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SIMULTANEOUS DETERMINATION OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES IN UMBILICAL CORD PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A convenient high-performance liquid chromatographic method for the simultaneous determination of caffeine and its N-demethylated metabolites in plasma is described. Separation is achieved by reversed-phase chromatography using a mobile phase consisting of 0.01 *M* sodium acetate buffer, pH 5.0—methanol—tetrahydrofuran (95:4:1) in conjunction with a μBondapak C_{18} column protected by a guard column containing Bondapak C_{18}/Corasil. With a flow-rate of 3 ml/min, levels in the region of 50 ng/ml for the dimethylxanthines and 100 ng/ml for caffeine can be determined by ultraviolet detection at 254 nm. The method was used clinically for measuring cord blood samples to provide information regarding fetal exposure to caffeine and its N-demethylated metabolites during late pregnancy.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) has been referred to as the most common drug of abuse in our society [1]. Although its pharmacologic and toxicologic effects are not fully understood the U.S. Food and Drugs Administration in 1980 advised pregnant women to reduce their caffeine consumption [2]. This was prompted by indications of decreased intra-uterine fetal growth, lower birth weight and skeletal abnormalities induced by the drug in animal studies [3—6]. However, in spite of the demonstrated teratogenicity of caffeine in animals [7, 8] the implications for humans are unclear since the mode and level of exposure, and metabolism of the drug differ widely [9]. Conflicting reports associating increased fetal risk with caffeine consumption during pregnancy [10, 11] only complicate the issue. The problem in these studies is compensating for the known adverse effects of cigarette smoking and alcohol

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consumption during pregnancy [12], in order to assess the effects of caffeine ingestion. Unfortunately, neither of the previously mentioned reports [10, 11] included details of circulating plasma caffeine levels to confirm the extent of coffee drinking or the degree of fetal exposure.

In studies aimed at providing such information, carried out in Vermont [13], Montreal [14] and London [15], transplacental passage of caffeine was assessed by measuring umbilical cord blood levels from unselected infants. These, however, did not attempt to measure the N-demethylated metabolites of caffeine namely, theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine). Since these compounds are also pharmacologically active [16] it would seem advantageous to be able to measure the individual metabolites and assess their contribution to the total xanthine level.

The increasing use of xanthines in the treatment of apnöea of prematurity [17, 18] and growing concern regarding fetal exposure to caffeine during pregnancy [2, 10, 19] indicate the need for a sensitive and specific method for the simultaneous determination of caffeine, theophylline, theobromine and paraxanthine in plasma. Previous high-performance liquid chromatographic (HPLC) assays have only been partially successful because some earlier workers [20, 21] appear to have overlooked the contribution of paraxanthine whilst Sved and Wilson's method [22] for mono- and dimethylxanthines cannot quantitate caffeine without further modification. More recently, however, improved HPLC assays have become available which will permit the simultaneous determination of caffeine and its N-demethylated metabolites in plasma [23—25].

This paper describes an alternative, more sensitive method based on the technique employed by Miksic and Hodes [26] for measuring theophylline in biological fluids.

TABLE I

EXTRACTION RECOVERIES OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES FROM 3% BOVINE SERUM ALBUMIN (n = 4)

Component	Standard solutions					Extracted samples
	Concn. (μg/ml)	Volume injected (μl)	Quantity on column (ng)	Mean peak height (mm)	C.V. (%)	Concn. (μg/ml)
Caffeine	10	30	300	76.38*	3.60	2
	10	90	900	109.17**	0.70	10
Theophylline	10	7.5	75	48.38*	2.29	0.5
	10	45.0	450	130.38**	0.73	5.0
Theobromine	10	7.5	75	57.13*	5.82	0.5
	10	45.0	450	159.67**	1.30	5.0
Paraxanthine	10	7.5	75	43.88*	2.85	0.5
	10	45.0	450	132.63**	2.21	5.0

*Detector sensitivity 0.01 a.u.f.s.
**Detector sensitivity 0.02 a.u.f.s.

EXPERIMENTAL

Reagents

Dichloromethane (laboratory reagent grade) and isopropyl alcohol, methanol, tetrahydrofuran, sodium acetate (Analar quality) were all purchased from BDH Chemicals (Poole, U.K.). Caffeine, paraxanthine, theophylline, theobromine and β-hydroxyethyltheophylline were supplied by Sigma London (Poole, U.K.).

Equipment

The Waters Assoc. (Hartford, U.K.) high-performance liquid chromatograph consisted of a constant volume Model M6000A pump in conjunction with a U6K injector and a Model 440 UV detector operating at 254 nm. The signal from the UV monitor was linked to a 10-mV Linseis Model LS 24/80/80 two-pen recorder adjusted to give a chart speed of 200 mm/h for the purpose of this assay.

Chromatography

The analytical column, a 30 cm × 3.9 mm I.D. μBondapak C_{18} (10-μm reversed-phase packing) together with a guard column containing Bondapak C_{18}/Corasil were supplied by Waters Assoc. This was used in conjunction with a mobile phase consisting of 0.01 *M* sodium acetate buffer, pH 5.0—methanol—tetrahydrofuran (95:4:1) which 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 3 ml/min producing a back pressure of approximately 25.5 MPa (3750 p.s.i.). The eluent was monitored at 254 nm with a detector sensitivity of 0.01 a.u.f.s.

Volume injected (μl)	Quantity on column (ng)	Mean peak height		C.V. (%)	Percent recovery corrected for volume losses
		Uncorrected (mm)	Corrected (mm)		
100	300	56.83	68.2*	4.68	89.29
60	900	81.08	97.3**	4.06	89.13
100	75	35.83	43.0*	2.91	88.88
60	450	104.00	124.8**	1.44	95.72
100	75	39.50	47.4*	7.05	82.97
60	450	126.50	151.8**	1.37	95.07
100	75	32.5	39.0*	2.67	88.88
60	450	98.75	118.5**	2.53	89.35

Procedure

A 300-μl aliquot of plasma containing 1200 ng of the internal standard (incorporated by adding 20 μl of a solution containing 60 μg/ml β-hydroxyethyltheopylline in ethanol) was extracted by shaking for 10 min with 6 ml of dichloromethane—isopropyl alcohol (90:10). Following centrifugation at 2000 rpm (approximately 600 *g*) for 10 min the organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 55°C. The residue was then reconstituted in 200 μl of mobile phase and 70—100 μl were injected onto the column.

Preparation of standard curves

A series of calibration standards were prepared in 3% bovine serum albumin (BSA). Each standard contained all four xanthine components. Concentrations of 2, 4, 6 and 10 μg/ml were adopted for caffeine whilst levels of 0.5, 1, 2.5 and 5 μg/ml were most suitable for paraxanthine, theophylline and theobromine. These standards were subjected to the previously described procedure alongside patient samples and graphs comparing the peak height ratio with the actual concentration of the xanthine present were then constructed.

Extraction recovery experiment

Samples were prepared in 3% BSA to give concentrations of 2 and 10 μg/ml for caffeine and 0.5 and 5 μg/ml for the dimethylxanthines representing values at the lower and higher extremities of the calibration graphs. These were subjected to the previously described extraction procedure; to facilitate quantitation of the extraction recovery, however, only a 5-ml aliquot of the original 6 ml of extractant was prepared for analysis. Extracted samples were injected and the peak heights measured; these were multiplied by 6/5 to adjust for the above volume differences giving rise to the corrected peak heights referred to in Table I. The corrected peak heights were compared with those obtained from injections of standard solutions containing 10 μg/ml caffeine and the dimethylxanthines in 3% BSA and the percentage recovery calculated.

RESULTS

The chromatogram obtained following injection of a solution of authentic components in 3% BSA is illustrated in Fig. 1. Caffeine, β-hydroxyethyltheophylline (internal standard), theophylline, paraxanthine and theobromine are well separated producing sharp, symmetrical peaks with retention times of 12.75, 8.55, 7.05, 6.45 and 4.35 min, respectively. Fig. 2 is a typical chromatogram of extracted xanthine-free plasma obtained from an adult female volunteer after abstaining from caffeine-containing beverages for five days. This trace clearly indicates the absence of endogenous components which could interfere with the quantitation of caffeine and the dimethylxanthines. The chromatogram shown in Fig. 3 is representative of the many extracted cord plasma samples. Peaks with retention times corresponding to caffeine, theophylline, paraxanthine and theobromine together with the internal standard can be observed.

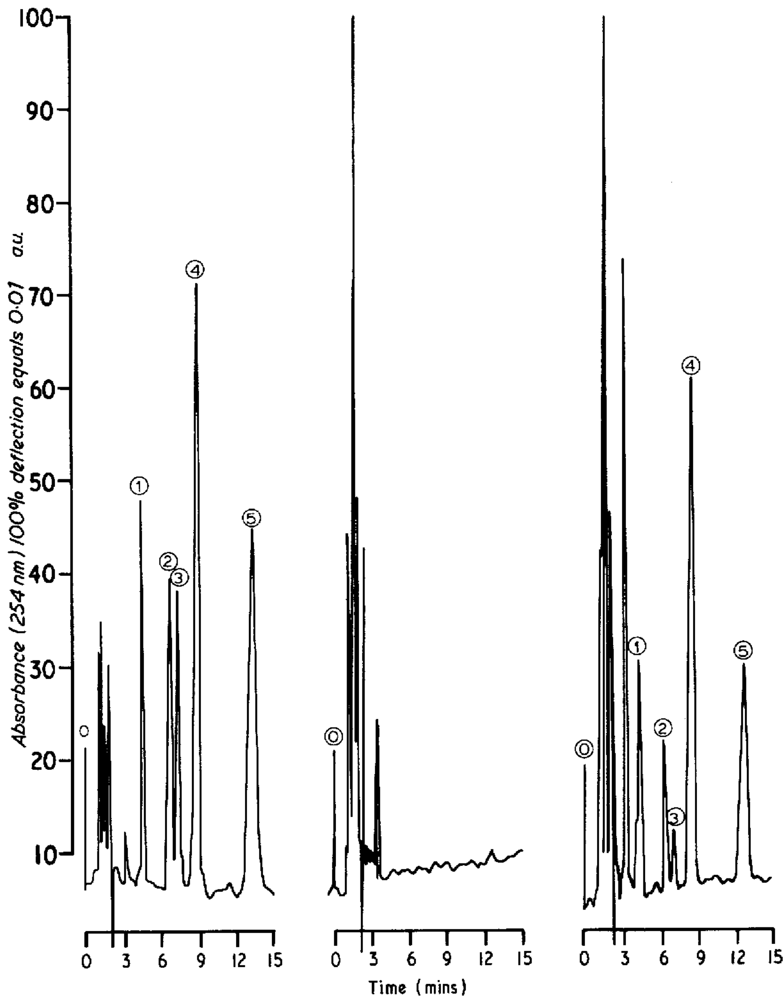

Fig. 1. Chromatogram of authentic components in 3% BSA. Peaks: 0 = injection; 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = β-hydroxyethyltheophylline (internal standard); 5 = caffeine.

Fig. 2. Chromatogram of extracted xanthine-free plasma obtained from an adult female volunteer after abstaining from caffeine-containing beverages for five days. Peaks: 0 = injection.

Fig. 3. Chromatogram of a typical extracted cord plasma sample obtained at delivery. Peaks: 0 = injection; 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = β-hydroxyethyltheophylline (internal standard); 5 = caffeine.

The adoption of 3% BSA as an alternative to plasma for calibration, recovery and storage studies was based on results (previously unpublished) obtained from comparisons using theophylline.

Theophylline was extracted from both plasma and 3% BSA at concentrations of 5 and 40 μg/ml. Recoveries of 91.85% and 93.01% with coefficients of variation (C.V.) of 9.66% and 6.85% were obtained for the low and high

TABLE II

EFFECTS OF STORAGE ON REPRODUCIBILITY OF MEASUREMENTS OBTAINED FROM SAMPLES PREPARED IN 3% BOVINE SERUM ALBUMIN

Samples stored at −20° C and assayed at weekly intervals up to six weeks followed by a final determination after six months.

Component	Concn. added (μg/ml)	Intra-batch variation*				Inter-batch variation**			
		Mean peak height ratio	Concn. determined	C.V. (%)	Recovery (%)	Mean peak height ratio	Concn. determined	C.V. (%)	Recovery (%)
Caffeine	2	0.36	1.90	3.10	95	0.35	1.85	9.83	92.5
	10	1.84	9.45	1.79	94.5	1.88	9.65	9.02	96.5
Theophylline	0.5	0.24	0.50	2.91	100	0.26	0.53	7.50	106
	5	2.44	5.0	1.60	100	2.49	5.1	4.63	102
Theobromine	0.5	0.33	0.505	9.82	101	0.33	0.505	7.10	101
	5	3.05	4.90	2.02	98	3.09	4.98	3.53	99.6
Paraxanthine	0.5	0.272	0.525	5.15	105	0.26	0.5	6.33	100
	5	2.45	4.85	1.03	97	2.49	4.93	3.44	98.6

*$n = 5$.
**$n = 11$.

plasma levels, respectively. Values for similar concentrations prepared in 3% BSA were 101.42% and 99.12% with respective coefficients of variation of 5.22% and 3.24%.

The effect of sample storage on reproducibility of results was also examined. Samples were prepared in both plasma and 3% BSA by adding aliquots of theophylline to give final concentrations of 5 and 40 μg/ml. These samples were stored at −20°C and assayed at weekly intervals for six weeks. The intra-assay results (i.e. replicate samples determined at zero time) gave mean plasma concentrations of 38.75 and 5.2 μg/ml with coefficients of variation of 1.46% and 2.61% for the high and low levels, respectively. Values obtained from equivalent samples prepared in 3% BSA were 40.25 and 5 μg/ml with respective coefficients of variation of 1.07% and 2.62%. The accumulated data obtained from the weekly analysis of replicate samples stored at −20°C over a period of six weeks were used to provide inter-assay results. The mean plasma concentrations were 39.5 and 5 μg/ml with coefficients of variation of 6.27% and 5.20% for the high and low levels, respectively. Similar sample concentrations made up in 3% BSA gave values of 40.75 and 5 μg/ml with respective coefficients of variation of 4.22% and 7.34%.

Calibration curves were obtained by comparing the peak height ratio (theophylline/internal standard) with the actual concentration of theophylline in spiked aliquots of plasma or 3% BSA. In both cases the relationship was linear over the concentration range 0–40 μg/ml. Slope values were 0.043 and 0.05 with correlation coefficients (r) of 0.984 and 0.993 for plasma and 3% BSA, respectively.

On comparison, the results obtained from these calibration, recovery and storage trials using theophylline samples prepared in both plasma and 3% BSA are very similar. This is a strong indication that, for the purposes specified above, 3% BSA is a suitable alternative to plasma. Since there is no reason to believe that caffeine or the other dimethylxanthines should behave differently, the assumption that 3% BSA can be used to obviate the need for xanthine-free plasma is not without foundation.

It is apparent from Table I that the extraction efficiency (in the region of 89 ± 7% for all components) and reproducibility (coefficients of variation $< 8\%$ in all cases) are independent of the xanthine concentration at the levels determined.

The effect of sample storage on reproducibility of results was also examined. Analyses were carried out on samples prepared in 3% BSA which concentrations were 0.5 and 5.0 μg/ml for the dimethylxanthines or 2 and 10 μg/ml in the case of caffeine. These samples were stored at −20°C and assayed at weekly intervals for six weeks followed by a final determination after six months. The findings (listed in Table II) indicate that intra-batch variation, i.e. the variation in results encountered when a batch of n replicate samples are assayed simultaneously on the same day, is generally very good (coefficients of variation $< 6\%$ with one exception). Furthermore, the inter-batch coefficients of variation are less than 10% for all the compounds measured. Clearly, with recoveries $> 92\%$ in all instances and good reproducibility (coefficients of variation $< 10\%$) for both fresh and stored samples, storage for up to six months at −20°C has had no serious adverse effect.

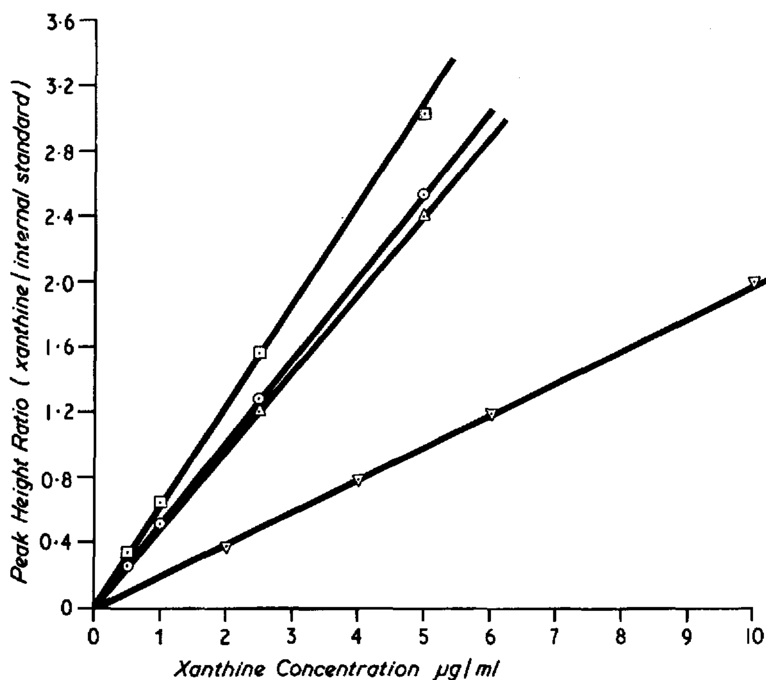

Fig. 4. Calibration graphs for caffeine and its N-demethylated metabolites. Samples prepared in 3% BSA. (▽) caffeine; (△) theophylline; (○) paraxanthine; (□) theobromine.

TABLE III

MEAN PLASMA CONCENTRATIONS OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES OBTAINED FROM UNSELECTED CORD BLOOD SAMPLES AT DELIVERY

Component	Mean concn.	S.D.	No. of patients (n)
Caffeine	2.85	2.309	113
Theobromine	1.05	1.253	111
Paraxanthine	0.67	0.476	113
Theophylline	0.287	0.174	113
Total (caffeine and dimethylxanthines)	5.146	3.227	111

Calibration curves were obtained by comparing the peak height ratio (xanthine/internal standard) with the actual concentration of xanthine in spiked aliquots of 3% BSA. The relationships were linear over the working range 0—5 µg/ml for the dimethylxanthines and 0—10 µg/ml in the case of caffeine as shown in Fig. 4. The correlation coefficients (r) and corresponding slope values are 0.98 and 0.197, 0.998 and 0.482, 0.999 and 0.501, 0.999 and 0.626 for caffeine, theophylline, paraxanthine and theobromine respectively (n = 24 in all cases).

The method was applied to measuring caffeine and its N-demethylated metabolites in 113 unselected cord blood samples obtained from The Leeds

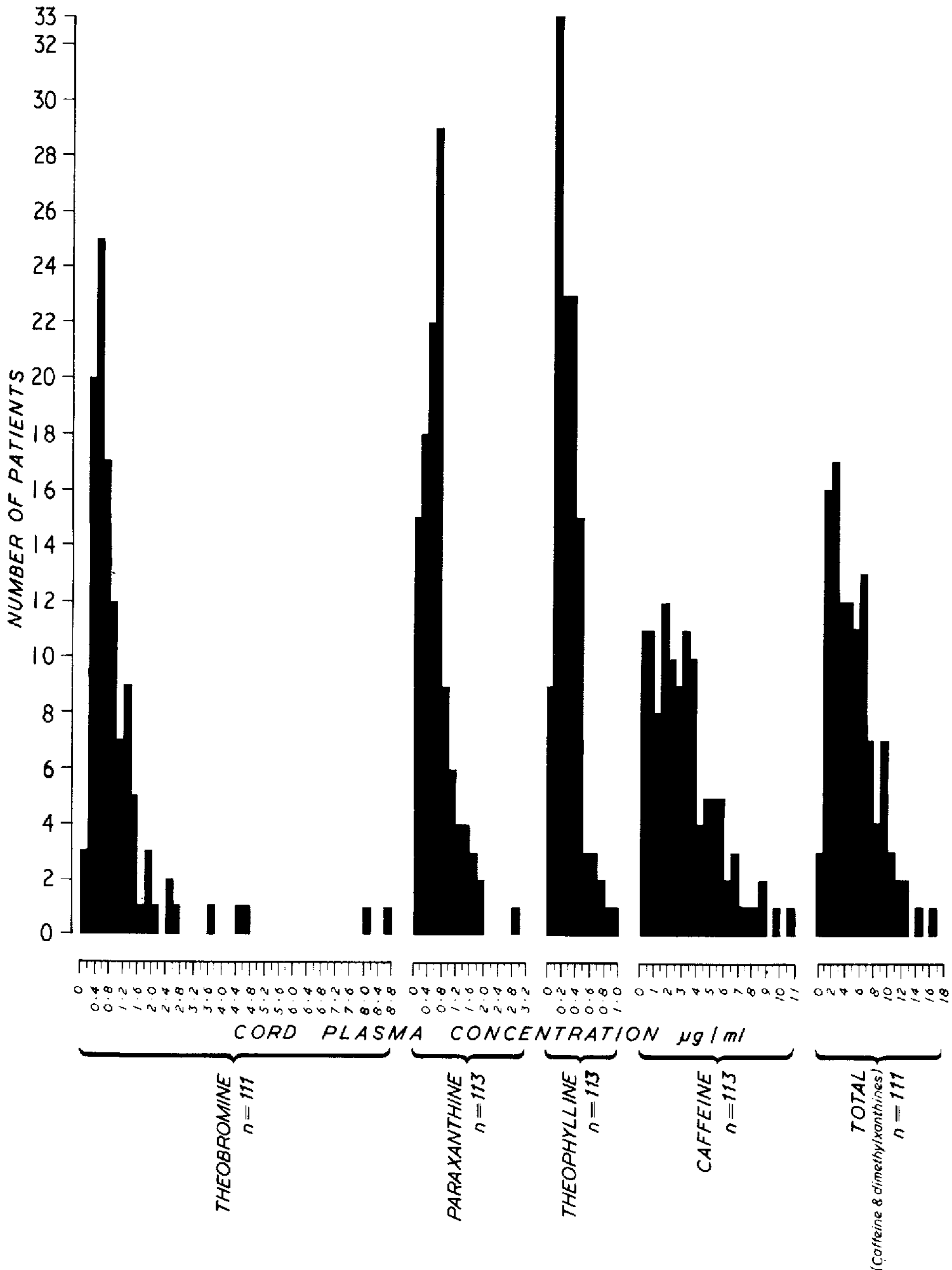

Fig. 5. Frequency distribution of cord plasma concentrations of caffeine and its N-demethylated metabolites among 113 unselected patients at delivery.

Maternity Hospital (Leeds, U.K.). The mean cord plasma concentrations were found to be 2.85, 0.29, 1.05, 0.67 and 5.15 μg/ml for caffeine, theophylline, theobromine, paraxanthine and total (caffeine + dimethylxanthines), respectively. These are summarised in Table III and the frequency distribution graphs for the individual components along with total levels are illustrated in Fig. 5.

DISCUSSION

In addition to the possibility of teratogenic effects [2], excessive caffeine ingestion has been associated with the development of cancers of the urinary tract [27, 28], coronary heart disease arising from hyperlipaemia [29] and anxiety states [30—32]. Clearly, caffeine is not the harmless substance once assumed and further investigation of its pharmacological and toxicological effects would seem justified. The method described is sensitive, selective, relatively quick and ideally suited to such investigations. The advantage of this method over earlier techniques [20—22] is readily apparent; by permitting the simultaneous determination of caffeine and all three N-demethylated metabolites in plasma, it provides additional information regarding circulating levels of pharmacologically active xanthines.

Comparison of the more recent HPLC assays [23—25] reveals similar analysis times (10—14 min) although completely different chromatographic conditions have been used to achieve separation. The technique employed by Van Aerde et al. [23] using normal-phase conditions provides the shortest analysis time (10 min), requires the smallest sample volume (100 μl of plasma) and will permit theophylline plasma concentrations as low as 200 ng/ml to be measured (value for caffeine not quoted). Tse and Szeto [24] used reversed-phase chromatography to achieve better sensitivity (100 ng/ml and 200 ng/ml for theophylline and caffeine, respectively) but with a much larger sample (500 μl of plasma) and a longer retention time (14 min). The ion-pair approach devised by Muir et al. [25] requires a similar sample volume to the previous method but the chromatographic separation is faster (10 min). Unfortunately, the detection limits of the assay were not indicated.

Although there is no improvement in the speed of the analysis, the present method is more sensitive than that of Tse and Szeto [24]. It is capable of detecting concentrations in the region of 100 ng/ml for caffeine and 50 ng/ml for the dimethylxanthines in only 300 μl of plasma compared with concentrations of 200 ng/ml and 100 ng/ml for caffeine and theophylline, respectively, in 500 μl of plasma using the latter technique.

There are probably numerous HPLC methods which may be suitable, or could be modified, for the purpose of simultaneously measuring caffeine and its N-demethylated metabolites in plasma. Many of these, however, have been primarily concerned with eliminating interferences in the estimation of individual xanthine components. The separation of paraxanthine from theophylline in biological fluids, for example, has attracted a lot of attention [26, 33—37]. Indeed, the present method and that of Tse and Szeto [24] are based on the conditions used by Miksic and Hodes [26]. The possibilities of some of these alternative methods are obviously limited; this is illustrated by the assay of Haughey et al. [38] for the determination of caffeine in plasma. Although the technique is very sensitive, separation of theophylline and paraxanthine is not achieved, making it unsuitable for the type of study reported here.

However, considering only those methods which are capable of measuring caffeine and its N-demethylated metabolites simultaneously [23—25], the present approach is clearly the most sensitive and has the additional advantage

of smaller sample requirement compared with the methods of Tse and Szeto [24] and Muir et al. [25].

This exploratory assessment of fetal exposure to caffeine and its N-demethylated metabolites in late pregnancy illustrates the clinical application of the technique. Mean cord plasma concentrations obtained from 113 unselected patients were 2.85, 0.29, 1.05, 0.67 and 5.15 μg/ml for caffeine, theophylline, theobromine, paraxanthine and total (caffeine and dimethylxanthines), respectively. The appreciably lower theophylline level probably reflects the lower dietary intake of this component compared with caffeine and theobromine [39]. The intermediate concentration established for paraxanthine supports an earlier observation that in human adults this is the major metabolite of caffeine [40].

On subjecting the data to linear-regression analysis, significant correlations between cord plasma concentrations of caffeine, theophylline and paraxanthine (at the 99% level of significance) were obtained. This seems to confirm that both dimethylxanthines are principally derived from the metabolism of caffeine [40]. Theobromine, however, only correlates with caffeine and paraxanthine at the 95% significance level and even less with theophylline, although a positive coefficient is still evident. This may be interpreted as an indication that the primary source of theobromine is dietary rather than metabolic and, since the major metabolites of caffeine in man are considered to be paraxanthine and theophylline [40], the comparatively higher mean plasma level of theobromine supports this view.

The mean caffeine level established in this study is approximately double previously published values found in Vermont and Marburg [13] and in Montreal [14] but compares favourably with that obtained in a London survey [15]. The latter study, which included an assessment of caffeine intake during pregnancy, showed that the daily consumption in pregnant British women was twice the value reported by Graham [39] in a survey of pregnant American women. The difference in mean cord plasma levels between the North American studies and the values obtained in England may be due, therefore, to national differences in dietary intake of caffeine during pregnancy.

These findings suggest that in Britain fetal exposure to caffeine and the pharmacologically active dimethylxanthine components may be much higher than previously indicated [13—15]. In Leeds, 46% of the babies were exposed to plasma levels in excess of the minimum concentration for respiratory stimulation by caffeine, which is 3 μg/ml, whilst total (caffeine and dimethylxanthine) plasma values exceeded this level in 67.6% of the cases. The significance of these elevated levels of circulating methylxanthines, in terms of effects on the outcome of the pregnancy, are unclear since they may not be associated with any adverse effects.

However, this technique is being used in continuing studies regarding fetal exposure to caffeine and its N-demethylated metabolites in late pregnancy in an attempt to identify relationships with parameters used to measure the successful outcome of pregnancy, e.g. birth weight, apgar score etc. These results will be reported on completion of the study.

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