CHAPTER 3

ANALYTICAL METHODS

3.1 INTRODUCTION

In this investigation, liposomes and LDPI formulations of INH and RFP were analyzed by physical characterization to determine the size, shape and lamellarity and chemical characterization to determine the drug entrapment efficiency, phosphatidyl choline and cholesterol content. The stability studies were conducted to determine the percent drug retained in LDPI over storage of 6 months period. *In vitro* drug diffusion studies followed by *in vivo* studies in rats were also carried out. The analytical methods employed in these investigations are discussed below.

3.2 Materials and Equipments

Material	Source
Isoniazid (INH)	S. D. Fine Chemicals, India
Rifampicin (RFP)	Gift samples from Cadila Pharmaceuticals, India.
Water (distilled)	Prepared in laboratory by distillation
Soyaphosphatidylcholine (S-100), Hydrogenated Soyaphosphatidylcholine (HSPC) and Hydrogenated soyaphosphatidylglycerol (SPG-3)	Gift samples from Lipoid, GmbH, UK
Stearylamine (SA)	Sigma Chemical Co. (USA)
2-Hydroxy-1-naphthaldehyde	Aldrich Chemical Co. (USA)
α-Tocopherol	E. Merck India Ltd., India
Cholesterol (CHOL), Dextrose monohydrate, Sucrose, Maltose, p-chloranil, ferric chloride hexahydrate, ferric chloride anhydrous, ammonium thiocyanate, sodium chloride, glacial acetic acid, hydrochloric acid, sulphuric acid, phosphoric acid, diethyl ether, ethyl acetate and acetone	S. D. Fine Chemicals, India
Chloroform, Methanol, Triton X-100	Qualigens, Mumbai
Ethanol	Govt. supply, Baroda
Nuclepore Polycarbonate membrane 2 μm 25mm	Whatman, USA
Trehalose	Sisco Research Laboratory, India
Rotahaler® Dry Powder Inhalation device	Cipla Ltd., India
Hydrolyzed gelatine	Gift samples from Nitta Gelatine, Japan.
Sorbolac– 400	Received as gift samples from Meggle, Germany
Pharmatose 325M	Received as gift samples from HMV, The Netherlands

Equipments	Source
Calibrated pipettes of 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, Funnels (i.d. 5.0 cm), beakers (250 ml) and other requisite glasswares	Schott & Corning (India) Ltd., Mumbai
Analytical balance	Precisa 205A SCS, Switzerland
pH meter	Systronics 335, India
Cyclomixer, three blade stirrer	Remi Scientific Equipments, Mumbai
Cooling Centrifuge	Remi C-24, Mumbai
Homogenizer, SD4C	Raliwolf Ltd. Mumabi
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
Stability oven	Shree Kailash Industries, Vadodara
UV-Visible Spectrophotometer,	Shimadzu UV-1601, Japan
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Water Bath ME10941	INCO, Ambala
Optical microscope with polarizer	BX 40, Olympus Optical Co. Ltd., Japan
Malvern particle size analyser	Malvern Master sizer 2000 SM, U K.
Scanning electron microscope	JSM-840 SEM, Jeol, Japan
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland
Karl fisher Autotitrator	Toshiwal Instruments (Bombay) Pvt. Ltd., Nasik

3.3 PHYSICAL ANALYSIS

The present study involves preparation of INH and RFP liposomal DPI formulations with neutral, positive and negative charge for pulmonary administration. Liposomes of these drugs prepared in this work were characterized by the physical analysis to determine the size, shape and lamellarity using the following analytical techniques.

3.3.1 Determination of shape and lamellarity of liposomes

The liposomes and LDPI of INH and RFP were diluted with water and examined under Olympus (BX 40F4, Japan) with polarizing attachment to study their shape and lamellarity at 1000X magnification. The lamellarity was confirmed by the presence of Maltese crosses in the photographs. The representative photographs were taken to confirm the results.

3.3.2 Determination of particle size of liposomes

Light scattering based on laser diffraction using Malvern, MasterSizer SM 2000k (Malvern Instruments Inc., UK). The apparatus consisted of a He-Ne laser (5 mw) and a sample holding cell of 50 ml capacity. Liposomes and LDPI were diluted with sufficient amount of water so that a 50 ml volume gives obscuration between 10-20%

as per the manufacturer's recommendation. The samples were stirred using a blade stirrer at 1000 rpm to keep the sample in suspension.

3.4 CHEMICAL ANALYSIS

The liposomes of INH and RFP prepared in this work were characterized by chemical analysis to determine the drug, PC and CHOL content in liposomes, to estimate drugs during stability studies, in diffusion medium, in bronchoalveolar lavage and in lung tissues. Calibration curves of the drug; PC and CHOL were prepared by the developed or reported analytical methods with suitable modifications when necessary to meet the need of this investigation. The various analytical methods used are described below:

3.4.1 Estimation of Phosphatidyl Choline

The Stewart assay (Stewart, 1980) was used for estimating phosphatidyl choline in liposomes. This method utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution.

3.4.1.1 Solutions

1. Ammonium ferrothiocyanate solution (0.1M) was prepared by dissolving 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double distilled water and making up the volume of the resulting solution to 1 litre.
2. Stock solution of phosphatidyl choline in chloroform (0.1mg/ml) was prepared by dissolving 50mg of phosphatidyl choline in 10ml of chloroform. 2ml of this solution was diluted 100 times to yield a solution of the required concentration.
3. Saturated sodium chloride solution: Sodium chloride was dissolved in distilled water with heating to form a supersaturated solution. This solution was then cooled to room temperature and filtered to give the required saturated solution.

3.4.1.2 Procedure for calibration curve

Suitable aliquots (0.1 – 1.5 ml) of the stock solution of phosphatidyl choline were transferred to 10ml centrifuge tubes. Appropriate quantities of chloroform were then added such that the total volume of the contents of the tubes was 3ml. To each tube, 2ml of ammonium ferrothiocyanate solution (0.1M) was then added. The contents of each tube were mixed by vigorous vortexing on a cyclomixer for 15sec. The tubes were then spun for 5min at 1800 rpm in a tabletop centrifuge. The lower, organic colored layer was then removed using a syringe and long needle (18 gauge) and transferred to a test tube. The absorbance of these solutions was measured at 485 nm

to the contents followed by vigorous vortexing on a cyclomixer for 30 sec and centrifugation at 1800 rpm for 5 min in a tabletop centrifuge. The lower chloroform layer was separated using a syringe and needle (18 gauge) and passed over a bed of anhydrous sodium sulphate into a 10ml volumetric flask. The process was repeated with a further 2ml and 1ml of chloroform. The chloroform layers were then pooled and the volume made up to 10ml with chloroform. To 0.5ml of this chloroform extract in a centrifuge tube, 2.5ml of chloroform and 2ml of 0.1M ammonium ferrothiocyanate solution was added. The contents were then subjected to the same procedure as detailed above for the standards (Section 3.2.1.2). Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidyl choline in the liposomes or supernatant using the regression equation.

3.4.2 Estimation of cholesterol

The Zlatkis, Zak and Boyle's method was used for cholesterol estimation. This method utilizes the ability of cholesterol in acetic acid to form a complex with ferric chloride and sulphuric acid (Zlatkis *et. al.,* 1953).

3.4.2.1 Solutions

1. Ferric chloride solution: A 0.05% w/v solution of ferric chloride hexahydrate in glacial acetic acid was prepared by dissolving 50mg of ferric chloride hexahydrate in 100ml glacial acetic acid.
2. Stock solution of cholesterol: A 0.5mg/ml solution of cholesterol in glacial acetic acid was prepared by dissolving 25mg of cholesterol in 50ml of glacial acetic acid.
3. Saturated sodium chloride solution: It was prepared in the same manner as for the estimation of phosphatidyl choline from liposomes (Section 3.2.1.1)

3.4.2.2 Procedure for calibration curve

Suitable aliquots of the stock solution of cholesterol (0.1 to 1ml) were transferred accurately into 10ml volumetric flasks. To each flask, 4ml of ferric chloride solution and 4ml of concentrated sulphuric acid was added. The contents were mixed, made up to the volume with glacial acetic acid and allowed to stand for 30 min. The absorbance of the resulting coloured solutions of the complex, formed between cholesterol, ferric chloride and sulphuric acid was measured at 550nm using Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length,

Table 3.2: Calibration for estimation of cholesterol (Zlatkis, Zak and Boyle's method)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed value
5	0.065 ± 0.001	0.069
10	0.154 ± 0.0021	0.155
20	0.344 ± 0.003	0.327
30	0.475 ± 0.004	0.499
40	0.695 ± 0.003	0.671
50	0.836 ± 0.004	0.843

Regression equation** Y= 0.0172X –0.0173

Correlation coefficient = 0.9966

*Mean of 6 values

3.4.3 Estimation of Isoniazid

Isoniazid in methanol shows strong absorbance in the ultraviolet region of the electromagnetic spectrum (Glenn A. Brewer, 1976).

3.4.3.1 Solutions

Stock solution of isoniazid (100µg/ml) was prepared by dissolving 10mg of isoniazid in 100ml of methanol.

3.4.3.2 Procedure for calibration curve

Suitable aliquots (0.4 – 2.0ml) of the stock solution of Isoniazid were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with methanol to give final concentrations of 4, 8, 12, 16 and 20µg/ml. The solutions were shaken well and their absorbance measured at 261nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.5 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.6 contains the optical characteristics for the solution of Isoniazid in methanol.

3.4.3.3 Stability and selectivity

Stability of the solutions of Isoniazid in methanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating Isoniazid was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes and LDPI to ascertain the selectivity of the method.

3.4.3.4 Estimation of isoniazid from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, methanol was added to break the liposomes and the volume was made up with methanol. The absorbance was measured at 261nm against a blank comprising of empty liposomes diluted with methanol in the similar manner. Triplicate estimations were made and the mean absorbance was determined. The amount of isoniazid in the liposomes or supernatant was then obtained using the regression equation.

Table 3.3 Calibration curve for isoniazid in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.E.M	Regressed Value
4	0.138 ± 0.001	0.136
8	0.277 ± 0.002	0.278
12	0.425 ± 0.002	0.420
16	0.545 ± 0.002	0.563
20	0.715 ± 0.002	0.705

Regression equation^{++}: Y = 0.0356X - 0.0068

Correlation coefficient = 0.9979

*Mean of 6 values

3.4.3.5 Estimation of INH from LDPI formulations

100 mg of Liposomal DPI formulation was accurately weighed and dissolved in 5 ml of saturated sodium chloride solution. 2ml of chloroform was added to the contents of the centrifuge tube followed by vortexing for 30 seconds and then centrifuging at 2750 rpm for 10 minutes. The upper chloroform layer was separated using a glass

solution was used for estimation of INH by the procedure as discussed previously (Section 3.4.3.2).

3.4.3.8 Estimation of INH in in-vitro deposition and in-vivo biological fluid and tissues

The modified USP **HPLC** method was used for the estimation INH in in-vitro deposition studies by Next generation impactor (NGI), biological fluid and tissue. The procedures are detailed as below.

Preparation of mobile phase buffer

Accurately weighed 0.44 g of sodium lauryl sulphate was dissolved in Milli Q water, and the volume was made up to 875 ml (0.05 % w/v) with Milli Q water.

Preparation of mobile phase

Mobile phase was prepared using mobile phase buffer: methanol with 2 g of sodium acetate in a ratio of 875:125.

Standard preparation

Accurately weighed 10mg of INH was dissolved and diluted using diluent to a concentration of 20 ppm.

Sample preparation

0.5 ml aliquot samples was transferred into pre-labelled tubes, to it 5 ml of acetonitrile was added and extracted for 10 minutes with 5 minutes pulse on vortexer. The samples are centrifuged at 2500 rpm for 5 minutes at 15°C and the separated organic phase was evaporated at 60°C under nitrogen gas and reconstituted with 200µl of mobile phase. The samples are then transferred into HPLC vial for analysis under the following chromatographic conditions.

Chromatographic conditions:

Column: Hypersil C_{18} BDS, 250 x 4.6mm, 5 µm

Flow rate: 1.0 ml

Wavelength: 262 nm

Injection volume: 20 µl

Retention time: 3.7 min

Run time: 10 min

Diluent: water: methanol (80:20)

3.4.4 Estimation of Rifampicin

Rifampicin in methanol shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.4.4.1 Solutions

Stock solution of RFP (100µg/ml) was prepared by dissolving 10mg of RFP in 100ml of methanol.

3.4.4.2 Procedure for calibration curve

Suitable aliquots (0.4 – 2ml) of the stock solution of RFP were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with methanol to give final concentrations of 4, 8, 12, 16, and 20µg/ml. The solutions were shaken well and their absorbance measured at 334nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.7 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.8 contains the optical characteristics for the solution of RFP in methanol.

3.4.4.3 Stability and selectivity

Stability of the solutions of RFP in methanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature. The above method for estimating RFP was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes and LDPI to ascertain the selectivity of the method.

3.4.4.4 Estimation of RFP from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, methanol was added to break the liposomes and the volume was made up with methanol. The absorbance was measured at 334nm against a blank comprising of empty liposomes diluted with methanol in the similar manner. Triplicate estimations were made and the mean absorbance was determined. The amount of RFP in the liposomes or supernatant was then obtained using the regression equation.

Table 3.4 Calibration curve for Rifampicin in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
4	0.124 ± 0.001	0.131
8	0.247 ± 0.006	0.239
12	0.349 ± 0.005	0.348
16	0.459 ± 0.006	0.456
20	0.560 ± 0.008	0.565

Regression equation^{++}: Y = 0.0271X + 0.0225

Correlation co efficient = 0.9988

*Mean of 6 values

3.4.4.5 Estimation of RFP from LDPI formulations

100 mg of Liposomal DPI formulation was accurately weighed and dissolved in 5 ml of saturated sodium chloride solution. 2ml of chloroform was added to the contents of the centrifuge tube followed by vortexing for 30 seconds and then centrifuging at 2750 rpm for 10 minutes. The upper chloroform layer was separated using a glass syringe with a long needle and transferred to 10ml volumetric flask after passing it through a bed of anhydrous sodium sulphate. The extraction procedure was repeated twice with 2ml portion of chloroform and the volume of the combined chloroform extract was made up to 10ml with chloroform. The remaining sodium chloride solution then subjected to the same procedure as discussed previously (Section 3.4.4.2).

3.4.4.6 Estimation of RFP retention and vesicle size determination in liposomal DPI formulations during stability studies

LDPI formulations of RFP samples from each batch at each Stability conditions like controlled room temperature (25°C ± 2°C), intermediate (30°C ± 2°C, 60 ± 5 % RH) and accelerated (40°C±2°C, 75±5% RH) conditions were withdrawn at definite time interval and hydrated with 5ml of distilled water for 30 minutes. The hydrated suspension was centrifuged at 3.3 x 10^6 x g for 15 minutes. The supernatant was analyzed for the RFP content by HPLC method as described in this chapter (Section

3.4.4.8). This will represent the amount of RFP leaked during storage stability of the both formulations at each stability condition.

The increase in vesicle size of RFP liposomes was determined from changes in vesicle diameter for both formulations of RFP prior to and after storage at controlled room temperature (25°C ± 2°C), intermediate (30°C ± 2°C, 60 ± 5 % RH) and accelerated (40°C±2°C, 75±5% RH) conditions. For determination of increase in vesicle size during stability at different storage conditions, samples were hydrated with 5ml of distilled water for 30 minutes and further processed to analyze the vesicle size as described in section 3.3.2.

3.4.4.7 Estimation of RFP in diffusion medium

The sample (RFP in diffusion medium at different time points) was mixed with 2ml saturated sodium chloride solution. 2ml of chloroform was added to the contents of the centrifuge tube followed by vortexing for 30 seconds and then centrifuging at 2750 rpm for 10 minutes. The upper chloroform layer was separated using a glass syringe with a long needle and transferred to 10ml volumetric flask after passing it through a bed of anhydrous sodium sulphate. The extraction procedure was repeated twice with 2ml portion of chloroform and the volume of the combined chloroform extract was made up to 10ml with chloroform. The remaining sodium chloride solution was used for estimation of RFP by the procedure as discussed previously (Section 3.4.4.2).

3.4.4.8 Estimation of RFP in in-vitro and in-vivo biological fluid and tissues

The modified USP **HPLC** method was used for the estimation RFP in in-vitro deposition studies by Next generation impactor (NGI), biological fluid and tissue. The procedures are detailed as below.

Preparation of mobile phase buffer

Accurately weighed 131.6 g of potassium hydrogen phosphate was dissolved in Milli Q water, to it 6.3 ml of orthophosphoric acid was added and the volume was made up to 1000 ml with Milli Q water.

Preparation of mobile phase

Mobile phase was prepared using water: acetonitrile: mobile phase buffer: 1M citric acid: 0.5M sodium per chlorate in a ratio of 510:350·100:20:20

Standard preparation

Accurately weighed 10mg of RFP was dissolved and diluted using diluent to a concentration of 20 ppm.

Sample preparation

0.5 ml aliquot samples was transferred into pre-labelled tubes, to it 5 ml of acetonitrile was added and extracted for 10 minutes with 5 minutes pulse on vortexer. The samples are centrifuged at 2500 rpm for 5 minutes at 15°C and the separated organic phase was evaporated at 60°C under nitrogen gas and reconstituted with 200µl of mobile phase. The samples are then transferred into HPLC vial for analysis under the following chromatographic conditions.

Chromatographic conditions:

Column: Hypersil C_8 BDS, 150 x 4.6mm, 5 µm

Flow rate: 1.5 ml

Wavelength: 254 nm

Injection volume: 50 µl

Retention time: 13 min

Run time: 20 min.

Diluent: water: methanol (50:50)

4.4.5 Estimation of water content and trapped volume

The water content was estimated by Karl Fischer method (Veego, India). Commercially available pyridine free reagent was used for the purpose of analysis. The reagent was standardized with known quantity of water (250mg). Before adding sample, 40ml of methanol was added into the titration vessel and titrated with the reagent to an audiovisual end point to consume any moisture that may be present.

Liposomal dispersion (5ml) after separation of unentrapped drug was centrifuged at 3.6 x 10^6 x g for 30 minutes to get a tight pellet. The supernatant was decanted off from it to remove every drop of excess fluid (including some liposomes if necessary). The pellets were solubilized in 0.1% Triton-X-100 in methanol (10ml). A small aliquot (0.5ml) was removed for the quantification of PC and CHOL (section 3.4.1 and section 3.4.2) and the remainder was used to obtain water content by Karl Fischer method.

3.5 RESULTS AND DISCUSSION

3.5.1 Estimation of phosphatidyl choline

The Stewart assay was used for estimating phosphatidyl choline in liposomes and in LDPI formulations (Stewart, 1980). This method is based on complex formation between ammonium ferrothiocyanate and phospholipids in organic solution. The complex in chloroform exhibits maximum absorbance at 485nm. The advantage of this method is that the presence of inorganic phosphate does not interfere with the assay. A disadvantage, however, is that this method is not applicable to samples where mixtures of unknown phospholipids may be present (New, 1990a). A correlation coefficient of 0.9979 (Table 3.1) indicated a linear relationship between absorbance and concentration of phosphatidyl choline taken for complex formation. Beer's law was found to be obeyed between 10 – 150 µg/3ml (Table 3.2). This high range is a reflection of the low absorptivity of the complex at the analytical wavelength. The data of regression analysis on the collected data along with the raw data are presented in Table 3.1. The regression equation obtained was $Y = 0.0028X + 0.0024$. The stability of the complex was ascertained by measurement of absorbance of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. Consequently, it is recommended that the absorbance be measured within an hour of sample preparation and this precaution was followed in all studies involved in this method. The selectivity of the method for phosphatidyl choline was established by estimating phosphatidyl choline in the presence of the other major constituents of the liposomes viz. cholesterol, Isoniazid, Rifampicin, lactose, sucrose, etc. at the levels at which these materials were included in the liposomes and LDPI formulations. None of the materials interfered in any way in the estimation of phosphatidyl choline when the Stewart assay was used for the purpose of estimation.

The method used for estimating phosphatidyl choline from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. A modified version of the Bligh-Dyer two-phase extraction method was used (New, 1990). Chloroform was used alone instead of chloroform: methanol mixture, recommended in the reported method, as it was found to give results comparable to these obtained with the original method. Also traces of methanol are reported to interfere with the final partition step in the estimation procedure (Stewart, 1980). As

chloroform was liable to form an emulsion with phosphate buffered saline, saturated with sodium chloride was added to prevent this. The chloroform layers were passed through a bed of anhydrous sodium sulphate to remove any traces of water. The chloroform extract so obtained was then made up to 10ml and a portion subjected to the procedure detailed for the estimation of phosphatidyl choline. Mass balance studies revealed good correlation between the amount of phosphatidyl choline added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of phosphatidyl choline in liposomes and LDPI formulations.

3.5.2 Estimation of cholesterol

The Zlatkis, Zak and Boyle method (Zlatkis *et. al.,* 1953) was used for estimating cholesterol in liposomes. Here, cholesterol in acetic acid forms a colored complex with ferric chloride and sulphuric acid, which exhibits maximum absorbance at 550nm. Linearity of the method was observed from a correlation coefficient of 0.9966 (Table 3.2). Beer's law was found to be obeyed between 5 – 50 µg/ml (Table 3.4). Regression analysis of the experimental data was performed and is tabulated along with the experimental data in Table 3.3. The regression equation obtained was Y = 0.0172 X + 0.0146. Monitoring of the absorbance of the solutions, used for preparing the calibration curve revealed that the color of the complex was retained as its original intensity for only 2h. The presence of the other constituents of the liposomes such as phosphatidyl choline, INH, RFP, Lactose, Sucrose, etc., at the same concentrations at which these materials were included in the liposomes and LDPI, did not interfere with the estimation of cholesterol. The method was used to estimate cholesterol in liposomes and supernatant obtained in the centrifugation step used for removing the unentrapped drug. The modified Bligh-Dyer two phase extraction method, described earlier (section 3.4.1.4) was used to extract cholesterol from liposomes. A portion of the chloroform extract, obtained by following the procedure detailed in the aforementioned section, was evaporated to dryness by heating on a water bath at 90°C. Care was taken that no chloroform splashed out of the volumetric flask during the process. The contents of the flask were then dissolved in glacial acetic acid before they were subjected to the procedure detailed earlier for the estimation of the cholesterol. There was good agreement between the amount of cholesterol estimated from liposomes and supernatant and that added initially when liposomes were being

prepared, indicating the suitability of the method for the estimation of cholesterol from these types of preparations.

Certain precautions were taken when the method is being used like sequence of reagent addition, solution transferring etc. The reagent addition sequence of addition of ferric chloride solution followed by concentrated sulphuric acid was added to the standard cholesterol solution or sample solution. If not the colour development was observed to be disturbed. If the solutions are shaken prior to measuring the absorbance, upon transferring to cuvettes, air bubbles are seen in the cuvette impending absorbance measurements. The solutions, therefore, was transferred carefully to the cuvette, only when they are devoid of air bubbles and that too with no turbulence. Also, the reagents used in the method (concentrated sulphuric acid and glacial acetic acid) are quite corrosive, hence were handled carefully.

3.5.3 Estimation of Isoniazid

INH in methanol yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 400nm. The scan showed absorption maxima at 261 nm. Linearity of the method was observed from a correlation coefficient of 0.9979 (Table 3.3). Beer's law was found to be obeyed between 4 – 20 µg/ml. Regression analysis of the experimental data was performed and is tabulated along with the experimental data in Table 3.3. The regression equation obtained was $Y = 0.0356X - 0.0068$.

The mean absorbance values of the methanolic solutions of INH at different concentrations at pre-selected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, INH is stable over a period of 24h in methanol.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of INH.

The method used to estimate the amount of INH entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of INH added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application. The same method was successfully used for the determination of INH during characterization procedures, stability studies and in vitro

diffusion studies. For determination of INH in in-vitro deposition studies by NGI and biological samples (Broncho alveolar lavage and lung tissues) HPLC method was used.

3.5.4 Estimation of Rifampicin

RFP in methanol yields a characteristic curve when scanned in the U.V. -Visible wavelength range between 200 to 600nm. The scan showed absorption maxima at 237, 334 and 475nm (Table 3.4). The analytical wavelength of 334 nm was selected as there was no interference of other material used in the formulation and used for further investigations. The regression equation was found to be Y = 0.0271X + 0.0225.

A correlation coefficient of 0.9988 (Table 3.4) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 4 and 20 µg/ml. Experimental and calculated values for the method are presented in Table 3.4. The mean absorbance values of the methanolic solutions of RFP at different concentrations at pre-selected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, RFP is stable over a period of 24h in methanol.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of RFP.

The method was used to estimate the amount of RFP entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of RFP added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application. The same method was successfully used for the determination of INH during characterization procedures, stability studies and in vitro diffusion studies. For determination of RFP in in-vitro deposition studies by NGI and biological samples (Broncho alveolar lavage and lung tissues) HPLC method was used.

3.6 REFERENCES

Arthur CC, Andrew PK, Cheung P and Florey K. (ed): Methotrexate in Analytical profiles of drug substances, vol. 5, Academic press Inc, New York, 1976; 283.

The Indian Pharmacopoeia , 3rd edn., Vol II. Controller of Publications, Delhi. 1985; A242 – A249.

New RRC. Miscellaneous methods in Liposomes: A Practical Approach. New RRC (ed.), Oxford University Press, Oxford, 1990; 252-266.

New RRC. Characterization of liposomes in Liposomes: A Practical Approach. New RRC (ed.), Oxford University Press, Oxford, 1990a; 105-161.

Shimada K, Matsuo S, Sadzuka Y, Miyagishima A, Nozawab Y, Hirota S and Sonobeb T. Determination of incorporated amounts of poly (ethylene glycol)-derivatized lipids in liposomes for the physicochemical characterization of stealth liposomes. Int. J. Pharm. 2000; 203: 255–263.

Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 1980; 104: 10-14.

Zlatkis A, Zak B and Boyle AJ. A new method for the direct determination of serum cholesterol. J. Lab. Clin Med. 1953; 41: 486-492.