Quantification of rimonabant in rat plasma by high performance liquid chromatography and its application to pharmacokinetic studies

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Abstract

A rapid, simple, and sensitive high performance liquid chromatographic method with UV detection was developed and validated for the determination of rimonabant (RMT) from rat plasma. The retention behavior of RMT and zolpidem tartrate (ZPT, internal standard-IS) as a function of mobile phase pH, composition and flow rate was investigated. Separation was developed on a reverse-phase Gemini C_{18} column (150mm×4.6mm i.d., 5µm particle size), using a mixture of methanol (MET): water (0.05% Triethylamine (TEA), pH-7 adjusted with ortho phosphoric acid) in the ratio of 75:25 (%v/v) at a flow rate of 1.0 ml/min with UV detection at 252 nm within 10 min, and quantified based on drug/IS peak area ratios. The plasma samples were prepared by using liquid-liquid extraction, yielding more than 97.86% extraction efficiencies. The calibration curve was linear (correlation coefficient of 0.9992) in the concentration range of 25-25000 ng/ml. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 6.91 and 22.70 ng/ml, respectively. Both the intra-day and inter-day precisions at four tested concentrations were below 1.42% R.S.D. The present method was selective enough to analyze RMT in rat plasma without any tedious sample clean-up procedure and can be successfully applied for estimating the pharmacokinetic parameters of RMT following oral administration of a single 5 mg RMT to white albino rats.

Keywords Rimonabant, RP-LC, Validation, LLE, Rat Plasma, Pharmacokinetic studies

Introduction:

Rimonabant (RIM) (structure shown in Fig 1) is a synthetic cannabinoid CB_1 receptor antagonist being developed for the treatment of multiple cardiometabolic risk factors, including abdominal obesity and smoking it is chemically [5- (4- Chlorophenyl) -1- (2, 4-dichlorophenyl) -4- methyl-*N*- (piperidin-1-yl) -1*H*- pyrazole-3-carboxamide, [$C_{22}H_{21}C_{l3}N_4O$] (Fig-1) is the first selective CB_1 receptor blocker developed by Sanofi-Aventis [1].

Endocannabinoids effect on the Ca^{2+} and K^+ ion channels results in a reduction of neuronal excitability and a suppression of neurotransmitter release. Additionally, binding to CB_1 receptor

results in blocking AC, which disrupts the conversion of ATP to cAMP, combined with the activation of MAPK, has an overall effect on gene expression [2].

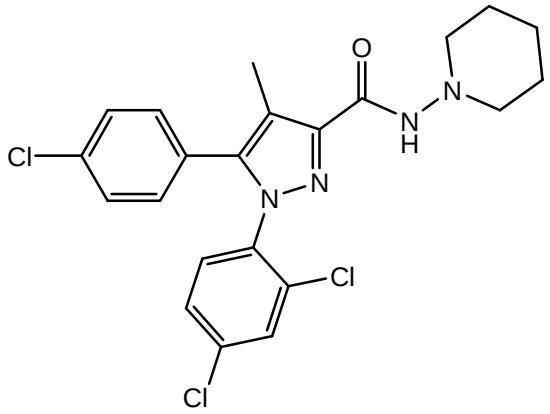


Fig-1 Structure of Rimonabant

The most frequent adverse events leading to drug discontinuation in these trials were mood-related disorders (depression, suicidal tendencies), which were the reason why the Food and Drug Administration did not approve rimonabant in the United States yet [3,4] and other effects like attenuation of effects of smoked cannabis [5,6], hepatoprotective functions [7,8], improve mitochondrial function [9], effects on behavioural satiety sequence in rats [10] and rimonabant may also play a role in treatment of cocaine addiction [11].

To our knowledge only few chromatographic methods have been published till date, quantification of rimonabant in human matrix has been reported in plasma only. Nirogi etal [12] reported an LC–MS/MS method for quantification of rimonabant in a short range of concentration (0.1–100 ng/mL) from 200µL of plasma, while therapeutic concentrations range from about 100 to 200ng/mL. McCulloch et al. also reported quantification of rimonabant in plasma [13], but after a single protein precipitation from 200µL of plasma. Another study reported quantification of rimonabant in mouse plasma using fused-core silica column [14], without any validation of the method. A stability indicating method in pure drugs [15] and a HPLC method for clinical study [16] and a liquid chromatographic method with UV detection in human plasma [17] has been reported.

In such cases it is important to develop newer analytical techniques in order to minimize batch to batch variation because quality alone controls the therapeutic value for the drugs.

Results and discussion

Rimonabant can be satisfactorily separated by reversed phase chromatography. Octylsilane (C_8) columns are similar to octadecylsilane (C_{18}). However, octylsilane columns are less retentive as compared to octadecylsilane. Majority of the ionizable pharmaceutical compounds can be very well separated on octadecylsilane reversed phase columns [18]. Hence, octadecylsilane was

selected. Rapid, sensitive and novel HPLC method for determination of RMT in rat plasma was optimized and validated.

Optimization of chromatographic conditions

Effect of mobile phase pH

With the aim of the optimization of mobile phase pH (6, 7 and 8), the remaining two factors were kept constant, i.e. mobile phase composition and flow rate. Observed chromatographic responses were plotted against respective pH. As shown in the Fig. 2(A), retention time increases with the increase in pH while asymmetry decreases. The number of theoretical plates as well as resolution between RMT and IS was maximum at pH 7. Moreover, the changes in peak width, capacity factor, separation factor and HETP are enumerated in Table 1. Looking at the importance of the different chromatographic parameters, pH 7 was found to be optimum.

Effect of mobile phase composition

The effect of mobile phase composition (i.e. ratio of methanol (MET): water (0.05% Triethylamine (TEA), pH-7 adjusted with ortho phosphoric acid) was studied at 65:35, 75:25 and 85:15, v/v levels) at pH 7 and the flow rate of 1 ml/min is shown in Fig. 2(B). Optimum retention of RMT and IS were obtained at 75:25, v/v level, which makes the method rapid, a one of the most desirable criteria. Satisfactory resolution and asymmetry values were achieved. An adequate theoretical plates (□12000) is indicative of a good column performance. As can be seen from Fig. 2(B), the resolution was poor at 85:15, v/v and a higher asymmetry was found at 65:35, v/v which indicates tailing of the peaks, but was <1.4 at 75:25, v/v. Other chromatographic parameters at different composition of mobile phase are listed in Table 1.

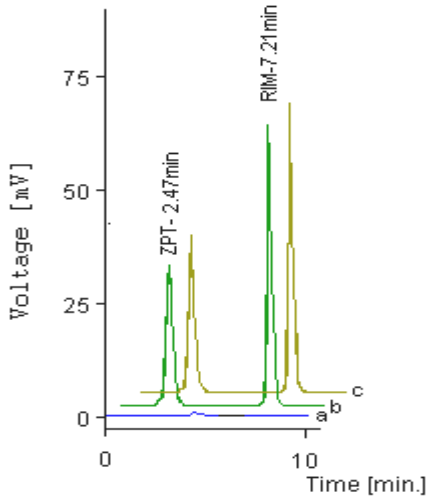


Fig-2 baseline devoid of samples (a), chromatograms of rimonabant (RIM-7.21min) and internal standard (ZPT-2.47min) (b), sample from formulation (c)

Effect of mobile phase flow rate

From Fig. 2(C), it can be observed that theoretical plates were highest at flow rate of 1 ml/min with asymmetry of less than 1.5. The change in flow rate had no significant effect on resolution while retention time decreased as the flow rate increased. The values of capacity factor and separation factor (Table 1) also indicate optimum flow rate of 1 ml/min.

Proposed chromatographic method

Looking at the different chromatographic parameters during the method development, the finally recommended mobile phase consisted of methanol (MET): water (0.05% Triethylamine (TEA), pH-7 adjusted with ortho phosphoric acid) in the ratio of 75:25, v/v. The best resolution and sensitivity of the method was obtained at 252 nm and mobile phase flow rate of 1 ml/min. Typical chromatogram at the optimized condition gave sharp and symmetric peak with retention time of 2.51 and 7.12 min for IS and RMT, respectively. Thus, within very short time the system became ready for the next sample injection without the need for additional wash time.

Validation of the proposed method

Calibration curve (linearity)

Calibration curve (peak area ratio of RMT to IS versus RMT concentration) in plasma was constructed by spiking seven different concentrations of RMT and fixed concentration of IS. The chromatographic responses were found to be linear over an analytical range of 25-25000 ng/ml and found to be quite satisfactory and reproducible with time. The linear regression equation was calculated by the least squares method using Microsoft Excel® 2007 program and summarized in Table 2. The correlation coefficient equals 0.9992, indicating a strong linear relationship between the variables.

The variance of response variable $S^2_{Y_x}$ calculated was 1.9634, indicates low variability between the estimated and calculated values. This further confirms negligible scattering of the experimental data points around the line of regression and good sensitivity of the proposed method. The variance of slope (S^2_b) and intercept (S^2_a) were obtained as 0.3761 and 0.4401, respectively. The calculated *t*-value for slope and intercept were reported in Table 2 and were less than tabulated *t*-values. This shows that the intercept is not significantly different from zero, indicating no interference in the estimations. Further the slope and intercept were within the confidence interval.

Table 1 Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters

S. No	Variable	Value	retention time (R_t, min)	Width (W, min)	Width @ 5% (W_5%, min)	Width @ 10% (W_{10}%, min)	Capacity factor (k')	HETP (h)
1	pH	6	7.35	0.2683	0.3657	0.3228	6.0020	0.0324
		7	7.12	0.2202	0.2677	0.2427	6.0179	0.0261
		8	7.02	0.2718	0.3794	0.3237	6.6188	0.0312
2	Mobile phase composition (v/v)	65: 35	7.89	0.3712	0.6871	0.5642	7.5595	0.0453
		75: 25	7.12	0.2202	0.2677	0.2427	6.0179	0.0261
		85: 15	5.28	0.3108	0.4421	0.3797	9.0721	0.0186
3	Flow rate (ml/min)	0.8	8.14	0.3987	0.4918	0.3464	7.2003	0.0217
		1.0	7.12	0.2202	0.2677	0.2427	6.0179	0.0261
		1.2	5.97	0.3212	0.3107	0.2680	4.9321	0.0247

Table 2 Spectral and statistical data for determination of rimonabant by proposed HPLC method

Parameters	Value
Absorption maxima, λmax (nm)	265
Linearity range (ng ml^{-1})	25-25000
Coefficient of determination (r^2)	0.9986
Correlation coefficient (r)	0.9992
Regression equation (Y[a])	$Y = 0.0694 \cdot x + 0.0063$
Slope (b)	0.0694
t_{cal}[b]	0.0093
Confidence interval[c]	−1.7145 to 1.7271
Intercept (a)	0.0063
t_{cal}[b]	0.0056
Confidence interval[c]	−1.5313 to 1.6502
Limit of detection, LOD (ng ml^{-1})	6.91
Limit of quantitation, LOQ (ng ml-^{1})	22.70

[a] $Y = a + bx$, where x is the concentration (µg/ml).

[b] t_{tab} = 2.57 for 95% two sided confidence interval for 5 degrees of freedom.

[c] Confidence interval was calculated at 95% two sided t value for 5 degrees of freedom.

Table 3 Summary of inter-day (n = 3) and intra-day (n = 6) precision and accuracy of the method in rat plasma

Nominal concentration (ng/ml)	Mean concentration found[a] (ng/ml)	S.D	Precision (RSD, %)	Mean accuracy[b](%)	t_{cal}[c]	Confidence interval (CI)
Inter- day (n=3)						
500	492.93	5.57	1.13	98.59	2.17	500 ± 8.26
1000	993.23	8.40	0.85	99.32	1.39	1000 ± 12.47
2500	2493.68	11.38	0.46	99.75	0.96	2500 ± 16.88
Intra-day (n=3)						
500	497.19	5.85	1.18	99.44	1.17	500 ± 6.13
1000	995.21	7.12	0.72	99.52	1.64	1000 ± 7.47
2500	2494.11	11.79	0.47	99.76	1.22	2500 ± 12.37

[a] Average of three and six determinations at three concentration levels for inter-day and intra-day respectively.

[b] All the mean accuracies were calculated against their nominal concentrations.

[c] $t_{cal} = |100 - R|\sqrt{n}/\text{R.S.D.}$, where t_{cal} is the calculated t value, n is the number of replicates, and R is mean accuracy. Tabulated t-value for 95% two sided confidence interval for 5 degree of freedom was (t_{tab}=) 2.57.

Accuracy and precision

Accuracy data in the present study ranged from 98.59 to 99.76% (Table 3) indicates that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of RMT, gave an R.S.D. below 9.79 (should be less than 15 according to CDER guidance for Bio-analytical Method Validation [19]), revealed that the proposed method is highly precise. Accuracy of the method was evaluated by using *t*-test at three concentration levels including the lowest quantifiable level. The *t*-values obtained for 500, 1000 and 2500 ng/ml were 0.66, 2.17 and 1.39 for inter-day whereas 0.74, 1.17 and 1.64 for intra-day, respectively. The *t*-value required for significance at 5% level at 5 degrees of freedom is 2.57, and the obtained values were well below this value. Thus no significant difference was observed between the amounts of drug added and recovered. Overall, the data summarized in Table 3, enables the conclusion that an excellent accuracy and high precision was obtained.

Sensitivity

The LOD and LOQ were found to be 6.91 and 22.70 ng/ml, respectively. When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies.

Specificity

Any potential interference (overlapping peaks) due to plasma endogenous components were within 2 min only (Fig. 3), later on there was no significant interference from blank plasma that affected the response of RMT and IS.

Stability

The spiked rat plasma samples stored at -4°C, were injected over a period of 1 month did not suffer any appreciable changes in assay value and meet the criterion mentioned above. Hence, the samples were stable during 1 month. The RMT was found to be stable in rat plasma after three freeze-thaw cycles.

Extraction efficiency

Extraction efficiency was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The extraction efficiency of RMT from rat plasma samples was satisfactorily ranged from 97.86 to 98.62% (R.S.D. was less than 2.05) at all three concentration levels, which confirm no interference effects due to plasma components. Recovery of IS was found to be 98.42% (R.S.D. = 1.47).

System suitability

System suitability tests, an integral part of a chromatographic analysis is used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis [20]. A system suitability test according to USP was performed on the chromatograms obtained from standard and test solutions to check different above mentioned parameters and the results obtained from six replicate injections of the standard solution are summarized in the Table 4.

Table 4 System suitability parameters

S. No	Parameters	RMT[a]	ZPT[a]
1	Retention time, R_t (min)	7.12	2.51
2	Area (mAU·s)	11.28	58.12
3	Capacity factor (*k'*)	6.37	2.32
4	Separation factor (α)	2.83	-
5	Theoretical plates (USP)	7254	7458
6	HETP (mm)	0.026	0.020
7	Resolution (R_s)	10.12	-
8	Asymmetry (A_s)	1.34	1.16

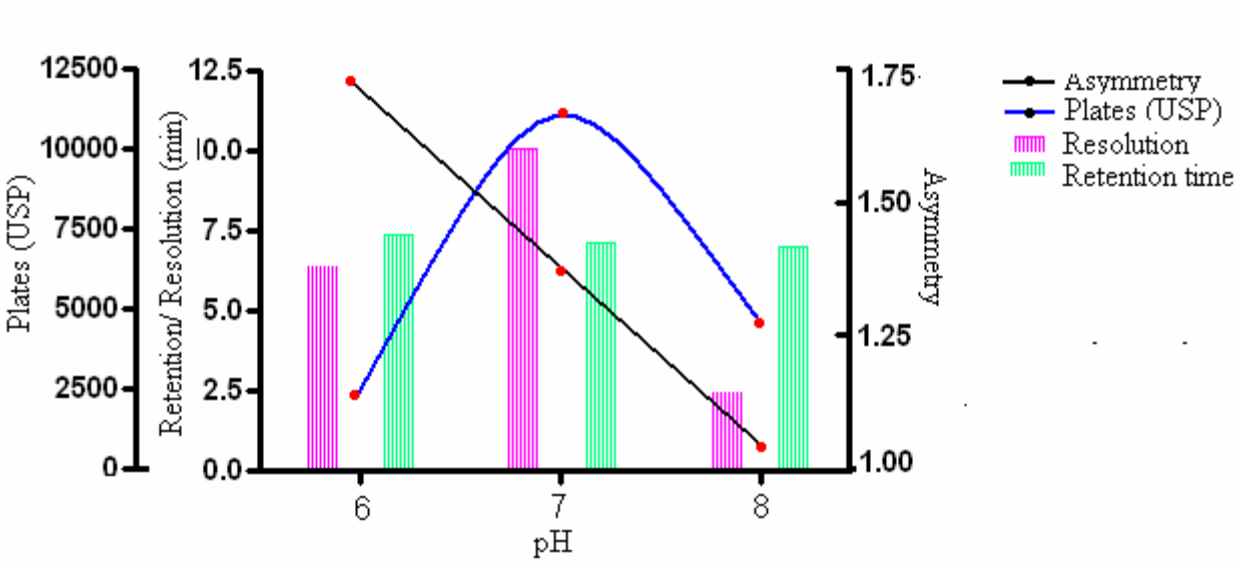
[a]average of six determination.

Table 5 Pharmacokinetic parameters of RMT after a single oral dose of 5 mg RMT to rats

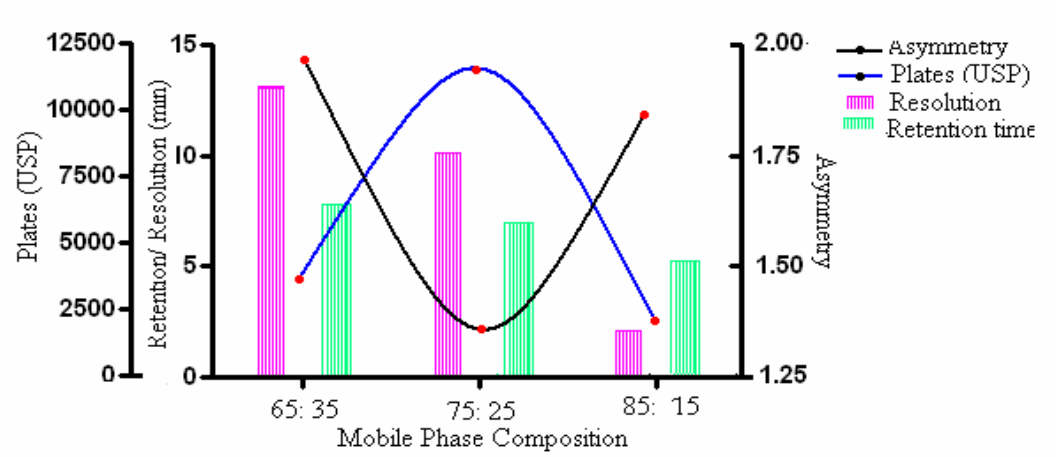
S. No.	Pharmacokinetic parameters	Observed value
1	Time required for maximum plasma concentration, T_{max} (h)	3.86±0.77
2	Maximum plasma concentration, C_{max} (ng/ml)	192.6±23.3
3	Plasma half life, $T_{1/2}$ (h)	186.2±13.6
4	Area under curve at 204 h, AUC (0→204) (ng h/ml)	71525±6845
5	Area under curve at infinite time, AUC(0→∞) (ng h/ml)	72014±5940

Pharmacokinetic Profile from rats

The developed method was applied to quantify RMT concentration in pharmacokinetic study carried out on rats. HPLC chromatogram of rat plasma is shown in Fig 3, which shows (A) typical chromatograms of blank rat plasma and (B) RMT in plasma after 60 min of drug administration. Representative mean plasma concentrations versus time profiles following a single oral administration of RMT to rats are presented in Fig 4. Various other pharmacokinetic parameters have been summarized in Table 5. The T_{max} and $T_{1/2}$ of RMT in the present study was similar, although the intake doses were different from those reported in literature [16].



(A)



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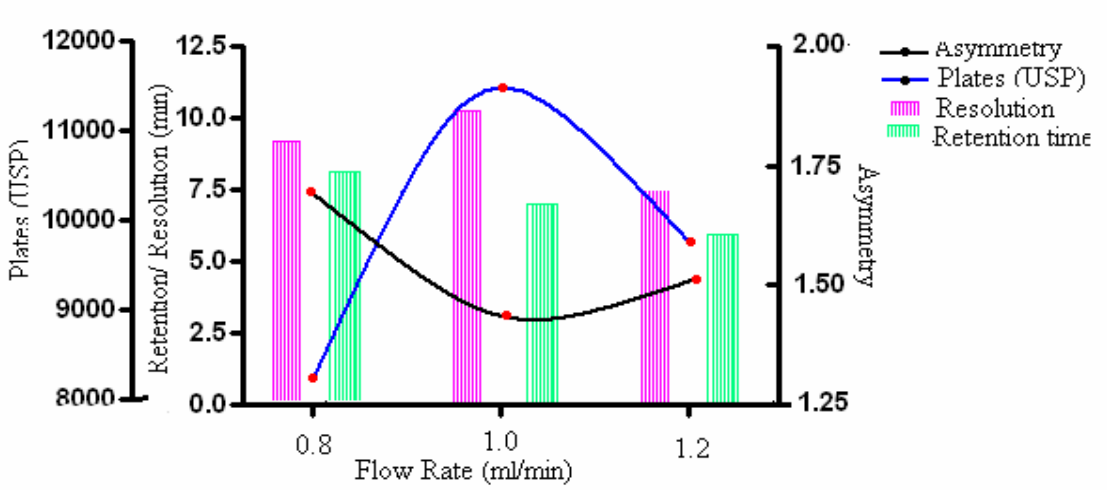

(C)

Fig 3. Effect of (A) mobile phase pH (6, 7, 8) at mobile phase composition of 75:25 and flow rate of 1.0 ml/min; (B) mobile phase composition (65:35, 75:25, 85:15) at pH 7 and flow rate of 1.0 ml/min; and (C) mobile phase flow rate (0.8, 1.0, 1.2 ml/min) at mobile phase composition of 75:25 and pH 7 on resolution, retention time, theoretical plates, and asymmetry

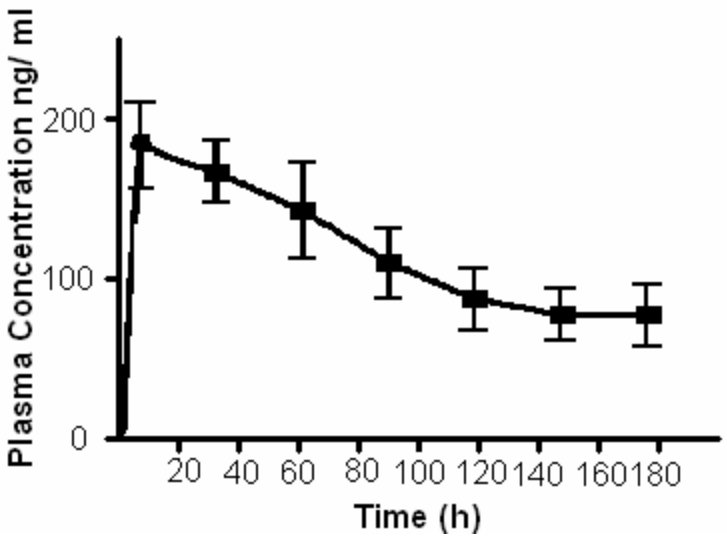

Fig 4 Mean (±S.D.) plasma concentration vs. time profile of rimonabant in rat plasma.

Materials and methods:

Chemicals and reagents

Rimonabant and zolpidem tartrate were obtained from Zydus research centre (Ahmadabad, India). Methanol HPLC grade was obtained from Qualigens Fine chemicals (Mumbai, India). Tri ethylamine (TEA) was purchased from Sisco Research Laboratories (Mumbai, India), ortho-phosphoric acid were obtained from Rankem (New Delhi, India). MilliQ water (Millipak® 40) was used through out the analysis. All the other chemicals used were of analytical grade.

Instrumentation

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) was composed of Class LC-10AT vp and LC-20AD Prominence solvent delivery module, a manual rheodyne injector with a 20µl fixed loop and a SPD-20A Prominence UV–visible detector. Separation was performed on a Gemini C18 column (paricle size 5µm; 150mm×4.6mm i.d.; Phenomenex, Torrance, USA) preceded by an ODS guard column (10 µm, 10mm×5mm i.d.) at an ambient temperature. Chromatographic data were recorded and processed using Spinchotech 1.7 version software (Spinchrom Pvt. Ltd., Chennai, India)

Chromatographic conditions

Analysis was isocratic at 1.0 ml/min flow rate with methanol (MET): water (0.05% Triethylamine (TEA), pH-7 adjusted with ortho phosphoric acid) in the ratio of 75:25 (%v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was premixed, filtered through a 0.2 µm membrane filter to remove any particulate matter and degassed by sonication before use. The absorbance of RMT was higher at 260 nm (Fig. 1) and further it was free from any interference. Hence, the eluted peaks were detected at 260 nm. A previous UV (200–400 nm) scanning was done in order to select the optimal absorbance wavelength. The sensitivity of the detector was set at 0.01 AUFS. The substance was quantified using its peak area ratio of RMT to IS. Prior to injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was required to remain below 1.0% on RMT/IS peak area ratio basis.

Preparation of solutions

A stock solutions of RMT (500 µg/ml) and ZPT (500µg/ml) were prepared in methanol and were stored at 2–8°C until used. Aliquots of these solutions were diluted stepwise with the mobile phase to obtain 30 µg/ml of both RMT and ZPT. This solution was used for the optimization of the proposed method.

The spiking solutions of RMT (10 µg/ml) and ZPT (10 µg/ml) were prepared by diluting the suitable aliquots of stock solutions with HPLC grade water. Suitable aliquots of the spiking solutions were spiked to plasma in order to obtain the RMT concentrations in the analytical range of 25 to 25000 ng/ml and that of ZPT to be 25000 ng/ml for calibration curve.

Optimization of chromatographic conditions

Sometimes, the effects of different chromatographic conditions on the instrumental responses create a situation where one has to compromise between different experimental variables in order to achieve the best chromatographic separation. Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers [17]. And therefore before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, compositions, and flow rate to check the retention time, shape, resolution, and other chromatographic parameters of RMT and IS peaks individually. From those experiments the mobile phase combination of MET and 0.05%TEA at pH 7 was found to be most suitable.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 6, 7 and 8 keeping the composition of mobile phase 75:25 and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 65:35, 75:25 and 85:15 with pH and flow rate kept constant at 7 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 75:25 and 7 respectively. Moreover, the effects of different level of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions. The apparent pH of the mixtures was adjusted to desired value using ortho phosphoric acid. Mobile phase was then filtered through 0.2µm membrane filter and sonicated before being used for chromatography.

Extraction of RMT from rat plasma

In order to investigate the practical applicability of the method in biological analysis, the present method was applied to the estimation of RMT from rat plasma. The sample was extracted from rat plasma using acetonitrile as extraction solvent which cause the precipitation of plasma proteins and extraction of the analyte. Blank plasma of white albino rat was spiked with known concentrated standards of RMT and IS vortex-mixed for 3min. There-after, the sample was added with 1ml of acetonitrile. The eluent was evaporated to dryness at 40°C under a stream of nitrogen. The dried extract was then reconstituted with 100µL of mobile phase, and a 20µLwas injected into the chromatographic system.

Validation of the proposed method

Once the chromatographic method had been developed and optimized, it must be validated. The validation of an analytical method verifies that the characteristics of the method satisfy the requirements of the application domain [21]. The proposed method was validated in the light of ICH Guidelines [22-24] for linearity, precision, sensitivity, and recovery. Consequently, the following were performed.

Calibration curve (linearity)

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of an analyte in the sample [25, 26]. Seven different concentrations of RMT with constant IS concentration were spiked to the blank plasma as described previously and calibration curve was constructed in the specified concentration range. The calibration plot (peak area ratio of RMT to IS versus RMT concentration) was generated by replicate analysis (n = 6) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

Accuracy and precision

Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows. Solutions containing three concentrations of the calibration curve, i.e. 500, 1000, and 2500 ng/ml were prepared. Six injections at each of the specified concentration levels were injected within the same day for repeatability, and over a period of 3 days (6 injections/ day) for reproducibility. Mean and relative standard deviation were calculated and used to judge accuracy and precision of the method. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Accuracy was calculated as the percent of ratio of RMT amount found to that of the actual.

Sensitivity

As per IUPAC [27] and ISO [28], the instrumental response sensitivity is the slope of the calibration line because a method with a large slope is better able to discriminate between small differences in analyte content. LOD and LOQ were determined according to following equation:

$$\text{LoD or LoQ} = \frac{ks_B}{S}$$

where k is a constant (3 for LOD and 10 for LOQ), sB is the standard deviation of the analytical signal, and S is the slope of the concentration/response graph.

Specificity

The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences, i.e. whether the compound elutes without any other interfering compounds or not [21]. The specificity of the method was determined by comparing the chromatograms obtained from the samples containing RMT and IS with those obtained from blank plasma. Five blank plasma samples from six lots of rabbit plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

Stability

Blank plasma was spiked with the known amount of RMT to achieve the concentration of 500, 1000, and 2000 ng/ml (n = 3) and stored at −4°C. The stability of these samples was checked for up to 1 month by comparing the results with fresh stock prepared on the day of analysis. Further,

the freeze–thaw (−20°C/room temperature) stability of the RMT spiked plasma samples were determined for three cycles. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision. No internal standard was added prior to the analysis.

Extraction efficiency

The recovery of an analyte is the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Different organic solvents (ethyl acetate, dichloromethane, acetonitrile and chloroform) were tried in the extraction step using SPE-cartridges, and acetonitrile proved to be the most efficient in extracting RMT from rat plasma and had a small variation in extraction recoveries over the concentration range. Spiked plasma samples were prepared in triplicate at three concentrations 50, 1000, and 2000 ng/ml of RMT and 2500 ng/ml of IS, and assayed as described above. The extraction efficiency of RMT was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of non-biological (unextracted) samples into the chromatographic system at the same concentration levels.

Application to study pharmacokinetics from rats

The method described above was applied to quantify the plasma concentration of RMT in a single-dose pharmacokinetic study conducted on six white albino rats. The protocol was approved by the Institutional ethical committee at SRM College of Pharmacy, India. The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals) guidelines. The rats weighing 150-200 g were housed with free access to food and water, except for the final 12 h before experimentation. After a single oral administration of 5 mg of RMT (Rimoslim tablets), 0.5 ml of blood samples were collected at 0, 1, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192 and 204 h time-points into heparinized collection tubes. The blood was immediately centrifuged (1900×*g*) for 10 min at an ambient temperature. The supernatant plasma layer was separated and stored at -20 ^{o}C until analyzed. The plasma samples were analyzed for RMT concentrations as described above. The total area under the observed plasma concentration-time curve (AUC) was calculated by using the linear trapezoidal rule. The maximum observed RMT concentration (C_{max}) and the time at which C_{max} was observed (T_{max}) and T½ were reported directly from the profile.

Conclusion

In the present work, a new rapid, simple and sensitive reversed phase HPLC method has been developed, optimized and validated for the estimation of RMT in rat plasma using UV detector and isocratic elution. Optimization showed that the mobile phase pH and composition are more crucial parameters to be controlled than flow rate for reproducible and quantitative estimation of the RMT. The short peak retention time of 7.12 min cuts down on overall time of sample analysis and thereby makes the method more cost effective. Method was found to be linear over an analytical range of 25-25000 ng/ml with LOD and LOQ of 6.91 and 22.70ng/ml, respectively.

The results of *t*-test applied to accuracy and precision data enabled the conclusion that an excellent accuracy and high precision was achieved. From the extraction efficiency data, the recovery of the active component was found to be quantitative. Selectivity of the method was demonstrated by the absence of any interfering peaks from other coexisting endogenous substances at the retention time of the drug as well as IS. Simple and reproducible sample extraction procedure along with reconstitution in minimum quantity of mobile phase offers the higher sensitivity (25ng/ml) for animal studies and was successfully applied for determination of RMT from rat plasma. In summary, the optimized chromatographic estimation of RMT with good resolution in a short time can be used for evaluating the bioavailability and also applied to routine therapeutic monitoring of the RMT.

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