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Note

Micro-method for the determination of caffeine and theophylline allowing direct application of biological fluids to thin-layer chromatography plates

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Theophylline (1,3-dimethylxanthine) is often used in children in the treatment of bronchial asthma. Levels of 10—20 μg/ml are considered to be "therapeutic serum concentrations" [1, 2]. The clearance and half-life show considerable variations from one patient to another. Thus, determination of theophylline serum concentrations is important in the avoidance of either ineffective or toxic serum levels. Contrary to theophylline, caffeine (1,3,7-trimethylxanthine) is rarely given therapeutically.

Recently the efficacy of theophylline and caffeine for treatment of apneic spells of prematures has been reported [3, 4]. Sufficient knowledge of pharmacokinetics, efficacy and toxicity of new drugs is mandatory, before their general clinical use, particularly in prematures, can be recommended. Multiple determinations of theophylline and caffeine serum concentrations for pharmacokinetic studies in prematures are only possible using a micro-method.

Existing spectrophotometric methods [5—7] for the quantitation of xanthines require relatively large amounts of serum (1—3 ml) and are of reduced specificity due to the interference of endogenous and exogenous substances. Some gas chromatographic [8—10] and high-pressure liquid chromatographic [11—13] methods, which require amounts of 0.05—1.0 ml serum, are known, especially for the determination of theophylline, but require extraction from biological material. Spectrodensitometric methods for theophylline after serum extraction are described [14, 15], but because large amounts of serum are required (1—5 ml), these methods are not suitable for use in prematures and children.

In the present paper a micro-method for the quantitative measurement of theophylline and caffeine in 5—10 μl biological fluid is described. Xanthine derivatives may be determined in nanogram amounts by their UV absorption, without extraction, after direct application of serum, saliva and urine to thin-

layer chromatography (TLC) plates. Using this method in pharmacokinetic studies some interesting results have been found, e.g. a markedly prolonged half-life of theophylline and caffeine in prematures as compared with older children.

EXPERIMENTAL

Materials

Reagent-grade chloroform, methanol, absolute ethanol, ethyl acetate and ammonia (25%) were obtained from Merck (Darmstadt, G.F.R.). The TLC plates were Kieselgel $60_{F_{254}}$ DC-Fertigplatten (20×20 cm, with a layer thickness of 0.25 mm; Merck). Theophylline and caffeine were used as DAB 7 substances and theobromine (3,7-dimethylxanthine) as Ph. Eur. II substance*. Stock solutions were prepared by dissolving 40 mg of theophylline or caffeine in 100 ml of methanol and 40 mg of theobromine in 100 ml of 0.2 *N* NaOH. Theophylline and caffeine standards of 25, 50, 100, 200 and 250 ng in 10 µl serum (plasma), saliva and urine, for the preparation of calibration curves, were obtained by appropriate dilutions of the stock solution. These reference standards were stored at −20° and used for 2 weeks. The stock solutions are stable at 4° for at least 2 months.

TLC

Six samples (two reference standards of 50 and 200 ng xanthine and four samples from patients) were applied to the TLC plate. The distance between the spots and to the side of the plate was about 2.5 cm and to the lower edge 1.5 cm. First, 10 µl of ethanol were spotted onto the plate with a micro-pipette (Brand, Wertheim, G.F.R.). Immediately afterwards, either 10 µl of plasma from a second micro-pipette, or 10 µl of saliva from a constriction pipette, were applied to the centre of the wet ethanol spot. After application of the last sample the plates were air dried for 20 min and subsequently developed. Urine (10 µl) was applied to the plate directly, without ethanol. The solvent system was chloroform—methanol (90:10), the development being carried out in a saturated tank (Desaga, Heidelberg, G.F.R.) with an elution time of 45—50 min and an elution distance of 15 cm. After TLC the plates were dried in an oven for 10 min at 100°. Subsequently caffeine (hR_F value: 44) and theophylline (hR_F value: 30) were marked under a UV lamp (254 nm) by their fluorescence quenching. For separation of theobromine and theophylline the solvent ethyl acetate—methanol—25% ammonia (80:20:10) must be used with an elution distance of 15 cm and hR_F values of 24 (theophylline), 43 (theobromine) and 53 (caffeine). Down to 50 ng of xanthines could be detected as fluorescence-quenching spots. Blanks of plasma, saliva and urine did not show UV absorption in the region of xanthines.

Spectrodensitometry

The UV absorption of the xanthine derivatives was measured using a dual-wavelength TLC scanner (CS-910; Shimadzu, Kyoto, Japan) in reflection mode

* DAB 7 = Deutsches Arzneibuch, Ausgabe 7; Ph. Eur. II = Pharmacopoea Europea, Band II.

with a sample wavelength of 273 nm and a reference wavelength of 315 nm. The zigzag scanning method with a light beam of 1.25×1.25 mm² was used. The integration range was 2 cm and the scan speed 5 mm/min. The integration zero point was adjusted by background correction and suppression in front of every spot. Absorption peaks and their integrals were simultaneously recorded by a two-pen recorder (Colora 1200-01, Lorch, G.F.R.) with aninput voltage of 50/50 mV and a paper speed of 1 cm/min. Each spot was measured twice. Storage of developed plates for up to two weeks did not change the results.

RESULTS AND DISCUSSION

When from 25 to 250 ng caffeine and theophylline in 10 µl plasma, saliva or urine were applied to TLC plates, linear calibration curves were obtained (Figs. 1 and 2). Thus, "therapeutic theophylline serum concentrations" of 6—14 µg/ml [3, 17] in apneic prematures and of 10—20 µg/ml in patients with asthma bronchiale [1, 2] could be determined in 10-µl samples. For concentrations of above 10 µg/ml xanthine, 5 µl biological fluid were sufficient for quantitation. Down to 1 µg/ml xanthine derivate could be determined by changing the scan speed.

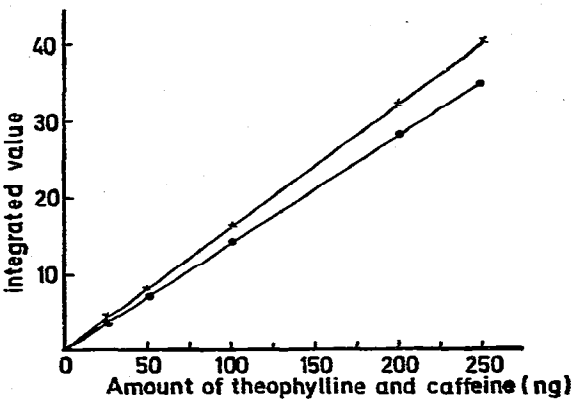

Fig. 1. Calibration curves for theophylline and caffeine. ×, caffeine (serum); •, theophylline (serum, saliva, urine) and caffeine (saliva, urine). 10 µl of biological fluid were applied to the TLC plate.

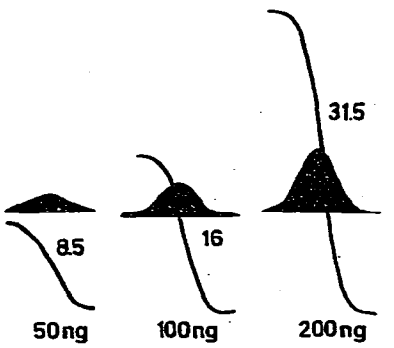

Fig. 2. Densitogram of 50, 100 and 200 ng caffeine after application of 10 µl serum with corresponding integral values of 8.5, 16 and 31.5.

In addition to the possibility of using micro-samples, the described method has the advantage of direct application of body fluids to TLC plates without prior extraction. Recently the determination of chinidine and salicylic acid, after direct application of serum and precipitation of proteins with ethanol on the TLC plate, has been described [16].

Theophylline and caffeine migrate completely from the serum and saliva after precipitation of the proteins, and are well separated. If the xanthines are applied in serum, somewhat lower R_F values are obtained compared with application in methanol, saliva or urine. Thus, for the quantitation of xanthines in serum, saliva or urine, the corresponding reference standards in the same bio-

logical fluid have to be run simultaneously. There was no interference by hemolysis. Hemoglobin was also precipitated by ethanol at the start point. The determination of xanthines was not influenced by higher bilirubin concentrations (e.g. 15 mg%). No interfering impurities, which could influence the measurement, were found after chromatography. Under a UV lamp (254 nm) a semi-quantitative analysis of the serum concentrations can be carried out by fluorescence quenching of the xanthines (e.g. in the case of intoxication). The quantitation of the xanthines in plasma, saliva or urine is subsequently carried out with the appropriate calibration curves (Fig. 1).

The xanthines could be determined with good accuracy. The coefficient of variation of 10- and 20-μg samples of theophylline per ml of serum on different plates was 2% (n=10) and for the same serum concentrations of caffeine was 3% (n=10). For 10 μg caffeine and theophylline in 1 ml saliva or urine a coefficient of variation of 2—3% was found (n=10). The possibility of using the zigzag scanning method is an important advantage of the TLC scanner CS-910 (Shimadzu) as compared with other densitometers. The UV absorption of the xanthines is cumulatively recorded and integrated by a micro-beam of 1.25× 1.25 mm^2 during zigzag motion of the TLC plate, whereas in the case of linear scanning a time-consuming and often difficult adjustment of the split appropriate to the different form and size of the spots is necessary. Moreover, a better accuracy is achieved with the zigzag scanning method.

Possible interference of some substances with the xanthine determination was investigated (Table I). All the xanthine metabolites have lower R_F values than caffeine and theophylline. Theobromine, which occurs in cacao and chocolate, is not well separated from theophylline by the solvent chloroform—methanol (90:10). A good separation is achieved with the solvent ethyl acetate—methanol—25% ammonia (80:20:10).

Since no extraction is needed with the described method, the determination

TABLE I

SUBSTANCES WHICH DO NOT INTERFERE WITH THEOPHYLLINE AND CAFFEINE DETERMINATION

Solvent: chloroform—methanol (90:10).

Drugs	Phenobarbital	Furosemide
	Spironolactone	Cephalotin
	Gentamicin	Atropine
	Ampicillin	Diazepam
	Diphenhydramine	Codeine
	Ephedrine	
Xanthine metabolites	1-Methylxanthine	3-Methyluric acid
	3-Methylxanthine	1-Methyluric acid
	7-Methylxanthine	1,3-Dimethyluric acid
	1,7-Dimethylxanthine	
Endogenous substances	Bilirubin	Hypoxanthin
	Hemoglobin	Uric acid
	Xanthine	

of the xanthine derivatives can be quickly performed. About 25 samples can be quantitated in 3—4 h.

We use the described method clinically for the control of serum concentrations and for pharmacokinetic studies in prematures with apnea. As an example, the theophylline serum concentrations during the treatment of an apneic premature are shown (Fig. 3). The serum concentrations were determined just before the next oral dose and 2 h later at the time of peak serum concentrations. The serum concentrations of 6—9.5 μg/ml are well within the "therapeutic range" of 6—14 μg/ml [3, 17].

As a second example, illustrating the pharmacokinetics of theophylline and caffeine in prematures the elimination from serum is described (Fig. 4).

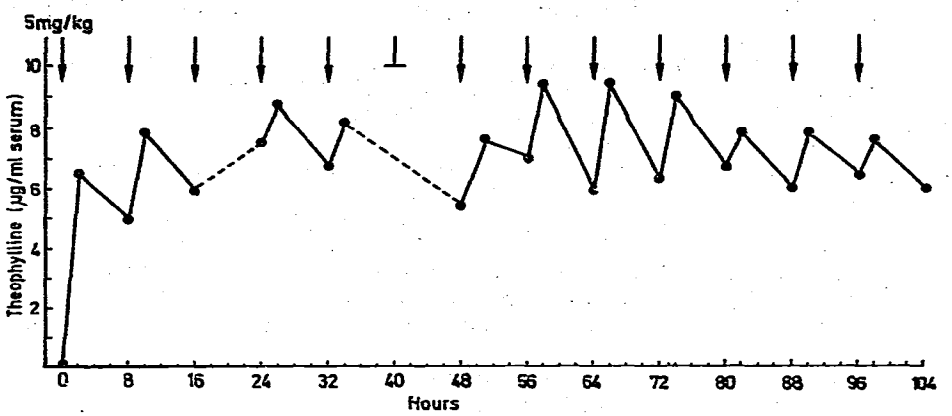

Fig. 3. Serum concentrations of theophylline in an apneic premature. Patient A: 1800 g, 0.5% theophylline solution, oral. Loading dose: 5 mg/kg. Maintenance dose: 2 mg/kg every 8 h (↓). One dose omitted (⊥).

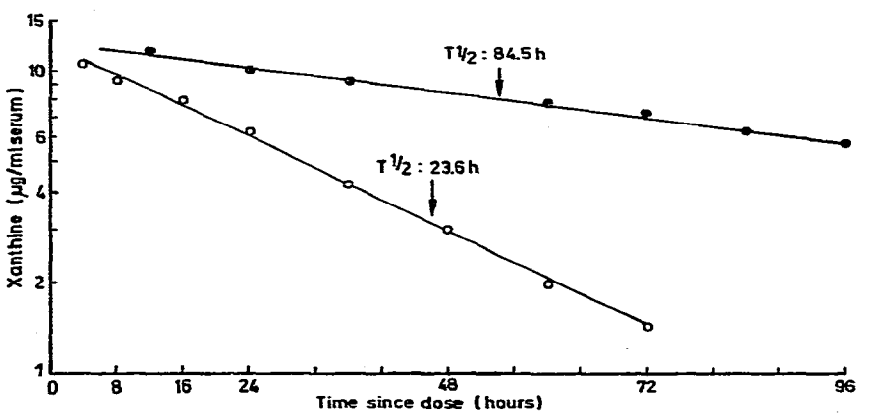

Fig. 4. Elimination of caffeine and theophylline from the serum of 2 prematures. Half-life ($T_{½}$) for theophylline and caffeine in adults: 3—5 hours. •, Patient P: 2000 g, single oral dose of 9 mg/kg caffeine (as caffeine citrate solution). ○, Patient P: 2000 g, 2 mg/kg theophylline (as 0.5% solution) oral every 6 h. Calculation of $T_{½}$ after steady-state-serum concentrations were attained and cessation of therapy.

Corresponding to a delayed elimination, the half-lives of theophylline (23.6 h) and caffeine (84.5 h) are markedly prolonged in comparison with adults. The extremely prolonged half-lives of theophylline and especially caffeine may be caused by immaturity of the demethylating liver enzymes, which metabolize the xanthine derivatives to ineffective methylxanthines and methyluric acids. Up to now the described micro-method has been successfully used by us for about 500 xanthine determinations in capillary blood, saliva or urine of prematures, children and adults.

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