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In Vitro Percutaneous Penetration Of Methotrexate

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IN VITRO PERCUTANEOUS PENETRATION OF METHOTREXATE

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Abstract of Dissertation

This research project was designed to investigate the effects of various physical and chemical parameters on percutaneous penetration of methotrexate. The potential of propylene glycol in water as a vehicle for topical delivery of methotrexate has been examined. A detailed examination of solubility, partition coefficient and pH parameters in the range of 2 to 6 pH units has been conducted with the goal of correlating their effects on skin penetration of methotrexate. The importance of physiochemical parameters such as solubility and partition coefficient and their aid in the development of a suitable delivery system for topical application has been explained. In vitro percutaneous absorption of methotrexate was examined across suitably characterized human cadaver skin samples.

The results of this investigation suggested that the stratum corneum formed the main effective barrier for penetration of topically applied methotrexate. The analysis of the penetration data revealed relatively high lag times even at the most optimal pH, indicative of the low amounts of drug penetrating during the initial hours. These observations might explain the unsuccessful clinical results seen with topical methotrexate.

At low pH values between 1 and 3, the protonation of the nitrogens possibly of the pteridine nucleus and also at the 10 position was found to retard skin penetration. Between pH values 3 and 5, the concentration of the unionized species present was optimal, contributing to penetration by passive diffusion. This was consistent with the improved rate of penetration and comparatively low values for lag times in this pH range. Relatively low drug solubility in this pH range in the vehicle examined might account for lower rate and extent of penetration than those observed in the pH range 5 to 6. Beyond pH 5, the concentration of unionized drug was very low, yet the lag times, and rate and extent of penetration were the highest observed. Relatively high drug solubility and gradually increasing contribution of shunt pathways probably accounted for this.

The results of this investigation emphasize the particular importance of in vitro skin penetration studies which should precede clinical trials. Based on the results of this investigation, it is suggested that optimal conditions for topical delivery of methotrexate should include a vehicle system with a pH of about 4 capable of dissolving the drug to the extent of 0.4% w/v or more while still retaining good partitioning characteristics for the skin.

DEDICATION

To my Dad.

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INTRODUCTION

Methotrexate, an antineoplastic agent, is also effective for control of recalcitrant psoriasis. It has been shown to selectively inhibit DNA synthesis in psoriatic epidermal cells, thus decreasing the mitotic activity (1,2). Though there is no longer any doubt about the efficacy of methotrexate in dermatology (3), the risk of short- and long-term systemic side effects has precluded the use of this drug for most patients. Injection of intralesional methotrexate into psoriatic plaques shows a dose-related decrease in mitotic activity accompanied by production of methotrexate 'damaged cells' beginning 2 hours after injection (4). These effects are similar to those found in psoriatic epidermis following systemic administration of methotrexate. The data suggest that methotrexate acts directly on the psoriatic plaques rather than systemically at a distant site. This finding along with the need to circumvent the toxic side effects of systemic administration of methotrexate on gastrointestinal tract, bone marrow, hair roots (5), and liver (6,7) has prompted a continuing search in the last two decades for safe topical delivery of methotrexate to the affected skin.

Current Status of Topical Methotrexate Therapy

Clinical trials of topical methotrexate therapy have unfortunately been uniformly disappointing.

Condit (8) in 1961 reported that very small amounts of methotrexate, 0.5 mg/kg, inhibit conversion of folic acid to folinic acid in mice. In addition the author also showed that topically applied methotrexate is absorbed through psoriatic lesions. Nurse (9) in 1963, failed to confirm the above findings following topical application of 0.5% methotrexate in a water-miscible ointment base on rabbit and human skin under occlusive and non-occlusive conditions. Nurse suggested that the body levels of folinic acid must be depleted by previous small parenteral doses of methotrexate before any local action can be demonstrated. Fry and McMinn (10) in 1967 reported varying degrees of remission of psoriasis following topical application of 0.2% methotrexate in an aqueous base. They proposed that the action of methotrexate appeared to be directly on the epidermal cells. According to these authors the variability in response was probably due to the different levels of folic acid reductase in the psoriatic skin. In addition, the authors disagreed with Van Scott and Reinertson's theory (11) that methotrexate acts at a distant site from the skin to have an effect on psoriasis. They also suggested that the negative results of Nurse (9) were probably due to the instability of the formulation used by Nurse. However Comaish and Juhlin (12) refuted the instability

theory while reporting no visible effect on psoriatic lesion when methotrexate was applied topically within seven days after preparation in aqueous cream, soft paraffin or 90% dimethylsulfoxide in water - even at concentrations 40 times greater than that used by Fry. These authors also concluded that rapid transit through the epidermis might have accounted for their inability to demonstrate epidermal labelling by autoradiography following topical application of methotrexate. In a subsequent article, Comaish (13) proposed that methotrexate might act on skin cells indirectly by its effect on the liver.

By the 1970-1980 decade, researchers became increasingly cognizant of the importance of pH and vehicle effects on percutaneous penetration. Furthermore, availability of radiolabeled methotrexate provided a more sensitive assay procedure by way of liquid scintillation counting techniques.

One of the first reports on percutaneous penetration of methotrexate was by Newbold and Stoughton (14) wherein the authors provided evidence for the in vitro penetration of methotrexate through hairless mouse skin and human skin. The concentrations studied were 0.5% and 2.5% methotrexate under controlled pH conditions. About the same time, Stewart and co-workers (15) reported rapid in vivo absorption of topically applied methotrexate through hairless mouse skin, but could not achieve comparable results through human skin. Certain dialkyl esters of methotrexate and 3',5'-dichloromethotrexate were synthesized and found to be inhibitors for

dihydrofolate reductase (16). Subsequent in vivo study by Weinstein and McCullough (17) demonstrated selective inhibition of psoriatic epidermal cells at lower concentrations of these drugs. In addition the ester derivatives were found to be more active than the parent compound, suggesting that the increased lipid solubility potentiated their activity. While investigating this and other possibilities, McCullough and co-workers (18) could not achieve more than 2% penetration when tested in vehicles containing 80% dimethylsulfoxide, 25% dimethylacetamide, 0.1% retinoic acid or 2.5% C-10 methylsulfoxide. More recently, Wallace and Barnett (19) have reported that parallel pathways exist for penetration of topically applied methotrexate. According to these authors the fraction of the drug penetrating through the shunt pathway increases as vehicle pH and ionization increases.

The surge in interest in the percutaneous penetration of methotrexate is evidenced by the increasing number of publications in recent years. However, the published results have often been contradictory and lacking in detailed examination of the various physicochemical factors. They point to a need to investigate the effect of various physicochemical factors such as lipid solubility, pH, ionization, vehicle on the percutaneous penetration of methotrexate. An understanding of such factors is essential to achieve predictability in therapy and hopefully to provide a solution to the contradictory opinions and varied viewpoints.

Solubility Characteristics and Percutaneous Penetration

The solubility characteristics of a substance greatly influence its ability to penetrate biological membranes. Assuming passive diffusion as the primary mechanism for transepidermal absorption of methotrexate, concentration of the unionized species and its lipid solubility would be expected to play dominant roles in controlling the overall rate and extent of absorption. The aqueous solubility of the drug would determine the concentration presented to the absorption site, while the partition coefficient would influence the rate of penetration across the absorption site. Since the solubility of methotrexate is a function of pH, comparison of pH-solubility profile with both pH-rate of penetration profile and solubility-rate of penetration profile could be very instructive.

pH of the Vehicle and pK_a of Drug

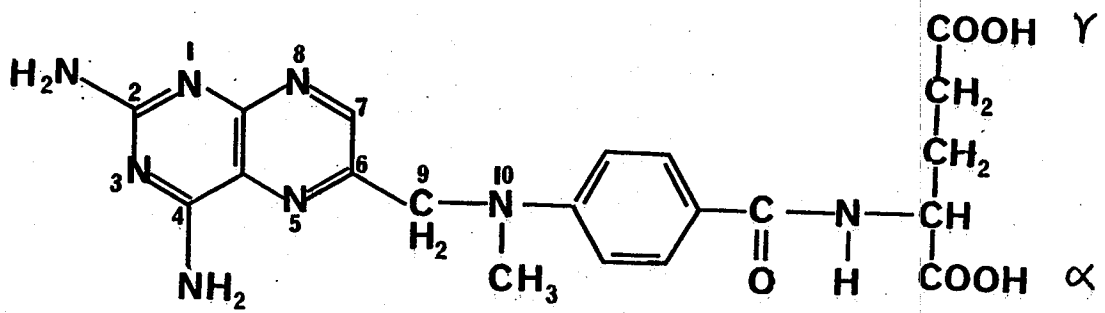
One of the main parameters affected by pH of the vehicle is the polarity of the diffusing molecule. Absorption by passive mechanism is maximized when the concentration of non-polar molecules is greatest, thereby positively affecting the diffusion gradient of drug across the membrane (20). The molecular structure of methotrexate presents a complicated situation for correlation of kinetic parameters with penetration rate. Methotrexate has five pK_a values, three of which 5.71, <-1.5, and 0.5 are associated with the protonation of nitrogens at 1, 5, and 10

positions respectively (21) while the other two correspond to the ionization of the α and γ carboxylic groups (Figure 1). Unfortunately, a review of the literature provides three different sets of pK_a values for the ionization of the α and γ carboxylic groups, namely 3.36 and 4.70 (21), 4.3 and 5.5 (22), and 4.8 and 5.5 (23). In view of the foregoing, the effects of pH on the concentration of unionized methotrexate have not been fully delineated. By using a saturated solution of the drug in the vehicle at different pH, the thermodynamic activity of drug could be maintained at its maximum so that the correlation, if any, between flux and effective concentration of drug in the vehicle could be examined.

Scope of the Present Study

As pointed out earlier, the published results on percutaneous penetration of methotrexate have often been contradictory and lacking in detailed examinatin of the various physicochemical factors.

This research project was designed to investigate the effects of various physical and chemical parameters on percutaneous penetration of methotrexate. The investigation might help define a suitable vehicle system for the topical delivery of methotrexate. A detailed examination of solubility, partition coefficient and pH parameters in the range of 2 to 6 pH units was conducted with the goal of correlating their effects on skin penetration of



4-Amino-N^{10}-methyl pteroylglutamic acid

Figure 1. Chemical structure of methotrexate.

methotrexate. *In vitro* percutaneous absorption of methotrexate was also examined across suitably characterized human cadaver skin samples.

It is hoped that this investigation will contribute to a better understanding of the percutaneous penetration behavior of methotrexate leading to the development of an effective topical dosage form.

THEORY

The design of a delivery system for the topical application of a drug presents a challenging opportunity for research pharmacists and dermatologists. Increased knowledge and understanding of the physical-chemical parameters of drugs and adjuvants along with a trial-and-error approach to formulation has worked remarkably well in developing solutions to problems related to drug stability, physical stability of formulation, irritation and sensitization properties, and aesthetic acceptability. In the past couple of decades, the focus of attention has shifted to correlating physicochemical parameters in an effort to optimize drug delivery from topical application. Although notable achievements have been made, the continuing effort has not yet realized any blueprint to insure optimal product (24). It is important to recognize that a variety of factors are involved in optimizing drug availability from topical dosage forms. At the same time, it should also be noted that a single topical product cannot be ideal, in terms of drug bioavailability, for every type of skin disease or for every patient. To quote J. J. Seelman (25), "My experiments have convinced me that there will probably never be such a thing as a universal ointment vehicle. On

the contrary, I believe that the more the problem is studied the more it will be realized that vehicles should be individualized in accordance with the drugs used, the therapeutic aim and special needs of the disorder to be treated."

Some of the important factors which influence percutaneous absorption are:

(a) Solubility of the penetrant in the vehicle,

(b) Partition coefficient of the penetrant between the vehicle and stratum corneum,

(c) Nature of the vehicle,

(d) pH of the vehicle and pK_a of the penetrant, and

(e) Molecular characteristics of the drug.

For a number of drugs, physical parameters such as solubility and partition coefficient have been shown to correlate well with the rate of percutaneous penetration. Solubility and partition coefficient information in conjunction with the effect of changes of pH on the thermodynamic activity of ionizable drugs have been utilized to develop predictive models for optimizing drug availability with limited success. Of course it should be borne in mind that when dealing with a nonhomogeneous complex membrane like human skin, deviations from such predicted models must be expected. A brief discussion of some of the parameters of relevance to the present investigation follows.

(a) Solubility of Drug in the Vehicle

The solubility of drug in the vehicle represents the concentration which is presented at the absorption site. When high concentrations of the penetrant are present in the donor phase, positive or negative deviations from Fick's law may occur as a consequence of the membrane changes induced or because the partition coefficient between the donor phase and skin membrane is not constant over the entire concentration range.

For a drug with limited aqueous solubility, a vehicle system comprising water as primary solvent with a mutually miscible cosolvent may often be utilized. If the cosolvent chosen is a solubilizer for the drug, the solubility could then be varied by altering the relative proportions of the primary solvent and cosolvent. Increased concentrations of drug in solution in the vehicle can be achieved by increasing the proportion of the cosolvent in the mixture. A representative plot of solubility against percent cosolvent in the mixture would then look as shown in Figure 2. Additionally, such data may also reveal whether the drug solubility has first-order dependence on the proportion of cosolvent present in each vehicle.

(b) Partition coefficient

Partition coefficient, defined by the equation,

$$P = C_s/C_v$$

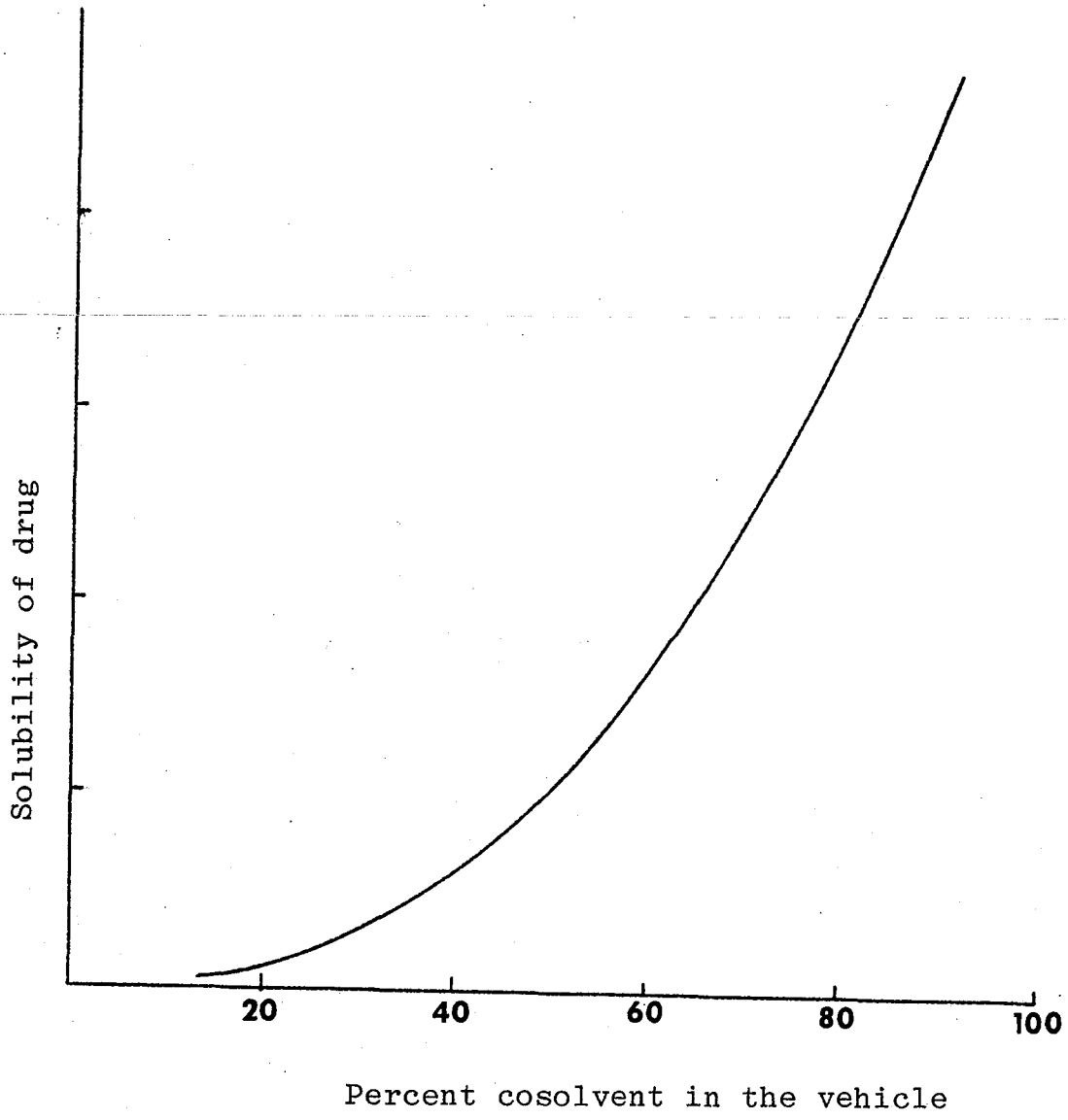


Figure 2. Representative plot of solubility of drug against percent cosolvent in the vehicle.

where C_s and C_v are the concentrations of the drug in the stratum corneum and vehicle respectively, can be regarded as an index of drug's relative affinity for the skin and the vehicle. Greater the value of P, the lower is the interaction between the drug and the vehicle. Conversely, a low P value indicates strong affinity between the drug and the vehicle and reflects the tendency of the drug to remain in the vehicle.

Due to difficulties involved in determination of solubility of drug in the stratum corneum, partition coefficients are usually obtained for some arbitrarily selected two-phase system. The drug is dissolved in the aqueous vehicle, while the oil phase simulates the stratum corneum. Though partition coefficients by this method cannot be exactly related to the rate of diffusion through the skin, good correlation has been shown for a number of compounds. For vehicles comprising a primary solvent and cosolvent, with cosolvent being a solubilizer for the drug, the partition coefficient-percent cosolvent profile (Figure 3) may mirror the solubility-percent cosolvent profile.

(c) Nature of the Vehicle

Vehicles do significantly affect the penetration of substances through stratum corneum (24). The physical characteristics of the vehicle are a major consideration in vehicle selection. Substances may be more rapidly

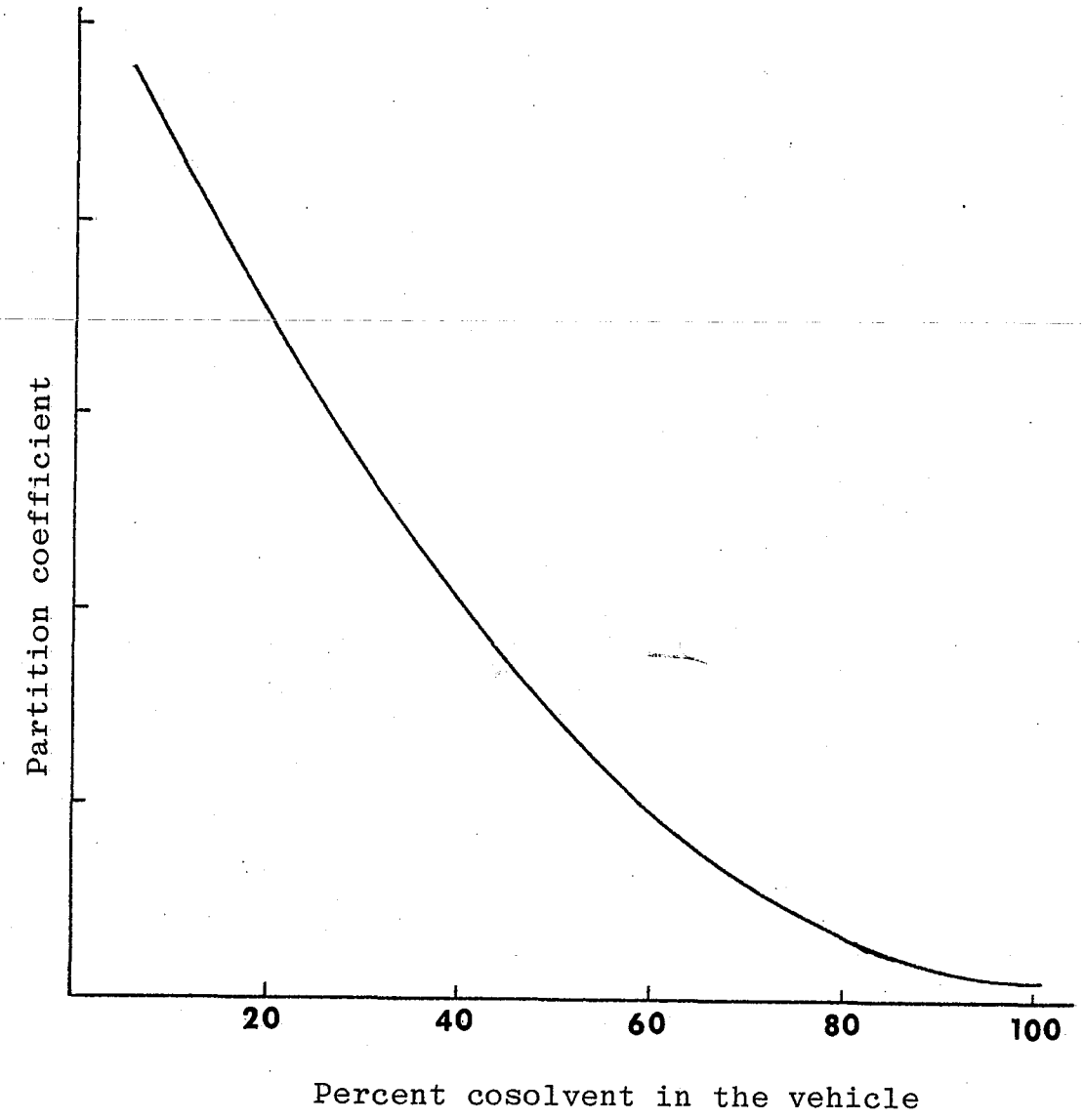


Figure 3. Representative plot of partition coefficient of drug against percent cosolvent in the vehicle.

released from vehicles having a low affinity for the penetrant, i.e. vehicles with relatively low solvent power for incorporated compounds may induce more rapid penetration (26). In general, a compound must be at least partially soluble in its vehicle so that it can be readily released into the receptor phase (skin barrier). High solubility may result in preferential retention of the drug in the vehicle.

The physical properties of vehicles are also important in the degree of occlusion they produce leading to water retention in the stratum corneum layer. The efficiency of various types of vehicles in aiding penetration can be reasonably explained on the basis of their effect on hydration of the stratum corneum or how the vehicle alters the activity of water in the stratum corneum and influences the stratum corneum-vehicle partition coefficient. Greases and oils are the most occlusive vehicles and induce the greatest hydration through sweat accumulation at the skin-vehicle interface (27). This can be accentuated by covering with occlusive bandages or plastic. Emulsions of the water-in-oil type are less occlusive than greases. Substances in the vehicle, such as humectants, which have a high affinity for water, would act in proportion to the relative humidity of the environment. If the latter is low, the humectant would tend to dehydrate the stratum corneum and decrease penetration.

Some vehicles have pronounced effect when applied to the skin surface. Certain nonaqueous bases promote penetration by producing structural change or chemical damage in the barrier layer (28). Dimethylsulfoxide, dimethylacetamide, and dimethylformamide are examples of these.

Low molecular weight volatile solvents such as ether, methanol, ethanol and acetone may enhance drug penetration. Substantial lipid extraction from stratum corneum cells, which leaves a more porous barrier, may be the likely explanation for this effect.

Choice of Optimal Vehicle

Diffusion of drug from the vehicle into the skin surface and subsequent penetration of drug through the stratum corneum are functions of partition coefficient of drug between the stratum corneum and vehicle and also of the rel-tive solubility of the drug in the vehicle (29.30). This concept of correlation of physical parameters such as solubility and partition coefficient with the rate of percutaneous penetration for several compounds can be utilized judiciously to predict the choice of optimal vehicle for subsequent in vitro penetration studies.

For a vehicle comprising a primary solvent and cosolvent, a representative plot as shown (Figure 4) of solubility and partition coefficient against percent cosolvent in the vehicle has a crossover point. It is

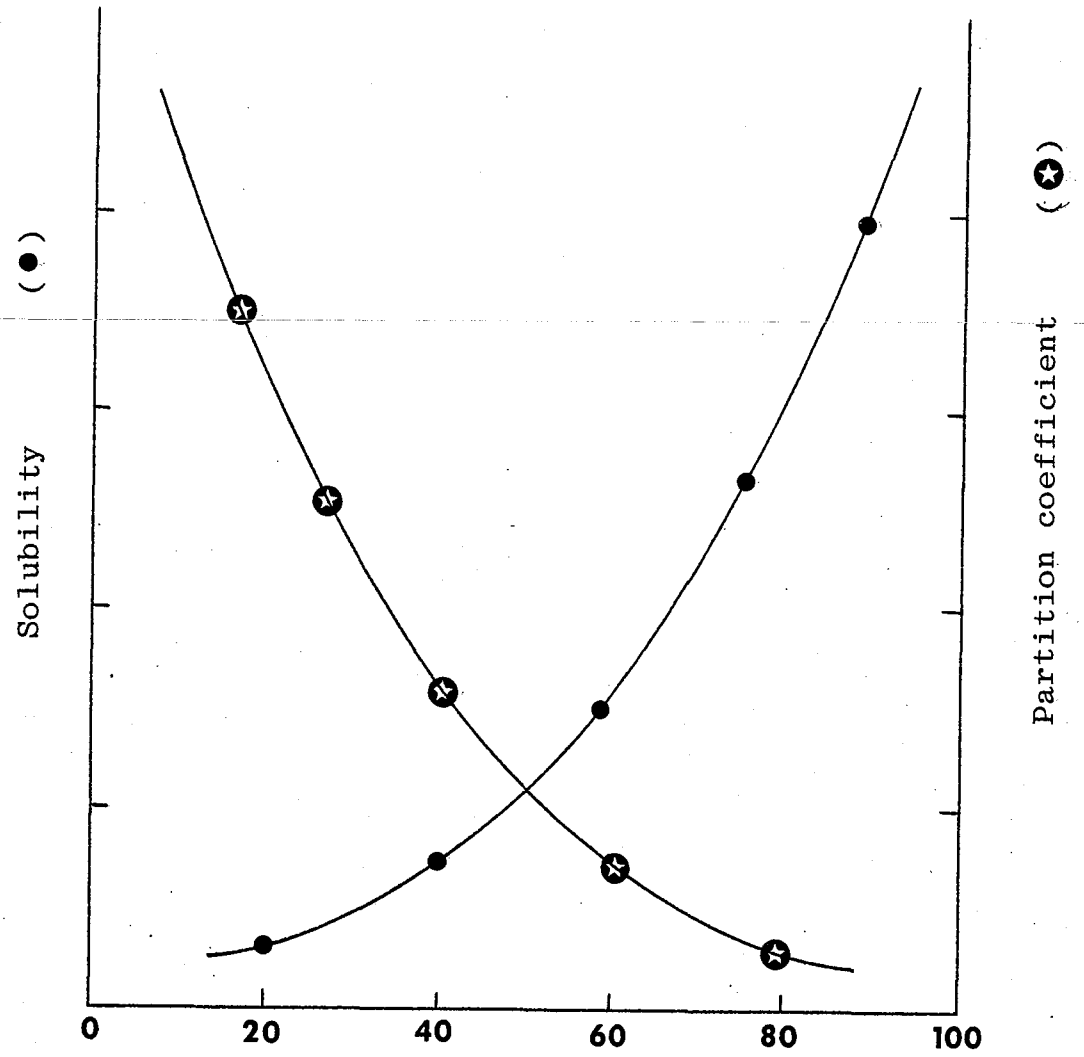


Figure 4. Representative plot of solubility and partition coefficient of drug against percent cosolvent in the vehicle.

evident from the plot, that for percentages of cosolvent to the left of the crossover point, even though the partitioning of the drug is high, the concentration that could be presented to the absorption site is relatively low. On the other hand, for vehicle compositions with percent cosolvent greater than that at the crossover point, even though the concentration is high, the partitioning value is too low so that the drug has a greater tendency to remain in the vehicle. Thus, at the crossover point an optim solubility and partition coefficient exists and an optimal vehicle composition is suggested.

This method of selecting a vehicle is arbitrary and suffers from one limitation. As can be visualized from the plot, an alteration in the dimension of axis on either the solubility scale or the partition coefficient scale would shift the crossover point correspondingly. But if one chooses to plot the maximum value of solubility on level with the maximum value of partition coefficient, then a symmetry exists wherein the curves mirror each other. The crossover point could then serve as an indicator of optimal vehicle composition.

(d) pH of the Vehicle and pK_a of Drug

The selection of one particular vehicle composition based on the results of solubility and partition coefficient parameters provides a good basis for investigation of the

effect of pH on the rate of percutaneous penetration. Assuming that passive mechanism is operative for transport of drugs through biological membranes, for ionizable drugs the matter of interest would be the concentration of the unionized species which is presented to the absorption site rather than the total concentration of the drug itself. Depending on the pK_a value of the drug, the pH of the vehicle would determine the degree of ionization.

Thermodynamic activity of a drug is the product of its concentration and activity coefficient in the vehicle. Rapid release of the drug is dependent on its high thermodynamic activity in the vehicle, as the direction of flow is always from the higher to lower thermodynamic potential. For specific concentrations of certain substances, it has been shown that thermodynamic activities may vary as much as 1000-fold from one vehicle to another (26). To aid in comparison of the results of <u>in vitro</u> percutaneous penetration studies at different pH, the thermodyanmic activity in each case can be maintained at maximum value by use of a saturated solution of the drug in vehicle.

<u>In vitro</u> percutaneous penetration studies at various pH values may reveal whether the flux has correlation to the total concentration presented or to the concentration of the unionized species or both.

Limitations of In Vitro Study

Reviewing studies on *in vitro* percutaneous absorption, one should always bear in mind the limitations of the experimental technique involved:

(a) The *in vitro* skin technique may not reflect the role of the skin *in vivo*;

(b) Predictions and conclusions are based on results of measurement for the steady-state rate with obvious difficulties for analyzing low concentrations during the initial few minutes;

(c) Normally, records for the sources of skin used are unavailable which makes it difficult to explain the variability in observed results;

(d) Information regarding age of the donor may not be available and age is an important criterion; and

(e) Skin from different sites of the body have different diffusional characteristics.

These limitations, while expressing the need for improvement in methodology, reflect the danger involved in comparing the work of various investigators. Improved *in vitro* techniques have been tried with a measure of success in correlating the various physical and chemical properties to the rate of percutaneous penetration. The main advantage of the in vitro technique is that it permits absolute control of the environment allowing the

demonstration of the importance of individual parameters towards percutaneous penetration of a given substance. Carefully conducted *in vitro* studies provide valuable information paving the way for *in vivo* experiments and further development of effective topical dosage forms.

EXPERIMENTAL I

The protocol for the experimental study was based on the theoretical aspects discussed in the preceding chapter. A preliminary investigation of the purity of methotrexate sample was conducted. Solubility of the drug in a series of propylene glycol-water mixtures was then determined. The partition coefficient of methotrexate in the two-phase isopropyl myristate/propylene glycol-water system was also examined. Based on the results of solubility and partition coefficient determinations, an optimum composition of propylene glycol in water was chosen for further investigation. The *in vitro* skin penetration profile of methotrexate from this optimum vehicle was studied. The results of this study were expected to provide greater insight into the penetration behavior of methotrexate from a propylene glycol-water system, and also establish a basis for subsequent investigation of the effect of pH on the *in vitro* penetration of methotrexate.

Assay Method

Analysis of samples for methotrexate was carried out by high-performance liquid chromatography by the method of Tong, Rosenberg, and Ludlum (31). The chromatograph

was equipped with a 6000-psi pump[1], a variable wavelength detector[2] and a loop injector.[3] A 30-cm long, 3.9 mm inner diameter stainless steel column[4] was used. Accurate determination of area under the curve was accomplished by use of an automated integrator system.[5] Injection volume was maintained constant with the aid of a gas-tight 800 series microliter syringe[6] which eliminated the need for use of a standard. The eluent was 85% 0.005 M/1 ammonium acetate[7] in water (pH 5.0) and 15% v/v acetonitrile.[8] The absorbance was recorded at 307 nm. The detector was set at 0.1 aufs and sensitivity at 0.02.

Determination of Purity of Methotrexate U.S.P.

Methotrexate U.S.P.[9] had 7.3% moisture (Karl Fischer). For purity determination, a small amount of methotrexate U.S.P. sample was dissolved in the solvent mixture used

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[1]Waters 6000-A pump, Waters Associates, Milford MA.

[2]Model 450, Variable Wavelength Detector, Water Associates, Milford MA.

[3]U6K Injector, Waters Associates, Milford MA.

[4]μ-Bondapak, Waters Associates, Milford MA.

[5]Data Module, Waters Associates, Milford MA.

[6]Hamilton Co., Reno NV.

[7]HPLC grade, J.T. Baker Chemical Co., Phillipsburg NJ.

[8]HPLC grade, J.T. Baker Chemical Co., Phillipsburg NJ.

[9]Lot 1260-A0922, Lederle Laboratories, Pearl River NY.

for elution. After dissolution, the solution was filtered through a 0.22μ filter.[10] Twenty μl of the sample was then injected on to the column. The column flow rate was set at 0.4 ml/min. The purity of the sample was calculated as the ratio of area under the curve for the peak corresponding to methotrexate to the total area under the curve for all the different peaks observed. The methotrexate peak was confirmed by spiking with an authentic sample.[11] Identification of the impurities was not pursued.

Solubility Studies

The solubility of methotrexate U.S.P. in a series of propylene glycol-water mixtures varying from 20% propylene glycol[12] in water to 80% propylene glycol in water was determined at room temperature ($22^{o} \pm 0.5^{o}$). An excess of methotrexate was added to 25.0 ml of propylene glycol-water mixtures in 50-ml amber-colored Erlenmeyer flasks with ground-glass stopper. A teflon-coated magnetic bar was placed in each of the flasks prior to flushing with nitrogen and capping them tightly. The flasks were then kept stirred on magnetic stirrers for a period of six days. Each determination was conducted in triplicate in a constant temperature room.

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[10] Swinnex-25, Millipore Filter Corp., Bedford MA.

[11] U.S.P. Convention Inc., Rockville MD.

[12] J.T. Baker Chemical Co., Phillipsburg NJ.

Prior to sampling, the stirring was stopped and the excess drug was allowed to settle. An aliquot was then filtered using a filter with 0.22μ filter paper. The first 5 ml of the filtrate was rejected in each case to avoid any discrepancy in the determination of solubility values due to possible adsorption of drug to the filter paper.

The concentration of methotrexate in the propylene glycol-water system was determined by high-performance liquid chromatography as discussed under assay method. Appropriate dilutions of the saturated solution of drug in the eluent were made prior to injection on to the column. A standard plot (Figure 5) of area under the curve against concentration for known amounts of the drug was determined.

Partition Coefficient

Preliminary experiments had shown that methotrexate reached equilibrium distribution in the two-phase isopropyl myristate[13]/propylene glycol-water system in four days. Therefore, 3 ml of a saturated solution of methotrexate in the various propylene glycol-water mixtures was agitated for four days with 3 ml of the respective propylene glycol-water saturated-isopropyl myristate in amber bottles. Blank flasks with 3 ml of saturated solution of methotrexate

[13]Ruger Chemical Co. Inc., Irvington NJ.

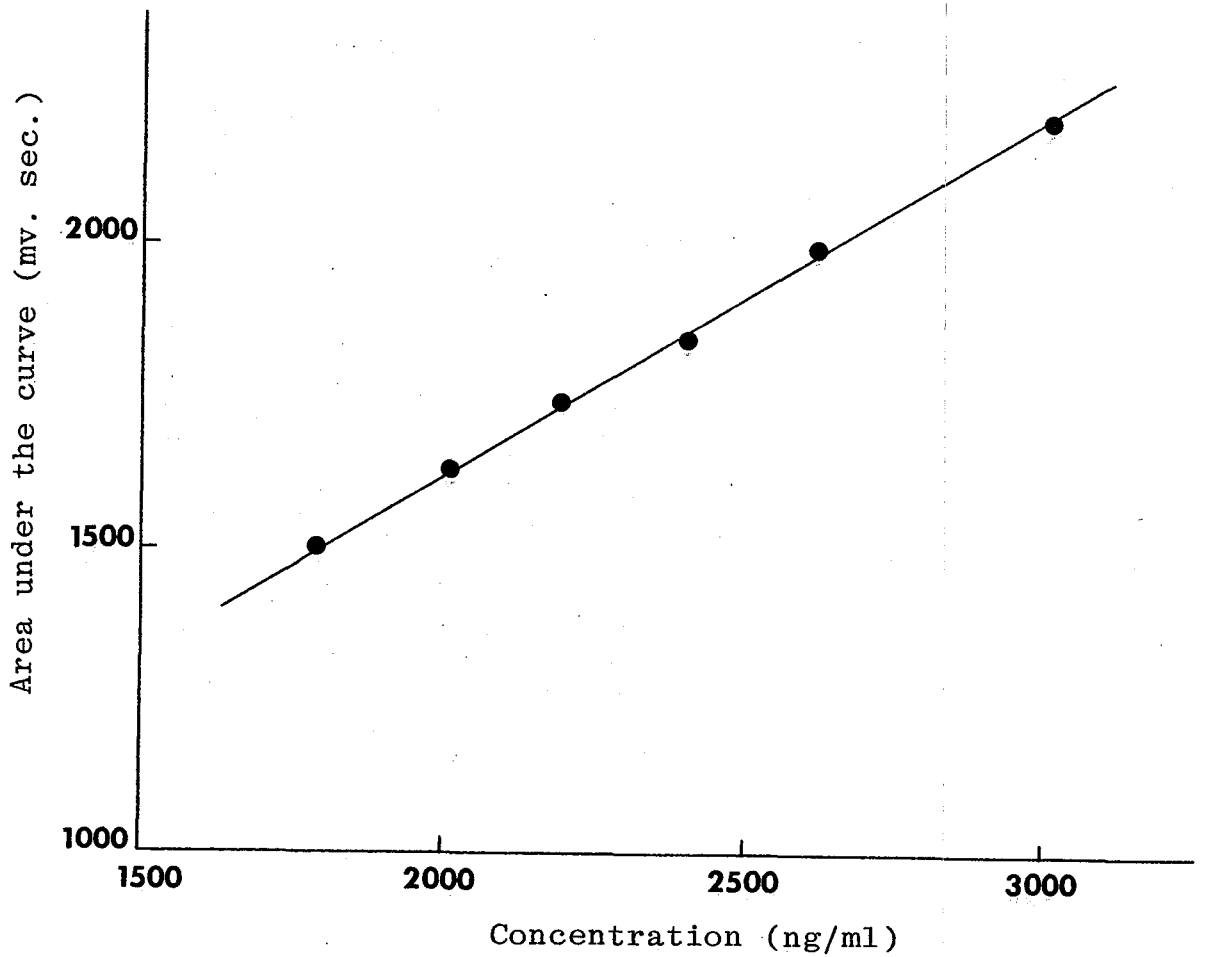

Figure 5. Representative HPLC standard plot of area under the curve against concentration for methotrexate U.S.P. dissolved in propylene glycol-water mixture.

in the various propylene glycol-water mixtures and no isopropyl myristate were also shaken alongside. The partitioning study was conducted at room temperature ($22^{o} \pm 0.5^{o}$) in a constant temperature room. After equilibration, the aqueous phase and oil phase were separated by centrifugation. The aqueous phase, after appropriate dilutions, was analyzed for methotrexate content. The concentration in the isopropyl myristate phase was deduced as the difference between the initial and final concentrations of drug in the propylene glycol-water phase. Partition coefficients were calculated as the ratio of isopropyl myristate to propylene glycol-water concentration of methotrexate.

Results and Discussion

Purity of Methotrexate U.S.P.

Chromatograms (Figure 6) of a solution of methotrexate in the mixed solvent 85% v/v, 0.005 M/1 ammonium acetate in water (pH 5.0) and 15% v/v acetonitrile showed six peaks. Peak IV was identified as that of methotrexate by method of spiking with an authentic sample. The retention time and area percent of these six peaks for four different injections of the sample is shown in Table I. The purity of methotrexate U.S.P. sample was calculated to be 95.51% anhydrous.

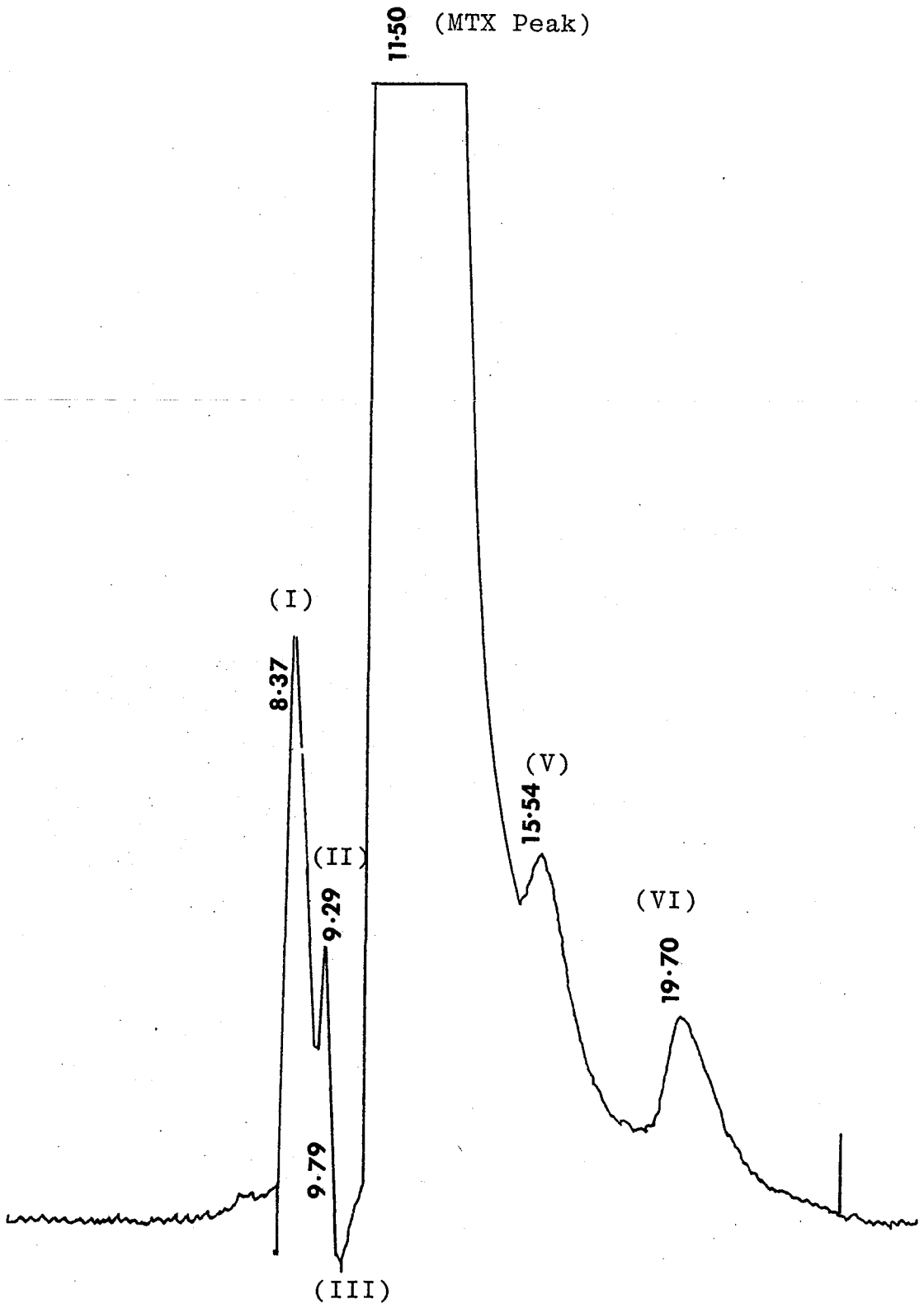

Figure 6. Chromatogram of methotrexate in 85% v/v 0.005 M/L ammonium acetate in water (pH 5.0) and 15% v/v acetonitrile.

TABLE I

Retention Time and Area Percent of Peaks for High-Performance Liquid Chromatogram of Methotrexate U.S.P. in 85% v/v 0.005 M/l Ammonium Acetate in Water (pH 5.0) and 15% v/v Acetonitrile

	Injection #	Peak I	Peak II	Peak III	MTX Peak	Peak V	Peak VI
Retention Time	1	8.80	9.60	10.13	12.10	16.53	21.23
	2	8.37	9.29	9.79	11.50	15.54	19.70
	3	8.37	9.25	9.70	11.45	15.58	19.70
	4	8.41	9.25	9.75	11.50	15.62	19.75
Mean Retention Time		8.49	9.35	9.84	11.64	15.82	20.10
Area Percent	1	0.73	0.12	0.10	95.17	1.50	2.36
	2	0.73	0.16	0.08	95.77	1.10	2.12
	3	0.78	0.12	0.14	95.54	1.31	2.07
	4	0.73	0.15	0.09	95.54	1.23	2.23
Mean Area Percent		0.74	0.14	0.10	95.51	1.29	2.20

Solubility Studies

The solubility of methotrexate U.S.P. in 20%, 40%, 50%, 60%, and 80% propylene glycol in water at $22^{\circ} \pm 0.5^{\circ}$ is shown in Table II, Figure 7. In addition the pH of the saturated solution of methotrexate in these vehicles is also listed. For determination of pH, a glass electrode with a Ag/AgCl internal reference electrode was used. Since a propylene glycol-water mixture is not purely aqueous, the pH is referred to as the apparent pH. As can be seen, the solubility increases with increasing concentration of propylene glycol. The attainment of equilibrium solubility in 3 days was confirmed by the fact that there was no difference in solubility values between the samples analyzed at the end of 3 and 6 days.

A semi-log plot of solubility against percent propylene glycol (Figure 8) showed a linear relationship. From the plot, solubility of methotrexate U.S.P. was found to be related to percentage of propylene glycol in the vehicle by the equation,

$$\ln S(\text{mg/ml}) = 0.0362\ (\%\ \text{propylene glycol}) - 2.4852$$

Partition Coefficient

The partition coefficient values of methotrexate in the two-phase isopropyl myristate/propylene glycol-water system for 20%, 40%, 50%, 60%, and 80% propylene glycol in water are shown in Table III, Figure 9. As the percentage

TABLE II

Solubility and pH of Methotrexate U.S.P. in Propylene Glycol-Water System

Propylene Glycol[a] %	Solubility[b] $22^{o} \pm 0.5^{o}$ mg/ml	pH[c] 22^{o}
20	0.1752 ± 0.0048	4.13
40	0.3052 ± 0.0115	4.18
50	0.5936 ± 0.0308	4.12
60	0.7444 ± 0.0202	4.27
80	1.4567 ± 0.0363	4.47

[a]All percentages are expressed as v/v.

[b]All solubility values are expressed as mean ± standard deviation of three determinations.

[c]Corning Model 125 pH meter with glass electrode and Ag/AgCl internal reference electroce.

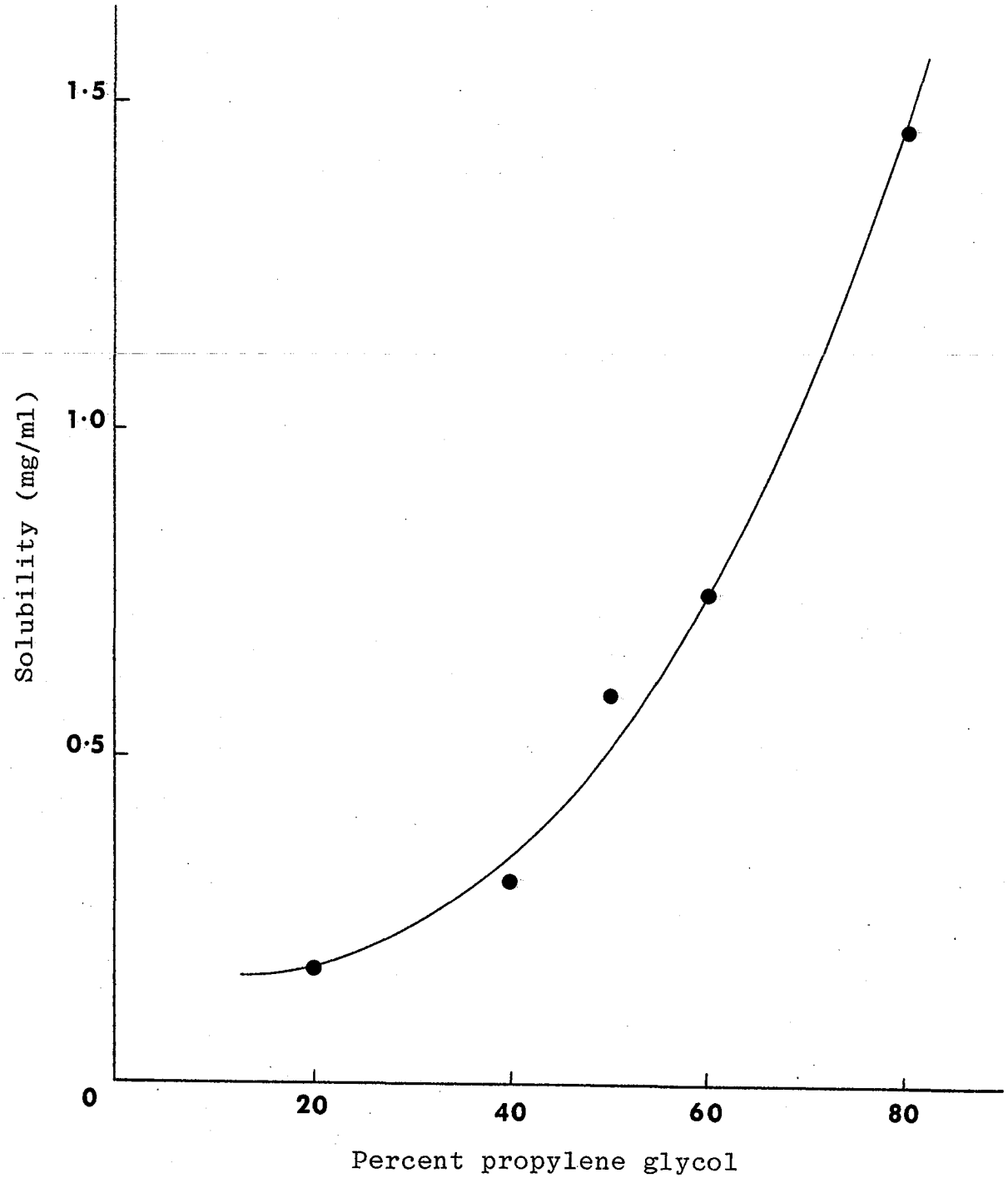

Figure 7. Plot of solubility of methotrexate U.S.P. against percent propylene glycol in propylene glycol-water system.

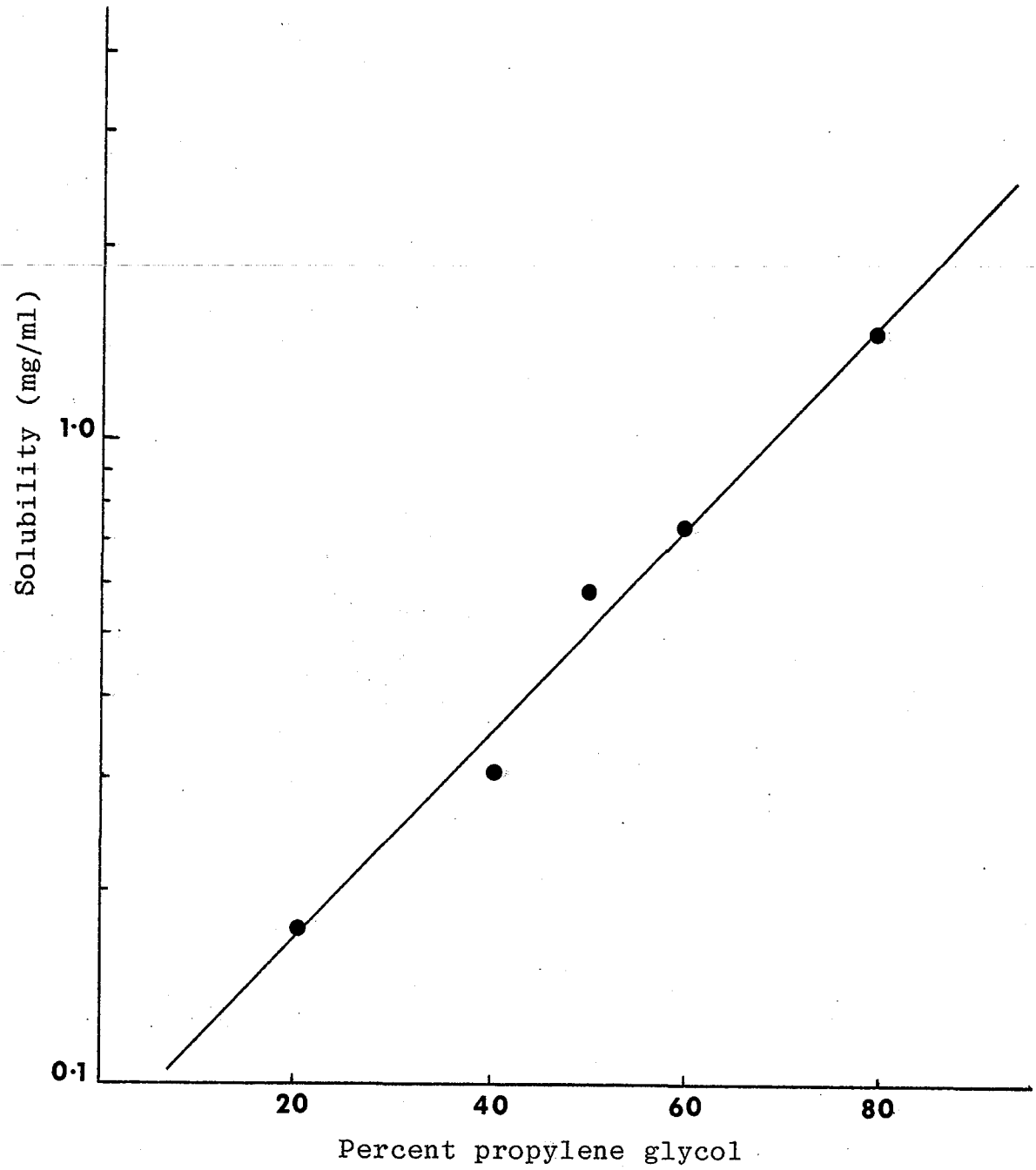

Figure 8. Semi-log plot of solubility of methotrexate U.S.P. against percent propylene glycol in propylene glycol-water system.

Table III

Partition Coefficient of Methotrexate in Isopropyl Myristate/Propylene Glycol-Water System at 22° ± 0.5°

Propylene Glycol[a] %	Partition Coefficient k_p ± 1 S.D.	Number of Determinations N
20	0.0454 ± 0.0067	4
40	0.0255 ± 0.0096	4
50	0.0203 ± 0.0037	2
60	0.0170 ± 0.0025	2
80	0.0140 ± 0.0005	3

[a]All percentages are expressed as v/v.

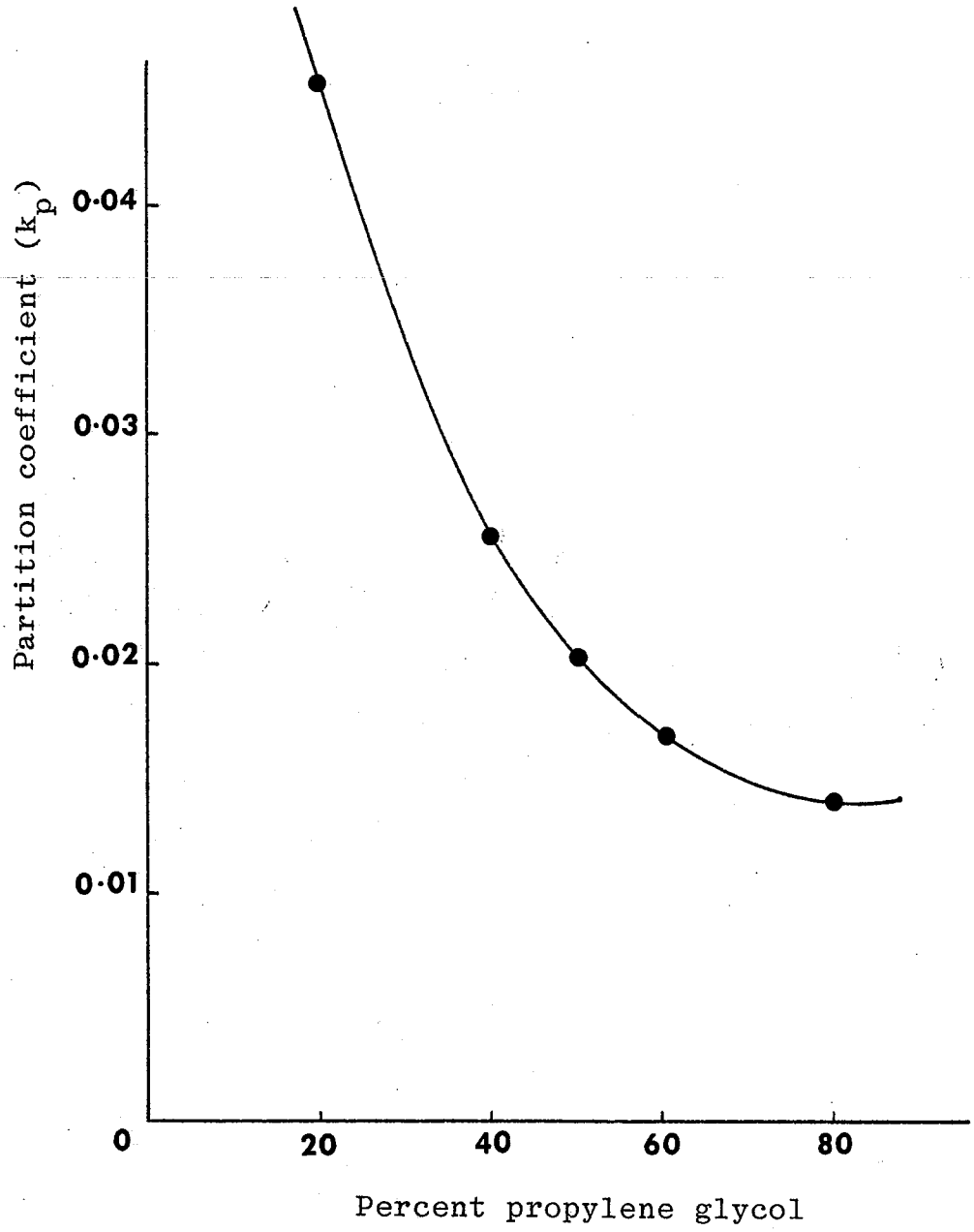

Figure 9. Plot of partition coefficient of methotrexate U.S.P. in isopropyl myristate/propylene glycol-water system against percent propylene glycol.

of propylene glycol in the vehicle was increased, the partition coefficient was found to decrease. This shows the increasing tendency of the drug molecules to remain in the vehicle with increasing fraction of propylene glycol in the vehicle.

A log-log plot of partition coefficient against percent propylene glycol in vehicle (Figure 10) showed a linear relationship. From the plot, the partition coefficient was found to be related to the percentage of propylene glycol in the vehilce by the equation,

$$\ln (k_p) = -0.8651 \ln(\% \text{ propylene glycol}) - 0.5002$$

Choice of Optimal Vehicle

For investigation of *in vitro* percutaneous penetration of methotrexate a vehicle composition of 50% propylene glycol in water was chosen.

Consideration of the advantages of presenting a reasonably high concentration of drug in the donor vehicle necessitated use of a higher proportion of propylene glycol in the vehicle. This along with the opposing fact that higher proportions of propylene glycol might cause the barrier to become less permeable (29) made the choice of 50% propylene glycol in water more practicable.

Additionally, the system appeared to provide a good balance between aqueous and non-aqueous phases. The results

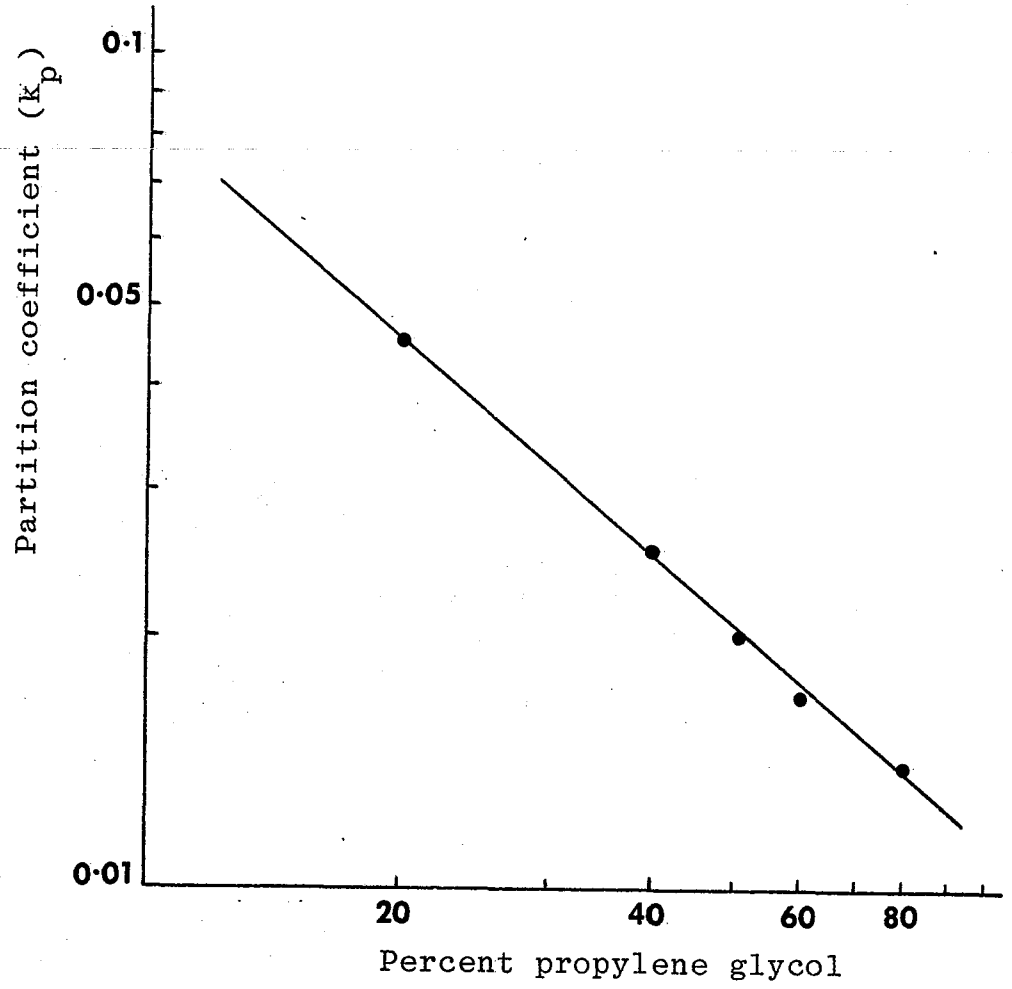

Figure 10. Log-log plot of partition coefficient of methotrexate U.S.P. for isopropyl myristate/ propylene glycol-water systems as a function of propylene glycol concentration in the glycol-water phase.

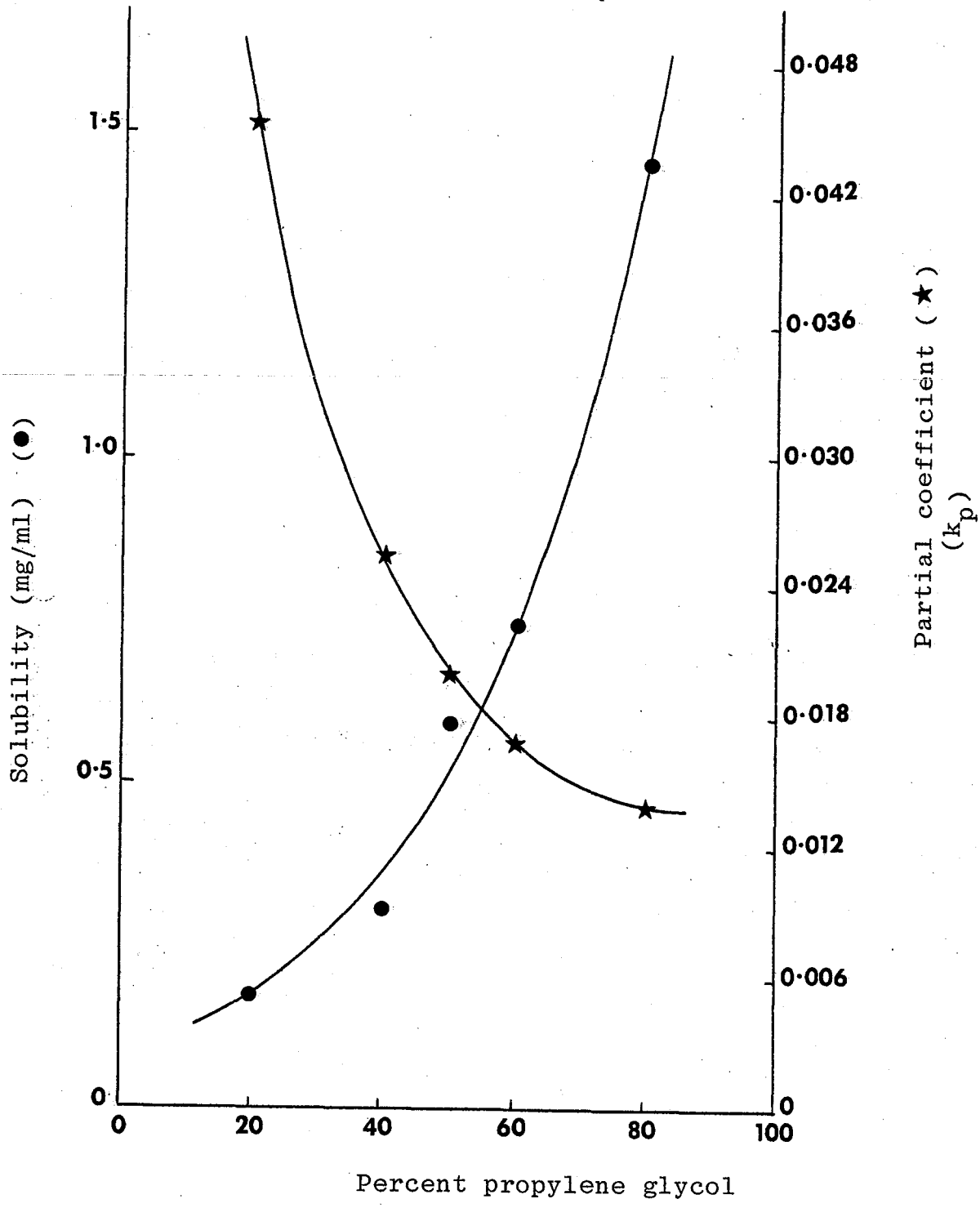

Figure 11. Solubility-partition coefficient-percent propylene glycol profile for methotrexate U.S.P. in propylene glycol-water system.

of the solubility-partition coefficient-percent propylene glycol profile (Figure 11) provides additional support to the selected vehicle composition.

EXPERIMENTAL II

Laboratory measurements of steady-state penetration rates and the experimental determination of the physical parameters affecting penetration rates provide valuable bench marks against which less well-controlled *in vivo* topical bioavailability studies can be compared.

All penetration studies were conducted using excised human skin since most animal skin models may not satisfactorily mimic human skin. The results of comparative evaluations of formulation effects on percutaneous absorption utilizing *in vitro* animal skin models may not be in agreement with those based upon human skin samples as was demonstrated by Chowham *et al*. (33).

Determination of In Vitro Penetration Rate and Distribution of Methotrexate

Preparation of Skin. All of the penetration experiments reported here utilize human abdominal skin obtained at autopsy. Immediately following incision, the skin was placed in a plastic bag and stored in a freezer for periods up to but not exceeding three months. This method of storage has been reported not to damage the skin (34). Before the experiment, the skin was allowed to thaw gradually to room temperature, following which the skin was

placed on a smooth dissection board with the epidermal surface flat in contact with the board. All subcutaneous fat was completely removed by a scalpel. From each specimen (the skin of a single donor), 6 to 8 pieces of suitable sizes were cut.

Skin Cell. Each piece of skin was mounted in a special glass cell as illustrated in Figure 12. The skin cell consisted of a lower glass chamber with a sampling port. A Teflon-coated magnetic bar placed at the bottom of the cell provided efficient mixing. The lower chamber was enclosed by a water jacket which allowed circulation of water at the selected temperature. The skin was placed in position on an O-ring between two ball joints of the top and bottom chambers, using a pinch type, ground-joint clamp. The diffusion area was 2.01 cm^2. The epidermal side of the skin was covered with Saran Wrap to provide occlusion. Normal saline[14] was pipetted into the skin cell bathing the dermal side. The sampling port was closed by a rubber closure and any air bubbles on the dermal side were carefully removed by slightly tilting the cell. Each cell was mounted on a magnetic stirrer. The temperature of the fluid in the lower chamber was maintained at $37^{\circ} \pm 0.5^{\circ}$ by circulating water from a constant temperature water

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[14]Abbott Laboratories, North Carolina IL.

[15]Haake-Model-FE, VWR Scientific Inc., San Francisco CA.

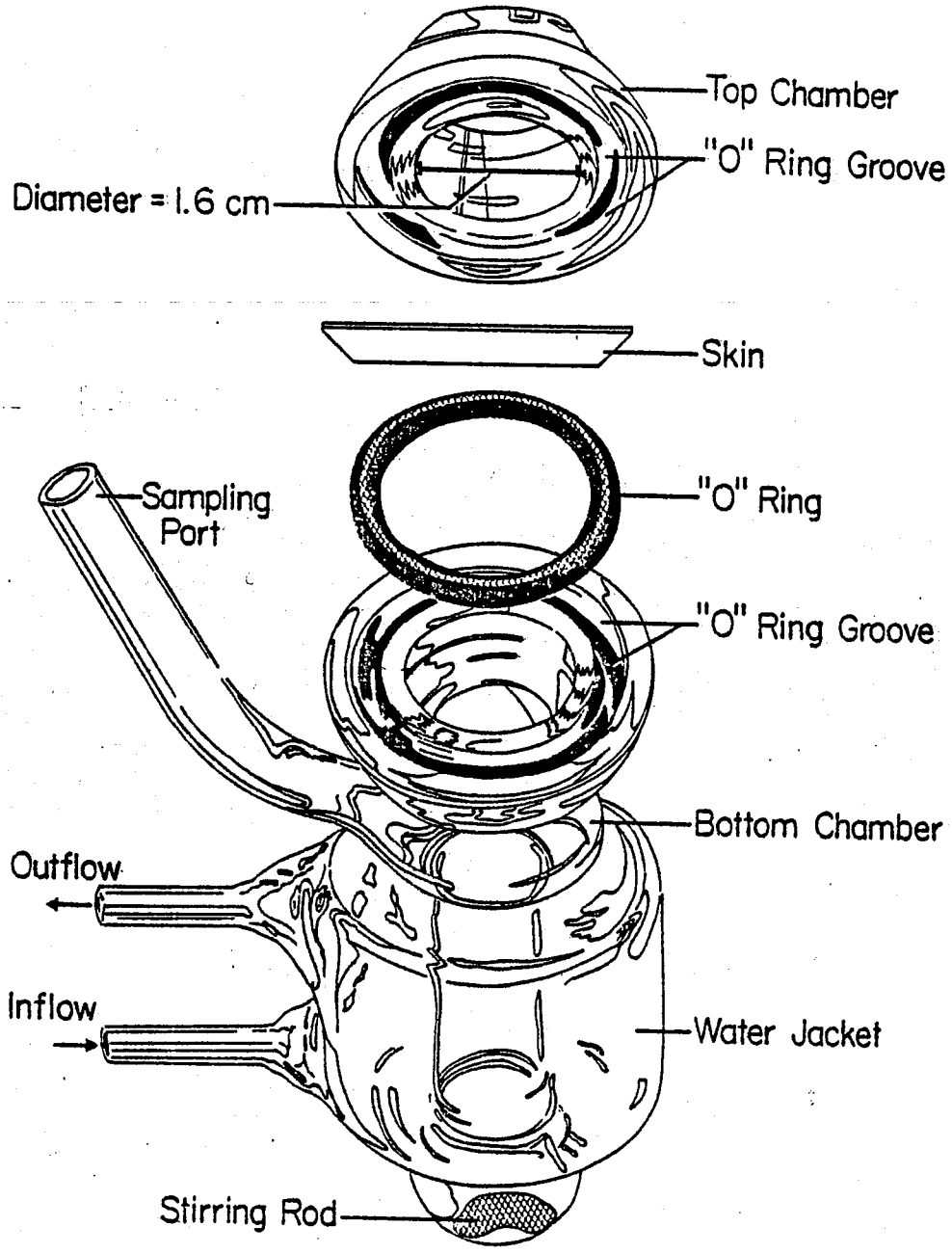

Figure 12. Diagramatic representation of the diffusion cell used in penetration studies.

circulator[15] through the jacket of each cell. This helped to simulate in vivo conditions.

After mounting, each piece of skin was allowed to stand for 4 hours before beginning the experiment. This allowed adequate time for equilibration with respect to the temperature and relative humidity of the environment. During the actual run, each cell was covered and the experiment carried out under minimum exposure to light in order to minimize photodecomposition of methotrexate.

A saturated solution of methotrexate U.S.P. in 50% propylene glycol-water was used as the donor phase. One ml of this solution was pipetted onto the epidermal side of the skin and the skin cell was covered with Saran wrap which provided good occulusion.

All experiments were carried out for a period of 14 days. No samples were withdrawn during the first 80 to 96 hours. Thereafter, at selected time intervals, preferrably once a day, the receptor solution from the bottom chamber was completely removed through the sampling port using a disposable syringe with its needle attached to a thin flexible plastic tubing. This allowed for a quick and complete removal of the receptor solution and refilling with fresh normal saline solution. The receptor samples

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[15]Haake-Model-FE, VWR Scientific Inc., San Francisco CA.

withdrawn were stored in the refrigerator until the end of the experiment. The samples were then assayed for methotrexate by high-performance liquid chromatography following appropriate dilutions with normal saline.

At the termination of a penetration experiment, the remaining solution on the donor side was removed. The epidermal surface was then washed of the remaining applied dose with three 1-ml portions of 50% propylene glycol in water. The washings were added to the original solution and the volume made up to 5 ml. This was then assayed for methotrexate after appropriate dilutions.

The skin was then removed from the cell and the circular portion of the skin in contact with the bathing fluid was cut out using surgical scissors. The epidermis and dermis were easily separated by means of a forceps and collected separately in two amber colored Erlenmeyer flasks. To the epidermal portion, 7.5 ml of 50% propylene glycol in water was added. The dermal portion was cut into very small pieces and 15 ml of 50% propylene glycol in water was added. The contents of both flasks were then stirred for a period of 48 hours. At the completion of this time interval, the solutions were assayed for methotrexate following appropriate dilutions.

All samples were filtered through a disposable 0.22 μ filter[16] prior to dilution (if necessary) and injected on

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[16]Gelman Sciences, Inc., Ann Arbor MI.

to the column. Analyzing samples from *in vitro* skin penetration experiments, it was necessary to modify the elution solvent mixture in order to overcome interference from peaks due to impurities from skin samples. The polarity of the solvent was increased by using a mixture of 90% v/v 0.005 M/l ammonium acetate in water (pH 5.0) and 10% v/v acetonitrile. This facilitated faster elution of the polar impurities. In order to decrease the retention time of methotrexate peak, the flow rate was increased to 0.9 ml/min. New standard curves were determined at the beginning of each analysis and checked after the completion of analysis. Knowing the areas under the curve for injection of different samples, the unknown concentrations were read from the standard curve.

Results and Discussion

The penetration data for saturated solution of methotrexate in 50% propylene glycol in water were analyzed by plotting Q (amount penetrated per unit area) against time. Based upon this plot, a regression analysis of the steady-state region of the penetration curve was carried out using the regression program ona TI-55 calculator. The regression line was extrapolated to time axis to establish the lag times as shown by the dotted lines in Figures 13, 14, 15, and 16. The penetration data obtained from the Q *versus* t plots are shown in Table IV. The

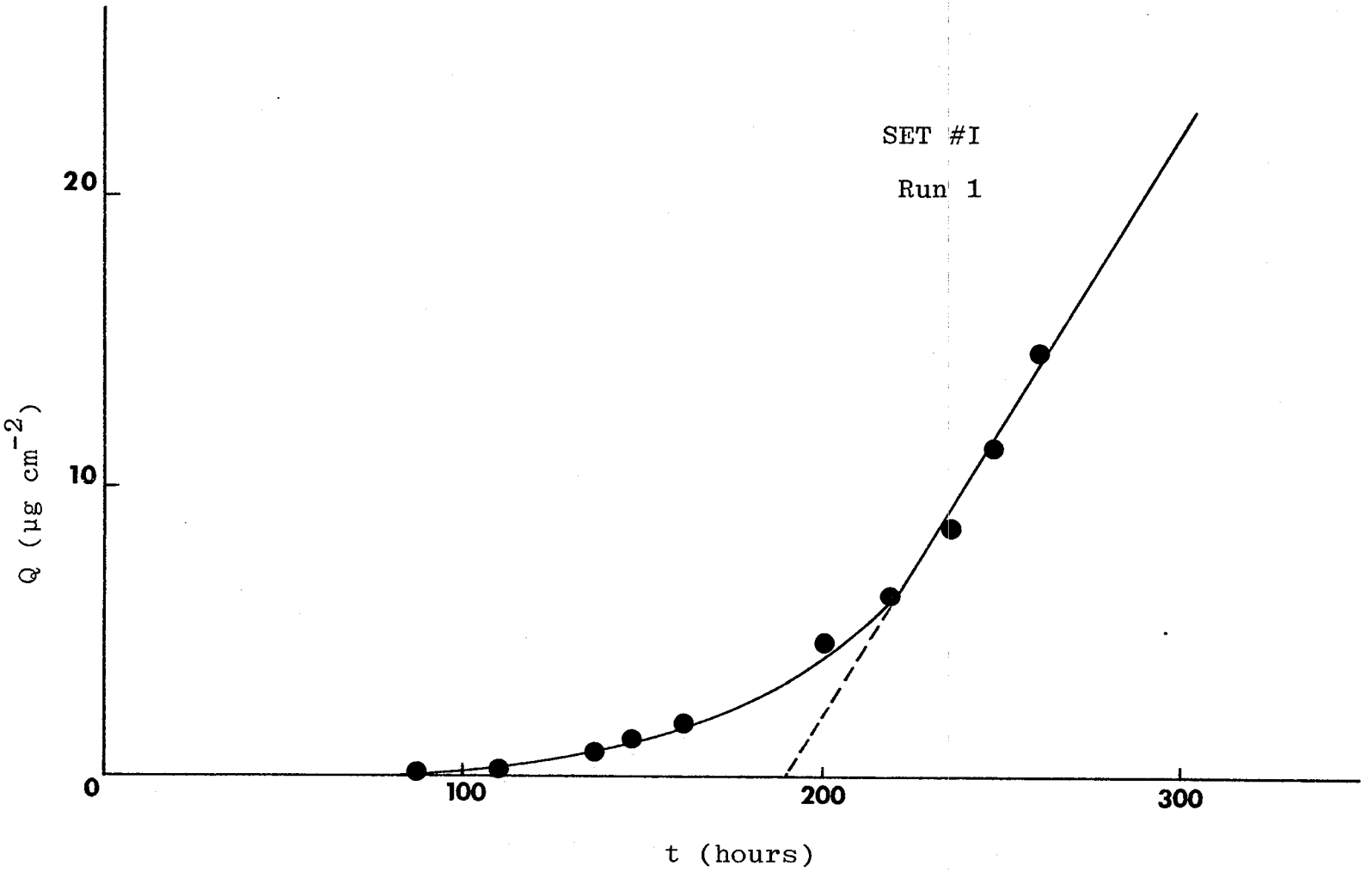

Figure 13. In vitro percutaneous penetration of methotrexate: Q versus t plot. SET I.

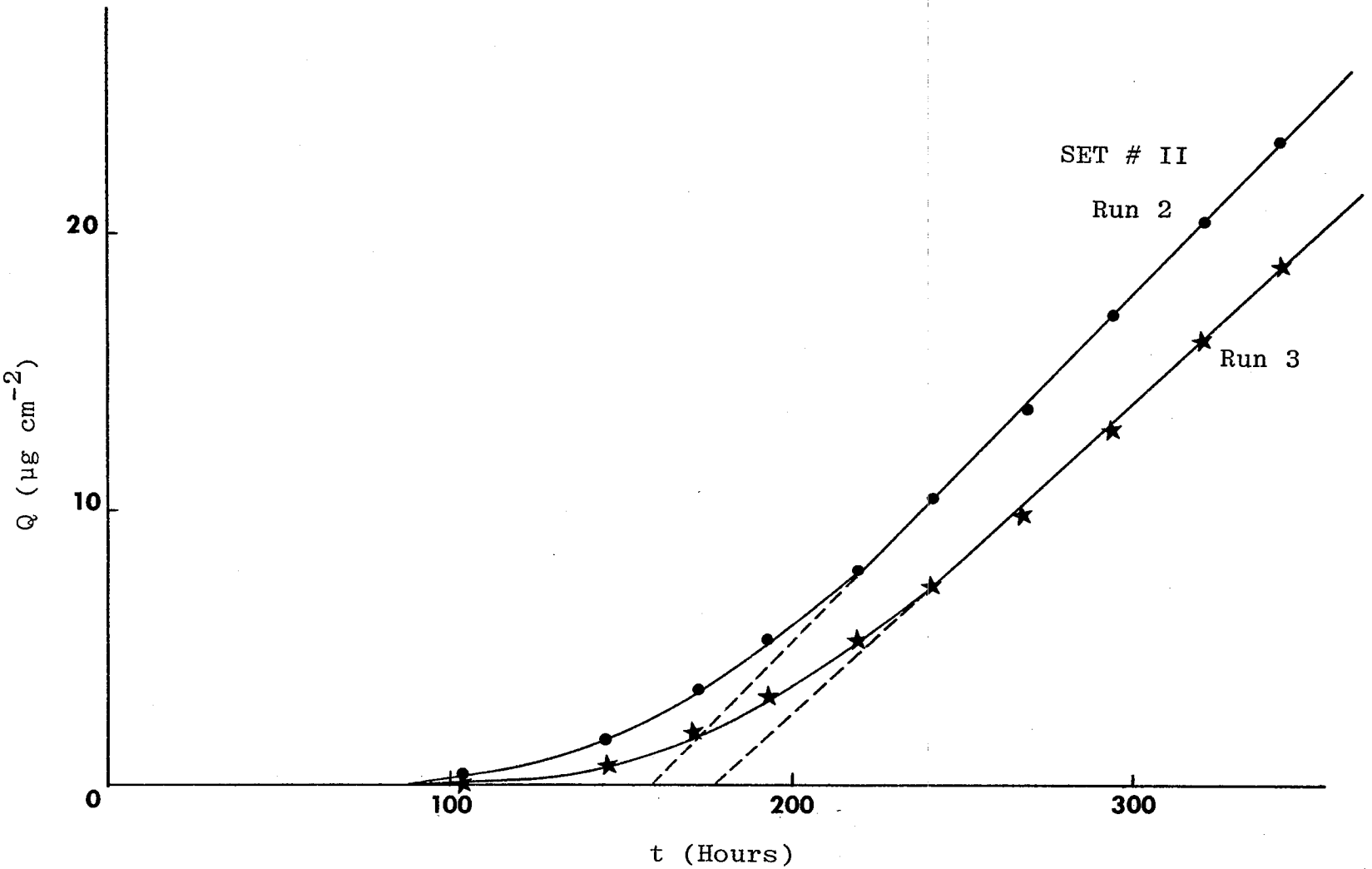

Figure 14. In vitro percutaneous penetration of methotrexate: Q versus t plot, SET II.

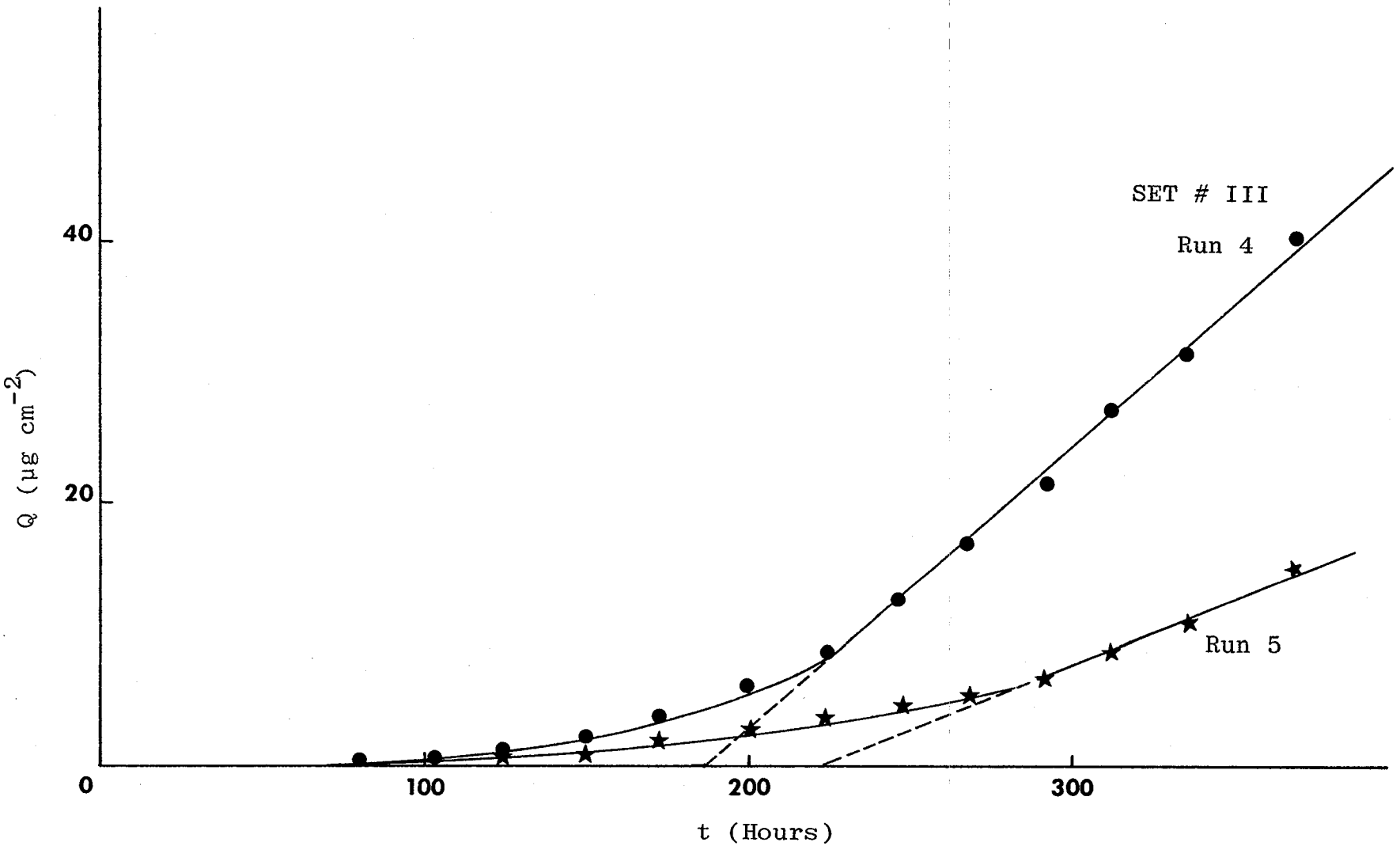

Figure 15. In vitro percutaneous penetration of methotrexate: Q versus t plot, SET III.

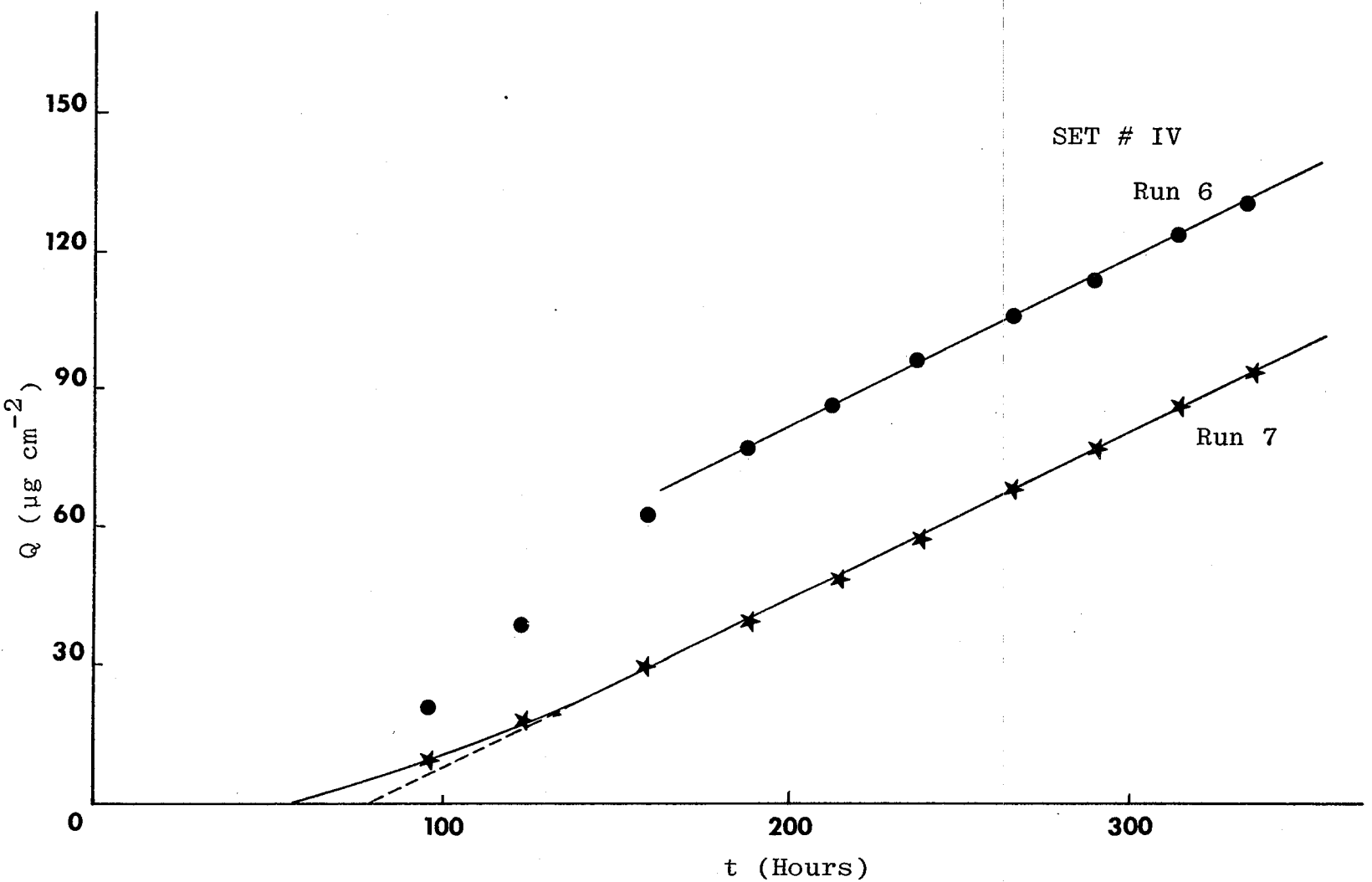

Figure 16. *In vitro* percutaneous penetration of methotrexate: Q *versus* t plot, SET IV.

TABLE IV

In Vitro Skin Penetration Data of Methotrexate

Set[a]	Run[b]	Applied Concentration µg/ml	Steady-State Penetration Rate[c] µg cm^{-2} hr^{-1}	Lag Time Hours	Correlation Coefficient[d]
I	1	627.1	0.1992	190	0.984
II	2	566.5	0.1272	158	0.999
	3	566.5	0.1122	176	0.998
III	4	566.6	0.2190	187	0.998
	5	566.5	0.1015	223	0.990
IV	6	627.1	0.3692	---	0.999
	7	627.1	0.3663	80	0.999

[a]Set numbers refer to skin specimens from different donors.

[b]Runs 2 and 3, 4 and 5, and 6 and 7 are duplicate runs.

[c]All steady-state values were computed from the regression line drawn from the data for each run by using a TI-55 calculator.

[d]Reported r values are for steady-state region of the penetration data.

distribution of methotrexate at the end of 14 days is shown in Table V.

From the results it is quite evident that methotrexate is absorbed to a widely variable extent by different excised human skin specimens. Even pieces of skin immediately adjoining one another may show considerable variation in the rate and extent of absorption. The percentage of the applied dose recovered from the dermis was nearly the same as that from the stratum corneum. Results are reported only from those experiments where the stratum corneum appeared grossly intact at the end of 14 days. Experiments in which the stratum corneum was damaged during the actual run, did not show much resistance to the passage of methotrexate. The concentration of the drug in the initial sample for such experiments was about 5 to 10 times that for duplicate runs where the stratum corneum remained intact throughout the course of the experiment. Also the total amount of drug penetrating at the end of the 14-day period was between 50 and 80% of the applied dose. The penetration profile for experiments with broken stratum corneum did not register a steady-state region but instead a gradual increase in the amount penetrating per unit area per unit time.

The results demonstrate that;

(a) The intact stratum corneum is probably the main effective barrier for passage of methotrexate through excised cadaver skin.

TABLE V

Relative Distribution of Methotrexate 14 Days After Application

Set[a]	Run[b]	Amount Penetrated	Washings of Epidermis	Applied Dose Recovered		Accountability
		%		% Epidermis	% Dermis	%
I	1	23.79	49.22	13.88	11.56	98.45
II	2	41.75	48.47	2.77	1.87	94.86
	3	33.56	54.27	3.45	1.76	93.04
III	4	14.34	62.85	1.65	2.16	81.00
	5	5.43	84.88	2.18	0.88	93.37
IV	6	45.72	44.58	1.03	2.26	93.59
	7	34.80	52.10	1.02	2.53	90.45

[a]Set numbers refer to skin specimens from different donors.

[b]Runs 2 and 3, 4 and 5, and 6 and 7 are duplicate runs.

(b) The diffusion of methotrexate through the stratum corneum is probably the rate-limiting step and not the release of the drug from 50% propylene glycol in water. Alternatively, the values for distribution of methotrexate at the end of the 14-day period could reflect relative solubilities rather than permeability differences. In view of the poor solubility of methotrexate in the stratum corneum as suggested by the low partitioning value, the transfer of drug from propylene glycol-water phase to the stratum corneum may be the slow-step, resulting in the retainment of the drug in the donor vehicle.

(c) The water-bearing tissues of dermis appear to offer the least resistance to the passage of methotrexate.

At present there appears to be no definitive explanation available for the wide variation in observed results. Biological variation, tissue health, bacterial action, the presence of drugs or their metabolites could all influence the uptake of pteridines in the skin.

Studies hitherto on percutaneous penetration of methotrexate (14, 18) as well as sodium salt of methotrexate (22) have been carried out for comparatively shorter periods of time, not exceeding 20 hours. On the other hand, the present investigation was conducted over 14 days. The results of the present work, especially the very high

lag times, clearly demonstrate the inability of methotrexate to reach steady-state levels over short periods of time. The penetration rates calculated for data generated from experimental runs over short time interval could lead one to erroneous conclusions. In fact, the rates thus calculated would be apparent steady state rates and reflect the slope of the 'non-linear' portion of the present penetration profile.

The high values of lag time indicate the very low amounts of drug penetrating during the initial few hours. If this is the case, lack of percutaneous penetration during the period of application could be the reason for clinical ineffectiveness of topically applied methotrexate.

EXPERIMENTAL III

Effect of pH on Solubility and In Vitro Percutaneous Penetration

The solubility of methotrexate in a series of 50% v/v aqueous propylene glycol over a pH range of 1 to 12 was determined and *in vitro* penetration of methotrexate from selected vehicles in the above series was examined. The results of these experiments were expected to provide meaningful information about the correlation, if any, of solubility and concentration of the dissolved unionized species with the rate of percutaneous penetration. The pH-rate of penetration profile was expected to give a greater insight into the pH dependency of the skin penetration behavior of methotrexate.

Solubility Studies

Aqueous media in the pH range 1 to 12 were prepared (Appendix I) using acetic acid[17], chloroacetic acid[18], hydrochloric acid[19], and potassium hydroxide.[20] The pH

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[17] J.T.Baker Chemical Co., Phillipsburg NJ.

[18] Eastman Organic Chemicals, Rochester NY.

[19] Matheson, Coleman and Bell, Norwood OH.

[20] Mallinckrodt, Inc., St. Louis MO.

values of the aqueous media were checked on a pH meter[21] equipped with a glass electrode and Ag/AgCl internal reference electrode. Equal volumes of propylene glycol and the various aqueous solutions were then mixed to give 50% v/v propylene glycol in aqueous medium. Saturated solutions of methotrexate were prepared in each of the above mixtures. Solubility studies were conducted in a constant temperature room at $22^{\circ} \pm 0.5^{\circ}$. The high-performance liquid chromatography procedure adopted for analysis of samples was the same as discussed in the preceding sections.

Results and Discussion

The solubility of methotrexate U.S.P. in the various 50% v/v propylene glycol in aqueous media is shown in Table VI, Figure 17. In the table is also listed the pH of the aqueous solutions, the apparent pH of 50% v/v propylene glycol-aqueous medium, and also the apparent pH of saturated solution of methotrexate in the various mixtures. A semi-log plot of solubility against pH showed a V-shaped curve with minimum solubility occurring around pH 4 (Figure 18). The higher solubility values of methotrexate in propylene glycol-acetic acid mixtures and propylene glycol-chloroacetic acid mixtures probably suggest some form of interaction of the drug with the respective aqueous medium. Hence for

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[21]Corning Model 125 pH Meter, VWR Scientific Inc., San Francisco CA.

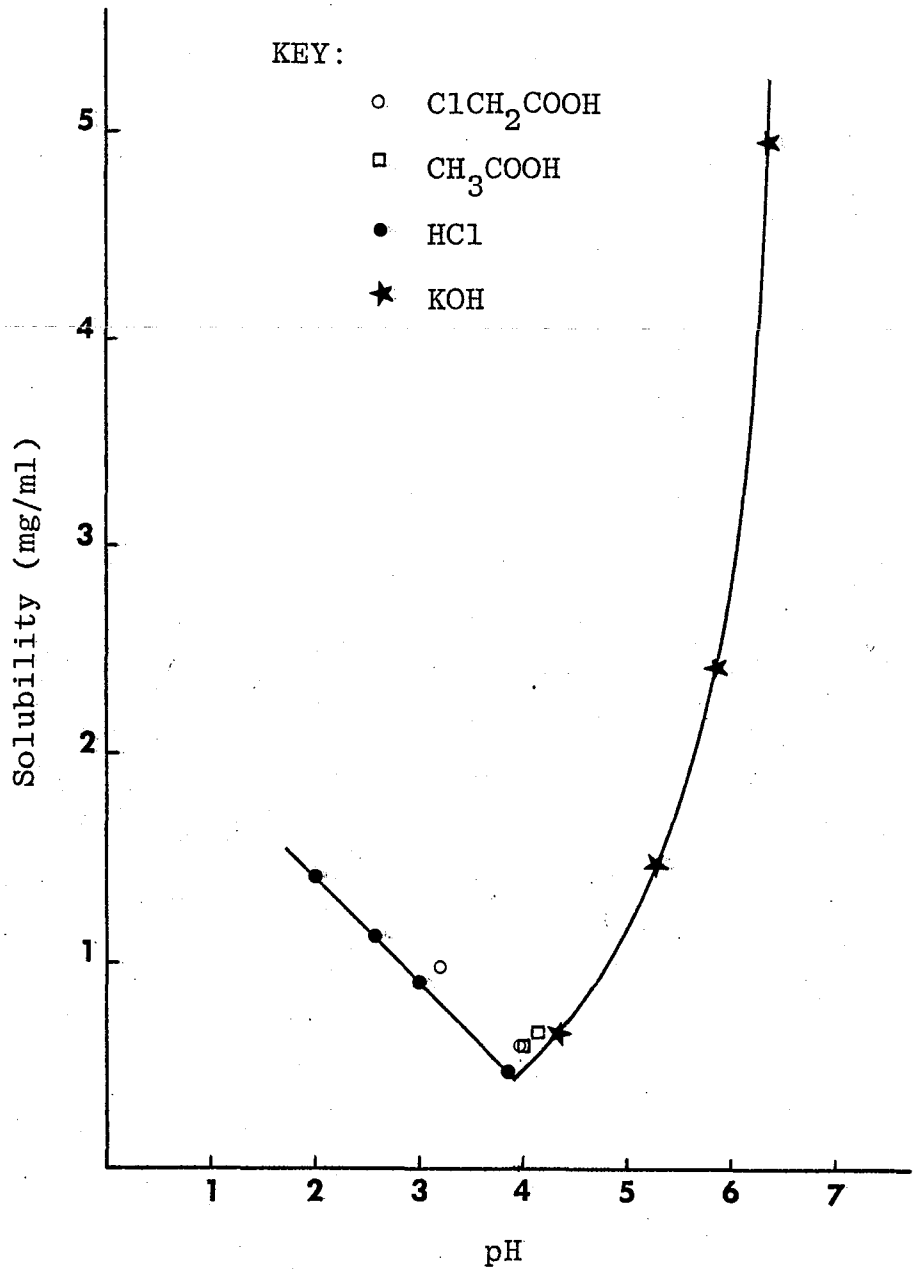

Figure 17. Plot of solubility of methotrexate U.S.P. against pH of saturated solution of the drug in 50% v/v propylene glycol-aqueous medium.

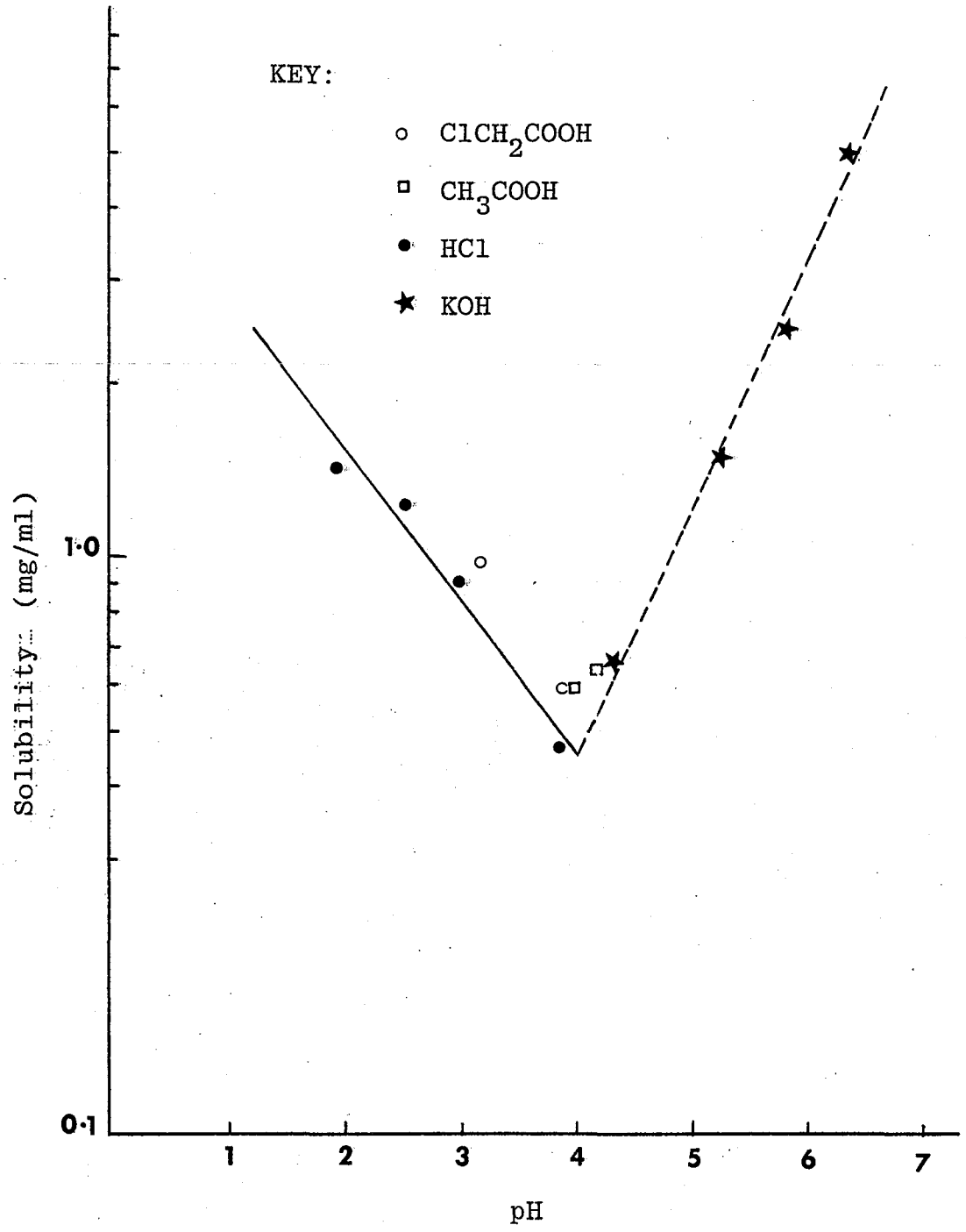

Figure 18. Semi-log plot of solubility of methotrexate U.S.P. against pH of saturated solution of the drug in 50% v/v propylene glycol-aqueous medium.

TABLE VI

Solubility and pH of Methotrexate U.S.P. in 50% v/v Propylene Glycol in Aqueous Medium

Aqueous Medium	pH			Solubility
	Aqueous Medium	50% v/v PG in Aqueous Medium	Satd. Soln. of MTX in 50% v/v PG-Aqueous Med.	$22^{o} + 0.5^{o}$ mg/ml
0.001 M Potassium Hydroxide	11.02	10.62	4.33	0.6536
0.01 M Potassium Hydroxide	12.12	11.78	5.29	1.4776
0.02 M Potassium Hydroxide	12.18	12.06	5.84	2.4188
0.05 M Potassium Hydroxide	12.56	12.48	6.34	4.9636
0.001 M Acetic Acid	3.94	4.47	4.17	0.6329
0.01 M Acetic Acid	3.39	3.80	4.00	0.5911
0.001 M Chloroacetic Acid	3.14	3.50	3.90	0.5912
0.01 M Chloroacetic Acid	2.50	2.85	3.19	0.9713
0.001 M Hydrochloric Acid	3.02	3.22	3.87	0.4633
0.005 M Hydrochloric Acid	2.36	2.53	2.98	0.8976
0.01 M Hydrochloric Acid	2.14	2.31	2.56	1.1211
0.03 M Hydrochloric Acid	1.72	1.88	1.98	1.4059

in vitro penetration studies these delivery systems were not employed.

In propylene glycol-hydrochloric acid mixtures, the solubility of methotrexate increased as the molar concentration of hydrochloric acid in the vehicle mixture was increased. A semi-log plot of molar concentration of hydrochloric acid against molar solubility of methotrexate gave a linear relationship with a correlation coefficient of 0.999 (Figure 19). From this plot, the solubility of methotrexate U.S.P. in 50% v/v propylene glycol-hydrochloric acid system was found to be related to the molar concentration of hydrochloric acid in the vehicle mixture by the equation;

$$\ln (M \text{ of } HCl) = 1626 \times (\text{Molar solubility of MTX}) - 8.5571$$

In the propylene glycol-potassium hydroxide solution system, the solubility of the drug increased as the molar concentration of potassium hydroxide in the vehicle mixture was increased. A plot of molar solubility of methotrexate U.S.P. against molar concentration of potassium hydroxide in the vehicle mixture showed a linear relationship with a correlation coefficient of 0.999 (Figure 20). From the plot, the molar solubility of methotrexate U.S.P. in 50% v/v propylene glycol-potassium hydroxide in water system was found to be related to the molar concentration of potassium hydroxide in the vehicle mixture by the equation;

$$\text{Molar Solubility of MTX} = 0.1930 (M \text{ of KOH}) + 0.0013$$

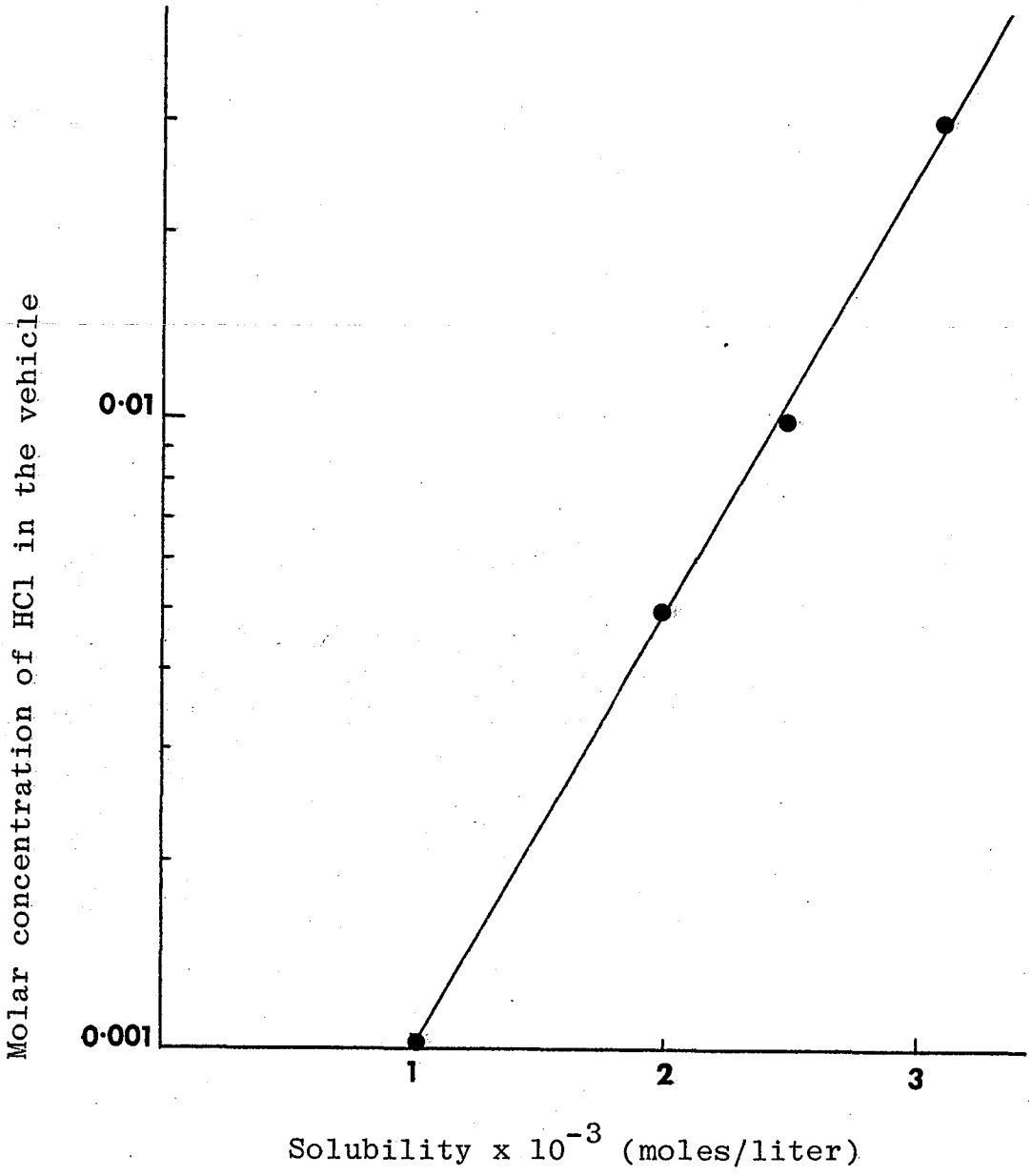

Figure 19. Semi-log plot of molar concentration of hydrochloric acid against molar solubility of methotrexate U.S.P. in 50% v/v propylene glycol-hydrochloric acid mixtures.

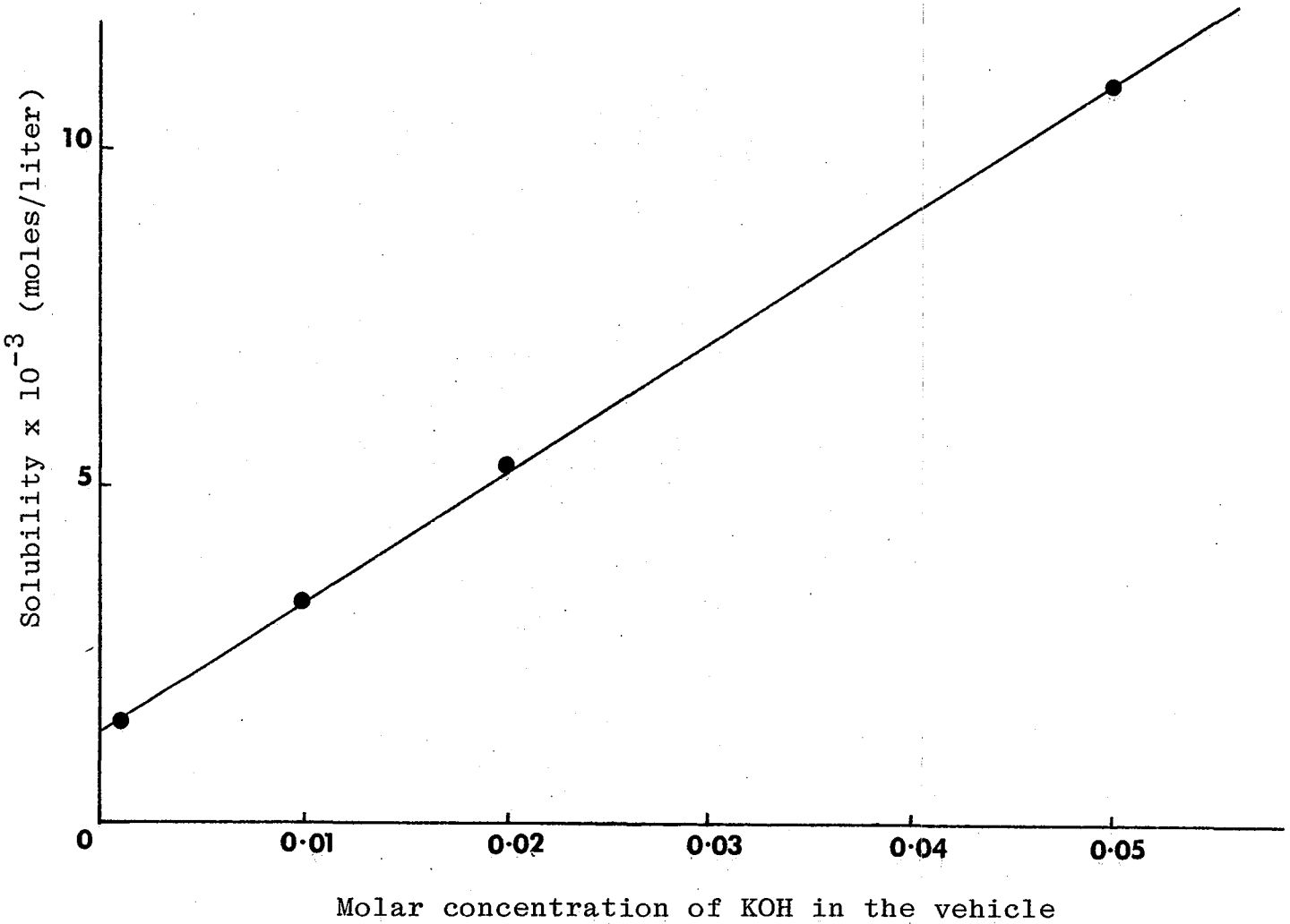

Figure 20. Plot of molar solubility of methotrexate U.S.P. against molar concentration of potassium hydroxide in 50% v/v propylene glycol-potassium hydroxide in water mixtures.

Effect of pH on In Vitro Percutaneous Penetration

If passive diffusion is the predominant mechanism for percutaneous penetration of methotrexate, the changes in vehicle pH would be expected to influence the in vitro percutaneous penetration rate of methotrexate through excised human cadaver skin, since transport by passive mechanism is maximized when the drug is present in the unionized form. A set of five saturated solutions of methotrexate in 50% v/v propylene glycol-aqueous media having pH values of 1.98, 2.98, 3.87, 5.29, and 6.34 were chosen for this study. A saturated solution of methotrexate in 50% v/v propylene glycol-water having a pH value of 4.12 was used as control. The procedure for in vitro skin penetration studies was similar to the one discussed in the preceding section. One ml of the respective solution was used as donor vehicle. By using a saturated solution, the thermodynamic activity of the drug was mainted at the maximum in each case. The penetration experiments were carried out for approximately 360 hours with minimal exposure to light because of the known photodecomposition of methotrexate. During the actual run, the cells were covered with Saran Wrap to insure occlusion. Samples were withdrawn once every 24 hours except for the initial 80 to 100 hours during which no sampling was undertaken. At the completion of the penetration experiments, the donor vehicle remaining on the epidermal side was recovered. The stratum corneum was separated from the

dermis and methotrexate from both these phases was extracted into the respective vehicle. Samples were analyzed by high-performance liquid chromatography. New standard curves were prepared and used for each set of skin penetration studies.

Results and Discussion

Considerable biologic variation exists among different skin specimens with regard to percutaneous absorption. The effect of this variable was minimized by utilizing skin pieces from the same regional site for each experiment. In all, three sets of penetration experiments were carried out. In each set, six skin cells were run - one for each pH. This limitation was dictated by the size of the skin samples available from the same site. The penetration data for saturated solutions of methotrexate in 50% v/v propylene glycol in aqueous media were analyzed by plotting Q (the amount penetrated per unit area) against time. Based upon this plot a regression analysis of the steady state region of the penetration curve was carried out and the rate and lag times were determined.

The penetration profiles for the three sets of experiments are shown in Figures 21, 22, 23. The penetration data obtained from the Q versus t plots for the three sets of experiments are listed in Tables VII, VIII and IX. The relative distribution and mass balance analysis of methotrexate at the end of 14 days were carried out for each set.

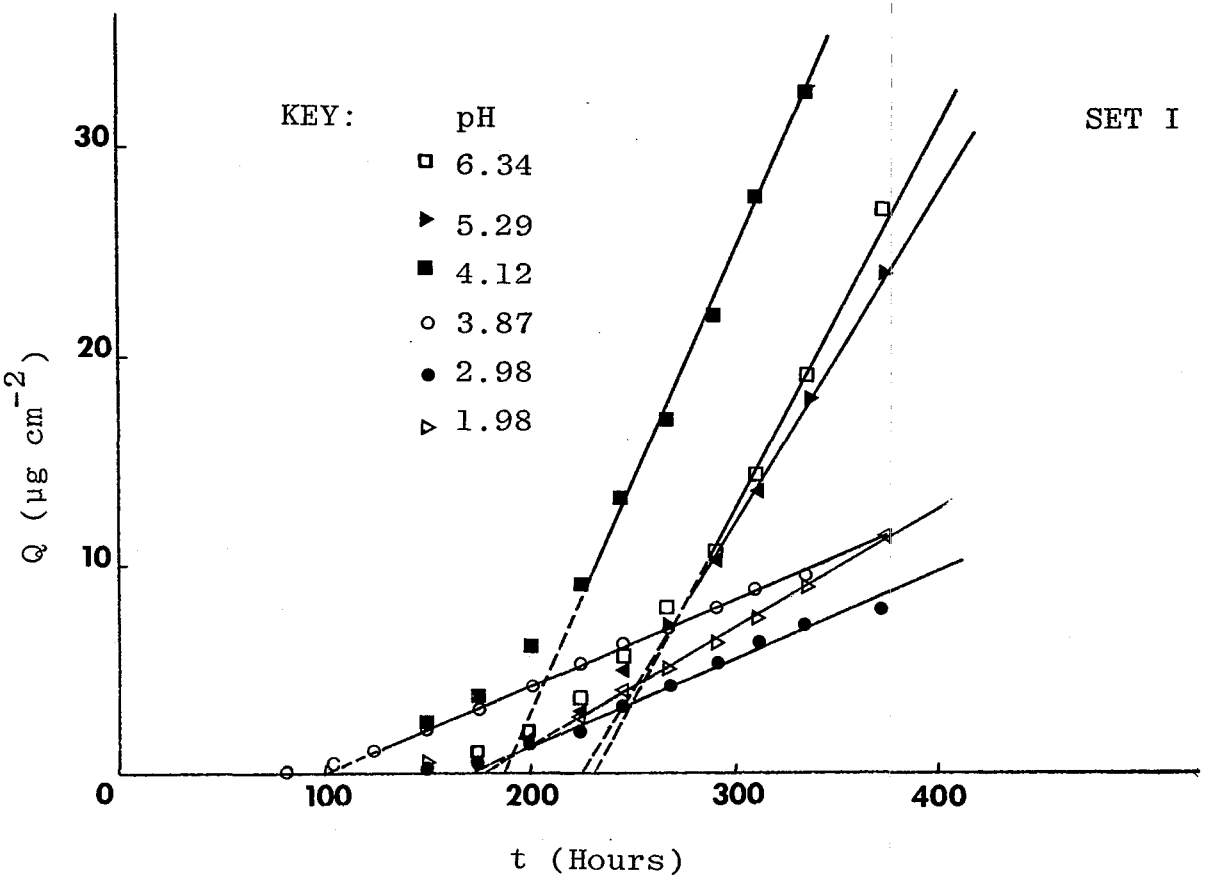

Figure 21. Effect of pH on <u>in vitro</u> percutaneous penetration of methotrexate: Q <u>versus</u> t plot, SET I.
NOTE: For pH 3.87, 5.29 and 6.34 the data plotted is Q x 10^{-1} <u>versus</u> t.

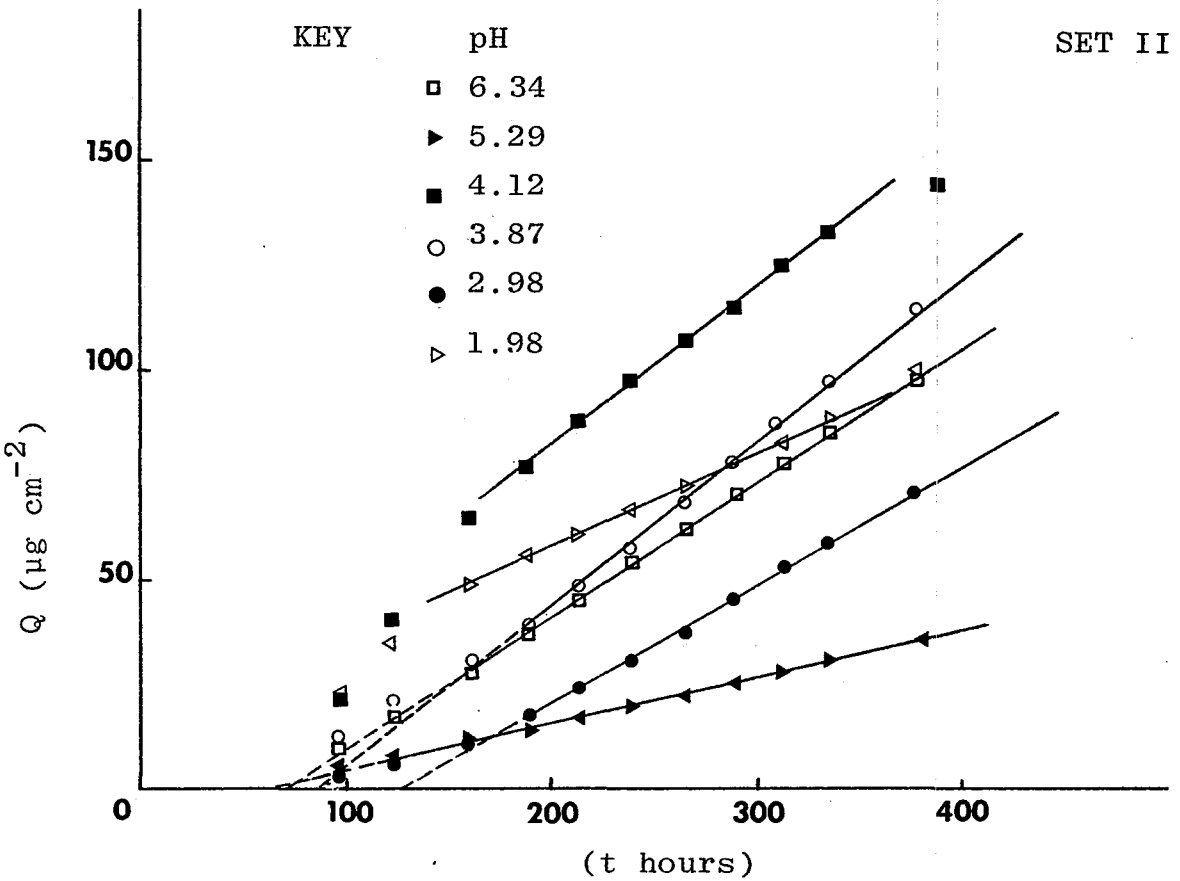

Figure 22. Effect of pH on *in vitro* percutaneous penetration of methotrexate: Q *versus* t plot, SET II.

NOTE: For pH 5.29 and 6.34 the data plotted is Q x 10^{-1} *versus* t.

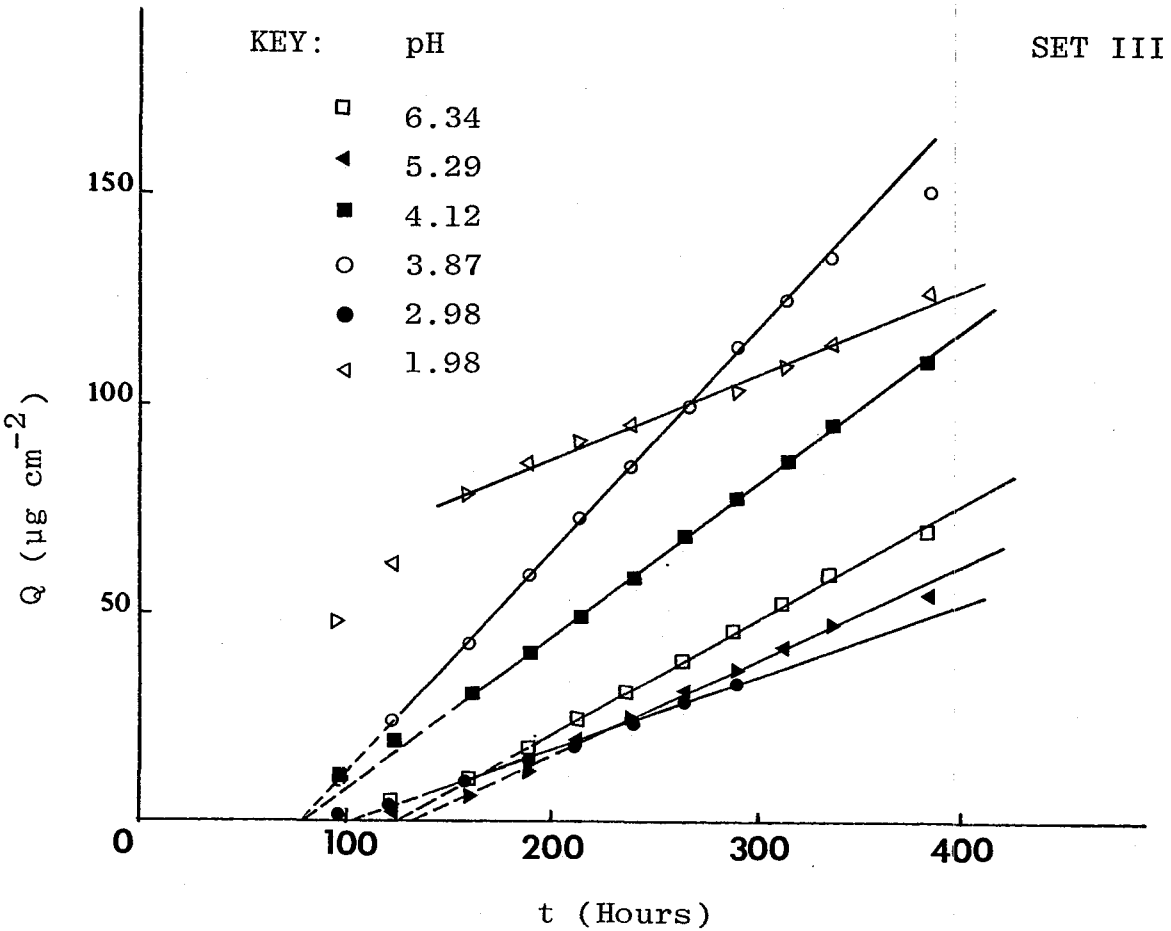

Figure 23. Effect of pH on in vitro percutaneous penetration of methotrexate: Q versus t plot, SET III.

NOTE: For pH 5.29 and 6.34 the data plotted is Q x 10^{-1} versus t.

TABLE VII

Effect of pH on *In Vitro* Percutaneous Penetration of Methotrexate
Penetration Data : SET I

pH	Concentration	Steady-State Penetration Rate[a]	Lag Time	Correlation Coefficient[b]
	µg/ml	µg cm^{-2} hr^{-1}	Hours	r
1.98	1405.9	0.0562	177	0.993
2.98	897.6	0.0423	172	0.992
3.87	463.3	0.4176	99	0.999
4.12	566.5	0.2190	187	0.998
5.29	1476.6	1.6150	226	0.998
6.34	4963.6	1.8214	231	0.994

[a]All steady-state values were computed from the regression line drawn from the data for each run at each pH by using a TI-55 calculator.

[b]Reported r values are for steady-state region of the penetration data.

TABLE VIII

Effect of pH on In Vitro Percutaneous Penetration of Methotrexate Penetration Data : SET II

pH	Concentration μg/ml	Steady-State Penetration Rate[a] μg cm^{-2} hr^{-1}	Lag Time Hours	Correlation Coefficient[b] r
1.98	1405.9	0.2182	-	0.999
2.98	897.6	0.3814	86	0.999
3.87	463.3	0.2721	125	0.999
4.12	627.1	0.3692	-	0.999
5.29	1477.6	1.1174	63	0.999
6.34	4963.6	3.1809	71	0.999

[a]All steady-state values were computed from the regression line drawn from the data for each run at each pH by using a TI-55 calculator.

[b]Reported r values are for steady-state region of the penetration data.

TABLE IX

Effect of pH on In Vitro Percutaneous Penetration of Methotrexate Penetration Data : SET III

pH	Concentration	Steady-State Penetration Rate[a]	Lag Time	Correlation Coefficient[b]
	µg/ml	µg cm^{-2} hr^{-1}	Hour	r
1.98	1405.9	0.2022	--	0.993
2.98	897.6	0.1747	103	0.997
3.87	463.3	0.5294	79	0.999
4.12	627.1	0.3663	80	0.999
5.29	1477.6	2.2737	130	0.999
6.34	4963.6	2.7434	124	0.999

[a]All steady-state values were computed from the regression line drawn from the data for each run at each pH by using a TI-55 calculator.

[b]Reported r values are for steady-state region of the penetration data.

The results are summarized in Tables X, XI, and XII.

Interpretation of Penetration Data

At low pH, between 1 and 3, the amount of methotrexate retained by the stratum corneum was quite high. This may be because, at low pH, methotrexate is apparently capable of ionizing further as a weak base, i.e. the nitrogens at positions 1 and 5 of the pteridine nucleus and also at position 10 assume a positive charge (Figure 1). This contribution of protonation of nitrogen in retarding penetration is consistent with the data for penetration rate and also the absolute amount penetrating at the end of 14 days. In spite of the higher concentration of drug in the donor vehicle at this pH range, the rate of penetration was slower when compared to the 3-5 pH range and lesser amounts penetrated at the end of 14 days. Thus protonation of nitrogens in the methotrexate molecule appears to hinder penetration by passive diffusion as well as through transfollicular route, strongly suggesting surface interaction with the stratum corneum.

In two out of the three sets of experiments carried out, the lag time at pH 3.87 was lower than at other pH values. The shorter lag time, indicative of the ease of penetration is most likely due to the higher fraction of unionized species present in the donor vehicle at pH 3.87 facilitating drug penetration by passive diffusion. Marked increase in the rate was seen when the pH of the donor

TABLE X

Effect of pH on Relative Distribution of Methotrexate 14 Days After Application
SET I

pH	Applied Concentration	Amount Penetrated		Washings of Epidermis	Applied Dose Recovered		Percent Accountability
	µg/ml	%	Absolute mg		% Epidermis	% Dermis	
1.98	1405.9	1.63	0.0230	94.08	0.16	0.63	96.51
2.98	897.6	1.75	0.0157	84.36	9.04	1.33	96.48
3.87	463.3	45.01	0.2085	53.65	1.70	3.37	103.74
4.12	566.5	14.34	0.0812	62.85	1.65	2.16	81.00
5.29	1477.6	32.56	0.4808	48.47	4.04	4.69	89.74
6.34	4963.6	10.84	0.5379	93.31	0.93	1.51	106.59

TABLE XI

Effect of pH on Relative Distribution of Methotrexate 14 Days After Application
SET II

pH	Applied Concentration	Amount Penetrated		Washings of Epidermis	Applied Dose Recovered		% Accountibility
	µg/ml	%	Absolute mg		% Epidermis	% Dermis	
1.98	1405.9	14.04	0.1973	32.50	25.90	5.44	75.92
2.98	897.6	25.57	0.2300	55.64	1.24	2.63	85.08
3.87	463.3	30.26	0.1402	67.26	1.19	2.34	101.10
4.12	627.1	45.72	0.2867	44.58	1.03	2.26	93.59
5.29	1477.6	48.74	0.7202	47.80	0.69	4.53	101.77
6.34	4963.6	35.21	1.8491	58.46	0.92	5.42	105.82

TABLE XII

Effect of pH on Relative Distribution of Methotrexate 14 Days After Application
SET III

pH	Applied Concentration	Amount Penetrated		Washings of Epidermis	Applied Dose Recovered		% Accountability
	µg/ml	%	Absolute mg		% Epidermis	% Dermis	
1.98	1405.9	18.14	0.0255	21.85	38.69	3.60	82.28
2.98	897.6	17.20	0.0154	61.66	3.08	2.46	84.40
3.87	463.3	65.56	0.3037	40.51	1.18	2.94	110.19
4.12	627.1	34.80	0.2183	52.10	1.02	2.53	90.45
5.29	1477.6	73.40	1.0845	18.85	0.75	4.30	97.30
6.34	4963.6	28.03	1.1024	68.02	1.31	6.15	103.51

vehicle was raised from 2.98 to 3.87. In spite of the increase in concentration of the drug at pH 4.12, the rate was slightly lower than at pH 3.87. The high values of percent amount penetrating in 14 days for the pH range 3 to 5, strongly suggests the major contribution towards penetration from passive diffusion with comparatively minimal effect from transfollicular and appendageal routes.

Between pH 4.12 and pH 5.29, a sharp rise in the penetration rate was observed while beyond pH 5.29 the rate of penetration appeared to level off. The fraction of the unionized species in the donor vehicle would be expected to be very low beyond pH 5. Hence, the high values for penetration rate above pH 5, consistent with the increased solubility of methotrexate, appear to suggest the increasing contribution of pore transport with increasing pH.

While these data suggest considerable variation between different skin specimens towards percutaneous absorption of methotrexate and in some cases variation even within the same skin specimens, a semi-log rate of penetration-pH profile (Figure 24) for the three sets of experiments revealed a definite trend. This is more convincingly demonstrated in a semi-log plot of the average value of the penetration rate for the three sets of experiments against pH (Figure 25).

At the chosen pH values, calculation of the fraction of methotrexate present as each species (Appendix II)--

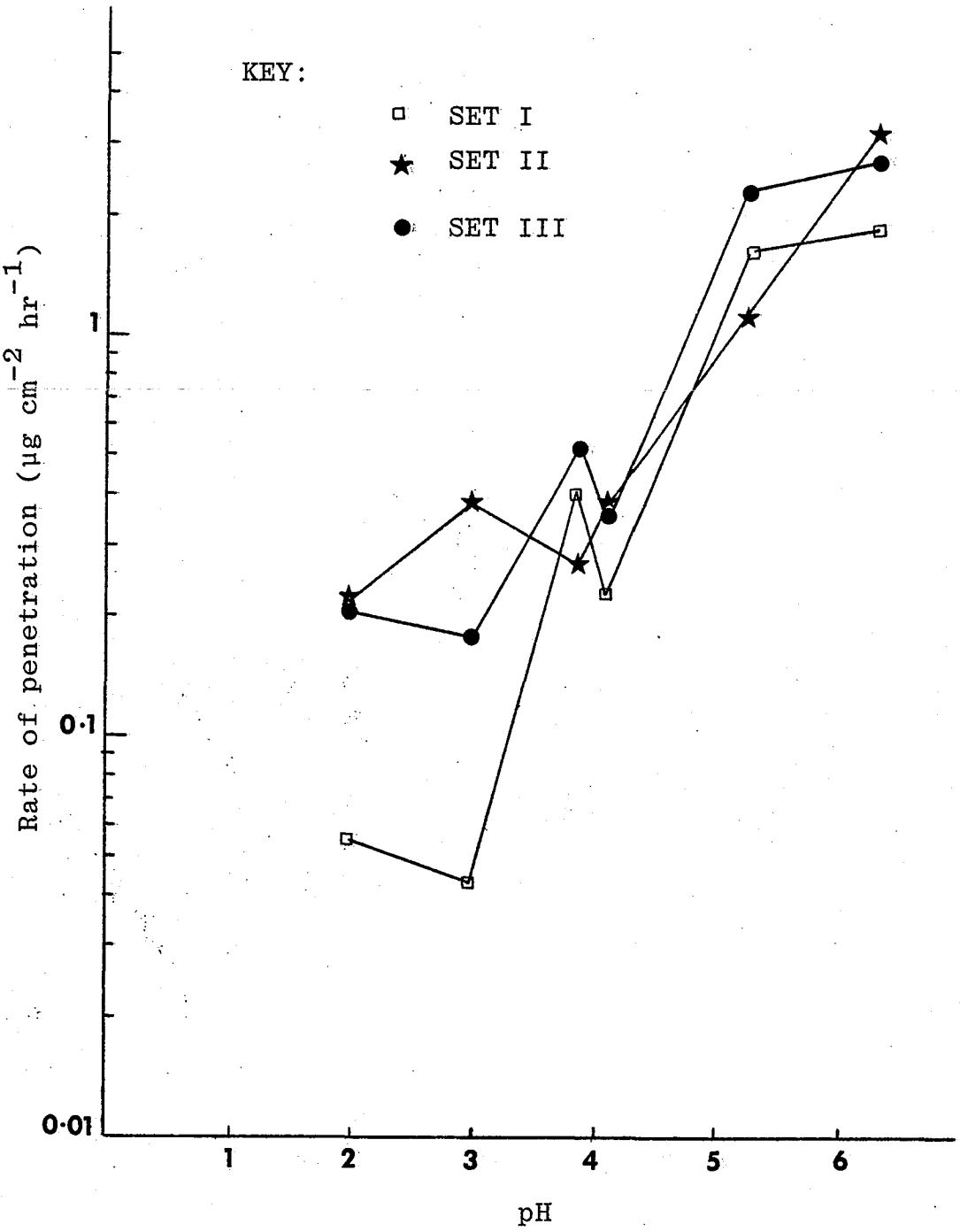

Figure 24. Semi-log plot of rate of penetration against pH for methotrexate absorption from saturated solutions of the drug in 50% v/v aqueous propylene glycol system.

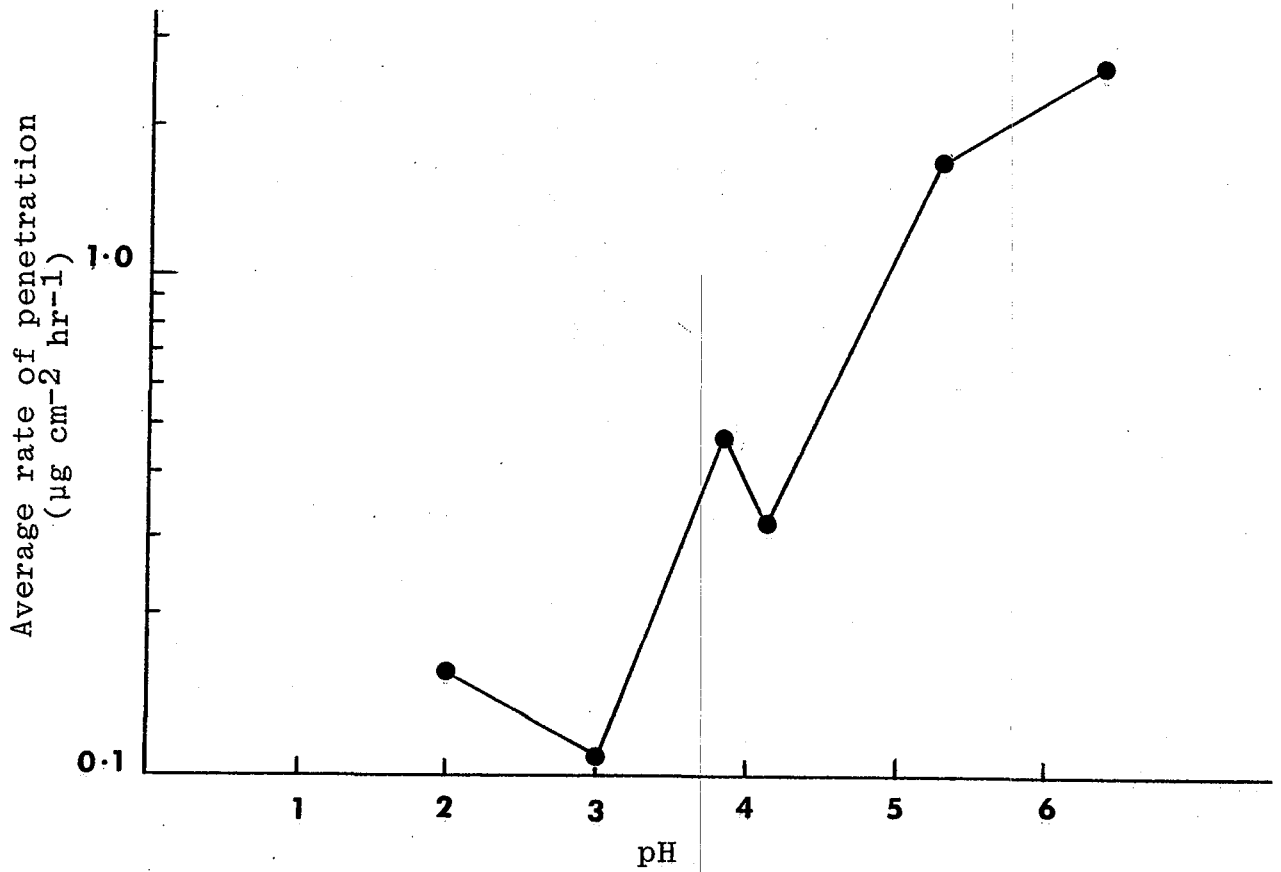

Figure 25. Semi-log plot of average rate of penetration against pH for methotrexate absorption from saturated solutions of the drug in 50% v/v propylene glycol in aqueous media.

unionized, monoprotic, and aprotic revealed no definite correlation of the rate of penetration with the fraction of any of the individual species. This was true for each of the three different sets of pK_a values reported in the literature for methotrexate. Of course it should be recognized that in propylene glycol-aqueous media, the pK_a values for methotrexate may be somewhat different from the hitherto reported literature values.

These findings suggested that at any given pH, the absorption of methotrexate from 50% v/v propylene glycol in aqueous medium might be dependent on the combined effect of transport by passive diffusion and transfollicular and appendageal routes. It is extremely difficult to delineate the individual contribution due to each of these factors. The salient features of the effect of pH on <u>in vitro</u> percutaneous penetration of methotrexate may be summarized as follows:

(a) At low pH, between 1 and 3, protonation of the nitrogens retards penetration;

(b) Concentration of the unionized species is optimal between pH 3 and 4, facilitating penetration by passive diffusion, but low solubility in this pH range limits transport; and

(c) With increasing pH, contributions from passive diffusion decrease while contributions from pore transport increase.

CONCLUDING REMARKS

Effectiveness of systematically administered methotrexate in psoriasis has prompted continuing effort towards therapy by topical route. However, controversy reigns over the clinical efficacy of this mode of therapy. Several theories, most of which are neither documented nor denied by experimental facts, have been proposed to explain the wide variation in clinical response to topical treatment with methotrexate.

A 'systemic site of action' has been cited as one of the possible reasons for unsuccessful results with topical therapy. Van Scott and Reinertson (11) in 1959, first suggested that pharamcological action of methotrexate in psoriasis might require initiation at a distant systemic site. Nurse (9) in 1963, proposed that a depletion of body stores of folinic acid may be necessary for methotrexate to act on psoriatic epidermal cells. Comaish (13) in 1969 hypothesized that methotrexate might act on skin cells indirectly by its effect on the liver. According to Cipriano *et al.* (35), inhibition of hepatic production of N^5-methyltetrahydrofolate rather than inhibition of endogenous production of these compounds by the epidermal cells might be important.

Too rapid dispersal from the site of application following intralesional injection has been proposed for the ineffectiveness through this route as well (9).

Inhibition of tritiated deoxyuridine incorporation in explants of human skin following local application of methotrexate as shown by Marks et al., (36) casts doubt on the theory that conversion products of methotrexate may be responsible for the effectiveness of systemically administered methotrexate.

Lack of percutaneous penetration has often been considered as one of the possible reasons for the clinical inefficacy of topical methotrexate. Comaish and Juhlin (12) reported the only quantiative estimate of percutaneously absorbed methotrexate following in vivo studies. According to these authors, 0.06 to 0.5% of the total topically applied methotrexate was recovered in the urine after 72 hours in four patients.

Apart from the fact that stratum corneum forms the main effective barrier for penetration of topically applied methotrexate (as confirmed by this study), there appears to be little or no consensus regarding mechanism of topical absorption of methotrexate. Unfortunately, research on topical methotrexate has not been methodical. Clinical trials have preceded adequate physicochemical and formulation studies leading to questionable predictions and postulations about the biochemical effects of methotrexate.

Impurities in the samples of methotrexate used and inadequate analytical techniques have further added to the confusion.

In the present work, two important physiochemical characteristics of methotrexate solubility and partition coefficient, have been examined and generated data have been utilized as an aid in the development of a delivery system for methotrexate. The potential of 50% v/v aqueous propylene glycol as a vehicle for topical delivery of methotrexate was investigated. Examination of solubility of the drug in a propylene glycol-water system revealed a logarithmically increasing relationship between solubility and percent propylene glycol. The partition coefficient of methotrexate when tested in an isopropyl myristate/propylene glycol-water system, was found to decrease with increasing concentrations of propylene glycol in the vehicle mixture.

The solubility and in vitro percutaneous penetration rates of methotrexate in 50% v/v propylene glycol-aqueous medium in the pH range 2 to 6 was also investigated. The solubility-pH profile showed a minimum at approximately pH 4.

The results of the in vitro penetration studies showed that at low pH values, between 1 and 3, a high percentage of the total applied dose was retained in the stratum corneum. This may be due to the possible protonation of the nitrogens at positions 1 and 5 of the pteridine nucleus

and also at position 10, leading to interaction with the stratum corneum. In the pH range 3 to 5, concentration of the unionized species was optimal. The high values for percent penetrating at the end of 14 days and comparatively low values for lag time strongly suggested the major contribution towards penetration was from passive diffusion with minimal effects through transfollicular and transappendageal routes. Beyond pH 5, the increase in penetration rate was attributed to the increase in concentration of methotrexate in the donor vehicle. The very high values for lag time beyond pH 5 were indicative of diminished contribution of passive transport relative to other pathways such as intercellular and transfollicular pathways. Interestingly, increased solubility at pH 5.29 and 6.34 did not contribute to low lag times--underscoring the importance of contribution from passive diffusion. The high lag times beyond pH 5 further raise the possibility of drug interaction with the skin.

No definite correlation was apparent between the penetration rates at various pH and the total concentration of the drug in the vehicle. Calculation of the fraction of different species of methotrexate present at each pH based on the pK_a values reported in the literature showed no correlation between the penetration rates and concentration of any of the three individual species present. The penetration rate-pH profile for experimental runs

was reproducible in spite of the wide variation between different skin specimens.

To provide adequate release, a vehicle must dissolve the drug, but not to the extent that the drug remains preferentially in the vehicle; the stratum corneum/vehicle partitioning must be adequate. The reults of this investigation reveal the complicated penetration behavior of methotrexate, at least from the vehicle system examined. Further, the results suggest that lack of percutaneous penetration and limited duration of clinical trials might as well have been the main reasons for the reported clinical ineffectiveness of topical methotrexate.

It is suggested that the optimal conditions for topical delivery of methotrexate should include a vehicle system with a pH of about 4 to 6 capable of dissolving the drug to the extent of 0.4% w/v or more while still retaining good partitioning characteristics for the skin. It would be worthwhile examining the <u>in vitro</u> skin penetration from such vehicle systems. The efforts along these lines might yet unlock the clinical potential of methotrexate in topical treatment of psoriasis.

APPENDIX I

Preparation of Aqueous Media

(A) Hydrochloric Acid Solutions:

Concentrated hydrochloric acid (11.9 N) was used for dilution.

pH 1.72 - 1.250 ml of concentrated hydrochloric acid diluted to 500 ml with distilled water to give approximately 0.03M hydrochloric acid.

pH 2.14 - 0.420 ml of concentrated hydrochloric acid diluted to 500 ml with distilled water to give approximately 0.01M hydrochloric acid.

pH 2.36 - 0.208 ml of concentrated hydrochloric acid diluted to 500 ml with distilled water to give approximately 0.005M hydrochloric acid.

pH 3.02 - 0.042 ml of concentrated hydrochloric acid diluted to 500 ml with distilled water to give approximately 0.001M hydrochloric acid.

(B) Potassium Hydroxide Solutions:

pH 11.02 - 0.056 g potassium hydroxide dissolved in 1000 ml distilled water to give approximately 0.001M potassium hydroxide in water.

pH 12.12 - 0.560 g potassium hydroxide dissolved in 1000 ml distilled water to give approximately 0.01M potassium hydroxide in water.

pH 12.18 - 1.120 g potassium hydroxide dissolved in 1000 ml distilled water to give approximately 0.02M potassium hydroxide in water.

pH 12.59 - 2.80 g potassium hydroxide dissolved in 1000 ml distilled water to give approximately 0.05M potassium hydroxide in water.

(C) Acetic Acid Solutions:

Glacial acetic acid (17.4 N) was used for dilution.

pH 3.39 - 0.575 ml of glacial acetic acid diluted to 1000 ml with distilled water to give approximately 0.01M acetic acid.

pH 3.94 - 0.0575 ml of glacial acetic acid diluted to 1000 ml with distilled water to give approximately 0.001M acetic acid.

(D) Chloroacetic Acid Solutions:

pH 2.50 - 0.945 g chloroacetic acid dissolved in 1000 ml distilled water to give approximately 0.01M chloroacetic acid in water.

pH 3.14 - 0.0945 g chloroacetic acid dissolved in 1000 ml distilled water to give approximately 0.001M chloroacetic acid in water.

APPENDIX II

Calculation of the Fraction of Unionized Methotrexate

To calculate the fraction of methotrexate present as each species - diprotic, unionized (α_2), monoprotic (α_1) and aprotic (α_0) the equilibria are (37):

$$[H^+]\ [MTX^-] = K_{a_1}\ [MTX] \qquad (1)$$

$$[H^+]\ [MTX^=] = K_{a_2}\ [MTX^-] \qquad (2)$$

The mass balance on methotrexate is:

$$C = [MTX] + [MTX^-] + [MTX^=] \qquad (3)$$

where C is the analytical concentration of methotrexate. Since $[H^+]$ and C are known, there are a total of three equations in three unknowns.

The fraction of unionized methotrexate present is the ratio of the concentration of the unionized species to the analytical concentration,

$$= \frac{[MTX]}{C} \qquad (4)$$

The reciprocal of the fraction of unionized drug can be calculated by dividing each term of equation (3) by $|MTX|$, thus;

$$\frac{1}{\alpha_2} = \frac{C}{[MTX]} = 1 + \frac{[MTX^-]}{[MTX]} + \frac{[MTX^=]}{[MTX]} \qquad (5)$$

$$\frac{1}{\alpha_2} = 1 + \frac{K_{a_1}}{[H^+]} + \frac{K_{a_1} K_{a_2}}{[H^+]^2} \qquad (6)$$

Thus α_2, the fraction of unionized drug can be calculated from its reciprocal using equation (6), the reported pK_a values and the measured pH of the solutions.

The fraction of monoprotic methotrexate present is the ratio of the concentration of the monoprotic species to the analytical concentration,

$$= \frac{[MTX^-]}{C} \qquad (7)$$

The reciprocal of the fraction of monoprotic species can be calculated by dividing each term of equation (3) by $[MTX^-]$, thus;

$$\frac{1}{\alpha_1} = \frac{C}{[MTX^-]} = \frac{[MTX]}{[MTX^-]} + 1 + \frac{[MTX^=]}{[MTX^-]} \qquad (8)$$

$$\frac{1}{\alpha_1} = 1 + \frac{H^+}{K_{a_1}} + \frac{K_{a_2}}{H^+} \qquad (9)$$

Thus α_1, the fraction of monoprotic drug can be calculated from its reciprocal using equation (9), the reported pK_a values and the measured pH of the solution.

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