Chapter-4

Materials and Methods

4. MATERIALS & METHODS

4.1. Chemicals and Reagents

All chemicals and reagents were of analytical grade and chemicals required for sensitive analytical works were obtained from S.D Fine chem. Ltd, HPLC grade and Millipore Mumbai.

4.2. Instrumentation

Shimadzu LC-20AT liquid pump, Instrument Mode SPD -20A Serial Number L20134301264LP, UV-Visible detector, Luna 5µ C_{18} RP-HPLC column (250 x 4.6mm, 5 µm, ID); 25 µL Hamilton injecting syringe Spinchrom software were used. Sartorius (CP225D) electronic balance was used for weighing the materials.

4.3. Chromatographic system and conditions

Shimadzu LC-20AT liquid pump, SPD-20A UV-Visible detector, a Luna 5u C_{18} RP-HPLC column (250 x 4.60mm 5 µm, ID), 25 µL Hamiltonâ injecting syringe and spinchrom software were used. Sartorius (CP225D) electronic balance was used for weighing the materials. Pure samples are Lamivudine Zidudine and Nevirapine, HPLC grade Methanol (S.D Fine chem. Ltd, HPLC grad), HPLC grade Millipore water (Direct Q3, Millipore) was used for preparing the mobile phase in the ratio of 89:11 (v/v).

4.4. Preparation of standard stock solutions

Accurately weighed 100 mg of Lamivudine, Zidovudine and Nevirapine standard were taken in separate 100 mL volumetric flask and dissolved with 70 mL of methanol in each volumetric flask and sonicated for 30 mins, and mobile phase was prepared by mixing of HPLC grade Methanol and Water in the ratio of 89:11(v/v). Mobile phase was simultaneously degassed and filtered through 0.45 µm membrane filter in a Millipore filtration assembly. Degassing was achieved by vacuum pump attached to the unit. Then diluted with the mobile phase to get 1 mg/mL standard stock solution and filtered through 0.45 µm filter.

4.4.1. Linearity for Lamivudine

Several aliquots of standard stock solutions 0.05 mL, 0.10 mL, 0.15 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.8 mL and 1.0 mL of were taken in different 10 mL volumetric flask and diluted up to the mark with methanol. Evaluation was performed with Ultra-Violet detector for Lamivudine, at 272nm. HPLC was operated and the prepared mobile phase was run through the column to equilibrate it. Hamilton syringe was thoroughly cleaned with HPLC grade methanol. When a stable baseline was obtained, 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Plots of concentration vs. area were prepared.

4.4.2. Linearity for Zidovudine

Several aliquots of standard stock solutions 0.25 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL and 3.5 mL of Zidovudine were taken in different 10 mL volumetric flask and diluted up to the mark with methanol. Evaluation was performed with Ultra-Violet detector for Zidovudine 266nm. HPLC was operated and the prepared mobile phase was run through the column to equilibrate it. Hamilton syringe was thoroughly cleaned with HPLC grade methanol. When a stable baseline was obtained, 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Plots of concentration vs. area were prepared.

4.4.3. Linearity for Nevirapine

Several aliquots of standard stock solutions 0.25 mL, 0.5 mL, 0.75 mL, 1.0 mL, 1.25 mL, 1.5 mL, 1.75 mL and 2.0 mL of Nevirapine were taken in different 10 mL volumetric flask and diluted up to the mark with methanol. Evaluation was performed with Ultra-Violet detector for Nevirapine at 284 nm. HPLC was operated and the prepared mobile phase was run through the column to equilibrate it. Hamilton syringe was thoroughly cleaned with HPLC grade methanol. When a stable baseline was obtained, 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Plots of concentration vs. area were prepared.

4.5.1. Application of the developed method to drug formulations for Lamivudine

For the estimation of Lamivudine in tablet dosage forms, 20 tablets were weighed separately and their average weight was calculated. Tablets were crushed in a mortar and pestle and passed through a fine sieve separately. A portion of the powder equivalent to 100 mg of the drug was accurately weighed and transferred to separate 100 mL volumetric flasks. The drug was dissolved in by adding HPLC grade methanol 70 mL to the volumetric flasks with constant stirring for 15 – 20 min on a magnetic stirrer. Volumes were made up to the mark with mobile phase and filtered through 0.45 µm filter. Several aliquots of the supernatant sample solutions 0.05 mL and 0.4 mL of Lamivudine was taken in different 10 mL volumetric flask and diluted up to the mark with methanol for each brand and 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Evaluation was performed with Ultra-Violet detector for Lamivudine at 272 nm. The solutions were then analyzed.

4.5.2. Application of the developed method to drug formulations for Zidovudine

For the estimation of Zidovudine in tablet dosage forms, 20 tablets were weighed separately and their average weight was calculated. Tablets were crushed in a mortar and pestle and passed through a fine sieve separately. A portion of the powder equivalent to 100 mg of the drug was accurately weighed and transferred to separate 100 mL volumetric flasks. The drug was dissolved in by adding HPLC grade methanol 70 mL to the volumetric flasks with constant stirring for 15 – 20 min on a magnetic stirrer. Volumes were made up to the mark with mobile phase and filtered through 0.45 µm filter. Several aliquots of the supernatant sample solutions 1.0 mL and 1.5 mL of Zidovudine was taken in different 10 mL volumetric flask and diluted up to the mark with methanol for each brand and 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Evaluation was performed with Ultra-Violet detector for Zidovudine, 266 nm. The solutions were then analyzed.

4.5.3. Application of the developed method to drug formulations for Nevirapine

For the estimation of Nevirapine in tablet dosage forms, 20 tablets were weighed separately and their average weight was calculated. Tablets were crushed separately in a mortar and pestle and passed through a fine sieve separately. A portion of the powder equivalent to 100 mg of the drug was accurately weighed and transferred to separate 100 mL volumetric flasks. The drug was dissolved in by adding HPLC grade methanol 70 mL to the volumetric flasks with constant stirring for 15 – 20 min on a magnetic stirrer. Volumes were made up to the mark with mobile phase and filtered through 0.45 µm filter. Several aliquots of the supernatant sample solutions 0.25 mL and 1.0 mL, of Nevirapine was taken in different 10 mL volumetric flask and diluted up to the mark with methanol for each brand and 20 µL of each prepared drug solutions were injected and the peak area reports thus obtained were recorded. Evaluation was performed with Ultra-Violet detector for Nevirapine at 284 nm. The solutions were then analyzed.

4.6. Method Validation

The method was validated according to **ICH & US FDA** guidelines.

4.6.1. Linearity and range

They were established along the standard curve. The regression coefficient, y-intercept and slope of the regression line were calculated for three drugs.

4.6.2. Precision

Three concentrations of the drug solutions were prepared and injected six times during the day. The area report thus obtained was recorded. The percentage RSD was then calculated for the areas to obtain the intraday variation.

4.6.3. Intermediate precision

Three concentrations of drug solutions were prepared and injected in triplicates on three different days. The area report thus obtained was recorded. The % RSD was then calculated for the areas to obtain the interday variation.

4.6.4. Method Precision

Drug substances weighed six times and analyzing as per the proposed method at two different concentrations.

4.6.5. Accuracy

Three concentrations of each drug solutions were prepared and injected in six times. The area reports thus obtained were recorded. The percentage recovery studies were then calculated.

4.6.6. Reproducibility/Ruggedness

Different analyst variations were seen at various concentrations. The results obtained were compared with RT and % RSD.

4.6.7. Specificity

The specificity of the developed method was seen by injecting the blank (methanol without standard drugs), solution containing drugs blended with excipients (centrifuged and supernatant diluted) and demonstrating that the result is unaffected by the presence of these excipients by comparison with the results obtained by injecting standard drug solutions.

4.6.8. Robustness – Effect of variation in the flow rate (±1 mL/min) and temperature (±1-5°C) were studied. Effect of variation in the mobile phase ratio also studied.

4.6.9. System suitability tests

System suitability parameters such as tailing factor, number of theoretical plates and HETP were calculated and seen that they lay within the acceptance limits according to guidelines.

4.6.10. LOD and LOQ

They were calculated according to the formula given in the ICH guidelines.

4.7. Stability Indicating Studies

4.7.1. Forced degradation studies for Lamivudine

1. Base Stressed Studies

Stock solutions of Lamivudine was prepared by dissolving 100 mg bulk drug in 100 mL volumetric flask added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL of 0.1N NaOH (basic hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 10 µg/mL and volume was made up to the mark with mobile phase. The sample was withdrawn at 1, 24, 48, 72, 96, 120 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD was calculated from the standard concentration of bulk drug.

2. Acid Stressed Studies

Stock solutions of Lamivudine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks separately and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted

with 10 mL of 0.1N HCl (acid hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 10 µg/mL and volume was made up to the mark with mobile phase. The sample was withdrawn for LAM at 1, 24, 48, 72, 96, 120 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

3. Oxidation Stressed Studies

Oxidative studies were performed on bulk drugs in 5% H_2O_2 at ambient conditions. Solution was prepared by dissolving 100 mg bulk drugs of Lamivudine in 100 mL volumetric flask and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL 5% H_2O_2 and kept for 30 minutes, further diluted with HPLC grade methanol. The solutions were filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 10 µg/mL and volume was made up to the mark with mobile phase. The samples were withdrawn for LAM at 1, 4, 6, 24, 48 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

4. UV- light stability studies

Stock solutions of Lamivudine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was filtered through 0.45 µm filter paper and kept for 30 minutes.

To study UV-stability of LAM was exposed to UV light to determine the effects of irradiation on bulk solution. The sample was withdrawn for LAM at 1, 2, 3, 4 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drugs.

5. Thermal Stress Studies

Stock solutions of Lamivudine, was prepared by dissolving 100mg bulk drug in 100mL volumetric flasks separately and added with 10mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was kept for 30 minutes and filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 10 µg/mL and volume was made up to mark with mobile phase. Humidity study was performed separately to accelerated conditions at 40^0 C, 50^0 C, 60^0 C, and 70^0 in the stability chamber. The samples were withdrawn

at 10, 20, 30 min intervals and periodically analyzed by developed HPLC method for LAM. The % degradation of drugs to be remained, retention time and % RSD was calculated from the standard concentration of bulk drug.

4.7.2. Forced degradation studies for Zidovudine

1. Base Stressed Studies

Stock solutions of Zidovudine was prepared by dissolving 100 mg bulk drug in 100 mL volumetric flask added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL of 0.1N NaOH (basic hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 10 µg/mL and volume was made up to the mark with mobile phase.

Then the solutions transferred into 10 mL volumetric flasks to prepare 25 µg/mL volumes were made up to mark with mobile phase of ZDV. The samples were withdrawn for ZDV at 1, 24, 48, 72 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

2. Acid Stressed Studies

Stock solutions of Zidovudine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks separately and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL of 0.1N HCl (acid hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to the mark with mobile phase. The sample was withdrawn for ZDV at 1, 24, 48, 72 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

3. Oxidative Stressed Studies

Oxidative studies were performed on bulk drugs in 5% H_2O_2 at ambient conditions. Solution was prepared by dissolving 100 mg bulk drugs of Zidovudine in 100 mL volumetric flask and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL 5% H_2O_2 and kept for 30 minutes, further diluted with HPLC grade methanol. The solutions were filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to the mark with

mobile phase. The samples were withdrawn for ZDV at 1, 2, 3, 6, 12, 13, 14, hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

4. UV- light stability studies

Stock solutions of Zidovudine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was filtered through 0.45 µm filter paper and kept for 30 minutes.

To study UV-stability of ZID was exposed to UV light to determine the effects of irradiation on bulk solution. The sample was withdrawn for ZDV at 1, 2, 3, 4, 5, 6, 7 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drugs.

5. Thermal Stress Studies

Stock solutions of Zidovudine was prepared by dissolving 100mg bulk drug in 100mL volumetric flasks separately and added with 10mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was kept

for 30 minutes and filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to mark with mobile phase.

Humidity study was performed separately to accelerated conditions at 40^0 C, 50^0 C, 60^0 C, and 70^0 in the stability chamber. The samples were withdrawn at 10, 20, 30 min intervals and periodically analyzed by developed HPLC method for ZID. The % degradation of drugs to be remained, retention time and % RSD was calculated from the standard concentration of bulk drug.

4.7.3 Forced degradation studies for Nevirapine

1 Base Stressed Studies

Stock solutions of Nevirapine was prepared by dissolving 100 mg bulk drug in 100 mL volumetric flask added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL of 0.1N NaOH (basic hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to mark with mobile phase. The sample was withdrawn at NEV at 1, 24, 48, 72 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD was calculated from the standard concentration of bulk drug.

2. Acid Stressed Studies

Stock solutions of Nevirapine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks separately and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL of 0.1N HCl (acid hydrolysis) and kept for 30 minutes, further diluted with HPLC grade methanol. The solution was filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to mark with mobile phase. The sample was withdrawn for NVP at 1, 24, 48, 72 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

3. Oxidation Stressed Studies

Oxidative studies were performed on bulk drugs in 5% H_2O_2 at ambient conditions. Solution was prepared by dissolving 100 mg bulk drugs of Nevirapine in 100 mL volumetric flask and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with 10 mL 5% H_2O_2 and kept for 30 minutes, further diluted with HPLC grade methanol. The solutions were filtered through 0.45 µm filter paper. Then the solution transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to mark the with mobile

phase. The samples were withdrawn for NEV at 1, 2, 3, 4, 5, 24 hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drug.

4. UV- light stability studies

Stock solutions of Nevirapine was prepared by dissolving 100 mg bulk drugs in 100 mL volumetric flasks and added with 10 mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was filtered through 0.45 µm filter paper and kept for 30 minutes.

To study UV-stability of ZID was exposed to UV light to determine the effects of irradiation on bulk solution. The sample was withdrawn for NEV at 1, 2, 3, 4, 5, 6, hrs intervals and periodically analyzed by developed HPLC method. The % degradation of drugs to be remained, retention time and % RSD were calculated from the standard concentration of bulk drugs.

5. Thermal Stress Studies

Stock solutions of Nevirapine was prepared by dissolving 100 mg bulk drug in 100mL volumetric flasks separately and added with 10mL of HPLC-grade methanol to dissolve the drug and then diluted with mobile phase up to the mark and then solution was kept for 30 minutes and filtered through 0.45 µm filter paper. Then the solution

transferred into 10 mL volumetric flask to prepare 25 µg/mL and volume was made up to mark with mobile phase.

Humidity study was performed separately to accelerated conditions at 40^0 C, 50^0 C, 60^0 C, and 70^0 in the stability chamber. The samples were withdrawn at 10, 20, 30 min intervals and periodically analyzed by developed HPLC method for NVP. The % degradation of drugs to be remained, retention time and % RSD was calculated from the standard concentration of bulk drug.

4.8. Application of the Developed Method to Pharmacokinetic Studies

Instrumentation

The dissolution rate studies of Lamivir, Zidovir and Nevimune from tablets were performed on a Veego, VDA-8DR USP dissolution apparatus.

Chemicals and reagents

Mobile phase consisted of methanol (S.D fine HPLC grade) and water (S.D fine HPLC grade) was used during analysis. For dissolution studies, working solution of 0.1N HCL [(s.d fine AR grade, pH 1.2)], 0.2M Potassium Dihydrogen Phosphate and 0.2 M Sodium Hydroxide [(s.d fine AR grade, pH 6.8)] and 0.2M Potassium Dihydrogen Phosphate and 0.2 M Sodium Hydroxide [(s.d fine AR grade, pH 7.2)] was used to mimic the gastric physiological condition.

Preparation of buffer solution at pH 1.2 (IP 2007)

Place 8.5 mL conc. HCl in a 1000 mL volumetric flask then added to make up volume up to mark with water.

Preparation of buffer solution at pH 6.8

Preparation of Potassium Dihydrogen Phosphate, 0.2 M

Dissolved 27.218 g of potassium dihydrogen phosphate and diluted with water to 1000 mL

Preparation of Sodium Hydroxide

0.2 M: Dissolved sodium hydroxide in water to produce a 40 to 60 percent w/v solution and allowed to standing. Taking precautions to avoid absorption of carbon dioxide siphon off the clear supernatant liquid and diluted with carbon dioxide-free water a suitable volume of the liquid to contain 8.0 g of NaOH in 1000 mL.

Phosphate Buffer at pH 6.8

Place 250 mL of 0.2 M potassium dihydrogen phosphate in a 1000 mL volumetric flask, added 112 mL of 0.2 M sodium hydroxide and then added water to make up to the volume.

Preparation of buffer solution at pH 7.2

Preparation of Potassium Dihydrogen Phosphate, 0.2 M

Dissolved 27.218 g of potassium dihydrogen phosphate and diluted with water to 1000 mL.

Preparation of buffer solution at pH 1.2 (IP 2007)

Place 8.5 mL conc. HCl in a 1000 mL volumetric flask then added to make up volume up to mark with water.

Preparation of buffer solution at pH 6.8

Preparation of Potassium Dihydrogen Phosphate, 0.2 M

Dissolved 27.218 g of potassium dihydrogen phosphate and diluted with water to 1000 mL

Preparation of Sodium Hydroxide 0.2 M

Dissolved sodium hydroxide in water to produce a 40 to 60 percent w/v solution and allowed to standing. Taking precautions to avoid absorption of carbon dioxide siphon off the clear supernatant liquid and diluted with carbon dioxide-free water a suitable volume of the liquid to contain 8.0 g of NaOH in 1000 mL.

Phosphate Buffer at pH 6.8

Place 250 mL of 0.2 M potassium dihydrogen phosphate in a 1000 mL volumetric flask, added 112 mL of 0.2 M sodium hydroxide and then added water to make up to the volume.

Preparation of buffer solution at pH 7.2

Preparation of Potassium Dihydrogen Phosphate, 0.2 M

Dissolved 27.218 g of potassium dihydrogen phosphate and diluted with water to 1000 mL.

Preparation of Sodium Hydroxide, 0.2 M

Dissolved sodium hydroxide in water to produce a 40 to 60 percent w/v solution and allowed to standing. Taking precautions to avoid absorption of carbon dioxide siphon off the clear supernatant liquid and diluted with carbon dioxide-free water a suitable volume of the liquid to contain 8.0 g of NaOH in 1000 mL.

Phosphate Buffer at pH 7.2

Place 250 ml of 0.2 M potassium dihydrogen phosphate in a1000 mL volumetric flask, added 173.5 mL of 0.2 M sodium hydroxide and then added water to make up to the volume.

***4.8.1 In-Vitro* Dissolution Studies from Tablet Dosage Form for Lamivudine**

Dissolution studies in tablets were carried out in 900 mL of 0.1N HCl (pH 1.2) gastric, Phosphate Buffer at pH 6.8 mouth pH, and pH 7.2 small intestine pH, according to the USP dissolution procedures the single entity products with the use of a USP paddle type of apparatus at a stirring rate of 50 rpm. The temperature of the cell was maintained at 37 ± 0.5°C. Aliquots of Lamivudine were withdrawn 5mL at predetermined time intervals of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min and replaced with equal volume of pre-warmed 0.1N HCl solution to maintain the sink condition. The sample solutions were diluted with methanol (HPLC grade) then the

samples was analyzed by using methanol and water (89:11, v/v) ratio and concentrations were determined at 272 nm for LAM, The retention time of LAM is 2.86 min. The cumulative percentage of drug released in the media was plotted against time in order to determine the release profile from the tablet formulations. The dissolution test data were obtained by averaging three parallel studies.

4.8.2 In-Vitro Dissolution Studies from Tablet Dosage Form for Zidovudine

Dissolution studies in tablets were carried out in 900 mL of 0.1N HCl (pH 1.2) gastric, Phosphate Buffer at pH 6.8 mouth pH, and pH 7.2 small intestine pH, according to the USP dissolution procedures the single entity products with the use of a USP paddle type of apparatus at a stirring rate of 50 rpm. The temperature of the cell was maintained at 37 ± 0.5°C. Aliquots of Zidovudine samples were collected predetermined time intervals of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min and replaced with equal volume of pre-warmed 0.1N HCl solution to maintain the sink condition. The sample solutions were diluted with methanol (HPLC grade) then the samples was analyzed by using methanol and water (89:11,v/v) ratio and concentrations were determined at 266 nm for ZDV. The retention time for ZID is 2.96. The cumulative percentage of drug released in the media was plotted against time in order to determine the release profile from the tablet formulations. The dissolution test data were obtained by averaging three parallel studies.

4.8.2 In-Vitro Dissolution Studies from Tablet Dosage Form for Nevirapine

Dissolution studies in tablets were carried out in 900 mL of 0.1N HCl (pH 1.2) gastric, Phosphate Buffer at pH 6.8 mouth pH, and pH 7.2 small intestine pH, according to the USP dissolution procedures the single entity products with the use of a USP paddle type of apparatus at a stirring rate of 50 rpm. The temperature of the cell was maintained at 37 ± 0.5°C. Aliquots of Nevirapine samples were collected predetermined time intervals of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 20, 22, and 24 min and replaced with equal volume of pre-warmed 0.1N HCl solution to maintain the sink condition. The sample solutions were diluted with methanol (HPLC grade) then the samples was analyzed by using methanol and water (89:11, v/v) ratio and concentrations were determined at 284 nm for NVP respectively. The retention time of NVP is 3.18 min. The cumulative percentage of drug released in the media was plotted against time in order to determine the release profile from the tablet formulations. The dissolution test data were obtained by averaging three parallel studies.

4 .9. Pharmacokinetic *In-Vivo* Studies

Experimental Animals

Male albino rats, weighing between 150-200 g was included in the study. Rabbits were divided as four groups A, B, C, D, and rabbits were housed in the departmental animal house at an ambient temperature of 25 °C, under a 12hour dark- 12 h ours light cycle, for the whole period of the study. Experiments were carried out according to the guidelines of the animal ethics committee of the college **Himalayan Pharmacy Institute IAEC-No- HPI/08/60/IAEC0059.**

4.9.1. *In-vivo* Pharmacokinetic animal Studies for Lamivudine

The application of the developed method in animal studies selected New Zealand White adult male rabbits, each weighing 3 to 3.5 kg were used in the experiments. The rabbits were fed balanced diet pellets and maintained on 12 h/12 h light/dark cycle in a temperature controlled room, at 20°C to 24°C before the experiment. The experimental procedures conform to the ethical principles (Sikkim, India) on the use of animals. The doses were chosen because of its comparability to human dosing and to allow comparison to animal studies. Oral dose of 22.5 mg/kg Lamivudine was administered to all the rabbits (four groups each four total n=16). Heparin injected at the dose of 800 units/kg from the stock (5000

units). Five min before the administration of the drug, because to avoid clotting of the blood samples. Blood samples were withdrawn from ear marginal vein at 0, 1, 2, 3, 4, 5, 7, 14 hrs post dose. The samples were stored at - 20°C and analyzed by the following method. Blood sample were centrifuged at 6000 rpm for 10 min at 0°C aliquots of plasma sample added with 1 mL of 10% TCA [Trichloroacetic acid] (Ruprecht et al., 1990). The centrifuge tubes are horizontally shaken for 10 min followed by centrifuging at 3000 rpm for 5 min at 0°C. The samples were allowed to pass through the silica gel Column bed with minimal suction. The column was further washed with methanol and bed was suctioned dry for 1 min and the organic phase was evaporated to dryness under a gentle stream of nitrogen at + 40°C (Dailly et al., 2004). The residue is reconstituted for 2 min by 1 mL methanol. Aliquots of 0.5 mL of plasma samples transferred to 5 mL volumetric flask and diluted with mobile phase up to the mark. The entire samples injected in HPLC and mobile phase (89:11 v/v) used from developed method.

4.9.3. *In-vivo* Pharmacokinetic animal Studies for Zidovudine

The application of the developed method in animal studies selected New Zealand White adult male rabbits, each weighing 3 to 3.5 kg were used in the experiments. The rabbits were fed balanced

diet pellets and maintained on 12 h/12 h light/dark cycle in a temperature controlled room, at 20°C to 24°C before the experiment. The doses were chosen because of its comparability to human dosing and to allow comparison to animal studies. Oral dose of 42 mg/kg Zidovudine was administered to all the rabbits (four groups each four total n=16). Heparin injected at the dose of 800 units/kg from the stock (5000 units). Five min before the administration of the drug, because to avoid clotting of the blood samples. Blood samples were withdrawn from ear marginal vein at 0, 1, 2, 4, 6, 8 hrs post dose. The samples were stored at - 20°C analysis and analyzed by the following method. Blood sample were centrifuged at 6000 rpm for 10 min at 0^0C aliquots of plasma sample added with 1 mL of 10% TCA (Trichloroacetic acid). The centrifuge tubes are horizontally shaken for 10 min followed by centrifuging at 3000 rpm for 5 min at 0^0C. The samples were allowed to pass through the silica gel column bed with minimal suction. The column was further washed with methanol, and bed was suctioned dry for 1 min and the organic phase was evaporated to dryness under a gentle stream of nitrogen at + 40^0C (Dailly et al., 2004). The residue is reconstituted for 2 min by 1 mL methanol. Aliquots of Zidovudine 0 .5 mL of plasma sample transferred to 10 mL volumetric flask and diluted with mobile phase

up to the mark. The entire samples injected in HPLC and mobile phase (89:11 v/v) used from developed method.

4.9.2. *In-vivo* Pharmacokinetic animal Studies for Nevirapine

The application of the developed method in animal studies selected New Zealand White adult male rabbits, each weighing 3 to 3.5 kg were used in the experiments. The rabbits were fed balanced diet pellets and maintained on 12 h/12 h light/dark cycle in a temperature controlled room, at 20°C to 24°C before the experiment. The doses were chosen because of its comparability to human dosing and to allow comparison to animal studies. Oral dose of 28 mg/kg Nevirapine were administered to all the rabbits (four groups each four total n=16). Heparin injected at the dose of 800 units/kg from the stock (5000 units). Five min before the administration of the drug, because to avoid clotting of the blood samples. Blood samples were withdrawn from ear marginal vein at 0, 1, 2, 3, 4, 5, 7, 14 hrs and 0, 1, 2, 4, 6, 8 hrs and 0, 1, 3, 5, 8, 24, 28, 32, 48, 52 hrs post dose. The samples were stored at - 20°C analysis and analyzed by the following method. Blood sample were centrifuged at 6000 rpm for 10 min at 0°C aliquots of plasma sample added with 1 mL of 10% TCA (Trichloroacetic acid). The centrifuge tubes are horizontally shaken for 10 min followed by centrifuging at 3000 rpm for 5 min at 0°C. The

samples were allowed to pass through the silica gel column bed with minimal suction. The column was further washed with methanol, and bed was suctioned dry for 1 min and the organic phase was evaporated to dryness under a gentle stream of nitrogen at + 40^0C (Dailly et al., 2004). The residue is reconstituted for 2 min by 1 mL methanol. Aliquots of 0.5 mL Nevirapine of plasma samples transferred to 5 mL volumetric flask and diluted with mobile phase up to the mark. The entire samples injected in HPLC and mobile phase (89:11 v/v) used from developed method.