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Original Article

STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF REPAGLINIDE IN RABBIT PLASMA

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ABSTRACT

Objective: A simple, selective and sensitive reverse-phase high-performance liquid chromatography (RP-HPLC) method to estimate repaglinide (REP) in rabbit plasma using rabeprazole (RAB) as an internal standard was developed and validated for various qualifications.

Methods: The chromatographic separation was performed on C_{18} (2) analytical column (5 µ, 250×4.6 mm) using acetonitrile: 0.05% trifluoroacetic acid in water (55:45, v/v) as mobile phase at the flow rate of 1 ml/min. Validation of the analytical method was performed as per ICH guidelines.

Results: The retention times of REP and RAB were found at ~4.3 and 5.1 min respectively, with adequate system suitability parameters (theoretical plates ≥3619, tailing factor ≤1.38, resolution factor 2.37). The method has linearity over a concentration range of 10 to 1000 ng/ml (r^2=0.9987). The results of accuracy (≥98.17%), intra-, inter-day precision (≤2.9%), recovery (101.21±2.09%) and process efficiency (99.77±3.74%) found satisfactory with no matrix effect. The analyte in samples were found stable up to 6 h, 3 freeze-thaw cycles and not more than 2 mo corresponding to bench-top, short and long term stability studies respectively.

Conclusion: The developed RP-HPLC method for estimation of REP in rabbit plasma was developed. The method was found to be rapid, cost-effective and accurate to estimate the REP from the sample matrix. The method can be a most useful tool for *in vivo* study of REP in the rabbit.

Keywords: HPLC-UV, Repaglinide, Bioanalytical, Validation, Rabbit plasma

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INTRODUCTION

Repaglinide (REP) is a novel carbomoxyl methyl benzoic acid derivative, also known as 2-ethoxy-4-[2-[[3-methyl-1-[2-(lpiperidinyl)-phenyl] butyl] amino]-2-oxoethyl]-benzoic acid (fig. 1) [1]. It is a novel short-acting secretagogue belongs to meglitinide class, used to regulate fasting as well as postprandial elevated blood glucose in patients under the treatment of type-II diabetes mellitus [2, 3]. It was understood from the literature that, REP reduces the hyperglycemia by stimulating pancreas through depolarization of β-cells via K^+_{ATP} channel blockade at plasma membrane, thereby ensue Ca^{2+}influx through voltage-dependent channels, that triggers the Ca^{2+}dependent exocytosis of insulin-containing granules [2-5].

To date, several analytical techniques have been reported to estimate REP from human plasma, involving competitive solid-phase enzyme immunoassay [6], high-performance liquid chromatography (HPLC) integrated with electrochemical [7], ultraviolet (UV) [8, 9], photodiode-array (PDA) [10], detectors. Gradient elution techniques for concurrent estimation of REP with other anti-diabetic agents from the biological matrix on HPLC using UV detection were reported [8, 9]. Furthermore, HPLC-tandem mass spectrometry (LC/MS/MS) methods were established to assess the REP from plasma and urine samples of human [10, 11], monkey [12], rat [13] and equine [14]. Though, Gandhimathi *et al.*, Berecka *et al.*, Joshi *et al.* and Soni *et al.* were reported HPLC methods to estimate the REP alone or combined with other drugs from pharmaceutical formulations but were not applied for biological samples [15-18]. However, all the above methods reported for REP estimation from biological matrix need of expensive instruments for gradient elution and for eluate detection, restrict the application of them in common research laboratories equipped with limited resources.

The principle objective of the present study was to establish a simple, selective and precised reverse phase-HPLC (RP-HPLC) integrated with ultraviolet (UV) detection method to quantify REP in rabbit plasma using rabeprazole (RAB) as an internal standard (IS). Further, an attempt was done to validate the developed analytical method for essential qualifications like selectivity, linearity, recovery, accuracy, precision and other suitable validation parameters.

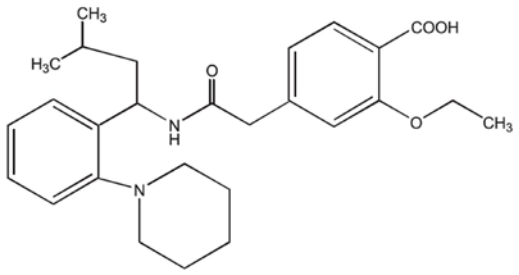

Fig. 1: Chemical structure of REP

MATERIALS AND METHODS

Chemicals and reagents

REP and RAB pure drug samples were acquired from KP Labs, Hyderabad, India. HPLC grade acetonitrile (ACN) and methanol were supplied by Rankem, Hyderabad, India. Trifluoroacetic acid (TFA) was supplied by SDFCL, Mumbai, India. HPLC grade water was used during the entire procedure. Heparin-coated blood sampling tubes were purchased from CML biotech (P) Ltd., Kerala, India.

Instrumentation and chromatographic condition

The HPLC (Shimadzu, Kyoto, Japan) system consists of a binary pump (LC-20AD), equipped with UV/Visible detector (SPD-A20), a Rheodyne injector port (7725i) with 20 µl loop volume and the data

acquisition was done by LC-solutions software. Weighing of materials was done with 0.1 mg precision balance (Denver Instruments, New York). Plasma samples processing was included vortex mixer, refrigerated centrifuge and deep freezer (Remi, India)

The chromatograms were developed on an analytical column C18 (2), 250×4.6 mm; 5 µ particle size (Luna®, Phenomenex) via isocratic elution by fixed composition of ACN:0.05% TFA in water (55:45, v/v) mixture as mobile phase, pumped with constant flow rate (1 ml/min) and 285 nm was set as detection wavelength for entire study. The composition of eluent was admixed freshly for total volume required, passed through a membrane filter (#0.45 µm) and degassed by ultrasonic bath sonication for 30 min precedent to each batch analysis.

Standard solutions and quality control samples

Individual primary stock solutions of REP and RAB (1 mg/ml) were prepared using methanol as solvent and kept at -20°C temperature to avoid decomposition of analyte on storage. The primary stock solution of REP was diluted appropriately with mobile phase mixture to obtain working standards (WS) in concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 µg/ml, similarly, a single WS solution of IS (5 µg/ml) was also prepared for entire study. The standards for calibration (CS) were made by spiking of individual rabbit plasma (180 µl) with each WS solution of REP (20 µl) to result in (10, 25, 50, 100, 250, 500 and 1000 ng/ml) plasma-drug mixture (200 µl) prior to analysis was done. Quality control (QC) standards of REP at 4 different levels at lower limit of quantitation (LLOQ), low QC (LQC; 3*LLOQ), medium QC (MQC; 50% of upper LOQ) and high QC (HQC; 90% of upper LOQ) were independently made at concentration level of 10 ng/ml, 30 ng/ml, 500 ng/ml and 900 ng/ml respectively as like method described for CS.

Extraction procedure

Preparation of samples for injection to develop the chromatogram was done as per extraction procedure reported earlier by Yin *et al.* and Ma *et al.*, with fewer modifications [19, 20]. To 200 µl rabbit plasma (blank/test/CS/QC) sample transferred in an eppendorf tube (2 ml) quadruple volume (800 ml) of ACN and 20 µl WS solution of RAB (5 µg/ml) was added as extraction reagent and IS respectively, capped and vortexed on a cyclomixture for 1 min. The mixture resulted was subjected to refrigerated centrifuge at 5000 g for 15 min at 4°C. The protein-free clear supernatant (500 µl) was shifted into a fresh eppendorf tube (1 ml) and evaporated to dryness in a vacuum concentrator. Reconstitution of the residue was done with mobile phase mixture (100 µl), capped, vortexed for a min and centrifuged again at 5000 g for 15 min at refrigerated condition (4°C). The particle-free clear supernatant (20 µl) was allowed to inject into the HPLC column to develop chromatogram.

Method validation

The validation approach is essential to demonstrate the competency of an analytical method for its purpose intended. The validation of the analytical method was conducted for essential qualifications like selectivity, linearity, accuracy, precision, LLOQ, matrix effect, recovery, performance of the method and stability of analyte were studied in accordance with guidelines of International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) guidelines for bioanalytical method validation [21, 22].

Selectivity

The blank chromatograms of plasma sample (n=3) were examined for probable interfering peaks at the detection time of REP and IS. In addition, chromatograms developed for rabbit plasma (n=3) spiked with IS alone and chromatograms of plasma spiked with both REP and IS were carefully examined for peak deriving and for significant interfering of each on other [22].

Linearity, range and lower limit of quantitation

Linearity was measured through ($1/x^2$) weighed linear regression equation (mean r^2; n=5) of calibration graph plotted employing seven nonzero concentrations of REP (between range 10 to 1000 ng/ml) spiked in rabbit plasma samples verses corresponding peak area ratio of REP:IS measured. Acceptance of the calibration curve was done when it passes good coefficient of correlation ($r^2 \geq 0.98$) and/or estimated precision (percentage coefficient of variation (%CV)) of CS fall within accepted limits (LLOQ: ±20%w/w and for all other CS levels within±15%w/w of theoretical/nominal concentration). LLOQ was defined as the least concentration of analyte present in plasma sample that could be determined with minimum accuracy (80% to 120%w/w) and precision (%CV) not more than (NMT) 20%w/w [10, 22].

Accuracy and precision

Accuracy (percentage relative error (%RE)) is the measure to which estimated value conforms to the theoretical/nominal amount of analyte exists in the sample matrix, was studied in 5 replicates of 4 QC levels (LLOQ: 10, LQC: 30, MQC: 500 and HQC: 900 ng/ml) estimated against freshly constructed calibration curve (10 to 1000 µg/ml). The assay of QC levels was repeated in 3 separate runs within a day and in 3 distinct days to determine intra-and inter-day precision (%CV) respectively. The acceptance limit for accuracy as well as precision was set as NMT±20%w/w for LLOQ and for all other QC level is NMT±15%w/w [22, 25].

Matrix effect (ME), extraction efficiency (EE) and process efficiency (PE)

All these parameters were studied using 3 sets of samples at 3 individual QC levels (LQC: 30, MQC: 500 and HQC: 900 ng/ml) along with IS in triplicate. Set 1: QC levels prepared in methanol, set 2: QC levels spiked into liquid extracts of blank plasma and set 3: samples extracted from plasma spiked with QC levels. The ME was determined by relating the assay of set 2 with set 1; recovery was estimated by comparing set 3 with set 2 and PE was calculated by comparing set 3 with set 1. The EE was assessed from percentage recovery should be consistent rather than high value [22, 23].

Stability studies

Stability of an analyte in the sample matrix is dependent on storage conditions and duration, chemical properties of an analyte and the matrix. Stability of REP in sample matrix was determined in triplicate, by assay of QC samples at 2 levels (LQC: 30 and HQC: 900 ng/ml) in all types of study. In bench-top stability study, the integrity of the REP and IS in wet state (blood and reconstituted sample) and dry state (extracted residue for reconstitution) samples were studied separately up to the average working period (6 h). Stress-induced stability was assessed by subjecting the QC (plasma) samples for 3 freeze-thawing (-25°C to 4°C) in a frequency of 1 cycle per day, in that the samples were kept at least 18-20 h in frozen state and 4-6 h thawing state in each cycle. The frozen QC samples were slowly thawed to room temperature at the end of each cycle and assayed to study the precision. In assessing long term stability, assay of the stored QC samples (-20°C) was done after thawing the samples to room temperature in order to determine the precision by end of 0, 1st, 2nd and 3rd months. In all type of stability studies 0 time/0 cycle samples were kept as control and to which other samples were compared to assess the significance of difference by one way ANOVA [22-27].

Statistical analysis

All the results of assay, validation parameters and *in vivo* animal pharmacokinetic parameters were expressed in mean±SD, %CV and %RE. Statistical significance of variations was studied by one way ANOVA done by data analysis tool pack, Microsoft excel® (2007).

RESULTS

Optimization of chromatographic condition

The chromatographic separation and elution of analyte was done by mobile phase consisting of ACN:0.05% TFA in water (55:45, v/v) in reverse phase analytical column (250×4.6 mm; 5 µ particle size). The retention times (R_ts) of REP and IS were found as ~4.3 and 5.1 min respectively. The system suitability parameters of the method were calculated as follows: theoretical plates for REP and IS were found as 4764 and 3619 respectively. Tailing factor was found as 1.16 and 1.38 for REP and IS respectively and the resolution between two drugs was found as 2.37.

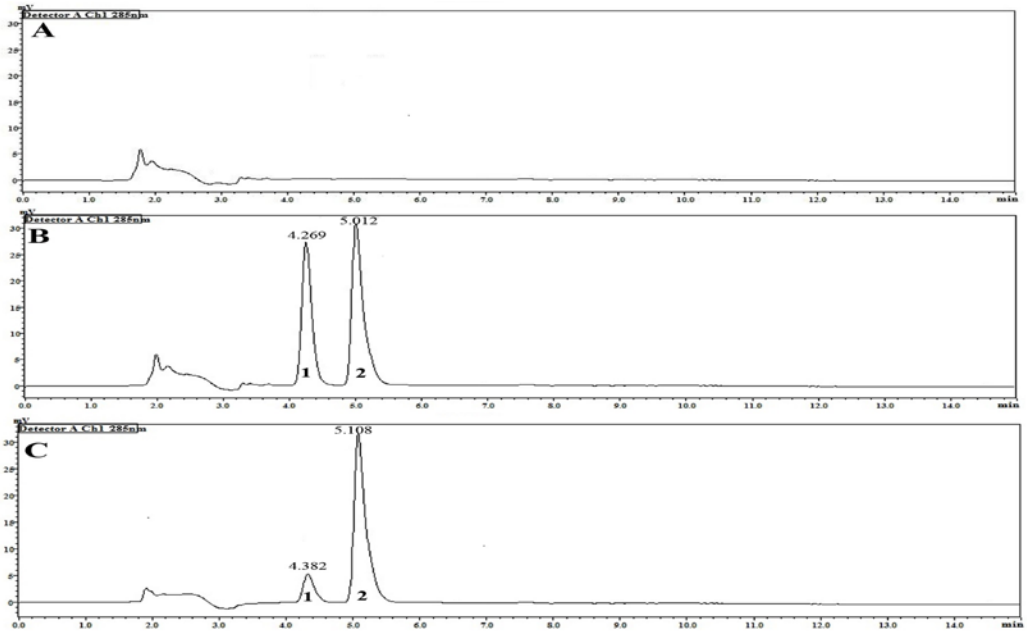

Fig. 2: Chromatograms of (A) blank plasma, (B) rabbit plasma spiked with 500 ng/ml concentration of REP and IS, (C) Plasma sample collected after 1 h following 4 mg oral dosing of REP and spiked with IS

Method validation

Selectivity

The chromatogram of blank rabbit plasma sample (fig. 2. A) was compared with the chromatogram developed for rabbit plasma spiked with REP and IS (fig. 2. B). Further, the chromatogram of rabbit plasma sample collected at 1 h after 4 mg oral dosing of REP with spiked IS was illustrated in fig. 2. C.

Linearity, range and lower limit of quantitation

The linear regression equation of calibration curve plotted was resulted as Y=1.415X+0.0022 with r^2 value of 0.9987 and the linearity was found as high in between concentration range of 10 to 1000 ng/ml. The LOD and LOQ value calculated from the regression equation of the calibration curve was found as 1.73 ng/ml and 5.23 ng/ml respectively. The practical LLOQ of REP in rabbit plasma sample was accepted as 10 ng/ml.

Accuracy and precision

The accuracy and precision were expressed as %RE and %CV (table 1). The assay results studied for accuracy were varied between 98.17±2.71%w/w to 101.39±2.35%w/w, with %RE was ranged from -1.82%w/w to 1.39%w/w. The result of intra-day precision (%CV) was found between 0.65%w/w to 2.9%w/w and the inter-day precision was found in the range of 0.49%w/w to 2.76%w/w.

Table 1: Results of accuracy and precision study (n=5)

Conc. spiked (ng/ml)	Assay (%w/w)	SD (%w/w)	RE (%w/w)	Conc. Found (ng/ml)	SD (ng/ml)	CV (%w/w)
Intra Day						
10	101.02	2.93	1.02	10.1	0.29	2.9
30	100.53	0.65	0.53	30.16	0.19	0.65
500	99.72	2.19	-0.28	498.6	10.96	2.19
900	98.73	2.2	-1.28	888.53	19.84	2.23
Inter Day						
10	101.39	2.35	1.39	10.14	0.24	2.32
30	100.68	0.49	0.68	30.2	0.15	0.49
500	98.17	2.71	-1.82	490.88	13.56	2.76
900	99.73	1.39	-0.27	897.61	12.54	1.39

Matrix effect (ME), extraction efficiency (EE) and process efficiency (PE)

The results of ME, EE and PE were determined by relating the assay of paired samples as described in the methods, was shown in table 2. All these three parameters were studied at QC levels 30, 500 and 900 ng/ml in triplicates and the mean results determined for ME was 98.61±3.95, EE was 101.21±2.09 and PE was 99.77±3.74 % w/w.

Table 2: Results of matrix effect, extraction efficiency and process efficiency

Drug conc. (ng/ml)	Assay of sample*			ME*	EE*	PE*
	Methanol	Unextracted	Extracted			
30	101.46±3.24	99.26±1.53	101.87±4.03	97.89±3.01	102.61±2.43	100.44±4.06
50	99.67±5.16	98.53±2.14	99.73±1.92	99.1±6.99	101.24±2.15	100.24±5.47
900	100.93±2.49	99.74±2.48	99.52±2.06	98.84±2.13	99.76±0.72	98.63±2.52
Mean**	100.68	99.17	100.37	98.61	101.21	99.77
%RSD**	3.39	1.88	2.7	3.95	2.09	3.74

*All the results represented as mean±SD (n=3); **All the results represented as mean±SD (n=9)

Stability studies

The bench-top stability study for the whole blood sample, dried and reconstituted samples after extraction was performed at 2 QC levels in triplicates and the results were shown in table 3. The results of stress-induced (freeze-thaw) and long term stability study (storing at -20 °C) were reported in table 4.

Table 3: Results of bench top stability study after 6 h (n=3)

QC Level	Conc. (ng/ml)	%Accuracy	%RSD	p-value
blood				
LQC	30	101.74	5.85	0.92
HQC	900	99.85	4.43	0.83
Reconstututed				
LQC	30	102.6	2.14	0.17
HQC	900	103.73	4.48	0.56
Dry state				
LQC	30	100.2	3.73	0.85
HQC	900	99.62	4.08	0.97

Table 4: Results of short term and long term stability study (n=3)

Short term (Stress induced) stability					Long term stability		
QC Level	**Conc. (ng/ml)**	**%Accuracy**	**% CV**	**p-value**	**%Accuracy**	**% CV**	**p-value**
Cycle 0					**0 Mo**		
LQC	30	99.89	3.66	-	101.02	4.57	-
HQC	900	100.37	1.75	-	100.91	2.75	-
Cycle 1					**1 Mo**		
LQC	30	99.31	10.94	0.93	98.76	5.66	0.62
HQC	900	95.53	8.91	0.41	99.79	3.03	0.66
Cycle 2					**2 Mo**		
LQC	30	98.99	9.22	0.88	97.72	6.29	0.49
HQC	900	98.2	7.05	0.63	95.45	5.53	0.19
Cycle 3					**3 Mo**		
LQC	30	99.81	9.74	0.99	72.43	15.5	0.015
HQC	900	98.06	10.93	0.73	69.64	17.24	0.012

DISCUSSION

The chromatographic condition was optimized for bio-analysis of REP in rabbit plasma using RAB as IS on RP-HPLC consisting with UV detection. In this process, several compositions between ACN and TFA (0.05% v/v in water) were tried as eluent to develop the chromatogram. Increased proportion of ACN in the mobile phase resulted in faster elution but the peaks of REP and IS were not completely separated. Reduction of ACN proportion in the mobile phase resulted tailing of peaks and it was found that mixture of ACN:0.05% TFA in water (55:45, v/v) as optimum mobile phase composition by which the REP peak clearly resolved from IS peak and solvent fronts. In order to develop a liquid-liquid extraction method, various solvents (ACN, Methanol, Dichloromethane and ethyl acetate) were used for extraction of REP and IS from plasma samples, among that ACN found to be most efficient than all others. This method was simple and efficient in the extraction of analyte and chromatogram developing when compared to earlier reported methods by Venkatesh *et al.* and Ruzilawati *et al.* [9, 10]. The system suitability parameters: theoretical plates (>3000), Tailing factor (<2) and resolution (>2) for the developed HPLC method were satisfactory to meet the ICH guidelines [21].

The chromatogram of blank rabbit plasma sample was compared with the chromatogram developed for rabbit plasma spiked with REP and IS. No significant endogenous noise peaks around the R_ts of REP and IS were found in chromatogram of blank plasma sample, justifies the selectivity of this analytical method. The calibration curve plotted using plasma concentration of REP (X) versus mean peak area ratio of REP:IS of triplicates (Y) has high linear (r^2=0.9987) with acceptable variability (≤9.8%) in the selected concentration range indicates the sensitivity of the method as good. All the results of accuracy (%RE) and Precision (%CV) were found less than ±1.82%w/w and ±2.9%w/w respectively were complied with the official guidelines (%RE and %CV NMT±20%w/w for LLOQ and for other QC levels NMT±15%w/w) confirms the method's accuracy and precision in estimation of analyte. The recovery of REP from plasma samples done by liquid extraction using ACN, shown better and consistent EE (99.76 to 102.61%w/w) than earlier reported by Venkatesh *et al.* (92.37 to 106.50%w/w) [8].

The bench-top stability was studied to find the influence of the maximum processing time on the integrity of analyte in the matrix. The REP is proved to be stable in the wet state as well as dry state samples during an average bench-top working period (6 h). Also, the chemical stability of REP in plasma was confirmed at the end of 3 stress cycles applied (p≥0.73) and significant stability of analyte was retained in the plasma up to 2 mo (p≥0.19) during storage at -20°C, beyond that the integrity of analyte in the sample matrix was insignificant (p≤0.015).

CONCLUSION

A simple and selective RP-HPLC-UV method to estimate REP from rabbit plasma using RAB as an IS was developed and validated for various qualifications per ICH and FDA guidelines. The method is selective to estimate REP from the sample matrix, has good linearity, accuracy, precision and stability over the therapeutic range. The liquid extraction procedure used in this method was simple, reproducible with good extraction efficiency and process efficiency. The method could be applied to estimate the plasma concentration of REP after the suitable route of administration in New Zealand white rabbit to study the *in vivo* pharmacokinetics of REP from the dosage forms.

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AUTHORS CONTRIBUTIONS

All the authors have equal contribution in this research work.

CONFLICT OF INTERESTS

The authors declared no conflict of interest

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