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DETERMINATION OF PLASMA THEOPHYLLINE BY STRAIGHT-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: ELIMINATION OF INTERFERING CAFFEINE METABOLITES

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SUMMARY

Several authors have recently reported interference in theophylline analysis by paraxanthine (1,7-dimethylxanthine), an important metabolite of caffeine. A method for the determination of theophylline in plasma is described, eliminating caffeine and related compounds by means of straight-phase high-performance liquid chromatography. The resulting procedure is sufficiently rapid, accurate and sensitive to be applied in routine monitoring of therapeutic levels in patients as well as for pharmacokinetic purposes. Although only 0.1 ml of sample is required, concentrations as low as 0.2 mg/l can be measured with acceptable precision. A brief comparative evaluation of this procedure with a radioimmunoassay is made.

INTRODUCTION

Theophylline and its derivatives are frequently used for treatment of acute and chronic bronchial asthma and apnea of the newborn. The low therapeutic index of the drug and the poor relationship between dosage regimens and

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plasma concentrations have in several cases caused severe intoxication, sometimes resulting in fatal accidents [1]. However, the significant correlation between plasma levels and clinical response allows, by careful monitoring of plasma drug concentrations, an adequate, individualized dosage schedule to be arranged [2].

A great variety of techniques for theophylline determination in biological fluids has been published, ranging from spectrophotometry [3,4], fluorimetry [5], gas chromatography [6—8] and thin-layer chromatography [9,10] to more recent methods such as radioimmunoassay [11], EMIT [12] and isotachophoresis [13].

Several publications emphasize the advantages of high-performance liquid chromatography (HPLC) with respect to sensitivity, specificity and the possibility of using microsamples. Most HPLC techniques for the determination of theophylline and related compounds differ from each other in sample preparation or in the chromatographic system used. The biological sample can be injected directly into the chromatograph [14,15], or after elimination of proteins by trichloroacetic acid [16], by solvent denaturation [17—21] or by molecular filtration [22]. These methods often involve expensive material, large sample volumes, or special care to maintain the characteristics of the analytical system.

Sample extraction prior to injection [23—31] offers, by optimising the pH and extracting solvent, the advantage of a higher specificity while evaporation and dissolution of the residue in a minimal amount of a suitable solvent adds to the sensitivity of the procedure. Most analyses are developed on bonded octadecyl reversed-phase systems with aqueous eluents containing acetate or phosphate salts to obtain a pH varying between 3.5 and 7 [18—27]. Good separation is also achieved on ion-exchange materials [15,28] or straight-phase systems [29,30].

Numerous workers have succeeded in eliminating interferences caused by compounds related to theophylline, originating from dietary or metabolic

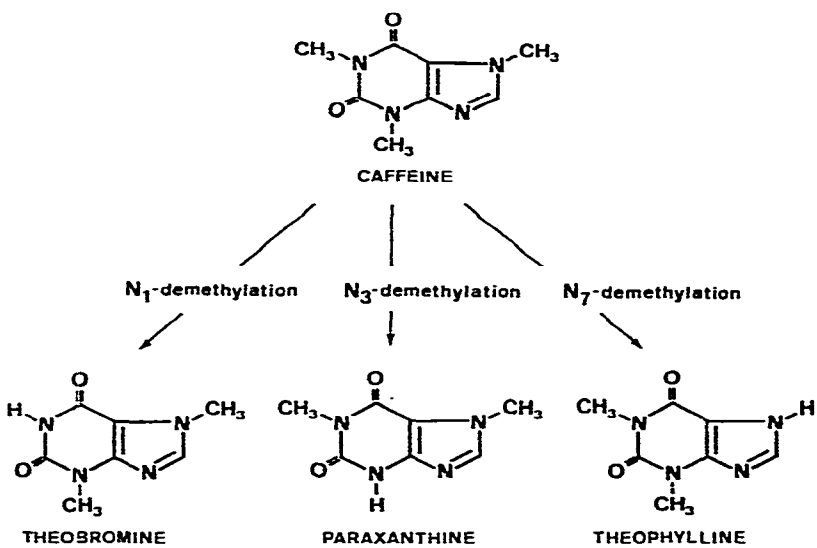

Fig. 1. Metabolic N-demethylation routes of caffeine in man [35].

sources. Nevertheless, the potential interference of paraxanthine (1,7-dimethylxanthine), an important metabolite of caffeine (Fig. 1), has been mostly overlooked or underestimated [32]. Observed by Thompson in 1974 [33], by Sved in 1976 [34] and identified mass spectrometrically by Midha [35], paraxanthine has a similar UV absorption and a retention time identical to that of theophylline in most reversed-phase systems. This often results in misleading elevations of serum theophylline values up to 3 mg/l. Assuming a therapeutic range of 5—20 mg/l theophylline serum concentration, the deviation from the true value can consequently reach 50% or more. Only a few investigators have proposed a suitable chromatographic procedure [36—41] to solve this problem.

In our laboratory we have developed a fairly rapid HPLC method using a straight-phase isocratic system on which the above-mentioned dimethylxanthines can be distinguished. Although only 100 μl of plasma is required, the limit of detection is as low as 0.2 mg/l with acceptable precision.

MATERIALS AND METHODS

Reagents

Chloroform was obtained from Riedel-De Haën (Seelze, Hannover, G.F.R.); formic acid was from Noury-Baker (Deventer, The Netherlands); theophylline was from Serva Feinbiochemica (Heidelberg, G.F.R.); 3-isobutyl-1-methylxanthine from Aldrich-Europe (Beerse, Belgium); 1,7-dimethylxanthine and theobromine from Sigma (St. Louis, MO, U.S.A.); and caffeine was from Knoll (Ludwigshafen, G.F.R.). Dioxane, 2-propanol and ammonium sulphate were purchased from E. Merck (Darmstadt, G.F.R.). All reagents were used as received.

Apparatus

HPLC analyses were carried out with a Waters Assoc. Model 6000 chromatography pump in conjunction with a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and an LC—UV variable-wavelength absorbance detector (Pye Unicam, Cambridge, Great Britain). An SP 4000 Chromatography Data System connected with an SP 4050 printer/plotter (Spectra Physics, Santa Clara, CA, U.S.A.) was used for quantitation of the results.

Chromatographic conditions

A 10 cm × 2.8 mm I.D. stainless-steel column (RSL, Eke, Belgium) was slurry-packed in a carbon tetrachloride—methanol mixture (70:30, v/v) with silica gel (RSil 5 μm; RSL). The slurry was compressed with light petroleum (b.p. 40—60°C) at 315 bar by means of an air-driven fluid pump (Haskel, Burbank, CA, U.S.A.). The solvent system consisted of chloroform—dioxane—formic acid (95.5:4.5:0.01, v/v), degassed in an ultrasonic bath and kept capped and stirred while in use. The flow-rate was 1.4 ml/min, corresponding to a pressure of 84 bar at the head of the column. All chromatograms were run at ambient temperature. The eluent was monitored continuously at 273 nm with a detector sensitivity setting of 0.04 a.u.f.s.

Extraction procedure

Equal volumes (100 μl) of plasma, saturated ammonium sulphate solution and internal standard solution (3-isobutyl-1-methylxanthine, 25 mg/l) are pipetted into a 18 × 100 mm polypropylene centrifuge tube and extracted by vortexing for 20 sec with 10 ml of a chloroform—2-propanol mixture (95:5, v/v). Following centrifugation (5 min at 800 *g* RCF*), the upper aqueous layer is removed by aspiration and 8 ml of the organic layer are transferred to a conical glass test-tube and evaporated to dryness at 60°C under a stream of dry nitrogen. The residue is stored at low temperature until analysis. After reconstitution in 50 μl of the mobile phase, 20 μl are injected into the chromatograph.

Preparation of standard curves

A 100-μl volume of a 4 mg/ml theophylline solution in 0.01 *N* hydrochloric acid was added to 19.9 ml of drug-free pooled blood-bank plasma, resulting in a stock solution of 20 mg/l. Theophylline concentrations ranging from 0.2—20 mg/l were prepared by further diluting with plasma. After incubation at 37°C for 14 h, all standards were extracted and analysed in duplicate by the described procedure.

Peak area drug/internal standard ratio was plotted against concentration and a straight-line fit of the data was made by linear regression analysis.

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms of serum samples collected before and after theophylline administration to a patient not withdrawn from caffeine-containing beverages. All constituents eluted in the order of increasing polarity, the retention times of the internal standard and theophylline being 4 and 8 min, respectively. The flow-rate and the percentage of dioxane in the mobile phase were dictated by a compromise between baseline resolution and chromatographic analysis time.

With the exception of one case, in which the nature of the interfering compound could not be elucidated, no drugs or naturally occurring endogenous substances interfered during the 5-month period in which we analysed a large number of samples collected from numerous patients. Considering the potential interference of theophylline metabolites, we used 5% 2-propanol in chloroform as the extraction solvent, which separated the drug from the methyluric acids but not from 3-methylxanthine [42]. The latter compound, however, as well as 1-methylxanthine, does not seem to be present in detectable amounts in plasma [43]. Nevertheless, no chromatographic response was obtained in vitro, presumably due to retention on the column.

Using the extraction solvent—salt combination [44] good extraction efficiency could be achieved (91.03% for theophylline and 99.8% for the internal standard). At a neutral pH, strongly basic and acidic material remains in the aqueous phase and most lipid and co-extractable material is excluded by the establishment of a lipid—protein interphase between the organic and the aqueous layer. Furthermore, the problem of a preliminary pH fluctuation connected with the use of an acetate buffer [45] is avoided.

*Relative centrifugal force.

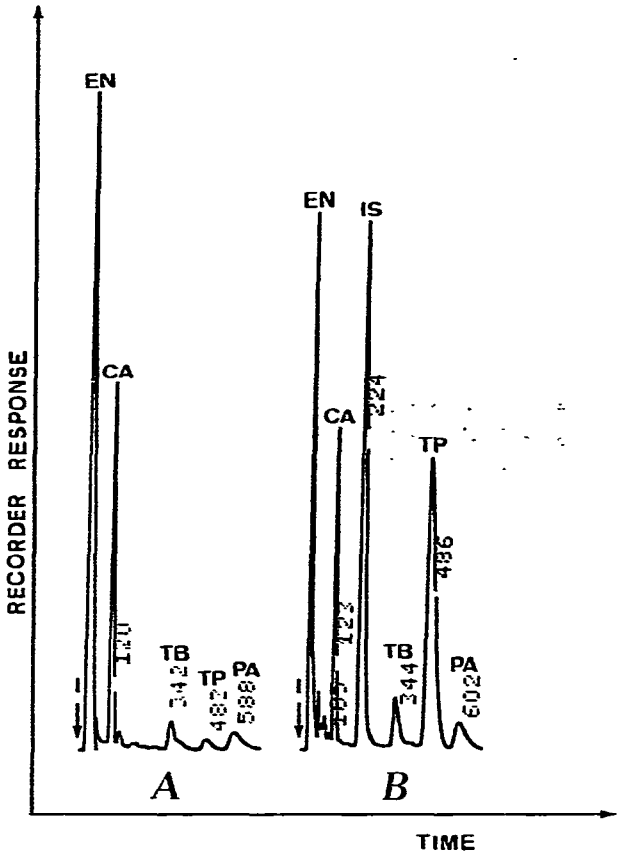

Fig. 2. Typical chromatogram of a blank serum from a non-fasting patient (A) and a serum sample after administration of theophylline (B). I = injection; EN = endogenous compounds; CA = caffeine; IS = internal standard; TB = theobromine; TP = theophylline; PA = paraxanthine. Retention times (sec) are printed at each peak.

Sensitivity and selectivity were increased by measuring at 273 nm, the optimum detection wavelength for theophylline. We found 10 ng to be the detection limit.

Excellent linearity ($r = 0.9999$) was noted in the relationship peak area drug/internal standard versus plasma theophylline concentrations ranging from 0.25 to 20 mg/l.

Small fluctuations in solvent strength, changes in adsorbant water content or ambient temperature variations cause shifting of the peaks. This can seriously affect the accuracy of the method when measuring peak height ratios. Therefore we preferred to use an automatic data-processing equipment from which direct digital read-out of peak areas was obtained.

As illustrated in Table I, systematically repeated calibration was not necessary. Kept in a tightly closed glass container at room temperature, the aqueous 3-isobutyl-1-methylxanthine solution proved to be stable for several months. Yet standard theophylline solutions were run periodically to evaluate the precision of the assay under routine conditions.

TABLE I

STATISTICAL EVALUATION OF THREE CALIBRATION CURVES CARRIED OUT AT 1-MONTH INTERVALS

	Intercept	Slope
Calibration curve 1	0.0008945	0.045249
Calibration curve 2	0.0003188	0.044660
Calibration curve 3	−0.008156	0.045267
Statistically expected coefficients	0.00012809	0.045059
Variation	0.127×10^{-5}	0.192×10^{-7}

An estimation of "within-run" variation was carried out by analysing spiked plasma samples containing 1 mg/l (C.V. = 2.5%, n = 5), 8 mg/l (C.V. = 2.2%, n = 5) and 15 mg/l (C.V. = 2.7%, n = 5). The accuracy of the method was further evaluated by directly comparing the results of 100 theophylline determinations, performed by the described procedure and by a radioimmunologic assay (RIA) in patient plasma specimens.

All RIA measurements were carried out with a Gammadab [^{125}I] Theophylline Radioimmunoassay Kit (Clinical Assays, Travenol Lab., Lessines, Belgium),

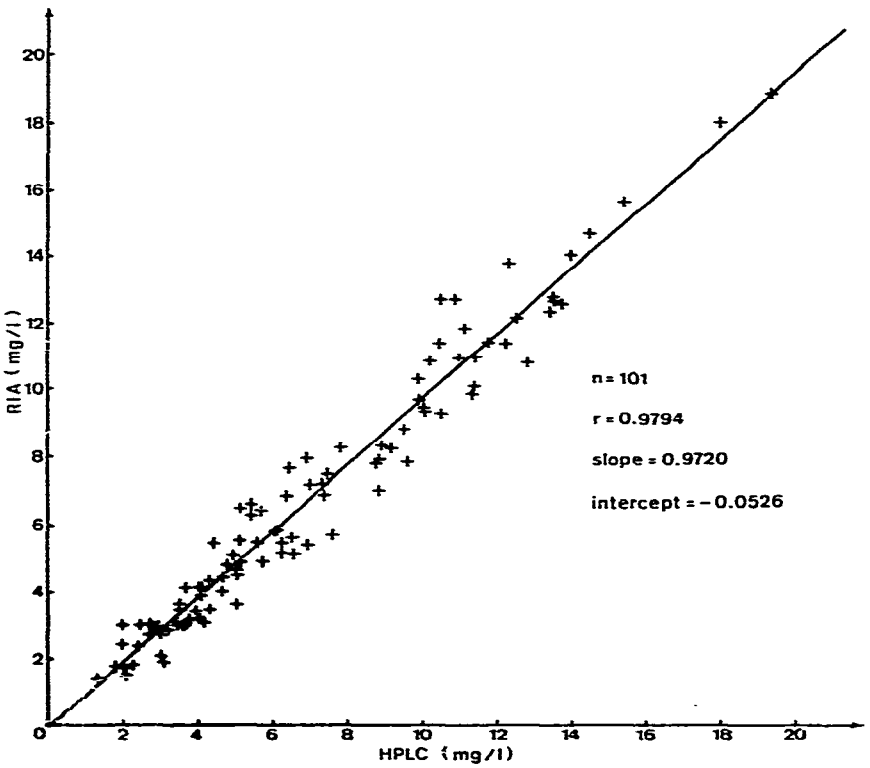

Fig. 3. Correlation of patient plasma readings as determined by the HPLC procedure and by RIA.

TABLE II

"BETWEEN-RUN" PRECISION (2-MONTH PERIOD): COMPARISON OF THE HPLC PROCEDURE AND RIA

Procedure	Theophylline plasma conc. (mg/l)	No. of samples (n)	Mean recovery (%)	Standard deviation (mg/l)	Coefficient of variation (%)
HPLC	8.0	16	101.0	0.27	3.3
RIA	15.0	54	97.3	0.73	5.0

following the directions provided by the supplying company. A calibration curve was established by linear regression analysis after logit/log transformation of the data ($r > 0.996$), and the final results were automatically calculated by a programmed desk-top calculator (Hewlett-Packard HP 9810A). As illustrated in Fig. 3, good correlation was found between the methods.

Although RIA offers the advantages of speed and specificity, its precision (Table II) and sensitivity (minimal detectable concentration = 2 mg/l) may be acceptable in routine monitoring of therapeutic plasma levels, but are hardly sufficient for pharmacokinetic purposes. Furthermore, the high cost and the need for consistent reliability of the reagents have to be taken into consideration as compared to the HPLC technique, which, on the other hand, involves more technician time and investment in rather expensive and complex chromatographic equipment.

CONCLUSIONS

By the HPLC method described in this paper, good separation is achieved of theophylline and its isomers, resulting in higher accuracy in routine monitoring plasma drug concentrations. Chromatographic conditions remained stable for a long period of time without special cleaning or maintenance procedures of the system. The small sample size, coupled with high sensitivity, allows plasma collection by capillary puncture, making the method attractive for pediatric and pharmacokinetic investigation where multiple sampling is mostly required.

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