Validated HPTLC Method for Simultaneous Estimation of Metformin Hydrochloride, Atorvastatin and Glimepiride in Bulk Drug and Formulation

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Abstract

This paper describes a new, simple, precise, and accurate 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 satisfactorily resolved 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.

Keywords: Thin layer chromatography; Densitometry; Validation and Quantification; Metformin hydrochloride; Atorvastatin; Glimepiride

Introduction

MET is chemically 1,1-dimethyl biguanide [1] (Figure 1). Despite its therapeutic benefits, the actual mode of action of Metformin is uncertain. It acts by activating AMP-activated protein kinase (AMPK) in liver cells, leading to increased fatty acid oxidation and glucose uptake by cells and decreased lipogenesis and hepatic glucose production. Its net effect is an improvement in insulin resistance.

ATV is (3R,5R)-7-(2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1H-pyrrol-1-yl)-3,5-dihydroxyheptanoic acid [2] (Figure 2). It is a HMG-CoA (3-hydroxy-3-methylglutaryl-coenzymeA) reductase inhibitor. This enzyme is involved in cholesterol biosynthesis by catalyzing the conversion reaction of HMG-CoA to mevalonate. The function of lowering the amount of cholesterol results in clearing the LDP (low-density lipoprotein) cholesterol in the blood by increased LDL receptors.

GLM chemically is 3-ethyl-4-methyl-N-[2-[4-[(4-methylcyclohexyl) carbamoylsulfamoyl]phenyl]ethyl]-2-oxo-5H-pyrrole-1-carboxamide [3] (Figure 3). GLM likely binds to ATP-sensitive potassium channel receptors on the pancreatic cell surface, reducing potassium conductance and causing depolarization of the membrane.

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depolarization stimulates calcium ion influx through voltage-sensitive calcium channels. This increase in intracellular calcium ion concentration induces the secretion of insulin.

Hence, the combination of MET, ATV and GLM extended release complement each other and provides reduction in plasma cholesterol along with glycaemic control thereby providing a comprehensive control of diabetes and associated dyslipidemia.

The pharmaceutical formulation used was TRIPILL 2, which was procured from Cipla Ltd. India whose label claim is Metformin Hydrochloride 500 mg, Atorvastatin 10 mg, Glimepiride 2 mg.

Metformin is an oral antihyperglycaemic drug used in the management of type 2 diabetes. It improves glucose tolerance in patients with type 2 diabetes, lowering both basal and postprandial plasma glucose. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization.

The primary mechanism of action of glimepiride in lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells. In addition, extrapancreatic effects may also play a role in the activity of sulphonylureas such as glimepiride.

Atorvastatin reduces total cholesterol, LDL-C and apo B in 2 patients with diabetic dyslipidemia. Atorvastatin also reduces VLDL-cholesterol (VLDL-C) and triglycerides and produces variable increases in high-density lipoprotein cholesterol (HDLC) and apolipoprotein A1. Atorvastatin reduces total cholesterol, LDL-C, VLDL-C, apo B, triglycerides, and non-HDL-C, and increases HDL-C in patients with isolated hypertriglyceridaemia. Atorvastatin also reduces intermediate density lipoprotein cholesterol (IDL-C) in patients with dysbetalipoproteinemia.

Today TLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, high sample throughput and the need for minimum sample preparation. The major advantage of TLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC thus reducing the analysis time and cost per analysis.

Literature review reveals that methods have been reported for analysis of MET, ATV and GLM by LC and HPTLC in combination with other drugs separately [4-14]. LC-MS-MS development and validation for simultaneous quantitation of MET, GLM and pioglitazone in human plasma have been reported [6]. Stability-indicating high-performance liquid chromatographic assay of atorvastatin with fluorescence detection has also been reported [7]. To date there have been no published reports on simultaneous quantitation of MET, ATV and GLM by HPTLC in bulk drug and in tablet dosage form. This present study reports for the first time the simultaneous quantitation of MET, ATV and GLM by HPTLC in bulk drug and in tablet dosage form. The proposed method is validated as per ICH Guidelines [15].

**Experimental**

**Materials**

Working standards of pharmaceutical grade MET (Batch No.: 3489/201), ATV (Batch No.: 5436/501) and GLM (Batch No.: 2895/213) were obtained as generous gifts from Cipla Limited, Patalganga (Maharashtra, India). They were used without further purification and certified to contain 99.96%, 99.98% and 99.99% (w/w) on dry weight basis for MET, ATV and GLM respectively. Fixed dose combination tablets (Brand Name: TRIPILL 2) containing 500 mg of MET, 10 mg of ATV and 2 mg of GLM were procured from Cipla Ltd. India. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

**Instrumentation**

The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample (Hamilton, Bonded, Switzerland) syringe on silica gel precoated aluminum plate 60F-254, plates, [20 cm × 10 cm with 250µm thickness; E. Merck, Darmstadt, Germany] using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1µL/s was used and the space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. The monochromator bandwidth was set at 20 nm, each track was scanned three times and baseline correction was used. The mobile phase consisted of water:methanol:ammonium sulphate (1:1:4 v/v/v) and 12 mL of mobile phase was used per chromatography run. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 40 min at room temperature (25°C ± 2) at relative humidity of 60% ± 5. The saturation time was kept more than an ideal time (30 min) because of less amount of organic solvent present in the mobile phase used for chromatography run. Each chromatogram was developed over a distance of 8 cm. Following the development the TLC plates were dried in a stream of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow rate in laboratory was maintained unidirectional (laminar flow, towards the exhaust). Densitometric scanning was performed using a Camag TLC scanner III in the reflectance absorbance mode at 237 nm and operated by CATS software (V 3.15, Camag). The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample amounts.

**Preparation of standard stock solutions**

Standard stock solutions with a concentration of 1000µg/mL were prepared in methanol for MET, GLM and ATV, respectively. From the...
standard stock solutions, diluted mixed standard solutions were prepared containing 100 µg/mL of MET, GLM and ATV, respectively. The stock solution was stored at 2-8°C protected from light.

Optimization of the HPTLC method

The TLC procedure was optimized with a view to develop a simultaneous assay method for MET, ATV and GLM respectively. The mixed standard solution (100 µg/mL of MET, 100 µg/mL of GLM and 100 µg/mL of ATV) were taken and 10 µL sample was spotted on to TLC plates and run in different solvent systems. Optimization of HPTLC method was very difficult in this case as MET was not moving at all. After many trials it was found that ammonium sulphate is necessary for movement of MET (Figure 4). Hence, water:methanol:ammonium sulphate was tried in different ratios. In order to reduce the less effect TLC chamber was saturated for 45 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 30 min for complete development of the TLC plate.

Validation of the method

Validation of the optimized TLC method was carried out with respect to the following parameters.

Linearity and range

From the mixed standard stock solution, 50 µg/mL of MET, 150 µg/mL of GLM and ATV respectively, 4 to 14 µL solution were spotted on TLC plate to obtain final concentration 200–700 ng/spot for MET, 600–2100 ng/spot for ATV and GLM. Each concentration was applied six times to the TLC plate. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves. Linearity of the method was studied by injecting six concentrations of the drug prepared in the mobile phase in triplicate into the system keeping the injection volume constant. The peak areas were plotted against concentrations to obtain the calibration graphs.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (200 ng/spot, 400 ng/spot and 600 ng/spot for MET and 600 ng/spot, 1200 ng/spot and 1800 ng/spot for ATV and GLM respectively) of the drugs six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation

Limits of detection (LOD) and quantitation (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for MET, ATV and GLM by spotting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of mixed standard solution of MET, ATV and GLM were performed from the standard stock solution in the range of 10–700 ng/spot. The samples were applied to TLC plate and the chromatograms were run and measured signal from the samples was compared with those of blank samples.

Robustness of the method

Following the introduction of small changes in the mobile phase composition (± 0.1 mL for each component), the effects on the results was examined. Mobile phases with different compositions, e.g. water:methanol:ammonium sulphate (1:1:4.1 v/v/v), (1:1:1.4 v/v/v), (1:1:1.4 v/v/v), were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 5%. The plates were prewashed with methanol and activated at 110°C for 2, 5, and 7 min respectively prior to chromatography. The time from spotting to chromatography and from chromatography to scanning was varied from ± 10 min. The robustness of the method was determined at three different concentration levels for 200 ng/spot, 400 ng/spot and 600 ng/spot for MET, 600 ng/spot, 1200 ng/spot and 1800 ng/spot for ATV and GLM.

Specificity

The specificity of the method was determined by analyzing standard drug and test samples. The spot for MET, ATV and GLM in the samples was confirmed by comparing the Rf and spectrum of the spot with that of a standard. The peak purity of MET, ATV and GLM was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (MET, ATV and GLM combination tablet) to which known amount of MET, ATV and GLM standard powder corresponding to 80, 100 and 120% of label claim had been added (standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Analysis of a marketed formulation

To determine the content of MET, ATV and GLM in conventional tablet (Brand name: TRIPILL 2, Label claim: 500 mg MET, 10 mg of ATV and 2 mg of GLM per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 500 mg of MET, 10 mg of ATV and 2 mg of GLM was transferred into a 100 mL volumetric flask containing 60 mL methanol, sonicated for 30 min with occasional shaking and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined (5000 µg/mL for MET, 100 µg/mL for ATV and 20 µg/mL for GLM). Then 1 mL of the above filtered solution was diluted to produce a concentration of 500 µg/mL for MET from which 1 µL of the spot was applied which gave final concentration of 500 ng/spot, for ATV and GLM the solution was used without dilution to produce a concentration of 100 µg/mL for ATV and 20 µg/mL for GLM. Then 10 µL for ATV and 40 µL for GLM were applied to a TLC plate which was developed in optimized mobile phase. The dilutions were done individually due to the large differences in LOD and LOQ values as well as label claim. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

Results and Discussion

The results of validation studies on simultaneous method developed for MET, ATV and GLM in the current study involving water:methanol:ammonium sulphate (1:1:4, v/v/v) as the mobile phase for TLC are given below.

Linearity

Linear relationships were observed by plotting drug concentrations against peak areas for each compound. MET showed linear response in the concentration range of 200, 300, 400, 500, 600, 700 ng/spot and for ATV and GLM the concentration range of 600, 900, 1200, 1500, 1800, 2100 ng/spot, respectively. The corresponding linear
regression equation was \( y = 3.6457 x + 165.76 \) for MET, \( y = 1.9106 x + 728.23 \) for ATV and \( y = 1.5361 x + 3533.1 \) for GLM with square of correlation coefficient \( R^2 \) of 0.9994 for MET, 0.9990 for ATV and 0.9994 for GLM respectively.

**Precision**

The results of the repeatability and intermediate precision experiments are shown in Table 1. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, as recommended by ICH guidelines.

**LOD and LOQ**

Signal-to-noise ratios of 3:1 and 10:1 were obtained for LOD and LOQ respectively. The LOD and LOQ were found to be 100 ng/spot and 200 ng/spot for MET, 500 ng/spot and 600 ng/spot for ATV and GLM respectively.

**Robustness of the method**

The standard deviation of peak areas was calculated for each parameter and the % RSD was found to be less than 2. The low values of the % RSD, as shown in Table 2 indicated the robustness of the method.

**Specificity**

The peak purity of MET, ATV and GLM was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., \( r (S, M) = 0.9973 \) and \( r (M, E) = 0.9981 \). A good correlation \( (r =0.9994) \) was also obtained between the standard and sample spectra of MET, ATV and GLM respectively.

**Analysis of a formulation**

Experimental results of the amount of MET, ATV and GLM in tablets, expressed as a percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets. Two different lots of MET, ATV and GLM combination tablets were analyzed using the proposed procedures (Table 4).

**Conclusion**

Introducing TLC into pharmaceutical analysis represents a major step in terms of quality assurance. The developed TLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for the analysis of MET, ATV and GLM as bulk drug and in pharmaceutical formulation without any interference from the excipients. It was concluded that the developed method offered several advantages such as rapid, cost effective, simple mobile phase and sample preparation steps and improved sensitivity made it specific, reliable and easily reproducible in any quality control set-up providing all the parameters are followed accurately for its intended use. It may be extended to study the degradation kinetics of MET, ATV and GLM, also for its estimation in plasma and other biological fluids. The proposed TLC method is less expensive, simpler, rapid, and more flexible than HPLC.

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**References**


