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Development and validation of a high-performance liquid chromatographic method for bioanalytical application with rimonabant

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ABSTRACT

A simple and feasible high-performance liquid chromatographic method with UV detection was developed and validated for the quantification of rimonabant in human plasma. The chromatographic separation was carried out in a Hypersil BDS, C_{18} column (250 mm × 4.6 mm; 5 μm). The mobile phase was a mixture of 10 mM phosphate buffer and acetonitrile (30:70, v/v) at a flow rate of 1.0 ml/min. The UV detection was set at 220 nm. The extraction recovery of rimonabant in plasma at three quality control (QC) samples was ranged from 84.17% to 92.64%. The calibration curve was linear for the concentration range of 20–400 ng/ml with the correlation coefficient (r^2) above 0.9921. The method was specific and sensitive with the limit of quantification of 20 ng/ml. The accuracy and precision values obtained from six different sets of QC samples analyzed in separate occasions ranged from 88.13% to 106.48% and 0.13% to 3.61%, respectively. In stability tests, rimonabant in human plasma was stable during storage and assay procedure. The method is very simple, sensitive and economical and the assay was applied to human plasma samples in a clinical (pharmacokinetic) study of rimonabant.

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1. Introduction

Rimonabant [N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] is a selective cannabinoid-1 receptor (CB1) antagonist used for treatment of obesity (Fig. 1B) [1]. Initially it was intended as anti-obesity and smoking cessation dual-purpose drug but later program has been discontinued [2,3]. Recently, the European Commission has granted marketing authorization for rimonabant. Rimonabant is currently under consideration for approval at the U.S. Food and Drug Administration (FDA) [4]. The anti-obesity potential of rimonabant was demonstrated in a phase 3 trial in obese patients, where significant reduction of body weight and triglyceride levels was observed in a 2-year study [5]. The drug is believed to interact with the G-protein coupled CB1 receptor in the brain and produces several biochemical consequences, including inhibition of Ca^{2+} channel [6]; activation of K^+ channels [7]; inhibition of adenylate cyclase [8]; and activation of mitogen-activated protein kinase [9]. The effect on Ca^{2+} and K^+ ion channels results in a reduction of neuronal excitability and a suppression of neurotransmitter release. The administration of rimonabant to rodents has been shown to reduce the rewarding/reinforcing behaviors of several drugs of abuse like heroin [10], cocaine [11], etc. So rimonabant may be used in the treatment of addiction. There is evidence that after administration of rimonabant to rodents show a marked decrease in food consumption, this indicates it as a potential drug for the treatment of obesity [1].

Very few methods have been published for the quantification of rimonabant in plasma. According to the best of our knowledge, no analytical method using HPLC/UV has been reported for the determination of rimonabant in plasma. A method was developed for the determination of rimonabant in human plasma by high-performance liquid chromatography–tandem mass spectrometry (LC/MS/MS) [12]. Only a few analytical methods using LC/MS/MS have been reported for the determination of rimonabant in rat and mouse plasma [13,14]. However, these techniques are not suitable because this equipment is not available in all clinical laboratories due to its high cost. In the present study, a simpler method has been developed and validated for the determination of rimonabant in human plasma by HPLC with UV detection. A corresponding pharmacokinetics study has been conducted with the developed method.

2. Experimental

2.1. Chemicals and reagents

Rimonabant was obtained from M/S Cosmas Pharmacals (Ludhiana, India) and glimepiride (internal standard (IS)) from M/S Swiss Garnier Life Sciences (Himachal Pradesh, India) (the structure of

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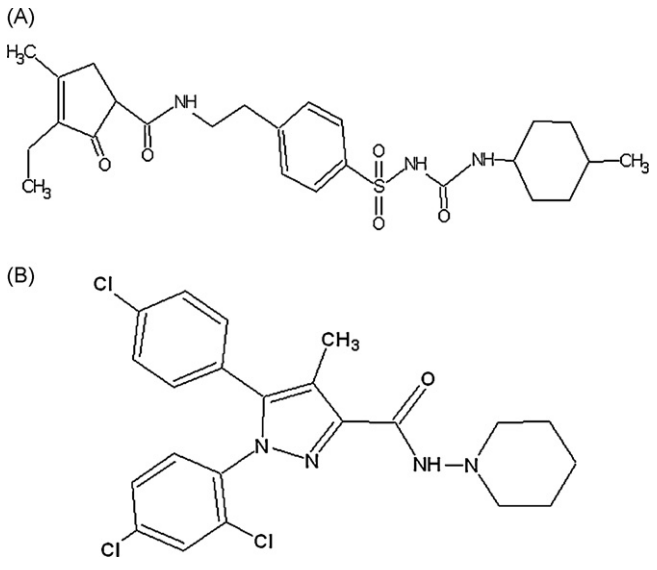

Fig. 1. Structural representation of (A) glimepiride (IS) and (B) rimonabant.

the compounds are given in Fig. 1A). Ethyl acetate and hexane of HPLC-grade were obtained from Merck (Mumbai, India). HPLC-grade water (resistivity of 18 M cm) generated from Milli-Q water purification system was used throughout the analysis. The blank human plasma with EDTA-K_3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

2.2. Instrumentation and chromatographic conditions

The HPLC apparatus consisted of a Shimadzu LC-20AT separation module and an SPD-20A UV detector (Kyoto, Japan). Detection and quantification were performed using Spinchrom software. RP-HPLC analysis was performed isocratically at room temperature using a Hypersil BDS, C_{18} (250 mm × 4.6 mm, 5 μm) column. The mobile phase consisted of a mixture of 10 mM phosphate buffer and acetonitrile (30:70, v/v) at a flow rate of 1.0 ml/min and a sample size of 50 μl was injected through the rheodyne injector system fitted with 50 μl fixed loop. The eluent was monitored with a UV detector set at 220 nm.

2.3. Preparation of stock and working solutions

Stock solutions of rimonabant and IS were prepared at concentration of 100 μg/ml in acetonitrile and methanol, respectively. Stock solutions were stored at −20 °C until they were used for working solutions by adding appropriate volume of mobile phase. Working solutions of different concentrations were prepared from above-mentioned stock solution afresh before use.

2.4. Preparation of calibration standards and quality control (QC) samples

Seven calibration standards in plasma at concentration of 20, 50, 100, 150, 200, 300 and 400 ng/ml were prepared by spiking appropriate aliquots of working solution of rimonabant in plasma. For internal standard the final concentration in plasma was 400 ng/ml. Four levels of QC samples at a concentration of 20 ng/ml (lower limit of quantitation, i.e. LLOQ), 60 ng/ml (low), 200 ng/ml (medium), and 320 ng/ml (high) were also prepared following the above method. QC samples were prepared in a 50 ml pool, then aliquoted into pre-labeled 2 ml polypropylene vials and stored at −20 °C.

2.5. Sample preparation

For calibration standards, an aliquot quantity of 0.9 ml plasma sample was taken in a 10 ml stopper test tube and 0.1 ml internal standard was added and mixed. To it 8 ml mixed solvent (ethyl acetate:n-hexane = 70:30, v/v) was added. The mixture was then mixed for 15 min followed by centrifugation at 5000 rpm for 20 min. 7 ml of organic layer was separated and evaporate to dryness at 40 °C under N_2 atmosphere. The residue was reconstituted in 200 μl of mobile phase, filtered through 0.22 μm membrane filter and 50 μl was injected into the HPLC system.

2.6. Validation of method

The method was validated for specificity, linearity, accuracy, precision, recovery and stability according to the principles of the FDA industry guidance [15].

2.6.1. Specificity

The specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This test was performed by analyzing the blank plasma samples from 6 different sources (or doners) to test for interference at the retention time of rimonabant and glimepiride (IS).

2.6.2. Linearity

The linearity of calibration curve for rimonabant was assessed in the range of 20–400 ng/ml in plasma samples. Standard samples of rimonabant and IS over the concentration range of 20–400 ng/ml and 400 ng/ml was prepared as described in Section 2.4, respectively. Peak area ratios of each analyte to IS were measured and the calibration curve was obtained from the least-squares linear regression (no weighing factor) presented with their correlation coefficients.

2.6.3. Accuracy and precision

Accuracy and precision were determined by analyzing six replicates of three QC samples (low-, medium-, and high-concentration) for consecutive 3 days. Accuracy (DEV) was determined as percent difference between the mean observed concentration and the nominal concentration for each QC sample [16,17]:

$$\mathrm{DEV}(\%) = \frac{[\text{nominal}] - [\text{observed}]}{[\text{nominal}]} \times 100$$

The precision of the assay was assessed by observing the concentration of 6 replicates of each QC sample for within-run precision (WRP) and 18 replicates of each QC sample for between-run precision (BRP). The between-run precision was defined as [16,17]:

$$\mathrm{BRP}(\%) = \frac{\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}}{\mathrm{GM}} \times 100$$

The within-run precision was calculated as

$$\mathrm{WRP}(\%) = \frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \times 100$$

where $\mathrm{MS_{bet}}$ = between-groups mean square, $\mathrm{MS_{wit}}$ = within-groups mean square, GM = grand mean, and n = represents the number of replicates within each validation run.

2.6.4. Extraction recovery

The extraction recovery of analyte at three QC samples was determined by measuring the peak area responses from plasma samples spiked with particular standard working solution of analyte before extraction with those from drug-free plasma samples

extracted and spiked with same concentration of analyte after extraction. The recovery of IS at concentration of 400 ng/ml was determined in the same way. The recovery of rimonabant was determined using six replicates of each QC sample.

2.6.5. Stability

The stability of rimonabant in plasma was evaluated with four studies; short-term, long-term and freeze–thaw stability study as well as stability in ambient temperature. Six replicates of three QC samples at concentration of 60 ng/ml (LQC), 200 ng/ml (MQC) and 320 ng/ml (HQC) was prepared and then subsequent HPLC analysis was carried out as described previously.

The QC samples were kept at room temperature for 24 h, extracted and then analyzed for short-term stability study.

The long-term stability study was carried out with plasma samples spiked with rimonabant, which were stored at −20 °C for 1 and 3 months, then extracted and analyzed.

The freeze–thaw stability study was evaluated by comparing the QC samples that had been frozen and thawed three times, with the plasma samples thawed once.

2.6.6. Pharmacokinetic study

The analytical method proposed and validated here was successfully applied in a pharmacokinetics study of rimonabant in healthy human volunteers. The study was approved by the ethics committee of Jadavpur University, Kolkata, India. It was an open, randomized, crossover study to asses relative bioavailability of rimonabant in twelve healthy male volunteers with mean age of 24.25 ± 4.65 years and mean weight of 56.17 ± 6.02 kg following single dose administration of rimonabant 20 mg tablet. Test preparation was rimonabant 20 mg tablet manufactured by M/S Cosmas Pharmacals (Ludhiana, India) and reference preparation was tablet Riosity (containing rimonabant 20 mg) manufactured by M/S Sun Pharmaceutical Industries Ltd. (Mumbai, India).

3. Results and discussion

3.1. Specificity

Fig. 2 shows the representative chromatograms of blank plasma, blank plasma spiked with rimonabant and IS, and volunteer plasma sample after 3 h of administration of an oral 20 mg dose of rimonabant. The IS and analyte was well resolved in this particular bioanalytical method. Moreover there is no chemical interaction between the analyte and IS. Total run time of the chromatogram is about 18 min and the retention time of drug and IS are 15.8 and 5.3 min, respectively. No interfering peaks at these retention times were found in the chromatogram obtained from blank plasma, as the area of analyte and internal standard did not differ in case of successive analysis of matrix from different individuals.

3.2. Linearity

The equation of the calibration curve was obtained by least-squares linear regression analysis of the peak area ratio of rimonabant to internal standard versus concentration. The calibration curve exhibits an excellent linearity with regression coefficient of 0.9921 (±0.0027, range: 0.9893–0.9947). The calibration equation shows with average slope 0.0027 (±0.0001, range: 0.0026–0.0029) and intercept 0.212 (±0.031, range: 0.192–0.248), respectively. Table 1 shows all back calculated values with excellent accuracy and precision.

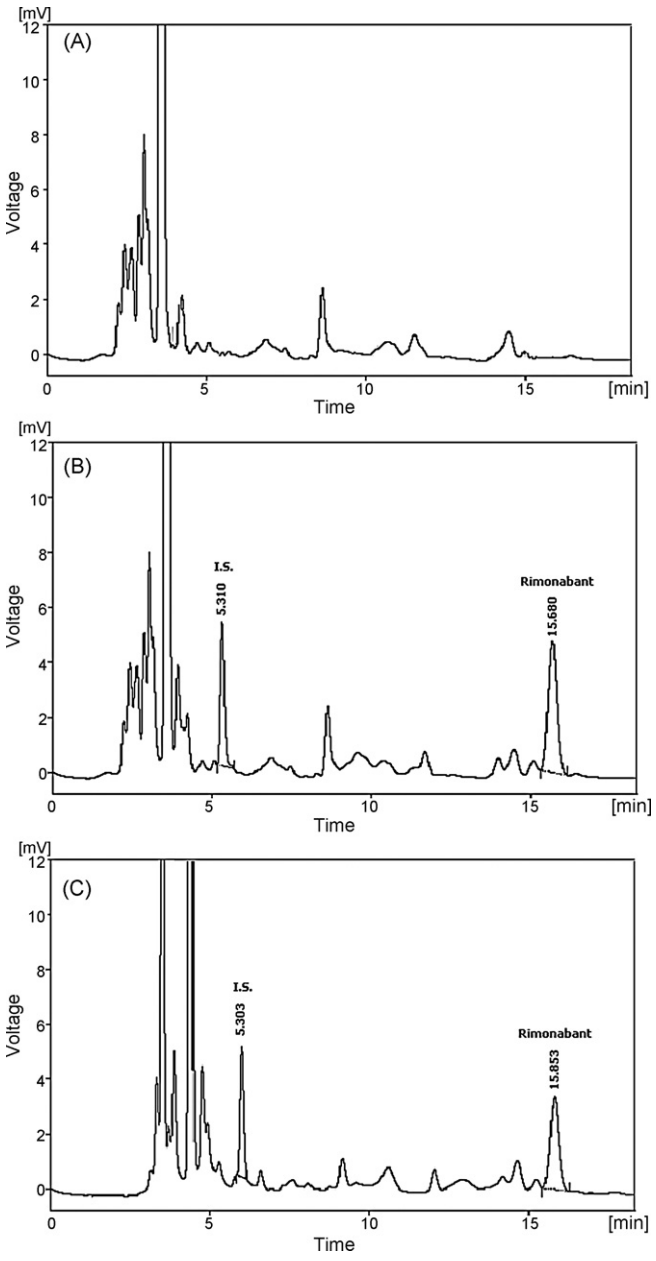

Fig. 2. Representative chromatogram of: (A) blank plasma. (B) Blank plasma spiked with drugs (200 ng/ml) and IS. (C) Volunteer plasma sample containing rimonabant (172.54 ng/ml).

3.3. Accuracy and precision

The between-run and within-run precision values of rimonabant for various concentrations ranged from 0.13% to 0.98% and 1.57% to 3.61%, respectively. At the same concentrations, the values for accuracy were also within acceptable limit. The precision and accuracy data of three QC samples are presented in Table 2.

3.4. Extraction recovery

The recoveries (mean) of rimonabant at low-(60 ng/ml), medium-(200 ng/ml) and high-(320 ng/ml) quality-control samples was 84.17%, 87.53% and 92.64%, respectively. The recovery (mean) of IS was very good by mix solvent of ethyl acetate:n-hexane and its value was 85.32% at the concentration used in the assay.

Table 1
Summary of calibration standards.

Conc. added (ng/ml)	Mean conc. found (ng/ml)	S.D.	CV (%)	RE (%)	*n*
20	20.45	1.19	5.81	+2.25	6
50	51.42	2.48	0.94	+3.69	6
100	102.18	2.95	1.16	+1.68	6
150	152.97	3.19	0.82	+1.62	6
200	198.61	3.98	0.80	−1.97	6
300	296.77	4.09	0.24	−1.10	6
400	405.92	5.68	0.19	+1.17	6

S.D. = standard deviation; CV (%) = coefficient of variation [(S.D./mean) × 100]; RE (%) = relative error [{(conc. found − conc. added)/conc. added} × 100]; *n* = number of replicates.

Table 2
Assessment of accuracy and precision from quality-control sample.

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	WRP (%)	BRP (%)	*N*
60.00	58.36	3.73	6.39	2.01	0.98	18.00
200.00	196.07	7.29	3.72	3.61	0.14	18.00
320.00	320.54	5.73	1.78	1.571	0.13	18.00

GM = grand mean; S.D. = standard deviation; DEV = percent deviation from nominal value; WRP = within-run precision; BRP = between-run precision; *N* = total number of replicate observation.

Table 3
Short-term and long-term stability data[a].

Storage condition	Low-QC (60 ng/ml)	Medium-QC (200 ng/ml)	High-QC (320 ng/ml)
3 freeze/thaw cycle	93.81 (5.81)	97.56 (1.98)	98.16 (2.91)
24 h ambient	94.758 (7.29)	98.41(1.32)	98.45 (3.15)
1 month frozen (−20 °C)	96.42 (6.24)	96.21 (2.11)	97.01 (3.81)
3-Month frozen (−20 °C)	95.13 (5.28)	95.42 (3.98)	96.72 (2.56)

[a] The data presented in this table are the percentage of measured value vs. theoretical value with CV in parenthesis (*n* = 6).

3.5. Stability

The stability of rimonabant in plasma was determined under various conditions according to the procedure described in the earlier section. Each stability test included six replicates of three levels of QC samples. All stability results for the analyte are presented in Table 3.

QC samples undergoing three freeze–thaw cycles gave coefficient of variance (%CV) ≤ 5.81 and an accuracy of 93.81–98.16%. QC samples storing at ambient for 24 h gave %CV ≤ 7.29 and an accuracy of 94.75–98.45%.

Long-term frozen storage stability was tested at 1 and 3 month after QC sample pools were prepared and stored at −20 °C. The 1 month stability data of all three QC samples showed an accuracy of 96.21–97.01% with %CV ≤ 6.24 and the 3-month stability data had an accuracy of 95.13–96.72% with %CV ≤ 5.28 in plasma.

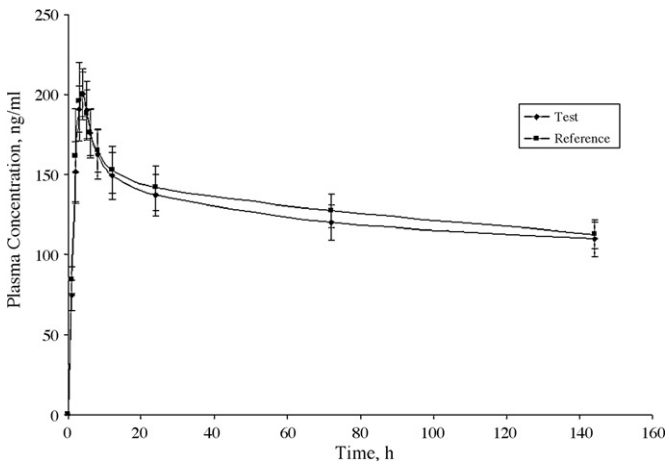

Fig. 3. Mean (±S.D.) plasma concentration vs. time profile of rimonabant in human plasma.

3.6. Application to clinical study

The above-mentioned bioanalytical method was used to determine the plasma concentration of rimonabant for a bioequivalence study. The mean (±S.D.) plasma level of rimonabant after the oral administration of a single dose 20 mg rimonabant tablet in 12 healthy human volunteers are shown in Fig. 3. The maximum plasma concentration (C_{max}) ranged from 211.28 ± 8.12 ng/ml at the time of 3.33 ± 0.89 h (t_{max}) and the elimination half-life ($t_{1/2}$) ranged from 185.25 ± 14.85 h.

4. Conclusion

The HPLC method described here for analysis of rimonabant in plasma is very simple, specific and sensitive. Few analytical methods using LC/MS/MS have been reported for the determination of rimonabant in rat and mouse plasma [13,14]. The LC/MS/MS method described by Nirogi et al. (LLOQ: 0.1 ng/ml and run time: 2.0 min) and Delyle et al. (LLOQ: 2.5 ng/ml and run time: 5.0 min) [12,18] is the only method for the analysis of rimonabant in human plasma. Though the methods had better sensitivity, but it requires more expensive instrumentation. The proposed method to analyze rimonabant in plasma by HPLC with UV detection (LLOQ: 20 ng/ml and run time: 18 min) happens to be first of its kind described so far in the literature. This new method will be of immense help for carrying out pharmacokinetic study of rimonabant in laboratories that lack sophisticated analytical instrument like LC/MS/MS.

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