2. INTRODUCTION

a. Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia due to deficiency in insulin secretion or insulin action (1). The hyperglycemia is accompanying with dysfunction, long-term damage, and failure of various organs, mainly the kidneys, eyes, heart, nerves and blood vessels as shown in Figure 1.

Figure 1: Type II diabetes complications (2)

The pathogenic processes are involved in the development of diabetes as shown in Figure 2. In T2DM, the body either produces insufficient amounts of insulin to meet the demands of the body or insulin resistance has developed. Insulin resistance developed when the muscle, liver and fat cells fail to respond to insulin, even when insulin levels are high. This will affect on fat cells to break down free fatty acids to produce energy; muscle cells are deprived of an energy source and liver cells fail to build up glycogen stores. These range from autoimmune destruction of
the β-cells of the pancreas with following insulin deficiency to abnormalities that result in resistance to insulin action (3-8).

Figure 2: Pathophysiology of T2DM (9)
Sign and symptoms of marked hyperglycemia as shown in Figure 3 include fatigue, weight loss, polydipsia, polyuria, polyphagia, and blurred vision. Diminishing of growth and exposure to certain infections may also accompany chronic hyperglycemia.
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Figure 3: Sign and Symptoms of T2DM (10)

Now a days DM is rapidly becoming a global health crisis. In India 64.5 million people are suffering from diabetes mellitus DM. The T2DM, consider today as a lifestyle disease, is usually accompanied with urbanization, stressful life, modernization change in lifestyle habits and caloric imbalance. Generally DM is the multiple chronic ailments, managing this disease, it's a big task. It can be treated by using allopathic drugs and herbal drugs.

b. Allopathic drug

There are varieties of glucose-lowering agents available in the market for the treatment of T2DM as shown in Figure 4. In that GLB and GLM come under the class of an oral sulfonylurea anti-hyperglycemic agents. These drugs are given once in a day because it has a long lasting effect. Membrane receptor is the major site of actions on pancreas β-cells, where it acts via ATP-regulated potassium channel to cause membrane depolarization and insulin get a release (11). Although side effects, including the risk of hyperlipidemia and hypoglycemia, are the main obstacles hindering achievement of glycemic targets. Over a period of time, patients may become gradually less responsive to treatment with oral hypoglycemic agents because of the decline of their diabetic state. Therefore, patients should be examined with regular clinical and laboratory evaluations, including glycosylated hemoglobin, blood glucose and lipid levels, to regulate the minimum effective dosage and to detect primary failure or secondary failure. The rate of primary failure will vary greatly depending upon patient adherence to food habits and life style. The drawback of currently available oral anti-diabetic agents either in terms of efficacy or safety tied to the arrival of the disease into worldwide frequency has been encouraged combination therapy that can manage T2DM more efficiently and safely.
c. Herbal drugs

The Indian earliest literature, reports more than 800 herbal plant species with anti-diabetic properties (13). Herbs are also known to provide alternative therapy in the inhibition of the diabetic complications, addition to cholesterol lowering action. Some of these herbs also have been confirmed to help in the re-development of β-cells and in overwhelming insulin resistance and there are variety of poly-herbal formulations available in the market which can be used as alternative therapy to treat the DM complications (14). The alternative therapies with anti-hyperglycemic effects are progressively required by patients with hyperglycemia. But herbs used for diabetes are possible to have the drawbacks of conventional drugs, potential herb-drug interactions expected in patients also receiving conventional anti-diabetic drugs. The scientific data on the interactions and extent of interactions make an important part of product monograph. All the product monographs and label information, detail extensively on drug-drug interactions, but it is not the same case with herb-drug interactions. The monographs do not provide adequate information to the prescriber with respect to the herb-drug interactions (15). This indicates that the scientific data collection regarding herb-drug interactions continues to be an antique part and remains as an unmet need in rationalizing the use of herbal drugs as related medications.
d. Bio-analytical method development and validation

To evaluate the pharmacokinetic parameters of allopathic drugs (GLB and GLM) by adopting the bio-analytical method and validations. Bioanalytical method is essential to be validated to establish their applicability for the planned use. Detailed and accurate bioanalytical method is desirable for assay of commercial dosage forms and pharmacokinetic studies. The choice of suitable techniques and applicable chromatography settings like column, mobile phase and type of detection were considered. In pharmacokinetic studies it is essential to develop bioanalytical method that covers concentrations of drugs in the body, hence a broad calibration concentration range is required (16). When it is necessary to analyze selected drug, bioanalytical method need to be developed for their analysis. Before analyzing the sample with HPLC or LCMS/MS, it is needed to have a clear sample without endogenous substances. The sort of pre-treatment primarily be influenced by the polarity of the sample. Various types of matrices have individuality and they differ in their protein, carbohydrate and fat contents. So an effective sample preparation technique is essential to get rid of all above substances.

The objective of validation of bioanalytical procedure is suitable for given plasma sample is reliable and reproducible for the proposed use. Many elements, for example, interference of matrix substances, selectivity, and stability were investigated.

(I) Sample preparation

Sample preparation is an important pre-analytical step in drug analysis, and includes isolation, clean up and concentration (or occasionally dilution) of samples. The sample preparation is important in bio-analysis because the biological samples contain many proteins and fatty matters which need to be processed before injecting into the column. The approaches of pre-treatment comprises

i. Protein precipitation

ii. Liquid-liquid extraction

iii. Solid phase extraction (17)

i. Protein precipitation

It is the most basic and minimum time-taking technique that is most regularly employed for bioanalysis. Protein precipitation involves denaturation (loss of tertiary and secondary nature) of proteins in the bio medium with precipitating substances like strong acid/base/heat or, most commonly, the use of a polar organic solvent like acetonitrile/methanol (18). Most of the bio analysis methods employ either 1:3 or 1:4 ratios of bio medium and precipitating agent followed by vortexing and centrifugation.

Centrifugation makes the denatured proteins into lumps, which get settled at the bottom and supernatant clear solution is separated and filtered for analysis. As precipitation results in major change in protein structure, the drug gets freely soluble in the polar organic solvent. However, this technique becomes very tedious process when it comes to handling a huge number of samples, especially in bio analysis or clinical bio analysis.
ii. Liquid-liquid extraction (LLE)

The principle of using two opposite polar solvents i.e. Aqueous and organic for extraction of analyte, is termed as solvent extraction (LLE) and it is one of the choices of technique for normal phase methods in bio analysis. Among the two phases the aqueous phase will dissolve the hydrophilic compounds and organic phase will dissolve hydrophobic substances. The organic phase extracted drugs samples are recovered back by evaporation of the solvent; while aqueous phase extracted drugs sample can be analysed as such. This type of extraction is simple, rapid and cost effective with respect to SPE (19). However, this procedure has certain margins like

- Poor reproducibility of results
- More amount of plasma sample is required
- Low selectivity (20, 21).
- Limits to certain type of compounds only (22).

Recently, several works were made to improve the boundaries associated with LLE.

iii. Solid Phase Extraction

This technique was developed to overcome the drawbacks of above two techniques i.e. Precipitation and solvent extraction. SPE principle of extraction is same as affinity-liquid chromatography (23). The sample is portioned between a solid support and liquid phase. The extracting substance should have more affinity towards solid phase. Endogenous/matrix compounds are removed from the stationary phase by one or several washing steps and then the components of interest are desorbed with suitable solvents. Intermolecular forces (i.e. hydrophobic interaction and ion forces) are mainly responsible for the extraction. It can be operated in both the phases (Reverse/Normal). The most common C18 cartridges (Sorbents) are coated on silica support. In bioanalysis, normally the reversed phase mode is used as the substances of interest, most often, is dissolved in an aqueous phase such as plasma or serum.

In General, the intentions of SPE remain

- Extraction of the analyte of interest from contaminants/interfering substances
- Elution of analyte at its retention time.

However, solid phase extraction can be operated in all the three phases like normal phase, reverse-phase and ion-exchange phase; but among all reversed phase is the most commonly used phase. Because of the complexity of various chemical compounds available in the market, the available extraction techniques were not appropriate all the time and hence there is a need for different approaches.

(II) Method validation according to USFDA guidelines

Validation is a supporting document, which gives assurance that the method is appropriate for the intended purpose. For critical studies like, pharmacokinetic study, complete validation document is required.

Complete bio analytical method validation is significant:

- When a novel bio analytical method is developed
- When analysing a new molecule
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- When extension of developed method for pharmacokinetic studies.

Bio analytical method development and validation include demonstrations of (24)

i. Selectivity
ii. Accuracy, precision and recovery
iii. Calibration curve
iv. Sensitivity and
v. Stability

i. Selectivity

Selectivity is the aptitude of the developed method to differentiate the analyte with the other endogenous elements in the sample.

ii. Accuracy, precision and recovery

The accuracy of a method defines the closeness of test results attained by the method to the actual value of the analyte. The precision describes the closeness of individual measures of analyte of a single homogeneous volume of plasma sample. The intra-assay precision and accuracy were estimated by analyzing six replicates containing drug at four different Quality control (QC) levels. The inter-assay precision was determined by analyzing the four levels of QC samples on four different runs.

iii. Calibration curve

A calibration curve is the association among instrument response and known concentrations of the analyte.

iv. Sensitivity

Sensitivity is defined as the lowermost analyte concentration that can be measured with acceptable accuracy and precision (i.e., LLOQ).

v. Stability

Stability tests were conducted to estimate the stability of drug in plasma samples under different conditions and using different concentrations.

- Stock solution stability
  Stock solution stability was implemented by observing the original stock solution, spiked stock solution and IS a stock solution at room temperature for 6 h. Then the samples were analyzed, processed and compared with the freshly prepared solutions.

- In-injector stability
  In-injector stability was implemented by observing the original stock solution, spiked stock solution and IS a stock solution at room temperature for 24 h. Then the samples were analyzed, processed and compared with the freshly prepared solutions.

- Bench-top stability
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Bench-top stability was implemented by observing the original stock solution, spiked stock solution and IS a stock solution at room temperature for 12 h. Then the samples were analyzed, processed and compared with the freshly prepared solutions.

- Freeze–thaw stability
  Freeze–thaw stability was implemented by freezing (-20°C) and thawing (room temperature) the stock solution, spiked stock solution and IS a stock solution till completion of three cycles. Then the samples were analyzed, processed and compared with the freshly prepared solutions.

- Freezer stability
  Freezer stability was implemented by observing the original stock solution, spiked stock solution and IS a stock solution at -20°C ± 10°C for 25 days. Then the samples were analyzed, processed and compared with the freshly prepared solutions.

c. In vivo herb-drug interaction studies in diabetic patients

Polypharmacy is a common phenomenon in the T2DM. In addition to the prescribed medications, patients add their own medications, especially the herbal home remedies and poly-herbal formulations with or without prescriber's notice (25). It is known that all ingested substances have the potential to interact. The significant and non-significant effects of an herbal drug in individual state which is compared with oral anti-diabetic drugs (26), which is usually related to the concentration of drug this is going to impact on the blood glucose, lipid levels and HbA1c. In combination state of herb-drug, concentrations of drug in blood are determined by the drug's absorption, distribution, metabolism, and excretion (ADME) (27). In some instances, understanding how to adjust a dose or dosage regimen in the individual state and combined state of herbal drug, or how to avoid herb-drug interactions. The possibility of herb-drug interactions affecting the safety and efficacy of the prescribed medicines and disturbing prevalence data on diabetes (28), we thought of exploring the effect of herb-drug interactions and rationality of the use of herbs along with oral antidiabetic medications.

Poly-herbal formulation (Mehagni) used in this study comprises of haridra, madhunasini, amalaki and ekanayakam, used as anti-diabetic drugs in T2DM Table 3. All these components of the poly-herbal formulation have proven anti-diabetic and anti-hyperlipidemia properties that help in reducing the complications of T2DM. Although commercially available poly-herbal formulation (Mehagni) is consumed by the patients with T2DM as an alternative therapy, its interaction with widely used anti-diabetic drugs like GLB and GLM have not been evaluated so far. Basically GLB and GLM are class sulfonylurea drug. However, these drugs were prescribed in pre-diabetic conditions in the dose range of 1mg, 2mg, 3mg up to 8mg based on the severity of DM. Sometimes to manage chronic conditions of DM, these drugs are prescribed with other oral anti-diabetic drugs. GLB and GLM can maintain the blood glucose level only, but if it comes to lipid profiles these drugs will not maintain the T-Chol level in blood, it’s the biggest drawback of these drugs. Based on this, the current study was carried out with an idea that, poly-herbal formulation might impact on the blood glucose level, lipid level and PK parameters of allopathic drugs (GLB and GLM). Till date, there are clinical studies determining the efficacy of individual constituents comprised in poly-herbal formulation (Mehagni), nevertheless, no studies have been reported regarding combination therapy with GLB and GLM.