following reasons.

1. Capacity-limited elimination of II occurs at plasma concentrations above 400 μg/ml (2). Both intravenous and oral administrations at 6.34 mmoles/kg gave plasma concentrations well above this threshold, while percutaneous applications of I gave plasma concentrations below this concentration. When capacity-limited elimination occurs, the AUC increases disproportionally with respect to dose. The presence of capacity-limited elimination after the intravenous dose in the present case artifactually led to a larger $AUC_{0\text{-}4,iv}$ value for comparison with values obtained for the percutaneous treatments. Thus, the relative $AUC_{0\text{-}4}$ values for the percutaneous absorption experiments are likely to underestimate F.

2. Percutaneous drug absorption is sometimes fairly protracted. Some I absorption might still take place after the 4-hr period monitored in the percutaneous experiments. This factor would also lead to underestimation of F using the relative $AUC_{0\text{-}4}$ values. The present data could not be used for calculation of AUC from time zero to infinity because significant uncertainties in elimination $t_{1/2}$ estimates are produced by nonlinear elimination kinetics and possible incomplete absorption after the percutaneous doses.

The present results, however, suggested that at least ~10% of the percutaneous I dose was absorbed. Mean plasma II concentrations up to 200 μg/ml were obtained following a 6.34-mmoles/kg topical dose. These concentrations approach those levels (~400 μg/ml) required for complete hypnosis in the rat. In fact, some animals in the percutaneous experiments were sufficiently sedated that continued ether anesthesia was unnecessary. It is clear that the pharmacological effects of I can be elicited through skin absorption.

Hair removal by a commercial depilating agent apparently increased the rate of percutaneous I absorption in the rat. Although hair removal by shaving was done as close to the skin as possible, the remaining hair stubs probably still posed a significant barrier for drug absorption. On the other hand, the depilating agent might have caused some damage to the skin epidermis. This methodological uncertainty is encountered whenever haired animals are used for skin absorption experiments.

(1) J. T. Lettieri and H.-L. Fung, *Res. Commun. Chem. Pathol. Pharmacol.*, **22**, 107 (1978).

(2) J. T. Lettieri and H.-L. Fung, *J. Pharmacol. Exp. Ther.*, **208**, 7 (1979).

(3) S. P. Bessman and W. N. Fishbein, *Nature*, **200**, 1207 (1963).

(4) A. S. Hunter, W. J. Long, and C. G. Ryrie, *Br. J. Anaesth.*, **43**, 620 (1971).

(5) G. D. DiVincenzo, M. L. Hamilton, C. J. Kaplan, W. J. Krasavage, and J. L. O'Donoghue, *Toxicol. Appl. Pharmacol.*, **44**, 593 (1978).

(6) S. T. Horhota and H.-L. Fung, *J. Pharm. Sci.*, **67**, 1345 (1978).

(7) *Ibid.*, **68**, 608 (1979).

(8) J. T. Lettieri and H.-L. Fung, *Biochem. Med.*, **20**, 70 (1978).

(9) J. L. Bruning and B. L. Kintz, "Computational Handbook of Statistics," 2nd ed., Scott, Foresman, Glenview, Ill., 1977.

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Theophylline Analysis by Reversed-Phase High-Pressure Liquid Chromatography: Elimination of Interferences

Keyphrases ◻ Theophylline—analysis, reversed-phase high-pressure liquid chromatography, elimination of ampicillin and 1,7-dimethylxanthine interference ◻ Smooth muscle relaxants—theophylline, reversed-phase high-pressure liquid chromatographic analysis, elimination of ampicillin and 1,7-dimethylxanthine interference ◻ High-pressure liquid chromatography—analysis, theophylline, elimination of ampicillin and 1,7-dimethylxanthine interference

To the Editor:

High-pressure liquid chromatography (HPLC) has been applied to theophylline analysis in biological fluids (1–5). HPLC methods have distinct advantages over traditional UV spectrophotometric methods (6, 7) such as reduced sample requirement, greater specificity, and shorter analysis time. However, several drugs and drug metabolites interfere in the HPLC analysis of theophylline (8–11). Of particular concern are the interferences caused by ampicillin, a commonly used antibiotic, and by 1,7-dimethylxanthine, an important metabolite of caffeine. 1,7-Dimethylxanthine is difficult to detect using ratios of peak heights obtained at two different wavelengths because of the similarity of its UV spectrum to that of theophylline. We have found elevation of serum theophylline values caused by 1,7-dimethylxanthine to be as high as 2 μg/ml. Such a discrepancy could cause misleading interpretation of pharmacokinetic data and would lead to inaccurate dosage regimen calculations.

We modified the method of Orcutt *et al.* (2) to eliminate interferences caused by ampicillin and 1,7-dimethylxanthine by using either a reversed-phase column with a 5-μm support or a radial compression module with a reversed-phase cartridge. A mobile phase of methanol–tetrahydrofuran–sodium acetate buffer provided the desired resolution.

The HPLC system was composed of a single pump[1], a loop injector[2], and a dual-wavelength UV detector with absorbance capability at 254 and 280 nm[3]. A precolumn[4] helped prolong column life. Two types of prepacked columns were used. One consisted of a steel, 25 × 0.5-cm column packed with octadecylsilane bonded to a 5-μm irregular support[5]. The other, intended for use in a radial compression unit, was a polyethylene column packed with octadecylsilane bonded to a spherical 10-μm silica support[6].

[1] Model 6000A, Waters Associates, Milford, Mass.
[2] Model U6K, Waters Associates, Milford, Mass.
[3] Model 440, Waters Associates, Milford, Mass.
[4] Co:Pell ODS, Whatman, Clifton, N.J.
[5] Partisil PXS 5/25 ODS, Whatman, Clifton, N.J.
[6] RCM with radial pak A, Waters Associates, Milford, Mass.

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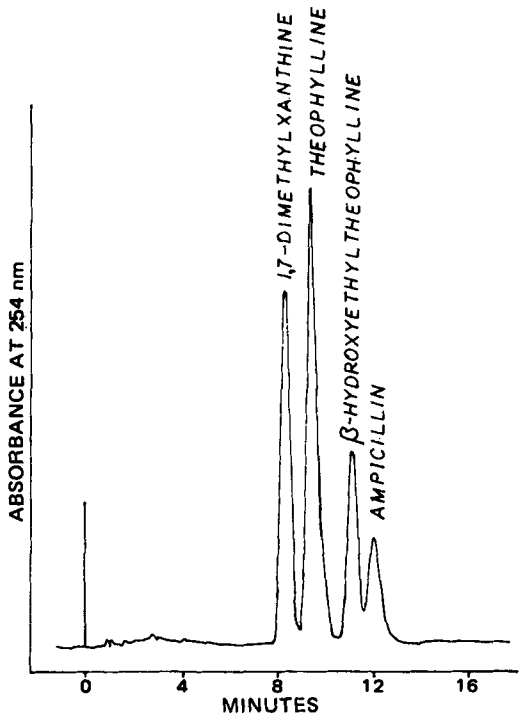

Figure 1—*Chromatogram of theophylline, ampicillin, 1,7-dimethylxanthine, and the internal standard, β-hydroxyethyltheophylline, on a radial compression, reversed-phase column. Conditions were: 6% methanol, 1.2% tetrahydrofuran in 0.01 mole of pH 5.0 sodium acetate/liter, flow rate of 3.0 ml/min, and detection at 280 nm, 0.01 aufs.*

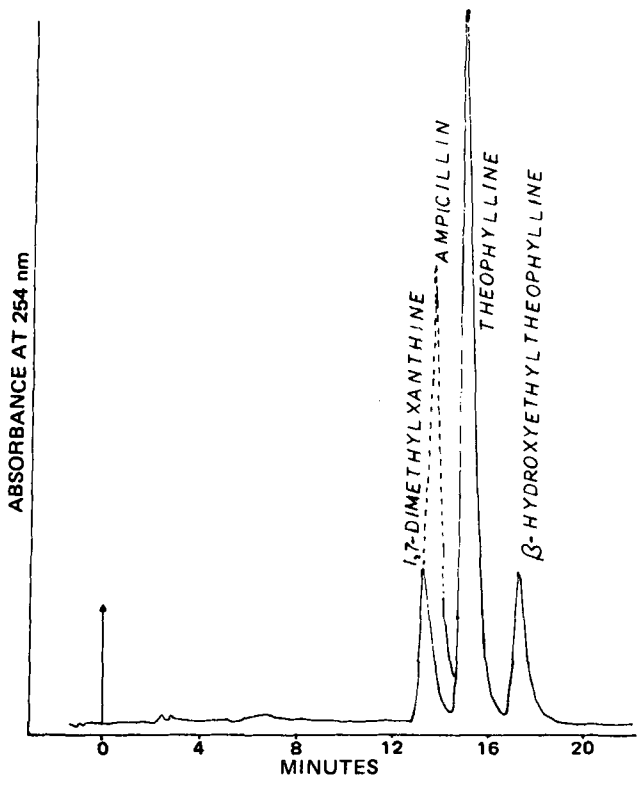

Figure 2—*Chromatogram of theophylline, ampicillin, 1,7-dimethylxanthine, and β-hydroxyethyltheophylline on a conventional column with a 5-μm support, reversed-phase column. Conditions were: 7% methanol, 1% tetrahydrofuran in 0.01 mole of pH 5.0 sodium acetate/liter, flow rate of 1.5 ml/min, and detection at 280 nm, 0.01 aufs.*

The mobile phase was made from 0.01 mole of sodium acetate/liter adjusted to pH 5.0 with acetic acid. This buffer was added to 60 ml of methanol and 12 ml of tetrahydrofuran to make 1 liter of eluent for use with the radial compression system. Eluents used with the 5-μm column were composed of 70 ml of methanol and 10 ml of tetrahydrofuran added to the sodium acetate buffer to make 1 liter. All chromatography solvents were filtered prior to use. Flow rates were 3.0 ml/min with the radial compression module and 1.5 ml/min with the 5-μm support column.

Blood and saliva samples were prepared by the method of Orcutt *et al.* (2) with the following modifications. The internal standard solution was 20–40 μg of β-hydroxyethyltheophylline/ml in 100% acetonitrile, and all samples were centrifuged at 11,500 rpm for 5 min after precipitation. Theophylline standards were prepared over the 2–50-μg/ml range in 0.01 mole of pH 5.0 sodium acetate/liter. These standards were diluted with an equal volume of internal standard solution and chromatographed. Peak height ratios (theophylline/β-hydroxyethyltheophylline) were used to define the standard curves.

The separation of theophylline, β-hydroxyethyltheophylline, ampicillin, and 1,7-dimethylxanthine obtained with the radial compression system is shown in Fig. 1. The chromatographic analysis time for theophylline was 12 min, and pressure did not exceed 1000 psi. Other substances that interfere with the analysis are acetazolamide, cephalexin, cefoxitin, and dyphylline. The major disadvantage of the radial compression module is the initial equipment cost. However, longer column life coupled with lower column cost when compared to conventional columns may counterbalance the initial expense. The system is easily interchangeable with most chromatographic equipment.

The separation of theophylline, β-hydroxyethyltheophylline, ampicillin, and 1,7-dimethylxanthine obtained with the 5-μm conventional column is shown in Fig. 2. The chromatographic analysis time for theophylline was 18 min. Pressures were ~3500 psi. Dyphylline caused interference, but acetazolamide did not. Although the high retention and resolution of the 5-μm support column provided excellent methylxanthine separation, analysis time was longer and pressures were much higher than in previously used 10-μm columns. The compatibility of pumping and injection systems with high pressures must be considered with these columns.

The two chromatographic systems provided excellent linearity with standard solutions over the concentration range examined ($R^2 = 0.9999$). No endogenous substances interfered in the analysis of the limited number of samples analyzed. The following methylxanthines and drugs were well resolved (resolution >2) from theophylline and the internal standard using either system: caffeine, theobromine, 1-methylxanthine, 1-methyluric acid, 1,3-dimethyluric acid, phenobarbital, cefamandole, cefazolin, cephalothin, and methicillin. The methods proposed for theophylline analysis offer the advantages of selectivity from two important interfering substances. In addition, the organic solvents used have less toxic properties and lower cost than acetonitrile. Care must be taken to prepare the mobile phase by a standardized method. Small fluc-

tuations in solvent strength can cause shifting of peaks and changes in peak height ratios. Resolution of theophylline and β-hydroxytheophylline can be optimized for each column by adjustments in the methanol concentration of ±2%.

(1) R. D. Thompson, H. T. Nagasawa, and J. W. Jenne, *J. Lab. Clin. Med.*, **84**, 584 (1974).
(2) J. J. Orcutt, P. P. Kozak, Jr., S. A. Gillman, and L. H. Cummins, *Clin. Chem.*, **23**, 599 (1977).
(3) R. F. Adams, F. L. Vandemark, and G. J. Schmidt, *ibid.*, **22**, 1903 (1976).
(4) L. C. Franconi, G. L. Hawk, B. J. Sandmann, and W. G. Haney, *Anal. Chem.*, **48**, 372 (1976).
(5) S. J. Soldin and J. G. Hill, *Clin. Biochem.*, **10**, 74 (1977).
(6) A. Schack and S. H. Waxler, *J. Pharmacol. Exp. Ther.*, **97**, 283 (1949).
(7) P. Jatlow, *Clin. Chem.*, **21**, 1518 (1975).
(8) C. A. Robinson, Jr., and J. Dobbs, *ibid.*, **24**, 2208 (1978).
(9) R. C. Kelly, D. E. Prentice, and G. M. Hearne, *ibid.*, **24**, 838 (1978).
(10) C. A. Robinson, Jr., B. Mitchell, J. Vasiliades, and A. Siegal, *ibid.*, **24**, 1847 (1978).
(11) J. W. Nelson, A. L. Cordry, C. G. Aron, and R. A. Bartell, *ibid.*, **23**, 124 (1977).

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6,6′-Azopurine, a Potent *In Vitro* Inhibitor of Rabbit Liver Aldehyde Oxidase

Keyphrases □ 6,6′-Azopurine—inhibition of rabbit liver aldehyde oxidase, *in vitro*, prevention of azathioprine cytotoxicity □ Enzyme inhibitors—6,6′-azopurine, inhibition of rabbit liver aldehyde oxidase, *in vitro*, prevention of azathioprine cytotoxicity □ Azathioprine—prevention of cytotoxicity by 6,6′-azopurine

To the Editor:

Hepatic aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) oxidizes many purines at the C_8-position (1–3) and has been implicated in the metabolism of azathioprine (3), methotrexate and its dialkyl and dichloro derivatives (4), formycin B (5, 6), cyclophosphamide, and the *N*-alkylphenothiazines (7, 8). Inhibitors of aldehyde oxidase and xanthine oxidase, either endogenous or therapeutically administered, may change the conversion rate of azathioprine into active and inactive metabolites. This factor becomes significant for the development of treatment schedules for the most favorable balance between therapeutic effect and drug toxicity.

We wish to report the finding of such an inhibitor. During investigations of oxoazopurine pharmacology (9, 10), we observed that 6,6′-azopurine disodium salt (I) was a potent inhibitor of rabbit liver aldehyde oxidase and also acted as a substrate for this enzyme. The oxidation of I by the enzyme resulted in the formation of a known potent xanthine oxidase inhibitor, 8,8′-dioxo-6,6′-azopurine (II) (9).

Table I—Identification of the Product of Enzymic Oxidation of 6,6′-Azopurine Disodium Salt (I) by Rabbit Liver Aldehyde Oxidase

Solvent System	R_f Values[a] Chemically Synthesized II	Enzymatically Synthesized II	I
Ethanol–pyridine–water (67:20:13)	0.17	0.16	0.26
n-Propanol–ammonia (concentrated)–water (60:30:10)	0.36	0.37	0.59
Ethanol–0.5 *M* ammonium acetate (5:2)	0.09	0.09	0.4

[a] Chromatographic analyses were carried out using Whatman chromatography grade 1 paper. Results shown are averages of triplicate determinations.

A modified literature method (1) was used to obtain a partially purified aldehyde oxidase preparation from rabbit liver homogenates containing 5.04 mg of protein/ml. It was assayed using a spectrophotometric method based on measuring the rate of N^1-methylnicotinamide chloride oxidation at 300 nm (1, 6). The conversion of N^1-methylnicotinamide chloride to its 2-pyrone was measured by monitoring the increase in absorbance at 300 nm upon addition of the enzyme at ambient temperature to reaction mixtures containing, in 3 ml: substrate (0.5, 1, 2.5, 5, or 10 m*M*), desired concentration of I ranging from 2.6 to 13 μ*M* in 5 m*M* pH 7.8 potassium phosphate buffer, and 0.005% edetic acid. The double-reciprocal plot (Fig. 1) of the inhibition of rabbit liver aldehyde oxidase showed that I was a competitive inhibitor of this enzyme with a k_i of 3.3×10^{-6} *M*. The k_m for N^1-methylnicotinamide chloride was 318 μ*M*.

For the enzymatic synthesis of II from I, the following procedure was used. Compound I, 2.5 mg, was dissolved in 10 ml of water to obtain a clear yellow solution. To this solution were added 7.5 ml of 0.05 *M* potassium phosphate buffer containing 0.005% edetic acid at pH 7.8 and 2.5 ml of aldehyde oxidase suspension (5.04 mg of protein/ml). A control reaction was carried out using the same reactants except that 2.5 ml of the buffer was added in the last step instead of the aldehyde oxidase suspension. Both reaction mixtures were incubated at room temperature for 15 min.

At the end of 15 min, both solutions were brought to a 50% ammonium sulfate saturation by adding 313 g of solid

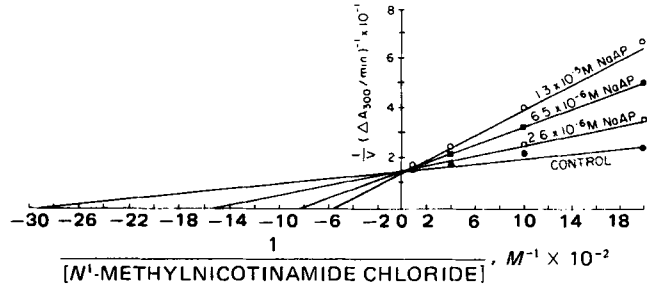

Figure 1—*Double-reciprocal plots of the* in vitro *inhibition of rabbit liver aldehyde oxidase by 6,6′-azopurine disodium salt (I, NaAP).*

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