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The Simultaneous Determination of Theophylline, Theobromine and Caffeine in Plasma by High Performance Liquid Chromatography

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A high performance liquid chromatographic procedure for the simultaneous micro-scale determination of theophylline, theobromine and caffeine in plasma is described. After a single dichloromethane extraction of 0.05 — 0.2 ml of acidified plasma, the evaporated residue is chromatographed on a reverse-phase gC-18) column. With a mobile phase of acetate buffer (pH 4) — acetonitrile (88:12) at a flow-rate of 2.0 ml/min., the three methylxanthines are separated within six minutes. Detection at 276-280 nm enables quantitation of 0.1 mg/l of drug in a 0.1 ml sample. The method is reproducible, correlates well with EMIT for plasma theophylline, and is applicable to the routine monitoring of both paediatric and adult patients as well as to metabolic studies.

THEOPHYLLINE (1,3-dimethylxanthine, TP) is a potent phosphodiesterase inhibitor which has found frequent and efficacious use as a bronchodilator in the treatment of obstructive airways disease. However, the narrow therapeutic index and the considerable individual variation in elimination associated with this drug generally requires the monitoring of plasma concentrations if the optimal therapeutic effect is to be attained[1]. Two other dietary xanthines, caffeine (1, 3, 7-trimethylxanthine, CA) and theobromine (3, 7-dimethylxanthine, TB), possess a similar range of pharmacological properties[2]. The use of CA has been advocated for the treatment of neonatal apnea[3].

Spectrophotometric procedures for the determination of xanthines in biological fluids are normally time-consuming and non-specific. In addition, they often lack sensitivity and have a large sample volume requirement[4]. Similarly, gas chromatographic assays for plasma TP frequently have poor sensitivity and long analysis times, and may require derivatisation of the drug prior to chromatography[5].

To overcome sensitivity problems, gas chromatograpic methodologies for the measurement of plasma CA need relatively large sample volumes[6] or quantitation by mass spectrometry[7]. A number of high performance liquid chromatographic methods have been reported[8] for the measurement of TP in biological fluids but these have generally not been suitable for the co-determination of CA and TB. The reverse phase high performance liquid chromatographic procedure described in this paper provides a rapid, sensitive and specific method for the simultaneous analysis of TP, CA and TB in plasma. Results of comparison studies with an enzyme multiplied immunoassay technique (EMIT) for TP are also presented.

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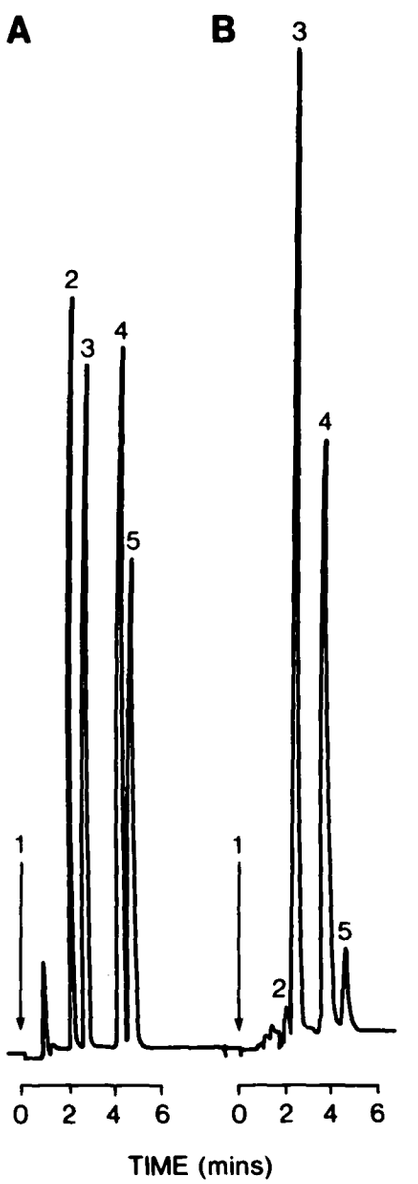

Fig. 1 — Chromatograms of plasma (0.1 ml) extracts, 0.05 AUFS.

A) Prepared standard containing 7.5 mg/l of TB, TP and CA.

B) Patient sample containing TB, TP and CA.

Peaks: 1, TB; 2, TP; 3, Internal standard; 4, CA.

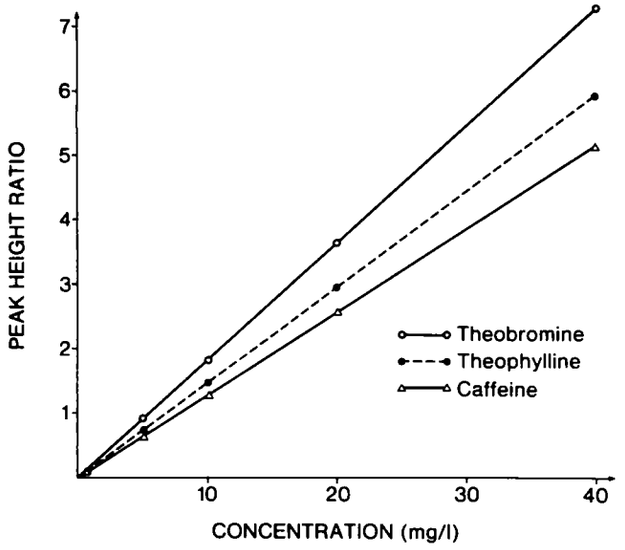

Fig. 2 — Typical calibration curve for TB, TP and CA.

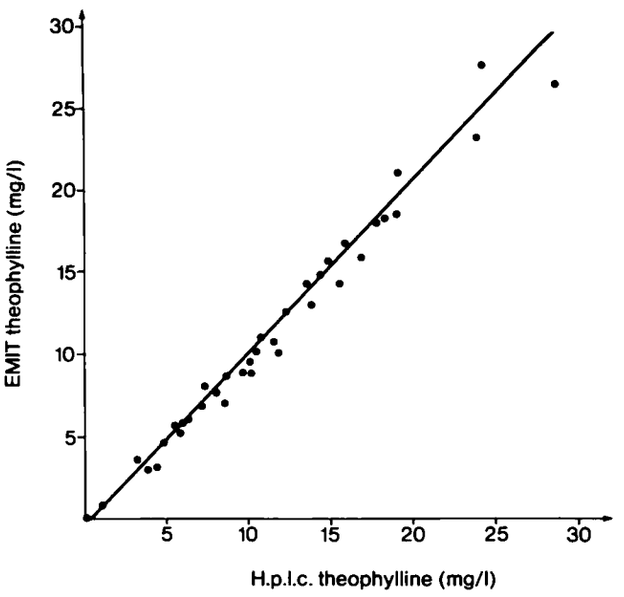

Fig. 3 — Correlation between plasma theophylline concentrations assayed by h.p.l.c. and EMIT.

Materials and Methods

Chemicals

Anhydrous theophylline, theobromine and caffeine were supplied by Hamilton Laboratories (Adelaide, South Australia). The internal standard, 8-chlorotheophylline, was obtained from ICN Pharmaceuticals Inc. (Irvine, CA. 92664). All other reagents and solvents were of AR grade.

High Performance Liquid Chromatography

The system used (Altex Scientific Inc., Berkeley, CA. 94710) consisted of a Model 905-42 injector, model 110 solvent delivery system and a model 155 variable wavelength detector operating at 276 nm. The instrument was fitted with a 15 cm x 4.6 mm (i.d.) reverse phase S5 ODS column (Edwards Instrument Co., Narellan, New South Wales) and operated at ambient temperature. The mobile phase was acetate buffer (10 mmol/l, pH 4) — acetonitrile (88:12) at a flow rate of 2.0 ml/min.

Enzyme Immunoassay

EMIT assays for TP were performed according to the instructions of the manufacturer (Syva Corporation, Palo Alto, CA. 94304). The apparatus used consisted of a Gilford Stasar III spectrophotometer, Syva model 1500 automatic pipetter-diluter and Syva model 2400 automatic timer-printer.

Extraction Procedure for Liquid Chromatography

To 0.05-0.20ml of plasma or serum in a stoppered glass tube was added an equal volume of 0.2 M HCl and 4.0 ml of dichloromethane containing the internal standard (8-chlorotheophylline, 50 μg/l).

After vortex mixing for 30 s the tubes were centrifuged at 3000 r.p.m. for 3 min. and the aqueous (upper) phase aspirated. The organic phase was transferred to a clean conical-tip glass tube and then evaporated (Vortex evaporator). The residue was redissolved in 25 μl of the mobile phase and a 20 μl aliquot injected into the chromatograph.

The standard curve was prepared by diluting 0.2 g/l aqueous stock solutions of TP, CA and TB with xanthine-free plasma to give final concentrations containing 0.5-40 mg/l. These samples were then treated like serum, as above. Unknown concentrations were determined by comparison of the methylxanthine/8-chlorotheophylline peak height ratios with those of the calibration curve.

Results and Discussion

Fig. 1 shows chromatograms obtained following this procedure for an extract of 0.1 ml of a plasma standard containing 7.5 mg/l of each of TP, TB and CA, and for 0.1 ml of a patient sample extract containing TP (12.5 mg/l), CA (1.2 mg/l) and TB (0.4 mg/l.) Sharp, symmetrical peaks with retention times of 2.1, 2.6, 4.1 and 5.0 mins are obtained for TB, TP, internal standard (8-chlorotheophylline), and CA respectively. Xanthine-free plasma gives no interfering peaks under these chromatography conditions.

Sample preparation by solvent extraction was adopted in preference to a method using direct injection of protein-precipitated plasma since a number of the cephalosporin antibiotics, in particular cefazolin and cephalothin, have been reported[9,10] to interfere with reverse phase liquid chromatographic assays of TP following the latter procedure. Furthermore, dichloromethane extraction of acidified plasma provides a convenient means for the simultaneous selective enrichment and concentration of the sample. The solvent extraction method has thus proved more sensitive than another technique using protein precipitation prior to the high performance liquid chromatographic assay of theophylline[11]. With photometric detection at 276 nm, the approximate absorption maximum for the methylxanthines in the mobile phase, as little as 0.1 mg/l of TP, TB and CA can be quantitated in a 0.1 ml sample by the method described here. It should be noted that detection at 280 nm may be used with little apparent loss in sensitivity.

Mean recoveries, calculated by comparing the peak height for extracted compound with that of an equal amount injected directly into the chromatograph, for samples containing 0.5-40 mg/l of TP, TB and CA were 82 ±3, 85 ±4.5 and 91 ±4% respectively. The recovery efficiency was essentially identical for samples extracted from equal volumes of plasma or water, thereby allowing for the use of aqueous standards.

Calibration curves for TP, TB and CA were linear in the range 0.5-40 mg/l and passed through the origin (Fig. 2). The reproducibility of the method at a given concentration was investigated by the analysis of replicates at concentrations of 1.0 and 20 mg/l of the three methylxanthines. Intra-assay coefficients of variation (n = 20) for TP, TB and CA were 4.1, 3.7 and 3.8% respectively at 1 mg/l, and 2.9, 3.0 and 2.7% respectively at 20 mg/l. Inter-assay coefficients of variation, measured over two months (n = 18), for a sample containing 5 mg/l of TP, TB and CA, were 4.3, 5.2 and 4.9% respectively. During over a year of operation, no drugs or metabolites were found to interfere with the determination of these compounds.

Forty patient plasma samples were analysed for TP by the method described here and by EMIT. The results are summarised in Fig. 3 which shows excellent agreement between the two procedures, the correlation coefficient being 0.98 and the regression slope essentially unity ($y = 1.03x - 0.51$). A similar correlation between a high performance liquid chromatographic assay using the protein precipitation procedure for sample preparation and EMIT has been demonstrated for TP[12].

Although the EMIT assays may be performed faster than chromatographic analyses, the present procedure has significant advantages in cost, sensitivity, versatility, reproducibility[13] and, with a chromatgraphy time of less than six minutes, is also comparatively rapid. Moreover, the method described has been validated for the determination of CA and TB in plasma and has been successfully applied to both routine patient monitoring (including neonates) and pharmacokinetic studies.

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