A Synopsis

On

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE ANTIDIABETIC POLYHERBAL FORMULATION.

Submitted to

Gujarat Technological University

For the Degree of

Doctor of Philosophy

In

Pharmacy

By

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A. TITLE OF THE THESIS AND ABSTRACT

 ölçüni: DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE ANTIDIABETIC POLYHERBAL FORMULATIONS.

Abstract

Diabetes mellitus is the most common endocrine disorder, affecting 16 million individuals in the United States and 200 million worldwide. Despite the use of advanced synthetic drugs for the treatment, use of herbal remedies is gaining higher importance because of synthetic drugs have drawbacks and limitations. Antidiabetic herbal formulations (AHF) are considered to be more effective for the management of diabetes. In recent days, different formulations are available which are used for management of Diabetes. So, present investigation was undertaken with a view to develop different analytical methods for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin, markers present in different antidiabetic Polyherbal formulations so as we can measure them in very precise manner. Most sensitive RP-HPLC method by applying QbD concept was developed and further LCMSMS study was performed to differentiate Curcumin from other Curcuminoids. HPTLC Method was developed by using less amount of solvents and with good resolution of peaks which is very helpful for early phase of formulation development. Chemometric Methods (CLS & ILS) were developed which are capable to determine constituents in presence of matrix very accurately. UV spectrophotometric Methods (Absorbance correction method and First order derivative) were developed as they are very much convenient and less time consuming and also useful for small scale industries. All developed methods were validated according to ICH guideline and statistical comparison was done by ONE WAY ANOVA method. Developed analytical methods can be widely used in recent era as this is a prime requirement for dossier submission and commercial acceptability.

B. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.[1]. Diabetes mellitus taking its place as one of the main threats to human health in the 21st century. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030.[2]. The increase in incidence in developing countries follows the trend of urbanization and lifestyle changes, perhaps most importantly a
"Western-style" diet [3]. Despite the use of advanced synthetic drugs for the treatment, use of herbal remedies is gaining higher importance because of synthetic drugs have drawbacks and limitations. Antidiabetic herbal formulations (AHF) are considered to be more effective for the management of diabetes. In recent days, different formulations are available which are used for management of Diabetes.

Plants synthesize substances that are useful for the maintenance of health in humans and other animals. Due to low toxicity and known pharmacological activity, herbal drugs have been popularly and extensively used for many centuries. Plants synthesize a variety of phytochemicals most of them are derivatives of a few biochemical motifs. All plants produce chemical compounds as part of their normal metabolic activities. These include primary and secondary metabolites [4]. “Health for All” is a dream and goal of WHO in which he gets successes somewhat and strives for more; but at the moment it has been proven that present pharmaceuticals are not successful in a satisfactory manner to offer general health benefits.

“Quality can be defined as the condition of a drug that is determined by its characteristics, purity, content, and supplementary chemical, physical and biological properties or by the built-up processes.” [5].

The term “herbal drugs” denotes plants or plant parts that have been converted into phyto pharmaceuticals by means of simple processes involving harvesting, drying, and storage. Hence they are capable of variation. This variability is also caused by differences in growth, geographical location, and time of harvesting.

Analysis of herbal drugs is a difficult task as compared to analysis of synthetic drugs because several problems not applicable to synthetic drugs influence the quality of herbal drugs and this is as given below [6-11].

- Herbal drugs are generally combination of many components.
- The active principle(s) is (are), in the majority cases mysterious.
- Selective analytical technique or reference compound could not exist commercially.
- Plant materials are chemically and naturally unpredictable.
- Chemo-varieties and chemo cultivars exist.
- The source and quality of the raw material is inconsistent.
- Adulteration and substitution is a burning problem.
Adulteration may be defined as mixing or substituting the original drug material with other spurious, inferior, defective, spoiled, useless other parts of same or different plant or harmful substances or drug which do not confirm with the official standards\textsuperscript{12}.

\textbf{Brief description on the state of the art of the research topic}

Traditional herbal therapies are prescribed by Ayurvedic and other complementary systems of medicines as remedies to many ailments in human and animals. A sizeable population in almost all developed countries uses at least one form of unconventional therapy including herbal medicines\textsuperscript{13-14}. These medicines are available as single or poly herbal preparations. Because of consumption of these herbal preparations by a large masses of developed as well as developing countries, there is a need to control and assure the quality of such preparations through systematic scientific studies including chemical standardization, biological assays and validated clinical trials\textsuperscript{15}. This importance of quality control and quality assurance of herbal products is driven by possible variations in the nature and content of constituents due to different times of harvesting and conditions of storage, processing and formulation methods.\textsuperscript{16-17}

In order to overcome all these difficulties and to develop more convenient, reliable and cost effective possible Analytical methods which can separate as many constituents as possible. The method development is to be extended to other marketed formulations in diabetes division, as this is a prime requirement for dossier and commercial acceptability.

\textbf{C. DEFINITION OF THE PROBLEM}

Literature Survey reveals that several methods such as Three HPTLC methods for simultaneous estimation of Gallic acid and Curcumin\textsuperscript{18-20}, Four HPLC method for simultaneous estimation of Gallic and Ellagic Acid\textsuperscript{21-24}, Four HPTLC methods for estimation of Gallic and Ellagic acid\textsuperscript{25-28}, One HPTLC method for estimation of Ellagic Acid and Curcumin have been reported\textsuperscript{29}. But, not a single UV, HPLC or HPTLC method is reported so far for simultaneous estimation of Gallic Acid, Ellagic Acid and Curcumin in Polyherbal Formulation. Hence, the aim of the present investigation was to develop analytical methods so the constituents present in Antidiabetic Herbal formulations can be detected in very precise manner without interference of other constituents.
D. OBJECTIVE AND SCOPE OF WORK

Overall Objective of Research are summarized as:

- To develop RP-HPLC Method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal antidiabetic formulations.
- To develop HPTLC Method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal antidiabetic formulations.
- To develop Chemometric Methods for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal antidiabetic formulations.
- To develop UV spectrophotometric Methods for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal antidiabetic formulations.
- All developed methods to be validated for specificity, linearity, accuracy, repeatability (Precision), ruggedness, limit of detection and limit of quantification, robustness and System suitability.
- To perform statistical comparison of developed methods.

- **Scope of Research work**
  Developed Methods for Polyherbal antidiabetic formulations can be effectively utilize in day to day analysis in Quality control laboratories and as many variations which are frequently observed in every Herbal formulations due to geographical variation and Season variation can be minimized.

E. ORIGINAL CONTRIBUTION BY THE THESIS

The entire work in this synopsis, as well as thesis is original. Extensive literature review was done to identify the challenges associated with the measurement of bioactive constituents in Polyherbal formulations. RP-HPLC method can be utilized as it is most sensitive method and HPTLC Method can be utilized at the time of early phase of formulation development. Developed Chemometric methods can be utilized to minimize interference from matrix. UV Spectrophotometric methods can be easily utilized by small scale industries.
F. METHODOLOGY OF RESEARCH, RESULTS / COMPARISONS

➢ High Performance Liquid Chromatography:
A Quality by Design (QbD) Approach for the Development of a High-Performance Liquid Chromatography for the Simultaneous estimation of Gallic Acid, Ellagic Acid and Curcumin from Antidiabetic Polyherbal Formulations.

Analytical Quality by Design (AQbD) approach was used for the Optimization of a high-performance liquid chromatography method for the simultaneous estimation of Gallic Acid, Ellagic Acid and Curcumin in Polyherbal Antidiabetic formulations. In case of the QbD concept, Assay by the help of Design of Experiments (DOE) and Response Surface Methodology (RSM) by Central Composite design (CCD), in order to obtain a good separation along with quantification of all compounds along with a minimum analysis time along with better resolution. A deep understanding of the Analytical Target Profile (ATP), followed by a risk assessment for variables that affect the efficiency of the method led to the development of a precise, accurate and cost-effective method. The separation was achieved on Agilent C18 column (25 cm, 4.6 mm, 5μm) using gradient mobile phase. Mobile Phase A: Water with % Formic acid and Mobile Phase B: Acetonitrile. The flow rate was 0.95 mL/min. Retention time of Gallic acid, Ellagic acid and curcumin were found to be 3.267, 4.633 and 12.527 minutes respectively. The assay was linear over the range of 2-14 μg/ml for Gallic Acid, 5-35 μg/ml for Ellagic Acid and 1-7 μg/ml for Curcumin. The intra-and inter-day precision were less than 2%, with accuracies between 98-102% of the true values. The correlation coefficient for Gallic acid, Ellagic acid and Curcumin were found to be 0.9975, 0.9994 and 0.9981 respectively.
Approximately 2% – 6% (w/w) of turmeric is curcuminoids. The curcuminoids contains 80% curcumin, 18% desmethoxycurcumin and 2% bisdesmethoxycurcumin. So in the chromatogram, we obtain 3 consecutive peaks for curcumin. To confirm the same, LCMSMS Analysis was performed [first peak having molecular ion peak at 307.08 m/z ratio. So it is of bisdesmethoxycurcumin (molecular weight: 308.333 g/mol); second peak having molecular ion peak at 337.08 m/z ratio. So it is of desmethoxycurcumin (molecular weight: 338.333 g/mol); third peak having molecular ion peak at 367.08 m/z ratio. So it is of curcumin (molecular weight: 368.333 g/mol)] and from the different molecular weights all three constituents can be identify. The method was successfully applied to the quantification of Gallic Acid, Ellagic Acid and Curcumin simultaneously in different Antidiabetic Polyherbal Formulations.

- **High Performance Thin Layer Chromatography:**

Simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in antidiabetic Polyherbal formulations using a validated High Performance Thin Layer Chromatographic Method:

A simple, Rapid, Accurate, Precise and Economic HPTLC Method is developed and validated as per ICH Guideline for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin. The separation was achieved on CAMAG HPTLC system, using winCATS Planar Chromatography manager software and Linomat V as a sample Applicator. TCL Silica gel 60 F254 (20*20) was used as Stationary Phase and Toluene: Ethyl acetate: Formic Acid (3: 3.5: 1 v/v) as a Mobile Phase. Detection was done at 300 nm using D2 Lamp and 50°C temperature was maintained throughout the day. Rf value of Gallic acid, Ellagic acid and Curcumin were found to be 0.59, 0.51 and 0.78. The linearity of Gallic Acid, Ellagic Acid and Curcumin was in the range of 20-400 ng/band, 50-1000 ng/band and 10-200 ng/band of Gallic acid, Ellagic acid and Curcumin respectively.
The correlation co-efficient (‘r’ value) for Gallic acid, Ellagic acid and Curcumin were found to be 0.9972, 0.9957 and 0.9981 respectively. Percentage recoveries obtained for Gallic acid, Ellagic acid and Curcumin in the range of 98.12 – 100.50 %, 98.69 – 101.14 % and 98.36 – 101.24 % respectively. Method can be validated as per ICH Q2R1 guideline. Method can be applied for routine estimation of Gallic acid, Ellagic acid and Curcumin in Antidiabetic Polyherbal formulations.

➢ Chemometric Methods:

- **Simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Antidiabetic Polyherbal Formulation by Classical Least Square (CLS) and Inverse Least Square (ILS) Method:**

In Chemometric Methods, first we have to prepare Calibration set to feed appropriate data to the software and validation set to validate that developed method.

Calibration Set: A training set consisting of 20 mixture solutions in the possible combinations containing 2 -20 μg/ml of Gallic acid 5-50 μg/ml of Ellagic acid & 1-10 μg/ml of Curcumin was used for Chemometric Calibrations. Randomly, take the mixture of all three markers and zero order absorbance spectra were measured & stored in the computer. To estimate the CLS and ILS models for the training set, the computer was fed with absorbance & concentration matrices at different wavelength range, then calculations were carried out with the use of software MATLAB R2015a.
Validation Set: Different mixtures of the three drugs were prepared by diluting different volumes of Gallic Acid, Ellagic Acid and Curcumin standard solutions in 10 ml measuring flask & diluting to volume with methanol.

In chemometric Methods like CLS, Value of coefficient was obtained by equation; \( A = K \times C \)

While In case of ILS, Value of coefficient was obtained by equation; \( C = P \times A \)

Where,

\( A = \) Absorbance

\( C = \) Concentration

\( K \) & \( P = \) Calibration coefficient

Following is the equation with coefficient values obtained by software.

<table>
<thead>
<tr>
<th>( C_{GALIC} )</th>
<th>( C_{ELLIC} )</th>
<th>( C_{CURCUM} )</th>
</tr>
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<tbody>
<tr>
<td>0.0029</td>
<td>0.0138</td>
<td>0.0028</td>
</tr>
<tr>
<td>0.0032</td>
<td>0.0149</td>
<td>0.0030</td>
</tr>
<tr>
<td>0.0035</td>
<td>0.0158</td>
<td>0.0032</td>
</tr>
<tr>
<td>0.0039</td>
<td>0.0166</td>
<td>0.0033</td>
</tr>
<tr>
<td>0.0044</td>
<td>0.0173</td>
<td>0.0033</td>
</tr>
<tr>
<td>0.0048</td>
<td>0.0180</td>
<td>0.0035</td>
</tr>
<tr>
<td>0.0053</td>
<td>0.0185</td>
<td>0.0036</td>
</tr>
<tr>
<td>0.0058</td>
<td>0.0185</td>
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</tr>
<tr>
<td>0.0062</td>
<td>0.0180</td>
<td>0.0037</td>
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<tr>
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<td>0.0172</td>
<td>0.0036</td>
</tr>
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<td>0.0034</td>
</tr>
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<td>0.0077</td>
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<td>0.0031</td>
</tr>
<tr>
<td>0.0080</td>
<td>0.0141</td>
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<tr>
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<td>0.0083</td>
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<td>0.0111</td>
<td>0.0022</td>
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<td>0.0083</td>
<td>0.0102</td>
<td>0.0020</td>
</tr>
<tr>
<td>0.0081</td>
<td>0.0093</td>
<td>0.0019</td>
</tr>
<tr>
<td>0.0078</td>
<td>0.0084</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

\[ \begin{bmatrix} C_{GALIC} \\ C_{ELLIC} \\ C_{CURCUM} \end{bmatrix} = \begin{bmatrix} 0.0029 & 0.0138 & 0.0028 \\ 0.0032 & 0.0149 & 0.0030 \\ 0.0035 & 0.0158 & 0.0032 \\ 0.0039 & 0.0166 & 0.0033 \\ 0.0044 & 0.0173 & 0.0033 \\ 0.0048 & 0.0180 & 0.0035 \\ 0.0053 & 0.0185 & 0.0036 \\ 0.0058 & 0.0185 & 0.0037 \\ 0.0062 & 0.0180 & 0.0037 \\ 0.0066 & 0.0172 & 0.0036 \\ 0.0070 & 0.0164 & 0.0034 \\ 0.0074 & 0.0157 & 0.0033 \\ 0.0077 & 0.0149 & 0.0031 \\ 0.0080 & 0.0141 & 0.0028 \\ 0.0082 & 0.0132 & 0.0026 \\ 0.0083 & 0.0121 & 0.0024 \\ 0.0083 & 0.0111 & 0.0022 \\ 0.0083 & 0.0102 & 0.0020 \\ 0.0081 & 0.0093 & 0.0019 \\ 0.0078 & 0.0084 & 0.0017 \end{bmatrix} \begin{bmatrix} A1 \\ A2 \\ A3 \\ A4 \\ A5 \\ A6 \\ A7 \\ A8 \\ A9 \\ A10 \\ A11 \\ A12 \\ A13 \\ A14 \\ A15 \\ A16 \\ A17 \\ A18 \\ A19 \\ A20 \end{bmatrix} \]
With the help of software we can get the concentration of the prepared validation set and from the same data, we can validate that Method. Accuracy of the Method can be determined from the result of Validation set. The precision was determined by means of a one way ANOVA including 10 replicates carried out on three successive days for Formulation. LOD and LOQ values were determined to check whether Linearity starting points for all constituents can be measured accurately and precisely or not.

The predictive ability of a model can be defined as RMSEP (Root Mean Square Error of Prediction). RMSEP summarizes both Accuracy and Precision. It is used for examining the errors in the predicted concentrations.

Developed Chemometric Methods can be utilized for further estimation of Gallic acid, Ellagic acid and Curcumin together in marketed Polyherbal Antidiabetic Formulations.

- UV Spectrophotometric Methods:
  - Simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Antidiabetic Polyherbal Formulation by Absorbance Correction Method:

A simple, accurate, precise and reproducible UV Spectrophotometric Method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin is developed and validated as per ICH.
The linearity of Method was investigated in concentration range of 2-20 μg/mL for Gallic acid, 5-50 μg/mL of Ellagic acid and 1-10 μg/mL of curcumin respectively. 4 μg/mL of Gallic acid, 10 μg/mL of Ellagic acid and 2 μg/mL of Curcumin were prepared and each solution were scanned between 200-800 nm.

At wavelength of 421 nm, only Curcumin showed absorbance. So, determination of Curcumin at this wavelength is possible. Ellagic Acid can be determine at 364.5 nm. Absorbance of Curcumin will be deducted from the Absorbance of Ellagic Acid at same wavelength. For Gallic acid, Absorbance is measured at difference of 2 wavelengths, i.e. 246 and 266 nm. At the difference of these 2 wavelengths, No interference of Absorbance of Ellagic acid & Curcumin as both shows same absorbance at these selected Wavelength. The result by Absorbance correction Method has been validated for Linearity, Accuracy, Precision, Ruggedness, LOD and LOQ according to ICH Q2 (R1) Guideline. Absorbance correction method is very much precise as % RSD was found to be less than 2. Method is also very accurate as % Recovery was found to be within the limit 98-102%. So, Developed Method can be utilized for simultaneous determination of Gallic acid, Ellagic acid and Curcumin in marketed Polyherbal Antidiabetic Formulations.
Simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Antidiabetic Polyherbal Formulation by First order derivative Spectrophotometry:

A simple, Accurate, precise and reproducible UV Spectrophotometric Method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin is developed and validated as per ICH guideline. 4 μg/mL of Gallic acid, 10 μg/mL of Ellagic acid and 2 μg/mL of Curcumin were prepared and each solution were scanned between 200-800 nm. Zero order spectra of drugs were converted to first order derivative spectra. Here in this Method, All drugs measured on a wavelength on which other 2 drugs having ZCP (Zero cross point). So, drugs can be measured without interference of other drugs. The linearity of Method was investigated in concentration range of 2-20 μg/mL for Gallic acid, 5-50 μg/mL of Ellagic acid and 1-10 μg/mL of curcumin respectively.

Gallic acid was determined on 255 nm where Ellagic acid and curcumin have zero absorbance. Ellagic acid was determined on 343 nm where Gallic acid and Curcumin have zero absorbance. Curcumin was determined on 452 nm where Gallic acid and Ellagic acid have zero absorbance. The result by First order derivative Spectroscopy has been validated for Linearity, Accuracy, Precision, Ruggedness, LOD and LOQ according to ICH Q2 (R1) Guideline and results were fall under the acceptance criteria according to guideline. So, Developed Method can be utilized for further estimation of Gallic acid, Ellagic acid and Curcumin together in marketed Polyherbal Antidiabetic Formulations.
G. ACHIEVEMENTS WITH RESPECT TO OBJECTIVES

- RP-HPLC method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin were developed using QbD approach (CCD as Experimental Design) and further confirmation of Curcuminoids by LCMSMS Analysis.
- HPTLC Method for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal Antidiabetic formulations were developed.
- Chemometric Methods (CLS and ILS) for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in Polyherbal Antidiabetic formulations were developed.
- UV Spectrophotometric methods (Absorbance correction and First order Spectroscopy) for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin were developed.
- All developed methods were validated for Specificity, Linearity, Accuracy, Precision, Robustness, Limit of Detection and Limit of Quantitation and for System suitability Parameters.
- Statistical comparison of Methods was performed by using one way ANOVA.

H. CONCLUSION

- RP-HPLC method have been developed and validated for estimation of Gallic acid, Ellagic acid and Curcumin using DAD as a detector and Gradient elution mode for mobile phase. Optimization of mobile phase pH, ratio of organic and aqueous phase are the critical part of method. For better Method development QbD approach was incorporated which contain CCD (Central composite Design) as Experimental Design. The Optimized Solution provided from software can be validated by applying same condition and by measuring % predicted error of the responses. Desirability can be calculated to prove better result assurance. Further, Curcumin is always available in Curcuminoids. Along with Curcumin; desmethoxycurcumin and bisdesmethoxycurcumin are also available. So, to confirm the Curcumin peak, LCMSMS scan was done. Shorter retention time of markers cuts down cost of experiment. Good resolution and specificity of Method made routine Analysis for measurement in Quality control laboratories.

- HPTLC method have been developed and validated for the estimation of Gallic acid, Ellagic acid and Curcumin using CAMAG Instrument with winCATS planar chromatography
manager as software. The advantage of Method are low cost of reagents, Rapid Analysis and good peak Shapes. Lower values of LOD and LOQ proves High Sensitivity method.

- Chemometric methods (CLS and ILS) can be developed and frequently used for complex matrix. It covers whole wavelength range and all possible concentration from the Linearity. So it is widely used for measurement of all three constituents very precisely when they all available in combination. Measurement of coefficient value from the matrix is the key part of Chemometric Methods. Low cost instrument and low interference of matrix play a key role for successful application of both methods in routine Analysis.

- UV spectrophotometric methods have been developed and validated for simultaneous estimation of Gallic acid, Ellagic acid and Curcumin in their combine Polyherbal dosage form. Low cost of Instrument and accurate and precise results are the key factor for applying UV Spectrophotometric Methods in routine Analysis.
I. COPIES OF PAPERS PUBLISHED AND A LIST OF ALL PUBLICATIONS ARISING FROM THE THESIS

➢ Published Papers


➢ Accepted Paper


➢ Presented Paper

J. REFERENCES:


24. B. Charrier, M. Marques, J.P. Haluk, 1992, “HPLC Analysis of Gallic and Ellagic Acids in European Oakwood (QuercusroburL.) and Eucalyptus (Eucalyptus globules.) ” Holzforschung, 46, 87-89, ISSN No. 1437-434X.


