A Review on Various Analytical Methods for Analysis of Metformin

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ABSTRACT

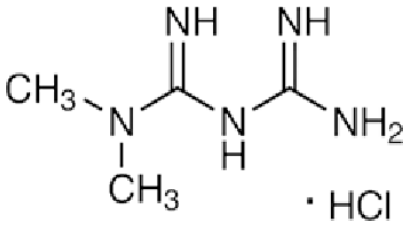
Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves. The most common is type 2 diabetes, usually in adults, which occurs when the body becomes resistant to insulin or doesn't make enough insulin. Type 1 diabetes, once known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin by itself. Oral hypoglycemic drugs are used only in the treatment of type 2 diabetes and metformin is a class of Biguanides. At the molecular level, metformin inhibits the mitochondrial respiratory chain in the liver, leading to activation of AMPK, enhancing insulin sensitivity (via effects on fat metabolism) and lowering cAMP, thus reducing the expression of gluconeogenic enzymes. Various analytical methods such as UV spectroscopic, high performance thin liquid chromatography (HPTLC), high pressure liquid chromatography (HPLC), reverse phase high performance liquid chromatography (RP-HPLC), ultra-performance liquid chromatography(UPLC), thin layer chromatography (TLC), liquid chromatography tandem mass spectroscopy, gas chromatography-mass spectroscopy, capillary electrophoresis (CE), spectrophotometric methods for determination of metformin as single and in combination with other drugs have been reported. In this review an attempt has been made to cover all the recent analytical methods which have been used for analysis of metformin.

Keywords: Metformin hydrochloride, Analytical methods, HPLC, HPTLC, UPLC, TLC.

INTRODUCTION

Metformin Hydrochloride, also known as 3-(diaminomethylidene)-1,1-dimethylguanidine hydrochloride, is an effective biguanide class oral antihyperglycemic medication. Metformin hydrochloride has long been considered the first-line medication for non-insulin-dependent diabetic mellitus (type II) blood glucose control. Metformin hydrochloride works by activating the enzyme AMP-activated protein kinase (AMPK), which reduces hepatic glucose synthesis (gluconeogenesis) and hence lowers blood glucose levels. It reduced glucose absorption in the intestine while improving insulin sensitivity, which improved peripheral glucose uptake and utilization. It disrupts the mitochondrial respiratory chain and increases anaerobic glycolysis for peripheral glucose utilization. It encourages weight loss rather than weight gain and is used to reduce the risk of macrovascular and microvascular complications in people with diabetes[1].

Chemical structure:



Side effects:

- Nausea
- Stomach upset
- Diarrhea
- Metallic taste
- Bloating
- Gas

Dosage: 500 mg or 800 mg of 2 or 3 times daily[2].

ANALYTICAL METHODS

There are several methods used to estimate metformin in pharmaceutical preparation and in human plasma such as UV, HPLC, HPTLC, TLC, UPLC, LC/MS, Capillary electrophoresis, methods have been reported, among these HPLC is the most widely used method for the analysis of metformin. In this review an attempt has been made to compile all the analytical methods which have been recently used for the analysis of metformin.

UV spectroscopic methods:

Various UV spectroscopic methods have been reported for determination of metformin hydrochloride in single and combination with other drugs. G. Mubeen et al. worked on simple and sensitive spectrophotometric method which has been developed and validated for the estimation of metformin hydrochloride in bulk and in tablet formulation. The primary

amino group of metformin hydrochloride reacts with ninhydrin in alkaline medium to form a violet color chromogen, which is determined spectrophotometrically at 570 nm. It obeyed Beer's law in the range of 8-18 µg/ml. Percentage recovery of the drug for the proposed method ranged from97-100% indicating no interference of the tablet excipients[3]. Ambadas R. Rote et al. developeda UV spectrophotometric method and validated for the estimation of metformin hydrochloride in tablet formulation. Metformin hydrochloride is determined spectrophotometrically at 232 nmusing distilled water as solvent. It obeyed Beer's law in the range of 2-10 µg/ml. Percentage recovery of the drug for the proposed method ranged from 102-105% indicating no interferenceof the tablet excipients. The proposed method was found to be accurate and precise for routine estimation of metformin hydrochloride in bulk and pharmaceutical formulation[4].

Reatul Karim et al. (2012) worked on a simple, economic, sensitive, precise and accurate UV spectrophotometric method which was developed and validated for quantification of metforminhydrochloride in bulk and in tablet dosage form. Adequate drug solubility and maximum assaysensitivity was found in 0.01N sodium hydroxide at 233 nm. Calibration graph constructed at 233nm was linear in concentration range of 1-25 µg/ml with correlation coefficient of 0.9998. The method was validated as per ICH guidelines in terms of linearity (within 1-25 µg/ml), accuracy (% recovery), precision (inter-day and intraday), specificity and robustness. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.2226 µg/ml and 0.6745µg/ml respectively[5]. M.S. Arayne (2009) reported a sensitive and accurate UV spectrophotometric method with multivariate calibration technique for the determination of metformin hydrochloride in bulk drug and different pharmaceutical formulations which has beendescribed. This technique is based on the use of the linear regression equations by using relationship between concentration and absorbance at five different wavelength. The results weretreated statistically and were found highly accurate, precise and reproducible. The method is accurate, precise (% recovery 102.50 ± 0.063, CV ≤ 0.56, r^2 = 0.997) and linear within the range1-10 µg/ml[6].

Parag. S. Mahadik et al. (2012) performed a simple, accurate, precise and cost effective UV-Vis spectrophotometric method for the estimation of metformin, in bulk and pharmaceutical dosage form. The absorption maxima of the drug was found to be 233 nm in 0.1N HCl: Distilled water (27:75). A linear response was observed in the range of 5-10 µg/ml witha regression coefficient of 0.999[7]. Kaushelendra Mishra et al. (2011) reported a simple, reproducible and efficient method for the determination of metformin hydrochloride (MET) was developed and validated. The analysis complied with beer's law in the concentration range of 8-13 µg/mL at 233 nm for MET. In ourstudy the validation of analytical method for determination of MET by UV in tablets formulation was performed in accordance the parameters including-system suitability, specificity, limit of quantification, limit of detection, linearity of response, accuracy, precision(reproducibility & repeatability), robustness (change of wavelength ± 2 nm)[8].

V. P. Patil et al. (2015) developed a simple and precise stability indicating UV spectrophotometric method for metformin hydrochloride in the bulk and tablet dosage form.

Separation of the drug from its degradation products was achieved by UV Spectrophotometric method using distilled water and scanned between 200 to 400 nm. Metformin hydrochloride was subjected to stress conditions such as hydrolysis (acid and base), oxidation, photolysis and thermal degradation. The maximum absorbance was found to be at 232.2 nm and found to be linear over the range 2-10 µg/ml with good correlation coefficient (r^2 = 0.998). The limits of detection and quantification were 0.5232 and 1.5856 µg/ml respectively[9]. Nishith Patel et al. studied a new, simple, accurate and sensitive UV-spectrophotometric absorption correction method has been developed for simultaneous determination of metformin hydrochloride and repaglinide in bilayer tablet dosage form utilizing concept of standard addition. The method is based upon determination of repaglinide at 299 nm where metformin hydrochloride shows zero absorbance& metformin hydrochloride at 208 nm in 0.1N HCl as a solvent & in phosphate buffer pH 6.8 repaglinide at 283 nm where metformin hydrochloride shows zero absorbance & metformin hydrochloride at 232.4 nm. Linearity was observed in range of 10-60 µg/ml and 1-6 µg/ml for metformin hydrochloride and repaglinide respectively. The correlation coefficient value was found to be 0.989-0.9998[10].

Audumbar Digambar Mali et al. (2015) worked on a simple, precise, economical, fast and reliable two UV methods which have been developed for the simultaneous estimation of metformin hydrochloride and glimepiride in bulk and pharmaceutical dosage form. Method A is Absorbance maxima method, which is based on measurement of absorption at maximum wavelength of 236 nm and 228 nm for metformin hydrochloride and glimepiride respectively. Method B is area under curve (AUC), in the wavelength range of 217-247 nm for metformin hydrochloride and 213-239 nm for glimepiride. Linearity for detector response was observed in the concentration range of 5- 25 µg/ml for metformin hydrochloride and 5-25 µg/ml for glimepiride. The accuracy of the methods was assessed by recovery studies and was found to be 100.23 % and 99.67 % for metformin hydrochloride and glimepiride respectively[11]. Chirag et al. (2014) reported two simple, precise and economical UV spectrophotometric methods have been developed for the simultaneous estimation of alogliptin benzoate and metformin hydrochloride in bulk and pharmaceutical dosage forms. Method A is simultaneous equation method (Vierodt's Method), which is based on measurement of absorption at 277 nm and 232 nm i.e. λ_{max} of alogliptin benzoate and metformin hydrochloride respectively. Method B is absorbance ratio (Q-analysis method) which is based on measurement of absorption at wavelength of 250 nm and 277 nm i.e. isoabsorptive point of alogliptin benzoate and metformin hydrochloride and λ_{max} of alogliptin benzoate respectively. Linearity was observed in the concentration range of 5-25 µg/ml for Alogliptin benzoate and 1-10 µg/ml for metformin hydrochloride. The accuracy of methods was assessed by recovery studies and was found to be within range of 98-102% for both alogliptin benzoate and metformin hydrochloride. The developed methods were validated withrespect to linearity, accuracy (recovery), and precision[12].

R.H. Majithia et al. (2020) carried out a simple, accurate, precise and economical Q-Absorption ratio spectrophotometric method was developed and validated for estimation of

anagliptin and metformin hydrochloride in synthetic mixture. Anagliptin and metformin hydrochloride showed an iso-absorptive point at 238 nm in distilled water. The second wavelength used was 233 nm which is λ_{max} of metformin hydrochloride in distilled water. The concentration of the drugs was determined by using ratio of absorbance at iso-absorptive point ($\lambda 1$ = 238 nm) and at the λ_{max} of metformin hydrochloride($\lambda 2$ = 233 nm). This method is linear for both drugs; in range of 2–12 μg/mL at $\lambda 1$ (r^2 = 0.999) and at $\lambda 2$ (r^2 = 0.9998) for anagliptin, and in the range of 5–30 μg/mL for metformin hydrochloride found at $\lambda 1$ (r^2 = 0.9995) and at $\lambda 2$ (r^2 = 0.9997). The % recovery was 100.42 –101.83 % of anagliptin and 99.94–101.63 % of metformin hydrochloride by standard addition method. The LOD was found to be 0.201 μg/mL and 0.262 μg/mL for anagliptin at $\lambda 1$ and $\lambda 2$ respectively. The LOD was found to be 0.320 μg/mL and 0.167 μg/mL for metformin hydrochloride at $\lambda 1$ and $\lambda 2$ respectively. The LOQ was found to be 0.610 μg/ mL and 0.794 μg/mL for Anagliptin at $\lambda 1$ and $\lambda 2$ respectively. The LOQ was found to be 0.972 μg/mL and 0.506 μg/mL for metformin hydrochloride at $\lambda 1$ and $\lambda 2$ respectively. The method was found to be precise as % RSD was less than 2.00 in Repeatability, Interday and Intraday precision for anagliptin and metformin hydrochloride. The % assay of analyte drugs in synthetic mixture was found to be 100.601% of anagliptin and 100.206 % of metformin hydrochloride which showed good applicability of the developed method[13].

Madhuri Ajay Hing et al. (2016) performed a simultaneous estimation of metformin and sitagliptin in marketed formulation using Q-Absorbance ratio method. In this spectroscopic method, 237 nm (λ_{max} of metformin) and 253.26 nm (iso absorptive point for other drugs) were selected for measurement of absorptivity. Both the drugs show linearity in a concentration range of 5-25 μg/ml for metformin and 0.5-2.5 μg/ml for sitagliptin at 237 nm and 253.26 nm respectively. Accuracy, precision and recovery studies were done by QC samples covering lower, medium and high concentrations of the linearity range. The relative standard deviation for accuracy, precision studies were found to be within the acceptance range (<2%). recovery of metformin and sitagliptin were found to be 99.73-101.16 % and 99.44-101.56 % respectively confirming the accuracy of the proposed method[14].

Sushil D. Patil et al. (2019) reported a new, simple, accurate and sensitive UV spectrophotometric the method have been developed for estimation of empagliflozin and metformin in combined tablet dosage form. The first method is absorbance corrected method (method A), the second method is area under curve (method B) and the third method is dual wavelength (method C). the first method A the empagliflozin determined at λ_{max} 224nm and metformin determined at λ_{max} 232nm and intercept at λ_{max} 203nm. The second method B wavelength length range 219-229 nm and 227-238 nm were selected to determined empagliflozin and metformin. In the third method C, empagliflozin was determined by plotting the difference in absorbance at 224-238 nm against the concentration of metformin. Similarly for the determination of metformin, the difference in absorbance at 232-244 nm against concentration of empagliflozin in combined formulation. The methods were validated by following the analytical performance parameters suggested by the International Conference on

Harmonization (ICH). Beer's law is obeyed in the concentration range of 2-10µg/ml for empagliflozin and 4-20 µg/mL for metformin by the following methods[15]. Mahesh Attimarad et al. (2021) studied the recent trend in green analytical chemistry is the development of greenanalytical methods using environmentally friendly solvents. Therefore, three ecofriendly manipulated UV spectroscopic techniques have been validated for the concurrent quantification of newly approved remogliflozin etabonate (REM) and metformin hydrochloride(MET) tablets using water as a solvent. The first method was established using first derivativeabsorption spectroscopic method by determining the peak amplitude at 233.0 nm for remogliflozin etabonate and 252.2 nm for metformin, a zero crossing of one the component. The second and third methods were based on the peak amplitude difference and first-order derivative absorption of the ratio spectra developed by the manipulation of scanned UV spectra. Remogliflozin etabonate and metformin showed good linearity in the series of 1–20 µg ml^{-1} and 2.5–35 µg ml^{-1}, respectively, by all three methods with an excellent correlation coefficient($r^2 \geq 0.998$)[16].

Manojkumar K. Munde et al. (2020) carried out four new UV spectrophotometric methods namely simultaneous equation, absorbance ratio, area under curve and first derivative (zero crossing) spectroscopic methods were developed and validated for simultaneous estimation of empagliflozin and metformin hydrochloride in bulk and tablet formulation. In simultaneous equation method, absorbance was measured at 224 and 232 nm for both the drugs. empagliflozin and metformin hydrochloride was estimated using 224 and 232 nm in absorbance ratio method. In Area under curve method both drugs were estimated at224 and 232 nm respectively. First derivative (zero crossing) method was based on the transformation of UV spectra in to first derivative spectra followed by measurement of first derivative signal at 224 and 232 nm for empagliflozin and metformin hydrochloride, respectively using 2 nm as wavelength interval and 1 as scaling factor[17].

HPTLC Chromatographic Methods:

Several high performance thin layer chromatography (HPTLC) methods have been reported for determination of metformin in single and combination with other drugs. HPTLC methods are widely used chromatographic methods for analysis of metformin in bulk and tablet dosage forms. Shweta Havele et al. (2010) worked on a simple and sensitive, HPTLC method which has been developed for the quantitative estimation of metformin in its single component tablet formulation. Metformin was chromatographed on silica Gel 60 F254 TLC plate using ammonium sulfate (0.5%): 2-propanol: methanol in the ratio of 8.0:1.6:1.6 (v/v/v) as mobile phase. Metformin showed R_f value of 0.50±0.03 was scanned at 238 nm using Camag TLC Scanner 3. The linear regression data for the calibration plot showed a good relationship with $r^2 = 0.999$. The method was validated for precision and recovery. The limits of detection and quantification were 95 and 200 ng/spot respectively[18]. A. Rajasekaran et al. (2014) carried out a simple, precise, rapid, selective, and economic high-performance thin layer chromatographic method which has been established for simultaneous estimation of Metformin Hydrochloride and Linagliptin in formulation. The chromatographic separation was performed on precoated silica gel 60 GF 254 plates with acetone-methanol-toluene-

formic acid 4:3:2:1 (v/v/v/v) as mobile phase. The plates were developed to a distance of 8 cm at ambient temperature. The developed plates were scanned and quantified at their single wave length of 259 nm. Experimental conditions such as band size, chamber saturation time, migration of solvent front, slit width, etc was critically studied and the optimum conditions were selected. The drugs were satisfactorily resolved with Rf 0.61 and 0.82 for metformin hydrochloride and linagliptin respectively. The calibration plot was linear between 400-2000 (ng/spot) and 20-100 (ng/spot)for metformin hydrochloride and linagliptin respectively. The limits of detection and quantification for metformin hydrochloride and linagliptin are 20 (ng/spot) and 10 (ng/spot) respectively[19].

C.H. Madhusudan Reddy et al. (2008) studied a HPTLC method for estimation of metformin HCl in single and in combination dosage form which was developed and validated by using mobile phase consisting of methanol: chloroform: ammonium acetate (6:3:1 v/v/v). Densitometric analysis of metformin hydrochloride was carried out in the absorbance mode at 236 nm. The linearity was found to be in the concentration range of 100- 300 ng/spot.The % recovery of metformin hydrochloride was found to be 100.24-101.58%, indicating no interference from the excipients in the method[20]. Asha ByjuThomas et al. (2011) reported stability indicating high performance thin layer chromatography (HPTLC) method which was developed and validated for determination of two anti-diabetic drugs, nateglinide and metformin hydrochloride in co-formulations. Study was performed on pre-coated silica gel HPTLC plates using chloroform: ethyl acetate: acetic acid (4:6:0.1 v/v/v) as the mobile phase. A TLC scanner set at 216 nm was used for direct evaluation of the chromatograms in the reflectance/absorbance mode. Method was validated according to ICH guidelines. The correlation coefficients of calibration curves were found to be 0.996 and 0.995 in the concentration range of 200–2400 and 500–3000 ng band^{-1} for nateglinide and metformin, respectively. The method had an accuracy of 99.72% for nateglinide and 100.08% for metformin hydrochloride. The method had the potential to determine these drugs simultaneously from dosage forms without any interference of the tablets excipients. Nateglinide and metformin hydrochloride were also subjected to acid, base, oxidation, wet, heat and photo-degradation studies[21].

Darshana K. Modi et al. (2012) worked on a simple, rapid, and precise high-performance thin-layer chromatographic (HPTLC) method for simultaneous estimation of two antidiabetic drugs, metformin hydrochloride and sitagliptin phosphate, in tablet dosage form which have been developed and validated. Chromatography was performed on silica gel 60 F_{254} plates with butanol : water : glacial acetic acid (6 : 2 : 2, v/v/v) as mobile phase. This system gave a good resolution for metformin hydrochloride (value of 0.35 ± 0.01) and sitagliptin phosphate (value of 0.75 ± 0.01). Detection and quantification were carried out at 227 nm. The linear regression data for the calibration plot showed a good relationship with r = 0.9995 and 0.9991 for metformin hydrochloride and sitagliptin phosphate, respectively. The method was validated for precision and recovery. The limits of detection and quantification were 13.05 and 39.56 ng/μL for metformin hydrochloride and 2.65 and 8.03 ng/μL for sitagliptin phosphate, respectively. The amounts of the drugs in the marketed formulation

were 99.86% and 98.91% for metformin hydrochloride and sitagliptin phosphate, respectively[22]. Keyur B. Ahir et al. (2013) performed a reversed phase high-performance liquid chromatography (HPTLC) method that has been established for simultaneous analysis of metformin hydrochloride and repaglinide. HPTLC method was developed using on precoated silica gel G60 F254 plates as stationary phase, using methanol: ammonium sulphate (0.25%) (pH-5.7) (2.5:7.5, v/v) as mobile phase. The plates were scanned at approximately 243 and 236 nm for HPLC and HPTLC both respectively. In HPTLC method both the drugs were resolved using proposed mobile phase and Rf value was found to be 0.34 for metformin and Rf 0.60 for repaglinide. The method was found to linear in the range 500-2500 ng/band for metformin, and 100-500 ng/band based for repaglinide respectively[23].

Jitendra PP, et al. (2020) reported a high Performance thin Layer Chromatographic Method that have been developed to quantify metformin hydrochloride and Alpha Lipoic Acid. Separation of both the drugs was carried out by using silica gel 60F254 plates. The mobile phase comprised of Toluene, Ammonium Acetate (4%), Ethyl Acetate (5:4:1 v/v/v). The detection wavelength was found to be 227 nm. The Rf values of metformin hydrochloride and alpha lipoic acid were found to be 0.28 and 0.65 respectively. The method was linear over concentration range 1500-7500 ng /band for metformin hydrochloride and 600-3000 ng / band for alpha lipoic acid. The developed method was validated according to ICH guidelines. Linearity, regression value, recovery and %RSD of intraday and interlay precision values were found within the limits and the method was found to be satisfactory. The developed HPTLC method was found to be simple, accurate and precise[24]. Sakhare et al. (2017) studied stability indicating high performance thin layer chromatography (HPTLC) method of analysis of metformin hydrochloride and Benfotiamine both as a bulk drug and in their combined formulation that has been developed. The basic aim of this method is to separate both the drugs by HPTLC and measure their spots at 249 nm. The separation was carried out on TLC aluminium sheets of silica gel 60F 254 using Benzene: Methanol: Triethylamine (8.5:1:0.5, v/v/v) as a mobile phase. Stability of MET and BENT was carried out by forced degradation study. Metformin hydrochloride and benfotiamine gave distinct and well defined peak at Rf 0.26 and 0.72, respectively. Calibration curves were linear in range of 500- 3000 and 75-450 ng/spot for metformin hydrochloride and benfotiamine, respectively. Method was successively applied to tablet formulation. Stability study shows that the chromatograms of samples degraded with acid, base, hydrogen peroxide, light and dry heat showed well separated spots of pure metformin hydrochloride and benfotiamine as well as some additional peaks at different Rf values. The HPTLC method was also able to selectively quantitate metformin hydrochloride and benfotiamine in presence of their degradation products obtained in forced degradation study. Hence, the method can be used as stability indicating[25].

Sunil R. Dhaneshwar et al. (2010) carried out a HPTLC method for simultaneous estimation of Metformin hydrochloride (MET), Atorvastatin (ATV) and Glimepiride (GLM) as the bulk drug and in tablet dosage forms. Chromatographic separation of the drugs was performed on aluminum plates precoated with silica gel 60 F 254 as the stationary phase and

the solvent system consisted of water: methanol: ammonium sulphate (1: 1: 4 v/v/v). Densitometric evaluation of the separated zones was performed at 237 nm. The three drugs were satisfactorilyresolved with Rf values 0.37 ± 0.02 and 0.59 ± 0.02, 0.75 ± 0.02 for MET, ATV, GLM respectively. The accuracy and reliability of the method was assessed by evaluation of linearity(200-700 ng/spot for MET, 600-2100 ng/spot for ATV and 600-2100 ng/spot for GLM), precision (intra-day% RSD was 0.54–1.23 and inter-day% RSD was 0.90–1.48 for MET, intra-day% RSD was 0.91–1.74 and inter-day% RSD was 0.56–1.52 for ATV and intra-day% RSD was 0.60–1.27 and inter-day% RSD was 0.96–1.48 for GLM), accuracy (99.66 ± 0.14 for MET, 98.46 ± 0.40 for ATV and 98.62 ± 0.39 for GLM), and specificity in accordance with ICH guidelines[26]. Bendale et al. (2017) reported a stability indicating high-performance thin layer chromatography method was developed and validated of vildagliptin (VIL) and metformin (MET) in pharmaceutical dosage forms. In the present study, system suitability test,stress study, alkali hydrolysis, acid hydrolysis, neutral hydrolysis, oxidative stress degradation, dry heat degradation, wet heat degradation, photodegradation study has been used. In this method, optimization by changing various parameters, such as organic solvent and the composition of the mobile phase, acid or base modifier used in the mobile phase; by varying one parameter and keeping all other conditions constant. 10 µl of the stock solution for MET (500 ng/band) and 2 µl of the stock solution for VIL (100 ng/band) were applied to TLC plates.The final solutions were applied on the HPTLC plates and these were developed as per the optimized densitometry conditions. From the spectra, it was observed that MET and VIL exhibited good absorbance at about 217 nm. Both the drugs showed degradation with additional peaks at Rf values of 0.16 for MET and with Rf values 0.81 for VIL respectively. The method was validated for linearity, precision, accuracy, limit of detection, limit of quantification, ruggedness, specificity, and robustness. Good separation was achieved by using the mobile phase Hexane: Methanol: Acetonitrile: Glacial Acetic Acid (2:3.5:2.5:0.2 v/v/v/v) with retardation factor (Rf) values of 0.22 ± 0.01 for MET and 0.73±0.02 for VIL[27].

Kumar Manikanta A et al. (2010) studied a high-performance thin layer chromatographic method for analysis of atorvastatin, glimipride and metformin in pharmaceutical dosage form. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of water: methanol: ammonium sulphate (3.5:3.5:12.6, v/v/v). This system was found to give compact spots for atorvastatin, glimipride and metformin (Rf value of 0.50 ± 0.01, 0.65 ± 0.01 and 0.33 ± 0.01). Densitometric analysis of atorvastatin, glimipride and metformin were carried out in the absorbance mode at 245nm. The linear regression data for the calibration plots showed good relationship with r^2 = 0.999 ± 0.001 from 100-700 ng for atorvastatin, r^2 = 0.998 ± 0.002 from 20-140 ng for glimipride and r^2 = 0.996 ± 0.001 from 100 -1500 ng for metformin, respectively. The methods were validated for precision, accuracy, ruggedness and recovery. The limits of detection and quantification were 20 and 80 ng per spotfor atorvastatin, 5 and 20 ng per spot for glimipride and 50 and 100 ng per spot for metformin,respectively[28].

HPLC Chromatographic methods

Several high-pressure liquid chromatographic (HPLC) methods have been reported for determination of metformin hydrochloride in single and combination with other drug. RP-HPLC method was developed by Ramesh Gugulotha et al. (2016) for reverse phase high performance liquid chromatographic (RP-HPLC) method for the analysis of Metformin Hydrochloride (MET) in bulk and tablet dosage form that has been developed and validated. This method was performed with a Symmetry C18 (4.6 × 150mm, 5µm) column with 60:40 (v/v) 50mM potassium dihydrogen orthophosphate buffer : methanol as mobile phase at a flow rate of 1.0 ml/min. UV detection at 262nm; MET was eluted with retention time of 1.694 min.The method was continued and validated in accordance with ICH guidelines. Validation revealed that the method is rapid, specific, accurate, precise, reliable, and reproducible. Calibration curve plots were linear over the concentration ranges of MET was 100-300 µg/mL. A limit of detection (LOD) was 0.15 µg/ml and limits of quantification (LOQ) was 0.5 µg/mL for MET[29]. In another study Nilesh Nikam et al. (2019) developed a simple and reproducible method for Metformin (MET) by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Metformin was separated on C18 column [4.6x250 mm, particle size 5µm], usingcombination of phosphate buffer with pH of 3.0 and methanol at the UV detection of 238nm. Isocratic elution of phosphate buffer with pH of 3.0 and methanol was used as a mobile phasewith various ratios and flow rates, eventually 30:70 v/v phosphate buffer with pH of 3.0 and methanol was being set with the flow rate of 1mL/min. The statistical validation parameters such as linearity, accuracy, precision, inter-day and intra-day variation were checked, assay studies of metformin were within 98% to 102% indicating that the proposed method can be adoptable for quality control analysis of metformin[30].

Chhetri et al. (2013) reported a reversedphase high performance liquid chromatographic method for the determination of metformin hydrochloride in bulk and dosage forms that has been developed and validated. The separation was carried out on a reversed phase C-18 column (250 mm x 4.6 mm, 5.0 µm) with UV detection at 233 nm. The mobile phase contained 34% acetonitrile and 66% aqueous phase. Aqueous phase contained 10 mM of monopotassium phosphate and 10 mM of sodium lauryl sulfate. Mobile phase pH was adjusted to 5.2. The mobile phase was run isocratically. The flowrate of the mobile phase was maintained at 1.3 ml/min. The linearity of the calibration curve was obtained in the concentration range of 2.5 to 20 µg ml^{-1} and coefficient of determination (r^2) was found to be 0.9985. The % RSD value for intraday and interday precision was below 1 which indicated that the method was precise. Limit of detection and limit of quantification were 0.1 and 0.3 µg/ml respectively[31]. Chengalva et al. (2016) developed an RP-HPLC method for simultaneous quantitative estimation of metformin hydrochloride and nateglinide in tablets and validate as per ICH guidelines. The method used a reverse phase column, Inertsil C18-ODS 3V (250×4.6 mm, 5 µm), a mobile phase comprising of phosphate buffer (pH 4.0): acetonitrile: methanol (30:60:10) flow rate of 1.0 ml/min and a detection wavelength of 221 nm using a UV detector. The developed method resulted in elution of metformin hydrochloride at 2.45 min and nateglinide at 4.21 min. The calibration curves were linear (r^2=0.999) in the concentration range of 60-140 µg/ml and 14.4-33.2 µg/ml for metformin hydrochloride and nateglinide respectively. The percentage recoveries were found to be 99.59-101.36 for metformin hydrochloride and 98.43-101.38 for nateglinide. The LOD was

found to be 2.18 µg/ml and 1.55 µg/ml for metformin hydrochloride and nateglinide respectively. LOQ was found to be 8.52µg/ml and 4.69µg/ml for metformin hydrochloride and nateglinide respectively[32].

Chandrabatla Varaprasad et al. (2015) performed RP-HPLC method using a PDA detector at 225 nm wavelength for simultaneous estimation of metformin and linagliptin in pharmaceutical dosage forms has been developed. The method was validated as per ICH guidelines over a range of 250-2500 µ g/mL and 1.25-12.5 µg/mL for metformin and linagliptin respectively. Analytical column W\ater's X-Bridge C18, 150 × 4.6 mm, five µ was used at a temperature of 30°C ± 0.5°C. Acetonitrile and 0.02M phosphate buffer (pH 5.0) in the ratio of35:65% v/v composition were used as mobile phase at a flow rate of 1.0 mL/min. Retention times of 1.6 and 4.6 min were obtained for metformin and linagliptin respectively. The percentage recoveries of metformin and linagliptin are 100.12% and 99.42% respectively[33]. In another study Nareddy Preethi Reddy et al. (2015) developed a new HPLC method for simultaneous estimation of metformin and canagliflozin in pharmaceutical dosage forms. Chromatography was carried out on an ODS 250mm, 4.6 mm, 5µm particle size with a isocratic mobile phase composed of buffer, acetonitrile and methanol at a flow rate of 1mL/min. The column temperature was maintained at 30°C and the detection was carried out using a PDA detector at 212 nm. Validation parameters such as system suitability, linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ), stability of sample and standard stock solutions and robustness were studied as reported in the International Conference on Harmonization guidelines. The retention times for metformin and canagliflozin and were 2.783 min and 3.781 min respectively. The percentage recoveries of metformin and canagliflozin were 100.1% and 100.2% respectively[34].

Prabhakar et al. (2014) carried out a simultaneous estimation of the metformin hydrochloride and repaglinide in tablet dosage form. Chromatogram was run through Devenosil ODS HG-5 RP C18(150mm: 4.6mm, 5µ). Mobile phase containing buffer, acetonitrile and methanol in the ratio of 20:80 was pumped through column at a flow rate of 1.0ml/min. Buffer used in this method was 0.01N potassium dihydrogrn phosphate, Optimized wavelength for metformin hydrochloride and repaglinide was 242 nm. Retention times of metformin hydrochloride and repaglinide were found to be 1.97 min and 4.34 min. %RSD of the metformin hydrochloride and repaglinide were found to be 1.55 and 1.22 respectively. %Recovery was Obtained as 100.008 and 98.08 for metformin hydrochloride and repaglinide respectively. LOD, LOQ values obtained from regression equations of metformin hydrochloride and repaglinide were 0.1, 0.3 and 0.15, 0.45 respectively. Regression equation of metformin hydrochloride is y = 210870x + 22039, and of repaglinide is y = 64545x + 2189. Regression co-efficient was 0.998[35]. In another study Serap Sağlık Aslan et al. (2017) reported a derivative spectrophotometric method and one HPLC method which were developed and validated for analysis of anti-diabetic drugs, repaglinide (RPG) and metformin hydrochloride (MET) in tablets. The spectrophotometric methods were based on zero-crossing first-derivative and fourth-derivative spectrophotometric method for simultaneous analysis of RPG (308 nm) and MET (267 nm), respectively. Linear relationship between the absorbance at λ_{max} and the drug concentration was found to be in the ranges of

5.0 - 50.0 µg·mL^{-1} for both RPG and MET. The quantification limits for RPG and MET were found to be 0.568 and 1.156 µg·mL^{-1}, respectively. The detection limits were 0.170 and 0.347 µg·mL^{-1} for RPG and MET, respectively. The second method is a rapid stability-indicating isocratic HPLC method developed for the determination of RPG and MET. A linear response was observed within the concentration range of 5.0 - 50.0 µg·mL^{-1} for both RPG and MET. The quantification limits for RPG and MET were found to be 1.821 and 1.653 µg·mL^{-1}, respectively. The detection limits were 0.601 and 0.545 µg·mL^{-1} for RPG and MET, respectively[36].

K. Sravana Kumari et al. (2020) development and validation performed on stability indicating RP-HPLC method for the simultaneous estimation of ertugliflozin pidolate and metformin hydrochloride in bulk and tablets was developed and validated. The separation of ertugliflozin pidolate and metformin hydrochloride was achieved isocratically on Kromasil C18 column (150 mm × 4.6 mm, 5 µm) using 0.1% ortho-phosphoric acid buffer (pH 2.7): acetonitrile (65:35% v/v) as mobile phase, pumped at a flow rate of 1 ml/min and column temperature of 30 ± 2 °C. HPLC grade water : ACN (1:1) was used as diluent. About 10 µl of standard solution of the drugs was injected, and the eluted analytes were detected at 224 nm. Metformin hydrochloride was eluted at 2.170 min and ertugliflozin pidolate at 2.929 min with a run time of 5.0 min. Linearity of the developed method was observed in the concentration range of 0.9375–5.625 µg/ml for ertugliflozin pidolate and 62.5–375 µg/ml for metformin hydrochloride with a correlation coefficient of 0.999 for both the drugs. LOD for ertugliflozin pidolate and metformin hydrochloride were 0.025 µg/ml and 0.87 µg/ml respectively. LOQ for ertugliflozin pidolate and metformin hydrochloride were 0.076 µg/ml and 2.63 µg/ml[37]. Gadapa Nirupa et al. (2012) worked on developing a single analytical method for estimation of individual drug from a multidrug composition which is a very challenging task. A simple, rapid, precise, and reliable reverse phase HPLC method was developed for the separation and estimation of three drugs glimepiride, pioglitazone and metformin in bulk drug mix and pharmaceutical dosage forms. estimation was carried out using Inertsil ODS-3V (250 mm × 4.6 mm, 5 μm) column; mobile phase consisting of acetonitrile, tetrahydrofuran, and buffer at pH 5; the flow rate of 1.7 mL/min and ultraviolet detection at 228 nm. All the three drugs were properly resolved having run time of 5 minutes, 3.9 minutes and 1.3 minutes for glimepiride, pioglitazone, and metformin, respectively[38].

Other Analytical methods:

LC-MS/MS method was developed by Mohammed Al Bratty et al. (2017) a new liquid chromatography tandem mass spectrometric method for simultaneous estimation of anti-hyperglycemic agents, metformin, linagliptin, sitagliptin and vildagliptin from human plasma was developed and validated as per ICH/USFDA guidelines. Chromatographic separations were achieved using Chromolith High Resolution RP-18e HPLC column (100 mm × 4.6 mm, macropores 1.15 µm) with an isocratic elution mode using the mobile phase composed of 0.01 M ammonium formate buffer (pH 3.0): acetonitrile (80:20 v/v). A flow rate of 0.4 mL min^{-1} was maintained throughout the analysis. Detection was performed by triple quadrupole MS fitted with ESI probe functioning in the positive ion MRM mode. Extractions of all the

drugs from plasma were carried out by acetonitrile crash technique. Alogliptin was used as an internal standard to minimize the error which could occur during analysis. The validation study data demonstrated that the new method is highly selective and sensitive (the limits of detection were 1.76, 1.94, 0.17 and 3.08 ng mL^{-1} for metformin, linagliptin, sitagliptin and vildagliptin, respectively). The %CV and %RE values were within the acceptable limit, <1% for most of the data, which indicate that the reported method was very precise and accurate. A linear calibration curve (correlation coefficient, $r^2 > 0.999$) was obtained at the concentration range of 0.5–400.0; 5.0–400.0; 10.0–500.0 and 0.5–40.0 ng mL^{-1} for metformin linagliptin, sitagliptin and vildagliptin, respectively. The extraction efficacy was evidenced from recovery study and all four analytes were found to be stable in plasma[39]. Bassam M. Ayoub et al (2017) developed a new LC–MS/MS method for determination of empagliflozin and metformin. Bridged Ethylene Hybrid C_{18} column (50 mm × 2.1 mm, 1.7 μm), isocratic elution based on 0.1% aqueous formic acid:acetonitrile (75:25, *v/v*) as a mobile phase, column temperature at 55°C and flow rate at 0.2 mL min^{-1} were used. The mass spectrometer was operated under multiple reaction monitoring mode using electrospray ionization by monitoring the transition pairs (precursor to product ion) of *m/z* 451.04–71.07 for empagliflozin and *m/z* 130.11–71.14 for metformin in the positive mode. The validation parameters were acceptable over concentration ranges of 5–1,000 ng ml^{-1} and 50–25,000 ng ml^{-1} for empagliflozin andmetformin, respectively[40].

P Venkateswarao Rao et al. (2021) studied the use of highly responsive simple liquid–liquid extraction method development using deuterated MET and deuterated ERT, LC-MS/MS method for gradation of MET and ERT in the rat plasma. The chromatographic condition involves isocratic mode using Waters XBridge C18 3.5 μ (150×4.6 mm) column. Mobile phase was 0.1% orthophosphoric acid and acetonitrile in the ratio of 80 : 20 v/v. Detection was carried out on a triple quadrapole MS employing electrospray ionization technique, operating multiple reactions, monitoring with the transitions of m/z 258.2→174.1, m/z 250.1→210.2, m/z 258.2→174.1, and m/z 260.3→210.2 for MET, ERT, deuterated MET, and deuterated ERT, respectively, in the positive ion mode. The method has been validated, and the linearity was observed in the range of 10–150 ng/ml and 0.1–1.5 ng/ml for MET and ERT, respectively. For intraday and interday %RSD, the values were found to be within the acceptable limits. Recovery studies for MET and ERT obtained, mean recovery of 99.5 and 98.6%, respectively[41]. Ucakturk, Ebru et al. (2013) developed a gas chromatography-mass spectrometry method for the determination of metformin in human plasma. A new, simple, specific gas chromatography-mass spectrometry method (GC-MS) was developed for the determination of metformin in human plasma. A number of derivatization approaches (silylation, acylation and methylation) were tested to derivatize metformin prior to GC-MS analysis. Derivatization of metformin was achieved by using N-methyl bis(trifluoroacetamide) (MBTFA). Several parameters such as reaction temperature and time were optimized, which affected the yield of the derivatization reaction. A trifluoroacetyl derivative of metformin was identified and quantified in selected ion monitoring mode (mass-to-charge ratio (m/z): 303). The method was fully validated using parameters such as specificity, carryover, linearity, limits of detection and quantification,

precision, accuracy, stability, recovery, robustness and ruggedness. Linearity was demonstrated over the concentration range of 100-3000 ng ml^{-1} with a coefficient of determination (r^2) above 0.996. The limits of quantification and detection were found to be 100 and 40 ng ml^{-1}, respectively. Intra- and inter-day accuracy and precision were within the acceptable limits (<15% for concentration points in the calibration range; <20% for the limit of quantitation). The developed method was successfully applied for the identification and quantification of metformin in the plasma of diabetic patients[42].

Regina Andayani et al. (2015) reported a simultaneous determination of two antidiabetic drugs, metformin hydrochloride and glibenclamide in pharmaceutical tablet formulations. A simple, rapid, precise, and accurate thin layer chromatography-densitometry (TLC-Densitometry) had been developed for the determination of mixed metformin hydrochloride and glibenclamide in tablet dosage forms. Normal phase thin layer chromatography plate (silica gel 60 F254) was used as stationary phase and methanol: water: glacial acetic acid (6:4:0.25) as mobile phase. This system gave a good resolution for metformin hydrochloride (Rf value of 0.52) and glibenclamide (Rf value of 0.78). Determination was done by densitometry in the absorbance mode at 237 nm and 300 nm for metformin hydrochloride and glibenclamide respectively. The method was validated for linearity, precision and accuracy. The linear regression data for the calibration plot showed a good relationship with r^2 = 0.999 and 0.996 for metformin hydrochloride and glibenclamide, respectively. Precision of the method were between 0.56- 2.02% for metformin hydrochloride and 0.08-1.30% for glibenclamide. Accuracy of the method was found to be 88.43-104.54% for metformin hydrochloride and 97.22-102.88% for glibenclamide. According to the results, this method was in accordance with good validation requirements[43]. Basavaiah et al. (2015) worked on a stability-indicating, robust, fast, and user friendly reversed-phase ultra-performance liquid chromatographic (UPLC) assay method which has been developed and validated for the analysis of metformin hydrochloride (MET). The drug was degraded under different stress test conditions prescribed by International Conference on Harmonization (ICH). The drug was well separated from degradation products using a reversed-phase (C-18) column (Waters Acquity BEH C18, 100 mm × 2.1 mm, 1.7 µm) and a mobile phase comprising of equal volumes of methanol and acetonitrile mixture (30%) and phosphate buffer with pH 3.2 (70%), which was delivered initially for 5 min. Other UPLC parameters were: flow rate, 0.20 mL min-1; detection wavelength, 230 nm and injection volume, 2 µL. The method was validated for linearity, precision, accuracy, specificity and selectivity. The linear regression analysis for the calibration curve showed a good linear correlation over the concentration range, 0.1–300 µg mL^{-1}, with regression coefficient, r^2 = 0.9999. The limit of detection (LOQ) and limit of detection (LOD) were 0.01 and 0.002 µg mL^{-1} respectively. The accuracy of the method was further ascertained by recovery studies via standard addition procedure and the recoveries obtained were 100.7 – 101.4%[44].

Sahu et al. (2017) carried out a validated stability indicating UPLC method for determination of metformin hydrochloride (MET) and empagliflozin (EMPA). The method used a reverse phase column, dikma C18 (50×2.1 mm, 1.8 µ), a mobile phase comprising of

phosphate buffer (pH- 3): methanol (30:70 v/v) flow rate of 1.0 ml/min and a detection wavelength of 240 nm using a photodiode array detector. The retention time was found to be 1.189 min and 1.712 min for MET and EMPA respectively. The proposed method was found to be having linearity in the concentration range of 500-2500 µg/ml for MET (r^2 = 0.989) and 5-25 µg/ml for EMPA (r^2 = 0.994), respectively. The mean % recoveries obtained were found to be 100.35-100.48% for MET and 99.80-101.30% for EMPA respectively[45]. Ben-Hander et al (2019) performed a method for the determination of metformin hydrochloride (MH) in pharmaceutical formulations by capillary electrophoresis with capacitively coupled contactless conductivity (C4 D) detection was investigated. The separation was achieved under normal polarity mode at 17.5°C,30 kV, hydrodynamic injection (50 mbar for 8 s) and using a bare fused silica capillary 72 cm × 75 µm i.d. (detection length, 10.5 cm from the outlet end of the capillary). The optimized background electrolyte consisted of 10 mM 2-morpholinoethanesulfonic acid and 10 mM histidine, pH 6.8. C4 D parameters were set at fixed amplitude of 100 V and frequency of 650 kHz. Under the optimum conditions, the method shows good linearity over the range of 10-30 µg mL^{-1} MH (r^2 =0.9971). Limits of detection and quantitation based on S/N ratio of 3 and 10 were 0.049 and 0.15 µg mL^{-1} , respectively[46].

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