

CHAPTER 5

SUMMARY AND CONCLUSIONS

The antineoplastic agents are used in the treatment of malignant disease when surgery or radiotherapy is not possible or has proved ineffective, as an adjuvant to surgery or radiotherapy or as the initial treatment. The two main groups of drugs used in the treatment of malignant diseases are the biological alkylating agents and the antimetabolites.

The present investigations were conducted on three of the most important antineoplastic agents viz. 5 Fluorouracil (5FU), Methotrexate (MTX) (antimetabolites) and Cyclophosphamide (alkylating agent).

The aim of the present investigation was :

1. Development of simple, selective and sensitive analytical methods for estimation of the selected drugs.
2. Accelerated stability studies in aqueous solution and semisolid bases were conducted for the selected drugs by using the newly developed analytical methods and comparing the data obtained with the reported methods.
3. Fabrication, optimisation and evaluation of liposomal dosage forms for selective targetting of 5FU and MTX to the skin.

5.1 DEVELOPMENT OF ANALYTICAL METHODS

5.11 Colorimetric Methods :

5.111 Colorimetric methods for 5FU

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

On the basis of detailed analysis of the chemical structure of 5FU, a number of simple, selective and sensitive colorimetric methods were developed.

a) Complexation of 5FU with metal ions :

5FU gave a yellowish green coloured complex with copper acetate in anhydrous chloroform-methanol medium (3:2) made alkaline with diethylamine solution. The absorption maxima of the coloured complex was found to be at 350nm. The calibration curve was rectilinear in the range of 0.4-11.0µg/ml. The sensitivity of the method was found to be 0.4µg/ml.

5FU also gave a purple coloured complex with cobalt acetate in anhydrous chloroform-methanol medium made alkaline with isopropylamine solution. The absorption maxima of the coloured complex was found to be at 570nm. The calibration curve was rectilinear within the range of 20-200µg/ml. The sensitivity of the method was found to be 10µg/ml.

The formation of coloured complex with divalent metal ions like-copper and cobalt may be due to the presence of the -CONHCO- moiety of 5FU.

No chromogenic reaction occurred with other metal ions like vanadium, manganese, ferric and nickel.

b) Coupling with diazotised aromatic primary amines :

5FU was reacted with several diazotised aromatic primary amines viz. sulfanilic acid, sulfanilamide, aniline, p-aminophenol, paraamino benzoic acid (PABA) anthranilic acid, o-phenylene diamine and p-anisidine.

Chromogenic reaction occurred between 5FU and o- and p-nitro aniline, sulfanilic acid, sulfanilamide, PABA and anthranilic acid in presence of glycerol and sodium hydroxide.

5FU reacted with diazotised o- and p-nitroanilines to give orangish yellow colour (absorption maxima at 450nm), and a red colour with diazotised sulfanilic acid, sulfanilamide, PABA and anthranilic acid (absorption maxima at 520nm in each case). The calibration curve was rectilinear in the range of 10-80µg/ml in the earlier case and 10-100µg/ml in the latter case. The sensitivity obtained was 5µg/ml in all cases.

c) Estimation of 5FU as mercury complexes :

The mercuric complex of compounds having pyrimidinedione nucleus are reported to form coloured compounds with dithizone and diphenyl carbazone solutions.

The complexation of 5FU with mercury occurred in aqueous medium at pH 8. The complex formed reacted with dithizone and diphenyl carbazone solution in chloroform medium to give a greenish red coloured complex (absorption maxima at 605nm) and red colour complex (absorption maxima at 560nm) respectively. The calibration curve was rectilinear in the range of the 5-100 µg/ml in both the cases. The sensitivity obtained was found to be 5 µg/ml for both the methods.

d) Estimation of 5FU - dosage forms by the proposed colorimetric methods :

5FU is available commercially as injection in usual strength of 500mg in 10ml, pH adjusted to 9 with sodium hydroxide solution. It is also available as 5% w/w cream.

5FU was estimated in the above dosage forms by the proposed colorimetric methods. The percentage recoveries of the drug from injection and cream by various methods are as follows :

Copper acetate method	-	98.95% - 99.98%
Cobalt acetate method	-	98.23% - 99.38%
Coupling with diazotised primary amines	-	98.95% - 99.91%
Dithizone method	-	98.86% - 99.41%
Diphenyl carbazone method	-	98.83% - 99.72%

The percentage recoveries obtained by the proposed colorimetric methods were compared with that of standard spectrophotometric method (I.P. and U.S.P. method)

On the basis of analysis of the drug from injection and cream it may be concluded that the method is reproducible and comparable with I.P. and U.S.P. methods respectively.

5.112 Colorimetric methods for MTX :

MTX is chemically N-(4-(2,4-diamino-6pteridinyl)-methyl) methyl amine benzoyl) glutamic acid.

The purity of commercially available MTX is about 85%. It is usually contaminated with closely related compounds. Hence the drug was purified by chromatographic procedure according to the method of Galleli, J.F., and Yokoyama, G.

a) Complexation of MTX with metals of first transition series :

No chromogenic reaction occurred between MTX and metal ions of first transition series viz. nickel, manganese, ferric, copper, ceric and cobalt ions.

b) Reaction of MTX with Folin-Ciocalteu reagent (F.C. reagent) :

A greyish blue colour developed on reaction of MTX with F.C. reagent in sodium carbonate medium. The greyish blue colour showed an absorption maxima at 760nm. The benzoyl glutamic acid moiety of MTX may be responsible for the colour development. The calibration curve was rectilinear in the concentration range of 2-50µg/ml. The sensitivity was found to be 2µg/ml.

c) Reaction of MTX with Nessler's reagent :

When MTX was reacted with Nessler's reagent, an intense yellow colour was formed which showed an absorption maxima at 430nm. The calibration curve was rectilinear in the range of 5-100µg/ml. The sensitivity was found to be 4µg/ml.

d) Reaction of MTX with nitric acid :

When MTX was subjected to nitration an intense yellow colour was obtained. Nitration occurred at boiling temperature. The absorption maxima of the yellow colour solution was found to be at 380nm. The calibration curve was rectilinear in the concentration range of 1-100µg/ml. The sensitivity was found to be 1µg/ml.

e) Reaction of MTX with hydroxylamine hydrochloride and ferric chloride :

4-hydroxypteridine obtained on alkaline hydrolysis of MTX was benzoylated. The benzoyl ester was estimated as ferric hydroxamate. A red colour complex was formed which showed an absorption maxima at 520nm. The calibration curve as rectilinear in the range of 100-500µg/ml. The sensitivity was found to be 75µg/ml.

f) Estimation of MTX in commercially available injection, tablets and synthetic mixtures :

MTX is available as injection of various strengths (250mg in 10ml), as tablets of strength 2.5mg/tablet. Two synthetic mixtures were prepared with commonly used excipients.

MTX was estimated in the above dosage forms by the proposed colorimetric methods.

The percentage recoveries obtained by various methods are as follows :

F.C. method	-	99.37% - 99.76%
Nessler method	-	99.82% - 99.91%
Nitric acid method	-	99.64% - 99.95%
Hydroxamate method	-	98.41% - 99.03%

The recoveries are within the pharmacoeplal limits (90-110%). The results indicate that the proposed colorimetric methods are simple, selective, sensitive and reproducible.

5.113 Colorimetric Methods for Cyclophosphamide :

Cyclophosphamide is chemically the monohydrate of 2-bis (2-chloro-ethyl) amino perhydro - 1,3,2 - oxaza phosphorinane 2 - oxide.

a) Reaction of cyclophosphamide with cobalt and ferrothiocyanate solution :

The orange red coloured complex formed between cyclophosphamide and ferrothiocyanate and blue coloured complex formed between cyclophosphamide and cobaltthiocyanate in chloroform showed an absorption maxima at 490nm and 625nm respectively. This may be due to the formation of ion pair complex between metal and the drug.

The calibration curve was rectilinear in the concentration range of 10-250µg/ml in case of cyclophosphamide ferrothiocyanate complex and 20-200 µg/ml in case of cyclophosphamide cobaltthiocyanate complex. The sensitivity was found to be 5µg/ml in the former case and 20µg/ml in the latter case.

b) Reaction of cyclophosphamide with picric acid :

Cyclophosphamide reacted with picric acid in chloroform medium to give yellow colour due to formation of picrate. The yellow coloured solution showed an absorption maxima at 410nm.

The calibration curve was rectilinear in the range of 20 - 200 µg/ml .

c) Estimation of cyclophosphamide in injection, tablets and synthetic mixtures :

Cyclophosphamide is available as injection of various strengths (100mg, 200mg, 500mg, 1g and 2g of the drug per vial) and as tablets of strength 25mg or 50mg/tablet. Two synthetic mixtures were prepared with commonly used excipients.

Cyclophosphamide was estimated in these dosage forms by proposed colorimetric methods. The percentage recoveries were obtained within the range 99.16% - 99.97% which were comparable with that of the reported nitroso method (99.35-99.92%).

The results indicate that the proposed methods are simple, rapid and reproducible.

5.12 Fluorimetric Method for 5FU :

5FU exhibits native fluorescence in aqueous alkaline medium. A rapid, simple and sensitive fluorimetric method was developed for 5FU in alkaline medium at different pH values. It was observed that maximum fluorescence was exhibited at pH 9. The excitation and emission wavelengths were found to be at 300nm and 400nm respectively.

The calibration curve was rectilinear in the concentration range of 0.05 - 8 µg/ml. 5FU was estimated in injection and cream by this method. The percentage recoveries were found to be 99.11% and 99.82% which were comparable with that of standard UV spectrophotometric method.

5.13 High Performace Liquid Chromatographic Method (HPLC) :

A HPLC method was developed using a simple solvent system consisting of water and methanol (80:20). The liquid chromatograph used was Water's Model with a fixed wavelength detector at 254nm. The flow rate was 1ml/minute and the retention time was found to be 3.38 for 5FU. Thymine was used as the internal standard which had a retention time of

4.49 under the same conditions. The calibration curve was rectilinear in the concentration range of 0.05-200µg/ml.

5FU was estimated in injection and cream by the HPLC method. The percentage recoveries were found to be 99.01% and 99.696% which were comparable with that of the standard spectrophotometric method (I.P. method).

5.14 In vitro Estimation of 5FU, MTX and Cyclophosphamide in

Human Plasma :

Human plasma was spiked separately with 5FU, MTX and cyclophosphamide (concentration 1mg/ml). In each case the drug was estimated by the proposed analytical methods. The percentage recoveries were found to be

5FU - 98.91% - 99.72%

MTX - 99.01% - 99.92%

Cyclophosphamide - 98.95% - 99.95%

On the basis of analysis of the data it may be concluded that the plasma components do not interfere with any of the proposed analytical methods.

5.15 In vivo Estimation of 5FU from Rat Blood :

Anaesthetised rats were injected with 5FU (50mg/kg body weight, i.v.). The blood samples were withdrawn at 5,15,30 and 60 minutes and analysed for the drug by the following methods.

- i) 5FU - copper acetate method
- ii) 5FU - diazotised sulfanilic acid method
- iii) 5FU - diphenyl carbazone method
- iv) Fluorimetric method

v) HPLC method

vi) UV method

The results indicate that, at the given dose 5FU could be estimated from rat blood by HPLC, fluorimetric and copper acetate method upto 60 minutes and by the UV method upto 30 minutes. However it was not possible to measure the blood levels by diazotised sulfanilic acid method and diphenyl carbazone method. This may be because at the given dose of 5FU the blood levels achieved do not fall within the sensitivity limits of these two methods. It may thus be concluded that the proposed analytical methods viz. HPLC, fluorimetric and copper acetate method may be used for pharmacokinetic studies of 5FU in animal models.

5.2 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 dosage forms.

5.21 Stability Studies of 5FU :

Stability studies of 5FU were carried out at pH 7.0, 8.0, 9.0 and 10.0 at 3 different temperatures viz. 25°C, 45°C and 60°C for 90 days. The percentage of drug remaining at regular time intervals was estimated by the following methods.

i) cobalt acetate method.

ii) diazotised sulfanilic acid method.

iii) HPLC method.

iv) UV method.

The first order degradation plots were constructed for each pH at different temperatures and the stability parameters viz. stability constant K , $t_{1/2}$, t_{90} and the activation energy E_a values were calculated.

The results obtained by the proposed methods were statistically compared with standard UV method using student's 't' test. No statistically significant difference was observed between the UV method and the proposed methods. It may therefore be concluded that the proposed analytical methods are stability indicating.

5.22 Stability Studies of MTX:

Stability studies of MTX were carried out at pH 1.2, 3.9, 5.0, 6.0, 7.0, 8.0 and 10.0 at 3 different temperatures 25°C, 45°C and 60°C for 90 days. The percentage drug remaining at regular time intervals was estimated by the F.C. method and UV method.

The first order degradation plots were constructed for each pH at different temperatures and the stability parameters viz. K , $t_{1/2}$, t_{90} and the activation energy (E_a) values were calculated.

No statistically significant difference was observed in the results of the stability studies by the proposed methods and the reported data. It may therefore be concluded that the UV method and F.C. method are stability indicating.

5.23 Stability Studies of 5FU and MTX in Semisolid Dosage Forms :

The stability of 5FU was studied in four different bases viz. hydrous emulsifying base (I.P), cetomacrogol cream (B.P), HPMC K4M gel base and carbopol gel base (0.15% w/w drug concentration) at 4°C and 25°C for 90 days.

The percentage drug remaining at regular time intervals was estimated by the following methods.

For 5FU :

- i) cobalt acetate method.
- ii) diazotised sulfanilic acid method.
- iii) UV spectrophotometric method.

For MTX :

- i) F.C. method.
- ii) UV spectrophotometric method.

The stability data obtained by UV method and the proposed methods are statistically similar. From these studies it may be concluded that 5FU and MTX are compatible with all four bases and that the proposed analytical methods can be used for estimation of drugs from semisolid dosage forms.

5.24 Stability Studies of Cyclophosphamide :

The stability studies of cyclophosphamide were carried out at pH 1.2, 2.0, 3.9, 7.0, 8.0 and 10.0 at temperatures 25°C, 45°C and 60°C over a period of 90 days. The percentage drug remaining at regular time intervals was estimated by ferrothiocyanate method.

The first order degradation plots were constructed for each pH at different temperatures and the stability parameters viz. K, $t_{1/2}$, t_{90} and activation energy values (E_a values) were calculated.

The results obtained by the proposed analytical method were compared with the reported data and they were found to be statistically similar. It may be concluded that the proposed method is stability indicating.

5.3 LIPOSOMES OF 5FU AND MTX FOR SKIN TARGETTING

Liposomes of egg lecithin and cholesterol were prepared by thin film technique and modified method of Szoka and Papahadjopoulos for 5FU and reverse phase evaporation and double emulsification method for MTX. Thin film technique for 5FU and reverse phase evaporation technique for MTX gave high efficiency of drug encapsulation. The loading of the drugs into the liposomes were optimised by varying the millimolar concentration of calcium chloride solution and molar ratio of egg lecithin and cholesterol.

Calcium chloride solution of 25mM strength gave the best results in terms of complete flocculation and drug entrapment. The ratio of egg lecithin and cholesterol also played an important role in drug loading. Maximum drug loading was achieved in liposomes formulated with 1:1 molar ratio of lecithin : cholesterol for both the drugs.

At 1:1 molar ratio of lecithin : cholesterol the millimolar concentrations of these were varied and it was observed that maximum drug loading was obtained at 0.381mM :

0.381mM for 5FU (27.5% entrapment) and 0.635mM : 0.635mM (45% entrapment).

The partical size analysis of the liposomes was carried out using optical microscopy. It was observed that the liposomes of 5FU were in the size range of 1.0 - 6.0µm while those of MTX were between 1.0-10.0µm.

The photomicrographs taken on transmission electron microscope seem to suggest the formation of multi lamellar vesicles for both the drugs.

The following composition of liposomes were subjected to in vitro permeation studies after incorporation into four semisolid bases viz. hydrous emulsifying base (I.P.) cetomacrogol cream (B.P.), HPMC K4M gel base, carbopol 941 gel base (drug concentration-0.15% w/w in each case).

For 5FU :

Lecithin mM	:	Cholesterol mM
0.381	:	0.381
0.761	:	0.381
0.381	:	0.761
0.635	:	0.635

For MTX :

0.635	:	0.635
1.269	:	0.635
0.635	:	1.269
1.269	:	1.269

The in vitro permeation studies were conducted across rat skin using a suitably modified vertical permeation apparatus.

The mean permeability coefficient values for each liposomal formulation of 5FU and MTX were calculated and statistically compared with those obtained for the corresponding plain drug formulation and the physical mixtures using ANOVA technique. It was observed that in all cases the liposomal formulations significantly decreased the permeation of 5FU and MTX through the skin. The rank order correlation for the reduction in permeability coefficient in the four bases in descending order may be given as

For 5FU :

HPMC K4M gel base > Carbopol gel base > Hydrous emulsifying
59% reduction 54% reduction base 41% reduction
> Cetomacrogol base
35% reduction

For MTX :

HPMC K4M gel base > Hydrous emulsifying base ~ Carbopol gel
68% reduction 66% reduction 66% reduction
base > Cetomacrogol base.
52% reduction

The in vitro data also suggests that the reduction in the permeation of 5FU and MTX does not seem to depend on the concentration of lipids present in the liposomes.

The reduction in permeation of liposomally encapsulated drug may be due to the fact that the lipid vesicles act as a barrier which prevent the penetration of the drug into the diffusion medium.

The stability studies of liposomal formulations indicate that at refrigeration temperature there is no significant change in the drug content and in vitro permeation profile of liposomal drug creams over a period of 90 days.

The efficacy of the liposomal formulations of 5FU and MTX were studied and compared with that of the corresponding plain drug creams on dinitrochlorobenzene induced contact allergic dermatitis in female guinea pigs. The scoring method of Higuchi et al was used.

From the in vitro permeation studies it was observed that amongst the gel bases HPMC K4M gel base and amongst the cream bases, hydrous emulsifying base gave the best results in terms of percentage reduction in permeation of 5FU and MTX. Hence the formulations in these bases were selected for in vivo study.

The percentage reduction in erythema at different time intervals upto complete recovery were calculated. The values of the percentage reduction for liposomal formulations and plain drug formulations of 5FU and MTX were compared using ANOVA technique.

In case of formulations of 5FU, complete recovery from erythema occurred within 72 hours with both liposomal drug cream and plain drug cream. When the values of the percentage reduction between the bases (hydrous emulsifying base and HPMC gel base) were compared no significant difference was observed.

In case of formulations of MTX complete recovery from erythema occurred within 48 hours with both liposomal and plain drug cream. When the values of the percentage reduction between the hydrous emulsifying base and HPMC gel base were compared no significant difference was observed.

When the percentage reduction in erythema at each time interval obtained for plain drug formulations and liposomal drug formulations were compared, the difference in the values were not statistically significant for both the drugs. This indicates that incorporation of either 5FU or MTX into liposomes did not alter the efficacy of these potent drugs. The results of the in vitro and in vivo studies indicate that liposomal formulations of 5FU and MTX significantly decrease the permeation of these drugs across the skin without compromising on their efficacy.

The concept of selective targetting of topically applied therapeutic agents, by incorporating into liposomes, to various cell types in the skin shows promise and warrants further studies. To make the technique successful it will be necessary to investigate the mechanism of transport of liposomes across stratum corneum, their interaction with various cell types in the skin and their metabolic fate within the healthy and diseased skin. Efforts should also be directed towards studying their long term stability as an acceptable dermatological vehicle and possible toxicity of the liposome-drug complex. The real potential of liposomes in intradermal drug delivery can be evaluated only by careful analysis of the results of such investigations.