Evaluation of a Glassy-Carbon Electrode for Amperometric Detection of Selected Methylxanthines in Serum After Their Separation by Reversed-Phase Chromatography

Eugene C. Lewis¹ and Dennis C. Johnson

We examined the relative merits of direct-current, pulse, and differential pulse amperometry at a flow-through, glassy-carbon electrode used in monitoring column chromatography. We give a rapid, simple procedure for determining theophylline (1,3-dimethylxanthine) in 20–100 μl of blood serum. Interference by 1,7-dimethylxanthine, a metabolite of caffeine, is compared for photometric and amperometric detection, and some advantages of combined photometric and amperometric detection are discussed.

Additional Keyphrases: combined photometry and amperometry • anodic electroanalysis • chromatovoltamogram • monitoring therapy • drug (theophylline) assay

Amperometric detection was first applied to column chromatography more than 25 years ago (1–3). Kemula used the dropping mercury electrode for this purpose for many years (4), but the technique did not receive general acceptance because of the difficulties associated with application of a liquid metal in a flow-through detector (5, 6). The chemical literature now contains more than 100 references to applications of amperometric detectors for liquid chromatography. We have prepared an annotated bibliography, which is available upon request. We also recommend the review by Kissinger (6).

Electroanalytical methods are frequently discounted by analysts as inconvenient or suited only for specialized applications. Frequently, results of an electroanalysis of simple solutions of analyte have been misused to justify more general applicability of the specific procedure. Analysts are naturally disenchanted when they discover that the procedure is suitable only for such synthetic samples. Much of our recent effort in the development of amperometric detection has focused on anodic electrode reactions to avoid the interference observed from reduction of dissolved oxygen in cathodic detection. Anodic detection requires the use of solid electrode materials and we must acknowledge that solid electrodes are susceptible to interferences resulting from surface adsorption of reaction products, particularly when anodic reactions of organic substances are studied. Hence, any proposed technique for anodic detection of specified substances must be validated for genuine samples before the development of the analytical procedure can be considered complete.

Solid electrodes that have become “fouled” by adsorption of material from the sample, or products of the electrode reaction, can often be reactivated by switching the electrode potential to a value suitable for desorption or electrolytic decomposition of the adsorbed material. The alternate application of the electrode potential at values for detection and cleaning can be consistent with the principles of pulse voltammetry. MacDonald and Duke (7) demonstrated the advantage of the pulse technique for maintaining the long-term stability of flow-through electrodes in the detection of p-aminophenol. They also reported the advantages of increased sensitivity and decreased dependence of the electrode current on variations of flow rate, as is expected for pulse amperometry applied to convective electrodes.

The anodic electrochemical reactions of purines, including methylxanthines, at carbon electrodes have been described in reviews by Dryhurst (8, 9). These reviews and the literature cited therein should be consulted for information regarding the mechanisms of the electrochemical reactions. The anodic half-wave potentials for the isomeric methylxanthines are similar in value, and determinations of methylxanthines in physiological fluids by amperometric techniques with constant applied potential (direct current) is predicted to require prior separations. Resolution of signals for electroactive species with similar values of half-wave potential can often be achieved by differential pulse amperometry when direct-current amperometry fails. Advantage of this fact for electrochemical detection in liquid chromatography has been demonstrated by Swartzfager (10) for synthetic samples. Described here is the investigation of direct-current, pulse, and differential pulse amperometric techniques for anodic detection of methylxanthines. Pulse amperometric detection was used for the chromatographic determination of theophylline in blood serum.

Methylxanthines have numerous pharmacological actions and are used therapeutically for their effects on cardiac muscle and the cardiovascular system, the central nervous system, the kidneys, and smooth muscles (11). Theophylline (1,3-dimethylxanthine) is used for symptomatic relief of asthma (12) and for treatment of apneas (13). Relief is rarely obtained in therapy when theophylline concentrations in the serum decline below 10 mg/liter. For concentrations exceeding 20 mg/liter, toxic reactions are more common (11). The biological half-life of theophylline varies widely among individuals and has been reported to range from 1.4 to 7.8 h for a group of children (14). The narrow therapeutic range and this large variation in half-life dictates that dosage be individualized in the management of therapy (14–16). A selective analytical method is required because the physiological sample may

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contain metabolites of dietary methylxanthines with properties similar to the unmethylated theophylline (17). Metabolites of methylxanthines are known to interfere in analyses based on liquid–liquid extraction with spectrophotometric measurement (18).

Reports have appeared describing the application of adsorption (19–21), ion-exchange (22, 23), normal phase (24), and reversed-phase liquid chromatography (16, 25–27) for determining theophylline in physiological fluids. In general, the claim is made that these analytical procedures for theophylline are free of interference from other methylxanthines and metabolites of methylxanthines. In most studies, 1,7-dimethylxanthine, a metabolite of caffeine, was not examined (17). Theophylline and 1,7-dimethylxanthine were recognized by Thompson et al. (22) to be only partly resolved by a procedure involving ion-exchange chromatography. There are no unequivocal reports of complete separation of these two isomers by reversed-phase chromatography. Orcutt et al. (27) recently suggested that adjustment of the composition of their acetate-buffered mobile phase to 5 parts of acetonitrile per hundred from 7 parts per hundred would obviate interference from a substance detected in randomly selected serum samples, which they presumed to be 1,7-dimethylxanthine. However, they did not report any attempt to separate theophylline from known 1,7-dimethylxanthine. Orcutt et al. reported a positive interference for nearly one-fourth of the blank samples of serum, ranging from 0.25 to 3.5 mg of apparent theophylline per liter.

Methods and Materials

Chromatography

The liquid delivery system was assembled from components available from commercial sources according to conventional designs (28). The delivery system for the mobile phase consisted of a Milton Roy "MiniPump" and a Model 709 "PulseDampener," both obtained from Laboratory Data Control, Riviera Beach, Fla. 33404. The mobile phase was sparged of dissolved air in the reservoir by compressed helium. A Nupro 2-μm filter made of sintered stainless steel and obtained from Hawkleye Fitting and Valve Co., Des Moines, Iowa, was placed between the pulse dampener and the sample injector to prevent particulate material from entering the chromatographic column. A Rheodyne Model 7120 loop injector (Larry Bell Associates, Hopkins, Minn. 55343) was used for sample injection. Sample loops were available with volumes of 10, 20, 50, 100, 151 and 220 μl. Chromatographic columns included a μBondapak C18 (column A) and a Bondapak C18/Corasil (column B), provided by Waters Associates, Milford, Mass. 01757. All high-pressure connections were made with no. 316 stainless-steel fittings. Low-pressure connections between the chromatographic column and detector were made with Teflon tubing (Altex Scientific Inc., Berkeley, Calif. 91740) and tube-end fittings (Larry Bell Associates).

Detectors

The glassy carbon detector (now generally available) was constructed by Pine Instrument Co. of Grove City, Pa. 16127, according to a design described elsewhere (5). The tubular electrode had an internal diameter of 1.09 mm and a length of 3.17 mm. The reference electrode was a Model 39270 saturated calomel electrode (SCE) with fiber junction, supplied by Beckman Instruments, Inc., Fullerton, Calif. 92634. The counter-electrode was a coil of platinum wire wrapped around the probe of the reference electrode.

A PAR Model 174A polarographic analyzer from Princeton Applied Research Corp., Princeton, N.J. 08540, was used for potentiostatic control in experiments with dc, pulse, and differential pulse voltammetry. A cycle time of 0.5 s was used for pulse and differential pulse detection. A potentiostat constructed in our laboratory, according to an established design (29, 30), was also used for detection at constant potential.

For photometric detection we used a Model 200 ultraviolet detector from Chromatronix, Inc., Berkeley, Calif. 94710, operated at 254 nm (mercury-vapor lamp).

Electrochemical Procedures

The electrode was preconditioned at the start of each experimental period by applying a potential more positive than the most positive value to be used for detection in subsequent experimentation. Generally, this preconditioning potential was 2 V. Functional groups that form on the surface of a glassy carbon electrode during anodic polarization are produced more readily at large positive potentials. Hence, baseline stability at the potential used for anodic detection was achieved more quickly when the electrode potential was first taken to a large positive value than if the electrode had not been preconditioned in the manner described.

The voltammetric response of the electroactive species was determined by measuring the peak current obtained at a constant value of electrode potential after the sample was injected into the chromatographic system. Peak currents were measured at potential values separated by convenient intervals within the potential range accessible at the glassy carbon electrode. After each incremental change in electrode potential, the background current was allowed to decay to a steady-state value before the next aliquot of the sample was injected. For synthetic samples containing a single electroactive species, the column could be removed from the chromatographic system, to increase the speed with which voltammetric data were obtained. The eluent used for chromatographic separations was also used as the diluent for the synthetic samples so that change in the background current caused by a change of the ionic character of the flowing stream was negligible. Voltammetric information for complex samples was obtained with the column in the chromatographic system.

Reagents

Water was distilled and de-ionized by passage through a column of Amberlite MB-3, a mixed-bed ion-exchange resin (Mallinckrodt, Inc., St. Louis, Mo. 63147). Acetonitrile was the "distilled-in-glass" reagent from Burdick and Jackson Laboratories, Muskegon, Mich. 49442.

Compounds (and their suppliers) were as follows: theophylline, theobromine, and uric acid (Matheson, Coleman and Bell), caffeine (Eastman Kodak Co.), and xanthine and 8-chlorotheophylline (Aldrich Chemical Co., Inc.). Adams Chemical Co. of Round Lake, Ill. 60073, supplied the following methylxanthines and metabolites: 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid, and 1,3-dimethyluric acid. Creatinine was furnished by Sigma Chemical Co. The 7-[2,3-dihydroxypropyl]theophylline, generically called "dyphylline," was generously provided by Dr. J. D. McCallister of Mallinckrodt, Inc. The 8-chlorotheophylline was found to be contaminated by a substance having the chromatographic retention time of theophylline. All other organic compounds yielded a single chromatographic peak and the purity was concluded to be acceptable for qualitative work. Only the purity of the theophylline was established by a standard method of assay.

Quinbrun capsules (Mead Johnson) containing theophylline were obtained from the Student Health Service of Iowa State University. Lyophilized Q-PAK Chemistry Control serum
(unassayed) was generously provided by Hyland Division of Travenol Laboratories, Inc., Costa Mesa, Calif. 92626.

In choosing the eluent for the chromatographic separation, we considered both the separation requirements and the necessity of high ionic conductivity for electrochemical detection. Eluent I consisted of 4 liters of de-ionized water, 56.8 g of sodium monohydrogen phosphate, and 55.2 g of sodium dihydrogen phosphate monohydrate, dissolved in a conical flask. After dissolution was complete, 200 ml of this phosphate buffer was withdrawn for use as sample diluent. To the remaining solution in the conical flask, 200 ml of acetonitrile was added. Eluent II was solution in 4 liters of de-ionized water of 55.2 g of sodium dihydrogen phosphate monohydrate and 210 ml of acetonitrile.

Procedure for Determination of Theophylline in Blood Serum

Place 50 µl of acetonitrile into a 15-ml conical centrifuge tube with a capillary pipet. Add 100 µl of serum and aspirate the liquid into both pipets several times, to ensure complete precipitation of protein. Allow the capillary pipets to remain in the centrifuge tube and add 0.85 ml of the phosphate-buffered diluent reserved in the preparation of eluent I. After mixing, rinse the pipets several times with the solution in the centrifuge tube (mix by gently blowing through the pipets by use of a conventional mouthpiece and tubing). Discard the pipets and centrifuge the tubes long enough to settle the precipitated protein. Aspirate the supernatant liquid into a 1.0-ml syringe, being careful not to aspirate any precipitate. There will be sufficient supernatant liquid for three injections. Inject the supernatant liquid into the chromatograph with a 220-µl sample loop. Use of a precolumn is strongly recommended as the best insurance against blockage of the column by any particulate matter that may be accidentally injected.

The sample preparation may be scaled down for pediatric specimens by adding 20 µl of serum to 20 µl of acetonitrile and subsequent addition of 0.36 ml of aqueous phosphate-buffered diluent after complete precipitation of protein. This procedure gives sufficient supernatant liquid for a single injection.

The pulse amperometric mode of detection was used for the analysis; the initial potential of the PAR-174A was set at 0.75 V and the detection potential was adjusted to 1.35 V by scanning 0.600 V more positive and then activating the "hold" switch. Hence, E<sub>i</sub> = 0.75 V and ∆E = 0.600 V.

Results

Comparison of Three Modes of Detection

We studied 1-methylxanthine as a representative member of the family of methylxanthines, to compare dc, pulse, and differential pulse amperometric detection. Figure 1 shows the curves for the current–potential response of 1-methylxanthine, determined for the flow-through electrode by the procedure just described.

A limiting current plateau was observed for dc amperometric detection in the range 0.8 < E < 1.3 V. The peak currents for the sequential injections of 1-methylxanthine were measured with respect to the background current. Hence, we could detect values of electrode potential in a region where background current from solvent decomposition was significant (E > 1.3 V). The net current for detection of 1-methylxanthine decreased dramatically from the limiting value for E > 1.3 V, probably because the effective surface area of the detector decreases as a result of the continuous formation of small O₂ bubbles. Similar behavior was observed for all other compounds in this study.

The limiting current obtained for pulse amperometric de-

tection is greater than that for dc amperometric detection, as expected on the basis of theoretical considerations.

Differential pulse amperometric detection offers the advantage of greater selectivity than dc or pulse amperometric detection, a selectivity that is increased as the pulse amplitude, ∆E, is decreased but with a concomitant decrease in sensitivity. Figure 1 shows response for differential pulse detection of 1-methylxanthine, for ∆E = 100 mV. The values of ∆I for differential pulse detection are plotted in Figure 1 as a function of E<sub>i</sub> + ∆E/2, where E<sub>i</sub> is the initial potential of the electrode before application of the pulse ∆E. The maximum values of peak current were 3.0, 1.3, and 0.6 µA for ∆E, equal to 100, 50, and 25 mV, respectively. The width of the peak response obtained for differential pulse detection, and measured at half the peak height for each value of ∆E, was about 0.10 V.

Calibration and Precision

The theophylline was assayed according to an official method (United States Pharmacopeial Convention) based on the Volhard titration (31). The average for seven determinations was 99.92% theophylline, with a standard deviation of 0.66%.

Figure 2 shows the current–potential response for theophylline and 1,7-dimethylxanthine obtained by dc amperometric detection. From the curve for theophylline, potential values were chosen for which sensitivity was expected to be a maximum for the detection of theophylline by dc, pulse, and differential pulse amperometry. Calibration curves (Figure 3) were constructed for the three modes of detection by injecting 100-µl aliquots of solutions of theophylline in the range of concentration 10<sup>−3</sup> to 10<sup>−2</sup> mol/liter.

The potential for dc amperometric detection was 1.30 V, and the resulting calibration curve is linear in the range 90 ng to 18 µg of theophylline. Noise in the baseline observed for the lowest concentrations of theophylline was oscillatory in nature, with a frequency equal to that of the reciprocating pump producing the flow of eluent. Background current in tubular electrodes proportional to the flow rate of solutions of elec-

![Fig. 1. Comparison of current–potential response for detection of 1-methylxanthine by direct-current, pulse, and differential pulse amperometry](image-url)
tro-inactive electrolytes has been observed in other work in our laboratory. We made no attempt to improve the effectiveness of the pulse damper on the pumping system.

The linear dynamic range shown in Figure 3 for differential pulse detection is not nearly as great as for dc amperometric detection. A requirement for obtaining a linear calibration curve by differential pulse detection is that the value of E1/2 for the analyte must remain constant. This fact has great significance for the application of differential pulse detection in liquid chromatography, because E1/2 would normally be set at a value nearly equal to the E1/2, so that sensitivity is nearly maximum. The value of E1/2 will vary with concentration for nonisometric and irreversible electrode reactions. Hence, the sensitivity for differential pulse detection will depend on concentration for those reactions. The mechanism reported by Hansen and Dryhurst (32) for oxidation of theophylline is complex, a dimeric product being formed. Thus, nonlinear response for the differential pulse technique should be expected.

The calibration curve for theophylline obtained by pulse amperometric detection is shown in Figure 3 for columns A and B. The difference in the linear ranges of the two curves for pulse detection is a result of differences in the values chosen for the potentiostatic parameters. For column A, E1 = 0.75 V and ΔE = 0.60 V, whereas for column B, E1 = 0.80 V and ΔE = 0.30 V. For achieving greatest linearity of the calibration curve by pulse detection, the electrode potential should have an initial value at which no faradaic reaction occurs and the final potential should correspond to a value well into the region of limiting current. A linear range over nearly four decades of concentration was obtained for E1 = 0.75 V and ΔE = 0.60 V.

In a separate study we compared the precision of the three modes of detection for repeated injections of 9.2 μg of theophylline (Table 1).

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The first peak in each series of injections was invariably larger than the subsequent peaks: 1% for dc, and 3–4% for pulse and differential pulse detection. The cause of this slight decrease in sensitivity is not known. If the time between consecutive injections was kept greater than 10 min, no decrease was observed. Perhaps products of the electrode reaction are slow to desorb from the electrode surface and so temporarily block active sites.

Separation of Methylxanthines

A solution was prepared containing the dietary methylxanthines, the metabolites of the dietary methylxanthines (17), and several additional compounds. Dyphylline, used for treatment of asthma, was included in the mixture because of it might be present in the serum of patients. 8-Chlorothephylline was included because of its common use as an internal standard for determinations of theophylline by liquid chromatography with photometric detection. Pharmaceutical preparations such as Dramamine, used for motion sickness, also contain 8-chlorothephylline. Uric acid, creatinine, and xanthine were included in the mixture because they are common components of physiological fluids. The actual quantity of each constituent of the mixture was not determined exactly, but was adjusted to produce similar photometric response. Figure 4 depicts a chromatogram for the synthetic mixture for column A and eluent I with photometric detection. Many constituents of the sample were resolved chromatographically. Theophylline and 1,7-dimethylxanthine were eluted together as peak 11. Theophylline and 1,7-dimethylxanthine were only partly resolved for eluent II (resolution = 0.6). Similarly, resolution was incomplete with use of the eluent suggested by Orcutt et al. (27).

A mixture containing each of the methylxanthines represented in Figure 4 except 1,7-dimethylxanthine was prepared, and aliquots of this mixture were successively injected into column A, to obtain a series of values of electrode potential for dc amperometric detection. We have named the plot of the chromatograms obtained as a function of detector potential (I-t-E) a "chromato-voltammogram." Such a plot is shown in Figure 5 for the mixture of methylxanthines. The possibility of tunable selectivity for the amperometric detector is clearly illustrated in Figure 5. At E = 1.40 V, each of the constituents
Fig. 4. Chromatographic separation of methylxanthines and selected urinary metabolites with photometric detection

Column A; eluent I; photometric detection at 254 nm. 1, uric acid; 2, creatinine; 3, 1-methyluric acid, 3-methyluric acid, and 7-methyluric acid; 4, xanthine; 5, 7-methylxanthine; 6, 1,3-dimethyluric acid; 7, 3-methylxanthine; 8, 1-methylxanthine; 9, theobromine; 10, 8-chlorotheophylline; 11, theophylline and 1,7-dimethylxanthine; 12, dyphylline; and 13, caffeine

Fig. 5. Chromato-voltammogram of methylxanthines at glassy carbon detector

1, 1.77 μg 7-methylxanthine; 2, 1.61 μg 3-methylxanthine; 3, 1.59 μg 1-methylxanthine; 4, 3.67 μg theobromine; 5, 4.09 μg theophylline; and 6, 7.27 μg caffeine. Detection by dc amperometry; column A; eluent I

of the sample was detected, whereas theobromine and caffeine were not detected at E = 1.15 V. An advantage of the detection of theophylline in this mixture at 1.15 V rather than at 1.40 V is that the rate of sample analysis may be increased. This results because caffeine, which has a retention time of about 30 min, will not interfere in the detection of theophylline in subsequent samples injected at 15-min intervals, even though caffeine from a previous injection may elute simultaneously with theophylline.

The selectivity of differential pulse amperometric detection
is much greater than for dc or pulse amperometric detection. This fact is illustrated for the mixture of methylxanthines in Figure 6. The values of \( E_1 \) and \( \Delta E \) for curve A in Figure 6 were selected to give optimum selectivity for theophylline. Note particularly that 1-methylxanthine, the most easily oxidized compound in the mixture, is scarcely detectable. Selectivity for theobromine and caffeine was optimized for curve B, and no peak was obtained for theophylline.

### Ratios of Response for Theophylline and 1,7-Dimethylxanthine

Theophylline and 1,7-dimethylxanthine could not be completely resolved under any of the chromatographic conditions investigated in this research. Two unresolved compounds can be accurately determined if a detector responds selectively to one of the compounds, or if two detectors having different ratios of sensitivity for the two compounds are used simultaneously. Preferably in our case, a selective detector would respond to theophylline and not to 1,7-dimethylxanthine. The response of each of the three modes of electrochemical detection was compared to the response of photometric detection at 254 nm and the results are summarized in Table 2.

The values of \( E_{1/2} \) for theophylline and 1,7-dimethylxanthine are very similar (see Figure 2) and complete resolution of the mixture is not expected to be satisfactory for dc or pulse amperometric detection. Resolution of the mixture is likewise not possible by photometric detection, even with variable wavelength, because of the similarity of the absorption spectra for the two compounds. Of the techniques compared, the largest ratio of response was obtained for differential pulse detection with \( E_1 = 1.175 \) and \( \Delta E = 100 \) mV. The peak height for theophylline was about sevenfold that for 1,7-dimethylxanthine. After coffee is consumed, the concentration of 1,7-dimethylxanthine in blood serum is sufficiently low that the resulting error in determination of theophylline in therapeutic concentrations by differential pulse detection would be trivial.

Differential-pulse amperometric detection can be accurately applied for analysis, in spite of the nonlinear response, if the calibration curve is carefully constructed over the range of concentration expected for the unknown samples. The available volume of sample is quite limited for the determination of theophylline in pediatric cases, and high sensitivity is necessary. Because pediatric patients do not consume caffeine, resolution of theophylline from the metabolite of caffeine is not a problem. We chose to apply pulse amperometric detection for these analyses because we have observed that the detection limit for pulse detection at solid electrodes is frequently lower than for differential-pulse detection, because the difference of the absolute values of two electrical signals cannot exceed the value of the larger of the two signals. The response for differential-pulse detection increases as \( \Delta E \) is made larger, with the limit being precisely the conditions for pulse detection, i.e., no faradaic reaction at \( E_1 \) and a transport-limited reaction at \( E_1 + \Delta E \).

The response ratio for pulse amperometric detection was observed to depend on the potentiostatic parameters chosen for the detection, as is illustrated in Table 2. Under the conditions recommended here for determination of theophylline in serum (\( E_1 = 0.750 \) V and \( \Delta E = 0.600 \) V), the response for theophylline exceeds that for 1,7-dimethylxanthine.

<table>
<thead>
<tr>
<th>Mode of detection</th>
<th>injected Theophylline</th>
<th>Peak</th>
<th>injected 1,7-Dimethylxanthine</th>
<th>Peak</th>
<th>Response ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photometric (254 nm)</td>
<td>18 ( \mu g )</td>
<td>0.56 ( \mu A^* )</td>
<td>18 ( \mu g )</td>
<td>0.53 ( \mu A )</td>
<td>1.1</td>
</tr>
<tr>
<td>Direct current (( E = 1.275 ) V)</td>
<td>18 ( \mu g )</td>
<td>6.3 ( \mu A )</td>
<td>18 ( \mu g )</td>
<td>4.4 ( \mu A )</td>
<td>1.4</td>
</tr>
<tr>
<td>Differential pulse (( E_1 = 1.175 ) V, ( \Delta E = 100 ) mV)</td>
<td>18 ( \mu g )</td>
<td>0.622 ( \mu A )</td>
<td>18 ( \mu g )</td>
<td>0.094 ( \mu A )</td>
<td>6.6</td>
</tr>
<tr>
<td>Pulse (( E_1 = 0.800 ) V, ( \Delta E = 0.300 ) V)</td>
<td>18 ( \mu g )</td>
<td>2.7 ( \mu A )</td>
<td>18 ( \mu g )</td>
<td>5.8 ( \mu A )</td>
<td>0.5</td>
</tr>
<tr>
<td>Pulse (( E_1 = 0.750 ) V, ( \Delta E = 0.600 ) V)</td>
<td>198 ng</td>
<td>0.559 ( \mu A )</td>
<td>198 ng</td>
<td>0.370 ( \mu A )</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>900 ng</td>
<td>2.26 ( \mu A )</td>
<td>900 ng</td>
<td>1.47 ( \mu A )</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* \( \mu A \), absorption units.
Table 3. Sensitivity Factor for Photometric and Pulse Amperometric Detection of Theophylline Comparison

<table>
<thead>
<tr>
<th>Amount injected (G), ng</th>
<th>Peak response (P), A</th>
<th>SD, A</th>
<th>CV, %</th>
<th>Sensitivity factor (P/G), ng⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Photometric (254 nm)</td>
</tr>
<tr>
<td>110</td>
<td>0.0072 (11)</td>
<td>0.00031</td>
<td>4.3</td>
<td>0.211</td>
</tr>
<tr>
<td>220</td>
<td>0.0146 (11)</td>
<td>0.00071</td>
<td>4.9</td>
<td>0.093</td>
</tr>
<tr>
<td>440</td>
<td>0.0293 (8)</td>
<td>0.00077</td>
<td>2.6</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pulse amperometric (E₁ = 0.75 V, ΔE = 0.60 V)</td>
</tr>
<tr>
<td>110</td>
<td>0.347 (10)</td>
<td>0.0126</td>
<td>3.6</td>
<td>0.250</td>
</tr>
<tr>
<td>220</td>
<td>0.624 (12)</td>
<td>0.0233</td>
<td>3.7</td>
<td>0.122</td>
</tr>
<tr>
<td>440</td>
<td>1.164 (8)</td>
<td>0.0302</td>
<td>2.6</td>
<td>0.088</td>
</tr>
</tbody>
</table>

* No. determinations in parentheses.

Table 4. Recovery of Theophylline in Serum

<table>
<thead>
<tr>
<th>Theophylline added mg/liter</th>
<th>Theophylline found, mg/liter</th>
<th>Mean recovery, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photometric (254 nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0 (5)</td>
<td>19.9</td>
<td>99.3</td>
<td>2.76</td>
</tr>
<tr>
<td>Pulse amperometric (E₁ = 0.75 V, ΔE = 0.60 V)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0 (9)</td>
<td>19.8</td>
<td>99.1</td>
<td>4.42</td>
</tr>
</tbody>
</table>

* No. determinations in parentheses.

Comparison of the Pulse Amperometric and Photometric Detection of Theophylline

Numerous aliquots of standard solutions of theophylline were injected for the purpose of comparing the sensitivity for photometric detection and pulse amperometric detection. The data are summarized in Table 3. For the purpose of comparison, a sensitivity factor is defined as the slope of the calibration curve divided by the standard deviation at that point. The sensitivity factors for the two modes of detection are equivalent for the experimental conditions used. The photometric detector is expected to have a sensitivity factor nearly three times greater than in Table 3 if the optimum wavelength of 273 nm were used rather than 254 nm (27). Nevertheless, both detectors have sufficient sensitivity to permit the determination of theophylline in serum samples of reasonable size (20–100 μl). Because the pulse amperometric detection compares favorably with ultraviolet detection for determination of theophylline under realistic conditions, there is every reason to suggest that ultraviolet detectors and electrochemical detectors form a beneficial partnership when samples contain two components that are not resolved chromatographically.

Determination of Theophylline in Blood Serum

The procedure described in a previous section for determining theophylline in blood serum was tested on reconstituted lyophilized serum samples having the same lot number. A small chromatographic peak was observed by photometric and pulse amperometric detection, with a retention time characteristic of theophylline and 1,7-dimethylxanthine. A small peak was also detected photometrically with a retention time characteristic of caffeine. Typical chromatograms obtained for pulse amperometric detection are shown in Figure 7 for the reconstituted serum blank and the serum after constitution with an aqueous standard containing 20.0 mg of theophylline per liter. The technique was calibrated by use of values for chromatographic peak height given by standard solutions of theophylline (Table 4). The chromatographic results for the lyophilized serum samples were all corrected for the blanks obtained for serum reconstituted with de-ionized water. The recovery was essentially quantitative for both techniques. The results for samples analyzed 27 h after reconstitution were virtually identical to results for samples analyzed immediately. Loss of theophylline due to protein binding was negligible.

To gain experience with "real" samples, a pharmacokinetic study of ingested theophylline was performed by one of us (E.C.L.). Beverages containing methylxanthines were excluded from the diet for a period of 18 h (with the consequent mental agony, since habitual consumption of coffee and cola was the normal dietary practice). After a fasting period of 8 h, a blood specimen was collected by venipuncture. Two Quinbron capsules containing a total dose of 300 mg of anhydrous theophylline and 180 mg of glyceryl guaiacolate (an expectorant) were taken orally. Quinbron is an extended-duration form of theophylline. Blood specimens were collected at appropriate intervals to map the pharmacokinetic response. The specimens were allowed to clot and were then centrifuged. Serum was removed and stored at 4 °C until the analysis. Theophylline in these serum samples was determined chromatographically using photometric detection as well as pulse amperometric detection.

The pharmacokinetic response for both modes of detection is shown in Figure 8. Apparently, restriction of caffeine for 18 h was not adequate, because the first specimen gave a small peak at the retention time of 1,7-dimethylxanthine, which was detected by both detectors. We doubt that this peak resulted from an endogenous constituent of serum, because (e.g.) no peak with this retention time was observed 70 h later by pulse amperometric detection. The response at 70 h by photometric detection was a mere 0.2 mg/liter, reported as theophylline. One observes from Figure 8 that the values determined by pulse amperometric detection are slightly less than for photometric detection during the early hours of the pharmacokinetic study by about 0.6 mg/liter. Admittedly, this value is within the error of the methods, but a bias is apparent. The pulse amperometric mode of detection is less susceptible to
interference from 1,7-dimethylxanthine, so the difference in the results for the two detectors is explained on the basis of the presence of a small amount of 1,7-dimethylxanthine.

We conclude that only 1,7-dimethylxanthine and theophylline will ordinarily be eluted together under the chromatographic conditions described here. No other serum constituent occurring in normal amounts will elute at this retention time. One may be assured that if the photometric and amperometric detectors are used in combination, any significant deviation of the results calculated from the data obtained by the two detectors is a consequence of consumption of caffeine, or perhaps some other drug not investigated here.

Discussion

Beverages containing caffeine and other methylxanthines should be withheld from patients treated with theophylline, because toxic reactions may occur from the combined effects of the therapeutic and dietary methylxanthines (12). This medical contraindication is fortunate in terms of the analytical determination. However, the ultraviolet photometric detector would detect any caffeine, warning the clinician of noncompliance by the patient, and the electrochemical detector would give a better estimate of actual theophylline concentration without need to repeat the determination on a new specimen. If only the electrochemical detector is used, the analysis time is nearly halved because there is no need to wait for the elution of caffeine before making the next injection of sample. If the photometric detector is used, one generally must wait longer between injections.

There is significant merit in the simultaneous application of two detection systems in series for monitoring a chromatographic effluent, e.g., pulse and differential-pulse detection, pulse and photometric detection, etc. With appropriate multiplexing techniques it is even possible simultaneously to perform pulse and differential-pulse detection with a single potentiostat and one flow-through electrode. Advantages of a dual-detection system include possible resolution of two components that are not separated by the chromatographic process, and greater analytical accuracy because each sample component is detected twice. The problem of chromatographic separation of 1,3- and 1,7-dimethylxanthine is serum samples will undoubtedly be solved ultimately by discovery of the appropriate chromatographic conditions. If this particular interference were the only one ever anticipated for serum samples, dual detection would not appear to be so important, but all possible interferences cannot be anticipated for each individual specimen in the clinical laboratory. Unanticipated interferences will be easily discovered with dual detection when the response ratio for two unresolved compounds differ for the two detection systems. As a member of such a partnership, amperometric detection holds significant promise for clinical analysis. The procedures described here have also been used to detect methylxanthines in urine (33). More than 30 peaks were observed in addition to the peaks for the methylxanthines, and so the present determination of the methylxanthines is only illustrative of many possible applications of amperometric detection. No significant loss of electrode activity was observed over the 90-min period for a single chromatogram for each urine sample.

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