obtained, facilitating more precise determination of proper dosage regimen.

We are grateful to Dr. Amadeo Pesce for his technical assistance and support, and we thank Lederle Laboratories for providing the purified compounds used during assay development.

References


Theophylline, Dyphylline, Caffeine, Acetaminophen, Salicylate, Acetylsalicylate, Procainamide, and N-Acetylprocainamide Determined in Serum with a Single Liquid-Chromatographic Assay

Ching-Nan Ou1,2 and Vicki L. Frawley1

We describe a single set of liquid-chromatographic conditions for assay of theophylline, dyphylline, caffeine, acetaminophen, salicylate, acetylsalicylate, procainamide, and N-acetylprocainamide in serum. The chromatographic system includes a Waters Associates μ-Bondpak C18 column and acetonitrile in 0.1 mol/L potassium phosphate buffer, pH 4.0 (9.75/90.25 by vol), as the mobile phase. Only 50 μL of serum is required, and drug concentrations as low as 0.5 mg/L can be detected. Absolute and relative analytical recoveries range from 95 to 101%. Day-to-day variation of the method is less than 6% for each drug. Linearity extends to 1 g/L for all drugs. Recycling of the mobile phase under pressure eliminates the need to prepare and de-gas solvents. The use of the single stationary and mobile phase provides a practical and economical approach to routine and urgent therapeutic drug monitoring.

Additional Keyphrases: drug assay • economics of laboratory operation • pediatric chemistry

The various methods for determination of therapeutic drugs in serum include the traditional ultraviolet spectrophotometry (1), gas-liquid chromatography (2, 3), "high-performance" liquid chromatography (HPLC) (4–9), and homogeneous enzyme immunoassays (EMIT®) (10–14). HPLC and EMIT have become the two most popular methods for therapeutic drug monitoring in both routine and stat clinical laboratories. Although EMIT gives quick results and can be easily automated for high-volume testing, not all drugs of clinical interest can be so assayed, and EMIT reagents are substantially more expensive than those used in other techniques. HPLC methods provide good sensitivity, accuracy, and precision and permit simultaneous analysis of several different drugs and their metabolites in the same sample. However, requirements for alterations in and subsequent equilibration of mobile phases for different drug assays detract from the efficiency and practicality of HPLC and shorten the useful lifetime of the analytical column.

Furthermore, concurrent measurement of parent drug and metabolites is often important in establishing an optimal regimen for each individual patient. For example, caffeine, produced through N-7 methylation of theophylline, is more effective than theophylline itself for treatment of apneas; therefore, both drugs must be monitored in the newborn receiving theophylline therapy (15–17). Similarly, proccainamide is metabolized to N-acetylprocainamide, which has the same therapeutic and toxic manifestations as procainamide (18–20).

Concurrent administration of theophylline and acetaminophen or salicylate for simultaneous control of asthma and fever is very common. Interference with theophylline measurement by acetaminophen or salicylate has been noted in most standard liquid-chromatographic methods (21–23). Thus development of an optimal chromatographic condition for separating theophylline and acetaminophen or salicylate is important, not only to avoid interference but also to permit simultaneous measurement of these drugs.

We devised a single set of HPLC conditions for concurrent analysis for theophylline, dyphylline, caffeine, acetaminophen, salicylate, acetylsalicylate, procainamide, and N-acetylprocainamide. Use of a single stationary and mobile phase permits results to be obtained quickly and consequently increases the efficiency and practicality of HPLC for drug monitoring.

Materials and Methods

Materials

Apparatus. For all HPLC analyses, we used a Model 110A pump (Beckman Instruments, Inc., Berkeley, CA 92634)
equipped with a Model 7125 injector (Rheodyne, Inc., Cotati, CA 94928), a Model 440 dual-beam ultraviolet-visible absorbance detector, and a μ-Bondapak C18 reversed-phase column (both from Waters Associates, Milford, MA 01757). A dual-channel chart recorder (Model 7130A; Hewlett-Packard, Palo Alto, CA 94304) monitored the separation profile at a chart speed of 12.7 mm/min. To evaluate peak areas and retention times we used an “Auto Lab System I” computing integrator (Spectra-Physics, Santa Clara, CA 95051).

*Drugs and standards.* Theophylline, β-hydroxyethyltheophylline, theobromine, 3-methylxanthine, caffeine, 8-chlorotheophylline, acetaminophen, salicylic acid, acetylsalicylic acid, procaïnamide, and N-acetylpseudoepioprocaïnamide were from Sigma Chemical Co., St. Louis, MO 63178; \( \text{N}^\text{50} \)-propionylprocaïnamide hydrochloride was from Aldrich Chemical Co., Milwaukee, WI 53201. All solvents were of reagent or HPLC grade.

Working standards were prepared by adding the stock solution of each drug (1 g/L) to doubly distilled water to give the following concentrations (mg/L): theophylline and caffeine, 10 and 20; dyphylline, 10 and 20; acetaminophen, 50 and 100; salicylate and acetylsalicylate, 100 and 300; procaïnamide and \( \text{N} \)-acetylpseudoepioprocaïnamide, 5 and 10 (in drug-free plasma instead of water). The working solutions of internal standards were: β-hydroxyethyltheophylline, 15 mg/L in acetonitrile, and \( \text{N} \)-propionylprocaïnamide, 10 mg/L in water.

Carbonate buffer (33 mmol/L) was prepared by adding 2.9 g of NaHCO₃ to 1 L of doubly distilled water and adjusting to pH 11.0 with NaOH solution.

*Mobile phase.* Monopotassium dihydrogen phosphate buffer (0.1 mol/L) was prepared in doubly distilled water and adjusted to pH 4.0 with phosphoric acid. The buffer was filtered through a 0.22-μm (pore size) filter (Millipore Corp., Bedford, MA 01730) to remove particulate matter. The mobile phase consisted of 97.5 mL of acetonitrile added to 902.5 mL of phosphate buffer (0.1 mol/L, pH 4.0).

**Procedures**

*Analytical.* Table 1 lists the extraction conditions for each drug. The extraction mixture is mixed for 30 s and the aqueous and organic phases then are separated by centrifugation for 3 min at 3000 × g. The major portion of the organic layer is transferred to a clean tube and dried under a stream of air or nitrogen. The residue is then reconstituted in 50 μL of methanol, and 20-μL aliquots of this solution are injected into the column. For measuring salicylate or acetylsalicylate, equal volumes of internal standard (β-hydroxyethyltheophylline in acetonitrile) and sample are mixed for 30 s and centrifuged

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**Table 1. Extraction Conditions**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Internal standard</th>
<th>Organic phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theophylline, dyphylline, caffeine</td>
<td>None</td>
<td>β-OH CHCl₃/2-propanol (95/5, by vol), 2 mL</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>HCl, 0.1 mol/L</td>
<td>β-OH Ethyl acetate, 3 mL</td>
</tr>
<tr>
<td>Salicylate, acetylsalicylate</td>
<td>None</td>
<td>β-OH None</td>
</tr>
<tr>
<td>Procaïnamide</td>
<td>NaHCO₃, 33 mmol/L</td>
<td>N-acetylpseudoepioprocaïnamide CHCl₃/2-propanol (95/5, by vol), 3 mL</td>
</tr>
</tbody>
</table>

*Serum, buffer, and internal standard: 50 μL each. β-OH, β-hydroxyethyltheophylline; NPPA, N-propionylprocaïnamide.*

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![Graph](image)

Fig. 1. Liquid chromatogram of standard mixture of 3-methylxanthine (3-MX), theobromine (THB), procaïnamide (PA), theophylline (THO), dyphylline (DP), acetaminophen (AC), \( \beta \)-hydroxyethyltheophylline (β-OH), \( \text{N} \)-acetylpseudoepioprocaïnamide (NPPA), salicylate (SA), caffeine (CA), 8-chlorotheophylline (8-CL), \( \text{N} \)-propionylprocaïnamide (NPPPA) and acetylsalicylate (ASA)

Mobile phase, acetonitrile/phosphate buffer (97.5/902.5 by vol)

at 13 000 × g for 5 min. The supernate (about 20 μL) is then directly injected into the column. On elution at 25 °C with the mobile phase, at a flow rate of 2 mL/min, the effluent is monitored at 254 nm.

The mobile phase can be recycled through 305 cm of 0.3 mm (i.d.) Teflon tubing, which produces sufficient back pressure (345 kPa, or 50 psi) to eliminate air-bubble formation in the flow cell. At the end of each day's chromatographic run, the column is cleaned with distilled water for 20 min, followed by methanol for 30 min, at a flow rate of 2 mL/min.

*Calculations.* We quantified each drug by comparing the peak area or peak height ratios for the standards, control, and serum samples.

*Analytical recoveries.* To assess true analytical recovery, we compared the peak area measured for a drug-added serum sample with the peak area measured for a standard solution injected. Relative recovery was calculated by comparing the quantities measured for drug-added serum with the actual quantities added.

**Results**

We processed an aqueous standard mixture of 3-methylxanthine, theobromine, procaïnamide, theophylline, dyphylline, acetaminophen, \( \beta \)-hydroxyethyltheophylline, \( \text{N} \)-acetylpseudoepioprocaïnamide, salicylic acid, caffeine, 8-chlorotheophylline, \( \text{N} \)-propionylprocaïnamide, and acetylsalicylic acid to evaluate the analytical performance of the chromatographic conditions. The resolution of these drugs is excellent except that dyphylline co-elutes with theophylline (Figure 1). The retention time of salicylate does not change as much as the other drugs when the acetonitrile concentration is changed; therefore, a decrease in acetonitrile from 97.5 to 95 mL/L produces overlapping between \( \text{N} \)-acetylpseudoepioprocaïnamide and salicylate.
Table 2. Analytical Recovery

<table>
<thead>
<tr>
<th></th>
<th>Conc, mg/L</th>
<th>Mean recovery, % (n = 5)</th>
<th>True *</th>
<th>Relative b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theophylline</td>
<td>20</td>
<td>97</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Diphylene</td>
<td>20</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>20</td>
<td>99</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>40</td>
<td>98</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Salicylate</td>
<td>50</td>
<td>99</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylate</td>
<td>50</td>
<td>100</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Procainamide</td>
<td>20</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>N-Acetylprocainamide</td>
<td>20</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* By comparison with peak area of standard solution. b By comparison of injected and measured quantities.

Analytical recoveries for the drugs tested ranged from 95 to 101% (Table 2), indicating that the method is accurately measuring these drugs in serum. In addition, the accuracy of the HPLC method for theophylline has been validated by an EMIT method used as described by Ou et al. (14).

Peak area ratio is linearly related to quantity of drug up to at least 1 g/L for the drugs studied. Therefore, no dilution is necessary in routine measurements done on clinical specimens.

Table 3 lists the day-to-day variation of the method for each drug. At subtherapeutic or therapeutic concentrations, the CV for all these drugs is <6%. Such precision and accuracy are achieved by having an optimized pH and mobile phase that produce good analytical resolution of each compound. As little as 0.5 mg of each drug per liter can be detected by this method.

Commonly used antiepileptic, antiarrhythmic, and anti-biotic drugs do not interfere. Ampicillin co-elutes with theophylline when a mobile phase with pH 4.0 is used (24, 25), and our results indicate that 33 mg of ampicillin per liter is equivalent to 1 mg of theophylline per liter in a direct-injection method as described by Orcutt et al. (21). However, with the extraction procedure we use, 714 mg of ampicillin per liter corresponds to 1 mg of apparent theophylline per liter. Sodium benzoate, an ingredient in a commercially available injectable caffeine preparation (caffeine and sodium benzoate injection, USP; Eli Lilly and Co., Indianapolis, IN 46206), elutes at 11 min and does not interfere with the drug measurements.

Discussion

The single chromatographic setup described here allows simultaneous analysis for eight different drugs and metabolites while maintaining good sensitivity, accuracy, and precision. The method is not subject to interference by the other drugs we tested except ampicillin. However, interference by ampicillin is almost completely eliminated under the extraction conditions described.

The separation capacity of the method permits multiple drug assay as well as simultaneous measurement of the parent drugs and their aforementioned metabolites.

Elbet et al. (26) used the same buffer and organic solvent with various compositions for the analysis of five cardiovascular drugs: procainamide, lidocaine, quinidine, disopyramide, and propranolol. Welch et al. (27) used a 60/40 (by vol) mixture of potassium dihydrogen phosphate (0.1 mol/L, pH 4.0) and acetonitrile to determine zomepirac in serum and plasma. Diphylene could be adequately separated from theophylline by increasing the acetonitrile concentration in the mobile phase from 97.5 to 80 mL/L. By using a rotary valve and combining these methods, one can measure at least 13 drugs with the simple acetonitrile/phosphate buffer system.

The chromatographic condition has been routinely used in our laboratory for a year and a half. A single column still has not lost its efficiency after more than 3200 sample injections.

In summary, the use of the single stationary and mobile phase provides both a practical and economical approach to therapeutic drug monitoring for the clinical laboratory. In addition, only 50 µL of serum is required for each analysis when the chromatographic conditions described are used, making it very suitable for monitoring drugs in a pediatric and neonatal population.

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References

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