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The liquid chromatographic determination of theophylline in untreated plasma by simple direct injection*

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Abstract: A liquid chromatographic method for determination of theophylline by direct injection of untreated plasma samples is described. Theophylline is detected without interference from related compounds such as paraxanthine.

A precolumn venting technique is used which considerably increases column life. The lifetimes of the separation columns are unaffected by plasma injections whereas the precolumn has to be changed after 140 injections (10 μl of plasma).

The peak purity of theophylline is examined spectrophotometrically.

Determinations are performed by external standardization with recoveries close to 100% with a precision better than 2.3% (RSD).

Keywords: *Reversed-phase liquid chromatography; theophylline; blood plasma; direct injection; precolumn venting.*

Introduction

Theophylline is a drug commonly used for the treatment of asthma and other pulmonary diseases. Because the difference between the therapeutic and the toxic concentration levels is small, therapeutic drug monitoring is essential and rapid, accurate determination is necessary.

Liquid chromatography and immunoassay are the most frequently used methods for the determination of theophylline [1].

The aim of this study was to develop a simple liquid chromatographic method for monitoring theophylline by direct injection of untreated plasma samples.

Numerous papers in the literature describe the liquid chromatographic determination of theophylline in biological fluids. Different sample pretreatment procedures aimed at removing plasma proteins have been used such as extraction [2, 3], protein precipitation [4, 5] and ultrafiltration [6, 7].

Direct injection of untreated plasma has also been reported [6, 8–13], but a problem has been that the chromatographic systems showed bad stability, e.g. losses of peak

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efficiency [8, 10], shifts of retention [10, 11] and increases of column back-pressure [6, 9–11]. These experimental difficulties and a lack of understanding of their cause may be the reason that the direct injection procedure has not found wide acceptance.

The use of protein-coated ODS silica as stationary phase [12] gave rise to very stable systems but with poor resolving power due to low peak efficiency.

A multicolumn technique, using direct injection into a pre-column packed with a gel permeation phase has been reported [13], but with no column stability data.

In the present study a precolumn venting technique, discussed in detail in a previous paper [14], has been used. With this technique experimental parameters such as retention, peak efficiency and column back-pressure can be controlled, resulting in a much improved performance during direct injections of plasma samples.

The procedure used in this study for determination of theophylline can easily be adapted to other compounds.

A common problem has been the resolution of theophylline from paraxanthine [1], a metabolite of caffeine [15] which is often present in blood plasma. Separations have previously been obtained by addition of tetrahydrofuran [16] or tetrabutylammonium salts [17, 18] to the mobile phase. In this study the retentions of theophylline, caffeine, their metabolites and related compounds were examined. By addition of *N,N*-dimethyl-*n*-octylamine (DMOA) to the eluent theophylline and paraxanthine were easily resolved.

The purity of the theophylline peak from plasma samples was examined by spectrophotometric methods, such as spectral fingerprinting and absorbance ratios using a multichannel detector [19].

Experimental

Chemicals

Theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), 3-methylxanthine, 1-methyluric acid, 1,3-dimethyluric acid and hypoxanthine (6-hydroxypurine) were purchased from Sigma Chemical Company (St Louis, MO, USA). Caffeine (1,3,7-trimethylxanthine) was of pharmacopoeial grade and *N,N*-dimethyl-*n*-octylamine (DMOA) (ICN-K&K Laboratories Plainview, NY, USA) was distilled. All other chemicals were of analytical grade.

Lichrosorb RP-8 (5 and 10 μm) was obtained from E. Merck (Darmstadt, FRG).

Apparatus

The pump used was a Model 711-47 Solvent Delivery System (LDC, Riviera Beach, FL, USA) and the injector a Rheodyne Model 7120 (Rheodyne Inc., Berkeley, CA, USA) equipped with a 10 μl loop. A three-port valve Model CV-3-HPax (Valco Instruments Co., Houston, TX, USA) was used as a venting valve. The detectors used were a LDC UV III Monitor operating at 254 nm and a multichannel rapid scanning spectrophotometric detector HP 1040 A (Hewlett-Packard, FRG) coupled to a personal computer HP-85 B (Hewlett-Packard, FRG). The 100 × 4.6 mm i.d. analytical columns were constructed from LiChroma tubing (316 stainless steel, Handy and Harman Tube Co., Norristown, PA, USA) with Altex (Altex, Berkeley, CA, USA) 2 μm stainless steel frits at both ends. The 10 × 3.2 mm i.d. precolumn was constructed similarly after modification of the end fittings [details to be published elsewhere]. Altex stainless steel frits were placed in both ends. A home-made pressure regulator [20] was used to regulate the back-pressure in the venting line when the precolumn venting technique was used.

Analytical method

A blood plasma sample was centrifuged to obtain a clear solution, 10 μl of which was injected into the precolumn (10 × 3.2 mm i.d., packed with 10 μm LiChrosorb RP-8) with the three-port valve in the waste position. After passage of 0.20 ml of eluent the valve was switched for elution of theophylline into the analytical column (100 × 4.6 mm i.d., packed with 5 μm Lichrosorb RP-8), the eluate of which was detected at 254 nm. The eluent consisted of methanol–phosphate buffer pH 6.0 (7:93, v/v) containing 7.8 mM of DMOA and was maintained at a flow-rate of 0.68 ml min^{-1}. The area of the chromatographic peak was measured and the concentration of theophylline obtained by comparison with external standards.

Procedures

Plasma samples, used for validation of the method, were prepared by adding 100 μl of a stock solution of theophylline to 900 or 1000 μl carefully mixed pooled blank plasma (obtained from University Hospital, Uppsala). Xanthine-free plasma, a gift from AB Draco, Lund, was injected to confirm that the eluting zone of theophylline was free from xanthines due to intake of xanthine containing foods or drugs. Specimens of theophylline containing plasma from adult and paediatric patients were kindly given from University Hospital, Uppsala. All plasma samples were stored frozen.

Prior to injection of the plasma it was carefully mixed and centrifuged at about 5000 ***g*** for 10 min.

Phosphate buffer pH 6.0 (ionic strength = 0.1) was prepared by diluting 73 ml 1.0 M sodium dihydrogen phosphate and 18 ml 0.5 M disodium hydrogen phosphate to 1000 ml with deionized water.

The separation columns were slurry-packed at 300 bar with a high-pressure pump with the stationary phase suspended in methanol. The precolumns were also slurry-packed using an ordinary LC pump with the packing suspended in a mixture of methanol and dichloromethane (1:1, v/v).

The column void volume was determined by injection of sodium nitrate.

External standards were prepared by dilution of a stock solution of theophylline with buffer. A standard curve was constructed by plotting the peak area, measured by triangulation, of the standards versus their concentration (mg l^{-1}).

Results and Discussion

Regulation of retention and selectivity

The phase systems used in this study were liquid–solid systems, with octyl silica as the solid phase. The retention in such systems may be regulated by the addition of uncharged organic modifiers, by the pH of the mobile phase and by the addition of hydrophobic ions acting as ion-pair reagents or as competitors with the solute for the binding-sites of the solid phase.

Theophylline has a weakly basic group with pK_a <1 and a weakly acidic group with pK_a = 8.6 [21]. It is thus uncharged in the range pH = 2–7.6. This restricts the possibility of regulating the retention of ion-pair reagents or pH. In order to change the retention of the uncharged form effectively by pH, the pH has to be raised above the pK_a value for the acidic group [22, 23]. Such pH values are impracticable owing to the low stability of silica-based supports at high pH [24].

By varying the content of methanol at pH 6 most of the xanthines were separated but no resolution between theophylline and paraxanthine was achieved.

Addition of DMOA to the eluent improved the selectivity of the separation (Fig. 1), the retentions of the anionic 1-methyluric acid and 1,3-dimethyluric acid (metabolites of theophylline and caffeine) increase with increasing concentration of the cationic DMOA due to ion-pairing in the stationary phase whereas the retention of the other xanthines (uncharged at pH 6) decreases with increasing concentration of DMOA. This is due to a competition of binding to the solid phase between DMOA and the solutes [25, 26]. The separation factor between theophylline and paraxanthine increased with concentration of DMOA and a suitable resolution was obtained with 8 mM of DMOA (Fig. 2).

Precolumn venting and column stability

The precolumn venting technique used [14] requires a small precolumn, 10 × 3.2 mm

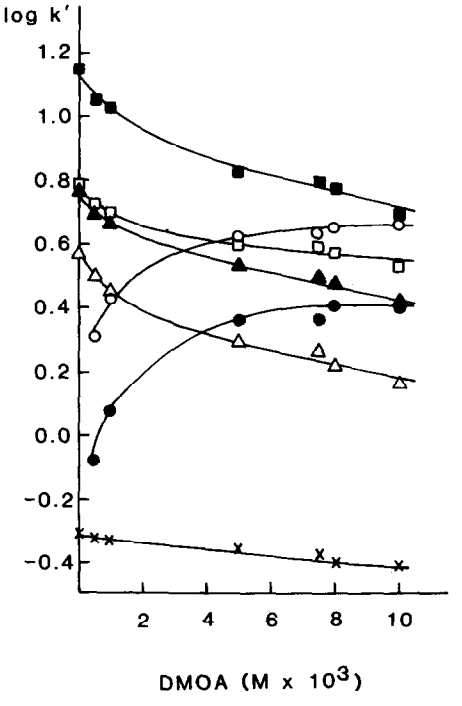

Figure 1
Retention (log k') of theophylline and related compounds in a methanol–phosphate buffer (pH 6.0) (10:90, v/v) eluent, determined as a function of *N*,*N*-dimethyl-*n*-octylamine concentration in the eluent. Key: × = hypoxanthine, ● = 1-methyluric acid, ○ = 1,3-dimethyluric acid, △ = theobromine, ▲ = paraxanthine, □ = theophylline and ■ = caffeine.

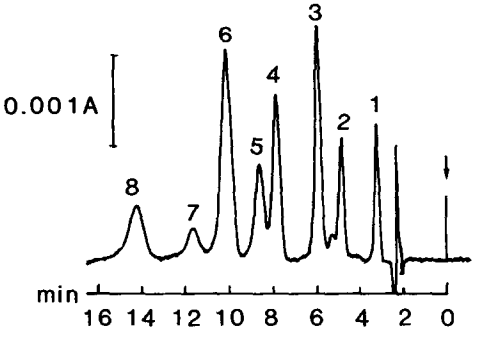

Figure 2
Chromatogram of theophylline and related compounds obtained according to the analytical method but with no venting. Key: (1) hypoxanthine (endogeneous compound); (2) 3-methylxanthine (metabolite of 6); (3) theobromine (natural xanthine); (4) 1-methyluric acid (metabolite of 6); (5) paraxanthine (metabolite of 8); (6) theophylline; (7) 1,3-dimethyluric acid (metabolite of 6) and (8) caffeine (natural xanthine).

i.d., and a three-port valve which is placed between the precolumn and the separation column.

During the injection of a sample the valve is set in the waste position and components eluting from the precolumn do not enter the separation column. Just before the components to be determined are eluted, the valve is switched to enable them to pass into the separation column. The effect of precolumn venting on the resulting chromatograms for patient plasma and xanthine-free blank plasma is shown in Fig. 3.

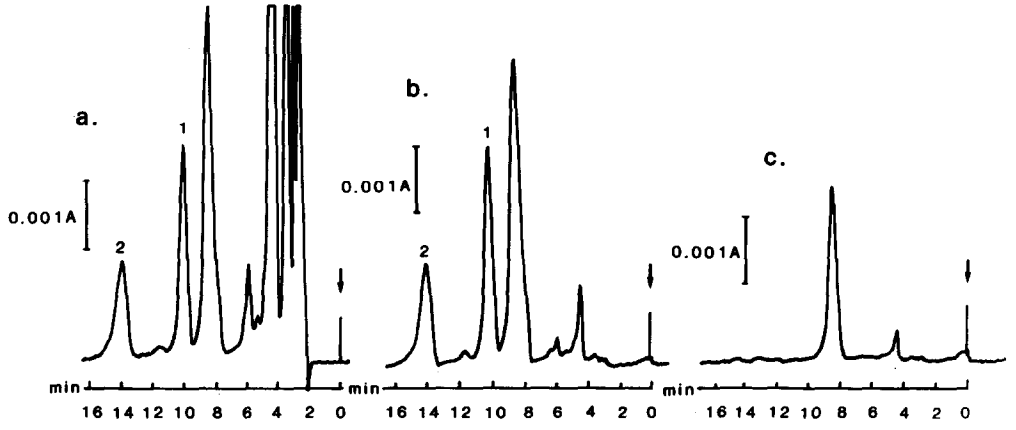

Figure 3
Chromatograms of plasma samples obtained according to analytical method. Key: (1) theophylline; (2) caffeine; (a) patient plasma, theophylline 5.5 mg l^{-1}, no venting; (b) sample as in a, precolumn venting 0.20 ml; (c) xanthine free blank plasma, precolumn venting 0.20 ml.

It has been found that the less retained plasma components (e.g. proteins) cause column deterioration [14]. The precolumn venting technique thus prevents these compounds from entering the separation column, the working life of which therefore is unaffected by the plasma injections. The precolumn–separation column combination is, however, affected by the plasma injections owing to deterioration of the precolumn. After a certain number of injections the peak efficiency decreases and the column back-pressure increases. The original conditions are restored by replacement of the precolumn.

The stability of the system depends on the eluent flow-rate, organic modifier content and the particle diameter of the packing in precolumn [14]. These parameters have been optimized to obtain as good stability of the precolumns as possible, i.e. 10 μm particles in the precolumn and a flow-rate of 0.7–1.0 ml min^{-1}.

With the chosen phase system 140 injections of 10 μl of plasma could be made before the initial values of the efficiency (N = 2200) and column back-pressure (45 bar) was changed by 10%, which is a reasonable limit for a change of the precolumn. It is possible to accept a larger change of these parameters, however, under these conditions standardization is necessary at closer intervals to ensure accurate quantitation.

Peak purity

The peak purity of theophylline was examined by comparing the UV spectrum corresponding to the peak maximum for a standard solution with that of a plasma sample (Fig. 4). The spectra are almost identical.

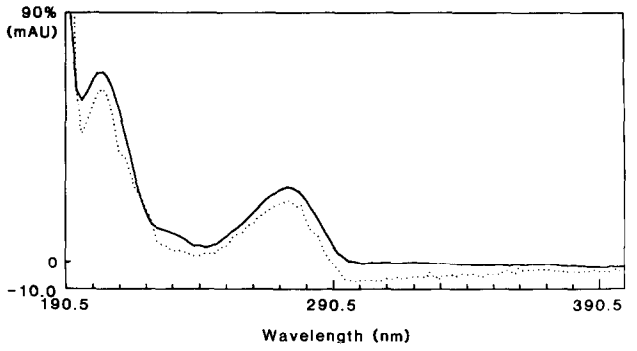

Figure 4
UV spectra of theophylline recorded at peak maxima. Key: theophylline standard 13.8 mg l^{-1}, range 40 mAU (———); theophylline in patient plasma 5.5 mg l^{-1}, range 16 mAU (. . . . . .).

Another method of checking peak homogeneity is to measure absorbance ratios. These can be obtained over the whole eluting zone of theophylline and should be constant if only one UV absorbing species is present. Wavelengths at the absorbance maxima of 204 and 272 nm and at the absorbance minimum of 243 nm were used. A typical patient's plasma sample (Fig. 5) showed that there was very small or no interference from other compounds in the eluted zone of theophylline. Using the absorbance ratio 204:272 some interference was indicated at the end of the eluting zone due to an unidentified compound which only absorbs at the lower wavelengths. This was confirmed by running a chromatogram of a blank plasma with detection at 204 nm. A small peak was detected. With a detection at 254 or 272 nm this interference was not apparent.

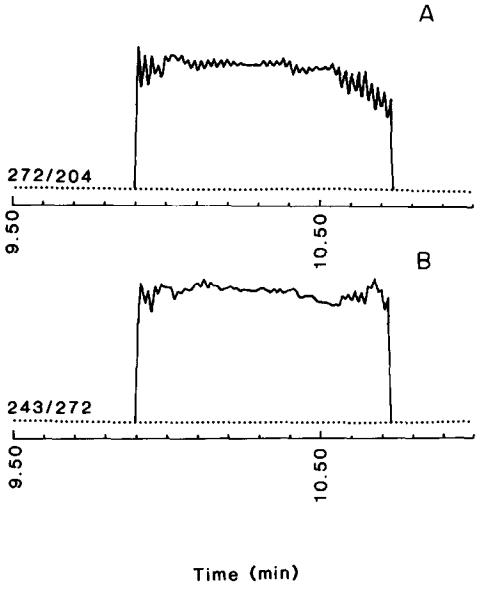

Figure 5
Absorbance ratios determined for the theophylline peak from a typical patient plasma sample chromatographed according to the analytical method. Key: (A) absorbance ratio 272/204 nm; (B) absorbance ratio 243/272 nm.

Quantitation

Quantitation was performed by external standardization. The calibration curve was linear over the range 1.16–27.21 mg l^{-1} with the fitting equation $y = 748.5x - 22.3$ (y = peak area in mm^2, x = concentration of theophylline in mg l^{-1}).

The correlation coefficient was 0.9999, and the standard deviation of the slope and intercept was 1.5 and 20.6, respectively.

Validation of precision and accuracy was made on blank plasma to which theophylline was added at three different concentrations (Table 1). The results showed that quantitation could be performed with high accuracy and precision.

Table 1
The inter-day and intra-day reproducibility as indicated by calibration data obtained over a period of four days

Added concentration of theophylline* (mg l^{-1})	Mean conc. (mg l^{-1})	Recovery (%)	RSD (%)	n
0.696	0.707	101.6	2.1	22
2.80	2.81	100.4	1.8	22
10.94	10.92	99.8	2.3	22

*The blank plasma used gave a small peak with the same retention as theophylline and corresponding to a concentration of 0.04 mg l^{-1} of theophylline. This peak was probably theophylline as the blank plasma chromatogram showed the presence of traces of caffeine for which theophylline is a metabolite [15].

An example of a separation of theophylline from metabolites and endogenous compounds (Fig. 3) is shown for a patient's blood plasma sample. One large endogenous peak was obtained in the chromatogram but it did not interfere with theophylline.

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