Journal of Chromatography, 342 (1985) 105—117
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2600

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES IN PLASMA USING SOLID-PHASE EXTRACTION

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(First received November 27th, 1984; revised manuscript received February 19th, 1985)

SUMMARY

An improved high-performance liquid chromatographic method for the simultaneous determination of caffeine and its N-demethylated metabolites in plasma is described. Excellent resolution of all components is provided by reversed-phase chromatography using a mobile phase consisting of 1% acetic acid—methanol (83:17) at a flow-rate of 2.7 ml/min, in conjunction with a Waters Assoc. Nova-Pak C_{18} column which was protected by a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak CN cartridge. Rapid extraction of caffeine and the dimethylxanthines from plasma was achieved using reversed-phase octadecylsilane bonded-silica columns (Bond-Elut C_{18}). With only 100 μl of sample, plasma levels in the region of 50 ng/ml for the dimethylxanthines and 100 ng/ml for caffeine can be determined using ultraviolet detection at 273 nm. The method has been used for measuring umbilical cord plasma samples to provide information regarding foetal exposure to caffeine and its metabolites and is also suitable for therapeutic drug monitoring of caffeine and theophylline levels in the treatment of neonatal apnoea.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is widely consumed and concern in recent years regarding the health consequences of the drug has stimulated considerable interest in assessing exposure to the methylxanthines by measuring levels in biological fluids.

Excessive caffeine consumption has been implicated in the aetiology of a variety of clinical conditions including coronary heart disease, myocardial infarction, cancers of the urinary tract, kidney and pancreas, anxiety states and

0378-4347/85/$03.30

fibrocystic breast disease. However, in an excellent review published recently Curatolo and Robertson [1] were of the opinion that the evidence in support of these associations is inconclusive. Furthermore, reports relating heavy coffee and caffeine consumption with lower birth weight, complications at delivery and birth defects which prompted the U.S. Food and Drugs Administration to advise pregnant women to reduce their caffeine intake [2], have not been corroborated in recent studies [3, 4]. The indication from these is that caffeine appears to have little effect on the outcome of pregnancy. Clearly, with so much conflicting evidence regarding the effects of caffeine consumption on health, there is considerable scope for further investigation of the pharmacology and toxicology of the methylxanthines.

The increasing use of caffeine and theophylline [1,3-dimethylxanthine) in the treatment of neonatal apnoea [5, 6] has also focussed attention on the methylxanthines. The metabolism and pharmacokinetic parameters of these drugs in neonates differ markedly from those observed in older children and adults [7, 8]. The elimination of caffeine at birth is slow [9, 10] because of diminished N-demethylation [8, 11] so that the drug is excreted in the urine largely unchanged [11]. In addition, theophylline is converted to caffeine in premature newborns [12, 13] and this unique metabolic route has been demonstrated with isotopically labelled drugs [14]. The same study showed that some caffeine originating from labelled theophylline is N-demethylated since paraxanthine and theobromine were found in urinary extracts as mixtures of labelled and unlabelled molecules. This confirms earlier observations of the interconversion of theophylline and caffeine in neonates by Bada et al. [15]. The presence of methylxanthines in the plasma of newborns also results from transplacental transfer of caffeine [16]. Indeed in a recent study [4], cord blood levels of caffeine and its N-demethylated metabolites were found to be higher than previously indicated [3, 16, 17]. Infants can also ingest caffeine in breast milk and measurable levels in breast-fed babies have been shown to reflect the caffeine content of their mothers milk [18]. Since caffeine and the dimethylxanthines theophylline, theobromine (3,7-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) are all pharmacologically active [19] it is essential, whether monitoring drugs in the treatment of neonatal apnoea or evaluating the health consequences of the methylxanthines, to be able to determine caffeine and its N-demethylated metabolites simultaneously in plasma.

Although a variety of analytical techniques have been applied to the analysis of caffeine and its metabolites in biological fluids, high-performance liquid chromatography (HPLC) now seems to be the most frequently used approach [4, 8, 20—37]. Some of these, however, have concentrated on the determination of caffeine or another of the methylxanthines and are unable to separate and quantitate all the metabolites [8, 23—29, 31, 32]. Many have been primarily concerned with eliminating interferences in the estimation of a single component exemplified by efforts to separate theophylline and paraxanthine, which tend to co-elute under many HPLC solvent conditions [8, 26, 29, 31, 32]. However, this problem is now well recognised and several successful methods for separating these two important caffeine metabolites have been established [4, 20—22, 30, 33—37]. More recently, improved HPLC assays which permit the simultaneous determination of caffeine and its

N-demethylated metabolites in plasma have been developed [4, 20—22]. The major disadvantage of these is that they all have relatively long retention times (10—15 min) and lengthy extraction procedures. The increasing use of shorter, more efficient columns to achieve rapid separations prompted us to evaluate the radially compressed Nova-Pak C_{18} column (Waters Assoc., Hartford, U.K.) for this purpose. Modification of the mobile phase used in our earlier method [4] was necessary but we have succeeded in reducing the analysis time to approximately 8 min whilst maintaining excellent separation of all the components. Furthermore, by using a variable-wavelength detector operating at 273 nm, we have increased the sensitivity so that the analysis can be carried out on significantly smaller sample volumes. Combining these improvements with a fast extraction procedure using reversed-phase octadecylsilane bonded-silica columns (Bond-Elut C_{18}), we have produced a rapid method for measuring caffeine and the dimethylxanthines in plasma which is suitable for both drug monitoring applications and investigations of the health consequences of these compounds.

MATERIALS AND METHODS

Reagents

Glacial acetic acid (Aristar quality), acetone (general purpose reagent grade), disodium hydrogen phosphate and phosphoric acid (Analar grade) were purchased from BDH Chemicals (Poole, U.K.). Caffeine, paraxanthine, theophylline, theobromine and β-hydroxyethyltheophylline were supplied by Sigma London (Poole, U.K.). Methanol (liquid chromatography grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Bovine serum albumin (BSA) was acquired from Armour Pharmaceutical (Eastbourne, U.K.).

Extraction columns and vacuum apparatus

Bond-Elut C_{18} columns, 1 ml capacity were used in conjunction with a special Vac-Elut vacuum apparatus to facilitate rapid sample processing. Both the Bond-Elut columns and the Vac-Elut vacuum apparatus are manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llandradach, U.K.).

Equipment

The Waters Assoc. high-performance liquid chromatograph consisted of a constant-volume Model 510 pump in conjunction with a U6K injector, a Lambda-Max Model 481 variable-wavelength LC spectrophotometer and a Model 730 data module.

Chromatography

The reversed-phase analytical column, a Waters Assoc. Nova-Pak C_{18} in Radial-Pak cartridge form (10 cm × 8 mm I.D.), was used in combination with a Waters Assoc. Z-Module RCSS system. This fully capped, 4-μm spherical, octadecylsilane bonded-silica column was protected by incorporating into the system a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak CN cartridge. The mobile phase consisting of 1% glacial acetic acid—methanol

(83:17) was filtered through a 0.45-μm Millipore filter (type HA) and degassed prior to use. Chromatography was performed at ambient temperature using a flow-rate of 2.7 ml/min which produced a back-pressure of approximately 11.6 MPa (1700 p.s.i.). The eluent was monitored at 273 nm with a detector sensitivity of 0.01 a.u.f.s. The Model 730 data module was programmed to operate with a chart speed of 198 mm/h.

Preparation of internal standard solution

The internal standard, β-hydroxyethyltheophylline, was dissolved in 0.1 *M* sodium phosphate buffer, pH 4, which was prepared by adding phosphoric acid to an aqueous solution of disodium hydrogen phosphate to achieve the desired pH before diluting to the final concentration (0.1 *M*). β-Hydroxyethyltheophylline (10 mg) was dissolved in 100 ml of 0.1 *M* sodium phosphate buffer, pH 4, to give a concentration of 100 μg/ml. Further dilution with 0.1 *M* sodium phosphate buffer, pH 4, provided the working internal standard concentration of 10 μg/ml. The internal standard was incorporated into the sample by adding a 100-μl aliquot of this solution to an equal volume of plasma, as described in the extraction procedure.

Procedure

Rapid extraction of caffeine and the dimethylxanthines from plasma was achieved using reversed-phase octadecylsilane bonded-silica columns (Bond-Elut C_{18}, 1 ml capacity). These were conditioned immediately prior to use in accordance with the manufacturer's instructions by drawing two column volumes (2 × 1 ml) of methanol followed by a similar volume of water through the column under vacuum. On releasing the vacuum, 100 μl of the plasma sample followed by 100 μl of the internal standard solution were loaded onto the column. After standing for approximately 1 min the sample was drawn through the column by re-applying the vacuum. The vacuum was then released, allowing a short equilibration period (2 min) before proceeding to the washing stage. Washing was accomplished by drawing two column volumes (2 × 1 ml) of water through the column under vacuum before eluting with 400 μl of acetone. The eluent was evaporated to dryness under nitrogen at 55°C and reconstituted in 150 μl of mobile phase. Aliquots of 15 μl were injected into the chromatograph.

Preparation of xanthine standards for calibration

Stock solutions of caffeine and theophylline (1 mg/ml in 1% acetic acid) were diluted further with 1% acetic acid to a final concentration of 100 μg/ml in each case. However, because of the limited solubility of theobromine in 1% acetic acid, this solution was prepared directly at the working concentration of 100 μg/ml. Paraxanthine was dissolved in 0.01 *M* sodium hydroxide to produce a concentration of 1 mg/ml and this was diluted further with 0.01 *M* sodium hydroxide to give a final concentration of 100 μg/ml. From these, two series of calibration standards were prepared in 2 ml of caffeine-free plasma or 3% BSA so that each calibrator contained all four methylxanthines. The concentration increments chosen for these calibration standards were 2, 4, 6 or 10 μg/ml in the case of caffeine and 0.5, 1, 2.5 or 5 μg/ml for each of the

three dimethylxanthines. These were extracted using the previously described procedure and, following analysis, graphs comparing peak height ratio with the actual concentration of the methylxanthine present were constructed.

Extraction recovery experiment

Samples were prepared in both caffeine-free plasma and 3% BSA by addition of authentic methylxanthines so that concentrations of 4 and 10 μg/ml for caffeine and 1 and 5 μg/ml for the dimethylxanthines were achieved. These concentrations were chosen to represent values at the lower and higher limits of the calibration graphs. The samples were then extracted using the previously described method and injected into the chromatograph. Measured peak heights were compared with those obtained from injections of standard solutions and the percentage recovery calculated.

RESULTS

Fig. 1A illustrates the chromatogram obtained following injection of a solution of authentic components in 3% BSA. Caffeine, β-hydroxyethyltheophylline (internal standard), theophylline, paraxanthine and theobromine are well separated with retention times of 7.98, 5.27, 4.62, 4.10 and 2.75 min, respectively. Comparison of these values with the retention times of potentially interfering compounds listed in Table I indicates that, with the exception of acetaminophen and acetazolamide, these commonly prescribed drugs and

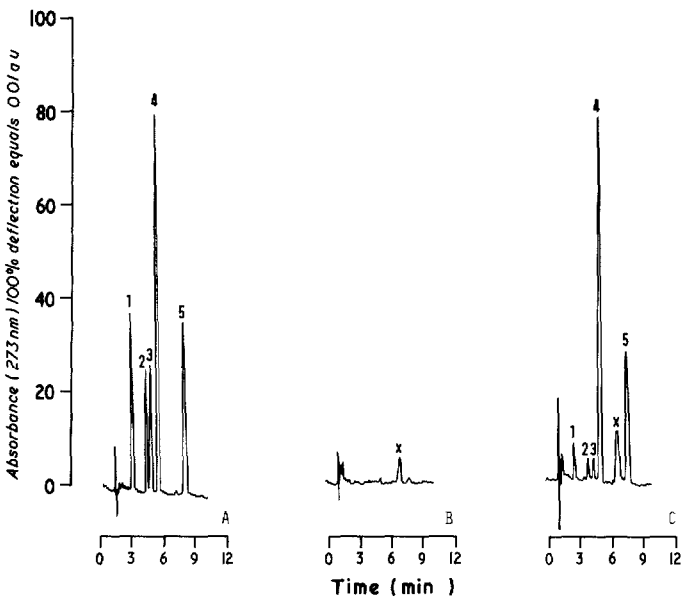

Fig. 1. (A) Chromatogram of authentic components in 3% bovine serum albumin. (B) Chromatogram of extracted xanthine-free plasma obtained from an adult male after abstaining from caffeine containing beverages for two weeks. (C) Chromatogram of a typical extracted cord plasma sample obtained at delivery. Concentrations determined (using a 15-μl injection) were 550, 550 and 480 ng/ml and 7.80 μg/ml for theobromine, paraxanthine, theophylline and caffeine, respectively. Peaks: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = β-hydroxyethyltheophylline; 5 = caffeine; X = endogenous component.

preparations used in paediatric medicine should not interfere with the quantitation of caffeine and its N-demethylated metabolites using this method. The chromatogram represented in Fig. 1B is that of extracted plasma obtained from an adult male patient after abstaining from caffeine-containing beverages for approximately two weeks. Although residual peaks corresponding to the previously mentioned methylxanthines are evident, this trace clearly indicates the absence of endogenous substances which might interfere with the estimation of the methylxanthines in plasma. Fig. 1C is a typical chromatogram of extracted cord plasma with peaks coresponding to caffeine, theophylline, paraxanthine and theobromine together with the internal standard, readily apparent. An unknown component, which occurs in all extracted cord plasma

TABLE I

RETENTION DATA OF SOME WIDELY USED DRUGS AND MEDICATIONS

Component	Retention time (min)	Component	Retention time (min)
Chlorothiazide	2.52	Benzyl penicillin	15.95
Theobromine	2.75	Trimethoprim	16.38
Acetaminophen	2.85	Acetylsalicylic acid	23.35
Acetazolamide	3.01	Salicylic acid	25.75
Paraxanthine	4.10	Phenobarbitone	35.72
Theophylline	4.62	Diazepam	—*
β-Hydroxyethyltheophylline	5.27	Carbamazepine	—
Caffeine	7.98	Cortisol	—
Vitamin K_1	9.09	Prednisolone	—
Cefuroxime	9.45	Prednisone	—
Ampicillin	11.56	Netilmicin	—
Sulphamethoxazole	12.32	Phenytoin	—
		D,L-α-Tocopherol	—

*Did not elute under the described conditions, i.e. no peaks observed up to 35 min.

TABLE II

EXTRACTION RECOVERIES OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES FROM PLASMA AND 3% BSA

Xanthine component	Concn. (μg/ml)	Plasma				3% BSA			
		Mean recovery (%)	n	S.D.	C.V. (%)	Mean recovery (%)	n	S.D.	C.V. (%)
Theobromine	1	87.09	5	3.33	3.82	92.04	4	3.99	4.33
	5	94.93	5	4.62	4.87	95.37	5	8.37	8.78
Paraxanthine	1	91.17	5	5.88	6.45	94.63	5	2.35	2.48
	5	87.15	5	5.73	6.58	88.87	6	7.26	8.17
Theophylline	1	82.27	4	5.53	6.73	84.23	4	0.64	0.76
	5	85.45	4	5.67	6.63	94.32	5	5.38	5.71
Caffeine	4	88.07	5	7.31	8.30	94.97	5	4.50	4.74
	10	89.10	5	5.19	5.82	88.78	5	3.40	3.83
β-Hydroxyethyl-									

samples, elutes between the internal standard and caffeine peaks with a retention time of 6.90 min. This may be unrelated to caffeine since it is prominent in the chromatogram obtained from caffeine-free samples (Fig. 1B) and in several others with very low methylxanthine concentrations.

In an earlier publication [4] we presented evidence supporting the use of 3% BSA, as an alternative to caffeine-free plasma, for calibration purposes in the analysis of caffeine and the dimethylxanthines. Since the technique reported here employs a different sample extraction procedure it was necessary to repeat the calibration, recovery and storage comparisons between plasma and 3% BSA using this new approach. It is apparent from Table II that the extraction efficiency (in the region of 89 ± 7% for all components) appears to be independent of the methylxanthine concentration and that comparable results are obtained using either plasma or 3% BSA. The reproducibility of the extraction is also consistent for both plasma and 3% BSA (coefficients of variation (C.V.) < 9% in all cases).

The effect of sample storage on reproducibility of results was examined by analysing replicate samples stored at −20°C for up to eight weeks. These were prepared in plasma and 3% BSA with concentrations of 1 and 5 μg/ml for the dimethylxanthines and 4 and 10 μg/ml in the case of caffeine. Following an initial analysis (zero time), samples were stored at −20°C and assayed at weekly intervals for four weeks prior to a final measurement after two months storage. With inter-batch coefficients of variation < 10% (with one exception) and recoveries in the range 104 ± 10% (see Table III) it is clear that storage at

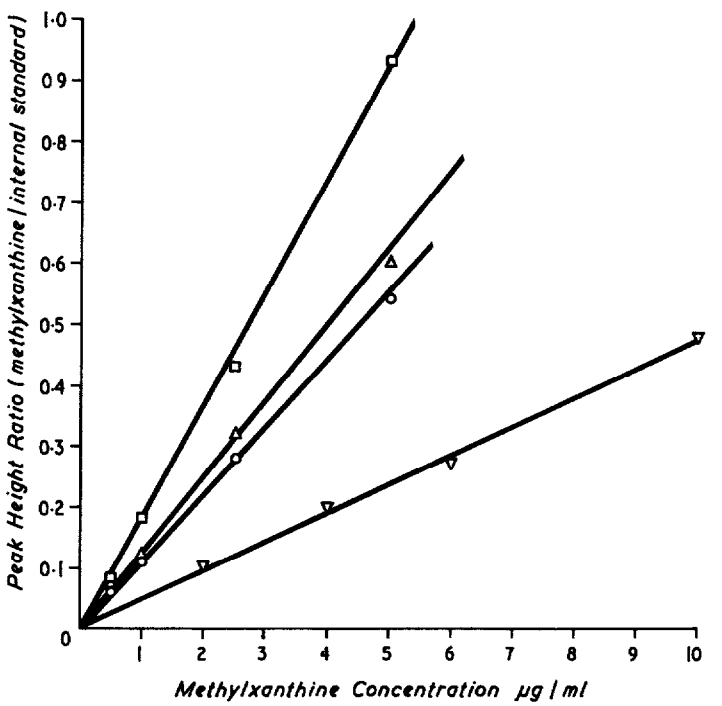

Fig. 2. Calibration graphs for caffeine and its N-demethylated metabolites (samples prepared in caffeine-free plasma). (▽) Caffeine; (△) theophylline; (○) paraxanthine; (□) theobromine.

TABLE III

EFFECT OF STORAGE ON REPRODUCIBILITY OF MEASUREMENTS OBTAINED FROM SAMPLES PREPARED IN 3% BOVINE SERUM ALBUMIN AND PLASMA

Xanthine component	Plasma				3% BSA			
	Concn. added (μg/ml)	Mean* concn. determined (μg/ml)	C.V. (%)	Recovery (%)	Concn. added (μg/ml)	Mean* concn. determined (μg/ml)	C.V. (%)	Recovery (%)
Theobromine	1	1.04	2.70	104.0	1	1.06	5.53	106.0
	5	4.90	5.55	98.0	5	5.18	8.05	103.6
Paraxanthine	1	1.13	7.46	113.0	1	1.06	10.28	106.0
	5	4.90	4.84	98.0	5	5.19	6.81	103.8
Theophylline	1	1.05	6.19	105.0	1	1.02	5.05	102.0
	5	4.72	5.45	94.4	5	5.06	6.05	101.2
Caffeine	4	3.99	7.38	99.75	4	4.07	8.71	101.75
	10	10.25	5.35	102.5	10	10.39	9.77	103.9

*$n = 6$.

−20°C for up to two months has had no serious effect. Furthermore, the close agreement between values obtained from equivalent samples prepared in plasma and 3% BSA is also readily apparent.

Calibration curves were obtained by comparing the peak height ratio (methylxanthine/internal standard) with the actual concentration of methylxanthine in spiked aliquots of plasma or 3% BSA. These relationships were linear over the working range 0—5 μg/ml for the dimethylxanthines and 0—10 μg/ml in the case of caffeine (see Fig. 2). The correlation coefficients (r) and slope values obtained from extracted plasma samples are 0.998 and 0.19, 0.997 and 0.10, 0.998 and 0.12, 0.987 and 0.05 for theobromine, paraxanthine, theophylline and caffeine, respectively. The corresponding values from calibration standards prepared in 3% BSA are 0.999 and 0.18, 0.994 and 0.11, 0.995 and 0.13, 0.996 and 0.05, respectively.

In addition to being used for monitoring caffeine and theophylline levels in the treatment of neonatal apnoea, this technique is also employed to provide information regarding foetal exposure to caffeine and its N-demethylated metabolites during the final stages of pregnancy. As the latter application constitutes part of an on-going study it is essential that the results obtained using this approach are comparable with those provided by our earlier method [4]. To demonstrate this, a series of samples were selected at random from those previously assayed for analysis by the procedure described here. The data from this comparison exercise comprise of 27 measurements of each of the four components by both original and new methods. As anticipated, there was a high correlation between the two methods for each component, giving the correlation coefficients listed in Table IV. Plots of "new method result" against "original method result" for each component suggested relations of the linear form:

new method result = $a + b$ (original method result)

The statistical analysis of this relationship, with both variables subject to experimental error, was done using the method described by Creasy [38] for which it is assumed that the experimental error standard deviations for each variable are equal and independent. The resultant estimate of the slope b and its 95% confidence interval are given for each component in Table IV. These slopes and confidence intervals are consistent with the hypothesis $b = 1$, i.e.

TABLE IV

STATISTICAL ESTIMATES FOR RELATION BETWEEN NEW AND ORIGINAL METHODS OF DETERMINING THEOBROMINE, PARAXANTHINE, THEOPHYLLINE AND CAFFEINE

Component	Correlation coefficient	Regression coefficient b		Intercept a	
		Estimate	95% Confidence interval	Estimate	95% Confidence interval
Theobromine	0.975	0.93	0.84—1.02	0.025	−0.003—0.053
Paraxanthine	0.957	0.96	0.85—1.09	0.007	−0.028—0.043
Theophylline	0.989	0.93	0.88—0.99	0.014	−0.006—0.033
Caffeine	0.990	1.04	0.98—1.10	−0.019	−0.134—0.098

the new and original methods have identical scaling for theobromine, paraxanthine and caffeine, and almost so for theophylline. Further investigation of the theophylline data showed that this minor discrepancy was entirely due to one outlier sample giving very high theophylline values by both methods and dominating the analysis. If this point is omitted the statistical analysis for theophylline gives $b = 1.05$, with 95% confidence interval of 0.93—1.19, entirely consistent with the hypothesis $b = 1$. Thus it is concluded that the new and original methods can be considered to have identical scaling for each of the four components.

Accepting $b = 1$, the best estimates of the intercept a and its 95% confidence interval were derived for each component by the standard statistical analysis of the paired differences between the new and original observations. These best estimates of a and their 95% confidence intervals are given in Table IV and are all consistent with the hypothesis $a = 0$, i.e. there is no bias between the new and original methods.

Thus the statistical analysis shows that the data is consistent with the hypothesis that, subject to random experimental error, the new and original methods give the same theobromine, paraxanthine, theophylline and caffeine results.

DISCUSSION

The method described for the determination of caffeine and its N-demethylated metabolites in plasma incorporates three modifications which serve to increase the speed of analysis and reduce sample requirement compared with our original approach [4].

Alteration of the chromatographic parameters by selecting a shorter, more efficient column (10 cm, Waters Assoc. Nova-Pak C_{18}, 4-μm spherical particles) and simplifying the mobile phase (1% glacial acetic acid—methanol, 83:17) provided excellent resolution of all four components and the internal standard in less than 8 min. Separation of paraxanthine and theophylline was achieved without any requirement for tetrahydrofuran in the mobile phase [4, 21, 33, 39]. Furthermore, the absence of buffered salt solutions from the mobile phase eliminates the need for pH adjustment and reduces the risk of pump- and column-related problems resulting from blocked frits and filters.

The speed and resolution of the chromatographic separation are also dependent on the proper choice of guard column. Preliminary investigations showed that the C_{18} Guard-Pak cartridge was not totally compatible with the analytical column, producing a marked loss of resolution and increasing retention times significantly. The CN Guard-Pak cartridge, by comparison, provided excellent resolution of all components with only marginal increases in retention values. Furthermore, Waters Assoc. have recently added a μBondapak C_{18} Guard-Pak cartridge to their range which exhibits properties similar to those of the CN Guard-Pak cartridge under the conditions described. Both are therefore suitable for use in this assay although the results presented here were obtained using the latter.

The use of solid-phase extraction columns (Bond-Elut C_{18}, 1 ml capacity) in preference to the liquid—liquid extraction procedure previously described

[4] considerably improved the ease and rapidity of sample processing. One of the rate-limiting steps using either method appears to be sample concentration by solvent evaporation, prior to reconstitution in mobile phase for analysis. The solid-phase technique described here necessitates evaporation of only 400 μl of acetone compared with 4 ml of dichloromethane—isopropyl alcohol (90:10) using the earlier method, thus reducing the time required for sample preparation by over 50%. Recovery of caffeine and the dimethylxanthines from plasma and 3% BSA is quantitative (see Table II). Extraction efficiencies are comparable with those obtained previously [4] and by Scott et al. [39] employing a similar liquid—liquid extraction approach.

The cost of solid-phase extraction columns can be minimised by repetitive use since regeneration can be effected by simply washing with two column volumes of a polar solvent (e.g. methanol) immediately following use. Indeed, Kabra et al. [40] reported recently that fifteen to twenty serum samples could be processed using the same extraction column without appreciable loss in extraction efficiency. We can confirm that these extraction columns may be used up to five times without seriously affecting performance, but we have not attempted to exceed this number.

The final modification involved changing the detector wavelength from 254 to 273 nm since the UV maximum for caffeine, theophylline and theobromine occurs in the proximity of 273 nm [41]. The effect of this combination of changes on sensitivity has been to reduce sample requirement from 300 to 100 μl of plasma whilst maintaining levels of detection in the region of 50 ng/ml for the dimethylxanthines and 100 ng/ml for caffeine.

Mean cord plasma levels obtained using this technique are 0.62, 0.57, 0.31 and 2.99 μg/ml for theobromine, paraxanthine, theophylline and caffeine, respectively. These compare favourably with earlier figures [4] although the theobromine level is significantly lower than previously indicated. However, as stated in the above publication, the primary source of theobromine appears to be dietary rather than metabolic. This discrepancy, therefore, could be entirely due to differences in the dietary habits of the two patient populations.

In a previous report [4] we compared methods for the simultaneous determination of caffeine and its N-demethylated metabolites in plasma [20—22]. However, two publications have appeared whilst this paper was being prepared so further comparisons will be justifiably restricted to these [39, 42]. The speed of the chromatographic separation of the present method compares favourably with recently reported assays [39, 42], in all cases retention times for the slowest eluting component are in the region of 7—8 min. The total analysis time of the method described, however, is greatly improved by rapid sample preparation. By comparison with our earlier procedure [4], which is very similar to the liquid—liquid extraction method used by Scott et al. [39], solid-phase extraction has yielded reductions in sample handling time in excess of 50%. Klassen and Stavric [42] also employed liquid—liquid extraction for their assay although they adopted an even longer, double-extraction approach without really improving recoveries.

In addition to providing a faster extraction procedure, the present assay is also slightly more sensitive, permitting the detection of plasma levels in the region of 50 ng/ml for the dimethylxanthines and 100 ng/ml for caffeine in

only 100 μl of sample. To attain this level of sensitivity, however, the injection volume has to be increased to 30 μl which necessitates reducing the amount of internal standard by 50% to maintain on-scale peak heights. Scott et al. [39] achieved detection limits of 100 and 200 ng/ml, respectively, whilst in the case of Klassen and Stavric [42] minimum levels of detection were between 100 and 300 ng/ml for all components.

Difficulties in readily obtaining caffeine-free plasma and evidence of residual peaks corresponding to caffeine and its N-demethylated metabolites in extracted samples (see Fig. 1B) prompted us to investigate the use of 3% BSA as an alternative for the preparation of calibration standards. The results obtained support evidence presented in an earlier publication [4] advocating the use of 3% BSA for calibration purposes and provide further justification for accepting this practice, which obviates the need for caffeine-free plasma in methods for the determination of caffeine and the dimethylxanthines.

The technique described is superior to our earlier method [4], which it has subsequently replaced, and provides an excellent tool for further studies concerning the disposition of caffeine and its N-demethylated metabolites in plasma.

ACKNOWLEDGEMENT

The authors wish to acknowledge the considerable help and advice provided by Dr. A. Baines of the Statistics Department, University of Leeds, U.K.

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