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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THEOPHYLLINE AND ITS DERIVATIVES WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Theophylline and its derivatives are determined by high-performance liquid chromatographic separation on a C_8 reversed-phase column with electrochemical detection. The analyte is oxidized at 1.24 V (*vs.* the standard calomel electrode) on a wax-impregnated graphite electrode using classical d.c. and current sampled d.c. modes, and the results obtained compared with those of a 254 nm ultraviolet (UV) detector. Polarographic detection offers greater sensitivity for theophylline than UV detection; further, interferences which hamper UV detection may be selectively eliminated with polarographic detection.

INTRODUCTION

Since 1936 theophylline (1,3-dimethylxanthine) has been used in drug therapy for the control of asthma[1]. To be effective the serum concentration of theophylline has to be in the therapeutic range of 10–20 mg/l of blood[2,3]. Several studies have been made involving high-performance liquid chromatographic (HPLC) separation and quantization of theophylline from blood serum and other biological fluids[4–11]. In most instances an internal standard such as 8-chlorotheophylline[5,11], monohydroxypropyltheophylline[7,8] or dihydroxypropyltheophylline[10] is used for quantitative purposes. The effluent was generally monitored with a 254-nm ultraviolet (UV) detector[5,7–11]; however, since the wavelength of maximum absorbance for theophylline is 273 nm, a considerable amount of sensitivity was sacrificed with this method of detection. Moreover, careful buffering of the aqueous mobile phase within strict pH regions[4,5,8–11] or use of a mixed solvent mobile phase[6,7] is required to assure complete resolution of the peaks for caffeine and the internal standard.

Hansen and Dryhurst[12] studied the electrochemical behavior of theophylline, theobromine and caffeine, and observed pH dependent, anodic oxidation waves for these molecules in the potential region 1.3–1.7 V *vs.* the standard calomel electrode (SCE). These data indicate that it should be possible to use an electrochemical detector to monitor the HPLC effluent for the presence of these molecules; in addition, selectivity may be enhanced through judicious choice of applied potential[13]. In this

potential range, mercury is not suitable as a working electrode; usable electrodes include the pyrolytic graphite electrode or the wax-impregnated graphite (WIG) electrode. A variety of electrochemical detectors with some form of carbon or graphite have been described in the literature[13–21] which would be applicable for use with the theophylline system.

EXPERIMENTAL

Instrumentation

A Milton-Roy minipump (Laboratory Data Control) with a pumping rate of 57 cycles/min is used as the pumping system. The pressure was monitored with a WICA diaphragm gauge. Samples were injected through a Reeve Angel septum type injector (Whatman) with a 10-μl syringe. The Altex LiChrosorb C_8, 10 μm, reversed-phase, 3.2 mm I.D. × 250 mm column was employed as the analytical column. The flow cell detector (Fig. 1) was connected to a Princeton Applied Research Corporation Model 174A polarographic analyzer. The WIG working electrode was prepared by soaking spectroscopic grade graphite (National Carbon, Union Carbide) in molten paraffin wax for 24 h. The Fisher saturated calomel electrode served as the reference electrode and platinum wire served as the counter electrode. Swagelok nylon fittings were used for the inlet and outlet ports, and to hold the working electrode in place. UV measurements were made using the Altex Model 152 UV detector.

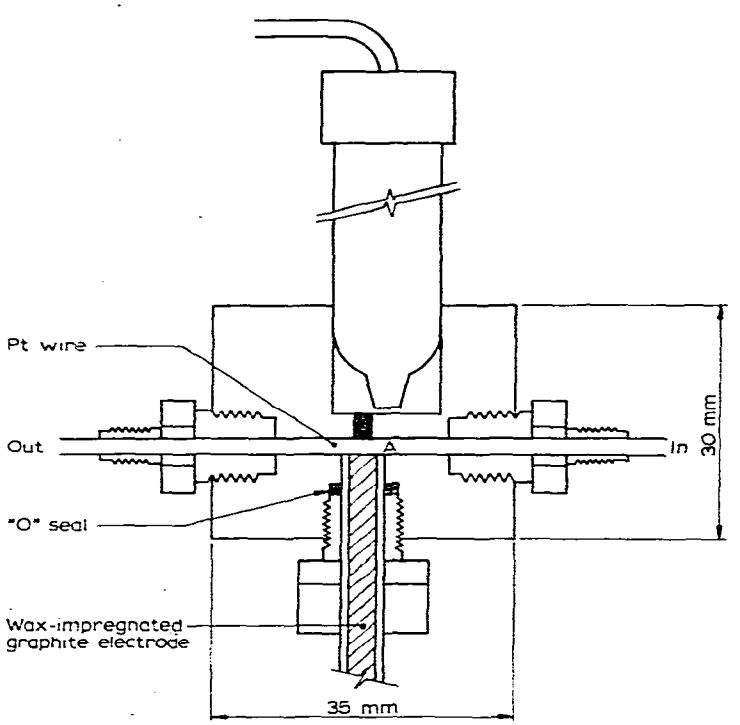


Fig. 1. Electrochemical detector flow cell. Material is plexiglass. Flow channel A has a 1.58-mm diameter.

Reagents

The mobile phase was a mixture of sodium acetate–acetic acid buffer (pH 4.0) and ethanol in a 92:8 ratio. The mobile phase was deaerated with an ultrasonic vibrator and vacuum. Theophylline, theobromine (3,7-dimethylxanthine), caffeine (1,3,7-trimethylxanthine) and 8-chlorotheophylline were purchased from Sigma (St. Louis, Mo., U.S.A.). Stock solutions of 1000 mg/l of theophylline, 400 mg/l theobromine, 1000 mg/l caffeine and 100 mg/l 8-chlorotheophylline with 1 *M* NaCl added are prepared by dissolving the solid material in distilled water.

Procedure

Preliminary work with the Altex UV detector indicated that the 92% sodium acetate–acetic acid buffer: 8% ethanol mixture gave the desired separation and eliminated tailing on the C_8 reversed-phase column. As shown in Fig. 2, this mobile phase also exhibits a clear electrochemical window for the observation of the oxidation of the molecules of interest.

Standard solutions of theophylline, 0, 5, 10, 15, 20, 30 and 40 mg/l were prepared by successive dilutions of the 1000 mg/l stock solution. These solutions were then diluted two-fold with the 100 mg/l 8-chlorotheophylline–1 *M* NaCl solution. Then 10 μl of each solution was injected using a modified stopped-flow injection technique. This technique involved stopping the pump, waiting until the column back pressure dropped to no more than 200 p.s.i., injection with a swift motion of the plunger and immediate restarting of the pump. The flow-rate was kept constant at 1.0 ml/min during each separation.

A modified Rabenstein and Saetre[22] design flow cell was used to monitor the effluent, where the WIG rod was substituted for the Hg pool type electrode. The WIG electrode had a diameter of 3 mm and a surface area of 7.07 mm^2. It was necessary to refinish the WIG electrode by polishing it with fine grade carborundum paper occasionally when the performance deteriorated. As an internal standard was used, each separation was self correcting for electrode performance in the short term. Further modifications of the flow cell involved moving the channel to the reference electrode to be opposing the working WIG electrode and cementing a glass frit in the channel. The reference electrode cavity was filled with saturated KCl solution. Also, a platinum wire counter electrode was epoxied into the wall of the flow cell. These changes were made in an effort to reduce the internal cell resistance. The combined changes in the flow cell design are presented in Fig. 1. The flow channel has a diameter of 1.58 mm and a total volume of approximately 14.7 μl, which is of the order of currently available UV flow cells.

Four methods of detection were used to monitor the effluent, namely, classical d.c., sampled d.c., and pulsed and differential pulsed modes. In the sampled d.c. mode the clock was set at 1 sec in order to correspond with the pump cycles. A potential of 1.24 V *versus* the SCE was applied across the cell which was under potentiostatic control. In addition, the effluent was monitored with the Altex Model 152 UV detector at 254 nm to facilitate comparison of our electrochemical detection method with previously reported UV detector based results. Since the wavelength of maximum absorbance for theophylline is 273 nm, an attempt was made to monitor the effluent at 280 nm using the Altex detector. However, the filter used to isolate

the 280 nm light limited the source intensity such that the theophylline detection limit was higher than at 254 nm.

Calibration curves of the theophylline, 8-chlorotheophylline peak height ratios *versus* concentration of theophylline for each detection method were constructed. At least four sets of determinations were made for each detection method.

RESULTS AND DISCUSSION

Current–voltage curves for theophylline and the mobile phase alone are shown in Fig. 2. The diffusion current displays a peak at an applied potential of 1.15 V *vs.* the SCE, well within the polarographic "window" for the mobile phase up to 1.3 V. Greater sensitivity towards theophylline was obtained, however, at an applied potential of 1.24 V rather than at 1.15 V. This potential permits oxidation of theophylline and 8-chlorotheophylline while selectively attenuating the response to the interferents, theobromine and caffeine, as their oxidation potentials are characteristically higher.

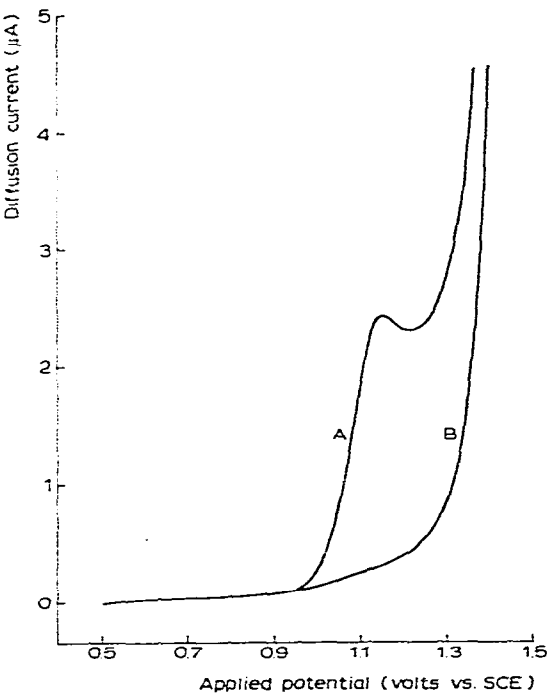
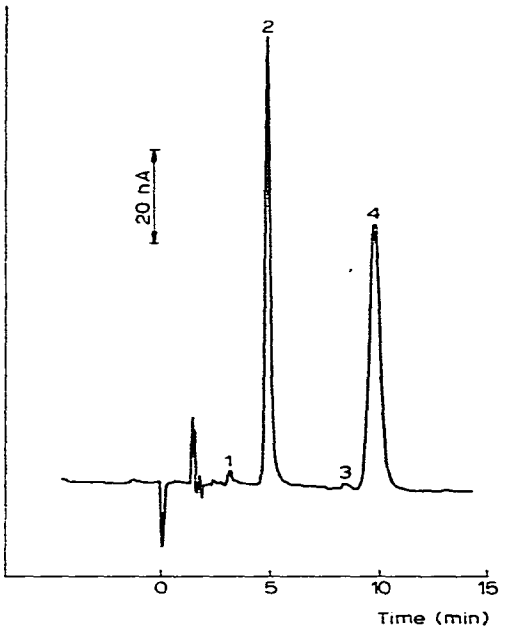


Fig. 2. D.c. current–voltage curve for theophylline (50 mg/l) (A) and mobile phase only (B); mobile phase, sodium acetate–acetic acid buffer (pH 4.0)/ethanol (92:8); scan rate, 5 mV/sec.

Figs. 3 and 4 show typical reversed-phase separations for a solution containing 20 mg/l each theophylline, theobromine and caffeine with 50 mg/l 8-chlorotheophylline as internal standard with electrochemical and UV detection, respectively. We note that the response of theophylline (relative to 8-chlorotheophylline) is enhanced with the electrochemical detector. Also, the response of theobromine



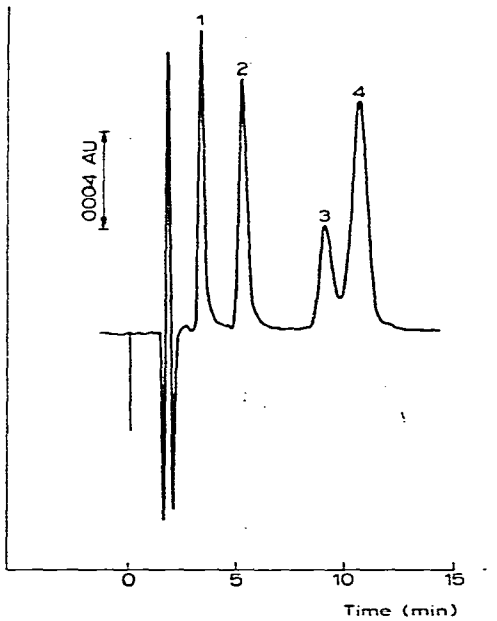


Fig. 3. Separation of theobromine (20 mg/l) (1), theophylline (20 mg/l) (2), caffeine (20 mg/l) (3) and 8-chlorotheophylline (50 mg/l) (4) with electrochemical detection in the classical d.c. mode at 1.24 V *vs.* SCE: LiChrosorb C_8, reversed-phase, 10 μm, 3.2 mm I.D. × 250 mm; mobile phase as in Fig. 2; flow-rate, 1.0 ml/min.

Fig. 4. Separation of same mixture as in Fig. 3, but with UV detection at 254 nm, experimental conditions as in Fig. 3.

and caffeine has been reduced considerably when the electrochemical detection method is compared with the UV detection of the same solution. The electrochemical detector offers baseline resolution for caffeine and 8-chlorotheophylline, which is not the situation with the UV detector. In previous studies[4–11] employing UV detection, it was necessary to carefully control the pH of the buffer in order to achieve resolution of the internal standard from other theophylline related compounds. Using the electrochemical detector the pH of the buffer mobile phase is not as critical, as complete resolution is not required.

Fig. 5 shows the calibration plot obtained using the UV detector. This calibration plot is similar to those already reported[4–11] with similar slope indicating the same relative sensitivity for theophylline and 8-chlorotheophylline. The relative deviation within the therapeutic range of 10–20 mg/l is less than ±0.5 mg/l.

With the two modes of electrochemical detection the calibration curves are also linear over most of the concentration range covered as shown in Fig. 5. However, the slopes of the plots are greater than was the slope in the UV method, indicative of more favorable sensitivity toward theophylline in each of the electrochemical modes.

There is a slight negative deviation from an Ilkovic relationship at high concentrations for the classical d.c. mode of detection. After several determinations the relative deviation is ±1.4 mg/l at a concentration of 40 mg/l, which is consider-

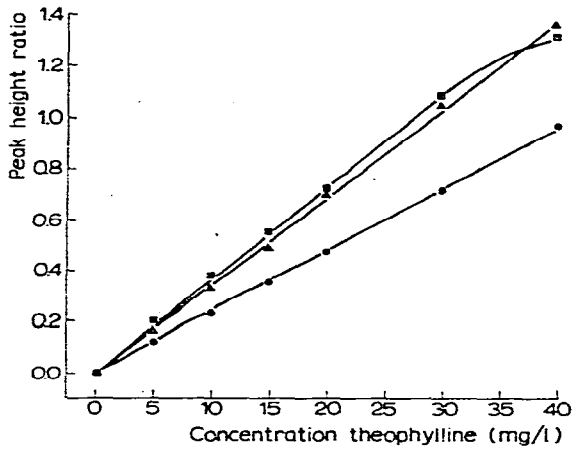


Fig. 5. Calibration plot of peak height ratio of theophylline and 8-chlorotheophylline. ●, UV detection at 254 nm; ■, classical d.c. mode at 1.24 V *vs.* SCE; ▲, current sampled mode at 1.24 V *vs.* SCE.

ably outside the therapeutic range anyway, and should not be cause for alarm. Within the therapeutic range the deviation is less than ± 1 mg/l, which compares favorably with the UV detection method already reported[5]. The sampled d.c. calibration plot is linear throughout the entire concentration range, and in the therapeutic region, the deviation is approximately ± 1 mg/l.

To this point, the detection limit using the polarographic detector is on the order 0.5 mg/l. In an effort to extend this limit, pulse and differential pulse measurements were pursued. However, background current due to the mobile phase, which is apparently not important in the other polarographic modes, caused saturation of the current to voltage converter in the differential pulse mode. If we could overcome this instrumental limitation, we expect a detection limit of 0.1 mg/l or 100 ppb* theophylline.

CONCLUSIONS

Electrochemical detection of theophylline and its related compounds from an HPLC separation is indeed feasible. The gain in sensitivity towards theophylline and selective attenuation of some troublesome interferences makes electrochemical detection attractive for routine analysis. Of the two modes of electrochemical detection described, the sampled d.c. mode offers greater sensitivity and more precision than does the normal (conventional) d.c. mode. Both offer more sensitivity and pH flexibility than does the standard UV mode.

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* The American billion (10^9) is meant.

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