CHAPTER 3

ANALYTICAL METHODS

3.1 INTRODUCTION

In this investigation, ALLP of TB and AMK were characterized for particle size, polydispersity, tap density, moisture content, crystallinity, shape, roundness, elongation ratio, fractal dimension, heterogeneity, clumpiness and drug content in the formulation. The stability studies were conducted to determine the drug content and aerosolization properties over storage of 6 months period. *In vitro* aerosolization properties studies followed by *in vivo* whole lung deposition, pulmonary clearance, alveolar macrophage uptake and pulmonary toxicity studies in rats were also carried out. The analytical methods employed in these investigations are discussed below.

3.2 MATERIALS AND EQUIPMENTS

Material	*Source*
Tobramycin sulfate (TB)	Gift samples from Sun Pharmaceuticals Industries Limited, Vadodara,India
Amikacin sulfate (AMK) and L-leucine	Gift samples from Alembic Limited, Vadodara, India.
Water (distilled)	Prepared in laboratory by distillation
Hydrogenated Soyaphosphatidylcholine (HSPC)	Gift samples from Lipoid GmBH, Ludwigshafen, Germany
Poloxamer 188	BASF, Ludwigshafen , Germany
6-Coumarin	Gift sample from Neelikon dyes, Mumbai, India
2,4,6-trinitrobenzene sulfonic acid	National chemicals, Vadodara,India
2,4-dinitrofluorobenzene, Tris(hydroxymethyl) aminomethane	Spectrochem,Mumbai, India
Glacial acetic acid, pyridine, potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, potassium hydroxide, sodium chloride, sodium hydroxide ,boric acid and D-mannitol	S.D.Fine chemicals, Mumbai, India
Methanol, sulphuric acid, isopropyl alcohol, acetonitrile, dimethyl sulfoxide	Qualigens chemicals, Mumbai, India
Hydroxypropylmethylcellulose	Colorocn Asia Limited, Goa, India
Ethanol	Govt. supply, Vadodara, India
Urethane	Sigma Chemical Co., USA
Tryphan blue	Fluka chemicals, USA
Lieshman's stain	Sigma-Aldrich Chemical Co., USA
Nuclepore Polycarbonate membrane 2 μm 25mm	Whatman, USA
Rotahaler® Dry Powder Inhalation device	Cipla Ltd., Mumbai, India
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	3K 30, Sigma Laboratory centrifuge, Osterode, GmBH.
Homogenizer, SD4C	Raliwolf Ltd. Mumabi
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
Cascade impactor	Graseby-Andersen, Atlanta, GA, USA
Laboratory spray dryer	LSD-48, JISL, Mumbai, India
Stability oven	Shree Kailash Industries, Vadodara
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Spectrofluorophotometer RF-540, Data recorder DR-3	Shimadzu, 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
Halogen moisture analyzer	HR 73 moisture analyzer, Mettler Toledo, Illinois, USA
HPLC system	Dionex HPLC with Chromleon 6.5 data processing software

3.3 PHYSICAL ANALYSIS

The present study involves preparation of light and large particles of TB and AMK suitable for pulmonary administration. ALLP 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. particle size, polydispersity, tap density, moisture content, crystallinity and morphological features.

3.3.1 Determination of Particle Size and Polydispersity

The particle size was determined by laser diffractometry (Malvern MasterSizer 2000 series, Malvern Instruments, Worcestershire, UK) using the Hydro 2000SM sampling

unit. The apparatus consisted of a He-Ne laser (5 mw) and a sample holding cell of 50 ml capacity. Each sample in sufficient quantity was dispersed in isopropyl alcohol so as to achieve obscuration range between 10-20%. Samples were kept under stirring using a blade stirrer at 1000 rpm to keep particles in suspended form and the measurements were recorded for volume mean diameter (VMD), which is related to the mass median diameter by the density of the particles (assuming a size independent density for the particles). The polydispersibility of powder was defined from span (Chew et al, 2002).

$$\text{Span} = \frac{[D(v, 90) - D(v, 10)]}{[D(v, 50)]} \quad \text{---------- Equation (3.1)}$$

Where D (v, 90), D (v, 10) and D (v, 50) are equivalent volume diameters at 90, 10 and 50% cumulative volume, respectively.

3.3.2 Determination of Tap Density

The powder density (ρ) was evaluated by tap density measurements. Assuming a perfect packing, the tap density of monodisperse spheres is approximately a 21% underestimate of the particle density due to the void spaces between particles. In case of polydisperse particles, the void spaces are reduced but this is probably counterbalanced by incomplete packing (Vanbever et al, 1999 and Bosquillon et al, 2004). The theoretical mass median aerodynamic diameter (MMADt) of individual particles was calculated based on the following definition:

$$\text{MMADt} = \sqrt{(\rho/\rho_1)} * d \text{ ------- Equation (3.2)}$$

Where $\rho_1 = 1$ g/ cm^3.

ρ = tapped density

d= geometric particle size

The tapped densities (ρ) were measured in a 10-ml glass measuring cylinder filled with fixed volume of powder. The tapped density was determined after 500 taps from a constant height.

3.3.3 Determination of Moisture Content

The weight loss on heating was analyzed by halogen moisture analyzer (HR 73 moisture analyzer, Mettler Toledo, Illinois, USA)). 0.5 g of the sample was placed on the pan and dried to constant weight at 50°C. The weight loss on heating was expressed as a percentage of the initial weight.

3.3.4 Determination of Crystallinity

The thermal properties were analyzed using differential scanning calorimetry (DSC) (DSC60, Shimadzu, Shimadzu Corporation, Japan) with TA- 60 WS work station. Thermograms were analyzed using shimadzu TA-60 software. An empty aluminum pan was used as the reference for all measurements. A sample (2-4 mg) of powder was placed in hermetically sealed aluminum pan and scanned at a rate of 10°C/min from 25°C to 300°C .The onset and peak temperatures and enthalpy of transition (ΔH) were determined for each peak.

3.3.5 Characterization of Morphological Features

The morphology was examined by scanning electron microscopy (SEM) (JSM-5610LV, JEOL, Japan). Samples were attached to sample stubs using double sided tape, and then viewed using an accelerating voltage of 15 kilovolt at the magnification of 250X to 5000X. Particle traits and surface topography were assessed using image analysis software (Image Proplus 5.0, Media Cybernetics, USA). Image analysis of the SEM pictures was conducted on a fixed area selected on the particle flat base in order to avoid tilting angle shadow effect. The traits such as roundness and elongation ratio were calculated as described below

Roundness: Reports the roundness of each object, as determined by the following formula: $(perimeter^2) / (4 * \Pi * area)$.

Elongation ratio: Reports the ratio between the Feret max (reports the longest caliper length) to the Feret min (reports the average caliper length) and were calculated from 16 caliper measurements at 6° intervals around the particle. These two measurements were not necessarily at right angles to each other.

Three dimensional surface plots, which describe the surface topography of particles, were drawn by scanning on the selected area of the image, an up and down, line showing the variability of gray level as a function of the position. Ten surface plots were drawn on each image and topographical features *viz.* fractal dimension, heterogeneity, and clumpiness; the descriptors of the texture of the surface were calculated and are defined as follows

Fractal dimension: Calculated as 1 minus the slope of the regression line obtained when plotting the log of the perimeter (using a particular stride) against the log of the stride length, as calculated with multiple starting points in the outline for the strides.

Heterogeneity: Reports the fraction of pixels that vary more than 10% from the average intensity of the object.

Clumpiness: Derived from heterogeneity measurement; the fractions of heterogeneous pixels remaining in an object after an erosion process reflecting the object texture.

3.4 CHEMICAL ANALYSIS

ALLP of TB and AMK prepared in this work were characterized by chemical analysis to determine the drug and phosphatidylcholine (PC) content in the formulation, *in-vitro* aerosol dispersion, estimation of drug content during stability studies, drug content in bronchoalveolar lavage and in lung tissues. Calibration curves of the drug PC and fluorescent marker (6-coumarin) 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 the present investigation. 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 on a Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length using a blank prepared in the same manner omitting the phospholipids.

The above procedure was repeated three times. Mean absorbance values along with standard deviation and the regression equation obtained are shown in Table 3.1.

3.4.1.3 Stability and Selectivity

Stability of the colored solutions, prepared above for the calibration curve of phosphatidyl choline, was ascertained by observing the changes in absorbance of the solutions over a period of 4 h. The selectivity of the method for phosphatidyl choline was investigated by carrying out the procedure detailed above in the presence of potential interferences such as L-leucine, poloxamer 188, hydrolysed gelatine, TB, AMK etc., at the levels at which these materials were included in the ALLP.

Table 3.1 Calibration for estimation of phosphatidyl choline (Stewart assay)

Concentration (µg/3ml)	*Mean Absorbance* ± S.D*
10	0.024 ± 0.002
20	0.057 ± 0.005
40	0.106 ± 0.004
60	0.158 ± 0.002
80	0.209 ± 0.003
100	0.270 ± 0.003

Regression equation** Y= 0.0027 X - 0.0007; Correlation coefficient = 0.9988

*Mean of 3 values

3.4.1.4 Estimation of Phosphatidyl Choline from Formulation

The Bligh-Dyer two-phase extraction method (New, 1990) was modified for estimating phosphatidyl choline from ALLP. Briefly, 0.1ml of ALLP dispersion was taken in a centrifuge tube and to this 2ml of chloroform was then added 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 3.4.1.2). Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidyl choline in the ALLP or supernatant using the regression equation.

3.4.2 Estimation of 6-coumarin

3.4.2.1 Procedure for Calibration Curve

Suitable aliquots (0.5 – 3.0 ml) of the stock solution of 6-coumarin in methanol were transferred to 10ml volumetric flask and volume was made with methanol. The percentage relative fluorescent intensity (RFI) of these solutions was measured at excitation wavelength of 458 nm and emission wavelength of 545 nm on a Shimadzu RF-540 Spectrofluorophotometer with data recorder DR-3. The instrument setting was low sensitivity, ordinate scale of 1x1 and abscissa of 1X 32. Above procedure was repeated thee times. Mean percentage RFI values along with the standard deviation are recorded in Table 3.2.

Table 3.2 Calibration for estimation of 6-coumarin

Concentration (ng/ml)	*Mean percentage RFI* ± S.D*
100	8.2 ± 0.115
200	16.3 ± 0.116
400	31.6 ± 0.100
600	47.6 ± 0.305
800	62.8 ± 0.305
1000	78.5 ± 0.346

Regression equation** Y= 78.266 X + 0.3392; Correlation coefficient = 0.9999
*Mean of 3 values

3.4.2.3 Estimation of 6-coumarin from Formulation

6-coumarin content in formulations was estimated by dispersing suitable quantity of powder in methanol; 6-coumarin was extracted into the methanol by shaking the dispersion for five minuets followed by filtration through 0.2μm filter. RFI of these solutions was measured at excitation wavelength of 458 nm and emission wavelength of 545 nm.

3.4.3 Estimation of Tobramycin sulfate

The modified USP HPLC method was used for the estimation TB in ALLP, biological fluid and tissue. The procedures are detailed as below.

Mobile phase - 2.0 g of tris(hydroxymethyl)aminomethane was dissolved in 800 ml of double distilled water. To this solution 20 ml of 1 N sulfuric acid was added and diluted with acetonitrile to obtain 2000 ml of solution. The solution mixed, allowed to cool, and passed through 0.2-μm filter.

2, 4-Dinitrofluorobenzene reagent - Solution of 2,4-dinitrofluorobenzene containing 10 mg per ml in alcohol was prepared.

Tris (hydroxymethyl) aminomethane reagent - Stock solution of tris(hydroxymethyl)aminomethane in water containing 15 mg per ml was prepared. In to a 200 ml volumetric flask, 40 ml of above stock solution was transferred and dimethyl sulfoxide was added into it. The solution was mixed and diluted further with dimethyl sulfoxide to volume, and mixed. The reagent was used within 4 hours (below 10°C, up to 8 hours).

Standard preparation - About 55 mg of TB was accurately weighed to a 50-ml volumetric flask, 1 ml of 1 N sulfuric acid and enough water was added to dissolve it, diluted with water to volume, and mixed. In to a second 50 ml volumetric flask, 10.0 ml of above solution was transferred and made up the volume with distilled water.

Derivatization procedure - To separate 50 ml volumetric flasks, 4.0 ml of each standard preparation, sample preparation, and of distilled water were transferred. To each flask 10 ml of 2, 4-dinitrofluorobenzene reagent and 10 ml of Tris (hydroxymethyl) aminomethane reagent were added and mixed thoroughly. Flasks with stoppers were placed in a constant temperature bath at 60 ± 2°C, and heated for 50 ± 5 minutes. At the end of 50 minutes flasks were removed from the bath, and allowed to stand for 10 minutes. Acetonitrile to about 2 ml below the 50 ml mark, was added and allowed to cool to room temperature, diluted with acetonitrile to volume,

and mixed. The solutions obtained were derivatized standard preparation, sample preparation, and the blank preparation, respectively. All solutions were heated at the same temperature and for the same duration of time and flasks were moved to and from the 60°C constant temperature bath at the same time.

Chromatographic conditions:

Column: Thermohypersil- C_{18}ODS, 250 x 4.6mm, 10 µm

Flow rate: 1.2 ml

Wavelength: 365 nm

Injection volume: 20 µl

Retention time: 18 min

Run time: 30 min.

3.4.3.1 Procedure for Calibration Curve

Suitable aliquots of the stock solution of TB were pipetted into 10ml volumetric flasks. To each flask 2 ml of 2, 4-dinitrofluorobenzene reagent and 2 ml of Tris (hydroxymethyl) aminomethane reagent were added and mixed thoroughly. Flasks with stoppers were placed in a constant temperature bath at 60 ± 2°C, and heated for 50 ± 5 minutes. At the end of 50 minutes flasks were removed from the bath, and allowed to allowed to cool to room temperature, diluted with acetonitrile to give final concentrations of 1,10,20,40,60,80,100,200,300 and 400 µg/ml and analysed in above explained HPLC system. The column effluent was monitored at 365 nm and at 0.005 absorbance units full-scale. The above procedure was repeated three times. Raw data was recorded in Table 3.3 along with standard deviation.

3.4.3.2 Stability and Selectivity

Stability of TB-complex in solvents, 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 TB was carried out in the presence of phosphatidyl choline, L-leucine, poloxamer 188 and other components of the ALLP to ascertain the selectivity of the method.

Table 3.3 Calibration for Tobramycin sulfate

Concentration (µg/ ml)	*Mean area(mAU)* ± S.D*
0	0
1	0.3288±0.001
10	4.8561±0.002
20	11.6719±0.001
40	23.281±0.002
60	33.8126±0.003
80	42.1011±0.002
100	51.8862±0.001
200	98.6723±0.004
300	147.765±0.005
400	194.3221±0.003

Regression equation** Y= 0.4843 X + 2.0214; Correlation coefficient = 0.9993

*Mean of 3 values

3.4.3.3 Estimation of Tobramycin sulfate from Formulation

TB content in formulations was estimated by dissolving equivalent quantity of powder containing 55 mg of TB in methanol; drug was extracted into the aqueous media by addition of equal volume of distilled water into it and incubated in water bath at 65°C for 30 minutes. The resultant system was centrifuged at 10,000 rpm for 10 minutes, supernatant was separated and volume was made up to 50 ml with distilled water. In to a second 50 ml volumetric flask, 10.0 ml of above solution was transferred and made up the volume with water. Sample was analysed in above explained HPLC system. The column effluent was monitored at 365 nm and at 0.005 absorbance units full-scale.

3.4.3.4 Estimation of Tobramycin sulfate from Biological Samples

Procedure for calibration curve – Lung tissue homogenate was treated with quantity sufficient of methanol to precipitate out the proteins. Samples were centrifuged at 5,000 rpm for 10 minutes at 10°C and supernatants were separated. Suitable aliquots of TB stock solution in water were added into pre-labelled 10 ml volumetric flask. To each flask, 100 µl of supernatant, 2 ml of 2, 4-dinitrofluorobenzene reagent and 2 ml of Tris (hydroxymethyl) aminomethane reagent were added and mixed thoroughly. Flasks with stoppers were placed in a constant temperature bath at 60 ± 2°C, and heated for 50 ± 5 minutes. At the end of 50 minutes flasks were removed from the bath, and allowed to allowed to cool to room temperature, diluted with acetonitrile to give final concentrations of 1,5,10,20,40,60,80,100,150 and 200 µg/ml and samples

were analysed in HPLC system. The column effluent was monitored at 365 nm and at 0.005 absorbance units full-scale. The above procedure was repeated three times. Raw data was recorded in Table 3.4 along with standard deviation. Estimation of TB from biological samples was carried out by treating lung tissue by similar procedure as explained above.

3.4.3.5 Stability and Selectivity

Stability of TB-complex in lung homogenate, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h stored at 2-8°C. Blank biological samples were analysed to ascertain the selectivity of the method.

3.4.3.6 Extraction Efficiency

The recovery of TB from lung homogenate was evaluated at low, medium and high concentrations of 1,100 and 200 μg/ml respectively. The recovery of TB from lung homogenate was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of the known amounts of standard solutions of TB. The overall extraction yields of TB in lung homogenate are shown in Table 3.5

Table 3.4 Calibration for Tobramycin sulfate in biological samples

Concentration (μg/ ml)	*Mean area(mAU)* ± S.D (Lung homogenate)*
0	0
1	0.4006±0.034
5	1.2407±0.06
10	4.4053±0.055
20	12.3516±0.018
40	21.5201±0.047
60	27.7168±0.102
80	37.203±0.213
100	44.4204±0.051
150	66.0633±0.322
200	92.4402±0.161

*Mean of 3 values

Regression equation for Lung homogenate Y= 0.4515 X + 0.6407; Correlation coefficient = 0.9968.

Table 3.5 Extraction efficiency of TB in lung homogenate (n=3)

Samples	*Concentration(μg/ml)*	*Recovered (%) ± S.D*
Lung homogenate	1	102±0.04
	100	98.1±0.05
	200	96.6±0.08

3.4.4 Estimation of Amikacin sulfate

The modified BP **HPLC** method was used for the estimation AMK in ALLP and biological samples. The procedures are detailed as below.

Mobile phase - Methanol -potassium phosphate buffer (70:30[vol/vol] pH 6.5) was prepared by mixture of 30 volumes of a 2.7 g/l solution of potassium dihydrogen phosphate adjusted to pH 6.5 with a 22 g/l solution of potassium hydroxide and 70 volumes of methanol. The solution mixed and passed through 0.2-μm filter.

2, 4, 6 -trinitrobenzene sulphonic acid reagent (TNB) - Solution of TNB containing 10 mg per ml in water was prepared.

Standard preparation - About 50 mg of AMK was accurately weighed to a 50-ml volumetric flask, enough water was added to dissolve it and volume was made up with distilled water.

Derivatization procedure - To separate volumetric flasks, 0.2 ml of each standard preparation, sample preparation, and of distilled water were transferred. To each flask 2 ml of TNB and 3.0 ml of pyridine were added, tightly closed and shaken vigorously for 30 s. Flasks with stoppers were placed in a constant temperature bath at 75°C, and heated for 45 minutes. At the end of 45 minutes flasks were removed from the bath, and allowed to cool for 2 minutes in cold water. 2 ml of glacial acetic acid was added and shaken vigorously for 30 s. The solutions obtained were derivatized standard preparation, sample preparation, and the blank preparation, respectively. All solutions were heated at the same temperature and for the same duration of time and flasks were moved to and from the 75°C constant temperature bath at the same time.

Chromatographic conditions:

Column: Thermohypersil- C_{18}ODS, 250 x 4.6mm, 10 μm

Flow rate: 1 ml/min

Wavelength: 340 nm

Injection volume: 20 μl

Retention time: 12 min

Run time: 20 min.

3.4.4.1 Procedure for Calibration Curve

Suitable aliquots of the stock solution of AMK were pipetted into 10ml volumetric flasks. To each 2 ml of TNB and 3.0 ml of pyridine were added, tightly closed and shaken vigorously for 30 s. Flasks with stoppers were placed in a constant temperature bath at 75°C, and heated for 45 minutes. At the end of 45 minutes flasks were removed from the bath, and allowed to cool for 2 minutes in cold water. 2 ml of glacial acetic acid was added and shaken vigorously for 30 s and were diluted with mobile phase to give final concentrations of 1,10,20,40,60,80,100,200,300 and 400 µg/ml and analysed in above explained HPLC system. The column effluent was monitored at 340 nm and at 0.005 absorbance units full-scale. The above procedure was repeated three times. Raw data was recorded in Table 3.6 along with standard deviation.

3.4.4.2 Stability and Selectivity

Stability of AMK-complex in solvents, 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 AMK was carried out in the presence of phosphatidyl choline, L-leucine, poloxamer 188 and other components of the ALLP to ascertain the selectivity of the method.

Table 3.6 Calibration for Amikacin sulfate

Concentration (µg/ ml)	*Mean area(mAU)* ± S.D*
0	0
1	1.4265±0.002
10	11.2994±0.003
20	13.966±0.001
40	30.1126±0.005
60	53.7583±0.003
80	76.5824±0.001
100	92.429±0.001
200	179.0124±0.004
300	256.8375±0.004
400	345.9833±0.006

Regression equation** Y= 0.8637 X + 1.5425; Correlation coefficient = 0.9987

*Mean of 3 values

3.4.4.3 Estimation of Amikacin sulfate from Formulation

AMK content in formulations was estimated by dissolving equivalent quantity of powder containing 50 mg of AMK in methanol; drug was extracted into the aqueous media by addition of equal volume of distilled water into it and incubated in water bath at 65°C for 30 minutes. The resultant system was centrifuged at 10,000 rpm for 10 minutes, supernatant was separated and volume was made up to 50 ml with distilled water. Sample was analysed in above explained HPLC system. The column effluent was monitored at 340 nm and at 0.005 absorbance units full-scale.

3.4.4.4 Estimation of Amikacin sulfate from Biological Samples

Procedure for calibration curve – Lung tissue homogenate was treated separately with quantity sufficient of methanol to precipitate out the proteins. Samples were centrifuged at 5,000 rpm for 10 minutes at 10°C and supernatants were separated. Suitable aliquots of AMK stock solution in water were added into pre-labelled 10 ml volumetric flask. To each flask, 100 µl of supernatant, 2 ml of TNB and 3.0 ml of pyridine were added, tightly closed and shaken vigorously for 30 s. Flasks with stoppers were placed in a constant temperature bath at 75°C, and heated for 45 minutes. At the end of 45 minutes flasks were removed from the bath, and allowed to cool for 2 minutes in cold water. 2 ml of glacial acetic acid was added and shaken vigorously for 30 s and were diluted with mobile phase to give final concentrations of 1,5,10,20,40,60,80,100,150 and 200 µg/ml and samples were analysed in HPLC system. The column effluent was monitored at 340 nm and at 0.005 absorbance units full-scale. The above procedure was repeated three times. Raw data was recorded in Table 3.6 along with standard deviation. Estimation of AMK from biological samples was carried out by treating lung tissue by similar procedure as explained above.

3.4.4.5 Stability and Selectivity

Stability of AMK-complex in lung homogenate, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h stored at 2-8°C. Blank biological samples were analysed to ascertain the selectivity of the method.

3.4.4.6 Extraction Efficiency

The recovery of AMK from lung homogenate was evaluated at low, medium and high concentrations of 1,100 and 200 µg/ml respectively. The recoveries of AMK from

lung homogenate was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of the known amounts of standard solutions of AMK. The overall extraction yields of AMK in lung homogenate are shown in Table 3.7

Table 3.7 Calibration for Amikacin sulfate in biological samples

Concentration (µg/ ml)	*Mean area(mAU)* ± S.D (Lung homogenate)*
0	0
1	1.6429±0.021
5	3.8409±0.032
10	10.5867±0.01
20	12.2203±0.064
40	26.042±0.44
60	51.9288±0.035
80	77.2331±0.024
100	91.0542±0.054
150	128.0837±0.014
200	176.3311±0.064

*Mean of 3 values

Regression equation for Lung homogenate Y= 0.8873 X - 1.1986; Correlation coefficient = 0.9943.

Table 3.8 Extraction efficiency of AMK in lung homogenate (n=3)

Samples	*Concentration(µg/ml)*	*Recovered (%) ± S.D*
Lung homogenate	1	97.8±0.06
	100	101.2±0.03
	200	96.2±0.08

3.5 RESULTS AND DISCUSSION

3.5.1 Estimation of Phosphatidyl Choline

The Stewart assay was used for estimating phosphatidyl choline in ALLP (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 0.9988 (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 – 100 μg/ml. This high range is a reflection of the low absorptivity of the complex at the analytical wavelength. The regression equation obtained was Y= 0.0027 X - 0.0007. 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 ALLP viz. L-leucine, poloxamer 188, TB and AMK etc. at the levels at which these materials were included in the ALLP. 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 ALLP 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 ALLP. Thus the method was found to be satisfactory for estimation of phosphatidyl choline in ALLP formulations.

3.5.2 Estimation of 6-coumarin

6-coumarin is used as fluorescent marker in ALLP. Estimation of 6-coumarin in the formulation is a rapid and sensitive method for characterization of in-vitro aerosol performance (Lombry et al, 2002). It emits fluorescence at excitation wavelength of 458 nm and emission wavelength of 545nm. Linearity of the method was observed from 100-1000 ng/ml with a correlation coefficient of 0.9999 (Table 3.2). The regression equation obtained was Y= 78.266 X + 0.3392. Monitoring of the RFI of the solutions, used for preparing the calibration curve revealed that the fluorescence of the solution was retained as its original intensity for 24h. The presence of the other constituents of the ALLP such as phosphatidyl choline, L-leucine, poloxamer 188, TB and AMK etc., at the same concentrations at which these materials were included in the ALLP, did not interfere with the estimation of 6-coumarin. The method was used to estimate 6-coumarin in ALLP by extracting it into the methanol as described earlier. There was good agreement between the amount of 6-coumarin estimated from ALLP and that added initially during preparation of ALLP.

3.5.3 Estimation of Tobramycin sulfate

Since TB has poor absorbance in UV and visible region, it is derivatized with absorbance enhancing agent 2, 4-dinitrofluorobenzene. Extraction of TB into aqueous media followed by derivatization shows absorption maxima at 365nm at retention time of 18 min. Selectivity of method was estimated by analysing the placebo samples treated in similar manner to that test samples and injected. There was no interfering peak appeared in the chromatograms of real samples. Stability of TB-complex was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature. The stability results were evaluated by comparing the subsequent results and with the standard (assuming initial assay value as 100%) and expressed as percent deviation. ANOVA studies of the results indicated no significant difference between the readings. Thus, TB-complex is stable over a

period of 24h. The calibration curve for TB was linear over the range from 1 to 400µg/ml. The calibration curve was calculated by linear regression method. The regression equation obtained was Y= 0.4843 X + 2.0214; Correlation coefficient = 0.9993.

Estimation of TB from lung homogenate was carried out by extraction of TB followed by derivatization. Linearity was observed over the range from 1 to 200µg/ml. The calibration curve was calculated by linear regression method. The regression equation obtained was Y= 0.4515 X + 0.6407; Correlation coefficient = 0.9968. The selectivity of the method was established by injecting blank biological samples, there was no interfering peak in the chromatogram of TB. Stability of the method was ascertained as explained above. The recovery of TB from lung homogenate was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of the known amounts of standard solutions of TB. Methanol was selected as the optimal extracting solvent which enabled recovery up to at least 90%.

3.5.4 Estimation of Amikacin sulfate

Since AMK has poor absorbance in UV and visible region, it is derivatized with absorbance enhancing agent 2, 4, 6 -trinitrobenzene sulphonic acid. Extraction of AMK into aqueous media followed by derivatization shows absorption maxima at 340nm at retention time of 12 min. Selectivity of method was estimated by analysing the placebo samples treated in similar manner to that test samples and injected. There was no interfering peak appeared in the chromatograms of real samples. Stability of TB-complex was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature. The stability results were evaluated by comparing the subsequent results and with the standard (assuming initial assay value as 100%) and expressed as percent deviation. ANOVA studies of the results indicated no significant difference between the readings. Thus, AMK-complex is stable over a period of 24h. The calibration curve for AMK was linear over the range from 1 to 400µg/ml. The calibration curve was calculated by linear regression method. The regression equation obtained was Y= 0.8637 X + 1.5425; Correlation coefficient = 0.9987.

Estimation of AMK from lung homogenate was carried out by extraction of AMK followed by derivatization. Linearity was observed over the range from 1 to

200µg/ml. The calibration curve was calculated by linear regression method. The regression equation obtained was Y= 0.8873 X - 1.1986; Correlation coefficient = 0.9943. The selectivity of the method was established by injecting blank biological samples, there was no interfering peak in the chromatogram of AMK. Stability of the method was ascertained as explained above. The recovery of AMK from lung homogenate was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of the known amounts of standard solutions of AMK. Methanol was selected as the optimal extracting solvent which enabled recovery up to at least 90%.

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