Chapter 3
Analytical Method Development

Analytical method development

3.1 Introduction

Analysis is an important and integral component in the formulation of any drug molecule and characterization of developed formulations. Analytical method development and validation play important role in the discovery, development and evaluation of drugs and pharmaceuticals in variety of samples like bulk powders, formulations, in vitro release samples, stability samples and biological samples. Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical, biopharmaceutical and clinical pharmacokinetic studies. If analytical method is not suitable and sensitive there is a possibility of erroneous results, which will lead to false conclusion. Thus there is a need of development of suitable and sensitive analytical method for estimation of drug in various samples. Though literature may provide methods for drug analysis, however such methods may not always be found suitable for the specific formulation development process. In such case it is essential to develop a need based simple, sensitive and cost effective analytical method for estimation of drug.

With the FDA's process analytical technology initiative, the current view of 'quality by design' is further strengthened by stating that the quality should be built in product and should not be inspected within. Drug control and regulatory agencies of several countries have recognized the importance of analytical science in product design and development and have released extensive guidelines on validation requirements. Although analytical validation requirements depend upon the type of analyte and analytical instrument, it broadly includes specificity and selectivity, linearity and range, accuracy and precision, sensitivity, reproducibility, and stability.

3.1.1. Analytical method validation

Analytical method has to be validated when it is necessary to verify whether its performance parameters are adequate for estimation of the drug. Analytical method must be validated, (a) when a new method is developed for a specific problem; (b) when indications of established method are changing with time; (c) when an established method is revised to include improvements or to extend it for another purpose; (d) when an established method is used in a different laboratory, or with different analysts or different instrumentation; (e) to prove the equality between two methods, i.e. a new method and a standard (1). Various validation

parameters have been suggested by various regulatory agencies. These include selectivity/specificity, accuracy, precision, linearity and range, LOD, LOQ, robustness and rugged-ness (2, 3).

3.1.1.1 Validation parameters

3.1.1.1.1 Specificity

Specificity is the ability of the method to measure the analyte response accurately in the presence of all potential sample components e.g. placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc. (1, 2). The analyte response in test sample is compared with the response of a solution containing only the analyte. Any contribution of the matrix to the analyte response leads to constant or proportional systematic error and such methods are referred as non-specific.

3.1.1.1.2 Linearity and range

The linearity of an analytical method refers to the ability to produce test results that are, either directly proportional to the concentration of analyte in the sample over the entire range of interest (2). Linearity of the method is evaluated by measuring the standard solutions response in the range of 50 to 150% of target concentration (1). Linearity of test results is assessed by calculation of a regression line by the method of least squares (4). Acceptability of linearity data is evaluated from the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient greater than 0.9999 is normally considered as evidence of acceptable fit of the data to the regression line, while the y-intercept should be less than a few percent of the response obtained for the analyte at the target level.

The range of an analytical method is the interval between the upper and lower levels over which acceptable accuracy, linearity, and precision are obtained (1). In practice, the range is determined using data from the linearity and accuracy studies. The range is normally expressed in the same units as the test results (e.g. percentage, parts per million) obtained by the analytical method. The range of the method is validated by confirming that the analytical method provides an acceptable degree of linearity, precision and accuracy when applied to samples containing concentration of analyte at the extremes of the specified range as well as within the range (4).

3.1.1.1.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of results obtained to conventional true value or an accepted reference value. The accuracy of the analytical method is

determined by using four main approaches (2, 3). The accuracy can be determined using a certified reference material and comparing the measured value with true value. A second approach compares the result of the proposed method with the result of a reference/another method whose accuracy and precision is known. Third approach involves a recovery study is performed in which the analyte is spiked by weight or volume into the matrix covering the entire linearity range followed by quantitation. The results are expressed as percent recovery. The fourth approach uses the technique of standard additions, which can also be used to determine recovery of spiked analyte. The accuracy is expressed as percentage bias, it may be helpful to evaluate whether the bias is because of random error alone. This is done by using the t-test to investigate whether the mean value differs significantly from the true value. If the deviation is significant, then the ratio of the deviation between the mean and measured results to the mean result is calculated as the estimate of bias (3).

3.1.1.1.4 Precision

The precision of an analytical method is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision is generally considered at three levels: repeatability, intermediate precision and reproducibility (1-3). The precision of an analytical method is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. The repeatability or intra-assay precision is obtained by repeatedly analyzing independently prepared homogenous samples under the same operating conditions over a short interval of time. Three concentrations at the low, medium and high range of calibration are performed and the % relative standard deviation (RSD) is calculated (3). Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. Intermediate precision is the precision obtained when analysis involves different days, different analysts, and different equipment in the same laboratory. The purpose of the determination of this precision is to detect the various factors within a single laboratory that's responsible for the variability of the results and to find a mechanism to control them (3). Reproducibility expresses the precision between different laboratories. When a method is transferred from one laboratory to another, it invariably encounters: different analysts, different in room temperature and humidity, different instruments, and also variations in the nature and quality of supplies, materials, consumables. Thus reproducibility must be done to answer these

variation. Statistical equivalence and analytical equivalence are used to judge the acceptability of results obtained from different laboratories.

3.1.1.1.5 Limit of detection

Limit of detection (LOD) is the lowest amount of analyte in a sample that can be consistently detected using the method but not necessarily quantified as exact value (2). Various approaches have been suggested to determine the detection limit. Visual evaluation used for non-instrumental methods, where the detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected (2). Other approach involves determination of the signal-to-noise ratio, which is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit (2). Also, standard deviation of the response and the slope is used for LOD determination. The LOD is calculated by using the relation $3.3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. The standard deviation of the response can be obtained by calculating the standard deviation of the y-intercept of the regression line (2).

3.1.1.1.6 Limit of quantitation

The limit of quantitation (LOQ) of an analytical method is the lowest concentration of analyte in sample which can be reliably quantitated with suitable precision and accuracy. The LOQ is determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. (2). Similar to LOD, the LOQ can be determined by different approaches. Visual examination used in case of non-instrumental techniques. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. LOQ is also determined by using relationship $10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. σ is obtained from the standard deviation of response of blanks or by residual standard deviation or regression line or the standard deviation of the intercept (2).

3.1.1.1.7 Robustness

The robustness of an analytical method is its capacity to remain unaffected by small variations in method parameters carried out purposely. The variations involve the change in pH of medium, composition of the medium etc. (2).

3.2 Analytical method for estimation of Netilmicin sulfate

Netilmicin is a semi-synthetic aminoglycoside antibiotic, synthesized by the alkylation of sisomicin (5). It is a wide spectrum antibiotic effective against most of the gram-negative and many gram-positive bacteria that are resistant to gentamicin, sisomicin, and tobramycin and has been found to be less toxic compared to these aminoglycoside antibiotics (6, 7). It is used as an active pharmaceutical substance in several ophthalmic and injectable products.

UV detection of netilmicin is limited by the highly polar basic nature and absence of a suitable chromophore. Netilmicin can be detected using direct UV detection at a low wavelength due to the presence of a double bond in its structure (8, 9). However, sensitivity is low. Derivatization yields higher sensitivity. Several methods includes derivatization of netilmicin have been reported to determine netilmicin in various pharmaceutical formulations and biological samples.

These methods includes Precolumn derivatization Liquid chromatography with dansyl chloride (10), o-phthalaldehyde (OPA) (11, 12), 1-fluoro-2,4-dini-trobenzene (13, 14), 11-fluorenylmethyl chloroformate (15), or OPA-b-mercaptopropionic acid (16), and postcolumn Liquid chromatography derivatization with OPA (17) followed by fluorescence or UV detection. Polarographic and voltametric methods to determine netilmicin have also been reported (18). Direct detection using ion-pair LC combined with MS (19), and pulsed electrochemical detection (PED) (20) have also been described. Due to the high cost and the need of volatile mobile phase constituents related to MS, PED was found to be the method of choice for the detection of aminoglycoside antibiotics (21).

However, these techniques are costly and time consuming. The literature survey revealed that none of the reported methods were suitable for the routine analysis of netilmicin in formulation and in vitro release sample. In general, method of analysis should be simple, rapid and cost effective apart from being sensitive, accurate, and precise. It has been found that there is no simple and precise UV-spectrophotometric method for estimation of netilmicin in bulk drug and formulations. Hence, aim of the present investigation is to develop a simple, sensitive, accurate,

reproducible, economical UV-spectrophotometric method and to validate according to standard guidelines.

3.2.1 Materials

Netilmicin Sulfate was provided as a gift sample by Samarth Life Sciences Pvt. Ltd. Mumbai, India. O-pthalaldehyde (OPA) and Thioglycollic acid were purchased from S. D. Fine chemicals; Mumbai. Methanol, Boric acid, Sodium hydroxide, Potassium dihydrogen orthophosphate, Magnesium chloride, Sodium chloride, Potassium chloride, Sodium phosphate, dibasic Sodium sulphate, Calcium chloride, Sodium acetate, Sodium bicarbonate, and Sodium citrate were purchased from Spectrochem Pvt. Ltd., Mumbai. Distilled water was used for reagents preparation.

3.2.2 Reagents

Potassium dihydrogen orthophosphate (0.2 M): Potassium dihydrogen orthophosphate (27.218 g) dissolved in 1000 ml distilled water (22).

Sodium hydroxide (0.2 M): Sodium hydroxide (8 g) dissolved in 1000 ml distilled water (22).

Phosphate buffer (pH 7.4: 50 ml of Potassium dihydrogen orthophosphate (0.2 M) and 22.4 ml of Sodium hydroxide (0.2 M) mixed together and volume was made up to 200 ml (22).

Sodium hydroxide (1 M): Sodium hydroxide (40 g) dissolved in 1000 ml distilled water (22).

Borate Buffer (pH-10.4): Dissolve 6.20 g of boric acid in 500 ml of water, adjust to pH 10.4 with 1 M sodium hydroxide and dilute with water to 1000 ml (22, 23).

OPA reagent: Weigh 400 grams of OPA and dissolve in 2ml methanol. Add 38ml borate buffer and 0.8ml thioglycollic acid and adjust the pH to 10.4. The solution is then heated at 60ºC for 15 minutes in a water bath. The reagent is now activated and ready to use. It is stable and can be stored in light resistant container for up to 2 days at temperature 2-8 ºC (23, 24).

Table 3.1: Composition of Simulated lung fluid (SLF) (25)

Chemicals	Concentrations (g/L)
Magnesium chloride	0.203
Sodium chloride	6.019
Potassium chloride	0.298
Sodium phosphate, dibasic	0.268
Sodium sulphate	0.071
Calcium chloride	0.368

Sodium acetate	0.952
Sodium bicarbonate	2.604
Sodium citrate	0.097
Purified Water	q.s. to 1 L

3.2.3 Ultraviolet (UV) Spectrophotometric method for Estimation of Netilmicin sulfate in Bulk and Formulation

3.2.3.1 Instrumentation

A double-beam UV–Visible spectrophotometer (Shimadzu 1800, Japan) with automatic wavelength correction and a wavelength accuracy of ±0.3 nm. The instrument is equipped with spectral bandwidth of 1 nm. Matched quartz cells (10 mm) was used for all absorbance measurements.

3.2.3.2 Selection of media

Different media were tried to develop a suitable UV-spectrophotometric method for the analysis of Netilmicin sulfate in formulations. Selection of media was based on sensitivity of the method, ease of sample preparation, cost and applicability of method. Based on applications media selected were phosphate buffer (PB) pH 7.4, Simulated lung fluid (SLF), Acetonitrile: Phosphate buffer (ACN: PB) (pH 7.4) (2:1).

3.2.3.3 Analytical method development for Netimicin sulfate

The analytical method (UV method) was developed for chemical characterization of drugs (Netilmicin Sulfate) in formulations. This method was also employed for determining % drug entrapment and during the stability studies of the formulations. These method will also be used in *in-vitro* release studies.

3.2.3.4 Preparation of Netilmicin Sulfate stock solution

A Stock solution of Netilmicin sulfate was prepared by dissolving 10 mg of Netilmicin sulfate in 10 mL of respective media to get final concentration of 1000 µg/mL.

3.2.3.5 Calibration curve

The absorbance maxima is determined by scanning 30µg/mL solutions of Netilmicin sulfate using UV-visible spectrophotometer. Calibration curve of Netilmicin Sulfate was developed in different solvent system i.e., phosphate buffer (pH 7.4), simulated lung fluid, and acetonitrile: phosphate buffer (pH 7.4) (2:1) after derivatization with O-Phthaladehyde (OPA) reagent. From

the stock solution suitable dilutions were made to obtain solutions of concentrations 10, 20, 30, 40, 50, 60 µg/mL. Briefly various concentrations of drug solution is mixed with 60µL OPA reagent and volume was made up to 2 mL with respective media. The samples are heated in water bath at 60ºC for 15 minutes and allowed to cool. Blank solution was prepared by adding 60 µL of reagent to 2 mL volumetric flask and making up the volume to 2 mL with respective media. Analysis was done by using UV-visible spectrophotometer at 335nm for measuring their absorbance. Further these methods were used for estimation of drugs at various stages of the studies.

3.2.3.6 Analytical method validation

The developed method was validated according to standard guidelines (1). Various validation parameter of the developed method were determined as per the standard guideline.

Specificity and selectivity of the proposed method was established by scanning a solution with drug concentration of 30µg/mL from the pure drug stock solution and drug with common excipients used in formulation. Both samples were scanned from 400 to 250 nm to check the any changes in the absorbance and spectrum of netilmicin sulfate in pure form and with excipients. Drug solution with and without various commonly used excipients (PLGA, Pluronic F68, DSPE-PEG etc.) in formulations were prepared and analyzed for any change in absorbance spectra of netilmicin.

For determining accuracy of proposed method, different quality control (QC) levels of drug concentrations [lower quality control sample (LQC) = 15 µg/mL, medium quality control sample (MQC) = 35 µg/mL and higher quality control sample (HQC) = 55 µg/mL] were prepared from stock solution and analyzed (n=3). Accuracy was assessed by calculating mean percentage recovery and percentage bias (% bias). % Bias was calculated as,

$$\% \text{ Bias} = [(\text{Actual conc.} - \text{Predicted conc.})/\text{Predicted conc.}] \times 100.$$

To provide additional support to accuracy, standard addition method was used. A known concentrations of pure drug (5 µg/mL) were added to a known pre-analyzed formulation sample and analyzed using proposed method (n=3) to check analytical recovery. The percent analytical recovery of the added pure drug was calculated as,

$$\% \text{ Analytical recovery} = [(Cv - Cu)/Ca] \times 100,$$

Where, Cv is the total drug concentration measured after standard addition, Cu is the drug concentration in formulation, and Ca is the drug concentration added to the formulation solution.

Repeatability was determined by analyzing different QC levels of the drug of the drug concentrations (n=9) as mentioned in accuracy. Inter and Intra-day variations was studied to determine intermediate precision of the proposed method. Inter–day variation study was carried out for 3 days (n=9). The percentage relative standard deviation of the predicted concentrations was taken as precision.

The limit of detection (LOD) and limit of quantification (LOQ) of netilmicin sulfate by the proposed method were calculated using standard deviation of intercept and slope of the regression equation.

Robustness of the developed method was determined by varying pH of the mediums.

3.2.3.7 Estimation from formulations

Commercially available injections of netilmicin sulfate (each containing 10 mg of drug) was diluted with suitable medium to prepare a 30 µg/mL concentration (n=3). Final solutions were evaluated by using UV spectrophotometer at 335 nm after derivatization with O-Phthaladehyde (OPA).

3.3 Analytical method for estimation of rhodamine B and 6-coumarin

The Spectrofluorimetric method was employed during preparation and optimization of rhodamine B and 6-coumarin loaded nanoparticles and for estimating distribution of rhodamineb B and 6-coumarin loaded nanoparticles in the pulmonary tissue & CFBE 41o- cells are discussed below. Media utilized to develop a Spectrofluorimetric method was Chloroform: Methanol (1:1) (26, 27).

3.3.1 Materials

Triton X 100, rhodamine B and 6-coumarin were obtained from Sigma, India. Sodium hydroxide, Methanol and Chloroform were purchased from Spectrochem Pvt. Ltd., Mumbai. Distilled water was used for reagents preparation.

3.3.2 Instrumentation

Shimadzu RF-1501 spectrofluorimeter (Japan), equipped with a 150 W xenon lamp and using 1.0- cm quartz cells.

Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) equipped with high Energy Xenon Flash Lamp.

3.3.3 Preparation of stock solutions

A Stock solution of rhodamine B and 6-coumarin (Shimadzu RF-1501 spectrofluorimeter) were prepared by dissolving each 1 mg of Rhodamine B and 6-Coumarin separately in 1 mL of Chloroform: Methanol (1:1) to get concentration of 1000 µg/mL. Which were further diluted to get final concentrations of 1000 pg/mL. Another stock solution (Synergy™ Mx Microplate Reader) was prepared by dissolving each 1 mg of Rhodamine B and 6-Coumarin separately in 1 mL of Chloroform: Methanol (1:1) to get concentrations of 1000 ng/mL.

3.3.4 Calibration curve

Calibration curve of rhodamine B and 6-coumarin was developed in Chloroform: Methanol (1:1). From the stock solution suitable dilutions were made to obtain solutions of concentrations 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000 pg/mL in Chloroform: Methanol (1:1). Blank solution was prepared by using respective media. Analysis was done by using Spectrofluorometer (RF-5301PC, Shimadzu) at excitation and emission wavelengths of 560 and 595 nm, respectively for Rhodamine B, while excitation and emission wavelengths of 458 and 505 nm, respectively for 6-coumarin.

Another calibration curve was obtained by using Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader. The rhodamine B and 6-coumarin stock solution were suitably diluted to obtain solutions of concentrations 20, 40, 60, 80, 100, 120, 140, 160 ng/mL in Chloroform: Methanol (1:1). Blank solution was prepared by using respective media. Analysis was done by using a microplate reader (Synergy MX, BioTek Instruments, Winooski, VT, USA) at excitation and emission wavelengths of 553 and 577 nm, respectively for rhodamine B, while at excitation and emission wavelengths of 430 and 485 nm, respectively for 6-coumarin. Further these methods were used for estimation of rhodamine B and 6-coumarin at various stages of the studies.

3.4 Estimation of plasmid DNA (pDNA)

The initial concentration of isolated plasmid DNA was determined by estimating absorbance at 260 nm in Tris buffer pH 8 using UV Visible Spectrophotometer (Schimadzu, Japan). Absorbance of 1 is considered as equivalent to concentration of 50 µg/mL (28). Along with the DNA, RNA also absorb at 260 nm. In same way, proteins absorb maximally at 280 nm but also show absorbance at 260 nm. The other contaminants such as phenol, chloroform used during DNA purification also interfere with DNA estimation. Therefore purity of the plasmid DNA

estimated by the ratio of absorbance at 260 to 280 nm. When the ratio is in between 1.8 to 2.0, plasmid is considered as pure. DNA is consider as pure. Higher and lower ratio indicates contamination with RNA and proteins respectively (29).

This pure DNA was used for preparation of nanoparticles. Estimation of the entrapped DNA in nanoparticles as well as DNA from release study was determined by using QuantiFluor™ dsDNA System (Promega). Analysis was performed as per protocol provided by Promega. The kit was supplied with 20X TE Buffer (pH 7.5), QuantiFluor® dsDNA Dye, 200X, Lambda DNA Standard, 100µg/ml.

3.4.1 Reagents

1X Tris EDTA (TE) buffer: 1X TE buffer prepared by diluting the 20X TE Buffer 20-fold with nuclease-free water and mixed.

QuantiFluor® dsDNA Dye working solution: It was prepared by diluting the 200X QuantiFluor® dsDNA Dye with 1X TE buffer.

Phosphate buffer saline (pH 7.4): Phosphate buffered Saline pH 7.4 was prepared by dissolving 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 ml.

Acetate buffer pH 5: Acetate Buffer pH 5.0: Dissolve 13.6 g of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1000 ml.

Lambda DNA stock solution: Suitable dilution was made with Phosphate buffer saline (pH 7.4) and Acetate buffer pH 5 to obtain a concentration 10 ng/µL.

3.4.2 Instrument

Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) equipped with high Energy Xenon Flash Lamp.

3.4.3 Calibration curve

A calibration curve was prepared by using the Lambda DNA Standard. The working standards were prepared by adding stock solution to the 96 multiwell plate containing 1X TE buffer, Phosphate buffer saline (pH 7.4) and Acetate buffer pH 5 buffer followed by addition of the 100 µL of 1X QuantiFluor® dsDNA Dye solution to obtain concentration of 10, 20, 30, 40, 50, 60, 70 ng/mL. Blank sample was prepared by using 1X TE buffer, Phosphate buffer saline (pH 7.4) and Acetate buffer pH 5 buffer. Further samples were incubated for 5 minutes at room temperature, protected from light. Analysis was done by using a microplate reader (Synergy MX,

BioTek Instruments, Winooski, VT, USA) at excitation and emission wavelengths of 504 and 531 nm, respectively.

3.5 Protein Assay

Pierce BCA Protein Assay Kit, Thermo Scientific was used to evaluate protein samples in cell lysate.

3.5.1 Reagents

BCA Reagent A: containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA Reagent B: containing 4% cupric sulfate

Albumin Standard: containing bovine serum albumin (BSA) 2mg/mL in 0.9% saline and 0.05% sodium azide.

BCA Working Reagent (WR): WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B).

3.5.2 Procedure

Standard solutions were made from the Albumin Standard stock solutions by dilution 10, 20, 30, 40, 50, 60 µL to 100 µL with lysis buffer followed by dilution with WR to 2 mL to obtain 10, 20, 30, 40, 50, 60 µg/mL. Blank solution were prepared in similar way without standard solution. The obtained solutions were mixed properly and incubated at 37 ^{o}C for 30 min. Samples were cooled to room temperature and absorbance was measured at 562 nm using UV-Visible spectrophotometer.

3.6 Results and discussion

3.6.1 Analytical method of estimation for netilmicin sulfate

Different media like Acetonitrile: Phosphate buffer pH 7.4 (2:1), Phosphate buffer (pH 7.4), Simulated Lung Fluid were evaluated for analysis of the netilmicin sulfate. The media were selected based on the sensitivity of the method, cost of solvents, ease of preparation and applicability of the method. Figure 3.1 shows the spectrums of Netilmicin sulfate in Acetonitrile:Phosphate buffer pH 7.4 (2:1), Phosphate buffer (pH 7.4), Simulated Lung Fluid (SLF) respectively. The λmax of netilmicin sulfate in these media was found to be 335 nm. Figure 3.2 shows the **c**alibration curve of Netilmicin sulfate in Acetonitrile: Phosphate buffer pH 7.4 (2:1), Phosphate buffer (pH 7.4), Simulated Lung Fluid at 335 nm. Apparent molar absorptivity of the netilmicin sulfate in Acetonitrile: Phosphate buffer pH 7.4 (2:1), Phosphate

buffer (pH 7.4), Simulated Lung Fluid (SLF) was found to be 3.43 x10^4, 2.69 x10^4, 2.86 x10^4 mol^{-1} cm^{-1}, respectively. Sandell's index represents the number of micro- or nanograms of the determinant per mL of a solution having an absorbance of 0.001 for the cell path length of 1 cm and is a suitable parameter for expressing and comparing the sensitivities of developed UV-spectrophotometric methods (30). Sandell's sensitivity coefficient of netilmicin sulfate was found to be 0.042, 0.053 and 0.049 µg/cm^2 in Acetonitrile: Phosphate buffer pH 7.4 (2:1), Phosphate buffer (pH 7.4), Simulated Lung Fluid (SLF) media, respectively (Table 3.3)

3.6.1.1 Calibration curve

For determining the encapsulation efficiency of netilmicin sulfate in nanoparticles calibration curve was prepared in Acetonitrile: Phosphate buffer (pH 7.4) (2:1). Absorbance values for different concentrations are shown Table 3.2. At all concentration levels the SD was low and the % RSD did not exceed 1.91. Linearity range was found to be 10-60 µg/ml (Figure 3.2). The linear regression equation in the Acetonitrile: Phosphate buffer (pH 7.4) (2:1) medium, at 335 nm was found to be {Absorbance= [0.0233× concentration (µg/ ml)] - 0.0152}; with a regression coefficient (R^2) of 0.9998.

For estimation of free drug and for determining the % drug release study, calibration curve was formed in Phosphate buffer (pH 7.4) and SLF. Absorbance values for different concentrations are shown in Table 3.2. Linearity range was found to be 10-60 µg/ml (Figure 3.2). The linear regression equation in the phosphate buffer (pH 7.4) medium, at 335 nm was found to be {Absorbance= [0.0196× concentration (µg/ ml)] - 0.0225}; with a regression coefficient (R^2) of 0.9994. The linear regression equation in the SLF, at 335 nm was found to be {Absorbance= [0.0201× concentration (µg/ ml)] - 0.003}; with a regression coefficient (R^2) of 0.9995.

3.6.1.2 Analytical method validation of netilmicin sulfate

The linearity range for netilmicin sulfate estimation was found to be 10-60 µg/mL (r^2 = 0.9994) in Phosphate buffer (PB) (pH 7.4), 10-60 µg/mL (r^2 = 0.9995) in Simulated Lung Fluid (SLF) and 10-60 µg/mL (r^2 = 0.9998) in Acetonitrile: Phosphate buffer (ACN:PB) (pH 7.4) (2:1) (Table 1). Lower values of parameters like standard error (S.E.) of slope and intercept (Table 3.1) indicated high precision of the proposed methods. Also, the mean slope and intercept values are within the 95% confidence interval. Goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated F-values (Table 3.3).

Absorption spectrum of pure drug sample was matching with the drug with excipients sample in all the selected media (Figure 3.3, 3.4, 3.5). The calculated t-values were found to be less than that of the critical t-value, indicating that statistically there was no significant difference between mean absorbance of solutions prepared from pure drug sample and drug with common excipients (Table 3.3). This indicates the selectivity and specificity of the proposed methods.

All three QC level showed an accuracy (% bias) ranging from -0.39 to 0.24 for Acetonitrile: Phosphate buffer pH 7.4 (2:1), -0.58 to 0.69 for Phosphate buffer (pH 7.4), -0.36 to 0.37 for Simulated Lung Fluid. The mean percent recovery value and their low SD values represent the accuracy of method (Table 3.4). Further validity and reliability of the methods was assessed by standard addition method (Table 3.5). In Acetonitrile: Phosphate buffer pH 7.4 (2:1) mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 100.57 (± 1.67), 102.45 (± 0.98), 99.00 (± 2.26) respectively. In Phosphate buffer (pH 7.4) mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 101.43 (± 3.24), 101.02 (± 1.43), 100.99 (± 2.35) respectively. In Simulated Lung Fluid mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 100.23 (± 2.50), 100.26 (± 1.73), 100.29 (± 1.22) respectively.

Precision of methods was determined by studying repeatability and inter-mediate precision. Repeatability of the methods was determined through % RSD value. It was ranging from 0.73 to 1.86, 1.10 to 2.23, and 0.98 to 1.65 in the Phosphate buffer (pH 7.4), Simulated Lung Fluid, Acetonitrile: Phosphate buffer pH 7.4 (2:1) medium, respectively (Table 3.4). Repeatability results indicates the precision of methods under the same operating conditions over a short period of time and inter-assay precision. Intermediate precision study depicts intra- and inter-day repeatability within laboratory. % RSD values were found low for intermediate precision, with the intra-day variation % RSD value was less than 1.82% and % RSD value of inter-day variation was less than 1.96% (Table 3.6). Lower % RSD values indicating the repeatability and intermediate precision of the method.

In Acetonitrile: Phosphate buffer pH 7.4 (2:1) Limit of detection (LOD) and Limit of quantification (LOQ) were found to be 0.9543 µg/ml & 2.8919 µg/ml, respectively. In Phosphate buffer (pH 7.4) Limit of detection (LOD) and Limit of quantification (LOQ) were found to be 1.022 µg/ml & 3.098 µg/ml respectively. While, in Simulated Lung Fluid Limit of detection

(LOD) and Limit of quantification (LOQ) were found to be 1.022 µg/ml & 3.098 µg/ml respectively.

The methods were found to be robust as variation of pH of phosphate by ± 0.2 units for Phosphate buffer (pH 7.4) and simulated Lung Fluid; while in case of Acetonitrile: Phosphate buffer pH 7.4 ratio of solvents was changed by 0.25 unit. Variation in these media did not have any significant effect on absorbance. The mean % recovery (± S.D.) were found to be 99.68 (± 0.76), 100.09 (± 0.29) and 99.98 (± 0.51) in the Phosphate buffer (pH 7.4), Simulated Lung Fluid, Acetonitrile: Phosphate buffer pH 7.4 medium, respectively (Table 3.3).

The assay values of Netilmicin sulfate injection were 100.83 ± 1.18, 100.75 ± 1.13, 103.03 ± 2.54 in the Phosphate buffer (pH 7.4), Simulated Lung Fluid, Acetonitrile: Phosphate buffer pH 7.4 medium, respectively (Table 3.7). The Student's t-values did not exceed the tabulated values indicating that no significant difference between assay values of formulations and the mentioned label claim value. This showed that the interference of excipient matrix is insignificant in estimation Netilmicin sulfate by proposed methods. The estimated drug content with low values of standard deviation indicates the precision of the proposed methods.

Table 3.2: Calibration data for estimation of Netilmicin sulfate at 335nm

Conc.	PB pH 7.4		SLF		ACN:PB pH 7.4	
(µg/ml)	Mean Abs[a] (± SD)	% RSD [b]	Mean Abs[a] (± SD)	% RSD [b]	Mean Abs [a] (± SD)	% RSD [b]
10	0.1713±0.0040	2.04	0.2144±0.0040	1.87	0.2436 ± 0.0040	1.65
20	0.3630±0.0076	1.83	0.4007±0.0047	0.82	0.4796 ± 0.0035	0.73
30	0.5673±0.0100	1.76	0.6030±0.0056	0.25	0.7216 ± 0.0030	0.42
40	0.7720±0.0065	0.84	0.8046±0.0040	0.08	0.9570 ± 0.0040	0.41
50	0.9660±0.0036	0.37	1.0011±0.0079	0.79	1.1703± 0.0328	1.91
60	1.1403±0.0177	1.55	1.2236±0.0227	1.85	1.4113 ± 0.0269	1.90

[a] Each value is mean of three independent determinations

[b] Percentage relative standard deviation

Table 3.3: Regression analysis parameter for estimation of Netilmicin sulfate at 335nm

Statistical parameter	**PB pH 7.4**	**SLF**	**ACN:PB pH 7.4**
Linearity (µg/ml)	10-60 µg/ml	10-60 µg/ml	10-60 µg/ml
Regression coefficient (R^2)	0.9994	0.9995	0.9998
Regression Equation	Absorbance = [0.0196× conc.(µg/ ml)] - 0.0225	Absorbance = [0.0201× conc.(µg/ ml)] - 0.003	Absorbance = [0.0233× conc.(µg/ ml)] - 0.0152
Confidence Interval for slope [a]	$1.876 \times 10^{-2} – 2.043 \times 10^{-2}$	$1.897 \times 10^{-2} – 2.131 \times 10^{-2}$	$2.121 \times 10^{-2} – 2.544 \times 10^{-2}$
Confidence Interval for intercept [a]	$- 4.884 \times 10^{-2} – 3.387 \times 10^{-3}$	$- 3.445 \times 10^{-2} – 4.0508 \times 10^{-3}$	$- 1.386 \times 10^{-2} – 4.416 \times 10^{-3}$
Standard deviation of intercept	0.607×10^{-2}	0.503×10^{-2}	0.6746×10^{-2}
Standard deviation of slope	1.94×10^{-4}	2.72×10^{-4}	4.921×10^{-4}
t- value for intercept [a] (tab =2.77)	2.42 (p-value 0.072)	2.42 (p-value 0.072)	2.42 (p-value 0.072)
F-value(tab)[b]	2.29×10^{-4} (3.68)[b]	8.03×10^{-4} (3.68)[b]	3.53×10^{-3} (3.68)[b]
Standard error of estimate	1.008×10^{-2}	1.000×10^{-2}	0.6717×10^{-2}
Limit of detection	1.022 µg/ml	1.428 µg/ml	0.9543 µg/ml
Limit of quantification	3.098 µg/ml	4.327 µg/ml	2.8919 µg/ml
System suitability	Molar absorptivity (1 mol^{-1} cm^{-1}) = 2.69×10^4 Specific absorptivity (ml/µg.cm) = 1.88×10^{-2} Sandell's Sensitivity (µg /cm^2) = 0.053	Molar absorptivity (1 mol^{-1} cm^{-1}) = 2.86×10^4 Specific absorptivity (ml/µg.cm) = 2.02×10^{-2} Sandell's Sensitivity (µg /cm^2) = 0.049	Molar absorptivity (1 mol^{-1} cm^{-1}) = 3.43×10^4 Specific absorptivity (ml/µg.cm) = 2.38×10^{-2} Sandell's Sensitivity (µg /cm^2) = 0.042
Selectivity t_{Cal} (t_{Crit})[a]	0.1697(4.3)	0.0348(4.3)	1.1061(4.3)
Robustness [c] (mean % recovery ± S.D.)	99.68±0.76%	100.09±0.29%	99.98±0.51%

[a] Calculated at 0.05 level of significance

[b] Theoretical value of *F* based on one-way ANOVA test at *P* = 0.05 level of significance.

[c] Performed by varying pH 6.8±0.1

Table 3.4: Accuracy data for Netilmicin sulfate

Medium	Level	Predicted Concentrations [a] (µg/ml)			Mean % Recovery [b] (±SD)	% Bias [c]
		Range	Mean [b] (±SD)	% RSD		
PB pH 7.4	LQC	14.83-15.39	15.10±0.28	1.86	100.69 ± 2.75	- 0.69
	MQC	34.52-34.83	34.79±0.25	0.73	99.42 ± 1.02	0.58
	HQO	54.11-56.11	54.84±1.18	2.15	99.72 ± 3.78	0.28
SLF	LQC	14.67-15.31	15.05±0.33	2.23	100.37 ± 2.77	- 0.37
	MQC	34.44-35.19	34.87±0.38	1.10	99.63 ± 1.43	0.36
	HQO	54.07-55.96	54.94±0.95	1.73	99.89 ± 2.53	0.10
ACN:PB	LQC	14.75-15.14	14.94±0.19	1.29	99.60 ± 1.99	0.39
pH 7.4	MQC	34.74-35.14	35.08±0.34	0.98	100.24 ± 1.24	- 0.24
	HQO	54.04-55.82	54.82±0.90	1.65	99.73 ± 1.65	0.26

[a] Predicted concentration is calculated from regression equation

[b] Each value is mean of three independent determinations

[c] Accuracy is given in % Bias

Table 3.5: Standard addition method for accuracy (n = 9)

Method	Drug in formulation (µg/mL)	Pure drug added (µg/mL)	Total drug found (µg/mL)	% Analytical recovery (±S.D.)
ACN:PB pH 7.4 (2:1)	14.92	5	20.23±0.33	101.57±1.67
	35.14	5	41.12±0.39	102.45±0.98
	54.85	5	59.25±1.35	99.00±2.26
PB pH 7.4	14.92	5	20.20±0.64	101.43±3.24
	35.14	5	40.55±0.57	101.02±1.43
	54.85	5	60.44±1.40	100.99±2.35
SLF	14.92	5	19.96±0.49	100.23±2.50
	35.14	5	40.24±0.69	100.26±1.73
	54.85	5	60.02±0.73	100.29±1.22

Table 3.6: Result of intermediate precision study

Medium	Level	Intra-day repeatability (% RSD) (n=9)	Inter-day repeatability (%RSD) (n=9)
PB pH 7.4	LQC	1.54	1.84
	MQC	1.51	0.85
	HQO	1.70	1.63
SLF	LQC	1.82	1.96
	MQC	0.99	1.06
	HQO	1.40	1.64
ACN:PB	LQC	1.53	1.20
	MQC	0.66	0.88
	HQO	1.26	1.13

Table 3.7: Determination of Netilmicin sulfate in marketed formulation

Commercial product	**Phosphate buffer (pH 7.4)**		**Simulated Lung Fluid**		**Acetonitrile: Phosphate buffer pH 7.4 (2:1)**	
	Amount found (mg) (±SD)	**% Assay (±SD)**	**Amount found (mg) (±SD)**	**% Assay (±SD)**	**Amount found (mg) (±SD)**	**% Assay (±SD)**
Netspan injection (10 mg)	10.01±0.11	100.83±1.18	10.09±0.11	100.75±1.13	10.12±0.25	103.03±2.54
t_{Cal} (t_{Crit})[a]	0.18 (4.3)		1.23 (4.3)		0.70 (4.3)	

[a] Calculated at 0.05 level of significance

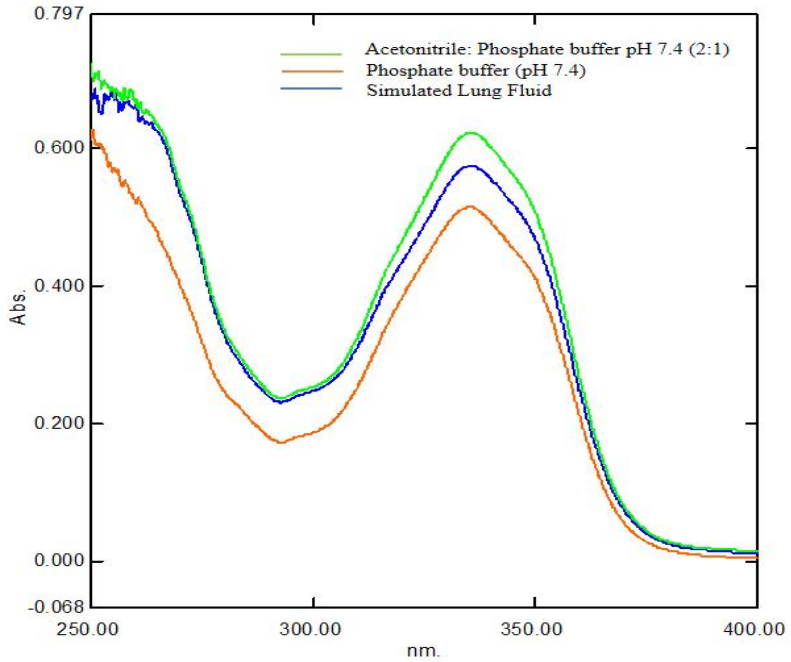

Figure 3.1: UV Absorption Spectrum of Netilmicin sulfate in Acetonitrile:Phosphate buffer pH 7.4 (2:1), phosphate buffer (pH 7.4), Simulated lung fluid.

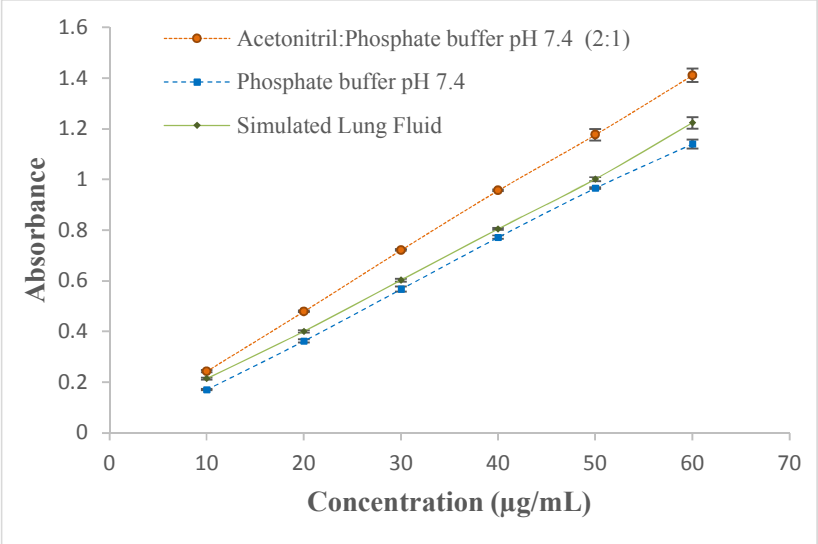

Figure 3.2: Calibration curve of Netilmicin sulfate in Acetonitrile:Phosphate buffer pH 7.4 (2:1), Phosphate buffer (pH 7.4), Simulated Lung Fluid at 335 nm

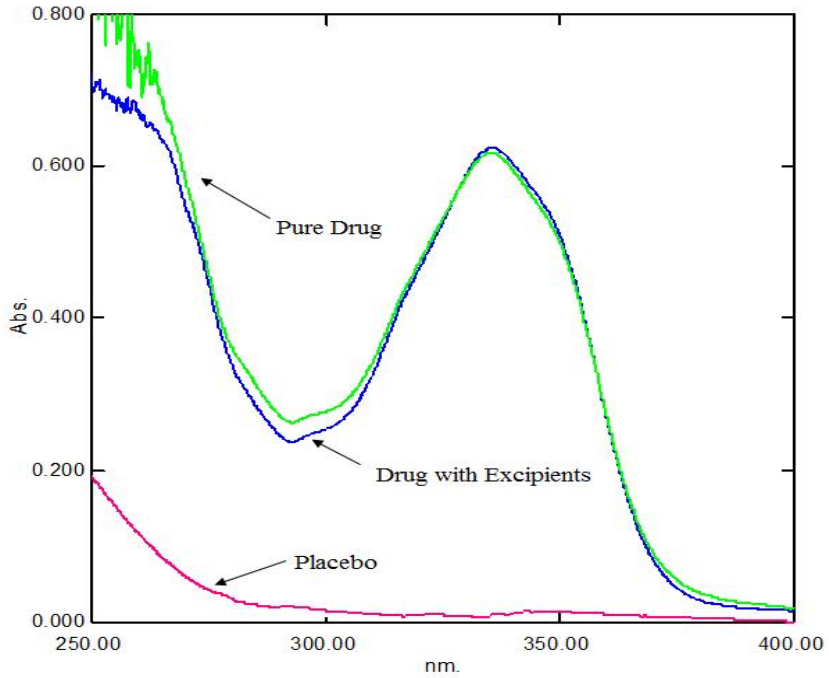

Figure 3.3: Overlaid spectra of pure drug and drug spectrum in presence of common excipients used in formulations in Acetonitrile:Phosphate buffer pH 7.4 (2:1)

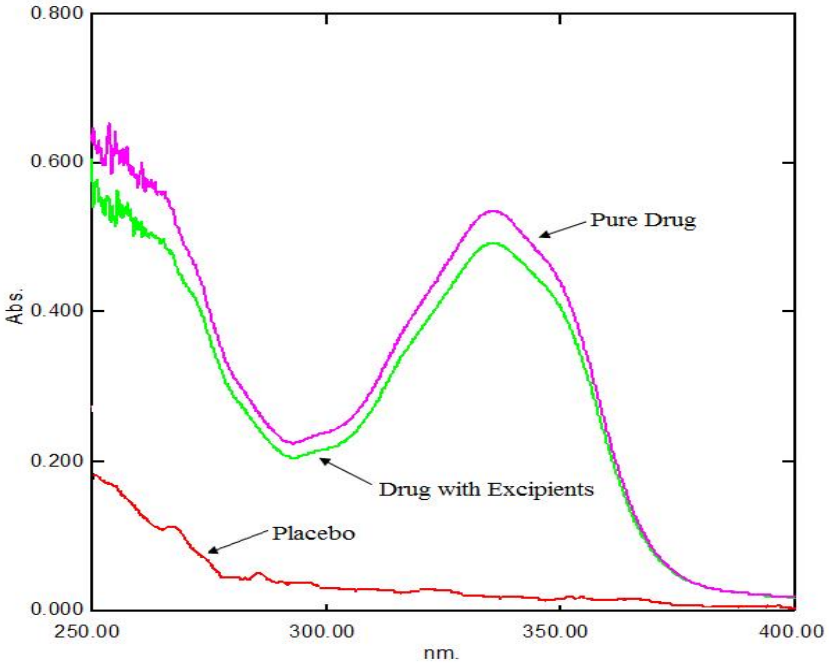

Figure 3.4: Overlaid spectra of pure drug and drug spectrum in presence of common excipients used in formulations in Phosphate Buffer pH 7.4

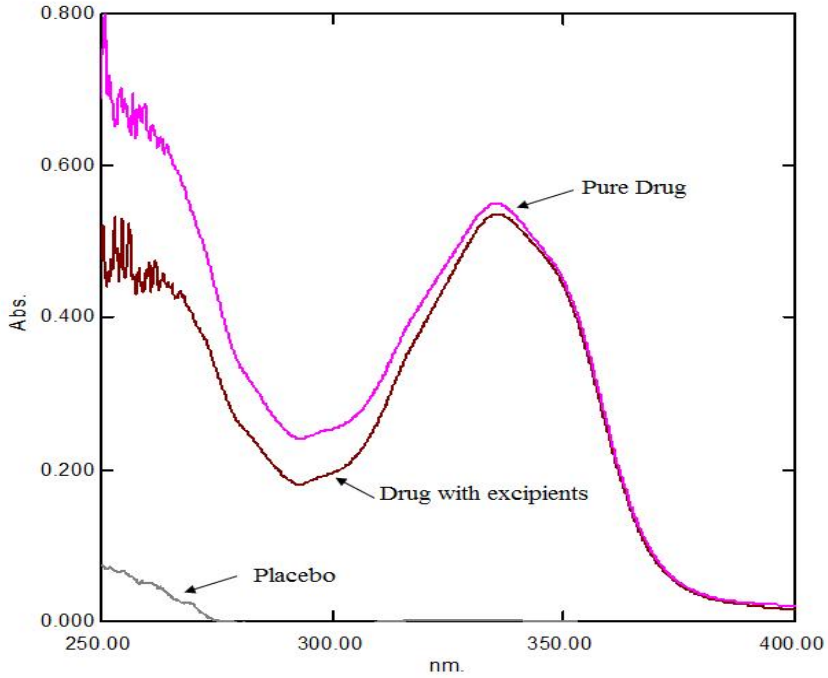

Figure 3.5: Overlaid spectra of pure drug and drug spectrum in presence of common excipients used in formulations in Simulated Lung Fluid

3.6.2 Analytical method for estimation of rhodamine B and 6-coumarin

The Spectrofluorimetric method was employed to estimate rhodamine B and 6-coumarin in nanoparticles and for estimating distribution of rhodamine B and 6-coumarin loaded nanoparticles in the pulmonary tissue & CFBE 41o- cells) (26, 27, 31). Calibration curve was developed in Chloroform:Methanol (1:1). Fluorescence intensity values obtained with Spectrofluorometer (RF-5301PC, Shimadzu) for different concentrations are shown in Table 3.8. At all concentration levels the SD was low and the % RSD did not exceed 7.28 for rhodamine B while that for 6-coumarin the % RSD did not exceed 8.69. Linearity range for rhodamine B was found to be 1000-8000 pg/mL (Figure 3.6) while, the linear regression equation was found to be {Fluorescence Intensity = [0.0703× conc. (pg/mL)] – 376.55}; with a regression coefficient (R^2) of 0.9981. Linearity range for 6-coumarin was found to be 1000-8000 pg/mL (Figure 3.6) while, the linear regression equation was found to be {Fluorescence Intensity = [0.0732× conc. (pg/mL)] – 25.17}; with a regression coefficient (R^2) of 0.9987.

Table 3.8: Calibration data for Rhodamine B and Coumarin 6 in Chloroform:Methanol (1:1) obtained with Spectrofluorometer (RF-5301PC, Shimadzu)

Conc. (pg/mL)	Rhodamine B		Coumarin 6	
	Mean Fluorescence intensity[a] (± SD)	% RSD [b]	Mean Fluorescence intensity[a] (± SD)	% RSD [b]
1000	436.74 ± 31.82	7.28	109.05 ± 9.48	8.69
2000	521.04 ± 19.25	3.69	170.92 ± 8.66	5.06
3000	591.49 ± 28.63	4.84	238.33 ± 18.39	7.71
4000	666.02 ± 20.78	3.12	308.33 ± 24.64	7.98
5000	731.12 ± 23.88	3.26	391.62 ± 13.76	3.51
6000	794.82 ± 16.67	2.09	461.32 ± 46.25	5.45
7000	856.80 ± 37.88	4.42	541.02 ± 36.53	6.75
8000	943.54 ± 31.87	3.37	615.61 ± 32.14	5.22
Linearity (pg/ml)	1000-8000 pg/mL		1000-8000 pg/mL	
Regression coefficient (R^2)	0.9981		0.9987	
Regression Equation	Fluorescence Intensity = [0.0703× conc.(pg/mL)] – 376.55		Fluorescence Intensity = [0.0732× conc.(pg/mL)] – 25.17	

[a] Each value is mean of three independent determinations

[b] Percentage relative standard deviation

Fluorescence intensity values obtained with Synergy™ Mx Microplate Reader for different concentrations are shown in Table 3.9. At all concentration levels the SD was low and the % RSD did not exceed 5.91 for rhodamine B while that for 6-coumarin the % RSD did not exceed 4.56. Linearity range for rhodamine B was found to be 20-160 ng/mL (Figure 3.6) while, the linear regression equation was found to be {Fluorescence Intensity = [248.52× conc. (ng/mL)] + 11906}; with a regression coefficient (R^2) of 0.9969. Linearity range for 6-coumarin was found to be 20-160 ng/mL (Figure 3.6) while, the linear regression equation was found to be {Fluorescence Intensity = [9584× conc. (ng/mL)] + 2404.2}; with a regression coefficient (R^2) of 0.9985.

Table 3.9: Calibration data for Rhodamine B and 6-Coumarin in Chloroform:Methanol (1:1) obtained with Synergy™ Mx Microplate Reader

Conc. (ng/mL)	Rhodamine B		6-Coumarin	
	Mean Fluorescence intensity [a] (± SD)	% RSD [b]	Mean Fluorescence intensity [a] (± SD)	% RSD [b]
20	17293.66 ± 1022.31	5.91	11260.20 ± 205.85	1.82
40	20765.00 ± 948.98	4.57	21730.40 ± 782.43	3.60
60	26643.00 ± 1346.26	5.05	32545.20 ± 1487.30	4.56
80	32923.66 ± 1213.27	3.68	41278.00 ± 1667.26	4.03
100	37196.00 ± 1470.84	3.95	49665.00 ± 1230.12	2.47
120	41137.00 ± 1937.84	4.71	59791.40 ± 2043.51	3.41
140	46453.33 ± 1605.31	3.45	68386.20 ± 1331.12	1.94
160	51767.66 ± 1802.35	3.48	78031.40 ± 2089.32	2.67
Linearity (ng/ml)	20-160 ng/mL		20-160 ng/mL	
Regression coefficient (R^2)	0.9969		0.9985	
Regression Equation	Fluorescence Intensity = [248.52× conc.(ng/mL)] + 11906		Fluorescence Intensity = [9584× conc.(ng/mL)] + 2404.2	

[a] Each value is mean of three independent determinations

[b] Percentage relative standard deviation

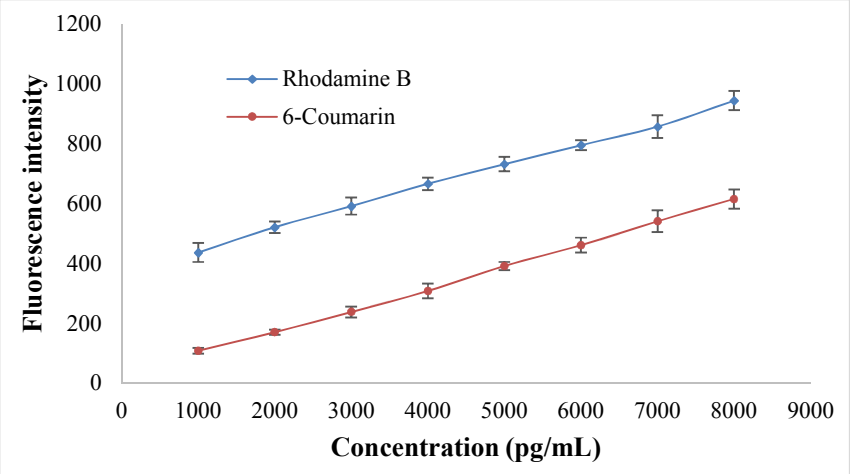

Figure 3.6: Calibration curve of Rhodamine B and 6-Coumarin in in Chloroform: Methanol (1:1) obtained with Spectrofluorometer (RF-5301PC, Shimadzu)

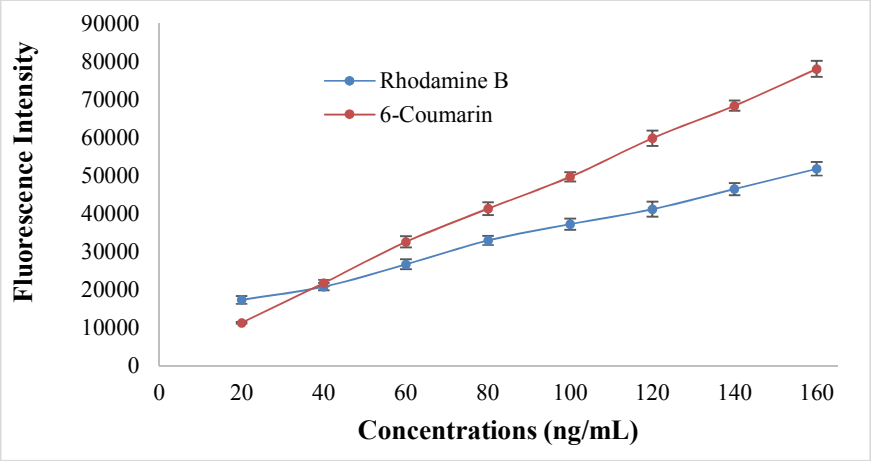

Figure 3.7: Calibration curve of Rhodamine B and 6-Coumarin in in Chloroform: Methanol (1:1) obtained with Synergy™ Mx Microplate Reader

3.6.3 Estimation of plasmid DNA (pDNA)

Isolation of plasmid from cell lysate needs a special attention as along with plasmid DNA, RNA and cell proteins are come as impurity. Phenol and chloroform are used for the purification of the plasmid DNA. Phenol also show absorbance at 260 nm, this may results in the wrong interpretation of DNA concentration. Thus to estimate accurate DNA the QuantiFluor® dsDNA System, Promega was used. The QuantiFluor® dsDNA System contains a fluorescent double-stranded DNA-binding dye (504nmEx/531nmEm) that enables sensitive quantitation of small amounts of double-stranded DNA (dsDNA) by using fluorescence plate reader. Based on the application difference media was used for development of calibration curve of the plasmid DNA. To estimate the entrapped DNA in NPs 1X TE buffer was used while to estimation of released DNA from the NPs PBS pH 7.4, and Acetate buffer pH 5 were used. (31)(28)Fluorescence intensity values for different concentrations are shown in Table 3.10. At all concentration levels the SD was low and the % RSD did not exceed 7.59 for 1X TE buffer, 8.82 for PBS pH 7.4 and 6.15 for Acetate buffer pH 5. Linearity range in all media was found to be 10-70 ng/mL (Figure 3.8) while, the linear regression equation was found to be {Fluorescence Intensity = [272.5 × conc. (ng/mL)] + 2477} with a regression coefficient (R^2) of 0.9997; {Fluorescence Intensity = [294.1× conc.(ng/mL)] + 834.02} with a regression coefficient (R^2) of 0.9994, {Fluorescence

Intensity = [279.79 × conc.(ng/mL)] + 431.5} with a regression coefficient (R^2) of 0.9992 in 1X TE buffer, PBS pH 7.4, and Acetate buffer pH 5 respectively.

Table 3.10: Calibration data for plasmid DNA in 1X TE buffer, PBS pH 7.4, and Acetate buffer pH 5

Conc. (ng/mL)	**1X TE buffer**		**PBS pH 7.4**		**Acetate buffer pH 5**	
	Mean Fluorescence intensity [a] (± SD)	**% RSD [b]**	**Mean Fluorescence intensity [a] (± SD)**	**% RSD [b]**	**Mean Fluorescence intensity [a] (± SD)**	**% RSD [b]**
10	5291.33 ± 402.09	7.59	3981.66 ± 351.18	8.82	3381.66 ± 208.16	6.15
20	7717.00 ± 229.05	2.96	6531.00 ± 562.04	8.60	5826.66 ± 322.43	5.53
30	10751.33 ± 735.99	6.84	9688.83 ± 537.64	5.54	8803.16 ± 287.33	3.26
40	13418.67 ± 384.46	2.86	12566.33 ± 568.62	4.52	11499.67 ± 450.92	3.92
50	16102.33 ± 881.81	2.26	15305.50 ± 926.94	7.36	14605.50 ± 360.55	2.46
60	18820.33 ± 773.89	4.68	18599.17 ± 577.35	3.10	17399.17 ± 503.32	2.89
70	21539.00 ± 773.89	3.59	21513.17 ± 750.55	3.48	19846.50 ± 854.40	4.30
Linearity (ng/ml)	10-70 pg/mL		10-70 ng/mL		10-70 ng/mL	
Regression coefficient (R^2)	0.9997		0.9994		0.9992	
Regression Equation	Fluorescence Intensity = [272.5 × conc.(ng/mL)] + 2477		Fluorescence Intensity = [294.1× conc.(ng/mL)] + 834.02		Fluorescence Intensity = [279.79 × conc.(ng/mL)] + 431.5	

[a] Each value is mean of three independent determinations

[b] Percentage relative standard deviation

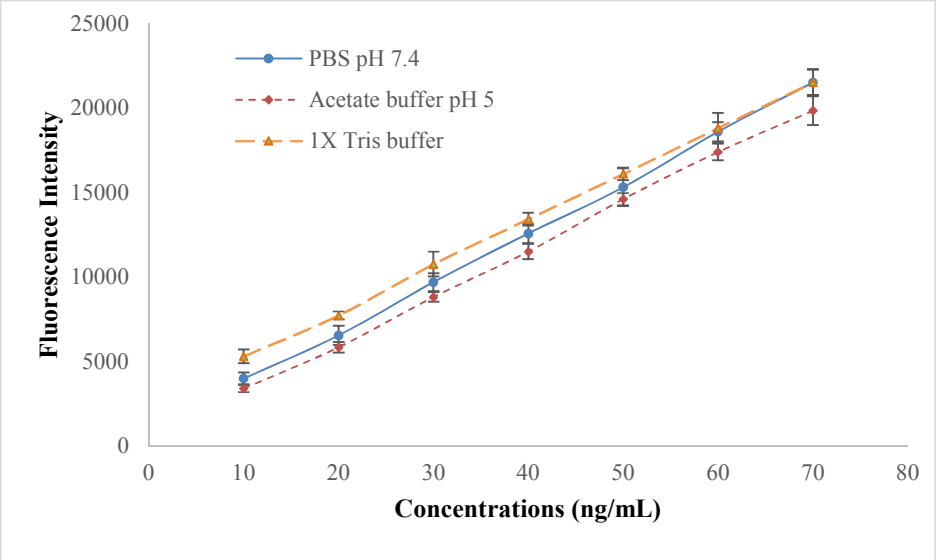

Figure 3.8: Calibration curve for plasmid DNA in 1X TE buffer, PBS pH 7.4, and Acetate buffer pH 5

3.6.4 Protein Assay

To estimate the proteins in cell lysate samples Pierce BCA Protein Assay Kit, Thermo Scientific was used. Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm. The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA. Studies with di-, tri- and tetrapeptides suggest that the extent of color formation is caused by more than the mere sum of individual color-producing functional groups. Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the BSA standard and assayed to develop a standard curve. Absorbance values for different concentrations are shown Table 3.11. At all concentration levels the SD was low

and the % RSD did not exceed 2.67. Linearity range was found to be 10-60 µg/ml (Figure 3.9). The linear regression equation was found to be {[0.0079 × conc. (µg/mL)] + 0.0665}; with a regression coefficient (R^2) of 0.9997.

Table 3.11: Calibration data for BSA

Conc. (ng/mL)	**1X TE buffer**	
	Mean Absorbance[a] (± SD)	**% RSD [b]**
10	0.143 + 0.003	2.09
20	0.224 + 0.006	2.67
30	0.304 + 0.006	2.23
40	0.379 + 0.004	1.18
50	0.463 + 0.015	3.23
60	0.534 + 0.006	1.12
Linearity (ng/ml)	10-60 ng/mL	
Regression coefficient (R^2)	0.9997	
Regression Equation	Absorbance = [0.0079 × conc.(µg/mL)] + 0.0665	

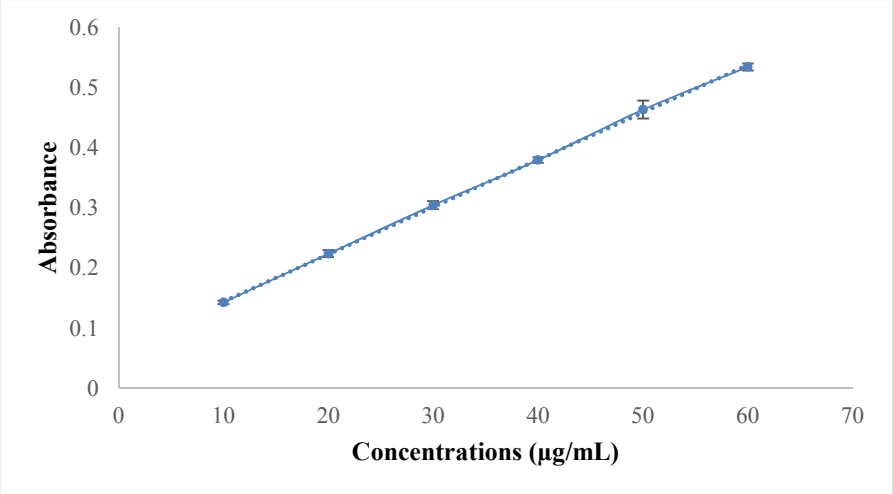

Figure 3.9: Calibration curve for BSA

3.7 Conclusion

The results and the statistical parameters demonstrate that the proposed methods for estimation of netilmicin sulfate were simple, rapid, specific, accurate, precise and inexpensive. Thus, these methods can be used for the determination of netilmicin sulfate either in bulk or in the dosage formulations without interference with commonly used excipients and related substances. The method for estimation of pDNA was also found simple, rapid, accurate, and precise. The methods for estimation of rhodamine B and 6-coumarin were also shown good linearity, accuracy and precision. So these methods can be used for further studies.

3.8 References

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