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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF THEOPHYLLINE IN BIOLOGICAL FLUIDS

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SUMMARY

A method has been developed to determine the level of theophylline in biological fluids. The sample is acidified and then extracted with a mixture of chloroform and isopropanol containing oxazepam as an internal standard. The methylxanthines are separated by high-pressure liquid chromatography using oxypropionitrile on a Porasil C column packing and quantitated in the effluent by UV absorption. This technique avoids special treatment of the plasma to eliminate caffeine and analyzes the materials at temperatures at which they are stable. The method is rapid, specific and precise.

INTRODUCTION

Methylxanthines have been pharmacologically important for many years and the effects of 1,3-dimethylxanthine (theophylline), 1,3,7-trimethylxanthine (caffeine) and 3,7-dimethylxanthine (theobromine) are well known. Caffeine and theobromine occur naturally in the diet, whereas theophylline is primarily a pharmaceutical agent used in the treatment of several disorders for its bronchodilating and diuretic effects.

The method most commonly used for analysis of the methylxanthines in biological fluids is that of Schack and Waxler, which was described in 1949[1], and is based on ultraviolet absorption following partial purification by differential extraction. The accuracy of this method is subject to the interference by other methylxanthines with similar absorption and extraction characteristics. Interference by caffeine in the determination of theophylline can be eliminated by degradation with heat in alkaline solution. This procedure still does not eliminate interference by theobromine. Although solutions of pure methylxanthines may be satisfactorily separated by gas–liquid chromatography[2], extractable endogenous substances in plasma or serum (Fig. 1) may interfere. The late appearance of these unknown compounds in the chromatogram limits the application of this method when large numbers of samples of biological fluids are to be analyzed.

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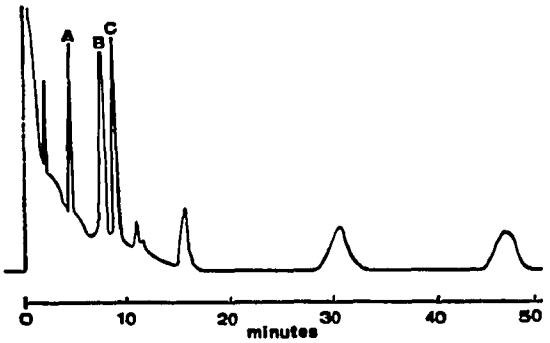

Fig. 1. Representative gas–liquid chromatographic separation of (A) 0.25 μg of caffeine, (B) 0.55 μg of theophylline and (C) internal standard (SC 4598) extracted from plasma. Peaks of unknown substances appear between 10 and 50 min. Operating conditions: packing, 1.5% OV-17 on Gas-Chrom Q; column temperature, 190°; injector temperature, 250°; detector temperature, 265°.

Wu and Siggia[3] described the separation of purine and strychnine alkaloids using high-speed liquid chromatography. This technique avoids the possible thermal degradation of these compounds and provides for the potential separation of all methylxanthines. These investigators did not, however, describe methods for the analysis of methylxanthines in biological fluids. We report here the precision and accuracy of a new high-pressure liquid chromatographic (HPLC) method for the analysis of theophylline in biological fluids.

MATERIALS AND METHODS

Apparatus

A Perkin-Elmer 1220 high-pressure liquid chromatograph with column temperature control and gradient generation accessories and a Nester Faust 1240 VI liquid chromatograph were utilized. A 250-μl injection loop was attached to the injection valve. A 4.2 mm × 2 m stainless-steel fitted column was packed with 36–75 mesh, permanently bonded oxypropionitrile on Porasil C, Durapak OPN (Waters Ass., Framingham, Mass., U.S.A.).

Reagents

Theophylline, nanograde chloroform and hexane (Matheson, Coleman & Bell, Kansas City, Mo., U.S.A.), caffeine and theobromine (Sigma, St. Louis, Mo., U.S.A.), 1-methylxanthine and 7-methylxanthine (K and K Labs., Plainview, N.Y., U.S.A.), analytical-reagent grade isopropanol (Mallinkrodt, St. Louis, Mo., U.S.A.) and oxazepam (generously supplied by Wyeth Labs., Philadelphia, Pa., U.S.A.) were used as received.

Procedure

One millilitre of serum from patients receiving theophylline or outdated blood bank plasma containing added theophylline was acidified with 0.8 ml of 1 *N* hydrochloric acid and extracted with 30 ml of chloroform–isopropanol (95:5) containing

0.02 mg of oxazepam. The extraction mixture was shaken at 75 oscillations/min for 15 min. After separation from the aqueous layer by pouring the specimens through Whatman 1PS phase separating paper, the organic phase was evaporated in an air stream to a volume of approximately 0.6 ml. Urine containing theophylline was prepared for analysis by passing 25-ml specimens over a 12 × 0.3 cm column containing Amberlite XAD-2 non-ionic polymeric adsorbent. The methylxanthines were eluted from this column with 25 ml of the chloroform–isopropanol mixture containing oxazepam. The eluate was evaporated to 0.6 ml. The injection loop of the liquid chromatograph was filled with the extract through a 2-μm metal frit filter.

Chromatographic operation

A Perkin-Elmer 1220 high-pressure liquid chromatograph was operated at a column temperature of 37° and a pressure of 500 p.s.i. One solvent chamber was filled with degassed hexane and the other with isopropanol. The solvent programmer was set for a linear 1% increase in isopropanol every 1.8 min and a flow-rate of 1.8 ml/min. Prior to sample application to the column, the initial isopropanol concentration was maintained at 14% and the gradient started as the specimen was injected. The gradient was reversed at 22% isopropanol to regenerate to the starting solvent composition, which occurred by the time the samples had cleared the UV detector. The column effluent was monitored with a UV detector at 270 nm with 0.02 O.D. full-scale sensitivity. Liquid chromatographs without gradient elution accessories may be operated with the same column to obtain equivalent separations as follows. Isopropanol (13% in hexane) was continuously degassed at 60° under reflux. The column oven was maintained at 37° and the flow-rate at 1.8 ml/min. The column pressure was 10–250 p.s.i., depending on the frit size used to retain the column packing. Column frits of 10 μm may be used for low-pressure operation if specimens are filtered before injection. To preserve the isopropanol–hexane mixture for this isocratic separation, the discharged effluent is cleared of contaminants by recycling through a 5-cm column of Florisil.

RESULTS

The separation of caffeine, theophylline and internal standard is shown in Fig. 2. A typical blank plasma extract, a blank containing only internal standard and plasma extract containing 10 μg of theophylline and 20 μg of internal standard are shown in Fig. 2. In several studies, the effluent containing the theophylline peak was collected and evaporated and the residue was derivatized and assayed by gas chromatography[2]. A single peak with a retention time similar to that of pure theophylline was obtained. The mean recovery from plasma of added theophylline was 93% over the 2.5–25 μg/ml range (Table I) with a coefficient of variation of 2.5%. Extraction of theophylline is pH dependent[4]. A minimum of 0.4 ml of 1 *N* hydrochloric acid per millilitre of plasma was necessary for maximal and reproducible extraction. Extraction with chloroform frequently leads to emulsion formation and erratic recovery of theophylline, which are prevented by the slow shaking speed.

The retention volumes of various methylxanthines using gradient elution are recorded in Table II. The retention volumes of theobromine, oxazepam, 1-methylxanthine and 7-methylxanthine are high under the conditions of the assay. Higher

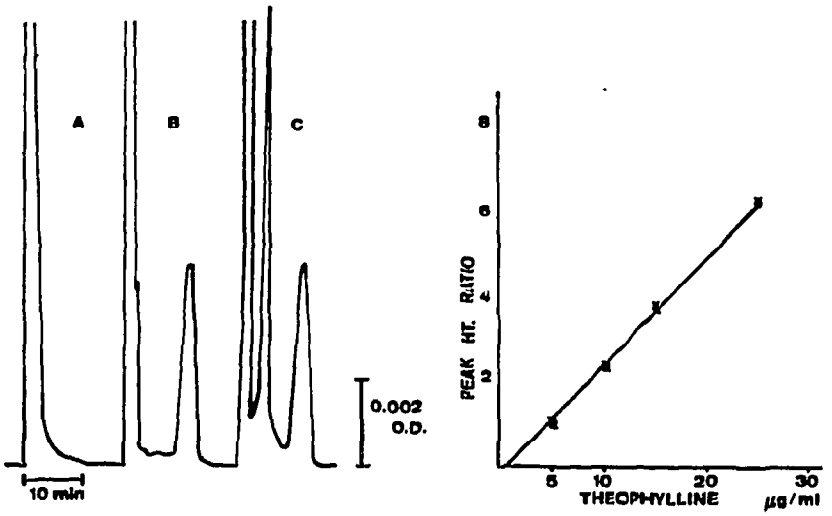

Fig. 2. Left: representative chromatography of (A) blank plasma, (B) blank plasma containing 10 μg/ml of theophylline and (C) internal standard. The chromatograph was operated isocratically with 13% isopropanol in hexane which was degassed at 60°. The column pressure was 25 p.s.i., temperature 37°, packing Durapak OPN and solvent flow-rate 1.8 ml/min. Right: relationship of peak height ratios of theophylline to internal standard (oxazepam).

TABLE I

RECOVERY OF THEOPHYLLINE FROM PLASMA

Added (μg)	*Found (μg) (n = 4)*		*Recovery (%)*
	Mean	*S.D.*	
2.5	2.3	0.4	92
5.0	4.5	0.2	90
10.0	9.4	0.8	94
15.0	13.6	1.0	91
20.0	18.4	1.4	92
25.0	24.2	1.6	97

TABLE II

RETENTION VOLUMES OF METHYLXANTHINES AND OXAZEPAM

Operating conditions: column packing, Durapak OPN; elution solvents: 14% isopropanol in hexane followed by a linear gradient of 1%/min; flow-rate, 1.8 ml/min; pressure, 500 p.s.i.; column temperature, 37°.

Substance	*Retention volume (ml)*
Theophylline	5
Caffeine	10
7-Methylxanthine	12.4
1-Methylxanthine	15.0
Oxazepam	24.0
Theobromine	27.5

isopropanol concentrations can be used to elute these substances more rapidly if they are of interest.

A series of benzodiazepines as well as analogs and homologs of methylxanthines (generously supplied by Searle, Chicago, Ill., U.S.A.) were tested as possible internal standards and found to be unsatisfactory except for oxazepam. Chlordiazepoxide and diazepam appear in the solvent front and do not interfere in the determination of theophylline. Flurazepam appears 10–15 min following the oxazepam peak. The flurazepam peak is broad and poorly resolved. When given in the usual therapeutic dosage, the above benzodiazepine drugs do not interfere in the determination of theophylline.

DISCUSSION

Recent studies by Khanna *et al.*[5] have shown that the rat can metabolize caffeine to theophylline. The results of Cornish and Christman[6] suggest that similar pathways may exist in man, although these investigators were not able to demonstrate the presence of theophylline as a metabolite. In studies in animals, it is possible to exclude interfering methylxanthines from the diet, but to assume a similar exclusion in studies in man may be inappropriate. Thus, in the analysis of the methylxanthines in man, the clear discrimination between theophylline and caffeine is extremely important. It has been possible to analyze methylxanthine solutions by gas chromatography[2], but in our experience endogenous substances interfere significantly with the determination of theophylline in plasma by this method.

Studies to circumvent these problems led to the development of the method reported here. The details of two types of chromatograph operations are recorded. The isocratic separation with 13% isopropanol in hexane appears to be temperature sensitive, whereas the separation by gradient elution appears to be more solvent sensitive. Both methods give satisfactory separation with a column temperature of 37° and either may be used following extraction of methylxanthines from acidified biological fluids with chloroform–isopropanol. The HPLC separation improved the specificity over previous methods while maintaining adequate precision. The method has the flexibility to determine any of the methylxanthines and the speed to analyze conveniently the most commonly occurring ones. This technique avoids the necessity for special treatment of the plasma to eliminate caffeine and analyzes the materials at temperatures at which they are stable. While it is possible that drugs administered to patients might interfere in the accurate determination of theophylline, we have seen no such interference of substances in the plasma of patients receiving a variety of drugs.

ACKNOWLEDGEMENT

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