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Research Article

DEVELOPMENT OF RP-HPLC METHOD FOR METFORMIN AND REPAGLINIDE IN RABBIT PLASMA

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ABSTRACT

A sensitive, specific and rapid high-performance liquid chromatography-ultraviolet spectroscopy method was developed and successfully validated to estimate the metformin and repaglinide in rabbit plasma. The solvent extraction method was used for metformin and repaglinide from serum by using ethyl acetate and 0.1N HCl. The mobile phase consists of acetonitrile: phosphate buffer pH 4.0 at 60:40 %v/v with 1% triethylamine at flow rate of 0.8ml/min and at fixed wavelength of 254nm. On ten minutes of run time, metformin was retention at 5.1 and repaglinide for 7.4min. The extraction efficiency was 98% for metformin and 95% for repaglinide. The intra-day and inter-day precision was in the terms of %RSD less than 1.76% for both the compounds. The developed method was validated and proposed method is useful for pharmacokinetics studies.

Keywords: Rp-Hplc method, Rabbit plasma, Metformin, Repaglinide.

INTRODUCTION

The combined use of metformin and repaglinide for type 2 diabetes mellitus was shown improved patient compliance by controlling the post prandial glucose levels and reaches normal glycemic levels[1]. Monotherapy with metformin, an oral anti diabetic agent is not sufficient to reach the target glycemic goals and multiple drugs may be necessary to achieve the basal glycemia. As per the biopharmaceutical classification system (BCS), metformin was belonged to class III, in terms it has high solubility in water and lower permeability to across the biological membranes, while the repaglinide belongs to class II. It has low solubility and higher permeability. The solubility profiles of both drugs can easily influence the chromatographic separations. Metformin showed single pKa value[2] at 11.5 and repaglinide3 showed two pKa values at 4.19 and 5.78 due to the zwitterionic crew. Until this decade, this combination for liquid chromatographic separation was not published. Metformin is the good therapeutic agent for type II diabetes mellitus and Hplc techniques for it were reported alone and combination with sulfonylureas[3], improvement of patient's compliance is more for combination of metformin and repaglinide rather than with sulfonylureas[4], these combinations are commercially available as tablet dosage forms. The Hplc estimation method for metformin in human plasma[5], ion-pair[6], and in microspheres and tablet dosage forms[7] were previously reported. Spectrophotometric study of metformin and repaglinide[8]and combination[9]of rosiglitazone and metformin were reported.

The combination of oral anti-diabetic agents depends on patient's clinical manifestations. Most of the doctors will choose metformin as the first choice of drug for the treatment of type II diabetes mellitus. Depend on clinical characteristics of the patients; failure monotherapy can switch to a combination of various anti diabetic agents. Adding of such agents to metformin, adequate controls the basal glycemia and post prandial glucose levels.

MATERIALS AND METHODS

Materials

Metformin (99.4% purity), chemically it is N, N-dimethylimidodiacarbonimidic diamide. Repaglinide (98.3% purity), chemically, (s)-2-ethoxy-(1-[2-[[3-methyl-1-[2- (1-piperidinyl) phenyl] butyl] amino]-2-oxoethyl] benzoic acid, were kindly supplied by aurobindo Pharma Ltd (hyd, India). De-ionized water obtained from a Millipore-Q water purification system (Millipore®, Mumbai). Methanol (RFCL limited, New Delhi, India), Lichrosolv® water, Lichrosolv® acetonitrile for chromatographic separations, were obtained from Merck (Merck, Mumbai, India). Solvent and sample filtrations were done by using Ultipor® N®66 0.2 µm for and 0.45 µm membranes, respectively.

Instrumentation

The shimadzu UFLC system consists of following components: Prominence® CBM-20A controller, gradient system with dual pumps LC-10 AT VP, SPD-10 A VP detector with Class-VP: V6.13 software with BDS Hypersil® column, C-18, 150mm×4.6mm i.d., particle size 5µm (Thermo® scientific, India) was used at fixed wave length 254nm. Sonicator was used for solubility and de-aeration (PCI analytics, Mumbai, India). Centrifuge was from REMI®, Mumbai, India. Waters symmetric® C18, i.d. 4.6×250 mm, 5 µm was used for check the accuracy of proposed method. Chromatographic conditions

The column was equilibrated for at least 45min with mobile phase consists of acetonitrile: phosphate buffer pH 4.0 (60:40) with 1% triethylamine. It was sonicated before the equilibration of column for 20min and followed by filtered through 0.2µm whatman membrane. Gradient elution technique was applied, and flow rate was 0.8ml/min at fixed wavelength at 254nm.

Preparation of standards

Stock solutions were prepared by dissolving appropriate concentration of metformin and repaglinide respectively, in methanol to yield a final drug concentration of 8400 µg/ml of metformin and 1100 µg/ml of repaglinide. Then stock solutions were mixed (50:50 %v/v) to obtain a combined working standard solution at 4200 µg/ml of metformin and 550 µg/ml of repaglinide. Working standards of 4200, 3360, 2520, 1680, 840, 420 µg/ml metformin and 550, 440, 330, 220,110 µg/ml repaglinide was prepared by dilution of the 4200/550 µg/ml combined standard solution.

Extraction procedure

The extraction of plasma samples using the following procedure: First, 200 µl of plasma sample was pipetted out into a 1.5 ml Eppendorf tube; thereafter, mixture of working standard solutions and 50.0 µl of ethyl acetate and 10.0 µl of 0. 1N Hcl was added. Metformin is water soluble, while repaglinide is insoluble in water, it has two pKa values due to zwitterionic behavior, and addition of hydrochloric acid gives ionization, results in improvement of water solubility. Due to hydrophobicity of the repaglinide, and it was impossible to dissolve directly into plasma. Both the compounds were easily dissolved in methanol: phosphate buffer pH 4.0. To the combined standard stock solutions, the subsequent plasma addition can cause protein precipitation, protein precipitation results in poor precision of analytical method. The mixture was vortex and 1.00 ml of methanol: phosphate buffer pH 4.0 was added. The mixed solution vortexes again subsequently centrifuged for 15 minutes at 10,000 g.

The supernatant present after centrifugation was transferred to a 1.5 ml Eppendorf tube, and evaporated to dryness at 65 ºC for 90 min. The dried sample was reconstituted in 200 µl of the mobile phase prior to analysis.

Method development

BDS Hypersil® column was equilibrated and tested by using methanol/water mixture at various compositions and flow rate at 1ml/min. Results in broad peaks with poorer resolution, then switched to methanol/potassium dihydrogen phosphate buffer, gives less broader peaks than first one at different ratios.

Later over to high polar solvent acetonitrile gives sharp peaks of metformin eluted first due to less affinity towards stationary phase and repaglinide at last. To optimize the separation, different fractions of acetonitrile and water tested, and optimum separation was obtained using 60% acetonitrile and 40% phosphate buffer pH 4 at flow rate[10] of 0.8 ml/min at fixed wavelength of 254 nm.

Method validation

Once the chromatographic method was developed, it must be validated to check the efficiency of proposed method with USP guidelines to determine the assay, linearity, accuracy, precision, sensitivity, specificity and recovery.

Calibration curve

Standard combinations of solutions were prepared by serial diluting with phosphate buffer pH 4. The linearity was determined between the 420-4200 ng/ml for metformin and 55-550 ng/ml for repaglinide daily constructed by repeated analysis for five times (n=5) and continued for three days (n=15).

Recovery, Precision, Accuracy

Recovery studies were conducted for extracted samples by applying the least square regression analysis for peak areas vs. concentrations. The standard solutions were covering the linearity between 55-550 ng/ml for repaglinide and 420-4200ng/ml for metformin. Each sample injected for five times. Accuracy was determined by injecting three samples of standard solutions were in the range of 50%, 100% and 150% of metformin/repaglinide for five times, by the same operator, same day and same equipment and by the different column to check specificity of analytical method.

Specificity and selectivity

The specificity of metformin and repaglinide retention times were investigated by repeated analysis. The interferences of endogenous compounds were identified and resolved in combination with sulphonylureas like gliclazide, glipizide, thiazolidinediones like pioglitazone. Non-sulfonylureas like nateglinide and mitiglinide were used in combination with metformin in the treatment of type II diabetes mellitus in five different blank plasma samples.

RESULTS AND DISCUSSION

Chromatograms

The retention times and capacity factors were at 5.1 ±0.23, 3.01±0.02 for metformin and 7.4 ±0.15, 4.35±0.04 for repaglinide. The affinity of metformin towards to mobile phase is less than the repaglinide, elutes faster than repaglinide depend on their solubilities, polarities and pH of the environment.

Linearity

The chromatographic analysis of metformin and repaglinide exhibited excellent regression values R2= 0.997 and R2=0.9995 over the concentration range of 420-4200 ng/ml and 55-550 ng/ml for metformin and repaglinide respectively. The three day daily analysis of five samples, resulting in the calibration curves were don't have a statistical significant in values of slope, regression and intercepts.

The assays showed the acceptable precision in the terms of %RSD, <5 and <3 for repaglinide and metformin respectively.

Recovery, precision and accuracy

Recoveries of metformin with repaglinide from extraction samples are 98 and 95% (n=5). The precisions of intraday analysis of five samples were in the terms of %RSD on the range over 0.17-0.68% for metformin in table 1 and 0.26-0.78% for repaglinide in table 2. The inter-day analysis on three consequent days resulted in the range of 0.85-1.70 % and 1.30-1.56 % for metformin and repaglinide respectively. The accuracy of proposed method was checked by using different column on intra-day and inter-day assays were shown in table 1 and table 2 for metformin and repaglinide.

Table 1: Accuracy and precision of metformin in rabbit plasma

BDS Hypersil® column C18					Waters symmetric® column C18				
Intra-day (µg/ml)	**Experiment (µg/ml)**	**SD**	**Accuracy (%)**	**Precision (%RSD)**	**Experimental (µg/ml)**	**SD**	**Precision (% RSD)**	**Accuracy (%)**	**n**
2.1	2.08	0.01	99.05	0.68	2.05	0.04	1.70	97.62	5
4.2	4.21	0.01	100.24	0.17	4.1	0.07	1.70	97.62	5
6.3	6.32	0.01	100.32	0.22	6.27	0.02	0.34	99.52	5
Inter-day (µg/ml)									
2.1	2.06	0.03	98.10	1.36	2.04	0.04	2.07	97.14	15
4.2	4.15	0.04	98.81	0.85	4.11	0.06	1.54	97.86	15
6.3	6.15	0.11	97.62	1.70	6.12	0.13	2.07	97.14	15

Table 2: Accuracy and precision of repaglinide in rabbit plasma

BDS Hypersil® column C18					Waters symmetric® column C18				
Intra-day (µg/ml)	**Experimental (µg/ml)**	**SD**	**Accuracy (%)**	**Precision (%RSD)**	**Experimental (µg/ml)**	**SD**	**Precision (% RSD)**	**Accuracy (%)**	**n**
2.75	2.72	0.02	98.91	0.78	2.73	0.014	0.51	99.27	5
5.5	5.48	0.01	99.64	0.26	5.45	0.035	0.64	99.09	5
8.25	8.3	0.04	100.61	0.43	8.23	0.014	0.17	99.75	5
Inter-day (µg/ml)									
2.75	2.69	0.04	97.82	1.56	2.69	0.042	1.57	97.81	15
5.5	5.39	0.08	98.00	1.43	5.16	0.240	4.55	93.81	15
8.25	8.1	0.11	98.18	1.30	8.01	0.169	2.10	97.09	15

Specificity and selectivity

The blank serum showed that no interference of endogenous and co-administered substances for elution on run time. The retention times were different for them not detected in the present chromatogram.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LODs of metformin and repaglinide were 135.6ng/ml and 18.15ng/ml respectively. LOQ of metformin and repaglinide were 420ng/ml and 55ng/ml respectively[11].

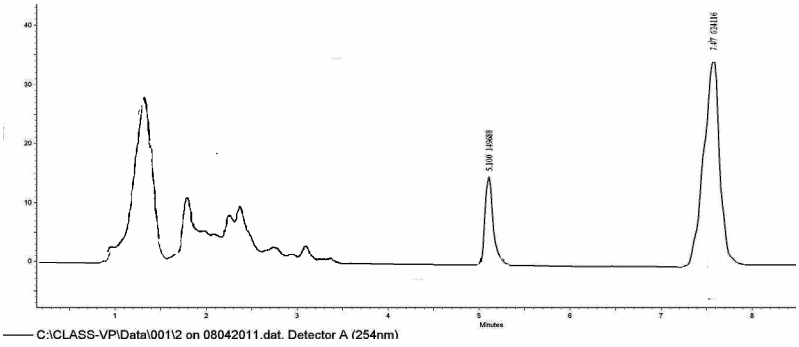

Fig 1: Chromatogram of metformin and repaglinide in rabbit plasma

CONCLUSION

Highly sensitive and specific analytical method was developed and validated for quantification of metformin with repaglinide in rabbit plasma samples. This analytical method was applicable to study of pharmacokinetic parameters in the research study of novel drug delivery systems in rabbit as an animal model. The specificity of this method was tested in five different sources, were analyzed. In fig.1 the combined peaks shows about drug free plasma. The chromatogram of fig.1 shows about retention times of 5.1 and 7.4 for metformin and repaglinide respectively. The current described Hplc method in rabbit plasma for metformin with repaglinide can be readily used for determination of pharmacokinetic parameters of novel drug delivery systems.

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