AN EVALUATION OF PROCEDURES FOR DETERMINING THE PROTEIN BINDING OF DRUGS

A THESIS SUBMITTED TO THE

FACULTY OF GRADUATE STUDIES OF THE

UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

BY

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 MARCH 1986

ISBN 0-315-34026-6

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. C.J. Briggs for initiating this project and for his patience and constant guidance throughout the preparation of this work. The help and constructive criticisms of Dr. K.J. Simons and Dr. J.W. Hubbard were highly appreciated. The technical assistance of Mrs. D. Keling and Mrs. D. Smith was highly appreciated. I am also grateful to Miss J. Unrau for helping in the typing of this thesis.

I would also like to thank Dr. J.W. Steele and all other members of the Faculty of Pharmacy, without whose help and cooperation my studies would not have been a success. The financial assistance extended to me by Parke Davis was highly appreciated. I am pleased to acknowledge the financial support provided by the Faculty and assistance from the Canadian Foundation for Pharmacy.

Last but not least, I am very grateful to all members of my family whose moral support and understanding made it all worthwhile.

ABSTRACT

The binding of sulfisoxazole (highly bound), theophylline (medium bound) and the cholinesterase-reactivating oxime, HI-6 (low bound), to bovine serum albumin was determined using conventional equilibrium dialysis (bags), equilibrium dialysis cells (Dianorm type) and ultrafiltration membrane techniques. The effect of varying temperature, drug concentration, pH and albumin concentration on the extent of binding was investigated. HPLC and U.V. spectrophotometry were compared to determine their reliability and applicability in the assay of HI-6 in aqueous media.

The effects of temperature and albumin concentration on percent bound sulfisoxazole, theophylline and HI-6 were found to be highly significant. Sulfisoxazole and theophylline binding was found to be dependent on pH of the medium in the basic range, whereas HI-6 binding was independent of pH. Percent bound sulfisoxazole decreased with increasing drug concentration. Theophylline and HI-6 binding were independent of drug concentration.

All three methods for determining protein binding gave significantly different results under identical conditions. Generally, ultrafiltration yielded higher values of percent bound drug than either the of equilibrium dialysis procedures.

The HPLC and spectrophotometric assay procedures for pure HI-6 in phosphate buffer were shown to give comparable results under the experimental conditions used. Although spectrophotometric analysis may not distinguish between HI-6 and some of its breakdown products, either technique for assay may be used with reasonable accuracy in routine day to day analysis of HI-6 if conditions utilized do not result in breakdown. However, HPLC should be used for long-term stability studies.

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AN EVALUATION OF PROCEDURES FOR DETERMINING THE PROTEIN BINDING OF DRUGS

1 INTRODUCTION

The interaction of drug molecules and other ligands with tissue and plasma proteins has been the subject of intensive study. For many years, it has been appreciated that the distribution, pharmacology and pharmacokinetics of drugs can be affected by this interaction.

The first major interaction between most drugs and body tissues occurs in the blood compartment, where the drugs can distribute unevenly between red cells, white cells, plasma proteins and plasma water. The main interaction in the blood compartment is due to the presence of a variety of plasma proteins which can bind the drug molecules. This binding occurs mainly through reversible forces such as ionic and hydrogen bonds, and Vanderwaals forces. Figure 1 is a schematic representation of drug distribution in the body tissues in relation to its activity and elimination:

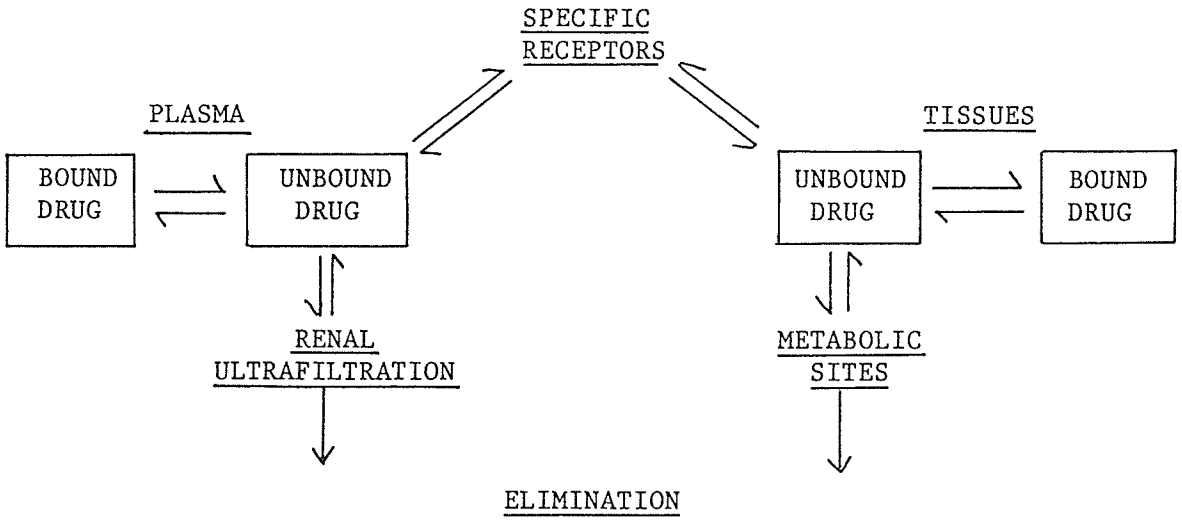


FIG. 1. The distribution of free and bound drug in the different body compartments.

Albumin is quantitatively the most important binding protein in the plasma, comprising approximately one half of the total plasma proteins. In a

normal healthy adult, albumin concentration in the plasma is approximately 4 g/100 mL, although lower levels may be found in circumstances such as pregnancy and certain disease states, such as renal and liver disease. At physiological pH, albumin has a net negative charge, but can interact with both anionic (acidic) and basic molecules, as well as neutral compounds. Many drugs, especially basic ones, also will bind to other plasma proteins such as $alpha_1$-acid glycoprotein (Borga, et al., 1977), and lipoproteins (Piafsky, 1980). There is evidence that globulins may have a very high affinity for certain compounds, notably the corticosteroids.

1.1 PHYSICAL AND CLINICAL SIGNIFICANCE OF PROTEIN BINDING

Binding of drugs to plasma proteins is an important pharmacological parameter. Frequently, it affects drug distribution and elimination (Keen, 1971; Jusko and Gretch, 1976), and the duration and intensity of the pharmacological response (Chignell, 1971). The high molecular weight of the plasma proteins prevents passage of bound drug molecules across the semi-permeable membranes of capillary walls. Only the free (unbound) drug is thought to be able to diffuse across biological membranes to interact with an effector site to produce a pharmacologic response, which may be either toxic or therapeutic. The actions of a large number of drugs including propranolol, phenytoin, coumarin anticoagulants and sulfonamides have been shown to be dependent on the free drug concentrations in plasma (Mcdevitt, et al., 1976; Anton, 1960). Quantitative variations in the protein binding of a drug can result in changes in clinical effectiveness due to an increase or decrease in the concentration of free drug available to produce a therapeutic response.

Restriction of the protein-bound drug in the intravascular space also ensures that only the free form of the drug is available for hepatic trans-

formation and/or renal excretion (Levy, et al., 1980). Consequently, the biological half-life of a highly bound drug is often longer than that for less bound compounds, resulting in a prolongation of the drug effects.

Two or more drugs may compete for the same binding site on a protein molecule, resulting in intra-individual variations in the concentration of unbound drug available for activity.

From these considerations, the need to be able to measure drug protein binding, particularly in plasma, and to understand its effects on drug disposition and action, is evident.

1.2 FACTORS AFFECTING PROTEIN BINDING OF DRUGS

The extent of binding to plasma components varies widely between drugs, and there may be considerable variation for a given drug within a group of individuals. Drug-protein binding may be influenced by four major factors:

1. The affinity between drug and protein
2. The concentration of drug
3. The concentration of protein
4. The presence of other substances which can compete for the binding site(s), or through an allosteric effect, alter drug binding.

Any or all of these factors can vary as a function of genetics, age, disease, other drug administration and environment.

Reduced plasma binding of many drugs, especially acidic and neutral drugs, is associated with hypoalbuminemia. A variety of diseases lead to a reduction in the plasma albumin concentration. This may result from the effect of the disease on the synthesis of albumin in the liver, as occurs in cirrhosis and viral hepatitis (Affrime and Reidenberg, 1975; Blaschke, 1976), and/or an increase in catabolism, as in the nephrotic syndrome (Gugler, et

al., 1975), surgical trauma, and burns. Impaired synthesis may also occur because of a reduction in the pool of amino acids available, due to malnutrition. Several authors have reported an increased incidence of toxic effects of drugs including prednisone, chlordiazepoxide and diazepam, in patients with reduced plasma albumin levels (Lewis, et al., 1971; Greenblatt and Koch-Weser, 1974). Qualitative variations in amino-acids, altering the conformation of the drug binding proteins can also occur in these disease states.

In acute phases of such inflamatory disorders as rheumatoid arthritis, Crohn's disease and stress, plasma concentrations of many globulins are elevated. These include $alpha_1$-acid glycoprotein, which is the major binding protein for many basic drugs, and accordingly, the plasma binding and total plasma concentration of these drugs are correspondingly elevated (Piafsky, et al., 1978; Piafsky, 1980).

One drug may influence the binding of another drug to plasma proteins. For this to occur, the second drug must be present in a concentration high enough to saturate the binding sites of the protein. Therefore, binding to albumin is only affected by such drugs as salicylates, phenylbutazone and sulfonamides, which are either given in very large doses or accumulate extensively during normal administration (Rowland, et al., 1973). Chronic administration of a drug may affect its own plasma binding, resulting in either an increase or a decrease in binding (Rowland, 1980). The mechanism for this interaction is not well understood, although decrease in binding of some drugs like phenylbutazone and disopyramide may be partially explained by the accumulation of metabolites which compete with the parent drug for common binding sites on the protein. Although in most instances competitive binding increases the free concentration of one drug, decrease in free concentration may occur as a result of this interaction; for example, the binding of pempidine

has been shown to be enhanced in the presence of chlorthiazide, and tetracycline increases the binding of both promazine and chlorpromazine (Rowland, 1980).

Various disease states result in changes in the electrolyte balance in the blood, which alters the binding of drugs, since the activity coefficient of the drugs changes. Also, there is a possibility of changes in the three dimensional structure of the proteins when the electrolyte balance is disturbed. Age of the patient can also have a significant effect on the binding of drugs to plasma proteins, since the blood volume and composition change with age. Chignell, et al. (1971) have demonstrated that sulfaphenazole has a reduced affinity for serum albumin of neonates and infants, compared with that of adults. There is evidence that the elderly may exhibit as much as 30 percent lower plasma albumin than young adults (Sereni, et al., 1968).

Binding of free fatty acids (FFAs) to albumin constitutes a major transport mechanism for fats in plasma. Higher than normal levels of FFAs have been observed in humans subjected to such conditions as exercise, fasting, sympathetic stimulation, bacterial infection, diabetes mellitus and hyperthyroidism (Feely, et al., 1981). Although most drugs and fatty acids appear to be binding at different sites on the albumin molecule, the elevated levels of FFAs can result in the displacement of drugs from their binding sites, due to allosteric changes induced on the albumin molecule by the fatty acids (Spector, et al., 1973; Wurtman and Fernstrom, 1976). Protein binding of certain drugs has been shown to be affected by heparin, a common anticoagulant injected into intravenous cannulas to keep them patent (Wood, 1979; Weigandu, 1979).

In principle, there is a limited number of binding sites on the protein. As the drug concentration in the plasma increases, the number of available

binding sites decreases, and the fraction of free (unbound) drug increases. In practice the fraction of free drug in plasma for most drugs administered in therapeutic doses is essentially constant over the entire therapeutic concentration range. However, for highly bound drugs such as sulfonamides and salicylates, which are also given in very large doses, saturation of binding sites may occur at therapeutic concentrations. In these cases, slight changes in the extent of binding can result in a significant change in the fraction of free drug in the body, and consequently, in the clinical effects of the drug.

1.3 METHODS OF STUDYING PROTEIN BINDING

A variety of techniques has been used to characterize the plasma binding of drugs in vitro. The most commonly employed methods include equilibrium dialysis, ultrafiltration, ultracentrifugation and gel filtration (Vallner, 1977). These are based on physically separating the unbound from the bound drug. Other non-separative, mainly spectroscopic methods, generally are used to examine the qualitative nature of the interaction. These methods have provided insights into the nature of the amino acid residues which participate in the binding process, structural characteristics which promote binding, and the intermolecular forces responsible for binding. The difference in the partition coefficient of a drug between plasma or water and an organic solvent, also has been used to predict changes in the extent of protein binding.

1.3.1 Equilibrium Dialysis

Equilibrium dialysis (Klotz, 1946) is the oldest and probably the most popular method for studying protein binding. The procedure involves confining the protein component within a semi-permeable membrane through which unbound drug molecules can diffuse freely. Usually, the protein sample and a drug-

free buffer solution are placed on either side of the membrane. Unbound drug in the protein solution passes through the membrane until equilibrium is reached, at which time the concentration of free drug in the dialysate is equal to that in the protein solution. By measuring the drug concentration on each side of the membrane, the fraction of unbound drug can be determined.

The devices used for equilibrium dialysis range from the conventional simple cellulose bags tied at both ends and immersed in buffer, to specially designed apparatus such as the micro-Plexiglass double cell technique. A dynamic dialysis method has been described by Meyer, et al. (1962) in which a single experiment yields binding data for a wide range of drug concentrations.

The Spectrum equilibrium dialyser (DIANORM) (Weder, et al., 1971), is a relatively new equilibrium dialysis technique which is replacing the conventional "knotted bag" method. The apparatus consists of a semi-permeable membrane sandwiched between two PTFE (Teflon) half cells. The advantages of this procedure over other equilibrium dialysis methods include speed, precision and low non-specific adsorption. The small dialysis volume and large membrane area ensure rapid attainment of equilibrium, which saves time and minimises possible protein denaturation. The cells are standardized, which ensures reproducibility of results, and machined with precision so that a leak-free seal is produced between the cell halves. PTFE (polytetrafluoroethylene) is relatively inert, and this ensures minimal loss of drug or protein due to non-specific adsorption. Filling and emptying the cells is easily done using pipettes, and 20 samples can be dialysed simultaneously. The cells may be rotated at various speeds, and the drive unit can operate uniformly within a temperature range of -10 to +65°C. Heat exchange is rapid due to the thin cell wall design of the dialysis cell.

1.3.2 Ultrafiltration

Ultrafiltration is another method used widely for studying drug-protein binding. It involves filtering a pre-equilibrated solution of drug and protein under pressure, through a dialysis membrane. A protein-free filtrate containing unbound drug is collected beyond the membrane (Rehberg, 1943). Knowledge of the initial concentration of the drug present and that in the ultrafiltrate allows calculation of the amount of drug bound to the protein. The pressure may be exerted by a column of mercury or by centrifugation.

This technique is faster and simpler than equilibrium dialysis, since the solution of free drug is a sample which was initially in direct contact with the protein solution. However, for precise quantitative studies, the method is inferior to equilibrium dialysis because of the continually changing protein concentration (Davison, 1971), and adsorption of protein onto the membrane surface.

Various modifications of the ultrafiltration technique involve the use of Centriflo membrane cones (Piafsky, 1977), and the microultrafiltration technique of Shah, et al. (1974). Diafiltration (Bixler, 1968), involves a more elaborate ultrafiltration cell in which the lost volume is continuously replaced, thereby avoiding protein concentration changes. A novel ultrafiltration technique which utilizes an immersible molecular separator, has been described by Briggs, et al. (1979). The separator consists of an immersible cartridge with a non-cellulosic ultrafiltration membrane covering a plastic core. The cartridge is immersed in the drug-protein solution and ultrafiltration is effected through a vacuum. The method has been reported to give protein binding results comparable to ultrafiltration membrane cones, for theophylline (Fedak, 1980).

1.3.3 Ultracentrifugation

The ultracentrifugation technique involves the use of centrifugation to separate the small unbound drug molecules from the high molecular weight drug-protein complex. Theoretically, the method is preferable to equilibrium dialysis and ultrafiltration since it does not disturb the equilibrium nor alter the unbound drug concentration. However, it is more laborious, and problems exist with plasma if the drug binds to the low-density lipoproteins, which rise to the top of the tube druing the centrifugation process.

1.3.4 Gel Filtration

Gel filtration is a simple technique based on trapping the unbound drug into the pores of a gel complex. However, problems may arise if excessive dissociation of the bound complex occurs. The method also requires very large samples, which limits its application in clinical situations where only small or limited numbers of blood samples can be withdrawn from the patient.

None of the methods used to measure protein binding has an absolute superiority over the others, each having some inherent problems which may influence the results. Equilibrium dialysis is the simplest, and still the most popular method, often being used as a reference method against which other methods are compared. Difficulties in interpretation of results from equilibrium dialysis arise if binding is dependent on drug and/or protein concentration. In this technique, the concentration of drug in the protein solution at equilibrium is lower than that initially present, partly due to diffusion of drug into the buffer solution used for dialysis. Also, dilution of the protein by water moving from the buffer solution into the protein as a result of osmotic gradient may occur. This phenomenon, commonly referred to as 'volume shift', recently has been reported to decrease the *in vitro* binding

of several drugs. The binding of cortisone and drugs such as ibuprofen, propranolol and diazepam has been shown to vary with serum protein concentration (Lockwood and Wagner, 1983; Lima, et al., 1981). The latter authors have reported that the extent of volume shift depends on the type of drug, dialysis time, area and molecular weight cut-off of the membrane used, membrane thickness, and the degree of protein binding of the drug. Verbeeck, et al. (1984) have reported a decrease in plasma protein binding of various drugs with time, a factor which was partly attributed to the continual dilution of the protein solution. The use of isotonic solutions or high concentrations of low molecular weight buffers (Sonnenberg, 1949), does not entirely prevent this osmotic dilution, because buffer and salt ions freely equilibrate into the protein compartment, and the osmotic pressure due to the protein still remains.

It has been suggested (Steinhardt and Reynolds, 1969) that the Donnan distribution may cause significant errors in the determination of plasma protein binding of weak electrolyte drugs by equilibrium dialysis. The presence of a charged macromolecule (protein) on one side of the membrane will alter the concentration of permeant ionized particles (drug), according to the Donnan equilibrium. Drug molecules of the same charge as the macromolecule will be constrained to the opposite side of the membrane. The use of high concentration of electrolytes to diminish this effect (Steinhardt and Reynolds, 1969; Van der Giesen and Wilting, 1982), may cause artefacts due to competitive ion-ion binding (Bowers, et al., 1984). It is possible to correct for significant Donnan distribution by calculating the effective Donnan ratio from the drug ionization and the distribution of a non-binding ion. Keen (1966) has used the net charge on the protein molecule to indirectly estimate the Donnan distribution ratio. A direct and more reliable method of quanti-

tating this ratio has been described by Suter and Rosenbusch (1977).

Other problems associated with equilibrium dialysis include non-specific adsorption of free drug to the dialysis apparatus, competitive binding by buffer ions, control of retentate pH, and protein leakage into the dialysate. The long time required for equilibration in most dialysis techniques (usually more than 12 hours) may allow decomposition of the drug-protein complex, or growth of microorganisms. Therefore, the use of adequate representative controls is essential when using this method to measure protein binding.

Although ultrafiltration appears to be more appropriate than equilibrium dialysis as routine methodology for estimating protein binding, it is not as readily adapted for large samples, and it requires somewhat more expensive apparatus. Polarization of the protein onto the membrane, and drug uptake by the membrane, are common problems encountered in ultrafiltration. Since a portion of the aqueous phase is forced away from the protein, the latter solution becomes more concentrated with time, thereby tending to increase binding. This problem may be minimized by collecting only a small fraction of the total sample volume (Witlam, et al., 1981), but applicability of this technique is limited by the sensitivity of the analytical procedures available for assaying the drug.

In view of the problems associated with the methods, it is difficult to choose a method that can be relied on to give correct protein binding values for all drugs. The likelihood of obtaining an accurate estimate is increased, however, if two or more techniques give comparable results.

The measurements of plasma drug levels has taken on an increased importance in the past few years. For highly bound drugs which also have narrow therapeutic indices, and/or show dose-dependent kinetics, slight changes in binding may cause in disproportionate changes in the free (unbound) drug,

which may result in significant changes in the therapeutic or toxic effects of the drug. There is an obvious need for a simple, rapid, convenient and reliable method for routine determination of the protein binding characteristics of these drugs.

1.4 PRESENTATION OF BINDING DATA

The binding of drugs to plasma proteins is a dynamic process, and may be considered to obey the laws of mass action (Meyer, et al., 1968; Koch-Weser, et al., 1976; Vallner, 1977). The process may be represented as:

$$D_F + P \underset{K_2}{\overset{K_1}{\rightleftharpoons}} PD.$$

Where P = Free protein concentration

D_F = Free drug concentration

PD = Drug-Protein complex concentration

K_1 and K_2 are the rate constants for association and dissociation, respectively. At equilibrium, the association constant, Ka is:

$$Ka = \frac{K_1}{K_2} = \frac{PD}{P.D_F}$$

The fraction of free drug in the plasma, that is $\frac{D_F}{D_F + D_B}$, where D_B = bound drug concentration, depends on the magnitude of Ka, the total concentration of drug and the protein concentration. The higher the affinity of drug and the protein, the greater the equilibrium association constant, and the smaller the plasma free drug concentration.

Several methods are available for presenting drug-protein binding data (Klotz, 1946; Scatchard, 1949). The simplest and perhaps the most useful presentation is calculating the fraction or percentage of drug bound, thus:

$$B = \frac{DB}{D_T} = \frac{D_T - D_F}{D_T}$$

Where B = Fraction of total amount of drug bound

D_T = Total amount of drug, and D_F and D_B are as described above. The percentage of drug bound is therefore:

$$B\% = \frac{D_B}{D_T} \times 100 = \frac{D_T - D_F}{D_T} \times 100$$

Conventional calculation of percentage binding is based on the initial drug concentrations with no corrections for non-specific binding to the equipment . This may lead to an overestimation of the quantity of drug bound to the protein. In some studies, corrections for non-specific binding are based on initial control runs where the drug is dialysed without the protein. The variability which occurs between replicates of the same experiment makes this method unreliable. Recently, Briggs, et al. (1980) described an improved control procedure where replicate controls corresponding to the test samples are dialysed under identical conditions, except that the protein is excluded from the control drug solutions. This procedures eliminates differences between conditions of test and control runs and any binding detected can be attributed to the protein present. The extent of binding can therefore be calculated from:

$$B = \frac{D_C - D_T}{D_C}$$

Where D_C = Amount or concentration of free drug in control samples

D_T = Amount or concentration of free drug in test sample

Similarly, the percentage bound may be calculated as:

$$B\% = \frac{D_C - D_T}{D_C} \times 100$$

For many drugs, fraction bound will alter with change in drug or protein concentration. The use of this form of presentation is limited unless the drug and protein concentrations at which the determinations are made are specified.

1.5 PROTEIN BINDING OF SPECIFIC DRUGS

Several methods have been used to study the protein binding of drugs, and it is a well established fact that the protein binding results of one drug under identical conditions may vary from method to method. Whereas many comparisons of different methods have been done using the same drugs, few comparisons of methods have been done using drugs with different binding characteristics under standard conditions. A number of factors, including the method of determining binding, will affect the binding results of any given drug. It is likely that the extent of these effects on drug-protein binding will be influenced by the affinity of the drug for the binding protein, and hence, the extent to which the drug is bound to the protein. It is important to know how the methods used to study protein binding, affects the binding results of different drugs with respect to their extent of binding.

1.5.1 Sulfisoxazole Protein Binding

Sulfisoxazole (sulfafurazole) is a short acting sulfonamide which is widely used in the treatment of bacterial infections. Like most other sulfa drugs, sulfisoxazole has been shown to bind preferentially to albumin (Anton, 1960; Zini, et al., 1976) and also to other plasma proteins. Reported binding at therapeutic concentrations range from 65% to about 90% using different techniques (Anton, 1969; Stock, et al., 1980).

Yacobi, et al. (1977), and Yacobi and Levy (1977) have reported signifi-

cant intersubject differences in the free fraction of sulfisoxazole in the plasma of rats and humans, respectively. In view of the role of protein binding in the therapeutic effectiveness of highly bound drugs, these differences could have significant therapeutic implications. Protein binding of sulfisoxazole has been shown to be affected in certain disease states (Levy, et al., 1976), and intersubject variations in the excretion and metabolism of this drug have been attributed to variations in protein binding. Only the free form of sulfisoxazole is biologically active (Anton, 1960). Improvement in the monitoring and therapeutic use of sulfisoxazole and other highly bound drugs will be achieved if a fast and convenient method is identified which can be relied on to give accurate values for the free concentration of the drugs in plasma.

1.5.2 THEOPHYLLINE PROTEIN BINDING

Theophylline is a xanthine derivative used in the symptomatic treatment of asthma and other related conditions. Theophylline has been shown to bind to plasma proteins to an extent of about 50 to 70% at therapeutic concentrations (Koysooko, et al., 1974; Briggs, et al., 1979; Fedak, 1980). The efficacy and toxic effects of theophylline have been shown to be related to its serum concentrations, and it has been suggested that the protein binding of theophylline may influence its clearance (Jusko, 1976).

Theophylline has a narrow therapeutic index, and it has been suggested that accurate determination of free plasma levels could be useful in optimizing the management of asthmatic patients with altered protein binding of this drug.

1.5.3 HI-6 (OXIME)

A. Introduction

The use of atropine plus an oxime such as PAM chloride, TMB-4 or toxogonin is an effective therapy against poisoning by most organophosphorous (OP) compounds (Lipp, et al., 1960). In the case of poisoning with soman (O-pinacolylmethylphosphonofluoridate), however, this therapeutic regimen is not effective for a number of reasons:

(a) Rapid aging of the inhibited acetylcholinesterase (AChE) enzyme

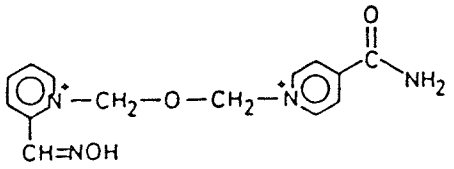
(b) Resistance of the aged enzyme to reactivation

(c) Poor lipid solubility of most of the conventional oximes prevents their entry into the central nervous system (CNS), a possible major site of action of soman

(d) Possible direct toxic biochemical effects of soman

There is considerable interest in the development of new oximes as therapeutic agents for treatment of poisoning by organophosphorous compounds which are resistant to conventional therapies. Some new bis-pyridinium type oximes originating from the laboratory of Professor Hagedorn in Germany, when combined with atropine have been shown to be effective antidotes to soman poisoning in mice (Kepner and Wolthuis, 1978; Oldiges and Schoene, 1970; Wolthuis, et al., 1976), dogs (Schenk, et al., 1976), and monkeys (Lipp and Dola, 1980). Of these compounds HI-6 1-(2-hydroxyimino-methyl-1-pyridinium)-1-(4-carbamoyl-1-pyridinium)-dimethyl ether dichloride, has been shown to be the least toxic and most efficacious antidote against soman poisoning (Clement, 1981).

B. Chemistry and Assay of HI-6



1-(2-hydroxyimino-methyl-1--pyridinium)-1-(4-carbamoyl-1-pyridinium) dimethyl ether dichloride)

FIG. 2. Chemical Structure of HI-6

HI-6 is a high molecular weight compound with the chemical structure shown in figure 2. It is a bis-quaternary ammonium salt consisting of two pyridine rings joined by an alkyl chain. It is readily soluble in water giving solutions of about pH 3.5 at 250 mg/mL.

Using various congeners of HI-6, Bogdan (1981) has shown that the oxime group in position 2 (relative to the nitrogen atom) of the first pyridinium ring is critical for reactivation of soman-inhibited AChE. The amide group in position 4 of the second pyridinium ring was found to be responsible for the reduced toxicity of HI-6.

Until recently, the quantitative determination of oximes in both aqueous and biological materials was performed by spectrophotometry under alkaline conditions. The procedure is based on the formation of a yellow anion with a characteristic wavelength of absorption, at higher pH values (Vlet, 1966). One major problem with this procedure for HI-6 analysis is that the drug is unstable in alkaline conditions, and unless assay conditions are standardized strictly, variation in assay results is inevitable. The solutions have to be assayed immediately to minimize degradation. The procedure also lacks specificity and may include measurement of metabolites and other breakdown products if biological materials are being used (Benschop, et al., 1979).

A second method of analysis based on a trans-oximation reaction with p-nitrobenzaldehyde has also been described (Johnson, 1968), but is less

commonly used. Other methods that have been used for HI-6 (oxime) assay include the acid hydrolysis technique of Askew (1956), and scintillation counting of radiolabelled HI-6 (Bodor, 1975).

The problem of specificity in HI-6 assay has been solved by chromatographic separation followed by assay. Because of the quaternary ammonium groups in the structures of the oximes, ion-pair high performance liquid chromatography (HPLC) provides a satisfactory method for their separation and assay. Perchlorate and n-alkane sulphonates are frequently used as counter-ions in the mobile phase, and usually, surfactants are added to decrease the tailing of peaks. Several HPLC methods for oximes have been described in the literature. Brown, et al. (1978) used a method with a mobile phase consisting of acetonitrile aqueous buffer with n-heptanesulphonate as counter-ions. The system described by Benschop, et al. (1980) utilises a silica gel stationary phase and an eluent of methanol and acetic acid buffer.

C. Pharmacology

Various authors have reported HI-6 reactivation of soman-inhibited AChE in vitro (Dejong and Wolring, 1980; Wolthuis and Kepner, 1978; Schoene, 1973). However, there is controversy regarding the actual site and mechanism of action of HI-6 in vivo. Whereas it was the general belief that AChE-activating potency of HI-6 against soman intoxication was due to its ability to cross the blood brain barrier (Metter and Wolthuis, 1968; Adams, et al., 1976), Clement (1981) found that inhibition of AChE in the CNS is not the primary lesion in the lethality produced by soman. He suggested that the beneficial action of HI-6 in soman-poisoned rats is due to reactivation of diaphragm AChE. Although some HI-6 gained entry into the brain, the concentration was not sufficiently high to reactivate completely the soman-inhibited AChE.

Besides its ability to reactivate phosphonylated cholinesterase, some other mechanism of action seems to be contributing to the therapeutic effects of HI-6 against soman poisoning. Several authors have reported ganglion-blocking activity (Lundy, 1978; Lundy and Tremblay, 1979), antinicotinic activity (Clement, 1979) and antimuscarinic activity (Kuhnen-Clausen, 1972; Amitai, et al., 1980). Thus, whereas reactivation of inhibited AChE appears to be the primary action of HI-6, it is possible that the other non-enzymatic pharmacological actions of HI-6 (which lack in the other oximes) may be important in its therapeutic action versus soman and other OP anticholinesterases.

D. HI-6 Protein Binding

Studies on the use of HI-6 against organophosphorus poisoning are still in the pre-clinical stages. No human clinical studies have been published, but investigations in animals have indicated that the drug has potential as an antidote for soman poisoning.

The pharmacokinetics of HI-6 are currently under investigation. HI-6 is a very water soluble compound, and this may limit its penetration into the brain and other sites of action. Studies in animals have indicated that the distribution of HI-6 in the body greatly influences its effectiveness through any of the possible modes of action. Hence, the extent of binding of the drug to plasma proteins, if significant, will affect this distribution, and consequently, its pharmacological effects. The therapeutic bis-onium derivatives oximes generally exhibit low binding to plasma proteins, but specific information is required regarding HI-6 binding. Unpublished sources (Marshall, 1983), have indicated that there is little binding to rat serum, but no further work has been reported in this respect.

1.6 OBJECTIVES OF THE STUDY

1.6.1 GENERAL OBJECTIVES

Protein binding is an important pharmacological parameter affecting the clinical effects of many drugs. A simple, rapid and convenient procedure is required for routine determination of plasma free drug levels. Many methods are available, but few comparisons of the methods have been made with specific drugs under identical conditions. The protein binding results obtained for a given drug by different methods are often varied. Unfortunately, there are no standard drug-protein solutions containing a known fraction of unbound drug to calibrate the apparatus. The likelihood of a correct assessment of the true value for protein binding is increased if two or more methods are shown to give comparable values for the same drug.

The general aim of this study is to evaluate two equilibrium dialysis procedures for determining protein binding, and to compare the results with those obtained by an ultrafiltration procedure. Equilibrium dialysis using the knotted cellulose bag technique, and the Spectrum equilibrium dialyser (Dianorm) will be compared with ultrafiltration using Centriflo membrane cones. The drugs for study will include sulfisoxazole (a highly bound drug), theophylline (a medium bound drug) and HI-6 oxime (a low bound drug). The final goal of the study is to be able to recommend suitable procedures that can be used routinely in the clinical laboratory, to accurately determine levels of free drug for most drugs, thereby providing information which could give a better guide to therapy.

1.6.2 SPECIFIC OBJECTIVES

A. Evaluation of Protein Binding Procedures

The suitability of three methods of determining protein binding will be assessed using three drugs of varying degrees of binding. The effect of the methods on the apparent extent of binding will be investigated with respect to:

1. Temperature variation
2. Variation of drug concentration
3. Variation of protein concentration
4. Variation of pH

1. Effect of Temperature on Binding

The effect of temperature variation on the extent of binding of sulfisoxazole, theophylline and HI-6 will be examined using three methods. The binding of some drugs including phenytoin (Hooper, et al., 1973), and barbiturates (Smith, et al., 1968), have been shown to vary with temperature. Fedak (1980) has reported a decrease in the binding of theophylline to bovine serum albumin, with increasing temperatures, using equilibrium dialysis (bags) or ultrafiltration membrane cones.

Most recent protein binding studies which have been reported were carried out at 37°C. However, some investigators have reported results from studies at non-physiological temperatures (Keen, 1966; Fedak, 1980). It is important to know whether temperature variation will affect binding before carrying out protein binding studies, because the choice of temperature may have a significant effect on the results obtained.

2. Effect of Drug Concentration on Binding

The effect of drug concentration on the extent of binding of the three drugs will be investigated using the three methods, and the results of the individual methods compared. Most studies report binding at therapeutic drug concentration. Briggs, et al. (1983) have reported a decrease in the binding of sulfisoxazole with increasing drug concentration, using knotted cellulose bags. The extent of binding of theophylline has been reported to be independent of initial drug concentration (Fedak, 1980), although significant differences were observed in the results of the three methods used to study protein binding.

Many factors, both endogenous and exogenous, influence the protein binding process, resulting in variations in the free drug levels in the blood. The therapeutic effects of sulfisoxazole (Anton, 1960), and theophylline are dependent on their free plasma levels. It is therefore desirable to know how drug concentration affects binding, and whether the results obtained are influenced by the degree of binding of the drugs and the technique used to measure binding.

3. Effect of Protein Concentration on Binding

The effect of varying protein (albumin) concentration on the extent of binding of sulfisoxazole, theophylline and HI-6 will be investigated using three methods under standard conditions. Many pathophysiological factors may alter plasma protein concentration through changes in the synthesis, catabolism or distribution of the protein (Rowland, 1980). A 25% decrease in albumin concentrations may result in doubling of the free concentration of phenytoin (Porter, 1975) with consequent fatal results. Knowledge of how free drug concentration varies with that of the protein may be useful in achieving

optimum therapy with many drugs, especially those highly bound drugs with a narrow therapeutic index.

4. Effect of pH variation on Binding

The majority of protein binding studies reported in the literature contain few references to the effect of pH on the binding characteristics. Bicarbonate and carbon dioxide are part of a physiological buffer system in serum, playing a key role in pH control. Brors, et al. (1983) have shown that there is a marked increase in pH during a dialysis time of three hours, and that the use of non-physiological buffers, even at high concentrations, failed to maintain a stable pH. pH-dependent binding in human serum has been reported for a number of drugs including warfarin (Wilting, et al., 1980), propranolol and fusidic acid (Henry, 1981), imipramine (Kristensen and Gram, 1982), and theophylline (Brors, et al., 1983). An increase in binding was observed with increasing pH for all the drugs except fusidic acid. Binding decreased with increasing pH in studies on the latter drug.

The pH is also important when considering formulation of certain dosage forms such as liposomes and proteinaceous microspheres. In these techniques, the pH may fall well outside the normal physiological range. This could result in changes in the loading of the dosage form and affect the release characteristics of the dosage form. It is therefore important in such cases to know how pH affects the behaviour of the drug and protein.

The implication of pH dependency for drug serum binding is that pH control may be essential when running in vitro binding studies of some drugs. Binding data obtained at non-physiological or unknown pH values may be irrelevant to in vivo conditions. Most conventional dialysis experiments take more than 12 hours. It is probable that pH effects (if any) will be more pro-

nounced in this method than the other methods which take less time for equilibration.

In the present study, the two equilibrium dialysis methods and ultrafiltration will be compared with respect to pH change. The effect on the degree of binding of the three drugs of this change will be assessed.

B. Comparison of HI-6 Assay Procedures

Several analytical methods have been described for the quantitation of oximes in biological fluids, including hydrolysis (with hydroxamine) of the deproteinized supernatant, and direct spectrophotometric measurement of absorption of the oximate anion at 355 nm (Ellin and Kondritza, 1959; Creasy and Green, 1959). Over the past few years, modern liquid chromatography, and specifically HPLC, has become established as the method of choice for solving many biochemical and clinical analysis problems associated with the spectrophotometric assay procedure. It is generally more specific than spectrophotometric methods, and separates the parent compound from breakdown products and metabolites. However, HPLC, besides being expensive, is time-consuming and requires more skilled personel. The spectrophotometric assay is easier and faster to run, and is less expensive. It may prove useful in the routine day to day clinical situation.

The present study will compare the suitability of the above two methods as techniques for quantifying HI-6 in aqueous media.

2. EXPERIMENTAL.

2.1 MATERIALS

(a) Instruments and Equipment

Conventional equilibrium dialysis was done using 1.0 cm diameter cellu-

lose dialysis tubing[5], with a molecular weight cut-off of 12,000 to 14,000. The bags were placed in dialysing buffer contained in 16 x 125 mm glass culture tubes and closed tightly with PTFE-lined screw caps. Samples were rotated using a model 340 Rotorack[5]. The Spectrum equilibrium dialysis unit consisted of a 20-cell equilibrium dialyser system[11] with a variable speed drive unit[11], and 4 cell carriers[11]. Standardized 60 mm diameter semi-micro PTFE cells[11] and stoppers[11] were used with Spectropor 4 membrane tubing[11]. Ultrafiltration was performed using type CF50A Centriflo membrane cones[2] with a molecular weight cut-off of 50,000. The cones were supported by a polyethylene conical support[2]. Samples were centrifuged in a refrigerated International Centrifuge[6]. The ultrafiltrate was collected in polycarbonate model CT1 tubes[2]. All studies above room temperature were carried out in a PSC model No. 6 temperature-controlled incubator[9]. Sulfisoxazole and HI-6 spectrophotometric assays were done on an LKB 4050 spectrophotometer[7].

The HPLC system used in theophylline and HI-6 assay consisted of a Waters U6K injector[12], a model M-45 solvent delivery system, a model 480 LC spectrophotometer and an Omniscribe model B 5117-51 recorder. The column used was a μBondapak C_{18}, 3.9 cm internal diameter x 30 cm long. The conditions for HI-6 assay were;

---

1. Aldrich Chem. Co., Milwaukee.
2. Amicon Corp., Danvers, Mass.
3. Ames Co. Division, Miles Laboratories Ltd., Rexdale, Ont.
4. Beckman Instruments, Fullerton, CA.
5. Fisher Scientific Co., New Fair Lawn, N.J.
6. International Equipment Co., Needham, Mass.

Column: μBondapak C_{18}

Mobile phase: 20% acetonitrile/80% Paired-Ion buffer

Wavelength: 295 nm

Chart speed: 0.5 cm/min

Flow rate: 1.5 mls/min

Sensitivity: 0.02 AUFS

The conditions for Theophylline assay were:

Column: μBondapak C_{18}

Mobile phase: 9% acetonitrile/91% 0.1M Na acetate, pH 4.0

Wavelength: 280 nm

Chart speed: 0.5 cm/min

Flow rate: 2.0 mls/min

Sensitivity: 0.02 AUFS

(b) Chemicals

Sulfisoxazole[10], theophylline[8], and beta-hydroxy ethyl theophylline (βHET)[8] were used as obtained from the manufacturers without further purification. The HI-6 used was custom prepared by the Defense Research Establishment Suffield (DRES 32). Purified toxogonin was used as internal standard for HI-6 protein binding studies. Spectrograde acetonitrile[5] was used in the HPLC assay procedures. Clinical grade dextran[10] was used in the HI-6 protein

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7. LKB Biochrom. Ltd., Rockford, Ill.
8. Pierce Chemical Co., Ill.
9. Precision Scientific Co., Chicago, Ill.
10. Sigma Chemical Co., St. Louis, Montana.
11. Spectrum Med. Ind., Anex, LA, California.
12. Waters Associates, Millford, MA.

binding studies. Bovine serum albumin powder (Cohn fraction V)[10] was also used.

Sodium nitrite, ammonium sulphamate, N-(1-naphthyl-ethyleneammonium dihydrochloride), tris(hydroxymethyl)aminomethane, acetic acid, sodium acetate, 1-heptane sulfonic acid, tetramethylammonium chloride, and concentrated glacial acetic acid were all Standard Laboratory Grade reagents (Fisher). Buffers were prepared with laboratory grade hydrated monobasic and dibasic potassium phosphate and phosphoric acid.

Protein leakage was checked using protein test reagent strips (Albustix)[3].

(c) Solutions

All solutions for HPLC were prepared using double distilled water filtered through 0.45 uM pore diameter membrane filters[6] (lot No. 106 16). All other solutions were made with unfiltered double distilled water.

Sodium acetate 2M was made by dissolving 164.05 g in one litre of water. acetic acid 2M was prepared by diluting 144.4 mL of concentrated glacial acetic acid with water to one litre. To make 0.1M sodium acetate buffer at pH 3.0, 95 mL of 2M acetic acid was mixed with 750 mL of water. The pH was adjusted using the 2M sodium acetate (by titration), and the volume made to 1 litre with double distilled water. To prepare 1 litre of paired-ion buffer at pH 3.0, 2.2 g of 1-heptanesulfonic acid, 50 mL of 0.1M sodium acetate buffer and 0.27 g of tetramethylammonium chloride were mixed with 300 mL of water, and the pH adjusted using concentrated glacial acetic acid. The volume was adjusted to 1 litre before the solution was filtered.

Mobile phase for HI-6 assay was made by mixing 800 mL of the buffer with 200 mL of acetonitrile. Mobile phase for theophylline assay was made by

mixing 5 of 2M sodium acetate with 90 mL acetonitrile and making up to the final volume of 1 litre with water.

The buffer used in the spectrophotometric assay of HI-6 was prepared by dissolving 6.36 g of tris(hydroxymethyl)aminomethane in water and making it to the final volume of 1 litre.

2.2 METHODS

2.2.1 ASSAY PROCEDURES

(a) Sulfixoxazole in Phosphate Buffer

Sulfisoxazole was assayed using the method of Bratton and Marshall (1939) for sulfonamides. The standard calibration curve was prepared using samples containing 1.0, 2.0, 5.0, 10.0 and 20.0µg/mL of sulfisoxazole in phosphate buffer. All drug samples for assay were treated as follows: To 2.5 mL of sample, 260µl of 0.1% sodium nitrite was added and left to stand for 3 minutes. Ammonium sulfamate (250µL 0.5%) was then added, followed by 250 µL of N-(1-naphthyl)ethylenediamine dihydrochloride (colour developing reagent) after 2 minutes. Absorbance was then read at 545 nm using an LKB model 4050 spectrophotometer. All samples, both test and control, were referenced to a buffer blank treated similarly.

(b) HI-6 in Phosphate Buffer

Spectrophotometric assay of HI-6 was performed using a modification of the procedure of Vlet (1966). Tris buffer (4.0mL) of was added to 1.0mL of assay sample, and the resulting solution mixed thoroughly, then subjected to a spectrophotometric assay at 355 nm. All samples were referenced to a buffer blank. Replicate samples containing 5.0, 10.0, 20.0, 25.0 and 50.0µg/mL were

used to prepare standard calibration curves.

HPLC assay of HI-6 was done using a modification of the procedure of Brown, et al. (1978). To every 1.0mL of HI-6 assay sample was added 25µL of 400µg/mL toxogonin (internal standard). The resulting mixture was mixed on a vortex for 30 seconds, then 10 to 20µL of the sample was injected onto the column.

(c) Theophylline in Phosphate Buffer

Theophylline was assayed by HPLC using a modification of the procedure of Orcutt, et al. (1978). 100µL of assay sample was mixed with an equal volume of 15µg/mL BHET (internal standard), and mixed on a vortex for 30 seconds. The samples were then centrifuged for approximately 2 minutes, after which 5 to 15µL of the sample was injected onto the column.

Standard calibration curves were made using samples containing 2.5, 5.0, 10.0 and 20.0µg/mL of theophylline in phosphate buffer.

2.2.2 DETERMINATION OF PROTEIN BINDING

(a) Equilibrium Dialysis (Knotted Bags)

Dialysis was done using 20 cm strips of dialysis tubing of 1.0 cm diameter, 4.8 nm pore width and a molecular weight cut-off of 12,000 to 14,000. Prior to use, the membranes were immersed in boiling water and stirred for approximately 2 hours as the water cooled. The tubing was then transferred to 70% methanol and stirred for another 30 minutes. It was then rinsed in double distilled water and soaked in dialysing buffer for 2 hours before use. Tubing not required immediately was refrigerated for not longer than 3 days.

In use, the tubing was drained of excess fluid then tied at one end with a double knot. 1.0mL each of drug solution and protein solution was intro-

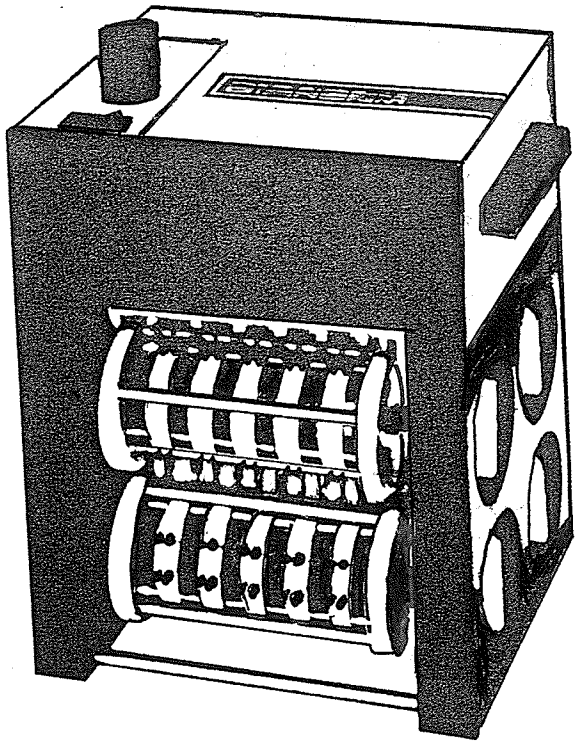
duced into the tubing through the open end which was then secured with a double knot. Both ends of the tubing were rinsed in double distilled water and buffer, then placed in glass culture tubes containing 4.0mL of dialysing buffer. The tubes were closed tightly with PTFE-lined screw caps. The samples were rotated on a Rotorack at 12 rpm for 24 hours. Preliminary studies had indicated that equilibrium was achieved at 16 to 20 hours, and no change in percent bound drug was noted for up to 36 hours.

After each dialysis, the dialysate was observed for protein leakage, both visually (presence of frothing), and using protein test reagent strips (Albustix). Samples were discarded if marked leakage of protein through the membrane had occured.

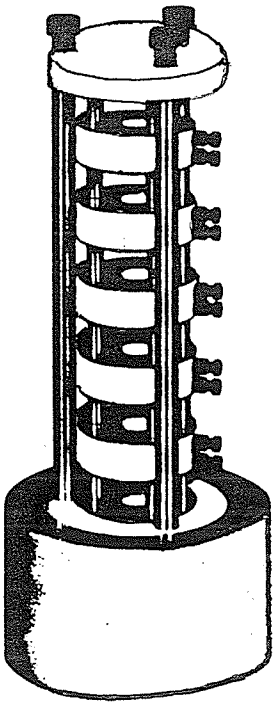
For each test sample dialysed, a corresponding control was also dialysed. The control sample was identical to the test sample except that the protein solution inside the membrane was replaced with an equal volume of buffer.

(b) Spectrum Equilibrium Dialysis (Dianorm)

In carrying out Spectrum equilibrium dialysis, PTFE semi-micro cells with a diameter of 6.0 cm and a total working volume of 1.36mL were used. Before being used for the first time, the cells were placed in a water bath at 30 to 40°C for 30 minutes, then all the stoppers were inserted immediately. Spectrapor 4 membrane with identical molecular weight and pore width specifications as described for the 'knotted bag' dialysis, was used. The 4.5 cm diameter tubing was cut into 6.0 cm length strips, soaked in water for 15 minutes, and then slit at both closed sides to produce single thickness membranes. The membranes were stirred in 30% ethanol for 20 minutes, rinsed in double distilled water, then placed in dialysing buffer for 2 hours before use.



(A) THE ASSEMBLED UNIT IN USE



(B) CELL CARRIER WITH ASSEMBLED CELLS

FIGURE 3. THE SPECTRUM EQUILIBRIUM DIALYSER

To carry out dialysis, a membrane strip was placed onto the female half cell which has a sealing lip. Pressure was applied at the edges to produce a smooth surface. The male half was then placed over the female half cell, uniformly sealing the membrane between the two Teflon components. The cells were then assembled in the cell carrier. To fill the cells, the assembled cell carrier was placed in a horizontal position with the filling holes adjacent to each other facing upwards. Keeping the third holes stoppered, 1.0mL of drug sample was introduced into one half cell, and an equal volume of albumin (test) or buffer (control) introduced into the other half, using a 5mL plastic syringe equipped with a 2-inch blunt end 23G needle. Care was taken not to puncture the membrane. The filled cells were stoppered and the cell carriers mounted into the drive unit (fig. 3a), and rotated at 12rpm at a constant temperature.

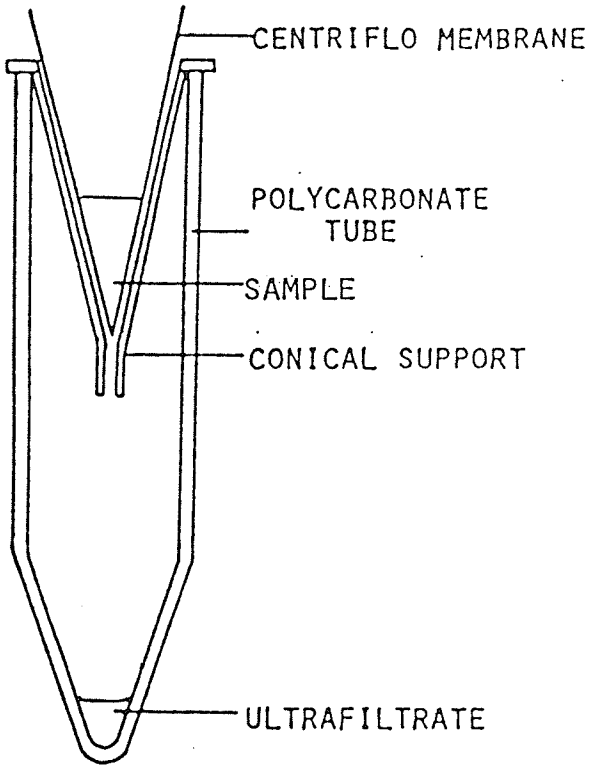
After 4 hours, the cell half containing the dialysate was drained through the third hole. A sample of the dialysate (0.5mL) was accurately pipetted into a clean tube and assayed for drug content. Before each assay, samples were tested for protein leakage using Albustix. Samples showing marked leakage were discarded.

For each test sample, a corresponding control was run in which the protein solution in the test sample was replaced with dialysing buffer.

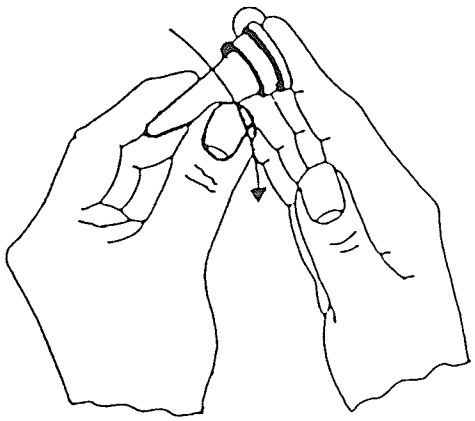
(c) Ultrafiltration

To prepare samples for ultrafiltration, double strength drug solution was combined with an equal volume of double strength albumin (test), or buffer (control) solution, and incubated at the working temperature, with constant agitation for two hours.

Ultrafiltration was carried out using type CF50A membrane cones with



(A) A UNIT IN USE



(B) INSERTING THE CONE

FIGURE 4. ULTRAFILTRATION MEMBRANE CONE APPARATUS.

approximately 95% retention for molecules with molecular weight above 50,000. Before use, the cones were soaked in double distilled water for at least one hour. Taking care not to scratch or crease the membrane interior, each membrane cone was placed into a polyethylene conical support, type CSIA, and the support rotated counterclockwise, pushing the cone down firmly until it was fully seated in the support with its tip protruding through the bottom (figure 4). The support was then placed in the top of a polycarbonate collecting tube, with the flange seated on the tube. The cones were centrifuged at 2,500 rpm for 10 minutes to remove excess water. The collecting tubes were replaced with dry ones, then 2.0mL of pre-equilibrated drug-protein sample was accurately pipetted into the dry cones and centrifuged at 1,500rpm and constant temperature for 15 minutes. These conditions had been shown to give an ultrafiltrate volume of not more than 0.15 mL, which was considered to give a minimal disturbance of the equilibrium.

After 15 minutes, a 0.1mL sample was accurately pipetted from the collecting tube and assayed for drug content. The rest of the collected sample was tested for protein leakage with Albustix. Samples that showed marked protein leakage were discarded and the leaking cones noted.

For each test sample, a corresponding control sample containing buffer in place of protein solution was prepared and run concurrently with the test samples.

The membrane cones were cleaned by soaking in a 5% sodium chloride solution for one hour, followed by several centrifugations and rinses in double distilled water. After the final centrifugation, the cones were stored either in the dry state or in 10% ethanol.

2.3 SPECIFIC PROTEIN BINDING STUDIES

2.3.1 The Effect of Drug Concentration on Binding

The effect of initial drug concentration on the binding of sulfisoxazole, theophylline and HI-6 by albumin was investigated using conventional equilibrium dialysis, Spectrum equilibrium dialysis (Dianorm) and ultrafiltration membrane cones. The study covered a sulfisoxazole and HI-6 concentration range of 50 to 250µg/mL, to encompass the possible range in human serum following a therapeutic dose. Drug concentrations of 50, 100, 150, 200 and 250µg/mL were used in both cases. Theophylline concentrations of 5, 15, 25, 50 and 75 µg/mL were used. In each study, the albumin concentration was constant at the approximate physiological concentration of 4.0% w/v and replicate test and control samples were prepared. The temperature and pH of the samples were kept constant at 37°C and 7.4 units, respectively. All drug and protein solutions were prepared initially at double the required strength, in pH 7.4 phosphate buffer. In the dialysis 'knotted bag' method, 1.0mL aliquot samples of drug and protein solutions were mixed in the dialysis tubing and dialysed against 4.0mL of buffer for 24 hours. Similar studies were done using the Dianorm and membrane cones as described. The "control calibration curve" procedure permitted direct comparison of assays with the standards run previously, allowing for the dilution by dialysate.

2.3.2 The Effect of Protein (Albumin) Concentration on Binding

The relationship between the concentration of protein and the extent of binding of sulfisoxazole, theophylline and HI-6 was studied over the protein concentration range of 1.0 to 8.0% w/v. Each of the drugs was investigated

using equilibrium dialysis (bags), the Spectrum equlibrium dialyser, and ultrafiltration membrane cones.

Theophylline binding was studied at a nominal concentration of 15ug/mL. Sulfisoxazole and HI-6 concentrations were kept constant at 200ug/mL. All solutions were made up in pH 7.4 phosphate buffer. Protein concentrations of 1.0, 2.0, 4.0, 6.0 and 8.0% w/v in phosphate buffer were used. The temperature of the samples was kept constant at 37°C.

For each protein concentration studied, replicate test and control samples were used. Equilibrium dialysis was performed in a temperature-controlled incubator for 24 hours with the bag technique and 4 hours with the Dianorm. Samples for ultrafiltration were pre-equilibrated at 37°C for two hours prior to ultrafiltration.

After appropriate equilibration times, samples were collected for assay of drug content. Protein leakage was tested for as described above.

2.3.3 The Effect of Temperature on Extent of Protein Binding

The effect of temperature on the binding of sulfisoxazole, theophylline and HI-6 to albumin was investigated in the range of 6 to 45°C (inclusive). Protein binding studies were done at 6°C, 25°C (room temperature), 37°C and 45°C, using conventional equilibrium dialysis, Spectrum equilibrium dialysis and ultrafiltration membrane cones.

In all the studies, protein concentration was kept constant at 4.0% w/v. Sulfisoxazole and HI-6 were used at a concentration of 200 ug/mL, while the theophylline concentration was 15 ug/mL throughout. All drug and protein solutions were made up in pH7.4 phosphate buffer. For each drug studied, the same stock solution of drug and protein was used throughout.

The 6°C study was done in a temperature controlled cold room. The 37°C and 45°C studies were carried out in a constant temperature incubator, while the 25°C study was run at room temperature.

Replicate samples were prepared for each temperature studied. A corresponding set of controls in which the protein solution in the test samples was replaced with buffer was run simultaneously with the test sample.

After the appropriate equilibration times, samples were removed and tested for protein leakage before being assayed for drug content.

2.3.4 The Effect of pH on Extent of Protein Binding

The effect of varying the pH of the drug and protein solutions on the extent of binding to albumin of sulfisoxazole, theophylline and HI-6 was examined. Equilibrium dialysis (bags), Spectrum equilibrium dialysis and ultrafiltration membrane cones were used to study the protein binding of the drugs.

The studies were conducted in 0.2M phosphate buffers of varying pH values ranging from 4.5 to 10.5 pH units. Buffers of pH 4.5, 5.8, 6.8, 7.4, 8.0 and 10.5 were prepared. For each pH value studied, drug and protein solutions were made in the appropriate buffers and used immediately. Samples of the stock solutions were reserved and analysed for the actual drug content.

Sulfisoxazole and HI-6 concentrations were kept constant at 200ug/mL. Theophylline concentrations of 15 ug/mL were used throughout. All dialysis studies were carried out at 37°C. Samples for ultrafiltration were incubated at 37°C for two hours prior to ultrafiltration at 37°C. The albumin concentration was kept constant at 4% w/v.

In all the experiments, test and control replicates were prepared. Samples were analysed for drug content after the appropriate equilibration times. Protein leakage was assessed as described above.

2.3.5 Comparison of HI-6 Assay Procedures

High performance liquid chromatography and the spectrophotometric assay

procedure were compared for their suitability as techniques for quantitation of HI-6 in aqueous media. Calibration curves were linear with standard solutions containing 5, 10, 20, 30, 40, 50 and 200μg/mL of HI-6 in pH7.4 phosphate buffer. Replicate samples for each concentration were assayed by HPLC and spectrophotometrically as described, and the results compared.

Studies were also done to determine whether there were any differences in the protein binding results obtained for HI-6 using the two methods of assay. Spectrum equilibrium dialysis and ultrafiltration membrane cones were used to study protein binding. The effect of varying temperature on the binding of HI-6 was investigated, as described, and the resulting samples assayed by HPLC and spectrophotometrically. An albumin concentration of 4.0% w/v was used. All solutions were made up in pH7.4 phosphate buffer.

In each study, replicate test and control samples were prepared. Protein leakage was tested for before each assay.

2.4 PROTEIN BINDING CALCULATIONS

A calibration curve was prepared from standard control values of varying drug concentrations for each of the procedures used to measure protein binding. This curve, which is referred to as the 'control calibration curve', (Briggs, et al, 1983) was used as a standard from which all test and control values were evaluated. It was used in order to account for the non-specific binding of the drug to sources other than the protein.

The percentage (%) drug bound was calculated for each of the control/test replicates by subtracting the test value from the control value and expressing the results as a percentage of the control, thus;

$$\% \text{ bound} = \frac{\text{control drug conc. } (\mu g/mL) - \text{Test drug conc. } (\mu g/mL)}{\text{control drug conc. } (\mu g/mL)} \times 100,$$

where the test drug concentration = Free (unbound) drug.

For each protein/drug system studied, mean percentages were calculated. An analysis of variance and Tukey's studentized range test were used to determine whether differences observed in the means of different systems were significant. Computer data analysis was done using the statistical analysis system (SAS) package. All tests were carried out at a 95% level of significance.

3. RESULTS AND OBSERVATIONS

3.1 ASSAY PROCEDURES

3.1.1 Sulfisoxazole in Phosphate Buffer

Numerical calibration curve data for sulfisoxazole in pH 7.4 phosphate buffer are presented in Table 1. The corresponding linear regression line is shown in Figure 5. The line fits the general equation $y = 0.0826X + 0.0305$, with a correlation coefficient of 0.9999. There is a linear relationship between the concentration of sulfisoxazole in phosphate buffer and optical density at 545 nm. Graphical and numerical 'control calibration curve' data obtained for sulfisoxazole using conventional equilibrium dialysis, spectrum equilibrium dialysis and ultrafiltration membrane cones, are presented in Figure 6 and Table 2. Binding data were calculated from the "calibration control" curves, which were in the concentration range of the standards, due to effective "dilution" by the dialysing solvent. A linear relationship between sulfisoxazole concentration and optical density was observed for each method, in the concentration range studied. Comparison of the linear regression parameters of the standard calibration curve of sulfisoxazole with those of the control calibration curves enabled calculation of the extent of non-specific binding of sulfisoxazole to the test apparatus (Table 9). All three

TABLE 1 CALIBRATION CURVE DATA FOR SULFISOXAZOLE IN PHOSPHATE BUFFER

Sulfisoxazole Conc. (µg/mL)	N	Absorbance at 545 nm ± S.E*
1.0	6	0.071 ± 0.002
2.0	6	0.138 ± 0.005
5.0	6	0.355 ± 0.005
10.0	6	0.639 ± 0.004
20.0	6	1.341 ± 0.01

*standard error of mean

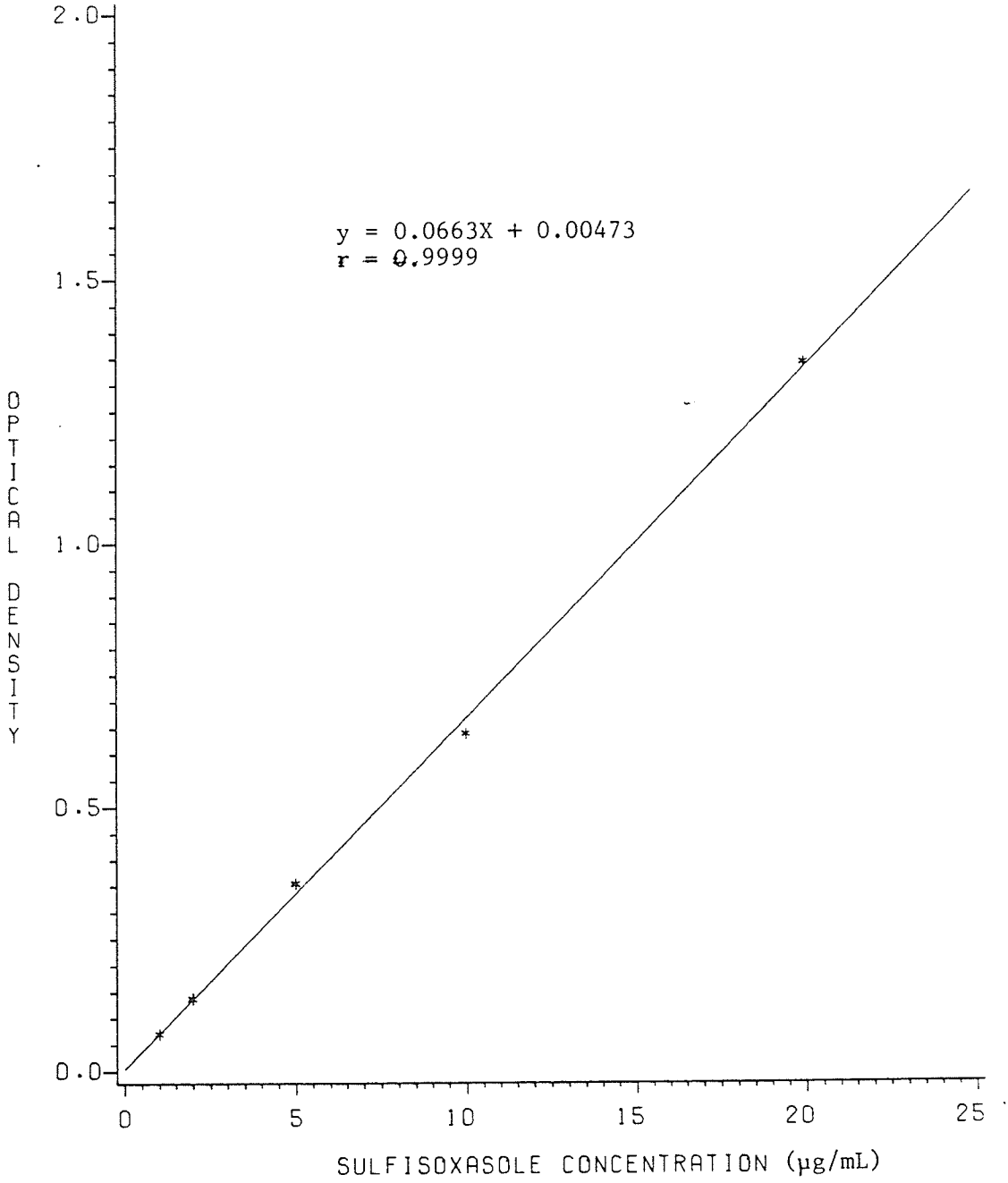


Figure 5. Calibration curve for sulfisoxazole in phosphate buffer.

TABLE 2 CONTROL CALIBRATION CURVE DATA FOR SULFISOXAZOLE

Method*	Sulfisoxazole Conc. (μg/mL)	N	Absorbance at 545 nm ± S.E
1	50	10	0.301 ± 0.01
1	100	10	0.613 ± 0.01
1	150	10	0.869 ± 0.02
1	200	10	1.144 ± 0.02
1	250	10	1.771 ± 0.01
2	50	10	0.304 ± 0.007
2	100	10	0.592 ± 0.02
2	150	10	0.963 ± 0.04
2	200	10	1.245 ± 0.04
2	250	10	1.566 ± 0.06
3	50	8	0.284 ± 0.02
3	100	8	0.592 ± 0.05
3	150	8	0.865 ± 0.02
3	200	8	1.139 ± 0.04
3	250	8	1.377 ± 0.05

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

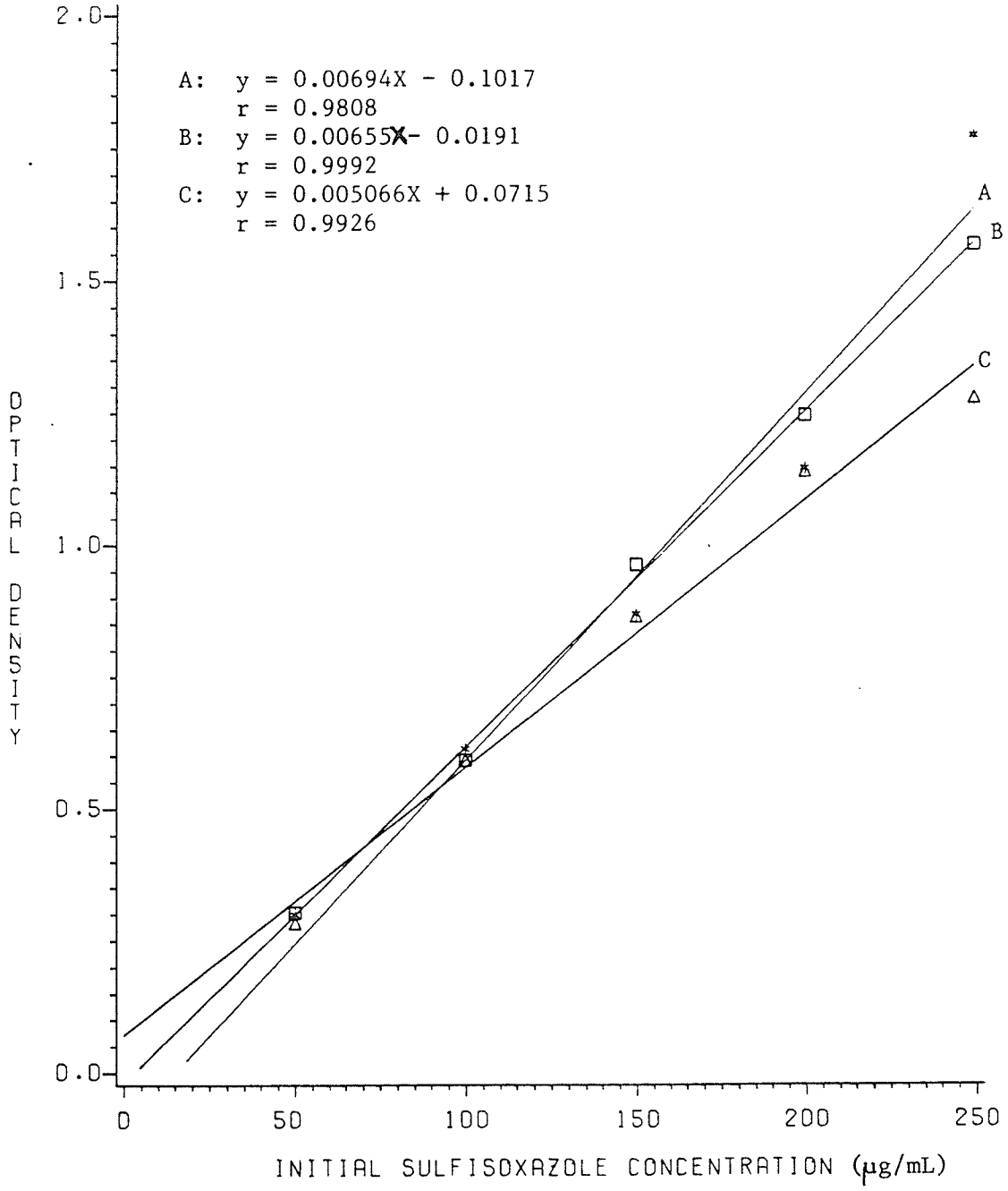


Figure 6. Control calibration curves for sulfisoxazole as determined by A-equilibrium dialysis (bags) (*), B-spectrum equilibrium (□) and C-Ultrafiltration membrane cones (△).

methods exhibited a high degree of non-specific binding of sulfisoxazole, although the Dianorm showed slightly lower binding than the other two methods. Conventional equilibrium dialysis and ultrafiltration showed comparable values (10.9% and 10.8%, respectively).

3.2.2 THEOPHYLLINE IN PHOSPHATE BUFFER

Figure 7 is an HPLC chromatogram showing the separation of theophylline and beta-hydroxyethyl theophylline (BHET) in pH 7.4 phosphate buffer. Both peaks were separate, distinct and reproducible, with approximate retention times of 4.6 minutes and 6.0 minutes respectively, for theophylline and the beta-hydroxyethyl derivative.

Theophylline calibration curve data are presented graphically in Figure 8. Numerical data are shown in Table 3. The data fit the linear regression equation $Y = 0.1256X + 0.0711$, with a correlation coefficient of 0.9994. In the concentration range standard, a linear relationshiop exists between the quantity of theophylline and BHET in phosphate buffer and their corresponding peak heights.

Control calibration curve data obtained for theophylline using equilibrium dialysis bags, the Dianorm and ultrafiltration, are presented in Table 4. Linear regression lines with their corresponding equations and correlation coefficients are shown in Figure 9. All three methods gave a linear relationship between theophylline and BHET concentrations in buffer, and their corresponding peak heights. Peak height ratios obtained using ultrafiltration were generally much lower than those obtained using the equilibrium dialysis methods, implying higher binding of theophylline to the ultrafiltration cones. Conventional dialysis resulted in the smallest degree of non-specific binding of theophylline (Table 9).

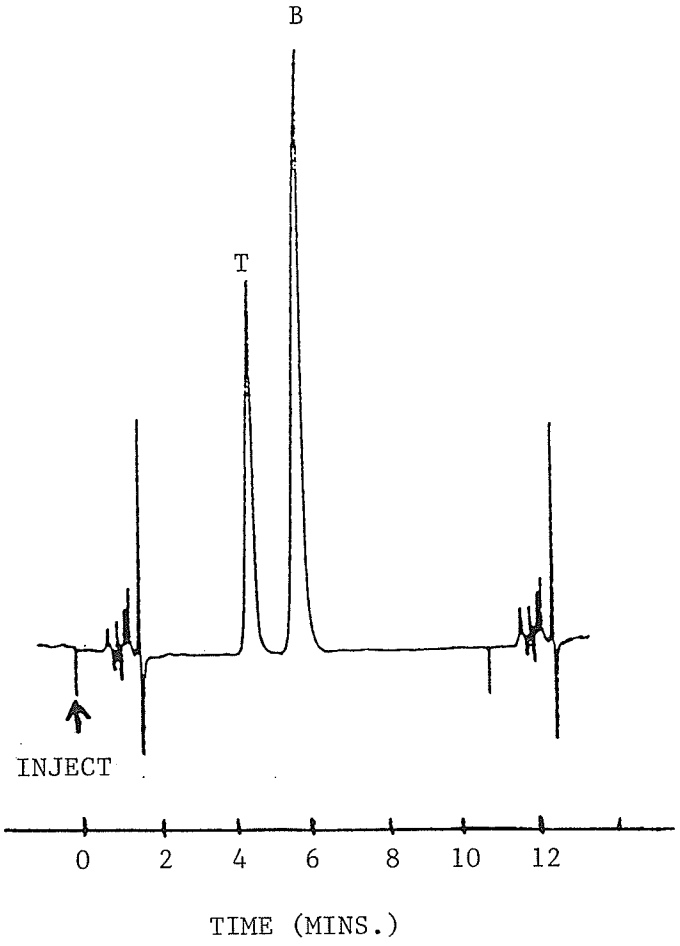


Figure 7. Chromatogram of a sample mixture of 15 μg/mL each of theophylline (T) and B-hydroxy ethyl theophylline (B).

TABLE 3 CALIBRATION CURVE DATA FOR THEOPHYLLINE IN PHOSPHATE BUFFER

Theophylline Conc. (µg/mL)	N	Peak Height Ratio* ± S.E
2.5	5	0.368 ± 0.01
5.0	5	0.685 ± 0.01
10.0	5	1.378 ± 0.02
20.0	5	2.563 ± 0.08

*Peak height ratio = Theophylline peak height/beta-hydroxyethyl theophylline peak height

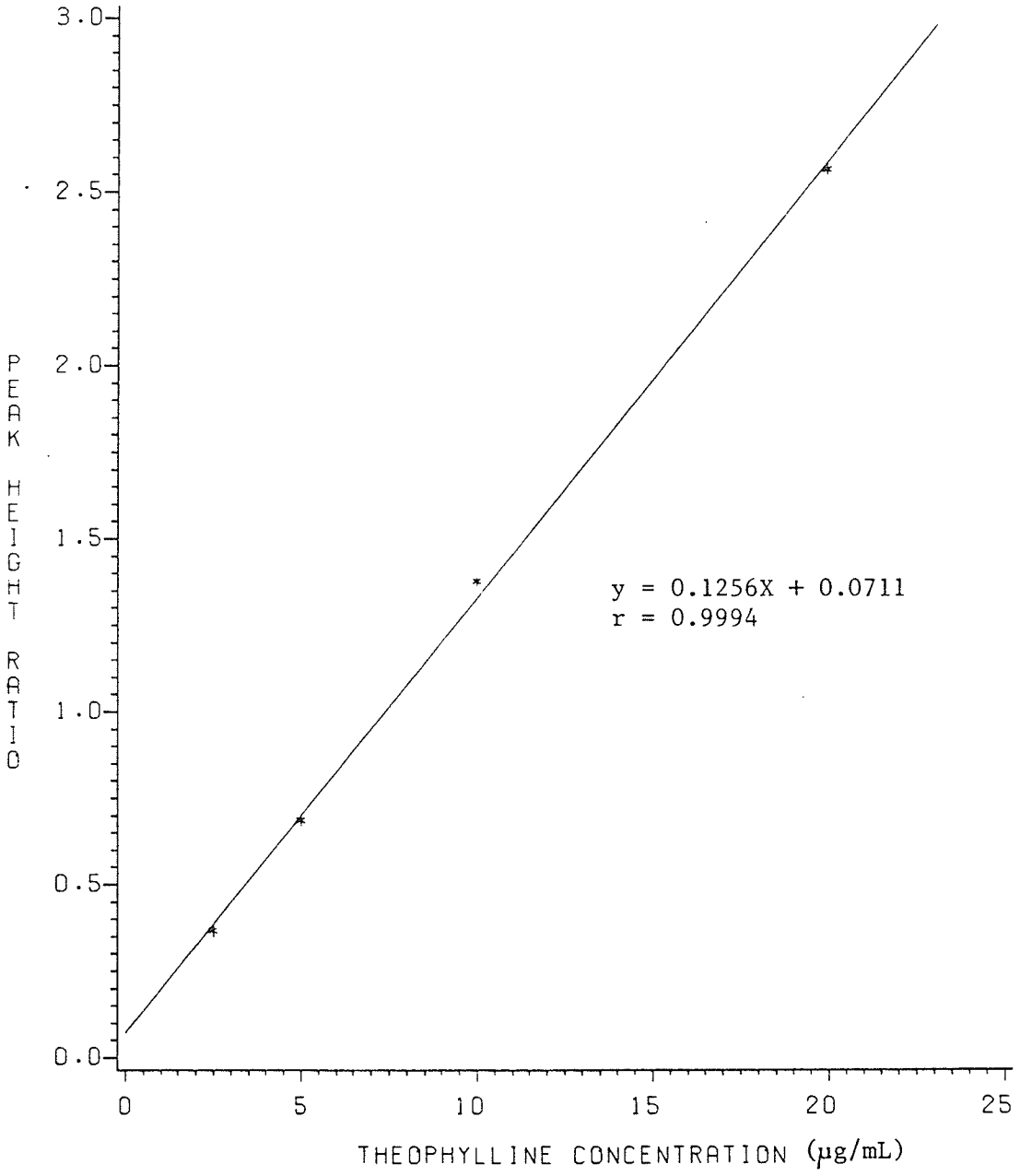


Figure 8. Calibration curve for theophylline in phosphate buffer.

TABLE 4 CONTROL CALIBRATION CURVE DATA FOR THEOPHYLLINE

Method*	Theophylline Conc.(µg/mL)	N	Peak Height Ratio ± S.E
1	5.0	10	0.198 ± 0.01
1	15.0	10	0.623 ± 0.08
1	25.0	10	1.072 ± 0.08
1	50.0	10	2.079 ± 0.08
1	75.0	10	3.058 ± 0.10
2	5.0	10	0.196 ± 0.03
2	15.0	10	0.585 ± 0.05
2	25.0	10	1.028 ± 0.08
2	50.0	10	1.983 ± 0.02
2	75.0	10	2.909 ± 0.18
3	5.0	8	0.191 ± 0.004
3	15.0	8	0.543 ± 0.06
3	25.0	8	0.984 ± 0.05
3	50.0	8	1.683 ± 0.08
3	75.0	8	2.465 ± 0.13

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

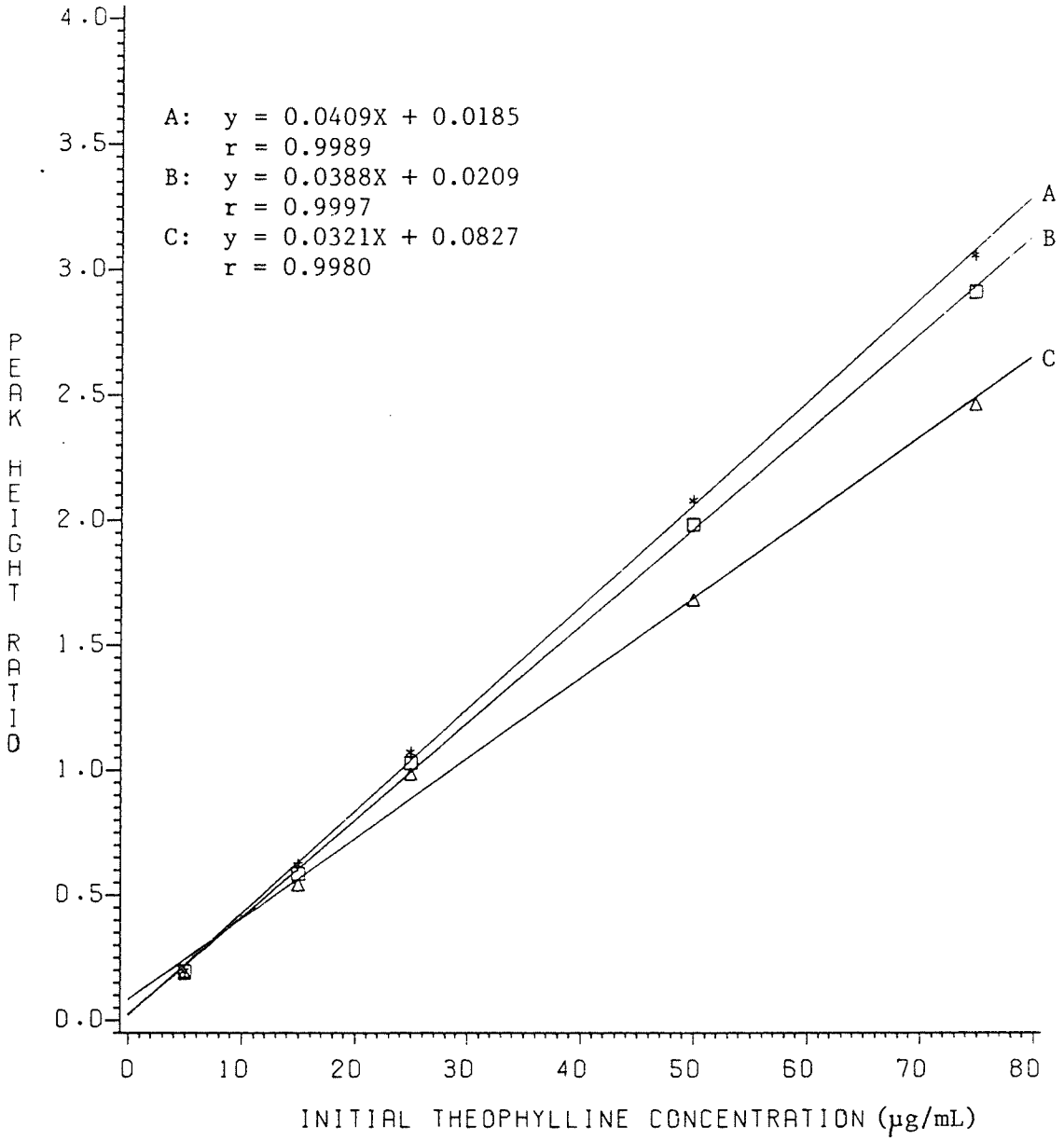


Figure 9. Control calibration curves for theophylline as determined by A-Equilibrium dialysis-bags (*), B-Spectrum equilibrium dialysis (□) and C-Ultrafiltration membrane cones (△).

3.1.3 HI-6 IN PHOSPHATE BUFFER

A sample HPLC chromatogram showing the separation of HI-6 and obidoxime (toxogonin) in pH 7.4 phosphate buffer is shown in Figure 10. Both peaks were separate, distinct and reproducible with approximate retention times of 7.9 minutes and 11.2 minutes, for HI-6 and toxogonin, respectively.

Spectrophotometric and HPLC calibration curve data for HI-6 in pH 7.4 phosphate buffer are shown in Tables 5 and 7, respectively. Graphical data are presented in Figures 11 and 12 with linear regression equations and respective correlation coefficients. It can be seen from the results that there is a positive linear relationship between the concentration of HI-6 in phosphate buffer and both optical density at 355 nm, and HPLC peak height ratios of HI-6 and toxogonin.

Spectrophotometric control calibration curve data for HI-6 obtained from equilibrium dialysis bags, Spectrum equilibrium dialysis and ultrafiltration membrane cone studies, are presented in Table 7 and Figure 13. Spectrum equilibrium dialysis and ultrafiltration HPLC control calibration data are shown ind Table 8 and Figure 14. A linear relationshiop was observed between HI-6 concentration in phosphate buffer and both optical density and peak heights ratio obtained in chromatographic separation.

All methods resulted in very little non-specific binding of HI-6 to the test apparatus (<2%).

3.2 PROTEIN BINDING STUDIES

The protein binding of HI-6 to bovine serum albumin in vitro at approximate physiological conditions was found to be around 5% when determined using ultrafiltration membrane cones. There was considerable variation between replicates, values of percent bound HI-6, ranging from less than 1 percent to

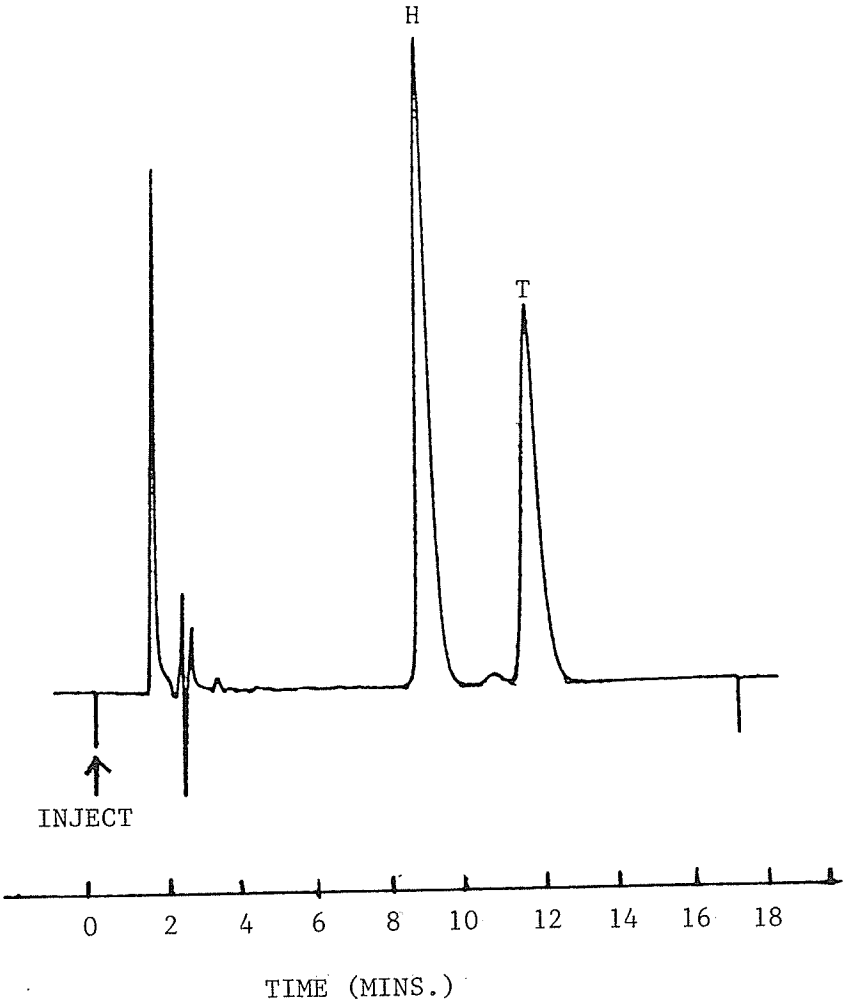


Figure 10. Chromatogram of a sample mixture of HI-6 (H) and Toxogonin (T).

TABLE 5 SPECTROPHOTOMETRIC CALIBRATION CURVE DATA FOR HI-6 IN PHOSPHATE BUFFER

HI-6 Conc. (µg/mL)	N	Mean Absorbance ± S.E*(355 nm)
10.0	6	0.081 ± 0.004
20.0	6	0.163 ± 0.005
30.0	6	0.238 ± 0.007
40.0	6	0.324 ± 0.007
50.0	6	0.398 ± 0.003

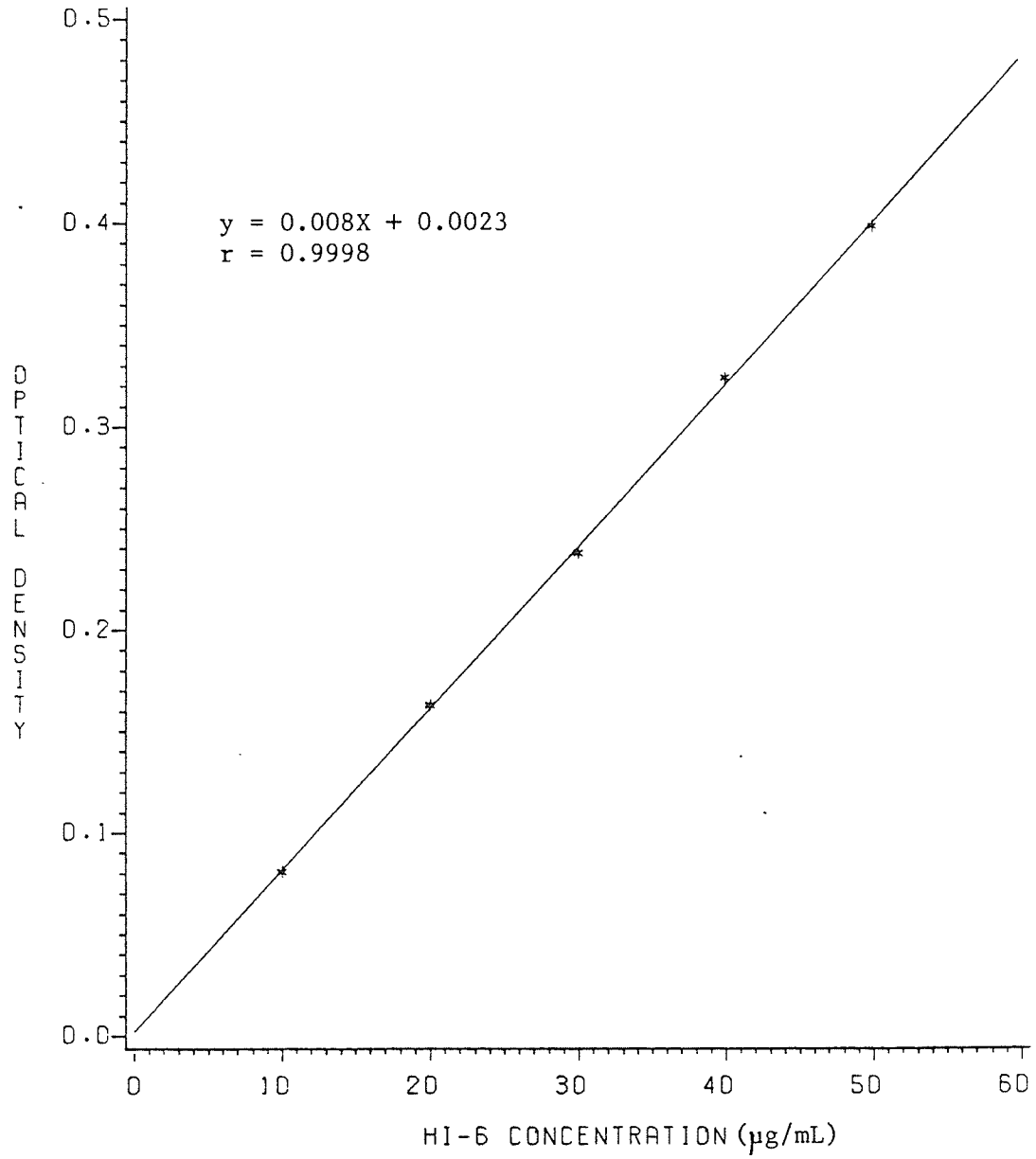


Figure 11. Spectrophotometric calibration curve for HI-6 in phosphate buffer.

TABLE 6 SPECTROPHOTOMETRIC CONTROL CALIBRATION CURVE DATA FOR HI-6

Method*	HI-6 Conc. (µg/mL)	N	Absorbance at 355 nm ± S.E
1	50	10	0.134 ± 0.007
1	100	10	0.269 ± 0.008
1	150	10	0.372 ± 0.01
1	200	10	0.519 ± 0.003
1	250	10	0.643 ± 0.02
2	50	10	0.136 ± 0.006
2	100	10	0.266 ± 0.01
2	150	10	0.375 ± 0.01
2	200	10	0.534 ± 0.02
2	250	10	0.675 1 0.02
3	50	8	0.137 ± 0.01
3	100	8	0.267 ± 0.02
3	150	8	0.361 ± 0.04
3	200	8	0.541 ± 0.004
3	250	8	0.696 ± 0.01

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

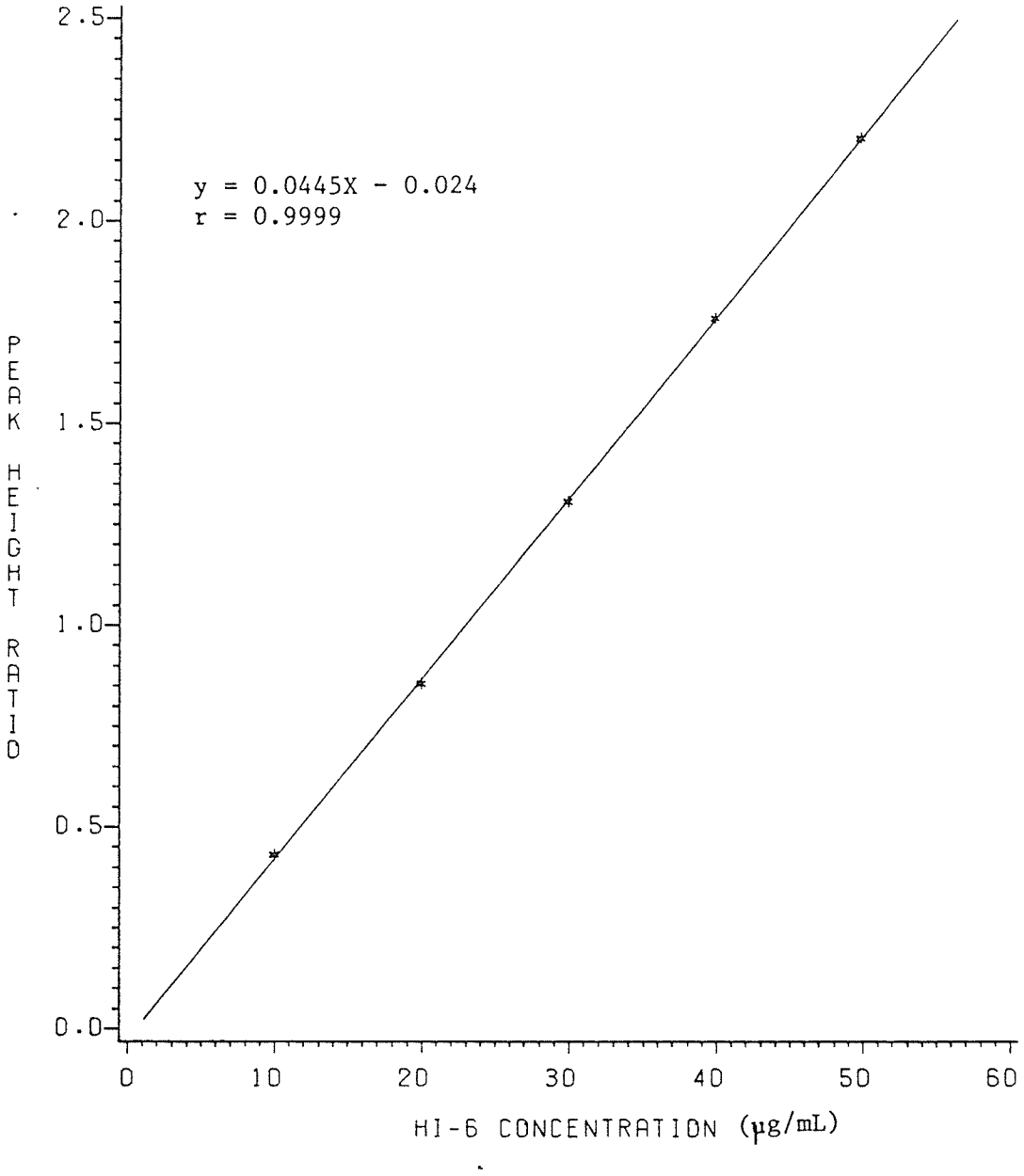


Figure 12. HPLC calibration curve for HI-6 in phosphate buffer.

TABLE 7 HPLC CALIBRATION CURVE DATA FOR HI-6 IN PHOSPHATE BUFFER

HI-6 Conc.(µg/mL)	N	Peak height ratio ± S.E
10.0	5	0.430 ± 0.03
20.0	5	0.854 ± 0.008
30.0	5	1.305 ± 0.009
40.0	5	1.758 ± 0.03
50.0	5	2.204 ± 0.02

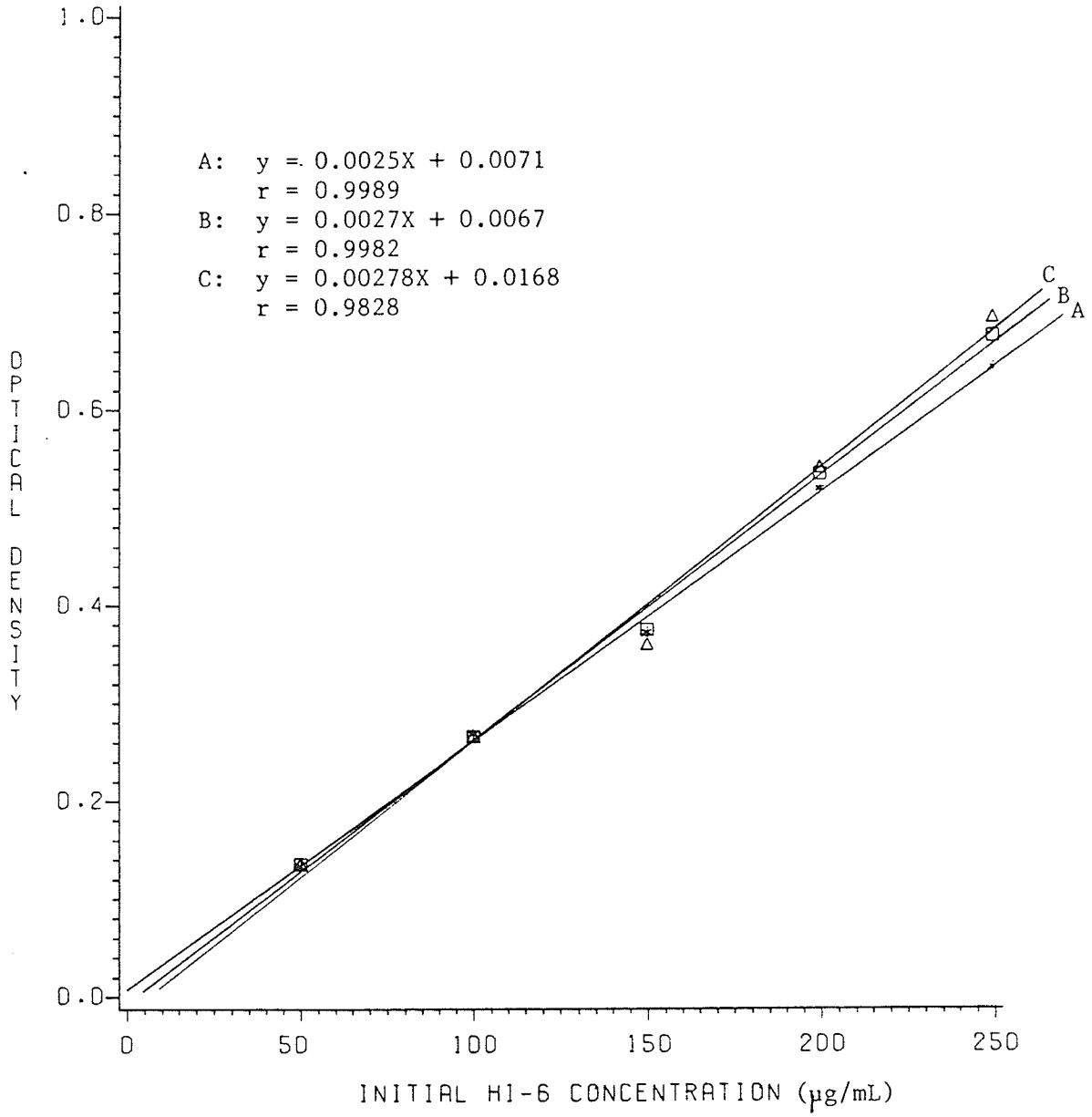


Figure 13. Spectrophotometric control calibration curves for HI-6 as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 8 HPLC CONTROL CALIBRATION CURVE DATA FOR HI-6

Method*	HI-6 conc. (µg/mL)	N	Mean peak height ratio ± S.E
2	50	10	0.713 ± 0.01
2	100	10	1.297 ± 0.009
2	150	10	1.962 ± 0.006
2	200	10	2.654 ± 0.003
2	250	10	3.419 1 0.003
3	50	8	0.695 ± 0.005
3	100	8	1.396 ± 0.04
3	150	8	2.108 ± 0.003
3	200	8	2.915 ± 0.06
3	250	8	3.429 ± 0.009

*Method 2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membranes curve

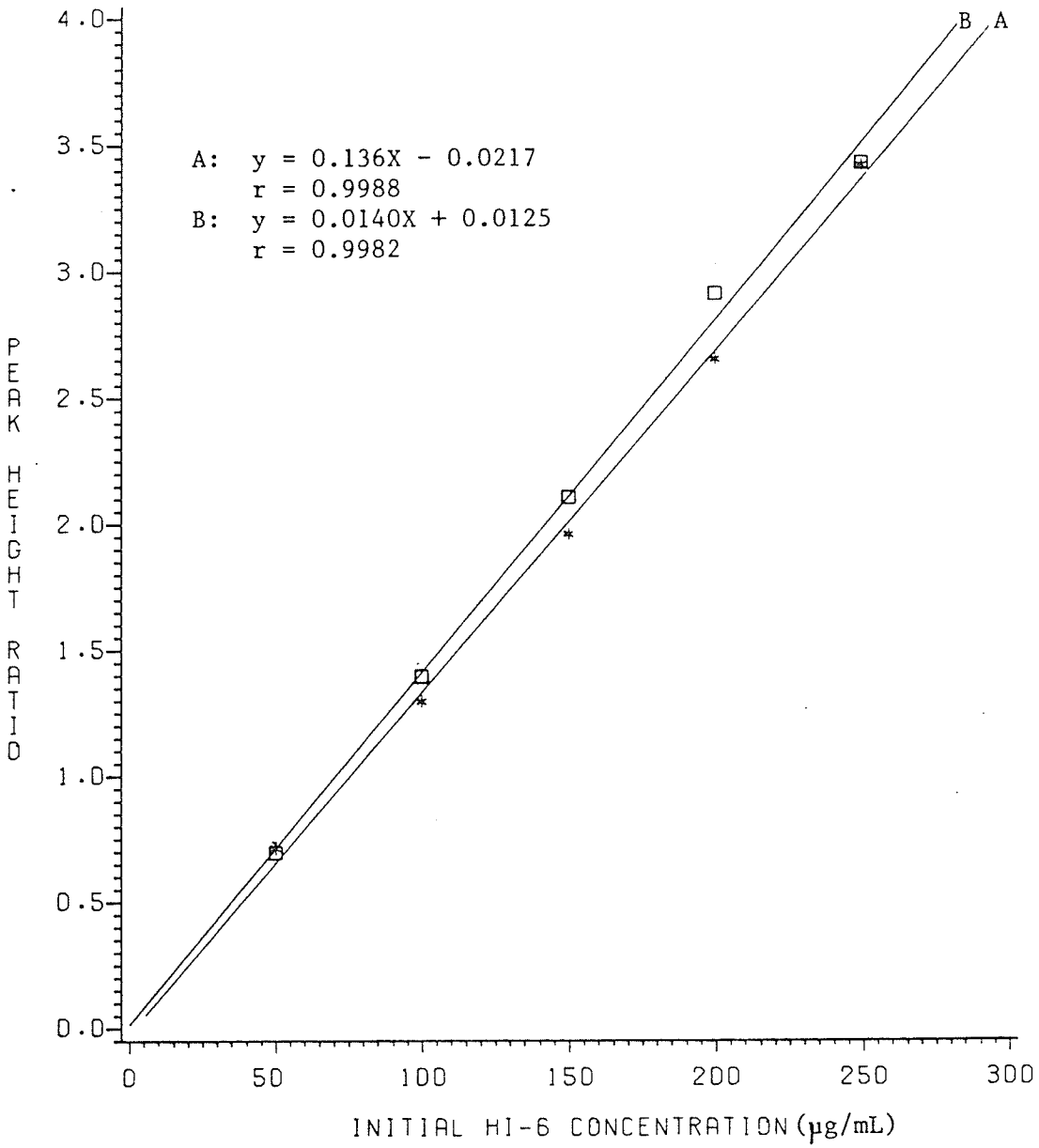


Figure 14. HPLC control calibration curves for HI-6 as determined by A-spectrum equilibrium dialysis (*), and B-Ultrafiltration membrane cones (□).

TABLE 9 NON-SPECIFIC BINDING OF SULFISOXAZOLE, THEOPHYLLINE AND HI-6 TO TEST APPARATUS AT 'THERAPEUTIC' CONCENTRATIONS

		METHOD		
Drug	Conc. (µg/mL)	Equilibrium dialysis (bags)	Spectrum equilibrium dialysis	Ultrafiltration Membrane Cones
Sulfisoxazole	100	10.9%	7.4%	10.8%
Theophylline	15	3.2%	7.5%	12.7%
HI-6	100	1.9%	1.3%	1.7%

Results are based on all determinations done at these concentrations during the study.

13.92 percent. Slightly lower values of percent bound HI-6 were obtained using Spectrum equilibrium dialysis, with a similar high degree of variability between replicates. The "signal to noise" ratio would greatly affect low readings.

It was observed that conventional equilibrium dialysis (bags), generally gave 'test' values that were higher than the corresponding 'control' values for HI-6. This made it impossible to calculate the extent of binding of HI-6 from the results obtained using this method. A sample of the test results obtained by varying HI-6 concentration from 50 µg/mL to 250 µg/mL is presented in Table 10. Similar results (not shown) were obtained for the temperature, pH and protein concentration studies using this method of studying protein binding.

In a further study, 2% dextran was added to the buffer of test sample to establish whether the anomaly in protein binding results of HI-6 could be abolished. The problem was suspected to be due to osmotic (osmosis-induced) volume shifts. The results of this study are presented in Table 11. Although there was a slight decrease in the test values, the change was not sufficient to correct the problem and allow calculation of binding.

For each of the three drugs the value for the percent bound drug as determined by the ultrafiltration technique generally was higher than that obtained by the equilibrium dialysis procedures. Conventional equilibrium dialysis most commonly produced the lowest values.

3.2.1 EFFECT OF DRUG CONCENTRATION ON EXTENT OF BINDING OF SULFISOXAZOLE, THEOPHYLLINE AND HI-6.

Numerical data for percent bound sulfisoxazole obtained at different drug concentrations, are presented in Table 12. Corresponding graphical data are presented in Figure 15. The data fit the general equations, $Y = -0.1453X + 106.19$; $Y = -0.1134X + 103.53$; and $Y = -0.0776X + 101.66$, with correlation

TABLE 10 EFFECT OF DRUG CONCENTRATION ON PROTEIN BINDING OF HI-6 AS DETERMINED BY EQUILIBRIUM DIALYSIS (BAGS)

HI-6 Conc.(µg/mL)	Control O.D* at 355 nm	Test O.D at 355 nm	Control-Test O.D
50	0.117	0.112	+0.005
50	0.109	0.134	-0.025
50	0.138	0.139	-0.001
50	0.122	0.132	-0.010
100	0.254	0.278	-0.024
100	0.207	0.290	-0.083
100	0.300	0.266	-0.034
100	0.278	0.289	-0.011
150	0.381	0.419	-0.038
150	0.356	0.408	-0.052
150	0.366	0.463	-0.097
150	0.380	0.441	-0.061
200	0.498	0.587	-0.081
200	0.518	0.562	-0.044
200	0.523	0.578	-0.055
200	0.518	0.605	-0.087
250	0.655	0.696	-0.041
250	0.647	0.677	-0.030
250	0.661	0.659	-0.002
250	0.652	0.690	-0.038

*optical density

TABLE 11 EFFECTS OF 2% DEXTRAN ON PROTEIN BINDING OF HI-6 AS DETERMINED BY EQUILIBRIUM DIALYSIS (BAGS) AT 200 µG/ML HI-6.

Control O.D* at 355 nm	Test O.D. (No dextran)	Test-Control O.D	Test O.D (+2% dextran)	Test-Control O.D
0.509	0.580	-0.071	0.499	0.010
0.486	0.579	-0.093	0.502	-0.016
0.492	0.512	-0.020	0.514	-0.022
0.493	0.587	-0.094	0.486	0.007
0.502	0.583	-0.081	0.508	-0.006
0.517	0.617	-0.100	0.497	0.020
0.498	0.493	-0.005	0.505	-0.007
0.500	0.561	-0.061	0.487	0.013
0.514	0.534	-0.020	0.503	-0.020

*O.D. = optical density.

coefficients of 0.9975, 0.9949, and 0.9872, for conventional equilibrium dialysis, the Dianorm and ultrafiltration, respectively. A negative linear relationship was observed between initial sulfisoxazole concentration and percent drug bound. Although the amount of sulfisoxazole bound to albumin increased with increasing initial concentration (Figure 16), percent bound drug decreased from 99.38% at 50 μg/mL initial sulfisoxazole, to 70.84% at 250 μg/mL, using conventional equilibrium dialysis. A similar trend was observed using the other two methods of studying binding.

The results of the analysis of variance showed that drug concentration and method of determining binding can affect the apparent percent-bound sulfisoxazole. Tukey's studentized range test on the data at the 95% level of significance was used to compare the results. For a given method of determining binding, all means of percent bound sulfisoxazole at the different drug concentrations were shown to be different from each other. Differences were also found between the means of percent drug bound determined by all three methods at higher sulfisoxazole concentrations. No significant difference was observed in the means obtained using Spectrum equilibrium dialysis and ultrafiltration at 50 μg/mL or 100 μg/mL (Table 13).

The results of percent bound theophylline at different drug concentrations are presented in Table 14 and Figure 17. Figure 18 is a plot of initial theophylline concentration versus the amount of drug bound. Linear regression parameters are also shown. The amount of theophylline bound was observed to increase with increasing initial theophylline concentration, although the fraction bound remained reasonably constant throughout the concentration range studied.

All three methods gave statistically different means for percent bound theophylline at each theophylline concentration studied (Tukey's test; P =

TABLE 12 EFFECT OF DRUG CONCENTRATION ON PROTEIN BINDING OF SULFISOXAZOLE

Method*	Initial Sulfisoxazole Conc.(µg/mL)	N	Mean % Bound ± S.E
1	50	10	99.38 ± 0.18
1	100	10	91.92 ± 0.22
1	150	10	83.49 ± 0.11
1	200	10	76.33 ± 0.33
1	250	10	70.84 ± 0.27
2	50	10	98.76 ± 0.45
2	100	10	91.87 ± 0.38
2	150	10	85.12 ± 0.32
2	200	10	81.05 ± 0.19
2	250	10	70.82 ± 0.46
3	50	8	98.82 ± 0.26
3	100	8	92.60 ± 0.39
3	150	8	89.44 ± 0.50
3	200	8	87.04 ± 0.31
3	250	8	82.19 ± 0.63

*1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

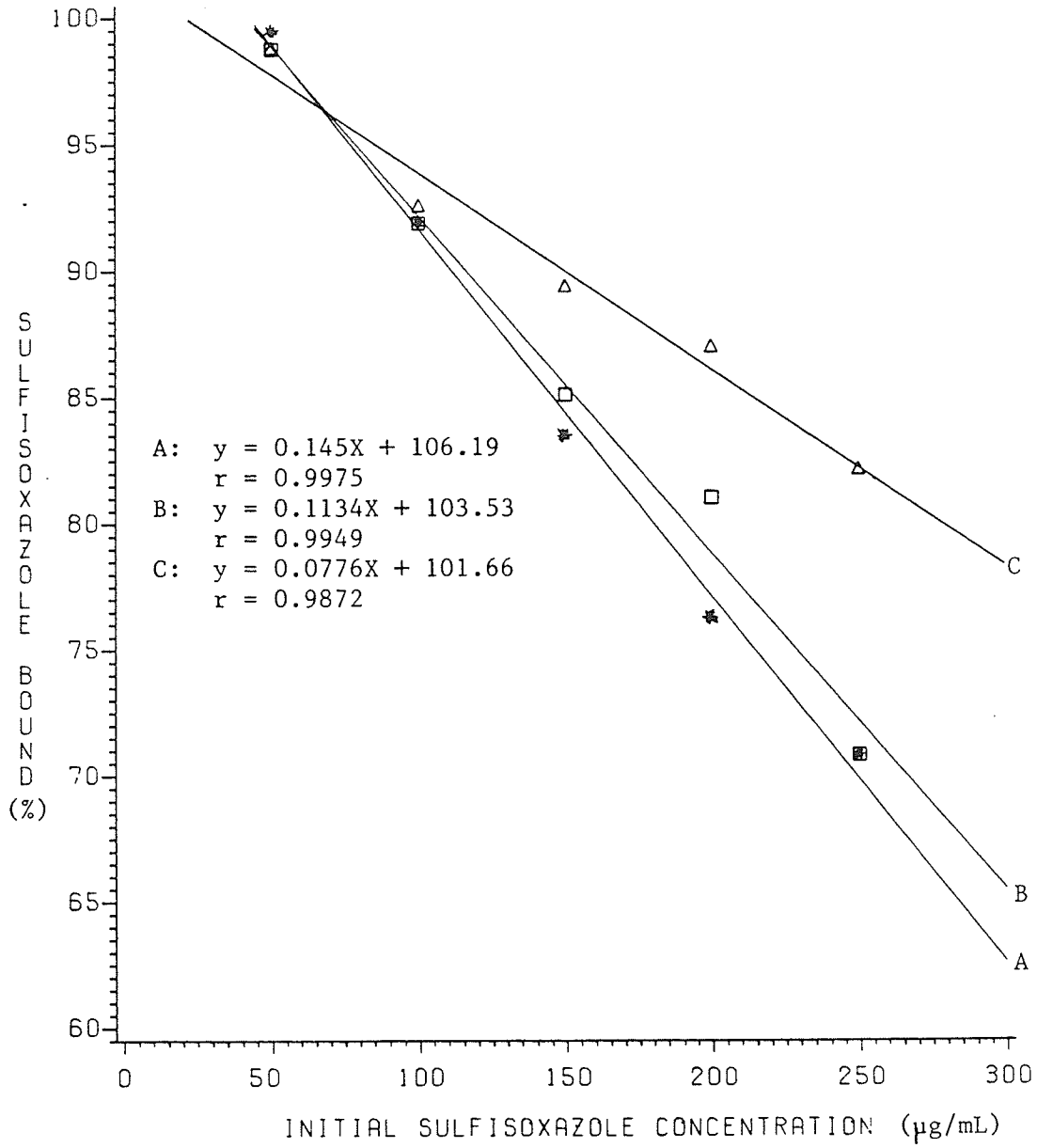


Figure 15. Effect of initial sulfisoxazole concentration on percent bound as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

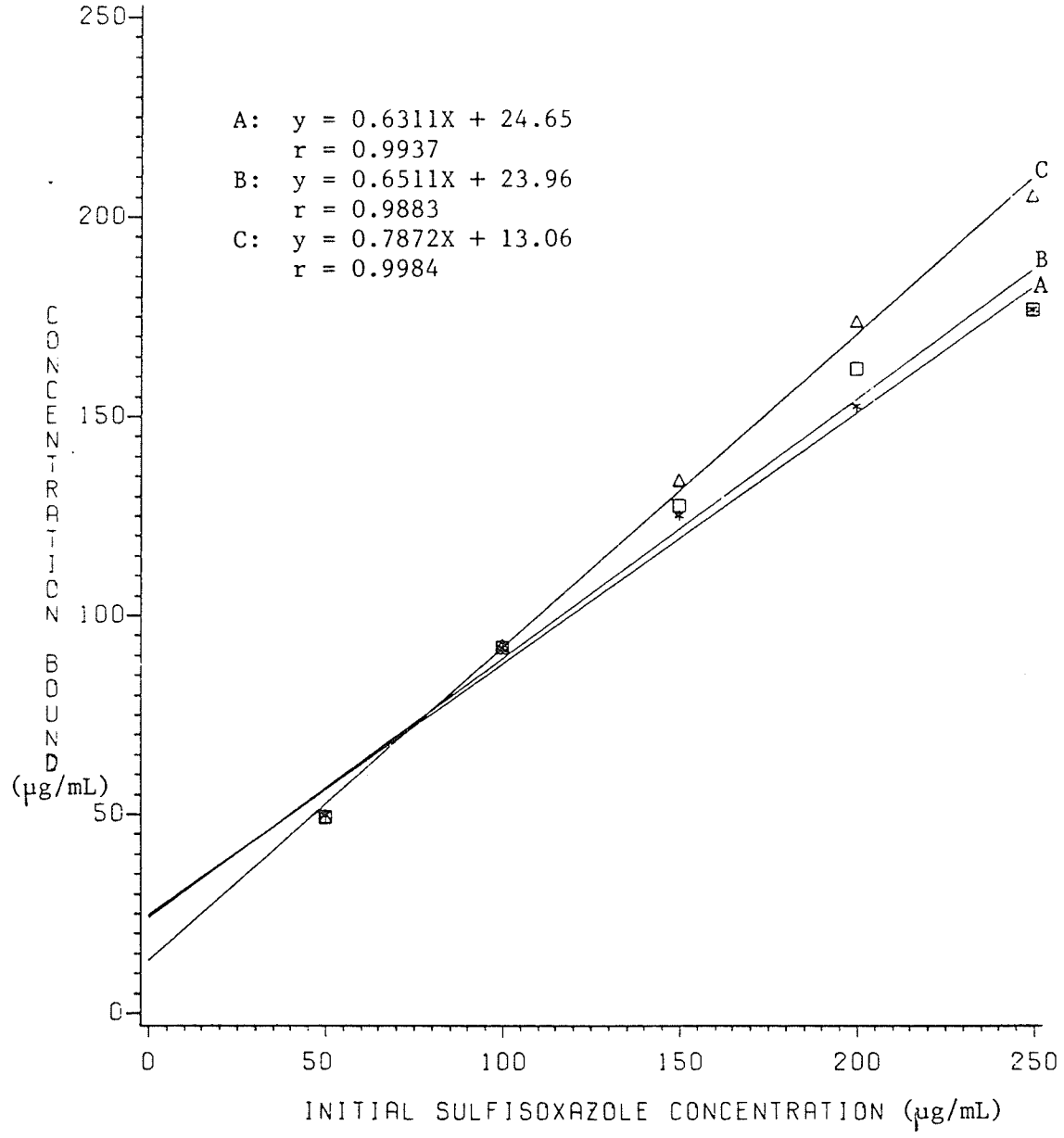


Figure 16. Sulfisoxazole concentration bound vs. initial sulfisoxazole concentration as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-ultrafiltration membrane cones (Δ).

TABLE 13 EFFECT OF DRUG CONCENTRATION AND METHOD ON PROTEIN BINDING OF SULFISOXAZOLE

	METHOD					
	Equilibrium Dialysis(Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
Sulfisoxazole Conc.(μg/mL)	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
50	10	99.39[a]*	10	98.76[a]	8	98.82[a]
100	10	91.62[b]*	10	91.87[b]	8	92.60[b]
150	10	83.49[c]*	10	85.12[c]*	8	89.44[c]*
200	10	76.33[d]*	10	81.05[d]*	8	87.04[d]*
250	10	70.84[e]*	10	75.82[c]*	8	82.19[e]*

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same drug concentration are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 14 EFFECT OF DRUG CONCENTRATION ON PROTEIN BINDING OF THEOPHYLLINE

Method*	Initial Theophylline Conc.(μg/mL)	N	Mean % Bound ± S.E
1	5	9	19.83 ± 0.72
1	15	10	18.93 ± 0.50
1	25	10	19.01 ± 0.38
1	50	10	20.21 ± 0.21
1	75	10	19.92 ± 0.47
2	5	10	26.93 ± 0.56
2	15	10	24.29 ± 0.56
2	25	10	23.63 ± 0.10
2	50	10	24.84 ± 0.75
2	75	10	24.83 ± 0.62
3	5	8	37.87 ± 0.29
3	15	8	39.60 ± 0.46
3	25	8	37.01 ± 0.35
3	50	8	36.16 ± 0.50
3	75	8	36.96 ± 0.26

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

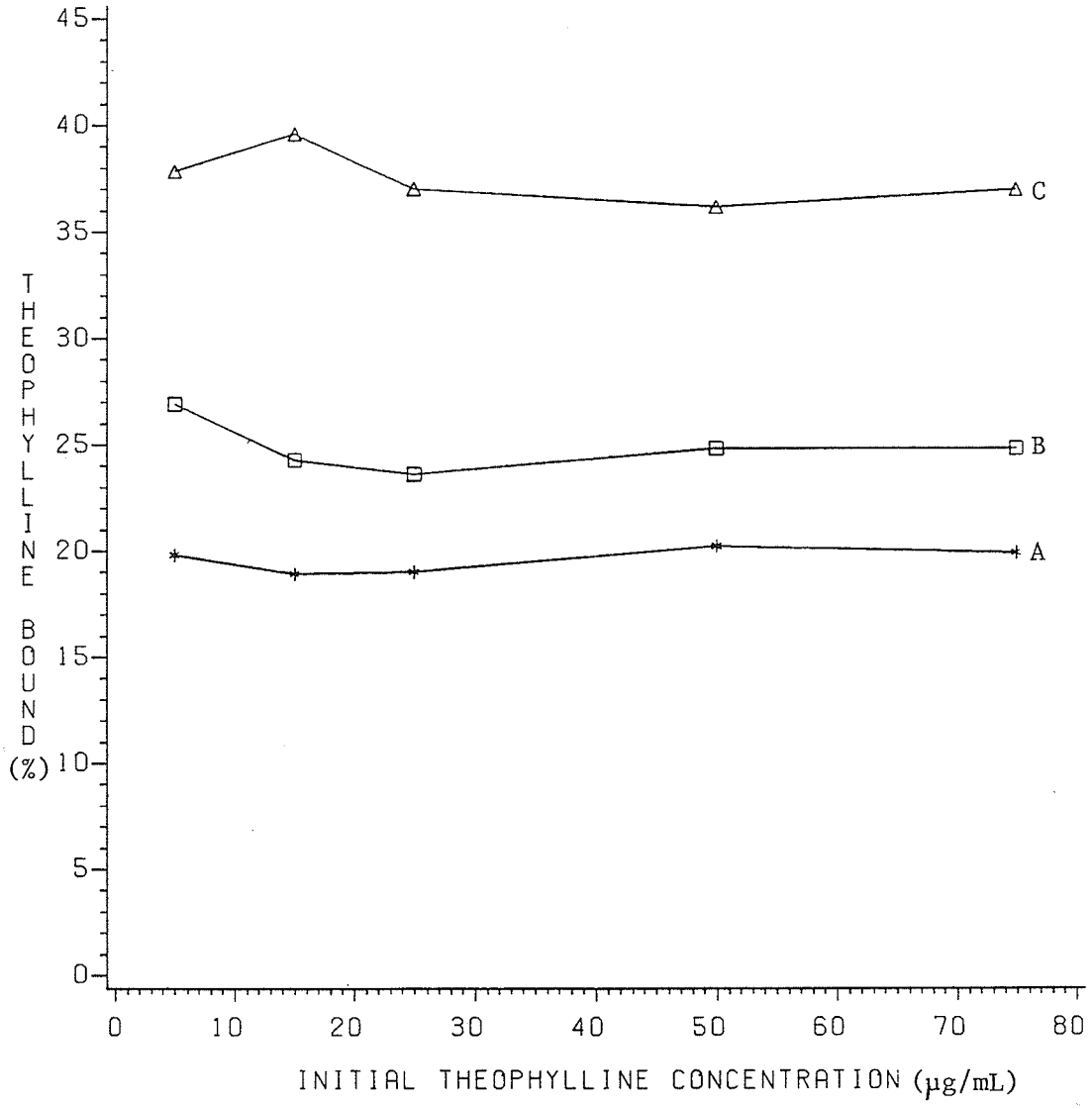


Figure 17. Effect of initial theophylline concentration on percent bound as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□) and C-Ultrafiltration membrane cones.(△)

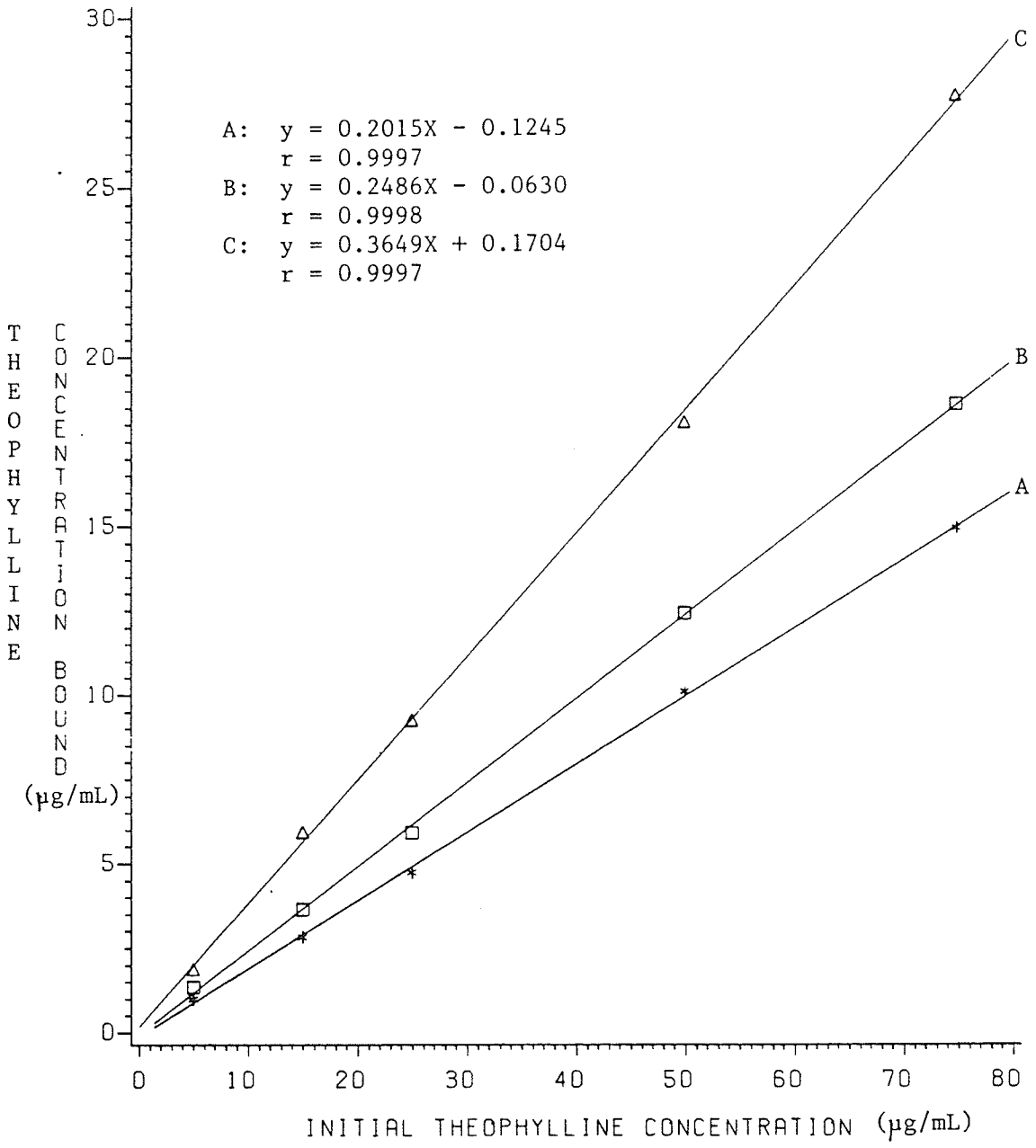


Figure 18. Theophylline concentration bound vs. initial theophylline concentration as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 15 EFFECT OF DRUG CONCENTRATION AND METHOD ON THE PROTEIN BINDING OF THEOPHYLLINE

	METHOD					
	Equilibrium Dialysis (Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
Initial Theophylline conc. (µg/mL)	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
5	10	19.53[a]*	10	26.93[a]*	8	39.87[a]*
15	10	18.93[a]*	10	24.29[b]*	8	39.60[a]*
25	10	19.01[a]*	10	23.63[b]*	8	37.01[b]*
50	10	20.21[a]*	10	24.84[b]*	8	36.16[b]*
75	10	18.92[a]*	10	24.83[b]*	8	36.96[b]*

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same drug concentration are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 16 EFFECT OF DRUG CONCENTRATION ON PROTEIN BINDING OF HI-6

Method*	Initial HI-6 Conc.(µg/mL)	N	Mean % Bound ±S.E
2	50	9	4.38 ± 1.10
2	100	10	3.69 ± 1.7
2	150	10	4.15 ± 0.92
2	200	10	4.48 ± 1.3
2	250	8	4.76 ± 1.2
3	50	8	5.62 ± 0.81
3	100	7	7.55 ± 1.4
3	150	8	7.48 ± 1.2
3	200	8	5.89 ± 0.6
3	250	8	6.52 ± 1.9

*Method: 2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

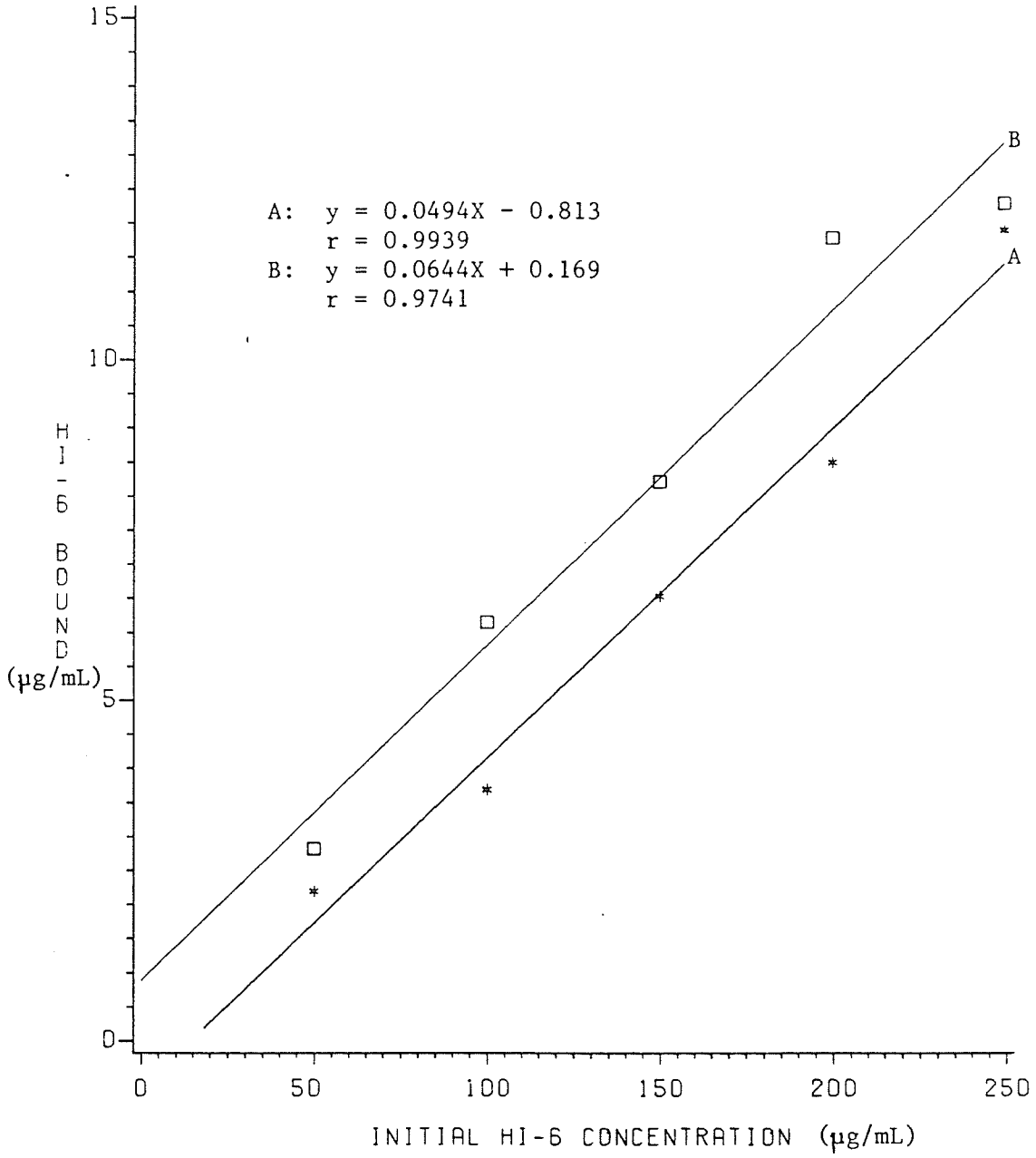


Figure 19. HI-6 concentration bound vs. initial HI-6 concentration as determined by A-Spectrum equilibrium dialysis (*) and B-Ultrafiltration membrane cones (□).

TABLE 17 EFFECT OF DRUG CONCENTRATION AND METHOD ON PROTEIN BINDING OF HI-6.

HI-6 Conc. (μg/mL)	Spectrum Equilibrium Dialysis N	Spectrum Equilibrium Dialysis Mean% Bound	Ultrafiltration membrane cones N	Ultrafiltration membrane cones Mean% Bound
50	9	4.38[a]	8	5.62[a]
100	10	3.69[a]	7	7.55[a]
150	10	4.15[a]	8	7.48[a]
200	10	4.48[a]	8	5.89[a]
250	8	4.76[a]	8	6.52[a]

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = 0.05.

Means of methods significantly different at the same drug concentration are indicated by *. Tukey's studentized range test P = 0.05.

.05). No significant difference was observed in the means obtained at the different drug concentrations using conventional equilibrium dialysis. Means of percent bound theophylline obtained using the Dianorm and ultrafiltration were found to be slightly but significantly higher at lower drug concentrations (15 μg/mL) than those at higher concentrations (Table 15). No difference was observed in the means of percent bound at concentrations higher than 15 μg/mL.

Numerical data for percent bound HI-6 at different initial drug concentrations as determined by Spectrum equilibrium dialysis and ultrafiltration, are presented in Table 16. Analysis of variance at the 95% level of significance indicated that for each method, there were no significant differences in the means of percent bound HI-6 at the different concentrations. No significant difference was observed between the methods at each of the drug concentrations studied (Table 17).

3.2.2 EFFECT OF TEMPERATURE ON EXTENT OF PROTEIN BINDING OF SULFISOXAZOLE, THEOPHYLLINE AND HI-6

The data obtained in the study of the effect of varying temperatures on the extent of binding of sulfisoxazole, theophylline and HI-6, by albumin, are summarized in Tables 18, 20 and 22, respectively. Graphical data are presented in Figures 20, 21 and 22. Linear regression parameters are also shown.

It can be seen that there is a negative linear relationship between temperature and percent bound drug for all the three drugs. There is a decrease in extent of binding of the drugs with increasing temperature. Analysis of variance showed that the effects of temperature and methods of measuring protein binding, were highly significant factors in determining the extent of binding of all three drugs. Tukey's studentized range test on the data at the 95% level of significance indicated that all means of percent

TABLE 18 EFFECT OF TEMPERATURE ON PROTEIN BINDING OF SULFISOXAZOLE

Method*	Temp. (°C)	N	Mean % Bound ± S.E
1	6	10	96.16 ± 0.81
1	25	10	89.48 ± 0.39
1	37	10	82.91 ± 0.72
1	45	10	80.66 ± 0.15
2	6	10	97.09 ± 0.61
2	25	10	88.87 ± 0.27
2	37	10	80.96 ± 0.11
2	45	10	78.24 ± 0.55
3	6	8	99.27 ± 0.20
3	25	8	86.20 ± 0.41
3	37	8	78.04 ± 0.36
3	45	8	72.05 ± 0.18

*Method: 1 = Equilibrium dialysis
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

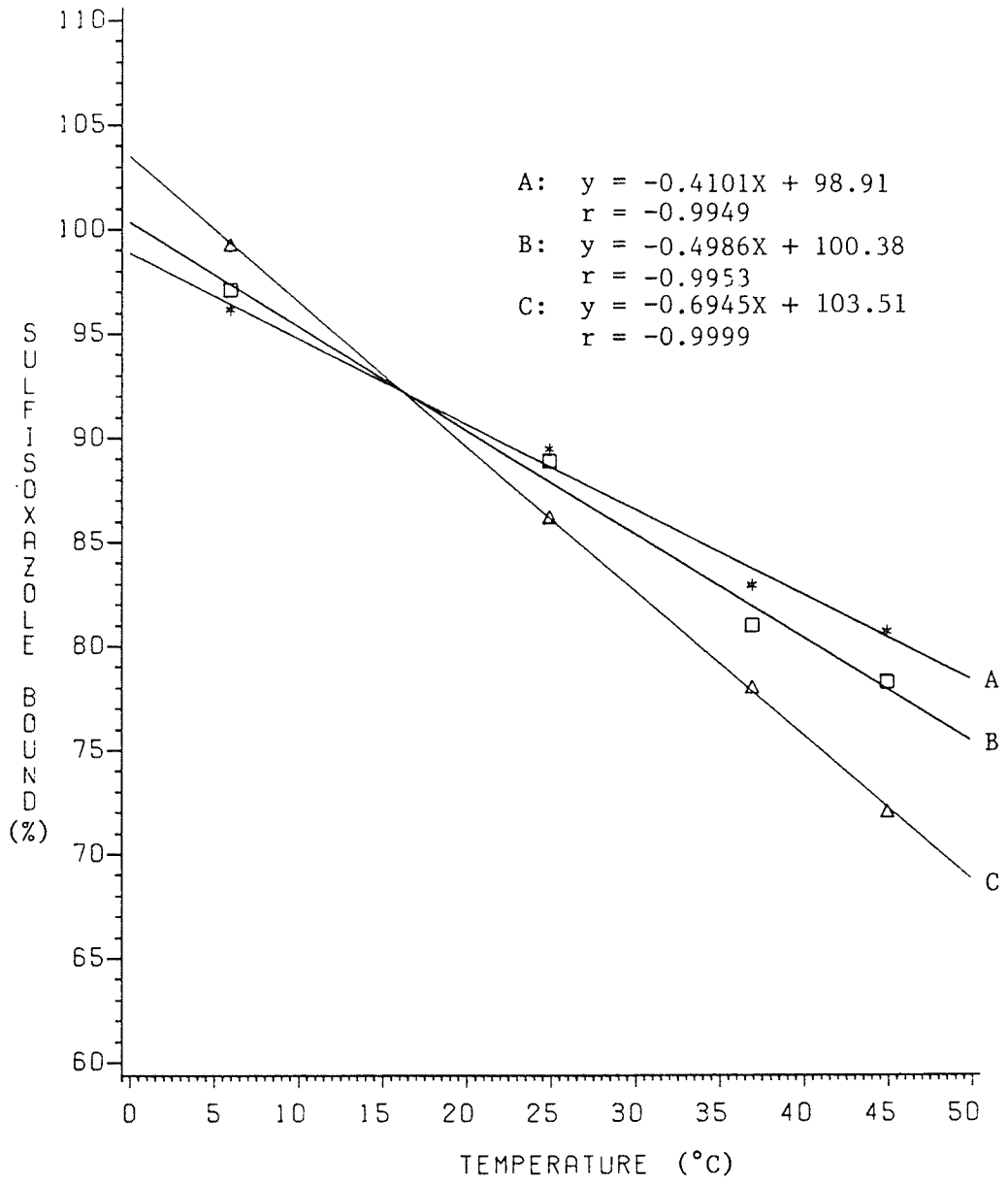


Figure 20. Effect of temperature on percent bound sulfisoxazole as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 19 EFFECT OF TEMPERATURE AND METHOD ON PROTEIN BINDING OF SULFISOXAZOLE

Temperature (°C)	METHOD: Equilibrium Dialysis (Bags) N	Mean% Bound	Spectrum Equilibrium Dialysis N	Mean% Bound	Ultrafiltration Membrane Cones N	Mean% Bound
6	10	96.16[a]	10	97.09[a]	8	99.27[a]*
25	10	89.48[b]	10	88.57[b]	8	86.20[b]*
37	10	82.91[c]*	10	80.96[c]*	8	78.04[c]*
45	10	80.66[d]*	10	76.24[d]*	8	72.05[d]*

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same temperature are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 20 THE EFFECT OF TEMPERATURE ON PROTEIN BINDING OF THEOPHYLLINE

Method*	Temp. (°C)	N	Mean Theophylline Bound ± S.E (%)
1	6	10	28.40 ± 0.64
1	25	10	20.82 ± 0.22
1	37	10	18.52 ± 0.50
1	45	10	10.19 ± 0.44
2	6	10	46.84 ± 1.02
2	25	9	32.89 ± 0.34
2	37	10	24.47 ± 0.56
2	45	10	21.33 ± 0.21
3	6	8	57.91 ± 0.58
3	25	8	42.87 ± 0.79
3	37	8	38.19 ± 0.52
3	45	8	36.36 ± 0.96

*Method: 1 = Equilibrium Dialysis (bags)
2 = Spectrum Equilibrium Dialysis
3 = Ultrafiltration membrane cones

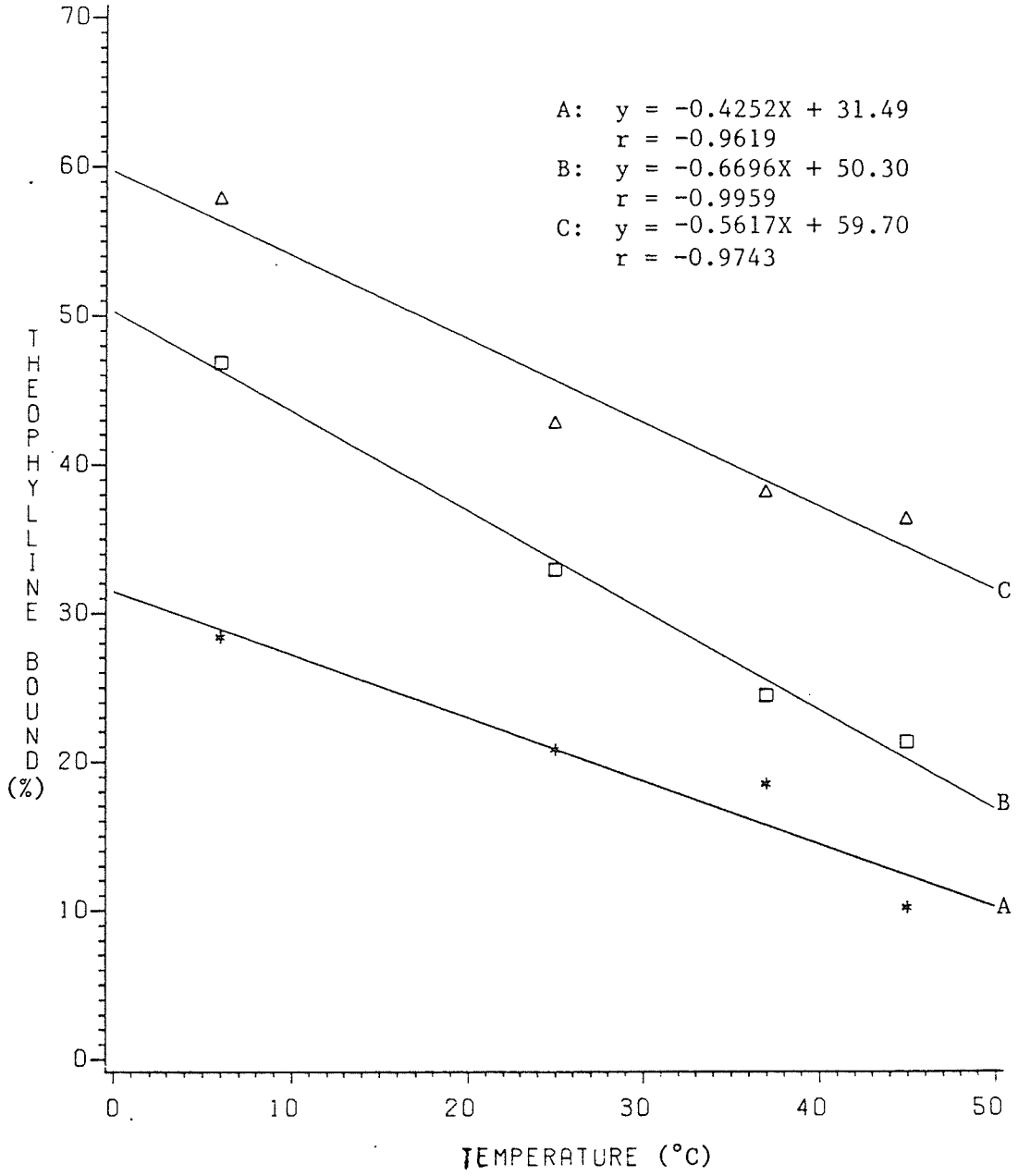


Figure 21. Effect of temperature on percent bound theophylline as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 21 EFFECT OF TEMPERATURE AND METHOD ON THE PROTEIN BINDING OF THEOPHYLLINE.

	METHOD					
	Equilibrium Dialysis(Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
Temperature (°C)	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
6	10	28.4[a]*	10	46.84[a]*	8	57.91[a]*
25	10	29.82[b]*	9	32.89[b]*	8	42.87[b]*
37	10	18.52[c]*	10	24.47[c]*	8	39.39[c]*
45	10	10.19[d]*	10	21.33[d]*	8	38.16[d]*

N = Number

Means with the same letter (per method) are not significantly different.

Tukey's studentized range test P = 0.05.

Means of method significantly different at the same temperature indicated by '*'. Tukey's studentized range test P = 0.05.

TABLE 22 EFFECT OF TEMPERATURE ON PROTEIN BINDING OF HI-6

Method*	Temp. (°C)	N	Mean % Bound ± S.E
2	6	9	5.86 ± 0.87
2	25	9	5.09 ± 0.98
2	37	9	4.57 ± 0.56
2	45	9	4.78 ± 0.78
3	6	8	7.01 ± 0.93
3	25	8	5.98 ± 0.88
3	37	8	5.71 ± 0.66
3	45	8	4.94 ± 1.46

*Method: 2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

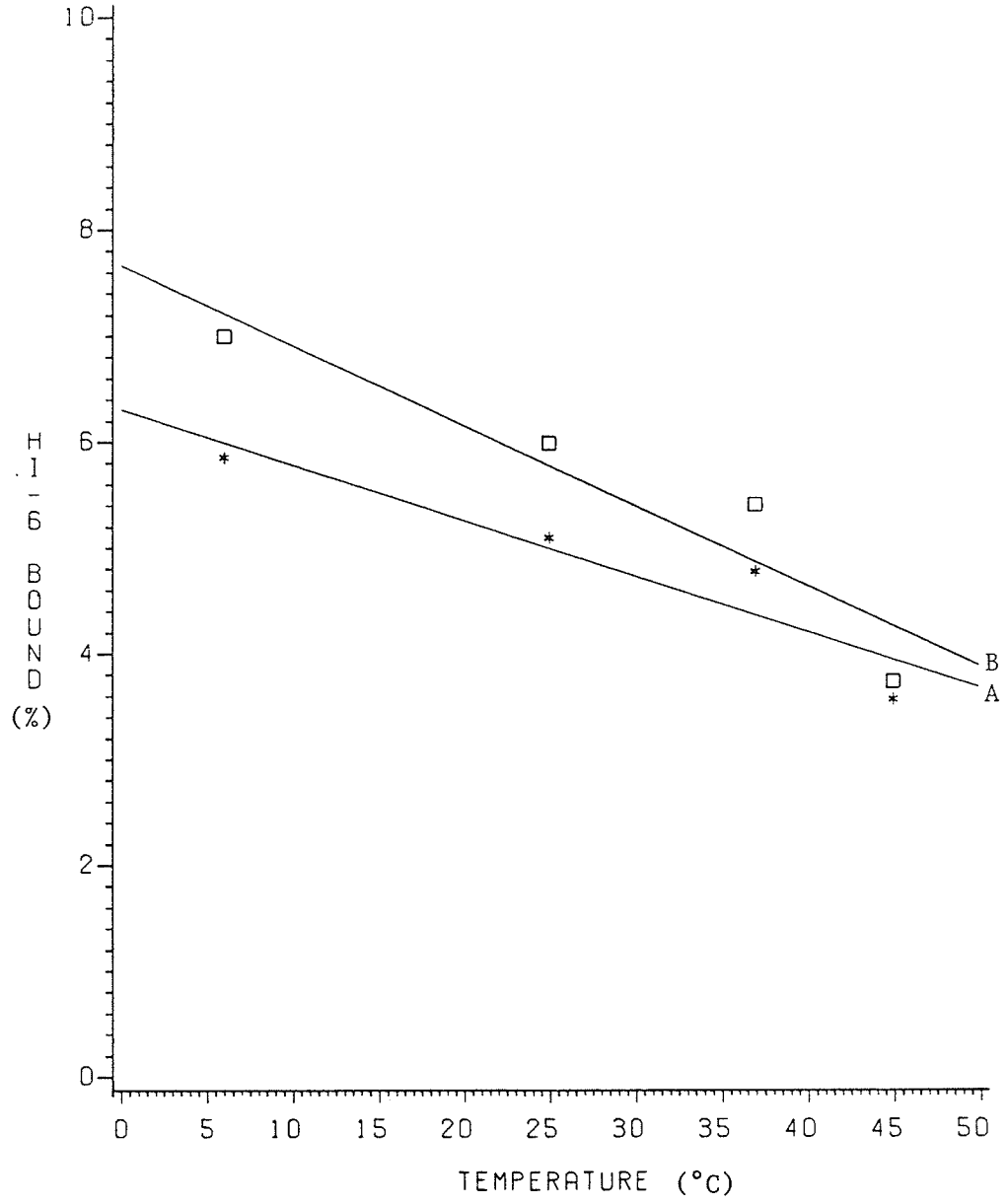


Figure 22. Effect of temperature on percent bound HI-6 as determined by A-spectrum equilibrium dialysis (*) and B-Ultrafiltration membrane cones (□).

TABLE 23 EFFECT OF TEMPERATURE AND METHOD ON PROTEIN BINDING OF HI-6

Temp. (°C)	METHOD: Spectrum Equilibrium Dialysis N	Spectrum Equilibrium Dialysis Mean% Bound	METHOD: Ultrafiltration membrane cones N	Ultrafiltration membrane cones Mean% Bound
6	9	5.86[a]	8	7.01[a]
25	9	5.09[a]	8	5.98[b]
37	9	4.57[a]	8	5.71[b]
45	9	4.78[a]	8	4.94[b]

Means with the same letter (per method) are not significantly different from each other. Tukey's test, P = 0.05.

Means of methods significantly different from each other at the same temperature are indicated by '*'. Tukey's studentized range test, P = 0.05.

bound sulfisoxazole and theophylline at the different temperatures were significantly different. Means of percent bound sulfisoxazole determined by ultrafiltration membrane cones were statistically different from those of the two equilibrium dialysis procedures at all temperatures (Table 19). No difference was observed between the latter two methods at 6°C and 25°C, but there were significant differences in the methods at the higher temperatures.

Significant differences in all three methods were noted for mean percent bound theophylline at all temperatures studied (Table 21). Differences between results obtained at all temperatures for a given method, were also statistically different at the 95% level of significance (Tukey's test, P = 0.05).

Although there was considerable variation in the results of percent bound HI-6 at the different temperatures, Tukey's test done at the 95% level of significance indicated that there were no significant differences between the means obtained using Spectrum equilibrium dialysis. No difference was detected between the means of percent bound HI-6 determined by ultrafiltration at 25°C, 37°C and 45°C. Percent bound HI-6 at 6°C was significantly higher than at the higher temperatures using this method (Table 23).

3.2.3 EFFECT OF pH ON THE EXTENT OF PROTEIN BINDING OF SULFISOXAZOLE, THEOPHYLLINE AND HI-6

A summary of the data obtained in the study of the effect of varying pH on the extent of binding of sulfisoxazole, theophylline and HI-6 is presented in Tables 24, 26, 28, and Figures 23, 24 and 25, respectively.

Analysis of variance indicated that pH and method of determining protein binding had a significant effect on the extent of sulfisoxazole and theophylline bound by albumin. A progressive decrease in the binding of sulfisoxazole was observed with increasing pH in the pH range of 6.4 to 10.5 units. Tukey's

TABLE 24 EFFECT OF pH ON PROTEIN BINDING OF SULFISOXAZOLE

Method*	pH	N	Mean % Bound ± S.E
1	4.5	10	90.62 ± 0.81
1	5.8	10	91.30 ± 0.36
1	6.8	10	90.65 ± 0.21
1	7.4	10	89.27 ± 0.42
1	8.0	10	86.60 ± 0.10
1	10.5	10	75.79 ± 0.59
2	4.5	10	92.68 ± 0.49
2	5.8	10	93.16 ± 0.56
2	6.8	10	89.28 ± 0.32
2	7.4	10	87.81 ± 0.11
2	8.0	10	87.23 ± 0.18
2	10.5	10	78.37 ± 0.64
3	4.5	8	92.99 ± 0.21
3	5.8	8	92.25 ± 0.33
3	6.8	8	92.16 ± 0.56
3	7.4	8	90.65 ± 0.21
3	8.0	8	87.74 ± 0.70
3	10.5	8	78.46 ± 0.52

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

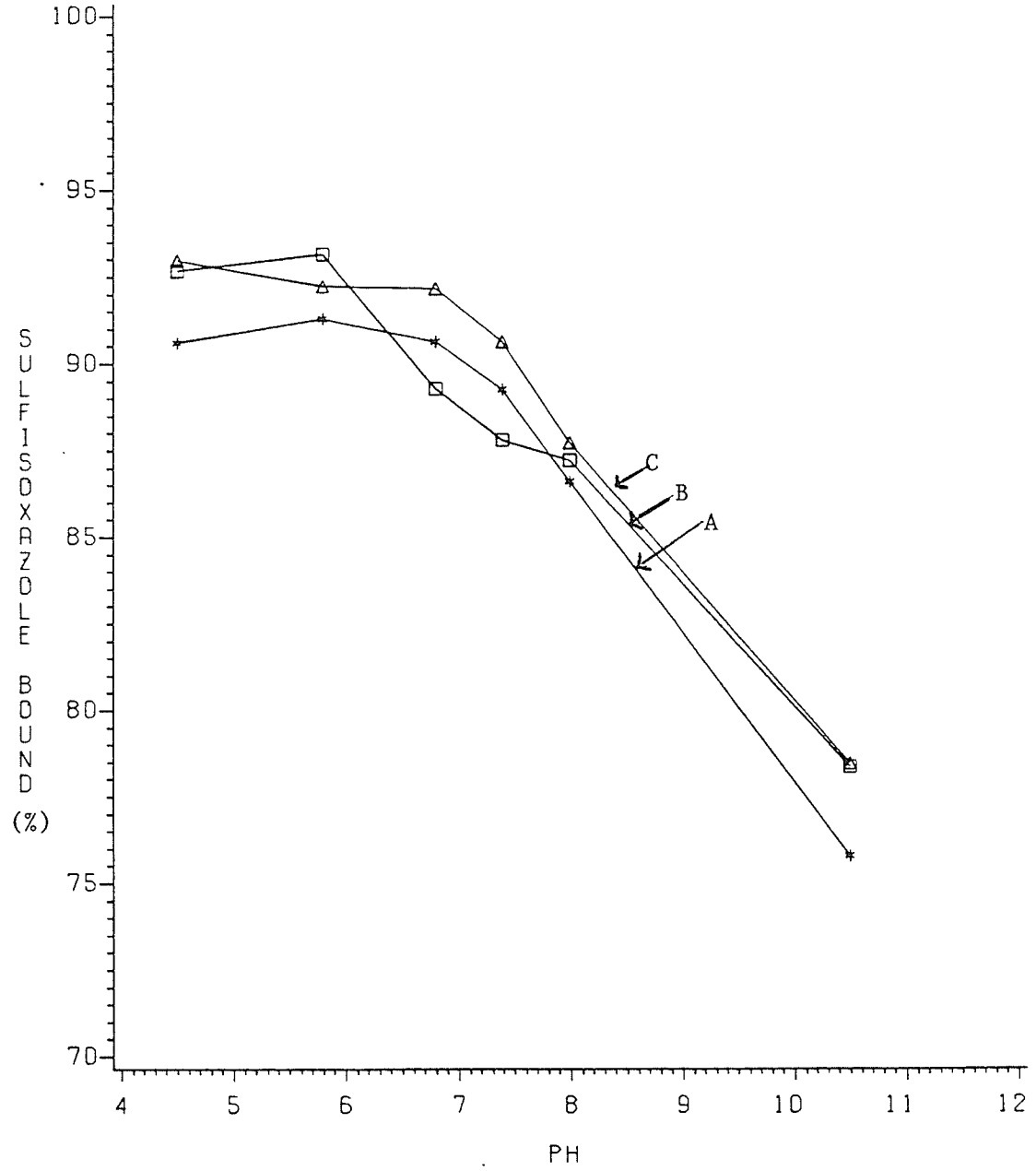


Figure 23. Effect of pH on percent bound sulfisoxazole as determined by A-equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis, and C-Ultrafiltration membrane cones (Δ).

TABLE 25 EFFECT OF pH AND METHOD ON PROTEIN BINDING OF SULFISOXAZOLE

	METHOD					
	Equilibrium Dialysis (Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
pH	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
4.5	10	90.62[a]*	10	92.68[a]	8	92.99[a]
5.8	10	91.30[a]*	10	93.16[a]	8	92.25[a]
6.8	10	90.65[a]	10	89.28[b]	7	92.16[a]*
7.4	10	89.27[b]	10	87.81[b]*	8	90.65[b]
8.0	10	86.60[c]	10	87.23[b]	8	87.74[c]
10.5	10	75.79[d]*	10	78.37[c]	8	78.46[d]

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same pH are indicated by '*'. Tukey's studentized range test P = .05.

studentized range test at the 95% level of significance indicated that the means of percent bound sulfisoxazole at different pH values in this range were statistically different from each other and from those at the lower pH values (Table 25). No significant difference was detected in percent bound sulfisoxazole between pH 4.5 and 6.8, inclusive. Means of percent bound sulfisoxazole determined by equilibrium dialysis bags were significantly different from those of the other two methods at the extreme low and high pH values, which are outside the normal plasma ranges.

A progressive increase in binding of theophylline was observed with increasing pH in the basic pH range (Table 26). Tukey's studentized range test at the 95% level of significance indicated that for each of the three methods, there was no significant difference in the means of percent bound theophylline below pH 6.8. Significant differences in theophylline binding were observed at all the higher pH values (Table 27). There were significant differences between methods at all pH levels studied. These differences were more pronounced at high pH, which could be important in formulating drug-loaded proteinaceous microspheres. Greater binding was observed using ultrafiltration than with dialysis methods.

HI-6 binding to albumin as determined by Spectrum equilibrium dialysis and ultrafiltration, remained relatively constant throughout the pH range studied. Analysis of variance done at the 95% level of significance indicated that there was no significant difference between the means of percent bound HI-6 at the different pH values. No statistically significant differences were observed between the means of percent bound HI-6 determined by Spectrum equilibrium dialysis and ultrafiltration membrane cones at all the pH values tested (Table 29).

TABLE 26 EFFECT OF pH ON PROTEIN BINDING OF THEOPHYLLINE

Method*	pH	N	Theophylline Bound (%) ± S.E
1	4.5	10	21.09 ± 0.33
1	5.8	10	19.64 ± 0.14
1	6.8	10	20.29 ± 0.81
1	7.4	10	22.82 ± 0.57
1	8.0	10	22.90 ± 0.28
1	10.5	10	29.14 ± 0.19
2	4.5	10	22.83 ± 0.36
2	5.8	10	22.66 ± 0.54
2	6.8	10	23.88 ± 0.18
2	7.4	10	24.47 ± 0.79
2	8.0	10	27.74 ± 0.55
2	10.5	10	40.25 ± 0.62
3	4.5	8	27.53 ± 0.19
3	5.8	8	27.16 ± 0.47
3	6.8	8	26.89 ± 0.71
3	7.4	8	39.38 ± 0.42
3	8.0	8	48.37 ± 0.68
3	10.5	8	57.12 ± 0.34

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

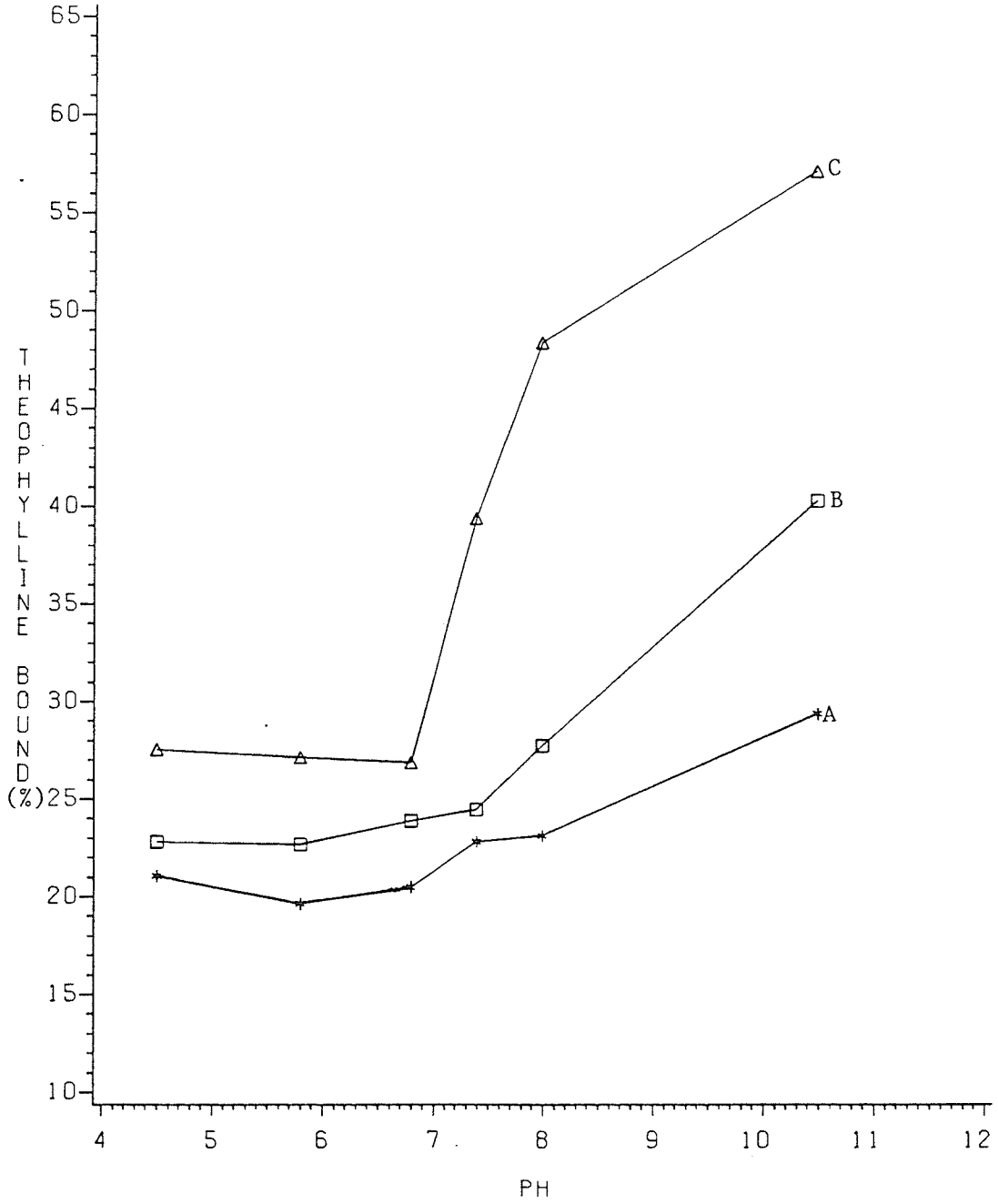


Figure 24. Effect of pH on percent bound theophylline as determined by A-equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 27 EFFECT OF pH AND METHOD ON PROTEIN BINDING OF THEOPHYLLINE

	METHOD					
	Equilibrium Dialysis (Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
pH	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
4.5	10	20.99	10	22.83[a]*	8	27.53[a]*
5.8	10	19.64	10	22.66[a]*	8	27.16[a]*
6.8	10	20.29	10	23.88[b]*	8	26.89[a]*
7.4	10	22.82	10	24.47[b]*	8	39.38[b]*
8.0	10	22.90	10	27.74[c]*	8	48.37[c]*
10.5	10	29.14	10	40.25[d]*	8	57.12[d]*

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same pH are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 28 EFFECT OF pH ON PROTEIN BINDING OF HI-6

Method*	pH	N	Mean % Bound ± S.E
2	4.5	10	3.96 ± 0.43
2	5.8	10	4.49 ± 0.72
2	6.8	9	4.54 ± 0.78
2	7.4	10	4.93 ± 0.45
2	8.0	10	5.69 ± 0.89
2	10.5	10	5.93 ± 0.64
3	4.5	6	5.33 ± 0.89
3	5.8	8	4.78 ± 0.85
3	6.8	8	4.55 ± 1.07
3	7.4	8	5.20 ± 0.89
3	8.0	8	5.43 ± 0.67
3	10.5	8	4.10 ± 0.51

*Method: 2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

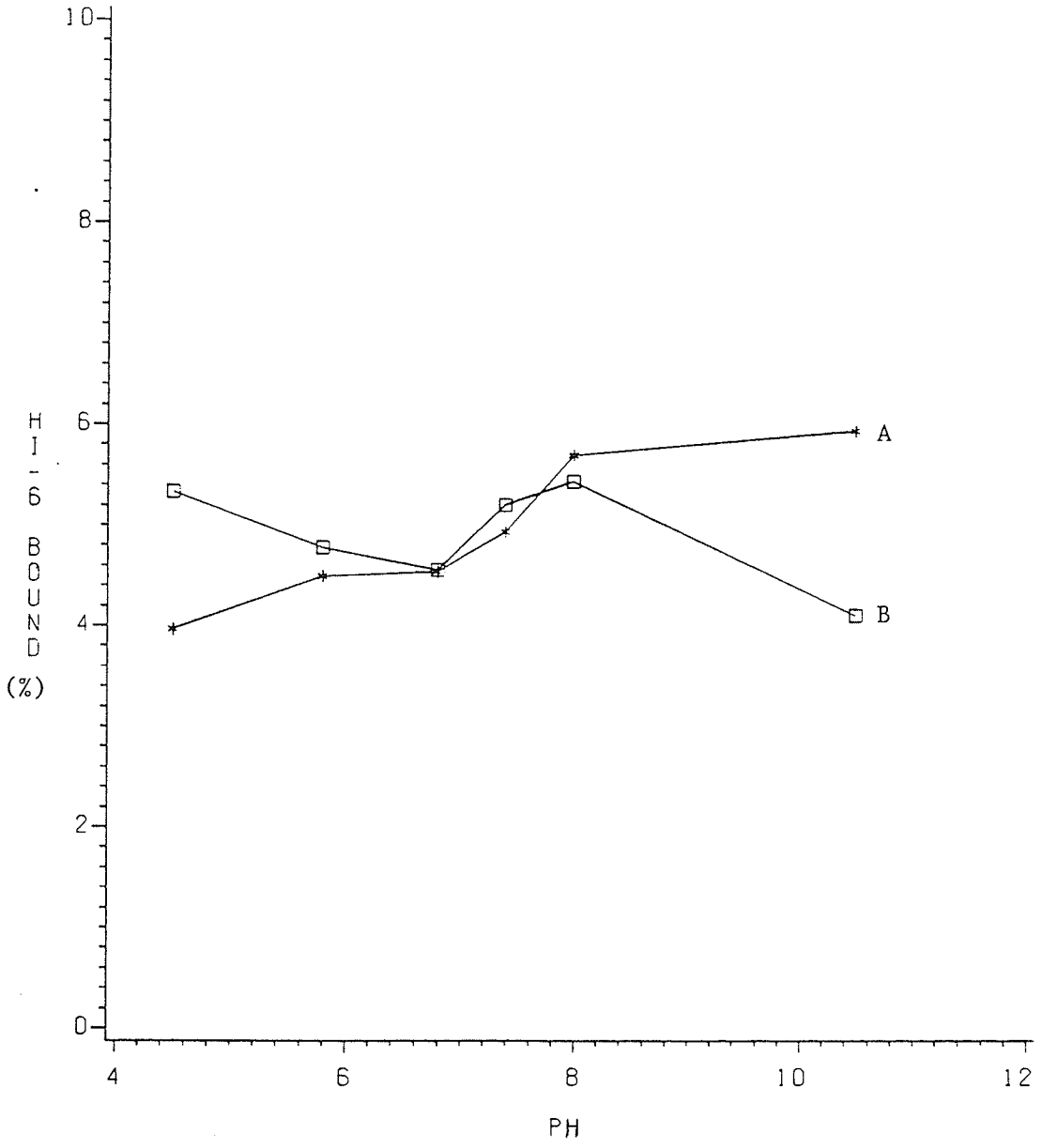


Figure 25. Effect of pH on percent bound HI-6 as determined by A-Spectrum equilibrium dialysis (*), and B-Ultrafiltration membrane cones (□).

TABLE 29 EFFECT OF pH AND METHOD OF DETERMINING BINDING ON PROTEIN BINDING OF HI-6

	METHOD			
	Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
pH	N	Mean% Bound	N	Mean% Bound
4.5	10	3.96[a]	6	5.33[a]
5.8	10	4.49[a]	8	4.78[a]
6.8	9	4.54[a]	8	4.55[a]
7.4	10	4.93[a]	8	5.20[a]
8.0	10	5.93[a]	8	4.10[a]

Means with the same letter (per method) are not significantly different. Tukey's studentized range test, P = 0.05.

Means of methods significantly different at the same pH are indicated by '*'. Tukey's studentized range test, P = 0.05.

3.2.4 EFFECT OF PROTEIN (ALBUMIN) CONCENTRATION ON EXTENT OF BINDING OF SULFISOXAZOLE, THEOPHYLLINE AND HI-6

Tables 30, 32 and 34 represent a summary of results obtained in the study of varying albumin concentration on the extent of binding of sulfisoxazole, theophylline and HI-6 by albumin. Graphical data are presented in Figures 26, 27 and 28, respectively.

For all three drugs, it was observed that as the concentration of albumin increased, the fraction of drug bound also increased, approaching a maximum at higher protein concentration. Although at low albumin concentrations, the binding of drugs increased rapidly with concentration of protein, an increase in albumin at higher concentrations produced only a small elevation in binding. For example, doubling the albumin concentration from 1.0% to 2.0% resulted in almost a 3-fold increase in the binding of theophylline (5.64% to 15.47%) when determined using equilibrium dialysis (bags). Increasing albumin concentration from 4% to 8% increased binding of the same drug by only about 30% (20.84% to 28.09%). The same trend was observed for the other drugs using all methods of studying binding. Generally, ultrafiltration gave higher values of binding than the equilibrium dialysis procedures, although this effect was more pronounced for the medium bound drug (theophylline) than the high and low bound drugs.

Analysis of variance was done to determine whether there were differences in the extent of drug bound at the different protein concentrations. The results showed that the effect of protein concentration on the binding of sulfisoxazole, theophylline and HI-6 was significant at the 95% level. Tukey's studentized range test indicated that all the means of percent bound sulfisoxazole and theophylline at the different protein concentrations were statistically different from each other at the 95% level of significance.

TABLE 30 EFFECT OF ALBUMIN CONCENTRATION ON PROTEIN BINDING OF SULFISOXAZOLE

Method*	Albumin Conc.(%w/v)	N	Mean % Bound ± S.E
1	1.0	10	29.76 ± 0.27
1	2.0	10	52.52 ± 0.16
1	4.0	10	75.86 ± 0.32
1	6.0	10	93.08 ± 0.51
1	8.0	10	98.78 ± 0.68
2	1.0	10	27.88 ± 0.42
2	2.0	10	51.63 ± 0.39
2	4.0	10	78.35 ± 0.22
2	6.0	10	89.38 ± 0.12
2	8.0	10	97.69 ± 0.41
3	1.0	8	24.50 ± 0.15
3	2.0	8	49.83 ± 0.28
3	4.0	8	74.12 ± 0.49
3	6.0	8	97.32 ± 0.21
3	8.0	8	99.22 ± 0.44

*Method: 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

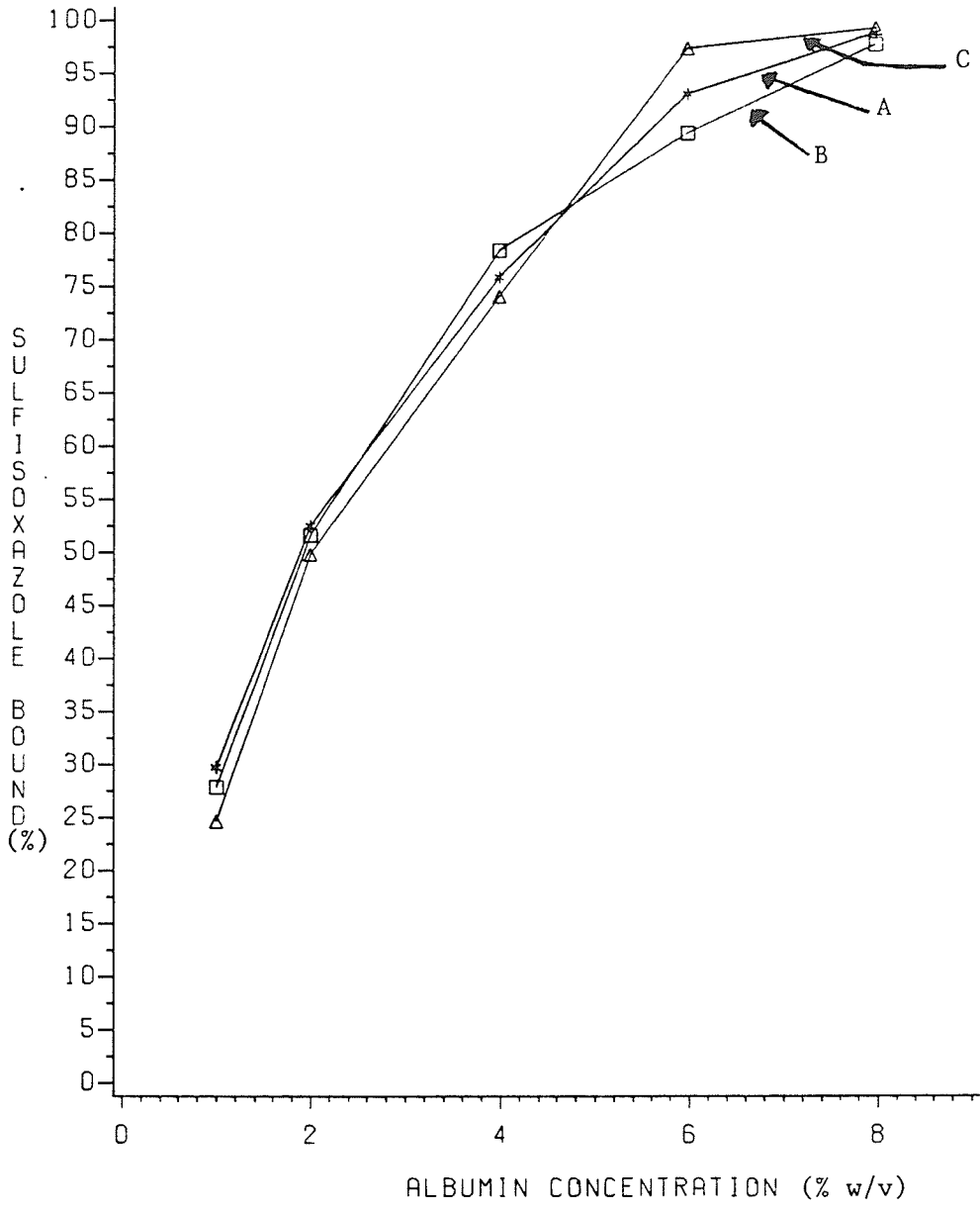


Figure 26. Effect of albumin concentration on percent bound sulfisoxazole as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (△).

TABLE 31 EFFECT OF ALBUMIN CONCENTRATION AND METHOD ON PROTEIN BINDING OF SULFISOXAZOLE

	METHOD					
	Equilibrium Dialysis (Bags)		Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
Albumin Conc. (%w/v)	N	Mean% Bound	N	Mean% Bound	N	Mean% Bound
1.0	10	29.76[a]*	10	27.88[a]*	8	24.60[a]*
2.0	10	52.52[b]	10	51.63[b]	8	49.83[b]
4.0	10	75.86[c]	10	78.35[c]*	8	74.12[c]
6.0	10	93.08[d]*	10	89.38[d]*	8	97.32[d]*
8.0	10	98.78[e]	10	97.69[e]	8	99.22[d]

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same albumin concentration are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 32 EFFECT OF ALBUMIN CONCENTRATION ON PROTEIN BINDING OF THEOPHYLLINE

Method*	Albumin Conc.(%w/v)	N	Mean % Theophylline Bound ± S.E
1	1.0	10	5.64 ± 0.48
1	2.0	10	15.47 ± 0.33
1	4.0	10	20.84 ± 0.72
1	6.0	10	23.60 ± 0.24
1	8.0	10	28.09 ± 0.91
2	1.0	10	7.92 ± 0.12
2	2.0	10	18.93 ± 0.29
2	4.0	10	24.43 ± 0.46
2	6.0	10	30.03 ± 0.56
2	8.0	10	35.22 ± 0.81
3	1.0	8	13.16 ± 0.30
3	2.0	8	34.42 ± 0.61
3	4.0	8	46.34 ± 0.28
3	6.0	8	51.93 ± 0.51
3	8.0	8	61.61 ± 0.59

* 1 = Equilibrium dialysis (bags)
2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

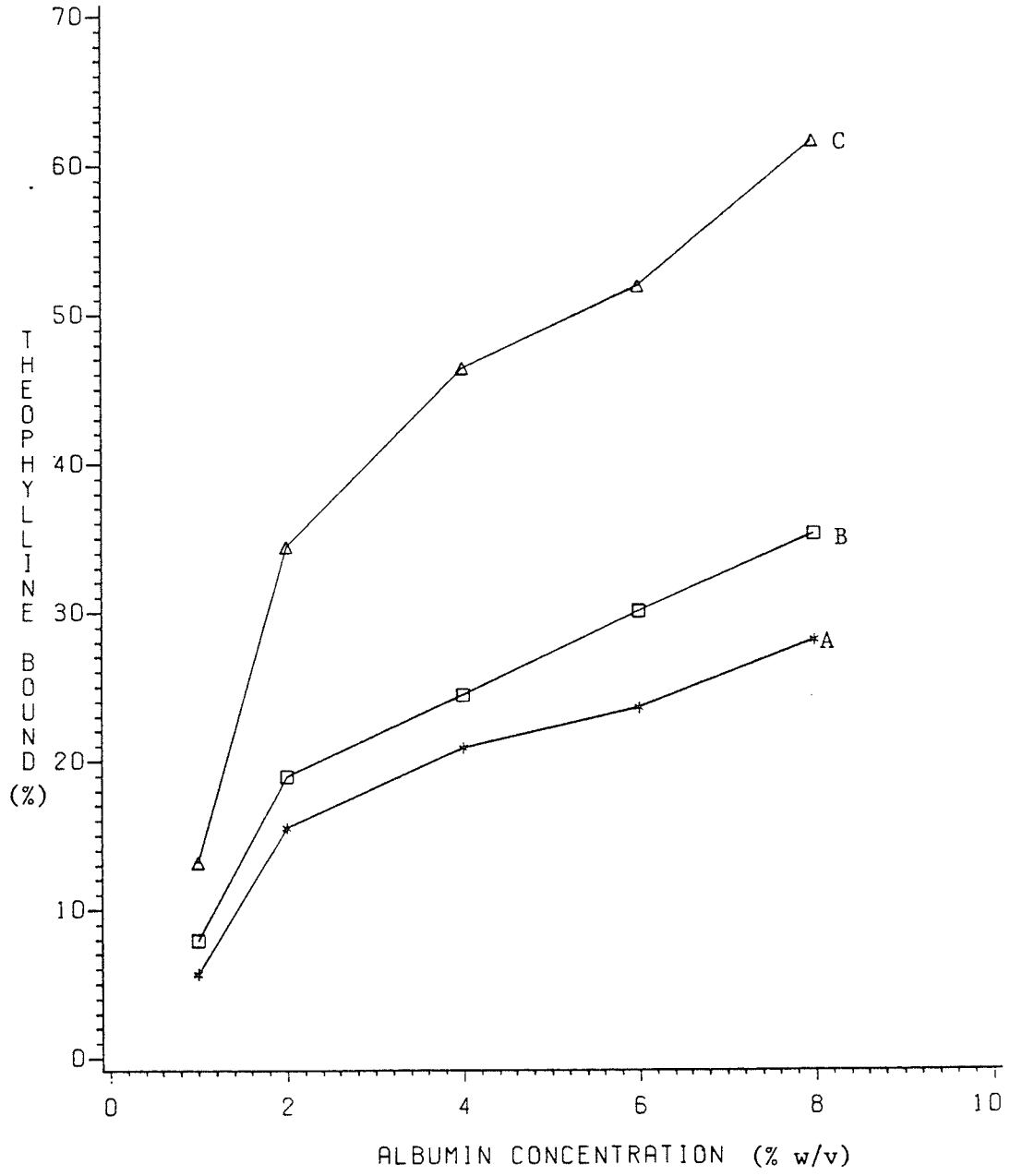


Figure 27. Effect of albumin concentration on percent bound theophylline as determined by A-Equilibrium dialysis (bags) (*), B-Spectrum equilibrium dialysis (□), and C-Ultrafiltration membrane cones (Δ).

TABLE 33 EFFECT OF ALBUMIN CONCENTRATION AND METHOD ON PROTEIN BINDING OF THEOPHYLLINE

Albumin Conc.(%w/v)	Equilibrium Dialysis(Bags) N	Mean Theoph. Bound(%)	Spectrum Equilibrium Dialysis N	Mean Theoph. Bound(%)	Ultrafiltration Membrane Cones N	Mean Theoph. Bound(%)
1.0	10	5.64[a]*	10	7.92[a]*	8	13.16[a]*
2.0	10	15.47[b]*	10	18.93[b]*	8	34.42[b]*
4.0	10	20.84[c]*	10	24.43[c]*	8	46.34[c]*
6.0	10	23.60[d]*	10	30.03[d]*	8	51.93[d]*
8.0	10	28.09[e]*	10	35.22[e]*	8	61.61[e]*

Means with the same letter (per method) are not significantly different. Tukey's studentized range test P = .05.

Means of methods significantly different at the same albumin concentration are indicated by '*'. Tukey's studentized range test P = .05.

TABLE 34 EFFECT OF ALBUMIN CONCENTRATION ON PROTEIN BINDING OF HI-6

Method*	Albumin Conc.(%w/v)	N	Mean % Bound ± S.E
2	1.0	9	1.06 ± 0.31
2	2.0	10	2.46 ± 0.65
2	4.0	10	4.86 ± 1.22
2	6.0	10	6.23 ± 0.74
2	8.0	10	7.76 ± 0.93
3	1.0	8	1.07 ± 0.34
3	2.0	8	3.48 ± 0.70
3	4.0	8	5.09 ± 0.50
3	6.0	8	6.62 ± 0.86
3	8.0	8	8.28 ± 1.02

*Method: 2 = Spectrum equilibrium dialysis
3 = Ultrafiltration membrane cones

All three methods showed significant differences in the means of percent bound theophylline at each protein concentration studied (Table 33) (Tukey's test, P = .05). Percent bound sulfisoxazole values obtained using the three methods were reasonably close although some differences between methods were detected, especially at the low protein concentrations (Table 31). At the physiological albumin concentration (4%), mean percent bound sulfisoxazole determined using the Dianorm was slightly but significantly different from those obtained by conventional dialysis and ultrafiltration.

Both ultrafiltration and Spectrum equilibrium dialysis showed an increase in the binding of HI-6 with increasing albumin concentration. Tukey's test done at the 95% level of significance indicated that for both methods, the means of HI-6 bound at 1% and 2% albumin concentration were not statistically different, but they differed from those at the higher albumin concentrations. No significant difference was detected in the means of HI-6 bound at 4%, 6% and 8% albumin.

No significant difference was detected between the two methods used to determine HI-6 binding at all albumin concentrations studied (Table 35).

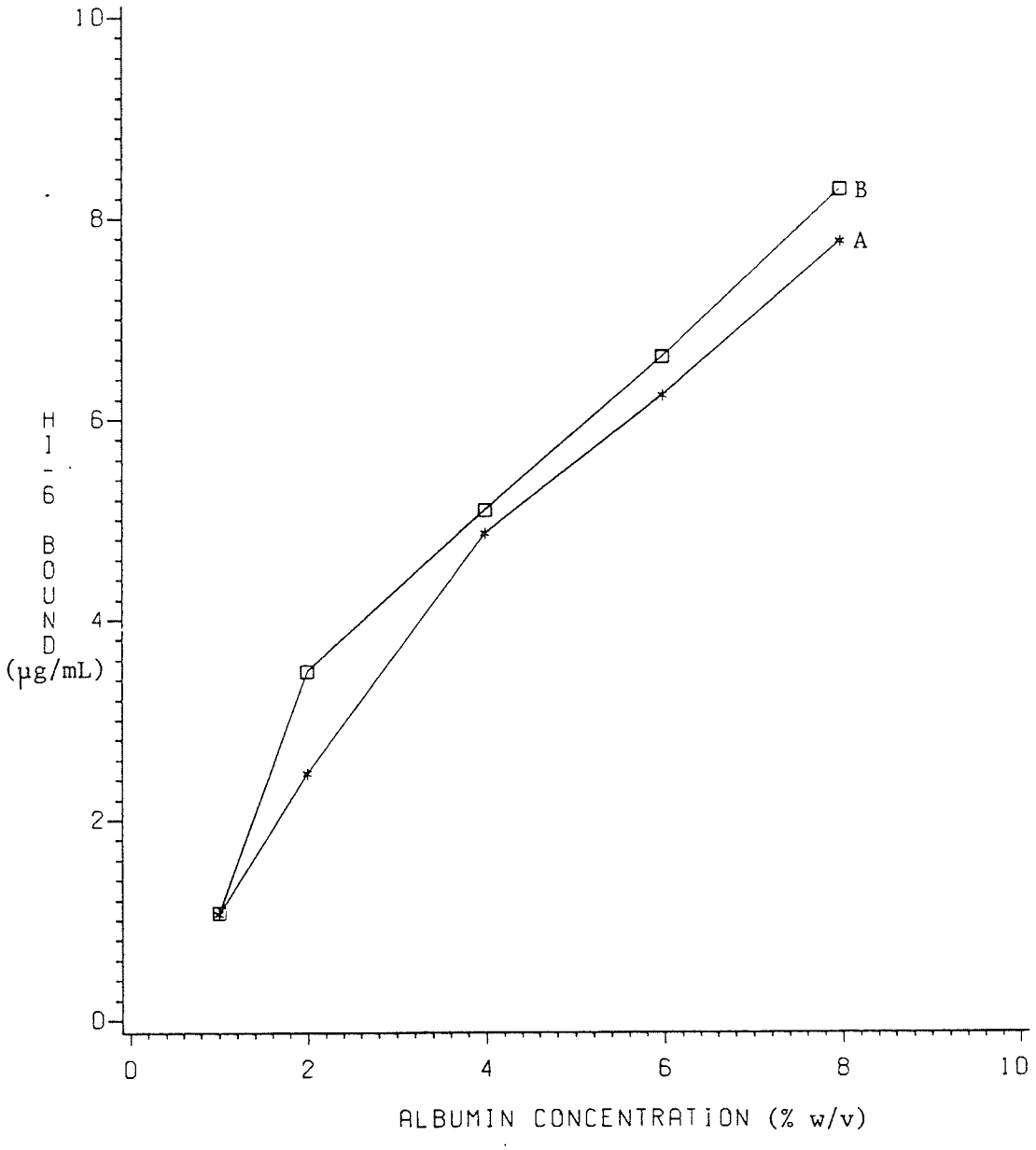


Figure 28. Effect of albumin concentration on percent bound HI-6 as determined by A-Spectrum Equilibrium dialysis, and B-Ultrafiltration membrane cones (Δ).

TABLE 35 EFFECT OF ALBUMIN CONCENTRATION AND METHOD ON PROTEIN BINDING OF HI-6

	METHOD			
	Spectrum Equilibrium Dialysis		Ultrafiltration Membrane Cones	
Albumin Conc.(%w/v)	N	Mean % Bound	N	Mean % Bound
1.0	9	1.06[a]	8	1.07[a]
2.0	10	2.46[a]	8	3.48[a]
4.0	10	4.86[b]	8	5.09[b]
6.0	10	6.23[b]	8	6.62[b]
8.0	10	7.76[b]	8	8.28[b]

Means with the same letter (per method) are not significantly different. Tukey's studentized range test, P = 0.05.

Means of methods significantly different at the same albumin concentration are indicated by '*'. Tukey's studentized range test, P = 0.05.

3.2.5 COMPARISON OF SPECTROPHOTOMETRIC AND HPLC ASSAY PROCEDURES FOR HI-6

Numerical data for a plot of spectrophotometric absorbance values of standard concentrations of HI-6 in phosphate buffer, versus corresponding HI-6/Toxogonin peak height ratios, obtained by HPLC assay of the same standard solutions are presented in Tables 5 and 7.

There is a strong linear correlation between HPLC HI-6 and toxogonin peak heights and spectrophotometric absorbance at 355 nm. Linear regression analysis yielded a straight line with the general equation;

PHR = 5.5974A - 0.0376, where PHR = peak height ratio, and A = absorbance. The correlation coefficient was 0.9997.

Protein binding results for HI-6 at different temperatures as determined by Spectrum equilibrium dialysis and ultrafiltration membrane cones, and assayed by both spectrophotometric and HPLC techniques, are presented in Table 36.

Analysis of variance was done to find out if the means of percent bound HI-6 obtained using the two assay techniques were significantly different under the conditions used in this study. No significant difference was detected between the two sets of results at the 95% level of significance (Table 37).

TABLE 36 PERCENT BOUND HI-6 AT DIFFERENT TEMPERATURES USING SPECTROPHOTOMETRIC AND HPLC ASSAY TECHNIQUES

			% Bound ± S.E	
Method*	Temp. (°C)	N	Spect. Assay	HPLC Assay
2	6	9	5.86 ± 0.87	5.03 ± 0.82
2	25	9	5.09 ± 0.98	4.92 ± 0.81
2	37	9	4.57 ± 0.56	4.89 ± 0.44
2	45	9	4.78 ± 0.78	4.91 ± 0.56
3	6	8	7.01 ± 0.93	7.39 ± 0.60
3	25	8	5.98 ± 0.88	5.16 ± 0.87
3	37	8	5.71 ± 0.66	5.42 ± 0.53
3	45	8	4.94 ± 1.46	5.05 ± 0.98

*Method: 2 = Spectrum equilibrium dialysis

3 = Ultrafiltration membrane cones

Means of assay methods significantly different at the same temperature are indicated by '*'. Tukey's studentized range test, P = 0.05.

DISCUSSION

The assay procedures for sulfisoxazole, theophylline and HI-6 in phosphate buffer showed adequate linearity, precision and accuracy within the concentration ranges studied. HPLC peaks for theophylline and HI-6 were separate, distinct and reproducible, and no breakdown of either drug was detected even at 45°C, or in the extreme pH ranges. It was shown that with standard concentrations of albumin, the protein did not interfere with either HPLC or spectrophotometric analyses of any of the drugs at the wavelengths used. Although slight protein leakage may affect the binding results, it was not a contributing factor to variability in results for quantitation of the compounds present.

One major problem with all procedures for separating free from bound drug is drug loss due to binding to the membranes or apparatus. Whereas HI-6 showed almost negligible binding to the test apparatus ($<2\%$), sulfisoxazole and theophylline exhibited a relatively high degree of non-specific binding at approximately therapeutic concentrations. Although conventional dialysis takes a much longer time than the Dianorm for equilibration, the large membrane surface area relative to the working volume in the latter method is probably the major contributing factor to the high degree of non-specific binding observed for theophylline. For both drugs, ultrafiltration gave the highest extent of non-specific binding. The reported figure of 19.8% retention of theophylline by CF25 Amicon cones (Fedak, 1980) is much higher than in the present study (12.35%). The author has also reported significant differences in the protein binding results of theophylline obtained using two types of Amicon membrane cones (CF25 and CF50). It is likely that the differences between our results and those reported by Fedak are due to differences in the binding characteristics of the ultrafiltration membranes used. Membrane

manufacturers have been modifying formulations and polymers in attempts to minimise non-specific binding. The precise formulation of the Amicon CF25 membrane polymer has been altered since the original batches of the cones were made.

The implications of these results are that in the absence of adequate representative controls to monitor non-specific binding, drug bound to the test apparatus may be considered to be due to protein binding, resulting in an overestimation of drug bound. Attempts to adjust dosages based on these results may be potentially hazardous. For drugs such as sulfisoxazole (Zini, et al., 1976), and diazepam (Stock, et al., 1980), which exhibit concentration dependent binding, the observed unbound fraction at any given initial concentration will not reflect the actual drug free at that concentration, since the drug available for protein binding is less than that originally present.

Zhirkov and Piotrovskii (1984) have demonstrated that several cardiovascular drugs at concentrations close to or even exceeding therapeutic concentrations, can bind extensively to dialysis and ultrafiltration membranes, including the CF50A Amicon cones. For some drugs such as verapamil, prazosin and nifedipine, the retention was so high that there was no detectable drug in the ultrafiltrate. Similar results have been reported for disopyramide using ultrafiltration (Hindering, 1974). Whereas non-specific binding may be of little clinical importance in certain drugs such as sulfonamides and salicylates, it may play a major role in contributing to errors in interpretation of the binding of drugs which have a low therapeutic index, and/or show concentration dependent binding. In such cases, the choice of method for determining binding is very important if clinically meaningful results are to be obtained.

Apart from a number of HI-6 studies where the results were very variable

and could not allow a clear distinction between the methods, significant differences were found between results obtained with all three methods of studying protein binding. Ultrafiltration generally gave higher values of percent drug bound than Spectrum equilibrium dialysis which in turn gave higher values than conventional dialysis. These discrepancies are due mainly to the dilution effects occuring in equilibrium dialysis resulting in a lower concentration of protein at equilibrium than was originally present. The time needed for equilibration may lead to changes in the binding characteristics of the proteins and a consequent dissociation of the drug-protein complex. Although ultrafiltration is more rapid than most dialysis procedures, ultrafiltration devices tend to be more susceptible to non-specific binding. The increase in protein concentration with time may also contribute to the high protein binding results in this method. Ultrafiltration and Spectrum equilibrium dialysis gave results which were close at approximate physiological conditions. Differences were more pronounced for the medium (theophylline) and low (HI-6) bound drugs than for the highly bound drug (sulfisoxazole). The physico-chemical properties of these three drugs differed considerably, and might affect the results obtained with a particular method.

Studies of HI-6 binding using equilibrium dialysis indicated that this is a poor choice of method for determining the binding characteristics of low bound drugs. Veerbeck, et al., (1984) have demonstrated that the effect of volume shifts in equilibrium dialysis is dependent on the extent of binding of the drug to the protein. Thus, whereas the same phenomenon as observed with HI-6 may be operating with the higher bound drugs, it may not be as easily recognized as with a low bound drug like HI-6. Ultrafiltration appears to be the method of choice for the determination of the protein binding of low bound

drugs since the problem of volume shift is avoided, provided the volume removed by filtration is minimised. When non-specific binding is not very extensive or when protein binding is not concentration dependent, the Dianorm is the best method of those tested for determining the protein binding of both highly and medium bound drugs.

Most sulfisoxazole protein binding studies have been done using equilibrium dialysis at approximately 100 to 200 µg/mL, which is considered as the average therapeutic concentration of this drug. Conventional dialysis (bags) and plexiglass equilibrium cell procedures have been used.

Sulfisoxazole binding to 4% albumin at 200 µg/mL and approximate physiological conditions ranged from 75.86 ± 0.33% to 89.27 ± 0.42% using conventional equilibrium dialysis; 74.12 ± 0.49% to 90.65 ± 0.25% using ultrafiltration. The values determined by conventional equilibrium dialysis are slightly higher than those of Anton (1960) who has reported a value of 68.0% using similar conditions. Higher binding values have been reported for sulfisoxazole using whole blood (Yacobi and Levy, 1979), and serum or plasma (Briggs, et al., 1983; Zini, et al, 1979). This difference between binding percentage measured in plasma and corresponding albumin concentration has been attributed to the binding of sulfisoxazole to plasma proteins other than albumin (Briggs, et al., 1983). Decreased sulfisoxazole binding has been reported in patients with impaired renal function (Yacobi and Levy, 1979).

Unlike sulfisoxazole, studies of theophylline binding to bovine serum albumin have been done using several methods, including conventional dialysis (Eichman, 1962), ultrafiltration (Briggs, et al., 1979), and immersible molecular separation (Briggs, et al., 1979). Fedak (1980) has demonstrated significantly different results for theophylline protein binding using the three methods under standard conditions.

Theophylline binding to albumin was found to be in the range of 18.93 ± 0.50% to 24.47 ± 0.56% using equilibrium dialysis (bags) and 38.19 ± 0.52% to 46.36 ± 0.28% as determined by ultrafiltration membrane cones. Theophylline binding measured using the Dianorm varied from 24.29 ± 0.56% to 27.74 ± 0.55%. These values are in close agreement with those reported by the above authors under similar conditions.

The low binding of HI-6 to albumin suggests that protein binding may be of little clinical importance in the therapeutic use of the drug. However, because the importance of other plasma proteins in the binding of HI-6 was not investigated, conclusions drawn from studies on binding to albumin alone should be interpreted with care. It was predicted that the highly polar nature of this drug would result in low binding to proteins and the present results with albumin are in accordance with this.

The observed decrease in the fraction of sulfisoxazole bound with increasing concentration of the drug implies that the number of binding sites on albumin available to this drug is limited. Zini, et al., (1976) have demonstrated that there is only one family of sulfonamide binding sites on human albumin, and this may be the case with bovine albumin. The significant increase in sulfisoxazole free fraction within the therapeutic range (8.08% at 100 μg/mL to 23.67% at 200 μg/mL using conventional equilibrium dialysis), demonstrates the importance of using pharmacologically realistic drug concentrations when studying the protein binding of this drug and others which show concentration-dependent binding, such as diazepam, valproic acid and dexamethasone. The high binding of sulfisoxazole, results in a situation in which any slight changes in binding could result in a significant change in the free fraction of the drug. Under normal circumstances, the increase in free fraction of sulfisoxazole is compensated for by an increase in the metabolism

of the drug (Levy, 1980), resulting in an overall minimal change in free fraction. However, this may not apply in patients whose elimination processes are compromised or in genetic slow drug metabolisers.

Theophylline binding to albumin as determined by conventional equilibrium dialysis showed binding to be independent of drug concentration. Fedak (1980) has reported similar results using the same procedure and ultrafiltration membrane cones. The observed concentration dependence of theophylline binding at concentrations below 15 μg/mL using ultrafiltration and Spectrum equilibrium dialysis could be artefactual, since there is proportionately higher non-specific binding of the drug to apparatus at lower drug concentrations. Any drug concentration dependence of the binding of HI-6 and other low bound drugs would be masked by the wide variations observed between replicates at the same concentration.

Both sulfisoxazole and theophylline binding showed a significant decrease of percent drug bound with increasing temperature using all procedures of determining binding. Sulfisoxazole binding at 37°C was observed to be 82.91 ± 0.72%, 80.96 ± 0.11% and 78.04 ± 0.36% respectively using conventional equilibrium dialysis, the Dianorm and ultrafiltration membrane cones. Respective values of theophylline binding were 18.52 ± 0.50%, 24.47 ± 0.56% and 38.19 ± 0.52%. All methods gave significantly different results from each other at this temperature.

Some authors have reported protein binding values of sulfisoxazole (Anton, 1960) and theophylline (Brors, et al., 1983), at room temperature. The observed temperature-dependence of the binding of sulfisoxazole and theophylline implies that protein binding studies done at non-physiological temperatures may not be relevant to the clinical situation. Protein binding results using conventional equilibrium dialysis obtained for sulfisoxazole and

theophylline at 25°C (89.48 ± 0.39%, and 20.82 ± 0.22%, respectively) indicate that results done at room temperature yield an elevation in protein binding results amounting to approximately 7.4%, and 11% of the amounts determined at 37°C for sulfisoxazole and theophylline, respectively. Corrections to these results based on calculated factors for conversion to give data equivalent to those which would be obtained at 37°C would be necessary for them to have clinical relevance.

Although no significant difference was observed in the binding of HI-6 at the different temperatures, the examination of the numerical data suggested a trend towards a decrease in the binding of HI-6 with increasing temperature. The very low protein binding of the drug plus the wide variability observed between replicates at any given temperature was the main reason for not detecting any differences in binding between temperatures.

All three drugs showed an increase in the extent of drug binding with increasing albumin concentration. Although at low albumin concentrations the drug binding increased rapidly, the increase became progressively gradual with further increases in albumin concentration beyond 4% w/v. Many authors, including Goldstein (1946) have reported possible polymerisation of albumin in solutions of concentrations greater than 2 g/litre. Such polymerisation may have two possible effects;

1. If albumin molecules are bound by sites other than those that bind the drug, there will be a fall in the number of binding sites due to steric blocking, although affinity would be preserved.
2. If albumin molecules bind on sites that bind the drug, then there will be a reduced affinity for the drug although the total number of binding sites would remain constant. The albumin binding would use some sites otherwise available to the drug.

The observed progressive decrease in the affinity of albumin for the drug implies that the latter mechanism may be responsible for the observed decrease in extent of binding at high albumin concentrations.

Although cases of hyperalbuminemia are rare, many pathophysiological conditions are associated with significantly decreased levels of albumin. In certain disease states, such as liver disease (Branch, et al., 1976), and the nephrotic syndrome (Gugler, et al.,1975), plasma albumin levels may fall to half the physiological concentration. Knowledge of how protein binding of a drug varies with changes in protein concentration may help to explain some of the observed drug-related toxic reactions which occur in some patients.

Many protein binding studies using albumin have been done at an original albumin concentration of 4 g/100 mL, which represents the average physiological concentration of this protein in plasma. Due to the dilution effects in conventional equilibrium dialysis, the protein concentration at equilibrium may be significantly lower than that originally present. The observed dependence of drug binding to albumin concentration implies that many of the reported values of protein binding using these procedures are an underestimation of the actual binding under physiological conditions. In addition, the relatively weak affinity of albumin at high concentrations permits binding to other circulating proteins which make it impossible to deduce binding percentages in plasma from in vitro percentages of binding to albumin. For drugs that bind to albumin as well as other plasma proteins, it may be more realistic to measure protein binding in plasma rather than isolated albumin.

The dependence on pH of binding in human serum has been reported for a number of drugs including warfarin, propranolol, fusidic acid, imipramine, quinidine, theophylline and sulfonamides. The binding of theophylline to albumin was shown to increase with increasing pH whereas sulfisoxazole binding

decreased with pH, within the basic pH range. It has been established that around neutral pH, bovine and human serum albumin exist in two conformational forms, the N- and B-forms (Wilting, et al., 1980). In phosphate buffer, pH 6.0 almost all the protein is in the N-conformation, whereas at pH 8, the beta-form is predominant. The N-B transition in albumin occuring between pH 6 and 9 is thought to be responsible for the observed effect of pH on binding. Katz and Klotz (1953) have shown that an increased number of sites becomes available for anionic and neutral ligands at pH values higher than to the isoelectric point of albumin. It has also been revealed that 150 "non-exchangeable" hydrogens exist in bovine serum albumin, but these hydrogens become completely exchangeable at the extreme pH values (<3.0 and >8.5). These findings are consistent with the observed increased binding of theophylline and other drugs, with increasing pH. The binding of sulfisoxazole, however, decreased with increasing pH. Henry et al., (1981), have also reported a decrease in the binding of fusidic acid with increasing pH. These differences may be attributed to differences in drug ionisation and to conformational changes in the albumin molecule that occur in this pH range.

The implication of pH dependence of drug serum binding is that binding data from experiments run at non-physiological or unknown pH values may be irrelevant to in vivo conditions. Most clinical binding studies are done on "spiked" serum. It has been shown that the pH of stored serum increases with time. It is therefore important to bring the pH of "spiked" serum back to the original whole blood pH before protein binding studies are done, if the extent of drug binding is pH-dependent.

In the past few years HPLC has become established as the method of choice for determining drugs in biological fluids. The spectrophotometric procedure used for HI-6, although simpler and more convenient, is less sensitive than

HPLC and its use may involve measurement of metabolites or other breakdown products. In a series of stability studies done in our laboratory (Keling and Purino, unpublished data), solutions of HI-6 in phosphate buffer assayed by U.V. spectrophotometry consistently gave higher values than identical solutions assayed by HPLC. After four days of storage of 250 mg HI-6 in solution at 95°C, the HI-6 remaining when assayed by spectrophotometry was 11.32 ± 0.31 mg/ml whereas no HI-6 was detected using the HPLC assay. The implication of these results is that the chromatographic procedure for HI-6 assay is more specific than the spectrophotometric method. A breakdown product appears to be interfering with HI-6 assay in the latter method.

A very strong correlation was observed between results of assay of non-degraded HI-6 using HPLC and spectrophotometry, even at concentrations as low as 5 μg/mL. No significant difference was detected between HI-6 protein binding values at different temperatures, obtained using the two methods of assay. The reason for the observed deviation of our results from those reported earlier may be the relatively milder conditions and shorter times of incubation in our study. Since it was not possible to obtain HI-6 protein binding values using conventional dialysis, it was not possible to compare the results of this method with those obtained using ultrafiltration and the Dianorm. Because of the longer time taken for equilibration in the former method, it is likely that some breakdown of HI-6 would have been detected, especially at 45°C. The influence of these breakdown products on protein binding measured for HI-6 is unknown. However, they could affect both the actual binding and the assay, leading to anomolous results, particularly if a spectrophotometric assay was used.

CONCLUSIONS

Measurement of free drug levels in monitoring therapy with highly bound drugs has taken on an increased importance in recent years. This is particularly relevant in drug displacement situations or when disease states cause modifications in plasma proteins or alterations in their concentration in plasma. For highly bound drugs which also have a narrow therapeutic index, slight changes in binding may result in disproportionate changes in the free (unbound) drug, and consequently, significant changes in the pharmacological effects of the drug. A simple, rapid and convenient procedure is required for routine determination of plasma free drug levels in the clinic.

The binding of sulfisoxazole, theophylline and HI-6 to bovine serum albumin was evaluated using conventinal equilibrium dialysis (bags), Spectrum equilibrium dialysis and ultrafiltration membrane cones. The extent of binding of all three drugs was shown to be directly related to temperature and albumin concentration. The binding of theophylline and sulfisoxazole also was affected by the pH of the medium. Percent bound sulfisoxazole decreased with increasing drug concentration, but theophylline and HI-6 binding was independent of initial drug concentration.

The protein binding results obtained by the three methods used each drug were significantly different from each other. Generally, for all three drugs, ultrafiltration gave the highest results for percent bound, whereas conventional dialysis gave the lowest. Ultrafiltration appears to be the method of choice for determining the protein binding characteristics of low bound drugs. Both Spectrum equilibrium dialysis and ultrafiltration may be used for medium bound drugs with reasonable accuracy, provided extensive binding of the drug to the test apparatus does not occur. Generally, the protein binding of highly bound drugs does not seem to be affected significantly by the method

used for separation.

HI-6 was found to bind to 4% bovine serum albumin to an extent of approximately 5% using ultrafiltration membrane cones. The spectrophotometric assay procedure for HI-6 gave results comparable with those obtained by HPLC. The former method of assay may be used in routine day to day analysis of non-degraded HI-6. However, it must be noted that the behaviour of HI-6 in biological fluids may be different from that observed in aqueous buffer, and any extrapolations should be interpreteted with caution. Similarly, the presence of breakdown products may interfere with the spectrophotometric assay of stored samples.

The results of this study demonstrate the importance of using standard physiological conditions when determining the protein binding of drugs, to obtain clinically relevant results. The method of determining binding has a significant effect on the results obtained, especially for very low bound drugs like HI-6, and to a lesser extent, medium bound drugs. For routine use, dialysis techniques are most suitable for medium and highly bound drugs. Although the technique influences results with low-bound drugs, the clinical significance of changes in the extent of binding of these drugs is minimal.

Precise, accurate data are much more important with highly bound drugs, particularly those with a low therapeutic index.

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Addenda

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