Journal of Chromatography, 232 (1982) 450—455
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1392

Note

High-performance liquid chromatographic method for the determination of diprophylline in human serum

N. PATERSON

Department of Chemical Pathology, University of Aberdeen, Foresterhill, Aberdeen (Great Britain)

(First received November 3rd, 1981; revised manuscript received June 21st, 1982)

Diprophylline [7-(2,3-dihydroxypropyl)theophylline, dyphylline] is a xanthine derivative which has been available for many years as a possible substitute for theophylline in the treatment of bronchospasm, and it has been suggested that it may show a lower incidence of side-effects than does theophylline [1]. The effectiveness of diprophylline therapy remains to be established [2] and blood level measurements are needed in conjunction with clinical investigations in order to establish appropriate dosage regimes which produce the desired therapeutic response.

In early studies of xanthine derivatives, the UV absorption method of Schack and Waxler [3] was used, but because of the poor specificity of this method, interpretation of results can be difficult, especially if dietary xanthine intake is not restricted. Specific gas chromatographic methods have been developed [4] and have been applied to pharmacokinetic studies in human volunteers [5]. These methods have good specificity and sensitivity but sample preparation can be complex or time-consuming. High-performance liquid chromatography (HPLC) has found wide acceptance in the measurement of serum theophylline levels [6—11] and has been applied to the measurement of diprophylline in human serum [12—14] and in serum and urine [15]. The direct injection of a trichloroacetic acid filtrate, as used by Valia et al. [14], results in a short column life and some interference by endogenous metabolites when a C_8 column is used, and the method of Simons and Simons [15] has low sensitivity.

The method described below was devised to allow routine measurement on a C_8 column of diprophylline in small serum samples (250 μl) from patients on a variety of other drugs and from volunteers participating in absorption studies.

0378-4347/82/0000—0000/$02.75 © 1982 Elsevier Scientific Publishing Company

EXPERIMENTAL

Materials and reagents

The following xanthine derivatives were purchased from Sigma, London, Great Britain: 1,3-dimethyluric acid, 8-chlorotheophylline, 3-methylxanthine, theobromine (3,7-dimethylxanthine), diprophylline [7-(2,3-dihydroxypropyl)-theophylline], 2-hydroxyethyltheophylline, theophylline (1,3-dimethylxanthine), proxyphylline [7-(2-hydroxypropyl)theophylline], caffeine (1,3,7-trimethylxanthine). All other reagents used were of analytical grade.

The internal standard used was theophylline at a concentration of 30 mg/l in water. It was prepared fresh daily by dilution from a 3 g/l aqueous stock solution.

The extraction solvent was chloroform—methanol (9:1, v/v).

Calibration standards were prepared by the addition of diprophylline to pooled bovine serum and were stored at −16°C. Peak height ratios of diprophylline and internal standard were plotted to give calibration graphs. Repeatability studies were performed on samples of pooled human serum to which diprophylline had been added.

Diprophylline absorption was studied in four adult male volunteers. After fasting overnight, 400 mg of diprophylline were taken by mouth, and 5-ml blood samples were taken at time zero, and at each half hour for 3 h then hourly until 6 h had elapsed. The samples were allowed to clot and the separated serum was stored at −16°C until analysis.

Samples from patients not taking diprophylline but on a variety of other drugs were residues of samples sent to this laboratory for a variety of tests.

Extraction procedure

Internal standard (50 μl) and 250 μl of sample were placed in a 10-ml conical glass centrifuge tube and vortex mixed. Anhydrous sodium sulphite (1.5 g) was then added and the tube capped and shaken vigorously for 30 sec with 2.5 ml extraction solvent. These steps were carried out with the minimum of delay to avoid the formation of a solid cake of sodium sulphite. After centrifugation at 1000 *g* for 5 min, the aqueous layer was discarded and the solvent filtered into a 10-ml conical centrifuge tube through a Whatman No. 1 filter paper which had been pre-wetted with chloroform. The filter paper was rinsed with 0.5 ml of chloroform. The filtrate was dried under nitrogen at 40°C, redissolved in 100 μl of dichloroethane and back-extracted into 100 μl of 0.1 *M* ammonium carbonate by vortex mixing for 10 sec. Centrifugation at 1000 *g* for 5 min produced a clear aqueous layer which was used for chromatography.

HPLC conditions

Chromatography was carried out on LiChrosorb RP-8, 10 μm, supplied by E. Merck, Darmstadt, G.F.R., packed at 300 bars in a 25 cm × 4 mm stainless steel column as a suspension in methanol. Eluant was delivered by an Altex 110 pump at 2.0 ml/min and absorbance of the eluate was monitored at 275 nm with an Hitachi 100-10 spectrophotometer using an Altex 8-μl flow cell with full-scale deflection set at 0.02. A Rheodyne 7125 syringe loading injection valve fitted with a 20-μl loop was used to introduce 10-μl samples.

The HPLC eluant was prepared by adding 250 ml of methanol to 750 ml of phosphate buffer prepared by adding 1.5 ml of 1 M KH_2PO_4 to 750 ml of glass-distilled water and adjusting to pH 3.0 with 0.9 M perchloric acid. The eluant was degassed ultrasonically and filtered before use. Chromatography was carried out at ambient temperature (18—22° C).

RESULTS

Representative chromatograms are shown in Fig. 1. Overall recovery was estimated by comparison of the peak heights found when known amounts of aqueous diprophylline standard were injected and the peak heights found when serum standards were carried through the entire procedure. The results shown in Table I are the mean of five measurements at each level. Calibration was achieved by plotting the peak height ratio of diprophylline and internal standard versus added diprophylline concentration and was linear to 20 mg/l with a linear regression equation $Y = 0.484X + 0.27$; the correlation coefficient was 0.9995. Although linearity extended beyond 20 mg/l, the peak height ratio was unacceptably high above this concentration and samples with higher concentrations were diluted with blank serum and re-extracted.

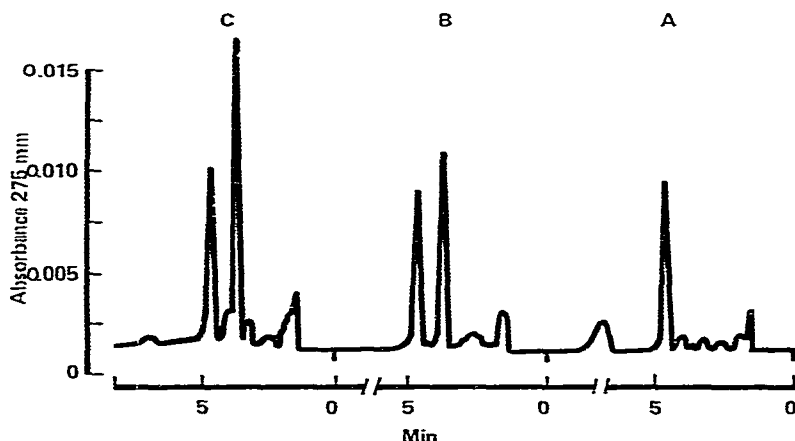

Fig. 1. HPLC of diprophylline standards and a human serum sample. (A) Blank human serum extract with added internal standard. (B) Serum standard containing internal standard and 5 mg/l diprophylline. (C) Patient's sample containing internal standard and 8 mg/l diprophylline. HPLC conditions as stated in the text.

Selectivity was studied in two ways. First, various xanthine derivatives were prepared as aqueous solutions and after injection of 0.1-μg amounts their retention times relative to diprophylline were measured. The results are shown in Table II. Second, in an attempt to exclude possible interference by a wide range of drugs and their metabolites, samples from patients on a variety of drugs but not taking diprophylline were extracted and chromatographed. No interferences were found for the following drugs: acetazolamide, amitriptyline, amylobarbitone, carbamazepine, diazoxide, disopyramide, ethosuximide, isoprenaline, nitrazepam, nortriptyline, oxazepam, paracetamol, pentobarbitone,

TABLE I

PERCENTAGE RECOVERY OF DIPROPHYLLINE ADDED TO HUMAN SERUM

Results shown represent the mean of five measurements at each level.

Concentration added (mg/l)	Calculated peak height* for 100% recovery (mm)	Measured peak height (mm)	Percentage recovery
2.5	18	16.5	92
5	36	35	97
10	72	71	99

*Based on the measured peak height after injection of known amounts of aqueous standard.

TABLE II

RETENTION TIMES OF SOME XANTHINE DERIVATIVES RELATIVE TO DIPROPHYLLINE

1,3-Dimethyluric acid	0.42
8-Chlorotheophylline	0.66
3-Methylxanthine	0.70
Theobromine	0.75
Diprophylline	1.00
β-Hydroxyethyltheophylline	1.22
Theophylline	1.33
β-Hydroxypropyltheophylline	1.90
Caffeine	2.11

TABLE III

WITHIN-BATCH AND BETWEEN-BATCH PRECISION OF DIPROPHYLLINE MEASUREMENTS AT THREE LEVELS OF DIPROPHYLLINE ADDED TO POOLED HUMAN SERUM

Concentration added (mg/l)	Within-batch (n = 10)		Between-batch (n = 15)	
	Mean ± S.D. (mg/l)	C.V. (%)	Mean ± S.D. (mg/l)	C.V. (%)
2.5	2.47 ± 0.13	5.3	2.32 ± 0.18	7.2
5.0	5.03 ± 0.24	4.7	4.95 ± 0.27	5.3
10.0	10.22 ± 0.51	5.0	9.95 ± 0.64	6.3

phenobarbitone, phenytoin, primidone, procainamide, salicylate, salbutamol, sulthiame.

The precision of the method within batch and between batch was assessed at three levels (Table III). The within-batch precision was measured by extracting and chromatographing ten replicate samples within one working day. The between-batch precision was measured over a period of 30—40 days.

Within-batch variation in the peak height of the internal standard was found to be less than ± 10% of the mean peak height.

Fig. 2 shows the mean serum levels found in four normal subjects following an oral dose of 400 mg of diprophylline. The calculated mean half-life was 2.3 h.

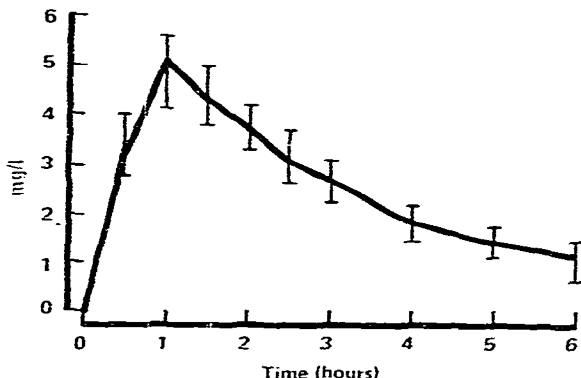

Fig. 2. Mean serum diprophylline concentration found in four subjects after taking an oral dose of 400 mg of diprophylline. The vertical bars indicate the range of concentrations found at each time.

DISCUSSION

Diprophylline has a solubility of 1 part in 10 of water and a correspondingly low solubility in organic solvents [16], so that many commonly used extraction techniques are unsatisfactory and sample preparation methods based on protein precipitation may have advantages. However, it was found in preliminary experiments that direct injection of trichloroacetic acid treated serum on to RP-8 columns resulted in a short column life and interference by endogenous serum constituents. Attempts to purify trichloroacetic acid treated serum by extraction into organic solvents led to low recoveries and loss of sensitivity. The difficulty of low solubility in organic solvents was overcome by the salting-out effect of the addition of sodium sulphite. If salting-out was not used, recoveries of approximately 10—20% were observed rather than the 92—99% recovery shown in Table I. The second extraction step was used to minimise the extraction of other drugs and of endogenous serum constituents. The value of this extraction is illustrated by the wide diversity of types of drug and their metabolites that were shown not to cause interference.

Theophylline was used as the internal standard. It has the required extraction, chromatographic and UV absorption characteristics and it is unlikely that a patient would be treated with these two xanthine derivatives simultaneously. The within-run repeatability of the internal standard peak height was always within ± 10%, so the presence of significant amounts of theophylline in any patient's sample would be indicated by a readily detectable increase in the internal standard peak height. If it is necessary to determine theophylline and diprophylline in the same sample, β-hydroxypropyltheophylline is a suitable alternative internal standard.

Most published work on the HPLC of xanthine derivatives makes use of C_{18} packing materials and acetonitrile-based eluants. In the present method, methanol is used as a much less toxic alternative to acetonitrile and the use of a methanol-based eluant and an octyl bonded silica packing allows good separation of theophylline and diprophylline from endogenous serum constituents. A single RP-8 column has been in routine use for 12 months with only occasional repacking of the top 1 mm being required to maintain resolution.

The sensitivity of the method allows the determination of 1 mg/l concentrations of diprophylline in serum with a coefficient of variation of $< 10\%$. Ten serum samples, three calibration standards and a quality control sample can be extracted and chromatographed in a 2.5-h period.

The results obtained on the four volunteers are in good agreement with previous published results. The short half-life of diprophylline and the low peak serum levels achieved after ingestion of doses comparable to the commonly used theophylline doses suggest that frequent large doses of this drug are likely to be required to achieve the deserved therapeutic effects. Studies on various doses and routes of administration in a variety of clinical situations are proceeding.

REFERENCES

1 P.V. Mancy, J.W. Jones, E.G. Gross and H.M. Korns, J. Amer. Pharm. Ass., 35 (1946) 266.
2 L. Hendeles and M. Weinberger, Drug Intell. Clin. Pharm., 11 (1977) 424.
3 J.A. Schack and S.H. Waxler, J. Pharmacol., 97 (1949) 283.
4 W.C. Butts, V.A. Raisys, M.A. Kenny and C.W. Bierman, J. Lab. Clin. Med., 84 (1974) 451.
5 F.E.R. Simons, K.J. Simons and C.W. Bierman, J. Allergy Clin. Immunol., 56 (1975) 347.
6 J.J. Orcutt, P.P. Kozak, S.A. Gillman and L.H. Cummins, Clin. Chem., 23 (1977) 599.
7 G.W. Peng, M.A.F. Gadala and W.L. Chiou, Clin. Chem., 24 (1978) 357.
8 Z.K. Shihabi, Clin. Chem., 24 (1978) 1630.
9 N. Weidner, J.M. McDonald, V.L. Tieber, C.H. Smith, G. Kessler, J.H. Laderson and D.N. Dietzler, Clin. Chim. Acta, 97 (1979) 9.
10 M.J. Cooper, B.L. Mirkin and M.W. Anders, J. Chromatogr., 143 (1977) 324.
11 J.W. Nelson, A.L. Cordy, C.G. Aron and R.A. Bartell, Clin. Chem., 23 (1977) 124.
12 P.K. Ng and R.A. Locock, Res. Commun. Chem. Pathol. Pharmacol., 26 (1979) 509.
13 L. Gisclon, K. Rowse and J. Ayres, Res. Commun. Chem. Pathol. Pharmacol., 23 (1979) 523.
14 K.H. Valia, C.A. Hartman, N. Kucharczyk and R.D. Sofia, J. Chromatogr., 221 (1980) 170.
15 K.J. Simons and F.E.R. Simons, J. Pharm. Sci., 68 (1979) 1327.
16 E.G.C. Clarke (Editor), Isolation and Identification of Drugs, Vol. 1, The Pharmaceutical Press, London, 1969, p. 318.