CHAPTER 3

ANALYTICAL METHODS

Liposomes have generated considerable interest in their utilization as carriers for targeting of drugs into lungs. The key point to grasp in considering the manufacture of liposomes is the spontaneous formation of phospholipid membrane as a result of unfavorable interactions between the phospholipid and water. Thus the emphasis in making liposomes is not towards assembling the membranes (which happens on its own accord), but towards getting the membranes to form vesicles of the right size and structure and to entrap materials with high efficiency and in such a way that these materials do not leak out of the liposomes once formed (New et al, 1990). The liposomes should result in prolongation of drug release and be clinically fit for its use in humans.

In this study, the liposomes & LDPI formulations of Amikacin and Amphotericin B 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 carried out to determine the percent drug retained in liposomes. In vitro drug diffusion studies and in vivo drug disposition studies were also carried out in artificial membrane and in rats. The analytical methods employed in this investigation are discussed below:

Materials

Material	Source
Amikacin Sulphate (AMK)	Nicholas Piramal India Ltd. Dhar, M.P.
Amphotericin B (AMB)	Ambalal Sarabhai Enterprise, Baroda, India.
Water (distilled)	Prepared in laboratory by distillation
Hydrogenated Soyaphosphatidylcholine (HSPC), Soyaphosphatidylcholine (S-100) and Hydrogenated soyaphosphatidylglycerol (SPG-3)	Gift samples from Lipoid (Germany)

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
Sorbolac– 400	Received as gift samples from Meggle, Germany
Pharmatose 325M	Received as gift samples from HMV, The Netherlands

Glasswares and Equipments

Apparatus	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

Centrifuge	Remi C-24, Mumbai
Homogenizer, SD4C	Raliwolf Ltd. Mumabi
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
Spectrofluorophotometer RF-540, Datarecorder DR-3	Shimadzu, Japan
Stability oven	Shree Kailash Industries, Vadodara
UV-Visible Spectrophotometer, U-2000	Shimadzu, Japan
Vacuum Pump F16	Bharat Vacuum pumps, Banglore
Water Bath ME10941	INCO, Ambala

3.1 Physical characterization of liposomes

The present study involves preparation of AMK and AMB liposomal DPI formulations with 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.

3.1.1 Determination of shape and lamellarity

The liposomes of these drugs 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 of were taken to confirm the results.

3.1.2 Determination of size

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 were diluted with sufficient amount of water so that 50 ml volumes 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.

Principle of working **:** It works on Mie's theory of laser light scattering. The IHSD (Intelligent High Speed Detector) manages the measurements. The sample and the hold circuit capture signals from the scattered light detector. This provides a "snapshot" of the scattered light. The timing of the measurement is controlled from the software.

The instrument settings used were as follows: Temperature 25°C; viscosity 0.01 poise; refractive index 1.33; scattering angle 90°; run time 30 sec; range 0-3000 nm.

3.2 Chemical characterization of liposomes

The liposomes of AMK and AMB 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:

3.3 Analytical methods for AMK liposomes

3.3.1 Preparation of calibration curve for the estimation of AMK

The colorimetric method was developed for the estimation of AMK is based on reaction between amikacin sulphate and p-benzoquinone in presence of alkaline borate buffer (pH 8) and heating solution at 70°C. Allowed to cool to room temperature, volume was made up and absorbance of the solution was measured at 350nm. The developed method was linear in the range of 10-60 μg/ml of amikacin sulphate solution.

3.3.1.1 Reagents

(i) Stock solution of AMK: A 1000 mcg/ml solution of AMK was prepared in distilled water.

(ii) Alkaline Borate buffer (pH 8.0):Alkaline borate buffer was prepared as per IP. To 50ml of 0.2M boric acid and potassium chloride solution in 200ml volumetric flask, 3.9ml of 0.2M Sodium hydroxide solution was added and volume was made up to 200 ml.

(iii) p-Benzoquinone Reagent:1mg/ml solution was prepared in water and used for the reaction. This reagent was prepared freshly.

3.3.1.2 Method

Aliquots of 0.1 ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml and 0.6 ml from the stock solution of AMK were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. To each flask was added 4ml of alkaline borate buffer (pH 8) and 1ml of reagent. Volume was made up to 10ml and solution was heated on water bath maintained at 70°C for 10 minutes and allowed to cool. The absorbance maxima (λ max) was determined using 30 µg/ml solution against the reagent blank on U-2000 UV-Visible spectrophotometer. The absorbance of all the prepared solutions was measured at the absorbance maxima, 350 nm, against a suitable blank. The readings were recorded in duplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.1 and the regressed calibration curve is shown in Fig. 3.1.

Table 3.1 Calibration curve for the estimation of AMK

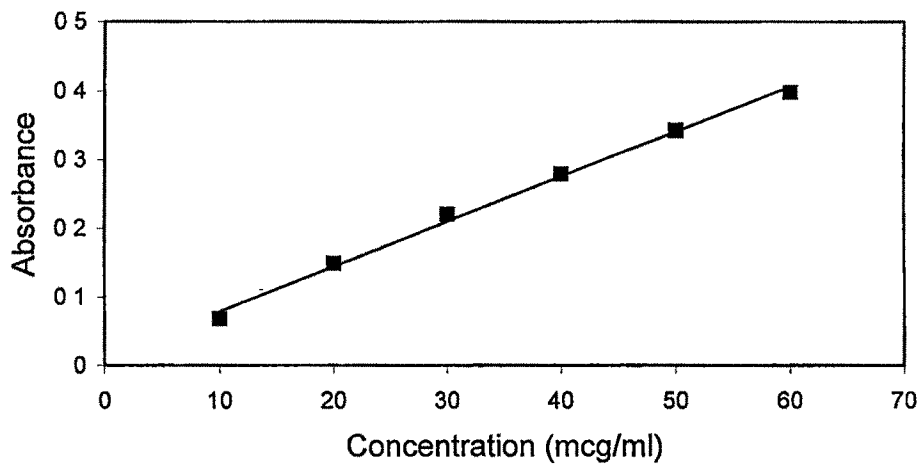
Concentration (µg/ml)	Absorbance* (± SEM)
10	0.068 (0.001)
20	0.149 (0.002)
30	0.220 (0.003)
40	0.279 (0.004)
50	0.343 (0.003)
60	0.398 (0.001)

r = 0.9977

Equation of the regression line is Y = 0.0065 x + 0.0137

* n = 6

Fig. 3 1 Regressed calibration curve for the estimation of AMK



3.3.1.3 Interference of formulation components

The interference of PC, Chol, lactose, negative charge inducer (SPG-3) and positive charge inducer (SA) in the estimation of AMK was determined by measuring the absorbance of the maximum used concentrations of PC, Chol, lactose, SPG-3 and SA at 350 nm against the reagent blank. The observations are recorded in Table 3.6.

3.3.2 Estimation of AMK in biological fluid and tissues

The spectrophotofluorometric method is based on the reaction of 2-hydroxy-1-naphthaldehyde with aminoglycoside antibiotic through their amino groups. The reaction exhibits maximum fluorescence intensity at 434nm after excitation at 366 nm. The developed method was linear in the range of 0.25-4 μg/ml of amikacin sulphate solution.

3.3.2.1 Reagents

(i) Stock solution of AMK: A 1000 μg/ml solution of AMK was prepared in water and then serially diluted as appropriate.

(ii) 2-Hydroxy-1-naphthaldehyde solution: 0.005 % w/v solution of 2-hydroxy-1-naphthaldehyde was prepared in acetone and kept in refrigerator.

(iii) Borate buffer pH 6.3: 0.2M boric acid solution was adjusted with 0.4M sodium hydroxide solution to the required pH i.e. 6.3.

3.3.2.2 Method

Aliquots of 25 μl, 50 μl, 75 μl, 0.1ml, 0.2ml, 0.3 ml and 0.4 ml from the stock solution of AMK were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. 1ml of 2-hydroxy-1-naphthaldehyde reagent and 5ml of borate buffer (pH 6.3) were added. The solution was heated in a water bath at 80°C for 30 minutes, cooled

and brought to volume with the same buffer solution. The relative fluorescence intensity was determined using 0.25 μg/ml solutions against the reagent blank on Shimadzu Spectrofluorophotometer. The relative fluorescence intensity of all the prepared solutions was measured at 434 nm (excitation at 366 nm), against a suitable blank. The readings were recorded in duplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.2 and the regressed calibration curve is shown in Fig. 3.2.

Reference:

EI-Shabrawy Y (2002) Fluorimetric determination of aminoglycoside antibiotics in pharmaceutical preparations and biological fluids. Spectroscopy letters, 35:99-109.

Table 3.2 Calibration curve for the estimation of AMK in biological fluids

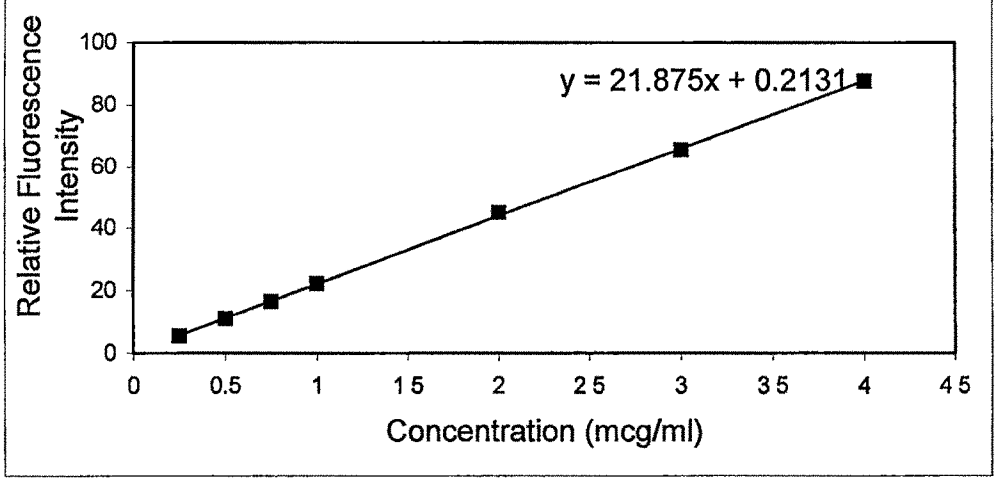
Concentration (μg/ml)	Relative fluorescence intensity* (± SEM)
0.25	5.48 (0.006)
0.5	11.03 (0.005)
0.75	16.42 (0.004)
1	22.16 (0.002)
2	44.95 (0.002)
3	65.47 (0.001)
4	87.54 (0.002)

r = 0.9998

Equation of the regression line is Y = 21.875x + 0.2131

* n = 6

Fig 3 2 Regressed calibration curve for the estimation of AMK in biological fluids



3.3.3 Preparation of calibration curve for the estimation of PC

Stewart assay (1980) was used wherein phospholipids forms a red colored complex with ammonium ferrothiocynate in organic solution.

3.3.3.1 Reagents

(i) Stock solution of PC: A 1000 μg/ml solution of PC was prepared in chloroform.

(ii) Ammonium ferrothiocynate solution, 0.1M: Accurately weighed 27.03 g of ferric chloride hexahydrate and 30.4 g of ammonium thiocyanate were transferred to a clean and dry 1000 ml volumetric flask and dissolved in sufficient amount of water. The volume was finally adjusted to 1000ml with water and was stored in an amber colored bottle at room temperature.

3.3.3.2 Method:

Aliquots of 0.1ml, 0.2ml, 0.3ml 0.4ml, 0.5ml, 0.6ml and 0.7ml from the stock solution of PC were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate calibrated centrifuge tubes. The volume in each tube was made up to 3ml with chloroform and to it was added 2ml of ammonium ferrothiocyanate solution. The content of each tube was vortexed on a cyclomixer for 30 seconds and then centrifuged at 2750 rpm for 10 minutes to separate chloroform layer. The chloroform layer was then removed using a glass syringe with long needle. The absorbance maxima (λ max) was determined by scanning 300 μg/3ml solution against the reagent blank on U-2000 UV-Visible Spectrophotometer. The absorbance of all the prepared solutions was then measured at the absorbance maxima, 485 nm, against reagent blank. The readings were recorded in duplicate and the experiment

Table 3.3 Calibration curve for the estimation of PC

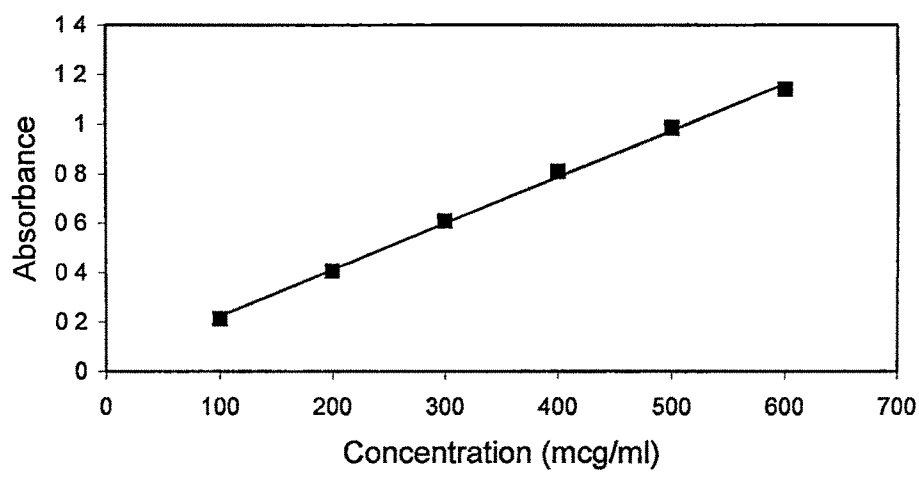
Concentration (µg/3ml)	Absorbance* (± SEM)
100	0.2132 (0.002)
200	0.4064 (0.002)
300	0.6074 (0.001)
400	0.8090 (0.002)
500	0.9850 (0.002)
600	1.1380 (0.001)
700	1.2136 (0.002)

r = 0.9933

Equation of the regression line is Y = 0.00086 x + 0.03790

* n = 6

Fig 3.3 Regressed calibration curve for the estimation of PC



was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.3 and the regressed calibration curve is shown in Fig. 3.3.

Reference:

Stewart, J. C. M. (1980), Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem., 104, 10-14.

3.3.4 Preparation of calibration curve for the estimation of Chol

Zlatkis, Zak and Boyle's method (Goel et al, 1988) was used wherein Chol in acetic acid forms a complex with ferric chloride and sulphuric acid.

3.3.4.1 Reagents

(i) Stock solution of Chol: A 500 µg/ml solution of Chol was prepared in glacial acetic acid.

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

3.3.4.2 Method

Aliquots of 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.2ml, 1.6ml and 2.0 ml from the stock solution of Chol were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. To each flask was added 4ml of the ferric chloride solution and 4ml of sulphuric acid and the volume was made up to 10ml with glacial acetic acid. The absorbance maxima (λ max) was determined by scanning 20 µg/ml solution against the reagent blank on U- 2000 UV-Visible Spectrophotometer. The absorbance of all the prepared solutions was then measured at the absorbance maxima, 550 nm, against reagent blank. The readings were recorded in duplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.4 and the regressed calibration curve is shown in Fig. 3.4.

Reference:

Goel BK (1988). Medical Laboratory Technology (Vol. III), Tata McGraw Hill, New Delhi, 1031.

Table 3.4 Calibration curve for the estimation of Chol

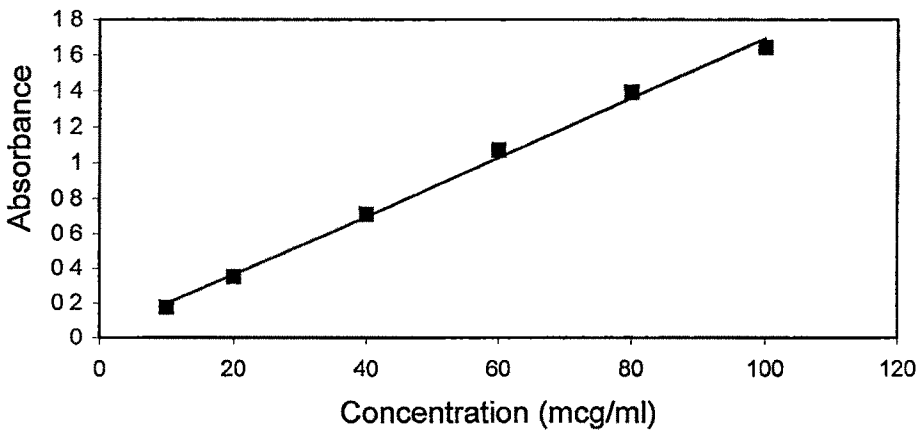
Concentration (µg/ml)	Absorbance* (± SEM)
10	0.1762 (0.006)
20	0.3514 (0.005)
40	0.7098 (0.004)
60	1.0719 (0.002)
80	1.3928 (0.002)
100	1.6424 (0.001)

r = 0.9975

Equation of the regression line is Y = 0.0166 x + 0.33

* n = 6

Fig 3.4 Regressed calibration curve for the estimation of CHOL



3.3.5 Estimation of AMK, PC and CHOL in liposomes

AMK, PC and CHOL were estimated in liposomes by the modified Bligh-dyer two-phase extraction procedure (New et al, 1990). The liposome after separation from the free drug was kept aside. AMK, PC and CHOL were estimated using the procedure given below in (a) for AMK, (b) for PC and (c) for CHOL.

(a) Accurately measured 0.5 ml of liposomal suspension was transferred to a calibrated centrifuge tube and the volume was made up to 2 ml with a 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 AMK as described in this chapter using p-benzoquinone as reagent (Section 3.3.1).

(b) The combined chloroform extract obtained in estimation of AMK was used for the estimation of PC as described in this chapter using Stewart assay (Section 3.3.3).

(c) Accurately measured 0.5 ml of liposomal suspension was transferred to a calibrated centrifuge tube and the volume was made up to 2 ml with a 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. 5ml of this chloroform extract was transferred into clean and dry 60ml separating funnel and washed using three 5ml portions of hydrochloric acid. 2ml of this chloroform extract was then transferred to a clean and dry 10ml volumetric flask and the solvent was evaporated to dryness. The contents of the flask were then analyzed for its CHOL content using the method described in this chapter by Zlatkis, Zak and Boyle's method (Section 3.3.4)

3.3.6 Estimation of AMK 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 was used for estimation of AMK as described in this chapter using p-benzoquinone as reagent (Section 3.3.1).

Table 3.5 Interference of excipients used in the formulations

Excipients	Absorbance at 350 nm	Absorbance at 410 nm	Peak area
HSPC/SPC	0.003	0.000	0.00
CHOL	0.001	0.000	0.00
SPG-3	0.001	0.001	0.00
SA	0.000	0.001	0.00
Lactose	0.002	0.002	0.00

3.3.7 Estimation of AMK retention and vesicle size determination in liposomal DPI formulations during stability studies

Both LDPI formulations of AMK, the samples from each batch at each temperature like freezer (-10°C ± 2°C), refrigerated (5°C ± 2°C), controlled room temperature (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 AMK content as described in this chapter (Section 3.3.1). This will represent the amount of AMK leaked during storage stability of the both formulations at each stability condition. The increase in vesicle size of AMK liposomes was determined from changes in vesicle diameter for both formulations of AMK prior to and after storage at freezer (-10°C ± 2°C), refrigerated (5°C ± 2°C), controlled room temperature (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. The hydrated suspension was centrifuged at 4.38 x 10^3 g for 90 second to sediment the

lactose particles. The supernatant was further processed to analyze the vesicle size as described in section 3.1.2.

3.3.8 Estimation of AMK in diffusion medium

The sample (AMK 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 AMK as described in this chapter using p-benzoquinone as reagent (Section 3.3.1).

3.3.9 Estimation of AMK in BAL and lung tissues

To 5ml of BAL recovered from rats, 5ml of 10% w/v trichloroacetic acid was added for deproteination. Blended on vortex mixer and centrifuged at 3000 rpm for 15 minutes and 1ml of protein free supernatant was transferred in to clean and dry 10ml volumetric flask and volume was made up with distilled water. This solution was further used for estimation of AMK as described in this chapter by spectrofluorometric method (Section 3.3.2).

Similarly to 5ml of lung tissue homogenate obtained from rats, 5ml of 10% w/v trichloroacetic acid was added for deproteination. Blended on vortex mixer and centrifuged at 3000 rpm for 15 minutes and 1ml of protein free supernatant was transferred in to clean and dry 10ml volumetric flask and volume was made up

with distilled water. This solution was further used for estimation of AMK as described in this chapter by spectofluorometric method (Section 3.3.2).

3.4 Analytical methods for AMB liposomes

3.4.1 Preparation of calibration curve for the estimation of AMB

The simple Spectrophotometric method is used for the estimation of AMB in which AMB was estimated in methanol: DMSO (1:1) mixture at 410nm. The developed method was linear in the range of 2-8 μg/ml of Amphotericin B solution.

3.4.1.1 Reagents

(i) Stock solution of AMB: A 1000 mcg/ml solution of AMB was prepared in Methanol: DMSO (1:1) mixture and then serially diluted as appropriate in the same solvent mixture. Each time the stock was prepared freshly.

3.4.1.2 Method

Aliquots of 0.2 ml, 0.3ml, 0.4ml, 0.5ml, 0.6ml and 0.8 ml from the stock solution of AMB (100 μg/ml) were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. Volume was made up to 10ml with methanol: DMSO (1:1) solvent mixture. The absorbance maxima (λ max) were determined using 4-μg/ml solution against the reagent blank on U-2000 UV-Visible spectrophotometer. The absorbance of all the prepared solutions was measured at the absorbance maxima, 410 nm, against a suitable blank. The readings were recorded in duplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.6 and the regressed calibration curve is shown in Fig. 3.5.

Reference:

Ruijgrok EJ, Vulto AG, Van Etten WM (2000) Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (Ambisome): Aerosol characteristics and In-vivo amphotericin B deposition in rats. J.Pharm.Pharmacol. 52: 619-627.

Table 3.6 Calibration curve for the estimation of AMB

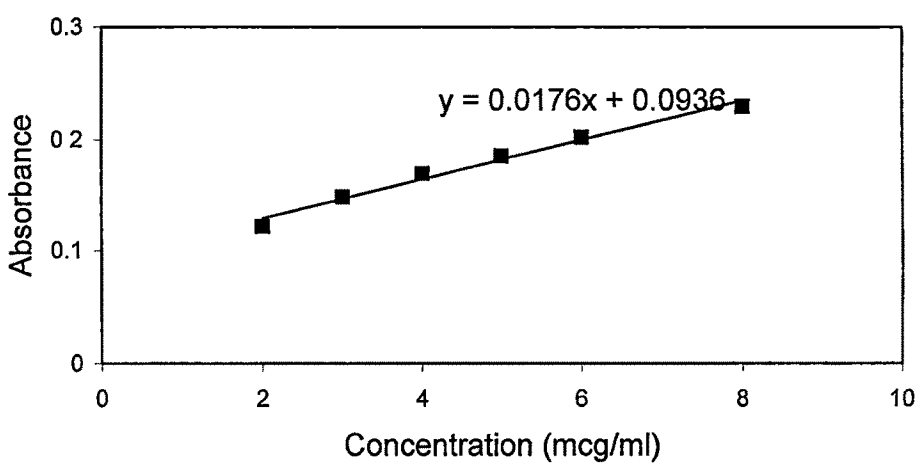
Concentration (µg/ml)	Absorbance* (± SEM)
2	0.1214 (0.001)
3	0.1485 (0.002)
4	0.1694 (0.001)
5	0.1853 (0.002)
6	0.2018 (0.003)
8	0.2295 (0.001)

r = 0.9910

Equation of the regression line is Y = 0.0176 x + 0.0936

* n = 6

Fig. 3.5 Regressed calibration curve for the estimation of AMB



3.4.2 Preparation of calibration curve for the estimation of AMB in biological fluids

The third order derivative Spectrophotometric method is used for the estimation of AMB in biological fluids like BAL and lung tissues. Samples are treated with acetonitrile and AMB was quantified on the basis of intensity of the peaks at 402 nm. The developed method was linear in the range of 12 - 1152 ng/ml of Amphotericin B solution.

3.4.2.1 Reagents

(i) Stock solution of AMB: A 1000 mcg/ml solution of AMB was prepared in DMF. The aliquots of this stock solution was further diluted with aceonitrile to give intermediate stock solutions (960 ng/nl), which in turn further diluted with acetonitrile: water (1.5:1) to give working solutions in the range of 12 – 1152 ng/ml. Stock solution was kept at –20°C and prepared fresh weekly, while working solutions were prepared fresh daily. All solutions were protected from light throughout the analysis.

3.4.2.2 Method

Aliquots of 0.125 ml, 0.25 ml, 0.5 ml, 1 ml, 2 ml, 3 ml, 4 ml and 5 ml from the stock solution of AMB (960 ng/ml) were accurately measured using suitable calibrated pipettes and transferred to clean and dry, separate, 10ml volumetric flasks. Volume was made up to 10ml with water. Normal spectra were obtained between 350 and 460nm at a scanning speed of 1500 nm/min on U-2000 UV-Visible spectrophotometer. Third order derivative spectra were produced by digital differentiation of the normal spectra using a derivative wavelength difference ($\Delta\lambda$) of 12.6 nm. Calibration curves were constructed by plotting values of intensity of the peak at 402 nm vs. concentration of each of working solution-

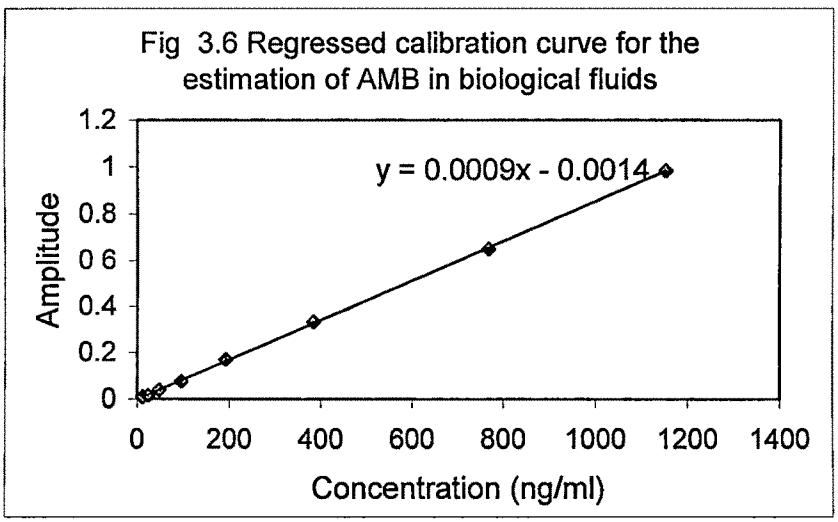
Table 3.7 Calibration curve for the estimation of AMB in biological fluids

Concentration (ng/ml)	Absorbance* (± SEM)
12	0.009 (0.001)
24	0.016 (0.001)
48	0.039 (0.002)
96	0.076 (0.001)
192	0.170 (0.001)
384	0.333 (0.001)
768	0.651 (0.001)
1152	0.985 (0.001)

r = 0.9996

Equation of the regression line is Y = 0.0009x - 0.0014

* n = 6



using water as a blank. The readings were recorded in duplicate and the experiment was repeated on 3 consecutive days using freshly prepared stock solutions each time. Mean values (n=6) along with the standard error mean are recorded in Table 3.7 and the regressed calibration curve is shown in Fig. 3.6.

Reference:

Botsoglou NA, Fletouris DJ, Papageorgiou GE, Florou-paneri P, Mantis AJ. (1996) Rapid determination of Amphotericin B in serum and urine by third-order derivative spectrophotometry. J. Pharm. Sci. 85:402-406.

3.4.3 Estimation of AMB, PC and CHOL in liposomes

AMB in liposomes was estimated directly by method described in previous section (3.4.1) and PC and CHOL were estimated in liposomes by the modified Bligh-dyer two-phase extraction procedure (New et al, 1990). The liposome after separation from the free drug was kept aside. AMB, PC and CHOL were estimated using the procedure given below in (a) for AMB, (b) for PC and (c) for CHOL.

(a) Accurately measured 0.2 ml of liposomal suspension was transferred to 10ml volumetric flask and dissolved directly in 5ml of methanol: DMSO solvent mixture and sonicated if necessary to dissolve. Volume was made up to mark with methanol: DMSO solvent mixture and AMB content was analyzed using the method described in this chapter (Section 3.4.1)

(b) Accurately measured 0.5 ml of liposomal suspension was transferred to a calibrated centrifuge tube and the volume was made up to 2 ml with a 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 combined chloroform extract obtained was used for the estimation of PC as described in this chapter using Stewart assay (Section 3.3.3).

(c) Accurately measured 0.5 ml of liposomal suspension was transferred to a calibrated centrifuge tube and the volume was made up to 2 ml with a 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. 5ml of this chloroform extract was transferred into clean and dry 60ml separating funnel and washed using three 5ml portions of hydrochloric acid. 2ml of this chloroform extract was then transferred to a clean and dry 10ml volumetric flask and the solvent was evaporated to dryness. The contents of the flask were then analyzed for its CHOL content using the method described in this chapter by Zlatkis, Zak and Boyle's method (Section 3.3.4)

3.4.4 Estimation of AMB from LDPI formulations

100 mg of Liposomal DPI formulation was transferred to 10ml volumetric flask and dissolved directly in 5ml of methanol: DMSO solvent mixture and sonicated if necessary to dissolve and filtered through 0.2 micron nylon filter. Volume was

made up to mark with methanol: DMSO solvent mixture and AMB content was analyzed using the method described in this chapter (Section 3.4.1).

3.4.5 Estimation of AMB retention and vesicle size determination in liposomal DPI formulations during stability studies

Both LDPI formulations of AMB, the samples from each batch at each temperature like freezer (-10°C ± 2°C), refrigerated (5°C ± 2°C), controlled room temperature (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 AMB content as described in this chapter (Section 3.4.1). This will represent the amount of AMB leaked during storage stability of the both formulations at each stability condition. The increase in vesicle size of AMB liposomes was determined from changes in vesicle diameter for both formulations of AMB prior to and after storage at freezer (-10°C ± 2°C), refrigerated (5°C ± 2°C), controlled room temperature (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. The hydrated suspension was centrifuged at 4.38 x 10^3 g for 90 second to sediment the lactose particles. The supernatant was further processed to analyze the vesicle size as described in section 3.1.2.

3.4.6 Estimation of AMB in diffusion medium

The sample (AMB in diffusion medium at different time points) was transferred to 10ml volumetric flask and dissolved directly in 5ml of methanol: DMSO solvent mixture and sonicated if necessary to dissolve. Volume was made up to mark with

methanol: DMSO solvent mixture and AMB content was analyzed using the method described in this chapter (Section 3.4.1).

3.4.7 Estimation of AMB in BAL and lung tissues

To 5ml of BAL recovered from rats, 5ml of 10% w/v trichloroacetic acid was added for deproteination. Blended on vortex mixer and centrifuged at 3000 rpm for 15 minutes and 1ml of protein free supernatant was transferred in to clean and dry 10ml volumetric flask and volume was made up with distilled water. This solution was further used for estimation of AMB as described in this chapter by third order derivative Spectrophotometric method (Section 3.4.2).

Similarly to 5ml of lung tissue homogenate obtained from rats, 5ml of 10% w/v trichloroacetic acid was added for deproteination. Blended on vortex mixer and centrifuged at 3000 rpm for 15 minutes and 1ml of protein free supernatant was transferred in to clean and dry 10ml volumetric flask and volume was made up with distilled water. This solution was further used for estimation of AMB as described in this chapter by third order derivative Spectrophotometric method (Section 3.4.2).

3.5 Results and discussion

AMK in liposomes was estimated by lysing the liposomes with saturated sodium chloride solution (modified Bligh-dyer method) and estimated by developed colorimetric method. The method used for the estimation of AMK is based on reaction between amikacin sulphate and p-benzoquinone in presence of alkaline borate buffer (pH 8) and heating solution at 70°C. Allowed to cool to room temperature, volume was made up and absorbance of the solution was measured at 350nm. The developed method was linear in the range of 10-60 µg/ml of amikacin sulphate solution (Fig. 3.1) and regression co-efficient was found to be 0.9977.

The color was stable up to 6 hours. The same method was successfully used for the determination of AMK during characterization procedures, stability studies and in vitro diffusion studies. For determination of AMK in biological samples (Broncho alveolar lavage and lung tissues) spectrofluorometric method was used by using 2-hydroxy-1-naphthaldehyde as fluorescence inducing reagent. Reaction was based on reaction of 2-hydroxy-1-naphthaldehyde with aminoglycoside antibiotic through their amino groups. The reaction exhibits maximum fluorescence intensity at 434nm after excitation at 366 nm. The developed method was linear in the range of 0.25-4 μg/ml of amikacin sulphate solution and regression co-efficient was found to be r = 0.9998 (Fig. 3.2).

A direct Visible Spectrophotometric method was used for the estimation of AMB at absorbance maxima of 410 nm using methanol: DMSO as solvent system to dissolve AMB. This method was linear in the range of 2-8 μg/ml of Amphotericin B solution (r = 0.9910) (Fig. 3.5). For the determination of AMB in biological samples (Broncho alveolar lavage and lung tissues), third order derivative Spectrophotometric method was used. Samples are treated with acetonitrile and AMB was quantified on the basis of intensity of the peaks at 402 nm. The developed method was linear in the range of 12 - 1152 ng/ml of Amphotericin B solution (r = 0.9996) (Fig. 3.6).

HSPC/SPC and CHOL content of liposomes were determined by separating them from liposomes by modified Bligh-dyer two phase extraction procedure and analyzing with an appropriate colorimetric method. HSPC/SPC is a good emulsifying agent. This emulsification was avoided by using saturated sodium chloride solution. PC content in liposomes as a whole was determined using Stewart assay. The absorbance maximum, determined by scanning a solution of

400 μg/3ml on U-2000 UV Spectrophotometer was found to be at 485 nm. The absorbance of all the prepared solutions was measured at the 485 nm. The developed method was linear in the range of 100 – 700 μg/3ml of PC solution (r = 0.9933) (Fig. 3.3). The absorbance of the solution was found to increase during first 5 minutes of preparation; hence, all the readings were taken 5 minutes after the completion of color development.

Zlatkis, Zak and Boyle's method was used for the estimation of CHOL. The absorbance maxima determined by scanning a solution of concentration 20 μg/ml on on U-2000 UV Spectrophotometer was found to be at 550 nm. The absorbance of all the prepared solutions was measured at the 550 nm. The developed method was linear in the range of 10 – 100 μg/ml of CHOL solution (r = 0.9975) (Fig. 3.4).

The interference of the formulation components like PC, CHOL, SA, SPG-3 and lactose in estimation of drugs was ascertained by analyzing the maximum used concentrations at corresponding wavelengths. The result indicates no significant differences ($P<0.05$) in the absorbance value suggesting no interference.

References:

New, R. R.C., (Ed), (1990), Liposome: A Practical Approach, Oxford University press, Walten Street, Oxford. p.108, 256,