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SIMULTANEOUS MICRODETERMINATION OF THEOPHYLLINE, CAFFEINE AND PHENOBARBITAL IN BLOOD COLLECTED ON PAPER

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SUMMARY

A sensitive and selective gas chromatographic method has been developed for the simultaneous determination of theophylline, caffeine and phenobarbital. Blood collection is performed by dropping 30 μl of blood onto a disc of a special paper. Vinbarbital is used for quantitation by the internal standard method. The chromatographic separation is performed on a 3% OV-17 column, after pentylation of the methylxanthine and internal standard, and the compounds are detected with a nitrogen-sensitive detector.

The sensitivity of the method allows the monitoring of theophylline therapy in premature newborns by the differential determination of caffeine and theophylline. The sampling method does not affect the accuracy and precision and is very suitable for the collection of small blood samples.

INTRODUCTION

The methylxanthines theophylline and caffeine are widely used for the prevention and treatment of apnea in the premature newborn. The metabolism

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and pharmacokinetic parameters of these drugs show important differences in premature newborns in comparison with those in older children or adults [1—6]. It has been shown that theophylline is converted into caffeine in premature newborns [7—10] and this original metabolic pathway has been demonstrated by use of molecules labelled with stable isotopes [11]. Caffeine has nearly the same pharmacological activity as theophylline, so it is necessary to measure both drug levels during theophylline therapy. Some neonatologists prefer to use caffeine instead of theophylline to treat apneoic attacks in the premature newborn [3]. As theophylline is a metabolite of caffeine, it is also important to measure both caffeine and theophylline plasma levels after caffeine administration. Therefore, in the drug monitoring of apneas in premature newborns, it is of utmost importance to measure the total methylxanthines plasma levels in order to ensure the efficacy of the treatment and to prevent toxicity.

The analytical method has to be able to separate theophylline from caffeine because the elimination rate constants of these two drugs are different in premature newborns. The mean half-life of theophylline is 30 h [9, 12] and that of caffeine is about 100 h [13]. Moreover, phenobarbital is often given at birth to prevent neonatal jaundice [14]. The half-life of this drug is very long in the newborn (200 h) [15], so it may be important to measure phenobarbital plasma levels simultaneously.

The lower and upper limits of the therapeutic range of plasma theophylline or caffeine concentrations are dictated by efficacy and toxicity. Available data [6] suggest that the range for theophylline is probably about 7—15 $\mu g \cdot ml^{-1}$. Plasma levels effective for the control of apnoeic spells can be lower, ranging between 3 and 5 $\mu g \cdot ml^{-1}$ [16, 17]. Plasma concentrations of caffeine as low as 3—4 $\mu g \cdot ml^{-1}$ can abolish apnea and regularize breathing patterns. Generally, therapeutic caffeine plasma levels range between 3 and 12 $\mu g \cdot ml^{-1}$ [18].

As many departments of neonatology do not have a laboratory available for assaying these drugs rapidly, we propose here a simultaneous method for the microdetermination of caffeine, theophylline and phenobarbital from blood collected on a special paper. This makes very easy the collection of blood by heel pricks from babies in incubators. Moreover, samples can be easily transported to a remote laboratory by letter mail.

EXPERIMENTAL

Materials

Theophylline, theobromine, caffeine and phenobarbital were purchased from Sigma (St. Louis, MO, U.S.A.). Tetramethylammonium hydroxide, N,N-dimethylacetamide, iodopentane, isopropanol, ethyl acetate and chloroform (analytical-reagent grade) were purchased from Merck (Darmstadt, G.F.R.) and used without further purification.

Stock 100 $\mu g \cdot ml^{-1}$ solutions of theophylline, caffeine, theobromine and phenobarbital in ethanol were prepared. The internal standard was vinbarbital, a barbiturate not often used in therapeutics. All stock solutions were stored at 4°C and used within 1 month. Working solutions (10 $\mu g \cdot ml^{-1}$) were prepared each day by dilution of the stock solution with ethanol. The extraction solvent

was chloroform—isopropanol (95:5). For extractions, blood was buffered with acetate buffer (pH 5.2) to improve the extraction efficiency.

A Schleicher & Schüll (Dassel, G.F.R.) Type 2992 paper was used for blood collection; 30 μl of blood were dropped into an 11 mm diameter circle printed on the paper and dried.

Extraction

A disc of 10 mm diameter containing the whole blood spot was stamped out, transferred into a 20-ml screw-capped centrifuge tube and rinsed with 300 μl of acetate buffer (pH 5.2) for 5 min. Then 20 μl of vinbarbital standard solution were added and thoroughly mixed, 1.5 ml of the extraction solvent were added and the blood was extracted for 1 min on a vortex mixer. After centrifugation, an aliquot of the organic phase was transferred into another centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C.

Derivatization of the N—H group was performed according to the reaction described by Greeley [19]. A 50-μl volume of N,N-dimethylacetamide and 20 μl of tetramethylammonium hydroxide solution (0.1 *M*) were added to the dry residue and shaked vigorously for 10 sec. Then 10 μl of iodopentane were added to the solution, which was shaken and allowed to stand at room temperature for 10 min. After centrifugation (2 min at 2000 *g*) the organic phase was transferred into a third centrifuge tube and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 50 μl of ethyl acetate and 1—3 μl were injected into the column for chromatographic analysis.

Standardisation

Various blood samples spiked with concentrations of caffeine, theophylline and phenobarbital ranging between 0.5 and 20 $\mu g \cdot ml^{-1}$ were prepared. These blood samples were dropped on to the paper and the spots dried, then 10 mm diameter discs, corresponding to 30 μl of blood, were stamped out of the blood spots and treated as described above.

Calibration graphs for each drug were obtained by plotting peak area ratio (peak area of drug / peak area of vinbarbital) against drug concentration.

Chromatographic separation

The gas chromatograph used was a Hewlett-Packard Model 5710A with a thermionic nitrogen—phosphorus-sensitive detector. The column used was a silanized glass tube (2 m × 1.8 mm I.D.) coated with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh). Separations were performed isothermally at 230°C (injector and detector temperature 250°C). Nitrogen was used as the carrier gas at a flow-rate of 30 $ml \cdot min^{-1}$. The output signal was integrated and the results were calculated using a Hewlett-Packard 3385A electronic integrator.

RESULTS

Chromatograms

A typical chromatogram of a standard mixture containing caffeine, theo-

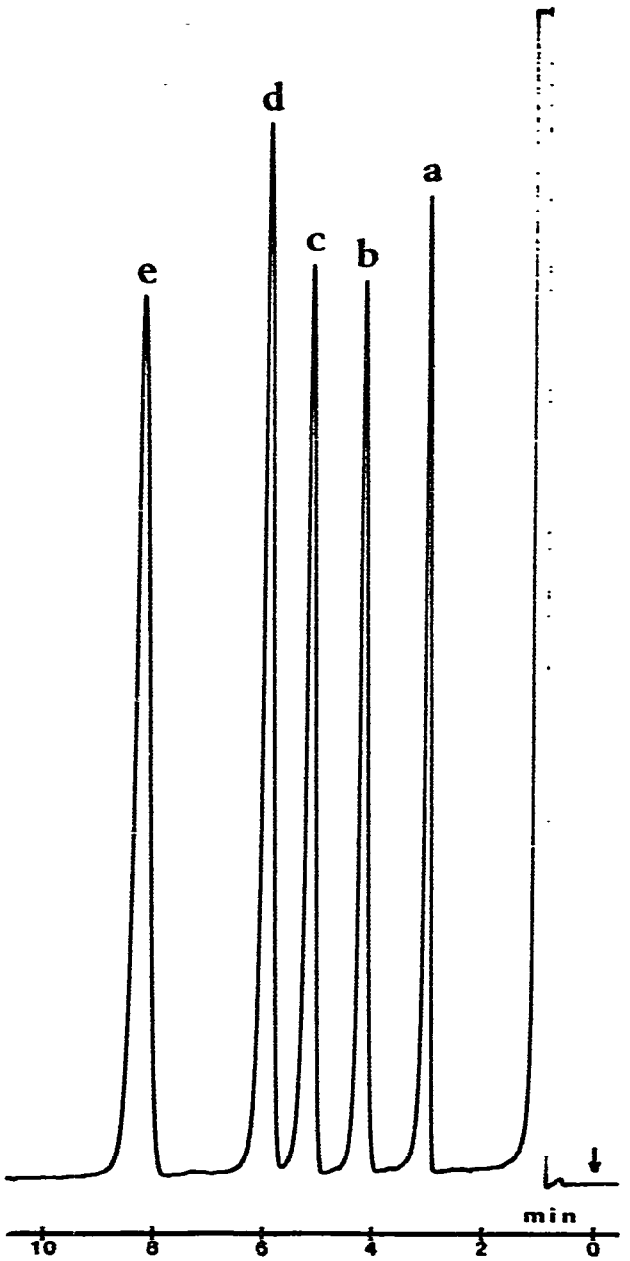

Fig. 1. Typical chromatogram of a standard mixture containing (a) caffeine, (b) vinbarbital, (c) theophylline, (d) paraxanthine and (e) phenobarbital.

phylline, paraxanthine and phenobarbital is shown in Fig. 1. Absolute and relative retention times of methylxanthines and various barbiturates are given in Table I.

Fig. 2 shows chromatograms of extracts of (A) a blood sample spiked with drugs and (B) the blood of a premature newborn treated with theophylline and

TABLE I

RETENTION TIMES OF METHYLXANTHINES AND BARBITURATES

Compound	Absolute retention time	Relative retention time
Caffeine	1.80	0.71
Allobarbital	1.80	0.71
Aprobarbital	1.92	0.76
Butalbital	1.92	0.76
Butobarbital	1.97	0.78
Amobarbital	2.10	0.83
Mebubarbital	2.35	0.93
Pentobarbital	2.35	0.93
Hexobarbital	2.40	0.95
Secobarbital	2.50	0.99
Vinbarbital	2.53	1.00
Theophylline	3.33	1.31
Paraxanthine	3.64	1.44
Theobromine	3.67	1.46
Brallobarbital	3.74	1.48
Mephobarbital	4.40	1.74
Phenobarbital	5.31	2.10
3-Methylxanthine	6.15	2.43
1-Methylxanthine	7.06	2.49
7-Methylxanthine	7.34	2.90

phenobarbital. Blood samples were collected on paper discs. Fig. 3 shows the chromatogram of a blank. The 3% OV-101 column is also suitable for the chromatographic separation of methylxanthines and barbiturates. On such a column the retention time of vinbarbital is greater than that of theophylline but the resolution is still very good.

Linearity

Concentrations were measured under the conditions described above, using blood samples spiked with concentrations of 0.5—20 $\mu g \cdot ml^{-1}$ of each drug and collected on paper. Standardization was performed using the internal standard method. A linear regression analysis of the three graphs (peak area ratio versus concentration) indicated an almost linear fit of the data:

caffeine: slope = 0.102; intercept = 0.022; $r = 0.998$;
theophylline: slope = 0.168; intercept = 0.020; $r = 0.999$;
phenobarbital: slope = 0.089; intercept = −0.002; $r = 0.999$.

Accuracy and repeatability

The accuracy and repeatability of the method in the therapeutic range were studied by measuring concentrations of blood samples spiked with 2, 5, 10 and 15 $\mu g \cdot ml^{-r}$ of each drug. Measurements were performed immediately on the whole blood, then 24 h and 8 days after collecting the same blood on paper and keeping the samples at room temperature. Five samples were assayed corresponding to each drug concentration. The results are given in Tables II—IV.

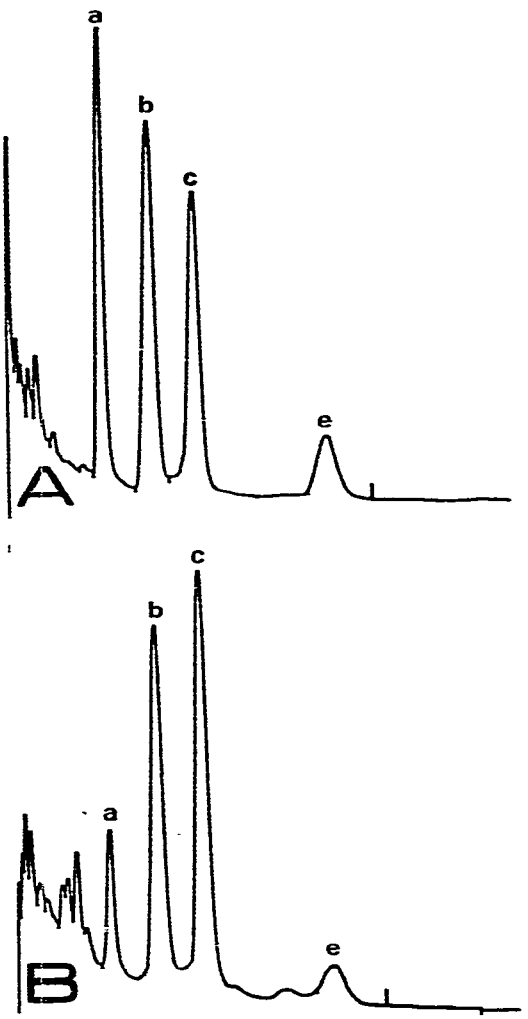

Fig. 2. (A) Chromatogram of an extract of blood collected on paper and spiked with (a) caffeine, (b) vinbarbital, (c) theophylline and (e) phenobarbital (10 $\mu g \cdot ml^{-1}$ of each). (B) Chromatogram of an extract of the blood of a premature newborn treated with theophylline and phenobarbital (collection on paper). Caffeine, 3.45 $\mu g \cdot ml^{-1}$; theophylline, 14.6 $\mu g \cdot ml^{-1}$; phenobarbital, 3.01 $\mu g \cdot ml^{-1}$.

Correlations between added and measured concentrations obtained from whole blood gave the following results:

caffeine: slope = 1.02 ± 0.0096; intercept = −0.063; r = 0.9991;
theophylline: slope = 1.01 ± 0.011; intercept = 0.078; r = 0.9987;
phenobarbital: slope = 1.02 ± 0.0106; intercept = −0.023; r = 0.9989.

The correlations between drug concentrations measured in whole blood and paper discs 24 h and 8 days after blood collection are given in Table V.

Fig. 3. Chromatogram of a blank.

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF BLOOD COLLECTED IN TUBES ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.17 ± 0.27	1.13	1.78
	10	10.20 ± 0.34	2.00	3.33
	5	5.24 ± 0.18	4.8	3.44
	2	1.98 ± 0.06	1.0	3.03
Caffeine	15	15.46 ± 0.25	3.07	1.62
	10	10.06 ± 0.25	0.60	2.49
	5	5.09 ± 0.11	1.80	2.16
	2	2.05 ± 0.10	2.50	4.76
Phenobarbital	15	15.23 ± 0.17	1.53	1.12
	10	10.43 ± 0.26	4.30	2.49
	5	5.16 ± 0.11	3.00	2.13
	2	1.91 ± 0.13	4.50	6.7

TABLE III

ACCURACY AND PRECISION OF THE ASSAY 24 HOURS AFTER COLLECTION OF BLOOD ON PAPER ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.19 ± 0.48	1.26	3.15
	10	10.23 ± 0.68	2.30	6.64
	5	5.16 ± 0.27	3.21	5.20
	2	2.04 ± 0.09	2.00	4.41
Caffeine	15	15.27 ± 0.42	1.50	2.75
	10	10.16 ± 0.74	1.60	7.20
	5	4.84 ± 0.36	3.20	7.40
	2	2.06 ± 0.13	3	6.30
Phenobarbital	15	15.25 ± 0.65	1.67	4.20
	10	9.77 ± 0.77	2.30	7.60
	5	5.19 ± 0.25	3.80	4.80
	2	2.08 ± 0.14	4	6.72

TABLE IV

ACCURACY AND PRECISION OF THE ASSAY 8 DAYS AFTER COLLECTION OF BLOOD ON PAPER ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.41 ± 1.1	2.63	7.1
	10	10.25 ± 0.83	2.50	8.09
	5	5.17 ± 0.35	3.40	6.76
	2	2.04 ± 0.12	2.00	5.8
Caffeine	15	15.61 ± 1.30	4.06	8.30
	10	9.65 ± 0.85	3.50	8.80
	5	5.13 ± 0.40	2.60	7.70
	2	2.04 ± 0.14	2.00	6.80
Phenobarbital	15	14.60 ± 1.0	2.61	6.80
	10	10.[illegible] ± 0.81	2.10	7.90
	5	5.11 ± 0.37	2.20	7.24
	2	2.07 ± 0.15	3.50	7.23

CONCLUSION

Blood levels of xanthines used to prevent apneas of premature newborns must be monitored with care because of the low therapeutic index of these drugs. This monitoring must be performed easily on very small blood samples by a specific and sensitive method. The choice of the internal standard is very important, especially for measurements of theophylline levels. Theophylline is

TABLE V

CORRELATIONS BETWEEN RESULTS FOR WHOLE BLOOD AND BLOOD ON PAPER DISCS

Correlations: A, whole blood versus paper discs 24 h after blood collection; B, whole blood versus paper discs 8 days after blood collection.

Correlation	Drug	Slope ± S.D.	Intercept	r
A	Theophylline	1.0009 ± 0.007	0.0022	0.9999
	Caffeine	0.9952 ± 0.019	−0.043	0.9996
	Phenobarbital	0.9757 ± 0.041	0.082	0.9981
B	Theophylline	1.016 ± 0.012	−0.078	0.9988
	Caffeine	1.001 ± 0.029	−0.070	0.9991
	Phenobarbital	0.943 ± 0.007	0.27	0.9993

methylated at the N_7 position by premature newborns to give caffeine. Caffeine can be present in the blood of babies in the first few days of life from placental transfer and afterwards as a result of breast feeding, so caffeine must not be used as an internal standard. Moreover, gas–liquid chromatographic methods involving methylation are not suitable because of the transformation of theophylline into caffeine. The method is also suitable for caffeine therapeutic monitoring. This methylxanthine is also widely used in the prevention and treatment of apneas of premature newborns [4, 18, 20]. Moreover, theophylline is one of the numerous metabolites of caffeine. Hence vinbarbital is a good internal standard; this barbiturate is not used in paediatric therapeutics, is quantitatively extracted (98%) and gives an N,N-dipentyl derivative that is easily chromatographed with a retention time between those of caffeine and theophylline.

The accuracy of the method is good when the results obtained for whole blood collected in tubes are compared with those for added drug concentrations. The accuracy and precision of the results obtained from blood collected on paper discs are also very good.

The accuracy remains nearly the same 24 h and 8 days after collection. The coefficient of variation increases from a mean of 3% for blood collected in tubes to 7% for blood collected on paper discs and assayed 8 days after collection for each of the three drugs.

Blood can be collected very easily on paper discs from premature babies in incubators. Hence it is possible to collect the very small amount of blood (30 µl) necessary for the drug assay and this technique is very practical for both collecting and sending the blood samples to a remote laboratory.

The method described has been used successfully for more than 1 year in pharmacokinetic studies and drug monitoring in premature newborns treated in various hospitals.

The sensitivity, specificity, accuracy and sampling technique make the proposed method very suitable for the complete therapeutic monitoring of the treatment of apneas of premature newborns.

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