Various novel analytical approaches were investigated and innovative methods were developed for analysis of drugs and pharmaceuticals selected for the study. For analysis of drugs, procedures entailing approaches of DOE and chemometric were employed. For getting statistically significant results, approaches for checking normal distribution of data, ANOVA, post hoc analysis as well as uncertainty calculations were done. For drugs, stability studies, degradation kinetics studies as well as bioanalytical studies were undertaken. For checking adulteration of herbals, various spectroscopic and chromatographic methods were employed. Also novel paper microfluidic device was fabricated and developed for checking adulteration of herbal pharmaceuticals and for analysis of biomarker which acted as a diagnostic device. The summary of entire work is as follows:

Chapter 3 encases the entire study regarding development of stability indicating analytical method for Cyproheptadine HCl (CPH) along with isolation, characterization and identification of major degradation product which is further bifurcated into section A and section B.

Section A: This section comprises of development of SIAM for CPH using OFAT (One factor at time) approach in isocratic mode of HPLC. The stability studies were done as per ICH guidelines for acid, base, neutral hydrolysis, oxidation, dry heat and photolytic stress conditions. An optimized HPLC method was finalized using sample comprising mixture of stress conditions and a simple method was developed by using acetonitrile-methanol-20 mM ammonium formate (pH 5.5 adjusted with 0.2% formic acid) (40:10:50, v/v/v) as the mobile phase. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000 psi respectively. The analysis was performed at ambient temperature with injection volume of 20 µL. The linearity range for analysis of CPH was obtained as 3-18 µg/ml. The separation was achieved on Hypersil BDS C_{18} column (250×4.6 mm, 5 µm particle size) at detection wavelength of 224 nm. The retention time of CPH was found to be 6.8 ± 0.07 min and the degradation products were observed at retention time of 2.8 min one each in acid and base hydrolysis conditions. In all other stress conditions although, degradation of drug was observed on the basis of decrease in peak area as compared to standard drug, no degradation product was observed in chromatogram. Many attempts of changing the polarity of mobile phase, scanning chromatogram into entire UV range by PDA detector were done but no degradation product other than acid and base stress conditions were observed. The developed method was validated as per ICH guidelines. The %RSD values for precision studies were below 2%, the % recovery for accuracy studies also stated the accuracy in the prescribed limits of 98-102%.

Section B: This section comprises of isolation of major degradation product of CPH formed in acid and base stress conditions. Forced degradation studies implicated one

degradation product each in acid and base condition, but as per LC/MS/MS analysis, they were inferred to be similar degradation products, thus isolation of only DP1 was carried forward. DP1 was formed as a major degradation product and it was isolated through conventional thin layer chromatography incorporating preparative technique using precoated silica gel plates for isolation. An optimized mobile phase for TLC procedure consisted of dichloromethane: toluene: Ethyl acetate: methanol in ratio of 60:10:10:20(%v/v/v/v). The TLC chamber was first saturated for 30 min with the optimized TLC mobile phase and the spots were identified under UV light in UV chamber using short wavelength UV light of 254 nm. The R_f value of standard CPH was found to be 0.9 and that of DP1 was found to be 0.4. The DP1 thus separated was isolated using preparative TLC technique and purified and recrystallised. This isolated DP1 was used for characterization of its structure using FTIR, NMR (Proton, C^{13}, DEPT and D_2O exchange), LC/MS/MS and DSC studies. Based on the spectral data the chemical name of isolated degradation product (DP1) at retention time of 2.8 min is 4-(dibenzo [1, 2-a: 1', 2'-e] [7] annulen-11-ylidene) which is hitherto unreported in literature.

Chapter 4 comprises of the study regarding development of various classical and chemometric assisted UV spectrophotometric methods in section A, HPLC methods for simultaneous estimation of Chlorhexidine gluconate (CHD) and Cetrimide (CET) in section B and HPLC method for estimation of Cetrimide (CET) in bulk and dosage form.

Section A: Various classical and chemometrics UV analytical methods comprising of bivariate and multivariate analytics were developed for combination of CHD and CET. Classical UV spectrophotometric methods developed were Vieordt's method, First Derivative spectroscopy method, Multicomponent analysis method, Absorption ratio spectra method, Mean centering of ratio spectra method and chemometric spectrophotometric methods developed were Classical least squares, Inverse least squares, Partial least squares, Principal component regression. For Vieordt's method, Multicomponent analysis method and mean centering of ratio spectra method, the λmax chosen was 260 nm and 217 nm for CHD and CET respectively. Calculations were based on simultaneous equation in Vieordt's method. The Multicomponent mode of UV 1700 was used in Multicomponent analysis by loading the calibration curve of mixture solutions into the system. In mean centering of ratio spectra method also the calibration curve of mixture solution is to be taken and then computations using molar absorptivity, mean centering (using software package Unscrambler X) are to be done. In first derivative spectroscopy method, CET was analyzed at 222 nm which was ZCP of CHD whereas CHD was analyzed at 275 nm which was ZCP of CET. The parameters Δλ and scaling factor were set to be 5. In absorption ratio spectra method, λ2-λ1 value is used for preparation of calibration curve for corresponding analyte after transformations for the method in UV probe software. 217-200 nm was used as λ2-λ1 for CET whereas 263-225 nm was used as λ2-λ1 for CHD calculations. Also four chemometric methods were

developed for combination of CHD and CET. For them calibration and validation sets were prepared and their absorbances were taken in wavelength range 220-260 nm with interval of 1 nm, thereby at twenty wavelengths. The calculations for CLS and ILS were done using software Matlab from Math works whereas for PLS and PCR they were done using software SAS JMP 13. For Chemometric methods, RMSEP and PRESS values were calculated. Parameters for all UV spectrophotometric methods and validation results were found to be falling within the specified limits as per ICH guidelines. Further statistical analysis using ANOVA and post hoc Tukey's test were done for checking the statistical significance of the results.

Section B: This section consists of development and validation of RP-HPLC method for CHD and CET drug combination. For development of optimized HPLC method for the drug combination, various trials for selection of appropriate mobile phase and chromatographic parameters were taken and finally a mobile phase consisting of phosphate buffer (pH 3): ACN: methanol in ratio of (15:30:55, v/v/v) was finalized giving resolved and symmetric peaks of CHD and CET. The detection wavelength was set at 210 nm, flow rate was set as 0.8 ml/min, separation was achieved using BDS Hypersil C-18 (250 mm x 4.6 mm, 5 μm) column and the analysis was carried out at ambient temperature. The optimized peaks in the chromatogram for CET and CHD were obtained at 3.20 and 3.80 min respectively. The developed HPLC method was validated as per ICH guidelines. Robustness study was done using DOE approach using Box Behnken design of Response surface methodology. On the basis of the results obtained from DOE study, it was concluded that deliberate, small variations in the chromatographic parameters set for the analysis of the drug combination did not have any significant impact on the results sustaining the validity of the developed HPLC method. For checking if the data obtained from chromatographic study are normally distributed, Anderson darling normality test was done confirming normal distribution of data and thus assuring integrity of method for precise and robust results.

Section C: Due to unavailability of RP-HPLC method for Cetrimide as per the literature survey, it was decided to develop an easy and validated RP-HPLC method for Cetrimide in bulk and pharmaceutical dosage form. For development of optimized method for CET various trials for selection of optimum mobile phase were taken and finally a mobile phase consisting of ammonium formate buffer 20 mm (pH=3, pH adjusted with formic acid): ACN: methanol (20:30:50, v/v/v) was selected for chromatographic study. The detection wavelength was set at 217 nm, flow rate was set as 1.0 ml/min, separation was achieved using BDS Hypersil C-18 (250 mm x 4.6 mm, 5 μm) column and the analysis was carried out at ambient temperature. The optimized peak in the chromatogram for CET was obtained 3.10 min. The developed HPLC method was validated as per ICH guidelines. For checking if the data obtained from chromatographic study are normally

distributed, Anderson darling normality test was done for confirming normal distribution of data and thus assuring integrity of method for precise and robust results.

Chapter 5 comprises of stability and degradation kinetics studies of Pomalidomide (POM). For getting optimized chromatogram of POM along with its degradation products, DOE approach was utilized. For getting optimized chromatogram, sample from all degradation stress conditions was mixed and then analyzed. Also the uncertainty in data of accuracy was checked by using total error approach. We have divided the study described above into two sections.

Section A: This section consists of development and validation of HPLC method for Pomalidomide and application of total error approach for checking uncertainty of data distribution. For development of optimized method for POM, various trials were taken for selection of optimum mobile phase and finally mobile phase consisting of 0.2% formic acid: ACN (pH=3), 60:40, v/v) was selected for chromatographic study. The detection wavelength was set at 225 nm, flow rate was set as 1.0 ml/min, separation was achieved using BDS Hypersil C-18 (250 mm x 4.6 mm, 5 μm) column and the analysis was carried out at ambient temperature. The optimized peak in the chromatogram for POM was obtained at 3.80 min. The linearity range for analysis of CPH was obtained as 10 - 60 μg/ml. The developed HPLC method was validated as per ICH and ISO 17025 guidelines. Total error approach was also applied for the results of accuracy in validation of assay method. Uncertainty profile for Pomalidomide was established where beta value was taken equal to 66.7% with a confidence level of 90%. The uncertainty contour falling within 1% acceptance criteria for accuracy assures validity of the developed HPLC method.

Section B: This section comprises of development of SIAM for POM using DOE approach in gradient mode of HPLC. Also, degradation kinetics study was done for each stress conditions applied. The stability studies were done as per ICH guidelines for acid, base, neutral hydrolysis, oxidation, dry heat and photolytic stress conditions. For fulfillment of the ATP (Analytical target profile) for the developed method which required symmetric peak of DP2 and good resolution between the peaks of drug substance and DP2, Central composite design (CCD) of Response surface methodology (RSM) was applied. On application of stress conditions, three DP's were obtained in acid, base hydrolysis and oxidation stress conditions. In neutral hydrolysis, photolytic and dry heat degradation conditions, degradation of drug was observed but no DP was obtained in the chromatogram. On application of experimental design, 20 analytical runs were taken as generated for the design. The responses for all runs were noted and then further analysis was for each response considered for the design. From 8 solutions obtained in the design space, best fitting for robust results was chosen as optimal for our study. The optimized chromatographic conditions used were A=Acetonitrile-0.2% and B=Formic acid (pH- 2.84) for mobile phase by establishing a gradient elution technique. Gradient set for mobile phase composition was (time/%B): 0.01/55, 2.6/62, 4/ 70, 5/60, 10/55, detection wavelength used was 225nm. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000 psi respectively. The analysis was performed at ambient temperature with injection volume of 20 μl. The mobile phase

was filtered through 0.2 µm disposable filters from Ultipore®, PALL life sciences (40 mm) and degassed by ultrasonic vibrations prior to use. Further degradation kinetic studies were done for each stress conditions at three levels of stressor viz., at variable elevated temperatures, at different time intervals and at variable strengths of stressor used for the study. These studies gave the order of reaction which can be used for the prediction of degradation rate constants, half lives, shelf lives and activation energy of the chemical reaction. After studying different parameters that affected the rate of the degradation it was concluded that the degradation rate is directly proportional to temperature, time interval and strength of stressor. Also it was inferred that POM follows first order kinetics for acid, base hydrolysis and oxidation stress conditions whereas it follows zero order kinetics for photolytic, dry heat degradation and neutral hydrolysis stress conditions. The developed method was further validated as per ICH guidelines and found out the system suitability parameters within the acceptance limits.

Chapter 6 consists of development of bioanalytical method for estimation of Cyproheptadine HCl in human plasma and its application in rat pharmacokinetic study. Sample preparation technique used for the study plays a significant role with respect to bioanalytical samples. The techniques tried by us for sample extraction were protein precipitation (PP), liquid liquid extraction (LLE) and solid phase extraction (SPE). Various solvent compositions and procedures were tried for extraction of drug from the biological matrix and finally LLE method comprising of ammonium formate buffer (pH=4) and n-hexane was finalized for our study giving >99% recovery of drug from the biological matrix. The method was easy, fast and didn't require any special equipment for extraction; also it gave better recovery than SPE technique. For development of chromatographic analytical method for CPH in human plasma, the composition of mobile phase consisted of 20 mM formate buffer: methanol: acetonitrile, pH-5.5, adjusted with 0.2%formic acid (50:10:40, v/v/v) as optimized for assay of drug in chapter 3. The detection wavelength was set at 224 nm, flow rate was set as 1.0 ml/min, separation was achieved using BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm) column and the analysis was carried out at ambient temperature. The optimized peak in the chromatogram for CPH was obtained at 6.80 min and of IS at RT of 4.70 min. The linearity range for analysis of CPH was obtained as 100-800 ng/ml. The developed HPLC method was validated as per ICH guidelines. Oxcarbazepine (OXZ) was used as internal standard for HPLC study. Stability studies were done for CPH. Bench Top Stability study, Freeze-Thaw Stability study, Room Temperature Stock Solution Stability study, Refrigerated stock solution stability study was done and its was inferred to be stable for analysis of biological study. Further pharmacokinetic study was done using Male Wistar Albino rats. The pharmacokinetic analysis inferred *Tmax* to be 4 hrs which helps in selection of dosage regimen for CPH. It also illustrates that a metabolite (M1) is formed on introduction of Cyproheptadine HCl to rats. The developed HPLC method was validated as per ICH guidelines and found out the system suitability parameters within the acceptance limits.

Chapter 7 encases development of chemometrics assisted spectrophotometric and DOE based HPLC method for checking the adulteration of phytopharmaceutical by their corresponding synthetic analogue. The study described above is presented into two sections.

Section A: This section comprises of development and validation of DOE based RP-HPLC method for checking adulteration of Sildenafil citrate (SIL), Verdenafil (VER), Tadalafil (TAD) in Ashwagandha (ASH) herbal tablets. We took herbal markers Withanolide A (WDA) and Withaferin A (WFA) as active constituents of Ashwagandha. As per literature WDA and WFA are one of the vital constituents of ASH for immunomodulatory and aphrodisiac activity. Thus, a chromatographic method consisting of 5 constituents (SIL, VER, TAD, WFA, WDA) needed to be developed along with interferences from other constituents present in ASH root powder such that the interference peaks of other constituents does not interfere with the main peaks of 5 components. Based on preliminary trials the required ATP for our study consisted on stable retention time of SIL and VER, asymmetry of SIL and VER as well as resolution between peaks of SIL and VER. All other constituents were well resolved and were giving symmetric peaks. For selection of factors which can have influence on the chromatographic method, CNX approach using cause and effect diagram was utilized which screened 6 factors having effect on the results specified in the ATP of our study. Then screening needed to be done of the 6 factors selected from CNX approach. For screening purpose, D-optimal screening design was utilized. Based on statistics, main effects plots, Pareto charts and residual plots, 4 factors namely, % organic phase at hold time (ml), Hold time in the gradient elution method (min)**,** %TEA in aqueous phase and pH of aqueous solvent in the mobile phase were screened for finally optimization of HPLC method. 2^4 full factorial design was used for optimization of HPLC method. Based on statistics showing factorial fit for responses, mathematical equation for the responses and plots for optimization namely contour plots, 17 solutions were obtained. On practical implementation of the solutions, the optimum design space was obtained for chromatographic method. A gradient chromatographic method was finalized falling in the design space. Mobile phase ratio of 65:35 (methanol: DDW added with 0.2% organic modifier TEA adjusted with formic acid for pH 6.5). The gradient elution followed (2min 65:35, 4min 75:25, 5.5min 80:20, 8min 65:35, 15min 65:35) scheme. The detection wavelength was set at 254 nm, flow rate was set as 1.0 ml/min, separation was achieved using Waters C_{18} (250 mm x 4.6 mm, 5 μm) column and the analysis was carried out at ambient temperature. The optimized peak in the chromatogram for SIL, VER, TAD, WFA, WDA was obtained at 4.0, 6.7, 7.3, 9.0, 11.3 min. The linearity range for analysis of SIL, VER, TAD, WFA, and WDA was obtained as 2-12 μg/ml. The developed HPLC method was used for differentiating between counterfeit, placebo and marketed samples and found that none of the marketed sample was adulterated with synthetic analogue, also method was specific as no analyte peak was observed in placebo samples and also was selective as deliberately prepared counterfeit samples in laboratory gave peaks for synthetic analogues as well as herbal markers. The developed HPLC method was validated as per ICH guidelines and found out the system suitability parameters within the acceptance limits.

Section B: This section consist of development of chemometric assisted analytical methods for checking adulteration of Sildenafil citrate, Verdenafil, Tadalafil in Ashwagandha herbal tablets using NIR, Raman and ATR data**.** Here we took 15 samples, 6 were marketed formulations of ASH tables, 5 were laboratory prepared counterfeit samples and 4 were placebo samples. We conducted their NIR, FTIR and RAMAN analysis. Their spectra were taken in zero order as well using SGolay derivatization into

first order for NIR and FTIR data and into second order for RAMAN data. The Exploratory data analysis (EDA) utilizing the chemometric techniques such as Principal component analysis (PCA) and Hierarchical cluster analysis (HCA), which reduce large complex data sets to simplified and interpretable views were applied. These views highlight the natural groupings in the data and show which variables most strongly influence those patterns. Based on the results it was concluded that a more clear distinction between different groups of samples was obtained by derivatizing the data. After derivatizing also more distinguished clusters were obtained by second order derivative Raman data. Also, a considerable distinction was obtained from FTIR data by zero as well as first derivative. The first two principal components were considered for development of models, the explained variance for each data source viz., Raman, NIR as well as FTIR for zero order and derivative data were also calculated. By combination of chromatographic and spectroscopic analysis, a detailed analytical profile for distinction of Counterfeit, Placebo and Marketed samples of ASH tablets was established.

Chapter 8 consists of smart phone based low cost and rapid estimation of analytes using paper microfluidic devices. Microfluidic is actually the science and technology of systems that process or manipulate small amounts of fluids, using channels with dimensions of tens to hundreds of micrometers. A new horizon for development of microfluidic devices have evolved which is paper based microfluidic devices for various applications. The fabrication of paper chip developed at our premises is very easy and economic. The fabrication of paper microfluidic device was accomplished using Whatman filter paper, laboratory parafilm and other miscellaneous constituents. The fabrication procedure used was same for both paper microfluidic devices developed by us. First we developed paper microfluidic device for analytical purpose for checking adulteration of Tadalafil (TAD) as adulterant in herbal Ashwagandha formulations. Another paper microfluidic device developed by us was for diagnosis of pernicious anemia. As per literature study, methyl malonic acid (MMA) is released as a biomarker in urine in patients suffering from pernicious anemia. Thus, for detection of MMA in urine a paper microfluidic device was developed. Both paper microfluidic devices developed by us were based on principle of colorimetry. The reagents specific for colorimetry reaction with the analytes were impregnated into the paper microfluidic device and then if the analyte is present in the sample, the color change infers the presence of analyte. The study described above is presented in two sections.

Section A: This section consists of estimation of Tadalafil as adulterant in herbal formulation by paper microfluidic device. As the principle for detection was based on colorimetry, first trials were taken for development of optimized spectrophotometric colorimetry method for estimation of TAD. Various trials using varied reagents were taken. Also the method thus developed had to be applied for application to paper microfluidic device. Finally analysis of sample using reagents 1N Na_2CO_3 with 0.05% $KMnO_4$ gave the best results. The concentration of reagents and volumes of reagents taken were varied for spectrophotometric as well microfluidic method. For spectrophotometric method 5 – 30 µg/ml was selected as the linearity range and 603 nm was chosen as the λmax for the study using UV spectrophotometer in visible range. For

paper microfluidic device method 125 – 1250 µg/ml was selected as the linearity range and analysis was carried out using photometrix app in android phone. The linearity range was varied for both methods as to balance the signal to noise ratio in spectrophotometric method and for achieving visibility of color change in paper microfluidic method. Both developed methods were used for checking their applicability in marketed sample of TAD and found to be specific. Both developed methods were used for differentiating between counterfeit, placebo and marketed samples of ASH and found that none of the marketed sample of ASH was adulterated with synthetic analogue, also method was specific as no color change was observed in placebo samples and also method was selective as deliberately prepared counterfeit sample gave color change for presence of TAD. Both methods were validated as per ICH guidelines.

Section B: This section encases estimation of MMA as a biomarker for diagnosis of pernicious anemia by paper microfluidic device. As the principle for detection was based on colorimetry, first trials were taken for development of optimized spectrophotometric colorimetry method for estimation of MMA. Various trials using varied reagents were taken. Also the method thus developed had to be applied for application to paper microfluidic device. Finally analysis of sample using reagents 0.5% FBB with ammonia gave the best results. The concentration of reagents and volumes of reagents taken were varied for spectrophotometric as well microfluidic method. For spectrophotometric method 10 – 60 µg/ml was selected as the linearity range and 526 nm was chosen as the λmax for the study using UV spectrophotometer in visible range. For paper microfluidic device method 50-10000 µg/ml was selected as the linearity range and analysis was carried out using photometrix app in android phone. The linearity range was varied for both methods as to balance the signal to noise ratio in spectrophotometric method and for achieving visibility of color change in paper microfluidic method. Application of both methods was checked on artificial urine prepared at laboratory premises spiked with MMA. Due to presence of MMA in urine samples, color change was observed for spectrophotometric as well as paper microfluidic device methods signifying applicability and selectivity of both methods. Both methods were validated as per ICH guidelines.