CHAPTER III

PREPARATION AND EVALUATION OF LIPOSOMES AND LIPOSOMAL GELS

3.1 INTRODUCTION

Liposomes are lipid vesicles consisting of one or more concentric phospholipid bilayers enclosing an aqueous space(1). They are most ideal as drug delivery systems because besides being biocompatible, biodegradable, non-toxic, non-antigenic and non-pyrogenic they have the ability to entrap hydrophobic agents in their lipid bilayer and hydrophilic agents in their aqueous interior. If liposomes are to be introduced into clinics, they should be prepared using validated procedures, characterized and studied for stability.

When liposomes are intended for dermal drug delivery, the product containing liposomes should be handled with greater care than other topical products. Microfluidics has listed a number of guidelines designed to avoid problems when using liposomes(2):

(i) Minimal mixing is recommended, since excessive shear can disrupt liposomes.

(ii) Formulations containing liposomes should contain less than 10% of glycols and glycerin and no surfactants which are likely to solubilise lecithin.

The above guidelines leave a liposome formulator with little options as regards the base used in topicals containing liposomes; the most suitable base being the gel base.

3.2 EXPERIMENTAL

3.21 Apparatus :

Spectrophotometers (Systronics UV-visible spectrophotometer 108 and Carl Zeiss Jena VSU2-P); rotary flash evaporator (Superfit); temperature controlled water bath (Superfit); cyclomixer, three blade stirrer and centrifuge (Remi); Microscopes (Carl Zeiss Jena, BH_2 Olympus microscope and Joel Jem 100 SX transmission electron microscope); Brookfield RVT Viscometer; formvar grid (Sigma); triple roller mill (Precision Scientific Company).

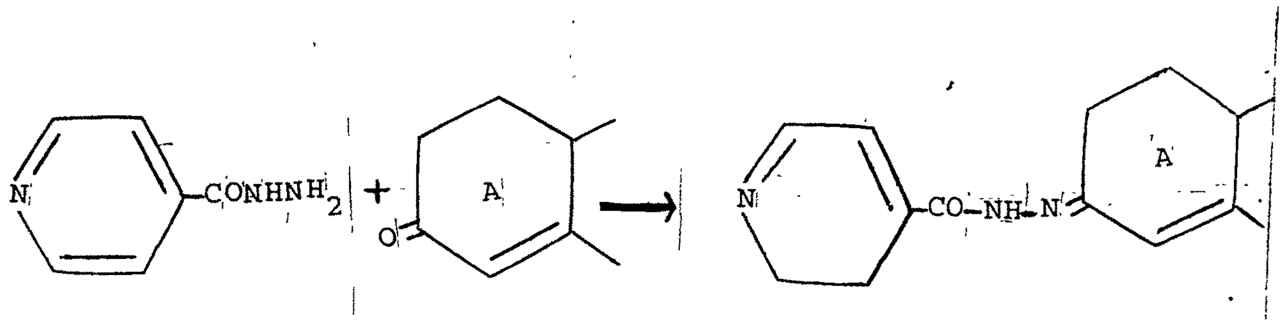
3.22 Materials :

Triamcinolone acetonide (TRMA) (gift sample from Sarabhai Chemicals); flucinolone acetonide (FLU) and clobetasol propionate (CLO) (gift samples from Gufic Labs.); isoniazid (Wilson Laboratories Ltd.), egg phosphatidyl choline (PC) (CSIR Centre For Biochemicals); cholesterol (CHOL) (s.d. Fine Chemicals) ; α- tocopherol (E. Merck); protamine (Sigma); ferric chloride hexahydrate, ammonium thiocyanate, ammonium molybdate, anhydrous sodium sulphate, sodium chloride, sodium hydroxide, methanol, chloroform, hydrochloric acid, glacial acetic acid, sulphuric acid (Qualigens); phenyl mercuric nitrate (BDH); methyl paraben, propyl paraben, calcium chloride fused, emulsifying wax, liquid paraffin, white petroleum jelly (National Chemicals); HPMC K4M (Methocel K4M), HPMC E4M (Methocel E4M) (Dow Chemical Company); Carbopol 941 (B F Goodrich Chemicals); poly vinyl alcohol (PVA) (CDH).

3.23 Colorimetric Method for the Estimation of the Drugs:

3.231 Principle :

Isoniazid gives a yellow colored hydrazone with Δ^4-3-ketosteroids in acidic medium which can be optimized for the estimation of such steroids(3).



Isoniazid A ring of a Δ^4 corticosteroid hydrazone

3.232 Reagents :

a) Isoniazid reagent : 500mg of isoniazid was dissolved in methanol and made upto 250ml with methanol. 0.63ml of hydrochloric acid was added and the solution further diluted to 500ml with methanol.

b) Stock solutions of TRMA, FLU and CLO : A 100µg/ml solution of the respective drugs was prepared in methanol.

3.233 Method(4) :

Appropriate aliquotes of the stock solutions of the drugs were taken into 25ml volumetric flasks so as to get a final drug concentration of 4,8,16,24, 32 and 40µg/ml in case of TRMA and 2,4,6,8,10,14,16 and 20 µg/ml in case of FLU and

CLO. Enough methanol was added to each flask so as to get 10ml of solution in each. To each flask was then added, 10ml of isoniazid reagent. All the flasks were then warmed in a water bath at 50$\pm$2^{O}C for 45 minutes. The flasks were then cooled and the volume made upto 25ml with methanol. The absorbance of these solutions was read at 410nm against a suitable reagent blank. All the analysis were repeated 6 times for all the drugs. The mean absorbance values are shown in Table 3.1 and 3.2 and the lines of regression are shown in Fig 3.1.

3.24 Colorimetric Method for the Estimation of PC :

3.241 Principle :

Phospholipids form a blood red colored complex with ammonium ferrothiocyanate in organic solution (Stewart assay).

3.242 Reagents :

(a) Ammonium ferrothiocyanate solution (0.1M): 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate were dissolved in distilled water and made upto 1 litre. The solution was stored in an amber colored bottle at room temperature.

(b) Stock solution of PC : A 100µg/ml solution of PC was prepared in chloroform.

3.243 Method(5a) :

Suitable aliquotes of the stock solution of PC (0.1,0.2,.....0.7ml) were transferred to calibrated centrifuge tubes. The volume in these tubes was made upto 2ml

TABLE 3.1

CALIBRATION CURVE FOR THE ESTIMATION OF TRMA.

Conc. (µg/ml)	* **Mean absorbance (±SEM)**
4	0.15 (0.005)
8	0.30 (0.002)
16	0.60 (0.003)
24	0.85 (0.004)
32	1.15 (0.003)
40	1.20 (0.010)

$r = 0.996$

Equation of the regression line is $y = -0.015 + 0.035x$

* n = 6

TABLE 3.2

CALIBRATION CURVES FOR THE ESTIMATION OF FLU AND CLO.

Conc. µg/ml	* **Mean absorbance (±SEM) for FLU**	* **Mean absorbance (±SEM) for CLO**
2	0.045 (0.002)	0.076 (0.004)
4	0.097 (0.011)	0.165 (0.004)
6	0.156 (0.005)	0.221 (0.008)
8	0.191 (0.007)	0.306 (0.004)
10	0.247 (0.009)	0.373 (0.005)
14	0.344 (0.008)	0.534 (0.006)
16	0.354 (0.005)	0.595 (0.008)
20	0.481 (0.008)	0.727 (0.007)

For FLU r = 0.999 and equation of the regression line is y=-0.001 + .024x

For CLO r=0.999 and equation of the regression line is y=-0.005 + 0.038x

* n = 6

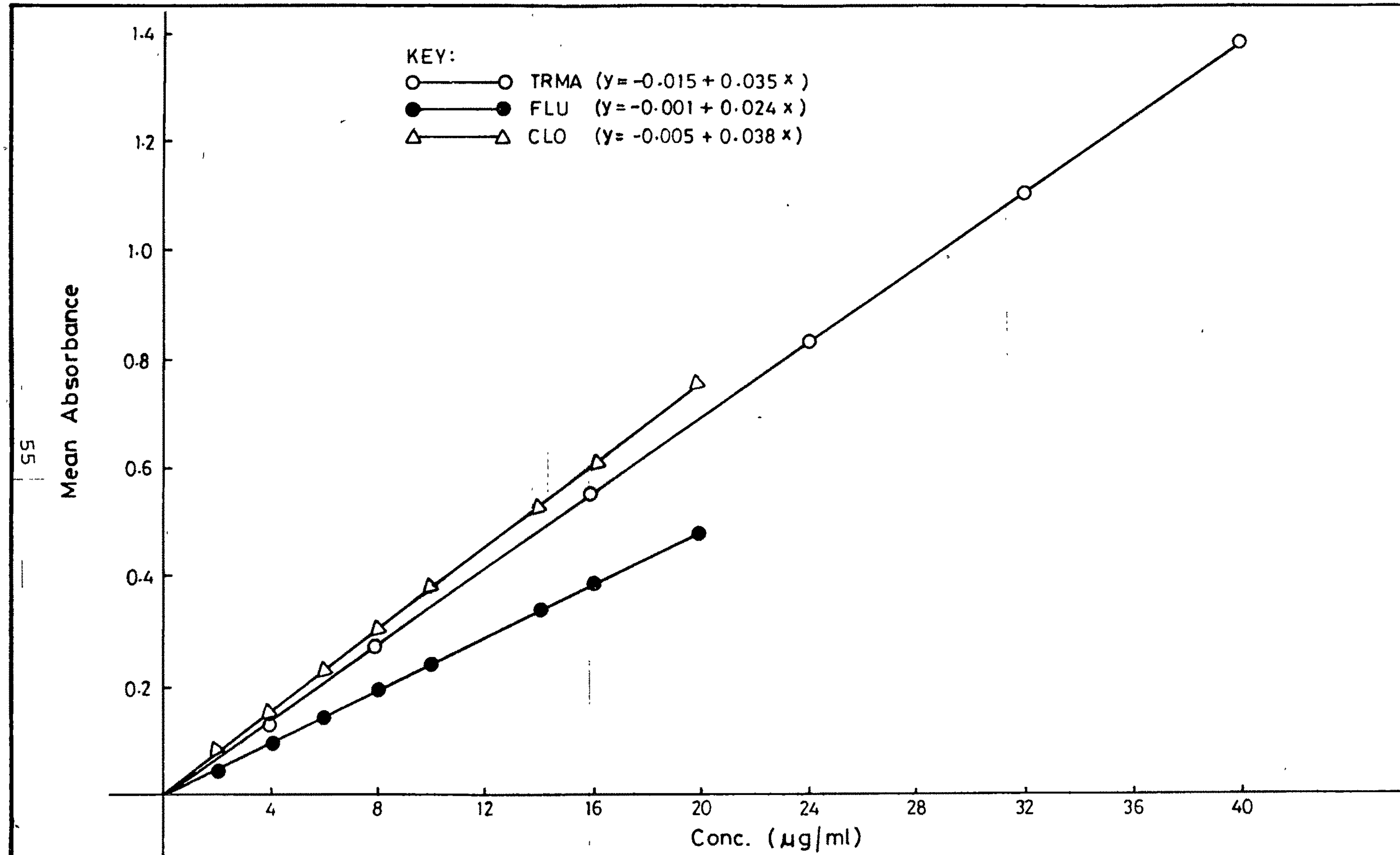


FIG. 3.1 : REGRESSED CURVES FOR THE ESTIMATION OF TRMA, FLU AND CLO (λ=410 nm).

with chloroform. To each tube was added, 2ml of the ammonium ferrothiocyanate solution and the contents vortexed on a cyclomixer for 2 minutes. The contents were then centrifuged at 2750 r.p.m. for 5 minutes to seperate the choloform layer. This layer was removed using a glass syringe with a long needle and its absorbance read at 485nm against a reagent blank. The method was repeated 6 times and the mean absorbance values are recorded in Table 3.3. The regressed calibration curve is shown in Fig.3.2.

3.25 Colorimetric Method for the Estimation of CHOL :

3.251 Principle :

CHOL in acetic acid forms a complex with ferric chloride and sulphuric acid (Zlatkis, Zak and Boyle's method).

3.252 Reagents :

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

(b) Stock Solution of Cholesterol : A 0.5 µg/ml solution of cholesterol was prepared in glacial acetic acid.

3.253 Method(6) :

Suitable aliquotes of stock solution of cholesterol (0.1,0.2....1ml) were transferred accurately into 5ml volumetric flasks. 2ml of ferric chloride solution and 2ml of sulphuric acid were added. The solutions were made upto volume with glacial acetic acid and the absorbance read, against a suitable reagent blank at 550nm. The method was

TABLE 3.3

CALIBRATION CURVE FOR THE ESTIMATION OF PC.

Conc. (μg/2ml)	* **Mean absorbance ($\pm$SEM)**
100	0.254 (0.004)
200	0.485 (0.004)
300	0.750 (0.003)
400	0.975 (0.006)
500	1.260 (0.008)
600	1.600 (0.008)
700	1.850 (0.020)

r = 0.999

Equation of the regression line is y = -0.175 + 0.003x

* n=6

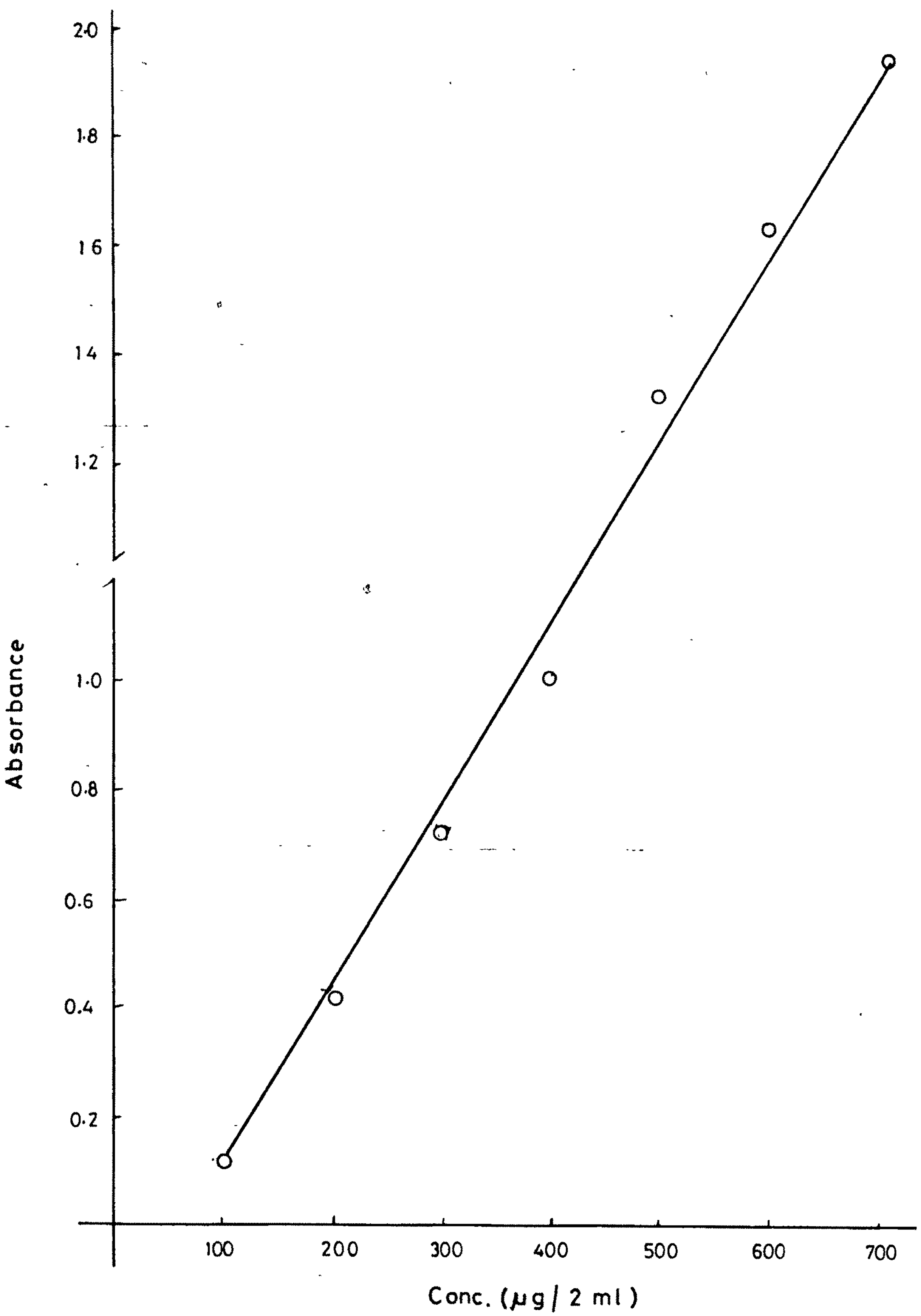


FIG. 3.2: REGRESSED CALIBRATION CURVE FOR THE ESTIMATION OF PC (λ max = 485 nm).

repeated 6 times and the mean absorbance values are recorded in Table 3.4. The regressed calibration curve is shown in Fig. 3.3.

3.26 Preparation of Liposomes:

3.261 Reagents :

Calcium chloride solution : A 25 mM solution of calcium chloride was prepared in distilled water.

3.262 Method :

Liposomes were prepared by using the lipid film hydration method (7). PC, CHOL and the drug, in the ratios as shown in Tables 3.5 and 3.6 were accurately weighed and then dissolved in chloroform-methanol mixture (2:1) in a 50ml round bottom flask. α -tocopherol, equivalent to 1% of the weight of PC taken, was added to the above solution and the solvent was evaporated, under vacuum, in a rotary flash evaporator, until a smooth, dry lipid film was observed. This film was hydrated with 10ml of calcium chloride solution and allowed to stand at room temperature for 1 hour. The round bottom flask was then shaken gently till the film contents got suspended in the solution. The liposomal preparation was then vortexed for 10 minutes to get a uniform dispersion which was allowed to hydrate in the refrigerator overnight. The preparation was then centrifuged at 2750 r.p.m. for 30 minutes. The supernatant was decanted and the pellet was resuspended in 5.0 ml of calcium chloride solution. This procedure was repeated twice. The residue of the last

TABLE 3.4

CALIBRATION CURVE FOR THE ESTIMATION OF CHOL.

Conc µg/ml	* Mean Absorbance (± SEM)
10	0.234 (0.008)
20	0.444 (0.009)
40	0.813 (0.008)
50	1.010 (0.009)
80	1.602 (0.007)
100	1.785 (0.009)

$r = 0.998$

Equation of the regression line is $y = -0.019 + 0.02x$

* n=6

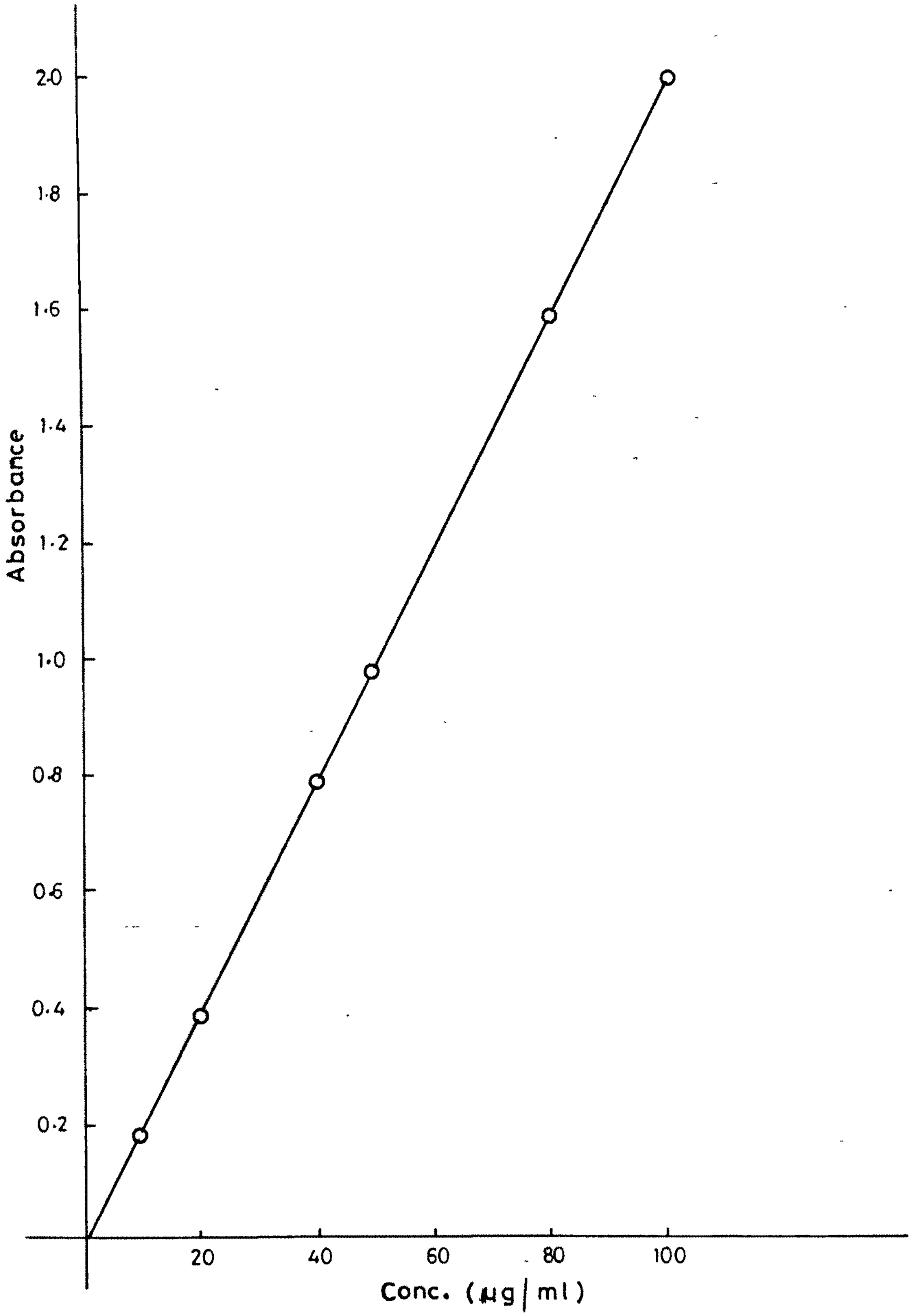


FIG. 3.3 : REGRESSED CALIBRATION CURVE FOR THE ESTIMATION OF CHOL (λmax = 550 nm).

TABLE 3.5

COMPOSITION OF THE VARIOUS BATCHES OF TRMA LIPOSOMES WITH RESPECT TO THEIR PC : CHOL AND TRMA : PC : CHOL RATIOS.

Batch	PC : CHOL Molar Ratio	TRMA : PC:CHOL Molar Ratio
TRMA1	4:1	1:0.6:0.152
TRMA2	4:1	1:0.72:0.182
TRMA3	4:1	1:0.91:0.23
TRMA4	2.7:1	1:0.91:0.34
TRMA5	2:1	1:0.91:0.46
TRMA6	1.34:1	1:0.91:0.68
TRMA7	4:1	1:1.81:0.46
TRMA8	2.7:1	1:1.81:0.68
TRMA9	2:1	1:1.81:0.91
TRMA10	1.32:1	1:1.81:1.37
TRMA11	1:1	1:1.81:1.83
TRMA12	4:1	1:3.62:0.92
TRMA13	2.6:1	1:3.62:1.37
TRMA14	2:1	1:3.62:1.85
TRMA15	1.32:1	1:3.62:2.75
TRMA16	1:1	1:3.62:3.62
TRMA17	4:1	1:5.43:1.37
TRMA18	2:1	1:5.43:2.75
TRMA19	1.32:1	1:5.43:4.12
TRMA20	1:1	1:5.43:5.43
TRMA21	1:1	1:10.86:10.86

TABLE 3.6

COMPOSITION OF THE VARIOUS BATCHES OF FLU AND CLO LIPOSOMES WITH RESPECT TO THEIR PC : CHOL AND DRUG : PC : CHOL RATIO.

Batch	PC : CHOL Molar Ratio	DRUG : PC : CHOL Molar Ratio
FLU1	8.33:1	1:2:0.24
FLU2	4:1	1:2:0.48
FLU3	1:1	1:2:2
CLO1	8.33:1	1:2:0.24
CLO2	4:1	1:2:0.48
CLO3	1:1	1:2:2

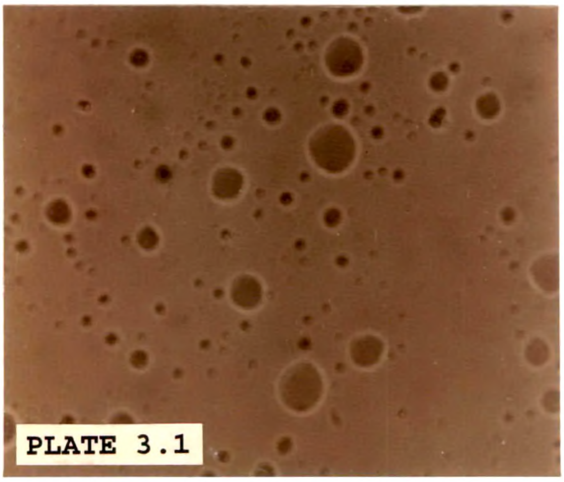


PLATE 3.1

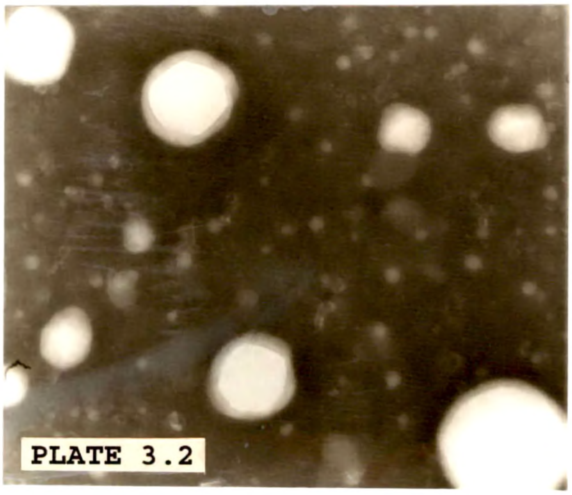


PLATE 3.2

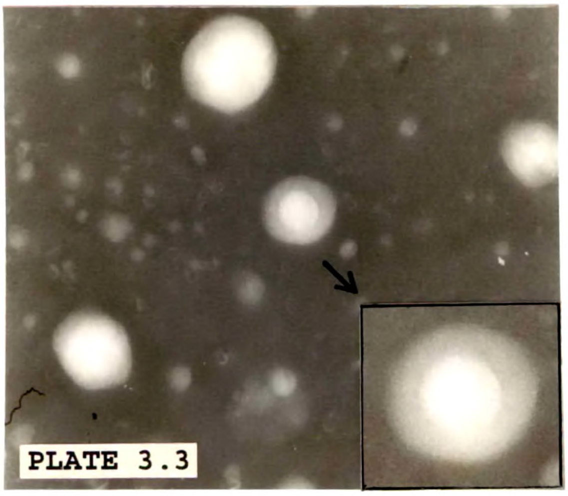


PLATE 3.3

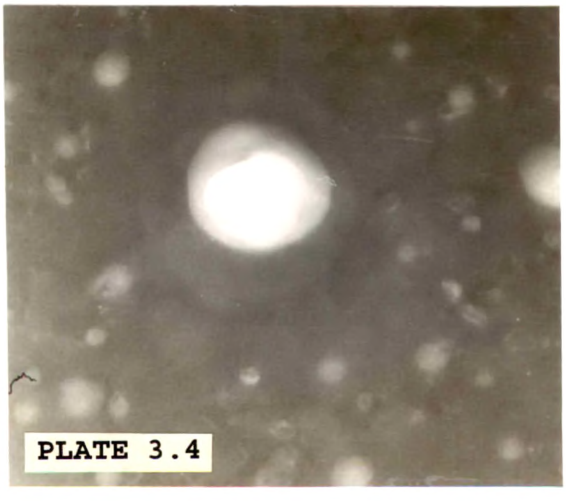


PLATE 3.4

centrifugation was resuspended in 2.0ml of calcium chloride solution and stored in the refrigerator till further use.

3.27 Characterization of Liposomes :

3.271 Physical Observations :

(a) Microscopic examinations : All the batches of the liposomes prepared were viewed under a Carl Zeiss Jena Microscope to study their shape. A representative batch (TRMA7) of liposomes was photomicrographed and one such photograph is shown in Plate 3.1.

(b) Transmission electron microscopy : Liposomes of batch TRMA7 were subjected to negative stain electron microscopy in order to get information regarding their lamellarity (multi-or unilamellar). A drop of liposomal suspension was put on a wax bed and a drop of 2% ammonium molybdate solution was added to it. The preparation was allowed to take up the stain for 5 minutes. The stained preparation was mounted on the surface of a formvar grid. Excess liquid was removed by adsorption onto a filter paper and the grid was then inserted into the assembly of a Joel Jem 100 SX transmission electron microscope. The preparation was observed under various magnifications and the photographs taken are shown in Plates 3.2 - 3.4

(c) Particle size analysis : Liposomes of batches TRMA1, TRMA7 and TRMA21 were subjected to particle size analysis using a BH_2 Olympus microscope (least count = 0.7nm) at a magnification of 100x. The frequency Vs particle size curve is shown in Fig. 3.4. The arithmetic mean diameter for each batch was calculated(8) and is shown in Table 3.7.

3.272 Chemical Analysis :

(a) Analysis of liposomes for % drug entrapped, PC and CHOL content : This was done using the modified Bligh-dyer two phase extraction procedure(5b). The prepared liposomal suspension was made upto 50ml with calcium chloride solution. 0.1ml of this liposomal dispersion was then transferred to a calibrated centrifuge tube and made upto 2ml with a saturated sodium chloride solution. 2ml of chloroform was added to the contents of the centrifuge tube followed by vortexing for 2 minutes and then centrifuging at 2750 r.p.m. for 10 minutes. The lower layer was seperated using a glass syringe with a long needle and transferred into a 10ml volumetric flask after passing it through a bed of anhydrous sodium sulphate. The extraction was repeated twice with 2,2 ml of chloroform and the combined chloroform extracts were made upto 10ml with chloroform. 5ml of this extract was transferred into a 25ml volumetric flask and the solvent was evaporated to dryness on a water-bath at 65°C. The contents of the flask were then analysed for the drug using the method described in Section 3.23. 2ml of the extract was transferred into a centrifuge tube and analysed for its PC content using the method described in Section 3.24

TABLE 3.7

PARTICLE SIZE ANALYSIS OF SELECTED BATCHES OF TRMA LIPOSOMES.

Size range (µm)	Mean of size range (d) µm	TRMA1		TRMA7		TRMA21	
		No. of particles in each size range (n)	nd	n	nd	n	nd
0.7-2.0	1.35	77	103.95	27	36.45	43	58.05
2.1-4.9	3.5	134	469.00	188	658.00	143	500.50
5.0-7.0	6.0	41	246.00	54	324.00	30	180.00
7.1-14.0	10.55	46	485.30	15	158.25	78	822.90
>14		2		16		6	
		$\Sigma n^*=298$	$\Sigma nd=1304.25$	$\Sigma n^*=284$	$\Sigma nd=1176.7$	$\Sigma n^*=294$	$\Sigma nd=1561.45$
		$\frac{\Sigma nd}{\Sigma n} = 4.38\mu m$		$\frac{\Sigma nd}{\Sigma n} = 4.14\mu m$		$\frac{\Sigma nd}{\Sigma n} = 5.13\mu m$	

$\frac{\Sigma nd}{\Sigma n}$ is the Arithmetic Mean diameter

* Σn does not include the particles > 14 µm in diameter.

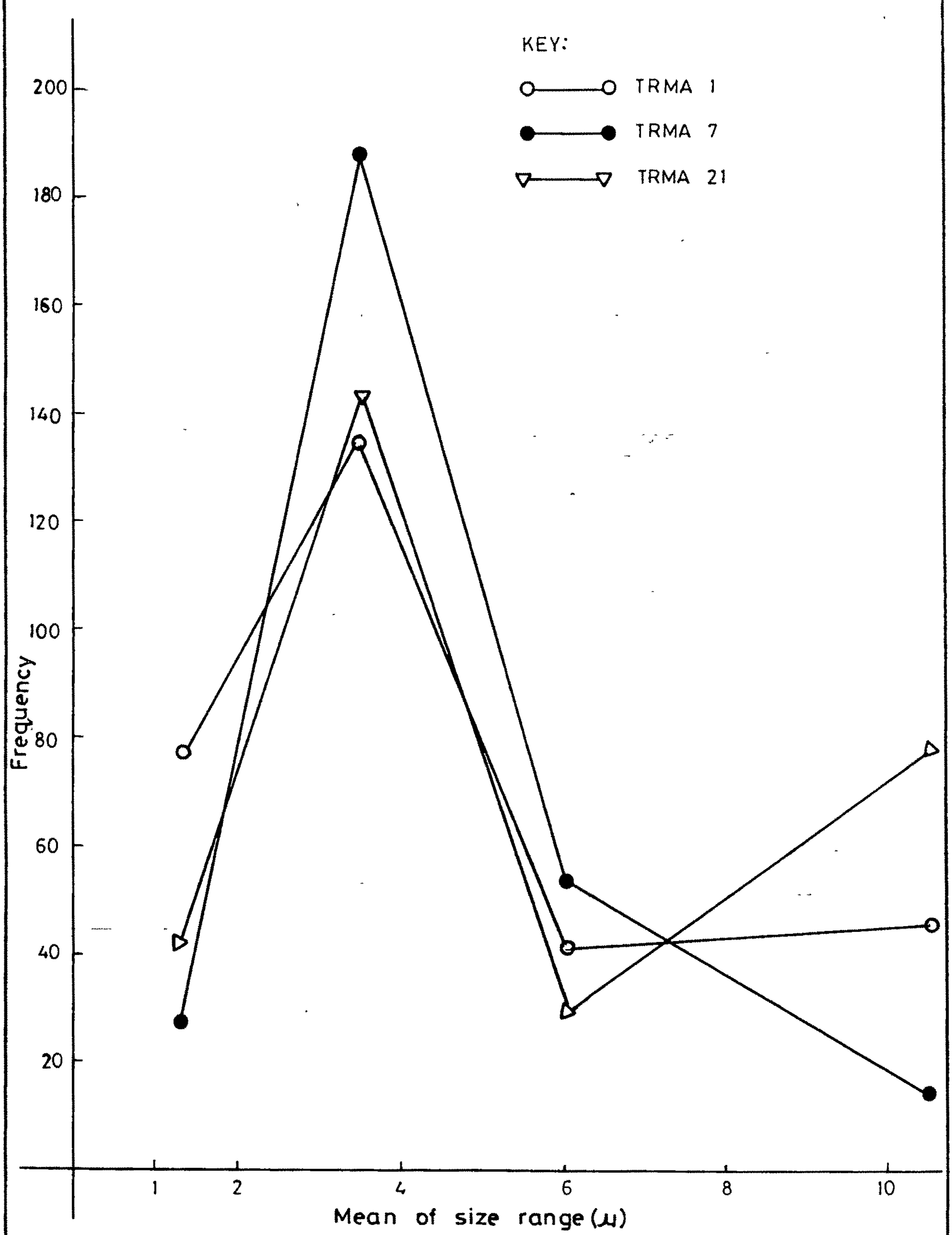


FIG. 3.4: PARTICLE SIZE DISTRIBUTION OF SOME LIPOSOMAL BATCHES OF TRMA.

while yet another 2ml was used for estimating the CHOL content using the method described in Section 3.25 after evaporating the solvent. All the analysis were carried out in triplicates. The results of the above analysis were used to calculate the % drug entrapped (Tables 3.8 and 3.9) and Drug : PC : CHOL ratio (Tables 3.5 and 3.6).

(b) Analysis of supernatant for drug content : The combined supernatant obtained during the preparation of liposomes of a batch, was analysed for its drug content in order to establish a material balance for the drug. 10ml of supernatant was saturated with sodium chloride and extracted successively with 10, 10, and 5 ml of chloroform. The chloroform extracts were transferred to a 25ml volumetric flask after passing them through a bed of anhydrous sodium sulphate. The solvent was evaporated to dryness on a water-bath at 65°C and the contents of the flask analysed for the drug using the method described in Section 3.23. All the analysis were carried out in triplicates and the mean values for the % of drug in supernatant for all the batches of liposomes prepared are shown in Tables 3.8 and 3.9.

(c) Protamine assay(5a) : This method was used to determine the % of unentrapped drug in the liposomal pellet after centrifugation. The property of protamine to get adsorbed on the surface of liposomes and cause their aggregation while leaving the unentrapped drug in the supernatant is used in this assay. To 0.1ml of

TABLE 3.8

DETAILS OF % DRUG ENTRAPPED, % DRUG UNENTRAPPED AND % DRUG IN SUPERNATANT DURING THE PREPARATION OF LIPOSOMAL BATCHES OF TRMA.

Batch	* **Mean % TRMA Entrapped**	* **Mean % TRMA Unentrapped #**	* **Mean % TRMA in supernatant**
TRMA1	47.14	0.10	50.86
TRMA2	70.77	0.04	26.44
TRMA3	64.30	0.08	34.72
TRMA4	72.80	0.06	25.20
TRMA5	85.13	0.09	14.00
TRMA6	77.64	0.10	20.36
TRMA7	91.42	0.04	7.40
TRMA8	74.65	0.04	20.35
TRMA9	96.66	0.03	1.78
TRMA10	88.10	0.03	9.21
TRMA11	93.62	0.05	2.48
TRMA12	88.13	0.06	7.98
TRMA13	80.20	0.05	15.42
TRMA14	85.00	0.08	12.36
TRMA15	77.60	0.04	20.21
TRMA16	89.40	0.09	5.32
TRMA17	89.60	0.09	6.28
TRMA18	95.40	0.08	2.21
TRMA19	86.15	0.05	10.23
TRMA20	89.65	0.08	7.21
TRMA21	88.50	0.09	9.01

(% drug entrapped + % drug in supernatant does not add upto 100% because of losses during the preparation procedure)

* n=3

% drug unentrapped refers to the results of the protamine test.

TABLE 3.9

DETAILS OF % DRUG ENTRAPPED, % DRUG UNENTRAPPED AND % DRUG IN SUPERNATANT DURING THE PREPARTION OF LIPOSOMAL BATCHES OF FLU AND CLO.

Batch	* **Mean % drug entrapped**	* **Mean % drug unentrapped #**	* **Mean % drug in supernatant**
FLU1	76.89	0.08	20.21
FLU2	88.29	0.07	5.48
FLU3	73.41	0.06	19.89
CLO1	75.20	0.06	18.21
CLO2	84.28	0.07	9.31
CLO3	72.21	0.07	23.62

(% drug entrapped + % drug in supernatant does not add upto 100% because of losses during the preparation procedure)

* n=3

% drug unentrapped refers to the results of the protamine test.

liposomal suspension in a calibrated centrifuge tube was added, 0.1ml of protamine solution (10µg/ml). After mixing the tube contents for 3 minutes with a glass rod, 3ml of saline was added and the contents centrifuged at 2750 r.p.m. for 30 minutes. 2ml of the supernatant was then seperated and extracted with 5,2 and 2ml of chloroform. The solvent from the combined chloroform extracts was evaporated and the extract analysed for drug, using the method described in Section 3.23. The analysis was done in triplicates and the mean % drug entrapped for each batch is reported in Tables 3.8 and 3.9.

3.28 Preparation of Gel and Cream Bases :

(a) Preparation of gel bases : The solid content in all these bases was fixed such that at room temperature the viscosity of the gel base was between 90,000-1 lac cps using a Brookfield RVT viscometer with C spindle at 2.5 r.p.m.

(i) HPMC gel bases : HPMC K4M and HPMC E4M gel bases were prepared by dispersing 50g of the respective powders slowly in 950ml of distilled water, containing 0.001% w/v of phenyl mercuric nitrate, under a triple blade stirrer. The mixture was allowed to stand overnight to free the gel from entrapped air.

(ii) Carbopol gel base : 8g of Carbopol 941 resin was stirred into 975ml of distilled water, containing 0.001% w/v phenyl mercuric nitrate, to form a thin, cloudy, lump free dispersion. To this dispersion, was added, very slowly and under continuous stirring, 20ml of a 10% w/v solution of sodium hydroxide to get a gel.

(iii) PVA gel : 200g of PVA was dispersed slowly in 800ml of distilled water, containing 0.001% w/v of phenyl mercuric nitrate, under a triple blade stirrer. The mixture was allowed to stand overnight to free the gel from entrapped air.

(b) Preparation of Cream Bases :

(i) Aqueous Cream base : This was prepared as per the procedure for preparing Hydrous emulsifying base I.P. 1966 except that instead of using chlorocresol, a combination of 0.15% w/w of m-paraben and 0.08% w/w p-paraben were used as preservatives.

(ii) Cetomacrogol Cream (B.P.) : This was prepared as per the procedure given for Cetomacrogol cream Formula B in B.P. 1980.

All the bases were stored in tightly closed amber colored glass bottles, at room temperature, till further use.

3.29 Preparation of liposomal and drug gels and drug creams :

All the gels used for in vitro studies contained 1% w/w of the drug while gels and creams of TRMA, FLU and CLO for in vivo studies contained 0.1% w/w, 0.025% w/w and 0.05% w/w of the respective drugs.

In the preparation of plain drug creams/gels, accurately weighed quantity of the drug was levigated with

1.0 gram of the base on a pill tile. This was then diluted in a geometric proportion with the base. The final product was passed through a triple roller mill.

In preparation of liposomal gels, liposomes obtained from 6 runs of the same batch were diluted to 100ml with calcium chloride solution. An aliquote equivalent to the quantity of the drug required to prepare 50g of the drug gel was transferred to a centrifuge tube and centrifuged pellet obtained was levigated with 1.0g of the gel base on a pill tile and then diluted in geometric proportion with the base.

The various gels and creams prepared are enlisted in Table 3.10 - 3.13. 50g of each gel/cream was prepared, packed in glass jars and stored in a refrigerator until further use.

3.2α Method of analysis of drugs from the topical formulations :

(a) HPMC gels : 100mg of the gel was accurately weighed and dissolved in 10ml of 1:1 methanol : dichloromethane. In case of the gels prepared for <u>in vitro</u> studies, 1 ml of this solution and in case of gels prepared for <u>in vivo</u> studies, the entire 10ml of this solution was estimated for its drug content using the method described in Section 3.23.

(b) Carbopol Gels : 100mg of the gel was accurately weighed and dissolved in 10ml of chloroform. In case of gels prepared for <u>in vitro</u> studies, 1ml of this solution and

TABLE 3.10

DESCRIPTION OF THE VARIOUS GELS OF TRMA PREPARED IN HPMC K4M GEL BASE.

Gel* Code	Content#
KTG	Plain TRMA
PMT (Physical mixture)	Plain TRMA
T_1	TRMA1
T_2	TRMA2
T_3	TRMA3
T_4	TRMA4
T_5	TRMA5
T_6	TRMA6
T_7	TRMA7
T_8	TRMA8
T_9	TRMA9
T_{10}	TRMA10
T_{11}	TRMA11
T_{12}	TRMA12
T_{13}	TRMA13
T_{14}	TRMA14
T_{15}	TRMA15
T_{16}	TRMA16
T_{17}	TRMA17
T_{18}	TRMA18
T_{19}	TRMA19
T_{20}	TRMA20
T_{21}	TRMA21

* All the gels used in *in vitro* studies contain 0.9-1.1% w/w of TRMA and when used for *in vivo* studies contain 0.9-0.11% w/w of TRMA.

\# Refer Table 3.1.

TABLE 3.11

DESCRIPTION OF THE VARIOUS FORMULATIONS OF TRMA PREPARED IN DIFFERENT BASES.

Formulation	Base	Content
KTG	HPMC K4M	Plain TRMA
KLG	HPMC K4M	TRMA7
ETG	HPMC E4M	Plain TRMA
ELG	HPMC E4M	TRMA7
CTG	Carbopol	Plain TRMA
CLG	Carbopol	TRMA7
PTG	PVA	Plain TRMA
PLG	PVA	TRMA7
ATC	Aqueous Cream base	Plain TRMA
CTC	CMC base	Plain TRMA

* All the formulations used in *in vitro* studies contain 0.9%-1.1% w/w of TRMA and when used for *in vivo* studies contained 0.09-0.11% w/w of TRMA.

\# The code has been devised as follows :

the 1st alphabet signifies the base;

the 2nd alphabet whether the drug is free(T) or in liposomes (L);

the 3rd alphabet whether the formulation is a gel (G) or a cream (C).

TABLE 3.12

DESCRIPTION OF THE VARIOUS FORMULATIONS OF FLU PREPARED IN DIFFERENT BASES.

Formulation Code*	Base	Content
KFG	HPMC K4M	Plain FLU
F_1	HPMC K4M	FLU1
F_2	HPMC K4M	FLU2
F_3	HPMC K4M	FLU3
# PMF	HPMC K4M	Plain FLU
CFC	CMC base	Plain FLU
AFC	Aqueous Cream base	Plain FLU

* All the formulations used in _in vitro_ studies contained 0.9-1.1% w/w of FLU and when used for _in vivo_ studies contained 0.0225-0.0275% w/w of FLU.

\# Gel containing physical mixture of PC, CHOL and FLU.

The formulation code has been devised as follows :

the 1st alphabet signifies the base;

the 2nd alphabet when the drug is free (F);

the 3rd alphabet whether the formulation is a gel (G) or a cream (C).

TABLE 3.13

DESCRIPTION OF THE VARIOUS FORMULATIONS OF CLO PREPARED IN DIFFERENT BASES.

Formulation Code*	**Base**	**Content**
KCG	HPMC K4M	Plain CLO
C_1	HPMC K4M	CLO1
C_2	HPMC K4M	CLO2
C_3	HPMC K4M	CLO3
# PMC	HPMC K4M	Plain CLO
CCC	CMC base	Plain CLO
ACC	Aqueous Cream base	Plain CLO

* All formulations used in <u>in vitro</u> studies contained 0.9-1.1% w/w of CLO and when used for <u>in vivo</u> studies contained 0.045-0.055% w/w of CLO.

\# Gel containing physical mixture of PC, CHOL and CLO.

The formulation code has been devised as follows :

the 1st alphabet signifies the base;

the 2nd alphabet when the drug is free (C);

the 3rd alphabet whether the formulation is a gel (G) or a cream (C).

in case of gels prepared for _in vivo_ studies, the entire 10ml of this solution was transferred into a 25ml volumetric flask. The solvent was evaporated to dryness over a water-bath at 65°C and the contents of the flask were analysed for drug by using the method described in Section 3.23.

(c) PVA Gels : 100mg of the gel was weighed accurately and dissolved in 10ml of distilled water. This solution was extracted thrice with 10, 5 and 5 ml of chloroform. The combined chloroform extracts were passed through a bed of anhydrous sodium sulphate into a 25ml volumetric flask. 2.5ml of this solution in case of gels prepared for _in vitro_ studies and the entire volume of the solution in case of gels prepared for _in vivo_ studies was evaporated to dryness on a water bath at 65°C. The contents of the flask were then analysed for drug by using the method described in Section 3.23.

(d) Creams prepared using Aqueous cream base and Cetomacrogol cream base: 100mg of the cream was weighed accurately and dispersed in 10ml of chloroform. The dispersion was passed through a bed of anhydrous sodium sulphate into a 25ml volumetric flask. The solvent was evaporated to dryness on a water-bath at 65°C and the contents were analysed for the drug by using the method described in Section 3.23.

Appropriate reagent blanks were used to eliminate interferences of the base in each case. Each gel/cream was analysed in triplicate using samples from 3 corners of the container. The gels/creams complied the assay if they contained between 90-110% of the amount claimed.

3.2x1 Stability Studies :

Stability studies were conducted for formulations which seemed promising following in vitro studies. 50g of each formulation was packed in a amber glass jar and stored at room temperature ($30\pm2^{\circ}C$) for a period of 45 days. The formulations were studied for their physical appearance, chemical assay and in vitro release profile at the prescribed sampling intervals. The results of the physical and chemical studies are shown in Tables 3.14 and 3.15 while those of in vitro release are shown in Chapter 4.

3.3 RESULTS AND DISCUSSIONS

As seen from Tables 3.1 and 3.2 and Fig 3.1, the colorimetric method used for analysis of the drugs is sensitive between 4-40 μg/ml for TRMA and 2-20 μg/ml for FLU and CLO. The sensitivity of this method decreases in presence of water and hence before using it for estimating the drug in samples, they were passed through a bed of anhydrous sodium sulphate.

The results of the Stewart assay are shown in Table 3.3. A linear relationship (r=0.999) exists between PC concentration and absorbance in the range of 100-700 μg/2ml as seen in Fig. 3.2. The results of the Zlatkis, Zak and Boyle's method for estimation of CHOL are shown in Table 3.4.

TABLE 3.14

DATA OF THE STABILITY STUDIES CONDUCTED ON SELECTED FORMULATIONS OF TRMA - PHYSICAL OBSERVATIONS.

Temperature of storage = Room temperature (32.0 $\pm$ 2.0^{o}C)

Formulation	Time		
	Initial	**15 days**	**45 days**
KTG	Off white; completely homogeneous	Off white; completely homogeneous	Off white; completely homogeneous
T_7	Straw yellow; completely homogeneous	Straw yellow; virtually homogeneous	Straw yellow; virtually homogeneous
T_8	Straw yellow; completely homogeneous	Straw yellow; virtually homogeneous	Straw yellow; very indistinct separation
T_9	Straw yellow; completely homogeneous	Straw yellow; distinct separation	Straw yellow; distinct separation with slight coalescence

TABLE 3.15

DATA OF THE STABILITY STUDIES CONDUCTED ON SELECTED FORMULATIONS OF TRMA - CHEMICAL ANALYSIS.

Temperature of storage = Room temperature (32.0 ± 2.0 °C)

Formulation	**% TRMA remaining**		
	Initial	**15 days**	**45 days**
KTG	100.00	99.90	99.69
T_7	100.00	99.81	100.10
T_8	100.00	100.00	100.10
T_9	100.00	100.10	100.10

A linear relationship (r=0.999) exists between the CHOL concentration and absorbance in the range of 10-100 μg/ml as seen in Fig. 3.3.

During the preparation of liposomes, it was ensured that the round bottom flask used was completely dry and the methanol used was dehydrated because the presence of even trace quantities of water, resulted in films, which adhered too firmly on the bottom of the flask, making their removal difficult by mere shaking with calcium chloride solution. Centrifuging the liposomal suspension for 30 minutes at 2750 r.p.m. resulted in a discrete liposomal pellet with an almost clear supernatant in most of the batches, indicating that the speed and duration of centrifugation were enough to sediment most of the liposomes.

Liposomes obtained by using the lipid film hydration method were off white and discrete indicating that little oxidation of the PC had taken place and that the strength of the calcium chloride solution used was sufficient to prevent the aggregation of liposomes. In the absence of an antimicrobial agent, the prepared liposomal suspensions were highly susceptible to microbial growth and hence they were used within 1-2 days of preparation.

Liposomes of all the batches when viewed under a Carl Zeiss Jena microscope (Mag. 40x and 100x), appeared almost spherical. A discrete dark outline marked the boundary of each liposome as can be seen from Plate 3.1. No free crystals of the drug were visible under the microscope in all the liposomal preparations indicating that centrifugation was an efficient means of seperating the unentrapped drug from the liposomes.

Multilamellar nature of the liposomes prepared was confirmed by the electronmicrographs of negatively stained samples presented in Plates 3.2-3.4.

Since batches TRMA1, TRMA7 and TRMA21 represented the lowest, middle and highest TRMA:PC:CHOL ratio of the liposomes prepared, these 3 batches were subjected to particle size analysis. From the frequency distribution plots shown in Fig. 3.4, it is clear that the particle size distribution of all the 3 batches of liposomes is normal i.e. symmetrical around the mean. Comparing the values of nd (Table 3.7) for each formulation, using ANOVA, the calculated F value (0.128) was less than the table F value (4.26) at $P<0.05$ indicating that the liposomal batches didnot differ significantly in their particle size distribution. The arithmetic mean diameters for the 3 batches studied are not significantly different indicating that the amount of PC and CHOL used for preparing liposomes does not alter the particle size distribution significantly.

PC is a good emulsifying agent and hence extraction procedures involving chloroform and water were rendered difficult. This emulsification was avoided by using a saturated sodium chloride solution. As is seen from Table

3.8, among the various liposomal batches of TRMA prepared, except for the batch TRMA1, all other batches showed greater than 70% entrapment. However, no clear pattern emerges as regards the % TRMA entrapped and TRMA:PC:CHOL ratio. Table 3.9 shows that more than 70% of FLU and CLO were entrapped in their liposomal batches prepared. The quantities of PC and CHOL obtained when the liposomal suspensions were analysed are not shown individually but the ratio of Drug:PC:CHOL in each batch prepared is shown in Table 3.5 and 3.6. When the supernatant was analysed for its drug content, a material balance was obtained between the amount of drug added in the preparation and the amount recovered after the preparation procedures, the loss being of the order of 5-6%. As is seen from Table 3.8 and 3.9, not more than 0.1% of the drug was in the free form in the final dispersion of the liposomes prepared.

The gel bases obtained by using 5% w/w of HPMC K4M and HPMC E4M and 20% w/w of PVA were translucent while that obtained by using 0.8%w/w of Carbopol 941 was transparent. Both the cream bases prepared were smooth and snow white.

During the preparation of the drug gels/creams, the levigation step was very crucial since the preparations contained very small quantities of the drug. Hence, mixing was continued till the % coefficient of variation in the assay readings was not more than 5% between 3 samples withdrawn from 3 different corners of the container.

From the results of the physical changes in the 3 formulations kept for stability studies (Table 3.14), formulation T_7 was found to be the best indicating that the a TRMA:PC:CHOL ratio of 1:1.81:0.46 may give a stable liposomal gel. Although the liposomes in formulations T_8 and T_9 seperated out in 15 days, they reverted back to their initial appearance when they were stirred with a glass rod. No significant changes occured in the % TRMA in the gels over the storage period in all the gels tested indicating that no loss of drug occurs during storage.

All the formulations discussed in this chapter were subjected to in vitro permeation studies.

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