THE SYNOPSIS

OF THE THESIS ENTITLED

DEVELOPMENT & VALIDATION OF ANALYTICAL METHODS FOR THE ESTIMATION OF ANTI-DIABETIC DRUGS

SUBMITTED TO

GUJARAT TECHNOLOGICAL UNIVERSITY, AHMEDABAD
MAY-2019

IN THE PARTIAL FULFILLMENT OF
DOCTOR OF PHILOSOPHY
IN
PHARMACY AND PHARMACEUTICAL SCIENCES

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1. INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is the most prevalent metabolic disease worldwide. Inadequate management and control of hyperglycemia in patients with T2DM may lead to the risk of developing complications over the long term due to chronic and progressive nature of the disease arising from pathophysiology of beta-cell dysfunction, insulin resistance and increased hepatic glucose output. Patients with T2DM often require a combination of therapeutic agents in order to achieve glycemic control over the long term [1-6].

Fixed-dose combination (FDC) therapies have been shown to improve adherence by reducing costs, pill burden, and the complexity of treatment regimen [8-10]. A treatment approach with a FDC that includes combination of anti-diabetic medications could be used to obtain adequate glycemic control in patients with type 2 diabetes. A combined formulation consisting of metformin, sitagliptin and glimepiride in a single tablet would potentially offer increased patient convenience and subsequent potential for increased therapeutic compliance and can be studied for the treatment of adults with inadequately controlled T2DM to improve glycemic control. A clinical trial was conducted for evaluation of sitagliptin in combination with metformin and sulfonylurea [13]. The aim of that clinical trial protocol was to determine the non-inferiority of the effectiveness of sitagliptin compared to a control group of patients treated with thiazolidinedione as add-on therapy, in low-income ethnic minority type 2 diabetic patients who are failing to maintain adequate control with maximal doses of metformin and a sulfonylurea agent.

Bio-analytical method development and validation of selected anti-diabetic drugs in human biological matrices using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)

Due to large inter-individual variability, therapeutic drug monitoring (TDM) has been recommended to ensure adequate blood levels and reduce the onset of severe side effects, especially when applied to early onset of diabetes. The sensitive bio-analytical methods can successfully be applied for the Pharmacokinetic (PK) and TDM analysis to obtain a more accurate quantification of patient exposure to anti-diabetic drugs. The developed methods can be applied to the analysis of the therapeutic drug levels of
selected drugs taken from diabetic patients to assess adherence to medications and to establish the relationship between drug levels and metabolic control of diabetes.

**Stability indicating method development and validation for the simultaneous estimation of various anti-diabetic drugs and their combinations**

Advantages of simultaneous stability studies are the identification of new degradation products, to understand mutual induction and/or inhibition of rates of degradation and to analyze the degradation products of both drugs. Various ultraviolet spectroscopic and high performance liquid chromatographic assay methods were reported for the estimation of metformin, sitagliptin, pioglitazone, glimepiride and simvastatin individually and in combination with other drugs [15-34]. All the above reported methods were based on the estimation of metformin, sitagliptin, pioglitazone, glimepiride and simvastatin alone or in combination with other drugs.

The degradation products were generated and successfully separated by the developed and validated high performance liquid chromatographic methods for the estimation of the selected anti-diabetic drug combinations.

These methods can be successfully applied for the determination of stability of the active pharmaceutical ingredients (APIs) during pre-formulation and formulation studies for the development of various fixed dose combinations of metformin, sitagliptin, pioglitazone, glimepiride and simvastatin in the laboratories.

2. **AIM OF THE PRESENTED WORK**

**Bio-analytical method development and validation of selected anti-diabetic drugs in human biological matrices using Liquid Chromatography-tandem mass spectrometry method (LC-MS/MS)**

Although lactic acidosis in some patients with a higher plasma metformin and its combinations and newer anti-diabetic drugs remains debatable, several studies have found such condition, making measuring their level become important to optimize the dosage and prevent the lactic acidosis and other severe side effects.
The aim of the study was to develop and validate the highly sensitive methods for the estimation of anti-diabetic drugs and their combinations in human biological matrices by high performance liquid chromatography- tandem mass spectrometry methods (LC-MS/MS) as per the US-FDA guidelines.

**Stability indicating method development and validation for the simultaneous estimation of various anti-diabetic drugs and their combinations**

The aim of the study was to generate the possible degradation products of metformin, sitagliptin, pioglitazone, glimepiride and simvastatin in various combinations by stress degradation and to successfully separate the degradation products from the analyte by the validated stability indicating reversed phase – high performance liquid chromatography (RP-HPLC) methods.

### 3. OBJECTIVE AND PLAN OF WORK

- Bio-analytical method development and validation of Ertugliflozin in human K2EDTA plasma using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)
- Bio-analytical method development and validation of Ertugliflozin in human dried blood spots using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)
- Bio-analytical method development and validation of Metformin, Sitagliptin and Simvastatin in human K2EDTA plasma using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)
- Method development and validation of a reversed-phase liquid chromatographic method (RP-HPLC) for the simultaneous estimation of metformin, sitagliptin, pioglitazone, and glimepiride in the presence of their degradation products
- Stability indicating method development and validation for the simultaneous estimation of Metformin, Sitagliptin and Simvastatin in bulk drug and in their marketed formulation.
• Method development and validation of a reversed-phase liquid chromatographic method for the simultaneous estimation of metformin, sitagliptin and glimepiride in the presence of their degradation products
• Stability indicating method development and validation for the simultaneous estimation of Metformin and Dapagliflozin in bulk drug and in their marketed formulation.
• Stability indicating method development and validation for the simultaneous estimation of Metformin and Canagliflozin in bulk drug and in their marketed formulation.
• Development And Validation Of Q-Absorbance Ratio UV-Spectrophotometric Method For Simultaneous Estimation Of Metformin And Canagliflozin And Metformin In Bulk And Combined Dosage Form

4. ORIGINAL CONTRIBUTION BY THE THESIS
The authors contributed for:
• The development and validation of highly sensitive methods for the estimation of anti-diabetic drugs in human biological matrices by LC-MS/MS
• The development and validation of stability indicating methods for the estimation of anti-diabetic drugs and their degradation products

5. METHODOLOGY OF RESEARCH AND RESULTS

Bio-analytical method development and validation of Ertugliflozin in human K2EDTA plasma using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)

Introduction: Ertugliflozin belongs to the class of potent and selective inhibitors of the sodium-dependent glucose co-transporters (SGLT), more specifically the type 2 which is responsible for about 90% of the glucose reabsorption from glomerulus. The authors developed and validated a novel, automated, highly sensitive LC-electrospray-MS/MS method for the quantification of ertugliflozin in human K2EDTA plasma.
**Methods:** Human K2EDTA plasma was spiked with different calibration and quality control concentrations of ertugliflozin. Eight hundred µL internal standard solution containing dapagliflozin-d5 at a concentration of 10 ng/ml in acetonitrile were added. The extracts were analyzed using LC-electrospray-MS/MS in combination. The mass spectrometer was run in the negative multiple reaction-monitoring (MRM) mode. The total run time was 4.0 min. Ertugliflozin and its internal standard dapagliflozin-d5 were separated on an agilent poroshell EC-C18, 2.7µm, 120 Å, LC Column 50 x 4.6 mm. The optimized method was validated as per the US-FDA guidelines.

**Results:** The retention times of ertugliflozin and internal standard dapagliflozin-d5 were 2.58 min and 2.69 min, respectively. Calibration curves were constructed by plotting the peak area ratios of the corresponding analyte and internal standard against nominal analyte concentrations in the range of 0.25-1000 ng/ml. Accuracy was within the limits of acceptance criteria (80 – 120 %). %CV for Inter and Intra-day precision was within the limits of acceptance criteria ≤ 20%. Extraction recovery for ertugliflozin in human K2-EDTA plasma using acetonitrile protein precipitation method was >80%. No interferences with other compounds extracted from EDTA plasma exceeding 20% of the MS/MS detector signal at the LLOQ were found.

**Bio-analytical method development and validation of Ertugliflozin in human dried blood spots using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)**

**Introduction:** Ertugliflozin belongs to the class of potent and selective inhibitors of the sodium-dependent glucose co-transporters (SGLT), more specifically the type 2 which is responsible for about 90% of the glucose reabsorption from glomerulus. The authors developed and validated a novel, automated, highly sensitive LC-electrospray-MS/MS method for the quantification of ertugliflozin in human dried blood spots.

**Methods:** The blood was spiked with different concentration levels for calibrators and quality controls for Ertugliflozin. The blood drops were applied to filter paper cards. Punches of 6.4 mm were removed from the cards. Four hundred µL of protein precipitation solution (methanol/0.2M ZnSO₄, 7:3, v/v) containing the internal standard
dapagliflozin-d5 at a concentration of 10 ng/mL were added. The extracts were analyzed using LC-electrospray-MS/MS and the mass spectrometer was run in the negative multiple-reaction-monitoring (MRM) mode. The total run time was 4.0 min.

**Results:** For the DBSs, the assay has a lower limit of quantification of 0.25 ng/mL for Ertugliflozin. The range of reliable response was 0.25-1000 ng/mL ($r > 0.99$). The accuracy was within 85-115% of the nominal concentration and the imprecision was < 20% that met predefined acceptance criteria. There were no significant carry-over, matrix effects or matrix interferences.

**Conclusion:** This assay allows for measurement of small volume blood samples without the need for an IV blood draw and can be successfully applied for pharmacokinetics studies and therapeutic drug monitoring in patients for the estimation of ertugliflozin.

**Bio-analytical method development and validation of Metformin, Sitagliptin and Simvastatin in human K2EDTA plasma using Liquid Chromatography-tandem mass spectrometry method (LC-MS/MS)**

**Introduction:** Although lactic acidosis in some patients with a higher plasma metformin and its combinations remains debatable, several studies have found such condition, making measuring their level become important to optimize the dosage and prevent metformin-associated lactic acidosis. The aim of the study was to develop and validate the highly sensitive methods for the estimation of metformin in presence of its marketed combinations sitagliptin or simvastatin in human K2EDTA plasma by high performance liquid chromatography-tandem mass spectrometry methods (LC-MS/MS) as per the US-FDA guidelines.

**Methods:** Human K2EDTA plasma was spiked with different calibration and quality control concentrations of metformin in combination with sitagliptin and simvastatin. Eight hundred µL internal standard solution containing metformin-d6, sitagliptin-d4 and simvastatin-d6 at a concentration of 10 ng/ml, each respectively in acetonitrile were added. The extracts were analyzed using LC-electrospray-MS/MS in combination. The mass spectrometer was run in the positive multiple-reaction-monitoring (MRM) mode. The total run time was 6.0 min. The analytes were separated on an Agilent Poroshell EC-C18, 2.7µm, 120 Å, LC Column 50 x 4.6 mm. The assay was validated as per the US-
FDA guidelines.

**Results:** The retention times of metformin, sitagliptin and simvastatin are 0.51 min, 3.39 min and 4.74 min respectively. Calibration curves were constructed by plotting the peak area ratios of the corresponding analyte and internal standard against nominal analyte concentrations in the range of 0.49-1000 ng/ml. Accuracy was within the limits of acceptance criteria (80 – 120 %). %CV for Inter and Intra-day precision was within the limits of acceptance criteria ≤ 20%. Extraction recovery for metformin, sitagliptin and simvastatin in human K2-EDTA plasma using acetonitrile protein precipitation method was >70% for each analyte. No interferences with other compounds extracted from EDTA plasma exceeding 20% of the MS/MS detector signal at the LLOQ were found. No relevant matrix effects were detected for analytes in the plasma.

**Conclusion:** This is the first report of highly sensitive and fully validated LC-MSMS method for the quantitation of metformin in combination with sitagliptin and simvastatin in human K2EDTA plasma. The method is simple, accurate, precise, reproducible, sensitive and selective and can be applied successfully in the pharmacokinetic studies and for therapeutic drug monitoring.

**Method development and validation of a reversed-phase liquid chromatographic method (RP-HPLC) for the simultaneous estimation of metformin, sitagliptin, pioglitazone, and glimepiride in the presence of their degradation products**

**Objective:** This study was designed to conduct forced degradation and validation studies for the simultaneous estimation of metformin, sitagliptin, pioglitazone, and glimepiride.

**Methods:** Analytes were separated on an Agilent XDB-C18, 150 × 4.6 mm, 5 µm column using an isocratic elution mode having mobile phase composition of 20 mM potassium dihydrogen phosphate buffer (pH 4.0):acetonitrile (65:35% v/v). Analytes were detected at a wavelength of 225 nm. The optimized method was validated as per the ICH Q2 guidelines.

**Results:** The retention times of metformin, sitagliptin, pioglitazone, and glimepiride were 3.47, 4.83, 5.83, and 9.44 min, respectively. The linearity was 25–100 µg/ml for
metformin, 2.5–10 µg/ml for sitagliptin, 1–4 µg/ml for pioglitazone, and 0.75–3 µg/ml for glimepiride. The correlation coefficient for calibration curves was >0.99, and accuracy was between 98 and 102% for each analyte. Inter- and intra-day precisions were calculated <2% relative standard deviation for each analyte.

**Conclusion:** A significant degradation was observed in the presence of acidic, basic, neutral, oxidative, and photolytic stress conditions. The method is simple, precise, accurate, robust, and reproducible and was able to successfully separate and quantify metformin, sitagliptin, pioglitazone, and glimepiride in the presence of their degradation products.

**Stability indicating method development and validation for the simultaneous estimation of Metformin, Sitagliptin and Simvastatin in bulk drug and in their marketed formulation**

**Objective:** A reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of Metformin, Sitagliptin, and Simvastatin in the presence of their degradation products.

**Methods:** Analytes were separated on a Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.5): acetonitrile: triethylamine (85:15:0.1 %v/v/v). Analytes were detected at a wavelength of 225 nm. A 20µL fixed-loop injector was used for the injection of the samples with a flow rate of 1.0 mL min$^{-1}$. The optimized method was validated as per ICH Q2 guidelines.

**Results:** The retention times of Metformin, Sitagliptin, and Simvastatin were 3.70 min, 5.10 and 6.84 min, respectively. The linearity was 25-100 µg/ml for Metformin, 2-8 µg/ml Sitagliptin and 2.5-10 µg/ml Simvastatin. The correlation coefficient for calibration curves of Metformin, Sitagliptin, and Simvastatin was> 0.99. Accuracy was 98-102% for each analyte. Inter and Intra-day precision was calculated < 2 %RSD for each analyte. Limit of detection (LOD) and limit of quantitation (LOQ) were within the limits of ICH-Q2 guidelines. The method was robust with % RSD values < 2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in
the flow rate.

**Conclusion:** Significant degradation was observed in the presence of acidic, basic, neutral, oxidative and photolytic stress conditions. The proposed RP-HPLC method is simple, precise, accurate, robust and reproducible and was able to successfully separate and quantify Metformin, Sitagliptin, and Simvastatin in the presence of their degradation products; this implies the stability indicating nature and specificity of the method.

**Method development and validation of a reversed-phase liquid chromatographic method for the simultaneous estimation of metformin, sitagliptin and glimepiride in the presence of their degradation products**

**Objective:** A reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of metformin, sitagliptin and glimepiride in the presence of their degradation products.

**Methods:** Analytes were separated on an Agilent XDB-C18, 150 x 4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 20 mM potassium dihydrogen phosphate buffer (pH 4.0): acetonitrile (65:35 %v/v). Analytes were detected at a wavelength of 225 nm. The optimized method was validated as per ICH Q2 guidelines.

**Results:** The retention times of metformin, sitagliptin and glimepiride were 3.46 min, 4.57 and 8.51 min, respectively. the linearity was 25-100 µg/ml for metformin, 2.5-10 µg/ml sitagliptin and 0.75-3 µg/ml glimepiride. The correlation coefficient for calibration curves was > 0.99 and accuracy was between 98-102% for each analyte. Inter and Intra-day precision were calculated < 2 %RSD for each analyte.

**Conclusion:** Significant degradation was observed in the presence of acidic, basic, neutral, oxidative and photolytic stress conditions. The method is simple, precise, accurate, robust and reproducible and was able to successfully separate and quantify metformin, sitagliptin and glimepiride in the presence of their degradation products.
Stability indicating method development and validation for the simultaneous estimation of Metformin and Dapagliflozin in bulk drug and in their marketed formulation

Objective: A reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of Metformin and Dapagliflozin in the presence of their degradation products.

Methods: Analytes were separated on a Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (40:60 %v/v). The analytes were detected at a wavelength of 255 nm. A 20µL fixed-loop injector was used for the injection of the samples with a flow rate of 1.0 mL min⁻¹. The optimized method was validated as per ICH Q2 guidelines.

Results: The retention times of Metformin and Dapagliflozin were 3.78 min and 5.74 min, respectively. The linearity was 5-20 µg/ml for each of Metformin and Dapagliflozin, respectively. The correlation coefficient for calibration curves of both Metformin and Dapagliflozin were >0.99. Accuracy was 98-102%. Inter and Intra-day precision were calculated <2 %RSD. Limit of detection (LOD) and limit of quantitation (LOQ) were within the limits of ICH-Q2 guidelines. The method was robust with % RSD values <2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in the flow rate.

Conclusion: Significant degradation observed in the presence of acidic, basic, neutral, oxidative and photolytic stress conditions. The proposed RP-HPLC method is simple, precise, accurate, robust and reproducible and was able to successfully separate and quantify Metformin and Dapagliflozin in the presence of their degradation product, which implies the stability indicating nature and specificity of the method.

Stability indicating method development and validation for the simultaneous estimation of Metformin and Canagliflozin in bulk drug and in their marketed formulation
Objective: Reversed phase high performance liquid chromatographic method was developed and validated for the simultaneous estimation of Metformin and Canaglifozin in the presence of their degradation products.

Methods: Analytes were separated on Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (50:50 v/v). Analytes were detected at 250 nm. 20µL fixed loop injector was used for the injection of the samples with the flow rate of 1.0 mL min⁻¹. The optimized method was validated as per ICH Q2 guidelines.

Results: The retention times of Metformin and Canaglifozin were 3.47 min and 5.28 min respectively. The linearity was assessed by analysis of combined standard solution in the range of 5-20 µg/ml for each of Metformin and Canaglifozin respectively. Correlation coefficient for calibration curves of Metformin and Canaglifozin were 0.999 and 0.999 respectively. Accuracy was calculated in terms of % recovery and was within the limits of 98-102%. Inter and Intra-day precision was calculated in terms of %RSD and was <2%. LOD and LOQ were within the limits of ICH-Q2 guidelines. Method was robust with % RSD values <2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in the flow rate.

Conclusion: Significant degradation was observed in presence of acidic, basic, neutral, oxidative and photolytic stress conditions. Proposed RP-HPLC method is simple, precise, accurate, robust and reproducible and was able to successfully separate, identify and quantify the Metformin and Canaglifozin in the presence of their degradation products implies the stability indicating nature and specificity of the proposed method.

Development And Validation Of Q-Absorbance Ratio UV-Spectrophotometric Method For Simultaneous Estimation Of Metformin And Canagliflozin And Metformin In Bulk And Combined Dosage Form

Objective: A rapid, simple, accurate and precise UV spectrophotometric Q-absorption ratio method was developed and validated as per the ICH-Q2 guidelines for the
simultaneous estimation of Metformin and Canagliflozin.

**Methods:** The method involved Q-absorption ratio analysis using two wavelengths obtained by the overlay spectrum of the Metformin and Canagliflozin, with one being the maximum absorbance wavelength of Canagliflozin (220 nm, 2) and the other being the iso-absorptive point of both drugs (250 nm, 1). The method was validated as per ICH guidelines.

**Results:** Linearity and range were determined by analyzing six independent concentration levels of calibration curve in the range of 5-17.5 µg/mL for Metformin and Canagliflozin respectively (n=3). The value of correlation coefficient was > 0.99 for each of Metformin and Canagliflozin at 220nm and 250 nm. Accuracy of the method was confirmed by recovery study from marketed formulation at three levels – 80,100 and 120% of standard addition; the accuracy ranged between 99.63 and 101.14%. Intra-day and inter-day precision of Metformin and Canagliflozin was determined for three concentrations 5, 10 and 17.5 µg/mL at 220 nm, 240 nm and 250 nm; and % CV was <1.0%.

**Conclusion:** The proposed UV spectrophotometric Q-absorption ratio method is rapid, simple, precise, reproducible and economical that can be successfully employed for the industrial analysis of the combination of Metformin and Canagliflozin.

6. ACHIEVEMENTS WITH RESPECT TO OBJECTIVES
Successful development and validation of analytical methods for the estimation of anti-diabetic drugs and their combination by LCMS/MS, HPLC and UV-Spectrophotometric methods

7. CONCLUSION
Bio-analytical method development and validation of selected anti-diabetic drugs in human biological matrices using Liquid Chromatography- tandem mass spectrometry method (LC-MS/MS)
• Our first LC-MS/MS assay was developed to estimate the levels of ertugliflozin in human K2EDTA plasma and in human dried blood spots. This method is the first report of a fully validated and highly sensitive LC-MS/MS method.

• Our second LC-MS/MS assay simultaneously quantifies metformin, sitagliptin and simvastatin in human K2EDTA plasma. This method is the first report of a fully validated and highly sensitive LC-MS/MS method for the estimation of metformin and its marketed combinations- sitagliptin or simvastatin. The method can be successfully used for the therapeutic drug monitoring studies and pharmacokinetic studies in patients.

**Stability indicating method development and validation for the simultaneous estimation of various anti-diabetic drugs and their combinations**

Proposed reversed phase high performance liquid chromatographic methods were able to successfully separate and quantify metformin, sitagliptin, pioglitazone, glimepiride and simvastatin simultaneously in mentioned combinations in the presence of their degradation products. This implies the stability indicating nature and specificity of the method. The developed validated stability indicating RP-HPLC method is simple, precise, accurate, robust and reproducible resolving all the degradation products from the analytes of interest. The method can be successfully used for the quantitative determination of metformin, sitagliptin, pioglitazone, glimepiride and simvastatin individually and in the mentioned combinations as well as the stability of the analytes during the pre-formulation studies. The developed methods were validated as per ICH guidelines and can be successfully applied in quality control divisions of pharmaceutical industries.

8. **LIST OF PUBLICATIONS**


• Presented this research work at American Association of Pharmaceutical Scientists
   • Presented this research work at American Association of Pharmaceutical Scientists (AAPS)- 2016 held at Denver, Colorado, USA
5. Method development and validation of a reversed-phase liquid chromatographic method for the simultaneous estimation of metformin, sitagliptin and glimepiride in the presence of their degradation products
   • Accepted manuscript in International Journal of Pharmacy and Pharmaceutical Sciences 2019
6. Stability indicating method development and validation for the simultaneous estimation of Metformin and Dapagliflozin in bulk drug and in their marketed formulation.
   • Accepted manuscript in International Journal of Pharmaceutical Science Review and Research

9. REFERENCES


