# CHAPTER 1
# INTRODUCTION AND LITERATURE SURVEY

## 1.1 INTRODUCTION

Cancer is the common term for all malignant tumours. It probably derives from the Latin word for crab, 'cancer' - presumably because cancer adheres to any part that it seizes upon in an obstinate manner like the crab(1). There are three primary modalities of cancer treatment. The vast majority of cancer cures occur through surgery, where a localised tumour is removed before dissemination or metastasis. Radiation therapy can cure certain localised malignancies. However, for disseminated cancer, chemotherapy is the mainstay of remission induction.

Over the years there has been a systematic approach to the development of cancer chemotherapy. Since 1955, over 700,000 compounds have been screened for antineoplastic properties. In 1945 there was only one drug known to be effective namely nitrogen mustard. Today there are nearly 50 chemotherapeutic agents used singly or in combination in the treatment of malignancy(2).

The two main groups of drugs used in the treatment of malignant diseases are the biological alkylating agents and antimetabolites. Nitrogen mustards (cyclophosphamide), ethyleneimine compounds (thio-tepa) and alkyl sulfonates (busulfan) are the main alkylating agents. The antimetabolites include the folate antagonists (methotrexate), pyrimidine antagonists (5fluorouracil) and purine antagonists (6-mercaptopurine)(3).

Despite extensive experimental investigations and numerous theoretical considerations, the overall success in routine cancer therapy has been nominal. The main reason being the non selectivity of the chemotherapeutic agents which invariably lead to dose dependent systemic toxicities, often warranting discontinuation of treatment. Hence, the development of techniques which could selectively deliver drug molecules to the diseased vasculature is one of the most exhaustively pursued areas of research in experimental pharmacology and therapeutics.

## 1.11 Research Envisaged

For the present investigation three of the most important antineoplastic agents have been selected. These are

1.  5 Fluorouracil (5FU).

2.  Methotrexate (MTX).

3.  Cyclophosphamide.

The objective of the research project is as follows :

1.  Development of simple, rapid, sensitive and selective analytical methods for the estimation of these drugs in bulk drug as well as formulations and biological fluids.

2.  Accelerated stability studies of some of these formulations.

3.  Determination of some kinetic parameters like reaction rate constant, half-life and activation energy values.

4. Fabrication, optimisation and evaluation of liposomal dosage forms of 5FU and MTX for selective targetting into the skin. It has been recently reported that topically applied liposomes may act as sustained release vehicles for chemotherapeutic drugs into the skin. It was also observed that liposomally entrapped drug was retained in the skin 2-3 folds more than the free drug while blood drug concentration was the opposite, the liposomal form providing lower drug concentration than the free form(4). This fact was exploited to develop liposomal dosage forms for topical application of 5FU and MTX. The topical use of 5FU and MTX is limited due to erratic absorption and systemic toxicities. Therefore it was proposed to incorporate 5FU and MTX into liposomes to localise these drugs into the dermal region and also to reduce their systemic toxicities.

## 1.12 Proposed Plan of Work

### 1.121 Development of analytical methods :

The detailed survey for analytical methods available for 5FU, MTX and cyclophosphamide revealed that their molecular structures still offer ample scope for development of new analytical methods. Hence a number of simple, selective and sensitive methods were developed on the following lines :

a) Complexation of 5FU, MTX, cyclophosphamide with metal ions.

b) Reactions of 5FU with diazotised primary amines.

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c)   Reaction of 5FU with dithizone and diphenyl carbazone.

d)   Development of fluorimetric method for 5FU in alkaline medium.

e)   Development of HPLC method for 5FU using water-methanol system.

f)   Reaction of MTX with folin ciocalteau reagent.

g)   Reaction of MTX with Nessler's reagent.

h)   Reaction of MTX with nitric acid.

i)   Reaction of benzoylated MTX with hydroxylamine hydrochloride and ferric chloride.

j)   Reaction of cyclophoshamide with picric acid.

k)   Estimation of 5FU, MTX and cyclophosphamide from blood plasma by proposed analytical methods.

1.122  Accelerated stability studies :

5FU, MTX and cyclophosphamide have well established stability profile. The validity of some of the proposed analytical methods was established by conducting accelerated stability studies for the above compounds in aqueous solutions at various pH values. Stability studies for 5FU and MTX were also conducted in various semisolid bases. The results were statistically compared with those obtained by the reported methods.

1.123  Liposomes of 5FU and MTX for skin targetting :

a)   Preparation of liposomal formulations of 5FU and MTX by a suitable method.

b)   Optimisation of parameters for maximum drug loading.

c)   Incorporation of  drug loaded liposomes into semisolid bases.

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d)   In vitro permeation studies using rat skin.

e)   Stability studies of liposomal formulations.

f)   In vivo studies of the effects of liposomal formulations
     of 5FU and MTX on dinitrochlorobenzene induced erythema
     in guinea pigs.

## 1.2   PROFILE OF 5FU

### 1.21   Description : (5)

1.211   Name, structural formula and molecular weight :

5 Fluorouracil (5FU), (Roche) is chemically 5-fluoro-
pyrimidine-2,4 (1H,3H) - dione.
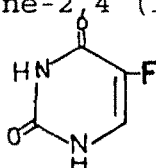


Molecular weight : 130.08

1.212   Appearance, colour and odour :

5FU is a white to practically white odourless,
crystalline powder.

1.213   Dosage forms :

Available as injection of strength 50mg in 1ml (I.P,
B.P and U.S.P) and as 5% cream (B.P and U.S.P).

### 1.22   Physical Properties :

1.221   Infrared spectrum : (6)

The I.R spectrum shows the principal peaks at 3124,
1716, 1657,1245 and 813cm$^{-1}$.

1.222   Ultraviolet spectrum : (5)

The UV spectrum of 5FU in acetate buffer (pH 4.7) in
the region 350-220 nm exhibits one maximum at 266 nm and one
minimum at 232 nm.

The UV spectrum in methanol shows maxima at 265nm which shifts to 210nm due to ketoenol tautomerism.

1.223  Fluorescence spectrum : (5)

The spectra measured in methanol solution of 5FU (1.0mg/ml) showed one excitation peak at 315nm and one emission peak at 391nm.

1.224  Melting range : (5)

The melting point lies between 282$^O$C and 283$^O$C.

1.225  Solubility : (7)

Sparingly soluble in water, slightly soluble in alcohol, practically insoluble in chloroform and solvent ether.

1.226  Dissociation constant : (5)

The pka's for 5FU have been determined spectrophoto-metrically to be 8.0±0.1 and 13.0±0.1.

**1.23  Stability Degradation (8,9) :**

5FU is stable in solutions which are not strongly basic (pH less than 9). When subjected to strongly basic conditions, 5FU is hydrolysed to urea, fluoride and an aldehyde. This hydrolysis is enhanced by increased pH and temperature. Some of the urea formed on hydrolysis reacts further giving ammonia and carbondioxide.

**1.24  Methods of Analysis :**

1.241  Thin layer chromatographic analysis : (10)

The TLC of 5FU has been studied in various developing solvents like ethylacetate, methanol, ether, 2-propanol, ethylacetate : methanol, ethylacetate : methanol : glacial

aceюpéc   acïd,   acetone:methanol,ethylacetate : water etc.,
on silicagel GF layers and visualised by short wave UV
radiation exposure.

1.242  Volumetric analysis : (5)

5FU may be assayed by dissolving in dimethylformamide
and titrating with 0.1N tetra-n-butylammonium hydroxide in
methanol to a blue end point using thymol blue as indicator.

1.243  Spectrophotometric analysis :

a)  UV spectrophotometry : (5)

Direct spectrophotometric analysis has been reported for

analysis of injections at 266nm in pH 4.7 acetate buffer.

b)  Colorimetry : (11)

A colorimetric method has been reported for 5FU with 2,4-

dinitro phenyl hydrazine solution.

c)  Fluorimetry : (12)

5FU has been derivatized with dansyl chloride at pH 10

and estimated from various pharmaceutical preparations.

d)  Fluorine analysis : (5)

The determination of organically bound fluorine and free

fluoride present in 5FU in bulk sample or injections have

been reported.

1.244  Gas liquid chromatographic analysis (GLC) : (5)

GLC has been carried out for 5FU using the following
instrumental conditions :

Column : 6ft. copper tubing

Column packing : 5% SE-30 on chromosorb G.

Column temperature : 250$^{\circ}$C

Carrier gas : nitrogen

Flow rate : 40 cc/minute.

Detector : Hydrogen flame ionization.

Various other methods have also been reported. (13,14,15)

1.245  High performance liquid chromatographic methods :

Various HPLC methods have been reported for estimation
of 5FU in bulk drug, dosage form, plasma and other body
fluids. Some of the methods have been listed in Table 1.1.

## 1.25  Mechanism of Action : (3)

5FU is a pyrimidine antagonist. It probably acts by
binding the enzyme thymidilate synthetase, thus preventing
the production of thymine, the basic component of DNA. It
also gets incorporated into RNA in place of uracil. It also
has immunosuppressant properties.

## 1.26  Absorption, Fate and Excretion : (3)

After i.v. injection, 5FU is cleared rapidly from the
plasma. It is distributed throughout body tissues and
fluids. It is converted to active metabolites within the
target cells itself. About 15% of the dose is excreted
unchanged in the urine. The remainder is inactivated
primarily in the liver. A large amount is excreted through
the respiratory tract.

## 1.27  Adverse Effects : (3)

The main adverse effects are bone marrow depression,
G.I. ulceration, severe diarrhoea and bleeding. Nausea,
vomitting, skin rashes, hyperpigmentation, alopecia, central
neurotoxicity and myocardial ischemia have been reported.

## TABLE 1.1

### SOME HPLC METHODS FOR ESTIMATION OF 5FU

| Sample 1 | Internal standard 2 | Column 3 | Mobile phase 4 | Detector 5 | Detection limit 6 | Reference 7 |
|---|---|---|---|---|---|---|
| 1. Serum | - | Bondapak $C_{18}$ | a) water<br>b) water-acetonitrile<br>14 : 1<br>7 : 3 | UV-254nm | 100-250ng/ml | 16 |
| 2. Serum | - | Bondapak $C_{18}$ | 50 mM dipotassium phospate pH 4.5-4.7 | UV-254nm | 0.1-10 µg/ml | 17 |
| 3. Serum | Thymidine | $C_{18}$ | 3-6% MeOH in o.o1M sodiumacetate pH 4 | UV-270nm | 0.05-10 µg/ml | 18 |
| 4. Serum | - | YWG-$C_{18}$ $H_{37}$ | 0.01 M tetrabutyl ammonium chloride | UV-254nm | 0.02-5 µg/ml | 19 |
| 5. Formulat - ions | Cytidine | Radial pak MBC$_{18}$ | 25mM - hexane-1-sulphonic acid | 270nm | 10-80 µg/ml | 20 |
| 6. Plasma | - | Column switching CPS-Hypersil and ODS-Hypersil | derivatised with 4-(bromomethyl)-7-methoxy coumarin aqueous 50% and 60% MeOH | Fluorescence excitation at 325nm and emission at 395nm | 0.01-10 µg/ml | 21 |
| 7. Serum | - | Bondapak $C_{18}$ | 0.02M NaH$_2$ PO$_4$.H$_2$O - pH 4.5 | UV-254nm | 0.1-20 µg/ml | 22 |
| 8. In 0.9% sodium chloride solution | - | Spherisorb | 2%tetrabutyl ammonium hydroxide to pH 6.0 with phosphoric acid | UV-313nm | 1.0-800 µg/ml | 23 |

contd...

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| 9. 5FU and its metabolites | - | Beckman ultra sphere ODS | Phosphate buffer-MeOH (19:1) tetrabutyl ammonium phosphate (pH 5.9) | UV-280nm | 10-30ng/ml | 24 |
| 10. Serum | - | Chrompack Li Chrosorb RP18 | 0.05M phosphate buffer pH 4.6 | UV-266nm | Upto 2000ng/ml | 25 |
| 11. Plasma | Iodouracil | Ultrasphere ODS | 0.02 M potassium dihydrogen phosphate pH 5.9 with 0.1mM tetrabutyl ammonium phosphate-MeOH (191:9) | UV-280nm | 0.01-5.0 µg/ml | 26 |
| 12. 5FU derivatised with 7(diethylamino)-3[4(iodoacetyl) aminophenyl] 4-methyl coumarin | - | Nucleosil sC$_{18}$ | 50% acetonitrile in 4mM potassium dihydrogen phosphate disodium hydrogen phosphate pH 7.5. | Chemilumine scence | 1000pg | 27 |
| 13. Body fluids | Bromo uracil | Supelcosil LC 18-5, ODS | Phosphate buffer pH 6.5-methanol | Photodiode 210-400nm array | low ng/ml range | 28 |
| 14. Vitreous gel | Flucytosine | C$_{18}$ | 0.05M phosphate buffer pH 3.5 | UV-254nm | 0.5-100 µg/ml | 29 |
| 15. Bodyfluids | Bromo uridine | Zorbax R | acetonitrile in 25mM ammonium phosphate | UV-270nm | 0.25µm in 20ul sample | 30 |
| 16. Serum | Bromouracil | YWG -C$_{18}$ H$_{37}$ | water: MeOH: acetate buffer pH 6.1 187:12:1 | UV-250nm | 0.05-50 µg/ml | 31 |

## 1.28   Therapeutic Uses  :  (32)

It is used alone or in combination in palliation of inoperable malignant neoplasms. It is often used with cyclophosphamide and MTX in the combination chemotherapy of breast cancer. A usual I.V. dose is 12mg/kg body weight. 5FU is also used topically as 5% cream or 1-5% solution in propylene glycol in the treatment of solar or actinic keratoses, dermatoses and, other tumours and pre malignant conditions of the skin. The white blood cell count should be determined during treatment with 5FU.

## 1.3   PROFILE OF MTX

### 1.31   Description :

1.311   Name, structural formula and molecular weight :  (33)

Methotrexate (MTX), (Lederle) is chemically N[4-(2,4-diamino-6-pteridinyl)-methyl] methylamine benzoyl] glutamic acid.
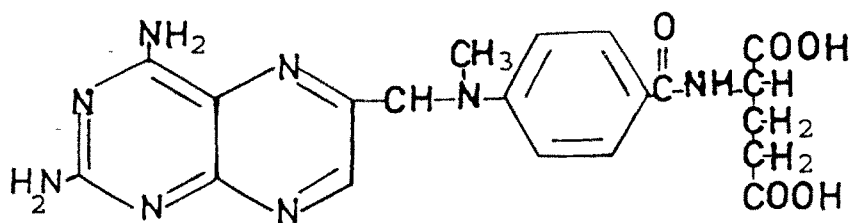


Molecular weight : 454.46 (33)

1.312  Appearance, colour and odour :   (33)

MTX is a bright yellow-orange, odourless powder.

<u>1.313 Dosage forms</u> :

Available as tablets of strength 2.5mg/tablet (I.P, B.P and U.S.P) and as injection of strength 25mg/ml (B.P. and U.S.P).

## 1.32 Physical Properties :

<u>1.321 Infrared spectrum</u> (IR) : (33)

The IR spectrum shows the principal peaks at 2.90 - 3.10, 3.00-4.00, 5.90-6.10, 6.20, 6.50-6.60 and 11.9µ.

<u>1.322 Ultraviolet spectrum</u> (UV) : (34)

The UV spectra of MTX is as follows :

| Solvent | $\lambda$ max |
|---|---|
| 0.1NHCl | 307,243 |
| 0.1MpH 6.7 tris buffer | 370,305,257 |
| 0.1N NaOH | 372,305,258 |

<u>1.323 Melting range</u> : (35)

MTX has a melting point range of 182-189°C.

<u>1.324 Solubility</u> : (36)

MTX is practically insoluble in water, alcohol, chloroform and ether. It is freely soluble in dilute solutions of alkali and alkaline carbonates, it is slightly soluble in dilute hydrochloric acid.

<u>1.325 Dissociation constant</u> : (37)

The basic pKa values for 2,4-diamino pteridine were < 0.5 and 5.32. The acidic pKa values for p-aminobenzoyl glutamic acid are 4.83 and 3.76. The pKa value assignable to diamino pteridinyl moiety is 5.60±0.03.

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## 1.33 Stability : (38)

Under strongly acidic aqueous conditions the amide is subjected to hydrolysis, yielding $N^{10}$-methyl-4-amino-4-deoxy pteroic acid and glutamic acid. Under highly alkaline aqueous conditions at elevated temperature, the principal decomposition products are $N^{10}$-methyl folic acid, $N^{10}$-methyl pteroic acid and glutamic acid. Photodecomposition of certain pterines is also reported.

## 1.34 Methods of Analysis :

### 1.341 Thin layer chromatography (TLC) :

Various TLC methods have been reported for separation of MTX from its degradation products. They are :

1.  Cation-exchange resin AG50wx4 with 15% $Na_2HPO_412H_2O$ pH8.5 buffer as developing solvent (39).

2.  Diethyl amino ethyl cellulose (DEAE) plate with 0.1M ammonium bicarbonate (pH 8.8) as developing solvent (40).

3.  Reversed phase TLC on silanised silica gel HF254 with citrate phosphate buffer (pH6.0) - MeOH (70:30) (41).

### 1.342 Column chromatography : (42)

A column chromatographic method has been reported for separation of MTX from its degradation products using DEAE cellulose column using ammonia-ammonium bicarbonate for elution.

### 1.343 Paper chromatography :

Descending paper chromatography has been reported for the assay of MTX (43,44).

## 1.344 Volumetric methods :

a) **Non-aqueous titration** : (33)

A method has been reported based on titration with sodium methoxide in pyridine to an azo-violet end point.

b) **Complex-formation titration** : (33)

This titration is based on complexation between $Ca^{2+}$ and glutamyl moiety of the drug.

## 1.345 Biological assay :

Several microbiological assays and enzymic assays have been reported for estimation of MTX (33).

## 1.346 Spectrophotometric methods :

a) **UV spectrophotometric method** : (45)

UV method reported, is based on measuring light absorbance of the drug at 303 in 0.1N NaOH.

b) **Colorimetry** : (45)

The reported colorimetric method is based on coupling diazotised MTX with 8-hydroxy quinoline in alkaline medium and measuring the absorbance at 450nm.

c) **Fluorimetry** : (46)

The fluorescence method reported is based on oxidation of the drug with potassium permanganate solution and the fluorescence is measured at 450nm at pH 7.0 with excitation at 370nm.

## 1.347 HPLC methods :

Various HPLC methods have been reported for estimation of MTX from formulations and body fluids some of which are given in Table 1.2.

TABLE 1.2

SOME HPLC METHODS FOR ESTIMATION OF MTX

| Sample | Internal standard | Column | Mobile phase | Detector | Detection limit | Reference |
|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 1. Bulk | - | anion exchange resin column AX- 107 | 3M-sodium percholrate 0.15-sodium hydrogen phosphate-0.15M sodium phophate -15ml acetonitrile | UV-254nm | 40-120 µg/ml | 38 |
| 2. Serum | - | Bondapak phenyl | 11% solution of acetonitrile in 0.15M sodium acetate buffer pH+4.6 | UV-303nm | 0.02-100 µg/ml | 47 |
| 3. Serum | - | $C_{18}$ | 5mM tetrabutyl ammonium phosphate in aqueous 25% MeoH | UV-302nm | 0.1-10 µg/ml | 48 |
| 4. Plasma | - | Bondapak $C_{18}$ | acetonitrile-MeOH-0.2M acetate buffer containing 0.03 MEDIA (63:84:853) | UV-305nm | 0.1-100 µg/ml | 49 |
| 5. Biological | - | Spherisorb ODS | 0.05M citrate phosphate buffer (pH3.2) MeOH- THF (20:4:1) | UV-303nm | 0.1-15 µg/ml | 50 |
| 7. Plasma | - | Bondapak $C_{18}$ | 20% methoenolic 2.5mM tetrabutyl ammonium nitrate (I) in 5mM sodium phosphate buffer pH 7.4-2.5mM followed by gradient elution with 30% methanolic (I) | UV-315nm | 40-100 ng/ml | 51 |

contd...

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| 8. Plasma | Theophylline | Lichrosorb RP18 | 0.05M ammonium acetate buffer acetonitrile.MeoH (174:13:13) pH 5.0 | UV-305nm | 0.2-50 µg/ml | 52 |
| 9. Bulk,separation from impurities | - | Nucleosil $C_{18}$ or Zorbax ODS | Phosphate-citrte buffe pH 5.7 acetonitrile(23:2) | UV-302nm | 0.1-0.2 mg/ml | 53 |
| 10. Serum | - | Lichrosorb RP-18 | MeoH-10mM acetate buffer pH 3.8 (11:39) | UV-305nm | 0.5-10 µg/ml | 54 |
| 11. Serum | Aminopterin | Ultrasphere ODS, RP-18 | 5% THF in 0.05M sodium dihydrogen phosphate pH 4.85) | UV-313nm | 0.01-10 µg/ml | 55 |
| 12. Serum | - | Separon Six $C_{18}$ | phosphate buffer (pH6.8) 4:1-MeOH | UV-305nm | 0.02 µg/ml | 56 |
| 13. Serum | - | Intersil ODS -2 | 25mm $H_2O_2$-8% acetonitrile in 25mM phosphate buffer pH 7.0 | Fluorimetric at 457nm (excitation at 379nm) | 1-10ng µg/ml | 57 |
| 14. Plasma and urine | - | Spherisorb 53 ODS2 | 0.1M sodiumphosphate (pH6.5) with 6% NNDMF and 0.2% of 30% $H_2O_2$ | UV-254nm | 100ng | 58 |

## 1.35 Mechanism of Action : (3)

MTX competitively inhibits the enzyme dihydrofolate reductase and prevents the formation of tetrahydro folate which is necessary for purine and pyrimidine synthesis. Thus the synthesis of DNA is inhibited and consequently the cell replication.

## 1.36 Absorption, Fate and Excretion : (3)

MTX is rapidly absorbed from the tract following low dose administration. However, absorption is retarded at high dose. It is completely absorbed following i.m. administration. It is distributed to various tissues and extracellular fluids. Only a very small amount crosses the blood brain barrier. It's plasma protein binding is 50%. MTX does not appear to undergo significant hepatic metabolism. It is mainly excreted unchanged in the urine. A small fraction is excreted into the bile.

## 1.37 Adverse Effects : (3)

Early signs of toxicity with MTX include leucopenia, thrombocytopenia, anaemia, ulceration of mouth, stomatitis and diarrhoea. Neurotoxic reaction, teratogenic effect and foetal deaths have been reported.

## 1.38 Uses and Administration : (32)

MTX is used in the management of acute lymphoblastic leukaemia and in the prophylaxis and treatment of meningeal leukaemia.

It is often used in combination with other neoplastic agents in the treatment of variety of malignant diseases. It is also of value in the treatment of psoriasis and other skin conditions like mycosis fungoides, dermatomyositis etc.

MTX may be given by mouth or by injection. The doses and regimens employed vary widely and may need to be adjusted according to the blood picture.

## 1.4   PROFILE OF CYCLOPHOSPHAMIDE

### 1.41   Description :

#### 1.411   Name, structure and molecular weight : (59)

Cyclophosphamide (Mead-Johnson) is chemically mono hydrate of 2-bis (2 chloroethyl) aminoperhydro -1,3,2-oxazaphospharinone 2-oxide.
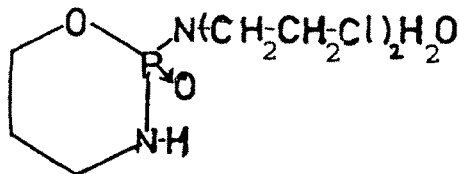


Molecular weight : 279.10

#### 1.412   Appearance, colour and odour : (59)

Fine, white, crystalline powder, odourless. It liquefies upon loss of its water of crystallisation.

#### 1.413   Dosage forms :

Available as injection for reconstitution of strength 100-200 mg. The product also contains 45 mg of sodium chloride per 100 mg of drug (I.P, B.P and U.S.P). Also available as tablets of strength 25 mg and 50 mg.

## 1.42  Physical Properties :

<u>1.421  I.R. Spectrum</u> : (60)

I.R. absorption spectrum has principal peaks at wave numbers 1225, 1044, 975, 1088, 945, 1128 $cm^{-1}$.

<u>1.422  Solubility</u>  : (59)

Soluble in water, freely soluble in alcohol and slightly soluble in solvent ether.

## 1.43  Stability Degradation : (61,62)

Cyclophosphamide is susceptible to spontaneous hydrolysis in aqueous solution and undergoes both specific acid and base catalysis at extreme pHs. A decrease in pH has been noted during hydrolysis and the rate is constant over the pH range of 2-10.

## 1.44  Methods of Analysis :

<u>1.441  Thin layer chromatography</u> (TLC) : (63)

Cyclophosphamide has been estimated by TLC using silica gel F254 plate with mobile phase $CHCl_3$-EtOH-glacial acetic acid (100:20:1) and visualised with 4-(-4-nitrobenzyl) pyridine reagent and analysed by densitometry.

Estimation of unchanged cyclophosphamide, its analogs and their metabolites in human urine by TLC has also been reported (64).

<u>1.442  Volumetric methods</u> :

a)  The method official in I.P. involves heating of drug with sulphuric acid and nitric acid, followed by treatment with citric acid-molybdic acid and quinoline solution.

The precipitate obtained is dissolved in alkali and the excess of alkali is estimated with hydrochloric acid using phenolphthalein-thymol blue solution as indicator.

b) In the other official method (65), drug is dissolved in sodium hydroxide in ethane 1,2 diol, refluxed, treated with silver nitrate-ammonium iron (III) sulfate solution and titrated with ammonium thiocyanate solution.

1.443 Spectrophotometric methods :

a) Colorimetric methods :

Various colorimetric methods have been reported for estimation of cyclophosphamide.

i) One method involves reaction of drug with hexa ammonium 4-amino 5-hydroxy naphthalene-2,7 disluphonic acid solution in acidic medium and after 24 hours the absorbance was measured at 830 nm against reagent blank (66).

ii) Aqueous cyclophosphamide was treated with 20% each of sodium nitrite solution, hydrochloric acid solution and the absorbance was measured at 350nm against reagent blank (67).

iii) The other method involves estimation of the drug with Υ- (p-nitro benzyl) pyridine at 540nm (68).

b) Infrared spectroscopic method : (69)

Cyclophosphamide was determined in tablets and injection by IR with KBr pellet and dehydrocholic acid as internal standard and sodium chloride cells.

<u>1.444</u>   <u>Gas</u> <u>chromatography</u> (GC) :

Various GC methods have been reported for estimation of cyclophosphamide in bulk, formulations and body fluids (70,71,72).

<u>1.445</u>   <u>HPLC</u> <u>methods</u> :

Various HPLC methods have been reported for estimation of cyclophosphamide, some of the methods are given in Table 1.3.

## 1.45  Mechanism of Action : (3)

Nitrogen mustards called as alkylating agents can transfer an alkyl radical to a suitable receptor site. These agents in neutral or alkaline solution, form ethylenimmonium cations which react with groups like amino, sulfhydryl, hydroxy or phosphate in the physiologically important molecules in the cells and render them unavailable for the normal metabolic reactions. Alkylating agents also inhibit DNA synthesis. They also have immunosuppressant action.

## 1.46  Absorption, Fate and Excretion : (3)

Cyclophosphamide is readily absorbed orally and widely distributed in the tissues. It undergoes activation in the liver to active metabolites including nor-nitrogen mustard and phosphoramide. Acrolein is also produced. It is principally excreted in urine.

## 1.47  Adverse Effects : (3)

The major dose-limiting effect is myelosuppression. Thrombocytopenia and anaemia may occur. Other reported

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TABLE 1.3

SOME HPLC METHODS FOR ESTIMATION OF CYCLOPHOSPHAMIDE

| Sample | Internal standard | Column | Mobile phase | Detector | Detection limit | Reference |
|---|---|---|---|---|---|---|
| 1. Bulk | Ifosfamide | Nucleosil $C_{18}$ | 41% methanol | R.I. detection | 1-10 µg/mL | 73 |
| 2. Serum | - | Bondapak $C_{18}$ | 2mm potasium phosphate buffer pH4- acetonitrile (71 : 29) | UV-195nm | 0.3mg/lt | 74 |
| 3. Bulk | ethylparaben | $C_{18}$ | Acetonitrile-water (3:7) | 200nm | - | 75 |
| 4. Whole blood and plamsa | - | ODS | Acetonitrile-10mM $KH_2PO_4$ (pH 6.8, 1:4) | 190nm | 0.3µg/ml | 76 |

adverse effects include alopecia, renal cystitis, GI disturbances, gonadal suppression and cardio toxicity.

**1.48 Uses and Administration : (32)**

Cyclophosphamide is widely used, often in combination with other agents in the treatment of a variety of malignant disease including Burkitt's lymphoma, Hodgkin's disease, acute and chronic lymphoblastic leukaemia, multiple myeloma and mycosis fungoids. The immunosuppressant properties of cyclophosphamide are used in Wegeners granulomatosis and in kidney transplantation. It is also used in the management of systemic lupus erythematosus, nephortic syndrome and rheumatoid arthritis.

Cyclophosphamide is given by mouth or i.v. injection. The dosage given depends on the malignant disease being treated. Regular blood counts are essential during therapy.

## 1.5 LIPOSOMES AS DRUG DELIVERY SYSTEM

**1.51 Liposomes as Carriers in Cancer Chemotherapy :**

Chemotherapy is a modality of cancer therapy that needs much improvement. Development of a new chemical entity is very costly and time consuming, but improvements in delivery of existing agents may yield more rapid clinical results. Water in oil emulsions (77-79), starch microspheres (80-82), ethyl cellulose microspheres (83,84), liposomes (85-90), polymethacrylate nanoparticles (91) etc., are notable examples of the systems which have been tested for their feasibility in targetted delivery of chemotherapeutic agents.

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Liposomes as a drug delivery system, offer great potential for improving cancer chemotherapy. A liposome is defined as a structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments. Discovered by Bangham in 1965, they are considered to be efficient therapeutic carriers of drugs (92). Depending on size, number of bilayers and multimembrane structure there are several classes of liposomes (93). Large multilamellar vesicles (MLV, size 0.05-10µm), Small unilamellar vesicles (SUV, 0.025-0.05µm), Reverse phase evaporation vesicles (REV, 0.5µm), Large unilamellar vesicles (LUV, 0.1µm). There are atleast 14 major published methods for making liposomes. MLV liposomes formed by original process used by Bangham involves rehydration of a dried lipid film with an aqueous phase. Molecules dissolved in original aqueous phase will be spontaneously entrapped in the aqueous spaces within the MLV and lipid soluble molecules that are added in the solvent phase will be incorporated in the vesicle membranes.

Liposomes can also be formed from w/o emulsions (94). Liposomes with large internal aqueous space and high capture can be prepared by reverse phase evaporation technique (95).

Much of the effort in liposomes research has been directed at tumour targetting. The effects of liposomal encapsulation have been studied with a wide variety of anticancer drugs, some of which are listed in Table 1.4.

## TABLE 1.4

**SUMMARY OF DEVELOPMENTAL STATUS OF ANTICANCER DURGS TESTED AS LIPOSOMAL FORMULATIONS.**

| Drug | In vitro | Animal testing | Clinical trials | Reference |
|------|----------|----------------|-----------------|-----------|
| **Plant derived products** | | | | |
| Etoposide | + | + | 0 | 96 |
| Vincristine/vin-blastine | + | + | 0 | 97,98 |
| **Antibiotics** | | | | |
| Bleomycin | + | + | I | 99,100 |
| Daunorubicin | + | + | III | 101,102 |
| **Antimetabolites** | | | | |
| 5-Fluorouracil | + | + | 0 | 103-107 |
| Methotrexate | + | + | 0 | |
| **Alkylating agents** | | | | |
| Chlorambucil | 0 | 0 | 0 | |
| Cisplatin | + | + | 0 | 108,109 |
| Carmustine | + | + | 0 | 110 |
| **Proteins** | | | | |
| Asparaginase | + | + | 0 | 111 |
| Interferons | + | + | 0 | 112 |

Symbols  0 = no report available
      + = positive results in vitro or in animal testing
      I = phase I clinical trials
    II = phase II clinical trials
   III = phase III clinical trials

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## 1.52 Liposomes -Topical Route of Administration :

Due to their high degree of biocompatibility liposomes were initially considered as delivery systems for intravenous delivery. Liposomes were first introduced in 1980 for topical drug delivery and since then have attracted considerable interest and generated many speculative claims concerning their potential utility both as a drug carrier and reservoir for controlled release of drugs within various layers of the skin. A number of clinical studies have now demonstrated the superiority of liposomal drug formulations over conventional delivery systems. In this respect, liposomal formulations have been successful in treatment of a number of dermatological diseases and disorders such as psoriasis, mycoses, idiopathic hirsuitism and cutaneous infections.

Mezei and Gulasekharam (113) reported that topical application of liposomal triamcinolone acetonide (TRMA) for five days resulted in a drug concentration in the epidermis and dermis four times higher than that obtained using a control ointment, while urinary excretion of the drug was diminished. They also compared the deposition of topically applied gels of free and liposomally entrapped TRMA in rabbit skin and found that application of gel resulted in a concentration of TRMA approximately five times higher in the epidermis and three times higher in the dermis than application of the free drug gel. The results of these studies suggested to them the inherent potential of liposomes as a selective drug delivery system for cutaneous application. A number of liposomal formulations containing

lipid-soluble drugs such as econazole, progesterone and minoxidil in multiple dose topical treatment have been demonstrated to provide a higher drug concentration, as compared with other delivery systems (in form of creams, lotions, ointments and pastes) (114).

Studies in nude mice (114) also demostrated that liposome encapsulated MTX can deliver four times more drug to the epidermis and concurrently reduce its subcutaneous absorption by two fold in comparison to the free drug applied together with empty liposomes.

In a clinical trial it was demostrated that psoriasis can be treated by topical application of liposomal entrapped MTX (114).

Liposomal encapsulated econazole and progesterone have also been shown to be superior over their conventional dosage forms in the treatment of patients suffering form mycoses and idiopathic hirsuitism (115).

In addition to the above roles liposomes may also be used for the protection of the skin (116). The above studies indicate that after incorporation into liposomes, both hydrophobic and hydrophilic drugs may be absorbed better into the skin.

Limited studies have been undertaken in order to explain the mechanism of liposome action on drug transfer into the skin. Following application of liposomes into the skin atleast two types of interactions seem possible (114).
1. Some intact vesicles can penetrate through the stratum corneum, perhaps, through the intercellular and appendagel

routes, hence they can act as reservoirs for drugs in the skin. 2. vesicles may fuse with lipid bilayers and/or corneocytes and as a result that may act as penetration enhancers.

The liposomal cream may produce a prolonged action on the skin than the conventional drug cream due to the following reasons : (117)-Fig-1

1.  In the conventional dosage form, the "free" drug should be released, diffused to the surface of the skin, dissolved (if it is not in solution form) before absorption into the horny layer takes place. The drug in the liposomal form should not be released, and if diffusion to the keratin layer is required, that is less of a problem, since the nature of the lipid vesicles makes that easy; the lipid vesicles are readily miscible with the skin surface lipid which can often serve as a barrier especially to lipid insoluble drugs.

2.  The drug should get through the horny layer which is the main barrier and often serves as a reservoir because of protein binding. The vehicle may have an occlusive effect that enhances hydration of the keratin layer, this in turn increases it's permeability. The liposomal form has an excellent potential for hydrating the horny layer, since the lipid vesicles create a lipid film, supplementing the skin surface lipids. Consequently the chance of increased permeability of the main barrier layer is greater with the liposomal form than that with
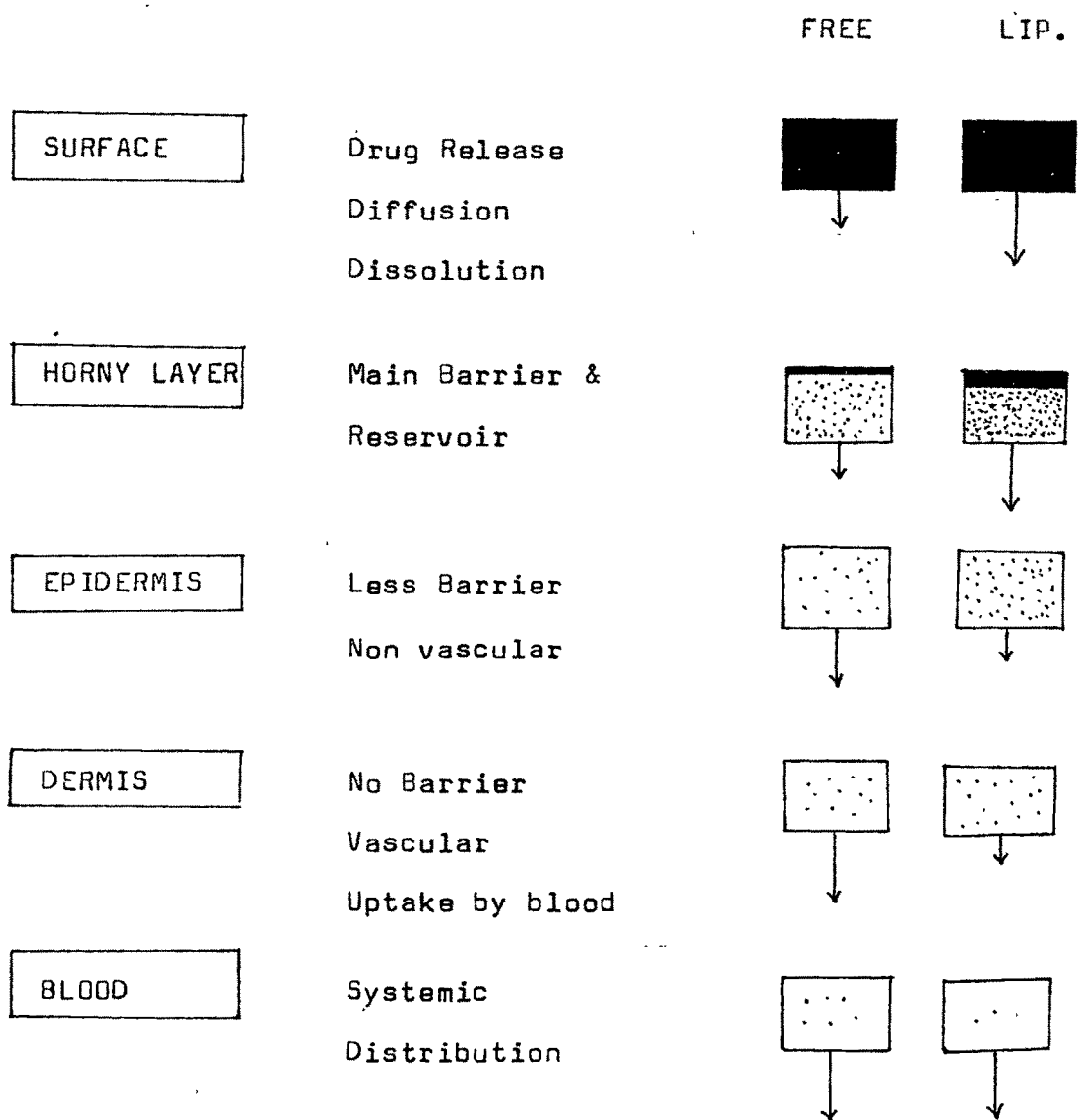
28

FIG.1 : SCHEMATIC REPRESENTATION OF THE MECHANISM & RATE
OF PENETRATION OF FREE AND (LIP) LIPOSOMAL DRUG.
THE DENSITY OF THE SPOTS INDICATE THE CONCENTRATION;
THE LENGHT OF THE ARROWS INDICATE THE MAGNITUDE OF
THE RATE OF DIFFUSION.

some of the conventional forms. The chance of protein binding is probably greater for the "free" drug than for the liposome encapsulated drug.

3. When the drug reaches the epidermis, the diffusion rate of the "free" form is expected to be higher than the liposome encapsulated form because of the difference in size. Since the free form is in the molecular state, the size of the penetrating drug is equivalent to the size of its molecule. The slower diffusion of lipid vesicles provides a longer residency time for the encapsulated drug.

4. Because the dermis is highly vascularized and because of a high concentration gradient, the "free" drug is quickly removed by the blood circulation. The larger liposomes, because of their size are not able to penetrate the blood vessels, therefore, the cutaneous clearance of liposomal drug is less than the "free" drug.

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