More Sensitive High-Pressure Liquid-Chromatographic Determination of Theophylline in Serum

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We present procedures for determining theophylline in 50 μl of serum. The drug is extracted into a small volume of solvent that contains an internal standard, 8-chlorotheophylline. The extract is analyzed by isocratic reversed-phase chromatography, with measurement of eluted theophylline at 273 nm. Day-to-day reproducibility within 5% is attainable for the concentration range 5–20 mg/liter. Other xanthenes and related metabolites do not interfere. Sensitivity is 1 mg/liter. The correlation coefficient, when results by a spectrophotometric procedure were compared, was 0.989. Amobarbital, secobarbital, phenobarbital, and diphenhydantoin do not interfere. Total analysis time for a single sample is 15 min.

Additional Keyphrases: ultraviolet spectrophotometry • pediatric chemistry • other xanthenes

The clinical response to theophylline may be better related to plasma concentration of the drug than to dosage (1). Spectrophotometric (2), gas-chromatographic (3), and liquid-chromatographic (4–6) procedures have been described for determining the drug in plasma. Particularly in extending the use of theophylline to the treatment of apnea in infants (7), one often needs to be able to determine theophylline on the smallest possible volumes of sample. Ultraviolet spectrophotometric methods require relatively large sample volumes, extensive manipulation and, preferably, background absorption corrections at a second wavelength to minimize potential interferences (2). Some gas-chromatographic methods (3) are sensitive and specific but require derivatization, which demands additional manipulation and expertise. The cation-exchange high-pressure liquid-chromatographic method of Weinberger and Chidsey (4) requires 0.1 ml of serum and does not utilize the optimum detection wavelength. The adsorption chromatography procedure of Sitar et al. (5) requires 0.5 ml of plasma and a large volume of extractant. The recent reversed-phase chromatographic method of Franconi et al. (6), requires a large volume of serum, a nonoptimum detection wavelength, and involves the risk of binding of the drug to a filtration membrane.

We present procedures, especially developed for use with pediatric samples, that exploit the microanalytical potential of high-pressure liquid chromatography for the determination of theophylline in 50 μl of serum. The theophylline is extracted into 200 μl of organic solvent containing an internal standard (to compensate for injection and extraction variations) and is analyzed by reversed phase chromatography. Eluted com-

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Materials and Methods

Instrument and Instrumental Conditions

We used a liquid chromatograph (Perkin-Elmer Model 601) with a Model LC 55 detector, a 10-mV recorder, and a reversed phase column, 0.25 m × 2.5 mm, packed with 13 μm (av particle diameter) octadecyl silica (Perkin-Elmer ODS-Sili-X-1).

Data reduction was done by use of the PEP II data processor (Perkin-Elmer). The column was eluted with a mixture of 480 ml of water, 10 ml acetonitrile, and 10 ml of a solution of acetic acid in water (1/99 parts by vol). The flow rate was 1.5 ml/min, the column oven temperature 55 °C. The detector was operated at 273 nm.

Reagents

We obtained theophylline, theobromine, and caffeine from Eastman Kodak, Rochester, N. Y. 14650, and 8-chlorotheophylline, 3-methylxanthine, hypoxanthine, xanthine, 1,3-methyl uric acid and β-hydroxyethyl theophylline from ICN Pharmaceuticals, Inc., Plainview, N. Y. 11803.

Acetonitrile, ultraviolet grade, distilled in glass, was from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442.

Chloroform, isopropanol and methanol, "Spectroquality."

Glacial acetic acid, reagent grade.

The extractant and internal standard consisted of equal volumes of chloroform and isopropanol, containing 20 mg of 8-chlorotheophylline per liter.

Procedure

Transfer 50 μl serum and 200 μl extractant to a 5-ml centrifuge tube. Mix vigorously for 15 s with a vortex-type mixer, and centrifuge at 2000 rpm for 2 min. Aspirate the organic (lower) phase and transfer it to a 10 × 75 mm tube, evaporate the solvent at 60 °C in a stream of air or nitrogen (flow rate, < 50 ml/min). Dissolve the residue in 20 μl of methanol and inject 10 μl into the liquid chromatograph.

Results and Discussion

We evaluated various chromatographic conditions by chromatographing a mixture of theophylline, theobromine, caffeine, and 8-chlorotheophylline, 0.1 μg of each in 10 μl
methanol, varying the composition of the mobile phase, the pH, and the column temperature.

Mobile-phase variation included acetonitrile/water ratios of 0/100, 2/98, 5/95, 10/90, and 20/80 parts by volume. Elution order of the compounds was unaffected, but retention times and resolution of theobromine and theophylline were affected. Although a mobile phase containing no acetonitrile will elute theophylline, added acetonitrile in the mobile phase shortens the retention time. With too-high proportions of acetonitrile (>6%) there is a corresponding loss of resolution of theobromine and theophylline.

The effect of column temperature was evaluated over a range 25 to 70 °C. Higher temperatures improve column efficiency, giving a higher theoretical plate count for theophylline as temperature is increased. The temperature we elected to use in the procedure is 55 °C. This is high enough to avoid ambient variation and gives adequate column efficiency for the analysis.

The effect of pH was most marked for the internal standard, 8-chlorotheophylline. We could adjust the retention time of this compound almost at will by adjusting the pH of the mobile phase. At pH 7.5 the retention time of the internal standard relative to theophylline is 0.43; at pH 6.5 it is 1.59; at pH 4.5 it is 1.45. We elected to use pH 4.5 routinely so that the internal standard eluted after theophylline, where the detector indicated the virtual absence of interference from normal components of serum. Also, theobromine and theophylline are best resolved at this pH.

The detector wavelength, 273 nm, is the absorbance maximum for theophylline. We compared absorbances at this wavelength and at 254 nm, the wavelength characteristically used in fixed wavelength detectors. Although 254 nm is usable, the absorbance is only about 35% of that at 273 nm (254 nm is on the ascending shoulder of a symmetrical peak; the corresponding point on the descending side is at 290 nm).

Under the finally-adopted chromatographic conditions, Figure 1 shows the resolution of the mixture of theobromine, theophylline, caffeine, and the internal standard (0.1 µg of each compound).

Quantitation. We used peak area ratios of theophylline to internal standard as the basis for quantitation. To determine linearity, we measured, in duplicate, sera containing added theophylline at concentrations of 1, 2, 5, 10, 20, or 25 mg/liter. The peak area ratios were calculated, the duplicates averaged, and peak area ratio was plotted vs. concentration. Figure 2 shows that the linearity of the plot was excellent over this range.

Patients' specimens. The procedure was evaluated for patients' sera. Figure 3 shows characteristic chromatograms of sera. The concentration of theophylline in the serum depicted in Figure 3B was calculated to be 7.6 mg/liter, that in serum 3C 15.1 mg/liter. These may be compared with a theophylline-free serum 3A. Both A and B show a peak with a retention time equal to that of caffeine.

Reproducibility. We obtained within-run CV data by analyzing 10 aliquots each of two sera containing ingested theophylline, processing 50-µl aliquots during one day. We evaluated day-to-day precision by taking aliquots of each serum on a daily basis for 20 working days. Table 1 gives both sets of data.

Accuracy. We evaluated the accuracy of the procedure by using sera from 15 patients who were on therapy with theophylline. With these, we did both the liquid-chromatographic procedure and a standard spectrophotometric procedure (2). These results are shown in Figure 4. The linear regression
Table 1. Reproducibility for Analyses of Serum Pools

<table>
<thead>
<tr>
<th></th>
<th>Under-run</th>
<th>Day-to-day</th>
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<tbody>
<tr>
<td>Mean concn, mg/liter</td>
<td>5.6</td>
<td>5.4</td>
</tr>
<tr>
<td>SD</td>
<td>0.21</td>
<td>0.26</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.7</td>
<td>4.8</td>
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</table>

*10 aliquots of each concentration.

*20 aliquots of each concentration.

**Background.** Ten sera from subjects on an unrestricted diet, but not ingesting theophylline from any known source, were processed to determine if any normal component(s) of serum in the extract interfered with the determination of theophylline. The background never exceeded the equivalent of 0.2 mg of theophylline per liter, and ranged downward to undetectable. Background values were obtained similarly any material with a retention time equivalent to that for the internal standard, and ranged from barely detectable (0.2 mg/liter) to undetectable.

**Interferences.** Uric acid, 3-methylxanthine, hypoxanthine, 1,3-methyl uric acid, and β-hydroxyethylxanthine were chromatographed under the conditions of the method. These compounds eluted close to the solvent front and so do not interfere. Their relative retention times (internal standard = 1.00) are: uric acid, 0.28; xanthine, hypoxanthine, and β-hydroxyethylxanthine, 0.33; and 3-methylxanthine, 0.38.

We studied drugs other than theophylline for potential interferences: primidone, phenobarbital, amobarbital, secobarbital, phenacetin, and diphenhydantoin. None eluted within 8 h, and so do not interfere. Dramamine®, a drug prescribed for motion sickness, contains 8-chlorotheophylline; if a patient has ingested this drug it would invalidate the analysis. Chelidonine (diphenyl) is incompletely resolved from theophylline, the retention time relative to the internal standard being 0.61 and 0.56, respectively. We subsequently evaluated a column packing consisting of 10-μm octadecyl silica (Perkin-Elmer ODS HPLC Sili-X 1), on which theophylline is completely resolved from theophylline, if the pH of the mobile phase is 4.6. Retention times relative to 8-chlorotheophylline are: theobromine, 0.42; theophylline, 0.58; diphenyl, 0.70; and caffeine, 1.24. We have not completed studies on patients' samples.

Several of the compounds studied for potential interference were not eluted from the column under the conditions described. This suggests the need to change the mobile phase to a higher concentration of organic solvent at regular intervals to elute compounds that may have been retained by the column. For this purpose, we use a 20-min elution of the columns with acetonitrite/water (5/50 by vol) before each day's analyses.

**Column stability.** Column stability, perhaps because of the generally mild analytical conditions and the chemically bonded liquid phase, is excellent. The useful life of a column is largely limited by mechanical phenomena such as settling of the packing material and a gradual increase of column back-pressure to exceed pumping capability. The first may be remedied by introducing additional packing into the dead volume at the top of the column. The second effect usually indicates clogging of a porous plug column closure; replacement of this generally reduces the back-pressure. In our laboratory several hundred analyses were done before column replacement was required.

In summary, the separation on the column optimizes the presentation of the analyte to the spectrophotometric detector. Extraction with suitable solvents, including back extraction, followed by wavelength-corrected spectrophotometry (2) would probably provide results as meaningful as those given by liquid chromatography (Figure 4). However, liquid chromatography offers particular advantages for microanalysis, monitors many potential interferences, is sparing of reagents, and offers economy of manipulation.

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Gas-Chromatographic Identification of Drugs in Gastric Aspirate Samples: A Rapid Screening Procedure for Emergency Toxicology

Fred P. Abramson

Gastric aspirate has not been used frequently in the identification of ingested drugs. Because there is only qualitative significance in such a sample, a new method is described that facilitates identification. A 1-ml gastric aspirate sample is extracted with 100 μl of chloroform and analyzed directly by gas chromatography. Seventeen drugs commonly involved in overdose cases are included in this 25-min procedure. The gastric drug screen has been applied to more than 500 patients, and results are described.

Although frequently neglected as an analytical medium, gastric aspirate appears to be well suited for use in emergency toxicology (1–3). Gastric lavage is usually done as part of the treatment of an overdose patient, hence, a gastric aspirate sample will be available from nearly every patient suspected of being an overdose case, with no additional effort. A procedure developed specifically for gastric aspirates offers advantages of both speed and simplicity over procedures for blood or urine, which hitherto have incidentally been applied to gastric aspirates.

Materials and Methods

Apparatus: We used three different gas chromatograph systems interchangeably: A Model GC-45 (Beckman Instruments, Fullerton, Calif. 92634); a Hewlett-Packard Model 5731A (Hewlett-Packard, Palo Alto, Calif. 94304); and a Varian Aerograph Model 2740 (Palo Alto, Calif. 94303). Each instrument contains a 180 cm x 2 mm i.d. Pyrex column packed with 8% SP-2250DA on 100/120 mesh Supelcoport (Supelco, Bellefonte, Pa. 16823) with a helium flow rate of 40 ml/min. The column temperature was programmed between 150 and 250 °C in 8 min. Injector temperature was 225 °C, detector temperature 275 °C. Each instrument is equipped with flame ionization detection and linear temperature programming. The third instrument is connected to a Model 21-491 mass spectrometer (Du Pont Co., Instrument Products Div., Wilmington, Del. 19898) with a Model 21-094 computer system attached. The gas chromatograph–mass spectrometer system was used in the development of the method and to confirm the identity of all gas-chromatographic peaks observed in the gastric aspirate extracts from the first 100 patients.

Procedure: Gastric aspirate, 1 ml is retrieved following filtration through coarse paper (Whatman No. 41 W&R Balston, Ltd., England) and placed in a 1-ml tapered-bottom reaction vial (Supelco). The particle-free liquid from viscous aspirates or those containing excessive debris may be more easily separated by brief centrifugation. One hundred microliters of an aqueous 2.0 mg/ml caffeine (USP, Eastman Kodak, Rochester, N. Y. 14650) solution is added as an internal control, both for extraction yield and retention time. Although this is not a quantitative process, control for extraction yield is necessary from the viewpoint of quality assurance. By requiring the caffeine yield to be adequate for each extraction, one eliminates gross errors. The pH is adjusted to 5–6 with use of pH test paper (p-Hydron 3-9; MicroEssential Labs, Brooklyn, N. Y.) by dropwise addition of either NaOH or HCl, 30 mmol/liter solutions. After the pH is adjusted, 100 μl of chloroform (Spectranalyzed; Fisher, Fair Lawn, N. J. 07410) is added to the reaction vial with a glass microliter syringe (Hamilton Co., Reno, Nev. 89510), the vial is capped, laid on its side, and rolled gently for 2 min. (This small amount of chloroform readily forms an emulsion if more vigorous agitation is used in this extraction; hence, the gentle rolling procedure.) Finally 2 μl of the chloroform layer is injected into the gas chromatograph.

Interpretation: The retention time is noted for any peak with an amplitude greater than 5 chart divisions. In the initial set-up of each instrument, the attenuation has been adjusted so that the injection of a 2-μg caffeine sample gives a nearly full-scale deflection. The caffeine standard peak is identified.