8.3 mg/L (range, 4.8 to 12.7 mg/L). These values demonstrate that the method can distinguish low concentration in vivo acetaminophen samples from drug-free samples. Reportedly, the maximum concentration in plasma is attained within 40 to 60 min of drug ingestion (23), but there is a large individual variation. The results we obtained are near peak concentrations and are slightly higher, as expected, than the results reported by Horvitz and Jatlow (20), which were 1.1 to 6.4 mg/L from individuals on chronic medication (tough concentrations).

We conclude that a silica column can clearly separate the structural isomers of acetaminophen. The use of two internal

<table>
<thead>
<tr>
<th>Table 2. Drugs Screened for Interference&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
</tr>
<tr>
<td>Amphetamine</td>
</tr>
<tr>
<td>8-Chlorotheophylline</td>
</tr>
<tr>
<td>Cocaine</td>
</tr>
<tr>
<td>Codeine</td>
</tr>
<tr>
<td>Desmethylmethem succinimide</td>
</tr>
<tr>
<td>Ethosuximide</td>
</tr>
<tr>
<td>Glutathimide</td>
</tr>
<tr>
<td>Meperidine</td>
</tr>
<tr>
<td>Metamphetamine</td>
</tr>
<tr>
<td>Methadone</td>
</tr>
</tbody>
</table>

<sup>a</sup> All drugs were added to drug-free serum and extracted. Group I drugs, 60 mg/L, did not interfere. Group II drugs, 50 mg/L, interfered with the quantitation of acetaminophen only if the inappropriate internal standard was used.

<sup>b</sup> Eluted at the position of the 2-acetaminophenol peak, did not affect quantitation if 3-acetaminophenol internal standard was used for calculations.

<sup>c</sup> Eluted at the position of the 3-acetaminophenol peak, did not affect quantitation by 2-acetaminophenol.

<sup>d</sup> Eluted as a small shoulder on the 3-acetaminophenol peak, but did not affect quantitation.
standards simultaneously adds no complexity to the procedure and affords greater versatility. If a serum constituent interferes with one internal standard, the other internal standard is still available for quantitation.

Column consistency and stability are excellent. The absorbent activity of the silica columns is controlled by using water-saturated chloroform in the mobile phase. The three different ZORBAX SIL silica absorption columns that we have used gave identical results. A column should be regenerated after about 150 injections, when one notices decreasing sensitivity and decreasing retention times. A column can be regenerated by washing with solvents of increasing polarity in the following sequence: water-saturated chloroform, acetone, methanol, water, methanol, acetone, and then water-saturated chloroform. The column is finally washed for 1 h with the mobile phase before a sample is injected. If the regeneration procedure is followed, the useful life of a column will extend to well over 500 injections of extracted samples.

References