CHAPTER-5
DISCUSSION OF THE RESULTS

5.1 Fludarabine:

The study was aimed to develop RP-HPLC method for Fludarabine in pharmaceutical dosage form. The initial trials were conducted based upon the peak symmetry and time reduction in the chromatographic analysis. The C18 column was selected to conduct the method development study based on the polarity of Fludarabine. All the trails were done using C18 kromasil (250 X 4.6mm, 5 µm) column. The mobile phase was assessed after conducting the various trails using the solvents of acetonitrile, methanol and phosphate buffer. The mobile phase of Methanol: Acetonitrile: Phosphate Buffer 50:20:30 (v/v) was used and the result showed a good peak shape with limits of system suitability. Wave length was detected at the absorbance of 265 nm. The optimized conditions are showed in table 4.1. The results of the specificity were showed there was no interference and co-elution of any other peaks with the retention of Fludarabine. The peak purity of Fludarabine and sample was found within the limit which proved that there was no interference w.r.t blank and placebo peaks. Hence the developed method was specific.

The method validation was done as per ICH guidelines. The linearity performed with six concentration levels ranging from 20-120µg/ml for Fludarabine from the table 3.2. Linearity graph was plotted by taking absorbance vs sample concentration. The results shown that response is linear from 20-120 µg/ml and linearity plot was given in figure 4.4. Inter day and intra day precision was performed and values found to be less than 2. In the intraday studies, (n=6) samples were prepared both standard and sample drugs peak areas and the %RSD were calculated. These results are seen in the table 4.4. Likewise interday precision also studied same concentrations of sample and standard drugs are prepared for three consecutive days peak areas and the percentage RSD were calculated the obtained results are seen in the table 4.4. The recovery of the method was studied on three concentrations of drugs percentage assay and %RSD was calculated the results are seen in the table 4.4. Hence the developed method is rapid, simple and statistically validated for its accuracy.

LOD and LOQ were found to be 0.05µg/ml and 0.16µg/ml respectively. This values conforms the sensitivity of the developed method. The formulation analysis of the method was expressed by using commercially available marketed formulation.
Diltiazem

This study involves development and validation of chromatographic method of estimation of Diltiazem in tablet dosage form as per ICH guidelines with a validated RP-HPLC method. Chromatography was performed on Zodiac RP Column C18 (150 mm x 4.6 mm i.d. 5µm) with mobile phase containing Methanol: Acetonitrile in the ratio of 90:10 (v/v). The flow rate was 1ml/min and eluent was detected at 245nm at ambient temperature. The selected and proposed chromatographic conditions were found satisfactory with retention time 3.02 min. Table 4.1 gives the optimized chromatographic conditions developed for the analysis of Diltiazem. Developed Method was validated as per ICH guidelines

Interday and Intraday precision was performed by calculating %RSD of six replicate standard injections and values found to be less than 2. Hence the developed method was precise.

Recovery was performed from 50, 100 & 150% levels of test concentration. The concentrations used to spike the target concentration were prepared for the freshly prepared standard solution by using standard procedure. At triplicate injections were given and were compared with the corresponding peak areas of standard for the determination of the % drug recovery. % RSD in each spiked level was calculated and was found to be 0.767 in 50%, 1.135 in 100% and 0.338 in 150% spiked levels. Results found to be within the acceptance criteria.

Robustness was performed by varying method parameters such as wavelength, pH of the mobile phase and mobile phase ratio etc. For the developed method the robustness study was carried out at concentration of 20µg/ml of Diltiazem. Each factor was changed at two levels (+1 and -1). Results found to be in between 0.29-1.43 (table 4.7). LOD and LOQ were found to be 0.05µg/ml and 0.16µg/ml respectively.

A marketed formulation (Cardizem-60mg) was used for the determination of the ability of the method for the estimation of drug product. 99.16% (table 4.10) assay was observed in the formulation analysis. The results obtained in this study are statistically significant and found within the acceptance limit. This simplified developed method was validated which reduces time, cost ultimately with better...
chromatographic separation as considered with the other available known methods. Therefore the method is suitable for its intended use.

5.3 Etoposide

To develop specific RP-HPLC method initially mobile phase, wave length, sample concentration and column were optimized and conditions were shown in table 4.21. The method was linear in the range of 5-45µg/ml. The equation was \( y = 6172.6 + 77861x \) with \( r^2 \) value 0.9989 which are within the acceptance criteria. The calibration curve was shown in figure 4.16. The proposed methods were validated as per the ICH guidelines.

The precision of the method was studied by preparing both standard and sample drugs. The peak area and percentage RSD were calculated for homogenous samples with in the day (intraday) and next consequent three days for interday precision. For each cases % RSD was calculated and was found to be 0.22 for interday and 0.30 for intraday precision. This was lying within the acceptable limit ±2.

Recovery was carried out by standard addition method of 50% 75 and 100% addition to standard, pre analyzed sample of 20µg/ml in triplicates. % recovery for each case was calculated and was found to be 98.14 to 101.70. This was found to be well within the acceptance criteria of 98-102%. Recovery results were shown in table 4.24.

Robustness was performed by changing different parameters like mobile phase ratio, mobile phase flow rate and detector wavelength. % change in the results was calculated and was found to be within the acceptance 2. This indicates that the proposed method is valid. Robustness results were shown in table 4.27.

The ruggedness was determined by carrying out the experiment with different analysts. Standard solution of Etoposide at 25µg/ml was prepared six times by different analysts and was analyzed using the optimized method. The %RSD of the peak area obtained was calculated. Percentage RSD was found to be 0.37. Results were found to be within the acceptance limit, less than 2. This confirms that the method was found to rugged. Results are tabulated in the table 4.28.

LOD value for Etoposide is found to be 0.02µg/ml and LOQ value for Etoposide is found to be 0.06µg/ml. The stability of the method was studied by analyzing the standard concentration for different time intervals and % stability was calculated. The method was stable at a time period of 24H (table 5.10). Hence the method was stable.
Formulation analysis was conducted for commercial tablets of Etoposide (E50). Peak area of the detector response was used to calculate % assay. The results (98.76%) indicated that there is good agreement with the labeled content. This confirms that the proposed method can be applicable for the estimation of Etoposide in bulk drug also.

5.4 Iloperidone

This study involves development and validation of chromatographic method of estimation of Iloperidone in bulk and in drug product as per ICH guidelines with a validated RP HPLC method. Chromatography was performed on Li Chrospher C18 Column (250 X 4.6 mm, 5μ) with mobile phase containing Acetonitrile: methanol and acetate buffer in a proportion of 50:30:20 (v/v). The flow rate was 0.9ml/min and eluent was detected at 215nm at ambient temperature. The selected and proposed chromatographic conditions were found satisfactory with retention time of 6.57 min. Table 4.32 gives the optimized chromatographic conditions developed for the analysis of Iloperidone.

Interday and Intraday precision was performed by calculating %RSD of six replicate injections of standard solutions and values found to be less than 2. Hence the developed method was found to be precise.

Recovery was performed on 50, 75& 100% levels of test concentration. The concentrations used to spike the target concentration were prepared for the freshly prepared standard solution by using standard procedure. At triplicate injections were given and were compared with the corresponding peak areas of standard for the determination of the % drug recovery. % RSD in each spiked level was calculated and was found to be 0.346 in 50%, 0.152 in 100% and 0.670 in 150% spiked levels. Results found to be within the acceptance criteria.

Robustness was performed making slight changes in method conditions like mobile phase, wavelength, pH. For the developed method the robustness study was carried out at concentration of 60µg/ml of Iloperidone. Each factor was changed at two levels (+1 and -1). Results found to be in between 0.42-1.84(table 6.8). LOD and LOQ were found to be 0.01µg/ml and 0.03µg/ml respectively.

A marketed formulation (Fanapt-2mg) was used for the determination of the ability of the method for the estimation of drug product. 99.47% (table 4.42) assay was observed in the formulation analysis. The results obtained in this study are statistically significant and found within the acceptance limit.
developed method was validated which reduces time, cost ultimately with better chromatographic separation as considered with the other available known methods. Therefore the method is suitable for its intended use.

5.5 Risperidone:

To develop specific RP-HPLC method initially mobile phase, wavelength, sample concentration and column were optimized. Risperidone is a non-hygroscopic powder only slightly soluble in organic solvents and it is practically insoluble in water and aqueous media. According to our observation, Risperidone was dissolved in methanol (MeOH) on the preparation of standard stock solutions process. Therefore, the solubility of Risperidone was tested simply in various organic solvents in the initial experiments and it was concluded that Risperidone was clearly soluble in methanol. Thus, it was decided to use the mixture of Methanol and water in different proportion on the separation of Risperidone through C18 column. A well-defined peak for Risperidone was observed at 3.83 min under the experimental conditions. Even though the column temperature is an important parameter on separation of organic compounds by HPLC, the effect of diluent is usually stronger than the effect of temperature on solute retention. It was seen that changing the column temperature between 30–40°C, changed the retention time of Risperidone not more than 5%. According to the results, it was obvious that Risperidone could be easily separated and analyzed less than 10 minutes in conditions given in Table 4.43. The UV Spectrum of the Risperidone in mobile phase shows clearly that 238nm is better to use in to observe the maximum absorbance while it is being prevented from interference coming from matrix components. The chromatograms of standard and tablet solutions and Risperidone solution degraded at high temperature taken under optimum conditions are given in Figure 4.25 and 4.27 respectively.

The system suitability test was applied to the chromatograms taken under optimum conditions to check various parameters such as column efficiency (plates) and peak tailing. The RSD of six consecutive injections at 60 µg/ml was found to be 0.19 (Table 4.45), and 0.36 (Table 4.46) for intraday and interday precision respectively.

The linearity was determined with nine concentration levels ranging from 20-100 µg/ml. Calibration curve was constructed for Risperidone standards by plotting the concentrations versus peak area ratios. The graph in fig 4.28 proved that the method was linear.
The method can estimate the amount of drug in pharmaceutical formulations 99.27% accurately. The method was stable up to a time period of 18H. The solutions prepared in the proposed method give results accurately with a time period of 18H. Hence the developed method was stable. The influence of the small change in the conditions also doesn’t influence the results of the method. Hence the method can be used for the routine analysis of Risperidone. The method was applied for the estimation of Risperidone in bulk samples. The results proved that the method was applied for the estimation of Risperidone in bulk samples. The chromatogram of bulk drug analysis was shown in figure 4.29.

5.6 Topotecan:

To develop specific RP-HPLC method initially mobile phase, wave length, sample concentration and column were optimized and conditions were given in table 4.53. The method was linear in the range of 10-40 µg/ml. The equation was $y = 5551x - 140$ with $r^2$ value 0.9995, Which are with in the acceptable limits.

The system suitability test was applied to the chromatograms taken under optimum conditions to check various parameters such as column efficiency (plates) and peak tailing. The RSD of six consecutive injections at 30 µg/ml was found to be 0.20 (Table 4.55), and 0.41 (Table 4.56) for intraday and interday precision respectively.

Robustness performed by changing different parameters like mobile phase ratio, detector wave length and flow rate. The % change in the results was observed that less than 2. This indicates that method is robust and results were given in table 4.58.

The ruggedness carried out by preparing 30 µg/ml standard Topotecan solutions six times by different analysts and was analyzed using the optimized method. The %RSD was found to be 0.55 confirmed that the method was rugged. Results were given in table 4.59.

Recovery was carried out by preparing standard solution method of 50%, 75% and 100% addition to standard, pre analyzed sample of 10 µg/ml in triplicates. % recovery for each case was calculated and found to be in the range of 98.48- 101.32%, found to be with in the acceptance criteria. Results were shown in table 4.57.

The method can estimate the amount of drug in pharmaceutical formulations 98.933% accurately. The method was stable up to a time period of 24H. The solutions prepared in the proposed method give results accurately with a time period of 24H.
Hence the developed method was stable. The influence of the small change in the conditions also doesn’t influence the results of the method. Hence the method can be used for the routine analysis of Topotecan.

5.7 Amlodipine:

An accurate RP HPLC method was developed for the quantification of Amlodipine in pharmaceutical formulations. During method development phase of analysis most suitable chromatographic conditions were developed for the determination of the drug. It was stated that the mobile phase composition of methanol: water: acetonitrile in the ratio of 60:20:20(v/v) was best suited, as it gave good separation and sharp peak and retention time of 5.53 min was best for the routine analysis of the drug in the tablet dosage form. The flow rate was maintained as 1.0 ml/min. The pH was maintained as 5.9. All the system suitability parameters were satisfied as tailing factor was obtained as 1.23, theoretical plates were 10318 and retention time was found to be 5.53 min.

The optimized chromatographic conditions were further checked for system suitability and specificity parameters. The standard chromatogram was checked for system suitable criteria. Theoretical plates 10319 and tailing factor was found to be 1.23. The results of the system suitability were found to be within the acceptance limit. The developed method was validated according to ICH guidelines. The different validation parameters such as linearity, precision, recovery, robustness, robustness, sensitivity and formulation assay were estimated.

Amlodipine tablet % of assay values is determined of commercially available dosage form. The method developed of shorter data acquisition time, specific and economical mobile phase. The method has linearity in the range of 60-120µg/ml. Each sample was injected into chromatographic system and peak response was noted. Reproducibility results were obtained by calculating the slope, intercept for standard plot. The method was robust as there was no significant change in the system suitability. The method was specific, linear and robust and can be used for regular analysis.

To analysis the Amlodipine various analytical methods have been proposed by various authors. Among them Hassan et al, Tatar, S et al, Bahrami, Gh et al, Alsarra Ibrahim A et al, Zarghi, A.et al was developed analytical methods for the estimation of Amlodipine in biological fluids like human plasma, serum and urine. Xu, Guojin et al developed a new analytical method for the estimation of Amlodipine
with relative substances. All these methods have there’re selective application for the estimation of amlodipine in there respective target. Few analytical methods were reported for the estimation of Amlodipine in pharmaceutical dosage form. The methods developed by Dong, Yu et al, Sharma, Amrish et al, Li, Basavaiah, Kanakapura et al, Shang, Fei et al, Avadhanulu, A.B et al developed methods contains phosphate buffer in the mobile phase. Though the usage of salt buffers will increase the response of the drug, it reduces the life time of the column and may precipitate in the passage route on long stand and also increases the pressure of the pump. The method developed by us doesn’t contain any salt buffers and the method will eliminate the negative impacts on the system that created by salt buffers. Leite, Helen D et al and Malesuik Marcelo Donadel et al developed method contains triethyl amine and Orthophosphoric acid respectively in the mobile and also need to adjust the pH of the mobile phase. The method developed by Li, Chengping et al was simple but have linearity range of 0.5-100µg/ml, but our method having a linearity range of 60-210µg/ml was found to be very high calibration range.

Hence the developed method was specific, accurate, precise rugged, robust and sensitive. Validation of the proposed procedures was carried out ac-cording to the ICH and USP guidelines. It was concluded that method can introduced for regular analysis of API and finished producs.

5.8 Diacerine and Glucosamine:

To develop HPLC method different trials are taken like flow rate, mobile phase ratio and pH were investigated. During optimization, prontosil RP-C18 column (250mm × 4.6 mm i.d.), 5µm was finalized with better resolution. After several trials the optimum mobile phase composition and flow rate were determined as Water: Methanol: OPA 40:40:20 (v/v) and 0.9ml/min. At these conditions Diacerine and Glucosamine were eluted at 6.15 and 8.12min respectively. Chromatograms were given in Figures 4.42, 4.43 and 4.44. The specificity of the method was performed by injecting the placebo, blank and standard solution. No peak was observed at the retention time of Diacerine and Glucosamine. Hence method found to be specific.

The correlation coefficients ($r^2$) were obtained as 0.998 for Diacerine and 0.999 for Glucosamine which shows method was linear.. The slope (m) and intercept (c) of the calibration curves were found as 13981 and 46780 for Diacerine; and 5130 and 48189 for Glucosamine respectively (Table 4.47, Figure 4.45 and 4.46).
Interday and Intraday precision was performed by calculating %RSD of six replicate injections of standard solutions and values found to be less than 2. Hence the developed method was found to be precise.

Recovery was performed from 50,100&150% levels of test concentration. The concentrations used to spike the target concentration were prepared for the freshly prepared standard stock solution of both the drugs using the standard procedure. The mean recoveries of Diacerine and glucosamine were in range from 98.2-99.44% and 99.273-99.789% respectively. The average percentage of recovery was found to be within the acceptable range. Hence the developed method was found to be accurate.

Standard solution of Diacerine at 7.5µg/ml and Glucosamine at 100µg/ml was used to determine the ruggedness. The %RSD was calculated for the peak area obtain in the ruggedness and the acceptable % RSD of 1.08 and 1.12 was obtained for Diacerine and Glucosamine respectively.

The method was found to be robust and the small variations in the analytical conditions don’t affect the results of the chromatogram. The method also found to be stable and the solutions prepared in the method were found to be stable and accurate results were obtained at a stability period of more than 18H. The method was also found to be sensitive. The limit of quantitation for Diacerine was 0.16µg/ml and for Glucosamine was 1.0 µg/ml.

This method can be applied for the routine analysis of Diacerine and Glucosamine in pharmaceutical formulations using a market available brand Arthcure D (Diacerine-50mg and Glucosamine- 750mg). The average % Assay values for Diacerine and Glucosamine were found to be 99.55 and 99.78 respectively. Hence the developed method can be applicable for the regular analysis of Diacerine and Glucosamine in formulations.

All the results shown that the method was precise, robust and accurate for the determination of Diacerine and Glucosamine. Therefore, the method can be used for routine analysis of Diacerine and Glucosamine.

5.9 Olanzapine and Fluoxetine:

To develop specific RP-HPLC method initially mobile phase, wavelength, sample concentration and columns were optimized and found to be Methanol: Phosphate Buffer 70:30 (v/v) with flow rate of 0.8ml/min having isocratic pump and
the pump pressure is ranging from 9.5±5MPa. Wave length was detected at the absorbance of 232nm. Reverse phase C-18 column was used for both the drugs Olanzapine and Fluoxetine. The peak areas were determined by the optimized method conditions. The typical chromatograms were given in Figure 4.50 and 4.51 for standard and sample solutions respectively.

The method was validated as per ICH guidelines. The linearity determined with six concentration levels ranging from 5-35µg/ml for Olanzapine and 10-70µg/ml for Fluoxetine respectively from the table 4.85. The calibration curve was plotted absorbance vs sample concentration. The results shown that response is linear from 5-35 µg/ml, and calibration curves shown in figure 4.52 and 4.53 for Olanzapine and Fluoxetine respectively. The precision was carried out by inter day and intraday variation studies. In the intraday studies, (n=6) samples were prepared both standard and sample drugs peak areas and % RSD were calculated. These results are seen in the table 4.87. Likewise interday precision also studied same concentrations of sample and standard drugs are prepared for three consecutive days peak areas and the % RSD were calculated the obtained results are seen in the table 4.88. The recovery of the method was studied on three concentrations of drugs percentage assay and % RSD was calculated the results are seen in the table 4.89 and 4.90. Hence the proposed method is simple, rapid and statistically validated for its accuracy.

LOD and LOQ were determined by using the lowest concentration of the standard drug. LOD of the drug is found to be 0.6µg/ml for Olanzapine and 0.3µg/ml for Fluoxetine respectively. The LOQ of Olanzapine was found to be 2.0µg/ml and 1.0µg/ml for Fluoxetine respectively.

Formulation analysis was conducted for commercially combined tablets (DEOTEN OX). Percentages found for Olanzapine and Fluoxetine were 99.35% and 98.38 respectively. The result of the assay indicated that the method could be adopted for the assay of Olanzapine and Fluoxetine without interference from the excipients used to produce these commercially combined tablets.