High-Performance Liquid-Chromatographic Determination of Theophylline in Plasma

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A high-performance liquid-chromatographic method is reported for monitoring theophylline in plasma. Samples are deproteinized with 2.5 volumes of acetonitrile and the supernates chromatographed on a reversed-phase column. Absorption at 275 nm is monitored. One can accurately measure 1.5 mg of theophylline per liter, in as little as 10 μl of plasma, and only about 7 min is required per sample.

No interference was found in plasma samples from asthmatic patients. This method is particularly useful for routine therapeutic monitoring of both pediatric and adult patients.

Additional Keyphrases: drug assay • pediatric chemistry • monitoring therapy • deproteinization techniques

Theophylline is one of the most frequently monitored drugs because of its relatively well-defined relationship between its concentrations in plasma and therapeutic and toxic responses. Therapeutic bronchodilatation activity is optimal in the 10–20 mg/liter range in plasma for adults, but toxic symptoms appear with increasing frequency when the concentration exceeds 20 mg/liter (1–3). Thus the dosage regimen often must be adjusted to maintain concentrations within this range (3). The many recent methods for theophylline assay (4–8) attest to the increasing practice of monitoring theophylline therapy, especially in emergency cases such as acute asthmatic attacks (2, 3).

In a recent report from this laboratory, advantages and disadvantages of many of the theophylline assay methods were briefly reviewed (7). In our previously reported method, small volumes of solvent were used to extract 0.1-ml aliquots of plasma or saliva and the extracts were chromatographed on an ion-exchange column (7). Although this method has given satisfactory service for over one year, plasma samples from certain patients gave chromatograms with interfering peaks. The modified assay method reported here overcomes this problem and is even faster and simpler.

Materials and Methods

Reagents
Theophylline, theobromine, caffeine, diphyllyline, ephedrine, and phenobarbital were obtained from Sigma Chemical Co., St. Louis, Mo. 63178; 8-chlorotheophylline and 3-methylxanthine from Aldrich Chemical Co., Milwaukee, Wis. 53233; and 1-methyl uric acid and 1,3-dimethyl uric acid from Adams Chemical Co., Round Lake, Ill. 60073. Acetonitrile, glass-distilled, was from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442.

Chromatographic Instrumentation and Conditions
A high-performance liquid chromatograph (Model 601, Perkin-Elmer Corp., Norwalk, Conn. 06856) equipped with sample injector (Glenco Scientific Co., Houston, Tex. 77007) was used. Separation was at ambient temperature on a reversed-phase column (ODS HC Sil-X-1; Perkin-Elmer), with the mobile phase acetonitrile/water (10/90 by vol) at a flow rate of 2 ml/min.

A second column (μ-Bondapak C18; Waters Associates, Inc., Milford, Mass 01757) with the mobile phase acetonitrile/water (6/94 by vol) at a flow rate of 3 ml/min was also used.

The distilled water we used was filtered (0.45-μm HAWP filter; Millipore Corp., Bedford, Mass. 01730) before mixing with acetonitrile.

Absorption was monitored at 275 nm with a spectrophotometer (LC 55; Perkin-Elmer). An attenuator (Perkin-Elmer) was coupled with the detector to allow fivefold sensi-
tivity enhancement. Peak height measurements from chromatograms were used for quantitation.

Assay Procedures

Aliquots of 10–100 µl plasma samples in 2.5-ml tapered centrifuge tubes or 13 × 100 mm disposable culture tubes were vortex-mixed with 2.5 volumes of acetonitrile for a few seconds, then centrifuged at about 2000 rpm for 1 min. Ten microliters of the clear supernate was injected into the column and chromatographed. Plasma blanks supplemented with theophylline (2.5 to 40 mg/liter) were prepared and assayed as described to establish standard curves.

Results

Figure 1 shows representative chromatograms of theophylline from a patient’s plasma sample and of a plasma blank. For this particular sample, separation on an ion-exchange column by the previously reported procedure (7) resulted in interferences (Figure 1a). Such samples, although encountered only occasionally, present some obvious difficulties. When this particular sample was chromatographed on the reversed-phase columns as described here, theophylline was separated from interferences (Figures 1b,c,e) whether the sample was prepared by the previous solvent extraction method (7) or by the acetonitrile deproteinization method. Theophylline was eluted from the ion-exchange column with a retention time of 4.3 min (7) and from the reversed-phase columns with retention times of 4.9 min (ODS HC Sil-X-1) and 5.3 min (µ-Bondapak C18). A plasma blank showed no peak that would interfere with the assay (Figure 1d).

The peak heights for theophylline are proportional to its concentrations in plasma as indicated by the good linearity of the standard curves. Figure 2 shows typical standard curves of theophylline in plasma chromatographed on a reversed-phase column (ODS HC Sil-X-1). Samples prepared by either the solvent extraction or the acetonitrile deproteinization methods gave standard curves with the relations $y = 1.036x - 0.087$ and $y = 0.315x + 0.082$, respectively. The lower limit for accurate measurement of theophylline in plasma is about 1.5 mg/liter by the deproteinization method. By the solvent extraction method as little as 0.2 mg/liter can be detected in a 0.1-ml sample. However, even the sensitivity of the solvent extraction method depends on the volume of the sample and the volume fraction of the reconstituted sample solution that is chromatographed. Despite these differences in the sensitivity by the two methods of sample preparation, essentially the same assay results were obtained (Figure 3).

Table 1 summarizes results of our assay-reproducibility studies. For intra-assay studies, replicate samples were analyzed in the same run. The coefficients of variation were 4.48 and 4.24% for concentrations of 5 (n = 25) and 20 mg/liter (n
Table 1. Reproducibility of Theophylline Assay

<table>
<thead>
<tr>
<th>Theophylline</th>
<th>Theophylline added to plasma</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/liter</td>
<td>20 mg/liter</td>
<td>n = 25</td>
</tr>
<tr>
<td></td>
<td>CV = 4.48%</td>
<td>CV = 4.24%</td>
<td>5.01 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>CV = 4.88%</td>
<td>CV = 7.53%</td>
<td>20.00 ± 0.85</td>
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= 25), respectively. For inter-assay studies, replicate samples were assayed during two months. The coefficients of variation were 4.88% and 7.53% for concentrations of 5 (n = 30) and 20 mg/liter (n = 30), respectively. Because no internal standard was used in the assays, standard curves based on two or three concentrations were routinely established for the assay of plasma samples to guard against error due to possible variations in the slopes of standard curves.

Plasma samples from patients being treated concurrently with phenobarbital and ephedrine showed no interference with the assays. To evaluate possible interferences by dietary xanthines and compounds related to theophylline, such compounds were co-chromatographed with theophylline on the two reversed-phase column systems. Theobromine and diphylline interfered in one system (ODS HC Sil-X-1) but not in the other (µ-Bondapak C18). In the first system these interferences, which may be resolved by use of other mobile phases, could be recognized by the appearance of peaks or shoulders around the base of the theophylline peak, especially when theobromine and diphylline concentrations exceed 5% of the theophylline concentration in the sample. Figure 4 shows a representative chromatogram of these compounds chromatographed in the second system. 8-Chlorotheophylline and 1.3-dimethyl uric acid showed similar retention times and eluted as a single peak (Figure 4) when co-chromatographed.

Ephedrine was not retained and phenobarbital had retention times of greater than 30 min in these systems. Although the plasma samples were prepared by protein precipitation for chromatography, the columns gave reproducible separations for at least eight months in routine use, with occasional clean-up with dimethyl sulfoxide or methanol.

Discussion

The reported modifications give better resolution of theophylline than did the previously reported ion-exchange column system (7). An incomplete separation of theophylline from plasma peak was observed when the supernate of trichloroacetic acid-deproteinized plasma was chromatographed on an ion-exchange column (8), but the interference was avoided when this same sample was prepared by solvent extraction (8).

Acetonitrile was used primarily as a deproteinizing agent in our sample preparation. At the plasma/acetonitrile ratio of 1/2.5 by volume, protein precipitation was essentially complete, and the supernate remained clear upon addition of trichloroacetic acid. Plasma proteins precipitated by acetonitrile adhered to the bottom wall of the glass tubes, leaving a clear supernate after mild centrifugation, in contrast to results with trichloroacetic acid and tungstic acid as precipitants, or with other water-miscible organic solvents such as acetone, methanol, and ethanol. Tungstic or trichloroacetic acids also co-precipitated drugs such as gentamicin from plasma. Deproteinization with acetonitrile may be superior to ultrafiltration (5), which sometimes is complicated by adsorption of drugs onto the filtering materials (9, 10). The peak height for theophylline in plasma was usually 2 to 4% higher than for an equal concentration of theophylline in water mixed with 2.5 volumes of acetonitrile, probably owing to volume changes when plasma proteins were precipitated. Therefore, the analytical recovery by this deproteinization method is essentially 100%.

Sample preparation by acetonitrile deproteinization simplifies and speeds the assay. Each assay requires only about 7 min from receipt of the plasma sample. Because only 10 µl of the supernate is injected and chromatographed, as little as 10 µl of plasma can be assayed. This small sample requirement is particularly valuable for monitoring pediatric patients. This method of sample preparation is applicable to analyses for creatinine, phenobarbital, salicylates, procainamide, chloramphenicol, and other drugs in plasma. Results of these studies will be reported elsewhere.

Although the added acetonitrile dilutes the theophylline in the sample, the lower limit of 1.5 mg/liter for accurate quantitation makes the present method suitable for therapeutic monitoring. To measure lower concentrations of theophylline, solvent extraction (7) can be used for sample preparation. Assay sensitivity for the solvent extraction method can be greatly enhanced when a larger proportion of the reconstituted extract is injected to the column.

References

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Improved Ultraviolet Spectrophotometry of Serum Theophylline

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We present an improved method for ultraviolet spectrophotometry of theophylline in serum. We studied various extraction techniques aimed at eliminating interferences from co-extractable serum constituents. In the resulting modified procedure, 1 ml of serum is required and a salt-solvent pair of ammonium sulfate and chloroform-hexane is used for extraction. The solvent forms the top phase after extraction, the lower phase after back-extraction, thereby permitting easy removal of the appropriate phase from culture tubes. The use of ammonium sulfate coupled with the added specificity of the extraction solvent results in an extract with low background absorption and a well-defined spectrum for the extracted theophylline.

In laboratories with no gas-liquid or high-performance liquid-chromatographic equipment, theophylline is usually determined by ultraviolet spectrophotometry. Such methods generally require 2–3 ml of serum, relatively large volumes of extracting solvent, and filtration of the solvent before the drug is back-extracted into an aqueous medium (1, 2). Here we describe a procedure for the determination of theophylline in 1 ml of serum with use of 16 × 150 mm culture tubes, and validate the use of a salting-out technique and a selective extraction solvent for the analysis.

Materials and Methods

Apparatus

We used an “Acta III” double-beam recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92630) for these studies.

Reagents

Theophylline, caffeine, and theobromine, anhydrous crystals, were obtained from Sigma Chemical Co., St. Louis, Mo. 63178.

Ammonium sulfate, granular, was from Mallinckrodt Inc., St. Louis, Mo. 63160.

Theophylline stock standards. Aqueous theophylline standards were prepared in concentrations of 100, 200, and 300 mg/liter. The standards, stored at 4 °C, were stable for at least six months.

Theophylline standards in plasma. For assessment of analytical recoveries, standard curves, and other studies we used outdated blood-bank plasma. For the standard curves, add 1 ml of plasma to each of a series of 16 × 150 mm (or 20 × 125 mm) culture tubes, add 100 µl of each theophylline stock standard, and mix on a vortex-type mixer for 15 s.

Procedure

Add a constant amount of granular ammonium sulfate (about 0.8 g in the spoon end of a porcelain spatula, Coors, 19:K) in sequence to the culture tubes containing 1 ml of patient's serum or plasma-based standards. Mix the contents of each tube on a vortex-type mixer for 15 s. Add 15 ml of chloroform/hexane (7/3 by vol) to each tube, cap the tubes with Teflon-lined caps, and hand-shake for 10 s to ensure that the caps do not leak and to facilitate uniform dispersion of serum. Then extract for 15 min on either an Eberbach shaker or a rotating-type mixer. Centrifuge (2000 × g, 5 min) to separate the phases and transfer 14 ml of the organic phase to a 16 × 150 mm culture tube. Add 3 ml of bicarbonate-carbonate buffer (pH 9.0, 0.1 mol/liter), prepared as described in reference 2, to each tube and back-extract the theophylline by mechanically shaking it for 10 min. After a 1-min centrifugation, transfer the aqueous (top) phase to a 1-cm quartz cuvet and scan in the wavelength range from 330 to 240 nm.