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Determination of caffeine, theophylline and theobromine in serum and saliva using high-performance liquid chromatography

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SUMMARY. A method is described for the measurement of theobromine, theophylline and caffeine in serum and saliva by high-performance liquid chromatography (HPLC). A chloroform/isopropanol extract (85:15, v/v) is evaporated to dryness and chromatographed on a 100 × 4·5 mm id Hypersil octadecylsilane column with UV detection at 280 nm. Theobromine, theophylline, caffeine and the internal standard proxyphylline are satisfactorily resolved with an elution system of acetonitrile/tetrahydrofuran/50 mM acetate buffer, pH 4·0, (4:1:95, v/v). No interference is observed from the presence of xanthine metabolites or any of a number of common drugs examined.

A good correlation was observed between the concentrations of caffeine in serum and in saliva suggesting that salivary measurements may be useful for the study of caffeine pharmacokinetics in man. Caffeine levels determined by the HPLC procedure described here agreed well with those obtained by a radioimmunoassay method. The method is also suitable for determining the xanthine content of beverages by direct injection of diluted samples.

The therapeutic use of theophylline and caffeine for the treatment of asthma and neonatal apnoea and the requirement for therapeutic drug monitoring by measurement of the drugs in serum and saliva are well established. Recent studies on the biotransformation of theophylline to caffeine in the neonate[1] have confirmed that during theophylline therapy, caffeine may accumulate to clinically significant concentrations indicating a requirement for the simultaneous measurement of both caffeine and theophylline in monitoring therapy. In addition, it has been suggested that measurement of these methylxanthines, especially caffeine, may be of value in detecting toxicity resulting from impaired metabolism or excessive dietary intake in the form of tea, coffee, chocolate, cocoa, cola-flavoured drinks and caffeine-containing analgesics.[2–4]

In recent years, high-performance liquid chromatography (HPLC) has been extensively applied to the specific analysis of theophylline or caffeine,[5–9] but the simultaneous measurement of the methylxanthines has received little attention. Foenander *et al.*[10] described a procedure. wherein dichloromethane extracts of serum were chromatographed on octadecylsilane column using 12% acetonitrile in acetate buffer, pH 4. However, in our experience, dichloromethane extraction produces a low recovery and the chromatography system is unable to resolve theophylline from 1,7-dimethylxanthine (paraxanthine), a metabolite of caffeine often present in biological solutions in significant concentrations. Improved specificity was shown in a normal phase procedure adopted by Arnaud and Welsch[11] for the measurement of theophylline, theobromine, paraxanthine and caffeine in serum and saliva. Samples were extracted using a chloroform/isopropanol mixture but their results show a considerable variation in recovery between the xanthines. Moreover, normal phase-chromatography systems are prone to rapid column deterioration.

The specificity problems encountered with reverse phase systems for methylxanthines can be improved by the incorporation of

tetrahydrofuran[12] or tetrabutyl ammonium chloride[13] into the mobile phase. In the present study, the use of tetrahydrofuran has been adopted to provide a means for simultaneous measurement of caffeine, theophylline and theobromine in serum and saliva. The correlation between caffeine levels in serum and saliva was investigated and the results obtained by HPLC analysis were compared with those obtained using a radioimmunoassay procedure.[14]

Materials and methods

Caffeine was purchased from BDH Chemicals Ltd, Poole, England, and theophylline, 7β-hydroxypropyltheophylline (Proxyphylline), 1-methyluric acid and 1,7-dimethylxanthine from Sigma London Chemical Co. Ltd, Kingston-upon-Thames, Surrey. All other xanthines were obtained from Adams Chemical Co., Round Lake, Illinois, USA.

Stock solutions (200 mg/l) of the xanthines were prepared in distilled water. The working standards of caffeine, theophylline and theobromine were made by further diluting the stock solution in horse serum (Wellcome Reagents Ltd, Beckenham, England) to give concentrations of 5–30 μg/ml. The internal standard, proxyphylline, was made up in 0·2 M HCl and used at a concentration of 4 μg/ml.

Analar grade chloroform, isopropanol, tetrahydrofuran (BDH Chemicals Ltd, Dorset, England) and HPLC grade acetonitrile (Rathburn Chemicals Ltd, Walkerburn, Scotland) were used throughout the study.

EQUIPMENT

An Applied Chromatography Systems (ACS) 750/03 reciprocating pump was used with detection at 280 nm by an ACS 750/11E UV monitor fitted with a 10 μl flow cell. Chromatography was performed using a 100 mm × 4·5 mm id stainless steel column containing Hypersil ODS (Shandon, London, UK). Samples were injected via a Rheodyne loop injector fitted with a 50 μl loop.

PROCEDURE

Serum or saliva (50–200 μl), internal standard (200 μl containing 800 ng proxyphylline in 0·2 M HCl) and 5 ml of chloroform/isopropanol (85:15) were vortexed for 30 seconds in a stoppered tube and then centrifuged for 5 minutes at 2000 rpm. A 4 ml aliquot of the organic phase was evaporated to dryness with a stream of air in a water bath at 40°C. The residue was dissolved in 100 μl of acetonitrile/tetrahydrofuran/50 mM acetate buffer, pH 4·0 (4:1:95, v/v), 50 μl of which was injected. The same solvent mixture was used as the chromatography eluent at a flow rate of 1·5 ml/min which produced a pressure drop of 60 bars. For quantitation the ratio of peak heights of the xanthines to those of the internal standard in chromatograms from serum or saliva samples were compared with those obtained when appropriate working standards were similarly processed.

Results

A mixture containing 4 μg/ml of theobromine, theophylline, caffeine and proxyphylline, respectively, was satisfactorily resolved by the chromatographic procedure described. The retention time of the methylxanthines together with a number of potentially interfering compounds, are shown in Table 1. A chromatogram of a spiked serum sample (8 μg/ml) is shown in Figure 1.

The recovery of theobromine, theophylline and caffeine from serum and saliva was calculated by chromatographing extracts before and after the addition of methylxanthines at concentrations of 4 μg/ml and 8 μg/ml. The mean recovery (± SD) of theobromine from serum was 90·8% ± 4·9, of theophylline, 94·1%

TABLE 1. Chromatography retention data

Compound	Capacity factor (k)	Retention time (min)
Theobromine	2·3	2·0
Theophylline	4·2	3·1
Caffeine	7·8	5·3
Proxyphylline	9·3	6·3
7-methyluric acid	0·5	0·9
7-methylxanthine	1·0	1·2
1-methyluric acid	1·3	1·4
3-methylxanthine	1·7	1·6
1-methylxanthine	1·8	1·7
1,3-dimethyluric acid	2·0	1·8
1,7-dimethyluric acid	3·2	2·5
1,7-dimethylxanthine	3·8	2·9
1,3,7-trimethyluric acid	5·0	3·6
Paracetamol	3·0	2·4
Phenobarbitone	—	—
Primidone	—	—
Phenytoin	—	—
Trimipramine	1·0	0·6

—: Indicates that the compound did not chromatograph under the conditions used.

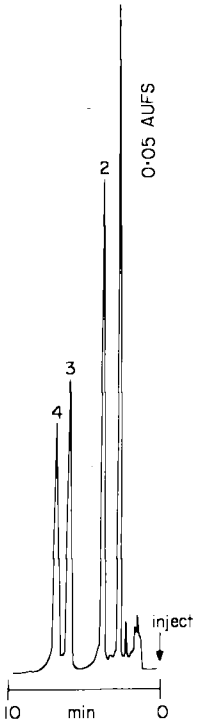

FIG. 1. Chromatogram of a spiked serum sample. Theobromine (peak 1), theophylline (peak 2), caffeine (peak 3) and proxyphylline (peak 4) were added to the sample at a concentration of 8 μg/ml each. Chromatographic conditions were the same as described in the text.

± 4·8 and of caffeine, 93·3% ± 8·8. Results obtained using saliva were similar, the values being 89·1% ± 2·7, 90·3% ± 1·9 and 92·9% ± 2·9 respectively.

Standard solutions over the range 1–30 μg/ml prepared in horse serum and assayed in duplicate showed a linear response; average coefficients of variation of duplicates over the whole range were 2·2% for theobromine, 2·64% for theophylline and 6·14% for caffeine. An extract of horse serum itself showed no significant interference. Of various solvents used for extraction of the methylxanthines from the sample, chloroform/isopropanol (85·15 v/v) was found to be the best (Table 2). Recovery from aqueous solution tended to be greater than from serum.

Within-batch precision was studied by replicate analysis (n = 5) of samples spiked with a mixture of theobromine, theophylline and caffeine at concentrations of 4 μg/ml for saliva and 8 μg/ml for serum. The saliva sample gave a mean ± SD of 4·4 ± 0·06 for theobromine, 4·29 ± 0·05 for theophylline and 5·09 ± 0·24 for caffeine. Corresponding values obtained by analysis of the spiked serum were 7·96 ± 0·20, 7·92 ± 0·21 and 8·05 ± 0·17. Between-batch precision was studied by the repeated analysis of two spiked serum samples 'A' and 'B' (Table 3). The values obtained with the sample 'A' were 4·89 ± 0·12 for theobromine, 4·53 ± 0·15 for theophylline and 4·65 ± 0·27 for caffeine. The corresponding values from 'B' were 8·35 ± 0·12, 8·19 ± 0·26 and 11·97 ± 0·39. The limit of detection was 100 ng/ml plasma for theobromine and theophylline and 200 ng/ml for caffeine.

The correlation between results obtained from caffeine determinations on serum and saliva samples collected simultaneously from 52 volunteers is shown in Figure 2. The correlation coefficient was 0·941 with a regression line, $y = 0{\cdot}710x - 0{\cdot}018$ where y is the value for saliva and x is the value for serum.

The results of caffeine determinations made on 42 plasma samples using an RIA procedure[14]

TABLE 2. Solvent extraction of some methylxanthines from serum and saliva

Solvent	Matrix	Percentage recovery		
		Theobromine	Theophylline	Caffeine
Chloroform/isopropanol (85:15, v/v)	Water	93·7	96·7	98·7
	Serum	90·8	94·2	93·3
	Saliva	89·1	90·3	92·3
Dichloromethane	Serum	66·2	66·1	58·8
Ethylacetate	Serum	64·6	81·8	77·5
Ether/dichloromethane (60:40, v/v)	Serum	46·5	69·5	76·7

Methylxanthines were measured as described in the text. Each value is the mean of five determinations carried out at concentration levels of 4 and 8 μg/ml of the matrix.

TABLE 3. Precision of the measurement of methylxanthines by the HPLC method

Sample	Mean + SD (μg/ml)		
	Theobromine	Theophylline	Caffeine
Within batch (n = 5):			
Saliva	4·4 ± 0·06	4·29 ± 0·05	5·09 ± 0·24
Serum	7·9 ± 0·20	7·92 ± 0·21	8·05 ± 0·17
Between batch (n = 4):			
Serum 'A'	4·89 ± 0·12	4·32 ± 0·15	4·65 ± 0·27
Serum 'B'	8·35 ± 0·12	8·19 ± 0·26	11·97 ± 0·39

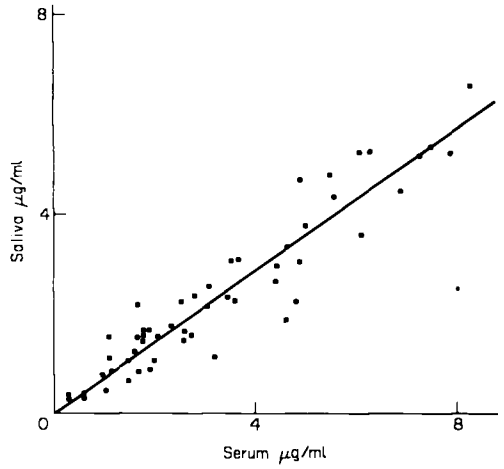

FIG. 2. Correlation between caffeine levels in serum and saliva. $y = 0{\cdot}71\,x - 0{\cdot}018$; $r = 0{\cdot}941$.

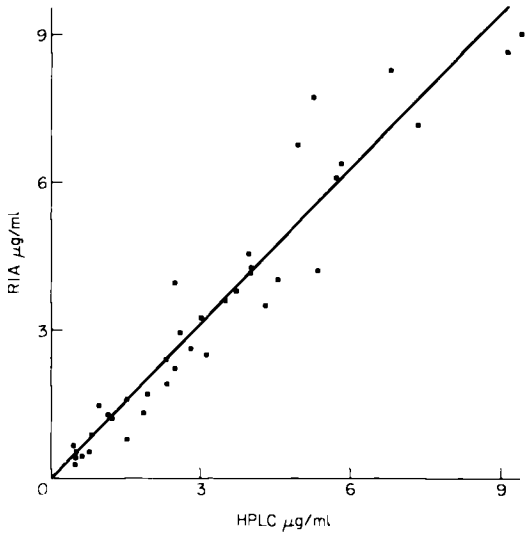

FIG. 3. Determination of serum caffeine by HPLC and radioimmunoassay. $y = 1{\cdot}04\,x - 0{\cdot}063$; $r = 0{\cdot}962$.

were compared with those obtained by HPLC; the correlation is shown in Figure 3. The correlation coefficient was 0·962 with a regression line, $y = 1{\cdot}04x - 0{\cdot}063$ where y is the value obtained by RIA and x the value by HPLC.

Discussion

Spectrophotometric methods for the measurement of theophylline and caffeine in biological fluids[15–17] require large sample volumes and are non-specific whilst gas chromatography procedures[18–20] have long analysis times and low sensitivity. Some of the obstacles to the more general application of methylxanthine measurements can be overcome by the use of radioimmunoassay[14] and enzyme immunoassay[21] techniques which are, however, unable to provide simultaneous estimation of the individual components of mixtures such as normally occur in biological fluids, nor do they provide any information about metabolites.

The method described here utilises the simplicity and reliability of reverse phase HPLC which overcomes the problem of column deterioration often encountered with normal phase procedures. The incorporation of tetrahydrofuran into the mobile phase results in improved specificity by resolving theophylline from 1,7-dimethylxanthine, a metabolite of caffeine. Sample preparation using organic extraction with a chloroform/isopropanol mixture has been adopted in preference to deproteinisation in order to overcome problems encountered with peak distortion[22] and interference from cephalosporin antibiotics.[23] The recovery values and precision of the extraction procedure was shown to be very satisfactory and results suggest that aqueous standards may be suitably employed as an alternative to those prepared in drug-free serum. Direct injection of saliva samples, di-

luted 1:4 with the internal standard, showed good reproducibility and results of caffeine analysis correlated well with those obtained using organic extraction ($r = 0{\cdot}995$). This did, however, lead to rapid column deterioration and organic extraction was subsequently adopted for routine use.

The good correlation observed between caffeine levels in serum and saliva indicates that analysis of saliva can be used to reflect concentrations in serum. The non-invasive nature of saliva sampling is of considerable advantage in therapeutic drug monitoring and pharmacokinetic studies, and the ability to perform at least 25 analyses in one working day makes the HPLC procedure highly suitable for these applications. HPLC can also be used to determine methylxanthine concentrations in beverages by direct injection of diluted samples into the system.

In our experience, the procedure described herein is robust and economical with no significant problems of late eluting peaks, column deterioration or interference from other drugs. Results of caffeine measurements made under a variety of clinical circumstances over the past 12 months are being prepared for publication.

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