Analysis for Underivatized Theophylline by Gas-Chromatography on a Silicone Stationary Phase, SP-2510-DA

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I describe a flame-ionization gas-chromatographic method for the quantitative analysis of serum theophylline without the need for derivatization. The analysis is performed isothermally on a new silicone stationary phase, 2% SP 2510-DA (Supelco). With this liquid phase, gas-chromatographic properties and sensitivities are comparable to methods requiring derivatization. A salt–solvent pair of ammonium sulfate and methylene chloride/hexane/acetic acid (80:20:0.1 by vol) is used for extraction. Ammonium sulfate greatly reduces co-extractable interferences from icteric, lipemic, and hemolyzed serum. The density of methylene chloride is adjusted with hexane so that the solvent is less dense than the salt-saturated serum and can be removed by decanting. Acetic acid, used to adjust the pH of the serum samples, is combined with the extraction solvent rather than being added individually to the serum samples. Theophylline values obtained by this method agree closely with those determined by enzyme immunoassay (correlation coefficient, 0.988).

Determination of theophylline has become routine in many clinical laboratories, and various gas-chromatographic methods are available for its analysis. Problems encountered when analyzing for theophylline as the free drug include low sensitivity, peak tailing, and long retention times (1, 2). Various types of derivatization procedures were introduced to eliminate these problems. With the on-column methylation methods, the xanthines cannot be distinguished, because theophylline and theobromine are both converted to caffeine (3, 4). Subsequent methods in which propyl (5), butyl (4, 6–8), and pentyl (9) derivatives were used have proved successful in resolving the xanthines. However, these methods have several deficiencies, including, in some cases, the need for temperature programming (3, 4, 6, 9) and the requirement for non-xanthine internal standards (2–8). Sample preparation requires too-many sometimes difficult steps, including two or three transfer, centrifugation, and evaporation steps, as well as several derivatizing reagents (4, 7–9). "High-performance" liquid chromatography has been used to analyze for theophylline; it does not necessitate the derivatization required in gas chromatography.

Recently a new gas-chromatographic column packing has been described that is sufficiently de-activated and has high resolution characteristics. The phase, SP 2510-DA, has been used to separate many of the anticonvulsants, barbiturates, and other acidic and neutral drugs (10). Another stationary phase, 2% SP 2110/1% SP 2510-DA, has also become available and with it the anticonvulsants can be separated under isothermal conditions.

In this study, I evaluated both of these phases, as well as several extraction procedures, for use in theophylline analysis. With these phases, derivatization is not required, yet the sensitivities and specificities characteristic of gas chromatographic methods involving derivatization are maintained. Several steps in the extraction procedure were eliminated by combining hexane and acetic acid with methylene chloride. The resulting extraction solvent eliminates the need to aspirate the aqueous phase before decanting and the need to acidify each serum sample before extraction.

Materials and Methods

Chromatography

I used a Model 5700A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Avondale, PA 19211). The glass columns, 2 m long × 4 mm i.d., were packed with either 2% SP 2510-DA or 2% SP 2110/1% SP 2510-DA on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823). The columns were conditioned by temperature programming from 25 to 250 °C (at 2 °C/min), at which the temperature was held for 16 h. Column, injector, and detector temperatures were 245, 300, and 300 °C, respectively. Nitrogen carrier gas flow rate was 50 mL/min.

Reagents

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

3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI 53233.

Ammonium sulfate (granular) was from Mallinckrodt, Inc., St. Louis, MO 63160.

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

For assessment of analytical recoveries, standard curves, and other studies, I used outdated blood-bank plasma. For the standard curves, I added 1 mL of plasma to each of a series of 16 X 150 mm culture tubes, added 100 μL of each theophylline stock standard, and mixed 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 Coors porcelain spatula, 19:k) in sequence to each culture tube containing 1 mL of patient's serum or plasma-based standards and mix on a vortex-type mixer for 15 s. To each tube add 10 mL of method.

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ylene chloride/hexane/acetic acid (80/20/0.1 by vol) containing 15 μg of the internal standard, 3-isobutyl-1-methylxanthine. 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 10 min on an Eberbach shaker. Centrifuge (2000 × g, 4 min) to separate the phases. Decant the organic phase into a 16 × 125 mm screw-cap conical centrifuge tube or transfer with a solvent transfer device (17). Place the tubes in a heating block set at 90 °C and evaporate the organic solvent in a stream of dry air. After the tubes have been removed and allowed to cool, add 50 μL of chloroform, vortex-mix, and cap. Inject 10 μL of the resulting solution into the gas-chromatograph.

Results and Discussion

Several studies were performed with the aim of selecting an extraction solvent that results in minimal interferences and high precision, that is compatible with the SP 2510-DA liquid phase, and that requires a limited number of steps. The extraction solvents evaluated included chloroform, methylene chloride, methylene chloride/hexane (8/2), and methylene chloride/hexane/acetic acid, (80/20/0.1) (Table I). I also studied the need for saturating serum with ammonium sulfate and for acidification of the serum before extraction. These studies included analysis of a pool of icteric, lipemic, and hemolyzed sera.

It is known that addition of ammonium sulfate to serum significantly reduces the amounts of coextractable material (12, 13). Extracts obtained with the solvents given in Table 1, when reconstituted in 50 μL of chloroform, were clear. When injected, the extracts produced stable baselines; peaks that would interfere with theophylline or the internal standard were absent (Figure 1). If ammonium sulfate was omitted, the extract had an intense yellow color and produced an unsuitable chromatographic baseline and several extraneous peaks which interfered with quantitation. The interferences were especially obvious with the icteric and lipemic sera. Two milliliters of a saturated solution of ammonium sulfate could be used in lieu of crystalline ammonium sulfate, but the chromatographic baselines were somewhat higher because more material was coextracted.

Because both chloroform and methylene chloride are more dense than the salt-saturated serum, the aqueous (top) layer has to be aspirated before the organic solvent is transferred. As a result, there can be some carryover of the aqueous material if the aspiration and solvent transfer is not performed carefully. To circumvent this problem and to eliminate the need for aspiration, hexane was added to methylene chloride to make the organic solvent phase less dense than the aqueous phase. The resulting phases can be easily separated by centrifugation and the organic (top) phase can be directly transferred by decanting or with a solvent-transfer device (17).

With chloroform as extraction solvent, the serum samples need not be acidified before extraction. However, with methylene chloride/hexane (8/2) acidification improved the analytical precision (Table I). Increased amounts of acid result in wider solvent fronts and several additional peaks. Evaporation time for the extraction solvent was approximately 15 min at 90 °C. Originally toluene/ethyl acetate (8/2 by vol) was used as an extraction solvent (14) because it results in very clean extracts (13); however, evaporation time was prolonged to 30 min. After evaporation, the contents should be dissolved in chloroform, because use of more polar solvents such as methanol results in a wider solvent front.

Figure 1 shows chromatographs resulting from the analysis of blank human plasma to which 10, 20, or 30 μg of theophylline and 15 μg of the internal standard have been added. Theophylline and the internal standard chromatograph at 3.7 and 4.2 min, respectively. The chromatographs were obtained on a 2 m × 4 mm i.d. column containing 2% SP 2510-DA. Similar chromatographs were obtained with a 2 m × 4 mm i.d. column containing 2% SP 2110/1% SP 2510-DA liquid phase. When a 1 m × 2 mm i.d. SP 2110/SP 2510-DA column was used, an unknown endogenous compound interfered with measurement of the internal standard. With the longer column (2 m × 4 mm i.d.) and a higher column temperature, the peak moved ahead of theophylline, as shown in Figure 1 (retention time, 1.6 min). The serum constituent did not interfere when a 1 m × 2 mm i.d. SP 2510-DA column was used; however, resolution of theophylline and internal standard was not entirely adequate for quantitation from peak-height ratios.

Table I compares the precision of the gas-chromatographic method with use of four extraction solvents. Analytical recoveries of theophylline with the solvents were similar and ranged from 80 to 85%. When a saturated solution of ammonium sulfate was used for extraction, recoveries were about

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**Table 1. Coefficient of Variation Obtained on Use of Various Extraction Solvents**

<table>
<thead>
<tr>
<th>Extraction Solvent</th>
<th>CV</th>
<th>Day-to-Day CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>4.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>5.7</td>
<td>—</td>
</tr>
<tr>
<td>Methylene chloride/hexane (8/2)</td>
<td>6.5</td>
<td>—</td>
</tr>
<tr>
<td>Methylene chloride/hexane/acetic acid (80/20/0.1)</td>
<td>4.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Based on analysis of 20 samples containing 20 mg of theophylline per liter.
5% lower than those obtained with use of crystalline ammonium sulfate. Theophylline values of 20 patients' samples analyzed by the gas-chromatographic method were compared to values obtained by enzyme immunnoassay methods. Both methods yielded an identical mean of 16.8 mg/L. The average per cent difference between results by the two methods was 5.8%, with a correlation coefficient, slope, intercept, and standard error of estimate of 0.998, 0.954, 0.750, and 1.261, respectively. The immunoenzymatic assays were performed with an ABA-100 (Abbott Diagnostics, South Pasadena, CA 91030). With this instrument, within-day and between-day CV's were 2.5 and 4.4%, respectively, but CV's obtained with another instrument and with other pipetting procedures ranged from 5 to 9%. The gas-chromatographic procedure is linear to a theophylline concentration of at least 30 mg/L.

The method is specific for theophylline in the presence of caffeine, theobromine, and 3-methylxanthine. Caffeine has a retention time of 0.6 min, theobromine about 1.6 min; and 3-methylxanthine does not elute. The following drugs do not interfere: n-isopropylethynorepinephrine (Isoetherine), prednisone, ephedrine, phenytoin, phenobarbital, primidone, ethosuximide, and 2-ethyl-2-phenylalanamide, a metabolite of primidone. Phenobarbital has a shorter retention time than theophylline and is effectively resolved on both phases. Carbamazepine could interfere with quantitation because its retention time is intermediate between that of theophylline and the internal standard. When six samples from the Antiepileptic Drug Level Quality Control Program were analyzed, the gas-chromatographic theophylline results were within 5% of those estimated by enzyme immunoassay. Abnormally high concentrations of cholesterol do not interfere. Palmitic, stearic, oleic, and linoleic acid were not present under the chromatographic conditions used in this study.

In summary, the extraction solvent described in this report, together with ammonium sulfate, results in a clean extract and good recoveries and reproducibilities. Furthermore, addition of hexane and acetic acid to methylene chloride conveniently eliminates several steps in the extraction process. Use of the described liquid phases considerably shortens the time required to analyze for theophylline and provides sensitivity comparable to the methods involving derivatization. With a 1-mL serum sample, 2.5 mg/L can be readily measured. For optimum results the phases should not be subjected to temperatures above that required for the analysis and wherever possible short columns and lower column temperatures should be used. The stationary phase, like other silicone phases, is sensitive to air at operating temperatures, so I suggest that the oven be cooled to ambient temperature before changing the septum and that the column should be flushed with nitrogen for at least 10 min before reheating the oven to operating temperature (15). I encountered no obvious problems from plasticizers, impure solvents, or column bleed in this study.

References