Serum Theophylline Analysis by High-Pressure Liquid Chromatography

Merle A. Evenson and Brenda L. Warren

We have developed and evaluated a rapid, high-pressure liquid-chromatographic method for theophylline in serum. Only 0.2 ml of serum is required for each determination, and the sensitivity of this method is 0.5 mg/liter. This method, involving liquid extraction and silica adsorption chromatography, provides adequate selectivity, accuracy, and precision for routine or research applications. Little sample preparation is required before chromatography. We found no endogenous or exogenous interferences. Use of \( \beta \)-hydroxypropyl theophylline as the internal standard provides reproducible results for this micro-scale method.

Additional Keyphrases: pediatric chemistry • adsorption • liquid extraction • internal standard • drug assay.

Theophylline, a stimulant and an alkaloid naturally occurring in tea leaves, is a drug which produces several pharmacological actions of therapeutic interest. A methylated xanthine (1,3-dimethylxanthine), it stimulates the central nervous system, acts on the kidney to produce diuresis, stimulates cardiac muscle, and relaxes smooth muscles. A most important use is to relax the smooth muscles of the bronchi in the bronchial constriction which occurs in asthma. Treatment with theophylline definitely increases vital capacity of the bronchi (1–6).

Slow intravenous injection and a quantitative analytical method for measuring drug concentrations in serum are highly desired to alleviate or minimize the adverse side-effects when theophylline is given intravenously (1, 7–9).

As shown by Jenne et al. (3), the metabolism of theophylline varies widely in rate, and a therapeutic dose for one patient may be toxic for another.

Therapeutic concentrations in plasma are about 10 mg/liter; concentrations in excess of 20 mg/liter may cause nausea, headache, palpitation, a decrease in blood pressure, and occasionally death (1, 10, 11).

Methods described in the literature for measuring theophylline in serum are by ultraviolet spectrophotometry, gas chromatography, and high-pressure liquid chromatography (HPLC). Schack and Waxler (12) describe an ultraviolet absorption method in which theophylline was extracted with chloroform and isopropanol, back-extracted with sodium hydroxide, and absorbance measured at 277 nm and 310 nm. The differential readings compensate for the blank. Gupta and Lundberg (13) developed a differential spectrophotometric method at 285 nm in which the same extract at two different pH's is used for the sample and reference solutions.

Johnson et al. (14) reported a gas-chromatographic procedure in which volatile butyl derivatives of theophylline are prepared and injected into the column. This method separated theophylline from theobromine and phenobarbital, two major interfering substances in classical methods. (Ed. note: see also Ferrier and Lear, this issue.)

Thompson et al. (15) developed an HPLC method for determining theophylline in serum and urine. The procedure involves preliminary separation of the xanthines on Aminex A-5, a cation-exchange resin column. Weinberger and Chidsey (16) adapted this method for use with pediatric samples.

Although the above HPLC methods are selective for theophylline, at least 27 min of instrument time is required for each injection.

Sitar et al. (17) present an HPLC method for theophylline in plasma, in which a column packed with silica is used. Their method requires 0.5 ml of plasma and at least 35 min of processing time before the evaporation step. Their method requires less than 10 min for chromatography and no interferences were observed. Manion et al. (18) describe a HPLC method for 1.0 ml of plasma, with organic phase extraction and chromatography on silica.

We have developed a HPLC method for the analysis of theophylline which requires only 8 min after injection onto the column. A liquid inlet pressure of 17.25 MPa (2500 pounds per square inch) and a flow rate of 0.4 ml/min are used with the column at ambient temperature. We also have eliminated the need for a pre-column by making a chloroform/isopropanol extract of 200 \( \mu l \) of serum (7–8 min processing time before evaporation) and injecting a 5-\( \mu l \) sample of the reconstituted extract. The mobile phase is 6% by vol absolute ethanol and 94% by vol chloroform/heptane/acetic acid mixture, the latter prepared by mixing 300 ml of water-saturated chloroform, 200 ml of heptane, and 400 \( \mu l \) of glacial acetic acid.

Materials and Methods

Equipment and Reagents

All HPLC analyses were performed with a Du Pont...
830 Liquid Chromatograph (Du Pont Instruments, Wilmington, Del. 19898) with a 254-nm ultraviolet detector.

Comparison data were obtained by a modified Schack and Waxler method developed by and used in our service toxicology laboratory, with use of a 402 UV-Visible Spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn. 06856).

A 10-μl syringe (cat. No. 701; Hamilton Co., Reno, Nev. 89504) was used to make injections directly into the column without interruption of flow.

For extractions, we used 15-ml centrifuge tubes with ground glass stoppers. A 200-μl adjustable “Pipetman” pipet (Rainin Instrument Co., Inc., Boston, Mass. 02215) was used for delivery of samples, standards, and controls. Ten-milliliter dispensers (Glenco Scientific, Inc., Houston, Tex. 77007) were used to deliver 8 ml of chloroform/isopropanol (see below) and 1 ml of acetate buffer (5 mol/liter, pH 6.5).

All reagents used were AR grade unless otherwise noted.

A “ZORBAX” SIL 25 cm × 2.1 mm (i.d.) column (Du Pont Instruments) pre-packed with 6–8 μm spherical silica particles was purchased.

Water-saturated chloroform (“Nanograde”) was prepared by adding 3.0 ml of distilled water per liter of chloroform and mixing with a magnetic stirrer for 2–3 h. Any undissolved water was aspirated from the top of the chloroform. The water-saturated chloroform was used in preparation of the mobile phase as well as in reconditioning the column.

Methanol and acetone for column reconditioning were prepared by adding 1.0 ml of distilled water per liter with thorough mixing.

Isopropanol, absolute ethanol, and heptane are used without prior treatment or special precautions.

The chloroform/isopropanol used for the extractions is a combination of 20 parts of non-water-saturated chloroform plus 1 part of isopropanol.

The 5 mol/liter acetate buffer was prepared from AR grade anhydrous sodium acetate. The pH was adjusted to 6.5 with 5 mol/liter acetic acid.

“Acetic methanol” (acetic acid/methanol, 5/95 by vol) was used for reconditioning the column.

“Basic methanol” was methanol adjusted to pH 8.0 with sodium hydroxide; it was used for reconditioning the column. (Care: a higher pH will cause rapid deterioration of the column packing.)

Standards and Controls

We used crystalline theophylline (cat. No. T1633; Sigma Chemical Co., St. Louis, Mo. 63178), theobromine (No. T-4500, Sigma), caffeine (Sandoz Pharmaceuticals, Hanover, N. J. 07936), and β-hydroxypropyl theophylline (β-HPT; C. H. Boehringer Sohn, Ingelheim im Rhein, West Germany; distributed in the U. S. by Henley and Co., New York, N. Y. 10004).

Stock 200 mg/liter standards of theophylline and β-HPT were prepared in distilled water.

Working standards of theophylline—5, 10, 20, 30, and 40 mg/liter—were prepared from the stock solution by dilution with distilled water.

A 30 mg/liter working standard of β-HPT was prepared from the stock solution and diluted with distilled water. Used as an internal standard, this is taken through the extraction procedure during sample preparation.

Extraction and Procedure

The sample is prepared as follows: Extract 200 μl of human serum samples, standards, or controls in the presence of the acetate buffer into chloroform/isopropanol. Evaporate 5 ml of the chloroform/isopropanol extract, redissolve in 50 μl of chloroform, and inject 5 μl onto the column. From the recorder chart, obtain peak-height measurements for theophylline and β-HPT and calculate the ratio of theophylline to β-HPT. Plot ratios for the standards and calculate sample values from the working curve (Figure 1).

The extraction is done as follows: Deliver 1.0 ml of acetate buffer at pH 6.5 into each 15-ml centrifuge tube. With the Pipetman pipet, deliver 0.2 ml of the 30 mg/liter β-HPT internal standard into each tube, followed by 0.2 ml of drug-free plasma pool. Pipet 0.2 ml of each standard solution into separate tubes and add the internal standard and the drug-free plasma pool. Deliver 0.2 ml of control pool, serum samples, and internal standard into the respective control and sample tubes. Vortex-mix each tube 2–3 s to thoroughly mix the buffered aqueous phases. Deliver 8.0 ml of chloroform/isopropanol into each tube and insert the ground-glass stopper. Vortex-mix each tube for 20 s,
then centrifuge for 5 min. Aspirate and discard the aqueous (top) layer from each tube, transfer 5.0 ml of the organic extract into thin-walled tubes, and evaporate it in a 60 °C water bath with dry air turbulence. Stopper the tubes until ready to inject the samples. Immediately before injection, pipet 50 µl of chloroform into the residue in the tube, stopper, and vortex-mix to reconstitute. With the Hamilton syringe, remove 5 µl of the reconstituted extract and inject it onto the column.

Buffers of pH 4.0, 6.5, and 8.0 were examined; the pH 6.5 buffer extracted the most theophylline: about 5% more than at pH 4.0 and about 20% more than at pH 8.0. The extraction coefficient doubled when isopropanol was added to the chloroform as compared to extracting with chloroform alone. We used the proportions of 20:1 for chloroform and isopropanol; proportions of 2:1 and 50:1 gave insignificant differences. About 90% of the theophylline is extracted by our protocol from either serum or aqueous standards.

Vortex-mixing times of 20 s, and 1, 2, and 4 min were evaluated; extraction was as complete in 20 s as in 4 min.

**Results**

**Precision and Accuracy**

A standard curve (theophylline, 5, 20, 40 mg/liter) was prepared with each set of extractions. The maximum variation in sensitivity observed is shown with the standard working curves in Figure 2. These curves were obtained on different days and represent the extremes in sensitivity changes. As a column ages, sensitivity decreases and the retention time increases. The intercept of the standard working curve will move up the y axis.

Within-run precision (Table 1) was determined on 10 separate extractions of 5 and 20 mg of theophylline per liter of serum. A non-serum-based standard in chloroform was also analyzed for within-run precision. The within-run coefficient of variation (CV) is 2.82% for 5 mg/liter and 1.04% for 20 mg/liter in serum. The CV for the 30 mg/liter theophylline in chloroform standard is 1.07%.

Between-run precision was established on two drug-free plasma pools. Theophylline had been added to concentrations of 10 and 20 mg/liter, respectively. Five-milliliter aliquots were frozen and a new tube of pool was used for each set of extractions. Between-run precision was also established for a 10 mg/liter standard.

A typical set of extractions includes the three standards, the two pooled plasma samples, and the human serum samples.

**Interferences**

A known negative serum blank shows peaks of less than 1% of full scale in the theophylline and β-HPT areas. Occasionally serum blanks with peaks with a re-

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**Table 1. Precision of HPLC**

<table>
<thead>
<tr>
<th></th>
<th>5 mg/liter serum</th>
<th>20 mg/liter serum</th>
<th>30 mg/liter CHCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>X</td>
<td>5.2</td>
<td>20.1</td>
<td>30.5</td>
</tr>
<tr>
<td>1 SD</td>
<td>0.14</td>
<td>0.21</td>
<td>0.32</td>
</tr>
<tr>
<td>CV</td>
<td>2.82%</td>
<td>1.04%</td>
<td>1.07%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Between-run</th>
<th>Pool A (10 mg/liter)</th>
<th>Pool B (20 mg/liter)</th>
<th>10 mg/liter aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>X</td>
<td>9.17</td>
<td>18.18</td>
<td>9.76</td>
</tr>
<tr>
<td>1 SD</td>
<td>0.49</td>
<td>0.64</td>
<td>0.29</td>
</tr>
<tr>
<td>CV</td>
<td>5.34%</td>
<td>3.52%</td>
<td>2.97%</td>
</tr>
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</table>

**Table 2. Analytical Recovery of Theophylline in Sera**

<table>
<thead>
<tr>
<th>Already In Serum, mg/liter</th>
<th>10 mg/liter</th>
<th>20 mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6 ± 0.5</td>
<td>18.2 ± 1</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>8.8 ± 0.5</td>
<td>18.6 ± 1</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>13.6 ± 0.7</td>
<td>23.8 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>6.6 ± 0.4</td>
<td>16.6 ± 1</td>
<td>26.4 ± 1</td>
</tr>
<tr>
<td>12.2 ± 0.7</td>
<td>21.8 ± 1</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>11.0 ± 0.6</td>
<td>20.8 ± 1</td>
<td>31 ± 1</td>
</tr>
</tbody>
</table>

**Table 2 (continued)**

<table>
<thead>
<tr>
<th>Plus theophylline</th>
<th>5 mg/liter</th>
<th>10 mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.0 ± 0.6</td>
<td>21.8 ± 1</td>
<td>26.8 ± 1</td>
</tr>
<tr>
<td>17.4 ± 0.6</td>
<td>22.6 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>23.0 ± 0.8</td>
<td>28 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>20.0 ± 0.7</td>
<td>24.6 ± 1</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>18.8 ± 0.6</td>
<td>23.6 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>19.4 ± 0.7</td>
<td>24.2 ± 1</td>
<td>29 ± 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X</th>
<th>98.3%</th>
<th>100.5%</th>
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</table>

**CLINICAL CHEMISTRY, Vol. 22, No. 6, 1976 853**
tention time like that of theobromine of up to 5% full scale will be observed, but this is of no consequence for this method.

The following drugs produce no peaks in the time interval from the caffeine peak (3.00 min) to the \( \beta \)-HPT peak (9.25 min): phenobarbital, diazepam, chlorpromazine, acetazolamide, furosemide, chlordiazepoxide, uric acid, 8-chlorotheophylline and dihydroxytheophylline. A peak for chloramphenicol has a retention time of 6.25 min, between theophylline (5.50 min) and theobromine (8.50 min), but does not interfere with either. Serum samples were analyzed before and after hemolysis and before and after addition of bilirubin; in neither case was the value obtained statistically different.

To evaluate the accuracy of the method we performed a series of standard addition (analytical recovery) studies. Two groups of serum samples were obtained, one with values near 10 mg/liter and another with values near 20 mg/liter. Each sample was individually analyzed with and without theophylline added. The sera with values near 10 mg/liter had added 10 and 20 mg/liter; the 20 mg/liter group had added 5 and 10 mg of theophylline per liter. Table 2 gives the recovery data. For the first group the recovery of added theophylline was 98.3% for the 10 mg/liter addition and 100.5% for the 20 mg/liter addition. For the second group the recovery was 97.3% for 5 mg/liter addition, 98.7% for the 10 mg/liter addition. Theophylline in the serum is evidently analytically indistinguishable from added theophylline.

We compared results for theophylline in human sera as measured by the HPLC and the spectrophotometric method. The data were obtained during seven months, with no more than 5 samples being analyzed each day. Duplicate extractions were conducted for the HPLC method, single analyses were done by the other method.

The spectrophotometric method is in routine use and is adequate for samples without interferences. These interferences are usually apparent from the spectra obtained. There are interferences with many samples, and this prompted us to develop the more specific present method.

The following statistical summary of the 56 comparisons and Figure 3 illustrate the comparison data for the individual samples. The values ranged from 0.0 to 41 mg/liter and the samples included specimens that were icteric, hemolyzed, or lipemic. The slope is 0.983, the \( y \)-intercept 0.278, Student’s \( t \)-test value is 0.413, the bias is 0.049, and the correlation coefficient is 0.995.

**Discussion**

Our method is accurate, precise, and rapid. It has several advantages over other HPLC methods: only 0.2 ml of serum is needed, sample-processing time is shorter, water-based rather than serum-based standards are used along with an internal standard, and we have found no interferences after seven months during which we analyzed more than 50 sera from a hospital population. Recovery studies indicate that the HPLC method is accurate. Moreover, in separate experiments, we determined that the signal obtained when the complete sample preparation protocol is followed is about 90% of that obtained with a direct injection of a standard solution. Precision is excellent, as shown by the within-run and between-run precision data.

Concentration and peak height are linearly related between 5 to 40 mg/liter, which amply covers the range of values found in hospital populations, but drops off rapidly between 40 and 50 mg/liter. Although sensitivity changes slightly from day to day, this is unimportant because a standard curve with an internal standard is established with each group of extractions.

Because of interferences commonly occurring with our present spectrophotometric method for theophylline, the comparisons were performed on sera samples which gave no obvious interferences by that method. Such screening will bias the comparison data. Evaluation of the comparison data shows that the two methods correlate quite well. The Student’s \( t \)-test value at the 95% confidence level when \( n \) is 25 is 2.06. For our method, 56 values were compared and gave a \( t \)-test value of 0.413. Thus there are considerably fewer than 5 chances in 100 that the difference between the values obtained by the two methods is statistically significant. The correlation coefficient gives an estimate of the reliability with which results of the two methods may be compared. In each instance when the spectrophotometric method gave higher results than the HPLC method, extraneous peaks occurred. These extra peaks were resolved and did not interfere with quantitation but did interfere with the spectrophotometric method.

A disadvantage of this HPLC method is the useful lifetime and the rate of deterioration of the column. This usefulness was extended by injecting acid methanol onto the column at the beginning of each day, or, with much use, every 4 h or after every 20 to 25 injections. Therefore, from our experience it is best to prepare not more than 25 extractions at one time.
Periodic reconditioning of the column was necessary after about every 200 to 250 injections. Reconditioning consisted of about 30 min pumping time for each of the following, in the sequence presented: water-saturated chloroform, acetone, methanol, water, methanol, acetone, and water-saturated chloroform. Following this sequence the mobile phase is pumped through the column for about 4 h or overnight. Indications that reconditioning is necessary are shown by increased peak widths, decreased sensitivity, and a non-zero intercept on the standard curve. We have been able to recondition the column twice after the initial conditioning but a third conditioning has not been successful.

Proportions of the mobile phase are critical to the separation, sensitivity, and retention time of the peaks. Ethanol has the most pronounced effect on the characteristics of the peaks. It is stored in the secondary reservoir under nitrogen. The primary reservoir contains the chloroform, heptane, and acetic acid mixture. By changing the proportioning potentiometer, the percentage of ethanol in the mobile phase can be altered. Increasing the percentage of ethanol will increase the sensitivity, shorten the retention time, and decrease the resolution. Increasing the concentration of heptane substantially above that suggested will decrease resolution. On the other hand, with no heptane, theophylline and theobromine peaks have nearly the same retention times. The acetic acid is added to increase sensitivity. However, too much acetic acid will result in an unstable baseline. The optimum proportions of the primary reservoir solutions are water-saturated chloroform, 300 ml; heptane, 200 ml; and acetic acid, 400 µl. The optimum mobile phase contains 6% of the secondary reservoir (ethanol) and 94% of the primary reservoir (chloroform/heptane/acetic acid).

For reproducible activity of the silica surface, it is necessary to use a water-containing mobile phase. Snyder and Kirkland (19) describe a convenient and reproducible method for adjusting the water content of nonpolar solvents. They recommend use of about 50% water-saturated mobile phase. By diluting the water-saturated chloroform with 200 ml heptane, we have attained a 60% water-saturated solution.

The available scale expansion is 80-fold that we are using. Thus the detection limit when the noise is low is a function of the scale expansion and the volumes of sample and organic phases used. We therefore believe that, realistically, the lower limit of detection for the scale expansion used here and the method discussed is 0.50 mg/liter.

In summary, this HPLC method for theophylline, like others in which organic phase extractions and adsorption chromatography on silica are used (17, 19), shows no endogenous or exogenous interfering substances. Further, this method has adequate linearity, precision, and accuracy in serum for the concentration range from 5 to 40 mg/liter. The high sensitivity allows the method to be useful for micro-scale samples usually obtained from pediatric patients.

We thank Gary Lenameyer, Michael Poquette, and Sherry Burney for the theophylline comparison data for the spectrophotometric method; Hanley and Co. for supplying the β-hydroxypropyl theophylline, 8-chlorotheophylline, and dihydroxytheophylline used in this method development; and the Clinical Chemistry Laboratory for supplying the statistical analysis of the comparison data. Loan of the Du Pont Model 830 high-pressure liquid chromatograph from the Instrument Products Division, E. I. du Pont de Nemours and Co. (Inc.), Wilmington, Del. 19889, is gratefully acknowledged.

References