Original Article

A Critical Review on Bioanalytical Methods Impurity Profiling, Degradation Study of Empagliflozin and Linagliptin

Dr. Durgaprasad Kemisetti[1], Mr. Amrit Kumar Rath[2]*, Prof. (Dr.) Sruti Ranjan Mishra[3]

[1]Associate Professor Faculty of Pharmaceutical Science Assam Downtown University
*[2]Associate Professor Department of Pharmaceutical Analysis Danteswari College of Pharmacy Borpadar, Raipur-road, Jagdalpur, C.G. 494221
[3]Professor cum Principal Danteswari College of Pharmacy

***Corresponding Author: -** Mr. Amrit Kumar Rath

Associate Professor Department of Pharmaceutical Analysis Danteswari College of Pharmacy Borpadar, Raipur-road, Jagdalpur, C.G. 494221
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Abstract

The development of sound Analytical method(s) is of supreme importance during the process of drug discovery, release to market and development, culminating in marketing approval. The objective of this paper is to review the method development, Impurity profile study, Degradation studies of the method for the drug production i.e Empagliflozin (EMP) and Linagliptin (LIN) from the developmental stage of the formulation to commercial batch of the product. Bioanalysis plays a vital role in drug discovery and development for the analysis of analytes (drugs, metabolites) in biological samples. To achieve a stable, reliable, and robust method one has to remove the interfaces from the sample matrix to improve bioanalytical system performance. This review provides an overview of the Impurity profile, Degradation study and various Analytical/Bio-Analytical method development of selected Anti-Diabetic drugs i.e EMP and LIN in single dosage form and their combination and a detailed investigation of different analytical techniques used for detection and quantification of these drugs on various matrices. Various techniques like spectroscopy, chromatography, electrochemical methods, and hyphenated techniques were reported to determine Anti-Diabetic drugs, either alone or in combination with other drugs, in different kinds of matrices like in bulk, existing dosage forms and also biological samples like plasma, serum, urine and myocardial tissue. Among various techniques used, HPLC, LC-MS systems are more commonly used because of their high sensitivity. This article summarizes the research works carried out since 2015 and intends to act as a handbook for future researchers.

Keywords: -Impurity, Degradation, Bioanalytical, HPLC, LC-MS, EMP, LIN, MET, Chromatography.

INTRODUCTION

Bioanalysis is the quantitative measurement of chemicals, pharmacological compounds, and their metabolic byproducts in a wide range of bio-samples. It is an integrated technique that has been employed in preclinical stages of drug-discovery to further support the clinical phases of drug discovery. However bioanalytical methods must be optimized, characterized, and validated according to United States Pharmacopeia (USP)/International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines to comply with the regulatory guidelines and acceptance criteria. Therefore, bioanalytical studies will provide an accurate and consistent estimation of drugs or metabolites in biological samples at a great level of sensitivity, selectivity, and specificity. In addition to this, bioanalysis quantitatively assesses the levels of drug candidates, their metabolites, endogenous biomarkers, etc.[1] In extremely complex biological matrices such as plasma, blood, urine, and tissues that are taken from various animal species (such as rodents, dogs, nonhuman primates, etc.) and humans. Bioanalysis supports discovery, nonclinical(tox), and clinical studies shows typical studies an integrated bioanalytical function would support. Bioanalytical data plays vital role in calculating pharmacokinetic parameters like bioavailability, bioequivalence, drug and metabolites exposure, clearance, their distribution into various body organs, correlation of pharmacokinetics (PK) effects and pharmacodynamics (PD) changes, etc[2] .Thus, throughout the research and drug discovery process, bioanalysis is essential for advancing therapeutic compounds from early discovery to regulatory filing and post market surveillance. Now a days bioanalytical scientists not only provide significant data but also actively engaging project/program go/no-go discussions, along with colleagues from other functional areas. While using analytical chemistry and instrumentation to provide reliable and accurate measurement is the most vital aspect of bioanalysis, knowledge from related disciplines such as biotransformation, pharmacokinetics, biology, pharmacology, etc. is invaluable for ensuring appropriate bioanalysis conduct[3].

1. EMPAGLIFLOZIN (EMP):-

EMP approved in 2014 represents a relatively new class of Anti-Diabetic oral drugs sold under the brand name Jardiance. Chemically, EMP belongs to the C-glucoside family. EMP is an inhibitor which is competitive, selective and potent which is having capacity for glucose reabsorption, which takes place in the proximal tubule of kidney and it also leads to increase the urinary glucose excretion. Among others, it is a medication used together with diet and exercise to treat type 2 diabetes. It offers advantages over sulfonylureas and can be administered as a substitute for metformin. It can be used together with other drugs like metformin or insulin. It is not advised for those with type 1 diabetes. It is ingested orally. Given recent trial evidence, is expected to soon receive a license to be used for patients with heart failure, irrespective of diabetic status. Jardiance can also be used in the treatment of coronary heart failure to reduce cardiovascular mortality[4].

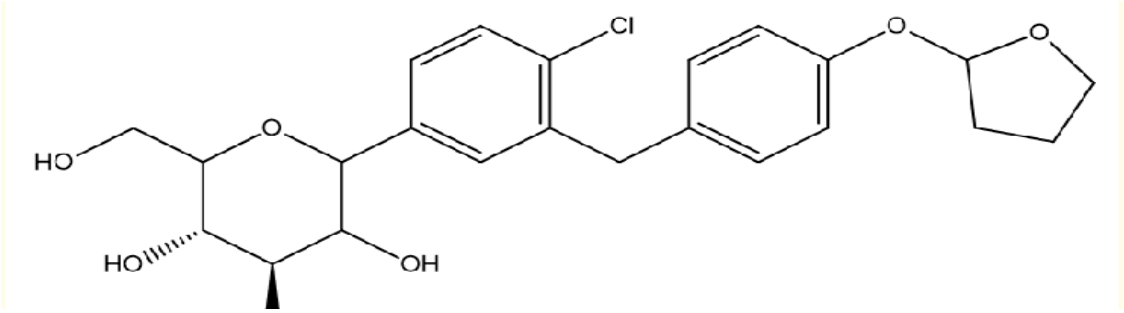

Figure-1:- Chemical Structure of EMP.

Mechanism of action: -

EMP works by inhibiting the Sodium Glucose Co-Transporter-2(SGLT-2) found in the proximal tubules in the kidney. Through SGLT-2 inhibition, EMP reduces renal reabsorption of glucose and urinary excretion of glucose.

2. LINAGLIPTIN (LIN) : -

Linagliptin (LIN) is a medication used to treat type 2 diabetes mellitus and is sold under the trade name Tradjenta. As a first-line drug, linagliptin is typically less recommended than metformin and sulfonylureas. It is combined with diet and exercise. It is not advised for those with type 1 diabetes. Linagliptin was typically well tolerated in clinical studies when used as monotherapy or in conjunction with other oral anti hyperglycemic medications. It had minimal to no negative effects on body weight and was generally linked to a really low prevalence of hypoglycemia [5].

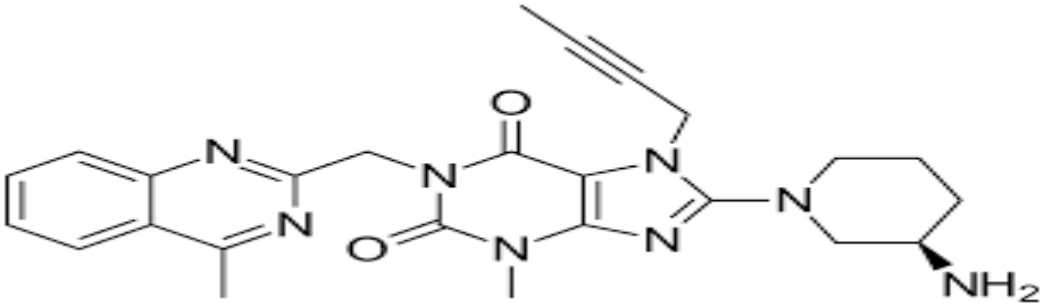

Figure-2:- Chemical Structure of LIN.

Mechanism of Action: -

LIN can be used to treat diabetes mellitus type 2 by Inhibitors of dipeptidyl peptidase 4 (DPP-4 inhibitors or gliptins) which is a class of oral hypoglycemics that block the enzyme dipeptidyl peptidase-4 (DPP-4). The FDA granted sitagliptin approval as the first drug in this class in 2006. Blood glucose levels are raised by glucagon while being decreased by DPP-4 inhibitors. DPP-4 inhibitors work by raising levels of creatin (GLP-1 and GIP), which inhibits the release of glucagon, which then boosts insulin secretion, slows down gastric emptying, and lowers blood glucose levels [6].

A. IMPURITY PROFILE: -

Identification of impurities must be closely monitored to meet the requirement in order to ensure the quality of pharmaceuticals. It's critical to comprehend the different types of impurities that can be present, and one must also determine the potential origins of these impurities. To track them, certain analytical techniques must be created. Impurities should normally be profiled to offer a standard for comparison. As adjustments are made to the synthesis, formulation, or production processes, even if only to make them better, new impurity may be seen. When hyphenated methods cannot reveal the structure of an impurity or when verification with authentic material is required, it may be necessary to isolate and characterize the impurity. The availability of a genuine material can also enable toxicological research and give a benchmark for ongoing drug product monitoring[7].

COMMON TERMS OF IMPURITIES: -

The presence of some impurities may not deleteriously impact drug quality if they have therapeutic efficacy that is similar to or greater than the drug substance itself. Even if a drug substance has an impurity with greater pharmacological or toxicologic capabilities, it can nevertheless be said that its purity has been compromised. Consequently, drug substance purity must be evaluated separately from these unfavourable extraneous components in order to guarantee that the patient is receiving an accurate dosage of the medicinal substance (e.g., inert, toxic, or pharmacologically superior impurities). Some of these phrases, like intermediates, indicate potential impurity sources, while others, like related products, seek to downplay any negative connotations. Let us review them individually

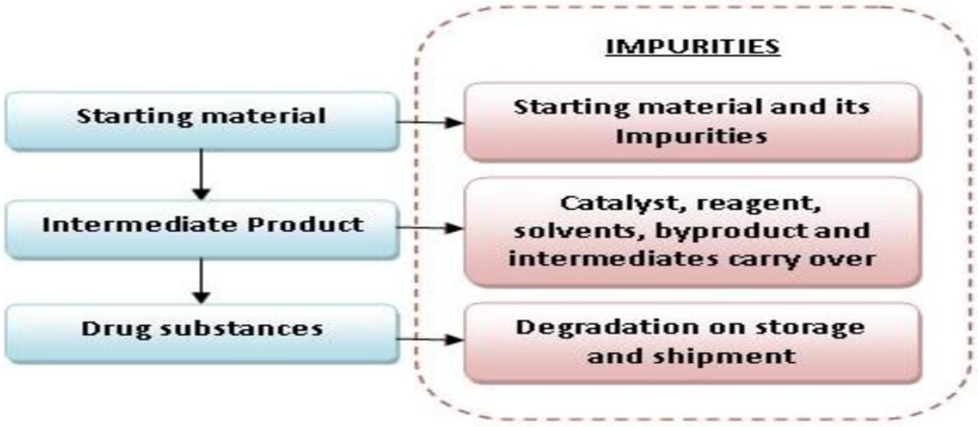

Figure-3 : Types of Impurities.

There are various terms associated with impurities are as follow,

- Starting material(s)
- Intermediates
- Penultimate intermediate (Final intermediate)
- By-products
- Transformation products
- Interaction products
- Related products
- Degradation products

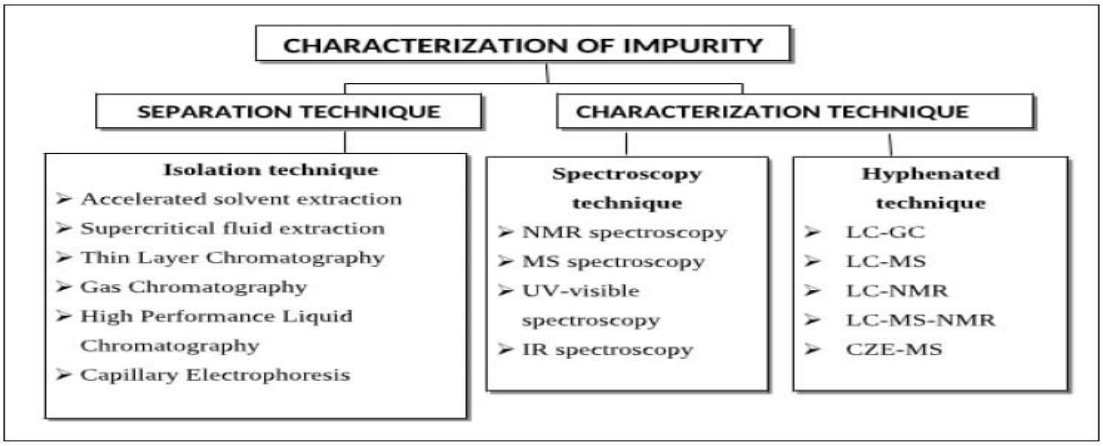

Figure 4 : - Characterization of impurities.

Impurities are typically characterized using the techniques described below: Matching retention data, MS, NMR, IR, UV. It is necessary to determine the impurity's content once it has been identified. A component must frequently have a signal that is at least twice as strong as the baseline or background noise in order to be detectable. For quantification of impurity, the multiple is set much higher. Initial estimations are generally done against the parent compound because in most cases the authentic sample of impurity is not available. When the authentic sample is available, it is important that it be used for estimations. If estimates show that a particular impurity content is larger than 0.1%, it must be classified in accordance with FDA and ICH. The Schematic of characterization of impurities mentioned in Fig-4 [8,9].

I. IMPURITY PROFILE OF EMPAGLIFLOZIN: -

A simple and fast analytical method with a low limit of quantification was developed and validated to determine EMP and its synthetic impurities using suitable parameters. These Parameters investigated for method validation, such as specificity, linearity, precision, and accuracy, presented results within the acceptable range. In addition, when EMP was subjected to UVC light for 24 hours and 2 M NaOH for 48 hours, the production of two degradation products distinct from the impurities investigated was noted. These molecules were identified contributing to the knowledge of the EMP impurity profile[8].

The sodium glucose cotransporter-2 (SGLT2) inhibitor EMP effectively improves glycemic control in adults with type 2 diabetes. Three unidentified impurities were found by LC analysis in pilot batches of EMP that ranged from 0.05 percent to 0.15 percent during production. Column chromatography and semi-preparative LC were used to separate these unidentified impurities from the crystallization mother liquor, and a thorough study of the data from HRMS, 1D-NMR (1H, 13C), and 2D-NMR (1H-1H COSY, HSQC, and HMBC) spectroscopy revealed their structures [10].

The reverse phase high performance liquid chromatographic method was used to identify and quantify four process-related impurities in the drug substance EMP. The analysis was conducted at a 55°C constant column temperature. Under acidic, alkaline, oxidative, photolytic, temperature, and humidity conditions, a forced degradation research was conducted. In terms of sensitivity, linearity, precision, accuracy, and robustness, the devised approach was validated. It can be used for routine stability testing and quality control analysis of EMP[11].

Because of the natural fluorescent behavior of EMP, an effective bioanalytical approach for EMP bioassay in rats' plasma was developed. Based on its high value of log P as 1.8, which accelerated the drug's migration from plasma to the organic layer, diethyl ether (DEE) was successfully used for better extraction of EMP from rats' plasma. After excitation at 226.5 nm, the relative fluorescence intensity for EMP was measured at emission (299.4 nm). During this process, protein precipitation and liquid-liquid extraction were assessed for the plasma extraction. Protein precipitation, however, did not produce the expected recoveries. In this investigation, it was unavoidable to encounter protein precipitation issues such as the prolonged evaporation and dryness phases, the quiet existence of contaminants in the samples, and the additional cleaning required[12].

II. IMPURITY PROFILE OF LINAGLIPTIN: -

During the LIN stress testing, one unidentified degradation product (impurity I) in an acidic environment was found in the HPLC. Using spectral data (MS, MS/MS, 1D, 2D, and infrared spectrum), it was then isolated, identified, and characterized before being subjected to mechanism analysis. The structural alerts of potentially genotoxic N-Acylated amino aryl and alkyl halide were found in impurity I, a degradation product of LIN, and impurity II, another process-related impurity. Therefore, a rapid and simple ultra-performance liquid chromatography method was introduced to simultaneously determine these two potentially harmful impurities in LIN at ppm levels. A convenient, simple UPLC method was created for the simultaneous determination of two PGIs (potential genotoxic impurities) of LIN at the trace level. One of the PGIs in particular was a brand-new compound called LIN's acidic degradation product. A potential formation mechanism was also put forth[13].

By using the high-performance liquid chromatography [HPLC] method, several related substances in the range of 0.05 percent to 0.15 percent were discovered during the development of the LIN process. To determine the molecular mass of these impurities, a liquid chromatography mass spectrometry [LC-MS] study was conducted. It was looked into where these impurities came from. These impurities were made synthetically, and IR, NMR, and mass spectrometry were used to characterize them. For further confirmation, they were co-injected. These impurities are

Table-1 :- Various impurities of Linagliptin .

SL NO.	IMPURITY'S	CHEMICAL FORMULA
1.	IMPURITY-I	7-Di(but-2-ynyl)-3-methyl-8-[(R)-3-(methylene amino) piperidin-1-yl]-1H-purine-2,6(3H,7H)-dione
2.	IMPURITY-II	8-[(R)-3-Aminopiperidin-1-yl]-7-[(E)-3-bromobut-2-enyl]-3-methyl-1-[(4-methylquinazolin-2-yl)methyl]-1H-purine-2,6(3H,7H)-dione
3.	IMPURITY-III	8-[(R)-3-Aminopiperidin-1-yl]-1-(but-2-ynyl)-3-methyl7-[(4-methylquinazolin-2-yl)methyl]-1H-purine-2,6(3H,7H)-dione
4.	IMPURITY-IV	8-[(S)-3-Aminopiperidin-1-yl]-7-(but-2-ynyl)-3-methyl-1-[(4-methylquinazolin2-yl)methyl]-1H-purine-2,6(3H,7H)-dione
5.	IMPURITY-V	8-[(R)-3-Aminopiperidin-1-yl]-7-[(E)-3-bromobut-2-enyl]-3-methyl-1-[(4-methylquinazolin-2-yl)methyl]-1H-purine-2,6(3H,7H)-dione
6.	IMPURITY-VI	7-(But-2-ynyl)-3- methyl-1-[(4-methylquinazolin-2-yl)methyl]-8-(piperidin-3-ylamino)-1Hpurine-2,6(3H,7H)-dione
7.	IMPURITY-VII	8-[(R)-3-Aminopiperidin-1-yl]-3-methyl-1,7-bis((4-methyl quinazolin-2-yl)methyl)-1H-purine-2,6(3H,7H)-dione
8.	IMPURITY-VIII	7-(But-2-ynyl)-8-(dimethylamino)-3-methyl-1-[(4-methyl quinazolin-2-yl)methyl]-1H-purine-2,6(3H,7H)-dione

All the above-mentioned impurities including in process and raw material related impurities, were linked to LIN. Based on LC-MS research, the likely structures of these impurities were identified. These impurities were then prepared synthetically and examined using 1H NMR, IR, and mass spectrometry. By co-injecting these pure samples of impurities into the HPLC analysis, the proposed structures of these impurities were verified. As a result, while making LIN, proper control of these impurities in the corresponding raw materials, intermediates, and final drug substance is necessary in order to comply with current regulatory requirements[14]. Numerous probable impurities in the range of 0.05 percent to 0.15 percent that were related to both the process and the raw materials were noticed during the development of the LIN process. During this study some thermodynamic models assessed how temperature and solvent composition functioned. In pure solvents, methanol had the highest mole solubility value at 313.15 K (3.02 103) and was followed by ethanol (2.31 103), acetonitrile (1.72 103), n-propanol (1.25 103), and isopropanol (1.253 103) (8.74 104). However, in mixtures of acetonitrile (w) and ethanol (1w), the solubility profiles increased monotonically as the temperature increases, reaching a maximum at w = 0.80 and then declining at each subsequent temperature. More importantly, during the process development, the change in the polarity of the mixed solvent aids in the separation of several related potential impurities related to the raw materials and processes [15].

B. REGULATORY REQUIREMENTS FOR PHARMACEUTICAL IMPURITY IDENTIFICATION

Impurities are divided into three categories by the ICH, FDA, and USP guidelines: organic impurities, inorganic impurities, and residual solvents. These impurities can come from a variety of sources, as shown in Fig. 4. A new drug substance's ability to control organic impurities depends on the impurities' Maximum Daily Dose and Total Daily Intake (TDI). Table 2. (Provides the ICH threshold for a new drug substance's control of organic impurities.) Organic impurities in a new drug substance at (or greater than) 0.05 percent or 0.1 percent require identification, depending on whether the MDD is higher or lower than 2g. The control of organic impurities in a new drug product are outlined in Table 3. To give low dose drug products more consideration, the identification thresholds for organic impurities in new drug products are divided into four groups based on the MDD. Since the MDD for the majority of new drug products is between 10 mg and 2 g per day, any impurities at 0.2 % would need to be found [9].

Table 2. Reporting, Identification, Qualification thresholds for impurities in a new drug substance according to ICH Q3A (R2) [16]

Maximum daily dose	**Reporting Threshold**	**Qualification Threshold**	**Identification Threshold**
≤ 2g/day	0.05%	0.15% or 1.0mg per day intake (whichever is lower)	0.10% or 1.0mg per day intake (whichever is lower)
>2g/day	0.03%	0.05%	0.05%

Table 3. Reporting, Identification, Qualification threshold for Degradation Products in New Drug Products according to ICH Q3B (R2) [17]

Reporting Thresholds	
Maximum Daily Dose	Threshold
≤ 1g	0.1%
> 1g	0.05%

Identification Thresholds	
Maximum Daily Dose	Threshold
< 1mg	1.0% or 5µg TDI, whichever is lower
1mg – 10mg	0.5% or 20µg TDI, whichever is lower
> 10mg – 2 g	0.2% or 2 mg TDI, whichever is lower
> 2 g	0.10%
Qualification Thresholds	
Maximum Daily Dose	Threshold
< 10 mg	1.0% or 50µg TDI, whichever is lower
10mg – 100mg	0.5% or 200µg TDI, whichever is lower
> 100mg – 2 g	0.2% or 3mg TDI, whichever is lower
> 2 g	0.15%

C. FORCED DEGRADATION STUDY

The forced degradation studies are used to facilitate the development of analytical methodology, to better comprehend the stability of the drug substance and the drug product, to identify the pathways for degradation and the end products of degradation, and more. This study will assist in obtaining the most stable formulation. Stability of the drug substance and the drug product is a crucial factor that can affect purity, potency, and safety. Due to changes in the stability of the drug, there may be a risk to the patient's safety from the formation of toxic degradation products. So the question for pharmaceutical scientists has been how much degradation is enough f or the purposes of validating chromatographic

assays, the degradation of drug substances between 5 and 20 % has been accepted as reasonable. According to some pharmaceutical scientists, the acceptable stability limit for small drug molecules is typically 90 % of the label claim, and 10 % degradation is ideal for use in analytical validation. The forced degradation study Chart is shown in Fig.5.[18]

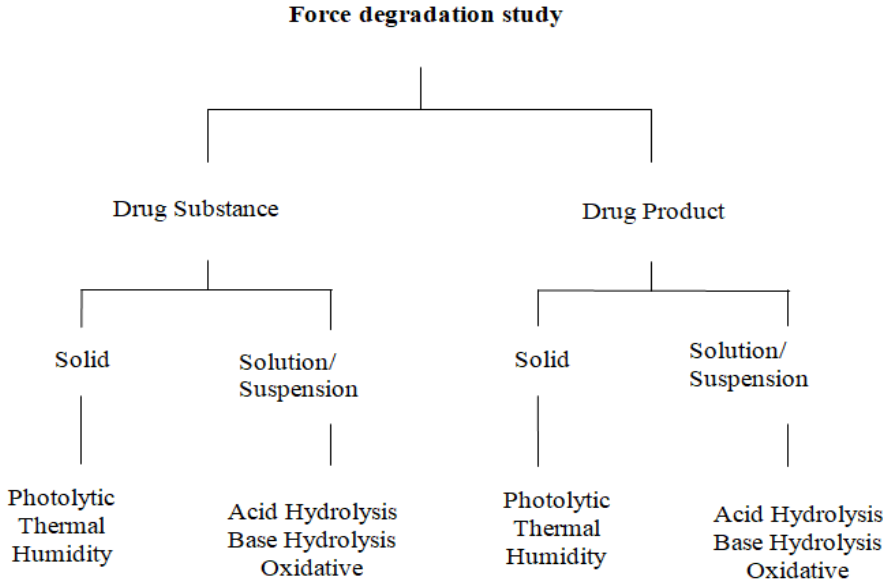

Figure-5: Classification of Forced Degradation study.

I. FORCED DEGRADATION STUDY OF EMPAGLIFLOZIN: -

For the purpose of estimating EMP in API and finished formulation in accordance with ICH guidelines, new analytical methods were developed in the current study. To identify the degraded products and their percentage, the study concentrated on conducting forced degradation studies for EMP. The author used various chromatographic parameters and it was observed at a UV wavelength of 232 nm. Based on the linear regression equation y = 61309 x – 8123 with a correlation coefficient of 0.9999, it was identified that the method was linear. performed according to the prescribed protocol for the validation parameters. Altering forced degradation studies, which can be examined using the LCMS method, may be helpful in identifying the various degraded products. The analyte is eluted quickly using this streamlined procedure while using less organic solvent [19].

An easy and quick analytical method with a low limit of quantification has been created and validated in order to determine EMP and its synthetic impurities. Using among the factors that were looked at to determine whether a method had produced results that were acceptable were specificity, linearity, precision, and accuracy. Furthermore, the formation of two degradation products distinct from the impurities investigated was observed when EMPA was exposed to UVC light for 24 hours and 2 M NaOH for 48 hours [8].

A high-performance thin-layer chromatographic method was developed and validated to allow for the simultaneous determination of EMP and LIN. The recently authorized Glyxambi tablet dosage form was successfully pharmaceutically analyzed using the suggested approach. LIN and EMP both provided satisfactory resolutions with RF values of 0.22 and 0.57, respectively. According to ICH recommendations, the method was validated. EMP and LIN were found to be subject to acid hydrolysis and alkaline degradation during stability testing. Because the method could successfully separate the drugs from their degradation products, it can be used as a stability indicating method. For quality control laboratories, the suggested validated stability indicating assay is suitable as a quick, simple, and affordable method for the sensitive determination of the drugs mentioned. When plates were scanned at 229 nm, the peaks were symmetrical in nature and there was no tailing to be seen [20].

For the determination of EMP, stability-indicating high-performance liquid chromatography (HPLC) and spectrofluorimetric methods were developed. Wet heat, oxidation, photodegradation, acid hydrolysis, and alkali hydrolysis were all applied to EMP. A kinetics study of the alkaline degradation pathway was conducted because it produced the majority of the end product under stress. The activation energies of the degradation process were calculated, and Arrhenius plots were constructed. HPLC was used to achieve isocratic chromatographic elution by applying synchronous mode and measuring in the $\Delta\lambda$ = 70 nm range at 297.6 nm, a spectro-fluorimeter was used to record the relative fluorescence intensity. For HPLC and spectrofluorimetric methods, the linearity ranges were discovered to be 5–50 g/ml and 50–1000 ng/ml, respectively [21].

II. FORCED DEGRADATION STUDY OF LINAGLIPTIN: -

A HPLC-DAD method for LIN quantitation in the presence of its tablet-bound degradation products carried out from the tablets, at 225 nm, the drug was identified. The method showed high linearity (r2 > 0.999) with CV% and % error of the mean <2% over the range of 1–50 µg/mL. The thresholds for quantification and detection were 0.3 and 1.0 µg/mL,

respectively. With a run time of 17 minutes, the LIN retention time was 11 minutes. LIN and its degradation products were kept apart under all the imposed degradation conditions. The technique was effective in measuring the amount of LIN in the tablets and studying its kinetics of forced oxidative, acidic, and alkaline degradation. [22].

For the simultaneous estimation of metformin hydrochloride and LIN in formulation, a high-performance thin layer chromatographic method has been developed on a precoated silica gel 60 GF254 plates, the mobile phase for the chromatographic separation was acetone-methanol-toluene-formic acid 4:3:2:1 (v/v/v/v). At room temperature, the plates were developed to a distance of 8 cm. The developed plates were scanned at their unique wave length of 259 nm, and the data were quantified there. The ideal experimental conditions were chosen after careful consideration of factors like band size, chamber saturation time, solvent front migration, slit width, etc. With Rf values of 0.61 and 0.82 for metformin hydrochloride and LIN, respectively, the medications were satisfactorily resolved. The full drug degradation pathway was identified using the stress degradation technique combined with the HPTLC method. Base hydrolysis: Base degradation with 0.1M, 1M, and 2M NaOH for 3 hours at 80°C led to the complete degradation of LIN and 59.03 to 80.27 % hydrolysis of Metformin (MET)with an additional peak for the degradation product being observed. Acid Hydrolysis: For 3 hours in an acidic environment (0.1M, 1M, and 2M HCl), severe hydrolytic degradation was seen. Degradation of MET ranged from 42.54 to 42.63 % and LIN degraded completely. Oxidation: Formulation performed oxidative degradation for three hours at 80°C with 3 % hydrogen peroxide. Under oxidative conditions, complete degradation of LIN and a degradation rate of 53.36 % of MET were noted. Photolysis: For 48 hours, photolysis was carried out on the formulation in the direct sunlight. There was a 23.43 % degradation of MET and a complete degradation of LIN [23].

In order to ensure the robustness of the method, a novel reverse phase-high performance liquid chromatography (RP-HPLC), stability indicating method was developed for the determination of LIN (LIN) and its related substances in LIN and metformin HCl (MET HCl) tablets., with minor modifications to the buffer and the column, to identify the m/z and fragmentation of the maximum unspecified degradation products, i.e., impurity-VII (7), impurity-VIII (8), and impurity-IX (9). To ensure a thorough understanding of LIN and its related degradation products as well as optimal performance throughout the product's lifetime, a proposed degradation pathway for the drug has been made based on the findings, and the synthesis of Impurity-VII (7) is also discussed [24].

For the purpose of determining LIN in tablet formulation and biological sample, an HPTLC method was developed and validated. On pre-coated silica gel 60 F254 plates, the chromatography was carried out with methanol: toluene 7:3 (v/v) as the mobile phase. In pure and biological samples, the calibration plots were linear between 50-300 ng/band and between 50-500 ng/band, respectively. Recovery studies were used to evaluate the proposed method's accuracy (% recovery was 100.38 percent for tablet samples and 99.99 percent for biological samples, respectively). During stability testing, LIN was observed to be vulnerable to oxidation, alkali hydrolysis, and acid hydrolysis (3 % H2O2). The technique allowed for quantitative selection. LIN in the presence of components and degradation products that are probably present in the biological matrix. Under conditions of acidic, alkaline, and oxidative stress, LIN was discovered to degrade. The method's ability to quantitatively measure LIN in the presence of degradation products supports its ability to indicate stability. The tablet sample's chromatogram after exposure to acid, alkali, and oxide stress conditions [25].

D. BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

The development and validation of bioanalytical methods is essential in the process of discovering new drugs. As the sponsors must submit preclinical studies, non-human pharmacology and toxicology studies, clinical pharmacology, bioavailability, and bioequivalence evaluations to regulatory authorities, it is necessary to develop and validate bioanalytical methods. The biological matrices used in the development of the bioanalytical methods include blood, serum, plasma, and urine[26]. The bioanalysis process involves several steps, from sample collection to sample analysis and data reporting, and it analyses the drugs, metabolites, and biomarkers in biological samples. In the bioanalysis, sample preparation is highly essential. Due to its high selectivity and high sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the technique of choice in bioanalytical laboratories for separation and detection. Before beginning bioanalytical work, it's important to have knowledge of the chemical structure and properties of the analytes. It is essential for the development of drugs. The validation of the bioanalytical method is crucial for supporting the license applications for new drugs or biologics[27].

1. The development and validation of bioanalytical methods
2. The chemical structure, pKa value, solubility properties, stability, and adsorption properties of the analyte are studied prior to the development of the bioanalytical method.
3. The following categories of developed and approved bioanalytical methods exist:
4. Sample preparation
5. Development of bioanalytical methods, establishment of assay protocols, and
6. Using a validated bioanalytical technique to analyze drugs.

I. BIOANALYSIS

The term "bioanalysis" refers to the process of identifying and measuring analytes in biological samples (blood, plasma, serum, saliva, urine, feces, skin, hair, organ tissue). In addition to detecting small molecules, such as drugs and metabolites, bioanalysis also identifies large molecules, such as proteins and peptides. The use of bioanalysis in drug development and discovery is well established [28],[29].

II. EXTRACTION TECHNIQUE IN DRUG BIOANALYSIS

The preparation of the sample is the first step, and common sample preparation techniques include solid phase extraction (SPE)., liquid-liquid extraction (LLE), and protein precipitation (PPT). The following are the standard sample preparation methods are PPT, LLE, SPE [30]. The SPE has a high efficiency, cost-effective, high reproducibility, and easy to operate. It is used for separating and concentrating of a trace analytes in biological samples [31], [32]. Various types of SPE techniques includes Reversed phase- solid phase extraction, Normal phase-solid phase extraction. Typical sorbents in NP-SPE are silica with polar functional groups (Si-CN, Si-NH2, Si-Diol and pure silica). The retention mechanism in this technique is based on hydrogen bonding between analytes and sorbent [33].

III. ION EXCHANGE-SOLID PHASE EXTRACTION

It is most selective method in SEE, based on acidic drugs can be isolated with quaternary amine bonded silica or Si-NH2 as an anion exchange, for basic drugs strong cation exchange, Si-SCX and week cation exchange, Si-WCX can be used for isolating the cationic analytes [34].

IV. SEPARATION AND DETECTION INSTRUMENTATION

Liquid chromatography-UV (LC-UV)

The high-performance liquid chromatography (HPLC) is commonly used technique in bioanalysis. The main detector used in HPLC is UV-visible detector. Due to the wide range of selectivity of HPLC column; it is applied for separation of the drug and many metabolites in different matrices [35].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Liquid chromatography-tandem mass spectroscopy is having high selectivity as an important tool in drug discovery. It has advantages to reduced analysis time [36].

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS)

Supercritical fluid chromatography-Tandem mass spectrometry (SFC-MS/MS)

As compared to the HPLC, SFC has some advantage like rapid separation without using hazardous organic solvents. The diffusion rate of solute in supercritical fluid is ten times greater than organic solvents in LC. This technique has higher flow rate and higher sample capacity for determination of different drugs and metabolites in biological fluids [37].

V. Validation Parameters

Various parameters like Linearity, Selectivity (specificity), Calibration model, Precision and repeatability, Intermediate Precision, Reproducibility, Limit of detection, Limit of quantification, Robustness & Stability should be checked for the method validation to ensure the accuracy and specificity of the analytical method [38].

Table-4 Methods of impurity profiling, forced degradation and bioanalytical for oral EMP.

Sl No.	DRUGS	Rt (min)	Impurity/ Degradation /Biological Matrix	INSTRUMENT	STATIONARY PHASE	MOBILE PHASE	DECTECTOR	CHROMATO GRAM RECORDED	RUN TIME	REFERENCE
1.	EMP		Impurity profile	UHPLC Shimadzu-Nexera x2	Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm)		QTOF-MS			8
2	EMP		Impurity profile	LC HRMS, 1D-NMR (1H, 13C), and 2D-NMR (1H-1H COSY, HSQC, and HMBC) spectroscopy	A reversed-phase ACQUITY BEH C18 column measuring 2.1 mm × 50 mm × 1.7 m					10
3	EMP		Impurity profile	RPHPLC	Inertsil C8 (250 mm x 4.6 mm, 5 µm) column	0.1 percent orthophosphoric acid and acetonitrile	UV DETECTOR	230 nm		11
4	EMP	-	Rat Plasma	Shimadzu Spectrofluorometer RF-6000	-	-	SPECTRO PHOTOMETER	299.4nm	-	12
5	EMP			RP-HPLC	ZORBAxC18 (250 x 4.6mm, 5 µm particle size)	acetate buffer and acetonitrile in a ratio of 60:40	UV	232 nm	2.57 min	19
6	LIN and EMP				Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm),			229 nm		20

7	EMP				Intersil® C18 column (150 mm 4 mm, 5 µm)	acetonitrile-potassium dihydrogen phosphate buffer pH 4, (50:50, v/v)	UV	225 nm		21
8	EMP	1.08 min	human plasma	LC-MS/MS	X Bridge C18 column (75 mm × 4.6 mm, 3.5 µ)	acetonitrile and 10 mM ammonium bicarbonate (70:30 V/V)	Mass detector	-	2.40 min	39
9	MET & EMP		human plasma	UPLC-MS/MS	BEH C18 Coloumn(50x2.1mm, 1.7 µm)	Formic acid(0.01%): Acetonitrile (70:30 v/v)	Mass detector	-	<3.0 min	33
10	EMP	5.470 min	Dosage Form	HPLC	Thermo Hypersil GOLD C18 column (250 ×4.6 mm, 5 µm pore size).	0.1% solution of trifluoroacetic acid and acetonitrile (70:30 v/v)	UV Detector	224 nm		40
11	EMP, LIN, Metformin	14.5min,3.min , 2.01min	Dosage Form	RP-LC	Phenomenex C18 column (250 mm × 4.6 mm, 5 µm)	Acetonitrile: Methanol: Water in a ratio (27: 20: 53, v/v/v) pH 4 adjusted with 1% Ortho-phosphoric acid	PDA	223 nm	18 min	41
12	LIN & EMP	6.447, 4.716 and 4.079 min	Human Plasma	HPLC-UV	C18 (250×4.6×5)coloumn	buffer: acetonitrile (68:32)	PDA	218 nm	10 min	42
13	MET, LIN & EMP	-	Dosage Form	RP-HPLC	Thermo Hypersil octa decyl silane (250 mm × 4.6 mm, 5 µm	0.043 M potassium dihydrogen orthophosphate buffer premixed 00with 0.05%v/v TEA (buffer pH 3.79 adjusted using orthophosphoric acid): methanol (34.4:65.6, v/v)	DAD	225 nm	-	31
14	EMP	5.473 min	Dosage Form	HPLC	Thermo Hypersil GOLD C18 column (250 × 4.6 mm, 5 µm pore size)	0.1% solution of trifluoroacetic acid and acetonitrile (70:30 v/v)	UV Detector	224 nm	-	40
15	EMP	2.57 min	Stress Degradation	RP-HPLC	ZORBAxC18 (250 x 4.6mm, 5µm particle size)	Acetate buffer: Acetonitrile in a ratio of 60:40% v/v	UV Detector	232 nm	6 min	19
16	EMP	2.1 min	Impurities degradation	RP-HPLC	Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm)	acetonitrile: water (50:50 v/v)	UV Detector	-	-	8
17	EMP	3 min	Organic impurities	LC	CLC-phenyl column (250 mm 4.6 mm, 5 mm)	acetonitrile/ water mixture (72 : 28)	PDA	230 nm	8 min	30
18	EMP	2.54 min	Dosage form	HPLC	Poroshell 120 EC-C18, 4.6×100 mm, 4 µm column	methanol/acetonitrile/0.1% OPA (75:20:5)	DAD	222 nm	10 min	43

Table 5 -Methods of impurity profiling, forced degradation and bioanalytical for oral LIN :-

SL NO	DRUGS	Rt (min)	PLASMA/BLOOD IMPURITIES	INSTRUMENT	MOBILE PHASE/ STATIONARY PHASE	COLUMN	DECTECTOR	CHROMATOGRAM RECORDED	RUN TIME	REFERENCE
1	LIN	11 min	Forced degradation	HPLC	methanol: water containing 0.3 % TEA, 40:60	C18 column		225 nm		22
2	LIN		Forced Degradation	HPLC		Zorbax SB-Aq 250 4.6 mm, 5 µm column	TOF/MS	225 nm		24
3	LIN	-	Plasma	RP-HPLC	0.3% TEA: methanol. (60:40 v/v) pH 4.5 adjusted with o-phosphoric acid	Primesil C18, 250 mm x 4.6 mm	PDA	292 nm	1.0 mL/min	26
4	LIN and MET	-	Plasma	HPLC-MS/MS	methanol: 10 mM ammonium for mate buffer (containing 0.2 % formic acid) in a ratio of (95: 5, v/v)	Symmetry® C18	tandem mass spectrometer	231nm	0.25 mL min 1	28
5	EMP, LIN and MET	14.5 min, 3.4 min and 2.01 min		RP–LC	Acetonitrile: Methanol: Water in a ratio (27: 20: 53, v/v/v) pH 4 adjusted with 1% Ortho-phosphoric acid as mobile phase	Phenomenex C18 column (250 mm×4.6 mm, 5 µm)	PDA	223 nm	1 ml/min	41
6	EMP and LIN	-	-	LC-MS/MS	ammonium acetate buffer and acetonitrile as the mobile phase.	XSelect HSS Cyano (50 × 2.1 mm, 3.5 µm) column using 2 mM	PDA	-		44
7	LIN	-	impurity	LC	A mixture of 0.1% formic acid with pH 3.5 (A) and acetonitrile (B) was used as the mobile phase	Thermo Scientific® RP-8 column (100 mm × 4.6 mm; 5 µm)	PDA	-	0.6 mL min 1	29
8	LIN			HPLC	75% methanol: 25% formic acid 0.1% pH 4.1	Zorbax Eclipse XDB C18 column	PDA	254 nm	1.0 mL min 1	38
9	Canagliflozin, EMP, LIN and MET			HPLC	dipotassium hydrogen phosphate buffer (0.05 M, adjusted to pH 6 using o-phosphoric acid): acetonitrile: methanol (50: 25: 25, v/v/v)	Agilent Eclipse C8 column	PDA	277 nm	1.5 ml/min	28
10	LIN	3.3 min.		HPLC	methanol: phosphate buffer (of pH 4.5) as the mobile phase at a ratio of 70:30 v/v	C18 column (150×4.6mm, packed with 5 µm particles)	PDA	241 nm	1mL/min	45
11	LIN and MET and internal standard (phenformin)	4.95, 15.41 min and 11.06 min		HPLC	acetonitrile and 0.01M di-potassium hydrogen phosphate buffer in ratio of 75:25 and adjusting pH 7.0 with orthophosphoric acid	Grace vyadyec genesis CN (150 × 4.6 mm, 4 µm)	UV	237 nm	1.0 mL min 1	27
12	LIN			HPLC	The acetonitrile and the 0/01% v/v formic acid buffer	Waters Reliant™ HPLC Columns (250 mm × 4.6 mm i.d., 5 µm particle size)	PDA	230 nm	mL min 2	35

13	EMP and LIN		HUMAN PLASMA	spectroflourimetric methods Jasco FP-6200 Spectrofluorometer (Tokyo, Japan) equipped with 150-Watt xenon lamp		Phenomenex C18 column (250 mm×4.6 mm, 5 µm)	emission wavelength 538 nm after excition at wavelength 469 nm	1.0 The regression plots were found to be linear over the range of 40–1200 ng/mL and 3–700 ng/mL for EMP and LIN	[34]
14	LIN			Perkin Elmer LS 45 luminescence spectrometer (United Kingdom) equipped with 150-W xenon arc lamp and 1 cm quartz cell. Slit width for both monochromators were set at 10 nm			The emission of the formed product was measured at 479 nm after excitation at 390 nm.		[46]
15	LIN	11 min		HPLC-DAD	(methanol: water containing 0.3% TEA, 40:60, pH 4.5)	C18 column	225 nm	1 mL/min	[33]
16	LIN and MET			HPLC	mixture of Ammonium phosphate buffer (pH 3.00), and methanol in the volume ratio of 40:60 v/v as mobile phase	n Inertsil ODS2, 150 mm x 4.6mm, 5µ as chromatographic column uv detector	233nm	0.800 mLmin-1	[47]
17	MET, LIN in EMP			RP-UPLC	mixture solution of % phosphate buffer (pH=3) and 60% acetonitrile as mobile phase	Kromasil C18 column (2.1 x 50 mm, 1.8µm) PDA	248 nm	0.6 mL/min	[26]
18	LIN			RP-HPLC	mixture of Phosphate buffer : methanol (50:50 v/v) used as mobile phase and the pH was adjusted to 3 with o-phosphoric acid	Phenomenex C18 (4.6 x 100 mm, 5 µm	238 nm	0.8 ml/min	[37]

CONCLUSION

The review of the analytical methods reported for EMP, and LIN showed that spectrophotometric, HPLC with UV- visible detector have been reported. So far only few papers have been published based on its assay in rat plasma. In general, for the determination of the drugs in biological samples, HPLC-MS is ideal for estimation. So, it is a greater option to estimate the EMP and LIN in a biological sample by HPLC-MS can be carried out. This review represents an overview of the current analytical methods for the determination, impurity profiling, stress degradation study of EMP and LIN in an active pharmaceutical ingredient, tablet, and pharmaceutical dosage forms.

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