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Note

Reversed-phase high-performance liquid chromatographic determination of caffeine and its N-demethylated metabolites in dog plasma

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Theophylline (1,3-dimethylxanthine) is a bronchodilator used extensively in the treatment of chronic obstructive pulmonary diseases. Because of the relatively narrow and well defined therapeutic index associated with this drug [1], monitoring of its circulating levels in patients has become a common and important clinical practice.

While numerous assay methods have been reported for theophylline analysis, few have shown the capability of simultaneous determination of this drug and other dietary xanthines such as caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), and their various metabolites. A first step in the metabolism of caffeine in man is N-demethylation, which may occur at either the 1, 3 or 7 position to form theobromine, 1,7-dimethylxanthine and theophylline, respectively, the last two being the major pathways [2]. Theophylline and 1,7-dimethylxanthine can be separated when ion-exchange [3, 4] or normal-phase [5, 6] chromatographic columns are used, but peak resolution is poor on most reversed-phase chromatographic columns [7—11]. Interference by 1,7-dimethylxanthine can be a serious concern in the determination of theophylline when the consumption of beverages containing caffeine is difficult to control. Recent developments to solve this problem include the use of ion-pair complexing methods coupled with reversed-phase high-performance liquid chromatography (HPLC) [12, 13]. While these assays are capable of separating theophylline and 1,7-dimethylxanthine, they often lack the speed and efficiency desired in the routine analysis of these compounds. Farrish and Wargin [12] reported retention times of 22 and 12 min, respectively, for theophylline and

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1,7-dimethylxanthine. The method of Muir et al. [13], which requires ion-pair complexing and extraction together with gradient-elution systems, has retention times of 17 min for 1,7-dimethylxanthine, 20 min for theophylline, and 30 min for caffeine, plus a 17-min re-equilibration time. The complexity of the five-step ion-pairing gradient and a partially sloping baseline are further drawbacks of this system.

Miksic and Hodes [14] used a reversed-phase column with a 5-μm support or a radial compression module with a reversed-phase cartridge to resolve theophylline and 1,7-dimethylxanthine. The retention time for theophylline is 18 min on the 5-μm reversed-phase column with a mobile phase (7% methanol, 1% tetrahydrofuran, in 0.01 *M* sodium acetate pH 5 buffer) flow-rate of 1.5 ml/min at 24.2 MPa (3500 p.s.i.) pressure. The analysis time is reduced to 12 min using the radial compression unit with a mobile phase (6% methanol, 1.2% tetrahydrofuran, in 0.01 *M* pH 5 sodium acetate buffer) flow-rate of 3 ml/min at approximately 6.9 MPa (1000 p.s.i.). However, the high initial equipment cost of the radial compression module may limit the accessibility of this method.

In the present paper, an improved method using reversed-phase HPLC for the simultaneous determination of caffeine and its N-demethylated metabolites theophylline, 1,7-dimethylxanthine and theobromine is described. The method was used to analyze plasma samples from a beagle dog after receiving multiple oral doses of caffeine.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Model 6000 pump, a Waters Model U6K universal injector, a Waters Model 440 UV detector, a 30 cm × 3.9 mm I.D. 10-μm μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.), a 7 cm × 2.1 mm I.D. precolumn (Co:Pell ODS, Whatman, Clifton, NJ, U.S.A.), and a 10-mV Fisher Series 500 recorder (Fisher Scientific, Pittsburgh, PA, U.S.A.) operated at a chart speed of 0.5 cm/min.

Chemicals

Ultrapure water, glacial acetic acid and tetrahydrofuran (HPLC grade, J.T. Baker, Phillipsburg, NJ, U.S.A.); methanol and acetonitrile (UV grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.); sodium acetate and trichloroacetic acid (certified ACS grade, Fisher Scientific); theophylline (Pfaltz and Bauer, Stanford, CT, U.S.A.); theobromine, 1,7-dimethylxanthine, β-hydroxyethyltheophylline and caffeine (Sigma, St. Louis, MO, U.S.A.) were all used as received.

Assay procedure

To 0.5 ml of plasma in a 2-ml centrifuge tube were added 0.05 ml of 40% trichloroacetic acid and 0.05 ml of an internal standard solution (200 μg/ml of β-hydroxyethyltheophylline in pH 5 buffer). After mixing on a vortex mixer for 30 sec, samples were allowed to stand for 5 min, followed by centrifugation at 1000 *g* for 10 min. A 25-μl aliquot of the supernatant was injected into the chromatograph.

The mobile phase for liquid chromatography was 0.005 *M* sodium acetate buffer adjusted to pH 5—methanol—acetonitrile—tetrahydrofuran (92.5 : 3 : 2.8 : 1.7, v/v). This solution was filtered through a 0.45-μm filter and degassed under vacuum with sonication. The chromatograph was operated at ambient temperature with a flow-rate of 1.5 ml/min at 10.4 MPa (1500 p.s.i.). The UV detector was set at 280 nm and 0.02 a.u.f.s.

A standard curve for each compound studied was prepared by assaying dog plasma samples containing caffeine, theophylline, 1,7-dimethylxanthine and theobromine at concentrations of 0.5, 1, 2, 4, 8, 16 and 32 μg/ml. All samples were analyzed in quadruplicate. Quantitation was achieved by the drug:internal standard peak height ratio method.

Experiment in the dog

A one-year-old male beagle dog weighing 11.4 kg was given a 100-mg tablet of caffeine (NoDoz, Bristol-Myers, New York, NY, U.S.A.) twice daily at 9:00 a.m. and 9:00 p.m. for 8 days. Serial venous blood samples were collected during the 12 h after the morning doses on days 1 and 8. The procedures for drug administration and plasma collection and storage were previously described in detail [15].

RESULTS

Assay

Chromatograms of control dog plasma and plasma spiked with the test compounds are shown in Fig. 1A and B, respectively, while Fig. 1C is the result of analysis of a plasma sample obtained from the dog 1 h after the morning dose of caffeine on day 8.

Complete peak resolution was achieved by the HPLC system in 14 min, with retention times of 5.1, 7.6, 8.3, 9 and 13.1 min for theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine, respectively. Other xanthines did not interfere. Plots of peak height ratios versus plasma drug concentrations over the range of 0.5—32 μg/ml were linear, with correlation coefficients better than 0.999 and *y*-intercepts not significantly different from zero for all compounds. The accuracy and precision of the method, determined by assaying plasma samples containing known quantities of the drugs, are shown in Table I. The limits of detection were 0.1 μg/ml for theophylline and theobromine, and 0.2 μg/ml for 1,7-dimethylxanthine and caffeine.

Plasma levels of caffeine and metabolites in the dog

Plasma concentration—time data after single and multiple oral doses of caffeine to the dog are presented in Fig. 2. Caffeine was rapidly absorbed from a single oral dose, reaching a peak plasma level of 10.9 μg/ml in 1 h. Elimination half-life calculated by linear regression of the 3—12 h data was 4.3 h. Theophylline and 1,7-dimethylxanthine appeared in plasma within 1 h, and slowly reached peak concentrations of 2.38 and 1.00 μg/ml, respectively, at 8 h after caffeine administration.

After repeated oral administration of caffeine, twice daily for eight days, there were slight increases in the peak plasma level (17.5 μg/ml) and half-life

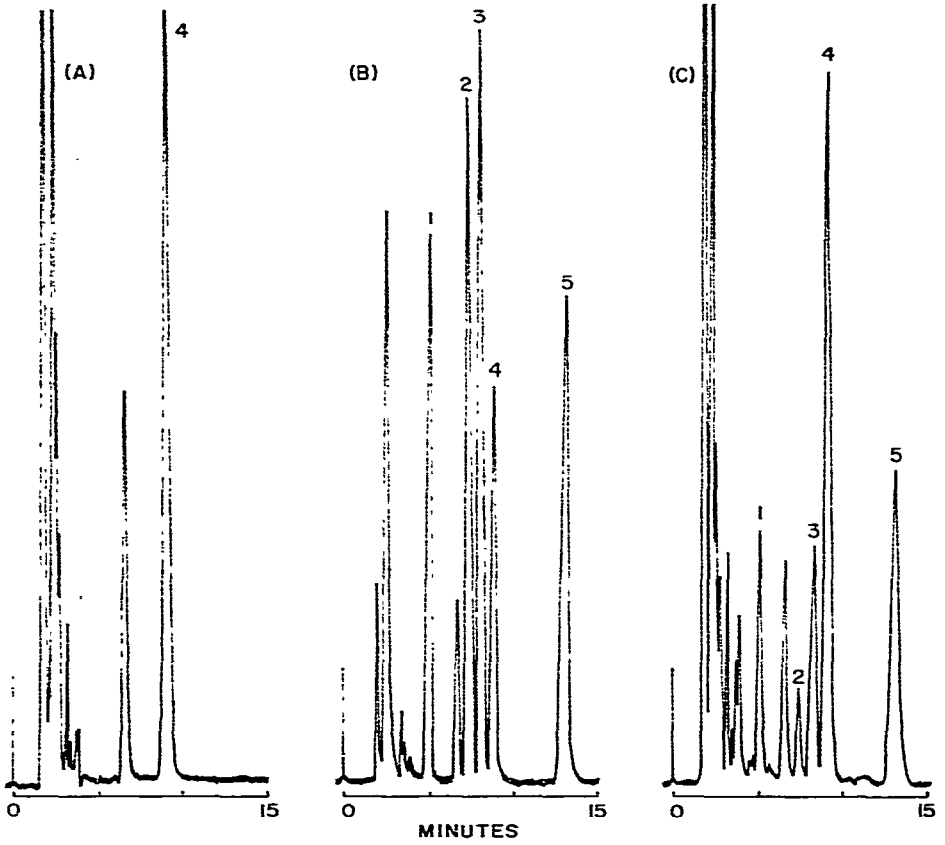

Fig. 1. Chromatograms of (A) control dog plasma with β-hydroxyethyltheophylline (internal standard); (B) plasma spiked with (1) theobromine at 16 μg/ml, (2) 1,7-dimethylxanthine, (3) theophylline, (5) caffeine, each at 32 μg/ml, and (4) β-hydroxyethyltheophylline at 20 μg/ml; and (C) plasma obtained from a dog 1 h after the morning dose on day 8 during a 100-mg caffeine twice daily regimen.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY

Concentration (μg/ml)	Theobromine		1,7-Dimethylxanthine		Theophylline		Caffeine	
	C_{obs}* (μg/ml)	C.V.** (%)	C_{obs} (μg/ml)	C.V. (%)	C_{obs} (μg/ml)	C.V. (%)	C_{obs} (μg/ml)	C.V. (%)
0.5	N.D.***	N.D.	0.47	6.38	0.54	5.56	0.62	12.0
1	0.84	0.59	1.10	0.27	1.12	6.79	1.15	10.4
2	N.D.	N.D.	2.14	1.87	2.13	3.29	2.11	6.16
4	4.04	0.71	4.05	0.25	4.05	1.98	4.03	3.47
8	N.D.	N.D.	7.85	0.38	7.82	1.41	7.86	2.54
16	16.3	0.63	15.8	2.86	15.6	3.46	15.8	1.08
32	31.8	1.16	32.1	0.78	32.2	1.83	32.1	1.71

*Mean observed concentration, $n = 5$.
**Coefficient of variation.
***N.D. = not determined.

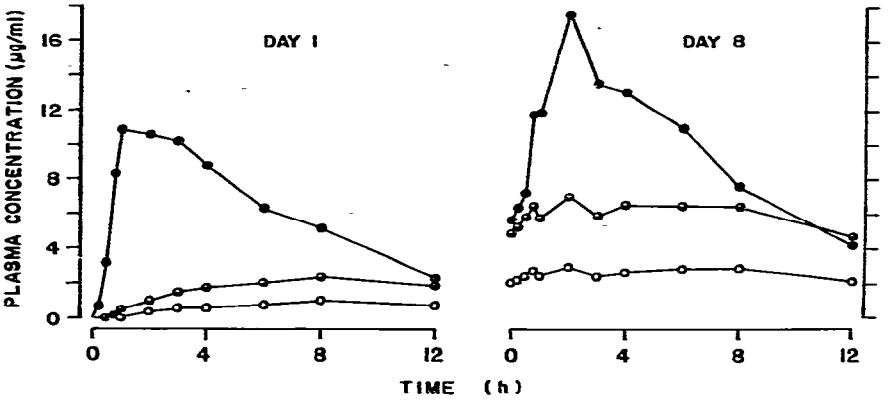

Fig. 2. Plasma concentrations of caffeine (•), theophylline (⊖), and 1,7-dimethylxanthine (○) after the morning doses on day 1 and day 8 during a 100-mg caffeine twice daily regimen.

(5.2 h) of the drug. On the other hand, significant accumulation of the metabolites was observed. Peak plasma concentrations were 6.99 μg/ml for theophylline and 2.98 μg/ml for 1,7-dimethylxanthine, both occurring at 2 h after dosing with caffeine.

Theobromine plasma concentrations similar to the levels of 1,7-dimethylxanthine were also observed. The data have been reported elsewhere [16].

DISCUSSION

In the present HPLC method, protein-precipitated plasma was injected directly into the liquid chromatograph. The simplicity of the method allowed quantitative analysis of small plasma samples (0.25—0.5 ml). This procedure is superior to solvent extraction methods which are more time consuming and frequently show low recovery and poor reproducibility due to emulsion formation. A regular 10-μm C_{18} column was used in the HPLC system, together with a precolumn to prolong column life and maintain maximum resolution.

Optimal resolution of theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine was achieved rationally by careful examination of the physicochemical properties and elution characteristics of these compounds in methanol, acetonitrile and tetrahydrofuran. The polarity of the xanthine analogues are, in descending order, theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine. Methanol, the most polar organic solvent used in the mobile phase, effectively accelerated the elution of the more polar interfering substances in the deproteinated plasma relative to the less polar xanthine analogues. Acetonitrile, a less polar solvent than methanol, increased the rate of elution of theophylline with respect to 1,7-dimethylxanthine. Similarly, tetrahydrofuran, the least polar of the three solvents, accelerated the elution of β-hydroxyethyltheophylline with respect to theophylline. The composition of the mobile phase was selected to give optimal resolution of the xanthine analogues in the shortest time possible.

Compared to the isocratic methods [14] and the ion-pair complexing methods [12, 13], the HPLC assay described here provides significant advantages in terms of speed and efficiency. It is sensitive and reproducible, and can be read-

ily applied to ordinary HPLC equipment without modification. The method was used successfully in the analysis of plasma from a dog during a multiple dosing experiment with caffeine, when significant accumulation (ca. three-fold) of both theophylline and 1,7-dimethylxanthine was observed. Higher steady-state levels of theophylline were noted in our previous paper [15], which was due apparently to interference by 1,7-dimethylxanthine. Nevertheless, the removal of the 7-methyl group appears to be the major metabolic pathway of caffeine in the dog, which is in agreement with previous observations in other laboratories [8].

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