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Serum theophylline measurement and method dependency

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SERUM THEOPHYLLINE MEASUREMENT AND METHOD DEPENDENCY

by

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THESIS

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ABSTRACT

Theophylline is invaluable in the treatment of bronchial asthma and some cardiorespiratory diseases in adults, and management of apnea and bradycardic spells in premature infants. Absorption and plasma clearance vary greatly due to age, weight, diet, smoking, and other drugs, leading to unpreductable plasma levels. Because both efficacy and toxicity are related to serum theophylline concentration, a sensitive and specific analytical method for its quantitation is essential.

We report studies of a modified EMIT® procedure (EMIT-MOD) for the Centrifichem 400® centrifugal analyzer (C/A). This method was compared with the following methods: a recommended EMIT procedure for the C/A (Syva, Palo Alto, CA), a modified extractive alkylation microprocedure for a gas-liquid chromatograph with nitrogen-phosphorus detection (GLC-NP) and a modified microscale reversed phase liquid chromatography method (RPLC) as a reference. The RPLC method involved protein precipitation and direct injection of the supernatant.

EMIT-MOD utilized 5-μL serum, 20-μL antibody reagent, 45-μL diluent and 350-μL of the diluted glucose-6-phosphate dehydrogenase (0.6:10 with 0.55 M Tris buffer) solution (30°C, T = 15s, ΔT = 60s, terminal mode). Within-run (N = 10) and between-run (8 days) precision studies showed coefficients of variation of 4.0% and 5.0%, 3.0% and 6.5%, at 10 and 20 mg/L respectively. Correlation studies with RPLC (N = 60), with GLC-NP (N = 100) and with Syva's EMIT procedure (N = 91) showed values of 0.98, 0.97 and 0.97, respectively. Theophylline was stable in frozen samples kept for 11 months at -10°C (N = 30, r = 0.98).

When comparing Syva's EMIT procedure with the RPLC reference method (N = 140, r = 0.96), 21 samples showed discrepancies of greater than 3 standard deviations Reanalysis of these sera using EMIT-MOD showed significant reduction in these disparities.

Four patients whose RPLC values were consistently lower (than EMIT, EMIT-MOD and GLC-NP) were all receiving ampicillin at the time the serum theophylline was drawn. Although this was not confirmed by *in vitro* experimental studies, other reports have implicated ampicillin and other antibiotics as interferences in RPLC methods involving protein precipitation.

EMIT-MOD is subject to fewer possible interferences than RPLC. As it reduces reagent consumption by 40% and eliminates excessive dilution and pipetting, it is faster, mor precise, convenient and economical that the inital EMIT-C/A method. EMIT-MOD is an ideal method for emergency and therapeutic monitoring of serum theophylline.

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INTRODUCTION

Physicians are regularly requesting theophylline analyses, as this drug has been recently reestablished for widespread therapeutic use. Theophylline is invaluable in the treatment of bronchial asthma in adults and the management of apnea and bradycardic spells in infants.

Consequently, the clinical chemistry laboratory plays an integral role in theophylline therapy. It has the responsibility of delivering fast, accurate, reproducible results, using the smallest, most convenient sample possible, at as low a cost as possible.

To best provide this service, the clinical chemist should be knowledgeable of the chemistry, pharmacological actions, therapeutic uses, and pharacokinetics of theophylline, as well as the available methodologies for its quantitation and the relative merits of each.

SOURCE AND CHEMISTRY OF THEOPHYLLINE

Theophylline (1,3-dimethylxanthine) is a derivative of xanthine (2,6-dioxypurane), which is structurally similar to uric acid. The numbering system is based on the parent nitrogenous base, purine (Figure 1). Theophylline (like caffeine and theobromine) is a naturally-occuring plant alkaloid, most commonly found in tea leaves.

Theophylline is only 1% soluble in aqueous solution, in which it is amphoteric and generally considered a weak or-

ganic acid, (pKa = 8.8; Clark, 1979). When the active proton at position 7 is released, a very stable anion is generated, as the negative charge on that nitrogen becomes evenly distributed around the "B" ring. Conversely, the 7-methyl group of caffeine prohibits acidic behavior of that molecule. The slightly basic nitrogen (N_9) on the "B" ring allows the back extraction of theophylline into very strong acid. The "A" ring nitrogens (N_1 and N_3) are neutral.

CELLULAR BASIS OF METABOLISM

The most significant action of theophylline is the competitive inhibition of phosphodiesterase, the enzyme whose activity maintains the normally low intracellular level of cyclic 3',5'-adenosine monophosphate (cyclic AMP). Cyclic AMP is an ubiquitous intermediate compound through which many hormones are thought to act, expecially in promoting glycogenolysis and lipolysis in some tissues. This inhibitory activity of theophylline is associated with the methyl substitutions on positions one and three (Brodie et. al., 1951; Cornish et. al., 1957; Beavo et. al., 1970).

A rise in cyclic AMP is usually associated with β-adrenergic reactions. Certain hormones, notably epinephrine, norepinephrine and glucagon, lead to elevated cyclic AMP levels, by activating the enzyme adenylate cyclase via specific receptor sites in various cell membranes. Adenylate cyclase then catalyzes the synthesis of cyclic AMP from ATP. Theophylline, however, increases cyclic AMP by inhibition

of phosphodiesterase, which functions to catalyze the destruction of cyclic AMP to 5'AMP. Thus, theophylline, the catecholamines and glucagon all have common pharmacological properties through their respective abilities to increase cyclic AMP. Furthermore, because of their different mechanisms of action, the effects are often potentiating.

Rasmussen (1970, 1974) has suggested that ionized intracellular calcium is an essential (perhaps terminal) signal for protein hormone action, because the action of such hormones (e.g. insulin) is inhibited in the absence of calcium. In a recent study, Gedik et. al., (1977) proved that calcium does play an important role in insulin release and that in patients with hypoparathyroidism, theophylline has a stimulatory effect on insulin secretion, even in the presence of hypocalcemia.

Another major area of biochemical and metabolic involvement includes the interrelationships of theophylline, calcium mobilization and skeletal muscle and neuromuscular transmission. The methylxanthines stregthen the contraction of isolated skeletal muscle through the intracellular translocation of ionized calcium. Certain levels of methylxanthines probably sensitize the calcium ion release mechanism of the sarcoplasmic reticulum of the muscle, such that the calcium ions are mobilized by the myoplasm. At a threshold level of myoplasmic free-Ca^{2+}, contraction occurs (Ritchie, 1975). Neuromuscular transmission is probably facilitated by increased release of acetylcholine (Ritchie, 1975).

PHARMACOLOGICAL ACTIONS

The three methylxanthines, theophylline, caffeine and theobromine share several pharmacological actions, but each compound differs in the intensity of each action. They all stimulate the central nervous system (CNS), relax smooth muscle, stimulate the cardiovascular system, activate skeletal muscle and neuromuscular transmission and act on the kidney to produce diuresis.

Although it is impossible to correlate each pharmacological action with a particular mechanism, it appears that the effects on smooth muscle, and the ability to potentiate catecholamines is associated with the cyclic AMP theory, while skeletal muscle and neuromuscular transmission relate more to calcium mobilization. The effects on the heart are complex and probably involve both mechanisms (Ritchie, 1975).

Theophylline is a less potent CNS stimulator than caffeine, but has considerable value as a specific respiratory stimulant.

Theophylline is indispensible in its ability to relax the smooth muscle of the bronchi which have been constricted by asthma. Its action on intestinal smooth muscle is exhibited by transient inhibition of motility following intravenous injection of aminophylline (Ritchie, 1975).

Theophylline works on all parts of the cardiovascular system. The direct myocardial action of theophylline briefly increases cardiac output. This inotropic effect is apparent at concentrations of 10 - 20 mg/L (Ogilvie, 1978).

Theophylline potentiates the cardiac inotropic response to β-adrenergic agonists (e.g. isoproterenol) and glucagon (Rall West, 1963; Marcus et. al., 1972), probably through cyclic AMP. Release of catecholamines following theophylline administration (Atuk et. al., 1967) partly explains this inotropic effect.

Theophylline is a vasodilator of both arterioles and veins of the coronary, pulmonary and general systemic circulation. By reducing pulmonary vascular resistance, asthma patients can increase perfusion to less-ventilated areas of the lung (Quimby et. al., 1958). In contrast, the effect upon cerebral vessels is vasodilation, resulting in decreased cerebral blood flow and oxygen tension (Skinhøj and Paulson, 1972; Magnussen and Høedt-Rasmussen, 1977).

Because of the various actions of theophylline, the systemic blood pressure is affected unpredictably. While stimulation of both the central vasomotor and direct myocardial functions tends to increase the blood pressure, central vagal stimulation and peripheral vasodilation favor a decrease. The net effect is generally a slight rise in blood pressure (Ritchie, 1975).

Less physiologically significant effects of theophylline may include intense but short-lived diuresis, decreased coagulation time and slight elevation of intermediary metabolism, especially glycogenolysis, gluconeogenesis and lipolysis (Ritchie, 1975).

THERAPEUTIC USES

Of its varied actions, theophylline exhibits its greatest effects in bronchodilation and circulatory vasodilation, therefore its primary indications are the treatment of respiratory and cardiovascular disease (Parker et. al., 1966a, b, 1967; Jezek et. al., 1970; Marcus et. al., 1972).

Theophylline is widely used in both acute therapy and chronic management of bronchial asthma patients. During asthma attacks, airway obstruction results from mucosal thickening, mucous plugging and bronchial smooth muscle constriction, but inflammatory edema and inspissated secretions may intensify the obstruction as the episode continues. Both children and adults show improvement in pulmonary vital capacity after achieving a therapeutic serum level. Theophylline has been reported to be effective in the treatment and prevention of apnea in the neonate (Shannon et. al., 1975; Aranda et. al., 1976; Giacoia et. al., 1976), as well as various other pulmonary disorders (Obstructive pulmonary disease, bronchitis, emphysema, Cheyne-Stokes respiration) (Dowell, 1965; Jezek et. al., 1970).

Because of the transient increase in cardiac output through direct myocardial stimulation, theophylline is a valuable adjunct in the emergency treatment of congestive heart failure (Parker et. al., 1966a). The primary function is relief of paroxysmal dyspnea associated with left heart failure (Udea et. al., 1967; Jenne et. al., 1977). Correlations between response and plasma level have not been estab-

lished because of the variability in theophylline clearance in such patients. Therefore it is indicated only until a cardiac glycoside can be instituted.

ADMINISTRATION OF THEOPHYLLINE

1. Available Formulations

There are over 200 forms of theophylline presently available in the United States (Hendeles, 1978; Weinberger, 1978) (Table 1). The large majority are for oral administration and marketed in fixed-dose combination with ephedrine, barbiturates, antihistamines or expectorants. According to Weinberger (1978), current data do not support the use of such combination products, although formulations containing such compounds should not interfere with bioavailability of theophylline (Welling et. al., 1976). Plain, uncoated, anhydrous tablets have virtually 100% bioavailability and absorption is almost as rapid as a solution (Hendeles, 1978). The number of available compounds and lack of standardization is confusing, but there is no particular advantage to any of the tablets or capsules (except cost) as they all undergo rapid dissolution (Weinberger et. al., 1978). The so-called salts of theophylline are essentially mixtures of the active drug plus an excipient, as theophylline does not form stable compounds with strong bases. These substances, Aminophylline tablets (theophylline ethylenediamine), Oxtriphyllin (choline theophylline), theophylline calcium salicylate or theophylline sodium glycinate, offer no

therapeutic advantage over plain theophylline tablets. They only create confusion as they are phantom products, i.e. a certain compound may contain only 60 - 85% theophylline. Sustained-release preparations offer potential advantages of longer dosage intervals and less fluctuation of serum levels if the absorption is reliable (Slo-Phyllin Gyrocaps, Theophyl S-R, and Theo-Dur). Other enteric-coated preparations have poor bioavailability (Hendeles, 1977b).

Certain liquid preparations of theophylline have alcohol, which does not enhance theophylline bioavailability (Koysooko et. al., 1975). The commonly-used parenteral form of theophylline is aminophylline (75 - 85% theophylline). The ethylenediamine serves only to increase the pH of the solution to dissolve a convenient amount of theophylline (Weinberger, 1978).

The only other acceptable form of theophylline is Somophyllin, a rectally-absorbed, solution-filled capsule. The bioavailability of each preparation is different, due to different amounts of anhydrous theophylline. Theophylline alone, is the active ingredient common to all these preparations, and the compound for which analysis is performed in the clinical laboratory.

2. Analogues and Derivatives

Dyphylline (dihydroxypropyltheophylline; glyphylline) is sometimes falsely advertised as a theophylline derivative. It is stable and therefore does not produce theophylline

in vivo or *in vitro*. Its pharmacokinetic characteristics are not well-known, however, its rate of elimination appears to be considerably faster than theophylline (Hendeles, 1978). This would probably create the same problems that theophylline shows when elimination rate is accelerated.

Dyphylline can be measured in the laboratory and can be isolated from theophylline and other methylxanthines. Under proper analytical conditions it should not interfere with theophylline.

When theophylline is given in combination with other β-adrenoceptor stimulants, whether in fixed-dose combination (e.g. ephedrine) or in combination with another drug (e.g. epinephrine, isoproterenol, solbutamol, terbutalene) synergistic efficacy and toxicity are apparent. Some studies report synergistic toxicity without clinically significant, additive therapeutic benefits (Weinberger and Bronsky, 1975). Other studies report additive bronchodilator effects.

Another adjunct in the treatment of bronchial asthma is cromolyn (disodium chromoglycate) which does not have bronchodilator properties, but prevents mast cell degranulation and the release of the mediators of immediate hypersensitivity (histamine, slow-reacting substance of anaphylaxis) (Meyers, Jawetz and Goldfein, 1976). Cromolyn has been reported as less effective than theophylline and furthermore, did not increase the total number of symptom-free days when combined with theophylline (Hambleton et. al., 1977).

3. Dosage

A wide range in dosage requirements for theophylline has been confirmed, depending on whether the indication is for an acute asthma attack or chronic management. A loading dose is mandatory for rapid relief of acute symptoms. In such instances the object is to provide a dose that will peak at the 10 - 15 mg/L range in the serum as quickly as possible. This will be acheived in most persons by a loading dose of 5 - 7.5 mg/kg as suggested by Mitenko and Ogilvie (1973b) and Hendeles et. al. (1976). If the serum theophylline concentration is not at or near zero at the time of administration of the loading dose, (due to previous therapy) the desired loading can be determined by the formula L.D. = D.S.C. - M.S.C./V_d. This avoids potential toxicity by overloading the patient. Ideally, a dose should be initiated, the plasma level measured 45 minutes later, and the dose readjusted (Hendeles, 1978; Weinberger, 1978).

In the course of chronic therapy, however, slow clinical titration is preferred to a rapid loading dose. Initial infusion rates should be based on the age, mean pharmacokinetic data and clinical status of the patient, but can be altered (based on serum levels) to achieve the final therapeutic rate. This rate is generally lower in adults (>17 years = 10 - 19 mg/kg/day), than in children (16 - 26

L.D.=loading dose; D.S.C.=desired serum concentration; M.S.C. =measured serum concentration; V_d=volume of distribution.

mg/kg/day) (Piafsky and Ogilvie, 1975; Zaske et. al., 1977; Weinberger and Hendeles, 1977; Wyatt et. al., 1978).

PHARMACOKINETICS

1. Absorption and Distribution

Absorption will be complete no matter which of the acceptable preparations is administered, but the time elapsed may differ. Intravenous absorption of theophylline is quicker than the oral or rectal routes (Truitt et. al., 1950; Isaksson and Lindholm, 1961; Lillehei, 1968). Oral administration is delayed by high fat or carbohydrate meals, but may be enhanced by a fasting state or high protein meals (Kappas et. al., 1977).

Once absorbed, theophylline is reversibly bound to plasma proteins in a percentage determined by the age and clinical status of the patient (Table 2). In healthy adults the fraction ofdrug bound in the serum is approximately 53 - 63% (Obilvie, 1978).

There is a wide variation in the half-life ($t_{\frac{1}{2}}$) of theophylline. The distribution phase (α) appears to occur within 30--45 minutes after an intravenous dose (Ogilvie, 1978), but the elimination phase (β) depends on the age, disease and other factors concerning the patient.

For theophylline, the concepts of half-life and dosage are intimately related. Because absorption of theophylline is rapid and is distributed quickly (α), the serum concentration provided by the initial loading dose is related to the

volume of distribution (V_d) or apparent space into which the drug diffuses. The V_d averages approximately 0.5 L/kg and shows little variability with respect to age, gender, smoking habits, or such diseases as asthma or pulmonary edema. As serum concentration equals dose divided by apparent volume of distribution ($C = D/V_d$) a standard loading dose (~5 mg/kg) will produce a serum concentration of 15 - 20 mg/L in most individuals.

Each 1 mg/kg of theophylline administered in any rapidly absorbed form, will result in an average 2 mg/L increase in serum theophylline concentration (Hendeles et. al., 1978). Exceptions to this concept of consistent volume of distribution include premature neonates and patients with hepatic cirrhosis, all of whom exhibit a larger volume of distribution (Aranda et. al., 1976). Alternatively, obese subjects exhibit a smaller apparent volume of distribution (Gal et. al., 1978). Patients with chronic obstructive pulmonary disease show a larger V_d if the disease is accompanied by arterial blood acidemia, but a lower V_d if arterial blood alkalemia is concurrent (Ogilvie, 1978).

2. Metabolism

Theophylline is eliminated from the body by biotransformation by the liver (subcellular microsomal fraction), in which it is partially demethylated and oxidized, followed by renal excretion. The major metabolites are 1,3-dimethyluric acid (~40%), 1-methyluric acid (~16%) and 3-methylxan-

thine (~36%), the last of which is active but 1 to 5 times less potent than the parent drug (Cornish and Christman, 1957; Jenne et. al., 1976; Perrson and Anderson, 1977).

The enzymes responsible for theophylline metabolism in the liver are not presently known but may include cytochrome P448 which can be induced by cigarette smoke (Ogilvie, 1978). Xanthine oxidase is not involved in theophylline metabolism, so there is no complete demethylation or subsequent rise in uric acid. Therefore, the drug is not contraindicated in gout.

3. Factors Affecting Plasma Clearance and Elimination

The elimination of theophylline (β phase) varies tremendously due to factors which influence the individual's ability to clear this drug from the plasma. Therefore, a close relationship between serum levels and plasma elimination rate (as opposed to V_d) is seen in chronic therapy. Furthermore, dosage requirements are different for each patient in continued therapy, i.e. patients with slower plasma-clearing abilities require less frequent dosage.

Age is a key factor in assessing plasma clearance of theophylline. Compared to asthmatic adults, neonates (treated for apnea) have decreased clearance values, while asthmatic children have values that are greater, but decrease to adult level by age 18 (Levy and Koysooko, 1975; Ellis et. al., 1976; Ginchansky and Weinberger, 1977). There is still some controversy as to whether old age per se, is associ-

ated with decreased plasma clearance values.

Gal et. al., (1978) stressed using the ideal body weight to calculate dosage in obese patients as their apparent V_d is reduced and mean elimination half-lives were slightly elevated.

Both high dietary carbohydrates and methylxanthines can lower plasma clearance values (Caldwell et. al., 1977; Kappas et. al., 1977). High protein, expecially charcoal-broiled meats appear to increase theophylline elimination (Kappas, 1978). There appears to be some minor biotransformation of caffeine to theophylline (~1 mg/L) in heavy coffee drinkers (Sved et. al., 1976), but this is unlikely to have any effect on overall theophylline clearance.

Frequent smoking (either tobacco or marihuama) definitely lowers plasma clearance of theophylline (Jenne et. al., 1975; Jusko, 1978; Jusko et. al., 1978; Pfeifer and Greenblatt, 1978), but this effect is reversible if the smoking is discontinued for several months (Hunt et. al., 1976).

Several diseases alter theophylline disposition. Patients with hepatic cirrhosis, acute pulmonary edema and congestive heart failure all tend to have much lower theophylline plasma clearances than subjects with normal organ function (Lawyer et. al, 1977; Mangione et. al., 1977; Piafsky et. al., 1977a, b).

Two macrolide antibiotics, Troleandomycin and erthyromycin, have been implicated in decreasing plasma theophylline clearance in patients with normal liver function Kozak

et. al., 1977; Weinberger et. al., 1977; Pfeifer et. al., 1978). Phenobarbitone (which may be combined with theophylline in certain preparations) has shown possible elevating effects on clearance values (Piafsky et. al., 1977c).

It is possible that theophylline may inhibit its own elimination because of mixed first order with Michaelis-Menton kinetics, which reduces plasma theophylline clearance. Although it had previously been assumed that theophylline elimination follows only a first order process, studies have indicated that with chronic administration of theophylline, the metabolite, 3-methylxanthine is first excreted by Michalis-Menton kinetics at a constant rate, followed by a period of apparent first order elimination (Caldwell, 1977). This phenomenon, which involves the reactions in the N-demethylation of theophylline, is not apparent in the absence of dietary methyxanthines or with single-dose administration of the drug (Hendeles et. al., 1977b; Weinberger and Ginchansky, 1977; Caldwell, 1977).

TOXICITY

Because of the numerous factors which can alter the rate of elimination of theophylline from the plasma, there is a constant possibility of toxicity. This is distinct from the minor caffeine-like side effects experienced in long term therapy before aquiring tolerance. The most frequently observed adverse effects include nausea, anorexia, vomiting, diarrhea, headache, nervousness and gastrointestinal bleed-

ing. Severe toxicity can cause seizures, cardiac arrhymias, and respiratory or cardiac arrest (Zwillich et. al., 1975; Jacobs et. al., 1976; Matthay et. al., 1976; Weinberger et. al., 1976; Hendeles et. al., 1977a, 1978). In rare instances deaths have occured in adults, all of whom suffered seizures prior to death (Zwillich et. al., 1975). It had previously been recommended that theophylline dosage could be increased until mild gastrointestinal effects occured, then decreased slightly to obtain the optimal therapeutic dose (Cupit and Powell, 1975). The assumption was that mild toxicity precedes seizures. Zwillich, however, found that in 7 out of 8 deaths due to theophylline, there were no other adverse effects prior to seizures. The possibility of toxicity in infants and children has been a constant concern due to reports implicating theophylline in seizures and even deaths (Weinberger, 1978).

RATIONALE BEHIND THEOPHYLLINE MEASUREMENT

Although there are recommended loading and maintenance doses, a far more dependable approach to theophylline therapy is the steadfast monitoring of plasma concentrations, and in so doing, many unnecessary deaths have been avoided.

The relationship between serum theophylline and toxicity is well documented. Serious adverse effects are rare at levels below 15 - 20 mg/L. Most serious complications are associated with levels above 40 mg/L.

The efficacy, as well as toxicity of theophylline is

clearly related to its serum concentration. It is evident that the bronchodilating effects (in patients with reversible airway obstruction or exercise-induced bronchospasm) (Bierman et. al., 1977; Pollock et. al., 1977), long term prevention of asthma symptoms and inotropic effects (Ogilvie, 1978) occur at 10 - 20 mg/L of theophylline.

RATIONALE BEHIND SALIVARY THEOPHYLLINE MEASUREMENTS

Quantitation of salivary theophylline as an alternative to serum monitoring, has aroused much recent interest. Several reports indicate the existance of a constant ratio between theophylline in salivary excretion and serum (S/P ratio), in simultaneously-drawn samples, from controls and/or patients (Table 3). If such a correlation does indeed exist, saliva might be substituted for serum in therapeutic monitoring of theophylline. The advantages are obvious. Saliva can be collected by non-invasive techniques at home without trained personnel.

Several investigators have reported proportional concentrations between saliva and serum theophylline, but have not been consistant in this finding. In these studies, saliva was stimulated by chewing paraffin and the mixed saliva was expectorated and collected in glass vials.

It appears that the S/P ratio is influenced by several intrasubject and intersubject factors including dose form and duration, sampling time, and partial adsorption of theophylline to the oral mucosa (Figure 3). Although most

drugs appear to be excreted in saliva by passive diffusion, perhaps theophylline is subject to some active transport mechanism as well (Danhof and Breimer, 1978). The S/P ratio seems higher in the adsorption phase than in the elimination phase. Because of inconclusive data, salivary theophylline determinations cannot yet be recommended in lieu of serum quantitation.

ROLE OF THE CLINICAL CHEMIST IN THEOPHYLLINE THERAPY

Careful monitoring of serum theophylline is essential to the welfare of the patient due to narrow therapeutic range and variable elimination rates. To fulfill the laboratory's obligations to the physician and the patient, the clinical chemist must consider:

Who are the patients requiring theophylline monitoring?

What is the nature of the sample?

How will these samples be quantitated?

The type of patient receiving theophylline may affect the outcome of its quantitation or the choice of methodology. If the patient requires emergency monitoring, a quick, reliable method is desirable and the specimen may need to be diluted. If the patient is a pediatric case, microsampling is of special concern.

The sample should be plasma or serum obtained during the steady state. Both peak (two hours after solid or solution forms, four hours after slow-releasing tablets) and trough levels (end of dosing interval) give useful informa-

tion. The possibility of other drugs in the serum or other interfering compounds should be considered.

The methodology for quantitation of theophylline depends largely on the capabilities of the personnel and the facilities of the laboratory. Due to the rapid expansion of pharmacokinetics and toxicology in recent years, the clinical chemist faces dozens of published theophylline methodologies from which to choose.

DESCRIPTION AND BRIEF EVALUATION OF AVAILABLE METHODOLOGIES

Essentially six basic methods can be considered for theophylline analysis in the clinical laboratory. They are: 1) ultraviolet spectrophotometry (UV), 2) thin-layer chromatography (TLC), 3) radioimmunoassay (RIA), 4) high-performance liquid chromatography (HPLC), 5) gas-liquid chromatography (GLC), and 6) enzyme immunoassay, with or without a centrifugal analyzer.

Other methods have been described which are not practical for most clinical laboratories, including high-pressure cation-exchange chromatography (Weinberger and Chidsey, 1975), electron-capture gas chromatography (Arbin and Edlund, 1975; Schwertner et. al., 1976), and gas-chromatography with mass spectrometry (Sheehan et. al., 1977).

1. Ultraviolet Spectrophotometry

UV methods are based on the procedure of Schack and Waxler (1949) and, subsequently, many modifications have

improved sensitivity and specificity (Gupta and Lundberg, 1973; Jatlow, 1975; Schwertner et. al., 1978) (Appendix).

UV methods are subject to many interferences, resulting in both falsely elevated and decreased theophylline values. Such substances include theophylline metabolites, theobromine, caffeine, free and substituted uric acid and other xanthines, allopurinol, tetracycline, prochloroperazine, phenobarbitone and other barbiturates, salicylates, acetominophen, sulfonamides, furosemide, thiamine, butazone, phenytoin, probenecid, dicoumerol, warfarin and others. Although some of these have been eliminated by methodological modifications (Frings et. al., 1975; Vasiliades and Turner, 1976; Banner et. al., 1977; Matheson et. al., 1977), many possible interferences still exist.

Most endogenous biological compounds absorb at lower UV wavelengths (180 - 200 nm), and only compounds with relatively large chromophores absorb at longer wavelengths (250 - 300 nm). Many drugs, however, may be present in the serum simultaneously and can absorb at higher UV wavelengths, even after solvent extraction procedures. Without chromatographic separation UV alone may not be sufficient to eliminate interferences.

UV methods require relatively larger samples (at least 1 mL) and large reagent volumes. Plasma blanks are variable, and sensitivity and specificity are inadequate compared to other methods. Therefore, UV cannot be recommended if another method is available. For the same reasons, UV

methods are not suitable for comparative analysis with newer methods.

2. Thin Layer Chromatography

Two quantitative thin-layer chromatographic methods have been described (Gupta et. al., 1977; Riechert, 1978) (Appendix).

Both methods claim sensitivity and specificity equal to more sophisticated chromatographic methods. They offer the particular advantage of allowing the simultaneous analysis of six samples, without using complicated techniques or instrumentation. The future of TLC in the determination of plasma drug levels looks promising. Recent advances in materials and methods to improve resolution and specificity have led to so-called high-performance thin-layer chromatography. This term has been used to describe methods already established for chlorpromazine and tricyclic antidepressants (Fenimore et. al., 1978). Further advantages include freedom from solvent restrictions as in HPLC and derivitization and column degradation as in GLC. Even more important is the static nature of the separated components, which can be easily quantitated compared to flowing column effluents.

TLC has generally been considered a semiquantitative procedure and is as much an art as a scientific technique. These methods might be acceptable in the near future, but require further testing before they can be recommended.

3. Radioimmunoassay

Radioimmunoassay is a possible choice for theophylline determination if a scintillation spectrometer is available.

Cook et. al. (1976) and Neese et. al. (1977) report similar micromethods using tritium-labelled theophylline (Appendix). RIA offers accuracy and precision at a relatively low cost, but introduces the hazard of radioactivity, and liquid scintillation spectrometry can be troublesome.

A commercial kit using ^{125}I-theophylline is now available from Clinical Assays (Division of Travenol Laboratories, Inc.). Gammadab® theophylline RIA kit affords the additional convenience of faster sample preparation and counting by more easily-automated gamma-ray spectrometry, although the cost of commercial reagents may be prohibitive.

As theophylline procedures using RIA or TLC are not as well established, and UV spectrophotometric assay are not quite commensurate with other methods, HPLC, GLC and enzyme immunoassay reflect the current status of theophylline quantitation. Therefore, the clinical chemist seeking a progressive method would choose from these.

4. High Performance Liquid Chromatography

In 1974, Thompson and Nagawasa reported a high-pressure cation-exchange chromatographic method for separating theophylline and its metabolites in serum and urine. More practical procedures were reported by Weinberger and Chidsey (1975) using Aminex A-5 cation exchange columns, and by

Jusko and Poliszcauk (1976) involving protein precipitation with trichoroacetic acid, and injection of the supernatant onto Dupont Zipax SCX columns. Although the lifespan of these columns is quite long, this type of method became obsolete with the advent of commercially-prepacked reversed phase columns.

Because of numerous advantages, HPLC, especially reversed-phase liquid chromatography (RPLC), may be considered the latest reference method for theophylline determinations. Small sample volume, simple extraction, control over chromatographic conditions, relatively short analysis time and excellent reproducibility and specificity, all contribute to the recent surge of acceptance of RPLC. The basic principles of RPLC are reviewed in the Appendix.

Many recent RPLC methods involve only a protein precipitation and no complex extraction or derivitization is necessary. Early reports indicated many drugs do not interfere with quantitation in such methods, but as RPLC became more common in clinical laboratories, the reports of drug interferences increased (Soldin and Hill, 1977; Kelly et. al., 1978; Robinson and Dobbs, 1978; Bond and Thornton, 1979). Any drug which remains in the supernatant (or even the aqueous phase after simple extraction) can co-elute with either theophylline or the internal standard, causing either false high or low results, respectively, when the peak height ratio method of quantitation is used (Figure 4). The probability of this occuring depends on the interrelation-

ships of the column packing, choice of solvent, hydrophobicity of the internal standard, pH of the plasma during precipitation and other drugs present in the patient's serum.

Although many RPLC methods have been reported (Table 6), the most widely adapted procedure for theophylline quantitation is that of Orcutt et. al. (1977). This method is used as the reference method for this work. In a small volume of serum (30 μL) the proteins are precipitated in one step by an ethanol solution containing the internal standard (β-hydroxyethyltheophylline). A 15 μL aliquot of the supernatant is injected onto a μ-Bondapak C-18 column and eluted with a solvent system of acetonitrile/potassium phosphate buffer. The peak height ratio method of quantitation is used.

Despite the increasing popularity of RPLC methods, its relative complexity and scarcity of trained personnel are still obstacles in its utility.

5. Gas Liquid Chromatography

Long before the development of RPLC, GLC was successfully used for therapeutic drug monitoring, and is still an excellent alternative (Table 7, Appendix).

Several methods analyzing nonderivatized theophylline have been reported (Chrzanowski et. al., 1974; Sheehan and Haythorn, 1976; Schwertner, 1979), but theophylline does not chromatograph well under these conditions due to peak tailing and long retention times. Schwertner, however, reports good results using a new stationary phase, 2% SP 2510-DA.

Derivitization helps produce narrow, symmetrical peaks by masking polar functions (e.g. the weakly acidic anionic organic acids with active protons, such as theophylline). This is accomplished by nucleophilic substitutions with an alkylating agent.

Most derivitizations are based on the flash methylation of active protons, first described by Macgee (1970) for diphenylhydantoin. or an alternative approach developed by Greeley (1974) for barbiturates, in which volatile alkyl derivatives were prepared before injection. Many published methods for theophylline analysis using GLC utilize one of these derivitization procedures (Appendix). It appears that regardless of which technique is used, the higher the number of carbons in the alkylating reagent, the better the resolution of closely-related compounds.

Extractive alkylation is an alkylating reaction that has been overlooked in clinical toxicology until very recently. In this technique, a chemical change induces extraction by ion-pair partitioning. The anionic substrate is extracted directly from aqueous solution into an organic solvent with a quaternary ammonium ion. An alkyl iodide in the organic solvent acts to catalyze the immediate and irreversible alkylation of the anion (Figure 5). The trialkyliodide is precipitated out of solution. Therefore, the compound can be extracted and derivitized in one simple procedure, which is far superior to the others.

Arbin and Edlund (1975) used a tortuous procedure to

prepare pentafluorobenzyl derivatives by extractive alkylation of theophylline for use with electron capture gas chromatography. Joern (1978) reported a simple, one-step extractive alkylation of theophylline using only 25 μL of serum, which forms pentyl derivatives using tetrahexylammonium hydrogen sulfate as the quaternary ammonium ion and iodopentane as the alkyl iodide.

Besides extraction, derivitization and sample size, internal standard and detection are critical parameters for GLC.

A problem in many gas chromatographic methods is the lack of a suitable internal standard, a chemical which is similar to the analyte in question, so that it can be extracted, derivitized and chromatographed with comparable efficiency. This chemical should not be a metabolite of the analyte or a compound which may be endogenous to the patient's serum. An ideal internal standard for theophylline assay is 3-isobutyl-1-methylxanthine (IBMX) (Figure 1) which is structurally similar to theophylline, but not likely to be found in patients' serum.

Most theophylline GLC methods use flame ionization detection (FID). The most recent development in GLC detectors is the alkali flame or nitrogen-phosphorus detector, which resembles the conventional FID in using hydrogen and air supply gases (but at much lower flow rates) with a high voltage collector (Figure 6). Alkali metal ions are emitted from an electrically-heated rubidium salt. These ions

selectively interact with and ionize nitrogen and phosphorus-containing compounds. Hydrocarbons, and hence most endogenous biological compounds, do not ionize efficiently by this process. The N-P detector is also 10 - 100 times more sensitive to drugs containing nitrogen and because of this selectivity, less rigorous cleanup extractions are needed.

Four methods for theophylline using N-P detection have been reported (Least et. al., 1976; Lowry et. al., 1977; Joern, 1978; Vinet and Zizian, 1979). If N-P detection is available, a modified extractive pentylation procedure is the ideal GLC method for theophylline and is the basis for the GLC procedure in this work.

6. Enzyme Immunoassay

Although both RPLC and GLC methods offer precise, accurate results, as well as a permanent picture of the analytes in the blood, they require sophisticated and expensive instrumentation and skilled personnel. EMIT® (Enzyme Multiplied Immunoassay Technique, Syva Corp.), which was first introduced in 1972 (Rubenstein et. al.) offers a unique alternative to chromatographic methods for theophylline quantitation. In this recently-popular technique, quantitation is based on competitive protein binding utilizing enzyme-linked theophylline (Appendix).

Standards and reagents are available in a kit as lyophylized powders. Reagent A contains substrate and antibody. Reagent B contains the enzyme-labelled theophylline.

With no separation step, a test is carried out in a few minutes by mixing a small quantity (50 μL) of plasma or serum with the two reagents.

Many studies confirm the accuracy, sensitivity and specificity of the EMIT® method (Legas and Raisys, 1976; Gushaw et. al., 1977; Chang and Bastiani, 1977; Koup and Brodsky, 1978).

7. Enzyme Immunoassay with a Centrifugal Analyzer

The ease and accuracy by which EMIT analyses can be performed have been drastica lly improved by applying them to the centrifugal fast analyzer (e.g. Centrifichem 400®, Union Carbide Corp.). This sytem, consisting of pipettor, analyzer and optional interfaced computer, permits precise, simultaneous spectrophotometric microanalysis, standardization and specificity.

The theophylline assay on the Centrifichem 400 uses the AUTO BLANK mode. This is an end-point mode for reactions with an initial lag phase, such that absorbance readings can be taken before a significant reaction has occured. This value, which represents a reagent and sample blank as well as each cuvette's variation from the Reference Cuvette (0) is stored in the memory and automatically subtracted from the final absorbance at a specified time. This difference in absorbance is printed and used to calculate the concentration of theophylline. Samples are flagged as abnormal if the total change in absorbance exceeds the linearity limit.

Although this is actually a kinetic rate reaction, because of the lag phase and stable linearity, it can be measured in the terminal mode. The entire assay procedure takes only a few minutes.

PURPOSE

To fulfill the requisites and responsibilities of instituting and maintaining a reliable analytical procedure, the clinical chemist must comprehensively assimilate, evaluate and compare methodologies. Only with such information can conclusions as to the usefulness, practicality, limitations cost and time of a method. Original modifications resulting in reduction of time, cost and sample size or increase in specificity, sensitivity and practicality can greatly benefit the clinician, laboratory and patient. By keeping informed as to the latest developments in clinical chemistry and therapeutic drug monitoring, and experimenting with new procedures and modifications, the clinical chemist can play an active role in patient care.

The purpose of this study was 1) to provide a concise, yet conclusive review of theophylline metabolism, usage and quantitation; 2) to evaluate and compare the best current procedures by performing extensive clinical studies using original modifications and 3) to seek and attempt to explain interferences which cause discrepancy in results between different analytical methodologies. This was accomplished by 1) evaluation and modification of the EMIT® theophylline

enzyme immunoassay, adapted for the Centrifichem 400® centrifugal analyzer; 2) evaluation and modification of a GLC theophylline procedure using a nitrogen-phosphorus detector and extractive alkylation and derivitization; 3) evaluation of an RPLC theophylline assay; 4) comparisons of all of these methods and 5) review of patients' madical records in cases of intermethod discrepancies.

METHODS AND MATERIALS

GENERAL EXPERIMENTAL DESIGN

We evaluated the performance of the EMIT® enzyme immunoassay for theophylline, adapted by Syva Corp. (Palo Alto, CA 94304) for the Centrifichem 400® centrifugal analyzer (Union Carbide Corp., Rye, N.Y. 10580). Reagents, standards and controls were a gift from Syva Corp. After performing precision, accuracy, and carry-over studies, comparative analysis with an RPLC method was done using patient specimens.

One hundred fifty serum samples were obtained from the Clinical Pharmacokinetics Laboratory at University of California, San Francisco Medical Center (San Francisco, CA 94143), which had been previously analyzed in that laboratory by RPLC. This method is considered the reference method for this work. It is based on the method of Orcutt et. al., (1977), involving protein precipitation of theophylline and injection of the supernatant.

One hundred of these specimens were analyzed for theophylline content on the Centrifichem 400. Studies involving linearity helped optimize this assay, after which 40 additional specimens were analyzed.

In order to resolve discrepancies between the RPLC and EMIT, a GLC-NP method was developed using the Hewlett-Packard 5840A gas chromatograph equipped with a nitrogen-phosphorus detector and a microprocessor. After optimizing the extraction and derivitization procedure and the chromatographic

conditions; precision, accuracy, linearity, detection limit and interference studies were performed. Theophylline concentration in 100 samples were quantitated by this method, 60 of which already had RPLC values and 40 of which were obtained at San Francisco General Hospital. There were then two methods by which the EMIT® assay on the centrifugal analyzer could be compared.

The EMIT theophylline assay was modified so that less reagents per test would be used (in order to reduce the cost) while maintaining precision, accuracy, and correlation with RPLC, GLC-NP and the original EMIT-centrifugal analyzer methods. This was accomplished by reducing the reagents in a constant proportion so that substrate, antibody and enzyme-labelled theophylline maintained optimal reactivity. Once again, linearity, precision and accuracy were determined. One hundred serum samples were analyzed by this modified procedure (EMIT-MOD), 60 of which had RPLC values, 91 of which had EMIT values and all of which had GLC-NP values.

All comparative analyses included calculations for correlation coefficients, and the linear regression line with slope and y-intercept, using a Texas Instruments programmable calculator. All samples with values differing from the reference value by three standard deviations or more were examined for obvious interferences, such as lipemia and hemolysis. The medical records of patients whose serum values consistantly disagreed with the reference method were examined for clues of possible interferences. For ampicillin, which

was implicated as a possible interference, experiments were initiated to confirm this possibility.

SPECIFIC METHODOLOGIES

1. Reversed Phase Liquid Chromatography (RPLC)

APPARATUS*: A solvent-delivery system of variable flow rate and pressure capacity to 6000 psi (Model M-6000A; Waters Associates, Milford, MA 91757), an injector with loop capacity of up to 2 mL (Model U6K, Waters Associates), a 30 cm X 4 mm i.d. column packed with the stationary phase, 10-μm diameter octadecyltricholoro-silane-coated silica beads (μ-Bondapak C-18, Waters Associates), an absorbance detector with dual channel capabilities for monitoring the eluent at 273 nm (Model S.F. 770; Schoeffel Co., Westwood, NJ 97657), and a dual-channel chart recorder (Omniscribe, Houston Instruments, Austin, TX 78753) were used for these determinations.

REAGENTS: Mobile phase; 5.0 X 10^{-4} M phosphate buffer was prepared by adding 1.0 mL of 1.0 M stock potassium phosphate buffer (in H_2O) up to 2 L with deionized water and adjusting to pH 4.0 with H_3PO_4, then filtering through millipore filters with aqueous paper. It is stable at least one year at 22°C. Eighty mL UV quality acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442) was added to 920 mL of the phosphate buffer, mixed and degased at reduced pressure for 5 minutes in an ultrasonic

*Apparatus at Clinical Pharmacokinetics Laboratory, University of California, San Francisco Medical Center.

bath. This was done daily.

Working internal standard was prepared by adding 1.0 mL stock internal standard [25 mg β-hydroxyethyltheophylline (Sigma Chemical Co., St. Louis, MO 63178) in 100 mL ethanol] to 9.0 mL ethanol for a final concentration of 25 mg/L. This was made fresh weekly.

Working theophylline standards were prepared by adding stock theophylline standard [15 mg theophylline (Sigma Chemical Co.) in 100 mL deionized water] to theophylline-free serum. A 15 mg/L standard was prepared by adding 200 μL of stock standard solution to 1.8 mL of theophylline-free serum. A 30 mg/L standard was prepared by adding 400 μL of stock standard solution to 1.6 mL of theophylline-free serum.

PROCEDURE: 200 μL of working internal standard and 200 μL of standard or sample were vortexed for ten seconds in microcentrifuge tubes, and covered with Parafilm®. After 15 minutes (to allow complete precipitation of proteins), they were centrifuged at 3000 rpm for 10 minutes. Fifteen μL of the supernatant was injected into the chromatograph, which is operated at the following conditions: solvent flow rate, 2.0 mL/min; spectrophotometer wavelength, 273 nm; recorder sensitivity, 0.02 absorbance units full scale; chart speed, 0.5 cm/min and pressure, 1800-2000 psi.

QUANTITATION: The ratio of the peak height of the internal standard to the peak height of the 15 mg/L standard, multiplied by the concentration of the standard equals a factor that remains constant for all subsequent samples.

The peak height ratio of theophylline to internal standard in the unknowns was then multiplied by this factor to give theophylline concentration in mg/L. Linearity was checked daily with the 30 mg/L standard (see Figure 4).

2. Enzyme Immunoassay (EMIT®) Adapted for the Centrifugal Analyzer (Centrifichem 400®)

APPARATUS: A Centrifichem 400 centrifugal analyzer (Union Carbide Corp., Rye, NY 10580), consisting of pipettor and analyzer equipped with a computer, was used with the following settings:

Pipettor controls	
Sample volume	7 µL
Sample & diluent	50 µL
Reagent volume	350 µL
Analyzer controls	
Filter	340 nm
Temperature	30°C
T_o	15 s
ΔT	45 s
Abnormal absorbance	2.0 U
Blank	Auto
Test mode	Term
Print out	ABS
Number of prints	1
Conc. factor/std. value	0000
Test code	00

A 100 µL micropipette (SMI, Berkeley, CA 94710) was also used in this study.

REAGENTS: All calibrators and controls were procured from Syva Corp., (Palo Alto, CA 94304).

EMIT Reagent A (antibody) and Reagent B (enzyme) were each reconstituted with 6.0 mL distilled water and allowed to stand at least 1 hour at ambient temperature (~25°C).

The EMIT® Buffer (0.55M Tris buffer) was prepared by diluting the concentrate up to 150 mL with distilled water.

Working Reagent A was prepared by mixing 1 part Reagent A plus 2 parts EMIT buffer. Working Reagent B was prepared by mixing 1 part Reagent B with 9 parts EMIT buffer. Both Working Reagents A and B must equilibrate 4 hours at room temperature or overnight at 2 - 8°C, and will be swirled and brought to room temperature before using. Excess working reagents may be stored in tightly-capped containers at 2 - 8°C for three days.

The EMIT serum theophylline calibrators (0, 2.5, 5.0, 10.0, 20.0, and 40.0 mg/L) were reconstituted with 1.0 mL distilled water each. The lyophilized serum controls (7.5 and 25.0 mg/L) were reconstituted with 2.0 mL distilled water each. Both calibrators and controls must equilibrate at least 1 hour at room temperature or overnight at 2 - 8°C.

PROCEDURE: All working reagents, calibrators, controls and samples should be at room temperature. The sample ring was loaded with sample cups containing distilled water in position #1, the zero calibrator in positions #2 and #3, followed by the calibrators (2.5 - 40.0 mg/L), controls and patient samples (serum or plasma) in duplicate. A last-sample plug was at the end. The sample ring was placed on the turntable with a cover to avoid evaporation and a clean, dry transfer disc in the center. Using the SMI pipette, 100 μL of Working Reagent A was dispensed into each sample well that was to receive a calibrator, control or sample.

Then the cycle button was depressed, and when the pipetting was complete, the transfer disc was carefully placed into the analyzer. After the run was complete, the disc was cleaned automatically.

QUANTITATION: A standard curve was plotted on special paper supplied by Syva. The $\Delta A - \Delta A_o$ value for each calibrator was plotted against its respective concentration in mg/L. ΔA_o is the average absorbance of the zero calibrator and ΔA is the value of the others, as read off the Centrifichem® printout. The curve was drawn as the best smooth curve, and was practically linear. If values for duplicates differ by more than 6 ΔA units, or if one calibrator is off the curve by more than 8 ΔA units, it must be repeated. Theophylline values were read off the curve to the nearest mg/L.

3. Modified Immunoenzymatic Method for the Centrifugal Analyzer (EMIT®-MOD)

The following changes in the original method were made:

1) Sample size: 5 μL.
2) Reagent A used with no dilution: 20 μL/well.
3) Working Reagent B: 0.6 mL diluted to 10 mL with EMIT buffer.
4) ΔT = 60s
5) Standard curve is drawn point-to-point.
6) Control: Therachem Tri-Level Anticonvulsant and Theophylline Control (Fisher Scientific Co., Orangeburg, NY 10962).

4. Gas-Liquid Chromatography with Nitrogen-Phosphorus Detection (GLC-NP)

Apparatus: A dual-column gas chromatograph equipped with both flame ionization and nitrogen-phosphorus (N-P) detectors was used (Model 5840A, Hewlett-Packard Co., Palo Alto, CA 94303). The software consists of an integrator to detect and measure peaks by means of a computer built into the processor, which can look backwards at stored data. The glass column, 6 ft. X 3 mm (o.d.) was packed with 3% OV-17 on 100/200 Chromosorb W-HP (Hewlett-Packard Co., Evandale, PA 19311). The chromatographic conditions were: oven temperature, 245°C; injector temperature, 275°C; detector temperature, 280°C; nitrogen carrier gas flow, 26.5 mL/min; hydrogen flow, 3 mL/min; air flow, 60 mL/min; chart speed; 0.5 cm/min; zero, 10% full scale; attenuation, 2 X 10^5; slope sensitivity, 0.3.

REAGENTS: The extraction reagent was prepared by dissolving 45 mg of tetrahexylammonium hydrogen sulfate (Regis Chemical Co., Norton Grove, IL 60053) in 10 mL of 0.1 M aqueous sodium hydroxide. This was good for at least three weeks at 4°C. The working internal standard solution was a mixture of 200 mL methylene chloride (reagent grade), 2.0 mL 1-iodopentane (Eastman-Kodak Co., Rochester, NY 14650) and 0.1 mL of a 1 g/L solution of 3-isobutyl-1-methylxanthine (Aldrich Chemical Co., Milwaukee, WI 53233) in methanol (reagent grade). This was stable at least two months at 4°C and provided a concentration of 20 mg/L. The cali-

brators provided with the EMIT® theophylline kit (Syva Corp.) were used as standards. These are lyophilized preparations of drug-free serum to which theophylline has been added to provide final concentrations of 0, 2.5, 5.0, 10.0, 20.0 and 40.0 mg/L. The control was 20 mg/L (Therachem Tri-Level Anticonvulsant and Theophylline Control). This was also used, diluted 1:2, with phosphate-buffered saline (PBS) pH 7.4. For the precision study, serum standards (8 and 22 mg/L) were prepared by adding drug-free serum to the 40 mg/L calibrator to achieve the desired final concentrations. Into the bottom of 16 X 100 mm disposable culture tubes, 100 μL of extraction reagent was pipetted. A volumetric pipette was used to deliver 1.0 mL of the internal standard solution to each tube. The contents of the tubes were vortexed for 15 seconds then centrifuged at 3000 rpm for 5 minutes. The tubes were carefully removed and gently twisted in a near horizontal position so that the lower solvent layer would break through the aqueous layer. The solvent was decanted into a disposable 12 X 75 mm culture tube, leaving the aqueous layer behind. The solvent was evaporated in a 56°C water bath under nitrogen. The residue was reconstituted with 50 μL of reagent grade n-hexane (Sigma Chemical Co.), 2 μL of which was injected into the gas chromatograph.

QUANTITATION: Concentrations were calculated by calibrating a 20 mg/L standard on the 5840A, which automatically distinguishes peaks from detector noise, determines beginning and end of peaks, measures areas and relative retention

times, corrects for baseline drift and skewed peaks and separates partially resolved peaks.

INTERFERENCE STUDIES INVOLVING AMPICILLIN

Two solutions of ampicillin were prepared (Bristol Laboratories, Syracuse, NY 12301) at 200 mg/L and 1 g/L using deionized water. Twenty-five μL of the 200 mg/L solution was added to 1 mL of serum containing 13 mg/L of theophylline for a final ampicillin concentration of 5 mg/L. This sample was run by RPLC and EMIT®-MOD. The 200 mg/L solution was analyzed by the RPLC method for theophylline.

The 1 g/L solution was used to prepare serum samples with 5, 10, 20, 40, 80, 100, and 200 mg/L of ampicillin, for analysis by EMIT-MOD, using serum that already contained 18 mg/L of theophylline.

A serum sample with ampicillin only (dose = 1 g/day), was spiked with a 1 g/L aqueous solution of theophylline (Sigma Chemical Co.) to provide a final concentration of 20 mg/L. This sample was analyzed for theophylline content by both RPLC and EMIT-MOD.

RESULTS

EMIT® - CENTRIFUGAL ANALYZER

STANDARDS: For the initial evaluation of the EMIT procedure, adapted to the Centrifichem 400®, a standard curve was drawn as the best-fit straight line using the calibrators and graph paper supplied by Syva Corp. Response was linear over the 2.5 - 40.0 mg/L range (Figure 8).

CONTROLS: The EMIT 10 μg/mL calibrator, and 7.5 and 25.0 mg/L controls supplied by Syva Corp. were used for within-run and between-run precision studies, respectively.

WITHIN-RUN PRECISION: Twenty-one replicated assays of the 10 μg/mL calibrator showed mean value plus standard deviation ($\bar{x} \pm$ S.D.) of 9.56 ±0.35 mg/L. The coefficient of variation (CV) was 3.63% (Table 8).

BETWEEN-RUN PRECISION: For the low (7.5 mg/L) and high (25.0 mg/L) controls on 12 and 8 days, respectively, results were 7.85 ± 1.01) mg/L, CV = 12.8% and 26.20 ± 3.54 mg/L, CV = 13.5% (Table 8).

ACCURACY AND RECOVERY: Ten serum samples containing known concentrations of theophylline were used to determine analytical recovery and accuracy of the EMIT and other methods. Samples were prepared by Syva Corp. and contained theophylline in concentrations ranging from 3 - 35 mg/L. The EMIT values were calculated as averages of duplicates.

Comparison of EMIT vs. the spikes samples (SPIKE) showed a correlation coefficient (r) of 0.99, a slope (m) of 0.92

and a y-intercept or bias (b) of 0.40 (Table 9). The percent recovery ranged from 90 to 100% with an average of 98%.

Accuracy of EMIT compared to RPLC (EMIT vs. RPLC), using the same known samples, showed r = 0.99, m = 1.05 and b = -0.33 (Table 9).

PATIENT DATA: Comparative analyses using 100 clinical serum samples previously quantitated by RPLC (EMIT vs. RPLC) showed r = 0.94, m = 0.89 and b = -1.26. When this data was recalculated using a smooth curve (essentially point-to-point) the results were r = 0.96, m = 0.91 and b = -0.66 (Table 10, Figure 9). These results were obtained at ΔT = 45 s (Figure 8).

EMIT®-MOD

STANDARDS: For the modified EMIT procedure (EMIT-MOD), the same serum calibrators were used to plot a smooth standard curve at ΔT = 60 s (Figure 10) on the graph paper supplied by Syva Corp. Response was linear over the 2.5 - 40.0 mg/L range.

CONTROLS: The Ortho Diagnostics Anticonvulsant and Theophylline control (18.8 $\pm$ 2.0 mg/L) was used for precision studies both undiluted and diluted 1:2 with phosphate-buffered saline (PBS), pH 7.4 (9.2 $\pm$ 0.8 mg/L).

WITHIN-RUN PRECISION: Ten consecutive assays of the Ortho control undiluted and diluted resulted in 18.35 $\pm$ 0.73 mg/L, CV = 4.0% and 8.8 $\pm$ 0.25 mg/L, CV = 2.8%, respectively (Table 8).

BETWEEN-RUN PRECISION: On 8 consecutive days, undiluted control (different lot than with-run study) showed 19.84 ± 0.99 mg/L, CV = 5.0%. Diluted control (8 consecutive days) showed 10.56 ± 0.69 μg/mL, CV = 4.5% (Table 8).

ACCURACY AND RECOVERY: Using the 10 samples of known concentrations, EMIT®-MOD vs. SPIKE showed r = 0.99, m = 0.90 and b = 0.42. The percent recovery ranged from 83-116%, averaging 97%.

Accuracy of EMIT-MOD compared to RPLC and EMIT was described by EMIT-MOD vs. RPLC (r = 0.99, m = 1.04 and b = -0.55) and EMIT-MOD vs. EMIT (r = 0.99, m = 1.01 and b = -0.32) (Table 9).

PATIENT DATA: Comparative analyses using patient samples resulted in EMIT-MOD vs. RPLC (r = 0.98, m = 1.05, b = -0.39, N = 60) (Table 10, Figure 11); and EMIT-MOD vs. EMIT (r = 0.97, m = 0.99, b = -0.41, N = 91) (Table 10, Figure 12).

REANALYSIS BY EMIT-MOD

All samples run by the initial EMIT method, having values equal to or greater than 3 standard deviations from the corresponding RPLC values, were reanalyzed by the EMIT-MOD method. Of the 21 samples, 14 exhibited values by the modified EMIT procedure that were much closer, and in agreement with their respective reference values. Eleven of the 14 were within 2 standard deviations of the RPLC value.

Of the 7 remaining reanalyzed specimens, 5 were within

1 mg/L of the initial EMIT value, 1 was within 2 mg/L and 1 was within 4 mg/L but all 7 still were quite discrepant from the RPLC values.

GLC-NP

Because of discrepancies found in evaluating the EMIT and EMIT-MOD methods, and in comparisons with RPLC and samples of known concentrations, the question of accuracy needed to be resolved. Therefore, to obtain a fourth value for each specimen, the GLC-NP procedure was developed. In addition to resolving discrepancies between EMIT®, EMIT-MOD and RPLC, GLC-NP was thoroughly evaluated as another possible method for routine theophylline quantitation.

The retention times of derivatized pure aqueous standards of theophylline and internal standard, 3-isobutyl-1-methylxanthine, were 3.85 and 4.85 minutes, respectively. Two samples of drug-free plasma, which had been negative for theophylline on the EMIT-MOD-Centrifichem 400® system, showed no endogenous interfering peaks at the afore-mentioned retention times.

STANDARDS: EMIT theophylline serum calibrators (0, 2.5, 5.0, 10.0, 20.0 and 40.0 mg/L) were used as standards. Further dilutions (1.0, 0.75, 0.50, 0.25 and 0.10 mg/L) of these standards were also used. Response was linear over 1.0 - 40.0 μg/mL range (Figure 13). Theophylline was detected below this range but without accuracy.

CONTROLS: For within-run precision studies, samples

were prepared at 8 and 22 μg/mL from drug-free plasma (and confirmed by EMIT®-MOD). Therachem Tri-Level Anticonvulsant and Theophylline Control (19.9 ± 1.2 mg/L) was used for between-run precision studies, both undiluted and diluted 1:2 with PBS (9.7 ± 0.5 mg/L).

INSTRUMENT PRECISION: Twenty consecutive analyses of the same derivatized 10 mg/L standard (value assigned by Syva Corp.) reflected instrument precision with 20.2 ± 0.72 μg/mL, CV = 3.5%.

WITHIN-RUN PRECISION: Studies at the 8 and 22 mg/L levels showed 8.53 ± 0.54 mg/L, CV = 6.3% (N = 15) and 22.40 ± 1.12 mg/L, CV = 5.0% (N = 14) respectively (Table 8).

BETWEEN-RUN PRECISION: For 13 analyses of high and low controls run on different days during a 3-week period, between-run precision was 20.22 ± 0.61 mg/L, CV = 2.8% and 10.21 ± 0.64 mg/L, CV = 6.2%, respectively (Table 8).

ACCURACY AND RECOVERY: Using the 10 samples with known concentrations, GLC-NP vs. SPIKE showed r = 0.99, m = 1.06 and b = 0.29. The percent recovery ranged from 100 - 122%, averaging 108%.

Accuracy compared to the other methods was described by GLC-NP vs. RPLC (r = 0.99, m = 1.16, and b = -0.06), GLC-NP vs. EMIT (r = 0.99, m = 1.08 and b = 0.57) and EMIT-MOD vs. GLC (r = 0.99, m = 0.90 and b = -0.35) (Table 9).

PATIENT DATA: Comparative analyses on patient samples are described by GLC-NP vs. RPLC (r = 0.99, m = 1.08, b = 1.10, N = 60) (Table 10, Figure 14), GLC-NP vs. EMIT

(r = 0.97, m = 1.02, b = 0.73, N = 79) (Table 10, Figure 15), and EMIT®-MOD vs. GLC (r = 0.97, m = 0.99, b = -0.82, N = 100) (Table 10, Figure 16).

INTERFERENCES: No interferences were seen from phenobarbital, diphenylhydantoin, ethosuxamide, carbamazepine or primidone, which were in the control at therapeutic levels.

STABILITY OF THEOPHYLLINE IN FROZEN SPECIMENS

Because of the time required to complete all of these studies, several months elapsed between the evaluation of the initial EMIT method and the development of the modified method. The stability of theophylline in spiked and patient serum stored at -10°C for 11 months was investigated. For the 10 samples of known concentration, which had been stored under these conditions, thawed and reanalyzed by EMIT, EMIT (frozen) vs. EMIT (fresh) showed r - 0.99, m = 1.05, and b = -0.58. Thirty thawed patient samples were reanalyzed and compared with their earlier EMIT values (prior to freezing) resulting in EMIT (frozen) vs. EMIT (fresh) (r = 0.98, m = 1.05 and b = -0.32). Therefore, theophylline is stable and recoverable for periods of at least 11 months in frozen serum. Subsequently, frozen samples were thawed and used in developing and evaluating the modified EMIT procedure and the GLC-NP procedure.

METHODOLOGICAL DISCREPANCIES

There were 7 previously-described specimens by which

EMIT® and EMIT-MOD values varied from the RPLC reference value by greater or equal to 3 standard deviations. Each of these 7 specimens had GLC values that also disagreed with RPLC. Therefore, these 7 specimens had RPLC values that were contrary to all other three methods.

GLC-NP values confirmed EMIT-MOD values in 12 of the 14 specimens in which EMIT-MOD resolved disagreements between EMIT and RPLC. The other 2 specimens, however, had GLC-NP values that agreed with the initial EMIT method instead. Two other GLC-NP values differed from all three methods.

The EMIT-MOD method had only 1 specimen whose value differed from all three other methods.

Only grossly hemolyzed or lipemic samples gave variable results. One sample had such an elevated concentration of theophylline, it had to be diluted 1:2 with PBS prior to analysis, and results were variable from method to method (Table 11).

INTERFERENCE WITH RPLC

The medical records of the 7 patients whose reference values could not be confirmed by any other method, were examined for possible interferences with the RPLC method.

Four patients had RPLC values that were lower than all three other methods, while three patients had RPLC values that were higher than the other values for theophylline. The medical records listed medications each person was receiving on the day the serum theophylline specimens in ques-

tion was drawn. In only three cases was a uric acid value available (7.0, 7.5, 7.0 mg% for patients # 6645, 4723 and 306, respectively). All 7 patients received Aminophylline as well as two or more additional medications (Table 12).

The only pattern that emerged was that all four patients (# 2939, 4723, 6438 and 7357, Table 12) whose RPLC was lower with respect to the other three methods, received ampicillin, (2 - 4 g/day). The three patients (# 306, 6645 and 2482) whose RPLC values were higher, did not receive ampicillin.

AMPICILLIN INTERFERENCE

To test the possible interference by ampicillin, with the internal standard in the RPLC method, a serum specimen was obtained from a medical technologist currently taking 250 mg of ampicillin 4 times daily (1 g/day) with no other medication. This serum was run by EMIT®-MOD and RPLC, spiked with theophylline to approximately 20 mg/L and rerun by both methods. Also a serum sample (recently run by RPLC, 13 mg/L) was run by EMIT-MOD, spiked with ampicillin to 5 μg/mL and rerun by both methods. Five mg/L is within the therapeutic range for ampicillin (Jawetz et. al., 1974).

The serum with ampicillin only, showed negative results by both methods. After being spiked with theophylline, the results were 19 mg/L for RPLC and 18 mg/L for EMIT-MOD, respectively. The serum with theophylline showed 13 mg/L before and after being spiked with ampicillin (Table 13, Figure 17).

In order to determine whether ampicillin interferes with EMIT®-MOD, an aqueous solution of ampicillin (200 mg/L) was analyzed and none was detected. Various levels of ampicillin (5, 10, 20, 40, 80, 100 and 200 mg/L) were added to a serum specimen which had been tested and retested for theophylline (18 mg/L). Ampicillin did not interfere at any level.

The same aqueous sample of ampicillin was run twice by RPLC. One to two mg/L of some substance was detected at the relative retention time for theophylline. There were no obvious interfering peaks around the internal standard (Figure 18).

DISCUSSION

A certain protocol should be followed to properly evaluate analytical methods for use in the clinical laboratory. The clinical chemist must comprehensively assimilate, evaluate and compare current methodologies. This involves an extensive review of the literature, to justify adoption of a method and to establish a working procedure. Next, the necessary equipment and instrumentation must be thoroughly inspected. The appropriate standards are obtained to determine linearity and sensitivity. Acceptable variability and reproducibility, as well as accuracy and analytical recovery must be analyzed. After a significant number ($N > 20$) of patient samples are analyzed by the proposed and established methods (preferably by split-sample analysis), a statistical evaluation can be made. Potentially interfering substances should be examined. Only at this point can a conclusion be drawn as to the overall worth of the method.

Occasionally, when an innovative methodology first becomes accessible, it is over-zealously embraced for clinical use before a thorough evaluation is complete. Despite early reports that no interferences could be found with RPLC methods utilizing protein precipitation techniques, such interferences can present a real problem in the therapeutic monitoring of theophylline. This is of great realistic concern to the clinician, chemist and patient alike; as other drugs, especially antibiotics, are frequently co-administered to

patients with severe respiratory disease and concurrent infection. Such a patient could suffer from an erroneous dosing. If the interference were with the internal standard, leading to falsely decreased theophylline quantitation, the possible adverse toxic effects could be especially harmful, should the dosage be increased based on this information.

In these RPLC methods, protein precipitation or dilution is followed by vortex-mixing and centrifugation, after which an aliquot of the supernatant is directly injected into the chromatograph. This is in contrast to methods involving preliminary extraction of theophylline into an immiscible organic solvent(s) (usually chloroform and isopropanol), followed by evaporation and reconstitution.

The advantages of such a method are numerous. The solvents are easily prepared and obtainable, extraction is simple, preparation and analysis time are short. In addition, the results are precise, sensitive and fast. But these qualities are of no avail if interferences with theophylline or the internal standard lead to erroneous results.

As RPLC methods were adopted more for clinical use, several reports of interferences followed (Table 14). As early as 1977, Soldin and Hill reported an RPLC method using the μ-Bondapak C-18 column (Waters Associates), 8-chlorotheophylline (8-CT) as the internal standard, but no extraction prior to injection. They described interference by ampicillin and methicillin near the theophylline peak. After modifying the procedure by including extraction with a 95/5

mixture of chloroform/isopropanol, the interference by these antibiotics disappeared. They also changed the internal standard to β-hydroxypropyltheophylline, which is more hydrophobic, and elutes after theophylline in this sytem. They noticed no interference from other drugs including gentamycin, clindamycin, penicillin G, kanamycin, cephaloridine, tetracycline and erythromycin.

Kelly et. al. (1978) reported interference with theohylline analysis by the cephalosporin antibiotics, cefazolin and cepalothin. Proteins were precipitated by an equal volume of acetonitrile, containing 8-CT, then the supernatant was injected into a Whatman Partisil 10 ODS reverse phase column. Fixed wavelength UV detection was at 280 nm. Cefazolin showed a retention time extremely close to theophylline and the two were inadequately resolved when both were present in the sample. Cephalothin interfered by appearing at the same time as the internal standard.

Shortly thereafter, Robinson et. al. (1978) also reported cephalosporin interference in the RPLC method described by Orcutt et. al. (1977) (using β-hydroxyethyltheophylline as the internal standard), although it was generally recognizable on the chromatogram.

Robinson and Dobbs (1978) also reported acetazolamide interference with theophylline using this method.

Clark (1979) developed a procedure to eliminate acetazolamide interference by adjusting the serum to pH 8.5 with sodium phosphate buffer, then extracting with chloroform/

isopropanol (9/1). Following vortex-mixing and centrifugation, the aqueous layer is aspirated and dried under air. It is reconstituted with methanol and an aliquot is injected. Because acetazolamide (pKa = 7.2) is a stronger acid than theophylline (pKa = 8.8), it nearly completely ionizes at pH .6 and is not extracted. Theophylline is mostly at this pH and is extractable.

Sometimes, even when a simple extraction is performed, interfering substances can co-extract and co-elute with theophylline. This was reported by Bond and Thornton (1979) for some trisulfapyrimidines (sulfadiazine, sulfamerazine, and sulfamethazine) in an RPLC method based on Adams et. al. (1976) in which theophylline and dyphylline are extracted by a 4/1 mixture of dichloromethane/isopropanol. Detection was by dual-wavelength monitoring at 254 and 280 nm. Attempts to eliminate this interference by adjusting the pH prior to extraction failed, as sulfadiazine was extracted at both acidic and basic pH. When the percentage of acetonitrile in the eluent was increased from 7 - 10%, the sulfadiazine eluted after theophylline and the internal standard, but theophylline and dyphylline no longer were resolved. Most laboratories do not receive requests for dyphylline analysis, nor is it typically administered simultaneously with theophylline.

The potential for such interferences in RPLC methods can be decreased by modifying one or more steps. Although ease and speed would be somewhat compromised, the RPLC

system would still be highly advantageous because greater specificity would be gained. The analytical factors which would affect ampicillin and other drugs' ability to interfere are column packing, solvent strength, and pH, isolation procedures, internal standard and UV wavelength used.

In the clinical laboratory it is often impractical to test for drug interferences due to the overwhelming number of possibilities. There appears to be no chemical similarity among the substances which show interference with the theophylline RPLC method except for ampicillin and cephalosporins, which have closely related ring structures (Figure 19). The clinical chemist can avoid unnecessary interferences by carefully evaluating the proposed method with a large number of samples (at least 50 - 100) and comparing with another current method (e.g. GLC-NP, EMIT with centrifugal analyzer) and not an older or less reliable procedure (e.g. UV spectrophotometry, TLC).

By performing a split-sample analysis on a large number of samples, using four different methodologies, certain patterns emerged that might otherwise have gone unnoticed. Seven samples showed RPLC values that were consistantly different by all other methods. Of the 4 patients whose RPLC values for theophylline were lower than the other methods, all four were receiving ampicillin. Of the three patients whose theophylline values were higher by RPLC than by all other methods, none received ampicillin.

Unfortunately, our interference studies did not confirm

ampicillin as the cause of decreased theophylline values in these patients. The results were quite perplexing, implying that the ability of a drug such as ampicillin to interfere in RPLC methods involves many interacting factors.

In order to cause falsely decreased theophylline values by RPLC, ampicillin would have to interfere with the internal standard peak. An aqueous solution of ampicillin (200 mg/L) showed no interference with the internal standard, but did show a 1 - 2 mg/L peak at the retention time for theophylline (Figure 17) plus an unidentified peak where biological "debris" generally elutes.

Ampicillin (α-aminobenzylpenicillanic acid) is hydrolyzed in aqueous solution at a temperature-dependent rate to the corresponding penicilloic acid (Figure 20). This compound would not chromatograph under the same conditions as ampicillin in vivo. In aqueous solution of ampicillin was two weeks old at the time of this experiment and had been stored at room temperature for most of this time. Actually, ampicillin may retain most of its activity for only 10 - 14 days at 25°C after which it declines drastically (Lorian, 1976). If decomposition of our aqueous solution had occurred, the compounds would not exhibit the same retention characteristics by the RPLC method.

The serum sample (13 mg/L of theophylline) which was spiked with 5 mg/L of ampicillin also showed no interference by RPLC. There was, however, unexplained peak tailing of both theophylline and the internal standard in these chromat-

ograms (Figure 15). The serum was spiked with the aqueous ampicillin solution shortly after it was prepared, but two weeks passed before the sample could be analyzed by RPLC.

The medical technologist who received ampicillin (1 g/day), displayed a very small serum concentration. Usually 3 - 6 g/day are prescribed, resulting in a serum level of 2 - 8 mg/L (Jawetz et. al., 1974). Therefore, due to this small concentration, no interference with theophylline (after adding 20 mg/L) could be expected. Even if she had received a larger dose, the behavior of ampicillin patients who are concomitantly receiving theophylline may be quite different from a patient receiving ampicillin only.

This still does not explain why only four persons out of the entire sampline (N = 140) displayed lower serum RPLC values. It is likely that more than four were receiving ampicillin at the time the serum theophylline was drawn. Little is known of the factors which influence the plasma clearance and elimination of ampicillin (diseases, other drugs, etc.), or the relationship between the time of dosage of ampicillin and the time of collection of the specimen.

Ampicillin is well-absorbed by mouth. Peak levels appear in 1 - 3 hours and detectable amounts persist for about 6 hours. It is excreted for the most part unchanged (50 - 80%) in the urine mainly by the tubules. In uremia, the neonatal period and patients over 60 years old, prolonged high serum levels (e.g. 25 mg/L) are caused by impairment of renal function (Hugo and Russell, 1977). Perhaps a

similar phenomenon caused prolonged elevated serum levels in the four patients studied here, enabling ampicillin to be detected.

Little else is known of ampicillin's pharmacokinetic behavior, expecially the chemical composition of metabolites. This is because the determination of antimicrobial drugs in biological fluids generally involves microbiological assays for their activity as opposed to measuring their concentrations as chemical entities. Therefore it is impossible to know (at this time) what form(s) (parent vs. metabolite) can be detected by RPLC.

A reversed phase method has been developed for amoxycillin and ampicillin (Vree et. al., 1978) for the purpose of obtaining pharmacokinetic information to aid in therapy. In three normal volunteers after 1250 mg oral doses of ampicillin, plasma concentrations of up to 11 mg/L were obtained, and a half-life of 1.5 hours was reported. Only 15% of the oral dose was recovered as the parent compound in the urine. This might suggest that a metabolite of ampicillin is detectable by RPLC in addition to or in lieu of the parent compound.

In an unpublished method by Tsuji, a μ-Bondapak C-18 column with acetonitrile/ammonium acetate buffer as the mobile phase (pH 6.0) was used to detect ampicillin at 254 nm (Tsuji, 1978-79). These conditions are quite similar to those in our RPLC theophylline method, in which ampicillin was thought to intefere.

The marriage of EMIT® enzyme-immunoassay and its extreme specificity, sensitivity and simplicity, with the centrifugal fast analyzer's incomparable precision, accuracy and speed, provides a superlative method for quantitating serum theophylline. Only one obstacle prevents it from being ideal, namely cost. Although less technologist time is required with this method, as opposed to GLC or RPLC, the cost per test may not be competitive. By modifying the use of EMIT reagents, such that less are used, but optimal proportions as well as performance remain constant, the test approaches an ideal method. EMIT-MOD offers less expensive and more precise results.

In the manual test, the reaction mixture is monitored with a digital recording spectrophotometer at 340 nm, that is equipped with a thermally regulated flow-cell at 30°C. The manual procedure uses 50 μL each of Reagent A and B per test. As 6.0 mL of each reagent is supplied per kit (approximately $175.00 per kit), this provides for 120 determinations. The cost of analysis per sample depends on the number of samples assayed with each standard curve, but has been estimated (Koup and Brodsky, 1978) as $4.00 per test minimally. This does not compare favorably with RPLC or GLC in which the cost may be $.50 or less per sample. Syva recommends running both the zero calibrator and all unknowns in duplicate, which further increases the cost.

The method for the Centrifichem 400® initially called for Reagent A to be diluted 1:3 with buffer, after which

100 μL was used per test. Working Reagent B was a 1:10 dilution of Reagent B, 350 μL of which was used per test. This results in actual consumption of 33 μL of Reagent A and 35 μL of Reagent B per test; or 171 tests per kit, which is already an increase from the manual method, but still rather expensive.

The objectives in trying to modify this method were to further decrease the amount of reagents and to simplify the assay.

The original Centrifichem® method called for a ratio of Reagents A to B of 0.9514 (33.3/35). This ratio reflected an optimal ratio, as A and B are standardized against each other. This ratio was adhered to in the modified method. Twenty μL of Reagent A was chosen as a possibility to be conveniently added to the sample well without prior dilution, using a micropipette. The direct pipetting eliminates error in dilution and also decreases the volume from 100 to 20 μL, with much less chance of splashing the antibody solution into the reagent well inadvertantly. In order to keep the A/B ratio constant, 21 μL of Reagent B was necessary per test. This was achieved by diluting Reagent B with buffer (0.6:10) and using 350 μL per test. This provides for 285 tests per kit, which is a substantial increase over the initial Centrifichem procedure. It also makes the cost competitive with other methods.

Besides a much lower cost, the EMIT®-MOD assay provides greater precision than the EMIT assay. Coefficients of vari-

ation for between-run precision dropped from around 13% (EMIT®) to 5% (EMIT-MOD). Three factors contributed to improved precision: less diluting of reagents, direct pipetting of Reagent A, and a more linear standard curve.

In the EMIT-MOD assay, only Reagent B is further diluted to use as a working reagent. By not diluting Reagent A, some variability is eliminated. Because Reagent A was diluted 1:3, it was consumed much faster than Reagent B and had to be diluted more frequently. Furthermore, direct pipetting of 20 μL of Reagent A allows more control in keeping all the antibody solution contained in its well prior to mixing in the rotor.

A standard curve was run, allowing printouts of ΔT every fifteen seconds, from 15s to 135s. At ΔT = 60s the shape of the curve became much more linear, while by 75s a loss of linearity was seen on the graph paper provided by Syva.

Forty additional specimens were run with ΔT extended to 60s. Results of the comparison of EMIT vs. RPLC for these specimens were r = 0.950, m = 0.929, and b = 0.592. Although the correlation did not improve by increasing ΔT to 60s, per se, the increased linearity of the curve insures more consistant and reliable results. Sixty seconds was also used for ΔT in the EMIT-MOD assay. Because absolute absorbance decreases as the amounts of reagents decrease, so does the difference (ΔA) between calibrators. Anything lower than 20 μL of Reagent A and 21 μL of Reagent B results in

absorbances too low to establish a standard curve at $\Delta T = 60s$.

A problem still remaining is the need to prepare Working Reagent B every three days, and to let it equilibrate at room temperature for 4 hours or overnight at 2 - 8°C; especially if emergency levels are requested.

The stability of Reagent B was studied. Two standard curves including controls were run using Working Reagent B that was 5 days old. The absolute absorbances were considerably lower than those using fresh Working Reagent B (same lot) but a linear standard curve was obtained and the controls were within their limits, indicating the reagent was still usable.

Although EMIT®-MOD appeared more precise and less costly, the question of accuracy needed to be resolved, as RPLC, EMIT, and EMIT-MOD did not always agree. Our GLC-NP method provided the necessary solution to this question and another alternative to compare with EMIT-MOD.

For laboratories experienced in gas chromatography, nitrogen-phosphorus detection provides a method that is competitive with both EMIT-MOD and RPLC. The N-P detector offers unique advantages in specificity and sensitivity over the standard flame ionization detector.

Optimizing the chromatographic conditions using the N-P detector is a fine art, that can only be achieved through much trial and error. The most important idea is that once chromatographic conditions are established, the equipment should be left as is, with gases flowing, voltage on and

detector temperature maintained. According to Hewlett-Packard, this does not destroy the alkali salt faster than if the apparatus were continually turned on and off. Adjustments must still be made every day. This is the only practical way to use this detector, as the chromatograph takes several hours to stabilize after it is turned on, although good chromatograms were not often obtainable for the first 24 - 36 hours.

Factors which affect stability and detector life are voltage, carrier gas, flow rates, temperature and column material. Two processes reduce collector lifetime: 1) irreversible loss of alkali material by overheating the collector or continually using a higher than necessary applied voltage, and 2) chemical coating of the surface of the alkali source, usually by silicon dioxide from column bleed (bente, 1977a).

Solvents can also present a problem. Although the N-P detector has a negligible response to most solvents (no solvent front), purity can be a problem as the detector is so sensitive to nitrogen or phosphorus impurities. Chlorinated solvents cause a reversible sensitivity loss and should be avoided if possible.

The most common problem with N-P detection is contamination from such seemingly innocuous substances as laboratory air contaminants, phosphate-containing detergents, H_3PO_4-treated columns or glass wool, Snoop® leak detector, cyano-substituted silicone columns (OV-225, XE 60), septums,

fingerprints and facial tissues (Bente, 1977a). When this occurs the emission current and hence sensitivity is reduced and the collector and jet must be cleaned.

Another potential problem is the interference of tributoxyethyl-phosphate, a placticizer found in butyl rubber stoppers of evacuated blood sampling tubes. Chrzanowski et. al., (1976) reported an unidentified substance that interfered with theophylline peaks on GLC-FID chromatograms. In vitro tests, using distilled water resulted in apparent theophylline levels as high as 40 - 53 mg/L after 60 minutes contact time with the rubber stopper, irrespective of whether an anticoagulant was used or which one. Others report of positive and negative interference by CORVAC serum separator tubes with GLC analysis (ASCP summary reports, 1978). The peak was found and identified by Least et. al., (1976) in serum collected Vacutainer® tubes, but did not interfere with theophylline or the internal standard, using N-P detection. The exact nature of this interference will vary depending on the choice of extraction, derivitization, internal standard and detection. Although there is great potential of a phosphate compound interfering with N-P detection, tributoxyethylphosphate did not interfere in the extractive procedure. This procedure for extraction and simultaneous derivitization surpasses any other method for preparing theophylline derivatives for GLC.

Prior to initiating this method, we tried an off-column pentylation (Least et. al., 1976) involving two centrifuga-

tion steps and two evaporation steps. The extra steps did not produce better results. The precipitate from the second centrifugation (the trialkyl ammonium iodide) did not form a compact pellet. Subsequently some of this material was aspirated along with the supernatant containing theophylline and injected into the column. This is exactly what prederivitization is supposed to avoid.

In contrast, the extractive pentylation was simple, inexpensive and produced excellent derivatives for GLC-NP analysis. Because of the large pentyl groups, closely related compounds can be resolved. Drugs such as cephalosporins, ampicillin and trisulfapyrimidines should not interfere in this method due to the extraction step, but if they did co-extract with theophylline, the pentylation would resolve them. No indications of antibiotic interference with GLC-FID or GLC-NP have been reported.

Depending on the needs and facilities of the clinical laboratory, any of the three methods (GLC-NP, RPLC, EMIT®-MOD) could provide reliable results on small volumes of serum.

GLC-NP offers a lower detection limit, good specificity, a sophisticated detection system and inexpensive reagent costs, in a relatively short time (about 30 - 40 minutes sample preparation time and 8 minutes per sample chromatography time). If a Hewlett-Packard 5840A gas chromatograph is used, results are quantitated rapidly by integration. Disadvantages are that equipment is expensive, flammable

gases are used at high temperatures, and the N-P detector takes time and patience to master. This method would be most advantageous in a laboratory performing dozens of GLC-NP analyses daily.

RPLC offers excellent linearity, sensitivity and specificity if an extraction step and the proper chromatographic conditions are used. Reagents are even less expensive and safer than GLC-NP, sample preparation is fairly fast and simple. The two major drawbacks are the pump and detector. The superb resolving capacity of the column compensates for UV spectrophotometry, which is inferior to GLC-NP detection systems. Because the apparatus is complex and expensive and there is such a wide range of control of chromatographic conditions, RPLC requires a highly-skilled operator. Both RPLC and GLC-NP are excellent for research and clinical studies because of the chromatogram itself, a permanent picture of components in the patient's serum.

For emergency and therapeutic monitoring of serum theophylline, the EMIT®-MOD assay on the Centrifichem 400® is the method of choice. It offers excellent sensitivity and specificity in only a few minutes. It is convenient to use, requires no special training and is relatively inexpensive. For laboratories that have a centrifugal analyzer, EMIT-MOD offers advantages not possible with other methods.

CONCLUSIONS AND SUGGESTIONS

Theophylline is indispensible in the treatment of bronchial asthma and other cardiorespiratory diseases in adults, as well as management of apnea and bradycardic spells in premature infants. Due to factors such as age, weight, diet, smoking, diseases and other drugs, the absorption and plasma clearance vary considerably from patient to patient, even when the same unit dosage is administered. This leads to unpredictable plasma levels. Because both efficacy and toxicity are related to plasma theophylline concentration (as opposed to dosage), the judicious clinical use of this drug is greatly facilitated by careful monitoring of serum levels. The therapeutic range is 10 - 20 mg/L. Toxic effects including mild to severe gastrointestinal and neurological disorders generally occur outside this range.

A sensitive and specific analytical method for the quantitation of theophylline is essential for following the pharmacokinetics in the body and for effectively controlling administration of the drug. Many methods have been described for monitoring serum theophylline, therefore choosing a suitable procedure can be quite perplexing. To judge the overall worth of a method, thorough evaluations regarding precision, accuracy, linearity, sensitivity and specificity must be available, as well as comparative analysis with other current methods.

Of the six basic methodologies which might be con-

sidered for theophylline determinations, only three fulfill the highest standards of analytical chemistry, namely, the EMIT® assay with the centrifugal fast analyzer, reversed phase liquid chromatography, and gas liquid chromatography with nitrogen-phosphorus detection and extractive alkylation. Statistical analyses show that the three are comparable and show excellent correlation with each other.

Of the other three methods, ultraviolet spectrophotometry is too cumbersome and nonspecific. Thin-layer chromatography is relatively long and undeveloped. Radioimmunoassay is expensive and involves an unnecessary hazard.

The EMIT assay on the Centrifichem 400® represents the epitomy of simplicity, sensitivity and specificity combined with speed, precision, and accuracy. The modified EMIT assay (EMIT-MOD) decreases reagent consumption such that this assay becomes competitive in cost with RPLC and GLC-NP. In addition, precision is improved and time is saved. The EMIT-MOD assay is reliable and affordable and may be of special interest to laboratories with a centrifugal fast analyzer, who wish to monitor serum theophylline but have previously lacked the analytical capability to do so.

The one drawback is the stability versus time problem of Working Reagent B, especially if emergency testing should be necessary. Once diluted, it does not equilibrate for 4 hours at room temperature or overnight at 2 - 8°C. Perhaps this could be achieved faster at a slightly warmer temperature. After 5 days storage, this working reagent results in

lower absorbances, but could probably be used more successfully after day 3 if the reaction time (T) were increased to 75 seconds.

Both chromatographic methods are sensitive and specific, as well as providing visual documentation of theophylline and other compounds in the plasma, which is valuable in pharmacokinetic studies.

Users of recent RPLC methods involving protein precipitation prior to injection have reported several incidences of interfering drugs which are not removed by this procedure. This could be resolved by one or more modifications including 1) performing a simple extraction prior to injection, 2) adjusting the pH of the extraction fluid, 3) changing the internal standard, or 4) varying solvent strength. Although this would add time and effort, the RPLC methods would still offer the advantages of simple preparation and injection at ambient temperature.

GLC quantitation has temporarily deferred to RPLC methods for theophylline, but with the advent of the N-P detector, these methods have once again become competitive for ease, sensitivity and specificity. This detector requires special attention and effort, but if kept stable, the results are excellent. It should only be considered in laboratories experienced in gas chromatography and analyzing a large number of procedures and specimens using GLC-NP.

Extractive alkylation has been overlooked in clinical procedures until recently. It offers great ease and speed

over other extraction and derivitization procedures, at a lower cost and with less harm to column packings.

Recent enthusiasm for RPLC methods over EMIT® and certainly over GLC, has been slightly hampered by reports of interferences due to incomplete isolation of theophylline from other drugs. This was evidenced by patient samples in this study whose RPLC value was significantly inaccurate in the accompaniment of ampicillin with theophylline therapy (though not substantiated by *in vitro* experimental studies). More studies are necessary to resolve the kinetics of ampicillin and its potential interference in RPLC methods.

Consequently, there is no perfect method for theophylline quantitation, but with careful evaluation, comparison and some ingenuity, the best of innovative, new techniques, and proven, yet flexible, established procedures together can provide several excellent alternatives.

Dosage form	Brand name	Manufacturer	Theophylline content (strength)
Tablet	Slo-Phyllin	Dooner	200 mg scored
			100 mg scored
	Theophyl-225	Knoll	225 mg scored
	Theolair	Riker	250 mg scored
			125 mg scored
Liquid-filled capsule	Somophyllin	Fisons	250 mg
			200 mg
			100 mg
Oral liquid	Elixophyllin pediatric suspension	Cooper	20 mg/mL
	Somophyllin syrup	Fisons	18 mg/mL
	Slo-Phyllin 150 syrup	Dooner	10 mg/mL
	Theophyl-225	Knoll	7.5 mg/mL
	Quibron elixir	Mead-Johnson	10 mg/mL
	Elixophyllin elixir	Cooper	5.3 mg/mL
Sustained release	Slo-Phyllin Gyrocaps	Dooner	250 mg
			125 mg
			60 mg
	Theo-Dur	Key	300 mg scored
			100 mg scored
Rectal solution	Somophyllin	Fisons	50 mg/mL
Intravenous solution	Aminophyllin USP	Searle	21 mg/mL

Table 1. Formulations of various theophylline preparations (adapted from J. Weinberg, J. Ped., Jan. 1978).

No. subj.	Population	Binding & (mean±SD)	Method	Reference
7	Healthy adults	59.0 ± 2.8	Equilibrium dialysis spectrophotometry	Koysooko et al. (1974)
21	Healthy adults	56.4 ± 3.8	Ultrafiltration ^{14}C-theophylline	Aranda et al. (1976)
21	Full term newborn	36.4 ± 3.8		
4	Nursing mothers	54.0 ± 5.7	Ultrafiltration HPLC	Yurchak and Jusko (1976)
4	Healthy adults	52.6 ± 3.8	Ultrafiltration ^{14}C-theophylline	Piafsky et al. (1977b)
57	Healthy adults	65.0 ± 6.0	Ultrafiltration HPLC	Mangione et al. (1978)
3	Hepatic cirrhosis	36.8 ± 4.1	Ultrafiltration ^{14}C-theophylline	Piafsky (1977b)
8	Hepatic cirrhosis	29.0 ± 16.0	Ultrafiltration HPLC	Mangionne et al. (1978)

Table 2. The binding of theophylline to human plasma proteins (theophylline concentration 10-20 mg/L at 37°C) (adapted from Ogilvie, Clin. Pharmacokinetics, 1978).

Method	S/P ratio	Dose	Samples	Investigator(s)
UV	0.52 ± 0.03	Single Dose	7	Koysooko et al. (1974)
UV	0.58	Steady State*	16	Levy et al. (1974)
GLC	0.49 ± 0.04	Single Dose	4	Shah & Riegelman (1974)
GLC	0.65	Steady State*	22	Johnson et al. (1975)
HPLC	0.67	Steady State*	12	Galant et al. (1977)

Table 3. Reported saliva/plasma (S/P) ratios for theophylline

Peak theophylline level (g/ml)	Approximate adjustment in total daily dose	Comment
5	100% increase	If patient is asymptomatic, consider trial off drug; repeat serum levels after adjustment
5-7.5	50% increase	
8-10	20% increase	Even if patient is asymptomatic at this level, an increased serum concentration may prevent symptoms during a viral URI, or heavy exposure to an inhalant allergen
11-13	Cautious 10% increase if clinically indicated	If patient is asymptomatic, no increase is necessary. If symptoms present during URI or exercise, increase as indicated
14-20	None	If 'breakthrough' in asthmatic symptoms present at end of dosing interval, change to sustained release product and repeat serum level
	Occasional intolerance requires 10% decrease	If side effects present, decrease total daily dose as indicated
21-25	10% decrease	Even if side effects are absent
26-30	25% decrease	Even if side effects are absent, omit next dose and decrease total daily dose as indicated. Repeat serum levels
31-35	33% decrease	
35	50% decrease	Omit next 2 doses, decrease as indicated and repeat serum levels

Table 4. Dose adjustment guided by serum concentration (adapted from Hendeles et al.,1978b).

	HPLC	GLC
SAMPLE-Nature	Liquid; soluble, nonionizable at acidic-neutral pH	Volatilizable: Gas, liquid or solid
Molecular weight	>100.....several million	<500
Preparation	protein precipitation or simple extraction; No derivitization	Solvent extractions; Derivitization
INTRODUCTION TO COLUMN	Injection system & high pressure pump which must pump solvent evenly	Directly through septum by syringe; affected by cleanliness & temp.
COLUMN-Stationary Phase	Prepacked, e.g. -Bondapak, C-18, octadecylsilica	Prepacked, e.g. 3% OV-17, 50% methyl, phenyl silicone on Gas Chrom Q
Mobile Phase	Polar (often aqueous) pH 2-7.5 only	Pure, inert N_2, He or H_2 gas; Flammable
CONTROL OF CHROMATOGRAPHIC CONDITIONS	Solvent strength Flow rate Temperature	Flow rate Temperature
THEORETICAL PLATES	100,000	300,000
DETECTION	UV Fluorescence	Thermal conductivity Flame ionization Electron capture Nitrogen-Phosphorus Mass spectrometry
SENSITIVITY	10^{-10}-10^{-12}g	10^{-12}g

Table 5. Comparison of HPLC and GLC

Investigator(s)	Sample (mL)	Isolation Procedure	Int. Std.	Column	Mobile Phase (1° solvent)	UV (nm)/ Quant.
*Manion et al. (1974)	1.0	extract $CHCl_3$/IPA	Oxaze-pam	Opn/PC	n-hexane	273/H
Sitar et al. (1975)	0.5	extract $CHCl_3$/IPA	None	V-Si 10	$CHCl_3$	273/S
Evenson & Warren (1976)	0.2	extract $CHCl_3$/IPA	BHPT	D-Zorbak-Sil	heptane/ acetate	254/S
Franconi et al. (1976)	1.0	membrane filter	None	W-PC 18	SAB	254/S
*Weddle & Mason (1976)	0.5	$(NH_4)_2SO_4$ ppt.	None	W-μPora-sil	hexane	280/S
Adams et al. (1976)	0.05	extract $CHcl_3$/IPA	8-CT	PE ODS-Sil-X-I	H_2O	273/H
Hill (1977)	0.05	extract $CHCl_3$	8-CT	W μ-Bon-dapak C18	H_2O	280/A
Soldin & Hill (1977)	0.025	extract $CHCl_3$	BHPT	W μ-Bon-dapak C18	SAB	254/A
Cooper et al. (1977)	0.1	cold per-chloric acid ppt.	None	W μ-Bon-dapak C18	MeOH	280/S

Table 6. HPLC methods for theophylline (continued on next page).

Investigator(s)	Sample (mL)	Isolation Procedure	Int. Std.	Column	Mobile Phase (1° solvent)	UV (nm)/ Quant.
Nelson et al. (1977)	0.05	CH_3CN ppt.	8-CT	Wh. Partisil 10 ODS	CH_3CN	280/H
Orcutt et al.	0.03	MeOH ppt.	BHET	W μ-Bondapak C18	SAB	254/H
Peng et al. (1978)	0.10	CH_3CN ppt.	None	W μ-Bondapak C18	H_2O	275/S
Petzold & Sood (1978)	0.03	CH_3CN ppt.	8-CT	W μ-Bondapak C18	SAB	254/H
Bond & Thornton (1979)	0.1	extract CH_2Cl_2/IPA	BHET	W μ-Bondapak C18	SAB	254 & 280 dual

* = Normal phase (others are reversed phase)
8-CT = 8-chlorotheophylline
BHPT = β-hydroxypropyltheophylline
BHET = β-hydroxyethyltheophylline
Opn/PC = oxoproprionitrile on Porosile
V = Varian
D = Dupont
W = Waters
PE = Perkin Elmer
Wh = Whatman
SAB = sodium acetate buffer
H = peak height ratios
S = standard curve
A = peak areas

Table 6. HPLC methods for theophylline (cont.)

Investigator(s)	Sample (mL)	Derivi-tization	Internal Standard	Column	Detec-tion
Koblansky et al.(1973)	1.0	On/Bu	None	17/GCQ	FID
Chrzanowski et al.(1974)	3.0	None	Pramoxine HCL	17/CWHP	FID
Shah & Riegelman (1974)	1.0	On/Pr	Thiobarbital	17/GCQ	FID
Arbin & Edlund (1975)	0.1	Off/Pfb	Theobromine	60/GCQ	EC
Dusci et al. (1975)	2.0	On/Me	Medazepam	225/HP	FID
Johnson et al.(1975)	1.0	On/Bu & Off/Bu	Alphenal & Aprobarbital	2259/Su	FID
Perrier & Lear (1976)	0.1	On/Bu	Amobarbital	17/CG	FID
Bailey et al.(1976)	2.0	On/Bu	IBMX	30/CWHP	FID
Schwertner et al.(1976)	1.0	Off/Pfb	IBMX	17/GCQ	EC
Least et al.(1976)	0.02	Off/Pe	IBMX	17/GCQ	FID
Sheehan & Haythorn (1976)	2.0	None	Cyheptamide	1/CWHP	FID
Abraham et al.(1977)	0.1	On/Et	MPPH	2250/Su	FID
Sheehan et al.(1977)	0.2	Off/Bu	IBMX	17/CWHP	MS
Lowry et al.(1977)	0.05	Off/Pe	IBMX	17/GCQ	NP
Joern (1978)	0.025	Ext/Pe	IBMX	17/GCQ	NP
Vinet & Zizian (1979)	0.1	On/Bu	IBMX	17,tpa/C-750	NP

(cont.)

Investigator(s)	Sample (mL)	Derivi-tization	Internal Standard	Column	Detec-tion
Schwertner (1979)	1.0	None	IBMX	2510/Su	FID

Off = off-column
On = on-column
Bu = Butylation
Me = methylation
Pr = Propylation
Pe = Pentylation
Pfb = pentafluorobenzylation
Ext = Extractive
IBMX = 3-isobutyl-1-methyl-xanthine

17 = 3% OV-17
60 = 3% XE-60
30 = 10% SE-30
1 = 3% OV-1
225 = 3% OV-225
2250 = 3% SP-2250
2510 = 2% SP-2510-0A

tpa = 1% terephthalic acid
GCQ = Gas Chrom Q
CWHP = Chromosorb WHP
HP = Chromosorb W
CG = Chromosorb G
C-750 = Chromosorb 750
Su = Supelcoport
FID = flame ionization detection
EC = electron capture
MS = mass spectrometry
NP = nitrogen-phosphorus

Table 7. GLC methods for theophylline.

Within-Run	$\bar{X}$ (μg/mL)	S.D. (μg/mL)	C.V.	N
EMIT	9.56	0.35	3.6	10
EMIT-MOD	8.80	0.25	2.8	10
	18.35	0.73	4.0	10
GLC-NP	8.53	0.54	6.3	15
	22.40	1.12	5.0	14
RPLC	15.40	0.56	3.6	10
Between-Run				
RPLC	15.70	0.75	4.7	5
EMIT	7.85	1.01	12.8	12
	26.20	3.54	13.5	8
EMIT-MOD	10.56	0.69	4.5	8
	19.84	0.99	5.0	8
GLC-NP	10.21	0.64	6.3	13
	20.22	0.61	3.0	13

Table 8. Precision studies.

Recovery	r	m	b	N	Average Recovery (%)
RPLC vs. "SPIKE"	0.99	0.90	0.68	10	100
EMIT vs. "SPIKE"	0.99	0.92	0.40	10	98
EMIT-MOD vs. "SPIKE"	0.99	0.90	0.42	10	97
GLC-NP vs. "SPIKE"	0.99	1.06	0.29	10	108
EMIT (frozen) vs. "SPIKE"	0.99	0.97	-0.18	10	97

Accuracy	r	m	b	N
EMIT vs. RPLC	0.99	1.05	-0.33	10
EMIT-MOD vs. RPLC	0.99	1.04	-0.55	10
GLC-NP vs. RPLC	0.99	1.16	-0.06	10
EMIT-MOD vs. EMIT	0.99	1.01	-0.32	10
EMIT-MOD vs. GLC	0.99	0.90	-0.35	10
GLC-NP vs. EMIT	0.99	1.08	0.57	10
EMIT (frozen) vs. EMIT (fresh)	0.99	1.05	-0.58	10

Table 9. Recovery and accuracy studies.

Method	r	m	b	N
EMIT vs. RPLC (best-fit curve)	0.94	0.89	-1.26	100
EMIT vs. RPLC (smooth curve)	0.96	0.91	-0.66	100
EMIT vs. RPLC	0.95	0.93	-0.59	40
EMIT-MOD vs. RPLC	0.98	1.05	-0.39	60
EMIT-MOD vs. EMIT	0.97	0.99	-0.41	91
EMIT-MOD vs. GLC-NP	0.97	0.99	-0.82	100
GLC-NP vs. RPLC	0.99	1.08	1.10	60
GLC-NP vs. EMIT	0.97	1.02	0.73	79
EMIT (frozen) vs. EMIT (fresh	0.98	1.05	-0.32	30

Table 10. Comparative analyses using patient serum.

Sample #	Description	RPLC (μg/mL)	EMIT (μg/mL)	EMIT-MOD (μg/mL)	GLC-NP (μg/mL)
4601	Grossly hemolyzed	31	27	34	35
6201	Grossly hemolyzed	30	27	21	18
5059	Severely lipemic	14	16	21	19
1254	Extremely elevated concentration	72	>80	100	>80

Table 11. Samples showing variable results by different methods of theophylline quantitation.

Patient	Diagnosis	RPLC μg/mL	GLC μg/mL	EMIT μg/mL	EMIT-MOD μg/mL	Medications
306	Emphysema/ bronchitis	23	18	18	19	Aminophylline Alupent inhaler Bronchobids
6645	Steriod-dependent asthma	16	12	10	12	Aminophylline Terbutalene Prednisone Methylprednisolone Alupent inhaler
2482	Chronic asthma	23	18	15	19	Aminophylline Metaproterinol Aldomet Beclamethasone Prednisolone Isupril inhaler
+ 2939	Chronic asthma	13	17	19	18	Aminophylline Prednisone Terbutalene Alupent inhaler *Ampicillin
+ 4723	Extrinsic allergic asthma	21	25	26	27	Aminophylline Terbutalene Isupril mist *Ampicillin
+ 6438	Chronic asthma	16	21	21	20	Aminophylline Terbutalene Methylprednisolone Bronkosol Iron sulfate Beclomethasone *Ampicillin
+ 7357	Asthma	16	19	20	22	Aminophylline Atorax Terbutalene Alupent Prednisone Norinyl *Ampicillin

+ = patients whose HPLC was lower than other methods

Table 12. Information from medical records of patients whose serum theophylline shows consistant discrepancy with RPLC when analyzed by three other methods.

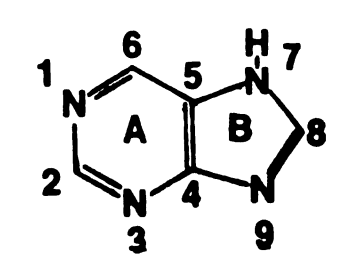
Sample Description	RPLC (μg/mL)	EMIT-MOD (μg/mL)
1. Serum with endogenous ampicillin (1 g/day = dose)	0	0
2. Serum #1 with 20 μg/mL theophylline added	19	18
3. Serum with theophylline	13	13
4. Serum #3 with 5 μg/mL ampicillin added	13	13
5. Aqueous solution of ampicillin 200 μg/mL	1.2	0

Table 13. Results of ampicillin interference study.

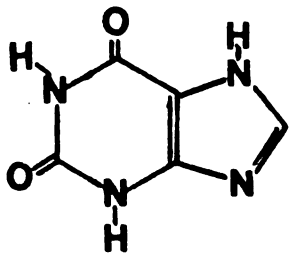
Investigator(s)	Interfering Drugs (location)	Pre-column Preparation	Int. Std.	Column	Mobile Phase	UV (nm)
Soldin & Hill (1977)	Ampicillin Methicillin (Theoph.)	Molecular filtration	8-CT	μ-Bondapak C-18 (Waters Assoc.)	90/10 NAB/CH_3CN 20 mM/L pH 4.0	254
Robinson et al. (1978)	Cephalothin (Int. Std.)	CH_3CN ppt.	BHET	μ-Bondapak C-18 (Waters Assoc.)	7/93 CH_3CN/NAB 10 mM/L pH 4.0	254
Kelly et al. (1978)	Cephalothin (Int. Std.) Cefazolin (Theoph.)	CH_3CN ppt.	8-CT	Partisil 10 ODS (Whatman)	90/10 NAB/CH_3CN 0.1 M pH 4.0	280
Robinson & Dobbs (1978)	Acetazolamide (Theoph.)	CH_3CN ppt.	BHET	μ-Bondapak C-18 (Waters Assoc.)	7/93 CH_3CN/NAB 10 mM/L pH 4.0	254
Bond & Thornton	Trisulfapyrimidines (Theoph.)	CH_2Cl_2/IPA 4/1 extraction	BHET	μ-Bondapak C-18 (Waters Assoc.)	7/93 CH_3CN/NAB 10 mM/L pH 4.0	254/ 280

CH_3CN ppt. = acetonitrile precipitation
8-CT = 8-chlorotheophylline
BHET = -hydroxyethyltheophylline
SAB = sodium acetate buffer
CH_2Cl_2 = dichloromethane
IPA = isopropanol
Theoph. = theophylline
Int. Std. = internal standard

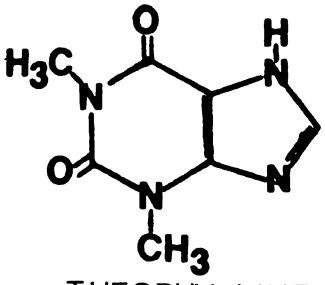
Table 14. Reported interferences in RPLC methods.



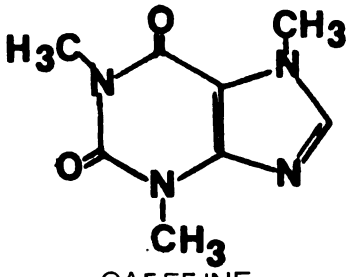
PURINE



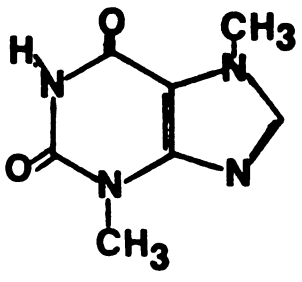
XANTHINE



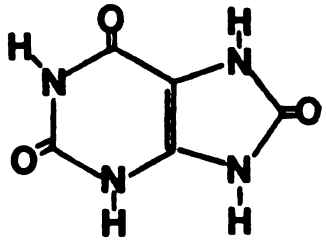
THEOPHYLLINE



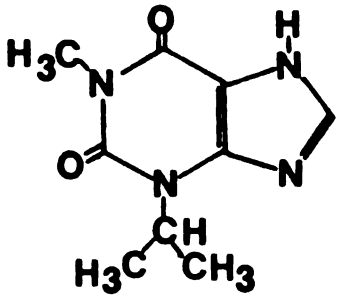
CAFFEINE



THEOBROMINE



URIC ACID



3- ISOBUTYL-1 -METHYLXANTHINE

Figure 1. Structures of important xanthine derivatives.

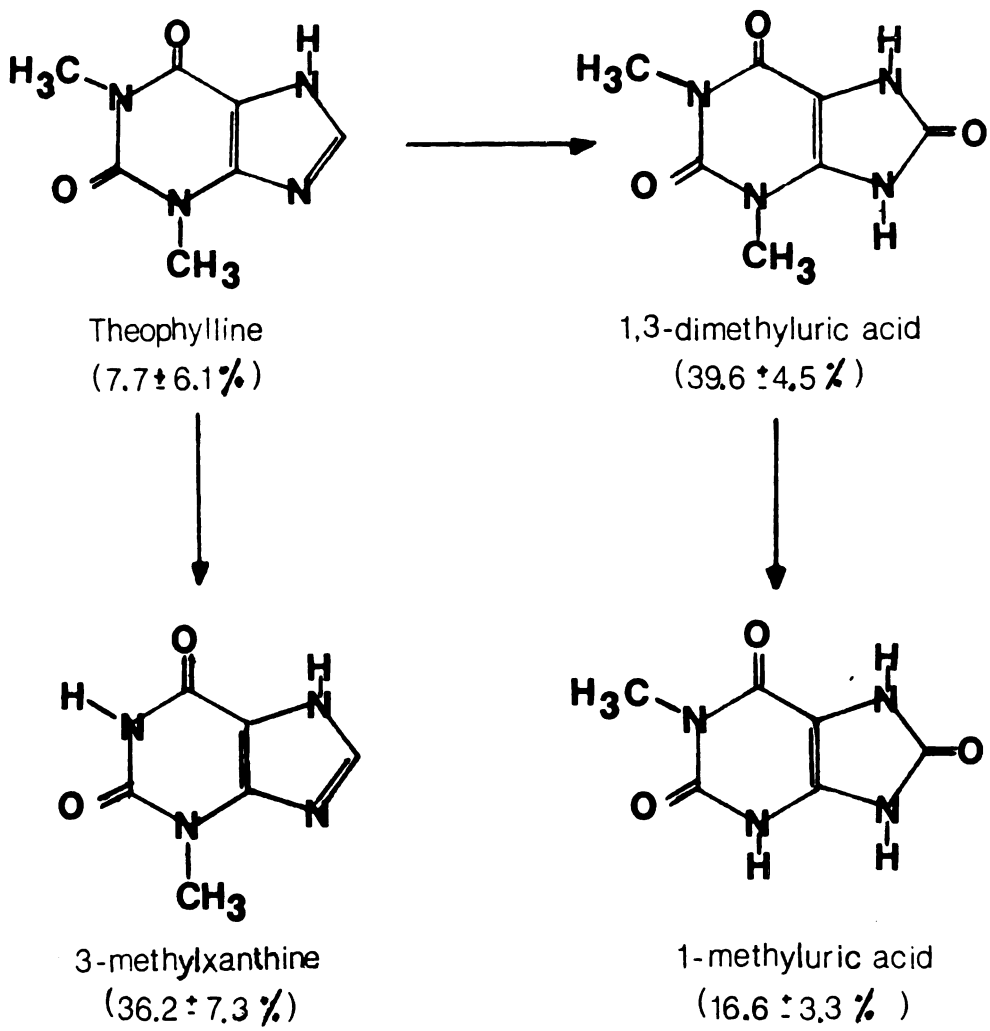


Figure 2. Proposed pathways for theophylline metabolism in man(adapted from Ritchie, 1975).

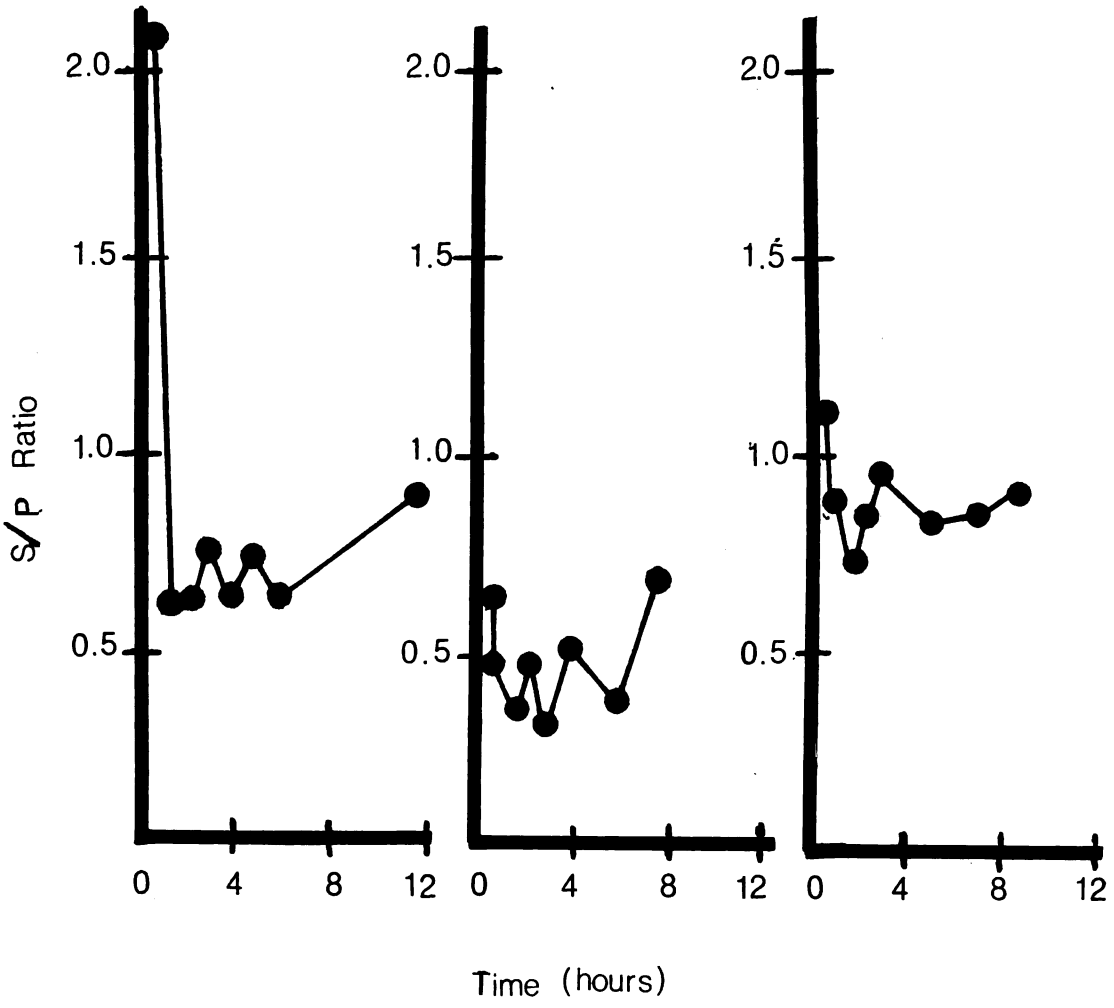
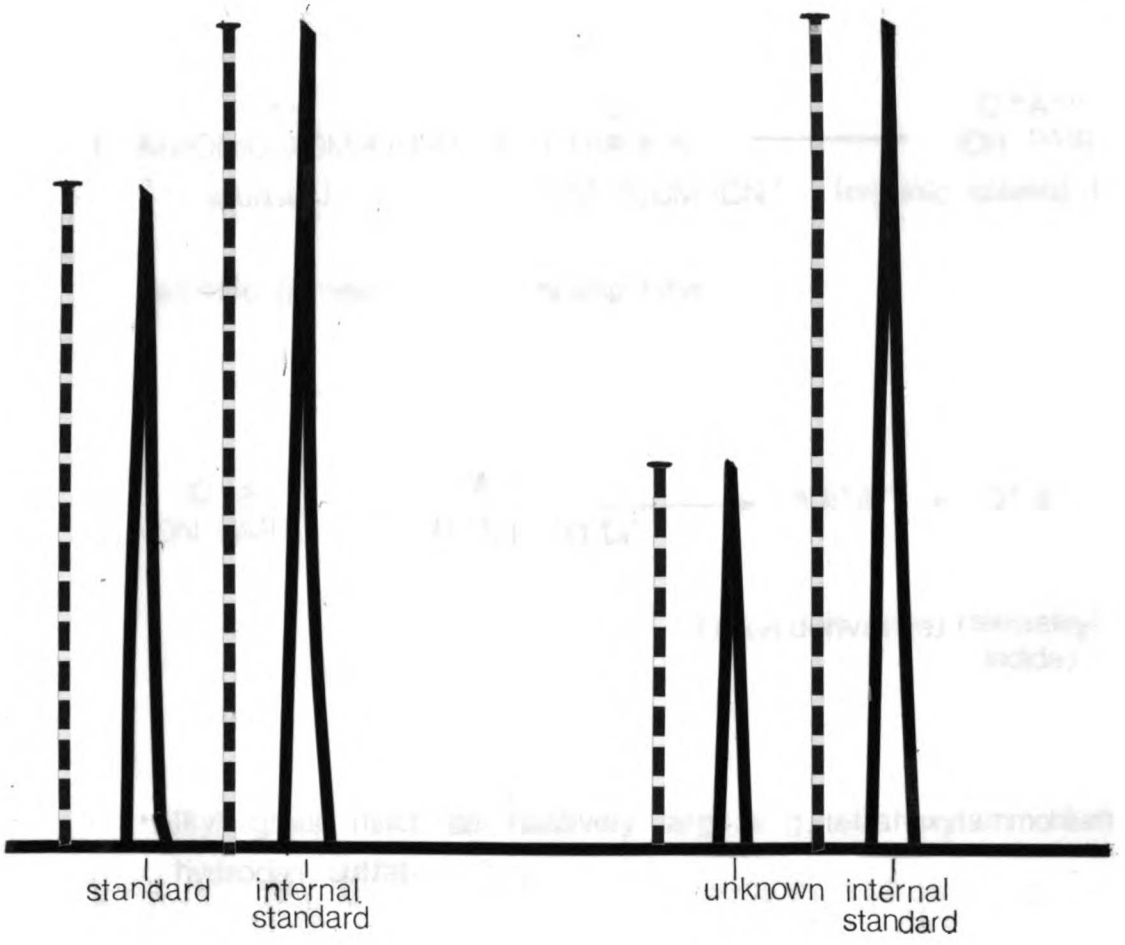


Figure 3. The saliva/plasma (S/P) ratio versus time after administration of an oral aqueous solution and two rectal formulations to one subject (adapted from Danhof & Breimer, 1978).



$$\frac{\dfrac{\text{peak height unknown}}{\text{peak height internal standard}}}{\dfrac{\text{peak height standard}}{\text{peak height internal standard}}} \times \text{concentration standard} = \text{concentration unknown}$$

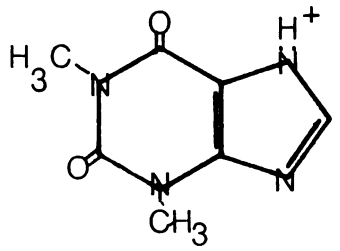
Figure 4. Schematic diagram showing quantitation by peak height ratios.

1. A^- ANIONIC COMPOUND (aqueous) + Q^+ TETRAALKYL-AMMONIUM ION* ⟶ Q^+A^- ION PAIR (organic solvent)

anionic nitrogen strong base

2. Q^+A^- ION PAIR + Alk^+I^- ALKYL IODIDE ⟶ Alk^+A^- (alkyl derivative) + Q^+A^- (tetraalkyl iodide)

*Alkyl group must be relatively large, e.g. tetrahexylammonium hydrogen sulfate.



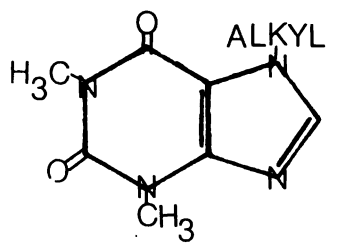


Figure 5. Extractive alkylation (ion pair partitioning).

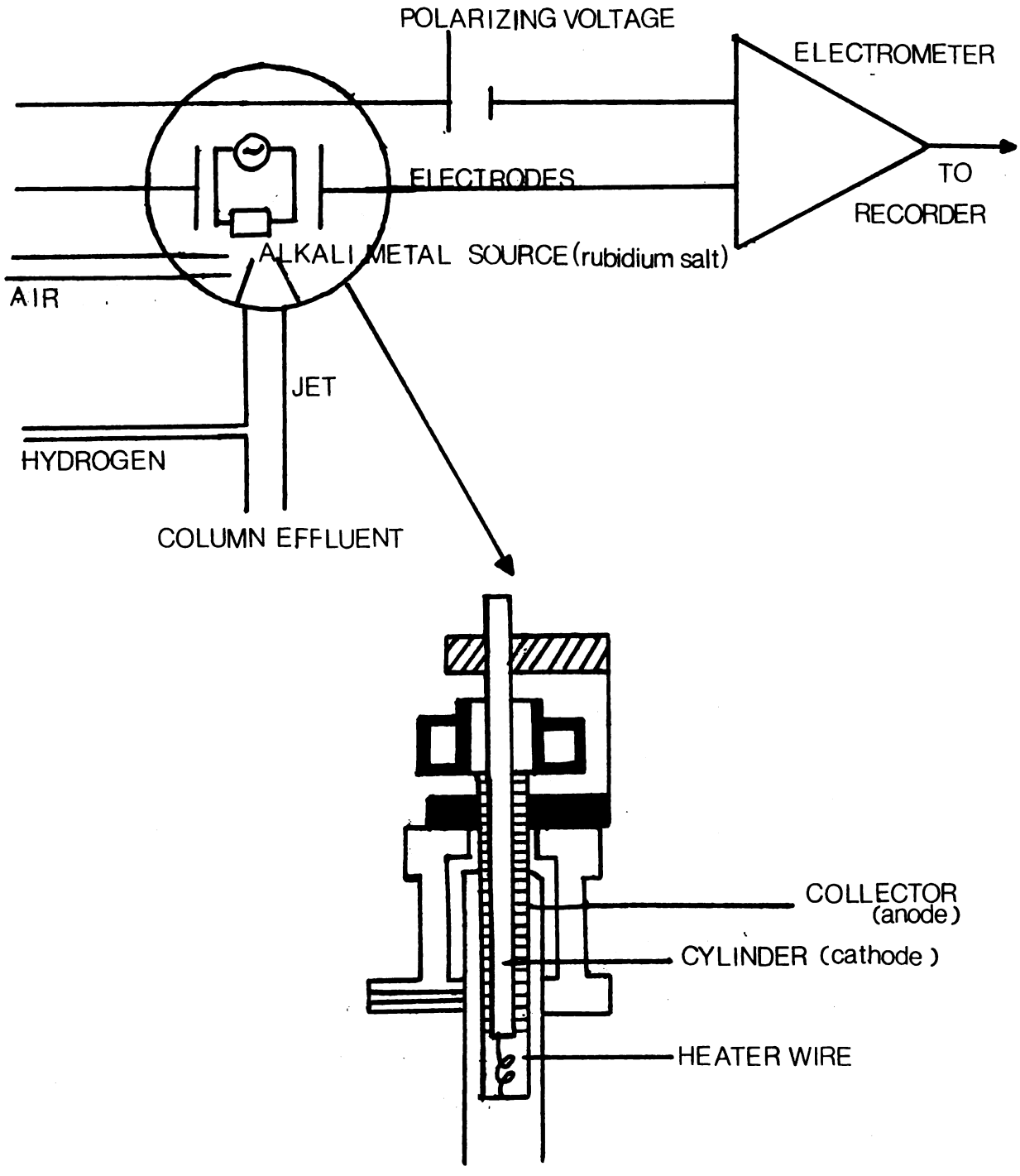
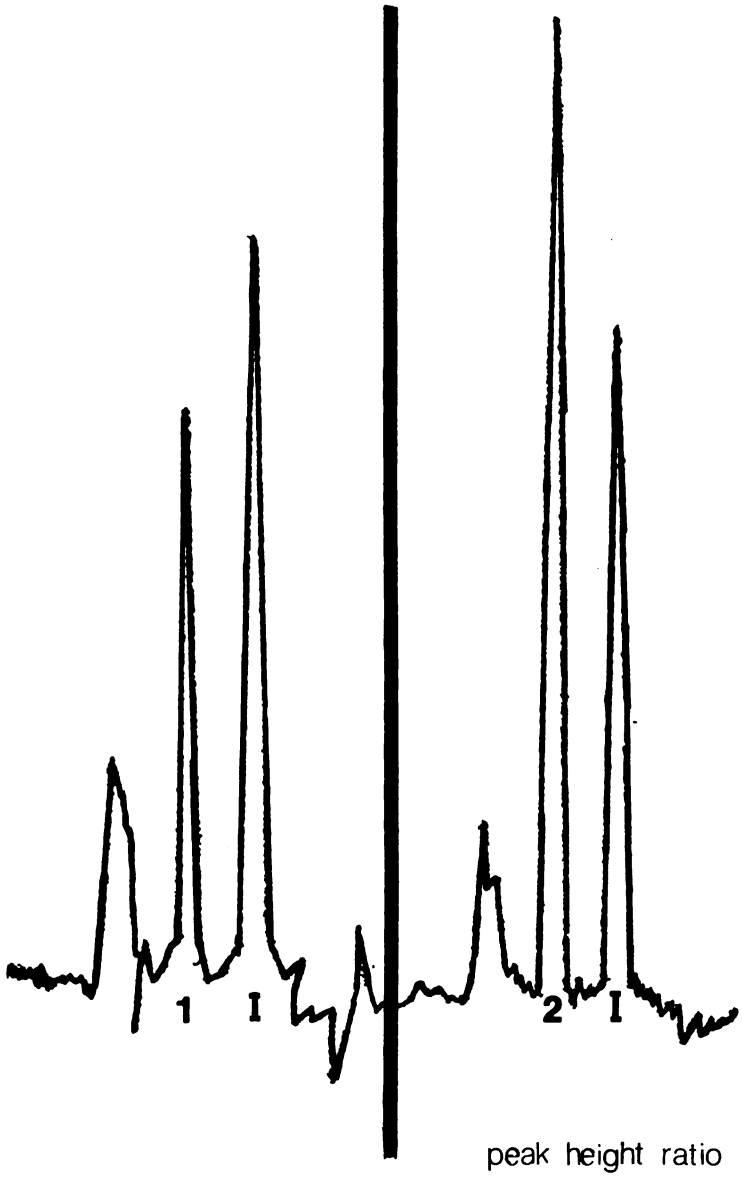


Figure 6. Nitrogen—phosphorus collector (adapted from Hewlett—Packard 5840A manual).



peak height ratio

1 / I	0.76
2 / I	1.49

1 = 15 mg/L standard
2 = 30 mg/L standard
I = 15 mg/L internal standard

Figure 7. Linearity of RPLC method for theophylline

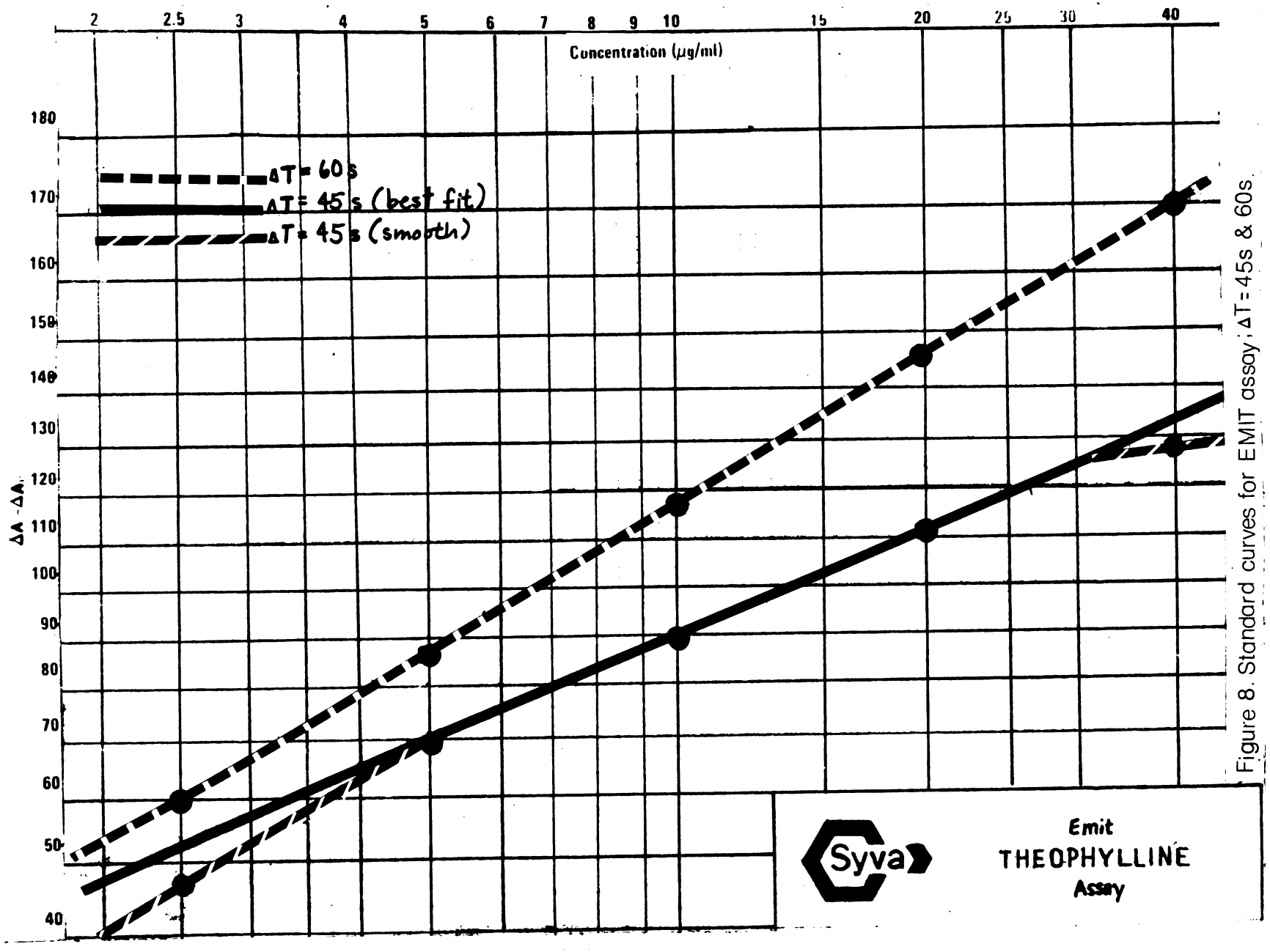


Figure 8. Standard curves for EMIT assay; ΔT = 45s & 60s.

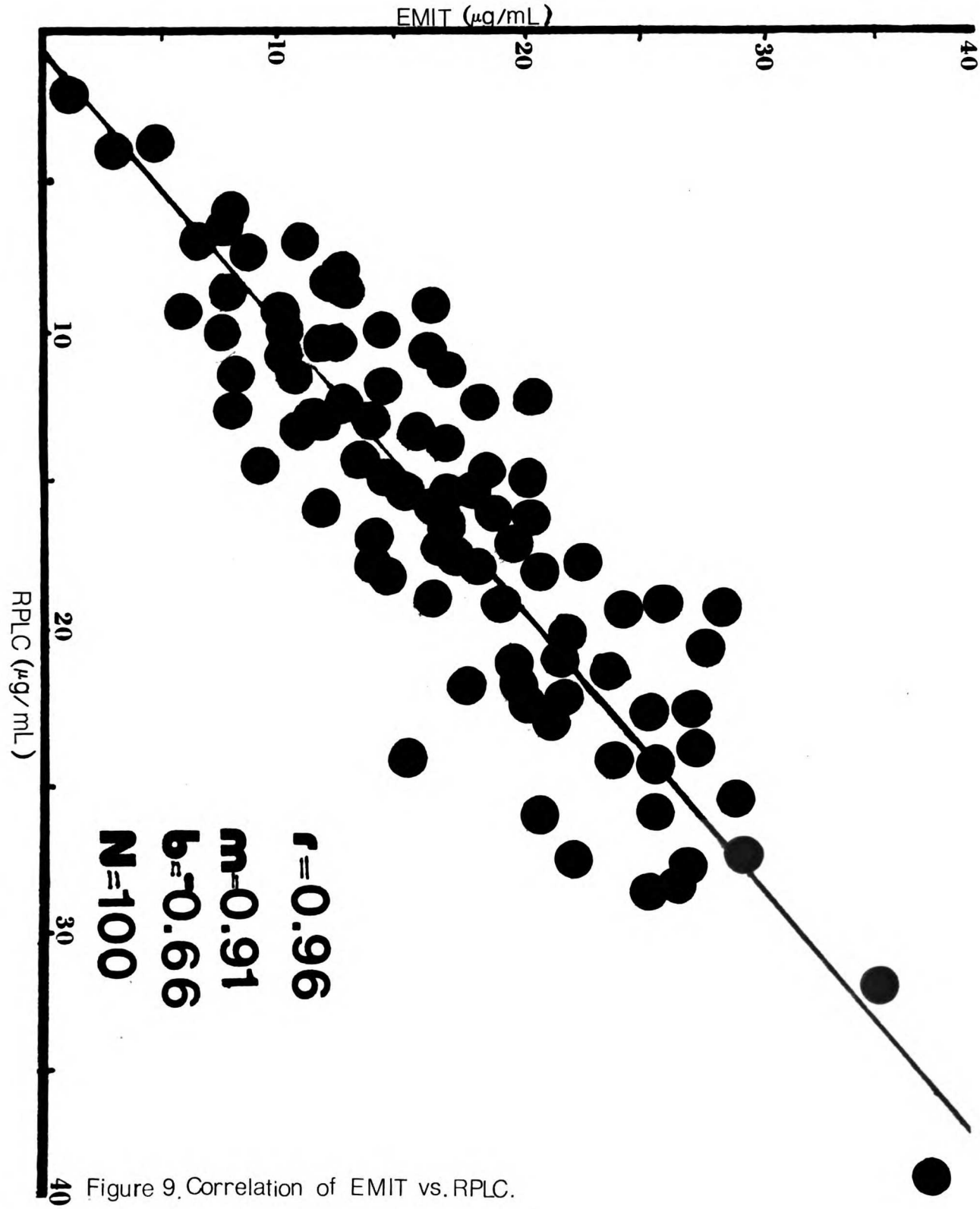


Figure 9. Correlation of EMIT vs. RPLC.

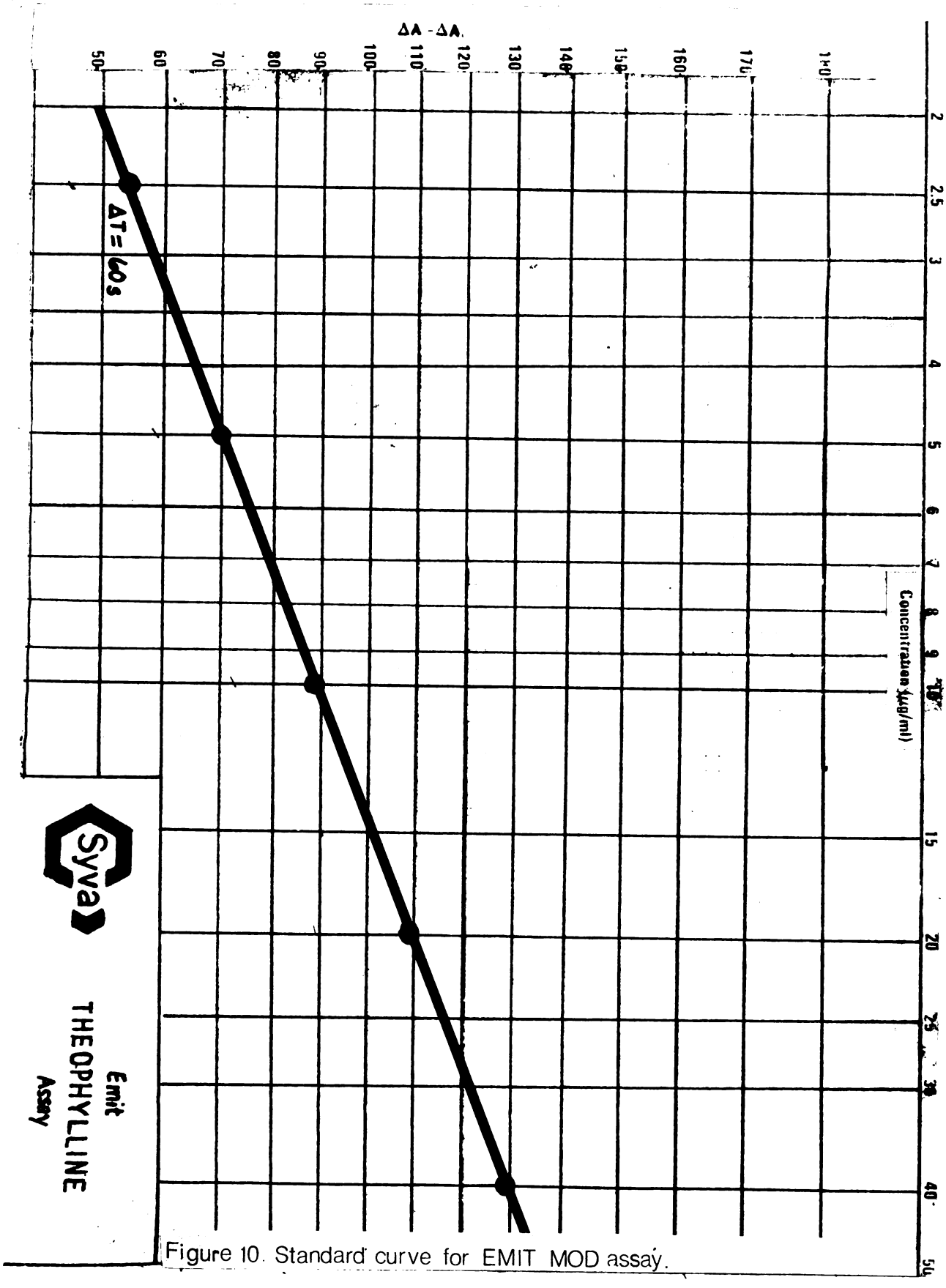


Figure 10. Standard curve for EMIT MOD assay.

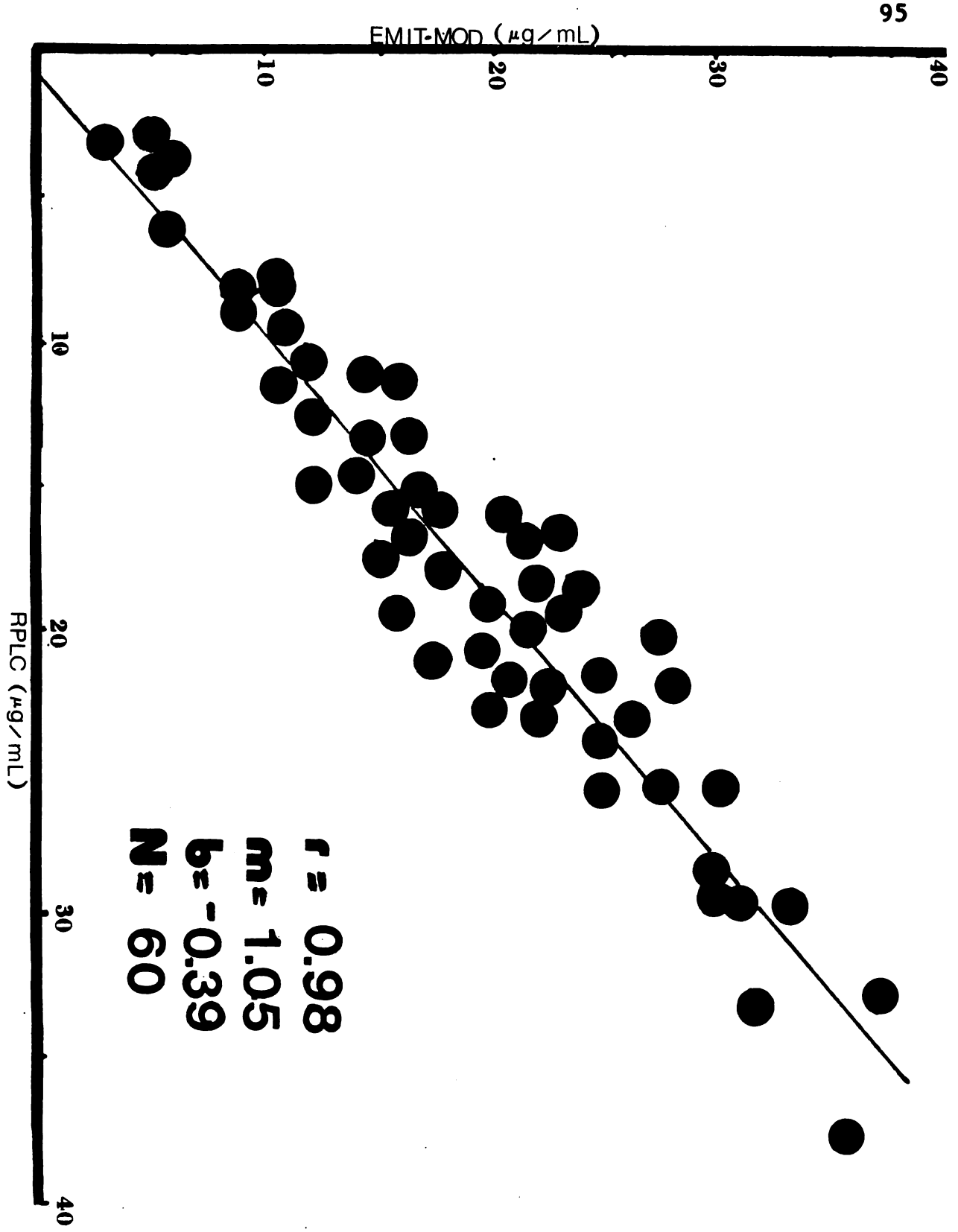
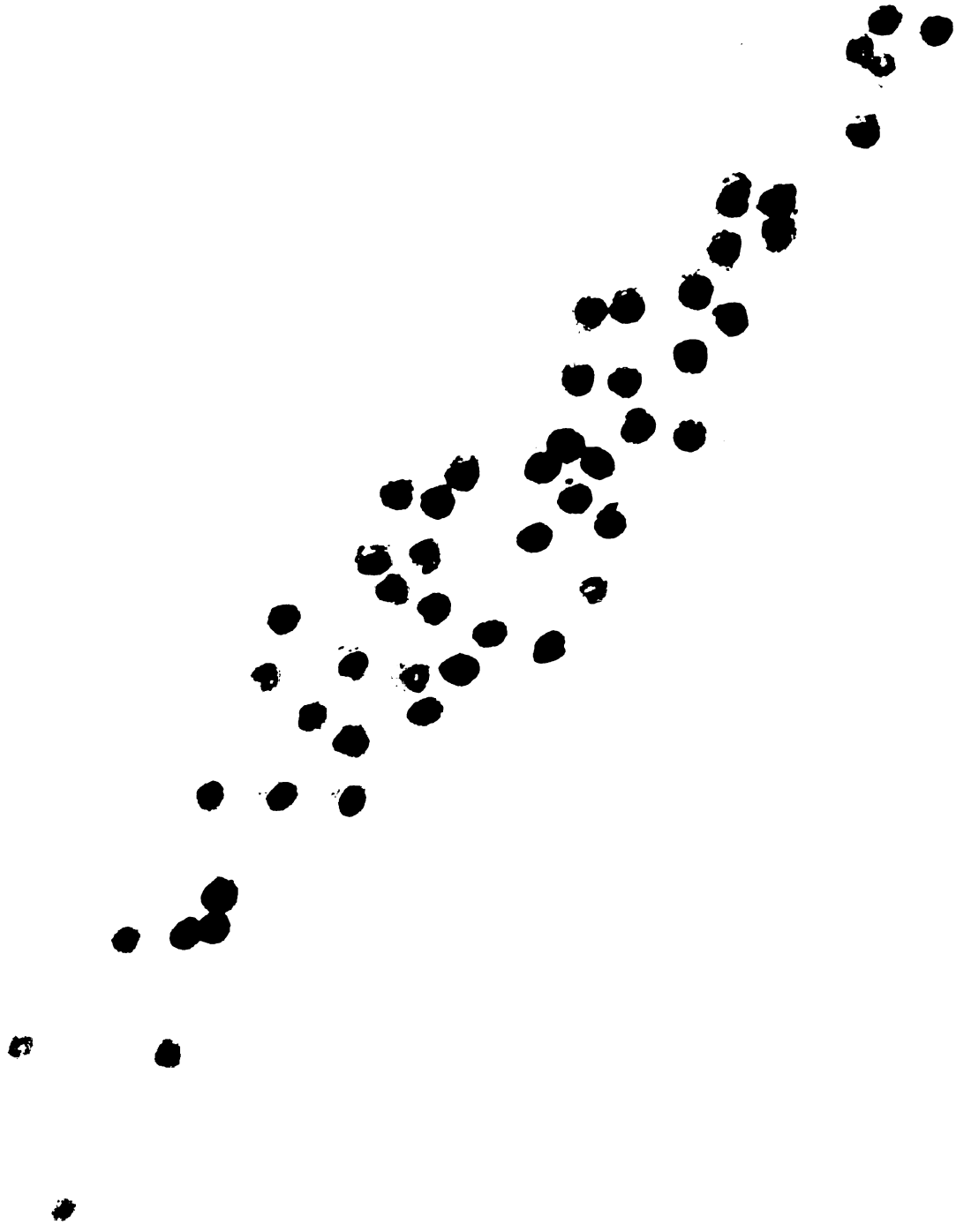


Figure 11. Correlation of EMIT-MOD vs. RPLC.



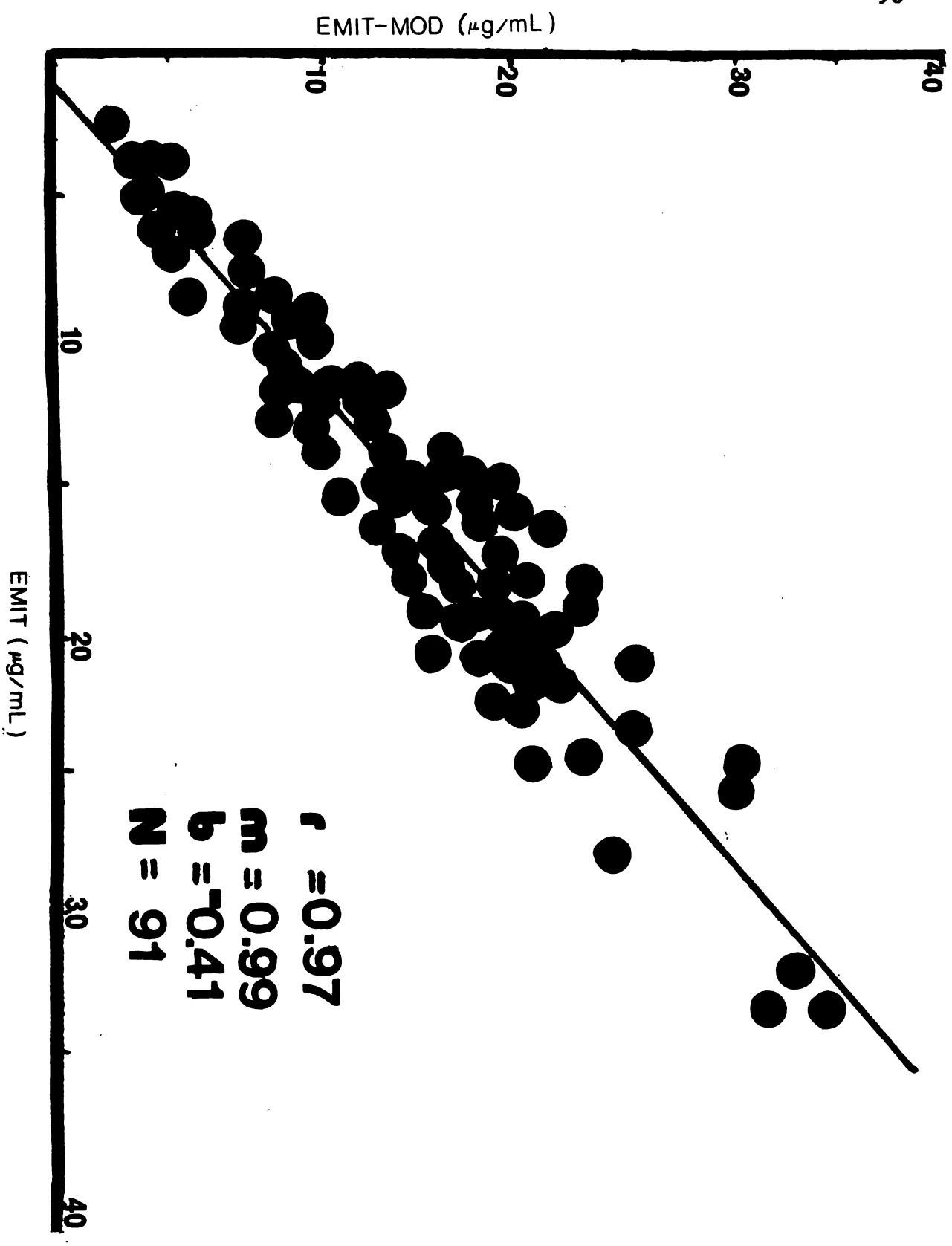
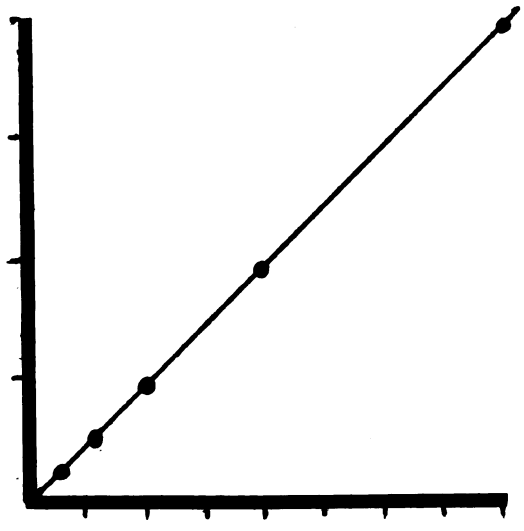


Figure 12. Correlation of EMIT-MOD with EMIT.



STANDARD (μg/mL)	AREA THEOPHYLLINE / INTERNAL STANDARD
2.5	0.12
5.0	0.23
10.0	0.44
20.0	0.94
40.0	1.98

Figure 13. Linearity of GLC-NP method for theophylline.

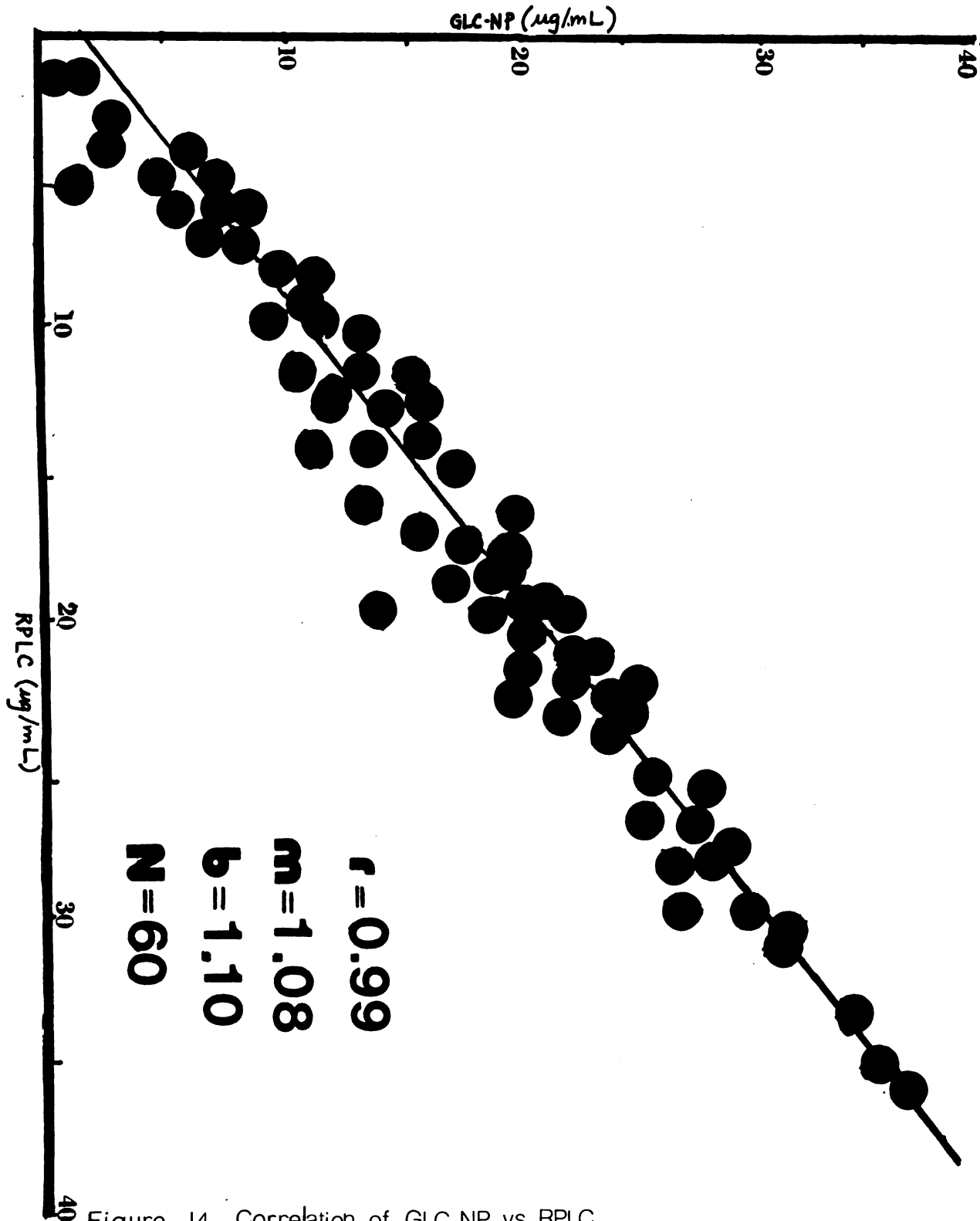


Figure 14. Correlation of GLC NP vs. RPLC.

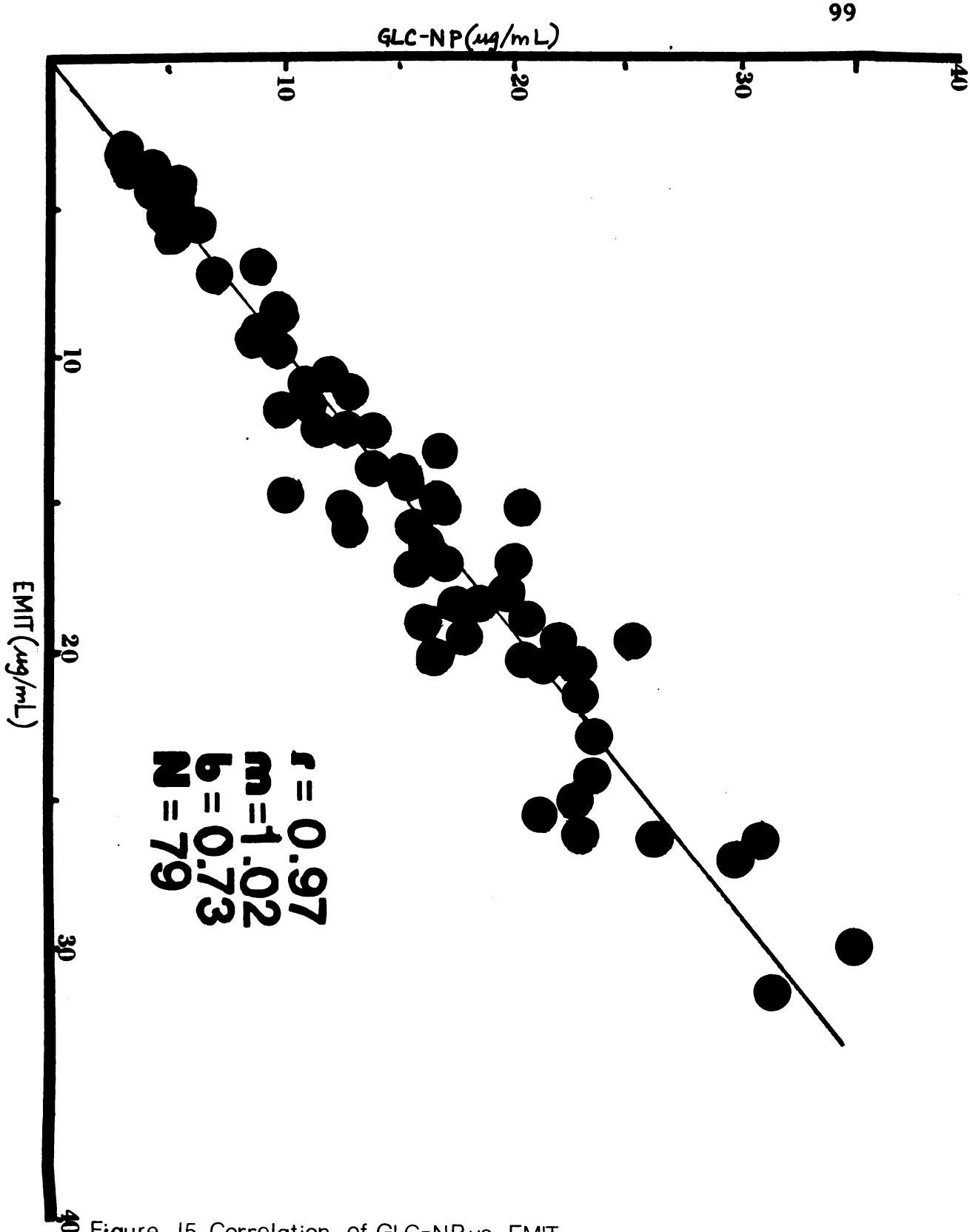
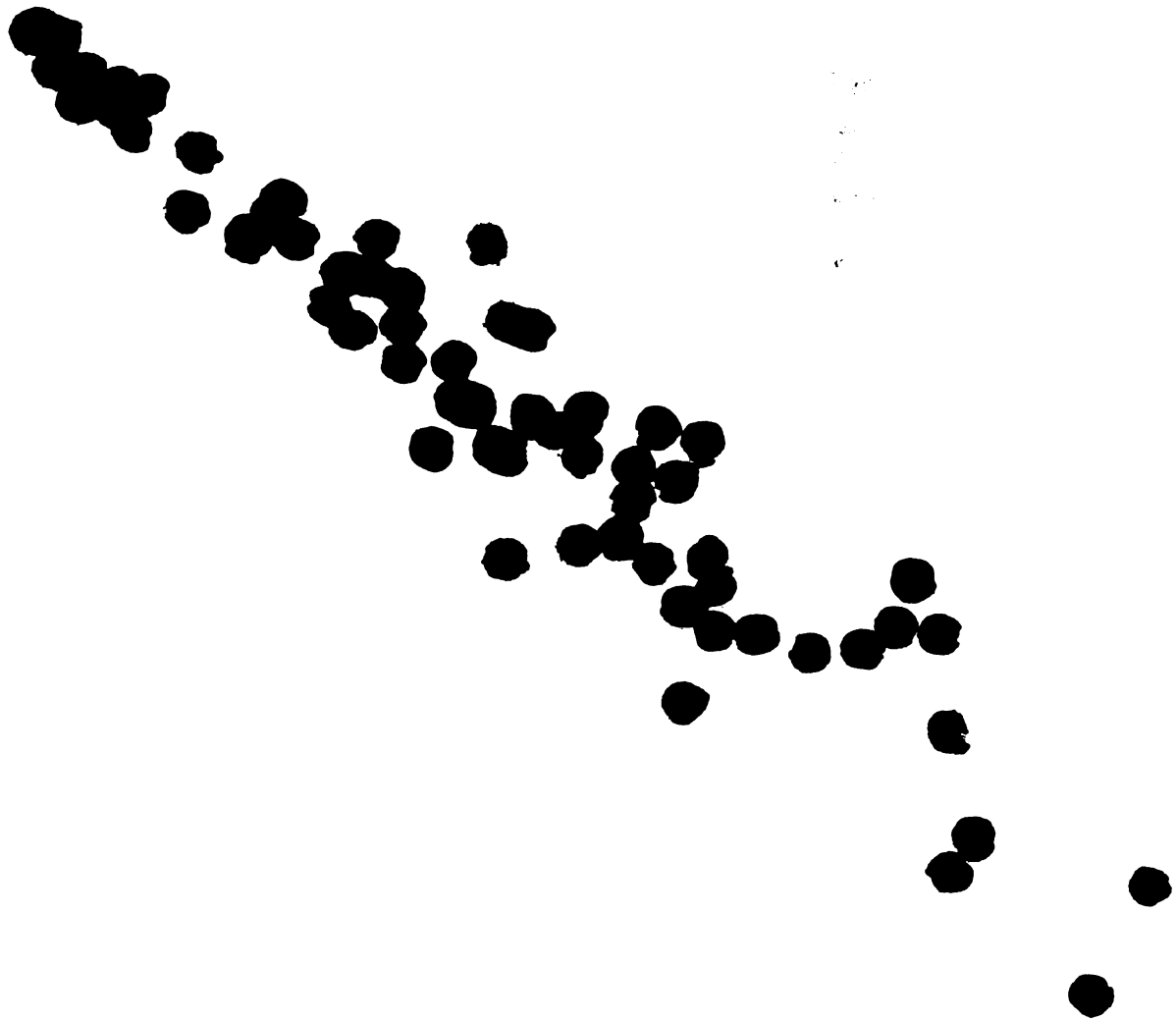


Figure 15. Correlation of GLC-NP vs EMIT.



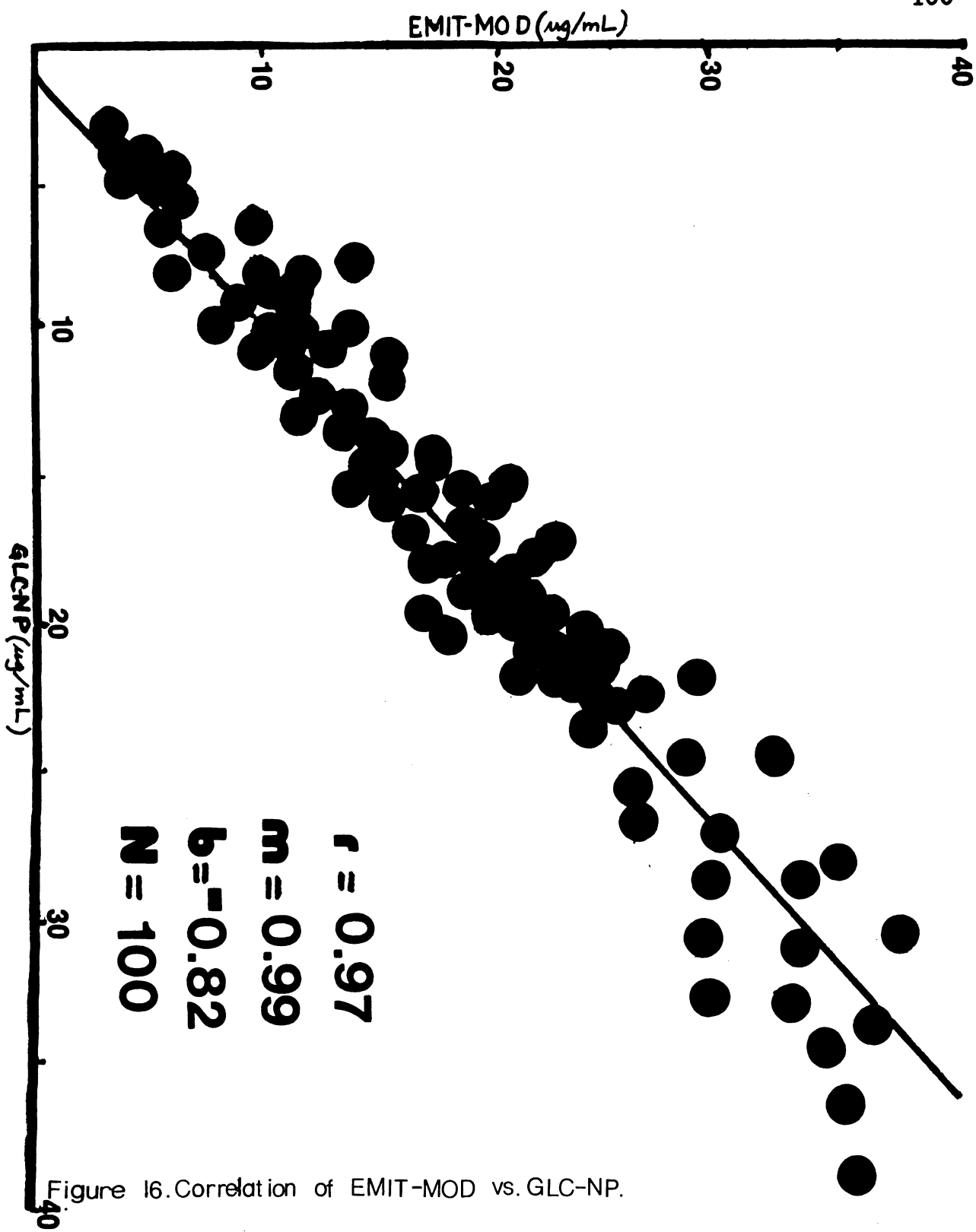
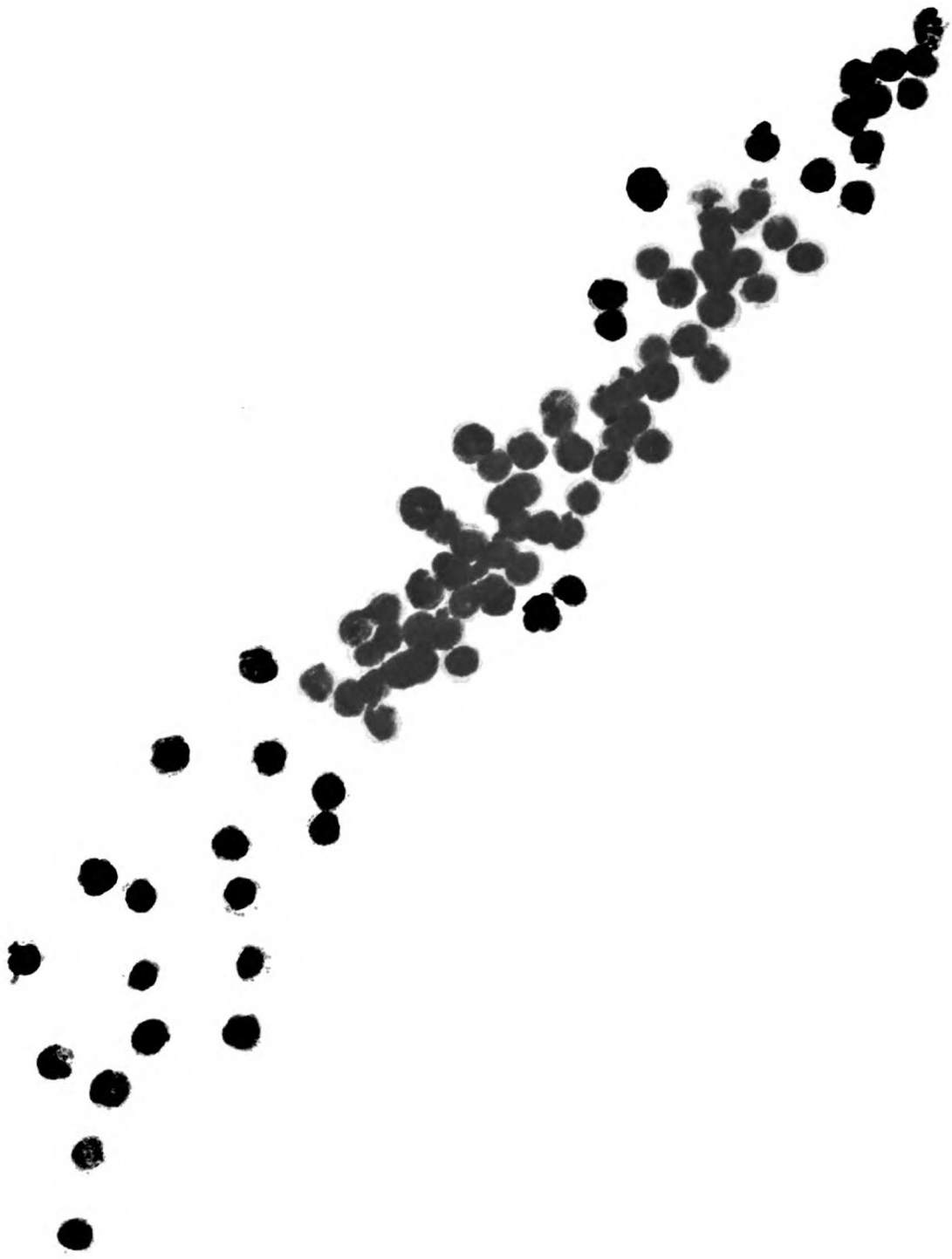
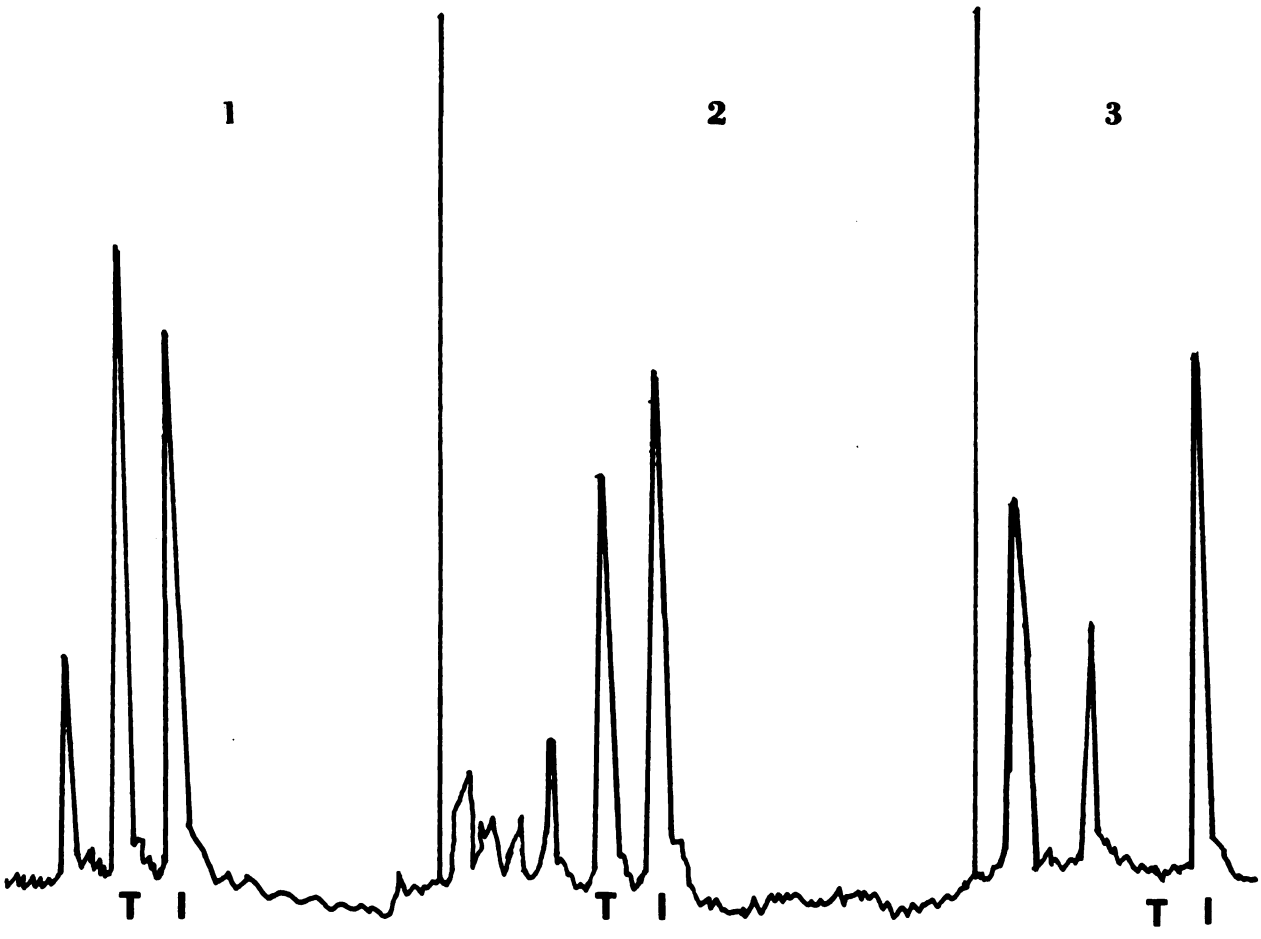


Figure 16. Correlation of EMIT-MOD vs. GLC-NP.





19 μg/mL* 13 μg/mL* 0 μg/mL*

* (RPLC)

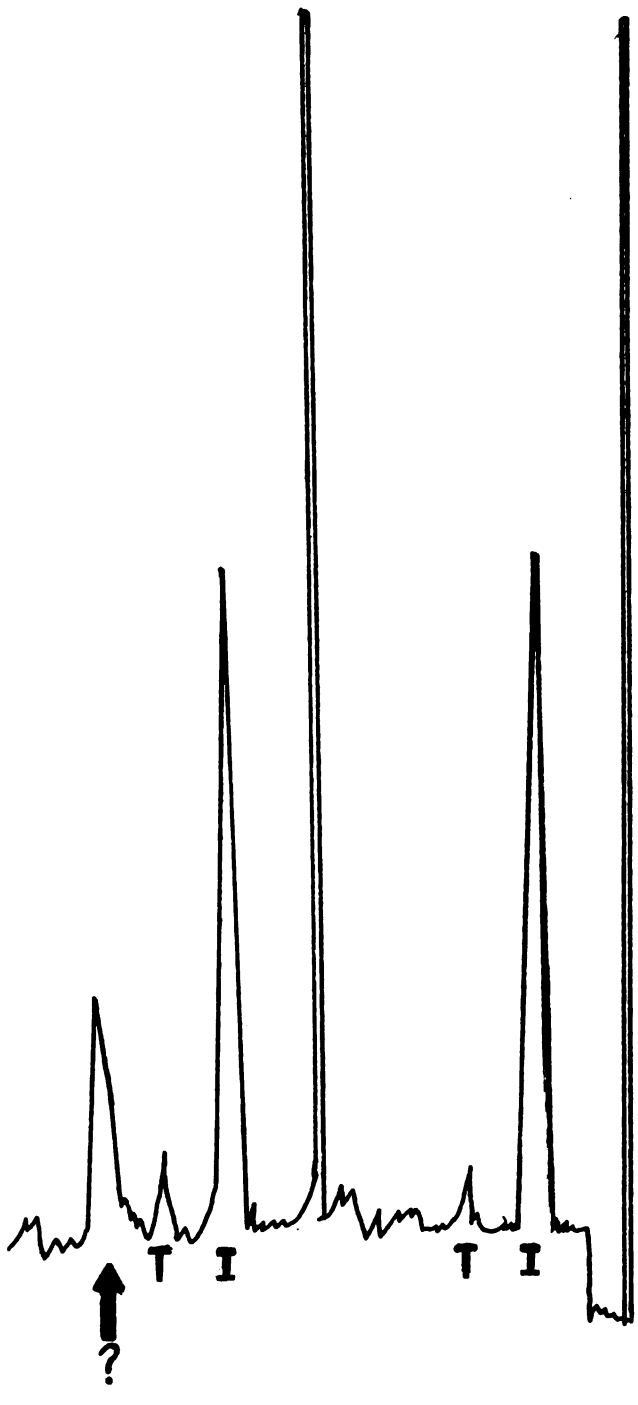
1 = 20 μg/mL theophylline added to serum with endogenous ampicillin.

2 = 13 μg/mL theophylline spiked to 5 μg/mL with ampicillin in serum.

3 = serum with endogenous ampicillin only.

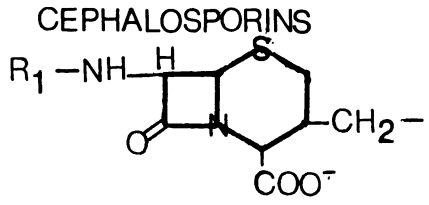
T = retention time for theophylline. **I** = retention time for internal standard.

Figure 17. Interference effects of ampicillin on theophylline analysis by RPLC.

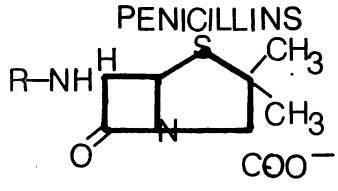


T = retention time for theophylline
I = retention time for internal standard

Figure 18. Aqueous solution of ampicillin (200 μg/mL) as analyzed by RPLC.

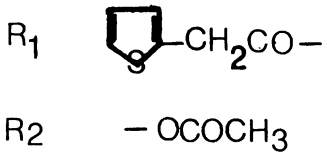


7–aminocephalosporanic acid

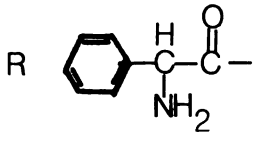


6–aminopenicillanic acid

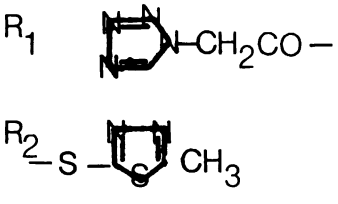
Cephalothin



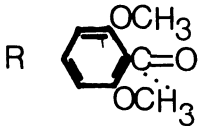
Ampicillin



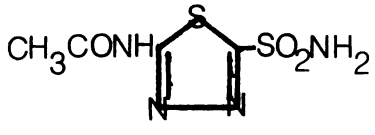
Cefazolin



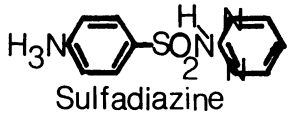
Methicillin



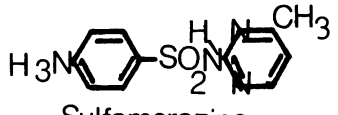
ACETAZOLAMIDE



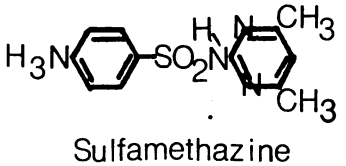
TRISULFAPYRIMIDINES



Sulfadiazine



Sulfamerazine



Sulfamethazine

Figure 19. Structures of compounds reported as interferences in RPLC methods for theophylline.

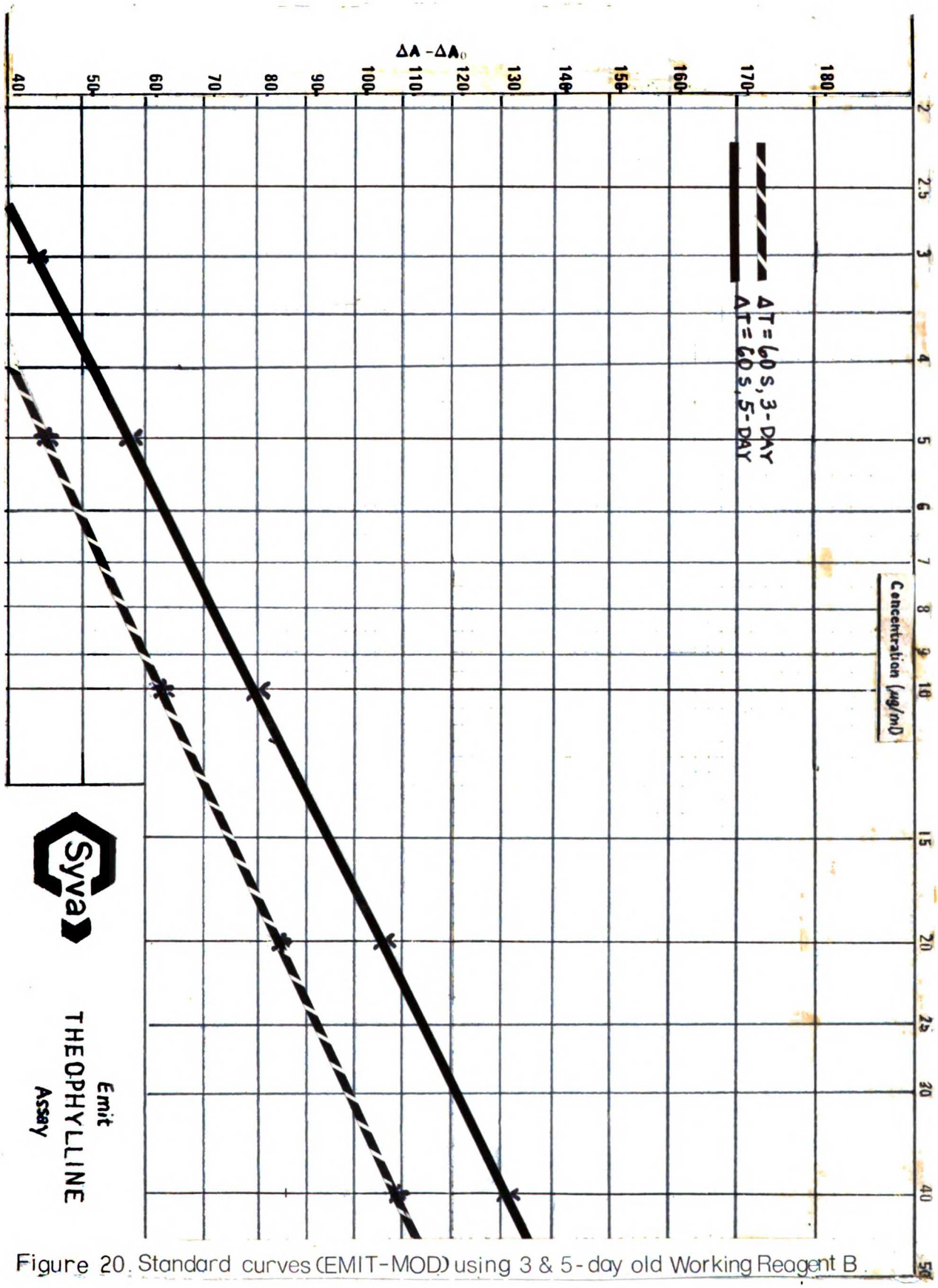


Figure 20. Standard curves (EMIT-MOD) using 3 & 5-day old Working Reagent B.

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APPENDIX

Further Description of Principles and Procedures Regarding Methodologies for Theophylline Quantitation

A. Ultraviolet Spectrophotometry

In UV methods, theophylline is differentially extracted into an organic phase, the re-extracted into an aqueous buffer and finally identified and quantitated by maximum UV absorption at 273 nm. Jatlow's (1975) modification of adjusting the pH of the final solution from 13 to 10, eliminated major interference from barbiturates. Subsequently, numerous modifications, including use of differential spectorphotometry (Gupta and Lundberg, 1973) in which a Na OH solution is scanned against an HCl solution, or adding ammonium sulfate to improve specificity (Schwernter et. al., 1978) have been tried.

B. Thin Layer Chromatography

Gupta et. al. (1977) extracted 0.5 mL of plasma, mixed with internal standard, dicholoromethane and sodium sulfate. The residue of the organic phase was dissolved in methanol and spotted on a silica gel plate into which a fluorescent indicator is incorporated. After drying, followed by placement in a solvent tank, theophylline and other compounds are separated by their relative solubilities and polarities, as the solvent moves through the gel by capillary action.

Plates are scanned by a densitometer and concentrations determined by integration.

In another procedure (Riechert, 1978), 10 μL of biological fluid was spotted directly onto the TLC plate. After drying at 100°C, theophylline and caffeine were marked under a UV lamp (254 nm) by their fluorescent quenching. UV absorption was measured using a dual wavelength scanner, and then integrated.

C. Radioimmunoassay

Neese et. al. (1977) reported a micromethod (2 - 5 μL sample) using tritium-labelled theophylline and serum theophylline compete for anitbody raised to 8-carboxytheophylline, conjugated (at the 8 position) to bovine serum albumin. This conjugation left the characteristic 1,3 and 7 positions of theophylline free, to insure the maximum specificity of the antibody. Theophylline metabolites and carreine show cross-reactivity, but are usually found in much lower concentrations in plasma. Separation of bound from free ligand was accomplished by ammonium-calcium sulfate precipitation. Radioactivity of the supernatant was counted by liquid scintillation spectrometry. A computer program constructed a log-logit curve, from which the log theophylline concentration was determined by interpolation. A similar method was simultaneously described by Cook et. al. (1976).

D. Reversed Phase Liquid Chromatography

RPLC is a type of liquid-liquid chromatography in which a stationary, nonpolar, N-alkyl liquid phase is chemically bonded to a solid, active silica support. The mobile phase is highly polar (in contrast to normal phase HPLC in which the mobile phase is less polar than the stationary phase). Analytes of varying polarity are partitioned between the mobile and stationary phases and are eluted in a general order of decreasing polarity (i.e. polar biological "debris" elutes quickly).

RPLC offers excellent control through manipulation of three parameters, solvent strength, temperature and flow rate. The basic components of the RPLC apparatus are the solvent system, pump, injector, column, detector and recorder.

Ability to control chromatographic conditions by altering the mobile phase is a major advantage in RPLC. Polar solvents remain pure and solubility of biological materials is easily achieved. Because of variable factors, such as viscosity, refractive index, UV cutoff, and polarity, an infinite number of solvent mixture can be created.

The pump is critical in that it must deliver the solvent at high pressure (e.g. 2000 - 3000 psi) at a constant flow rate. Pump failure is a common source of problems.

The injector is necessary to overcome the high pressures. Calibrating injector loops are available to deliver precise amounts to the column.

The columns are often run at ambient temperature. Their

bonded alkyl phases have weak surface energies which permit rapid analysis and re-equilibration. Column performance (resolving power) and efficiency are high.

The major difficulty in RPLC is the detector. Although fluorescent and electrochemical detectors have been used, the most common is still UV detection (either fixed or variable wavelength), which is not as sensitive as other methods.

Two main qualities affect reverse-phase retention properties, polar interactions and hydrophobic effects. Ofganic modifiers (e.g. acetonitile) are added to the main solvent to change the mobile phase plarity, so that compounds are resolved based on functional group polarity and hence, solubility differences. Acids or bases can change mobile phase pH, therby altering solubility by degree of ionization. Nonionized compounds chromatograph better, as secondary equilibria are less likely to influence separation and cause peak tailing. Due to instability of the bonded phase outside the pH range 2 - 7.5, only acidic or neutral compounds (which are nonionized under such conditions) chromatography well by RPLC.

RPLC is also a good discriminator of the nonpolar portions of molecules and therefore partly of molecular size, as evidenced by hydrophobic phenomena. Due to strong 3-dimensional intermolecular hydrogen bonding of the solvent, a high cohesive energy density produces a high surface tension, in which solutes experience a hydrobic force

that "squeezes" them out of the mobile phase, permitting retention by the stationary phase.

E. Gas Liquid Chromatography

GLC is gas liquid partition chromatography in which an inert solid support is coated with an active liquid stationary phase whose polarity depends on the analyte in question. Samples must be flash-volatilized at high temperatures so that they can be carried onto the column in the gas mobile phase. The basic components of the GLC system are carier gas, injection port, column, detector and recorder (Table 5). The carrier gas (usually nitrogen, helium or hydrogen) must be inert, dry, pure and delivered at a constant rate and pressure. The injection port, which is the weakest link in the system, vaporizes the samples and introduces them onto the column. Analytes are partitioned between the carrier gas and liquid phase (usually a substituted silicone oil) and are eluted in a general order of increasing molecular weight. The detector is the strength of GLC. Most commonly used for drug assays are flame ionization (FID), and nitrogen-phosphorus (N-P) detectors, which impart great sensitivity and selectivity to GLC analyses.

The derivitization procedure of MacGee (1970) uses tetramethylammonium hydroxide (TMAH) as the alkylating agent. Any compound with anionic nitrogens containing active protons can be combined with a tetraalkylammonium hydroxide to produce tetraalkylammonium salts. These quaternary amine

salts are injected onto the column and by pyrolysis at high temperatures the alkyl derivatives (and trialkyl-amines) are thermally eliminated, then detected. This technique has been reported in the flash-heater methylation (Dusci et. al., 1975), ethylation (Abraham et. al., 1977), propylation (Shah and Riegelman, 1974) and butylation (Koblansky et. al., 1973; Johnson et. al., 1975; Perrier and Lear, 1976; Bailey et. al., 1976; Vinet and Zizian, 1979) of theophylline. These methods are deleterious to the column by stripping the liquid phase off and reacting with the column. Linearity is lost after few injections.

In the procedure devised by Greeley (1974), soluble tetraalkylammonium salts are formed by mixing the anionic drug with a strong organic base (tetraalkylammonium hydroxide) and N-N-dimethylacetamide, a protophillic substance which provides a highly polar environment and increases ionization of the weak acid. These soluble salts are combined with analkyl iodide which reacts rapidly to form the alkyl derivative, which is then injected onto the column. Although this method involves extra steps, it eliminates column deterioration due to high pyrolysis temperatures and precipitating ammonium salts. Theophylline methods utilizing precolumn derivitization include off-column butylation (Johnson et. al., 1975) and pentylation (Least et. al., 1976; Lowry et. al., 1977).

F. Enzyme Immunoassay (EMIT®)

In this technique, based on competitive protein binding, theophylline is covalently bound to a bacterial glucose-b-phosphate dehydrogenase (G6PDH) near the enzyme's active site, so that interaction of the theophylline-enzyme complex with specific antibody to theophylline, decreases the enzyme's activity. Free drug in the standard or patient specimen competes with the enzyme-labelled drug for antibody and thereby decreases the antibody-induced inactivation of the enzyme. Enzyme activity is proportion to the concentration of free drug and is measured spectrophotometrically by an absorbance change at 340 nm due to the enzyme's catalytic action on the substrate, glucose-6-phosphate and the concomitant conversion of NAD^+ to NADH.

G. Enzyme Immunoassay with Centrifugal Analyzer

On the Centrifichem 400®, the pipettor measures and transfers microliter quantities of sample, diluent and reagent (B) into a Teflon® transfer disc, which has 30 radial cavities, each contoured to provide two separate wells (outer for sample and diluent, inner for reagent) and each in alignment with its own cuvette (Reagnet A is pipetted by hand). When the disc is loaded and sealed, the rotor accelerates within 3 seconds to approximately 960 revolutions per minute. Centrifugal force moves the small volumes of liquid together and the reaction begins immediately pulling the solution into the cuvette. Centrifuged liquids

are free of air bubbles, sediment, have a flat meniscus and are well-mixed due to a short burst of air forced through during the first 2.5 seconds.

The optical system acts as an effective double beam-in-time spectrophotometer. The tops and bottoms of the cuvettes are made of optical quartz, which pass under a focused light beam. This, in turn, passes through the reaction mixture to an interference filter, which isolates the correct wavelength. The transmitted light is converted to electric signals by the photomultiplier tube (PMT) such that the resulting voltage is a logarithmic analog of light absorbance in each cuvette. The PMT signal is converted to a digital absorbance which is visually displayed on a cathode ray tube (CRT), in which each cuvette is seen as a vertical bar. The printer will provide a printout in absorbance units or concentration depending on the type of test. After the test, the cuvettes are flushed and dried automatically and are ready for reuse.