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Research Article

DEVELOPMENT AND VALIDATION OF A HPLC METHOD FOR THE DETERMINATION OF METFORMIN HYDROCHLORIDE, NATEGLINIDE AND PIOGLITAZONE HYDROCHLORIDE IN MULTI COMPONENT FORMULATION

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ABSTRACT

A simple, rapid, and precise reverse-phase high-performance liquid chromatographic method for simultaneous analysis of Metformin hydrochloride, Nateglinide, and Pioglitazone hydrochloride in a tablet dosage form has been developed and validated. Chromatography was performed on a 25 cm × 4.6 mm i.d., 5-mm particle, C18 column with 85:15 (v/v) methanol: 20 mM potassium dihydrogen phosphate buffer as mobile phase at a flow rate of 1.2 ml/min. UV detection at 227 nm; Metformin hydrochloride, Nateglinide, and Pioglitazone hydrochloride were eluted with retention times of 2.15, 3.787, and 4.57 min, respectively. The method was validated in accordance with ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible. Calibration plots were linear over the concentration ranges 50-250 mg/ml for Metformin hydrochloride, 3.0-15.0 mg/ml for Nateglinide, and 2-10 mg/ml for Pioglitazone hydrochloride. Limits of detection were 0.03, 0.22, and 0.008 mg/ml and limits of quantification were 0.09, 0.67, and 0.02 mg/ml for Metformin hydrochloride, Nateglinide, and Pioglitazone hydrochloride, respectively. The high recovery and low coefficients of variation confirm the suitability of the method for simultaneous analysis of the three drugs in tablets. Statistical analysis proves that the method is suitable for the analysis of Metformin hydrochloride, Nateglinide, and Pioglitazone hydrochloride as a bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of three drugs and also for its estimation in plasma and other biological fluids.

Keywords: HPLC, Method Development, Method Validation, Calibration Curve, Nateglinide.

INTRODUCTION

Metformin, [Mt] chemically [1,1-dimethyl biguanide hydrochloride] (**Figure 1**) [1]. It acts by suppressing excessive hepatic glucose production and improving glucose clearance, its predominant effect is to decrease fasting plasma glucose. It is the most well-known member of the biguanide group, regarded as the main compound in mixed therapies, and is always used in high doses of about 500 or 850 mg. Nateglinide [Ng] is an oral

hypoglycaemic agent which lowers the blood glucose level by stimulating the pancreatic β-cells to secrete insulin [2]. Chemically, it is 1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(p-tolylsulfonyl) urea (**Figure 2**). Pioglitazone hydrochloride (Pg) is chemically designated as 5-[[4-[2-(5-Ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4-thiazolidinedione (**Figure 3**). It is a member of the thiazolidinedione group. The combination of Metformin hydrochloride, Nateglinide, and Pioglitazone is used in pharmaceutical preparations. This combination however, is not present in any official pharmacopoeia. In this respect, a method for the analysis of this combination is needed. In the scientific literature, analysis of Mt, Ng and Pg has been reported as individual ingredients and in

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combination with other compounds. Analytical methods have included estimation of Mt [3-8], Ng[9-11], Pg individually [12]. And in two component formulations of Pg and Mt have been analyzed in combination by [13-20]. Simultaneous HPLC analysis of Metformin with gliclazide [21] in combinations with other drugs have also been reported [22-31]. Because no chromatographic method for simultaneous analysis of Mt, Pg, and Ng in a combined dosage form has yet been reported, it was essential to develop a chromatographic method for simultaneous estimation of all the three drugs in a tablet formulation. The method described is rapid, economical, precise, and accurate and can be used for routine analysis of tablets. It was validated as per ICH norm [32-34].

Experimental

Materials and Methods

Pharmaceutical grade working standards Pioglitazone [Pg] (batch no. 2088581), Nateglinide [Ng] (batch no.2980007), Metformin HCl [Mt] (batch no. 1997418) were obtained from Sunpharma Laboratories, Dewas, India. All chemicals and reagents were of HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

Instrumentation

The LC system consisted of a pump (model jascoPU1580, intelligent LC pump) with auto injecting facility (AS-1555 sampler) programmed at 20 μ l capacity per injection was used. The detector consisted of a UV-Vis (Jasco UV 1575) model operated at a wavelength of 220 nm. The software used was jascoborwin version 1.5, LC-Net II/ADC system. The column used was HiQSil C18HS (250mm \times 4.6 mm, 5.0 μ m) Kya Technologies Corporation. Different mobile phases were tested in order to find the best conditions for separation of Mt, Ng and Pg. The mobile phase contained methanol: 20mM potassium dihydrogen phosphate (85:15, v/v) and the flow rate was maintained at 1.2 ml/min UV detection was carried out at 227 nm. The mobile phase and samples was filtered using 0.45 μ m membrane filter. Mobile phase was degassed by ultrasonic vibrations prior to use. All determinations were performed at ambient temperature.

Standard Solutions and Calibration Graphs for Chromatographic Measurement

Mt, Ng and Pg were weighed accurately and separately transferred to 10 mL volumetric flasks. All the drugs were dissolved in HPLC-grade methanol to prepare 1000 μ g/ml standard stock solutions. Calibration standards at five levels were prepared by appropriately mixed and further diluted stock standard solutions in the concentration range of 50-250 μ g/ml for Mt and 3-15 μ g/ml for Ng and 2-10 μ g/ml Pg. Samples in triplicate were made for each concentration and peak areas were plotted

against the corresponding concentrations to obtain the calibration graphs.

Sample Preparation

For the analysis of a tablet dosage form, 20 tablets were weighed individually and their average mass was determined. Then, the tablets were crushed to a fine powder. The powder amount equivalent to 500 mg of Mt, 30 mg of Ng and 15 of Pg were transferred to a 25mL volumetric flask and dissolved in 25 mL of HPLC-grade methanol, sonication was done for 15min with swirling. After sonication, The solution was filtered through a Whatman filter paper (#41). Before the assay of tablet formulations, 6 replicate aliquots (each 20 mL in volume) of the appropriately diluted tablet stock solution were sonicated for 15 min, then injected into the chromatographic system, and analyzed quantitatively. The analysis was repeated six times. The possibility of excipient interference with the analysis was examined.

Optimization of HPLC Method

The HPLC procedure was optimized with a view to develop a simultaneous assay method for Mt, Ng and Pg respectively. The mixed standard stock solution (200 μ g/ml of Mt, 12 μ g/ml of Ng, 6 μ g/ml of Pg) injected in HPLC. Different ratios of methanol and potassium dihydrogen phosphate buffer at different pH and molarities were tried.

METHOD VALIDATION

The method was validated according to the ICH guidelines [19]. The following validation characteristics were addressed: linearity, accuracy, precision, and specificity, limits of detection and quantitation and robustness.

Linearity and Range

Linearity of the method was studied by injecting the mixed standard solutions in the concentration range of 50-250 μ g/ml for Mt and 3-15 μ g/ml for Ng and 2-10 μ g/ml Pg injected six times into the LC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

The precision of the proposed method was evaluated by carrying out six independent assays of test sample. RSD (%) of six assay values obtained was calculated. Intermediate precision was carried out by analyzing the samples by a different analyst on another instrument.

Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantitation (LOQ) for the procedure were performed on

samples containing very low concentrations of analytes under the ICH guidelines. By applying the visual evaluation method, LOD was expressed by establishing the minimum level at which the analyte can be reliably detected. LOQ was considered as the lowest concentration of analytes in standards that can be reproducibly measured with acceptable accuracy and precision.

Robustness and System Suitability

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ± 0.1 ml/min), mobile phase composition (methanol ± 5 ml). These chromatographic variations were evaluated for resolution between Mt, Ng and At.

Solution Stability

To assess the solution stability, three different concentrations of Mt, Ng and Pg (2, 4 and 6 $\mu\text{g/ml}$) were prepared from sample solution and kept at room-temperature for 8 days. These solutions were compared with freshly prepared standard solutions.

System Suitability

The system suitability parameters with respect to theoretical plates, tailing factor, repeatability and resolution between Mt, Ng and Pg peaks were defined.

Specificity

Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the Mt, Ng and Pg. For determining selectivity of the method, a powder blend of typical tablet excipients containing lactose monohydrate, mannitol, maize starch, povidone K₃₀, citric acid anhydrous granular, sodium citrate, natural lemon and lime flavor and magnesium stearate was prepared and analyzed. All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks.

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (Mt, Ng and Pg combination tablets) to which known amounts of Mt, Ng and Pg 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 chromatograms in optimized mobile phase. These mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recoveries (%), RSD (%) were calculated.

Analysis of Marketed Formulation

The marketed formulation was assayed as described above. The peak areas were measured at 227 nm and concentrations in the samples were determined using multilevel calibration developed on the same LC-system under the same conditions using linear regression analyzed for Mt, Ng and Pg in the same way as described earlier.

Table 1: Linear regression data for the calibration curves ^a

Compound	Linearity ($\mu\text{g/ml}$)	$y = A + Bx$		r^2
		A	B	
Mt	50-250	94169	97790	0.999
Ng	3-15	6186.x	-250.0	0.999
Pg	2-10	36611	39561	0.999

^a n = 6; r^2 , coefficient of correlation.

Table 2. Intra and Inter Day Precision of HPLC method^a

Compound	Repeatability		Intermediate	
	Mean% assay	% R.S.D.	Mean% assay	% R.S.D.
Mt	101.07	0.27	100.90	0.11
Ng	100.81	1.72	99.15	0.80
Pg	100.05	0.47	99.09	1.38

^a n = 6, (2, 6, 10 $\mu\text{g/ml}$ for Pg 3, 9, 15 $\mu\text{g/ml}$ for an Ng 10, 30, 50 $\mu\text{g/ml}$ for Mt)

Table 3. Statistical Analysis of Parameters Required For System Suitability Testing of the Proposed HPLC Method

Parameters	Mt	Ng	Pg
Theoretical plates	39625.65	2390.16	3765.46
Resolution	-	6.43	2.41
Peak asymmetry	1.44	1.34	1.27

% R.S.D.	0.08	0.03	0.06
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Table 4. Robustness Testing^a

Chromatographic Factors ^a	Level	Retention time, t_R (min)			Resolution (Rs)			Asymmetry (As)		
		Mt	Ng	Pg	Mt	Ng	Pg	Mt	Ng	Pg
Flow rate (ml/min)	1.1	2.27	3.93	5.01	0	6.01	2.39	1.15	1.46	1.38
	1.2	2.15	3.78	4.57	0	6.43	2.41	1.04	1.34	1.27
	1.3	2.01	3.70	4.00	0	6.49	2.48	1.01	1.30	1.25
% of methanol	83	2.23	3.98	4.89	0	6.04	2.40	1.19	1.66	1.18
	85	2.15	3.78	4.57	0	6.43	2.41	1.04	1.34	1.27
	88	2.08	3.10	4.13	0	6.67	2.86	1.09	1.50	1.55

^a n = 6**Table 5. Stability of drugs in sample solutions^a**^a (n = 6) Average of three concentrations (2, 6, 10 $\mu\text{g/ml}$ for Pg 3, 9, 15 $\mu\text{g/ml}$ for Ng and 10, 30, 50 $\mu\text{g/ml}$ for Mt).

Parameters	Mt	Ng	Pg
% R.S.D	0.74	0.04	1.10

Table 6: Recovery studies^a

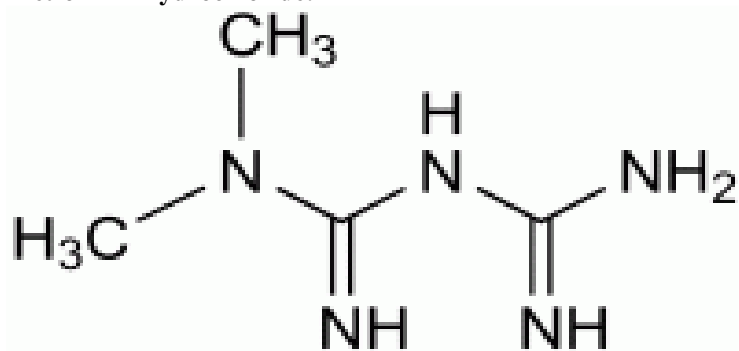
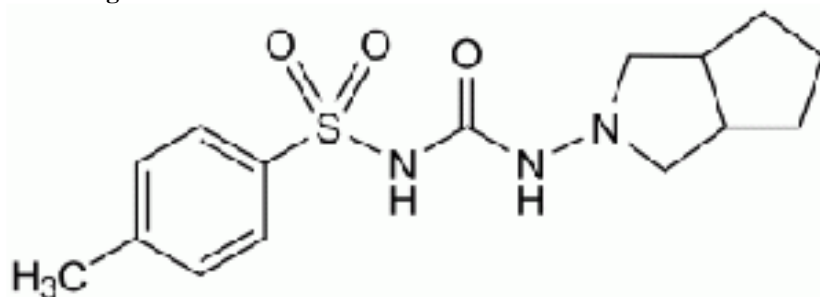
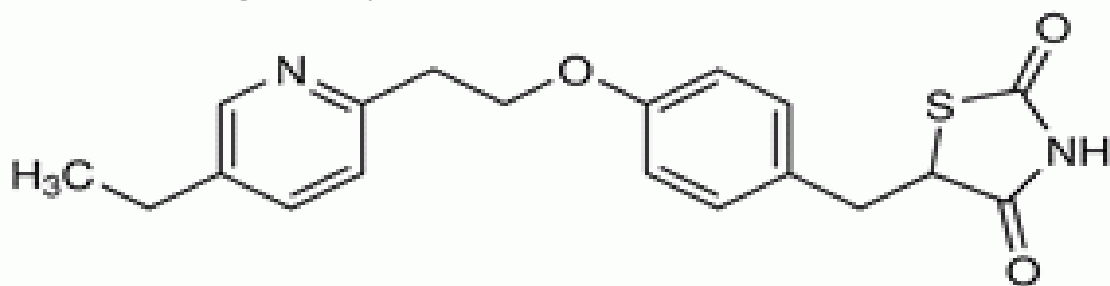
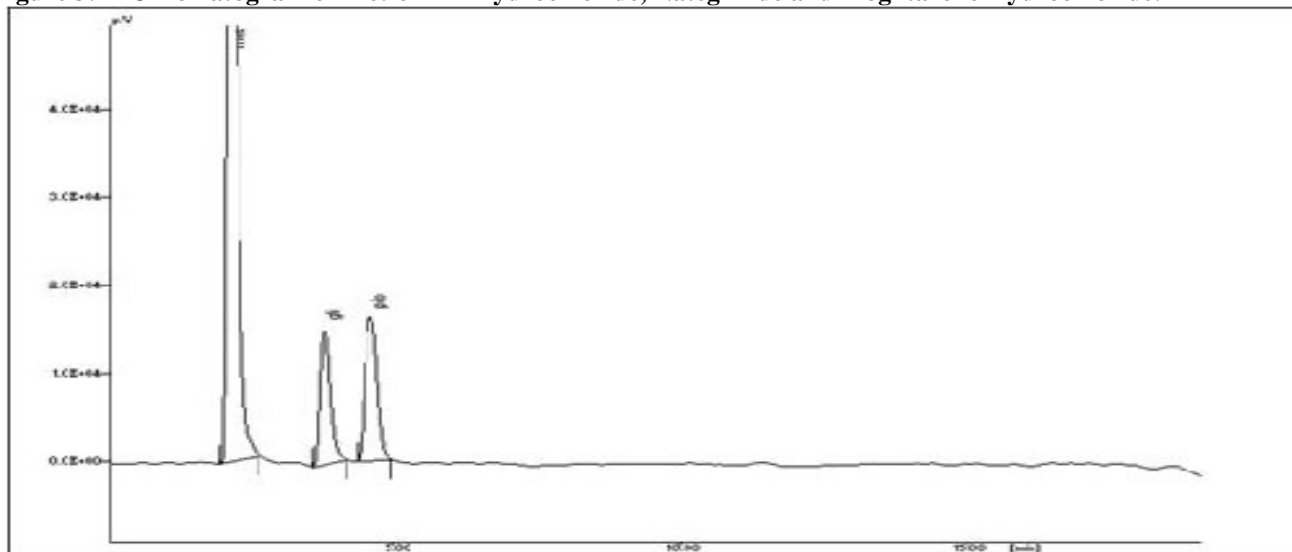
Label claim	Amount of drug added (%)	Total amount mg	Amount recovered, mg \pm % RSD	% Recovery
Pg 15 mg	80	27	26.74 \pm 0.09	99.04
	100	30	29.85 \pm 0.06	99.51
	120	33	32.88 \pm 1.02	99.66
Ng 30 mg	80	54	53.78 \pm 1.75	99.61
	100	60	59.94 \pm 1.97	99.90
	120	66	66.01 \pm 0.08	100.02
Mt 500 mg	80	900	900.18 \pm 0.12	100.03
	100	1000	1004 \pm 0.11	100.41
	120	1100	1098.24 \pm 0.04	99.84

^an = 6**Table 7 Applicability of the HPLC method for the analysis of the pharmaceutical formulations**

Sample	Label claim (mg)	Drug Content (%)	% R.S.D.
Pg	15	99.03	0.15
Ng	30	99.19	0.72
Mt	500	99.88	0.08

Table 8. Summary of Validation Parameters

Parameter	Mt	Ng	Pg
Linearity range ($\mu\text{g/ml}$)	50-100	3-15	2-10
Correlation coefficient	0.999 \pm 0.09	0.999 \pm 0.18	0.999 \pm 0.06
Limit of detection ($\mu\text{g/ml}$)	0.03	0.22	0.008
Limit of quantitation ($\mu\text{g/ml}$)	0.09	0.67	0.02
Recovery (n = 6)	100.03	99.84	99.40
Precision (% R.S.D.)			
Repeatability	0.27	1.72	0.47
Inter day	0.11	0.80	1.38
Robustness	Robust	Robust	Robust

Figure No. 1: Structure of Metformin Hydrochloride.**Figure No. 2: Structure of Nateglinide.****Figure No. 3: Structure of Pioglitazone Hydrochloride.****Figure 3: A Chromatogram of Metformin Hydrochloride, Nateglinide and Pioglitazone Hydrochloride.**

RESULTS AND DISCUSSION

Method Development and Optimization

The HPLC procedure was optimized with a view to develop a suitable LC method for the analysis of Mt, Ng and Pg in fixed dose combined dosage form. Initially methanol and water in different ratios were tried. But Mt gave broad peak shape, so water was replaced by potassium dihydrogen buffer (20 mM), and mixture of methanol and potassium dihydrogen phosphate buffer in different ratios were tried. It was found that methanol: potassium dihydrogen phosphate buffer (20 mM) in ratio of 85: 15, v/v gave acceptable retention time (RT 2.15 min for Mt, 3.78 min for Ng and 4.575 min for Pg), plates, and good resolution for Mt, Ng and Pg at the flow rate of 1.2 ml/min) Illustration 4.

Validation

Linearity

Linearity was evaluated by analysis of working standard solutions of Mt, Ng and Pg of five different concentrations. The range of linearity was from 50-250 µg/ml for Mt and 3-15 µg/ml for Ng and 2-10 µg/ml Pg. The regression data obtained are represented in Table 1. The result shows that within the concentration range mentioned above, there was an excellent correlation between peak area and concentration of each drug.

Precision

The results of the repeatability and intermediate precision experiments are shown in Table 2. The developed method was found to be precise, with RSD values for repeatability and intermediate precision

LOD and LOQ

The LOD and LOQ values were found to be 0.03 and 0.09 µg/ml for Mt. 0.22 and 0.67 µg/ml for N, and 0.008 and 0.02 µg/ml for Pg.

Specificity

Injections of the extracted commonly used excipients were performed to demonstrate the absence of interference with the elution of the drugs. These results demonstrate that there was no interference from other materials in the tablet formulation; therefore, confirm the specificity of the method Table 3.

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System Suitability

System suitability parameters such as the number of theoretical plates, HETP and peak tailing are determined. The results obtained are shown in Table 4.

Robustness of the Method

To ensure the insensitivity of the developed HPLC method to minor changes in the experimental conditions, it is important to demonstrate its robustness. None of the alterations caused a significant change in resolution between Mt, Ng and Pg, peak area, R.S.D., tailing factor and theoretical plates Table 5.

Solution Stability Studies

Three different concentrations of Mt, Ng and Pg, 2, 4 and 6 µg/ml were prepared from sample solution and stored at room temperature for 8 days. They were then injected into the HPLC system and no additional peak was found in the chromatogram indicating the stability of Mt, Ng and Pg in the solution Table 6.

Recovery Studies

Good recoveries of the Mt, Ng and Pg were obtained at various added concentrations for the tablets as shown in Table 7.

Analysis of a Commercial Formulation

Experimental results of the amount of Mt, Ng and Pg in tablets, expressed as a percentage of label claims were in good agreement with the label claims there by suggesting that there is no interference from any of the excipients which are normally present in tablets. Fixed dose combination tablets were analyzed using the proposed procedures Illustration 12. The data of summary of validation parameters are listed in Table 8.

CONCLUSION(s)

The new HPLC method described in this paper provides a simple, convenient and reproducible approach for the simultaneous identification and quantification that can be used to determine Metformin hydrochloride, Nateglinide, Pioglitazone hydrochloride in routine quality control.

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