Journal of Chromatography, 380 (1986) 431—436
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

CHROMBIO. 3156

Note

Simple and sensitive method for the simultaneous determination of enprofylline, theobromine, paraxanthine, theophylline and caffeine using high-performance liquid chromatography

N. GRGURINOVICH

Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, South Australia 5042 (Australia)

(First received October 21st, 1985; revised manuscript received March 5th, 1986)

Enprofylline (3,7-dihydro-3-propyl-1*H*-purine-2,6-dione) is a new xanthine derivative currently undergoing clinical investigation as a bronchodilator. It has been shown that enprofylline is five times more potent than theophylline as a bronchodilator in both animal and human experiments [1, 2], and that enprofylline is also free of the central nervous system (CNS) stimulatory side-effects of theophylline [1, 3, 4].

In patients receiving enprofylline for obstructive airways disease it is likely that they will also have theobromine, theophylline, caffeine and paraxanthine present in their blood. These methylxanthines, except for paraxanthine which is the major metabolite of caffeine, are found in a variety of foods and beverages, and are all pharmacologically active [4]. In addition, theophylline is used therapeutically as a bronchodilator and significant amounts of this particular methylxanthine may be present in those patients considered for enprofylline therapy. The presence of theophylline, caffeine, theobromine and paraxanthine could exert an additional effect to enprofylline. The nature and degree of these effects is dependent on the xanthine and its plasma concentration and it would be desirable to measure the individual drug contributions by measuring the plasma concentration of each methylxanthine and enprofylline.

In the past theophylline, theobromine, paraxanthine and caffeine have been measured by methods using gas chromatography or high-performance liquid chromatography (HPLC) [5—9]. To date only one HPLC method has been published for enprofylline [10] and no method has been reported for the simultaneous measurement of the methylxanthines and enprofylline.

0378-4347/86/$03.50

Adaptation of previously published methods for the analysis of either the methylxanthines or enprofylline by HPLC were tried in order to give a method for the simultaneous determination of these compounds. These attempts were unsuccessful because not all components were adequately resolved [7, 10] or not all components could be extracted by the sample preparation procedure [10]. A method using *n*-tetrabutylammonium hydroxide (TBAH) in the mobile phase reported good separation of the methylxanthines [8]. A similar method using TBAH in the mobile phase was adopted by this investigator and initially gave good separation of all the components but abandoned owing to rapid deterioration of column efficiency.

The method described in this paper uses an isocratic HPLC system with a simple solvent extraction technique to purify the sample for analysis and gives baseline separation of the methylxanthines and enprofylline. The method is sensitive (< 0.1 mg/l depending on the xanthine), reproducible, has a low sample volume requirement (0.2 ml) and a total chromatography time of 13 min making it suitable for the therapeutic monitoring of enprofylline or for research purposes where greater sensitivity is required.

Reagents

Dichloromethane, isopropanol, tetrahydrofuran, sodium dihydrogen phosphate, disodium hydrogen phosphate and orthophosphoric acid were supplied by Ajax Chemicals (Sydney, Australia), and were analytical-reagent grade. Caffeine, theophylline, paraxanthine, theobromine, *p*-phenoxyacetic acid and TBAH (40%, w/w) were supplied by Sigma (St. Louis, MO, U.S.A.). Enprofylline and the internal standard (3,7-dihydro-1-ethyl-3-(2-hydroxypropyl)-1*H*-purine-2,6-dione, D4126) were supplied by Astra Pharmaceuticals (Sydney, Australia). Horse serum (not inactivated) was supplied by C.S.L. (Melbourne, Australia).

Equipment and chromatography

A Waters HPLC system was used which consisted of a Model 440 fixed-wavelength detector fitted with a 280-nm filter, an M6000 solvent delivery system, a WISP automatic sample injector and a dual-pen recorder.

A Regis octyl Hi-Chrom reversed-phase column, 5 μm particle size, 25 cm × 4 mm, was used. The mobile phase consisted of a 3% tetrahydrofuran solution containing 10 m*M* disodium phosphate with the pH adjusted to 6.5 using dilute phosphoric acid. The mobile phase was filtered through a Millipore 0.45-μm filter under reduced pressure before use. The solvent flow-rate was 2.0 ml/min, the a.u.f.s. setting was 0.1 and the pen settings on the chart recorder were 2 and 10 mV for full scale deflection. No guard column was used and the chromatography was performed at ambient temperature.

Standards and reagents

Stock solutions of enprofylline, theobromine, paraxanthine, theophylline and caffeine were prepared in distilled water each having a concentration of 1000 mg/l. These solutions were stable for at least six months at room temperature. Combined aqueous working standards containing 200, 100, 50, 25, 10 and 5 mg/l were prepared weekly and these were diluted 1:9 with horse

serum daily to give serum standards containing 20, 10, 5, 2.5, 1.0 and 0.5 mg/l. The internal standard was prepared by dissolving 0.088 g of this substance in 100 ml of methanol. A 0.1-ml aliquot of this solution was diluted to 1 l with dichloromethane—propanol (95:5).

The phosphate buffer required in the extraction step was prepared by dissolving 1.56 g of sodium dihydrogen phosphate (dihydrate) and 2.82 g of disodium hydrogen phosphate in 20.0 ml of distilled water giving a solution with a pH of approximately 7.2.

Method

To labelled 15-ml culture tubes fitted with PTFE-lined screw caps were added 0.2 ml of sample or standards, followed by 0.1 ml of phosphate buffer (pH 7.2) and 6.0 ml of dichloromethane—propanol (95:5) containing the internal standard. The contents of the tube were vortex-mixed for 1 min and then centrifuged at 1500 *g* for 5 min to separate the aqueous and organic layers. The upper aqueous layer was aspirated and discarded and the organic layer decanted into a clean dry conical centrifuge tube. The organic solvent was removed using a vortex evaporator and the residue reconstituted in 0.2 ml of mobile phase. Thorough reconstitution was achieved by vortex-mixing the tube contents for 1 min. An aliquot (0.06—0.12 ml) was injected onto the column.

RESULTS AND DISCUSSION

In this method horse serum is used to prepare standards instead of human serum or plasma because it is difficult to obtain the latter in sufficient quantity completely free of xanthines. When human xanthine-free plasma is extracted according to the method described no endogenous interfering peaks are obtained (Fig. 1A) and is similar to the chromatogram for blank horse serum (Fig. 1B). Fig. 1C is the chromatogram of a 1.0 mg/l standard prepared in horse serum. Theobromine, paraxanthine, theophylline, caffeine, enprofylline and the internal standard have retention times of 2.5, 4.1, 5.0, 7.8, 10.4 and 13.0 min, respectively. Fig. 1D is the chromatogram of a patient sample showing the presence of enprofylline and the other methylxanthines.

In the analysis of methylxanthines the separation of paraxanthine and theophylline is always a problem. In the past investigators have used small percentages of tetrahydrofuran as a modifier in mobile phases containing methanol or acetonitrile [7] to give near baseline separation of these components. Also, mobile phases containing the ion-pairing reagent TBAH have been used to give complete separation of theophylline and paraxanthine in addition to the separation of all the other xanthines and their metabolites [8]. The method developed here uses an octyl, 5 μm particle size, reversed-phase column with a mobile phase containing 3% tetrahydrofuran to give complete baseline separation of the desired components. An alternative system using a Waters phenyl reversed-phase column (10 μm particle size, 30 cm × 3.9 mm) and a mobile phase containing an ion-pairing reagent was developed. A mobile phase containing 5% isopropanol—10 m*M* sodium acetate—3 m*M* TBAH with the pH adjusted to 7.0 gave good separation of the components except for enprofylline

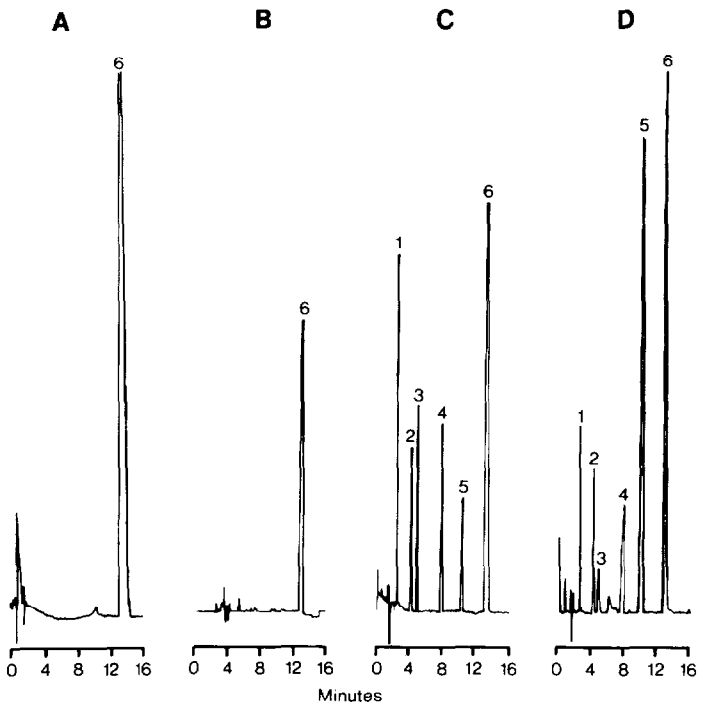

Fig. 1. Chromatograms of extracted horse serum and patient plasma showing enprofylline, the methylxanthines and internal standard. (A) Human plasma blank; (B) horse serum blank; (C) horse serum containing 1.0 mg/l each of enprofylline and the methylxanthines; (D) patient sample. Peaks: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = caffeine; 5 = enprofylline; 6 = internal standard, D4126.

and the internal standard. An alternative internal standard, phenoxyacetic acid, is used and the phosphate buffer solution used in the extraction procedure is prepared in a 4% solution of the ion-pairing reagent. This is necessary in order to obtain quantitative extraction of phenoxyacetic acid. The method using the ion-pair mobile phase, although sensitive, had a disadvantage in that the ion-pairing reagent caused the column to degenerate rapidly. With time, increasing amounts of the ion-pairing reagent were required in the mobile phase to maintain baseline separation of all the components in the chromatogram. Eventually the column could not give the required separation of the xanthines without the total chromatography time becoming too long, approximately 25 min. With the method initially described no changes in the concentration of the components of the mobile phase has been made over the four months of operation, during which time 500 samples have been analysed. This indicates good chromatographic stability and column life and is therefore the method of choice for the determination of enprofylline, theobromine, paraxanthine, theophylline and caffeine.

The recovery of each xanthine at each of six calibration concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 mg/l) was determined in triplicate. The mean (n = 18) recoveries for enprofylline, theobromine, paraxanthine, theophylline and caffeine over the calibration range 0.5—20.0 mg/l were 103, 95.1, 97.4,

TABLE I

WITHIN- AND BETWEEN-DAY EVALUATION OF PRECISION

Compound	Concentration added (mg/l)	Concentration found (mean ± S.D.) (mg/l)	n	Coefficient of variation (%)
Within-day				
Theobromine	5.0	4.86 ± 0.099	10	2.05
	0.5	0.53 ± 0.011	7	2.02
Paraxanthine	5.0	4.93 ± 0.083	10	1.66
	0.5	0.50 ± 0.025	7	5.09
Theophylline	5.0	4.89 ± 0.097	10	1.97
	0.5	0.49 ± 0.016	7	3.27
Caffeine	5.0	5.08 ± 0.099	10	1.97
	0.5	0.53 ± 0.01	7	1.79
Enprofylline	5.0	4.71 ± 0.104	10	2.20
	0.5	0.48 ± 0.016	7	3.40
Between-day				
Theobromine	5.0	5.08 ± 0.296	16	5.83
	0.5	0.51 ± 0.032	16	6.22
Paraxanthine	5.0	5.01 ± 0.236	16	4.72
	0.5	0.49 ± 0.029	16	5.87
Theophylline	5.0	4.98 ± 0.302	16	6.07
	0.5	0.50 ± 0.026	16	5.21
Caffeine	5.0	5.02 ± 0.240	16	4.78
	0.5	0.50 ± 0.031	16	6.21
Enprofylline	5.0	4.85 ± 0.257	16	5.29
	0.5	0.49 ± 0.025	16	5.10

97.5 and 90.7%, respectively. The mean recovery of the internal standard was calculated from five measurements and found to be 85.2%.

Horse serum was spiked with enprofylline and the methylxanthines to give quality controls containing 0.5 and 5.0 mg/l of each component. These were analysed on a within-day and between-day basis to evaluate precision and reproducibility (see Table I). The coefficients of variation were all less than 7.0% (between day) and 5.0% (within day).

A six-point calibration curve is used for every set of analyses. The peak-height ratios (peak height of compound/peak height of internal standard) of each component in each standard are plotted against concentration and give a straight line calibration curve for each compound. Linear regression analysis is performed on each set of calibration data and the following linear equations were obtained for a typical data set: enprofylline, $y = 0.318x - 0.036$ ($r = 0.999$); theobromine, $y = 0.833x - 0.038$ ($r = 0.999$); paraxanthine, $y = 0.419x - 0.053$ ($r = 0.999$); theophylline, $y = 0.533x - 0.059$ ($r = 0.999$), caffeine, $y = 0.361x - 0.044$ ($r = 0.999$).

The limit of determination for the method is 0.05 mg/l for theobromine and theophylline and 0.10 mg/l for paraxanthine, enprofylline and caffeine. However, by reducing the amount of internal standard added and choosing a more sensitive setting of the chart recorder or detector the limit of determination can easily be improved. Thus, this method is sensitive and specific for

enprofylline and the methylxanthines and is highly reproducible making it useful for research purposes or therapeutic monitoring of this compound.

REFERENCES

1 C.G.A. Persson and G. Kjellin, Acta Pharmacol. Toxicol., 49 (1981) 313.
2 C.G.A. Persson, I. Erjefalt and J.-A. Karlsson, Acta Pharmacol. Toxicol., 49 (1981) 317.
3 E. Lunell, N. Svedmyr, K.-E. Andersson and C.G.A. Persson, Eur. J. Clin. Pharmacol., 22 (1982) 395.
4 C.G.A. Persson, J.-A. Karlsson and I. Erjefalt, Life Sci., 30 (1982) 2181.
5 H.A. Schwertner, Clin. Chem., 25 (1979) 212.
6 M. Kowblansky, B.M. Scheinthal, G.D. Cravello and L. Chafetz, J. Chromatogr., 76 (1973) 467.
7 R. Hartley, J.R. Cookman and I.J. Smith, J. Chromatogr., 306 (1984) 191.
8 K.T. Muir, J.H.G. Jonkman, D.S. Tang, M. Kunitani and S. Riegelman, J. Chromatogr., 221 (1980) 85.
9 T. Foenander, D.J. Birkett, J.O. Miners and L.M.H. Wing, Clin. Biochem., 13 (1980) 132.
10 E. Lunell, O. Borga and R. Larsson, Eur. J. Clin. Pharmacol., 26 (1984) 87.