Our assay allows accurate determinations of serum concentrations down to 1.0 mg/liter (peak height in chromatograms 4 mm; maximal baseline fluctuation 1.5 mm). It is thus sensitive enough for clinical use, and appears suitable for pharmacokinetic studies.

We thank Waters Associates AB, Gothenburg, Sweden, for putting the chromatograph at our disposal.

References


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Spectrophotometry of Theophylline-7-acetic Acid and Theophylline

James A. Owen and Kanji Nakatsu

A spectrophotometric assay procedure is described for individually measuring theophylline-7-acetic acid and theophylline in 2.0 ml of serum. Absorbance is a linear function of concentration over a range of 0.5–50.0 mg/liter for theophylline-7-acetic acid and 0.5–40 mg/liter for theophylline. No endogenous or exogenous compounds were found that interfere with theophylline-7-acetic acid determinations. As with other spectrophotometric theophylline assays, theobromine and, to a slight extent, phenobarbital interfere with theophylline determinations.

Additional Keyphrase: drug assay

In practice, theophylline is not a particularly easy drug to use because of its physical and pharmacological properties. It is poorly soluble in water; furthermore, it is frequently associated with gastric upset and palpitations, and sometimes causes convulsions. In an attempt to obviate these problems, many theophylline derivatives have been tested. One of these analogs, theophylline-7-acetic acid, was synthesized by Baise in 1949 (1) and is currently marketed as an alternative to theophylline. This acidic analog is often combined with piperazine to form a neutral preparation for both oral and parenteral administration. This preparation produces little or no gastric irritation when given orally, or pain on injection (2). On the other hand, there is little information on its therapeutic effectiveness, the concentration required in plasma, and its kinetics (2,3). Before an attempt can be made to gather kinetie data it was necessary to develop an assay for theophylline-7-acetic acid because this drug cannot be assayed by the spectrophotometric theophylline assay procedure of Schack and Waxler (4). We describe here a specific spectrophotometric assay in which the serum concentrations of both theophylline-7-acetic acid and theophylline are individually determined. The method requires 2.0 ml of serum and is reliable at concentrations as low as 1 mg/liter.

Materials and Methods

Equipment. Absorbance data were obtained with a Coleman Model 124UV-Visible spectrophotometer (Perkin-Elmer Corp., Maywood, Ill. 60153) scanning from 310 to 250 nm, connected to a Recordall Series 5000 chart recorder (Fisher Scientific, Whitby, Ontario). For dispensing volumes of 1.0 ml or less, "Pipetman" (Mandel Scientific Co., Ville St. Pierre, Quebec H8R 1A3) adjustable pipets were used. "Repipet" dispensers (Labindustries, Berkeley, Calif. 94710) were used for volumes greater than 1.0 ml.

Chemicals and Reagents. All reagents, reagent-grade unless otherwise noted, were used without pretreatment or special precautions, and can be stored at room temperature, except for citrate buffer, which is stored at 4 °C.

The following chemicals were used in preparation of standards and controls; theophylline (as aminophylline), caffeine, theobromine (Sigma Chemical Co., St. Louis, Mo. 63178), theophylline-7-acetic acid (Adams Chemical Co., Round Lake, Ill. 60073), phenobarbital (Allen and Hanburys, Toronto), and 3-methylxanthine (Aldrich Chemical Co., Milwaukee, Wis. 53233).

Extraction and analytical procedure. The extraction is done as follows: Pipet a 2.0-ml aliquot of sample or standard
Table 1. Extraction Efficiency with Use of Various Saturated Salt Solutions

<table>
<thead>
<tr>
<th>Salt</th>
<th>Relative efficiency of extraction of theophylline-7-acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>0.78</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.78</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.80</td>
</tr>
<tr>
<td>KCl</td>
<td>0.87</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.89</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.98</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.00</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Into a 30-ml centrifuge tube, followed by 1.5 ml of a saturated NaCl solution and 15 ml of chloroform/1-butanol/coned HCl (84.5/15/0.5 by vol). Cap the tubes with a Teflon-lined screw cap, shake for 15 min, and centrifuge (250 × g, 6 min). Separate the phases by pouring the contents of each tube into funnels lined with Whatman no. 1PS phase-separating paper (Fisher Scientific) and collect the filtered organic phase into 30-ml centrifuge tubes. To the organic phase add 2.0 ml of citrate buffer (0.1 mol/liter, pH 5.5), cap the tubes, shake them for 15 min, and centrifuge as before. Separate the phases as before and collect the theophylline-containing organic phase. After the flow of the organic phase has stopped, transfer the funnel to a new centrifuge tube, puncture the filter paper, and drain the aqueous phase, which contains the theophylline-7-acetic acid, into the tube.

To the aqueous fraction add 5.0 ml of chloroform/isopropanol (95/5 by vol), cap, shake for 15 min, and centrifuge (5 min, 250 × g). Transfer the aqueous (upper) layer to a cuvet.

To back-extract, add to the organic fraction 2.0 ml of 0.1 mol/liter NaOH, cap, shake for 15 min, and centrifuge (250 × g, 5 min). Remove 1.0 ml of the aqueous phase, add 50 μl of a 2.0 mol/liter solution of NH₄Cl, mix vigorously for 2–3 s, and transfer this solution to a cuvet.

Absorbance at 275 nm was determined from an absorbance scan over 250–310 nm. Background absorbance was accounted for by the use of a suitable extract of control serum in the reference cuvet.

Results and Discussion

Standard curves for theophylline-7-acetic acid (0.5, 1.0, 5.0, 10.0, 20.0, and 50.0 mg/liter) and serum were linear with slopes of 0.023 A/mg per liter and 0.016 A/mg per liter, respectively. The curve passed through zero and through or very close to all other points; the regression coefficient in both cases was 1.0. Between-run reproducibility was determined by assaying 20 times during 6 weeks a serum sample that contained an added 20 mg of theophylline-7-acetic acid and 10 mg of theophylline per liter. This standard sample was kept at 4°C and was assayed for theophylline-7-acetic acid and theophylline. Assayed values (mean ± 1 SD) for theophylline-7-acetic acid and theophylline were 20.76 ± 0.64 and 9.85 ± 0.48 mg/liter, respectively. It can be seen that this method is accurate and precise. Analytical recovery was 83% for theophylline-7-acetic acid, 80% for theophylline.

Because spectrophotometric assays are inherently susceptible to interference from other compounds, we tested the above method with several other common drugs present. No interference with theophylline-7-acetic acid determinations was produced by high concentrations (20 mg/liter) of phenobarbital, caffeine, theobromine, or theophylline. Although 20 mg of 3-methylxanthine, a metabolite of caffeine and theophylline (5), per liter results in a 2 mg/liter error in determining theophylline-7-acetic acid concentrations, the low 3-methylxanthine concentrations (3–4 mg/liter), even during theophylline administration (6), preclude any appreciable interference.

As is the case for other spectrophotometric assays for theophylline, potential interfering compounds include 3-methylxanthine, phenobarbital, and theobromine. However, 3-methylxanthine is poorly extracted in the theophylline procedure (<10%) and is not bothersome. Falsely increased absorbance values owing to the presence of phenobarbital are decreased to acceptable values by using the method of Jatlow (7). By lowering the pH of the theophylline fraction from 13 to 10 with NH₄Cl before reading, the absorbance maximum for phenobarbital is shifted from 255 to 240 nm. As is the case for other spectrophotometric assays for theophylline, theobromine is co-extracted and can lead to falsely high values.

To maximize the extraction efficiency of theophylline-7-acetic acid, we examined various salt solutions and organic solvent compositions. To select the best salt, saturated solutions of different salts were used in the extraction of a theophylline-7-acetic acid standard with chloroform/isopropanol (95/5). The results are shown in Table 1. Because drug recovery varied little when 1.0–2.0 ml of the NaCl solution was used, we chose a mid-range volume of 1.5 ml for routine use. With use of NaCl as the salt, chloroform plus five volumes of each of the following organic solvents per 100 volumes was tested for extraction efficiency: methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 1-pentanol, t-pentanol, 1-octanol, and ethylene glycol. Of these, 1-butanol was the most favorable. Subsequent testing revealed that chloroform/1-butanol (85/15) was the optimal mixture.

Both theophylline-7-acetic acid and theophylline are extracted in the first extraction, so a selective back extraction is used to remove all of the theophylline-7-acetic acid from the filtered organic phase. Back extraction with a pH 5.5 buffer gives 100% recovery of theophylline-7-acetic acid, and only 5% extraction of theophylline. Any theophylline extracted into the pH 5.5 buffer is removed with 5.0 ml of chloroform/isopropanol.

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References