CHAPTER 4

LIPOSOMES OF 5FU AND MTX FOR SKIN TARGETTING

4.1 INTRODUCTION

Liposomes have aroused considerable interest in their probable utilisation as carriers for targetting of drugs into various layers of the skin. Reformulating the existing ointment, cream or lotion forms to liposomal forms could lead to improvement in both efficacy and safety of the topically applied drug and enhance patient compliance(1-8).

5FU is used topically in actinic keratosis, psoriasis, keratoacanthoma, superficial basal cell and squamous cell carcinoma of the skin in upto 5% cream. MTX is used systemically in treatment of psoriasis and allergic contact dermatitis(9). Reports are available on the topical application of MTX in psoriasis. However, the topical route is often found to be cumbersome, inefficient and leading to systemic toxicities(10,11).

The aim of the present investigation was to utilise liposomes

a) as drug carriers for topical application of 5FU and MTX.
b) as localizers to minimise drug clearance from the skin thereby decreasing the systemic toxicities.

4.2 EXPERIMENTAL

4.21 Apparatus and Equipments :

The apparatus and equipments used for the preparation and evaluation of liposomes of 5FU and MTX include rotary flash evaporator (Superfit, India), triple blade stirrer,

vortex mixer, centrifuge, magnetic stirrer and bath sonicator (Remi, India), glass permeation tubes (o.d=2cm), (Borosil, India).

4.22 Materials :

Egg phosphatidyl choline (Centre for Biochemicals, CSIR, India), cholesterol extrapure (s.d. fine chemicals, India), ∝-tocopherol (E. Merck, India) ferric chloride anhydrous, calcium chloride fused, ammoniumthiocyanate, disodiumhydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, sodium sulphate, glacial acetic acid, concentrated sulphuric acid, chloroform, methanol (Qualigens, India), purified water (I.P), nitrogen cylinder.

All chemicals used were of A.R. grade and were used as such without further purification.

4.23 Analytical Methods :

4.231 Estimation of lecithin : (12)

Lecithin estimation was done by Stewart assay method. In this method, the ability of lecithin to form a complex with ammonium ferrothiocyanate in organic solution is utilised.

(a) Reagents :

(i) Ammonium ferrothiocyanate solution (0.1M) :

27.03g of ferric chloride and 30.4g of ammonium thiocyanate were dissolved and diluted to 1000 ml with water.

(ii) Stock solution of lecithin :

A 0.1% w/v solution of egg lecithin was prepared in chloroform.

(b) Calibration curve for lecithin

To suitable aliquots of lecithin solution (0.1, 0.2...0.7ml), different volumes of chloroform (1.9, 1.8....1.3ml) were added followed by 2ml of ammonium ferrothiocyanate solution. The mixture was vortexed for 2minutes, centrifuged for 2 minutes at 3750g and the absorbance of the choloroform layer was read at 485nm against reagent blank. The mean of six absorbance values are recorded in Table 4.1 and Fig. 4.1. The calibration curve was linear in the range of 100-700µg/ml.

4.232 Estimation of cholesterol : (13)

Cholesterol was determined by colorimetric method. The method used here is that of Zlatkis, Zak and Boyle in which cholesterol in acetic acid solution gives a red colour with ferric chloride and sulphuric acid.

(a) Reagents :

(i) Stock solution of cholesterol : A 0.05% w/v solution of cholesterol was prepared in glacial acetic acid.

(ii) Ferric chloride solution : A 0.05% w/v solution of ferric chloride was prepared in glacial acetic acid.

(b) Calibration curve for cholesterol :

Aliquots of stock solution of cholesterol (0.1,0.2...1ml) were transferred accurately into separate 5ml volumetric flasks, 2ml of ferric chloride solution and 2ml of concentrated sulphuric acid were added. The solutions were mixed and made upto volume with glacial acetic acid where necessary. Absorbance of the resulting solutions were measured at 550nm against the reagent

TABLE 4.1

CALIBRATION CURVE OF EGG LECITHIN BY AMMONIUMFERRO THIOCYANATE METHOD

Aliquot ml	$CHCl_3$ added ml	Concentration in µg/ml	Mean absorbance (± S.D.)
0.1	1.9	100	0.254 (0.003)
0.2	1.8	200	0.505 (0.004)
0.3	1.7	300	0.752 (0.011)
0.4	1.6	400	0.995 (0.003)
0.5	1.5	500	1.353 (0.003)
0.6	1.4	600	1.544 (0.009)
0.7	1.3	700	1.853 (0.007)

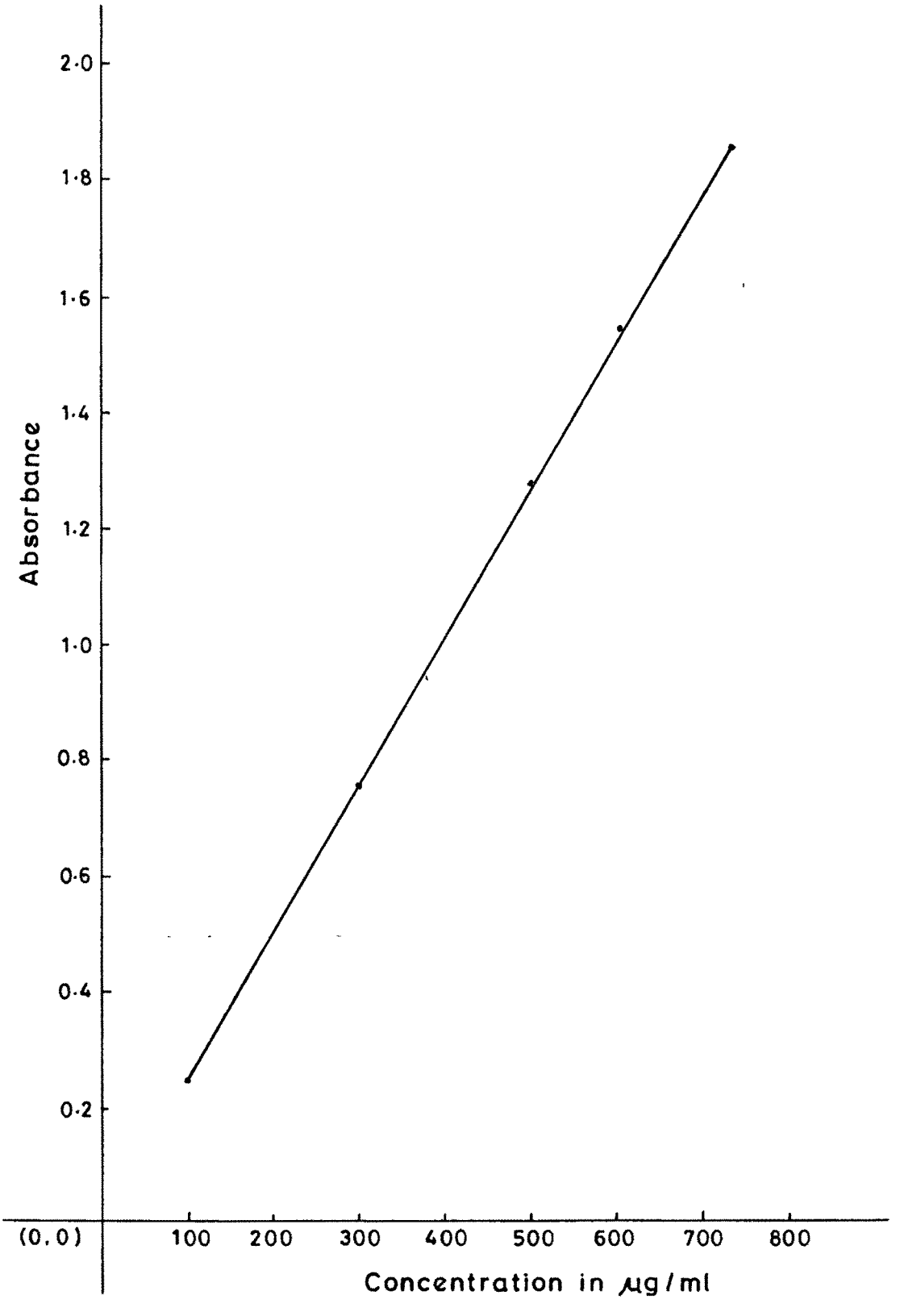


FIG. 4.1: CALIBRATION CURVE OF EGG LECITHIN BY FERROTHIOCYANATE METHOD

TABLE 4.2

CALIBRATION CURVE OF CHOLESTEROL

Conc. in µg/ml	Mean absorbance (± S.D.)
5	0.121 (0.003)
10	0.234 (0.004)
20	0.444 (0.007)
40	0.813 (0.005)
50	1.012 (0.007)
80	1.602 (0.013)
100	1.785 (0.063)

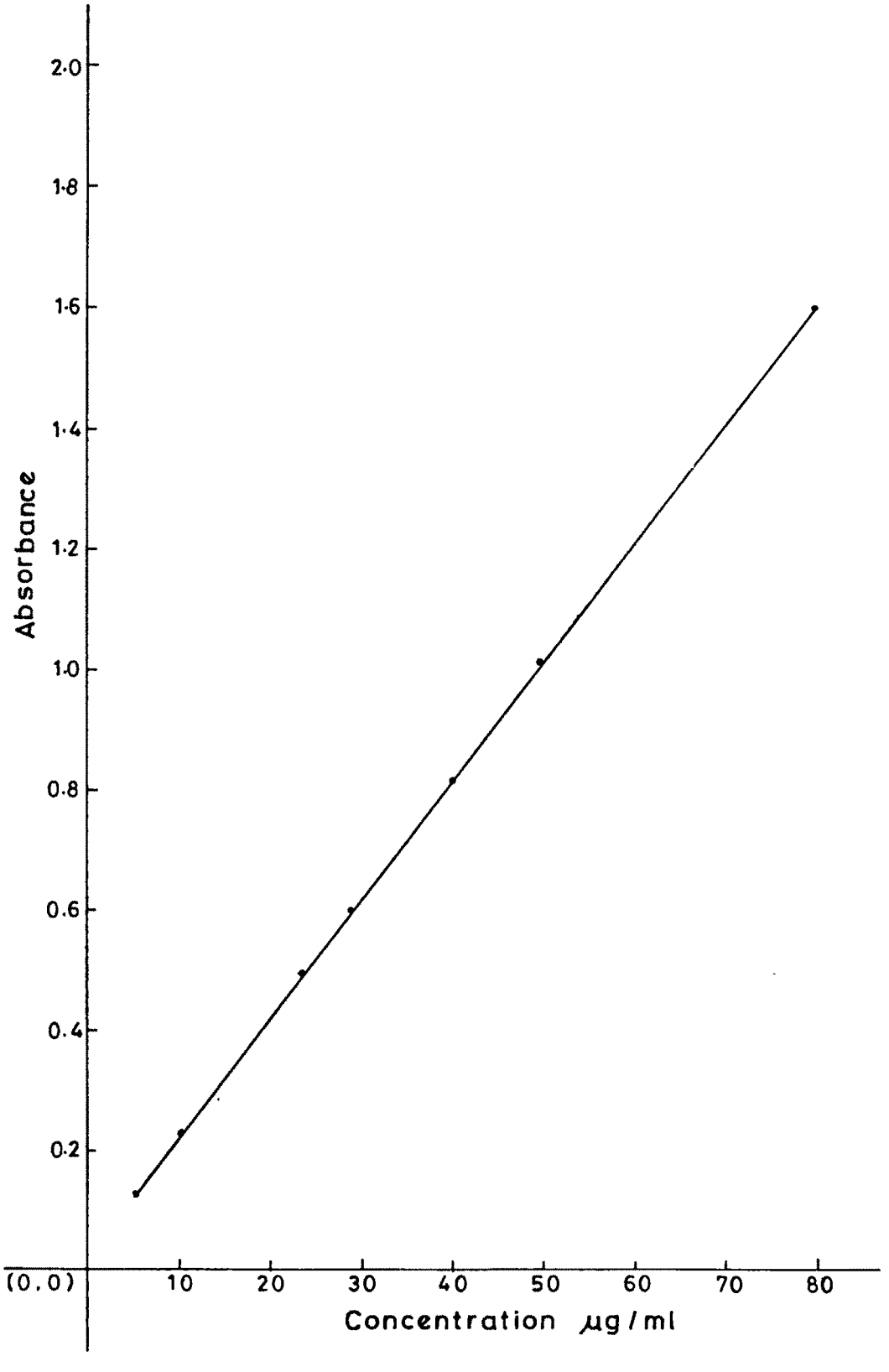


FIG. 4.2 : CALIBRATION CURVE OF CHOLESTEROL

TABLE 4.3

CALIBRATION CURVE OF 5FU AND MTX IN PBS

Conc. in µg/ml	Mean absorbance (± S.D.)	
	5FU λ= 266nm	MTX λ= 305nm
1	0.067 (0.002)	0.068 (0.003)
2	0.131 (0.012)	0.123 (0.007)
4	0.267 (0.007)	0.251 (0.009)
5	0.323 (0.005)	0.312 (0.012)
10	0.671 (0.004)	0.639 (0.017)
20	1.303 (0.007)	1.282 (0.007)
30	1.947 (0.008)	1.893 (0.013)

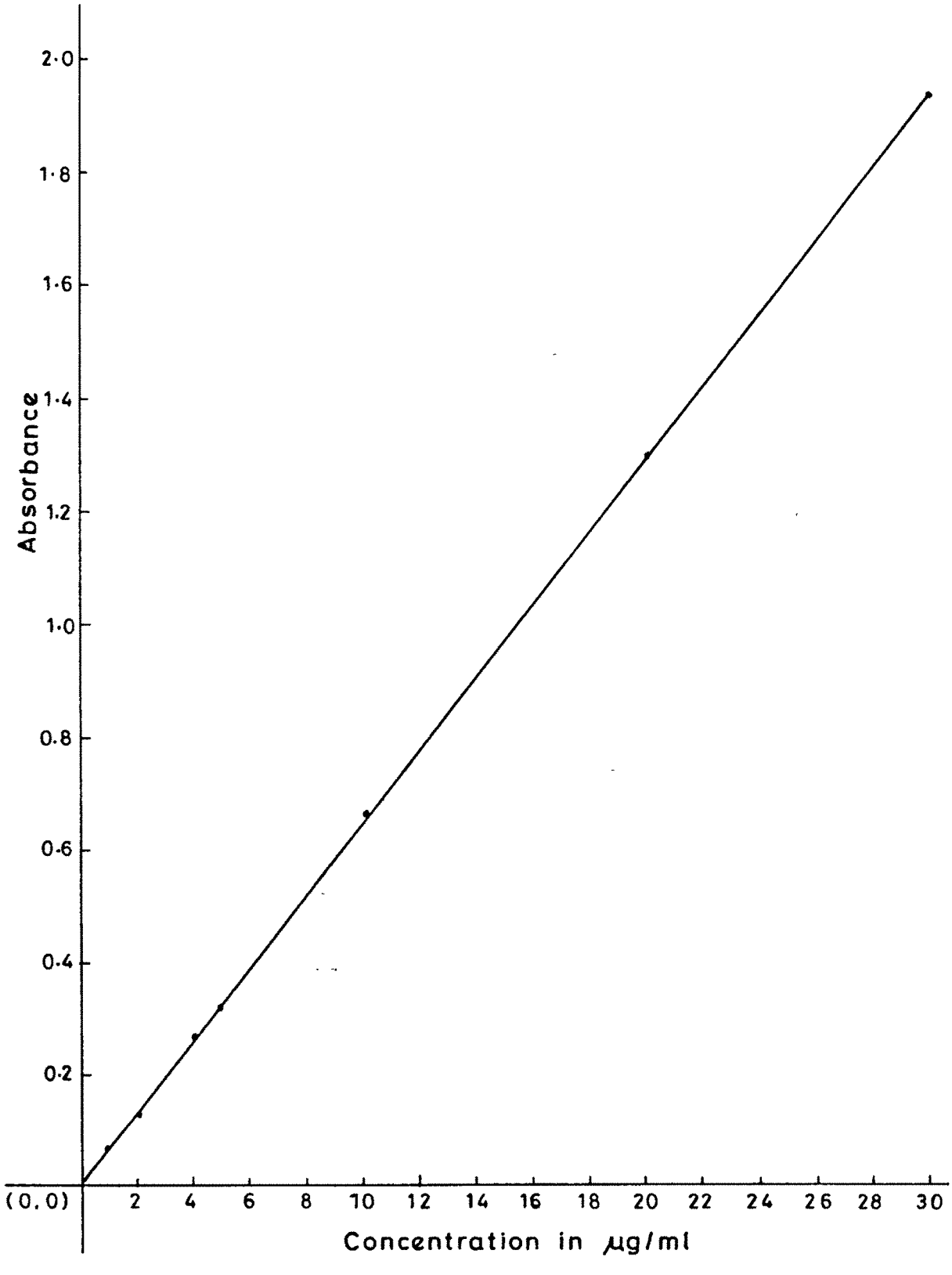


FIG. 4.3 : CALIBRATION CURVE OF 5FU IN PBS

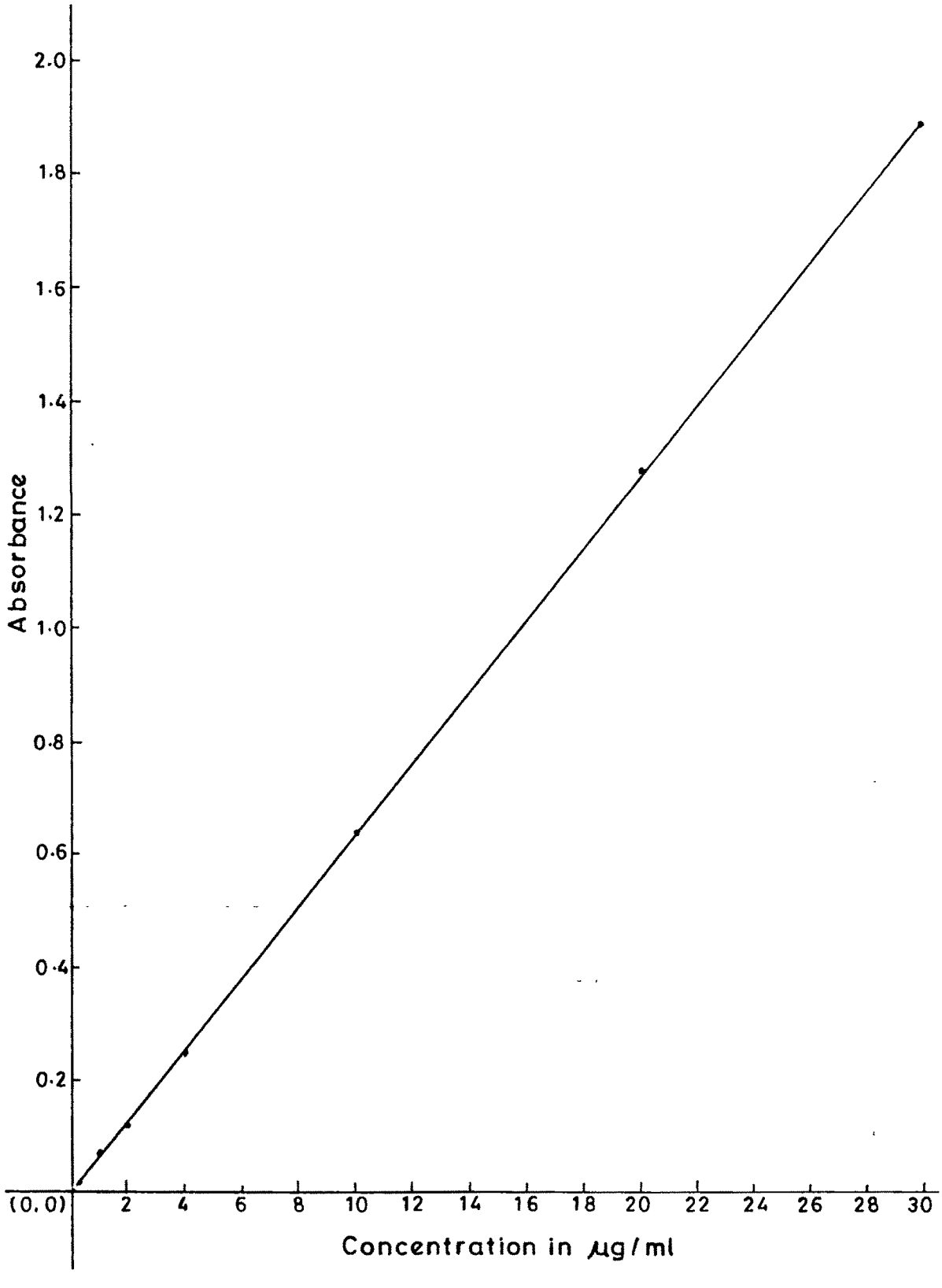


FIG. 4.4 : CALIBRATION CURVE OF MTX IN PBS

blank. The observations are recorded in Table 4.2 and Fig. 4.2. The calibration curve was linear in the rangeof 5-100µg/ml.

4.233 Calibration curves for 5FU and MTX in pH 7.4 phosphate buffered saline (PBS) :

For each of these drugs a common procedure was adopted. 10mg of the drug was weighed into a 100ml volumetric flask, dissolved and diluted to volume with PBS. Aliquots of the drug solutions (0.1,0.2,...3ml) were accurately transferred to separate 10ml volumetric flasks and made upto volume with PBS. The absorbance values were measured at 266nm for 5FU and 305nm for MTX. The mean of six absorbance values for 5FU and MTX are given in Table 4.3 and Figs. 4.3 and 4.4 respectively. Calibration curves were linear in the range of 1-30µg/ml in both the cases.

4.24 Preparation of Liposomes of 5FU and MTX :

Two procedures were used for preparation of liposomes of 5FU viz. thin film technique and modified method of Szoka and Papahadjopoulous. For MTX double emulsification method and reverse phase evaporation technique were used.

4.241 Reagents :

(a) Calcium chloride solution :

Solutions of 10,25,50 and 100 millimolar concentrations of calciumchloride were prepared in water.

(b) Stock solution of ∂-tocopherol :

A 0.1% w/v solution of ∂-tocopherol was prepared in chloroform.

(c) Stock solutions of 5FU and MTX :

A 2mg/ml solution of each of 5FU and MTX were prepared in PBS.

4.242 Liposomes of 5FU :

(a) Thin film technique : (14)

The required amount of egg lecithin and cholesterol were weighed into a 100ml round bottom flask and dissolved in 10ml of 2:1 chloroform : methanol mixture. 0.1ml of ℓ-tocopherol solution was added and the organic solution was evaporated at room temperature on a rotary flash evaporator under reduced pressure. Thus a thin film of lipid was deposited on the inner wall of the flask. Liposomes were prepared by adding to the flask 5ml of 25mM calcium chloride solution containing a suitable aliquot of 5FU solution. The liposomal suspension was vortexed for 2 minutes and left overnight at 4°C for swelling of liposomes.

(b) Modified method of Szoka and Papahadjopoulous : (15)

The required amount of egg lecithin and cholesterol were weighed into a 100 ml round bottom flask and dissolved in 10ml of 2:1 chloroform-methanol mixture, 0.1ml of ℓ-tocopherol solution was added. After addition of 5ml of 25mM calcium chloride solution, the mixture was sonicated on a bath sonicator for 5 minutes at room temperature. The organic solvent was then removed on a rotary flash evaporator. The liposomal suspension was left at 4°C overnight for swelling of liposomes.

4.243 Liposomes of MTX :

(a) Double emulsification method : (16)

5ml of 25mM calcium chloride solution containing a suitable aliquot of MTX solution was taken in a two necked flat bottom flask placed on a magnetic stirrer. Through one neck of the flask a tube was passed connected to a nitrogen cylinder. Through the other neck, 5ml of lipid solution containing required amounts of lecithin, cholesterol and ∝ -tocopherol were injected from a 10ml glass syringe through a 22 gauge hypodermic needle into the aqueous solution. The mixture was stirrerd vigorously and the organic solvent was removed completely by a strong jet of nitrogen. The liposomal suspension was left overnight at 4°C for complete swelling of liposomes.

(b) Reverse phase evaporation method : (17)

The required amounts of egg lecithin and cholesterol were weighed into a 100 ml round bottom flask and dissolved in 10ml of 2:1 chloroform-methanol mixture. 0.1ml of ∝-tocopherol solution was added and the organic solvent was evaporated off to form a thin film of the lipid. The thin film was redissolved in 15ml of 2:1 chloroform-methanol mixture in which reversed phase vesicles were formed. To this solution, 5ml of 25mM calcium chloride solution containing a suitable aliquot of MTX solution was added and the mixture was sonicated on bath sonicator for 5 minutes. The organic solvent was removed under reduced pressure at room temperature on a rotary flash evaporator. The solvent removal was

continued until all foaming ceased. The liposomal suspension was left at 4°C overnight for swelling of liposomes.

4.244 Separation of free drug :

The unentrapped drug in all the cases was removed by repeated centrifugation for 15 minutes at 3750g and washings with PBS. The washings were monitored for the drug content spectro photometrically at 266nm and 305nm for 5FU and MTX respectively. After the final wash the liposomal pellet was resuspended in 10ml of 25mM calcium chloride solution. The liposomal suspension was analysed for the entrapped drug, lecithin and cholestrol content by the procedures described below.

4.245 Characterisation of Liposomes :

1. Estimation of lecithin, cholesterol, 5FU and MTX from liposomes :

For the estimation of lecithin, cholesterol, 5FU and MTX from liposomes the following steps were carried out :

a. To 1ml of liposomal suspension in a 10ml centrifuge tube, 1ml of saturated sodium chloride solution and 2ml of chloroform were added.
b. The contents were vortexed for 2 minutes and centrifuged at 3750g to separate the two phases.
c. The lower chloroform layer was withdrawn using a glass syringe with a long needle and transferred to a 10ml volumetric flask through a cotton plugged funnel containing anhydrous sodium sulphate.

d. To the aqueous layer from step (c) 2ml of fresh chloroform was added and steps (b) and (c) were repeated twice.

e. The volume of the combined chloroform extracts was made upto 10ml with chloroform.

f. For estimation of lecithin, a suitable aliquot from step (e) was taken and colour was developed as per the procedure given under calibration curve of lecithin (section 4.231b).

g. For estimation of cholesterol, a suitable aliquot from step (e) was taken and evaporated to complete dryness on a water bath. The residue was dissolved in 1ml of glacial acetic acid and colour was developed as per the procedure given under calibration curve of cholesterol (section 4.232b)

h. For estimation of 5FU from 5FU liposomes, a suitable aliquot of the aqueous layer remaining at the end of step (d) was accurately withdrawn and transferred to a 5ml volumetric flask, volume was made up with PBS and the concentration was determined spectrophotometrically from the calibration curve of 5FU in PBS (section 4.233, Fig. 4.3, Table 4.3).

i. For estimation of MTX from MTX liposomes, a suitable aliquot of the aqueous layer remaining at the end of step (d) was withdrawn and transferred to a 5ml volumetric flask, volume was made up with PBS and the concentration was determined spectrophotometrically from the calibration curve of MTX in PBS (Section 4.233, Fig.4.4, Table 4.3).

2. Determination of size distribution of liposomes :

The size distribution of liposomes of 5FU and MTX was determined using Olympus BHA, Japan microscope. The results are given in Table 4.4.

3. Photomicrographs :

To study the shape of liposomes 0.381 : 0.381, 0.635:0.635 mM ratio of lecithin : cholesterol batches of liposomes of 5FU and MTX respectively were photomicrographed using Carl Zeiss Jena Model photomicroscope. The photographs are shown in plate 1.

4. Electron Microscopy :

In order to determine the lamellarity of the liposomes electron micrographs of liposomal batches containing 0.381:0.381, 0.635:0.635 mM ratio of lecithin: cholesterol for 5FU and MTX respectively were taken using Joel Jem 100sx Transmission Electron Microscope. The liposomes were stained using negative staining technique with 2% ammonium molybdate solution. The photographs are shown in plates 2 and 3.

4.246 Effect of ionic strength of calcium chloride, lipid composition on the encapsulation efficiency of 5FU and MTX liposomes :

The thin film method was used for preparation of 5FU liposomes and reverse phase evaporation method for MTX liposomes.

(a) Effect of ionic strength of calcium chloride :

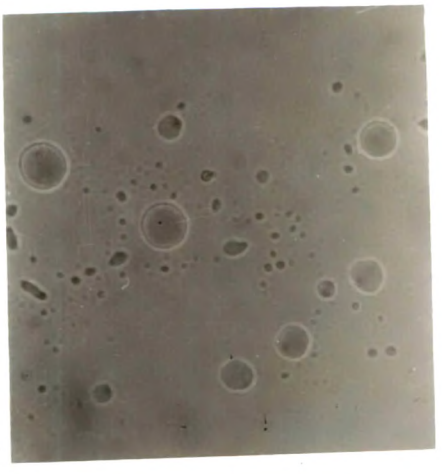
For 5FU, the liposomes were prepared by hydrating the thin film with 5ml of different millimolar concentrations

TABLE 4.4

PARTICLE SIZE DISTRIBUTION OF LIPOSOMES OF 5FU AND MTX

Size range μm	Mean size range μm	No. of particles in each size range 5FU (n_1)	MTX (n_2)	n_1d	n_2d	Mean diameter $\frac{\leq nd}{n}$ 5FU	MTX
0-2	1	35	20	35	20		
2-4	3	165	132	495	390		
4-6	5	91	120	455	600	3.49 μm	4.03 μm
6-8	7	9	26	63	182		
8-10	9	-	2	-	18		

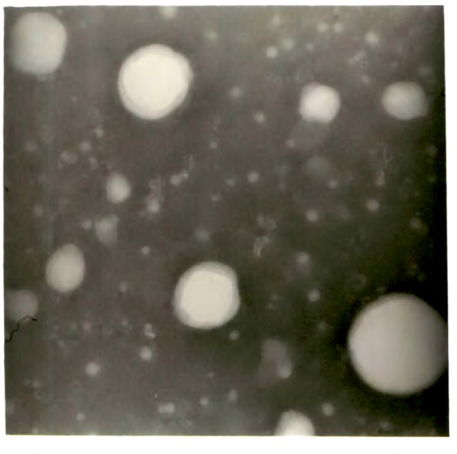
5FU PLATE - 1 MTX



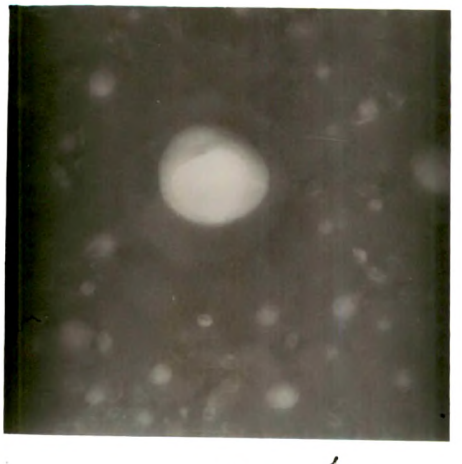
a 200X

b 200X

PLATE - 2 5FU LIPOSOMES



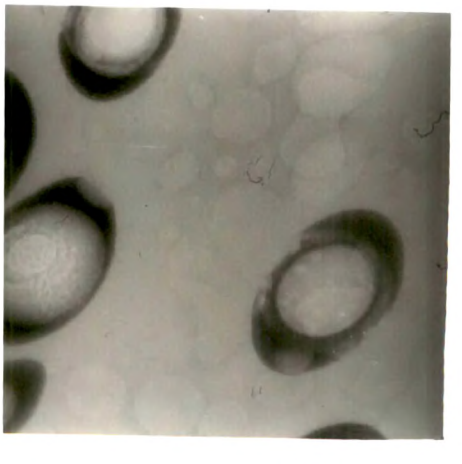
a 4.0×10^4X



b 8.0×10^4X

PLATE - 3 MTX LIPOSOMES

a 1.0×10^5X



b 1.2×10^5X

of calcium chloride. The observations are recorded in Table 4.5 and shown in Fig. 4.5.

For MTX, the reverse phase vesicles formed by redissolving the lipid were treated with 5ml of different millimolar concentrations of calcium chloride solution.

The observations are recorded in Table 4.5 and shown in Fig. 4.5

(b) Effect of lipid composition :

Egg lecithin and cholesterol were taken in various molar ratios and the liposomes of 5FU and MTX were prepared. The effect of lipid composition on encapsulation efficiency of 5FU and MTX are recorded in Table 4.6 and shown in Fig. 4.6.

4.247 Incorporation of liposomes of 5FU and MTX into cream bases :

To study the effect of semisolid bases on the in vitro permeation and in vivo efficacies, the liposomes of 5FU and MTX were incorporated separately into four different bases viz. hydrous emulsifying ointment base I.P, cetomacrogol cream base B.P., HPMC K4M gel base and Carbopol 941 gel base. The gel bases were prepared as per the procedure given in Chapter 3. (section 3.251). The gel bases had a viscosity of about 1 lakh cps.

A common procedure was adopted for incorporation of liposomes of 5FU and MTX into the different bases.

The selected batches of liposomal suspension of both 5FU and MTX were centrifuged and the supernatant was decanted. The liposomal pellet was weighed and required

TABLE 4.5

EFFECT OF IONIC STRENGTH OF CALCIUM CHLORIDE ON ENTRAPMENT OF 5FU AND MTX.

Millimolar ratio of lecithin : cholesterol - 0.127 : 0.127

Calcium Chloride mM	% entrapment	
	5FU	MTX
10	4.20	5.12
25	9.17	9.00
50	9.09	9.20
100	5.25	8.10

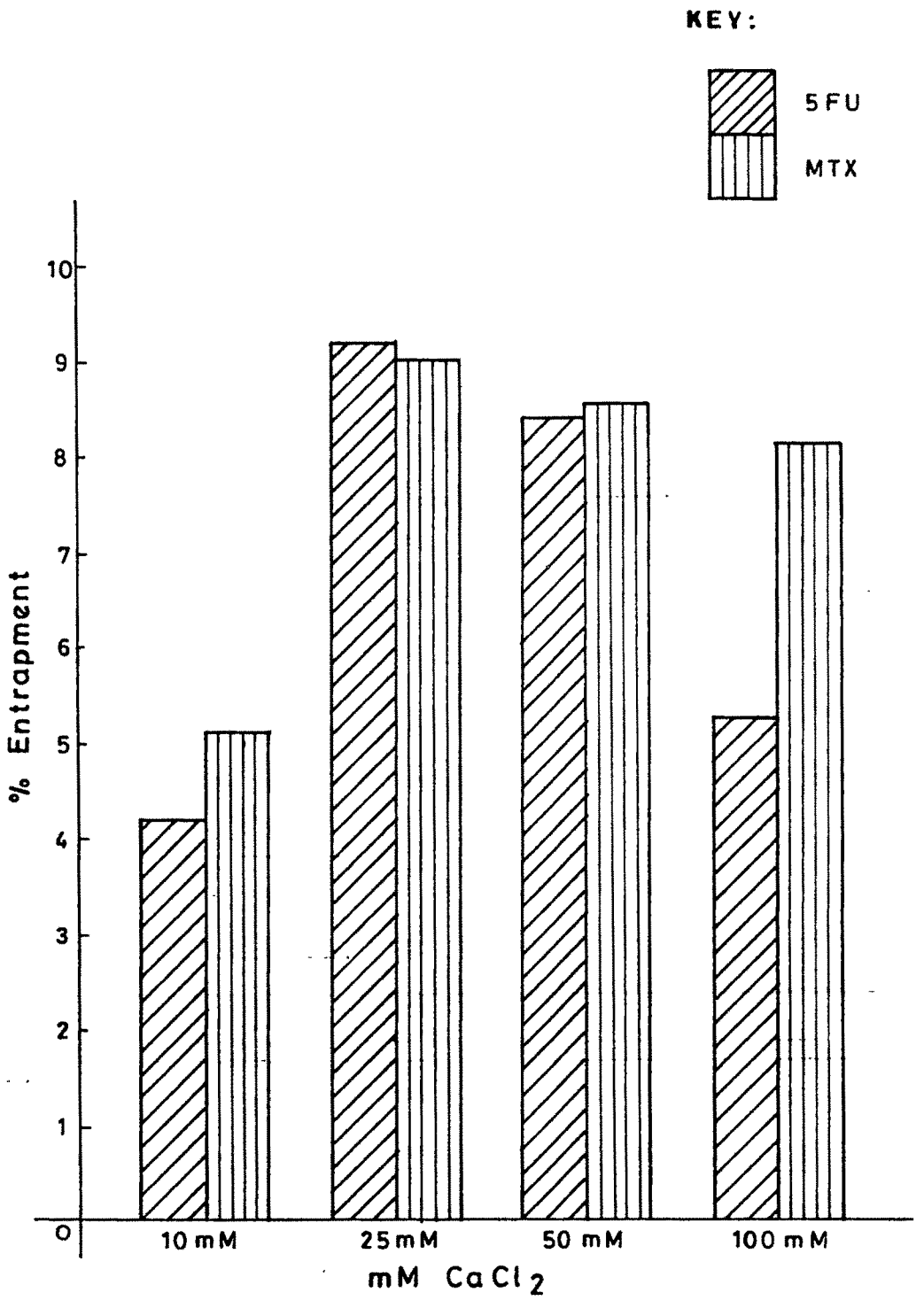


FIG. 4.5: EFFECT OF IONIC STRENGTH OF CALCIUM CHLORIDE ON % ENTRAPMENT OF 5FU AND MTX

TABLE 4.6

EFFECT OF MILLIMOLAR RATIO OF LECITHIN AND CHOLESTEROL ON PERCENTAGE ENTRAPMENT OF 5FU AND MTX.

Millimolar ratio of lecithin : cholesterol	% entrapment	
	5FU	MTX
0.127 : 0	0.49 incomplete flocculation	0.75 incomplete flocculation
0.127 : 0.032	1.23	2.01
0.127 : 0.064	3.13	3.53
0.127 : 0.096	8.07	7.55
0.127 : 0.127	9.17	9.01
0.127 : 0.255	9.14	9.02

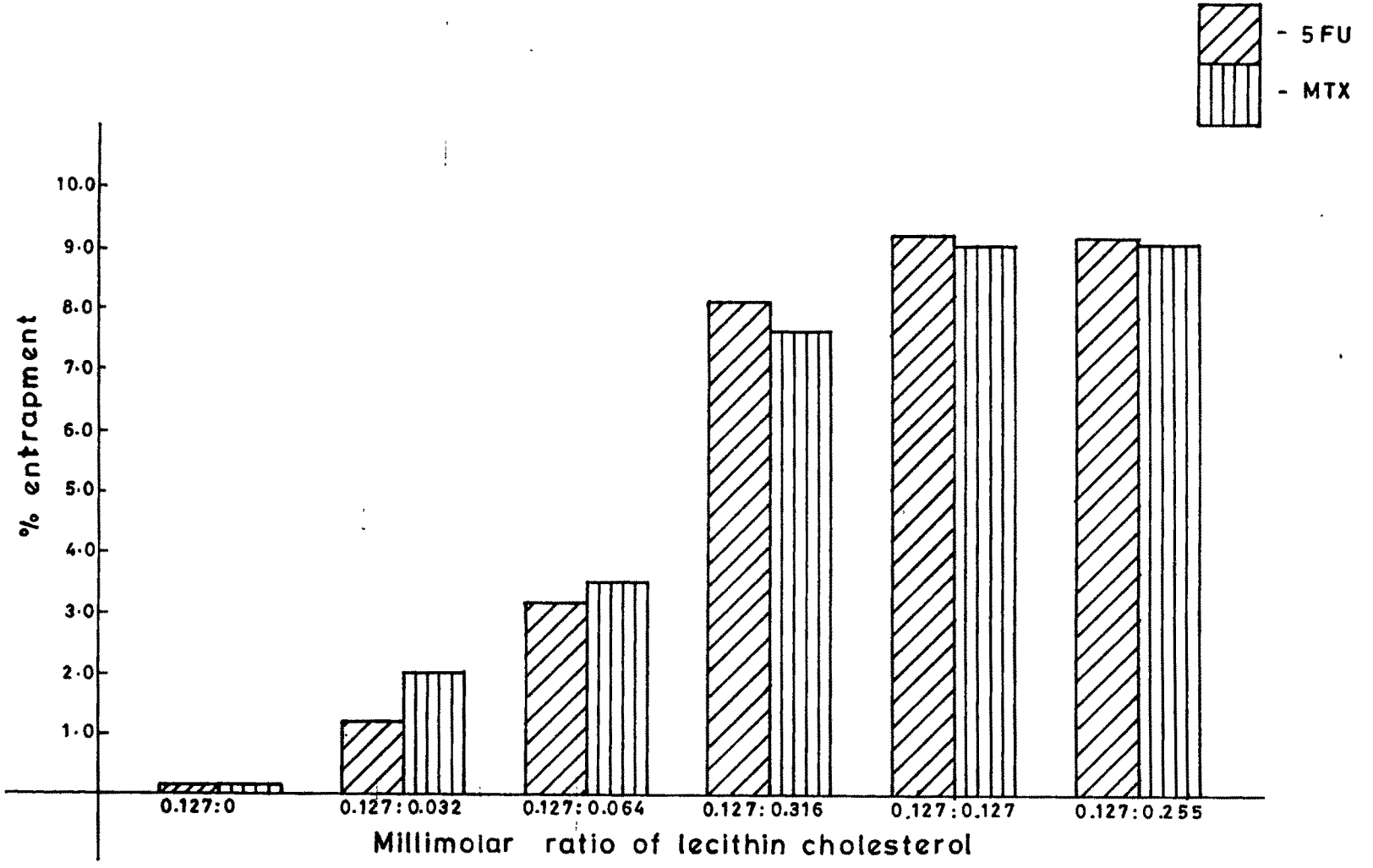


FIG. 4.6 : EFFECT OF MILLIMOLAR RATIO OF LECITHIN AND CHOLESTEROL ON % ENTRAPMENT OF 5FU AND MTX

amount of the base was added to give a cream containing 0.15% w/w of the drug. The liposomes were uniformly distributed into the base by gentle mixing on a pill tile. The liposomal creams were assayed for the drug content by the procedure given in chapter 3 (section 3.243). The composition of various liposomal preparations are given in Tables 4.7 and 4.8 for 5FU and MTX respectively.

4.25 In-vitro Permeation Studies :

The in vitro permeation studies of the liposomal formulations of 5FU and MTX were carried out on rat skin using a vertical type of in vitro permeation apparatus.

(a) Preparation of rat skin :

The skin from freshly sacrificed rats were excised, carefully shaved and defatted by ether treatment, washed with PBS and used for the permeation studies.

(b) In vitro permeation study :

The permeation study was carried out on a vertical type of membrane permeation system. The system consisted of a hollow glass tube open at both ends with outer diameter (o.d) of 2cms and length of 6cms. 400mg of each formulation was applied on the epidermal surface of the rat skin which was tied to one end of the permeation tube. The tube was dipped flush to the surface of 25 ml of PBS placed in the receptor compartment maintained at 37.5 $\pm$ 0.5^{o}C and stirred at 50rpm with a magnetic stirrer (Fig. 4.7, plate 4). Care was exercised to remove any air bubbles between underside of the skin and solution in the

TABLE 4.7

LIPOSOMAL FORMULATIONS OF 5FU IN VARIOUS BASES

Drug concentration - 0.15 % w/w

Base	Ratio of lecithin : Cholesterol (mM)				
	0:0	0.381:0.381	0.761:0.381	0.381:0.761	0.635:0.635
Hydrous emulsifying base I.P.	FA0	FA1	FA2	FA3	FA4
Cetomacrogol cream B.P.	FB0	FB1	FB2	FB3	FB4
HPMC K4M gel base	FC0	FC1	FC2	FC3	FC4
Carbopol 941 gel base	FD0	FD1	FD2	FD3	FD4

TABLE 4.8

LIPOSOMAL FORMULATIONS OF MTX IN VARIOUS BASES

Drug Concentration - 0.15 % w/w

Base	Ratio of lecithin : cholesterol (mM)				
	0:0	0.635:0.635	1.269:0.635	0.635:1.269	1.269:1.269
Hydrous emulsifying base I.P.	MA0	MA1	MA2	MA3	MA4
Cetomacrogol emulsifying cream B.P.	MB0	MB1	MB2	MB3	MB4
HPMC K4M gel base	MC0	MC1	MC2	MC3	MC4
Carbopol 941 gel base	MD0	MD1	MD2	MD3	MD4

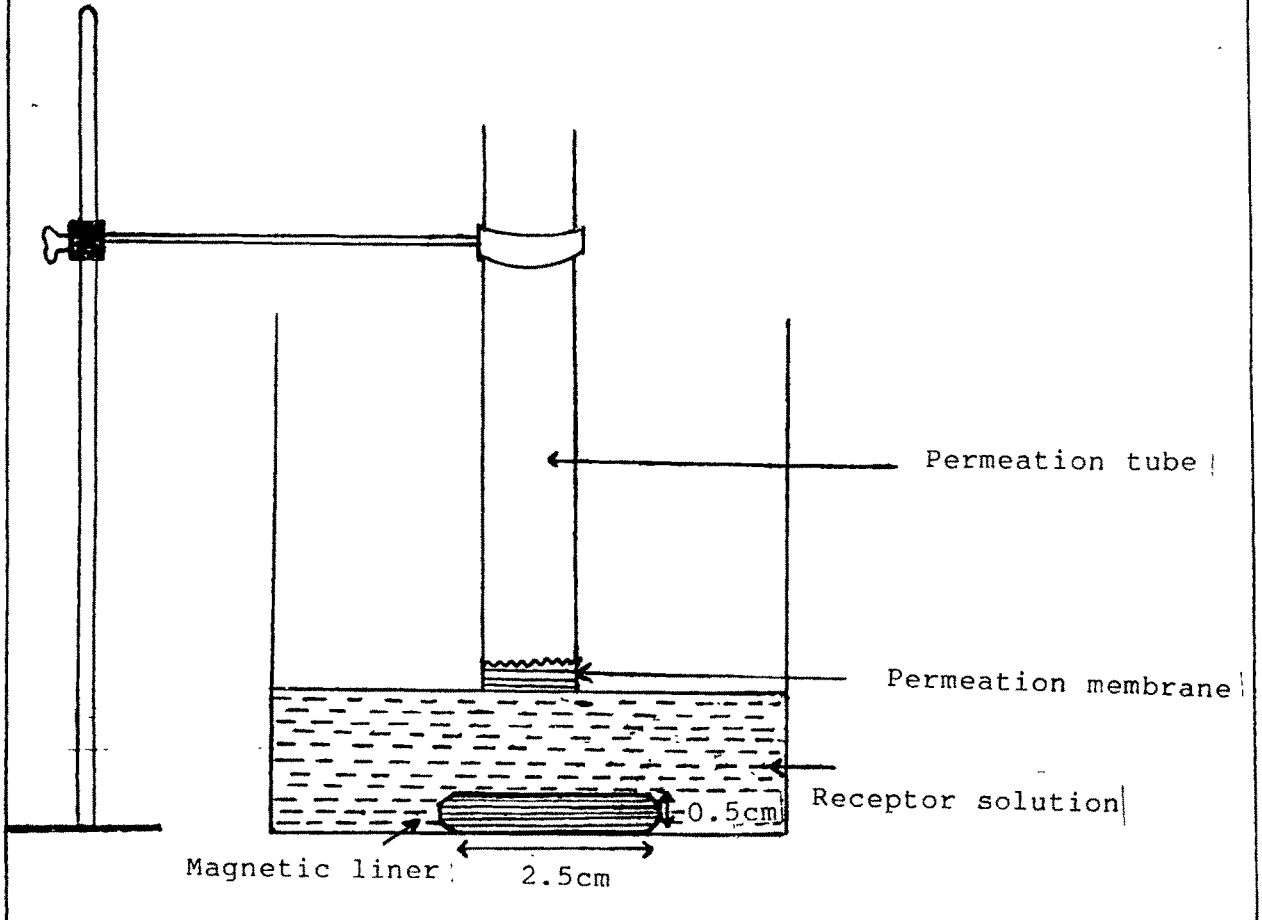
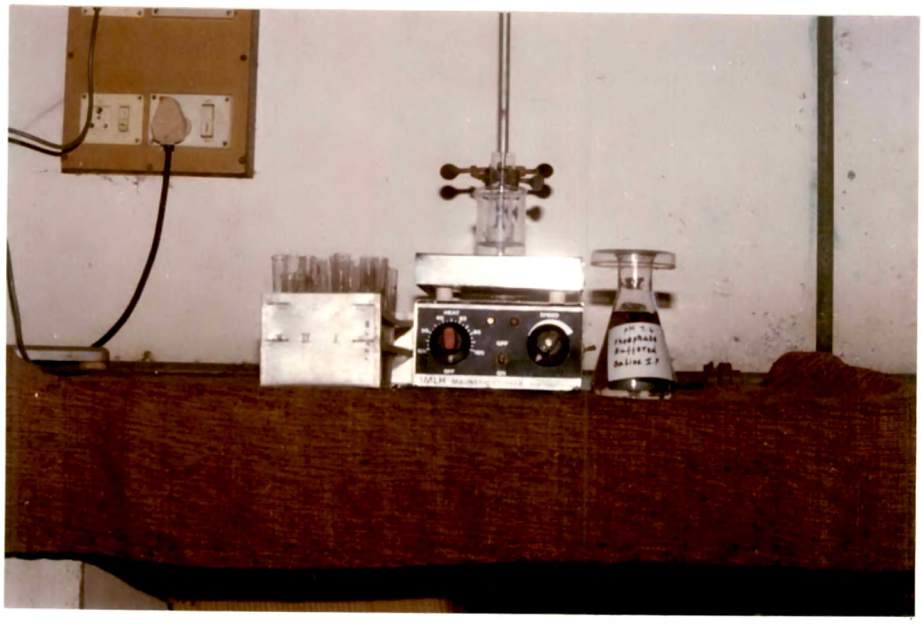


FIG 4.7 IN VITRO PERMEATION APPARATUS

PLATE - 4

IN VITRO PERMEATION APPARATUS



receptor compartment. 2ml aliquots were withdrawn at each sampling time interval from receptor cell and the concentration of 5FU and MTX were determined spectrophotometrically by the procedure given under calibration curve for 5FU and MTX in PBS (Section 4.233). Negative and positive blanks were run simultaneously to ensure noninterference of skin leachings and the components of the base. All permeation runs and sample analysis were carried out six times over a period of six hours.

The mean of the cummulative percentage of 5FU and MTX diffusing across the rat skin at each sampling time point were determined and the data are recorded in Tables-4.9 and 4.10 respectively and shown graphically in Figs.4.8a to 4.9b.

4.26 Stability Studies :

The stability of the liposomal formulations of 5FU and MTX was evaluated in four semisolid bases viz. hydrous emulsifying ointment base I.P, cetomacrogol cream base (B.P), HPMC K4M gel base and carbopol gel base. THe bases were prepared as per the procedure given in chapter 3 (Section 3.241).

4.261 Preparation of the liposomal cream for stability studies :

A common procedure was followed for the preparation of all the formulations.

TABLE 4.9

MEAN CUMULATIVE PERCENTAGE PERMEATION OF 5FU ACROSS RAT SKIN

Time in minutes	MEAN CUMULATIVE % PERMEATION OF 5FU (± S.D.) Formulation No.																							
	FA0	FFA1	FA1	FA2	FA3	FA4	FB0	FFB1	FB1	FB2	FB3	FB4	FC0	FFC1	FC1	FC2	FC3	FC4	FD0	FFD1	FD1	FD2	FD3	FD4
60	20.13 (0.117)	19.14 (0.195)	12.55 (0.121)	11.68 (0.231)	11.63 (0.312)	10.72 (0.112)	11.27 (0.932)	10.72 (0.912)	7.42 (0.751)	7.48 (0.695)	7.47 (0.799)	7.18 (0.717)	15.27 (0.972)	14.55 (0.732)	5.77 (0.712)	5.89 (0.995)	5.84 (0.719)	5.91 (1.213)	16.05 (1.057)	15.25 (0.912)	6.99 (0.715)	6.89 (0.715)	6.90 (0.193)	6.78 (0.975)
120	39.58 (0.312)	37.56 (1.011)	23.52 (0.732)	21.72 (0.712)	21.81 (0.732)	20.11 (0.932)	20.30 (0.937)	19.29 (0.923)	13.35 (0.999)	13.25 (0.514)	13.44 (1.595)	12.93 (0.315)	28.22 (0.765)	26.81 (0.512)	10.38 (1.131)	10.53 (0.912)	10.50 (0.912)	10.63 (0.975)	27.29 (0.993)	25.89 (0.755)	12.82 (0.999)	12.62 (1.677)	12.65 (1.592)	12.43 (1.091)
180	42.24 (1.312)	39.87 (1.375)	25.03 (1.011)	23.37 (0.923)	23.26 (1.621)	21.45 (1.013)	38.34 (1.412)	36.39 (1.012)	25.22 (0.973)	25.82 (0.614)	25.38 (1.512)	24.42 (1.214)	49.18 (0.632)	46.62 (0.932)	19.61 (0.971)	19.85 (0.795)	19.84 (1.912)	20.08 (1.117)	41.31 (1.312)	39.16 (0.115)	18.65 (0.712)	18.7 (0.913)	18.40 (2.691)	18.98 (1.117)
240	50.48 (0.397)	47.80 (1.213)	29.79 (0.712)	27.32 (1.397)	27.62 (0.951)	25.47 (0.732)	45.11 (0.195)	42.77 (0.554)	29.67 (1.112)	29.33 (0.497)	29.86 (0.959)	28.73 (0.395)	58.26 (0.713)	55.17 (0.639)	23.07 (1.111)	23.46 (2.012)	23.34 (1.173)	23.63 (0.195)	53.57 (2.312)	50.75 (0.619)	24.48 (0.497)	24.13 (1.317)	24.15 (3.12)	23.75 (0.919)
300	53.25 (0.539)	50.58 (0.705)	32.93 (1.312)	30.61 (1.011)	30.52 (1.011)	28.56 (0.732)	47.36 (0.977)	44.85 (0.497)	31.18 (0.931)	30.61 (1.011)	31.36 (1.012)	30.18 (0.951)	61.80 (1.381)	58.59 (0.395)	24.23 (1.319)	24.63 (0.935)	24.51 (0.913)	24.81 (0.817)	57.91 (0.975)	54.95 (0.356)	26.81 (1.395)	26.42 (1.421)	26.45 (1.017)	25.99 (1.375)
360	53.29 (0.613)	50.63 (0.551)	33.06 (0.791)	31.86 (0.932)	31.98 (0.932)	29.49 (0.932)	49.62 (1.312)	47.09 (1.013)	32.64 (0.595)	32.36 (1.412)	32.85 (0.915)	31.59 (0.199)	62.49 (1.213)	59.37 (0.563)	25.38 (1.137)	25.69 (1.113)	25.68 (0.691)	25.99 (1.316)	60.29 (1.011)	57.09 (0.316)	27.97 (0.919)	27.56 (0.575)	27.60 (1.512)	27.12 (0.95)

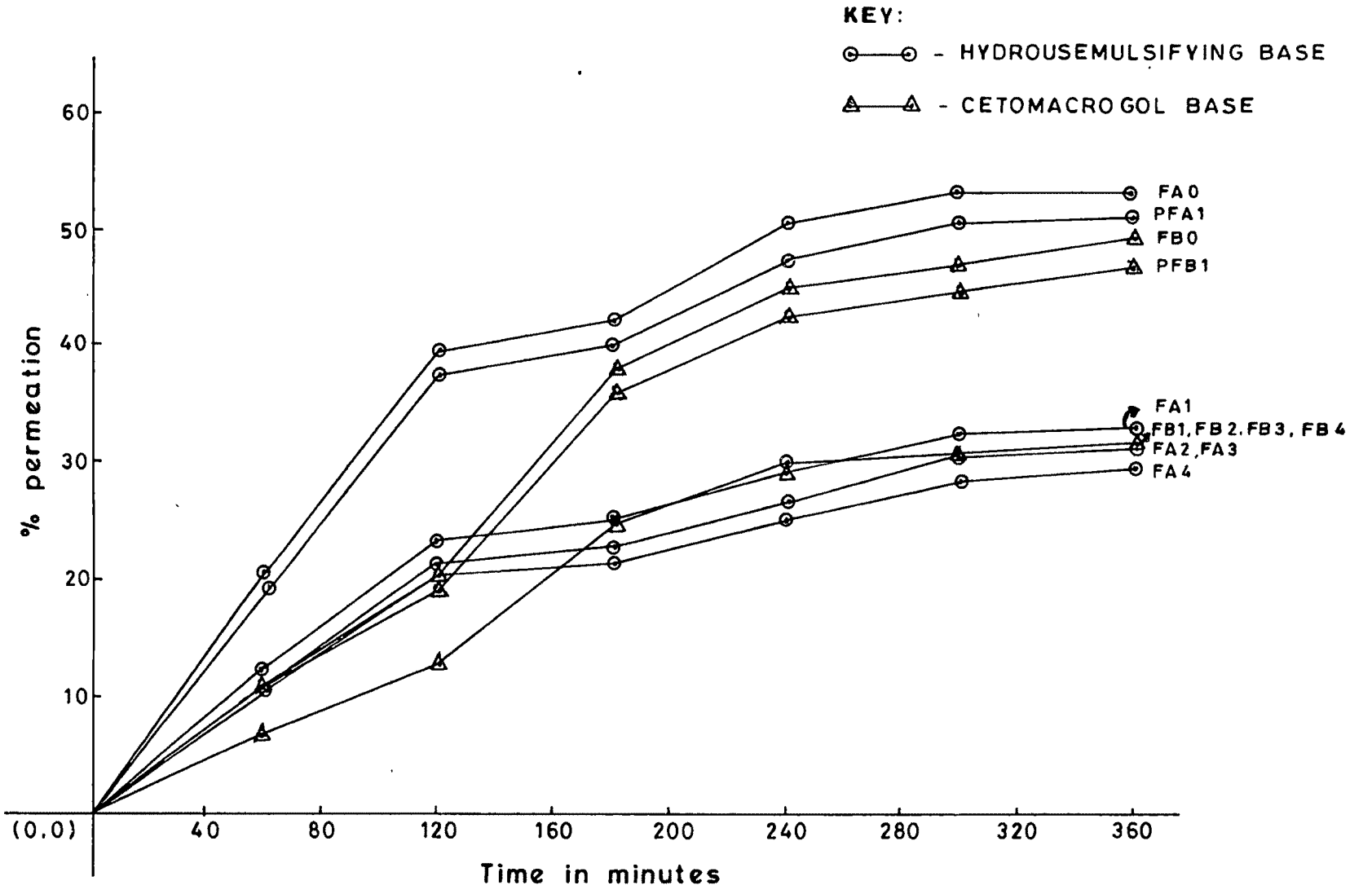


FIG. 4.8(a) : MEAN CUMMULATIVE PERCENTAGE PERMEATION OF 5FU ACROSS RAT SKIN

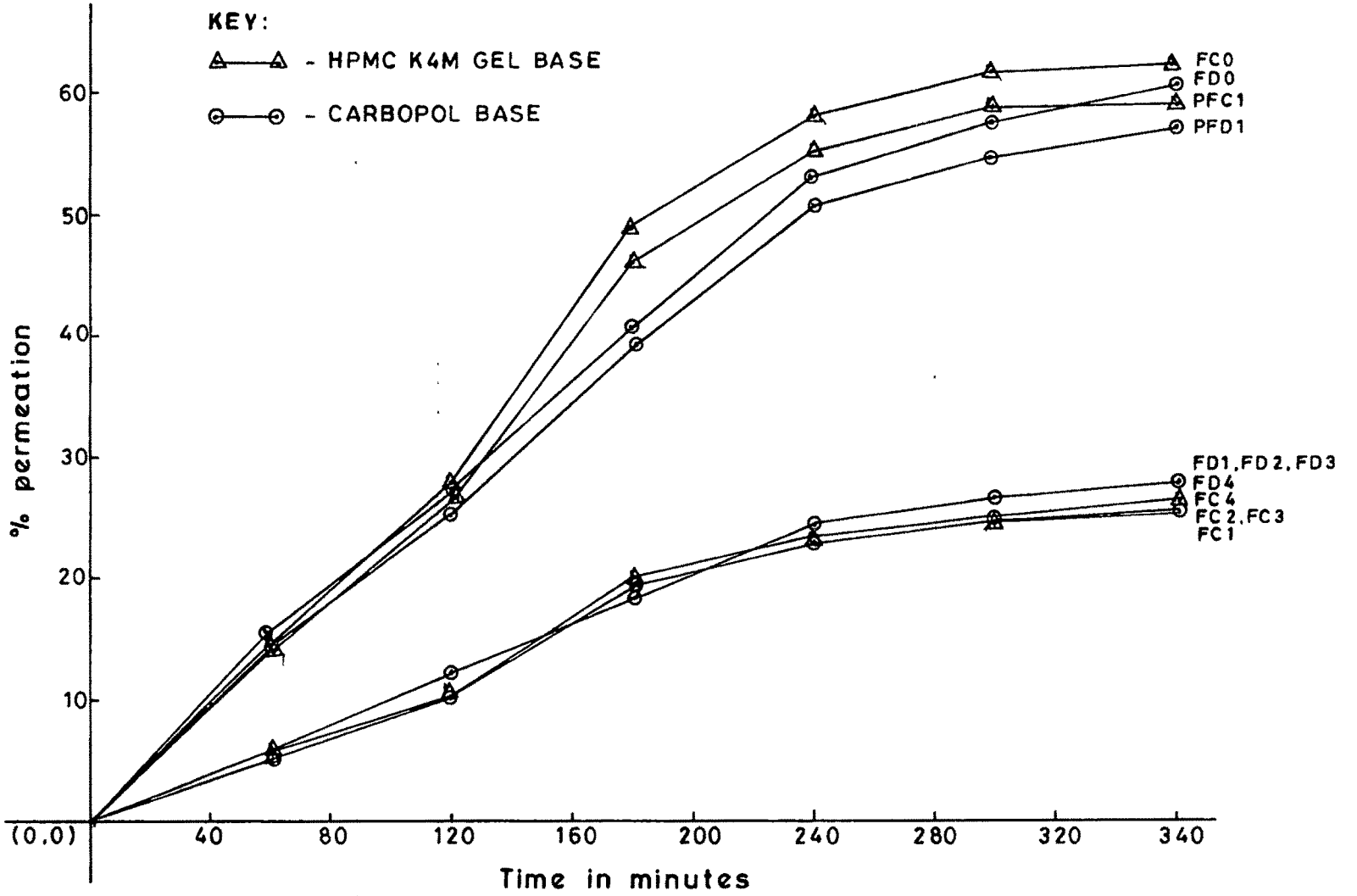


FIG. 4.8(b): MEAN CUMMULATIVE PERCENTAGE PERMEATION OF 5FU ACROSS RAT SKIN

TABLE 4.10

MEAN CUMULATIVE PERCENTAGE PERMEATION OF MTX ACROSS RAT SKIN

Time in minutes	MEAN CUMULATIVE % PERMEATION OF MTX (± S.D.) Formulation No.																						
	MA0	FMA1	MA1	MA2	MA3	MA4	MB0	FMB1	MB1	MB2	MB3	MB4	MC0	FMC1	MC1	MC2	MC3	MC4	MD0	FMD1	MD1	MD2	MD3	MD4
60	12.76 (1.177)	12.92 (0.973)	3.99 (0.975)	5.49 (0.975)	4.49 (1.312)	3.79 (0.712)	9.04 (1.012)	8.59 (0.497)	4.10 (1.412)	5.02 (0.979)	4.75 (1.011)	4.41 (0.197)	8.51 (0.712)	8.08 (0.812)	2.53 (0.171)	2.94 (0.717)	2.61 (1.000)	2.49 (0.275)	7.03 (0.117)	6.73 (0.815)	2.29 (0.275)	3.01 (0.377)	2.52 (0.675)	2.25 (0.139)
120	24.37 (0.959)	23.13 (0.871)	7.97 (1.391)	10.98 (1.635)	8.98 (0.989)	7.62 (1.132)	12.75 (0.179)	12.22 (0.614)	5.93 (1.312)	7.25 (1.132)	6.87 (0.132)	6.36 (0.512)	22.08 (0.732)	21.90 (0.395)	7.60 (0.917)	8.82 (0.171)	7.84 (0.917)	7.44 (0.515)	20.58 (0.915)	19.53 (0.397)	6.89 (1.327)	9.03 (1.011)	7.57 (0.997)	6.74 (1.312)
180	30.07 (1.351)	28.57 (0.857)	9.63 (0.979)	11.83 (0.732)	10.90 (1.117)	9.23 (0.717)	17.37 (0.975)	16.48 (0.517)	7.75 (1.117)	9.48 (0.875)	8.98 (1.312)	8.32 (1.132)	35.26 (0.675)	33.43 (0.515)	10.77 (0.191)	12.50 (0.919)	11.11 (0.917)	10.54 (0.275)	28.91 (1.011)	27.41 (0.412)	9.76 (0.197)	10.79 (0.917)	10.73 (0.732)	9.55 (2.137)
240	33..28 (1.027)	31.56 (0.832)	10.89 (1.542)	12.87 (1.017)	12.19 (0.975)	10.31 (0.319)	19.01 (1.011)	18.06 (1.112)	8.66 (0.979)	10.59 (0.667)	10.04 (0.913)	9.30 (0.142)	42.25 (0.715)	41.01 (0.732)	13.31 (0.237)	15.44 (1.011)	13.75 (1.007)	13.02 (0.731)	39.17 (1.721)	37.07 (0.913)	12.06 (0.721)	13.39 (0.312)	13.25 (0.713)	11.79 (0.713)
300	37.33 (1.321)	35.77 (0.945)	11.96 (1.113)	13.46 (1.001)	13.47 (0.659)	11.39 (0.777)	19.59 (1.432)	18.61 (0.915)	8.67 (1.329)	10.65 (1.363)	10.13 (1.132)	9.31 (0.795)	47.55 (0.115)	45.03 (0.945)	14.57 (0.979)	16.91 (0.717)	15.04 (0.919)	14.26 (0.471)	39.24 (0.275)	37.17 (0.817)	12.64 (0.317)	14.05 (0.365)	13.89 (0.612)	12.36 (0.695)
360	38.26 (1.617)	36.23 (0.959)	12.52 (0.975)	13.97 (0.517)	14.11 (1.017)	11.94 (0.715)	22.58 (1.395)	21.38 (0.853)	10.03 (1.137)	11.17 (0.675)	11.62 (1.312)	10.77 (0.515)	50.27 (0.617)	47.51 (0.599)	15.21 (1.021)	17.65 (0.531)	15.69 (0.145)	14.88 (0.717)	40.25 (0.325)	38.24 (0.421)	13.21 (1.312)	14.57 (0.997)	14.52 (1.312)	12.96 (1.012)

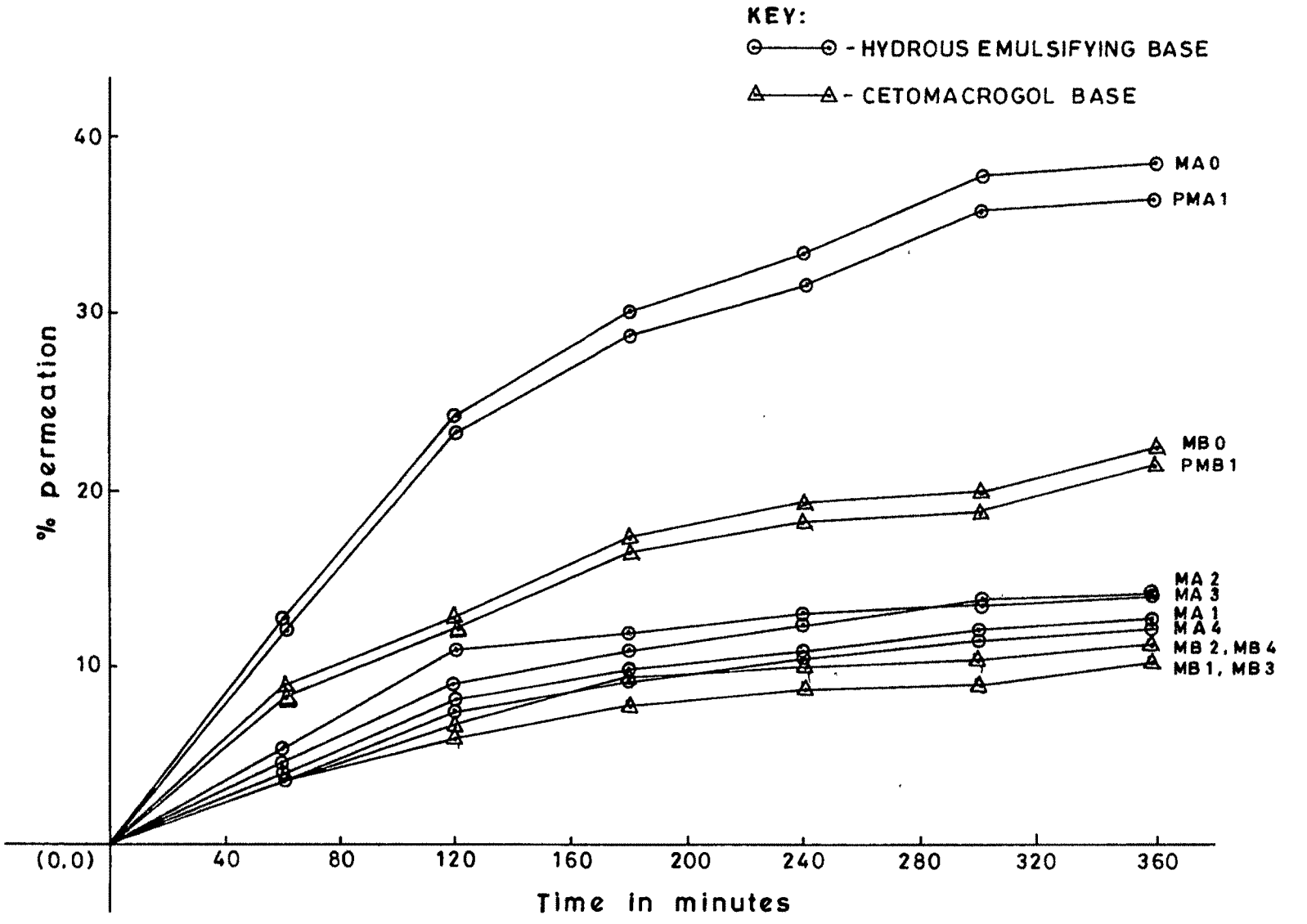


FIG. 4.9(a): MEAN CUMMULATIVE PERCENTAGE PERMEATION OF MTX ACROSS RAT SKIN

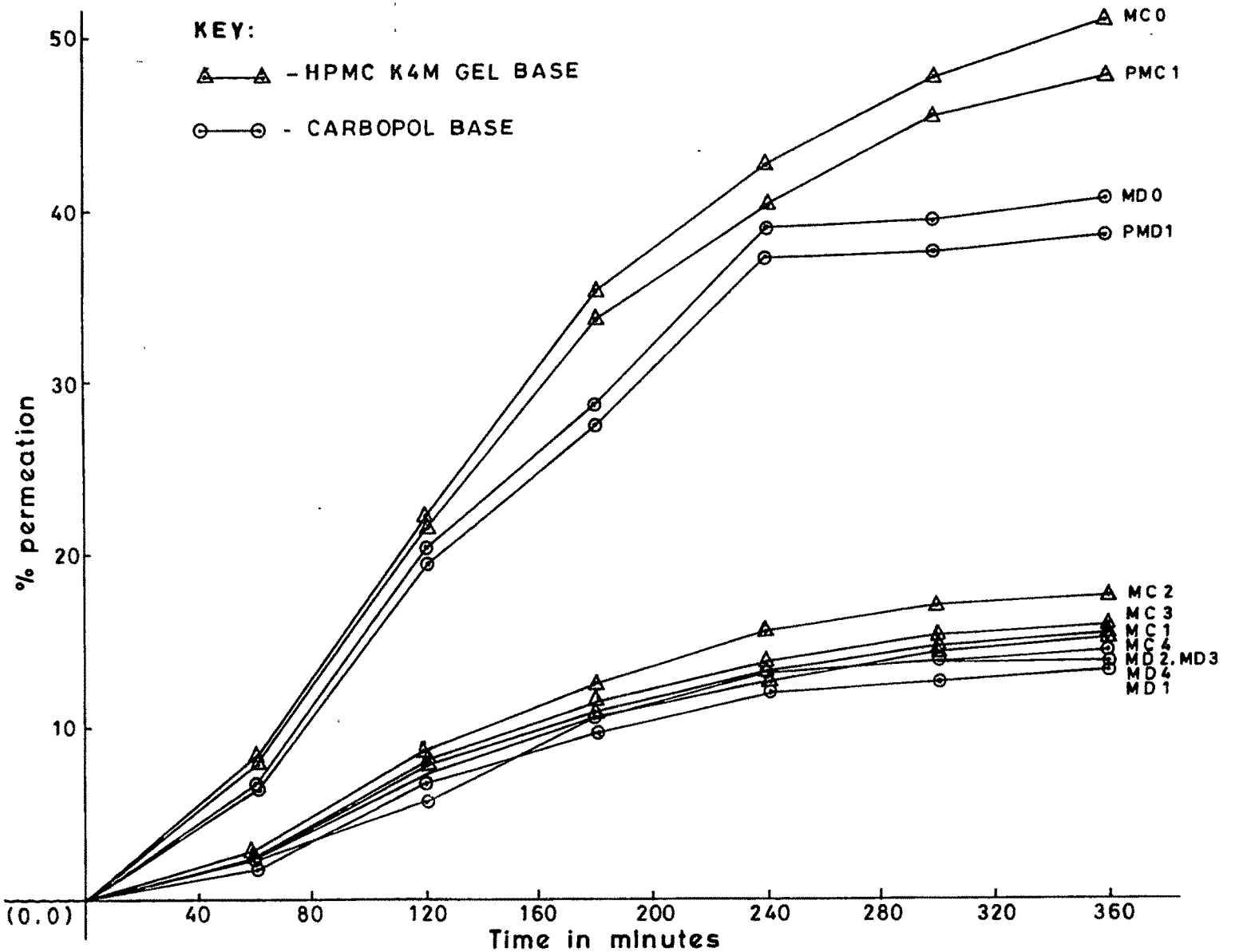


FIG. 4.9(b): MEAN CUMMULATIVE PERCENTAGE PERMEATION OF MTX ACROSS RAT SKIN

A quantity of liposomes equivalent to 45mg of drug (5FU/MTX) was weighed accurately and levigated with 1.0g of the base on a pill tile. This was then diluted in geometric proportion to a final weight of 30g. Each of the creams were assayed for initial concentration of drug (5FU/MTX) in triplicate by the procedure described in chapter 3 (section 3.243).

Then each of the liposomal cream was filled in lacquered aluminium tubes (5g/tube) and stored at refrigeration temperture ($4^{o}C$). At each sampling point, one tube was withdrawn, the drug content was determined in triplicate as per the procedure described in chapter 3 (section 3.243) and the in vitro permeation study was conducted thrice using the rat skin as per the procedure described in section 4.25. The results of the percentage drug (5FU/MTX) remaining is given in Table 4.17. The in vitro permeation data at each time interval for both 5FU and MTX liposomal formulations are given in Tables 4.18 and 4.19 respectively.

4.3 RESULTS AND DISCUSSION

Egg yolk lecithin was selected as the model phospholipid because of it's widespread use, easy availability and ease of handling.

Maximum encapsulation efficiency was achieved by Bangham's thin film technique for 5FU and by reverse phase evaporation technique for MTX (Table 4.11). Hence these two methods were adopted for all further investigations.

TABLE 4.11

EFFECT OF METHODS OF PREPARATION ON PERCENTAGE ENTRAPMENT OF 5FU AND MTX IN LIPOSOMES.

Millimolar ratio of lecithin : cholesterol - 0.127:0.127

S.No	Method of preparation	% entrapment	
		5FU	MTX
1.	Thin film technique	9.16	-
2.	Modified method of szoka and Papahadjopoulous	7.91	-
3.	Double emulsification method	-	6.85
4.	Reverse phase evaporation	-	9.01

From Table 4.5 and Fig. 4.5 it may be inferred that the ionic strength of calcium chloride solution seems to play a role in flocculation of liposomes and entrapment of the drug. Calcium chloride solution of 10mM concentration resulted in incomplete flocculation of liposomes. Solutions of 25mM and 50 mM gave the best result in terms of complete flocculation and drug entrapment. 100mM concentration of calcium chloride solution resulted in lowering of the drug loading. All further batches of liposomes were prepared using 25mM concentration of calcium chloride.

The ratio of lecithin : cholesterol also played an important role in the drug loading (Table 4.6 and Fig. 4.6). In liposomes formed without cholesterol it was observed that drug entrapment was low and the pelleting of liposomes was incomplete. As the cholesterol concentration was increased, the percentage of the drug entrapped increased and complete pelleting of the liposomes was possible. This may be due to the fact that cholesterol has the ability to render the bilayer structure more rigid, thereby preventing the leaching of the drug from the vesicles(18). Maximum drug loading was achieved in liposomes formulated with 1:1 molar ratio of lecithin : cholesterol for both 5FU and MTX.

At 1:1 molar ratio of lecithin : cholesterol, the effect of different concentrations of the lipids on loading of 5FU and MTX are recorded in Tables 4.12 and 4.13 respectively and shown in Fig. 4.10.

As the millimolar concentration of lecithin : cholesterol was increased from 0.127 : 0.127 to 0.381 : 0.381

TABLE 4.12

EFFECT OF MILLIMOLAR RATIO OF LECITHIN AND CHOLESTEROL ON PERCENTAGE ENTRAPMENT OF 5FU

Millimolar ratio of lecithin : cholesterol	% of 5FU entrapped
0.127 : 0.127	9.17
0.253 : 0.253	15.33
0.381 : 0.381	27.50
0.508 : 0.508	27.42
0.635 : 0.635	27.40
0.761 : 0.381	27.51
0.381 : 0.761	27.60

TABLE 4.13

EFFECT OF MILLIMOLAR RATIO OF LECITHIN AND CHOLESTEROL ON PERCENTAGE ENTRAPMENT OF MTX

Millimolar ratio of lecithin : cholesterol	% of MTX entrapped
0.127 : 0.127	9.00
0.253 : 0.253	15.03
0.381 : 0.381	28.24
0.508 : 0.508	35.11
0.635 : 0.635	45.23
1.269 : 1.269	43.51
0.635 : 1.269	45.16
1.269 : 0.635	43.02

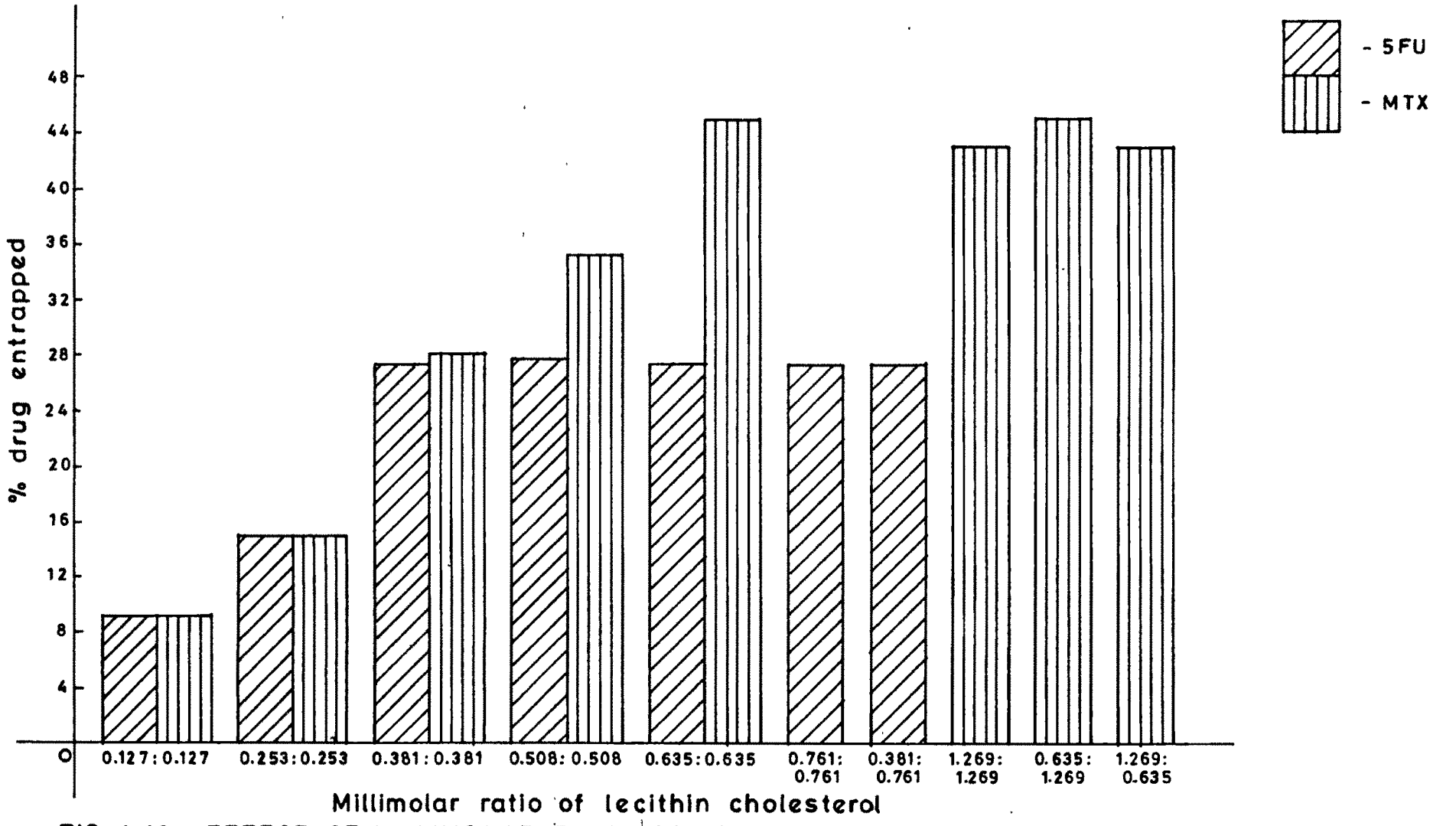


FIG. 4.10 : EFFECT OF MILLIMOLAR RATIO OF LECITHIN AND CHOLESTEROL ON PERCENTAGE ENTRAPMENT OF 5FU ANT MTX

the percentage entrapment of 5FU increased three folds (i.e., from 9.17% to 27.50%). However further increase in lipid concentration did not result in higher drug loading. In case of liposomes of MTX a five fold increase in percentage drug entrapment was achieved by increasing the millimolar concentration of lipids from 0.127 : 0.127 to 0.635:0.635 (i.e., from 9.0% to 45.0%). Above this no significant increase in drug loading was observed.

Although varying the millimoar ratio of lecithin : cholesterol from 0.381:0.381 (1:1) to 0.761:0.381 (2:1) or 0.381:0.761 (1:2) for 5FU liposomes and from 0.635:0.635 (1:1) to 0.635:1.269 (1:2) or 1.269:0.635 (2:1) for MTX liposomes did not have any significant effect on drug loading, liposomal formulations with these composition of lecithin and cholesterol were prepared to evaluate the effect of lipid concentration on the in vitro permeation of 5FU and MTX across the rat skin.

Particle size :

From Table 4.4 it is seen that particle size distribution of liposomes prepared using thin film technique lie between 1.0-6.0μm while those prepared using reverse phase evaporation technique lie between 1.0-10.0μm.

From plate 1 it is confirmed that the liposomes prepared were spherical in shape.

From the transmission electron micrographs it is seen that the liposomes are multilamellar. The electron micrographs of MTX appear elliptical which may be due to improper placement of the grid in the sample compartment of the electron microscope.

The results of assay of 5FU and MTX from all formulations are recorded in Table 4.14. It was found that in all cases the percentage recovery was within 98-100%.

In vitro release of 5FU and MTX from liposomal formulations :

The mean cummulative percentages of 5FU and MTX released at each sampling time point are recorded in Tables 4.9 and 4.10 and shown in Figs. 4.8 and 4.9 respectively. For each formulation the mean permeability coefficient P(cm/sec) was calculated from the formula(19).

$$P(cm/sec) = \frac{1}{C_D A} \cdot \frac{dc}{dt}$$

Where P = permeability coefficient in cm/sec.

C_D = donor side concentration ($\mu g/cm^3$)

A = area of diffusion (cm^2)

$\frac{dc}{dt}$ = slope of the receptor side concentration-time profile ($\mu g/sec$).

The values of the mean permeability coefficient for the formulations of 5FU and MTX are recorded in Tables 4.15 and 4.16 and shown in Figs. 4.11 and 4.12 respectively.

Permeability coefficient values obtained for liposomal formulations in each base were compared statistically using ANOVA with those obtained for the plain drug and the physical mixture in the same base. The permeability coefficient values between different formulations in the same base and different bases were also compared statistically using ANOVA technique. The differences were considered significant at $P<0.05$.

TABLE 4.14

ASSAY OF 5FU AND MTX FROM FORMULATIONS

Formulation No.	Mean Assay (%) (± S.D)	Formulation No	Mean Assay (%) (± S D.)
FA0	98.95 (0.980)	MA0	99 72 (0.851)
FA1	99.75 (0.910)	MA1	98 57 (0.361)
FA2	100.11 (0.551)	MA2	99.86 (0.715)
FA3	98.99 (0 24)	MA3	98.57 (0.361)
FA4	100.05 (0.651)	MA4	99.90 (0.651)
FB0	98.99 (0 241)	MB0	98.98 (0.851)
FB1	99.57 (0.392)	MB1	99.75 (0 601)
FB2	99.23 (0.741)	MB2	100.11 (0.851)
FB3	99.89 (0.542)	MB3	98.95 (0.980)
FB4	99.54 (0.544)	MB4	99.75 (1 001)
FC0	99.54 (0.751)	MC0	98.99 (0 241)
FC1	98.75 (0.251)	MC1	99 57 (0 360)
FC2	99.72 (0.951)	MC2	99.85 (0.712)
FC3	98.84 (0.751)	MC3	99.29 (0.341)
FC4	100.05 (0.551)	MC4	98.35 (0.518)
FD0	99.55 (0.851)	MD0	98.92 (0.612)
FD1	98.67 (0 365)	MD1	99.75 (0.541)
FD2	98.98 (0.821)	MD2	98.71 (0.921)
FD3	99.34 (0.551)	MD3	99.65 (0.021)
FD4	99.54 (0.751)	MD4	99.21 (0.215)

TABLE 4.15

PERMEATION COEFFICIENT AND PERCENTAGE REDUCTION IN PERMEATION FOR VARIOUS FORMULATIONS OF 5FU

Formulation No.	Permeation coefficient cm/sec	% Reduction permeation (as compared to plain cream)
FA0	1.96×10^{-4}	-
PFA1	1.86×10^{-4}	5.10
FA1	1.22×10^{-4}	37.76
FA2	1.17×10^{-4}	40.31
FA3	1.18×10^{-4}	39.79
FA4	1.08×10^{-4}	44.89
FB0	1.83×10^{-4}	-
PFB1	1.74×10^{-4}	5.62
FB1	1.20×10^{-4}	34.43
FB2	1.19×10^{-4}	34.97
FB3	1.21×10^{-4}	33.88
FB4	1.16×10^{-4}	36.61
FC0	2.30×10^{-4}	-
PFC1	2.18×10^{-4}	5.30
FC1	9.35×10^{-5}	59.35
FC2	9.47×10^{-5}	58.83
FC3	9.47×10^{-5}	58.83
FC4	9.58×10^{-5}	58.35
FD0	2.22×10^{-4}	-
PFD1	2.10×10^{-4}	5.30
FD1	1.03×10^{-4}	53.60
FD2	1.02×10^{-4}	54.05
FD3	1.02×10^{-4}	54.05
FD4	9.99×10^{-5}	55.00

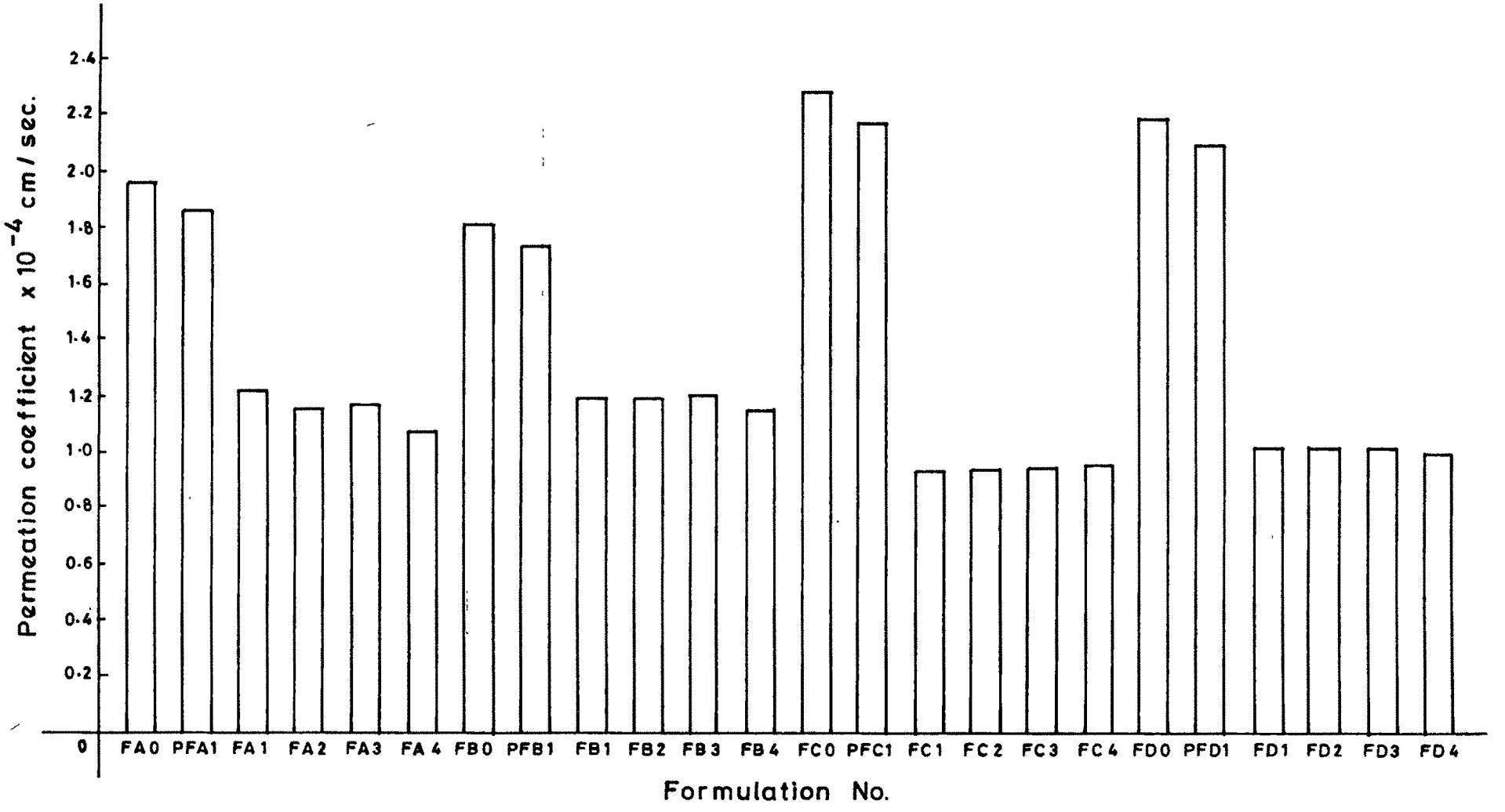


FIG. 4.11 : PERMEABILITY COEFFICIENT VALUES FOR FORMULATIONS OF 5FU

TABLE 4.16

PERMEATION COEFFICIENT AND PERCENTAGE IN PERMEATION REDUCTION FOR VARIOUS FORMULATIONS OF MTX

Formulation No.	Permeation Coefficient cm/sec	% Reduction (as compared to plain cream)
MA0	1.41×10^{-4}	-
PMA1	1.34×10^{-4}	5.00
MA1	4.61×10^{-5}	67.30
MA2	5.15×10^{-5}	63.48
MA3	5.20×10^{-5}	63.12
MA4	4.40×10^{-5}	68.79
MB0	8.32×10^{-5}	-
PMB1	7.89×10^{-5}	5.10
MB1	3.69×10^{-5}	55.65
MB2	4.12×10^{-5}	50.48
MB3	4.28×10^{-5}	48.55
MB4	3.96×10^{-5}	52.40
MC0	1.85×10^{-4}	-
PMC1	1.75×10^{-4}	5.35
MC1	5.61×10^{-5}	69.68
MC2	6.51×10^{-5}	64.81
MC3	5.78×10^{-5}	68.76
MC4	5.48×10^{-5}	70.38
MD0	1.48×10^{-4}	-
PMD1	1.39×10^{-4}	5.57
MD1	4.87×10^{-5}	67.09
MD2	5.37×10^{-5}	63.72
MD3	5.35×10^{-5}	63.85
MD4	4.78×10^{-5}	67.70

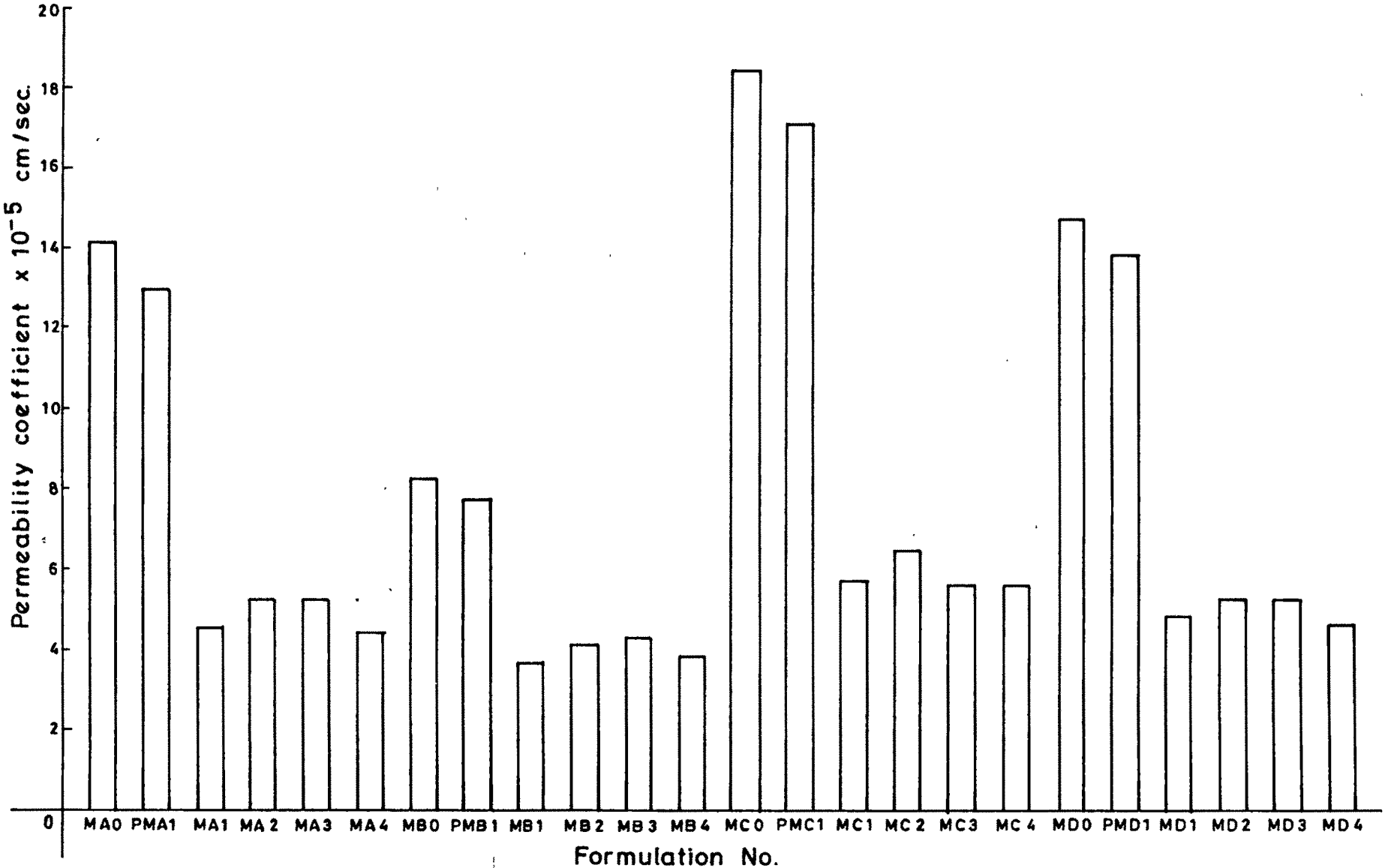


FIG. 4.12: PERMEABILITY COEFFICIENT VALUES FOR FORMULATIONS OF MTX

The percentage reduction in permeability coefficient in case of liposomal formulations as compared to plain drug was calculated from the formula :

$$\text{Percentage reduction } R = \frac{Po - P}{Po} \times 100$$

Where Po = permeability coefficient of control formulation.

P = permeability coefficient of liposomal formulation.

The values of R are recorded in Tables 4.15 & 4.16 and shown in Figs. 4.13 & 4.14 for 5FU and MTX respectively.

From the permeability coefficient data and the R values the following inferences can be drawn :

1. The physical mixture of lecithin : cholesterol : drug (0.381:0.381:0.021mM for 5FU, 0.635:0.635:0.009 mM for MTX) did not have any significant effect on the permeability coefficient as compared to the plain cream.
2. Liposomal formulations significantly decrease the permeation of 5FU and MTX through the skin.
3. In case of hydrous emulsifying ointment base, formulations of 5FU and MTX (formulations FA1-FA4 and MA1-MA4) gave approximately 41% and 66% reductin in permeability coefficient values respectively.
4. Liposomal formulations of 5FU and MTX in cetomacrogol base (formulations FB1-FB4 and MB1-MB4) gave approximately 35% and 52% reduction in permeability coefficient values respectively.
5. In case of the gel bases, the liposomal formulations in HPMC base (formulations FC1-FC4 and MC1-MC4) gave on an average 59% and 68% reduction in permeability coefficient

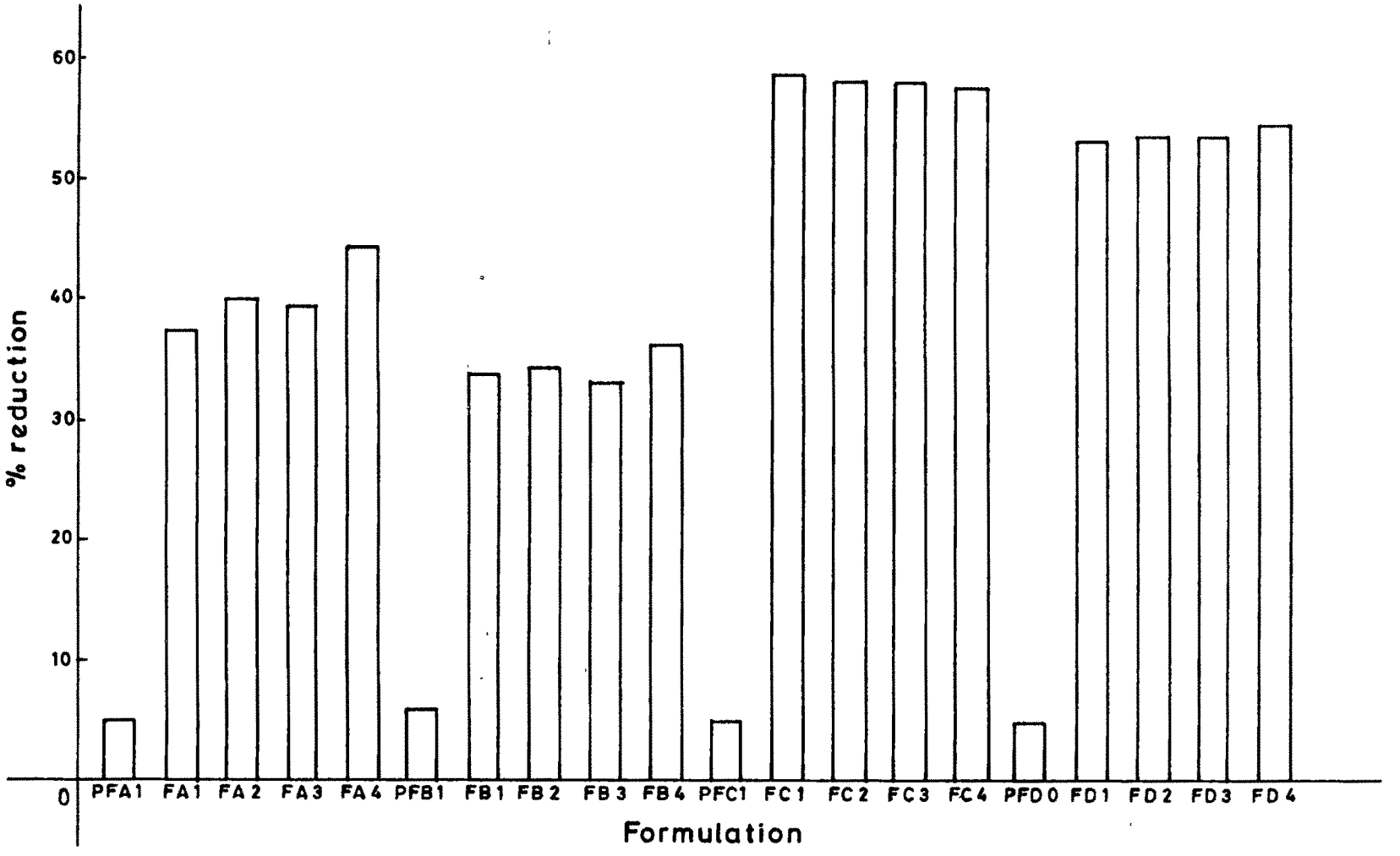


FIG. 4.13: PERCENTAGE REDUCTION IN PERMEABILITY COEFFICIENT VALUES FOR LIPOSOMAL FORMULATIONS OF 5FU

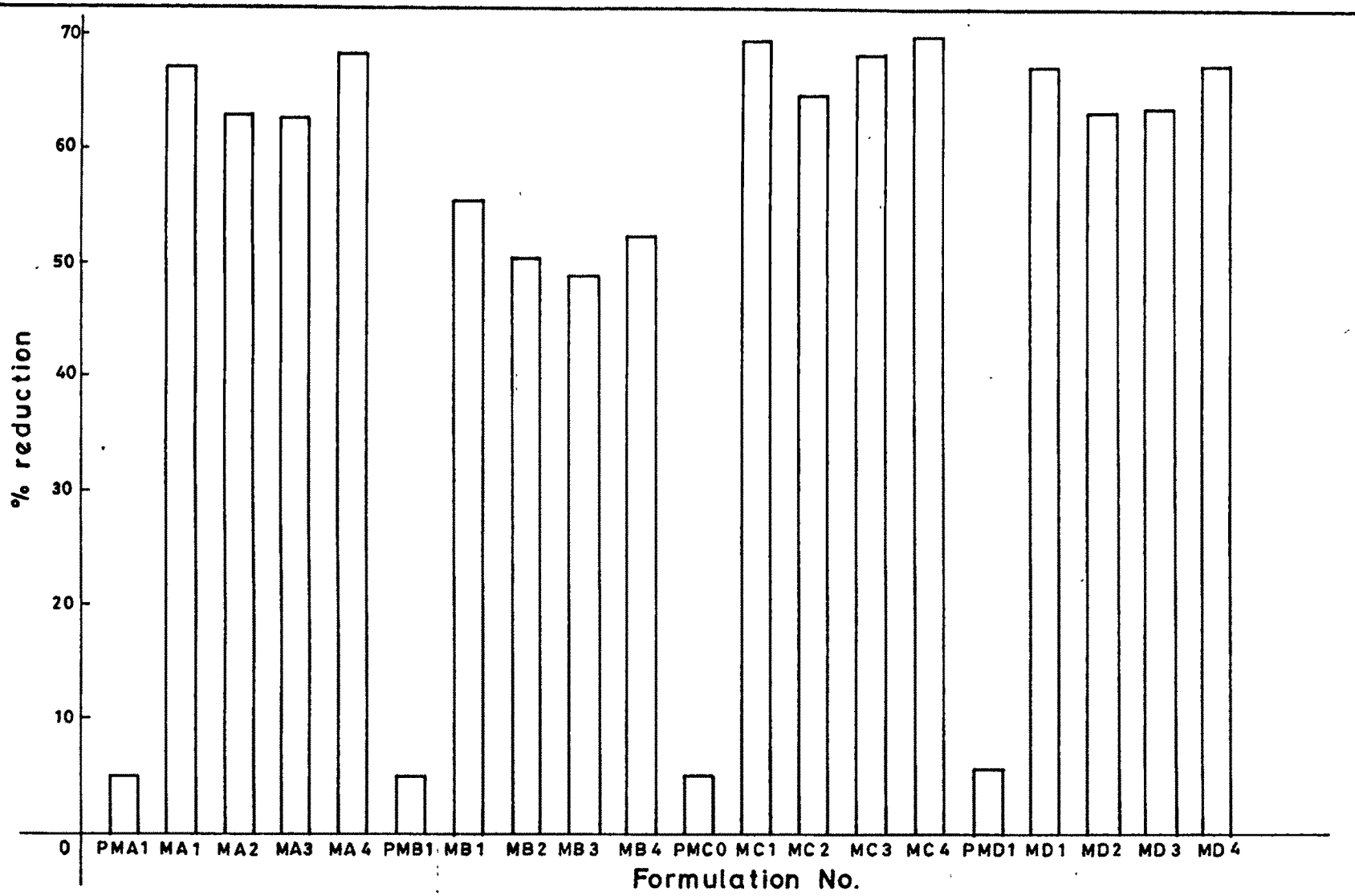


FIG. 4.14: PERCENTAGE REDUCTION IN PERMEABILITY COEFFICIENT VALUES FOR LIPOSOMAL FORMULATIONS OF MTX

values for 5FU and MTX respectively. While in carbopol base (formulations FD1-FD4, MD1-MD4) 54% reduction was observed for 5FU and 66% reduction was observed for MTX.

6. From the permeability coefficient data, it can be observed that the in vitro permeation of 5FU and MTX does not seem to depend on the concentration of lipids present in the liposomes.

7. When the values of permeability coefficients for 5FU and MTX were compared within the bases, it may be observed that the least significant effect of liposomes on in vitro release of the drug was in case of the cetomacrogol base (FA1-FA4 and MA1-MA4). On the otherhand the most significnt effect was observed in case of HPMC gel base (formulations FC1-FC4, MC1-MC4). This may be due to the fact that the permeability coefficient values for plain drug in cetomacrogol base is the least amongst all the bases while that of HPMC base is maximum.

8. The rank order correlation for the reduction in the permeability coefficient in the descending order may be given as :

For 5FU :

HPMC K4M base > carbopol base > hydrous emulsifying ointment base > cetomacrogol emulsifying base.

For MTX :

HPMC gel base > hydrous emulsifying ointment base ≃ carbopol base > cetomacrogol emulsifying base.

From the results it may be observed that encapsulatoin of drug into the liposomes significantly reduces the

TABLE 4.17

STABILITY DATA OF 5FU AND MTX LIPOSOMES IN DIFFERENT FORMULATIONS AT $4^{o}C$

Time in days	% Drug remaining undergraded (± S.D)							
	Hydrous Emulsifying Base		Cetomacrogol cream		HPMCK4M base		Carbopol base	
	FAI	MAI	FBI	MBI	FCI	MCI	FDI	MDI
0	100.00 (-)	100.00 (-)	100.00 (-)	100.00 (-)	100.00 (-)	100.00 (-)	100.00 (-)	100.00 (-)
1	99.85 (0.721)	99.93 (0.713)	99.88 (0.754)	99.92 (0.312)	99.91 (0.435)	99.91 (0.851)	99.99 (0.897)	99.92 (0.754)
15	99.75 (1.321)	99.85 (0.581)	99.81 (0.871)	99.65 (0.871)	99.73 (0.545)	99.86 (0.754)	99.82 (0.813)	99.76 (0.341)
30	99.52 (0.931)	99.73 (0.397)	99.20 (1.012)	99.42 (0.932)	99.69 (0.917)	99.63 (0.999)	99.76 (0.674)	99.21 (0.751)
90	99.36 (0.999)	99.29 (0.345)	98.99 (0.795)	99.01 (0.872)	99.01 (0.893)	99.52 (0.875)	99.54 (0.915)	99.20 (0.813)

TABLE 4.18

EFFECT OF TIME ON PERMEATION PROFILE OF 5FU FROM VARIOUS SEMISOLID BASES

Time in minutes	Time in days: 0				1				15				30				90							
	MEAN CUMULATIVE % PERMEATION OF 5FU (± S.D.)																							
	Formulation No.																							
	FA1	FB1	FC1	FD1	FA1	FB1	FC1	FD1	FA1	FB1	FC1	FD1	FA1	FB1	FC1	FD1	FA1	FB1	FC1	FD1	FA0	FB0	FC0	FD0
60	12.55 (0.131)	7.42 (0.139)	5.77 (0.672)	6.99 (0.707)	12.52 (0.751)	7.39 (0.917)	5.78 (0.213)	6.97 (0.012)	12.53 (0.255)	7.45 (0.312)	5.83 (0.392)	7.01 (0.175)	12.49 (0.114)	7.47 (0.123)	5.79 (0.279)	7.00 (0.321)	12.54 (0.322)	7.42 (0.219)	5.79 (0.355)	6.98 (0.312)	20.13 (0.413)	11.27 (0.231)	15.27 (0.195)	16.05 (0.332)
120	23.52 (0.212)	13.35 (0.217)	10.38 (0.311)	12.82 (0.492)	23.63 (0.329)	13.39 (0.717)	10.19 (0.392)	12.79 (0.531)	23.72 (0.175)	13.52 (0.329)	10.54 (0.333)	12.95 (0.134)	23.59 (0.529)	13.27 (0.379)	10.27 (0.635)	12.75 (0.411)	23.52 (0.511)	13.34 (0.437)	10.42 (0.712)	12.91 (0.335)	39.58 (0.532)	30.30 (0.391)	28.22 (0.279)	27.29 (0.566)
180	25.09 (0.713)	25.22 (0.591)	19.61 (1.001)	18.65 (0.617)	25.11 (0.712)	25.31 (0.593)	19.72 (0.297)	18.73 (0.625)	25.47 (0.723)	25.37 (0.777)	19.75 (0.345)	18.89 (0.713)	25.11 (0.912)	25.22 (1.011)	19.65 (0.532)	18.65 (0.792)	25.13 (0.832)	25.43 (0.911)	19.71 (1.011)	18.75 (1.212)	42.24 (0.795)	38.34 (0.632)	49.18 (0.811)	41.31 (0.911)
240	29.79 (0.791)	29.67 (0.741)	23.07 (0.491)	24.48 (0.542)	29.99 (0.611)	29.57 (0.932)	23.15 (1.011)	25.00 (0.832)	29.65 (0.695)	29.71 (0.713)	23.02 (0.911)	24.23 (0.897)	29.83 (1.011)	29.73 (2.132)	23.11 (0.932)	24.53 (0.795)	29.91 (1.131)	29.71 (0.895)	23.02 (1.311)	24.23 (0.597)	50.48 (0.832)	45.11 (0.595)	58.26 (0.623)	53.57 (0.715)
300	32.93 (1.013)	31.18 (0.975)	24.23 (1.412)	26.81 (0.634)	32.93 (0.911)	31.17 (0.713)	24.25 (0.975)	26.92 (0.675)	33.95 (0.777)	31.28 (0.813)	24.35 (1.011)	26.91 (0.653)	32.85 (1.223)	30.99 (0.915)	24.17 (0.835)	26.77 (0.399)	32.99 (0.915)	31.24 (0.456)	24.35 (1.323)	26.92 (1.311)	53.25 (0.895)	47.36 (0.654)	61.80 (0.817)	57.91 (0.777)
360	33.06 (0.975)	32.64 (1.215)	25.38 (0.817)	27.97 (0.735)	33.09 (1.112)	32.65 (0.815)	25.38 (2.011)	27.97 (0.817)	33.17 (1.223)	32.75 (1.513)	25.42 (0.911)	27.97 (0.854)	33.04 (1.311)	32.65 (0.679)	25.27 (0.854)	27.87 (0.921)	33.12 (0.715)	32.73 (0.954)	25.45 (1.254)	28.02 (0.921)	53.29 (0.795)	49.62 (0.654)	62.49 (1.044)	60.29 (1.129)

TABLE 4.19

EFFECT OF TIME ON PERMEATION PROFILE OF MIX FROM VARIOUS SEMISOLID BASES

Time in minutes	Time in days: 0				1				15				30				90							
	MEAN CUMULATIVE % PERMEATION OF 5FU (± S.D.)																							
	Formulation No.																							
	MA1	MB1	MC1	MD1	MA1	MB1	MC1	MD1	MA1	MB1	MC1	MD1	MA1	MB1	MC1	MD1	MA1	MB1	MC1	MD1	MA0	MB0	MC0	MD0
60	3.98 (0.311)	4.10 (0.615)	2.53 (0.541)	2.29 (0.492)	3.75 (0.312)	4.21 (0.323)	2.49 (0.217)	2.23 (0.349)	3.93 (0.471)	4.13 (0.431)	2.39 (0.321)	2.31 (0.521)	3.98 (0.202)	4.12 (0.212)	2.57 (0.551)	2.31 (0.075)	3.93 (0.313)	4.09 (0.432)	2.57 (0.173)	2.33 (0.191)	12.76 (0.351)	9.04 (0.197)	8.50 (0.177)	7.03 (0.213)
120	7.97 (0.514)	5.93 (0.611)	7.60 (0.625)	6.89 (0.411)	7.92 (0.297)	5.99 (0.524)	7.75 (0.451)	6.92 (0.297)	7.89 (0.332)	5.99 (0.414)	7.57 (0.431)	6.79 (0.221)	7.96 (0.432)	5.94 (0.441)	7.62 (0.617)	6.92 (0.421)	7.97 (0.232)	6.01 (0.732)	7.59 (0.279)	6.93 (0.376)	24.37 (0.231)	12.75 (0.311)	22.08 (0.175)	20.58 (0.392)
180	9.67 (0.454)	7.75 (0.211)	10.77 (0.395)	9.76 (0.759)	10.01 (0.315)	7.87 (0.195)	10.89 (0.329)	9.85 (0.591)	9.79 (0.379)	7.82 (0.252)	10.68 (0.421)	9.72 (0.691)	9.65 (0.613)	7.73 (0.377)	10.81 (0.421)	9.83 (0.317)	9.71 (0.311)	7.69 (0.271)	10.69 (0.311)	9.93 (0.421)	30.07 (0.117)	17.37 (0.192)	35.20 (0.295)	28.91 (0.327)
240	10.81 (0.391)	8.66 (0.322)	13.31 (0.455)	12.06 (0.175)	10.93 (0.912)	8.73 (0.378)	13.41 (0.477)	12.27 (0.727)	10.91 (0.759)	8.71 (0.334)	13.29 (0.321)	12.03 (0.594)	10.83 (0.432)	8.71 (0.912)	13.35 (0.542)	12.11 (0.492)	10.92 (0.327)	8.62 (0.631)	13.27 (0.713)	12.21 (0.414)	33.28 (0.281)	19.01 (0.751)	42.25 (0.295)	39.17 (0.723)
300	11.95 (0.991)	8.67 (0.432)	14.57 (0.512)	12.64 (0.632)	12.01 (0.231)	8.79 (0.397)	14.61 (0.335)	12.69 (0.592)	11.95 (0.532)	8.72 (0.411)	14.53 (0.321)	12.65 (0.517)	11.99 (1.001)	8.75 (0.732)	14.61 (0.612)	12.69 (0.327)	11.93 (0.395)	8.65 (0.614)	14.49 (0.429)	12.72 (0.725)	37.73 (0.321)	19.59 (0.514)	47.55 (0.455)	39.24 (0.245)
360	12.52 (0.715)	10.03 (0.733)	15.21 (0.412)	13.21 (0.259)	12.49 (0.432)	10.12 (0.479)	15.31 (0.511)	13.25 (0.354)	12.61 (0.611)	10.13 (0.712)	15.24 (0.412)	13.23 (0.331)	12.61 (0.613)	10.11 (0.321)	15.23 (0.521)	13.26 (1.011)	12.60 (1.007)	9.97 (1.312)	15.12 (0.717)	13.31 (0.813)	38.26 (1.541)	22.58 (1.011)	50.27 (0.921)	40.25 (0.954)

permeation of the drug across the skin. This may be due to the fact that lipid vesicles act as a barrier which prevents the penetration of the drug into the diffusion medium. This decreased permeation of 5FU and MTX through the skin is advantageous since the undersirable systemic effects of these cytotoxic drugs may be reduced considerably.

From the stability data (Table 4.17) it may be inferred that the liposomal semisolid dosage forms of 5FU and MTX are stable at $4^{o}C$ over the period of 90 days.

The data of the cummulative percentage of drug released for each of the formulation (Table 4.18 and 4.19) indicate that the in vitro permeation profile of the liposomal drug creams was not altered over the period of 90 days.

4.4 In Vivo Methods

In the final analysis, to gain a full insight into the percutaneous absorption process of a drug in man, the permeation studies should be determined in him. However, the approach is often fraught with experimental and ethical difficulties. Hence a persistent theme in work on percutaneous absorption is the development of suitable animal models which correlate adequately to man(20).

5FU is used in the treatment of actinic keratosis, psoriasis, superficial basal cell carcinoma, allergic dermatitis. MTX is used in the treatment of psoriasis and allergic contact dermatitis. Various useful models to induce dermatitis are known. The most common methods include UV erythema method, dinitrochlorobenzene induced allergic

dermatitis, croton oil induced dermatitis etc.(21) In the present investigation the efficacy of liposomal formulations of 5FU and MTX was studied by dinitrochlorobenzene induced allergic dermatitis in guinea pigs(22).

4.41 Experimental :

4.411 Reagents and chemicals :

Dinitrochlorobenzene (DNCB) (Koch light laboratories, England) acetone (Qualigens, India), alcohol (Alembic Chemical Works, India).

Dinitrochlorobenzene solution (DNCB) : 5%v/v

A 5% v/v solution of DNCB was prepared in alcohol-acetone mixture (2:1).

4.412 Formulations :

On the basis of the results of in vitro studies the following formulations were selected for in vivo evaluations.

For 5FU : FA1, FC1, FAO, FCO

For MTX : MA1, MC1, MAO, MCO, where FA1, MA1, FC1 and MC1 are liposomal formulations of 5FU and MTX in hydrous emulsifying ointment base and HPMC K4M gel base respectively.

FAO, FMAO and FCO, MCO are plain drug formulations of 5FU and MTX in hydrous emulsifying ointment base and HPMC K4M gel base respectively.

4.413 Selection, preparation of animals and design of in vivo studies :

Female guinea pigs fed on standardised diet, with an average weight of 300-400 g and having no skin diseases were selected. Those guinea pigs which gave prominent erythematous reaction to DNCB reagent with full recovery after 7 days were

used. The selected guinea pigs were divided into groups of three for each set of formulations. Prior to an in vivo run, the back of each animal was carefully shaved. A 24 hours period was allowed to elapse to give the skin time to heal from any minor injuries.

Six circular areas, each of 2cm diameter with 1cm gap were marked on the shaved surface. Each of the circumscribed area was then painted with DNCB reagent (0.25ml). The solution was allowed to evaporate and the application was repeated for three consecutive days. Full erythema with edema developed within twenty four hours after the third application. On each guinea pig, 400mg of liposomal formulation, plain drug cream, liposomal blank and plain cream blank were applied as per the design shown in Fig. 4.15.

The test sites were compared and evaluated at 0,2,4,6,8,24,48,72 hours 1 and 2 weeks after application of the formulation. The study was conducted in a complete cross over design with four days wash out period after complete recovery between two in vivo runs. The intensity of erythema was evaluated at each time interval by three unbiased observers. For scoring of the erythema the method of Higuchi et al., was followed (23) (Table 4.20). The skin surface of guinea pig prior to application of DNCB (score 0) and 24 hours after the last application of DNCB (score 4) are shown in plates 5 and 6 respectively.

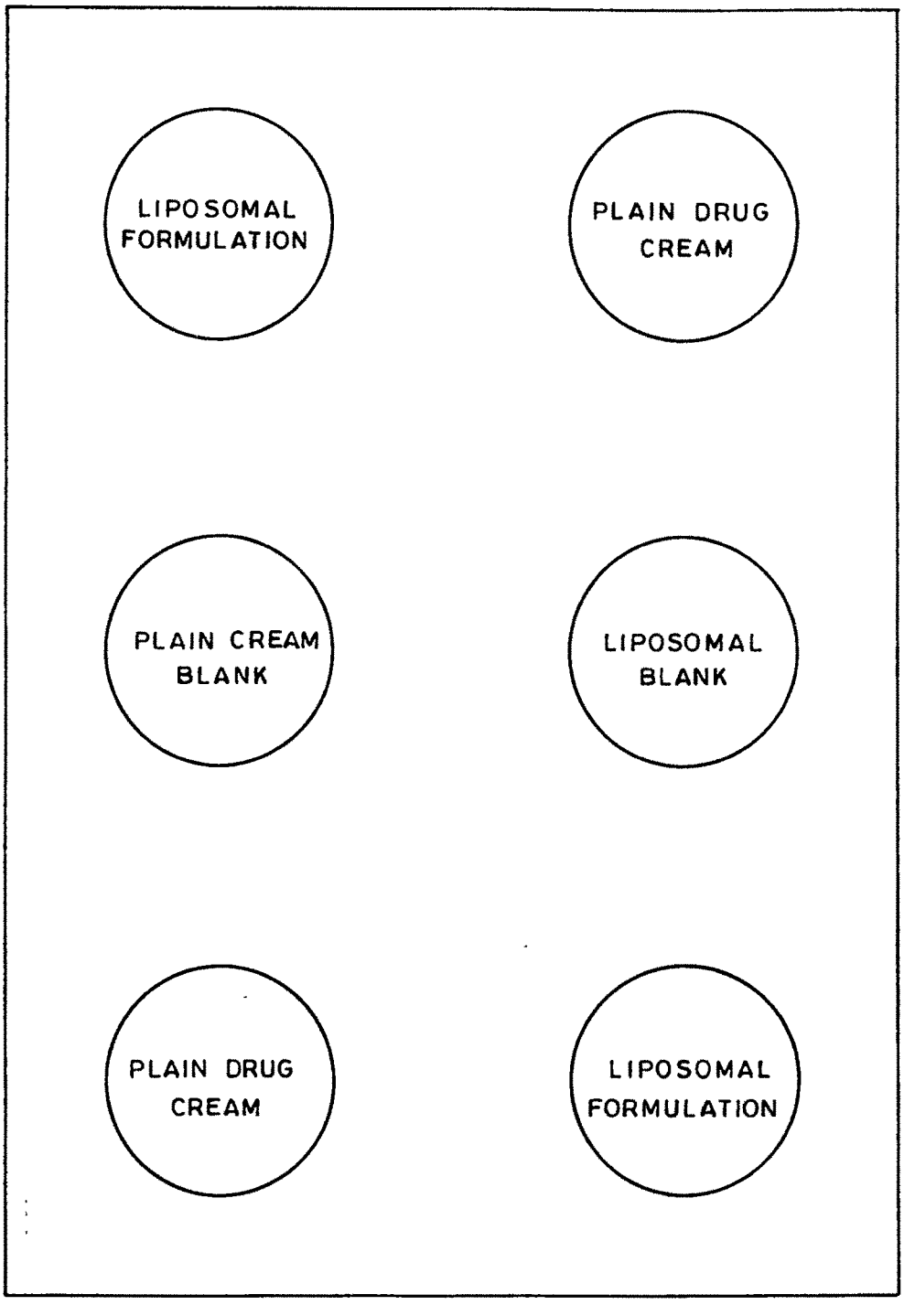


FIG. 4.15: SCHEME FOR APPLICATION OF FORMULATIONS ON DNCB INDUCED ERYTHEMA IN GUINEA PIGS

TABLE 4.20

SCORING PATTERN FOR DNCB INDUCED ERYTHEMA IN GUINEA PIGS

Score	Description
0.0	No evident erythema
1.0	Slight and partial redness.
2.0	Clear erythema over only a part of spot.
3.0	Full circle of definite redness.
4.0	Evident edema.

PLATE-5

SKIN SURFACE PRIOR TO DNCB INDUCED ERYTHEMA (Score 0).

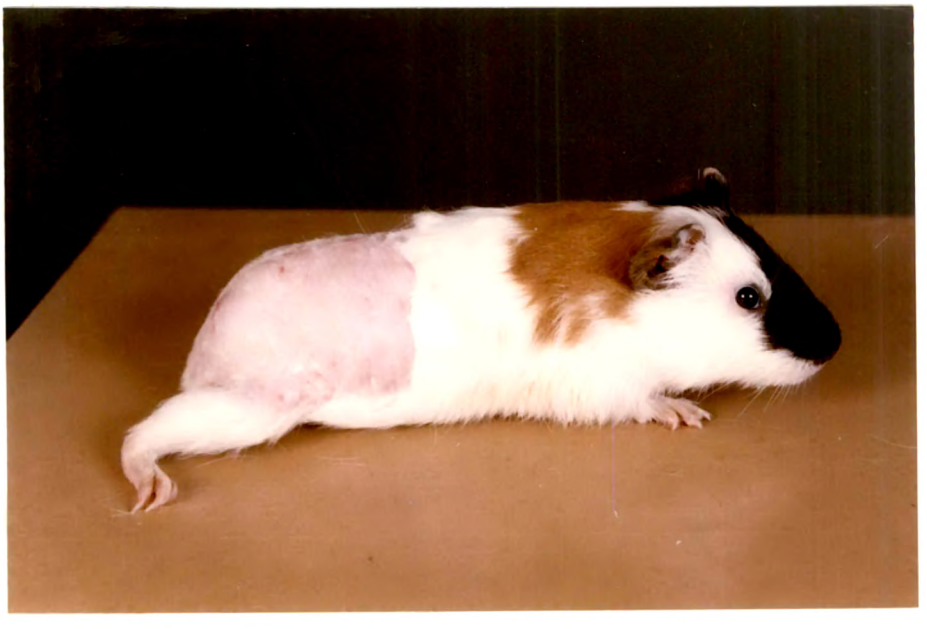
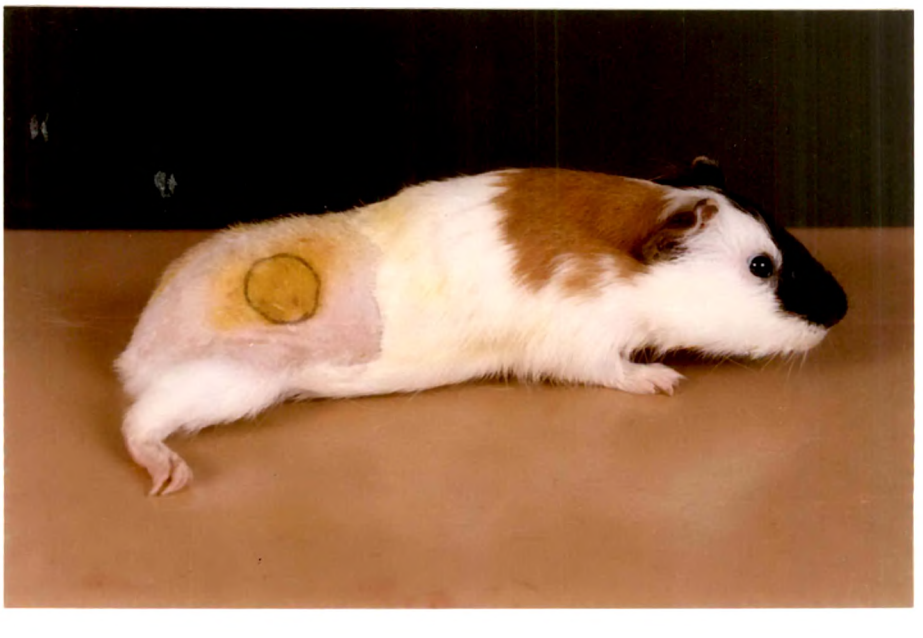


PLATE-6

SKIN SURFACE FOLLOWING DNCB INDUCED ERYTHEMA (Score 4).



4.42 Results and Discussion :

On the basis of the in vitro results it may be observed that amongst the gel bases, HPMC K4M base and amongst the cream bases, hydrous emulsifying base gave the best results in terms of percentage reduction in permeation of 5FU and MTX across rat skin. Hence these bases were selected for the in vivo study.

From the in vitro permeation profile data for the formulations in the individual bases (FA1-FA4, FC1-FC4, MA1-MA4 and MC1-MC4), it may be observed that the lipid concentration does not seem to play any significant role in reducing the drug permeation across the skin in case of 5FU and MTX. Hence liposomal formulations FA1, FC1, MA1 and MC1 were selected as model formulations to study the effect of 5FU and MTX on DNCB induced contact allergic dermatitis in guinea pigs. The effect was compared with that obtained by applying the corresponding plain cream (FA0, FC0, MA0 and MC0) and liposomal blank.

The results of the average erythema scores obtained from each individual run for both 5FU and MTX formulations are given in Tables 4.21-4.24. The percentage reduction in erythema at 4,8,12, 24, 48 and 72 hours were calculated using the formula :

$$\% \text{ reduction in erythema} = \frac{\text{Initial score-Score at time t}}{\text{Initial Score}} \times 100$$

The values of the percentage reduction for 5FU and MTX formulations are recorded in Tables 4.25 and 4.26 and shown

TABLE 4.21

MEAN ERYTHEMA SCORES OBTAINED FOR FORMULATIONS OF 5FU IN HYDROUS EMULSIFYING BASE.

Formulation No.	Score (± S.D.) Time in hours									
	0	2	4	6	8	24	48	72	168 (1 week)	336 (2weeks)
Hydrous emulsifying cream blank	4.00 (0.20)	4.00 (0.22)	4.00 (0.18)	4.00 (0.21)	4.00 (0.22)	3.66 (0.219)	3.66 (0.238)	2.66 (0.22)	0.6 (0.06)	0 -
Liposomal blank in hydrous emulifying cream base	4.00 (0.16)	4.00 (0.19)	4.00 (0.20)	3.85 (0.28)	3.85 (0.25)	3.66 (0.28)	3.66 (0.29)	2.16 (0.19)	0.5 (0.05)	0 -
FA0	3.83 (0.18)	3.66 (0.18)	3.16 (0.21)	2.83 (0.19)	2.50 (0.19)	1.16 (0.10)	1.05 (0.11)	0.83 (0.05)	0 -	0 -
FA1	3.66 (0.15)	3.66 (0.26)	3.16 (0.24)	3.16 (0.25)	2.66 (0.21)	1.16 (0.12)	1.06 (0.11)	0.83 (0.10)	0 -	0 -

TABLE 4.22

MEAN ERYTHEMA SCORES OBTAINED FOR FORMULATIONS OF 5FU IN HPMC K4M GEL BASE

Formulation No.	Score (± S.D.) Time in hours									
	0	2	4	6	8	24	48	72	168 (1 week)	336 (2 weeks)
HPMCK4M cream blank	4.00 (0.16)	4.00 (0.18)	4.00 (0.17)	4.00 (0.19)	4.00 (0.20)	3.83 (0.20)	3.66 (0.28)	2.66 (0.24)	0.83 (0.09)	0 -
Liposomal blank in HPMCK4M gel base	4.00 (0.15)	4.00 (0.16)	4.00 (0.12)	4.00 (0.19)	4.00 (0.19)	3.76 (0.19)	3.56 (0.27)	2.50 (0.20)	0.50 (0.05)	0 -
FC0	3.83 (0.13)	3.66 (0.18)	3.16 (0.21)	2.83 (0.22)	2.16 (0.18)	1.50 (0.15)	1.16 (0.12)	0.83 (0.09)	0 -	0 -
FC1	4.00 (0.12)	3.83 (0.19)	3.33 (0.25)	3.06 (0.24)	2.50 (0.23)	1.66 (0.17)	1.36 (0.12)	1.0 (0.11)	0 -	0 -

TABLE 4.23

MEAN ERYTHEMA SCORES OBTAINED FOR FORMULATIONS OF MTX HYDROUS EMULSIFYING CREAM

Formulation No.	Score (± S.D.) Time in hours									
	0	2	4	6	8	24	48	72	168 (1 week)	336 (2 weeks)
Hydrous emulsifying cream blank	4.00 (0.08)	4.00 (0.12)	4.00 (0.11)	4.00 (0.15)	4.00 (0.16)	3.66 (0.21)	3.66 (0.22)	2.65 (0.19)	0.60 (0.07)	0 -
Liposomal blank in hydrous cream base	4.00 (0.12)	4.00 (0.13)	4.00 (0.20)	3.85 (0.25)	3.85 (0.26)	3.66 (0.29)	3.66 (0.29)	2.16 (0.19)	0.50 (0.05)	0 -
MA0	4.00 (0.11)	3.66 (0.21)	3.40 (0.26)	2.83 (0.24)	2.66 (0.25)	1.50 (0.14)	0.83 (0.09)	0 -	0 -	0 -
MA1	3.83 (0.12)	3.66 (0.27)	3.33 (0.28)	2.66 (0.24)	2.50 (0.23)	1.33 (0.16)	0.83 (0.10)	0 -	0 -	0 -

TABLE 4.24

MEAN ERYTHEMA SCORES OBTAINED FOR FORMULATIONS OF MTX IN HPMC K4M GEL BASE.

Formulation No.	Score (± S.D.) Time in hours									
	0	2	4	6	8	24	48	72	168 (1 week)	336 (2 weeks)
HPMCK4M gel base	4.00 (0.04)	4.00 (0.10)	4.00 (0.14)	4.00 (0.15)	4.00 (0.19)	3.83 (0.22)	3.66 (0.27)	2.66 (0.22)	0.83 (0.083)	0 -
Liposomal blank in HPMC gel base	4.00 (0.05)	4.00 (0.11)	4.00 (0.15)	4.00 (0.13)	4.00 (0.20)	3.76 (0.28)	3.56 (0.26)	2.50 (0.20)	0.50 (0.04)	0 -
MC0	3.83 (0.07)	3.66 (0.18)	3.33 (0.27)	2.83 (0.22)	2.50 (0.25)	1.50 (0.16)	0.83 (0.04)	0 -	0 -	0 -
MC1	3.83 (0.03)	3.66 (0.29)	3.26 (0.25)	2.66 (0.23)	2.50 (0.22)	1.33 (0.13)	0.83 (0.09)	0 -	0 -	0 -

TABLE 4.25

PERCENTAGE REDUCTION IN ERYTHEMA FOR FORMULATIONS OF 5FU

Formulation No.	% reduction in erythema Time in hours				
	4	8	24	48	72
Aqueous Cream blank (ACB)	0.00	0.00	8.50	8.50	33.50
Liposomal blank in aqueous cream base (LACB)	0.00	3.75	8.50	8.50	46.00
FA0	17.50	34.72	69.71	72.58	78.32
FA1	13.70	27.32	68.30	71.04	77.32
HPMC K4M gel base blank (HB)	0.00	0.00	4.25	8.50	33.50
Loposomal blank in HPMC K4M gel base (LHB)	0.00	0.00	6.00	11.00	37.50
FC0	17.49	43.60	60.84	69.71	78.33
FC1	16.75	37.50	58.50	66.00	75.00

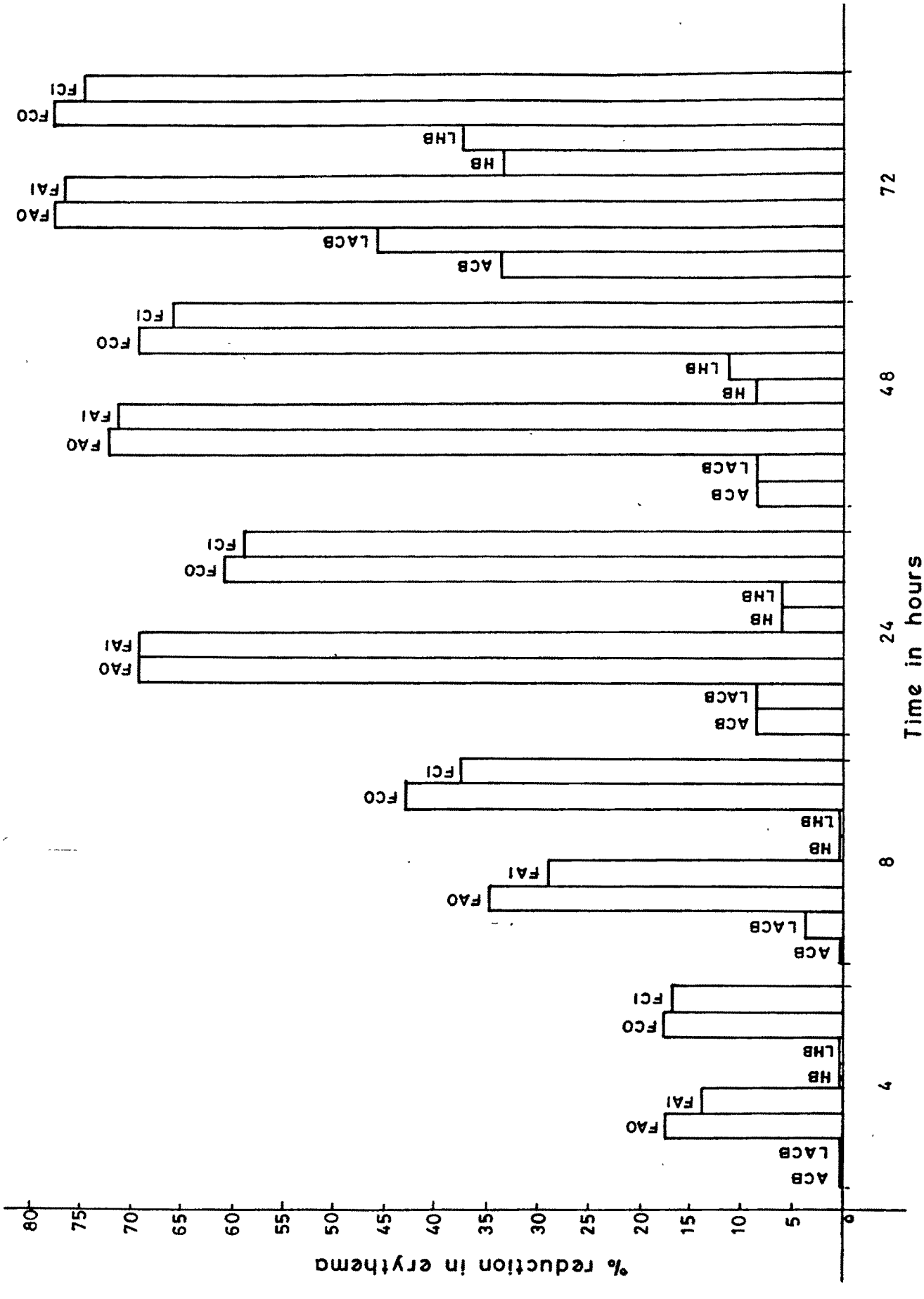


FIG. 4.16: PERCENTAGE REDUCTION IN ERYTHEMA OBTAINED FOR FORMULATIONS OF 5FU

TABLE 4.26

PERCENTAGE REDUCTION IN ERYTHEMA FOR FORMULATIONS OF MTX

Formulation No.	% reduction in erythema Time in hours				
	4	8	24	48	72
Aqueous Cream blank (ACB)	0.00	0.00	8.50	8.50	33.50
Liposomal blank in aqueous cream base (LACB)	0.00	3.75	8.50	8.50	46.00
MAO	15.00	33.50	62.50	79.25	100.00
MA1	13.05	34.73	65.27	78.32	100.00
HPMC K4M gel base blank (HB)	0.00	0.00	4.25	8.50	33.50
Liposomal blank in HPMCK4M gel base (LHB)	0.00	0.00	6.00	11.00	37.50
MCO	13.05	34.73	60.83	78.32	100.00
MC1	14.88	34.73	65.27	78.89	100.00

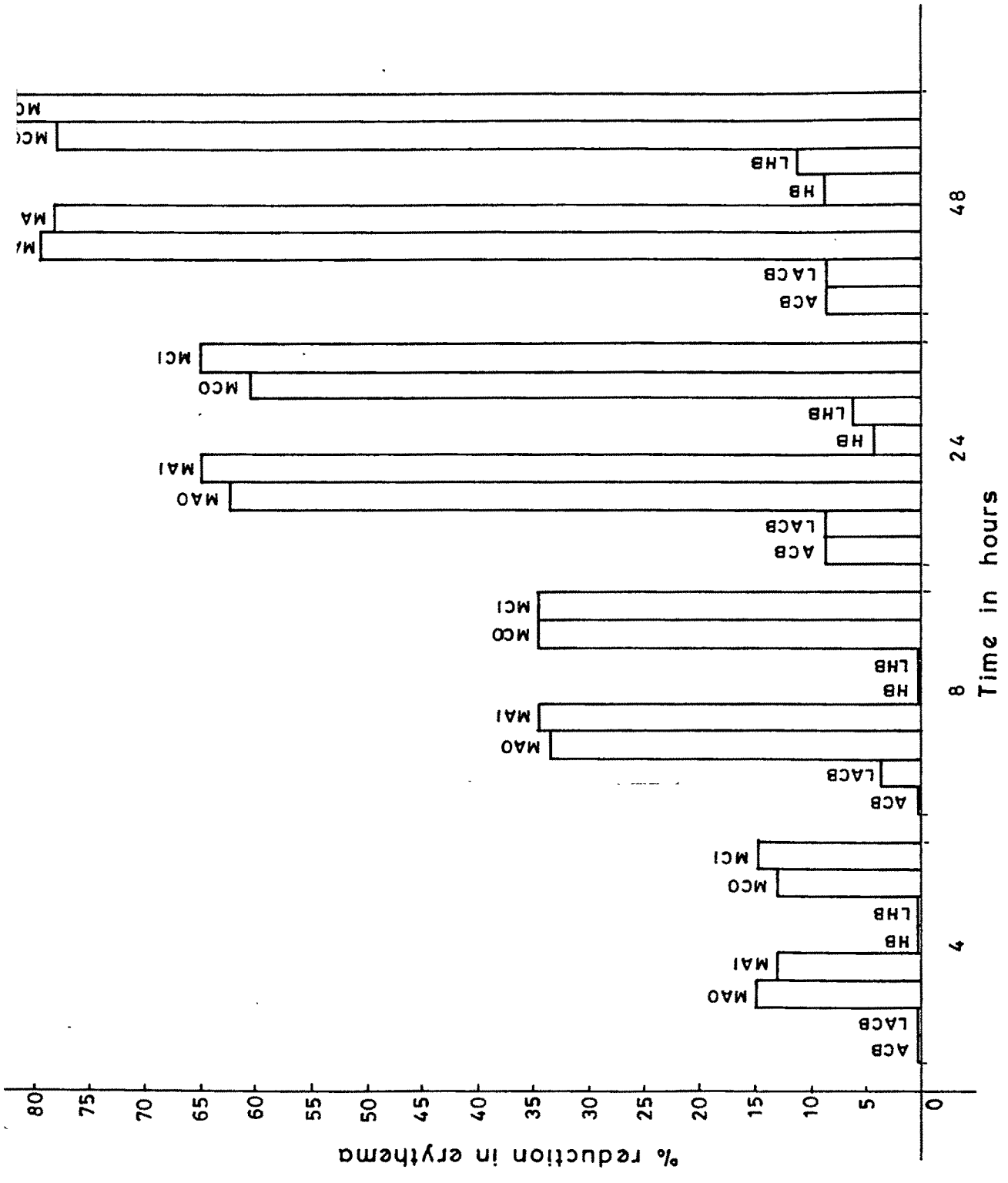


FIG. 4.17: PERCENTAGE REDUCTION IN ERYTHEMA OBTAINED FOR FORMULATIONS OF MTX

in Figs. 4.16 and 4.17 respectively. These values were compared using ANOVA technique.

Significant reduction in erythema was considered at greater than 10% reduction while complete recovery was considered at greater than 75% reduction in erythema.

From the results of the in vivo studies the following inferences can be made :

1. In case of the plain cream blank and liposomal cream blank, no significant reduction in erythema was observed in any of the cases. Complete recovery from erythema was observed only after 7 days.
2. In case of the formulation of 5FU in hydrous emulsifying ointment base 17.5% and 13.7% reduction in erythema were observed for the plain drug cream (FA0) and liposomal drug cream (FA1) respectively. Approximately 70% recovery occured within 24 hours in case of both the formulations. Complete recovery (> 75%) was observed within 72 hours.
3. In case of formulation of 5FU in HPMC K4M gel base, 17.5% and 16.8% reduction in erythema were observed in plain drug cream (FC0) and liposomal drug cream (FC1) respectively. Approximately 60% recovery occured within 24 hours in case of both the formulations. Complete recovery (>75%) was observed within 72 hours.
4. In case of the formulations of MTX in aqueous cream base 15.0% and 13.05% reduction in erythema were obtained for the plain drug cream (MA0) and liposomal drug cream (MA1) respectively. Approximately 60% recovery occured within

24 hours in case of both the formulations and complete recovery (>75%) occured within 48 hours.

5. In case of formulations of MTX in HPMC K4M gel base 13.05% & 14.88% reduction in erythema were observed in plain drug cream (MCO) and liposomal drug cream (MC1) respectively. Approximately 60% recovery occured within 24 hrs. in case of both the formulations. Complete recovery (>75%) was observed within 72 hours.
6. When the percentage reduction in erythema at each time interval obtained for plain drug creams (FAO, FCO, MAO, MCO) and liposomal formulations (FA1, FC1, MA1, MC1) were compared using ANOVA technique, the difference in the values were not statistically significant ($P<0.05$).

The in vivo studies indicate that semisolid formulations of 5FU and MTX are effective in the treatment of DNCB induced erythema in guinea pigs. On comparing the liposomal formulation with the plain drug formulation it was observed that both the formulations had comparable efficacy. Thus incorporation of either 5FU or MTX in liposomes did not alter it's efficacy.

The results of the in vitro and in vivo studies indicate that the liposomal formulations of 5FU and MTX significantly decrease the permeation of these drugs across the skin without compromising on the efficacy of these potent drugs. From this it may be concluded that the liposomal creams may have an advantage over the plain drug cream in terms of reduced systemic adverse effect.

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