Determination of Acetaminophen Concentrations in Serum by High-Pressure Liquid Chromatography

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We describe a method for determination of serum acetaminophen concentrations in serum by reversed phase high-pressure liquid chromatography. The homolog N-proponyl-p-aminophenol was used as an internal standard. The procedure, which requires only a single extraction with diethyl ether, can be optimized to be linear over the ranges of 10 to 100 or 1 to 20 mg/liter. Within-run CV was 1.2%; between-run CV was 4.4% and 4.9% at two different concentrations. Many commonly used drugs were tested and found not to interfere. The procedure is simple and rapid enough for use on an emergency basis in cases of overdosage, and can be optimized for measurement of either therapeutic or toxic concentrations.

Additional Keyphrases: drug assay • monitoring therapy • detecting overdosage • toxicology • reversed-phase chromatography • emergency procedure

The widely used analgesic drug acetaminophen (e.g., Tylenol, McNeil) is associated with significant hepatotoxicity in cases of overdosage. Hepatotoxicity has been shown to be correlated with serum drug concentrations and in particular with biological half-life (1). Recently there has been some interest in the use of sulfhydryl compounds, such as cysteamine and methionine (2), in the treatment of severe cases of overdosage. These compounds are not without hazard, and even if active therapy of this kind is not contemplated, serum concentration measurements are important clinically in determining if a significant overdosage has actually occurred, and in estimating prognosis and the need for serial liver function studies or other monitoring. Thus there is a need for a simple and rapid method for the determination of serum acetaminophen concentrations in overdosage emergency situations.

Colorimetric (3, 4) and ultraviolet spectrophotometric (5) methods for determination of serum acetaminophen concentrations are useful in evaluating overdosage, but are relatively insensitive. Gas chromatography of underivatized acetaminophen (6) is subject to difficulties with adsorption and tailing. Gas-chromatographic methods involving silylation or alkylation (7, 8) are sensitive and specific, but technically demanding. Methods involving ion-exchange high-pressure liquid chromatography (9-11) require chromatography times of 60 min or longer per sample, or are otherwise not suitable for clinical use. One method, in which a column containing silica was used (12), was simple and rapid, although an internal standard was not included. We prefer reversed-phase chromatography for its greater versatility in the clinical laboratory and its much smaller consumption of organic solvents.

We have developed a method for determining serum acetaminophen concentrations by reversed-phase high-pressure liquid chromatography. The use of a reversed-phase column containing octadecylsilane-coated silica permits compounds such as acetaminophen to be separated without first forming a derivative. This procedure is simple and rapid enough for use in emergency situations, and yet sufficiently sensitive to easily measure concentrations in serum after therapeutic doses.

Materials and Methods

Apparatus

A Model 601 liquid chromatograph (Perkin-Elmer Corp., Norwalk, Conn. 06856) with a Perkin-Elmer LC55 variable-wavelength detector was used in these studies. The chromatograph was equipped with a 4.6 mm × 25 cm column containing Partisil-10 ODS reversed-phase silica (Reeve-Angel, Whatman, Inc., Clifton, N. J. 07014).

Reagents and Standards

Acetaminophen was purchased from Sigma Chemical Co., St. Louis, Mo. 63178. A stock solution (10 g/liter) was prepared by dissolving 100 mg in 10 ml of methanol. Working standards were prepared by appropriate dilution of the stock standard in drug-free plasma.

N-Propionyl-p-aminophenol, the internal standard, was synthesized in our laboratory from p-aminophenol and propionic anhydride by standard chemical procedures (13). p-Aminophenol hydrochloride (2.9 g) was dissolved in about 25 ml of water. Propionic anhydride, 3 ml, and 10 ml of sodium propionate (2 mol/liter, prepared by neutralizing 7.5 ml of propionic acid in about 30 ml of water to pH 6-7 with 10 mol/liter NaOH and
diluting to 50 ml) were added, and the reaction mixture was stirred for 10 min. The reaction mixture was alkalinized to pH 9–10 with 10 mol/liter NaOH, saturated with NaCl, and extracted twice with 30-ml portions of ethyl acetate. The extracts were combined, washed with saturated NaCl solution, and dehydrated by filtering through Na2SO4. The ethyl acetate was evaporated under a stream of air in a water bath at 50–60 °C, and the residue was recrystallized twice from a mixture of water and ethanol.

A stock solution of internal standard (2 g/liter) was prepared by dissolving 20 mg of N-propionyl-p-aminophenol in 10 ml of methanol. The working internal standard (5 mg/liter) was prepared by adding 0.25 ml of the stock internal standard to 100 ml of diethyl ether just before use.

Acetonitrile (ultraviolet grade) was purchased from Burdick & Jackson Laboratories, Muskegon, Mich. 49442.

Other chemicals were reagent grade.

Liquid Chromatographic Procedure

Serum or working standards (0.5 ml) were placed in 15-ml screw-cap tubes with Teflon-lined caps. Solid NaCl (0.25–0.5 g) and 5 ml of working internal standard in diethyl ether were added to each tube. The tubes were extracted by shaking them 50–100 times by hand, and briefly centrifuged to separate the phases. The ether phase was transferred to a conical centrifuge tube, and the ether was evaporated under a stream of air at room temperature. The residue was dissolved in 50 μl of methanol, and 5 μl of this was chromatographed. The column was eluted with a 50 ml/liter solution of acetonitrile in potassium phosphate (0.1 mol/liter, pH 2.7) at a flow rate of 1.0 ml/min. Oven temperature was maintained at 40 °C. The column effluent was monitored at 250 nm. Samples were quantified by measuring the ratio of the maximum absorbance or peak height of acetaminophen to that of the internal standard.

The specific chromatographic conditions may need to be slightly modified to optimize them for the particular column one uses. For example, we found that with a new column it was necessary to increase the concentration of acetonitrile to 60 ml/liter and the temperature to 50 °C to obtain resolution and retention times comparable to those on the column previously used.

Results and Discussion

Acetaminophen had a retention time of 5.1 min under the chromatographic conditions used, and the internal standard a retention time of 6.2 min. Figure 1 shows a typical chromatogram of a standard, blank, and patient sample. Peak-height ratio and concentration were linearly related over the range from 10 to 100 mg/liter. A within-day precision study, with use of multiple aliquots of a 20 mg/liter standard (n = 11), showed a CV of 1.2%. Between-day precision studies (n = 10), with use of samples of plasma with added acetaminophen, yielded CV’s of 4.4 and 4.9% at mean concentrations of 12 and 70 mg/liter, respectively.

By decreasing the concentration of working internal standard to 1 mg/liter, increasing the injection volume to 10 μl, and increasing the recorder sensitivity, the linear range could easily be extended down to 1 to 20 mg/liter. Standard curves in this range showed a mean positive intercept of 300 μg/liter. This intercept value was not detectable in the 10 to 100 mg/liter range, and extractions of blank plasma or serum yielded no viable peak in the acetaminophen region. Extractions of plasma to which acetaminophen had been added in concentrations of 100 and 200 μg/liter gave detectable drug peaks, but no attempt was made to establish linearity or reproducibility at these very low concentrations.

Analytical recovery of acetaminophen was estimated by extracting a plasma standard without added internal standard and reconstituting the residue in methanol containing a known amount of internal standard. Similarly, recovery of the internal standard was estimated by extracting blank plasma with working internal standard and reconstituting the residue in methanol with added acetaminophen. The analytical recoveries of acetaminophen and the internal standard, as compared to unextracted standards, were 65 and 80% respectively. These incomplete recoveries are, of course,
corrected for by the use of extracted standards to establish the calibration curve.

Two specimens from patients with acetaminophen overdoses assayed by this method and by the differential ultraviolet spectrophotometric method of Routh et al. (5) yielded respective concentrations of 39 and 85 mg/liter by liquid chromatography and 36 and 96 mg/liter by the spectrophotometric procedure. Concentrations of acetaminophen in serum samples from patients receiving therapeutic dosages of this drug ranged from 1.1 to 6.2 mg/liter. These concentrations were below the range we are able to measure accurately by the spectrophotometric procedure.

To further confirm that the peak representing material recovered from patients’ samples with retention time equal to that of acetaminophen was in fact acetaminophen, we did a stop-flow ultraviolet scan of this peak and compared it to a similar scan of an acetaminophen standard. Figure 2 shows the spectra to be virtually identical, further validating the specificity of this method.

Of the various potentially interfering drugs tested under the chromatographic conditions used, only two drugs were found to have retention times similar to acetaminophen or the internal standard. Hydrochlorothiazide emerged from the column at the same time as acetaminophen, and sulfadiazine had a retention time similar to that of the internal standard. Both of these drugs were partly extracted, but hydrochlorothiazide in the usual doses is unlikely to reach concentrations measurable by this method, and sulfadiazine is used very infrequently. The following drugs were tested and found not to interfere: phenacetin, p-aminophenol, salicylic acid, caffeine, theophylline, theobromine, allopurinol, furosemide, tolbutamide, chlorpropamide, sulfisoxazole, sulfamethoxazole, tetracycline, chlorpromazine, imipramine, chlor Diazepoxide, diazepam, phenytoin, and various barbiturates.

High-pressure liquid chromatography on a reversed-phase column is a practical and specific method for determining serum acetaminophen concentrations. The method as described is optimized to measure concentrations typical of those found in overdosages, but with the minor modifications described the range is easily extended downward to measure concentrations found after therapeutic dosages. This procedure can equally well be performed on a liquid chromatograph equipped with a fixed-wavelength detector operating at the mercury-arc wavelength of 254 nm. As the spectrum in Figure 2 suggests, sensitivity could be further increased by using a detection wavelength of about 195 nm. Specificity and freedom from interferences at this wavelength would have to be re-established if this were to be done, but this increase in sensitivity should not be necessary for most applications. This procedure is simple and rapid enough for emergency use in overdose situations, and yet is also sufficiently sensitive to be suitable for use in metabolic studies after therapeutic dosages.

This study was supported by United States Public Health Service grant no. NIDA-10294.

References