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SIMULTANEOUS DETERMINATION OF DIPROPHYLLINE, PROXYPHYLLINE AND THEOPHYLLINE IN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective and reliable high-performance liquid chromatographic assay for the simultaneous determination of diprophylline, proxyphylline and theophylline is described. The method involves a single extraction procedure followed by separation on an ODS reversed-phase column using a ternary solvent system. The assay is sufficiently rapid and sensitive to be applied for pharmacokinetic studies as well as for routine monitoring of patient's serum after therapeutic doses of the combined preparation. The practicability and utility of the proposed method is demonstrated in a pharmacokinetic study on four healthy volunteers.

INTRODUCTION

The methylxanthine derivatives diprophylline [7-(2,3-dihydroxypropyl)-theophylline] and proxyphylline [7-(2-hydroxypropyl)-theophylline] have therapeutic properties similar to those of theophylline and can be used for the treatment of obstructive lung diseases. There is some evidence that a combined preparation of these agents with theophylline (Neo-Biphylline^{R}) may exhibit less frequent adverse side-effects than an equivalent dose of theophylline alone [1, 2].

Although guidelines for the monitoring of these xanthines during therapy have not yet been developed, it can be expected that serum concentration measurements will be required in the same situations as proposed for theophylline [3]. Several methods exist to measure theophylline in biological fluids, the most popular being spectrophotometric assay [4], enzyme immunoassay [5], gas chromatography [6] and high-performance liquid chromatography (HPLC) [7—17]. In situations, however, where a combination of

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different xanthines is used, immunological methods are not suitable and, therefore, only chromatographic methods can be applied to measure these drugs simultaneously.

In the present study we describe a sensitive and selective HPLC method, using an isocratic reversed-phase system, to determine diprophylline, proxyphylline and theophylline in serum or plasma. By using this method, the pharmacokinetics of these three methylxanthines have been studied in four healthy volunteers after an oral administration of this drug combination.

MATERIALS AND METHODS

Ragents

All reagents were analytical grade. Proxyphylline and diprophylline were obtained from G. Streuli (Uznach, Switzerland). Theobromine was from Siegfried (Zofingen, Switzerland) and 8-chlorotheophylline from Aldrich (Milwaukee, WI, U.S.A.). All other reagents were purchased from E. Merck (Darmstadt, G.F.R.).

Chromatographic conditions

Analyses were performed on an HPLC system consisting of a Constametric II pump (Milton Roy, Philadelphia, PA, U.S.A.) and a Tracor Model 970 variable-wavelength absorbance detector (Tracor, Austin, TX, U.S.A.) set at 274 nm and 0.04 a.u.f.s. A 25 cm × 4.6 mm I.D. Ultrasphere 5-μm ODS reversed-phase column (Beckman, Berkeley, CA, U.S.A.) was used together with a 5 cm × 3.2 mm I.D. precolumn dry-filled with 30—38 μm Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The system was operated at 40°C at a flow-rate of 1.5 ml/min developing a pressure of about 170 bar. The mobile phase consisted of 0.01 *M* sodium acetate buffer (pH 5.2)–acetonitrile–methanol (91:6:3, v/v), and was degassed and filtered before use.

Sample preparation

Serum (0.5 ml) or plasma from patients and calibration standards were transferred to a 12-ml glass-tube followed by 0.2 ml of 0.1 *M* phosphate buffer, pH 7.0. Proteins were precipitated by addition of 3 ml of 2-propanol, containing 1.5 μg of 8-chlorotheophylline as internal standard. The mixture was intensively stirred for 5 sec at 40,000 rpm using a high-speed dental micromotor (Bien-Air, Bienne, Switzerland) equiped with a Teflon mixing head. After centrifugation for 2 min at 3500 *g* the supernatant was transferred to a conical glass-tube and evaporated at 60°C under a stream of nitrogen. The residue was dissolved in 50 μl of methanol and 10 μl were injected using a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.). All analyses were performed in duplicate.

Preparation of standard curves

For the standard curves stock solutions of theophylline, proxyphylline and diprophylline were made in water–ethanol (80:20, v/v). These solutions were further diluted with drug-free human serum to give final concentrations of 0.25, 1, 2, 4, 8, and 12 μg/ml. Peak height ratios between drug and internal

standard were plotted against drug concentrations and analysed with a linear regression method yielding straight lines for all three components.

Recovery and reproducibility

In order to estimate the analytical recovery, serum samples were spiked with 10 μg/ml theophylline, proxyphylline, diprophylline and 8-chlorotheophylline, and extracted as before. Peak height ratios of these samples were then compared with samples to which the same amounts of the three components were added after the preparation procedure just before injection. Within- and between-day reproducibility and accuracy were determined by analysing ten samples containing 1.5 and 10 μg/ml of the three components, on the same day and on ten different days.

Stability of serum samples

In order to assess the stability of theophylline, proxyphylline and diprophylline, fresh serum samples were stored at 4°C or 21°C, respectively, and assayed after 0, 1, 2, and 7 days.

Drug administration and sample collection

Four healthy volunteers (two males and two females) aged 23—32 years and weighing 51—82 kg received an oral dose of 300 mg of proxyphylline, 300 mg of diprophylline and 200 mg of theophylline dissolved in 40 ml of water. Blood samples were collected immediately before and at 5, 10, 20, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18, and 24 h after administration. After centrifugation plasma was stored frozen at −20°C until assayed.

RESULTS AND DISCUSSION

A number of solvents at various pH values has been evaluated for the HPLC analysis to give an optimal resolution of the xanthine analogues in the shortest time possible. With the ternary solvent system finally used and, due to the high resolution power of the 5-μm ODS column, the desired separation could be achieved within 16 min (Fig. 1). The pH of the solvent had to be carefully controlled as the retention time for diprophylline was very sensitive to small changes of pH. The retention times of the various compounds are listed in Table I. The interference of the caffeine metabolite 1,7-dimethylxanthine (paraxanthine) could be minimized, whereas in most of the previous published studies using a reversed-phase separation system, paraxanthine either could not be separated from theophylline or was not investigated at all [7—14]. However, some authors have reported a good separation of paraxanthine, using either a straight-phase HPLC system [16] or reversed-phase ion-pair gradient elution [17]. In order to reduce the viscosity of the mobile phase and, as a consequence, to reduce the back-pressure, the column temperature was maintained at 40°C during the HPLC analysis. Because we used only a single extraction step, a precolumn was found essential to prolong the life of the analytical column. The precolumn was replaced after 60 injections.

The analytical recoveries for theophylline, proxyphylline and diprophylline were between 94.7% and 96.0%, and for the internal standard, 8-chloro-

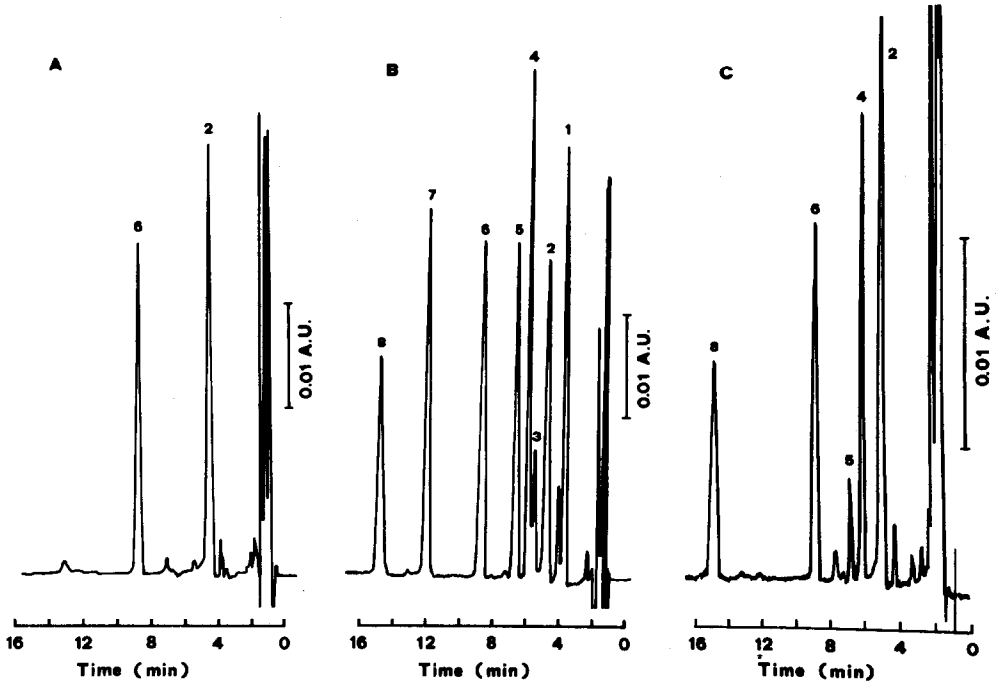

Fig. 1. Chromatograms of (A) control serum, (B) serum spiked with a mixture of xanthines, and (C) serum obtained after a single dose of 300 mg of diprophylline, 300 mg of proxyphylline and 200 mg of theophylline. 1 = Theobromine, 2 = unknown serum constituent, 3 = paraxanthine, 4 = theophylline, 5 = diprophylline, 6 = internal standard (8-chlorotheophylline), 7 = caffeine, 8 = proxyphylline.

TABLE I

RETENTION TIMES OF THE INVESTIGATED XANTHINE DERIVATIVES

Conditions of separation were as described in the text.

Xanthine derivatives	Retention time (min)
Theobromine	3.8
Paraxanthine	5.7
Theophylline	5.95
Diprophylline	6.7
8-Chlorotheophylline	8.85
Caffeine	12.1
Proxyphylline	15.1

theophylline, this recovery was 90.1%. Standard curves for all three xanthines are displayed in Fig. 2. They are strictly linear with coefficients of correlation better than 0.999.

Within-day and between-day reproducibility and accuracy for serum samples containing 1.5 and 10 μg of diprophylline, proxyphylline and theophylline per ml was excellent as summarized in Table II. Based on a signal-to-noise ratio of

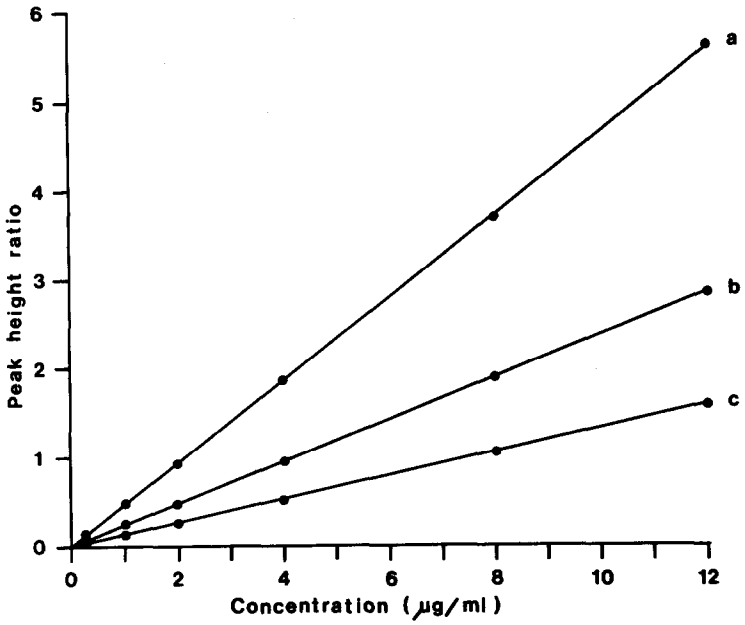

Fig. 2. Standard curves of theophylline (a), diprophylline (b) and proxyphylline (c) using 8-chlorotheophylline as internal standard.

TABLE II

PRECISION AND ACCURACY OF THE HPLC METHOD FOR THE DETERMINATION OF THEOPHYLLINE, DIPROPHYLLINE AND PROXYPHYLLINE IN PATIENT'S SERUM

Compound	Serum conc. (μg/ml)	Within-day (n = 10)			Day-to-day (n = 10)		
		Mean conc. (μg/ml)	C.V.* (%)	M.E.** (%)	Mean conc. (μg/ml)	C.V.* (%)	M.E.** (%)
Diproxyphylline	1.5	1.56	1.28	4.0	1.50	2.67	0
	10	10.1	0.89	1.0	9.96	1.6	0.4
Proxyphylline	1.5	1.53	1.31	2.0	1.48	2.03	1.3
	10	10.1	1.88	1.0	9.89	1.11	1.1
Theophylline	1.5	1.56	0.64	4.0	1.50	1.33	0
	10	9.97	0.90	0.3	9.95	1.11	0.5

*C.V. = coefficient of variation.
**M.E. = mean error.

3:1, the detection limits were 0.2 μg/ml for diprophylline, 0.25 μg/ml for proxyphylline and 0.1 μg/ml for theophylline.

The stability of all three investigated xanthines in serum at 4°C or room temperature was good. For up to seven days no significant reduction of the serum concentration could be observed. This is of practical importance, as

often the turn-around time for patient samples might be extended, especially over weekends.

The utility of the proposed HPLC method could be demonstrated by a pharmacokinetic study in four healthy volunteers. A dose of 300 mg of diprophylline, 300 mg of proxyphylline and 200 mg of theophylline was given orally to each of the participants. In Fig. 3 an example of the concentration—time course of one volunteer is displayed. After a very rapid absorption of the oral solution, proxyphylline and theophylline serum concentrations could be followed for at least 25 h, and the more-rapidly eliminated diprophylline for about 8 h. The serum concentrations were fitted to a one-compartment open model for oral dosage and the corresponding pharmacokinetic parameters were calculated on a Hewlett-Packard HP 85 desktop computer using the G-PHARM pharmacokinetic program developed by Gomeni and Gomeni [18]. The pharmacokinetic data for all three xanthines are listed in Table III. There is no evidence that the pharmacokinetics are influenced by administering these three xanthines together, as the calculated parameters were similar to those published before for single administration of each of these drugs [19—21]. The following antibiotics and other drugs sometimes used concomitantly with xanthines in patients with chronic bronchitis did not interfere with the HPLC method: carbenicillin, cefoperazone, cephacetril, penicillin G, diphenylhydantoin, phenobarbital and heparin. Only cefoxitin had a retention time similar to that of the internal standard.

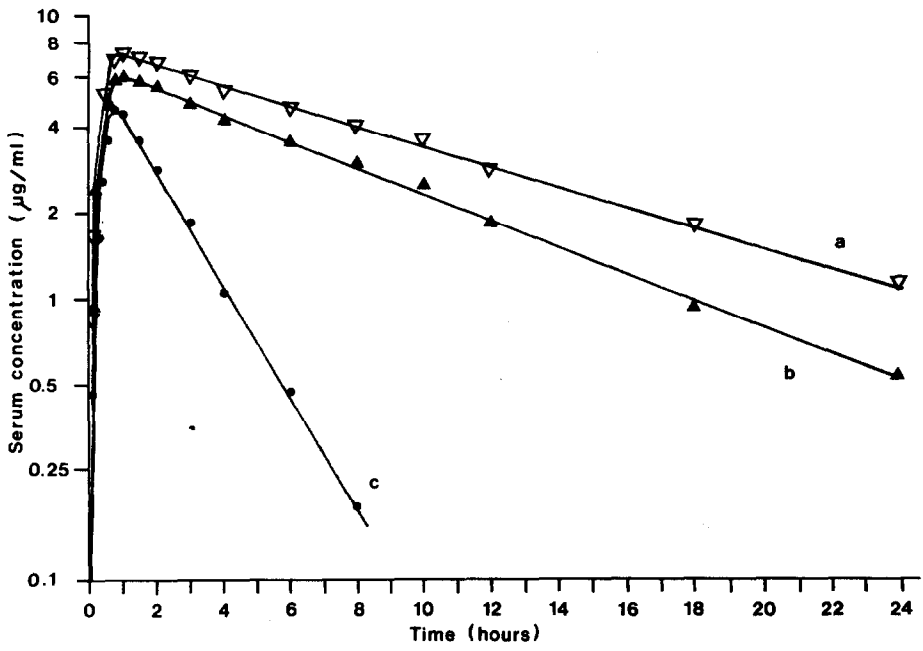

Fig. 3. Serum concentration—time curves after oral administration of 200 mg of theophylline (a), 300 mg of proxyphylline (b) and 300 mg of diprophylline (c).

TABLE III

PHARMACOKINETIC PARAMETERS* CALCULATED FROM SERUM CONCENTRATIONS AFTER ORAL ADMINISTRATION OF 300 mg OF DIPROPHYLLINE, 300 mg OF PROXYPHYLLINE AND 200 mg OF THEOPHYLLINE TO FOUR HEALTHY VOLUNTEERS

Values are expressed as mean ± S.D.

	Compound		
	Diprophylline	Proxyphylline	Theophylline
k_a (h^{-1})	2.47 ± 0.56	2.48 ± 1.30	2.14 ± 1.22
$t_{½a}$ (h)	0.29 ± 0.07	0.33 ± 0.16	0.39 ± 0.16
C_{max} (mg l^{-1})	4.55 ± 0.42	6.87 ± 1.53	5.97 ± 1.27
T_{max} (h)	1.25 ± 0.61	1.12 ± 0.55	1.50 ± 0.58
β (h^{-1})	0.348 ± 0.057	0.080 ± 0.003	0.105 ± 0.007
$t_{½\beta}$ (h)	2.03 ± 0.30	8.69 ± 0.28	6.64 ± 0.46
Cl** (l h^{-1} kg^{-1})	0.295 ± 0.044	0.051 ± 0.003	0.048 ± 0.019
V_β** (l kg^{-1})	0.862 ± 0.079	0.637 ± 0.041	0.464 ± 0.014

*k_a = absorption rate constant, $t_{½a}$ = absorption half-life, C_{max} = maximum serum concentration, t_{max} = time to reach C_{max}, β = elimination rate constant, $t_{½\beta}$ = elimination half-life, Cl = total body clearance, V_β = apparent volume of distribution.
**Cl and V_β are calculated assuming total absorption of the drugs [19, 22, 23].

CONCLUSION

With the highly selective HPLC method proposed in this study it is possible to determine diprophylline, proxyphylline and theophylline simultaneously in patient serum after therapeutic doses. The assay is fast, simple and reliable and is, therefore, very suitable for the routine laboratory. In addition, due to its sensitivity, this method can also be used for pharmacokinetic studies.

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