

Method Development and Validation of Metformin and Repaglinide in Rabbit Plasma by RP-HPLC

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Summary

A sensitive, specific and rapid high-performance liquid chromatography-ultraviolet spectroscopic (HPLC-UV) method was developed and flourishingly validated to estimate the metformin and repaglinide in rabbit plasma. The solvent extraction method was used for obtaining metformin and repaglinide from serum, using ethyl acetate and 0.1N hydrochloric acid. The mobile phase includes acetonitrile: phosphate buffer pH 4.0 at 60:40% v/v with 1% triethylamine at the flow rate of 0.8 mL/min and at fixed wavelength of 254 nm. On ten minutes of run time, metformin was retained at 5.1 and repaglinide at 7.4 min. Extraction efficiency was 98% for metformin and 95% for repaglinide. The intra-day and inter-day precision was, in terms of relative standard deviation (RSD), less than 1.76% for both compounds, in the same column and 4.55% in different columns. The developed method was validated, and this proposed method can be used further for pharmacokinetics studies.

Key Words: RP-HPLC method, plasma, metformin, repaglinide.

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RP-HPLC ile Tavşan Plazma Metformin ve Repaglinide Yöntem Geliştirme ve Validasyon

Özet

Hassas, spesifik ve hızlı yüksek performanslı sıvı kromatografi- ultraviyole spektroskopisi (HPLC-UV) yöntemi tavşan plazmasındaki metformin ve repaglinide tayini için geliştirildi ve valide edildi. Çözücü olarak etil asetat ve 0.1N hidroklorik asitin kullanıldığı ekstaksiyon yöntemi ile serumdan metformin ve repaglinide geri alındı. Hareketli faz olarak asetonitril:fosfat tamponu pH4.0, 60:40 (h/h), ile %1 trietilamin 0.8 mL/dakika akış hızında ve 254 nm sabit dalga boyu kullanıldı. 10 dakikalık çalışma süresinde metformin 5.1 dakika ve repaglinide 7.4 dakika alıkondu. Ekstraksiyon verimi metformin için %98, repaglinide için %95'dir. Her iki bileşik için gün içi ve günler arası kesinlik, bağıl standart sapma (BSS) cinsinden, aynı kolonda %1.76 dan, farklı kolonlarda %4.55 den küçüktür. Geliştirilen yöntem valide edildi ve bu önerilen yöntem ileriki farmakokinetik çalışmalarda kullanılabilir.

Anahtar Kelimeler: RP-HPLC yöntemi, plazma, metformin, repaglinide.

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INTRODUCTION

The combined use of metformin and repaglinide for type 2 diabetes mellitus has shown improved patient compliance by controlling the post prandial glucose levels and reaching the basal glycemia (1). Monotherapy with metformin, an oral anti diabetic agent, is not sufficient to reach the glycemic goals and multiple drugs may be necessary to reach the basal glycemia. As per the biopharmaceutical classification system (BCS), metformin belongs to class III; as it has higher solubility in aqueous solutions and lower permeability to cross 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 chromatographic separations. Metformin showed single pKa value (2) of 11.5 and repaglinide (3) showed two pKa values of 4.19 and 5.78, due to the zwitterionic group. Until this decade, this combination for liquid chromatographic separation has not been reported. HPLC techniques have been reported only for metformin in human plasma (4), urine (5), pharmacokinetics study in men (6), by ion-pair HPLC method (7) and in combinations with sulfonylureas like pioglitazone (8), with gliclazide in human plasma (9), and with glyburide (10). Estimation of repaglinide in human plasma (11, 12) was done by electrochemical method (13). Improvement of patient's compliance has been reported for combination of metformin and repaglinide, rather than with sulphonylureas (14), simultaneous estimation of nine anti-diabetic drugs (15), six antidiabetic drugs in human plasma (16) and pharmacokinetic study in men (17). These combinations are commercially available as tablet dosage form under the brand name of Prandimet® (18). HPLC estimation method for the metformin in human plasma (19), and in microspheres and tablet dosage forms (20) were previously reported. Spectrophotometric study of metformin and repaglinide (21) and combination of rosiglitazone (22) and metformin were also reported. Combining the use of oral anti-diabetic agents depends on patient's clinical manifestations. Most of the doctors will choose metformin as their first choice for the treatment of type II diabetes mellitus. Depending on clinical characteristics of patients; monotherapy can change over to the combination of various

anti diabetic agents. Adding non-sulfonylureas to metformin, the basal glycemia and post prandial glucose levels are adequately controlled.

The scope of this work was to develop a simple and sensitive method for determination of repaglinide and metformin in rabbit plasma using RP-HPLC with ultra-violet spectrophotometric detection. Within the context of this goal, an optimization of separation conditions with spectrophotometric detection was performed.

MATERIALS and METHODS

Materials

Metformin (99.4% purity), chemically it is *N*, *N*-dimethylimidodicarbonimidic diamide. Repaglinide (98.3% purity), chemically, (*S*)-(+)-2-ethoxy-4-[2- (3-methyl-1- [2- (piperidin-1-yl) phenyl] butyl amino) -2-oxoethyl] benzoic acid, were kindly supplied by Aurobindo Pharma Ltd (Hyd, India). De-ionized water obtained was 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). Solvents and sample were filtered using Ultipor® N®66 0.2 µm and 0.45 µm membranes, respectively.

Instrumentation

Shimadzu HPLC system, which consisted of the following components: Prominence® CBM-20A controller, gradient system with dual pumps of LC-10AT VP, SPD-10A VP detector with Class-VP: V6.13 software and BDS Hypersil® column, C-18, 150mm×4.6mm i.d, particle size 5µm (Thermo® scientific, India) was used at a fixed wave length of 254 nm. A bath sonicator was used for solving and degassing solvents, and mobile phases (PCI analytics, Mumbai, India). The centrifuge used was from REMI® Electrotechnik Pvt Ltd, Vasai, India. Waters symmetric® C₁₈, i.d, 4.6 × 250 mm, 5 µm was used to check the ruggedness. The column was equilibrated for at least 45min with a mobile phase consisting of acetonitrile: phosphate buffer pH 4.0, 0.1M (60:40) with 1% triethylamine. Mobile phase was sonicated before the equilibration of column for 20min and

followed by filtration through 0.2µm membrane. Gradient elution technique was applied, and flow rate was set at 0.8mL/min at a fixed wavelength of 254 nm. Standard stock solutions were prepared, dissolving appropriate concentrations of metformin and repaglinide in methanol respectively, to yield a final drug of 8400 µg/mL metformin and 1100 µg/mL repaglinide concentrations. Then the stock solutions were mixed (50:50 %v/v) to obtain a combined working standard solution composed of 4200 µg/mL of metformin and 550 µg/mL of repaglinide. Working standards of 4200, 3360, 2520, 1680, 840, 420 µg/mL of metformin and 550, 440, 330, 220, 110, 55 µg/mL of repaglinide were prepared by diluting 4200/550 µg/mL combined standard stock solution.

Extraction procedure

The plasma samples were extracted using the following procedure: First, 200 µl of plasma sample was pipetted out into a 1.5 mL of Eppendorf tube; thereafter, mixture of working standard solutions and 50 µl of ethyl acetate and 10µL of 0.1N HCl were added. Metformin is water soluble, while repaglinide is insoluble, it has two pKa values due to zwitterionic behavior, and addition of hydrochloric acid gives ionization, resulting in improvement of water solubility. Due to hydrophobic characteristic of the repaglinide, it is impossible to dissolve it directly in the plasma. Both compounds were easily soluble in a mixture of (40:60 %v/v) methanol: a phosphate buffer of pH 4.0. Subsequent additions of plasma can lead to protein precipitation, resulting in poor precision of the analytical method. The mixture was vortexed and 1mL of methanol: phosphate buffer of pH 4.0 was added. The mixed solution was vortexed again, and subsequently centrifuged for 15 minutes at 10,000 ×g. The supernatant present after the centrifugation was transferred to a 1.5 mL of Eppendorf tube, and evaporated to dryness at 65 °C for 90 min. The dried sample was reconstituted with 200µL of the mobile phase prior to analysis.

Method development

BDS Hypersil® column was equilibrated earlier and tested using methanol/water mixture at various compositions at a flow rate of 1mL/min. It resulted in broad peaks with poorer resolution, and then was

switched over to methanol/potassium dihydrogen phosphate buffer; which gave less broad peaks than the first one. To optimize this separation, different fractions of acetonitrile and water tested, and the optimum separation was attained using 60% of acetonitrile and 40% of phosphate buffer of pH 4 at a flow rate of 0.8 mL/min and with a fixed wavelength of 254 nm.

Method validation

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

Calibration curve

Standard combinations of stock solutions were prepared by serial diluting with phosphate buffer of pH 4. The linearity was determined to be between 420-4200 mg/mL for metformin and 55-550 ng/mL for repaglinide, attained by repeated analysis for five times (n=5) a day, and continued for three days (n=15).

Recovery, precision and accuracy

Recovery studies were conducted for extracted samples, 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 was injected for five times. Accuracy was determined by injecting three samples of the standard solutions 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 a 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 on long run time and resolved in combination. Sulfonylureas like gliclazide, glipizide, thiazolidinediones like pioglitazone; non-sulfonylureas like nateglinide were used in combination with metformin in the treatment of type II diabetes mellitus in five different blank plasma samples.

RESULTS and DISCUSSIONS

Chromatograms

The retention times and capacity factors were 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 the mobile phase is less than the repaglinide; eludes faster than repaglinide depending on their solubilities, polarities, and pH of the environment.

Linearity

The chromatographic analysis of metformin and repaglinide exhibited excellent regression values of $R^2 = 0.997$ and $R^2 = 0.9995$ over the concentration ranges of 420-4200 ng/mL and 55-550 ng/mL respectively. The three day daily analysis of five samples, resulting in the calibration curves don't have a statistical significant in values of slope, regression and intercepts. The assays showed an acceptable precision in the terms of %RSD, <5 and <3 for repaglinide and metformin, respectively.

Recovery, precision and accuracy

The recovery rates 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 a range of 0.85-1.70 % and 1.30-1.56 % for metformin and repaglinide, respectively. The accuracy (ruggedness) of proposed method was checked using a different column on intra-day and inter-day assays, and are given in Table 1 and Table 2 for metformin and repaglinide.

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 not to be detected in the present

Table 2. Accuracy and precision of repaglinide in rabbit plasma samples.

BDS Hypersil® column C18				Waters symmetric® column C18			
Intra-day (µg/mL)	Experiment (µg/mL) ± SD*	Accuracy (%)	Precision (%RSD)*	Experiment (µg/mL) ± SD*	Accuracy (%)	Precision (% RSD)*	n
2.75	2.72±0.02	98.91	0.78	2.73±0.014	99.27	0.51	05
5.50	5.48±0.01	99.64	0.26	5.45±0.035	99.09	0.64	05
8.25	8.30±0.04	100.61	0.43	8.23±0.014	99.75	0.17	05
Inter-day (µg/mL)							
2.75	2.69±0.04	97.82	1.56	2.69±0.042	97.81	1.57	15
5.50	5.39±0.08	98.00	1.43	5.16±0.240	93.81	4.55	15
8.25	8.10±0.11	98.18	1.30	8.01±0.169	97.09	2.10	15

*SD=Standard Deviation, %RSD=Percentage Relative Standard Deviation

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2.75	2.72±0.02	98.91	0.78	2.73±0.014	99.27	0.51	05
5.50	5.48±0.01	99.64	0.26	5.45±0.035	99.09	0.64	05
8.25	8.30±0.04	100.61	0.43	8.23±0.014	99.75	0.17	05
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5.50	5.39±0.08	98.00	1.43	5.16±0.240	93.81	4.55	15
8.25	8.10±0.11	98.18	1.30	8.01±0.169	97.09	2.10	15

*SD=Standard Deviation, %RSD=Percentage Relative Standard Deviation

chromatogram. A system suitability test was performed by six replicate injections of standard mixture verifying the following parameters: resolution of neighbour peak is more than 3, %RSD of each peak retention time is less than 0.3% and %RSD of each peak area is less than 1.5%.

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

The LODs of metformin and repaglinide were 135.6 ng/mL and 18.15ng/mL, respectively. LOQ of metformin and repaglinide were 420ng/mL and 55ng/mL, respectively.

CONCLUSION

A highly sensitive and specific analytical method was developed and validated for quantification of metformin with repaglinide in rabbit plasma samples. The specificity method was tested in five different sources, and was analyzed. The chromatogram of Figure 1 shows the blank plasma and Figure 2 shows the 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 such drugs, in floating drug delivery systems.

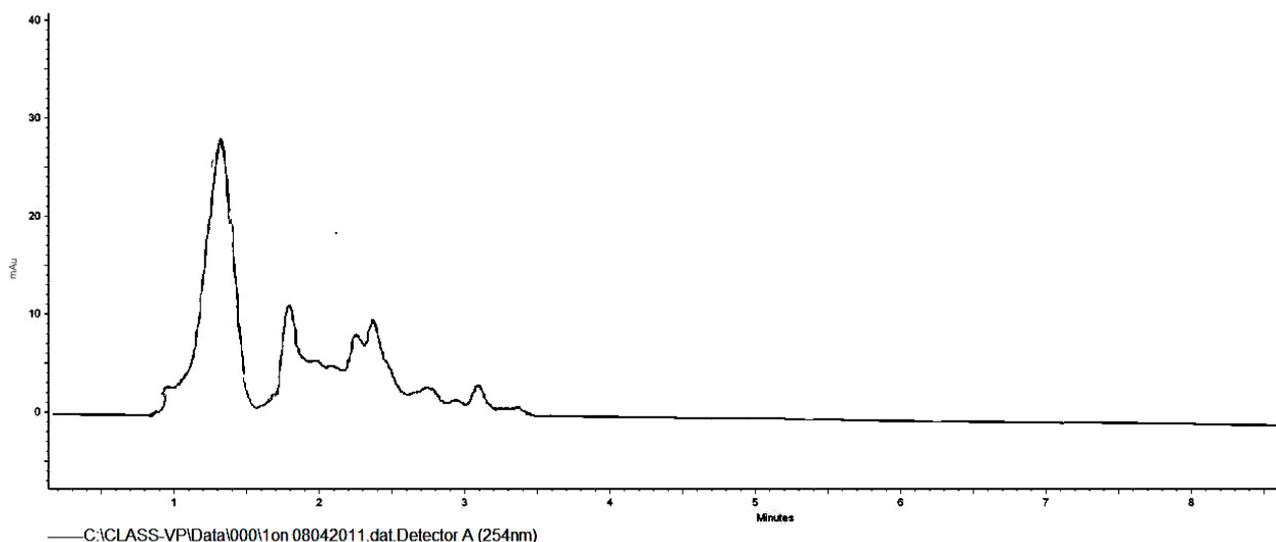


Figure 1. Chromatogram of blank plasma

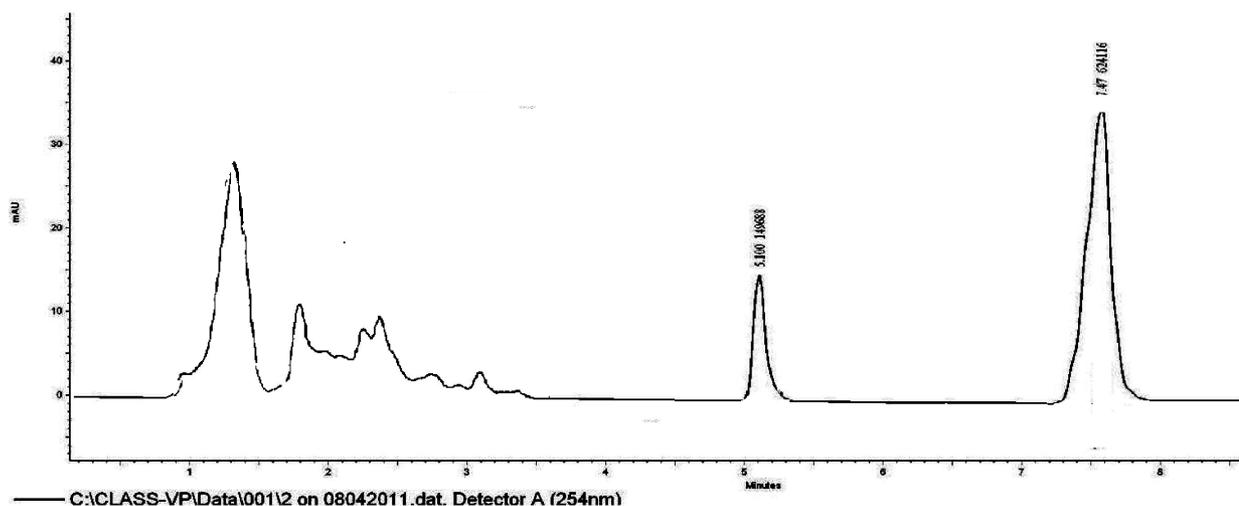


Figure 2. Sample chromatogram of metformin and repaglinide in rabbit plasma

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