A SYNOPSIS OF THE THESIS ENTITLED

INVESTIGATIONS ON NOVEL ANALYTICAL APPROACHES FOR SOME DRUGS AND PHARMACEUTICALS

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INTRODUCTION

The development of the pharmaceuticals brought a revolution in human health. These pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceuticals risky to be administered thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role. From the stages of drug development to marketing and post marketing, analytical techniques play a great role, be it understanding the physical and chemical stability of the drug, impact on the selection and design of the dosage form, assessing the stability of the drug molecules, quantitation of the impurities and identification of those impurities which are above the established threshold essential to evaluate the toxicity profiles of these impurities to distinguish these from that of the API, when applicable and assessing the content of drug in the marketed products. The analysis of drug and its metabolite which may be either quantitative or qualitative is extensively applied in the pharmacokinetic studies.[1]

1. Novel analytical methods development:

} In present era, market is floated with various combinations dosage forms and the number is increased day by day. These multicomponent formulations are gaining interest due to greater patient acceptability, increased potency, multiple action, fewer side effects, and quicker relief. Therefore, it is desired that these formulations meet the entire standards related to their quality, safety, and efficacy. This can only be possible if different newer analytical techniques are available for their determination. Different UV spectrophotometric methods are used in simultaneous multicomponent analysis. Such methods are based on recording and mathematically processing absorption spectra. Examples of such spectrophotometric methods include, simultaneous equation method, difference spectrophotometry, derivative spectrophotometry, absorbance ratio spectra, derivative ratio spectra, double divisor ratio spectra derivative method, successive ratio derivative spectra, Q absorbance ratio method, isobestic point method, absorpitivity factor method, dual wavelength method, ratio subtraction method, mean centering of the ratio spectra, absorption factor method and multivariate methods etc. [2, 3, 4]

} Chemometrics is also a major developed novel branch which is widely employed in various pharmaceutical fields. Basically it is combination of chemical data along with mathematical and statistical treatment of the data using various mathematical models and softwares. The developmental of various computational complexities along with the widespread growth of spectroscopy has used chemometrics for better results of the data obtained in spectroscopy. Examples of some of the chemometrics methods include Inverse least square, Partial least square, Classical least square, Principal component regression.

} Nowadays a major focus of pharmaceutical research is based on development of more effective dosage forms of existing drugs. For this purpose more focus on novel drug delivery systems is going on. To effectively design and exploit drug delivery systems, the underlying characteristic of a dosage form must be understood from the characteristics of the individual formulation components, to how they act and interact within the formulation, and finally, to how this formulation responds in different biological

environments. To achieve this, there is a wide range of novel analytical techniques that can be adopted to understand and elucidate the mechanics of drug delivery and drug formulation. Such methods include e.g. spectroscopic analysis; thermal investigations, surface analytical techniques, particle size analysis, rheological techniques, methods to characterize drug stability and release, and biological analysis in appropriate cell and animal models, [13] chromatographic techniques like HPLC, HPTLC, size exclusion chromatography, ion exchange chromatography etc.

} The HPLC methods developed need to be stability indicating as during the shelf life of product if potency is needed to be checked stability indicating analytical methods (SIAM) aid in more thorough and successful analysis. The main goal of SIAM's is the establishment of stability of drug substances and products by providing information about the conditions of stress testing. Several synonyms have been used in literature for stress testing which are stress studies, forced degradation studies, stress decomposition studies and forced decomposition studies. Although, in industry these studies have been in practice for along time, but was mandated with the advent of ICH guidelines. If drug is prone to degrade in certain conditions like photolytic, hydrolytic or oxidisable, its degree of degradation needs to be known by studying the degradation kinetics of the drug. Degradation kinetics helps in better understanding of degradation behaviour of drug substance and its interactions with excipients and thus aids in better development of formulation. The majorities of degradation reactions of pharmaceuticals take place at finite rate and are chemical in nature. Solvents, concentration of reactants, temperature, pH of the medium, radiation energy and presence of catalyst are the important factors that affect these reactions. The order of the reaction is characterized by the manner in which the reaction rate depends on the reactant concentration. The degradation of most pharmaceuticals is classified as Zero order, first order or Pseudo first order, although the compounds may degrade by complicated mechanisms and the true expression may be of higher order or be complex and non integer.[12]

} The International Conference on Harmonization (ICH) guideline indicates stress testing to determine the intrinsic stability of the molecule. The procedure infers by establishing degradation pathway in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedure used. The ICH guidelines 'stability testing of new drug substances and products' Q1A requires that stress testing should be carried out to elucidate the substance. The ICH guidelines Q3B entitled 'Impurities in New Drug Products emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of the degradation product. It is also required that analytical method should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug products.

} Also there is immense need for establishing the impurity profiling of drug substance to circumvent the possible deleterious effects of impurities on the health status of the society. Impurity profiling describes the account of maximum possible types of identified or unidentified impurities present in any drug substance. These impurities may be drug substance related impurities, process related impurities or stability related impurities. Stability related impurities include degradation products formed due to stress degradation conditions, interactions of drug substance with excipients. Various regulatory authorities like ICH, USFDA have specified limits for presence of these impurities in drug substance as presence of these impurities above the specified limits may influence the bioavailability, safety and efficacy of drug substance.[11]

2. Detection of counterfeit or fake drugs by spectroscopic techniques and application of novel chemometric approaches for it estimation

} In recent years, the emergence of counterfeit medicines has become a serious problem on the national and international level. In most developed countries due to effective regulatory systems and control on pharma market, the incidence of counterfeit medicines is less as compared to the developing countries especially in Asia where many counterfeit medicines are produced, and in countries like Africa, where poverty and loosed regulatory oversight make the existence of counterfeit products easier[5]. In recent years due obsession for ayurvedic medicines and phytopharmaceuticals, their demand have increased tremendously. Thus, enormous market has been flourished for herbal medications for life style treatments. Due to ever increasing demand, various pharmaceutical manufacturers have also plunged into phytopharmaceutical manufacturing. But the major drawback of ayurvedic medicines is that it takes long time intervals for curbing the ailment or for the therapeutic effect and this drawback invites a major limitation for its use. To, overcome the drawback an illegal practice of counterfeiting the herbal medicines with their corresponding synthetic analogues have emerged to a great extent in recent past. The synthetic analogues are added without consideration of its lethal doses and other side effects thus exposing society to a great extent of danger from the counterfeited herbal medicines. Thus, WHO, FDA and EMA have proposed many guidelines for maintaining the quality of herbal medicines. Various methods have been reported for estimation of synthetic phosphodiesterase inhibitors in pharmaceutical preparation using techniques like HPLC [6], LCMSMS [7]and XRF [8] etc.

} "A counterfeit medicine is one which is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products and counterfeit products may include products with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient active ingredient or with fake packaging." [9]

} For the specific detection of counterfeit drugs, spectroscopic techniques are preferred because they are fast and need only a little sample preparation or no preparation at all. Raman spectroscopy, near infrared spectroscopy (NIR) are the widely use for this purpose. Fourier transform infrared spectroscopy (FTIR) has also been used. However recently these three techniques are often used with the aid of chemometric techniques for detecting counterfeit drugs owing to the benefits of the later. [10]

3. Development of novel paper based microfluidic devices for analytical and diagnostic applications

} 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. The working principle of micro fluidic devices is based on capillary action of fluids containing the constituent to be detected through the porous micron and submicron particles layer which is invaginated by various techniques and its contains the reagent having reaction with constituent to be detected. [14] Microfluidic devices for diagnostic applications have reached into the very critical areas of diagnosis like for gene sequencing, protein analysis, pathogen detection and many others. In development of diagnostic microfluidics, principle of sandwich enzyme immunoassay with a unique mono-mono antibody combination specific against the antigen to be analysed is deployed. The microfluidics developed for diagnostic applications are also cited as point of care tests (POCT's). Also electrochemical and chromatographic principles have been investigated for burgeoning of diagnostic microfluidics nevertheless enzyme immunoassay based assays stay the primary

choice for aforesaid. Whereas for analytical studies paper based micro fluidic devices (μPADs) utilizing fast lithographic activation of sheets (Flash technology), colorimetry, florescence analysis, electrochemical sensing as well as paper chromatography have been resorted. [15] Also newer refinements like digital microfluidics and polymer based multichannel microfluidic systems for chromatographic applications have been flourished for analytical applications of microfluidics. Microfluidic devices for antibiotic assay and for various other microbiological applications are also reported fostering new pathways for microbiological studies. Colorimetry, floroscence and electrochemical based microfluidics for detection of counterfeits have also flourished to a great extent in the near past paving a new facet for microfluidics in regulatory area of pharmaceutical industry. Tremedous novel advancements also have been reported for polymer based microfluidics for formulation and development applications. They have the advantage of being portable and inexpensive which proves them to be a boon for the remote areas where the state of the art laboratory facilities are limited. [16]

OBJECTIVES OF THE STUDY

The specific aims of the work undertaken were:

1. Development and validation of novel analytical methods for some drugs for their routine analysis.
2. To develop novel chemometric and HPLC assisted analytical methods for checking the adulteration of phytopharmaceuticals.
3. To develop novel paper based microfluidic devices as rapid diagnostic and analytical tools.

METHODOLOGY:-

1. **Development and validation of novel analytical methods for some drugs for their routine analysis.**

Part I A: Exploration of various classical and chemometric assisted UV spectrophotometric methods for estimation of chlorhexidine gluconate (CH) and cetrimide (CET) in bulk and its formulation

Various methods developed under the said heading are as follows:

1. Vieordt's method

} If a sample containing two absorbing drug (X and Y) and each drug absorbs at each other's λmax, then it may possible to determine both drugs by the technique of simultaneous equations. Solutions with concentrations 30-180 μg/ml (for Cetrimide) and 3-18 μg/ml (for Chlorhexidine gluconate) were prepared and scanned between 200 to 400 nm. Analytical wavelength selected for CET was 217 nm and for CH was 260 nm

2. First Derivative spectroscopy method

} The first derivative transformation was done of zero order spectra and zero crossing point (ZCP) 275nm of CET for detection of CH was taken and ZCP 222nm of CH was taken for detection of CET.

3. Multicomponent analysis method

} The multi-component quantitation mode analysis is the mode in which the concentration of each constituent is determine by using absorption spectrum of the mixed sample with pure standards or standards made up of multiple constituent components. The two input wavelengths selected were 217nm and 260nm.

4. Absorption ratio spectra method

} In this method, the spectra of mixture are divided by the spectra of any one analyte (CET), for analysis of other corresponding analyte (CH) in the mixture. Then in this modified spectra two wavelengths are selected (λ1=200nm and λ2=217). Now λ2-λ1 will give absorption ratio spectra value for analyte CH. This way for CET also, absorption ratio spectra value is found out. The two wavelengths selected for CET are (λ1=225nm and λ2=263). Here λ2-λ1 will give absorption ratio spectra value for analyte CET. The above stated is done for whole calibration range. The relationship is linear and thus applied for analysis of unknown samples. λmax for CH was chosen as a difference of 217 nm and 200 nm while for CET it was chosen a difference of 263 and 225 nm. The linearity range selected for CH was 3-18 µg/ml and for CET was 30-180 µg/ml.

5. Mean centering of ratio spectra method

} Mean centering of ratio spectra method is an improvement for resolution of two analytes in a mixture. Also it eliminates the need for preliminary steps like derivatising the sample and also S/N ratio is improved in it. In this method first the analysis of mixture (Am) in entire calibration range was done at a λmax of CET (217 nm). This Am values are then divided by molar absorptivity (α_{CH}=505446 1/mol/cm). This Am/α_{CH} values for entire calibration range are then mean centered using software package Unscrambler X, version 10.5 and fed to model for analysis of CET. This way for CH also Am/ α_{CET} is done (where λmax of CH is 260 nm and αCET= 364450 1/mol/cm) and then mean centered and fed for analysis of CH. These values can now act as a predictor for future analysis of unknown sample.

} Chemometric methods are one kind of multivariate analysis i.e. considering more than one variable at a time. Here, we are considering absorbance at 20 different wavelengths (220 to 260 nm with interval of 1.0 nm) – 20 variables in contrast to other univariate methods described earlier where absorbance at only one wavelength is considered (Absorbance matrix 'A')

4 different methods developed under the said heading were:

1. Classical Least Squares
2. Inverse Least Squares
3. Principal Component Regression
4. Partial Least Squares or Projection to Latent Structures

The developed UV spectrophotometric methods were found to be valid, simple, rapid, accurate, precise and specific and sensitive for estimation of Chlorhexidine gluconate and Cetrimide. The sample recoveries for all methods were in good agreement with their respective label claims, which suggested non-interference of formulation additives

in its estimation. Hence, the developed methods could be successfully applied for estimation of Chlorhexidine gluconate and Cetrimide in bulk and its marketed formulation. Statistical analysis was done using One way Anova, Tukey HSD test and Scheffe test.

Part I B: Application of QBD and statistical analysis for validation of RP-HPLC method for chlrohexidine gluconate and cetrimide in its bulk and pharmaceutical dosage form and development of simple UV and RP-HPLC method for estimation of Cetrimide in its bulk and dosage form

} In this part, development of analytical method for simultaneous estimation of Chlorhexidine gluconate and Cetrimide by RP-HPLC method was carried out. The chromatographic conditions were successfully optimized for the separation of Chlorhexidine gluconate and Cetrimide by using Hypersil BDS C_{18} column (4.6×150mm) 5µ, flow rate of 0.8 ml/min, mobile phase ratio of (30:55:15 v/v/v) ACN: methanol: phosphate buffer (KH_2PO_4 and K_2HPO_4) phosphate pH 3 (pH was adjusted with orthophosphoricacid) and detection wavelength used was 210nm. The retention times were found to be 3.10 mins and 3.9 mins for Cetrimide and Chlorhexidine gluconate respectively. The % purity of Chlorhexidine gluconate and Cetrimide was found to be 99.92% and 100.45 % respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1). The linearity study of Chlorhexidine gluconate and Cetrimide was found in concentration range of 3 µg-18µg and 30µg-180 µg. DOE was applied in validation for checking robustness of the developed method using Box-Behnken design and also normal distribution of data was verified by Anderson-Darling normality test. The method developed was found to be specific, selective, and robust and can be applied for routine analysis of marketed formulation in laboratory premises.

} Also, development of analytical method for estimation of Cetrimide by RP-HPLC method was carried out. The chromatographic conditions were successfully optimized for the detection of Cetrimide by using Hypersil BDS C_{18} column (4.6×150mm) 5µ, flow rate of 1 ml/min, mobile phase ratio of (50:30:20 v/v/v) ACN: methanol: ammonium formate buffer pH 3 (pH was adjusted with formic acid) and detection wavelength used was 217nm. The retention time was found to be 3.10 min for Cetrimide. The % purity of Cetrimide was found to be 100.066. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1). The linearity study of Cetrimide was found in concentration range of 30µg-180 µg. Normal distribution of data was verified by Anderson-Darling normality test. The method developed was found to be specific, selective, and robust and can be applied for routine analysis of marketed formulation in laboratory premises.

Part I C: Development of QBD based stability indicating analytical method, degradation kinetics of Pomalidomide and recovery analysis for validation using a risk based approach

} The objective of this work was to develop stability indicating analytical method for estimation of Pomalidomide and its degradation products by RP-HPLC method. For

acquiring resolved and symmetric peaks of drug substance and degradation products during the stability study DOE was applied. The chromatographic conditions selected for the separation of Pomalidomide and its degradation products included use of Hypersil BDS C_{18} column (4.6×150mm) 5µ, flow rate was 1.0 ml/min, gradient set for mobile phase composition was %ACN(A) and 0.2%Formic acid (B), pH- 2.84 (pH was adjusted with formic acid with time interval ratio of 0.0-2.84 min(45:55 %v/v, A:B), 2.85-3.5 min(38.53:61.47%v/v, A:B), 3.6-4min(30:70%v/v, A:B), 4.1-5 min (40:60%v/v, A:B), and 5.1-10min (45:55 %v/v, A:B) , detection wavelength used was 225nm. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1). The method showed linearity in range of 10 µg/ml - 60 µg/ml. Total error approach was applied for accuracy of the developed method using uncertainty profile taking Beta value as 66.7% at 90% Confidence interval. Degradation kinetics study was undertaken for acid, base hydrolysis, peroxide degradation, photolytic as well as thermal dry heat induced degradation. Elucidation of degradation pathways, determination of order of reactions, half-life as well as shelf life was possible due to the study. The method developed was found to be specific, selective, and robust and can be applied for routine analysis of marketed formulation in laboratory premises.

Part I D: Impurity profiling of Cyproheptadine HCl in its bulk and dosage

In this study, impurity profile was determined for Cyproheptadine HCl. A novel LC-PDA method was developed for analysis of Cyproheptadine HCl in its bulk and dosage form. ICH prescribed Stress degradation was carried out to study the degradation behaviour of Cyprohepatadine HCl. From the stress degradation study, 2 major degradation products were found out in acid and base hydrolysis condition. For characterization of major degradation products, LC-MS/MS analysis was done which indicated a same impurity in both acid and base hydrolysis as per m/z values of the degradation products. The isolation of impurity was carried out by preparative TLC technique. Then for further characterization ^{1}H NMR, ^{13}C NMR,^{135} DEPT NMR, D_20 exchange, IR and DSC analysis of the impurity was carried out which inferred the impurity to be 4-(5Hdibenzo [a, d] - cyclohepten-5-ylidene). A stability indicating RP-HPLC method for identification and quantification of CPH and CPH degradation product I was developed and validated as per ICH Q2 (R1) guideline. The method was simple, sensitive, accurate and fast which is applicable for the assay of CPH and estimation of CPH degradation product I.

Part I E: Bionalytical method development for Cyproheptadine HCl in human plasma and application to rat pharmacokinetic study

} In bionalytical method development sample preparation is a technique which is used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. For this step 3 methods were tried as follows:

[i] For Protein precipitation the solvents tried were Acetonitrile, Methanol, ACN:MeOH (50:50), Trichloroaceitic acid, Perchloric acid

[ii] For Liquid Liquid Extraction the solvents tried were NaoH, n-pentane,n-hexane, MTBE, Ethyl acetate, Formate buffer

[iii] For Solid phase extraction the cartridges tried were Oasis HLB SPE cartridges (Waters), Orochem SPE Cartridges and C18 Supelco cartridges

} For development of analytical method, Cyproheptadine HCl was estimated in human plasma after liquid liquid extraction using 20 mm formate buffer and n-hexane as extracting solvents and oxcarbazepine as internal standard. For separation of Cyproheptadine HCl from plasma components, Hypersil BDS C_{18} column (250×4.6 mm i.d, 5µ particle size) at ambient temperature, 224 nm as detection wavelength and acetonitrile, methanol and 20 mM ammonium formate (pH 5.5 adjusted with 0.2% formic acid) (40:10:50, v/v/v) as the mobile phase and at a flow rate of 1 ml/min was used. This newly developed method showed good calibration curve in the concentration range of 100 – 800 ng/ml with excellent correlation coefficient (r^2>0.998) and giving recovery more than 99%. The %RSD for both intraday and interday was less than 2%. The newly developed and validated HPLC-PDA method was easy, fast and effectively utilized for pharmacokinetic studies in rats after oral administration of Cyproheptadine HCl.

Part II- To develop novel chemometric assisted and HPLC analytical methods for checking the adulteration of Phytopharmaceuticals

The main objective of the study is to develop methods for checking the adulteration of synthetic analogues Sildenafil citrate, Verdenafil and Tadalafil in Ashwangandha Herbal tablets.

Part II A: Exploration of spectroscopic techniques like NIR/Raman/ATR spectroscopy along with the Chemometric approach for checking the adulteration of synthetic analogues like Sildenafil citrate, Verdenafil and Tadalafil in Ashwangandha Herbal tablets.

} The objective of this work was to establish analytical techniques for identification of counterfeit medicines in herbal drugs. The herbal drug taken into consideration for the study was Withania Sominfera (Ashwagandha), vital constituent of herbal aphrodisiac formulations which is prone to be adulterated with synthetic constituents like sildenafil, verdenafil and tadalafil. Spectroscopic techniques like Near Infra red spectroscopy, Fourier transform Near Infra red spectroscopy and Raman spectroscopy analytics were carried out for elucidation of counterfeits in authentic herbal formulations. Statistical analysis of the data were carried out using chemometrics principles where methods like principal component analysis and hierchial cluster analysis statistical models were developed to differentiate between the authentic, counterfeit and placebo samples. The above stated methods are very easy to follow as no sample preparation is needed in any of them. Direct powder samples are utilized for the analysis. All samples (counterfeit, original and placebo) for each drug were first measured by the FTIR. The scan covered the range (4000-600cm-1) nm (about 2 nm increments). The spectrum of each sample was an average of five scans. A matrix

of 15×1763 (15 samples and 1763 wavelengths) was formed which was subjected to chemometric modelling. The samples were then measured by NIR. The scan covered the range (700-2500cm^{-1}) at about 8 nm increments. The spectrum of each sample was an average of five scans. A matrix of was 15×100 (15 samples and 100 wavelengths) was formed in study which was subjected to chemometric modelling. The samples were analysed by Raman spectrometer using a laser of 785 nm wavelength for excitation. The Raman signals scanned over a range of 700-1800 cm^{-1} Raman shift with 30 seconds of integration time. The spectrum of each sample was an average of ten scans. A matrix of 15×1100 (15 samples and 1100 wavelengths) was formed in study which was subjected to chemometric modelling. Every drug formulation has a unique spectral fingerprint in the NIR, Raman and IR spectra that identifies the brand of the drug. Incorrect formulations containing foreign or substitute ingredients can put the patient's life under risk. Taking this basic concept to compare the entire spectra, chemometric techniques like PCA and HCA were used to detect fine differences in the spectra. The PCA and HCA were also performed by applying the Savitzky-Golay derivative method for more clear distinction between different groups. The spectroscopic analysis thus done was able to implicate satisfactory distinction between the CF, FM and PL samples. Raman spectroscopic analysis along with data manipulation by conducting Savizky Golay second order derivatization proved to be best method for clear distinction between the three different groups of samples in comparison to other spectroscopic analysis carried out in our study. The spectroscopic analytical profile thus developed can be easily extended for real world samples and thus provide a thorough testing profile for analysis of counterfeits if any found in Withania Somnifera authentic samples.

Part II B: Development and validation QBD based RP-HPLC method for checking adulteration of Sildenafil citrate, Verdenafil, Tadalafil in Ashwagandha Herbal tablets

} The objective of this work was to establish chromatographic method for identification of counterfeit medicines in herbal drugs. The herbal drug taken into consideration for the study was Withania Sominfera (Ashwagandha), vital constituent of herbal aphrodisiac formulations. The preliminary chemical constituents in Withania Somnifera are Withaferin A and Withanolide A. For immediate effect these herbal medications are prone to be adulterated with synthetic constituents like Sildenafil, Verdenafil and Tadalafil by some avaricious money grubbing herbal manufacturers. For its authentification, a 5 component QBD based HPLC method was developed, in which CNX approach was utilized for preliminary investigations; D-optimal screening design was utilized for screening of significantly affecting factors whereas full factorial design was utilized for optimization of HPLC method. For development of HPLC method, various method parameters needed to be considered based on response component attributes which can have an effect on system suitability of HPLC method for which CNX approach was utilized. Then considering the factors which could have a significant impact on the HPLC method, QBD was applied for screening the factors which would have a significant impact on the system suitability

parameters on the HPLC method. Also preliminary trials were taken for understanding the pattern for chromatographic analysis which showed that WDA and WFA were satisfactorily separated from the peaks of SIL, VER and TAD. Also TAD showed a significant resolved and symmetric peak from the other two active ingredients, whereas optimization for stable retention time of SIL and VER, asymmetry of SIL and VER and also on the resolution between peaks of SIL and VER needed to be worked out. For screening purpose D-optimal screening design was utilized considering seven factors namely pH of mobile phase, Hold time 2, % of Organic ratio at end of Hold time 2, % of TEA, Wavelength, Vol of sample injected, and Flow rate whereas the five responses which were considered included Resolution between the peaks of SIL and VER, Retention time of SIL and VER, Asymmetry of SIL and VER. Based on statistics the factors significantly affecting the method were screened and then considering the four screened factors, pH of mobile phase, Hold time 2, % of Organic ratio at end of Hold time 2, % of TEA optimization of method was done utilizing 2^4 full factorial design. Based on statistics, 17 solutions were obtained. On practical implementation of the solutions, the optimum design space was obtained for chromatographic method. A gradient chromatographic method was finalised falling in the design space. Solvent A consists of methanol and solvent B consists of aqueous phase. Aqueous phase consist of 0.2% organic modifier TEA, pH 6.5 adjusted with formic acid. The gradient for solvent A was 65%; 2min, 75%; 4min, 80 %; 8 min, 65%; 15 min mounting up 100 % for the counterpart solvent B, Wavelength maxima of 254 nm, flow rate of 1 ml/min. For HPLC analysis the column used was Waters C_{18} column (250×4.6 mm, 5 µm particle size). The optimized chromatogram for the 5 component system was achieved by the method. Using the optimized method, analysis was undertaken of 6 marketed formulations, 4 placebo samples and 5 counterfeit samples. None of the marketed formulations of Withania sominfera showed the peaks of probable adulterants SIL, VER and TAD. The placebo samples did not show peaks of WDA, WFA as well as SIL, VER and TAD. However, the laboratory prepared counterfeit samples showed peaks of deliberately added counterfeits for analysis in ground if at all found in real world samples. Thus by chromatographic analysis, a detailed analytical profile for distinction of Counterfeit, Placebo and Marketed authentic samples of Withania somnifera was established.

Part III –Development of novel paper based microfluidic devices as rapid diagnostic and analytical tools

Part III A: Development of paper microfluidic based qualitative and semi quantitative method of Tadalafil citrate as adulterant in herbal formulation visually and by utilization of a photometrix app of android phone.

} Fabrication: First fabrication of simple paper microfluidic chip needed to be done for which various hydrophilic and hydrophobic materials were tried and finally a procedure comprising of Parafilm M, Whatman filter paper no.1 along with the detecting agents was utilized for fabrication of our microfluidic chip. The chip developed was impregnated with the reagents responsible for the colorimetric detection of analytes.

- Analysis: Qualitative and semi quantitative testing of analyte (Tadalafil) was done on fabricated microfluidic device. Experimental studies initiated with UV spectrophotometric analysis of Tadalafil citrate. In UV range Tadalafil has wavelength maxima of 284 nm. But our approach was to develop a colorimetric method because we wanted to extrapolate the method for paper microfluidic device. Trials were undertaken utilizing various solvents and reagents for development of a specific and prominent colour reaction for qualitative detection of Tadalafil. Finally a specific and prominent colour reaction for Tadalafil was obtained utilizing 0.1% KMnO4 in IPA and 5N NaOH. The colour developed after the chemical reaction was cyan having wavelength maxima of 603 nm. The study was done at laboratory basis using UV 1700 instrument as well as on the paper microfluidic chip developed as stated previously. For UV analysis data manipulation was done using UV probe software and Microsoft excel which gave satisfactory calibration curve for the sample. Whereas data manipulation for results obtained on paper microfluidic chip was done using photometrix app available free in play store of android phones. Semi quantitative analysis was done and it gave satisfactory results. Also, results of assay utilizing both methods gave satisfactory results.
- Thereby, qualitative and semi quantitative analysis of Tadalafil using paper microfluidic device was achieved using above approach.

Part III B: Development of paper microfluidic based qualitative and semi quantitative method for methyl malonic acid as a biomarker in urine for diagnosis of Vitamin B_{12} deficiency anemia visually and by utilization of photometrix app of android phone.

WORK TO BE DONE

1. To develop a visual qualitative method for identification of Methyl malonic acid (MMA) as a biomarker in urine for diagnosis of Vitamin B12 deficiency anemia.

2. To develop semi quantitative method for analysis of Methyl malonic acid (MMA) using photometrix app of android phone.

3. Thesis writing and compilation

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