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# WARFARIN-PHENYLBUTAZONE: A DRUG INTERACTION

by

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B.A., University of California, San Diego, 1971

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

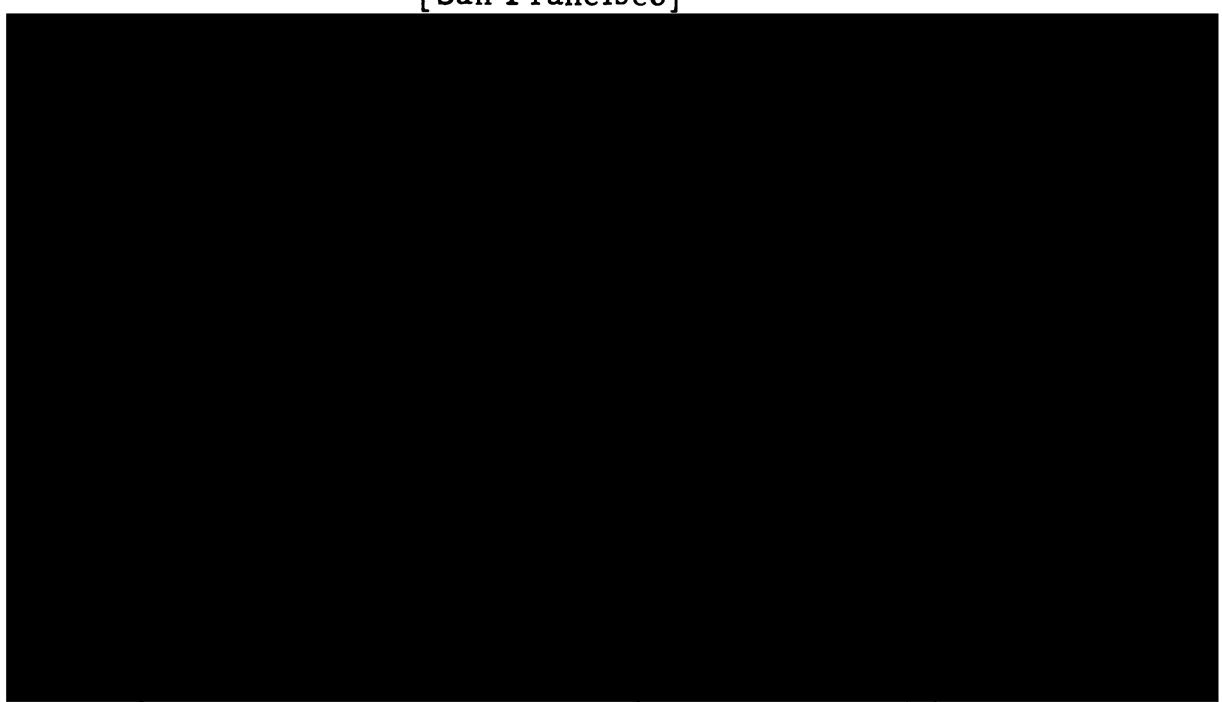
in

Comparative Pharmacology and Toxicology

in the

GRADUATE DIVISION

[San Francisco]



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Degree Conferred: . . . . . .

I dedicate this Master's Thesis

to

my parents and my fiancee´

## ACKNOWLEDGMENTS

I wish to express my gratitude to those who assisted me in this project

First to Dr. Rowland, under whose supervision and guidance, this project was initiated and completed. He provided many hours of assistance in all areas of this project--initiation, planning and experimental design, evaluation and interpretation, and preparation of this manuscript.

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# ABSTRACT

A basic premise in pharmacology is that the response to a drug is determined by its unbound concentration in plasma. Upon development of a sensitive, radioisotope assay, in which measurement of unbound warfarin is obtained, in vitro determinations of the fraction unbound of warfarin at therapeutic plasma concentrations were made using equilibrium dialysis of human plasma. The affinity constant of warfarin in human plasma at 37°C and pH 7.4 was measured to be $2.0 \times 10^5$ $M^{-1}$, with 1.2 binding sites to plasma albumin.

Comparison of in vitro data to that of in vivo data demonstrated that the binding of warfarin in the absence of exogenous displacing agents were similar. However, in the presence of phenylbutazone, there is a discrepancy between the two sets of data, suggesting a mechanism other than simple displacement from albumin by phenylbutazone.

A study in which a multiple dose regimen of warfarin was given in the absence and presence of phenylbutazone was performed. Daily measurement of plasma and urinary levels of warfarin and its metabolites, along with the unbound concentration of warfarin in plasma, suggests that the interaction and subsequent potentiation of warfarin hypoprothrombinemia by phenylbutazone is the inhibition of warfarin's metabolism, and not displacement of warfarin from its albumin binding sites.

# INTRODUCTION

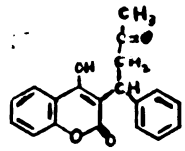
## Warfarin.

### History.

The coumarin anticoagulants, as have many drugs, were first isolated from natural sources, that of fermented sweet clover hay. In the early 1920's, a serious disease plagued cattle on the praires of North Dakota and Alberta, Canada. Cattle were bleeding to death from a strange malady, diagnosed as a new type of hemorrhagic septicaemia, or black leg (30). This was in line with the traditional thinking that diseases must be due to a pathogenic organism. This was not the case, however, for Schofield in 1922 described this malady as sweet clover disease. When cattle or sheep ate the spoiled clover hay, there was a progressive diminution of the clotting power of the blood and the resultant hemorrhages were usually fatal. Schofield assumed the hay contained a toxic substance that interfered with the normal blood-clotting mechanism of the animals. The toxic agent was presumably produced by the action of microorganisms during the curing process. In 1933, Link and his co-workers continued the study of this unusual phenomenon, its pathology, physiology, and biochemistry. Finally, in 1939, they identified the agent causing this disease: 3,3′-methylenebis-(4-hydroxy coumarin), commonly known as dicumarol and synthesized it in 1940. Link later synthesized warfarin in 1944, and it was recommednded for use as a rodenticide in 1948. In 1951, a man who attempted suicide with warfarin, survived and clinical trials established its safety in man in 1953 (39). Sodium warfarin is now the most popular oral anticoagulant in the United States.

Chemistry and Structure-Activity Relationship.

The basic feature of the coumarin derivatives is the 4-hydroxycoumarin moiety; 1-(4′-hydroxy-3′-coumarinyl)-1 phenyl-3 butanone being the scientific notation for warfarin, whose structure is:



Warfarin is an anionic acid with a $pK_A$ of 4.8. It is a lipid soluble drug at pH 7.4 since it is primarily unionized and easily passes through lipid membranes.

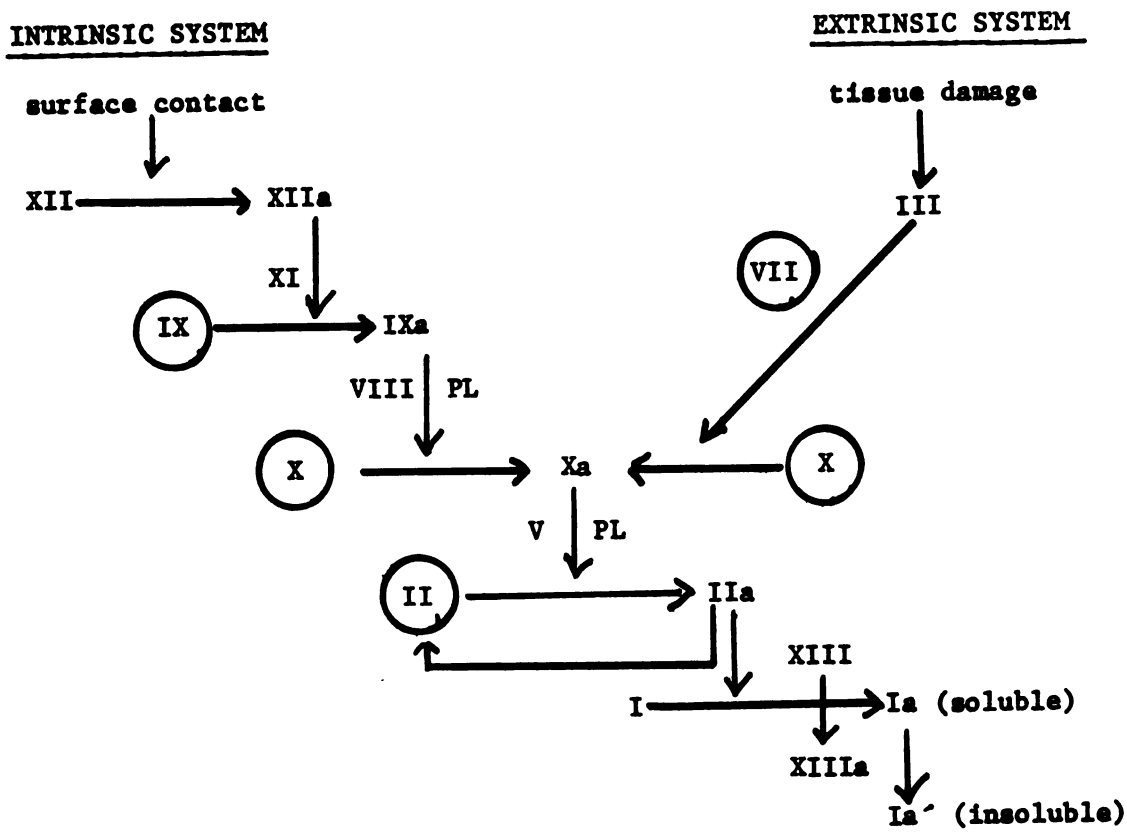
The correlation of the chemical structure with anticoagulant activity has been studied by Link and others ( 30 ). The essential requirements for anticoagulant activity appear to be an intact 4-hydroxycoumarin residue and a hydrogen or a hydrocarbon substituent at the 3-position. The effects of substituents in the 3-position and on the benzene rings are important for activity ( 1 3).

Pharmacology.

The only major pharmacologic action of warfarin and the other coumarin anticoagulants is the inhibition of the blood clotting mechanism. All coumarin anticoagulants act similarly, with differences being mainly quantitative rather than qualitative. However, in order to understand the pharmacologic action of warfarin, one must examine the blood clotting mechanism (Figure 1) . The prevention and control of abnormal bleeding requires the integration of a number of reactions.

Thrombin (IIa)does not exist in normal circulating blood, but appears in the plasma during clotting and is the agent that acts on fibrinogen (I). It is transferred to fibrin monomers (Ia), which are transferred by a thrombin activated factor XIII to form covalently bounded fibrin monomers (Ia′). Thrombin is activated from its precursor, the proenzyme plasma prothrombin (II), and can inhibit its own formation (1 3 ). Thrombin activation occurs either

Figure 1




**Legend:**

I. Fibrinogen
Ia. Fibrin monomers non-covalently bonded
Ia´ Fibrin covalently bonded
II. Prothrombin
IIa. activated Prothrombin
III. Tissue Thromboplastin
IV. Calcium (not shown but required for most of the reactions)
V. Proaccelerin
VI.
VII. Proconvertin
VIIa. activated Proconvertin
◯. Vitamin K dependent factors

VIII. Antihemophilic factor
IX. Plasma Thromboplastin Component (PTC)
IXa. activated PTC
X. Stuart-Prower Factor
Xa. activated Stuart-Prower Factor
XI. Plasma Thromboplastin Antecedent (PTA)
XII. Hageman Factor
XIIa. activated Hageman Factor
XIII. Fibrin Stabilizing Factor
XIIIa. activated Fibrin Stabilizing Factor
PL. Platelet Phospholipid

Scheme of the Blood Clotting Mechanism

through an intrinsic or an extrinsic pathway. The intrinsic pathway is the route in which all the necessary factors are present in the circulating blood beginning with surface contact, although the chemical or physical property that determines surface activation has not been determined. The intrinsic mechanism integrates the interaction of V, VIII, IX, X, XI, and XII,platelet factors and calcium ions. The extrinsic pathway begins following tissue damage which releases tissue thromboplastin (III) which interacts with factor VII in the presence of calcium ions to activate X. Activated factor X (Xa) interacts directly with V, and a phospholipid to convert prothrombin (II) to thrombin (IIa).

The synthesis and release of clotting factors II (prothrombin), VII (proconvertin), IX (Christmas), and X (Stuart-Prower), are vitamin K dependent and their abnormally low plasma concentration is often described as hypoprothrombinemia. The potency of Vitamin K on the synthesis of these factors varies considerably among individuals (39 ). The mechanism of Vitamin K on the production of these factors has not been established, with the current theories recently reviewed (4,10,12,39).

The normal regulation of clotting factors, are released at rates that maintain their normal plasma concentration by balancing catabolic losses. After a loading dose of warfarin, the factor with the shortest half-life declines first with the others following accordingly - factor VII, IX, X, II, with half-lives of 6, 20, 40 and 60 hours respectively(25,31). Warfarin and other coumarin anticoagulants affect only the rate of synthesis of the clotting factors; they do not alter the rate of catabolism of them. The reduced activity of these factors slows the generation of thrombin and fibrin and reduces hemostatic effectiveness (39 ). The kinetics of warfarin's hypo prothrombinemic effects have been reported by Nagashima et. al. (38 ).

The hypoprothrombinemic response is quantitated by means of the one-step prothrombin time, the thrombo test, or the prothrombin and proconvertin test ( 26).

Pharmacokinetics.

The biological response of warfarin is dependent upon its availability, its rate of absorption into the body and its rate of elimination, primarily by the liver. Warfarin is usually administered orally as the sodium salt, but since this compound is highly soluble in aqueous media, it is equally effective on oral, intramuscular, intravenous, and rectal administration. When given orally in man, the amount absorbed was reported to range from 78% to 100% of the dose in eight subjects, as compared to the area under the curve of an intravenous dose ( 6 ). Warfarin absorption started soon after administration and was completed by approximately two hours after dosing with the biological response having a latent period of approximately 12 hours. This time interval is followed by a rapid increase in prothrombinemic activity which reaches a maximum in 36 to 72 hours (6,39).

Warfarin is administered clinically as a racemate with the S(-) isomer 3 to 6 times more potent than the R(+) isomer in man(5,28). Once in the blood, warfarin is highly bound solely to the plasma albumin (43 ). Warfarin bound to albumin is inactive and not subject to biotransformation and excretion as is the unbound drug ( 26). Warfarin is transported to the liver where it undergoes metabolism, which is thought to occur in the microsomal, cytochrome P-450 dependent fraction ( 22 ). The isomers differ in their route of metabolism, with S-Warfarin oxidized primarily to 7-hydroxy warfarin and secondarily reduced to the SS alcohol ($Alcohol_2$) and oxidized to 6-hydroxy warfarin; whereas R-warfarin is oxidized solely to 6-hydroxy warfarin and reduced to RS alcohol ($Alcohol_1$). The schematic of the proposed

metabolic routes is shown in Figure 2. These two pathways appear to be noncompetitive (29 ).

The aromatic hydroxyl metabolites then undergo glucuronide conjugation, excretion into the bile, deconjugation in the intestinal tract and are largely reabsorbed into the blood and excreted into the urine as unconjugated, hydoxylated metabolites ( 1 ). There is little unchanged warfarin found in the urine, due to reabsorption by the kidney tubule.

The isomers of warfarin are eliminated by different metabolic routes and have different rates of elimination from the plasma. Breckenridge (5 ), Lewis (29 ), Levy (28 ), all found that there was an increased elimination rate for S(-) warfarin than for R(+) warfarin with no apparent change in volume of distribution (5 ). This increased clearance of S warfarin was true for both single and multiple dosing studies (5 ).

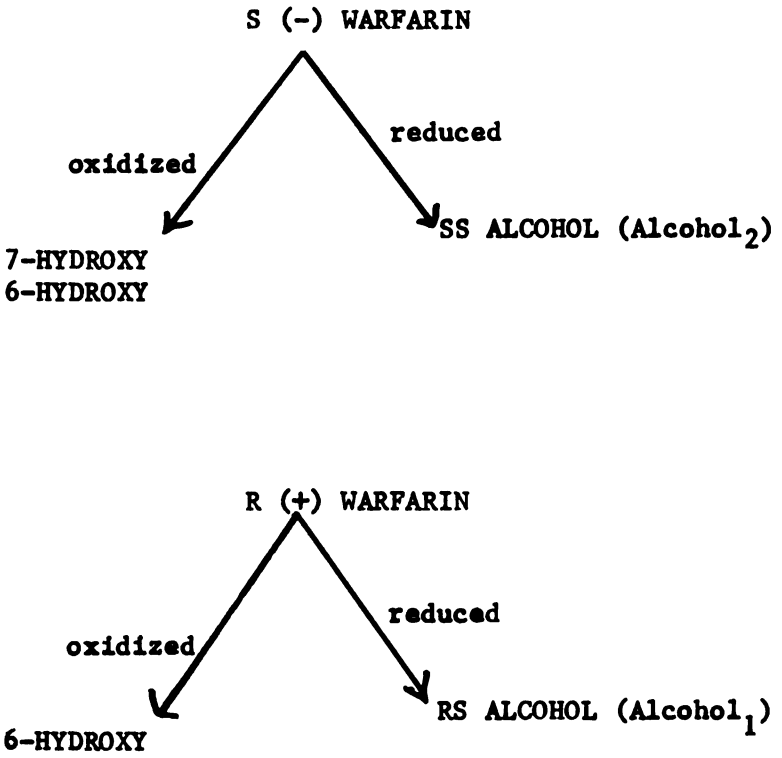
Following the cessation of warfarin therapy, the clotting mechanism returns to normal operation in 4 to 5 days depending upon the concentration of warfarin present at the time of cessation of therapy (39 ).

Drug-Protein Binding.

As early as 1878, Langley (27 ) suggested that drug-cell combination, and hence, the actions and effects of drugs, were probably governed by the law of mass action. This view was extensively developed by A.T. Clark in the 1920's, and it has remained the keystone of most theories of drug action.

Goldstein's classic review of interaction between drugs and proteins surveyed and summarized a rather large literature and clearly emphasized the potential importance of protein binding with respect to the behavior of drugs(19). Following this review, which increased an awareness of the significance of protein binding, many investigators were stimulated to study

Figure 2.




The Proposed Metabolism
of
R and S Warfarin (29)

and to reexamine the various aspects of the phenomenon. Scatchard (49 ) posed five fundamental guideline questions for those investigating drug-protein interaction: "How many? How tightly? Where? What of it? Why?" Research in this field, in attempting to answer the questions posed by Scatchard, moved in two directions:1) studies oriented to a fundamental physical-chemical understanding of the phenomenon which determined binding mechanisms, conformation of drug and protein association constants and other similar information, and 2) studies directed toward a more qualitative nature, experimentally and/or theoretically assessing the importance of protein binding as it relates to the action and uses of specific drugs (36 ).

Meyer and Guttman (36 ) followed the format of Goldstein (19 ), and summarized important observations of drug interaction reported since Goldstein's report in 1949 and discussed the newer experimental techniques.

Albumin is a protein, with a molecular weight of more than 69,000, which has a complex tertiary and quaternary structure consisting of more than 200 reactive residues, both cationic and anionic in nature (15 ). Because of the reactive nature of albumin, a number of important factors affect the structure, and thus, the capacity and nature of albumin binding. Temperature, the pH of the solution, ionic strength, dielectric constant of the medium, all have primary effects on the albumin conformation. In addition, the concentration of the drug being bound and the presence of other drugs or endogenous compounds that bind to albumin have an effect on the ability of the drug to bind to albumin(7,53). The above factors are important variables that must be considered when discussing and examining the drug-protein behavior.

Warfarin Protein Binding.

O'Reilly has presented a number of publications investigating the

thermodynamics of warfarin binding to albumin (43 ). Initial studies on binding to plasma protein were performed with the addition in vitro of the anticoagulant drugs to Cohn fractions of plasma proteins(42 ), while most studies employed crystalline human albumin (43 ). O'Reilly demonstrated , using continous-flow electrophoresis, that warfarin was bound solely to the albumin fraction ( 43). Subsequently, equilibrium dialysis experiments were performed demonstrating warfarin binding to albumin. Using 0.4% albumin solution (57 μM) at 27°C and pH 7.4, the warfarin concentration was varied from 2 to 400 μg/ml (6 μM to 1200 μM) and O'Reilly reported that warfarin at 27°C and pH 7.4 had an association constant of 2.3 x $10^5 M^{-1}$, and two binding sites, the second, weaker binding site, occurring at very high, non-physiologic concentrations.

Drug Interaction.

In the last two centuries, drugs have been the primary agent used to treat disease. Reactions between drugs may not have mattered 200 years ago when many of the agents were pharmacologically inert. William Withering in 1785, in his clinical description of the advocated treatment for "dropsy", suggested use of a wide variety of drugs (34 ). It is felt that then, there was a psychological appeal in prescribing not just one, but a combination of medically proven ingredients. This appeal was difficult to resist because of the inference that'more drugs are better than less', a conclusion far from the truth (34 ). Even now, this psychological appeal for the use of more than one drug to treat a problem plays a major role in drug therapy.

The pharmacologic action for any single drug may be understood but

there is no reason to assume the effect of a drug combination will be equal to the sum of the effects of the drugs administered individually (46 ).

A drug interaction might be broadly defined as any reaction between one drug and another substance within or outside of the body. Due to a wide range of factors such as age, disease, drug dosage and individual idiosycrasy, the actions of many drugs may be modified so that what appears as an acute interaction in one individual may be relatively mild or even absent in another (47 ).

Drug interactions can be classified as belonging within particular phases of drug activity: (1) pharmaceutical phase (involving the release of drug from the pharmaceutical preparation and its possible chemical incompatibility with other ingredients; (2) the pharmacokinetic phase (interactions are mediated by changes in drug absorption, binding, metabolism and excretion); (3) pharmadynamic phase (interactions principally mediated at specific sites of drug action). Stockley's classification is somewhat arbitrary as any drug interaction may occur in any or all of the categories(54).

Further investigation into any particular area is possible due to the large number of publications now available; however, it is not possible to list all of them. There are a number of publications that discuss drug interactions in general to which one could refer: Rowland (46 ), Azarnoff ( 3 ), Stockley (54 ), MacGreggor ( 34), Morrelli and Melmon (37 ), Prescott (44 ), Rosenoer and Gill ( 45), Hansten(20 ), and Martin (35 ).

There are numerous interactions between warfarin and other drugs. Hansten(20 ) and Koch-Weser (26 ) both summarize the apparent mechanism for each interaction with warfarin.

The most discussed interaction with warfarin is displacement by other drugs. <u>In vitro</u> studies have shown that a number of acidic drugs

including phenylbutazone(2,52), oxyphenbutazone(18,21),trichloroacetic acid(50) and diazoxide ( 50) have significantly displaced warfarin from albumin binding sites at concentration usually seen under therapeutic conditions. Many of these drugs potentiate the anticoagulant effect of warfarin (2,16,18,21,50), and thus, the proposed mechanism of action is one of displacement of warfarin from albumin.

Warfarin-Phenylbutazone Interaction.

The anti-inflammatory agent, phenylbutazone, is among the most effective potentiators of excessive hypoprothrombinemia causing serious hemorrhage which occurs in patients who have been clinically treated with coumarin and phenylbutazone. The interaction was recognized eight years after the release of phenylbutazone as a drug(26 ). This interaction is not unique for phenylbutazone, which has a diversity of effects on drug metabolism, excretion and protein binding when administered with other drugs.

The metabolism of drugs has been retarded, enhanced, or both following phenylbutazone or its analogues.(8,9,29). Also several effects on renal function have been produced by phenylbutazone and its analogues, including inhibition of tubular reabsorption or tubular secretion (17, 57 ).

The most reported interaction by phenylbutazone is the displacement of other drugs from albumin. This displacement has been reported *in vitro* ( 2,52 ) and has been associated with a change in the action of the displaced drug *in vivo* ( 2,26).

The mechanism of interaction between phenylbutazone and warfarin has been assumed to be a competitive inhibition of warfarin binding to albumin, thus increasing the concentration of unbound warfarin. This is responsible for the markedly enhanced hypoprothrombinemic action (39). Potentiation of the warfarin-induced hypoprothrombinemia appears to be

universal and may be seen following only one day of phenylbutazone therapy. It also causes a reduction in the dose of warfarin needed by 50 to 75% (26). Lewis has proposed another mechanism, one of a metabolic inhibition of warfarin by phenylbutazone (29).

## STATEMENT OF THE PROBLEM

One of the most common and toxic drug interactions is that of warfarin and phenylbutazone. Despite the rather large volume of literature acknowledging the occurence clinically, little investigation has been done to investigate its mechanism in man. Thus, the purpose of this work is to investigate the nature and mechanism of the warfarin-phenylbutazone interaction in man. This requires the development of an assay for the measurement of the fraction unbound of warfarin at therapeutic levels and its use in the evaluation of the importance of drug displacement in vivo in the warfarin-phenylbutazone interaction in man.

By using a multiple dose regimen for coadministration of warfarin and phenylbutazone the investigation of the interaction will proceed under usual clinical conditions, that of concurrent administration of both drugs. Thus, the resultant interpretation may have a more valid foundation in which to propose a mechanism of interaction in man.

# EXPERIMENTAL-EQUIPMENT AND MATERIALS

## Table I.

### EQUIPMENT

Beckman Expandomatic pH Meter

Beckman High Speed Ultracentrifuge Model L2-65B with SW-56 Ti Rotor

Beckman Spinco Model 152 Microfuge

Cahn Gram Electrobalance (Ventron Instruments Comp., Paramount, Ca.)

Cary Dual Beam Spectrophotometer Model 15

Centro Lab Jack

Constant Temperature Oven (Heraeus Hanan, W.Germany)

Dianorm Equilibrium Dialysis System (Inno. Med., Zurich, Switzerland)

Horizontal Shaker

International Centrifuge Model UV

International Table Top Centrifuge Model HN

Packard Tri-Carb Liquid Scintillation Counter Model 3375

Perkin-Elmer Spectrofluorometer Model 203

Shortwave Ultraviolet Mineral Light Model UVS-12 (Ultraviolet Products San Gabriel, Ca.)

TLC plate holders, solvent holders and tanks (Eastman)

Vortex (Clay-Adams)

Mettler Balance Model P-120

Table II.

MATERIALS AND SUPPLIES

Aquasol scintillation cocktail (New England Nuclear)

Beckman glass scintillation vials

Beckman Microfuge micro test tubes

Beckman polyallomer centrifuge tubes #328874

Butazolidin 100mg (Geigy)

Coumadin 5 mg (Endo)

Disposable Tuberculin syringes, 1 ml

Eastman no. 6061 Silica gel thin layer chromatography plates

Flint glass culture tubes 10x75 and 12x75 mm (Kimble)

Folin-Ciocalteau Phenol Reagent (Fisher Labs)

Mephyton 5mg (Merck, Sharp and Dohme)

Pederson micropipettes

Phenylbutazone (Geigy)

Polypropylene tubes

Precision spectrosil UV cells with a 1cm pathlength

Prescription bottles, 200ml

Screw-cap vials, one dram

$^{14}$C-Toluene ($4.17 \times 10^5$ dpm/ml) and $^{3}$H-Toluene ($3.17 \times 10^5$ dpm/ml)
International Chemical and Nuclear Corp.

Vacutainers--heparinized (143 USP units/tube)

Visking tubing 0.025 mm (Van Waters)

Warfarin (R,S, and RS) from Endo and $^{3}$H,$^{14}$C- and $^{14}$C-warfarin from Trager

## ANALYTICAL METHODS

### Protein Binding Methodology.

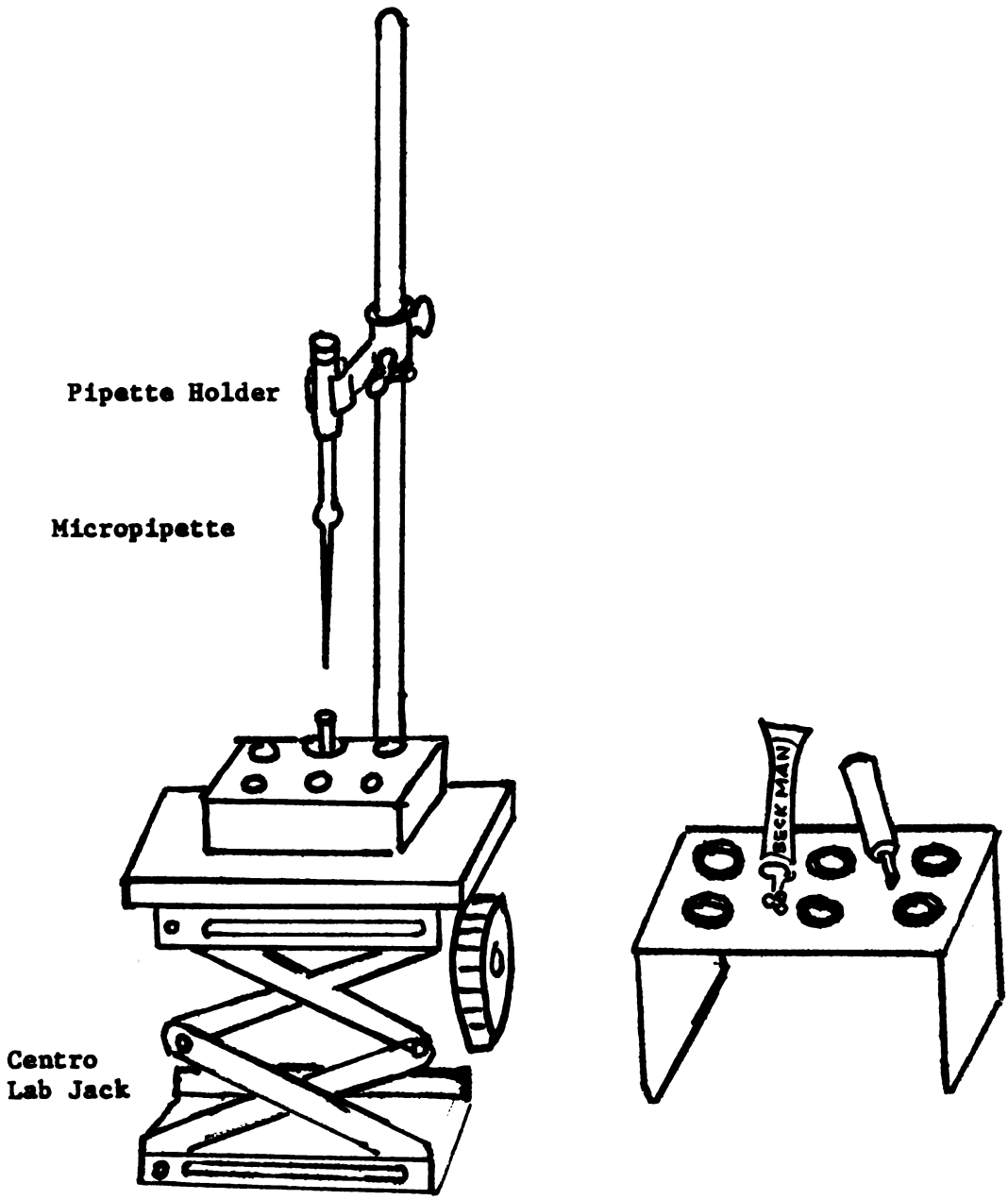
Two methods, high speed ultracentrifugation and equilibrium dialysis, were used for determining the binding of warfarin to plasma proteins.

#### Ultracentrifugation.

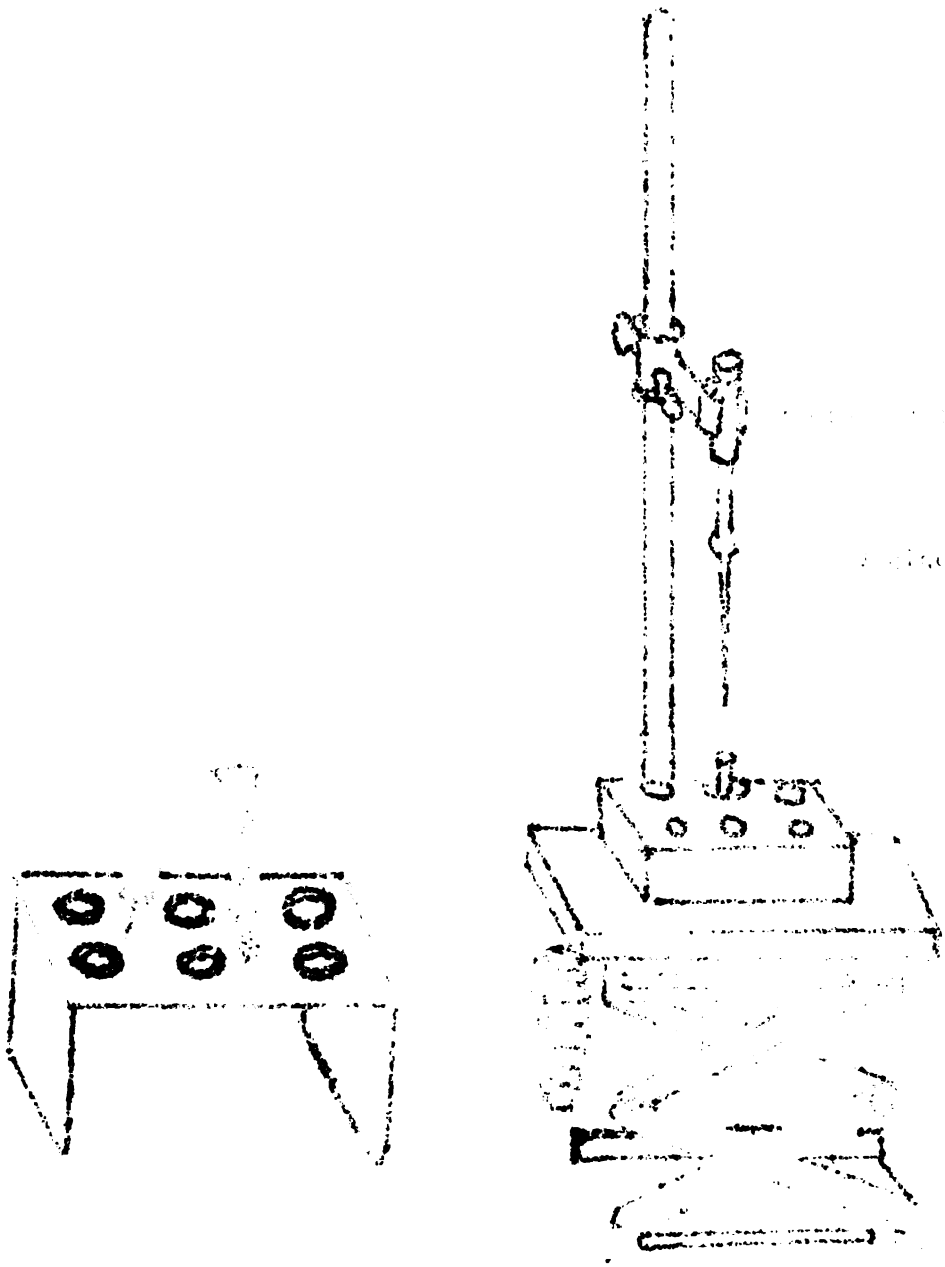
The plasma samples were carefully pipetted into 7/16 by 2 3/8 inch Beckman polyallomar centrifuge tubes, preventing the formation of air bubbles in the tubes. The tubes were placed in the swing buckets, filled 1/8 inches from the top (approximately 4.3 ml per sample), and counter-balanced within 50 mg of each other on a Mettler P-120 balance. The buckets were sealed, placed on the rotor and incubated in a constant temperature oven at 37°C for 2 hours prior to centrifugation. The rotor is then placed in the Beckman 12-65B ultracentrifuge with centrifugation at 37°C proceeding for 24 hours at 55,000 rpm (400,000 x g). Following centrifugation, the tubes were carefully removed from the buckets and placed in a wooden block with pre-drilled 1/2 inch holes. The block is placed on a Centro Lab jack and moved vertically until the tip of the Pederson micropipette is just below the surface of the first tube (figure 3). Samples of the clear supernatant of 100 µl or 200 µl are removed by simultaneously twisting the screw-top of the pipette holder and moving the jack upward, thus keeping the pipette tip just below the floating layer of lipoproteins while sampling. The aliquot is then placed into a Beckman glass scintillation vial. The unbound fraction of drug, $\alpha$, was computed by

$$\alpha = \frac{\text{plasma-water cpm/ unit volume}}{\text{plasma cpm/ unit volume}}$$

Figure 3.




An Illustration of the Apparatus for Removal
of
Aliquots from the Ultracentrifuge Samples



A second aliquot of 100 µl is placed in a 400 µl Beckman microfuge tube for a protein determination.

Equilibrium Dialysis.

The second method used to determine the protein binding of warfarin was that of equilibrium dialysis using the Dianorm Equilibrium Dialysis System (56 ). This system consists of 20 teflon cells, each consisting of two 1 ml half-cells separated by a Visking cellulose membrane, which can be run simultaneously.

Visking dialysis tubing, 13/16 inches flat width by 0.025 mm, was cut into 2-inch segments and soaked for at least 15 minutes in distilled water. The tubing was then cut along the folds into two pieces and put into 30% ethanol for 30 minutes. These membranes were repeatedly rinsed with distilled water, allowed to stand 15 minutes, rinsed again, and finally stored in distilled water until used. The membranes are blotted with tissue and placed in Krebs-Ringer Improved buffer (11 ), pH 7.4, prepared the day of the experiment. The buffer is discarded after 20 minutes, and new buffer is added to the membranes. The membranes are blotted with tissue paper to remove excessive buffer, just prior to insertion between the half-cells.

After assembly of the cells with the membranes and supports, they are ready to be filled with the appropriate plasma samples and buffer. Prior to injection of the samples into the cells, an aliquot is removed by a Pederson micropipette from each of the plasma samples and placed in a glass scintillation vial, to be used in the protein binding determinations. Then 1 ml plasma samples are injected into one of the half-cells by a disposable 1 ml tuberculin syringe with a 1/2 inch blunt needle while the buffer is simultaneously injected into the other half-cells. Each cell is done individually using a new syringe with the needle rinsed in buffer for each new

plasma sample. The cells were sealed with teflon plugs and this procedure continued until all the cells had been filled. The cells in the supports were then mounted on drive units and the apparatus was placed in a 37°C constant temperature water bath. The cells rotated at 10 rpm perpendicular to the membranes allowing continuous mixing of the cell contents. Dialysis continued for 6 hours unless otherwise noted. After 6 hours, the cells are removed from the water bath, each half-cell was quickly emptied using a new tuberculin syringe and needle, and the contents transferred to a labelled disposable culture tube. Using a Pederson micropipette, 100 µl aliquots from the plasma half-cell samples and 500 µl aliquots from the buffer (plasma-water) half-cell samples are placed in glass scintillation vials for protein binding determination. The fraction of unbound drug was computed by

$$\alpha = \frac{\text{buffer cpm/unit volume}}{\text{initial plasma cpm/unit volume}}$$

In addition a 100 µl aliquot is taken from the buffer samples and placed in Beckman microfuge tubes to be used in protein determinations for membrane leakage.

The cells were then rinsed, soaked overnight in distilled water, and then rinsed with methanol and acetone and allowed to dry.

Analysis of Warfarin and Metabolites.

Warfarin and the metabolites were extracted from the plasma and urine[1] samples, separated chromatographically, and quantitated. Each

---

[1]Urine samples following the above protocol, had a fluorescent yellow contaminant that did not allow measurement of 6-hydroxy warfarin. In order to alleviate this problem. A modification in which a two-dimensional TLC system using the same solvents as above was developed. The protocol for the two-dimensional system was the same as above except for modifications shown in brackets [ ].

sample was processed in duplicate. A standard of plasma or urine containing a known amount of warfarin, 6-hydroxy warfarin, 7-hydroxy warfarin, warfarin alcohol$_1$ and warfarin alcohol$_2$ was processed simultaneously.

Two ml of plasma or urine were acidified with 1.0 ml of 1.5 N HCL and extracted with 4.0 ml 1,2-dichloroethane (ethylene dichloride, EDC) in a polypropylene tube by agitation on a horizontal shaker for ten minutes. The tube was centrifuged at 10,000 x g, the aqueous and solid phases were discarded, and 3.0 ml of the EDC were transferred to a glass tube. While the EDC was evaporating in a stream of $N_2$, the sides of the tube were washed down 4-5 times with 100-200 µl of acetone. To the dried residue at the bottom of the tube, 25 µl of acetone was added. This was applied quantitatively as a 1.5 cm band [2.5 cm from the side and bottom] on a silica gel-G thin-layer chromatography (TLC) plate. The tube was rinsed an additional two times with acetone, which was added to the spot of application of the TLC plate.

The large spots of application were collected by chromatographing the TLC plates with methanol to a point 3 cm above the site of application. The plates were examined under short-wave ultraviolet light and the collection with methanol was repeated until all material with blue fluorescence had been collected at the methanol collection front. The TLC plates were cut off 2 cm below the methanol line. The plates were developed initially in chloroform:ethyl acetate:acetic acid 150:50:1 to a point 10 cm above the methanol collection line [5-10 cm from the top of the plate]. The plates were dried, cut off at a level of the methanol collection front, [rotated 90°, and a second methanol front was developed. The plates were then] developed a second time with t-butanol:benzene:$NH_4OH$: water 90:40:18:6 to a point approximately 15 cm [10-16 cm] above the methanol line. By this means, five fluorescent bands were resolved which corresponded to the

the standards: Rf .79 [.65,.54)] for warfarin, .69 [.53,.49)] for alcohol$_1$, .61 [.41,.50)] for alcohol$_2$, .53 [.29,.44)] for 6-hydroxy warfarin, and .23 [(.30,.34)] for 7-hydroxy warfarin. The bands were outlined with pencil, cut out, and each was placed in a 10 by 75 mm flint glass culture tube.

Warfarin, alcohol$_1$ and alcohol$_2$ were quantified proceding as follows: Each compound was eluted with 1.5 ml of acetone to the tube for 20 minutes. The acetone was transferred to a clean flint glass culture tube. The fluorescence of the solution was measured at 405 nm with the excitation wavelength of 345 nm, using a Perkin-Elmer model 203 spectrofluorometer. The fluorescence of each sample was remeasured after the addition of one drop of 3N HCl. The net fluorescence of the sample (the difference between the pre-acid and post-acid readings) was compared to the net fluorescence of the standard and the concentration of the compound was calculated from this ratio.

The fluorescent band containing 7-hydroxy warfarin was eluted for 20 minutes with 1.5 ml of n-butanol. The solvent was decanted into a clean flint glass culture tube. Three drops of concentrated HCl were added to the n-butanol, the tube vortexed and allowed to stand for one hour. The solutions were mixed, and the fluorescence was measured at 365 nm with an excitation wavelength of 330 nm. The fluorescence was measured for an n-butanol blank which was subtracted from all values. The concentration of 7-hydroxy warfarin in the sample was calculated by comparing the net fluorescence of the sample to that of the 7-hydroxy warfarin standard.

The band containing 6-hydroxy warfarin was eluted with 1.5 ml of Spectro-grade methanol for twenty minutes. The methanol was transferred to a clean flint glass culture tube, and the fluorescence was measured at 390 nm, with excitation at 325 nm. The concentration was calculated by

comparing the fluorescence of the sample to that of the 6-hydroxy warfarin standard.

The relationship between concentration and fluorescence was linear over a range from less than 10 ng to over 1000 ng per ml plasma or urine (29). Quenching, observed at much higher concentrations was obviated when indicated, by diluting both the sample and the standard with the appropriate solvent. The reproducibility of replicate determinations of more than 85% of the samples was within 15% or 15 ng of the average of the two or three determinations.

Protein Determination.

A modification was made of the Lowry method ( 33 ), a trichloroacetic acid precipitation, and used in the procedure so as to eliminate materials which might react with the Folin-Ciocalteau reagent.

The assay proceeds as follows: 100 µl of the plasma water or supernatant is added to a 400 ul Beckman microfuge tube, followed with 150 µl of 15% trichloroacetic acid. Each tube is vortexed for 15 seconds, allowed to stand 15 minutes, vortexed well again and then centrifuged 5 minutes in a Beckman Spinco Microfuge. Being careful not to remove any of the precipitate, the supernatant is removed using a #23 x 2 inch B-D Special Needle attached to a 1 cc disposable tuberculin syringe. The tips of the microfuge tubes containing the precipitate and remaining aqueous medium, are cut with a razor blade, and transferred to a 12 x 75 mm disposable culture tube. One ml of freshly prepared solution, of 2% sodium carbonate in 0.10 N NaOH and that of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate--mixed 50 parts to 1 part, was used to wash the precipitated protein from the

microfuge tube into a 12 x 75 culture tube. The tube is gently shaken and allowed to stand 10 minutes and repeated for an additional 10 minutes, in order to permit digestion of the precipitate. While vortexing lightly, 100 µl of the freshly prepared reagent D (2N Folin-Ciocalteau Reagent was added and mixed with equal parts water) and shaken for 15-20 seconds. Allow to stand 1 hour and then determine the absorbance at 750 nm read on a Cary 15 Spectrophotometer.

Standards must be run with the unknown samples. A 0.01 dilution of the plasma used to obtain the plasma water, is made with 0.9% sodium chloride. This stock dilution is further diluted 0.75, .5, .25, 0.1 with the 0.9% sodium chloride. These samples along with the stock dilution and an 0.9% sodium chloride blank constitute the standards. The resultant calibration curve is reproduced in Figure 4, wherein the absorbance is plotted against the dilutions of the original plasma sample. The protein determinations of the samples will thus be expressed as a percentage of the protein in the original plasma sample.

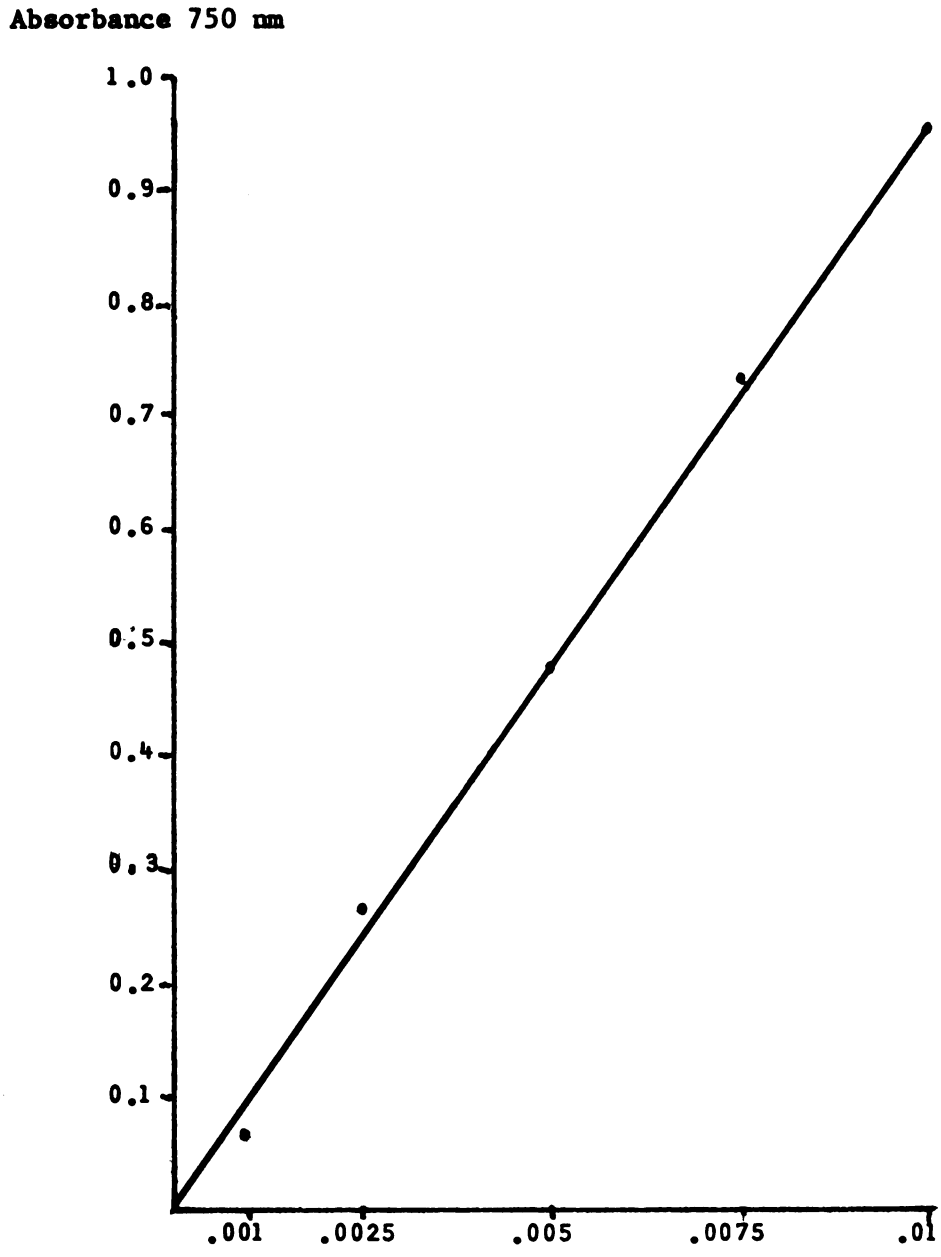
Phenylbutazone Determination.

Phenylbutazone concentrations were determined by the method of Jähnchen and Levy ([24]). A typical standard curve is reproduced in Figure 5.

Prothrombin Time Determination

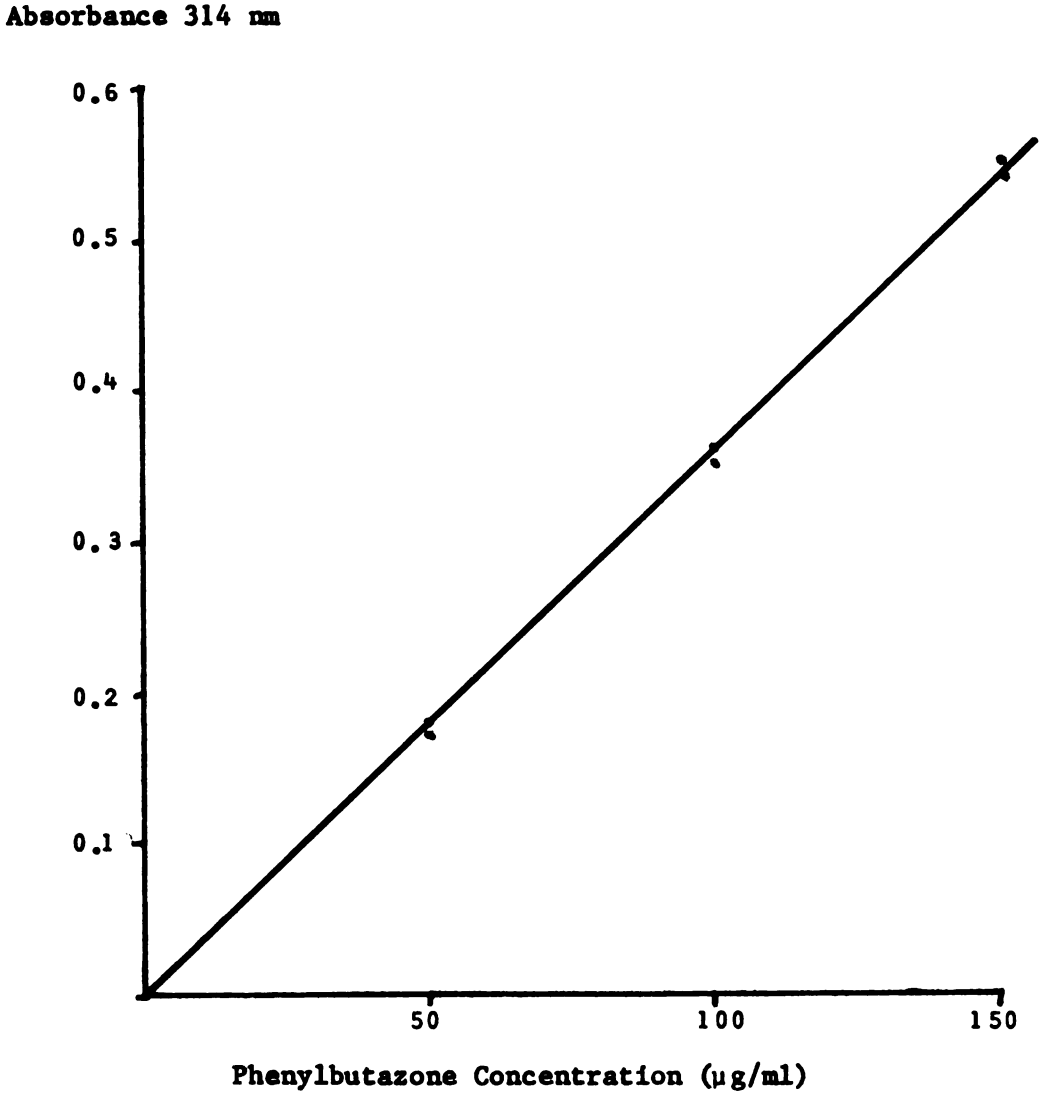
Prothrombin time was determined by Clinical Lab, UC Moff tt Hospital using an Electra-600 Automatic Photometric Clot Detection system.

Figure 4.




A Reproduction of a Lowry Protein Standard Curve
Using a
Serial Dilution of the Original Plasma as the Standard

Figure 5.




A Reproduction of A Standard Curve Obtained
for
Determination of Phenylbutazone Concentration

Electrophoretic Analysis of Plasma Proteins.

The protein fraction determinations on plasma samples were done on cellulose acetate using a Beckman Microsome-Model 15B and Clifford Densitometer (23). This procedure was performed by Cancer Research Institute Clinical Laboratory, UCSF.

The serum in which $^{3}H$-,$^{14}C$-warfarin had been added was electrophoresed on cellulose acetate. The resultant protein bands were cut into strips, placed in scintillation vials in which 10 ml of Aquasol had been added and were counted in a Beckman Liquid Scintillation Counter.

Sample Preparation.

The radiolabelled warfarin, unlabelled warfarin and phenylbutazone were weighed on a Cahn electrobalance, put into appropriately labelled one dram screw-cap vials, dissolved in 50-200 µl of absolute ethanol. An appropriate volume of 1M phosphate buffer, pH 7.4, was added to the samples in order to obtain the desired concentration. The phosphate buffer maintains the pH of each drug solution. The samples were frozen after use. The phenylbutazone solution was not used if it was more than 3 or 4 days old.

All plasma samples were prepared from blood from healthy male volunteers unless noted otherwise. The blood, collected in heparinized (143 USP units) Vacutainer tubes, is centrifuged using a table-top model centrifuge for 30 minutes at 2500 rpm. The plasma is then used for immediate experimentation or frozen for future experiments. After obtaining fresh plasma, or thawing the frozen plasma in a beaker of water at 37°C, an aliquot of radiolabelled warfarin, whose volume does not exceed 2% of the volume of the plasma sample, is added to the sample. The pH is adjusted to 7.4 on a Beckman Expandomatic pH Meter by gassing the plasma samples with $CO_2$.

The samples are then incubated at 37°C until ready to be placed into the experimental appartus.

Radioactivity Measurements .

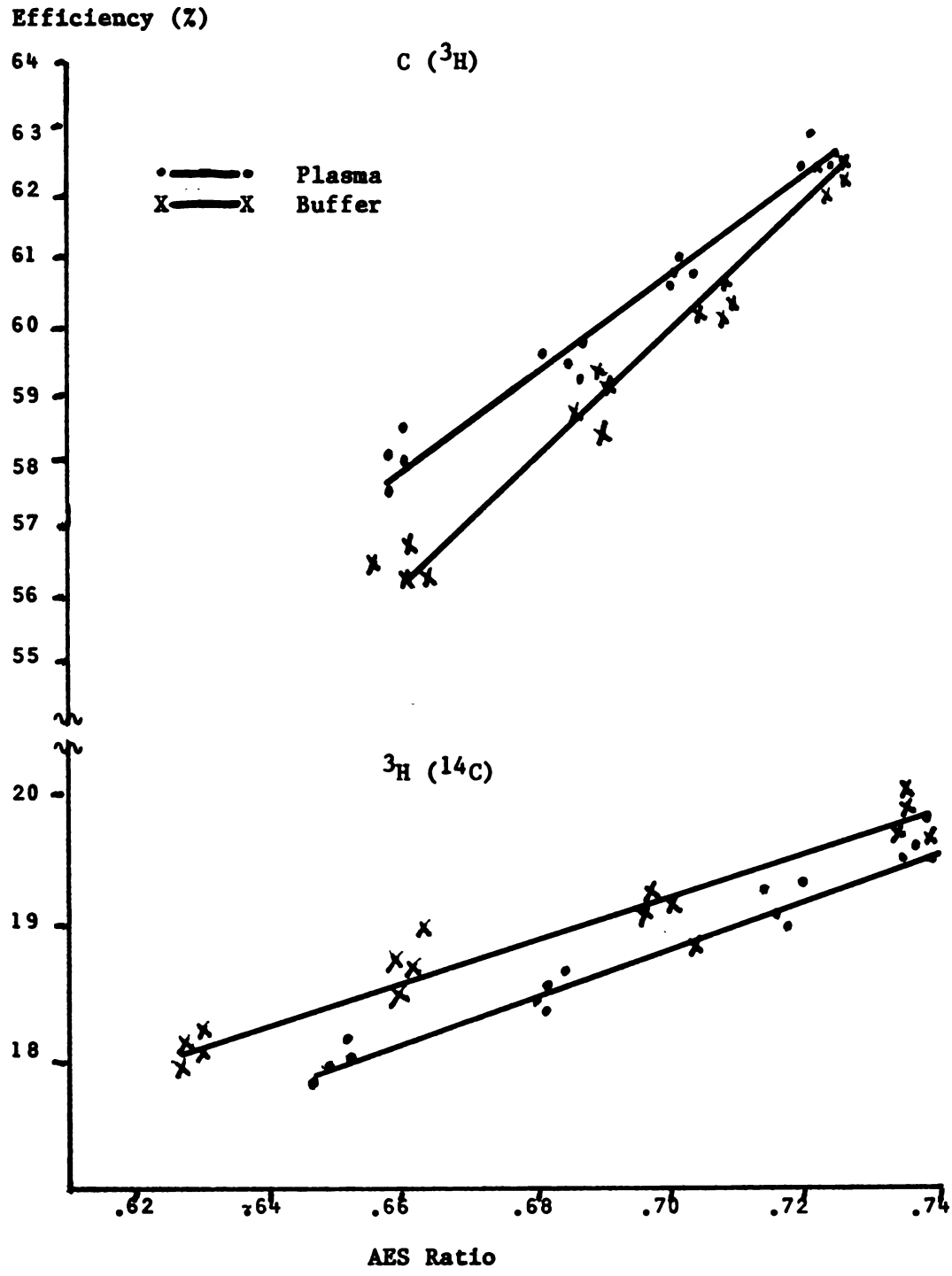
Each aliquot was measured using a micropipette and put into a Beckman glass scintillation vial, to which 10 ml Aquasol was added. The samples were counted 2 to 4 times for 5 to 50 minutes on a Packard Tri-Carb Liquid Scintillation Counter. There was a plasma and buffer blank for each counting cycle. The efficiency of the scintillation counting was determined by an automatic external standard (AES) procedure. A duplicate series of vials containing known amounts(dpm) of either $^{14}C$- or $^{3}H$-Toluene in Aquasol were counted along with a blank. To one of the series was added either additional plasma or buffer to act as a quencher. The vials were then recounted and lower AES ratios and cpm values were obtained for the quenched samples. A calibration curve for the double labelled experiments (Figure 6) and for the $^{14}C$- labelled experiment (Figure 7) were obtained by plotting the AES ratio against the efficiency (cpm/dpm) for each of the standards. Therefore, from each sample's AES ratio and cpm value, the dpm value and, thus, the efficiency could be obtained.

Protocol for Human Experiments.

Chronic administration of both warfarin and phenylbutazone was undertaken in a normal 25 year old white male under the guidance of a hematologist involved in warfarin research, and approved by the Committee on Human Experimentation at UCSF.

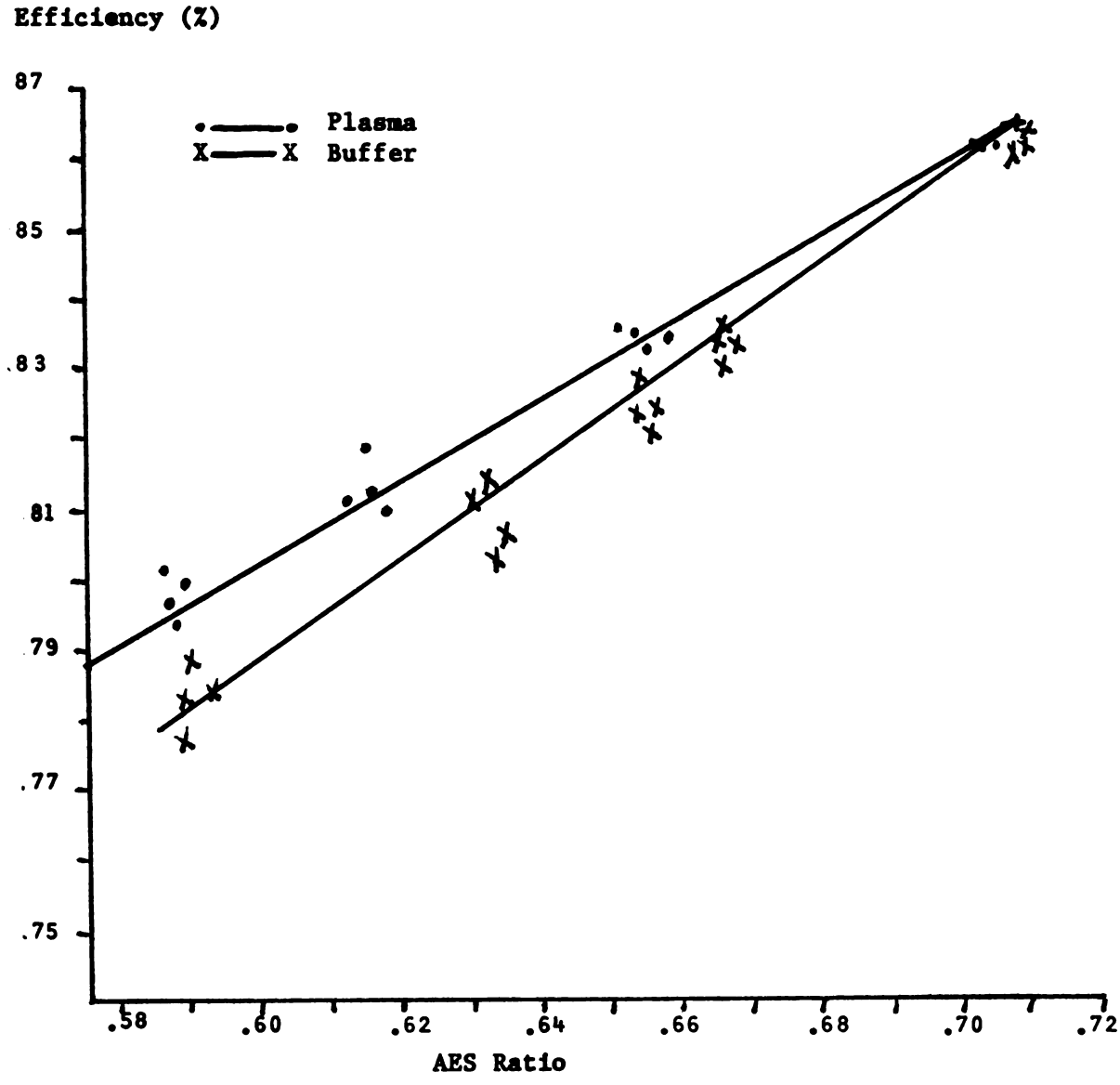
The individual was verified to be in a normal state of health through a preliminary physical and hematological examination. The latter of which included a white cell and differential count of the blood to determine a

Figure 6.




The Quenching Curve for Experiments Using Double-Labelled Warfarin, in which Human Plasma and Phosphate Buffer Act as Quenching Agents

Figure 7.




The Quenching Curve for Experiments using Single-Labelled $C^{14}$ Warfarin, in which Human Plasma and Phosphate Buffer Act as Quenching Agents

safe baseline and a prothrombin time determination. The study involved the collection of blood samples and 24 hour urine samples. Following two days of control sampling, a loading dose of 30 mg Coumadin$^{R}$, the sodium salt racemate of warfarin, was administered orally along with 10 mg of Mephyton$^{R}$ (Vitamin K) to prevent hemorrhaging. Subsequent to the loading dose, 10 mg of Coumadin and 10 mg of Mephyton were administered daily on an empty stomach according to the schedule in Table III. Urine was collected once the steady state level of warfarin was reached and continued daily until the end of the study. On the twelfth day of warfarin administration (11 March), 100 mg of Butazolidin$^{R}$ (a commercial preparation of phenylbutazone) was taken orally three times a day to simulate a normal clinical dosage regimen. After 10 days the phenylbutazone was discontinued while warfarin and Vitamin K were continued daily. After 10 more days, warfarin was discontinued. Following the study period, blood samples were collected every other day and urine was collected for one week.

Blood samples consisted of 20-30 ml of blood drawn on the appropriate days and placed in heparinized (143 USP units) Vacutainers, centrifuged, with the plasma frozen until the end of the experiment , for the purpose of subsequent analysis. Urine was collected for 24 hour periods, kept in a plastic container in the refrigerator until the following day when its volume was measured and a 150 ml aliquot was put into 200 ml prescription bottle and frozen.

Prothrombin times were done approximately every 10 days as indicated in Table III, with a total protein and fractional analysis done weekly(Table IV). A Hematocrit was obtained and hemoglobin determination was done periodically. Once on phenylbutazone, a white cell and differential count was done twice weekly along with a urinalysis and reticulocyte count twice in the 10 days in order to monitor for potential phenylbutazone complications and toxicity.

Table III.

MULTIPLE DOSE REGIMEN SCHEDULE

| Date | Days After Warfarin Commencement | Time of Drug Administration | | Time of Sample Collection | | Lab. Test |
|---|---|---|---|---|---|---|
| | | Warfarin | Phenylbutazone | Blood | Urine | |
| 25 Feb. | | | | | Blank | PTT and PA |
| 26 | | | | | Blank | |
| 27 | | | | Blank | | |
| 27 | 0 | 10:30 | | 10:30 | | |
| 28 | + 1 | 7:00 | | | | |
| 1 March | 2 | | | | | |
| 2 | 3 | 7:15 | | 10:30 | | PTT and PA |
| 3 | 4 | 8:30 | | | | |
| 4 | 5 | 6:30 | | | | |
| 5 | 6 | 7:00 | | 10:40 | | |
| 6 | 7 | 7:45 | | | | |
| 7 | 8 | 8:15 | | 10:35 | | |
| 8 | 9 | 6:30 | | | 11:00 | |
| 9 | 10 | 7:00 | | 10:35 | 9:00 | PA |
| 10 | 11 | 8:30 | | | 8:30 | |
| 11 | 12 | 7:30 | 12:45,19:45 | | 8:00 | |
| 12 | 13 | 8:15 | 9:15,13:00,18:30 | 10:30 | 11:30 | |
| 13 | 14 | 6:30 | 7:15,13:15,19:30 | 8:30 | 8:30 | WCD |
| 14 | 15 | 8:15 | 9:15,13:30,19:30 | 11:15 | 8:30 | |
| 15 | 16 | 7:15 | 7:45,15:30,3:00* | 11:30 | 11:15 | WCD |
| 16 | 17 | 8:15 | 8:15,14:15,2:00* | 11:15 | 10:30 | PTT and PA |
| 17 | 18 | 7:15 | 7:15,15:30,19:30 | 10:30 | 8:30 | |
| 18 | 19 | 7:00 | 9:00,13:45,18:45 | 10:45 | 9:30 | |
| 19 | 20 | 7:45 | 7:45,14:15,20:45 | 10:45 | 9:30 | |
| 20 | 21 | 8:15 | 8:45,13:45,23:00 | 10:45 | 10:30 | WCD |
| 21 | 22 | 7:30 | 7:30,12:30 | 9:45 | 10:00 | |
| 22 | 23 | 7:00 | | 11:00 | 9:30 | PA |
| 23 | 24 | 8:30 | | 12:15 | 8:30 | PTT |
| 24 | 25 | 8:15 | | | 7:45 | |
| 25 | 26 | 7:30 | | | 8:30 | |
| 26 | 27 | 7:15 | | | 8:15 | |
| 27 | 28 | 8:30 | | 11:30 | 8:45 | |
| 28 | 29 | 8:00 | | 10:30 | 8:15 | |
| 29 | 30 | 8:15 | | 10:45 | 7:45 | PA |
| 30 | 31 | 8:45 | | 11:00 | 8:15 | |
| 31 | 32 | 7:45 | | 10:45 | 8:30 | |
| 1 April | -1 | | | | 8:45 | |
| 2 | -2 | | | 10:30 | 9:15 | |
| 3 | -3 | | | | 7:00 | |
| 4 | -4 | | | 11:15 | 9:15 | |
| 5 | -5 | | | | 8:30 | |
| 6 | -6 | | | 9:30 | 9:00 | PA |

*Drug taken following day at time noted.

WCD=White Cell Count and Differential

PTT=Prothrombin Time

PA= Protein Analysis

Table IV.

ELECTROPHORETIC PROTEIN ANALYSIS

ALBUMIN CONCENTRATION

| Plasma Date | Albumin Concentration (grams/ 100 ml plasma) |
|---|---|
| 23 Feb. | 4.0 |
| 2 March | 4.3 |
| 9 March | 4.5 |
| 16 March | 4.1 |
| 22 March | 4.4 |
| 29 March | 4.2 |
| 6 April | 4.3 |
| | $4.26 \pm 0.17$ ($\bar{X} \pm SD$) |

## RESULTS AND DISCUSSION

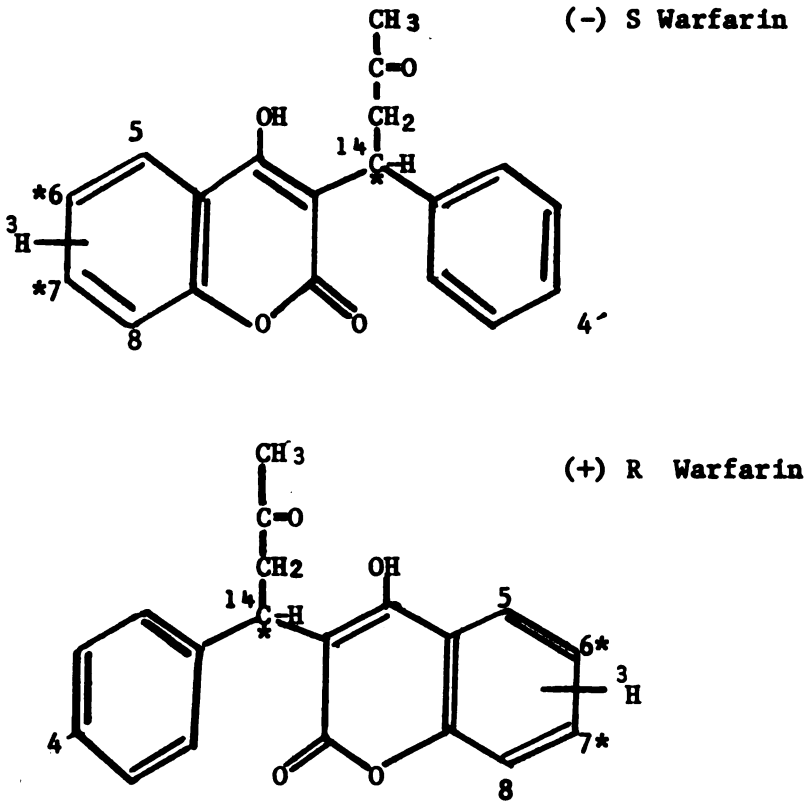
Protein Binding, Validation of Method.

The radioactive warfarin was a gift from Dr. William Trager, University of Washington School of Pharmacy, who synthesized both labelled compounds, the double labelled $^{3}H,^{14}C$-warfarin (17.9μCi$^{14}C$/mg) and the single labelled $^{14}C$-warfarin (4.19 μCi/mg). Both the double labelled and the single labelled warfarin samples contained the $^{14}C$ specifically at the benzl carbon. In addition, the double labelled warfarin contained $^{3}H$ in the coumarin nucleus, probably in the 7-position and possibly in the 6-position (Figure 8).

Initially, the double labelled warfarin was used in our binding studies to confirm the radioactive purity of the drug. Comparison of the ratio of the $^{3}H$ and $^{14}C$ channels of warfarin radioactivity in plasma prior to the experiment with that of the plasma water after the experiment, demonstrates that the ratio was similar before and after the experiment. It was concluded that the radioactivity in the plasma water was free warfarin and not a radioactive impurity or a fragment of the warfarin molecule (Table V).

Unfortunately, in order to get a reasonable count above the background level in the plasma water aliquot, it was necessary to use 5-10 μg/ml of $^{3}H$-,$^{14}C$-warfarin. It therefore was necessary to prove that the addition of this amount of labelled warfarin did not alter the binding of lower, therapeutic levels of warfarin. High speed ultracentrifugation was the method used to investigate the problem. With use of this method, however, two assumptions are made: first, that the binding equilibrium of the drug

Figure 8.




The radioactive labelled positions of the $^{3}H$ C-Warfarin, $^{14}C$ at the benzl carbon and $^{3}H$ at the 6- and 7-position of the aromatic ring. The C warfarin is labelled only at the benzl carbon.

| Length | |
|---|---|
| 0- | |
| 0.2- | |
| 0.4- | |
| 0.9- | |
| 1.4- | |
| 1.9- | |
| 2.4- | |
| 2.9- | |
| 3.4- | |
| 3.9- | |

Table V.

DETERMINATION OF RADIOACTIVE PURITY OF LABELLED WARFARIN

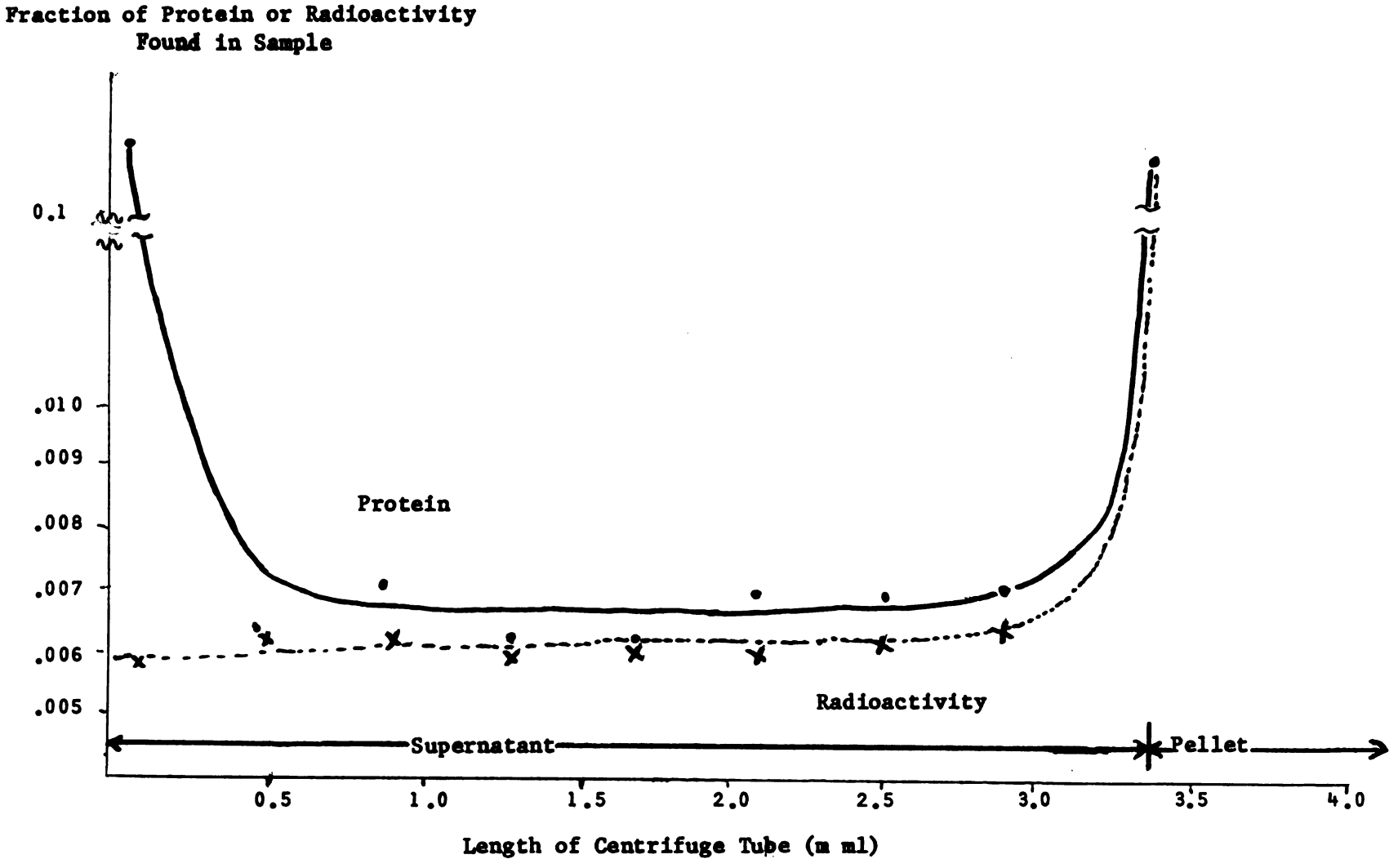
BY MEANS OF $^{3}H/^{14}C$ CHANNEL RATIO

| Plasma (Length down the tube in ml) | ratio | Plasma Water Length | Ratio | Blank Plasma Length | ratio |
|---|---|---|---|---|---|
| 0-0.2 | 3.7 | 0.2-0.4 | 3.6 | 0-0.2 | .56 |
| 0.2-0.4 | 3.2 | 0.6-0.8 | 3.6 | 0.4-0.6 | .50 |
| 0.4-0.9 | 3.7 | 1.0-1.2 | 3.5 | 0.8-1.0 | .35 |
| 0.9-1.4 | 3.6 | 1.4-1.6 | 3.2 | 1.2-1.4 | .53 |
| 1.4-1.9 | 3.6 | 1.8-2.0 | 3.3 | 1.6-1.8 | .48 |
| 1.9-2.4 | 3.6 | 2.2-2.4 | 3.6 | 2.0-2.2 | .50 |
| 2.4-2.9 | 3.5 | 2.6-2.8 | 3.3 | 2.4-2.6 | .53 |
| 2.9-3.4 | 3.4 | 3.0-3.2 | 3.7 | 2.8-3.0 | .55 |
| 3.4-3.9 | 3.7 | 3.4-3.6 | 3.5 | 3.2-3.4 | .53 |
| 3.9-4.15 | 3.4 | | | | |
| | 3.5±0.2 | ($\bar{X}$±SD) | 3.5±0.2 | | 0.5±0.1 |

and plasma proteins during centrfugation, is not altered from the actual binding equilibrium found in plasma; second, that measurement of radioactivity in the plasma water or supernatant of the centrifuged plasma, is free drug and, thus, no protein is present. If protein was present, that protein could have bound drug associated with it, thus, invalidating the measurement. Following ultracentrifugation, the protein-drug equilibrium is not altered based upon thermodynamic considerations ( 55). The second assumption, that no protein was present with bound drug, was demonstrated by the following experimental analysis for protein.

Plasma samples were centrifuged for 24 hours and then aliquots for protein and radioactivity content were obtained in the following manner. Using a micropipette, alternating aliquots were withdrawn beginning at the top of the tube proceding down to the concentrated protein layer. Figure 9 illustrates the results. The radioactive profile remains constant for the first 3.0 ml of the tube, while the protein profile illustrates a high amount of protein at the top of the cell and then diminished protein until the bottom of the tube is reached. The protein profile can be explained by formation of 3 protein gradients during centrifugation. Two of the gradients, one of high density proteins, albumin and gamma globulins, and a second consisting of low density lipoproteins, have increasing protein concentration as one proceeds toward the bottom of the tube. The third gradient, comprised of chylomicra and very low density lipoproteins ( less than 1.006), form an opalescent, lipid-like top layer that floats on the supernatant ( 32). Thus, this gradient has decreasing protein concentration as one proceeds toward the bottom of the tube. Examining the protein and radioactivity curves in figure 9, it is concluded that since there is not an increase in radioactivity at the top of the tube, while a high concentration of protein is present, warfarin must not bind to the chylomicra or very

Figure 9.




A Profile of Protein and Radioactivity in Centrifuged Samples

low density lipoprotein. Thus, the measured radioactivity in the plasma water must be unbound drug.

It is then necessary to determine to which plasma proteins does warfarin bind. Cellulose acetate electrophoresis of $^{3}H,^{14}C$-warfarin in serum demonstrated that the binding of warfarin was solely to the albumin fraction of plasma and that this is in good agreement with the finding of others (43). Due to molecular weight and size consideration, the albumin is known to be centrifuged to the bottom of the tube, which substantiates the assumption tha the radioactivity measured in the supernatant is only unbound warfarin.

Now it is possible to investigate if any changes occur to the binding equilibrium of therapeutic levels of warfarin and albumin upon the addition of labelled warfarin. In a series of experiments using outdated citrated blood bank plasma, labelled warfarin was varied from 5-20 µg/ml and the unbound fraction of the plasma warfarin was measured. The fraction unbound, $\alpha$, did not significantly vary between 2-20 µg/ml (Table VI). Since therapeutic levels of warfarin range from 1-5 µg/ml (orally), the addition of 10 µg/ml of labelled warfarin to therapeutic concentrations of warfarin in plasma would not alter the binding characteristics of the drugs.

Due to the length of the experiment (24 hours), the large quantity of plasma needed (10 ml) for duplicate wafarin binding determinations, with the maximum number of determinations possible (6) per run on the ultracentrafuge, a second method was employed, that of equilibrium dialysis. With the acquisition of the Dianorm Equilibrium Dialysis System, equilibrium dialysis provided many advantages over ultracentrifugation - a quicker result due to short dialyzing times, an increase (to 20) in the number of determinations that could be run simultaneously, and the need of only 2.3 ml of plasma for duplicate determinations of binding characteristics--thus prompting further

Table VI.

FRACTION UNBOUND OF WARFARIN

AS A FUNCTION OF PLASMA CONCENTRATION

| Experiment | Warfarin Plasma Concentration (μg/ml) | Fraction Unbound |
|---|---|---|
| 2972 | 2.5 | .00781 |
| | 10 | .00816 |
| | 15 | .00902 |
| 3172 | 2 | .00812 |
| | 5 | .00760 |
| | 10 | .00847 |
| | 15 | .00885 |
| 1182 | 2.5 | .00950 |
| | 5 | .00965 |
| | 10 | .01040 |
| | 20 | .01060 |

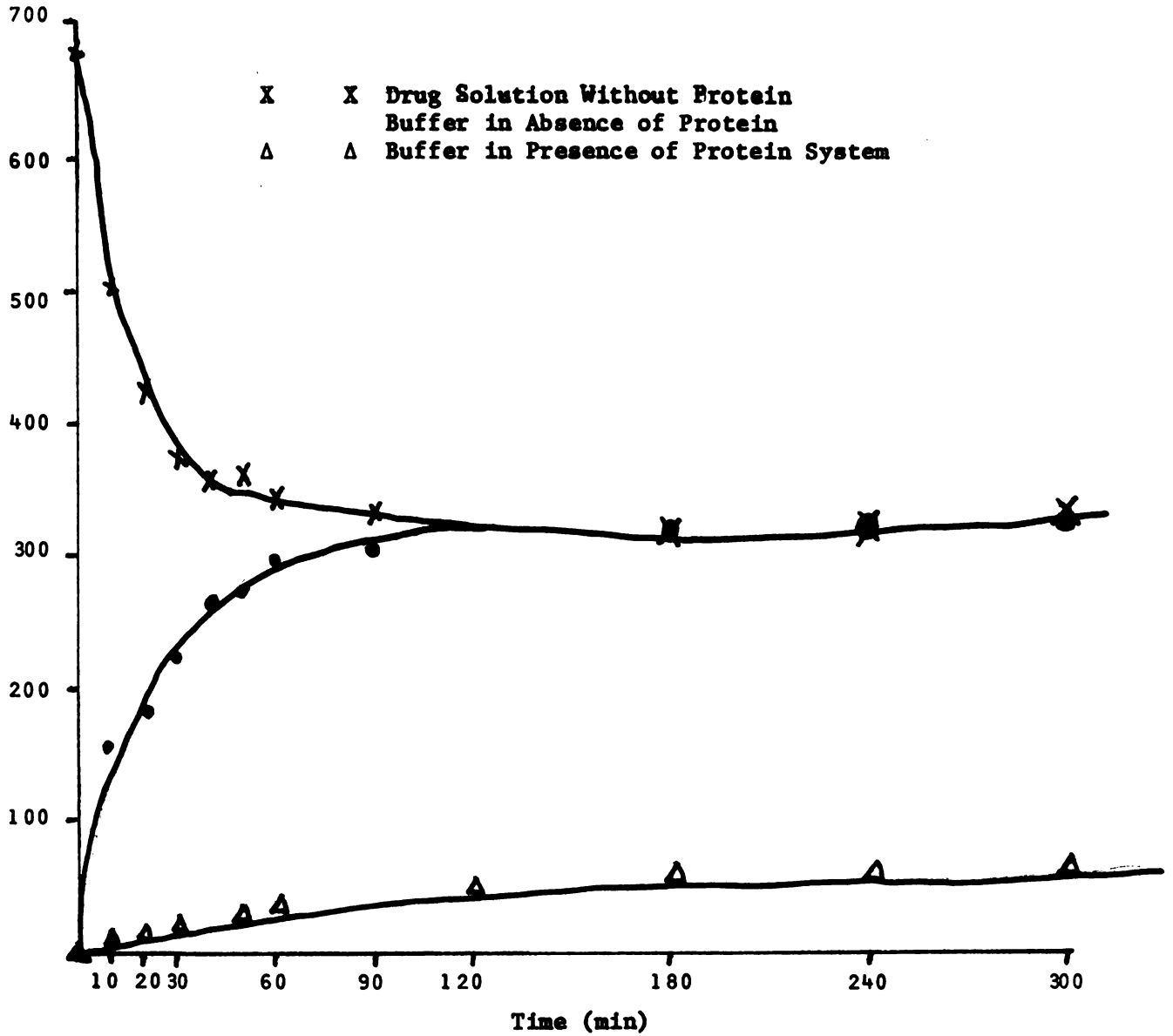
level of significance--Not Significant for all 3 studies

binding experiments to be evaluated with this Dianorm system.

In order to validate this method, a number of questions must be answered: (1) How long is required to attain equilibrium in this system? (2) How much drug binds to the membrane and to the cells themselves? (3) Is the temperature of the dialyzing solutions the same as the water bath, and how long is required to reach thermal equilibrium? (4) Is there any protein leakage across the membrane?

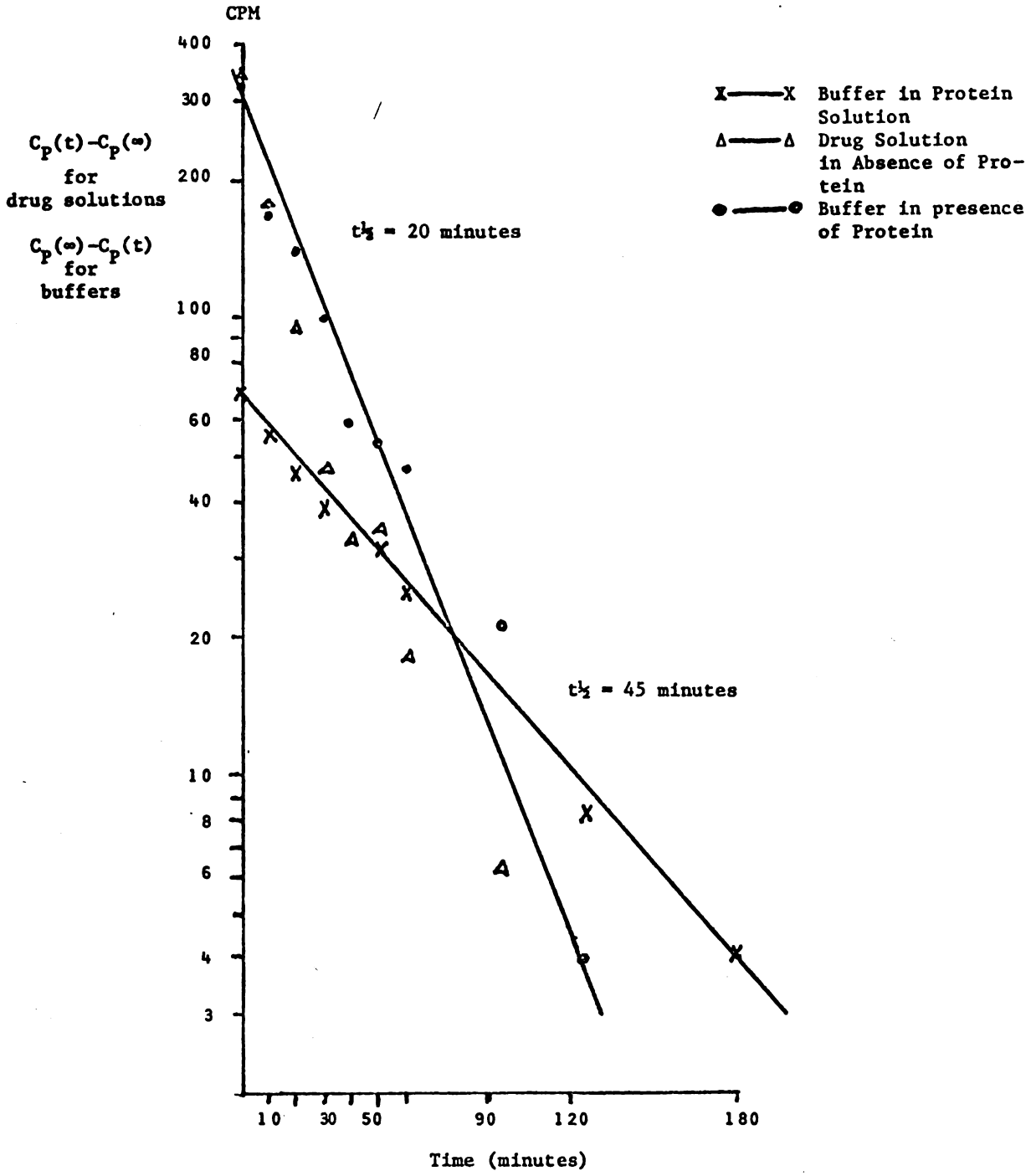
By examining the dialysis system at various time points, it was determined that equilibrium is reached in two or three hours in the absence of protein, and in approximately five hours in the presence of undiluted plasma protein (Figure 10). A relationship has been developed between the time required to reach equilibrium and the protein content of the solution to be dialyzed. Plotting the log of the difference of the drug concentration at each time point, Cp(t), minus the drug concentration reached at equilibrium, $Cp_\infty$, for the disappearance of the drug from the plasma or drug solution to the buffer with time (t); and plotting $\log(Cp_\infty - Cp(t))$ for the appearance of drug in the buffer with time, provides a decay curve for both the protein free and plasma protein studies (Figure 11). Examination of the curves reveals that the protein free study has a slope about twice that of the plasma protein study (i.e., $t_{1/2} \approx 20$ min. vs. $t_{1/2} \approx 40$ min.). This is in agreement with the theoretical analysis of the rate of approach to equilibrium during dialysis in the presence and absence of protein (Appendix A). When the fraction bound to the protein, $\beta$, approaches one, theory predicts the ratio of the slopes should approach two (48).

In order to determine how much drug binds to the cell walls and membrane, a comparison of the drug concentration at equilibrium with the drug concentration initially is made. Without any binding to the cells or membrance, the equilibrium value would be one-half the initial value.




**Determination of the Time to Equilibrium
using the
Dianorm Equilibrium Dialysis System in the Presence and Absence of Protein**

Figure 11.




Determination of the Half-Life for the Dianorm System to Reach Equilibrium in the Presence and Absence of Protein

By comparing the equilibrium value with one-half of the initial value, one obtains the amount bound to the cell components from the difference of two values. By dividing this difference by the initial value, the fraction of free drug bound to the cell components is determined. From Figure 10, it is shown that there is approximately 3-4% of the free drug bound to the membrane and cell components, which may be within experimental error. Since the cells are teflon and inert to binding of most ligands (56), drugs that bind to the system components probably bind to the membrane. A number of measurements were made to determine the amount of labelled drug binding to the membrane. The level of counts, apparently associated with the membrane, fell within the experimental error of the method. The evidence, therefore, suggests that only a neglible amount of the free drug binds to the cell components or membrane.

The temperature of the dialyzing solutions reached thermal equilibrium within $\pm$.1 $^{o}$C of the water bath in 10 minutes (56). Since the time of the experiment is considerably longer than this equilibration time the equilibration time is negligible and does not interfere with the drug equilibrium.

Measurement of protein content of the buffer half-cell solution using the modified Lowry procedure had an average value less than 0.01% of the protein originally in the plasma, which is negligible.

One problem encountered with this system was that a diminution of radioactivity per unit volume of plasma compared to the radioactivity initially in the plasma resulted. Since this was coupled with an increase of the plasma half-cell volume from 1.0 ml to approximately 1.2 ml following dialysis, it appeared that this diminution of radioactivity was simply a dilution phenomenon. To determine if such was the case, a series of plasma samples were measured for protein concentration and radioactivity per unit volume both

prior to and following equilibrium dialysis. In Table VII a comparison of the ratio obtained, (final value/initial value), i.e., .873 ±.013 (mean ±SD), for protein and .871 ±.004 for radioactivity, with no significant difference between the two effects is illustrated. It was then concluded that this phenomenon was a dilution effect which could easily be corrected.

Having done protein binding studies by both high speed ultracentrfugation and equilibrium dialysis, a comparison of the two methods would be appropriate in order to determine if both methods gave similar binding data for warfarin.

To freshly heparinized plasma was added the labelled warfarin at a final concentration of 10 µg/ml. The plasma was divided into two pools. To one was added phenylbutazone at a final concentration of 100 µg/ml. Then each pool, that of labelled warfarin and that of phenylbutazone and labelled warfarin were again divided into two aliquots - one for the ultracentrifugation experiment and one for the equilibrium dialysis experiment. The pH was adjusted to pH7.4 with $CO_2$ (gas) for each sample. Following the experiments, aliquots of equal volume for both experiments were counted for radioactivity and the unbound drug fraction, $\alpha$, was computed (Table VIII). As can be seen in the table, despite the similarity of values, there is a significant difference ($p<.001$) between the values obtained by each method. No explanation for this difference can be offered since the protein and drug concentration, pH, ionic strength, and temperature in each system were the same. Loss of $CO_2$ in the ultracentrifuge might account for the difference, however, such a loss was not detected. Attempts to check these results against ultrafiltration were unsuccessful as warfarin bound tightly to the membrane to a degree that rendered the results meaningless.

Table VII.

COMPARISON OF PROTEIN AND RADIOACTIVITY DILUTION EFFECTS

| Sample | Dilution Effect | |
|---|---|---|
| | Protein | Radioactivity |
| | $\frac{\text{(Sample plasma)}}{\text{(Initial plasma)}}$ | $\frac{\text{(Sample plasma)}}{\text{(Initial plasma)}}$ |
| 1 | .880 | .869 |
| 2 | .827 | .887 |
| 3 | .910 | .876 |
| 4 | .900 | ,875 |
| 5 | .844 | .859 |
| 6 | .875 | .861 |
| | .873±.013 ($\bar{X}$±SD) | .871±.004 |

level of significance--Not Significant

Table VIII.

COMPARISON OF EQUILIBRIUM DIALYSIS AND ULTRACENTRIFUGATION

| Plasma Drug Concentration | Equilibrium Dialysis | | Ultracentrifugation |
|---|---|---|---|
| (μg/ml) | fraction free | | fraction free |
| 10 warfarin | .0057 | | .0048 |
| | .0057 | | .0048 |
| | .0059 | | .0047 |
| | .0059 | | |
| | .0059 | | |
| | .0058 ± .0001 | ($\bar{X}$±SD) | .0048±.0001 |
| 10 warfarin + 100 phenylbutazone | .0118 | | .0085 |
| | .0108 | | .0086 |
| | .0108 | | .0082 |
| | .0110 | | |
| | .0109 | | |
| | .0111 ± .004 | ($\bar{X}$±SD) | .0084 ± .002 |

level of significance $p < .001$

Analysis of Warfarin and Metabolites in Urine

Despite the usual clean separation of warfarin and its metabolites by the method of Lewis (29), infrequently nonspecific yellow or blue spots appear creating assay problems. In the samples examined in this study, such a situation occurred, for it was not possible to measure the metabolite 6-hydroxy warfarin due to nonspecific interference. A modification of the one dimensional-2 solvent TLC system was developed, that is, a two dimensional TLC system using the same solvents running 90° from each other. This system allowed measurement of the 6-hydroxy metabolite. Figures 12-15 are reproductions of the separation of warfarin and its metabolites using this two dimensional system.

The two dimensional system was compared with the one dimensional system using standard solutions assayed in each system (Table IX). No significant difference was detected between the two sets of standards. The recovery of warfarin and its metabolites was considered to be the same in both systems.

A second analysis was performed by plotting the values obtained from one dimensional versus two dimensional systems for warfarin and each of its metabolites (Figures 16-19) .

These figures do not disclose any systematic discrepancy between the systems. Therefore, the results were also studied to determine if phenylbutazone administration might affect the assay of warfarin or its metabolites. Figure 20 is a plot of the ratio of the treatments against time for warfarin and each of its metabolites. The slopes of concentration versus time plots of alcohol$_1$ and alcohol$_2$ remain ostensibly constant; whereas, the difference in the warfarin and 7-OH plots are somewhat scattered, but cannot be attibuted to the presence of phenylbutazone.

Table IX.

COMPARISON OF URINE STANDARDS

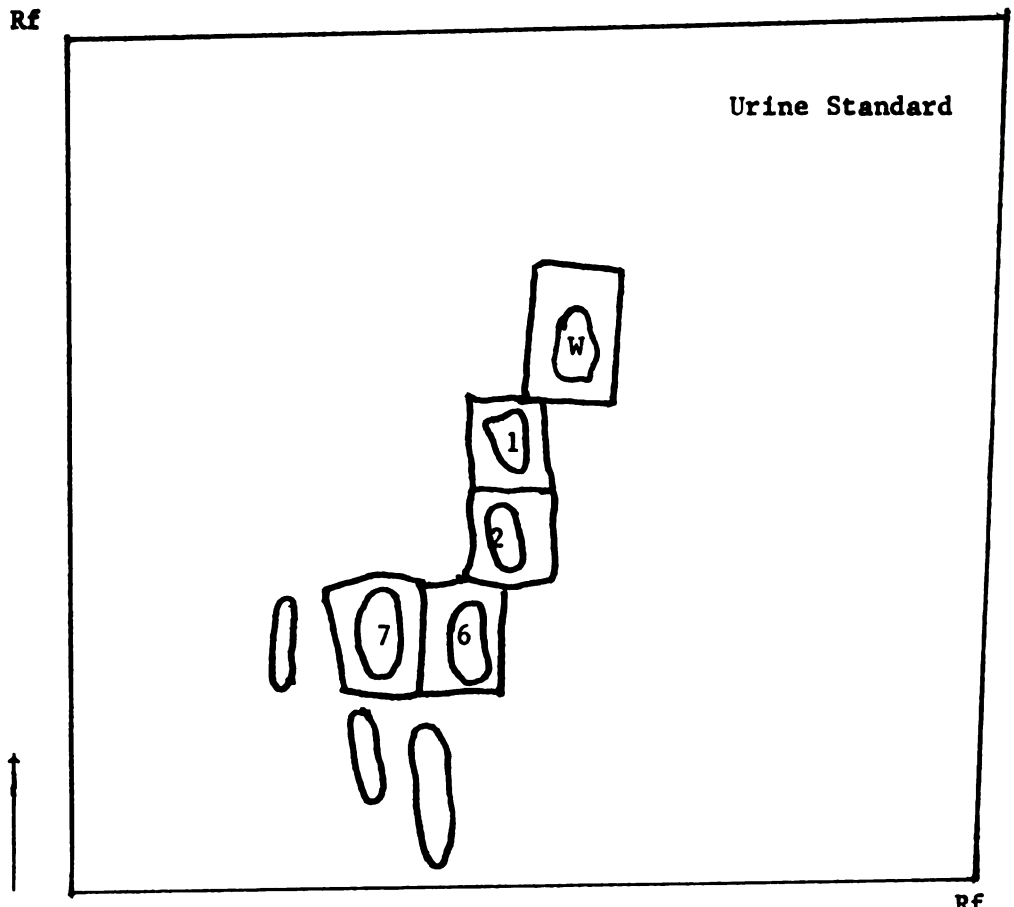
IN ONE DIMENSIONAL VS TWO DIMENSIONAL THIN LAYER CHROMATOGRAPH ASSAYS

| One Dimensional Urine Standard | | | | | | Two Dimensional Urine Standard | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| W | 1 | 2 | 6 | 7 | | W | 1 | 2 | 6 | 7 |
| 18.7 | 15.6 | 8.0 | 9.4 | 55.2 | | 20.4 | 16.3 | 10.9 | 9.7 | 51.0 |
| 20.1 | 17.3 | 10.8 | 8.7 | 40.5 | | 20.1 | 16.5 | 11.4 | 6.9 | 51.4 |
| 19.1 | 16.7 | 10.0 | 9.0 | 52.2 | | 19.5 | 15.9 | 10.6 | 8.1 | 50.7 |
| 18.9 | 15.6 | 10.6 | 8.8 | 33.0 | | 24.5 | 17.2 | 6.8 | 9.4 | 46.0 |
| 22.5 | 19.2 | 12.9 | 10.9 | 54.9 | | 20.9 | 17.9 | 9.7 | 10.1 | 40.8 |
| 21.0 | 23.0 | 12.5 | 9.3 | 63.0 | | 23.8 | 18.8 | 11.2 | 8.1 | 42.3 |
| 20.7 | 19.1 | 11.1 | 9.8 | 48.5 | | 24.3 | 17.3 | 10.7 | 11.3 | 57.7 |
| 20.3 | 16.7 | 10.4 | 8.9 | 45.7 | | 23.8 | 19.1 | 11.5 | 10.0 | 52.0 |
| 18.5 | 16.6 | 10.8 | 10.3 | 40.5 | | | | | | |
| 23.6 | 16.2 | 9.9 | 8.9 | 45.6 | | | | | | |
| 24.3 | | | | | | | | | | |
| 20.7 ± 2.0 | 17.6 ± 2.3 | 10.7 ± 1.4 | 9.4 ± 0.7 | 47.9 ± 8.8 | ($\bar{X}$) ± (SD) | 22.9 ± 2.6 | 17.4 ± 1.2 | 10.4 ± 1.5 | 9.2 ± 1.4 | 49.0 ± 5.6 |

Level of Significance--Not Significant for each pair of standards

W= fluorescent units per 1.0 µg/ml of warfarin
1= fluorescent units per 0.2 µg/ml of alcohol$_1$
2= fluorescent units per 0.2 µg/ml of alcohol$_2$
6= fluorescent units per 1.0 µg/ml of 6-hydroxy
7= fluorescent units per 0.5 µg/ml of 7-hydroxy

Figure 12.




1st Direction ↑

Chloroform : Ethyl Acetate : Acetic Acid
150:50:1

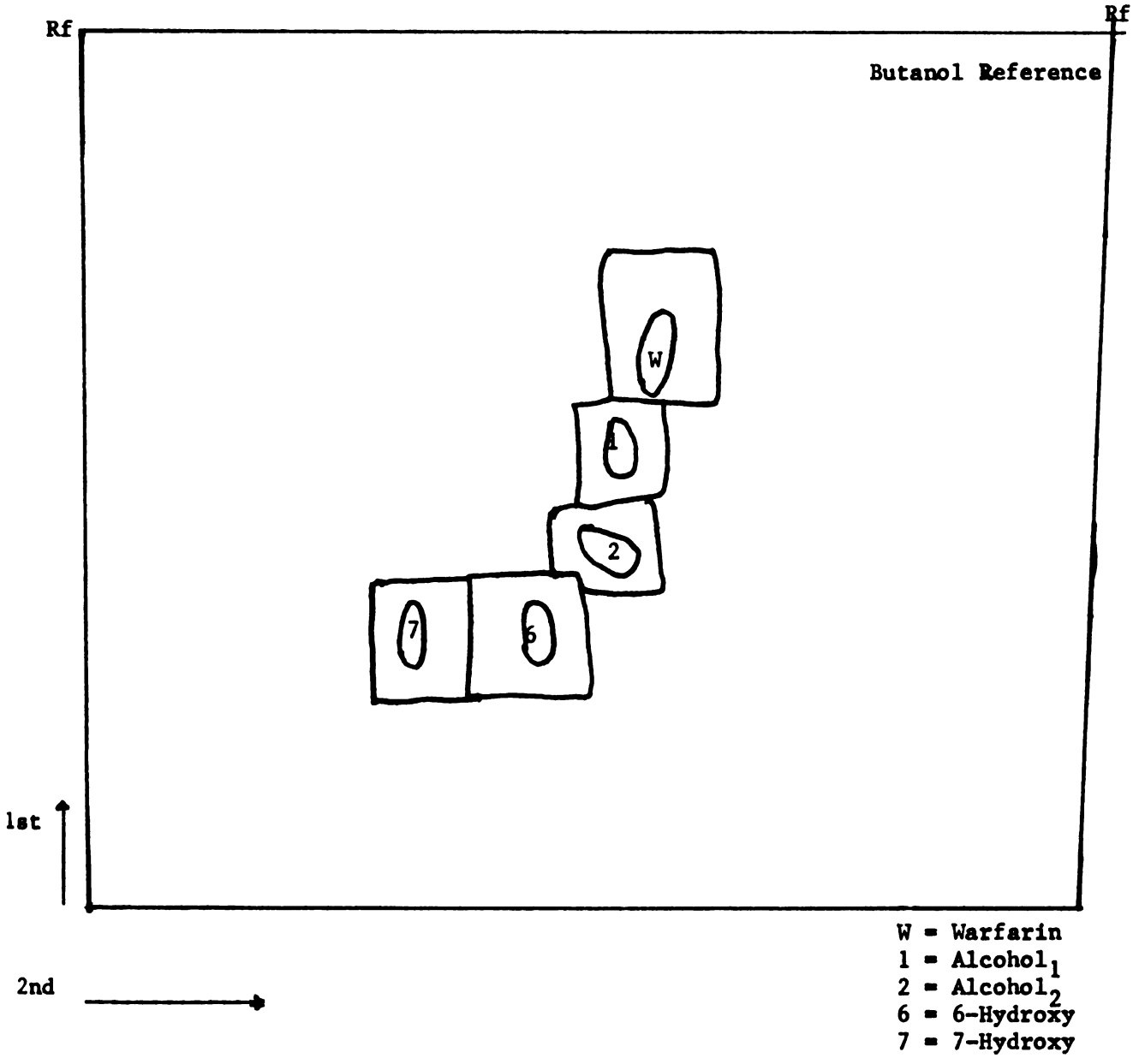
2nd Direction ⟶

t-Butanol:Benzene:$NH_4OH$:$H_2O$
90:40:18:6

W = Warfarin
1 = $Alcohol_1$
2 = $Alcohol_2$
6 = 6-Hydroxy
7 = 7-Hydroxy

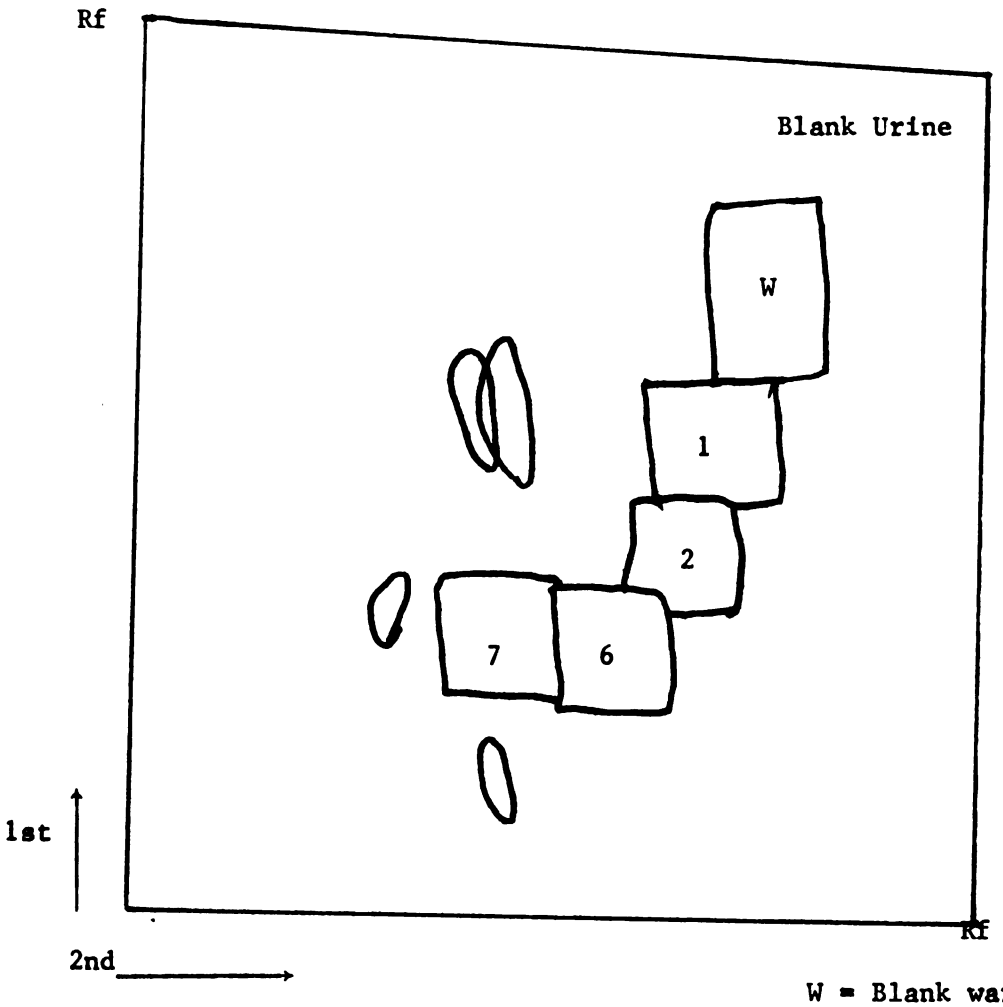
A Reproduction of the 2-Dimensional Thin Layer Chromatography For a Urine Standard

Figure 13.




A Reproduction of the 2-Dimensional TLC System
for the
Butanol Reference
This is to Check the Assay System if Problems Arise with the Urine Standard

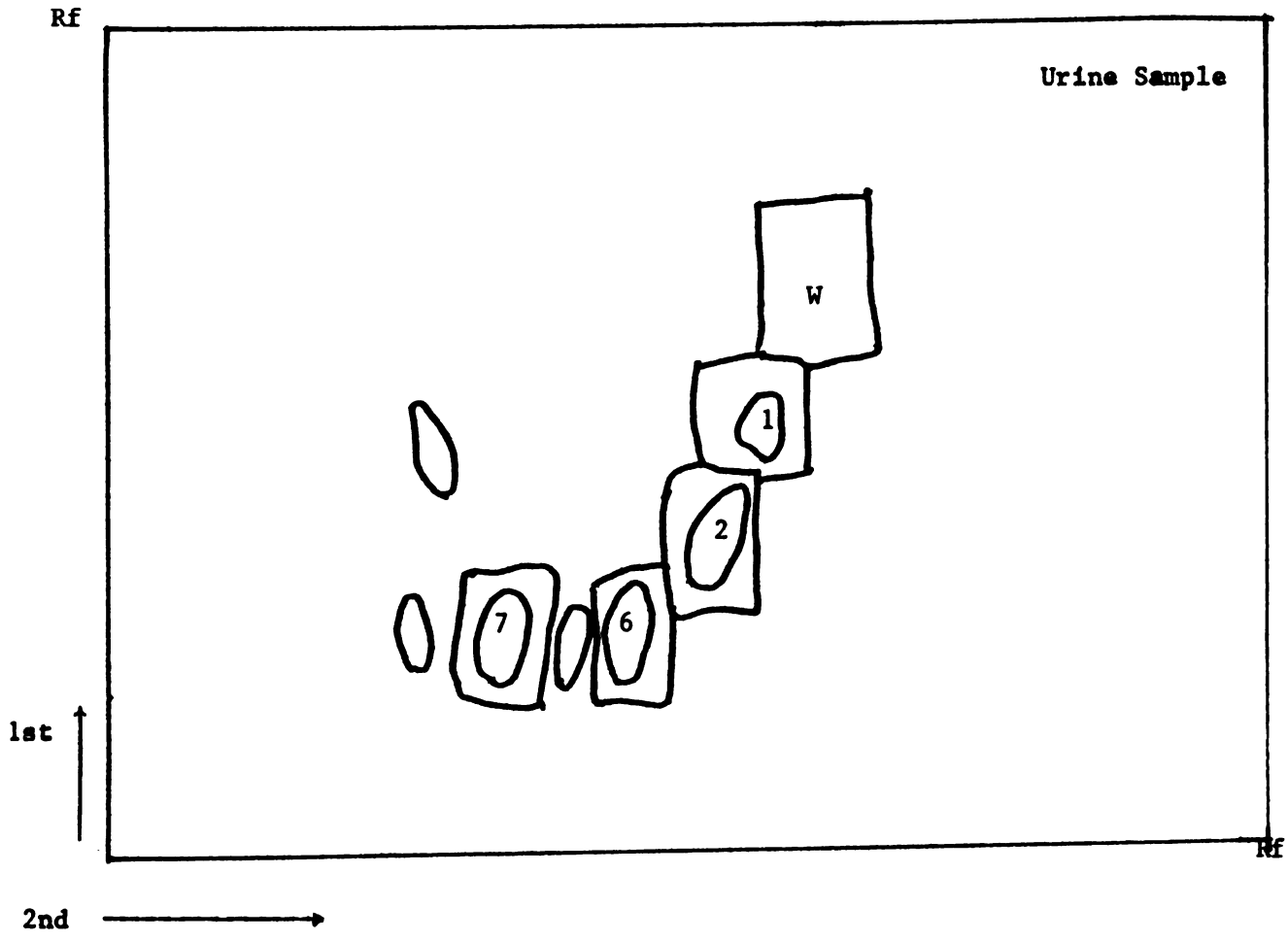
Figure 14.




W = Blank warfarin area
1 = Blank alcohol$_1$ area
2 = Blank alcohol$_2$ area
6 = Blank 6-hydroxy area
7 = Blank 7-hydroxy area

A Reproduction of the 2-Dimensional TLC for a Urine Blank.
The Blank Areas on the Plot Represent the Areas Where Warfarin and its Metabolites Would Be Found if Present.
This Provides Background Values for the Assay.
There are Present Other Fluorescent Spots not Corresponding to Warfarin or its Metabolites.

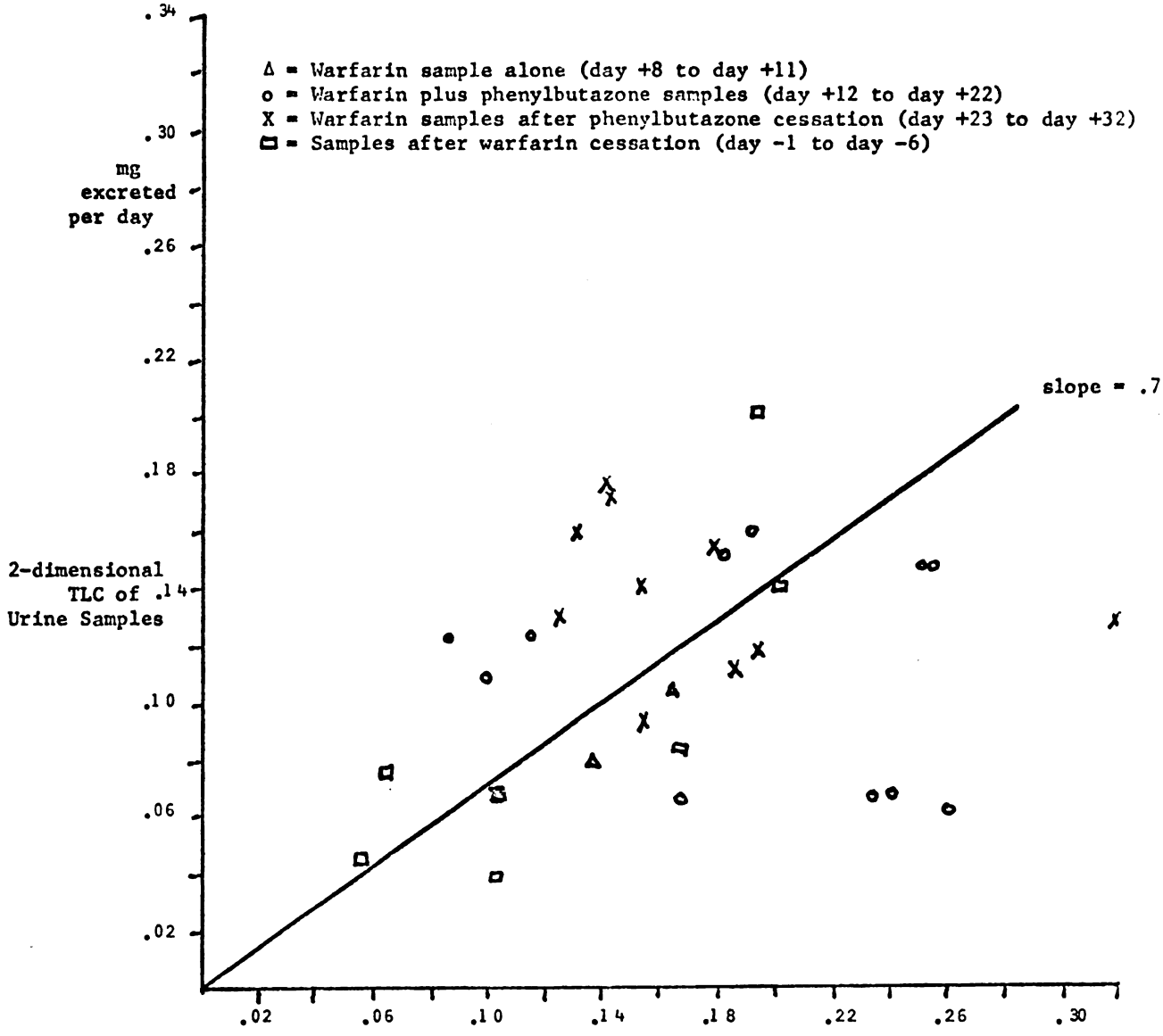
Figure 15.




W = Warfarin
1 = Alcohol$_1$
2 = Alcohol$_2$
6 = 6-Hydroxy
7 = 7-Hydroxy

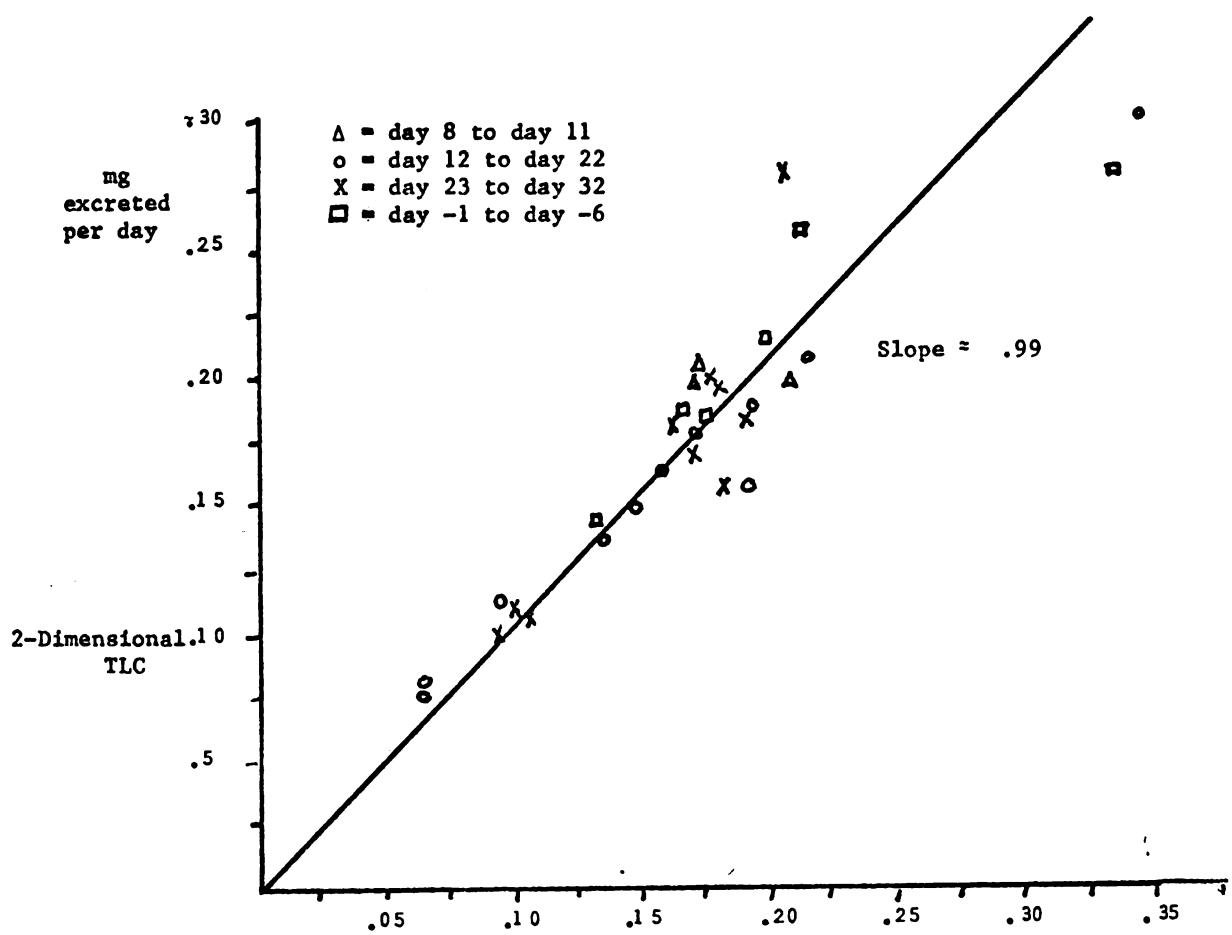
A Reproduction of a Urine Sample Using The
2-Dimensional TLC System.
The Circled Figures Represent the Areas of Fluorescence,
But the Spots Were Cut Out as the Above Rectangles.

Figure 16.



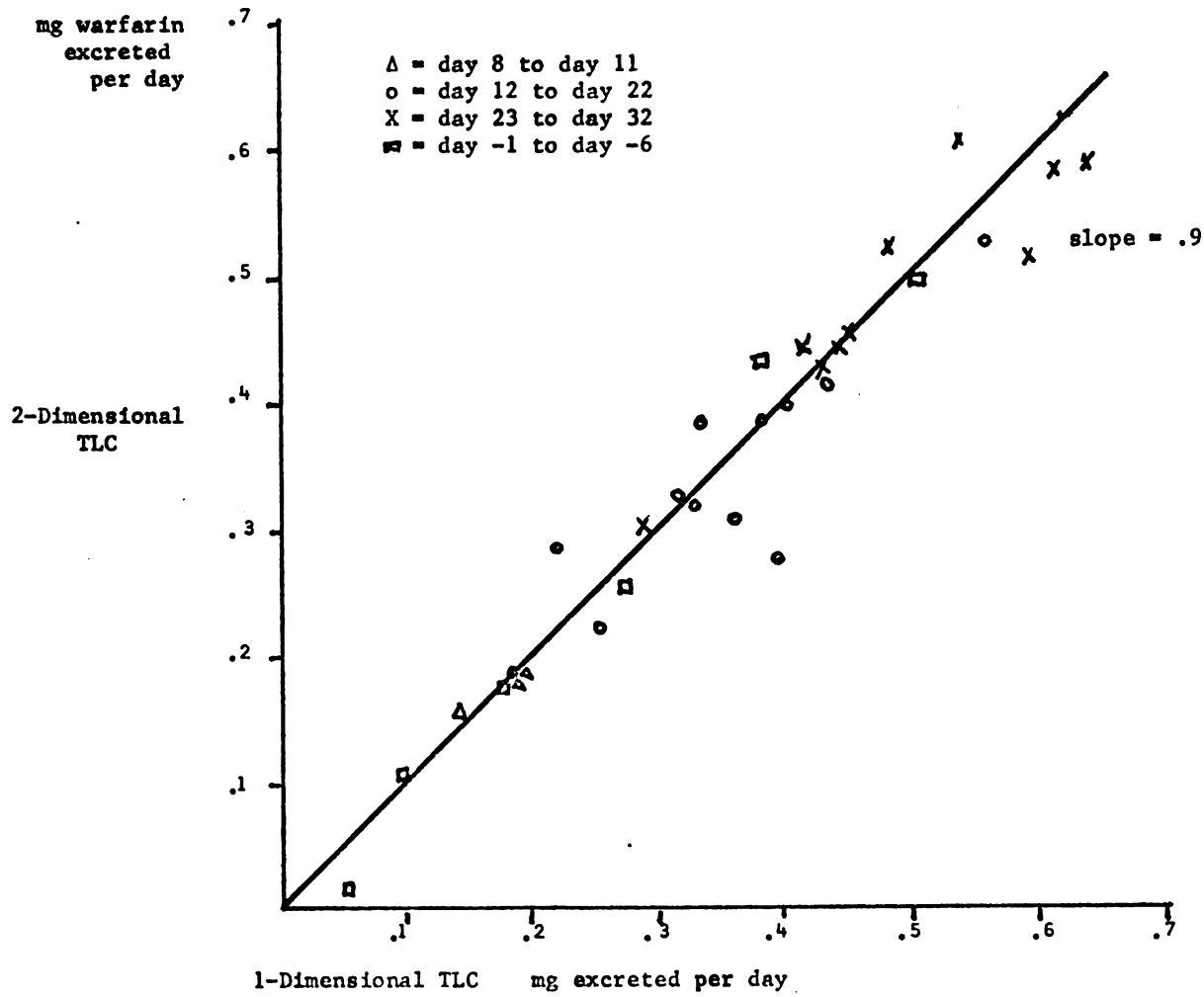

Comparison of 1-Dimensional TLC
with
2-Dimensional TLC
On Urine Samples for Warfarin

Figure 17.



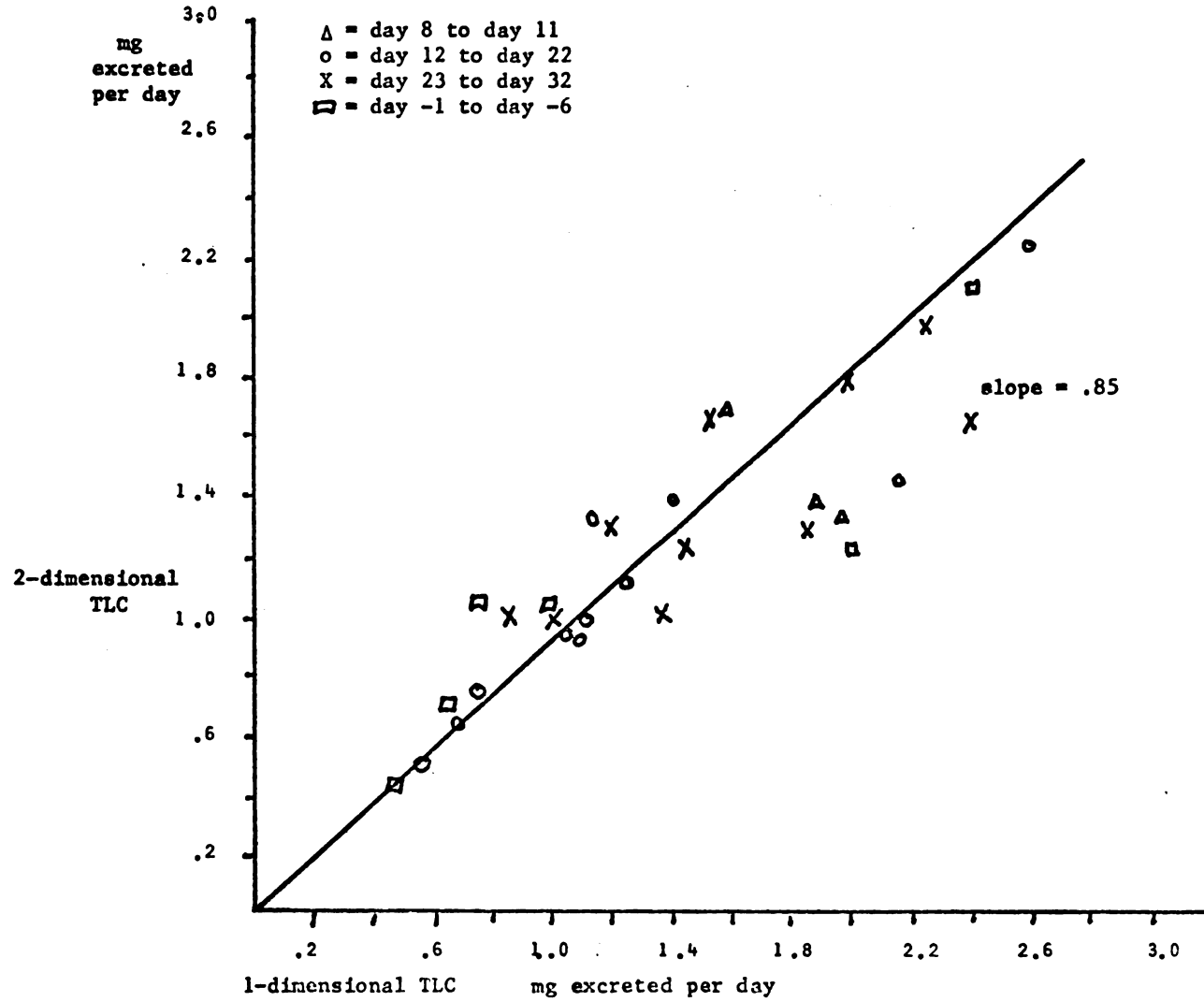

Comparison of the 1-Dimensional TLC
versus
The 2-Dimensional TLC on Urine Samples for Alcohol$_1$.

Figure 18.



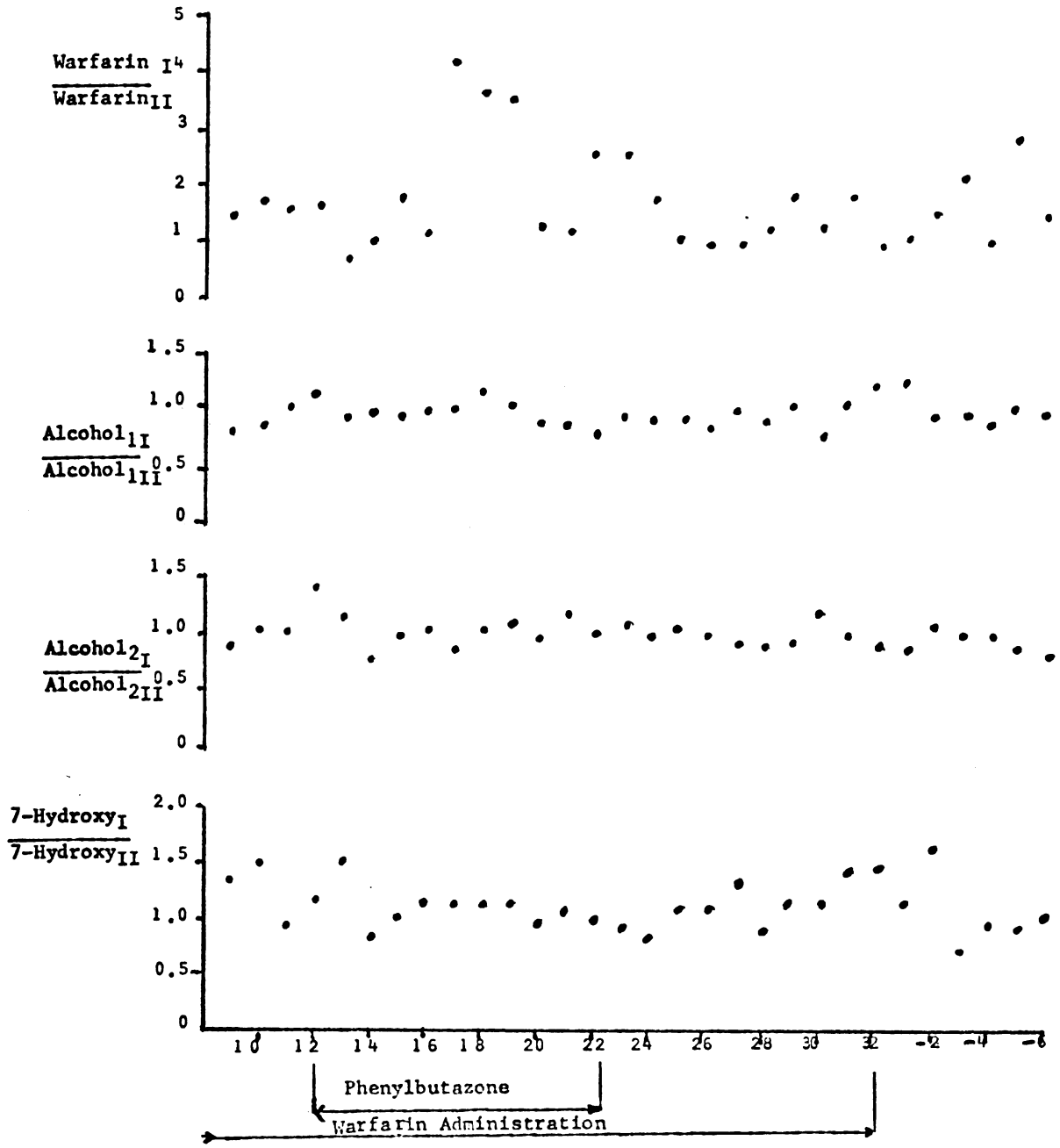

Comparison of 1-Dimensional TLC
with the
2-Dimensional TLC on Urine Samples for Alcohol$_2$

Figure 19.




Comparison of 1-Dimensional TLC
with
2-Dimensional TLC on Urine Samples for 7-Hydroxy Warfarin

Figure 20.




Comparison of Ratio of 1-Dimensional TLC (I) / 2-Dimensional TLC (II) with Time in Order to Determine if Any Effect of Phenylbutazone is Present

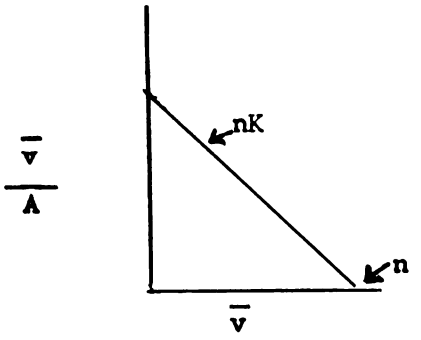
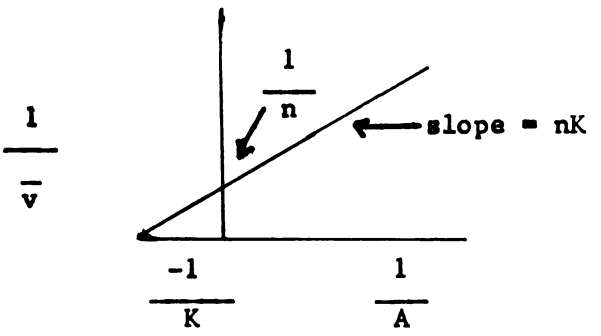
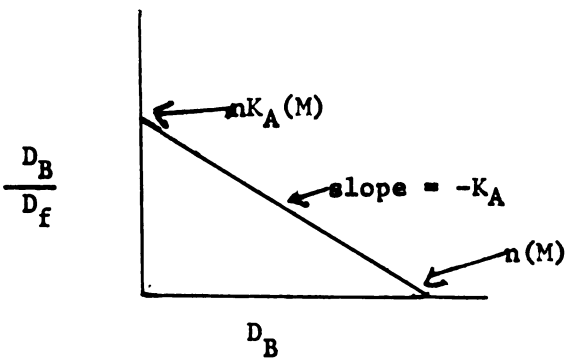
As expected, a two dimensional system was found to minimize effects from spurious contaminants, increase separation and possibly reduce interference for the higher warfarin and 7-hydroxy metabolite levels assayed in the one dimensional TLC system. Most important was that the recovery in both methods was similar. The two dimensional system of analysis was utilized in the remaining studies.

Analysis of Protein Binding Data.

The binding of small molecules to proteins is a function of the drug and protein concentration and temperature. Thus, describing binding data in terms of percent free of warfarin may be misleading( 19). It is preferable to define the binding through an equilibrium constant, the association constant $K_a$, and a capacity term, n, the number of binding sites for the small molecule on the protein.

There are a large number of methods for treating and presenting the results of drug-protein investigation. Three of these are shown in Figure 21. The Reciprocal plot and the Scatchard plot are the most common methods used today. Since these plots are directly derived from mass-law considerations, one assumes that activities can be represented by concentrations and that all sites within a class are equivalent in binding affinity and mutually independent. The reciprocal plot is $1/\bar{\nu}$ ($\bar{\nu}$ = the moles of small molecules per moles of protein in the system) plotted as a function of 1/A ( A = the moles of unbound small molecules) from which estimates of n and K are made from the slope and ordinate intercept. The Scatchard plot, $\bar{\nu}/A$ as a function of $\bar{\nu}$, allows estimation of n and nK by extrapolation to the abscissa and ordinate respectively. Curvature of such plots is usually indicative of the existence of more than one class of binding sites. The linearization of the data by the reciprocal plot requires use of a weighting function. Since

Figure 21.
Plots
**Comparison of Three Linear Transformations for Drug-Protein Binding Data With the Predicted K and n Values Given by Each Transformation**

| | | Association Constant $K$ ($\times 10^5 M^{-1}$) | Binding Sites $N$ |
|---|---|---|---|
| Scatchard |  | 1.99 | 1.2 |
| Reciprocal |  | 2.01 | 1.2 |
| Rosenthal |  | 2.00 | 1.2 |



$D_B$ = moles of drug bound to protein
$D_f$ = moles of drug unbound
$\bar{v}$ = $\frac{\text{moles of drug bound}}{\text{moles of protein}}$
A= moles of unbound (free) drug

Scatchard plot does not suffer from this disadvantage, it is the graph of choice. Reciprocal and Scatchard plots cannot be constructed if the nature and amount of protein in the experimental system is not known. Rosenthal proposed a plot (Figure 21) in which the moles of drug bound is divided by the moles of unbound drug as a function of the moles of bound drug, that is, $D_b/D_f$ as a function of $D_b$. It is independent of protein concentration (Pt) and allows estimation of nKPt, nPt, and K from the ordinate intercept, abscissa intercept, and slope respectively. If more than one protein or more than one class of binding sites on a single protein are involved in the binding, curvature of the plot results.

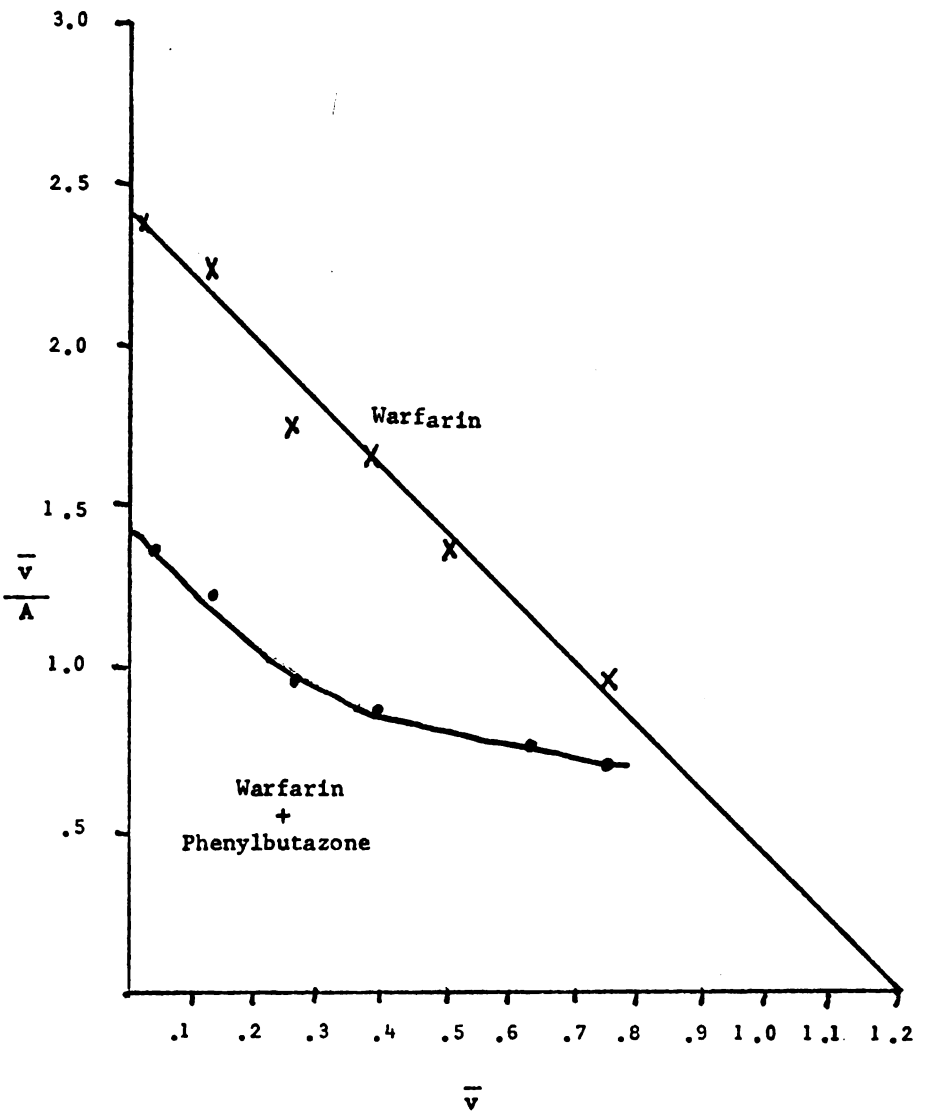
As seen in Table VI, up to 20 µg/ml of warfarin resulted in no significant increase in the unbound fraction of warfarin. In order to obtain an affinity constant of binding for warfarin, it was necessary to vary the concentration of warfarin from 5 to 150 µg/ml. The warfarin aliquots were added to heparinized plasma having an albumin concentration of 4.4 grams/100ml ($6.38 \times 10^{-4}$M). The plasma is adjusted to pH 7.4 and incubated at 37°C.

The resultant data was converted to a Scatchard-type plot (Figure 22). The association constant, $K_A$, and the number of binding sites, n, are reported in Figure 21, which compares the $K_A$ and n values from the three linear transformations. The average value of the three for $K_A$[2] is $2.0 \times 10^5 M^{-1}$ and for n is 1.2 sites.

---

[2]O'Reilly reports the association constant for warfarin as the ordinate intercept, nK, or $K_1$, the association constant for the first anion bound to albumin ( 41). At pH 7.4 and 37°C, O'Reilly reports $K_1$ equalling $2.17 \times 10^5 M^{-1}$. Using this convention, the data expressed above would exhibit a value $K_1$ of $2.39 \times 10^5 M^{-1}$.

Figure 22.




Scatchard Plot for Warfarin X——X and for Warfarin in Presence of Phenylbutazone •——•

Phenylbutazone Competition to the Binding of Warfarin.

Having established the binding pattern for warfarin in human plasma, studies using both the ultracentrifugation and equilibrium dialysis measured the effect in vitro of phenylbutazone on warfarin binding. Two types of competitive experiments were performed, one in which the warfarin concentration was varied with a fixed concentration of phenylbutazone; the other experiment held the warfarin concentration constant while varying the concentration of phenylbutazone.

With the phenylbutazone held constant while warfarin was varied, one is able to calculate the apparent association constant of warfarin in the presence of a displacing agent, $K_A'$. At a phenylbutazone concentration of 100 µg/ml ($3.25 \times 10^{-5}$M) in heparinized blood the warfarin concentration was varied from 5 to 150 µg/ml ($1.62 \times 10^{-5}$ to $4.87 \times 10^{-4}$M). At a pH of 7.4 and a temperature of 37°C, the $K_A'$ for warfarin was determined to be $9.0 \times 10^4$ $M^{-1}$, which differs from that of O'Reilly ( 2 ). His plot is linear, yet, examining the Scatchard plot for warfarin in the presence of phenylbutazone (Figure 22), one notices a curved line result, due either to more than one protein involved or more than one unequal binding site on the same protein. Since it is known that warfarin has a second, lower affinity binding site on albumin (43 ), this latter interpretation probably best explains the data. In addition, O'Reilly et. al. ( 2 ) have used this apparent association constant for warfarin in the presence of phenylbutazone to predict the affinity constant for phenylbutazone to albumin, using an equation of Edsall and Wyman ( 14) : $K_B = 1/B(K_A/K_A' - 1)$, in which $K_A$ is the association constant for labelled warfarin; $K_A'$ is the warfarin association constant in the presence of phenylbutazone; $K_B$ is the apparent association constant of phenylbutazone for albumin; and B is the molar concentration of

phenylbutazone. They are incorrect in the use of this equation. Upon reviewing the Edsall and Wyman reference, it is obvious from their derivation from mass-action law, that the B in the equation represents the unbound or free phenylbutazone contentration. Thus, without measuring unbound concentrations the apparent association constant of phenylbutazone cannot be obtained.

The second experiment examined the interaction of a fixed concentration of warfarin, either 5 or 10 μg/ml, with varying concentrations of phenylbutazone from 25 to 300 μg/ml ($8.12 \times 10^{-5}$ to $9.76 \times 10^{-4}$M). The experiment was performed using both equilibrium dialysis and ultracentrifugation. Attempts to plot the data, the free warfarin levels versus phenylbutazone concentrations, resulted in a non-linear plot (Figure 23).

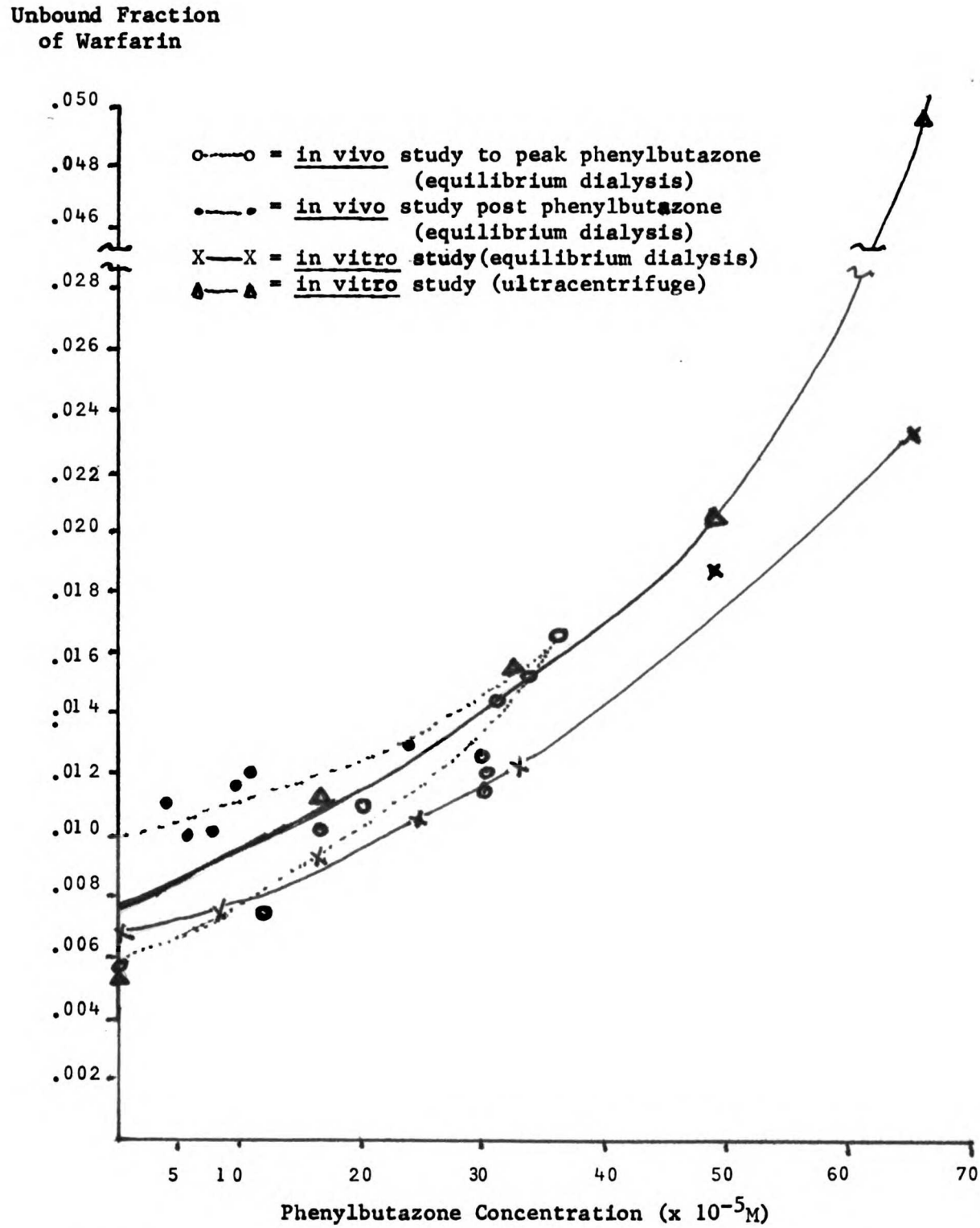
A final study was performed to determine if the degree of phenylbutazone displacement was different between R and S warfarin. 100 μg/ml of R, S, RS warfarin were added to heparinized plasma in addition to 100 μg/ml of phenylbutazone. 2.0 ml of the plasma supernatant recovered after ultracentrifugation was measured for warfarin by the method of Lewis (29 ). The results (Table X), illustrates no difference between the levels of displaced drug, either R,S, or RS warfarin, in the presence of phenylbutazone.

Fraction Free Levels of Warfarin in Human Volunteers.

A preliminary study of the fraction free or unbound levels of warfarin in human subjects was undertaken. The measurements occurred in samples following a single oral dose of warfarin (.75 mg/kg) either alone or following steady state phenylbutazone administration.

Adding a known amount of $^{14}C$-warfarin, which did not alter the unbound concentration of the drug initially present in the plasma, to the plasma samples obtained during the control studies (warfarin alone) and warfarin-

Figure 23.




Curvilinear Relationship Between Phenylbutazone Concentration and Unbound Fraction of Warfarin for Both In Vivo and In Vitro Studies Using the Equilibrium Dialysis and Ultracentrifuge

Table X.

COMPARISON OF WARFARIN ISOMER BINDING IN PLASMA

IN THE PRESENCE OF PHENYLBUTAZONE

| Warfarin | Unbound Concentration (μg/ml) |
|---|---|
| R (+) | 5.88 |
| S (-) | 5.75 |
| RS (±) | 5.35 |

The Warfarin plasma concentration equalled 100 μg/ml in all cases

Phenylbutazone plasma concentration equalled 100 μg/ml

phenylbutazone studies. These preliminary studies (Table XI) demonstrated that *in vivo*, phenylbutazone (or a metabolite) displaced warfarin from plasma albumin two to threefold. In one volunteer, five samples were obtained with time following a single dose of warfarin at steady state phenylbutazone levels. Unfortunately no control was obtained, but there appears an increasing trend of the fraction free of warfarin with time (Table XI).

In order to relate the *in vitro* binding of warfarin to albumin and its displacement by phenylbutazone, with the *in vivo* data from the study of the coadministration of both drugs, an experiment was designed to correlate the data.

Using blank plasma samples taken from one subject prior to administration of warfarin and phenylbutazone, 5.0 μg/ml of $^{14}C$-warfarin was added to the plasma. Then two concentrations of phenylbutazone, 50 and 100 μg/ml, obtained *in vivo*, were added to the plasma samples. Following equilibrium dialysis, the values for the fraction free of warfarin obtained in this *in vitro* study were compared to data obtained *in vivo*. As can be seen in Table XII, there was no difference between the *in vivo* and *in vitro* determinations of the fraction free of warfarin alone. However, there is a difference, although not significant ($p < .1$), between the levels obtained in the presence of phenylbutazone *in vitro* and *in vivo*. One might hypothesize that there are slight differences in binding *in vivo* than can be predicted by simple displacement from *in vitro* studies.

## Coadministration of Warfarin and Phenylbutazone in a Multiple Dose Regimen.

A more thorough study was then performed involving the daily collection of blood and urine samples on a multiple dose regimen involving the oral coadministration of warfarin and phenylbutazone. The protocol discussed

Table XI.

EFFECT OF PHENYLBUTAZONE PRETREATMENT ON THE FRACTION FREE OF WARFARIN IN PLASMA OF HUMAN SUBJECTS

| Subject | Phenylbutazone Concentration (μg/ml) | Fraction Unbound Warfarin | |
|---|---|---|---|
| | | Warfarin Control | After Phenylbutazone |
| J.I. | 73.5 | .0039 | .0100 |
| S.S. | 78.0 | .0034 | .0118 |
| R.A. | 80.5 | .0038 | .0080 |

| Subject | Date of Sample Collections | | |
|---|---|---|---|
| R.L. | 9-21,9-22 | 89.0 | .0074 |
| | 9-23,9-24 | 92.5 | .0085 |
| | 9-30,10-1,10-4 | 93.0 | .0096 |
| | 10-5 to 10-8 | 86.7 | .0100 |

Table XII.

COMPARISON OF IN VIVO AND IN VITRO WARFARIN BINDING LEVELS

IN THE ABSENCE AND PRESENCE OF PHENYLBUTAZONE

| Study | | Phenylbutazone Plasma Concentration (µg/ml) | Warfarin Plasma Concentration (µg/ml) | Warfarin Fraction Free |
|---|---|---|---|---|
| **In Vivo** | | | | |
| | Date | | | |
| Warfarin | (+10) | 0 | 3.986 | .0048 |
| Warfarin | (+14) | 51.5 | 3.230 | .0086 |
| + Phenylbutazone | (+21) | 96.3 | 2.344 | .0142 |
| **In Vitro** | | | | |
| Warfarin | | 0 | 5.0 | .0046 |
| Warfarin | | 50 | 5.0 | .0078 |
| + Phenylbutazone | | 100 | 5.0 | .0108 |

Level of Significance $p < .1$

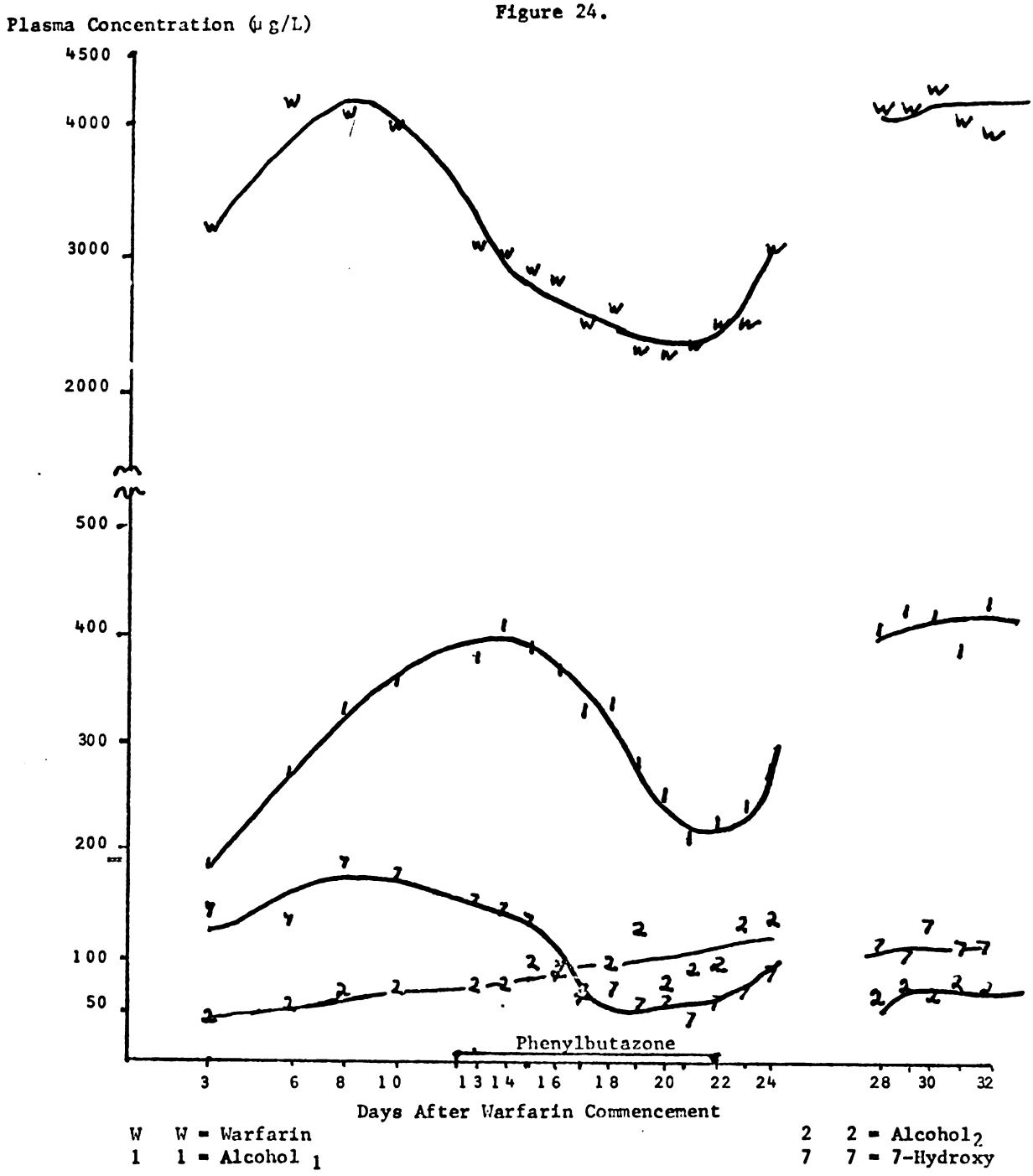
earlier involved the oral administration of 10 mg warfarin daily following a loading dose of 30 mg, with 10 mg a day of Vitamin K. After 12 days and and steady state levels were obtained, 100 mg of phenylbutazone was then given three times a day for a period of 10 days. Phenylbutazone was then stopped, but warfarin was continued for an additional 10 days. Blood samples and urine collecting were continued for 6 days following the end of warfarin administration.

It was not possible to follow the pharmacologic response of warfarin in the presence of phenylbutazone due to the resultant toxic response of warfarin . The pharmacologic potentiation of warfarin action following phenylbutazone administration has been documented ( 2 ) and will not be examined in this study.

The plasma data are shown in Figure 24 and Table XIII. After approximately six days there was an attainment of steady state levels for warfarin and its 7-hydroxy and alcohol$_2$ metabolites. Measurement of the 6-hydroxy warfarin levels were not possible due to interference in the assay. Interestingly, the plasma level of alcohol$_1$ was still increasing after 10 days, of which no explanation can be offered. However , the data clearly indicate that the concomitant administration of phenylbutazone suppresses the plasma level of warfarin. It also reduces the level of the alcohol$_1$ and the 7-hydroxy metabolites and apparently causes an increase in the levels of the alcohol$_2$ metabolite. These all return to their pre-phenylbutazone levels on withdrawal of the compound.

The data obtained from the protein binding studies on warfarin before, during and after phenylbutazone administration are shown in Figure 25 and Table XIV. It can be seen that during the phenylbutazone administration the plasma levels (free and bound) of warfarin was reduced. Yet the concentration of free warfarin increased from a mean of 0.023 µg/ml before

Figure 24.




Plasma Concentration of Warfarin and Metabolites

Table XIII.

WARFARIN AND METABOLITE PLASMA CONCENTRATIONS

| Days After Warfarin Commencement | Warfarin (μ g/ml) | $Alcohol_1$ (μ g/ml) | $Alcohol_2$ (μ g/ml) | 7-Hydroxy (μ g/ml) |
|---|---|---|---|---|
| 3 | 3.240 | 0.186 | 0.038 | 0.146 |
| 6 | 4.173 | .268 | .050 | .134 |
| 8 | 4.070 | .328 | .066 | .192 |
| 10 | 3.986 | .366 | .064 | .180 |
| 13 | 3.087 | .377 | .068 | .152 |
| 14 | 3.230 | .407 | .068 | .146 |
| 15 | 2.887 | .384 | .094 | .133 |
| 16 | 2.822 | .364 | .093 | .095 |
| 17 | 2.616 | .327 | .066 | .064 |
| 18 | 2.772 | .330 | .087 | .068 |
| 19 | 2.295 | .276 | .124 | .052 |
| 20 | 2.269 | .247 | .072 | .060 |
| 21 | 2.344 | .209 | .084 | .042 |
| 22 | 2.500 | .224 | .088 | .056 |
| 23 | 2.553 | .239 | .128 | .066 |
| 24 | 3.048 | .272 | .134 | .086 |
| 28 | 4.011 | .404 | .060 | .112 |
| 29 | 4.115 | .423 | .072 | .100 |
| 30 | 4.261 | .417 | .067 | .132 |
| 31 | 3.972 | .381 | .078 | .108 |
| 32 | 3.863 | .432 | .072 | .112 |
| -2 | 2.344 | .426 | .059 | .090 |
| -4 | 1.142 | .317 | .026 | .071 |
| -6 | .664 | .163 | .004 | .016 |

Figure 25.

Changes of Warfarin Plasma Concentration, Unbound Warfarin Plasma Levels, Unbound Fraction of Warfarin with Phenylbutazone Plasma Concentration

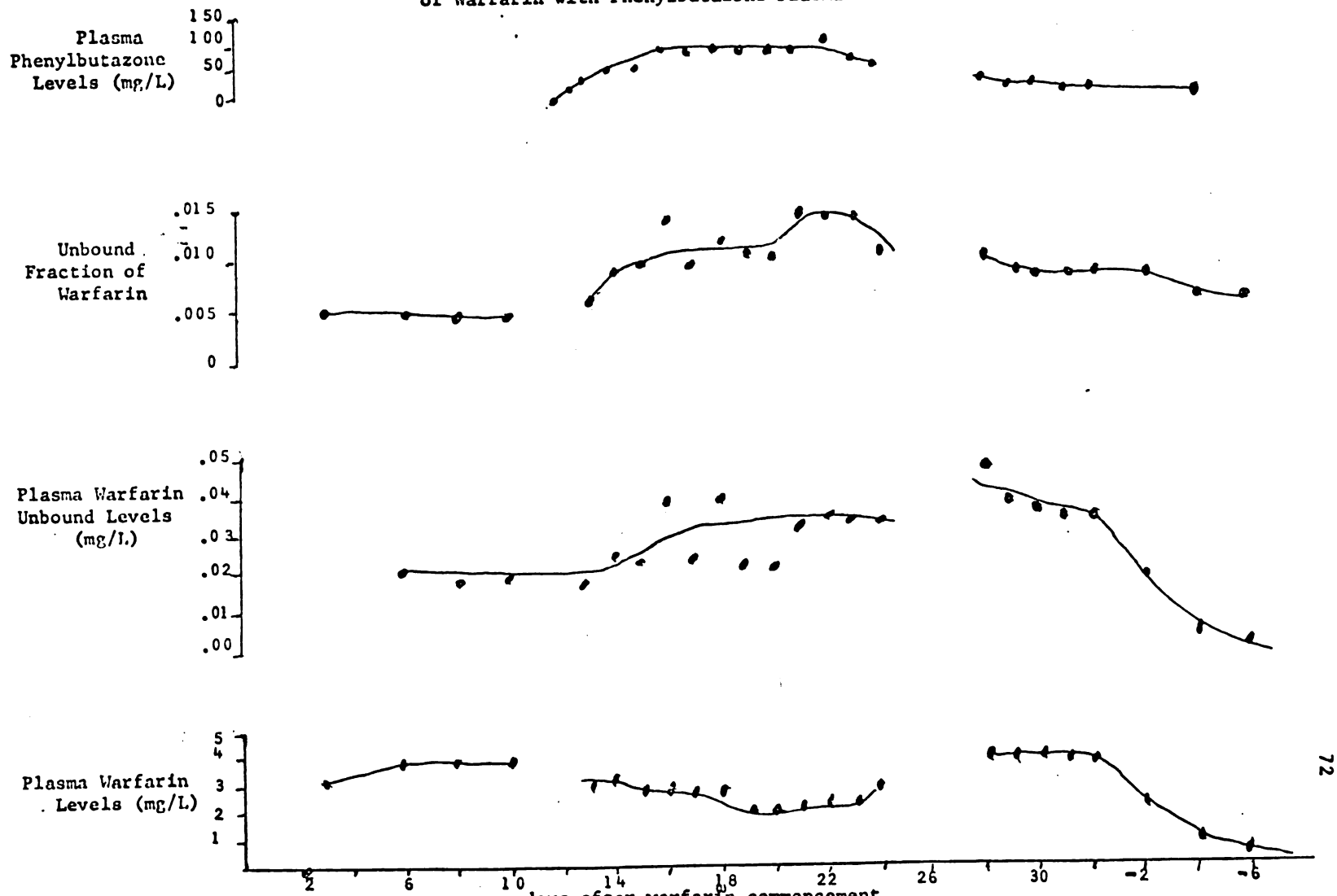


Table XIV.

UNBOUND FRACTION OF PLASMA WARFARIN

| Days After Warfarin Commencement | Fraction Free |
|---|---|
| 3 | .0054 |
| 6 | .0049 |
| 8 | .0047 |
| 10 | .0048 |
| 13 | .0059 |
| 14 | .0086 |
| 15 | .0093 |
| 16 | .0137 |
| 17 | .0093 |
| 18 | .0115 |
| 19 | .0103 |
| 20 | .0101 |
| 21 | .0141 |
| 22 | .0142 |
| 23 | .0134 |
| 24 | .0106 |
| 28 | .0101 |
| 29 | .0093 |
| 30 | .0086 |
| 31 | .0086 |
| 32 | .0088 |
| -2 | .0085 |
| -4 | .0069 |
| -6 | .0068 |

phenylbutazone to ≃ 0.040 μ g/ml during phenylbutazone administration.

In describing drug displacement, one should examine the situation when both the drug and the displacer are given on a multiple dose regimen, since this is common in drug therapy. The changes in the unbound concentration ($C_f$) depends on whether drug clearance depends on $C_f$ or total plasma concentration ($C_p$).

With a constant rate of drug administration ($R^o$) at the steady state plasma concentration, the total clearance of the drug from plasma by renal and metabolic processes, CL, is related to the steady state plasma concentration as follows:

$$R^o = CL(C_p)ss \qquad (1)$$

Correcting for protein binding:

$$R^o = CL \cdot \frac{(C_f)ss}{\alpha} \qquad (2)$$

where α is the unbound fraction of the drug. The renal clearance of a drug cleared by glomerular filtration of course depends on the $C_f$. One can justifiably presume that the metabolic clearance by the liver is also solely dependent on $C_f$ such that

$$CL = (CL_{max}) \cdot \alpha \qquad (3)$$

where $CL_{max}$ is the clearance from the unbound drug.

Displacement by increasing α increases clearance. Substituting equation 3 into 2, we obtain

$$R^o = CL_{max}(C_f)_{ss} \qquad (4)$$

This means that at steady state, when the drug entering the body equals the drug leaving, the unbound concentration should be constant and independent of the degree of protein binding. The displacing agent, therefore, affects the total plasma concentration, but the plasma free concentration at steady state should be unchanged.

In those cases where the displacing agent accumulates slowly relative to the elimination kinetics of the displaced drug, or at steady state; then the unbound concentration and presumably, the pharmacologic effect, should remain constant and apparently unaffected by the displacing agent. Thus, simple displacement does not appear to offer a valid explanation of the increase in the unbound levels of warfarin. However, a change in unbound warfarin could occur if phenylbutazone, in addition to displacing warfarin, also inhibits its metabolism.

The mechanism of warfarin metabolism and the interaction of phenylbutazone has been investigated by Lewis et. al. ( 29 ) and has been discussed earlier. Figure 1 includes a schematic of the proposed metabolic routes for the R- and S- warfarin.

Table XV includes the data on the urine studies. It depicts the rather low total recovery at steady state of the administration of warfarin, 85-90% of which is found as 6-hydroxy warfarin and 7-hydroxy warfarin. Studies have shown that there was no detectable conjugated products of warfarin metabolites in the urine ( 1 ) or significant drug in the feces ( 1 ).

One can calculate renal clearance of warfarin on the basis of the available data. The accuracy of renal clearance calculation of unbound warfarin in the plasma is based upon a number of presumptions. Since a large volume of plasma was required to carry out the protein binding analysis, only one blood sample was taken daily. While usually taken at the same time of the day, there is no information to confirm that this represents the mean plasma concentration. The estimation of the fraction free imposes a potential experimental error. The urinary excretion rate also has to be determined by estimating the concentration of free warfarin excreted per

Table XV.

WARFARIN AND METABOLITE EXCRETION IN THE URINE

| Days After Warfarin Commencement | +9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|
| Warfarin (mg/day) | .0344 | .0396 | .0523 | .0775 | .0621 | .0625 | .0744 | .0790 | .0318 | .0336 |
| $Alcohol_1$ (mg/day) | .2063 | .2002 | .2002 | .3038 | .1808 | .1626 | .1940 | .1367 | .1490 | .1578 |
| $Alcohol_2$ (mg/day) | .1552 | .1800 | .1843 | .2776 | .2232 | .2844 | .3234 | .3203 | .3827 | .4168 |
| 6-Hydroxy(mg/day) | .8946 | 1.7093 | 1.6156 | 1.4036 | 1.1211 | 1.2549 | 1.2384 | 1.0200 | .9996 | .9126 |
| 7-Hydroxy(mg/day) | 1.3792 | 1.3295 | 1.6870 | 2.7279 | 1.445 | 1.3278 | 1.3944 | .9313 | .9888 | .9471 |
| Amount Recovered(mg/day) | 2.67 | 3.46 | 3.74 | 4,79 | 3.03 | 3.09 | 3.22 | 2.49 | 2.55 | 2.47 |

Table XV. (cont.)

WARFARIN AND METABOLITE EXCRETION IN THE URINE

| Days After Warfarin Commencement | +19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|---|---|---|---|---|---|---|---|---|---|---|
| Warfarin (mg/day) | .0336 | .0767 | .0451 | .0333 | .0649 | .0562 | .0656 | .0803 | .0867 | .0695 |
| $Alcohol_1$ (mg/day) | .2080 | .1166 | .0766 | .0810 | .1953 | .1008 | .2002 | .1222 | .1148 | .1838 |
| $Alcohol_2$ (mg/day) | .5258 | .3856 | .3055 | .3946 | .5880 | .4510 | .5882 | .4247 | .4442 | .6088 |
| 6-Hydroxy(mg/day) | 1.0297 | .8479 | .6082 | .6899 | 1.0293 | .9644 | 1.2968 | .9868 | .9002 | 1.1534 |
| 7-Hydroxy(mg/day) | 1.1126 | .7592 | .5151 | .6588 | 1.2887 | 1.0169 | 1.7934 | .9984 | 1.0292 | 1.6587 |
| Amount Recovered(mg/day) | 2.91 | 2.19 | 1.55 | 1.86 | 3.18 | 2.59 | 3.94 | 2.61 | 2.58 | 3.68 |

| | |
|---|---|
| | |
| | |
| | |

Table XV. (cont.)

WARFARIN AND METABOLITE EXCRETION IN THE URINE

| Days After Warfarin Commencement | +29 | 30 | 31 | 32 | -1 | -2 | -3 | -4 | -5 | -6 |
|---|---|---|---|---|---|---|---|---|---|---|
| Warfarin (mg/day) | .0597 | .0770 | .0476 | .0883 | .1080 | .0712 | .0422 | .0379 | .0192 | .0225 |
| $Alcohol_1$ (mg/day) | .1692 | .2814 | .1849 | .1565 | .2844 | .2175 | .1860 | .2631 | .1840 | .1449 |
| $Alcohol_2$ (mg/day) | .5217 | .5082 | .4401 | .3034 | .4296 | .2559 | .1786 | .1862 | .1068 | .0667 |
| 6-Hydroxy(mg/day) | .9785 | 1.0990 | .9849 | .8976 | 1.6911 | 1.0720 | .6808 | .6634 | .4716 | .3026 |
| 7-Hydroxy(mg/day) | 1.2323 | 1.9712 | 1.6589 | 1.2802 | 2.2338 | 1.2144 | 1.0534 | 1.0264 | .7140 | .4412 |
| Amount Recovered(mg/day) | 2.96 | 3.93 | 3.32 | 2.73 | 4.74 | 2.82 | 2.14 | 2.17 | 1.49 | .97 |

day. Being a weak acid with a high partition coefficient,the urinary excretion of warfarin will be sensitive to urinary pH, It will, therefore, vary with the food being eaten and other factors affecting urinary pH. Estimation of the amount excreted of course presumes a complete collection of the urinary samples, the aliquot and multiplication by the volume of urine collected. The relative accuracy of the assay value, therfore, affects the estimate of the mean daily rate of urinary excretion. Finally, to estimate the renal clearance, one must decide what plasma water concentration to use in estimating the clearance. One alternative is to use the glomerular mean concentration obtained from two day's plasma values. The calculated value is then measured to be the water concentration of warfarin at the midpoint in the urine collection. Dividing this value into the mean urinary excretion rate results in the estimate of the renal clearance (Table XVI). Being subject to all the potential sources of error, one is justified in anticipating a certain degree of scatter.

Since the protein binding estimates were not made for the warfarin metabolites, one is not able to calculate the clearance for their unbound fraction. O'Reilly (40,41) has studied the protein binding of the metabolites, but in diluted plasma at alkaline pH and at 27°C. All three of these factors affect the protein binding and make it impossible to extrapolate his values to physiological conditions. Table XVII lists the uncorrected renal clearance values for the metabolites. The $alcohol_1$ and the $alcohol_2$ are nevertheless quite surprising since there is a tenfold difference in their values. From their similar structure one might expect that their protein binding characteristics should be similar. A possible explanation might be renal cortical metabolism with one of the compounds or the formation of the alcohol in the kidney. This would affect the clearance value very markedly.

Lewis reported S-warfarin has a shorter half-life, and thus, greater

Table XVI.

THE CORRECTED RENAL CLEARANCE OF WARFARIN

| Days After Warfarin Commencement | Unbound Warfarin Concentration (mg/L) | Corrected Renal Clearance (L/day) |
|---|---|---|
| 9 | 0.0193 | 1.78 |
| 10 | .0192 | 2.06 |
| 13 | .0225 | 2.76 |
| 14 | .0274 | 2.28 |
| 15 | .0323 | 2.30 |
| 16 | .0307 | 2.57 |
| 17 | .0308 | 1.03 |
| 18 | .0304 | 1.11 |
| 19 | .0232 | 1.45 |
| 20 | .0275 | 2.79 |
| 21 | .0342 | 1.32 |
| 22 | .0348 | .96 |
| 23 | .0339 | 1.91 |
| 28 | .0423 | 1.64 |
| 29 | .0375 | 1.59 |
| 30 | .0354 | 2.19 |
| 31 | .0341 | 1.40 |
| 32 | .0298 | 2.96 |

Table XVII.

UNCORRECTED RENAL CLEARANCE OF WARFARIN METABOLITES

| Days After Warfarin Commencement | $Alcohol_1$ (L/day) | $Alcohol_2$ (L/day) | 7-Hydroxy (L/day) |
|---|---|---|---|
| 9 | 0.580 | 2.43 | 7.54 |
| 10 | .546 | 2.81 | 7.54 |
| 13 | .461 | 3.28 | 9.70 |
| 14 | .412 | 3.56 | 9.55 |
| 15 | .519 | 3.48 | 12.5 |
| 16 | .396 | 4.11 | 11.9 |
| 17 | .454 | 5.04 | 15.0 |
| 18 | .523 | 4.01 | 16.1 |
| 19 | .797 | 5.59 | 19.9 |
| 20 | .514 | 4.94 | 15.2 |
| 21 | .355 | 3.55 | 10.7 |
| 22 | .351 | 3.72 | 10.8 |
| 23 | .766 | 4.49 | 17.2 |
| 28 | .445 | 9.22 | 15.7 |
| 29 | .405 | 7.56 | 10.7 |
| 30 | .705 | 7.06 | 16.6 |
| 31 | .455 | 5.89 | 15.1 |
| 32 | .364 | 4.47 | 12.1 |

disposition rate than R-warfarin. Following administration of phenylbutazone with a single oral dose of warfarin, there were changes in the metabolic pathway of warfarin. S-warfarin disappearance from the plasma was now much slower than that of R-warfarin. Lower plasma levels of 7-hydroxy warfarin resulted while RS-alcohol (alcohol$_1$) and SS-alcohol (alcohol$_2$) dropped. Interpretation of changes in metabolite levels must involve both the changes in rate of formation and the rate of elimination (via further metabolism and excretions) of each metabolite being examined. Multiple dosing regimens, in which drug concentration reach steady-state levels, allows valid interpretations of changes in drug metabolite levels, and, thus, drug metabolism.

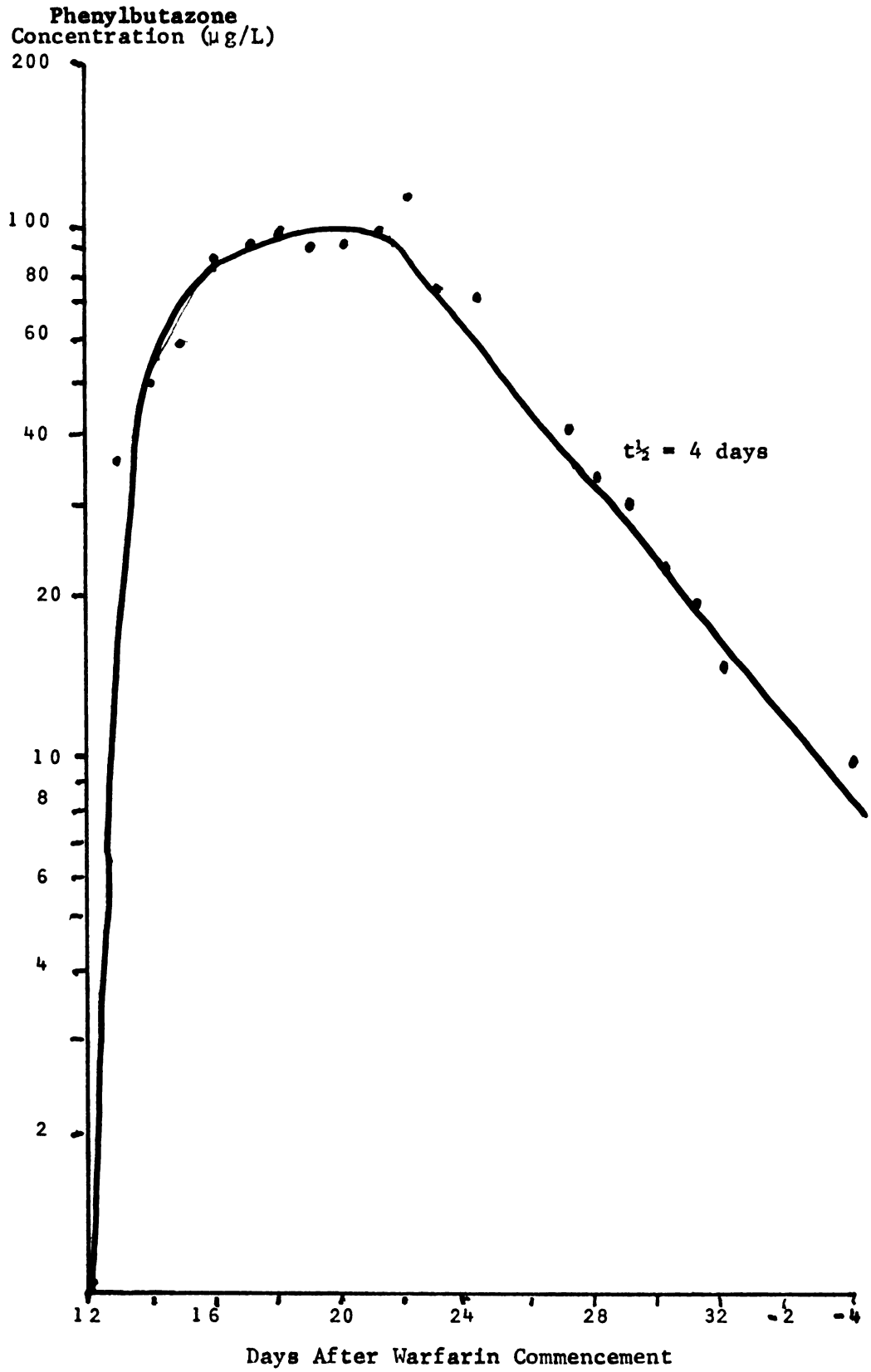
Following the end of phenylbutazone administration, phenylbutazone plasma levels declined with a half-life of about 4 days (Figure 27), and the warfarin returned to pretreatment levels as did all of the metabolites except for the SS-alcohol, which increased above phenylbutazone levels (Figure 24). We have no explanation for this.

The fall off curves for warfarin and its metabolites are given in Figure 26. The half-life of warfarin was estimated to be about 54 hours (Figure 26) with the elimination rate constant $K_d$ = 0.013 hours $^{-1}$. One can therefore attempt an estimate of the volume of distribution, $V_d$, based on the following

$$R^o = K_d V_d (C_p)_{ss} \qquad (5)$$

Since the intake was 10 mg/day and warfarin was absorption of 100% has been demonstrated ( 6 ), and the steady state levels of warfarin was 4 mg/L, with the $_d$ determined as above, then the volume of distribution was about 7.94 liters and a corrected clearance of $K_dV_d/\alpha$ or about 500 liters/day, when $\alpha$ = .005.

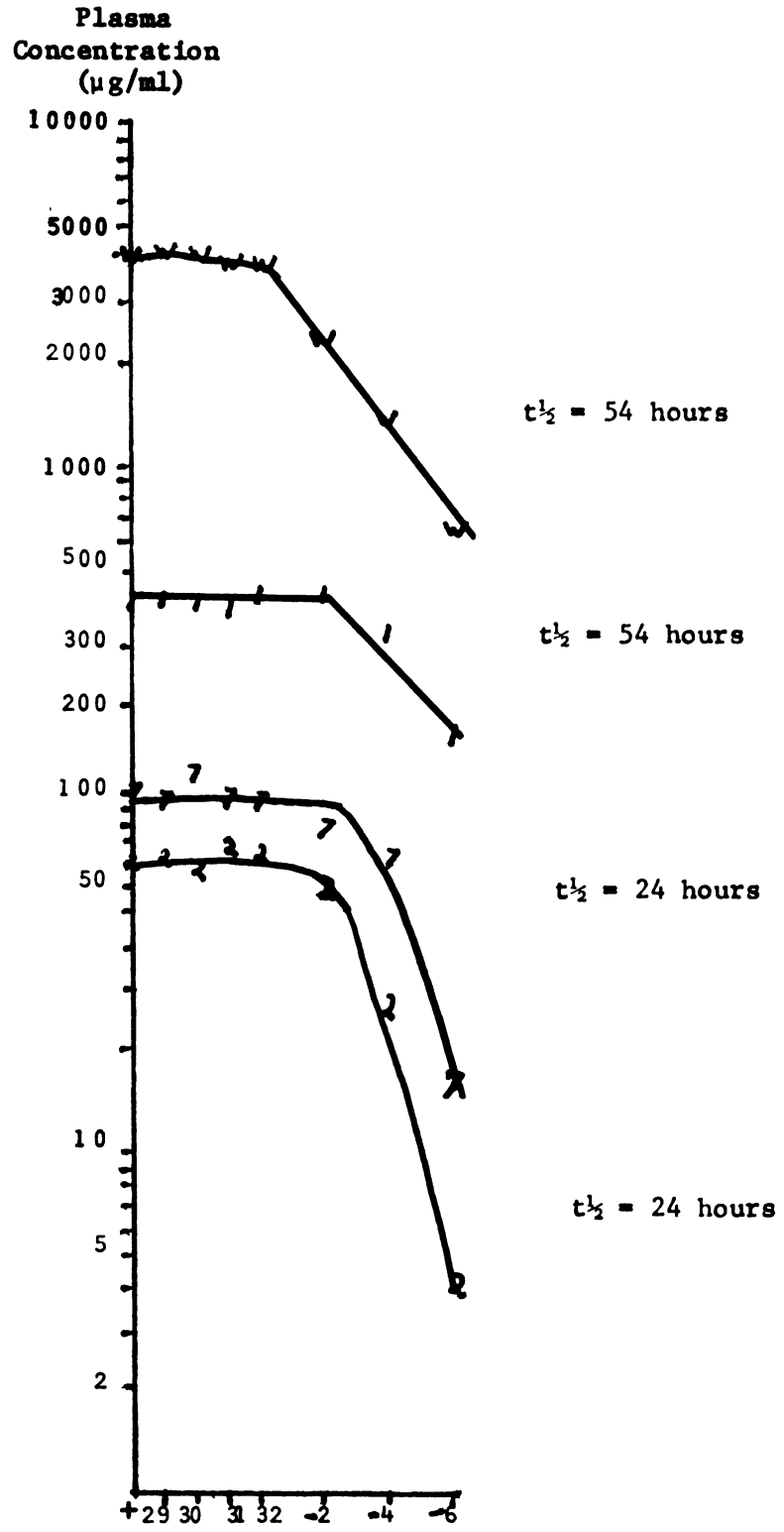
Figure 27.




Plasma Concentration of Phenylbutazone

The data in Figure 26 leads to the conclusion that alcohol$_1$ (RS) has the same decay characteristics of warfarin. One may gain the impression from Figure 26 that 7-hydroxy warfarin and alcohol$_2$ (SS) are being eliminated faster than warfarin. The more rapid elimination of 7-hydroxy warfarin and alcohol$_2$ may be explained by the fact that both of these metabolites are products of S warfarin, whereas alcohol$_1$ is a product of R warfarin. Lewis and others have shown that S warfarin is eliminated more quickly than R warfarin. Thus, the formation of 7-hydroxy warfarin and alcohol$_2$ and their subsequent elimination would be predicted to occur at a faster rate than the elimination of alcohol$_1$ and a mixture of R and S warfarin.

Figure 26.




Warfarin and Metabolite Plasma Disappearance Curves

## SUMMARY AND CONCLUSION

A logical premise in pharmacology is that the response to a drug is determined by its unbound concentration in plasma. Despite this premise, many investigators assume that the response is a direct function of the total drug concentration. For instance, research workers adjust antibiotic activity in accordance with total concentration of drug rather than free drug concentration. Thus, the clinical efficacy of the two drugs may be quite different, despite equivalent total drug concentrations, unless appropriate corrections are made. It is hoped that more investigators will examine the relationship between unbound drug concentration and response and thus, will attach a useful relationship between free drug and drug response.

The elimination of a drug from the body is dependent on the degree of protein binding. For drugs which solely are eliminated by the glomerular filtration of the unbound drug, or for drugs whose liver clearance is dependent upon the unbound drug concentration, then clearance is dependent upon the unbound drug concentration. At steady state, an interacting drug displaces the bound drug and temporarily increases the unbound drug concentration in the plasma. This produces a transient increase in drug clearance with return to the normal clearance levels. Therefore one can assume that at plateau, the unbound concentration of drug and the pharmacologic response should only show a transient change, if any. Over the longer time period of chronic administration the pharmacologic response should appear independent of the degree of displacement. If at steady state, there is an increase in drug response following administration of a second drug, drug displacement

cannot account for this action for long term changes in pharmocologic response. Alternative explanations must be sought. The results reported in this study suggest an inhibition of metabolism by phenylbutazone.

This study of the warfarin-phenybutazone interaction demonstrates the need to measure not only the changes in the warfarin plasma concentrations but to measure both the total and unbound concentrations of warfarin and its metabolites in plasma and the compounds in the urine. For racemic drugs, such as RS warfarin, measurements of the individual enantiomers concentrations is most important if they have unequal pharmacological potency.

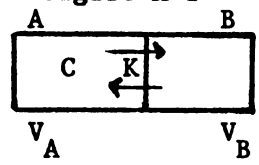
In addition, the use of a multiple dose regimen, allows examination of drug interaction under clinical conditions, and allows determination of drug mechanisms. As in the case of the warfarin-phenylbutazone interaction, metabolic action on warfarin by phenylbutazone was clearly illustrated by changes in metabolite levels, which were not readily apparent following single dose studies. With no observable changes in the corrected clearance of warfarin one can conclude that the major effect of phenylbutazone is one of interference of warfarin metabolism.

# APPENDIX

## PROTEIN BINDING

Examining the system described below in figure A-1, in which there are

figure A-1




two compartments, A and B, with volumes, $V_A$ and $V_B$ respectively. A small molecule C is initially present only in A but is permeable into B, with the equilibrium between A and B desribed by the constant K. Now let us examine two cases, one in the absence of protein and the other in the presence of protein but in only one of the two compartments.

No Protein

The amount of C in Compartment A with time can be described by equation A-1 in which the concentration of A, $C_A$, times the volume $V_A$ is equal to the equilibrium constant K times the difference of the concentrations of C in B and A.

$$V_A \frac{dC_A}{dt} = K (C_B - C_A) \qquad \text{(A-1)}$$

The amount of C in compartment B can be described similarly by

$$V_B \frac{dC_B}{dt} = K (C_A - C_B) \qquad \text{(A-2)}$$

These two differential equations can be solved using La Place Transforms. Equations A-3 and A-4 describe the transforms of these differential equations:

$$s\bar{C}_A - C_A(0) = \frac{K}{V_A}\bar{C}_B - \frac{K}{V_B}\bar{C}_A \qquad \text{(A-3)}$$

$$s\bar{C}_B = \frac{K}{V_B}\bar{C}_B - \frac{K}{V_B}\bar{C}_B \qquad \text{(A-4)}$$

Rearrangement of A-3 and A-4 produce A-5 and A-6 respectively:

$$\bar{C}_A\left(s+\frac{K}{V_A}\right) - \frac{K}{V_A}C_B = C_A(0) \qquad \text{(A-5)}$$

$$-\frac{K}{V_B}\bar{C}_A + \left(s+\frac{K}{V_B}\right)\bar{C}_B = 0 \qquad \text{(A-6)}$$

Solving for $\bar{C}_B$ in both equations and setting them equal to each other, and then solving for $\bar{C}_A$ one obtains equation A-7:

$$\bar{C}_A = \frac{C_A(0)\left(s+\frac{K}{V_B}\right)}{s\left(s+\frac{K}{V_A}+\frac{K}{V_B}\right)} \qquad \text{(A-7)}$$

Solving the La Place transform of $\bar{C}_A$, one obtains equation A-8:

$$C_A = C_A(0)\left[\frac{\frac{K}{V_B}}{\frac{K}{V_A}+\frac{K}{V_B}} + \frac{\frac{K}{V_A}}{\frac{K}{V_A}+\frac{K}{V_B}}\, e^{-\left(\frac{K}{V_A}+\frac{K}{V_B}\right)t}\right] \qquad \text{(A-8)}$$

Rearrangement and simplification of A-8 results in the following:

$$C_A = \frac{C_A(0)}{\frac{K}{V_A}+\frac{K}{V_B}}\left[\frac{K}{V_B} + \frac{K}{V_A}\, e^{-\left(\frac{K}{V_A}+\frac{K}{V_B}\right)t}\right] \qquad \text{(A-9)}$$

Letting time approach infinity in equation A-9, that is letting the system approach equilibrium, one obtains:

$$C_A(\infty) = \frac{C_A(0)}{\frac{K}{V_A}+\frac{K}{V_B}} \cdot \frac{K}{V_B} \qquad \text{(A-10)}$$

Simplification results in

$$C_A(\infty) = C_A(0)\,\frac{V_A}{V_A+V_B} \qquad \text{(A-11)}$$

At any time, the amount of C in compartment B can be described by:

$$V_B C_B = V_A C_A(0) - V_A C_A \qquad \text{(A-12)}$$

that is the concentration of C in compartment B with a volume $V_B$ being equal to the amount of C in compartment A initially minus the amount in compartment A at any time. Substituting A-9 for $C_A$ in equation A-12 results in:

$$C_B = \frac{V_A}{V_B}\left[C_A(0) - \frac{C_A(0)}{\left(\frac{K}{V_A} + \frac{K}{V_B}\right)}\left[\frac{K}{V_B} + \frac{K}{V_A}\, e^{-\left(\frac{K}{V_A} + \frac{K}{V_B}\right)t}\right]\right] \qquad \text{(A-13)}$$

Rearrangement and simplification results in:

$$C_B = \frac{V_A}{V_B} C_A(0)\, \frac{K}{V_A}\, \frac{1}{\frac{K}{V_A}+\frac{K}{V_B}}\left[1 - e^{-\left(\frac{K}{V_A} + \frac{K}{V_B}\right)t}\right] \qquad \text{(A-14)}$$

and therefore

$$C_B = C_A(0)\, \frac{V_A}{V_A + V_B}\left[1 - e^{-\left(\frac{K}{V_A}+\frac{K}{V_B}\right)t}\right] \qquad \text{(A-15)}$$

Substituting $C_A(\infty)$ for $C_A(0)\, \frac{V_A}{V_A + V_B}$ from A-11, one obtains

$$C_B = C_A(\infty)\left[1 - e^{-\left(\frac{K}{V_A} + \frac{K}{V_B}\right)t}\right] \qquad \text{(A-16)}$$

Rearranging equation A-9 and substituting $C_A(\infty)$ from equation A-11 results in

$$C_A = C_A(\infty)\left[1 + \frac{V_B}{V_A}\, e^{-\left(\frac{K}{V_A} + \frac{K}{V_B}\right)t}\right] \qquad \text{(A-17)}$$

Now subtracting $C_A(\infty)$ from both sides of equation A-17 and simplifying,

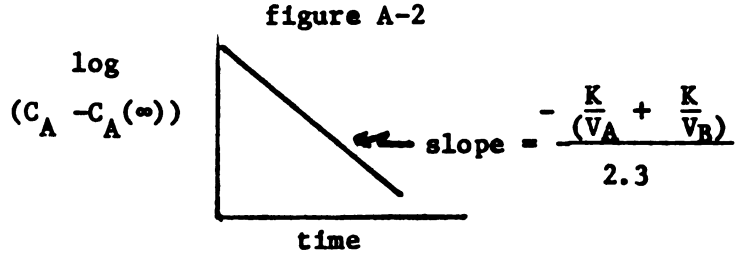
$$C_A - C_A(\infty) = C_A(\infty)\,\frac{V_B}{V_A}\; e^{-(\frac{K}{V_A} + \frac{K}{V_B})t} \quad . \qquad \text{(A-18)}$$

Taking the log of both sides of the equation, one obtains

$$\log\left[C_A - C_A(\infty)\right] = \log\left[C_A(\infty)\frac{V_B}{V_A}\right] - \frac{(\frac{K}{V_A} + \frac{K}{V_B})t}{2.3} \quad . \qquad \text{(A-19)}$$

which is in the form $y = b + mx$. Plotting on semi-log paper $(C_A - C_A(\infty))$ versus time, results in a straight line with a slope of $-\frac{(\frac{K}{V_A} + \frac{K}{V_B})}{2.3}$ (figure A-2).

figure A-2




Multiplying equation A-16 by (-1) and adding $C_A(\infty)$ to both sides of the equation results in

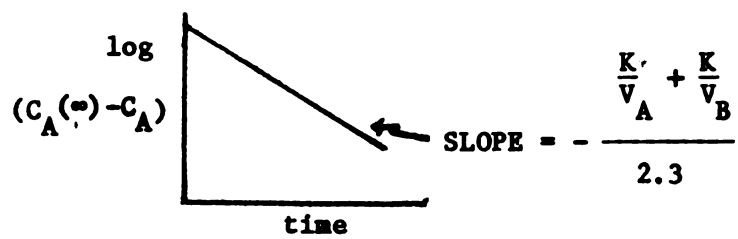
$$C_A(\infty) - C_B = C_A(\infty) - C_A(\infty)\left[1 - e^{-(\frac{K}{V_A} + \frac{K}{V_B})t}\right] \qquad \text{(A-20)}$$

This simplifies to

$$C_A(\infty) - C_B = C_A(\infty)\; e^{-(\frac{K}{V_A} + \frac{K}{V_B})t} \qquad \text{(A-21)}$$

Taking the log of both sides and plotting the resultant equation on semi-log paper as was done for A-19, one obtains a straight line with a slope equal to that found for A-19 (figure A-3).

figure A-3

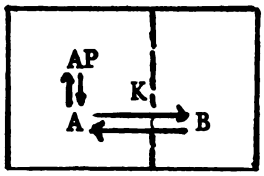


Thus the loss of C from compartment A into B must proceed to equilibrium at the same rate as does increasing C in compartment B.

Protein

The case for protein in one compartment is depicted in figure A-4.

figure A-4




Where A is a small drug or molecule in compartment A and is associated with protein (P). Only the unbound drug (A) is permeable into compartment B and is related by the equilibrium constant K.

The amount of A present in compartment A , equal to the concentration of A, both bound to P and unbound, times the volume of compartment A; is also equal to the difference in the concentration in B minus that in A times the equilibrium constant.

$$V_A \left(\frac{d(C_{AP}+C_A)}{dt}\right) = K\ (C_B - C_A) \qquad \text{(A-22)}$$

Similarly the concentration of B is given by

$$V_B \left(\frac{dC_B}{dt}\right) = K\ (C_A - C_B). \qquad \text{(A-23)}$$

The total amount in the system is equal to the dose which is equal to

$$\text{Dose} = V_A(C_{AP}+C_A) + V_B C_B \qquad \text{(A-24)}$$

Since there is an equilibrium between the free A and P with the bound complex, $C_{AP}$, as illustrated in A-25:

$$C_A + C_P \underset{k-1}{\overset{k_1}{\rightleftharpoons}} C_{AP} \qquad \text{(A-25)}$$

This can be expressed as an equilibrium expression

$$K_A = \frac{k_1}{k_{-1}} = \frac{(C_{AP})}{(C_A)(C_P)} \quad . \qquad \text{(A-26)}$$

If one assumes that $C_P$ is approximately equal to $C_{P\ total}$ and substituting $C_{P\ total}$ for $C_P$ in A-25, rearrangement results in

$$K_A\, C_{P\ total} = \frac{C_{AP}}{C_A} = \frac{\beta}{\alpha} \qquad \text{(A-27)}$$

where $\alpha = 1 - \beta$. Rearranging,

$$C_{AP} = C_A \frac{\beta}{\alpha} = C_A \left(\frac{\beta}{1-\beta}\right) \quad . \qquad \text{(A-28)}$$

Adding $C_A$ to both sides and simplifying

$$C_{AP} + C_A = C_A \left(\frac{1}{1-\beta}\right) \quad . \qquad \text{(A-29)}$$

Substituting $C_{AP} + C_A$ of A-29 into A-22, one obtains

$$\frac{V_A}{1-\beta}\left(\frac{dC_A}{dt}\right) = K\,(C_B - C_A) \qquad \text{(A-30)}$$

In order to solve this differential, La Place Transforms is used, forming

$$s\bar{C}_A - C_A(0) = (1-\beta)\,\frac{K}{V_A}\,(\bar{C}_B - \bar{C}_A) \quad . \qquad \text{(A-31)}$$

Rearrangement results in

$$\bar{C}_A\left(s + (1-\beta)\,\frac{K}{V_A}\right) - (1-\beta)\,\frac{K}{V_A}\,\bar{C}_B = C_A(0) \quad . \qquad \text{(A-32)}$$

Similarly, $V_B \frac{dB}{dt} = K\,(C_A - C_B)$ (A-33)

with the La Place transform

$$s\bar{C}_B = \frac{K}{V_B}\,(\bar{C}_A - \bar{C}_B) \quad . \qquad \text{(A-34)}$$

Rearranging A-34 to the following

$$-\bar{C}_A\,\frac{K}{V_B} + \left(s + \frac{K}{V_B}\right)\bar{C}_B = 0 \qquad \text{(A-35)}$$

Substituting $\bar{C}_A$ from A-32 into A-35 , and solving for $\bar{C}_B$ one obtains the following

$$\bar{C}_B = \frac{C_A(0)\,\frac{K}{V_B}}{s\left(s+\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}\right)} \qquad \text{(A-36)}$$

Solving the La Place Tranform for $\bar{C}_B$, one obtains the following equation:

$$C_B = \frac{C_A(0)\frac{K}{V_B}}{\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}}\left[1 - e^{-\left(\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}\right)t}\right] \qquad \text{(A-37)}$$

Allowing time to go to infinity, the equation simplifies to

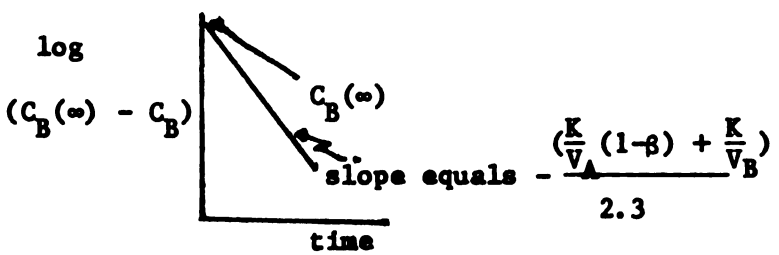
$$C_B(\infty) = \frac{C_A(0)\frac{K}{V_B}}{\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}} = \frac{C_A(0)\;V_A}{V_B(1-\beta)+V_A} \qquad \text{(A-38)}$$

Substituting $C_B(\infty)$ from equation A-38 back into A-37 one obtains

$$C_B = C_B(\infty)\left[1- e^{-\left(\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}\right)t}\right] \qquad \text{(A-39)}$$

Taking the log of both sides and plotting on semi-log paper, one obtains a straight line with a slope of $\frac{K}{V_A}(1-\beta)+\frac{K}{V_B}$), see figure A-5.

figure A-5



Now comparing the slopes of the log of the disappearance curves from the no-protein case, where slope equals $-K\left(\frac{1}{V_A} + \frac{1}{V_B}\right)$, to that with protein, where slope equals $-K\left(\frac{1-\beta}{V_A} + \frac{1}{V_B}\right)$. Letting $V_A = V_B$, then the slope of the case without protein becomes $-\frac{2K}{2.3\,V_A}$, whereas the slope for the case with protein becomes $-\frac{K}{2.3\,V_A}(2-\beta)$. If one assumes that for highly bound drugs, $\beta$ is very close to the value of 1, then the slope of a system without protein equals twice the slope of a system with protein.

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# TABULATION OF DATA

EXPERIMENT 17·5·2

(Table V. p.35, Figure 9. p.37)

DETERMINATION OF RADIOACTIVE PURITY OF LABELLED WARFARIN
AND
COMPARISON OF RADIOACTIVITY AND PROTEIN PROFILES

| Blank | | | | | Pre-Centrifuged Warfarin Plasma Control | | | | Plasma Water Sample | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Length down tube (ml) | Gross $^{3}H$ | CPM $^{14}C$ | $^{3}H/^{14}C$ ratio | Fraction of initial protein | Length down tube (ml) | Gross $^{3}H$ | CPM $^{14}C$ | $^{3}H/^{14}C$ ratio | Length down tube (ml) | Gross $^{3}H$ | CPM $^{14}C$ | $^{3}H/^{14}C$ ratio | Fraction of initial protein |
| 0 -0.2 | 14.9 | 26.4 | .564 | >.01 | 0 -0.2 | 283.0 | 77.5 | 3.7 | 0.2-0.4 | 184.8 | 42.7 | 3.6 | .0093 |
| 0.4-0.6 | 12.5 | 25.0 | .500 | .0070 | 0.2-0.4 | 281.2 | 86.7 | 3.2 | 0.6-0.8 | 157.0 | 43.2 | 3.6 | .0061 |
| 0.8-1.0 | 10.1 | 29.1 | .347 | .0068 | 0.4-0.9 | 671.5 | 181.0 | 3.7 | 1.0-1.2 | 157.9 | 45.7 | 3.5 | .0068 |
| 1.2-1.4 | 14.0 | 26.4 | .530 | .0072 | 0.9-1.4 | 662.2 | 183.7 | 3.6 | 1.4-1.6 | 145.5 | 45.9 | 3.2 | .0078 |
| 1.6-1.8 | 12.2 | 25.5 | .478 | .0070 | 1.4-1.9 | 681.6 | 189.1 | 3.6 | 1.8-2.0 | 157.5 | 47.9 | 3.3 | .0064 |
| 2.0-2.2 | 12.4 | 25.0 | .496 | .0073 | 1.9-2.4 | 672.5 | 184.7 | 3.6 | 2.2-2.4 | 157.7 | 44.4 | 3.6 | .0060 |
| 2.4-2.6 | 13.1 | 24.8 | .528 | .0089 | 2.4-2.9 | 671.1 | 189.6 | 3.5 | 2.6-2.8 | 150.0 | 45.0 | 3.3 | .0068 |
| 2.8-3.0 | 12.9 | 23.5 | .548 | >.01 | 2.9-3.4 | 668.4 | 197.4 | 3.4 | 3.0-3.2 | 176.7 | 47.8 | 3.7 | >.01 |
| 3.2-3.4 | 12.6 | 23.8 | .529 | >.1 | 3.4-3.9 | 678.9 | 182.6 | 3.7 | 3.4-3.6 | 5612.4 | 1610.6 | 3.5 | >.1 |
| | | | | | 3.9-4.15 | 314.6 | 93.6 | 3.4 | | | | | |

UNBOUND FRACTION FOR WARFARIN AT VARYING PLASMA CONCENTRATIONS

**(Table VI. p.39)**

Experiment 29·72

| Sample (μg/ml) | Plasma Water Gross CPM | | Volume | Plasma Gross CPM | | Volume | Unbound Fraction of Warfarin | |
|---|---|---|---|---|---|---|---|---|
| Blank | 13.8 | 28.3 | 200 μl* | 15.3 | 27.1 | 100 μl* | | |
| 2.5 | 94.8 | 51.0 | | 5333.1 | 1445.7 | | .00762 | .00801 |
| 10 | 304.3 | 111.6 | | 18218.6 | 5022.4 | | .00748 | .00834 |
| 15 | 626.5 | 201.2 | | 34589.9 | 9456.1 | | .00886 | .00917 |

Experiment 31·72

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Blank | 11.0 | 27.6 | 200 μl* | 12.1 | 26.5 | 100 μl* | | |
| 2 | 71.4 | | | 3731.6 | | | .00812 | |
| 5 | 138.8 | 64.3 | | 8441.8 | 2434.8 | | .00758 | .00702 |
| 10 | 255.1 | 98.5 | | 14593.1 | 4165.1 | | .00837 | .00857 |
| 15 | 490.7 | 100.7 | | 26901.5 | 7607.5 | | .00892 | .00898 |

Experiment 11·82

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Blank | 30.3 | 26.9 | 500 μl** | 25.5 | 24.5 | 100 μl* | | |
| 2.5 | 178.6 | 73.6 | | 3215.6 | 977.4 | | .00929 | .00981 |
| 5 | 358.7 | 110.5 | | 6339.7 | 1898.5 | | .0104 | .00892 |
| 10 | 725.1 | 232.1 | | 13514.8 | 3932.9 | | .0103 | .0105 |
| 20 | 1355.6 | 423.9 | | 24425.0 | 7629.5 | | .01076 | .01044 |

*All plasma water samples are 200 μl
All plasma samples are 100 μl.

**All plasma water samples are 500 μl.

Experiment 5.34 Equilbrium Dialysis in Absence of Protein
(Figure 10 and 11 pp.41-42)

| Time (min) | Buffer CPM | | $C_p(\infty)-C_p(t)$ | Solution CPM | | $C_p(t)-C_p(\infty)$ |
|---|---|---|---|---|---|---|
| | Gross | Net | | Gross | Net | |
| 0 | 20.5 | | 326.5 | 699.0 | 678.5 | 346.2 |
| 10 | 168.4 | 157.9 | 108.6 | 522.9 | 502.4 | 170.1 |
| 20 | 203.0 | 182.4 | 144.1 | 444.5 | 424.0 | 91.7 |
| 30 | 244.7 | 224.2 | 102.3 | 393.4 | 372.8 | 40.5 |
| 40 | 287.3 | 266.8 | 59.7 | 379.8 | 359.2 | 26.9 |
| 50 | 292.6 | 272.1 | 54.4 | 382.1 | 361.6 | 28.3 |
| 60 | 319.8 | 279.2 | 47.3 | 365.3 | 344.8 | 12.5 |
| 90 | 326.0 | 305.0 | 21.0 | 353.4 | 332.8 | |
| 180 | 343.0 | 322.4 | 4.1 | 341.8 | 321.3 | |
| 240 | 345.6 | 325.0 | 1.5 | 344.1 | 323.6 | |
| 300 | 347.0 | 326.5 | | 352.8 | 332.3 | |

Volume of Buffer and Solution Aliquots 200 µl

Experiment 18·3·4 Equilibrium Dialysis in Presence of Protein

| Time (min) | Buffer | |
|---|---|---|
| | Gross CPM | Volume |
| 0 | 28.2 | 500 µl* |
| 10 | 41.1 | |
| 20 | 50.4 | |
| 30 | 59.4 | |
| 40 | ++ | |
| 50 | 66.6 | |
| 60 | 71.7 | |
| 120 | 90.2 | |
| 180 | 94.1 | |
| 300 | 91.8 | |
| 1380 (23 hours) | 85.4 | |

* All buffer samples have volumes of 500 µl

++ sample lost

## PHENYLBUTAZONE PLASMA CONCENTRATIONS

(Figure 25 p. 72 and Figure 27 p.83)

| Sample (Days After Warfarin Commencement) | Phenylbutazone Concentration in Plasma | | |
|---|---|---|---|
| | Run 1 | Run 2 | Mean |
| Blank | 0 | 0 | |
| 6 | 0 | 0 | |
| 13 | 43. | 31. | 37. |
| 14 | 50. | 53. | 51.5 |
| 15 | 61. | 59. | 60. |
| 16 | 85. | 90. | 87.5 |
| 17 | 102. | 85. | 93.5 |
| 18 | 102. | 91. | 96.5 |
| 19 | 101. | 86. | 93.5 |
| 20 | 89. | 98. | 93.5 |
| 21 | 106. | 91. | 98.5 |
| 22 | 113. | 113. | 113. |
| 23 | 77. | 75. | 76. |
| 24 | 76. | 72. | 74. |
| 28 | 34. | 34. | 34. |
| 29 | 33. | 28. | 30.5 |
| 30. | 25. | 20. | 22.5 |
| 31 | 22. | 17. | 19.5 |
| 32 | 15. | 15. | 15. |
| -4 | 14. | 6. | 10. |

TOTAL URINE EXCRETION ASSAYED BY THE 1-DIMENSIONAL TLC

(Figures 16-20)

| Date* | Vol Total | Warfarin | | Alcohol$_1$ | | Alcohol$_2$ | | 6-Hydroxy$^{++}$ | 7-Hydroxy | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (ml) | conc(ng/ml) | total(mg) | conc($\frac{ng}{ml}$) | total(mg) | conc($\frac{ng}{ml}$) | total(mg) | | conc($\frac{ng}{ml}$) | total(mg) |
| +9 | 905 | 57 | .0511 | 190 | .1719 | 155 | .1407 | | 2083 | 1.8847 |
| 10 | 720 | 95 | .0684 | 240 | .1724 | 260 | .1877 | | 2719 | 1.9577 |
| 11 | 830 | 99 | ,0822 | 245 | .2034 | 232 | .1926 | | 1914 | 1.5886 |
| 12 | 1220 | 103 | .1257 | 281 | .3434 | 320 | .3904 | | 2633 | 3.2123 |
| 13 | 1010 | 43 | .0429 | 169 | ,1707 | 324 | .2571 | | 2138 | 2.1594 |
| 14 | 940 | 62 | .0583 | 168 | .1579 | 230 | .2169 | | 2160 | 1.1161 |
| 15 | 1200 | 106 | .1272 | 152 | .1824 | 264 | .3162 | | 1177 | 1.4124 |
| 16 | 1020 | 94 | .0959 | 132 | .1346 | 319 | .3254 | | 1064 | 1.0853 |
| 17 | 980 | 133 | .1308 | 151 | .1483 | 339 | .3322 | | 1137 | 1.1143 |
| 18 | 920 | 131 | .1205 | 199 | .1826 | 471 | .4333 | | 1168 | 1.0746 |
| 19 | 1220 | 96 | .1165 | 176 | .2147 | 456 | .5563 | | 1029 | 1.2553 |
| 20 | 1190 | 77 | .0916 | 83 | .0982 | 321 | .3814 | | 622 | .7402 |
| 21 | 940 | 53 | .0494 | 68 | .0639 | 380 | .3572 | | 590 | .5546 |
| 22 | 900 | 93 | .0837 | 71 | .0644 | 444 | .3996 | | 748 | .6732 |
| 23 | 1410 | 113 | .1586 | 127 | .1784 | 453 | .6387 | | 855 | 1.2051 |
| 24 | 1050 | 89 | .0935 | 85 | .0893 | 428 | .4489 | | 819 | .8560 |
| 25 | 1300 | 48 | .0624 | 136 | .1768 | 471 | .6123 | | 1527 | 1.9851 |
| 26 | 1010 | 65 | .0657 | 100 | .1015 | 424 | .4282 | | 1073 | 1.0832 |
| 27 | 1020 | 70 | .0714 | 109 | .1107 | 405 | .4131 | | 880 | .8976 |
| 28 | 1710 | 45 | .0761 | 95 | .1617 | 314 | .5380 | | 906 | 1.5494 |
| 29 | 1410 | 69 | .0966 | 120 | .1692 | 342 | .4815 | | 1012 | 1.4262 |
| 30 | 1400 | 64 | .0896 | 147 | .2051 | 423 | .5922 | | 1606 | 2.2484 |
| 31 | 1340 | 58 | .0777 | 143 | .1910 | 329 | .4409 | | 1789 | 2.3973 |
| 32 | 960 | 74 | .0710 | 190 | .1828 | 300 | .2880 | | 1937 | 1.8595 |
| -1 | 1800 | 54 | .0981 | 136 | .3357 | 209 | .3762 | | 1440 | 2.5920 |
| -2 | 1565 | 66 | .1025 | 126 | .1948 | 174 | .2723 | | 1284 | 2.0095 |
| -3 | 1240 | 67 | .0831 | 134 | .1662 | 144 | .1779 | | 610 | .7558 |
| -4 | 1115 | 29 | .0318 | 190 | .2119 | 163 | .1818 | | 881 | .9828 |
| -5 | 800 | 64 | .0512 | 217 | .1736 | 118 | .0944 | | 823 | .6580 |
| -6 | 850 | 33 | .0276 | 155 | .1318 | 62 | .0531 | | 547 | .4645 |

* Date expressed as days after warfarin commencement

++ 6-Hydroxy metabolite not measured by this assay

TOTAL URINE EXCRETION ASSAYED BY 2-DIMENSIONAL TLC SYSTEM

(Figures 16-20 pp.53-57 Table XV. pp.76-78)

| Date * | Vol Total | Warfarin | | Alcohol$_1$ | | Alcohol$_2$ | | 6-Hydroxy | | 7-Hydroxy | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (ml) | conc(ng/ml) | total(mg) | conc(ng/ml) | total(mg) | conc(ng/ml) | total(mg) | conc(ng/ml) | total(mg) | conc(ng/ml) | total(mg |
| +9 | 905 | 38 | .0344 | 228 | .2063 | 172 | .1552 | 989 | .8946 | 1524 | 1.3792 |
| 10 | 720 | 55 | .0396 | 278 | .2002 | 250 | .1800 | 2374 | 1.7093 | 1846 | 1.3295 |
| 11 | 830 | 63 | .0523 | 242 | .2009 | 222 | .1843 | 1946 | 1.6156 | 2032 | 1.6870 |
| 12 | 1220 | 63 | .0770 | 249 | .3038 | 227 | .2776 | 1151 | 1.4036 | 2236 | 2.7279 |
| 13 | 1010 | 61 | .0621 | 179 | .1808 | 221 | .2232 | 1061 | 1.1211 | 1432 | 1.4459 |
| 14 | 940 | 71 | .0625 | 173 | .1626 | 303 | .2844 | 1335 | 1.2549 | 1412 | 1.3278 |
| 15 | 1200 | 62 | .0744 | 162 | .1940 | 270 | .3234 | 1032 | 1.2384 | 1162 | 1.3944 |
| 16 | 1020 | 78 | .0790 | 134 | .1367 | 314 | .3203 | 1000 | 1.0200 | 913 | .9313 |
| 17 | 980 | 32 | .0318 | 152 | .1490 | 391 | .3827 | 1020 | .9996 | 1009 | .9888 |
| 18 | 920 | 37 | .0336 | 172 | .1578 | 453 | .4168 | 992 | .9126 | 1030 | .9471 |
| 19 | 1220 | 28 | .0336 | 171 | .2080 | 431 | .5258 | 844 | 1.0297 | 912 | 1.1126 |
| 20 | 1190 | 64 | .0767 | 98 | .1166 | 328 | .3856 | 710 | .8479 | 638 | .7592 |
| 21 | 940 | 58 | .0451 | 81 | .0766 | 325 | .3055 | 647 | .6082 | 548 | .5151 |
| 22 | 900 | 37 | .0333 | 90 | .0810 | 440 | .3946 | 766 | .6898 | 732 | .6588 |
| 23 | 1410 | 46 | .0649 | 139 | .1953 | 417 | .5880 | 730 | 1.0293 | 914 | 1.2887 |
| 24 | 1050 | 53 | .0562 | 96 | .1008 | 430 | .4510 | 918 | .9644 | 968 | 1.0169 |
| 25 | 1300 | 50 | .0656 | 154 | .2002 | 447 | .5882 | 998 | 1.2974 | 1380 | 1.7934 |
| 26 | 1010 | 79 | .0803 | 121 | .1222 | 421 | .4247 | 977 | .9868 | 988 | .9984 |
| 27 | 1020 | 85 | .0867 | 113 | .1148 | 435 | .4442 | 893 | .9002 | 1009 | 1.0292 |
| 28 | 1710 | 41 | .0695 | 107 | .1838 | 356 | .6088 | 674 | 1.1534 | 970 | 1.6587 |
| 29 | 1410 | 42 | .0597 | 120 | .1692 | 370 | .5217 | 694 | .9785 | 874 | 1.2323 |
| 30 | 1400 | 55 | .0770 | 201 | .2814 | 363 | .5082 | 785 | 1.0990 | 1408 | 1.9712 |
| 31 | 1340 | 35 | .0476 | 138 | .1849 | 329 | .4401 | 735 | .9849 | 1238 | 1.6589 |
| 32 | 960 | 92 | .0883 | 163 | .1565 | 316 | .3034 | 945 | .8976 | 1334 | 1.2802 |
| -1 | 1800 | 60 | .1080 | 158 | .2844 | 241 | .4296 | 940 | 1.6911 | 1241 | 2.2338 |
| -2 | 1565 | 45 | .0712 | 139 | .2175 | 163 | .2559 | 685 | 1.0720 | 766 | 1.2144 |
| -3 | 1240 | 34 | .0422 | 150 | .1860 | 144 | .1786 | 549 | .6808 | 849 | 1.0534 |
| -4 | 1115 | 34 | .0379 | 236 | .2631 | 167 | .1862 | 595 | .6634 | 920 | 1.0264 |
| -5 | 800 | 24 | .0192 | 230 | .1840 | 133 | .1068 | 589 | .4716 | 892 | .7140 |
| -6 | 850 | 26 | .0225 | 170 | .1449 | 78 | .0667 | 356 | .3026 | 519 | .4412 |

*Expressed as days after warfarin commencement

EXPERIMENT
21·7·3

DETERMINATION OF THE ASSOCIATION CONSTANT FOR WARFARIN

**(figure 22 p.61)**

| Sample | Buffer | | Plasma | | Unbound Fraction of Warfarin |
|---|---|---|---|---|---|
| | Volume | Gross CPM | Volume | Gross CPM | |
| Blank | 500 µl* | 22.0 | 100 µl* | 22.5 | |
| 5 µg/ml of warfarin | | 127.3 | | 3257.5 | .0065 |
| | | 128.4 | | | .0066 |
| 25 µg/ml | | 141.5 | | 3298.3 | .0073 |
| | | 128.5 | | | .0066 |
| 50 µg/ml | | 164.2 | | 3316.7 | .0086 |
| | | 171.7 | | | .0090 |
| 75 µg/ml | | 183.1 | | 3442.7 | .0094 |
| | | 181.1 | | | .0093 |
| 100 µg/ml | | 209.5 | | 3337.1 | .0113 |
| | | 210.9 | | | .0114 |
| 150 µg/ml | | 284.4 | | 3328.3 | .0159 |
| | | 294.4 | | | .0165 |

*All buffer samples are 500 µl.
All plasma samples are 100 µl.

EXPERIMENT 20·8·3 DETERMINATION OF APPARENT ASSOCIATION CONSTANT FOR

WARFARIN IN PRESENCE OF PHENYLBUTAZONE

**(Figure 22 p.61)**
**(Figure 23 p.67)**

| Sample | Buffer | | Plasma | | Unbound Fraction of Warfarin |
|---|---|---|---|---|---|
| | Volume | Gross CPM | Volume | Gross CPM | |
| **Blank** | 500 µl** | 54.4 | 100 µl** | 35.7 | |
| Warfarin 5 µg/ml | | 149.1 | | 3756.4 | .0050 |
| Warfarin 5 µg/ml | | 241.9 | | 3746.6 | .0095 |
| +Pb* | | 242.0 | | | .0095 |
| W 25 + Pb | | 253.6 | | 4057.8 | .0098 |
| | | 263.8 | | | .0106 |
| W 50 + Pb | | 316.1 | | 4134.8 | .0127 |
| W 75 + Pb | | 364.4 | | 4096.7 | .0152 |
| | | 346.5 | | | .0143 |
| W 100 + Pb | | 382.1 | | 4041.8 | .0162 |
| | | 405.5 | | | .0174 |
| W 150 + Pb | | 470.2 | | 4020.9 | .0207 |
| | | 503.4 | | | .0224 |

**All buffer samples are 500 µl
All plasma samples are 100 µl
*Pb (phenylbutazone) concentration equals 100 µg/ml

UNBOUND FRACTION OF WARFARIN IN HUMAN VOLUNTEERS

(Table XI. p.63)

| Sample | Unbound Fraction of Warfarin $^{3}H$ | Unbound Fraction of Warfarin $^{14}C$ |
|---|---|---|
| R.A. | | |
| Warfarin | .0032 | .0033 |
| | .0046 | .0037 |
| Warfarin + Phenylbutazone | .0081 | .0079 |
| S.S. | | |
| Warfarin | .0034 | .0033 |
| Warfarin + Phenylbutazone | | |
| 6 | .0113 | .0105 |
| 7 | .0124 | .0114 |
| 8 | .0133 | .0122 |
| J.I. | | |
| Warfarin | | |
| 1 | .0044 | .0042 |
| 2 | .0050 | .0042 |
| 4 | .0036 | .0030 |
| 5 | .0043 | .0038 |
| 6 | .0052 | .0036 |
| Warfarin + Phenylbutazone | | |
| 1 | .0100 | .0088 |
| 3 | .0083 | .0077 |
| 4 | .0074 | .0070 |
| 5 | .0089 | .0082 |
| 6 | .0087 | .0066 |

IN VITRO DISPLACEMENT OF WARFARIN BY PHENYLBUTAZONE

(Figure 23 p.67)

Experiment 5'112

| Sample | Plasma Water Gross CPM $^3H$ | Plasma Water Gross CPM $^{14}C$ | Plasma Water Volume | Plasma Gross CPM $^3H$ | Plasma Gross CPM $^{14}C$ | Plasma Volume | Unbound Fraction of Warfarin | |
|---|---|---|---|---|---|---|---|---|
| Blank | 29.8 | 22.4 | 200 µl** | 29.8 | 22.4 | 100 µl** | .0057 | .0057 |
| Warfarin* | 173.3 | 84.1 | | 12690.3 | 5455.3 | | | |
| Warfarin + Phenylbutazone Concentration | | | | | | | | |
| +50 | 273.3 | 125.3 | | 10667.8 | 4625.7 | | .0114 | .0112 |
| +100 | 367.6 | 167.3 | | 10876.8 | 4693.0 | | .0156 | .0155 |
| +150 | 448.8 | 213.6 | | 11035.7 | 4772.0 | | .0204 | .0201 |
| +200 | 1127.5 | 494.9 | | 11260.7 | 4842.7 | | .0502 | .0488 |

* Warfarin concentration in all samples equals 10 µg/ml

**Plasma Water concentration in all samples equals 200 µg/ml

Plasma concentration in all samples equals 100 µg/ml

WARFARIN BINDING CHARACTERISTICS

OF THE COADMINISTRATION STUDY
(Figure 25 p.72 Table XIV. p.73)

| Sample (Days After Warfarin Commencement) | Unbound Fraction of Warfarin in Plasma | | | |
|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | Mean |
| 3 | .0054 | | .0054 | .0054 |
| 6 | .0049 | | | .0049 |
| 8 | .0057 | | .0036 | .0047 |
| 10 | .0046 | | .0050 | .0048 |
| 13 | .0047 | | .0071 | .0059 |
| 14 | .0070 | | .0102 | .0086 |
| 15 | .0073 | | .0112 | .0093 |
| 16 | .0152 | | .0123 | .0137 |
| 17 | .0100 | | .0087 | .0093 |
| 18 | .0114 | | .0116 | .0115 |
| 19 | | .0120 | .0085 | .0103 |
| 20 | .0085 | | .0118 | .0101 |
| 21 | .0136 | | .0145 | .0141 |
| 22 | .0128 | | .0155 | .0142 |
| 23 | .0116 | | .0153 | .0134 |
| 24 | | .0106 | | .0106 |
| 28 | | .0092 | .0109 | .0101 |
| 29 | | .0093 | | .0093 |
| 30 | .0065 | | .0106 | .0086 |
| 31 | | .0077 | .0094 | .0086 |
| 32 | | .0076 | .0100 | .0088 |
| -2 | | .0085 | | .0085 |
| -4 | .0061 | | .0076 | .0069 |
| -6 | | .0068 | | .0068 |

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