Separation and Quantitation of Theophylline and Paraxanthine by Reversed-Phase Liquid Chromatography

To the Editor:
Theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) are both metabolites of caffeine, a xanthine derivative ingested from such dietary sources as coffee, tea, cocoa, and some soft drinks (1). Theophylline and paraxanthine are well separated when ion-exchange (2) or normal-phase (3) chromatographic columns are used, but have identical retention times on reversed-phase columns (4–10). At least two investigators using reversed-phase columns have noticed that there is a small "background" contribution by plasma at the retention time of theophylline (4, 9). This background may be paraxanthine. As much as 8 to 10% of a dose of caffeine may be excreted in the urine as paraxanthine (11). Orcutt et al. (10) noted that in serum samples containing significant quantities of caffeine (but no theophylline) paraxanthine could interfere by the equivalent of as much as 3.5 mg of theophylline per liter. Because xanthine intake is difficult to control in the clinical setting, theophylline concentrations in plasma are likely being overestimated with analytical techniques that involve reversed-phase columns. We present a reversed-phase liquid chromatographic method that allows for the separation and quantitation of paraxanthine and theophylline, and incorporates the use of tetrabutylammonium chloride, an ion-pair reagent.

We used a liquid chromatograph (Model 7000; Micromeritics Instrument Corporation, Norcross, GA 30093) with injector valve (Model 710; Micromeritics) and a variable wavelength detector (Chromonitor 785; Micromeritics) were used. The column (Spherisorb ODS; Laboratory Data Control, Div. of Milton Roy Co., Riviera Beach, FL 33404) was 250 x 4.6 mm (i.d.) and was prepacked with a spherical silica coated with octadecyltrichlorosilane (5-μm diameter). Detector output was monitored by a recorder (Fisher Recordall Series 5000; Omnistribe Recorder, Houston Instrument, Austin, TX 78753) and an integrator (Varian Instrument Division, Palo Alto, CA 44303). Flow rate was 1.0 mL/min, column temperature 50 °C, and detector wavelength 280 nm.

The mobile phase was a mixture of methanol ("Certified HPLC Grade"; Fisher Scientific Co., Fair Lawn, NJ 07410) in 50 mmol/L sodium acetate buffer, 10/90 by vol, containing 10 mmol of tetrabutylammonium chloride (Eastman Kodak Co., Rochester, NY 14650) per liter; the pH was adjusted to 4.2 with glacial acetic acid.

We prepared standard solutions of paraxanthine (Fluka AG, Buchs SG), theophylline (Sigma Chemical Co., St. Louis, MO 63178), and dyphylline (Mallinckrodt, Inc., St. Louis, MO 63160) in methanol. Concentrations for paraxanthine were 0.01 and 0.1 g/L; for theophylline 0.01, 0.1, and 0.25 g/L; for dyphylline 0.2 g/L. The extraction procedure is a modification of that of Adams et al. (4). We used a larger volume of plasma (500 μL) with consequent scale-up of extractant and addition of 200 μL of phosphate buffer (pH 4.0, 2 mol/L) to the plasma before extraction with chloroform/isopropanol (50/50 by vol). We prepared standard curves for paraxanthine and theophylline simultaneously with five samples at each of five plasma concentrations of the two xanthines: 1, 5, 6, 8 and 10 mg/L for paraxanthine and 1, 5, 10, 15, and 25 mg/L for theophylline. Standard curves for both theophylline (slope = 0.096, y-intercept = -0.019, r² = 0.998) and paraxanthine (slope = 0.079, y-intercept = 0.024, r² = 0.998) were linear. Coefficients of variation were less than 3% at concentrations exceeding 5 mg/L and less than 10% at 1 mg/L.

Blank plasma was supplemented with theophylline (10 mg/L), paraxanthine (6 mg/L), and dyphylline (20 mg/L) to establish the retention times and assure resolution of the three compounds. In a separate study, an apparently healthy adult man drank two 8-oz. cups (total, about 480 mL) of coffee 30 min apart. A plasma sample was drawn 30 min after the second cup. The subject was then given a 300-mg oral dose of theophylline (SloPhylline Syrup; Dooner Laboratories). We obtained a second plasma sample 2 h later. Samples were assayed in duplicate. We also analyzed the post-theophylline plasma sample without addition of tetrabutylammonium chloride.
chloride to the mobile phase.

Separation of paraxanthine and theophylline by use of various mobile phases of acetonitrile or methanol in buffer was attempted to no success. Only with 10 or fewer milliliters of methanol per liter of eluent did we achieve any degree of separation of these two xanthines; this separation was not baseline and was not useful for quantitative purposes. Addition of tetrabutylammonium chloride, however, resolved them from each other.

The chromatogram for plasma drawn before theophylline administration but after caffeine ingestion appears in Figure 1A. The paraxanthine and theophylline peaks are both present as caffeine metabolites. Post-theophylline plasma concentrations of paraxanthine (1.2 mg/L) and theophylline (9.5 mg/L) were determined from the chromatogram in Figure 1B. Exclusion of tetrabutylammonium chloride resulted in failure to resolve theophylline and paraxanthine (Figure 1C). The theophylline peak represents a concentration equivalent to 11.4 mg of theophylline per liter, which approximates the sum of the paraxanthine and theophylline concentrations determined in Figure 1B.

This assay is especially useful for determination of theophylline when the consumption of beverages containing caffeine is difficult to control. The degree of interference by paraxanthine is not easily assessed, in that it is dependent upon the time of caffeine intake relative to the time of plasma sampling for theophylline, and upon the dose of caffeine. Consequently, paraxanthine may be present throughout a theophylline dosing interval. We found that the theophylline in a single subject was overestimated by about 15% when two cups of coffee were followed by a 300-mg oral dose of theophylline. The potential for overestimation will be greater at lower theophylline concentrations. A semiquantitative assay technique may also help to explain the recent reports (8, 12) that theophylline biological half-lives as estimated from measurements by "high-pressure" liquid chromatography are longer than those obtained by other techniques.

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References

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Alcohol Dehydrogenase Activity in Various Buffers

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<tr>
<th>Substrate</th>
<th>Buffer</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Ethanol,</td>
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<td>bicarbonate 100</td>
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<td>NAD*</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>AMPdiol</td>
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<td></td>
</tr>
<tr>
<td>1-propanol</td>
<td>(AMP)</td>
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The AMP buffer is included because it is commonly used for measuring LD activity. The pH of these assay mixtures was 9.0, as recommended for LD; AD activity is even greater at higher pH values.

Although little is known about the clinical conditions under which AD appears in serum, we have observed that significant AD activity can be present in certain pathological situations. In these instances, use of the recommended buffers will result in increased apparent LD activity. What is more, in several sera from patients treated with AD, a sixth band was observed on LD electrophoresis on either cellulose acetate or agarose. This isoenzyme was AD, as confirmed by using bicarbonate buffer, and ethanol and NAD* as substrates. Its position on the electrophoretogram is more cathodic than that of LD isoenzyme 5 and it is observed because the enzyme reacts with the buffer as a substrate and results in the formation of insoluble tetratolium dye in a similar mechanism to LD.

In conclusion, although we cannot suggest suitable alternatives to the