Therefore, the half-life of elimination by metabolism, obtained from the urinary excretion plots, represents the half-life for elimination of probenecid during the terminal excretion phase and is represented as such in Table II.

It can be seen from Figs. 2 and 3 that the time required for elimination of the dose is extended appreciably as the dose is increased. This appears to be due to a prolonged absorption of the drug. The apparent absorptive phase was about 10 hr for the 0.5-g dose and increased to 30–40 hr for the 2.0-g dose. The curvature in the sigma-minus plots at the 2.0-g dose suggests some type of saturation. In this connection, it is interesting to examine the excretion of the major metabolite, probenecid acyl glucuronide.

The maximum excretion rate was roughly constant at about 20 mg/hr over 5–30 hr for the 2.0-g dose for both subjects. Data from Subject 1 are presented in Fig. 4. For the 1-g dose, the maximum excretion rate was also about 20 mg/hr over 5–20 hr. For the 0.5-g dose, the excretion rate reached a maximum of approximately 15 mg/hr about 5 hr after medication and immediately began to decline.

The other metabolites exhibited similar plateaus in their excretion rate plots as shown in Figs. 5–7, where the excretion rates of the mono-*N*-propyl, carboxylic acid, and secondary alcohol metabolites are also seen to plateau at about 10–15 mg/hr. This parallel can be seen in Table I, which demonstrates that the fractions of the drug recovered in urine in the form of these metabolites are about equal. This apparent saturation could result from a single rate-limiting step in a sequential process of metabolism as mentioned earlier. Saturation of urinary excretion offers another possible explanation, but the results of Perel *et al.* (2) indicate that urinary excretion is not saturated.

The poor solubility of probenecid in water suggests a more likely explanation. The solubility of probenecid in water at 37° was determined to be about 50 μg/ml. Therefore, higher doses of the drug administered in solution possibly could have precipitated in the GI tract. The presence of insoluble probenecid in the GI tract would cause the absorption process to be dissolution rate limited (zero order), making the drug available for metabolism at a constant rate until all precipitated drug redissolved. Blood level data, as well as excretion data following intravenous administration of probenecid, would be useful in further resolving the mechanism of absorption, distribution, and elimination of the drug.

REFERENCES

(1) A. M. Guarino, W. D. Conway, and H. M. Fales, *Eur. J. Pharmacol.*, **8**, 244(1969).

(2) J. M. Perel, R. F. Cunningham, H. M. Fales, and P. G. Dayton, *Life Sci.*, **9** (Part I), 1337(1970).

(3) K. Sabih, C. D. Klaassen, and K. Sabih, *J. Pharm. Sci.*, **60**, 745(1971).

(4) W. D. Conway and S. K. Melethil, *ibid.*, **63**, 1551(1974).

(5) P. G. Dayton, T. F. Yu, W. Chen, L. Berger, L. A. West, and A. B. Gutman, *J. Pharmacol. Exp. Ther.*, **140**, 278(1963).

(6) P. G. Dayton, S. A. Cucinell, M. Weiss, and J. M. Perel, *ibid.*, **158**, 305(1967).

(7) W. D. Conway and S. Melethil, *J. Chromatogr.*, **115**, 222(1975).

(8) Z. H. Israeli, R. F. Cunningham, and P. G. Dayton, *Fed. Proc.*, **30**, 226(1971).

(9) I. M. Weiner, J. A. Washington, and G. H. Mudge, *Bull. Johns Hopkins Hosp.*, **106**, 333(1960).

(10) J. M. Perel, P. G. Dayton, T. F. Yu, and A. B. Gutman, *Eur. J. Clin. Pharmacol.*, **3**, 106(1971).

(11) B. K. Martin, *Br. J. Pharmacol. Chemother.*, **29**, 181(1967).

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Rapid Determination of Theophylline in Human Plasma by High-Pressure Liquid Chromatography

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Abstract □ A rapid, specific, high-pressure liquid chromatographic method for the determination of theophylline in plasma was developed. The procedure is fast enough (21 min from receipt of blood to reporting value) to be used for emergency determinations. The sensitivity, precision, and accuracy are sufficient for routine monitoring of therapeutic levels in patients. The assay is specific enough to be valid in the presence of caffeine and theobromine. Metabolites of theophylline as well as a number of drugs do not interfere with the assay.

Keyphrases □ Theophylline—high-pressure liquid chromatographic analysis, plasma □ High-pressure liquid chromatography—analysis, theophylline, plasma □ Relaxants, smooth muscle—theophylline, high-pressure liquid chromatographic analysis, plasma

Many patients receive theophylline for the prevention and treatment of asthmatic attacks. Several investigations showed good correlation between the plasma concentrations of theophylline and the improvement of pulmonary function in asthmatic patients (1–3). Recent reviews of pharmacokinetic control of drug therapy (4, 5) suggested that monitoring of blood theophylline levels can contribute significantly to the control of therapy of asthmatics. Unfortunately, these benefits are of limited application to the emergency care situation. Present methods of analysis either lack specificity or are too time consuming to contribute to the rapid adjustment of theophylline levels in the patient admitted for acute asthmatic attack where a subtherapeutic theophylline level may or may not be a contributing cause.

The method of theophylline analysis most widely used at present is that of Schack and Waxler (6). This procedure is not suited to monitoring theophylline therapy in many emergency situations since caffeine, theobromine, and other dietary xanthines that may be present in the patient are not distinguished from theophylline. It is used where patients can be placed on

dietary restrictions for at least 24 hr prior to analysis. Gupta and Lundberg (7) used differential spectrophotometry to measure blood theophylline levels. No data were given on the effects of caffeine and theobromine on their assay.

Several assays have been reported that are specific enough to measure theophylline in the presence of caffeine, theobromine, and other interfering substances. These assays can be evaluated further by estimating the time required for a duplicate assay (T_d). This time should be the total time from receipt of a whole blood sample to reporting of results.

Shah and Riegelman (8) reported a GC method that distinguished theophylline from its metabolites and from caffeine. They used a separation procedure involving four extraction steps so that a T_d of 1 hr or more can be estimated from their data. Thompson *et al.* (9) described a high-pressure liquid chromatographic (HPLC) method for the determination of theophylline and its metabolites in blood and urine. Theophylline was well separated from caffeine, theobromine, and the metabolites of theophylline, caffeine, and theobromine. Analysis time was decreased by the finding that diluted plasma could be injected directly on column. On the other hand, the retention time of theophylline on the column was 20 min so the T_d might have been about 1 hr. Finally, Manion *et al.* (10) used a reversed-phase, gradient elution, HPLC method for theophylline. Since the chromatographic run lasted about 30 min, the T_d might have been more than 1 hr.

Thus, assays have been developed that are specific enough to determine blood theophylline levels in the presence of dietary xanthines and their metabolites. A need remains, however, for a method that is not only specific but fast enough to be used in emergency treatment. This paper describes a method with a T_d of 21 min. The method has been shown to measure accurately theophylline levels in the presence of caffeine, theobromine, theophylline metabolites, and a number of drugs often present in patients taking theophylline.

EXPERIMENTAL

Materials—UV grade hexane[1], 2-butanol[1], methanol[1], USP theophylline reference standard[2], theobromine[3], caffeine[4], and 3-methylxanthine[5] were used as obtained.

Apparatus—A high-pressure liquid chromatograph[6] equipped with a "septumless" injection port and a fixed wavelength (280 nm) UV absorption detector was used. The detector was operated at 0.01 absorbance unit full scale for most samples.

Chromatographic Parameters—The mobile phase contained 1% distilled water, 5% methanol, and 25% 2-butanol in *n*-hexane. The mobile phase was pumped at 3.33 ml/min at 25° through a stainless steel column (30.48 cm × 4 mm i.d.) packed with a high efficiency absorption packing[7]. It was found that connecting 25 cm of 0.25-mm i.d. tubing to the output of the UV detector flowcell made it unnecessary to "degas" the mobile phase. Aliquots (150 μl) of the extraction solution were injected directly on-column with a 250-μl syringe[8] using a stop-flow technique.

[1] Burdick and Jackson Laboratories, Muskegon, Mich.
[2] U.S.P.C. Inc., Rockville, Md.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Eastman Organic Chemicals, Rochester, N.Y.
[5] CYCLO Chemical Corp., Los Angeles, Calif.
[6] Model 4100, Varian Aerograph, Walnut Creek, Calif.
[7] μ-Porasil, Waters Associates, Milford, Mass.
[8] Model 725, Hamilton Co., Reno, Nev.

Table I—Separation of Theophylline from Other Compounds by HPLC[a]

Compound	Quantity of Solution[b] Injected: Volume, μl	Quantity of Solution[b] Injected: Concentration, μg/ml	Retention Time, min
Theophylline	10	25	2.9
Caffeine	10	50	5.9
Theobromine	100	10	7.4
3-Methylxanthine	100	0.5	3.5
1-Methyluric acid	100	5.0	6.6
1,3-Dimethyluric acid	100	Saturated	9.1

[a] μ-Porasil, conditions as described in the text. [b] Extraction solvent described in text.

Analytical Procedure—Two 0.5-ml aliquots of each plasma sample to be analyzed were placed in 15-ml screw-topped centrifuge tubes. Each tube received 0.3 ml of saturated ammonium sulfate solution and 5.0 ml of 40% 2-butanol in *n*-hexane. The two tubes were vortexed together for 30 sec and then centrifuged for 2 min at 3400 rpm. After centrifugation, 150 μl of the supernate from the first tube was drawn up into a 250-μl syringe[8] and injected into the chromatograph.

Six minutes after the first injection, 150 μl from the second tube was injected. Four minutes after the second injection, the chromatogram was removed and the peak heights were measured in millimeters. The peak heights were converted to plasma theophylline concentrations by comparison to a standard curve.

Preparation of Standard Curve—A theophylline stock solution of 0.500 μg/μl was prepared by dissolving a weighed sample of drug standard in methanol. Aliquots of 5, 10, 15, 20, 25, and 30 μl were added to 15-ml screw-topped centrifuge tubes. After the methanol was evaporated with a nitrogen stream at ambient temperature, 0.5 ml of pooled human plasma was added to each tube. The tubes were vortexed to dissolve the theophylline and then extracted and analyzed by the described procedure.

Fifteen analyses were done at each concentration: 5, 10, 15, 20, 25, and 30 μg/ml. The heights of the chromatogram peaks were measured in millimeters, and the average and standard deviation of the peak heights were taken at each concentration. A straight-line fit of the data was made by linear regression analysis, and the correlation coefficient was determined.

Evaluation of Accuracy and Precision—The accuracy and precision of the method were evaluated by performing 60 duplicate analyses on spiked samples of human plasma. Fifteen duplicate analyses were performed at each concentration: 5, 10, 15, and 20 μg/ml. The spiked samples were prepared by adding appropriate aliquots of the 0.50-μg/ml stock solution to 15-ml centrifuge tubes, evaporating the methanol in a nitrogen stream, and then dissolving the theophylline in 0.5-ml aliquots of plasma from selected plasma samples.

The 60 plasma samples used were left over from blood samples originally drawn for analyses of phenobarbital, phenytoin, quinidine, procainamide, or salicylate from patients known not to be receiving theophylline preparations. After preparation, the spiked samples were analyzed as described, and the mean and standard deviation of the measured values were determined.

Evaluation of Interferences—The retention times of caffeine, theobromine, 3-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid were determined by injection of solutions of these drugs in the extraction solvent into the liquid chromatograph. The volumes and concentrations injected, as well as the retention times determined, are given in Table I. Possible interferences from quinidine, primidone, phenobarbital, phenytoin, salicylic acid, aspirin, diazepam, chlordiazepoxide, and ephedrine were evaluated by performing the theophylline analysis on samples of plasma from patients receiving these drugs but not theophylline. In each case, the patient sample had therapeutic levels of the drug being evaluated for possible interference.

RESULTS AND DISCUSSION

Figure 1*b* shows the chromatogram for a single 150-μl injection of the extract of a plasma sample to which 15 μg/ml of theophylline had

Table II—Accuracy and Precision of Theophylline HPLC Assay

Theophylline Added, μg/ml	Theophylline Found[a], μg/ml	SD, %
5	5.09	4.54
10	9.89	2.43
15	14.98	2.42
20	19.78	2.07

[a] Mean of 15 assays, each done in duplicate.

been added. The method described under *Analytical Procedure* was used. Figure 1*a* shows the chromatogram of a 150-μl injection of a plasma sample that was extracted in exactly the same way but with no added theophylline. The peaks and baseline variations due to the addition of 150 μl of extraction solvent plus components extracted from plasma are seen to be complete prior to the theophylline peak.

The theophylline peak heights measured on the chromatograms were plotted against the concentration of theophylline in the plasma standard solutions in preparing a standard curve. When 15 plasma samples at each concentration (5, 10, 15, 20, 25, and 30 μg/ml, *i.e.*, a total of 90 determinations) were assayed, the line obtained by linear regression was described by $y = 8.21x - 2.52$ with a correlation coefficient of 1.00. Additional plasma standards were run from time to time over 4 months, during which 43 theophylline determinations were made on patient samples. The slope and intercept of the standard curve did not vary more than 5% during this period.

The accuracy and precision of the method are shown in Table II. The low percent standard deviations over the clinical range (10–20 μg/ml) show that the method is precise enough for normal clinical analysis problems. The higher percent standard deviation at 5 μg/ml suggests that the method might not be suitable for more exacting research studies in the subtherapeutic concentration range.

The retention times of a number of different substances that might interfere with theophylline determinations are given in Table I. All of these compounds were well separated from theophylline. In clinical samples, a small peak was often seen at the 3-methylxanthine position. Another small, broad peak was frequently seen at the caffeine position. Peaks corresponding to 1-methyluric acid and 1,3-dimethyluric acid were not seen, and they probably were not extracted.

A number of drugs that might be taken by patients also taking theophylline were tested for interference with the theophylline assay.

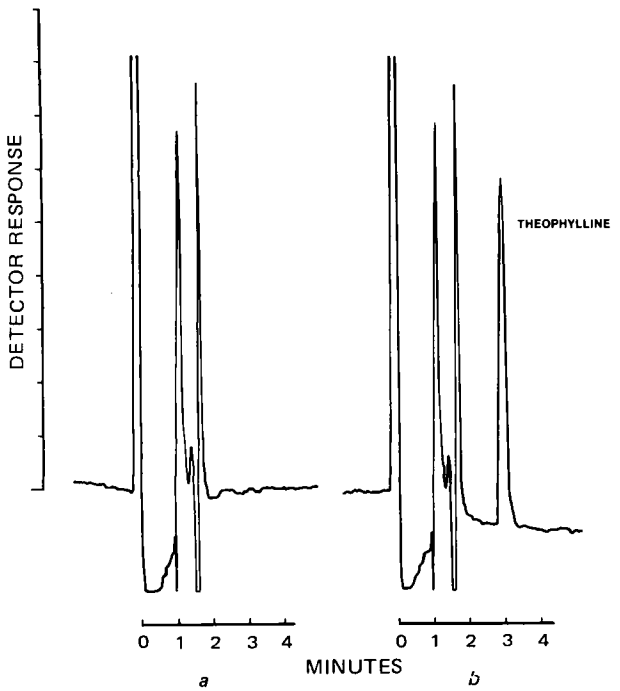

Figure 1—*High-pressure liquid chromatograms of human plasma extracts. Key:* a, *control plasma; and* b, *plasma containing 15.0 μg of theophylline/ml.*

Table III—Total Time Required for Duplicate Analysis of Whole Blood Samples for Theophylline[a]

Analytical Step	Time Required, min
Blood centrifugation	5.0
Pipetting of plasma and reagents	1.0
Vortexing	1.0
Centrifugation to separate phases	2.0
Handling of extract until first injection	1.0
Chromatographic time from first injection until second injection	6.0
Chromatographic time from second injection until removal of chart	4.0
Measuring peaks and calculation	1.0
Total time	21.0

[a] Conditions as described in the text.

Plasma samples from patients having therapeutic levels of phenobarbital, phenytoin, primidone, acetylsalicylate, ephedrine, diazepam, or chlordiazepoxide but no theophylline were analyzed. The samples were extracted and chromatographed in exactly the same way as for a theophylline analysis. In none of these cases was there a peak that would have given a false theophylline value. In some cases, this result was due to the fact that the potentially interfering drug or its metabolites did not extract under the assay conditions.

Table III illustrates that this quantitative assay can be done in 21 min from receipt of blood sample to reporting of data. Several measures were taken to attain this speed. Pipetting time was decreased by use of a transfer pipet[9] for plasma and for saturated ammonium sulfate. A dispenser[10] was used for the extraction solvent. The mobile phase velocity was the maximum the pump[6] would deliver. A mobile phase composition was chosen that gave the shortest retention time for theophylline which still allowed resolution of the theophylline peak from the 3-methylxanthine peak.

Quantization by peak height without an internal standard minimized the calculation time. The combination of normal-phase chromatography with an organic solvent extraction step minimized turnaround time since substances more polar than theophylline that would have been retained longer on the column were mostly eliminated by the extraction step. Finally, it is seen in Table I that the retention times of any caffeine or theobromine in the first extract would cause them to come off the column after injection of the second extract. In practice, if the second extract was injected 6 min after the first, the caffeine and theobromine peaks from the first extract came off the column before any theophylline in the second extract and did not interfere.

All of these timesaving steps were used in the analyses in which the accuracy and precision data were obtained. Thus, an analysis fast enough for emergency determinations has accuracy and precision sufficient for the routine monitoring of theophylline therapy.

REFERENCES

(1) P. A. Mitenko and R. I. Ogilvie, *N. Eng. J. Med.*, **289**, 600(1973).

(2) R. H. Jackson, J. I. McHenry, F. B. Moreland, W. J. Raymer, and R. L. Etter, *Dis. Chest*, **45**, 75(1964).

(3) R. Maselli, G. L. Casal, and E. F. Ellis, *J. Pediat.*, **76**, 777(1970).

(4) "Clinical Pharmacokinetics," G. Levy, Ed., APhA Academy of Pharmaceutical Sciences, Washington, D.C., 1974.

(5) V. Marks, W. E. Lindup, and E. M. Baylis, *Adv. Clin. Chem.*, **16**, 47(1973).

(6) J. A. Schack and S. H. Waxler, *J. Pharmacol. Exp. Ther.*, **97**, 283(1949).

(7) R. C. Gupta and G. D. Lundberg, *Anal. Chem.*, **45**, 2403(1973).

(8) V. P. Shah and S. Riegelman, *J. Pharm. Sci.*, **63**, 1283(1974).

(9) R. D. Thompson, H. T. Nagasawa, and J. W. Jenne, *J. Lab. Clin. Med.*, **84**, 584(1974).

[9] Model 0010-29 Biopette, Schwarz/Mann, Orangeburg, N.Y.

[10] A 10-ml Repipet, Labindustries, Berkeley, Calif.

(10) C. V. Manion, D. W. Shoeman, and D. L. Azaroff, *J. Chromatogr.*, **101**, 169(1974).

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Nitrofurantoin Solubility in Aqueous Urea and Creatinine Solutions

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Abstract □ Experiments were carried out to determine the effect of urea and creatinine on the solubility of nitrofurantoin in water at different temperature and pH conditions. The addition of urea to aqueous media increased nitrofurantoin solubility up to a maximum concentration level and then decreased solubility at higher urea concentrations. The amount of urea needed to bring about maximum nitrofurantoin solubility was dependent on temperature and ranged between 1.75 and 2.50%. Spectral studies suggest a possible interaction between urea and nitrofurantoin molecules. Nitrofurantoin solubility increased with an increasing creatinine concentration ranging from 0.05 to 1.6%. Spectral studies indicate a strong interaction between creatinine and nitrofurantoin molecules in solution. The combined effect of urea and creatinine on the solubility of nitrofurantoin could account for the absence of crystalluria with this drug, even though unusually high concentrations in urine have been reported.

Keyphrases □ Nitrofurantoin—solubility in aqueous solutions, effect of urea and creatinine, varying temperature and pH □ Urea—effect on aqueous solubility of nitrofurantoin, varying temperature and pH □ Creatinine—effect on aqueous solubility of nitrofurantoin, varying temperature and pH □ Solubility—nitrofurantoin in aqueous solutions, effect of urea and creatinine, varying temperature and pH

Nitrofurantoin, 1-[(5-nitrofurfurylidene)amino]-hydantoin, is an antibacterial agent widely used to treat urinary tract infections. It is a weak acid (pKa 7.2) possessing relatively low aqueous solubility characteristics. Solubility at 37° was reported to be 190 mg/liter in distilled water (1) and 125 mg/liter in pH 4.8 water (2). Bates *et al.*[1] (3) reported the solubility of this drug in water at pH 1.12 and 7.20 to be 154 and 374 mg/liter, respectively.

Nitrofurantoin concentrations in urine ranged from 250 to 500 mg/liter after 200 mg was administered every 6 hr to an 80-kg patient (4). Urine concentrations of 200–400 mg/liter were reported (5), and maximum urine nitrofurantoin concentrations ranged from 158 to 372 mg/liter in nine normal individuals, each of whom received a 100-mg tablet of drug every 4 hr for four doses (6). Although unusually high concentrations of nitrofurantoin are found in the urine, no case of crystalluria associated with nitrofurantoin therapy has been reported. These observations suggest that substances normally present in urine might affect the aqueous solubility of nitrofurantoin.

Preliminary findings indicated that urea initially increased nitrofurantoin solubility at low urea concentrations and then decreased solubility at higher urea concentrations (7). In the present study, an attempt was made to ascertain the effect of urea and another urine component, creatinine, on nitrofurantoin solubility at various temperatures and pH conditions.

EXPERIMENTAL

Materials—Nitrofurantoin[2] was used as received without further purification. Nitromethane[3] was spectrophotometric grade; urea, methyl alcohol, citric acid, creatinine[4], hydrochloric acid, disodium phosphate, and potassium chloride were reagent grade. A high molecular weight quaternary ammonium hydroxide solution[5] was diluted with absolute methanol to 0.04 *M*.

Equipment—A rotating apparatus[6], capable of holding multiple samples, was immersed in a water bath kept at constant temperature by a controlled-temperature circulating pump[7]. The pH values of aqueous systems were measured using a pH meter[8].

Preparation of Solution—McIlvaine's buffer solutions (pH 3–7) (8) were prepared by mixing appropriate volumes of 0.1 *M* citric acid and 0.2 *M* disodium phosphate solutions in deionized water.

Stock solutions of 0.2 *M* potassium chloride and 0.2 *M* hydrochloric acid were prepared with deionized water. Appropriate portions of the two were mixed and diluted to make the hydrochloric acid buffer solutions (pH 1.2–2.0). A 0.1 *M* hydrochloric acid solution was used for the pH 1.12 medium.

Appropriate amounts of urea and creatinine were dissolved in deionized distilled water or in various pH buffer solutions to make urea solutions ranging from 0.25 to 10% and creatinine solutions ranging from 0.05 to 2.0%.

Solubility Studies—Excess nitrofurantoin (~50 mg) was added to 40 ml of the appropriate test solution in a screw-capped bottle of 45-ml capacity. The tightly closed container was placed in a water bath at various temperatures (24, 30, 37, and 45 ± 0.1°) and rotated

[1] T. R. Bates, School of Pharmacy, State University of New York at Buffalo, personal communication. A lower value was erroneously reported in Ref. 3.

[2] A pure sample (Lot. E3769) was supplied by Eaton Laboratories, Norwich, N.Y.

[3] Aldrich Chemical Co., Milwaukee, Wis.

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.

[5] Hyamine, Sigma Chemical Co., St. Louis, Mo.

[6] Menold, Lester, Pa.

[7] Haake E52, Berlin-Lichterfeid, West Germany.

[8] Photovolt-digicord, W. H. Curtin & Co.