

**A VALIDATED REVERSE PHASE-HPLC METHOD FOR QUANTIFICATION OF RISPERIDONE IN RAT PLASMA BY PDA DETECTOR****Vineet Dubey* and Tulsi Ram Saini**

Industrial Pharmacy Research Lab, Department of Pharmacy, Shri G. S. Institute of Technology and Science, Indore, India-452003.

***Corresponding Author: Vineet Dubey**

Industrial Pharmacy Research Lab, Department of Pharmacy, Shri G. S. Institute of Technology and Science, Indore, India-452003.

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ABSTRACT

A reverse phase-high performance liquid chromatographic method was developed using photo-diode array detector for the quantification of risperidone in rat plasma. The Hypersil BDS Phenyl column (4.6 mm × 250 mm, 5 µm) was used for separation with a mixture of acetonitrile and 0.01 M sodium dihydrogen phosphate buffer (pH 6) as mobile phase in 60:40 ratio at a flow rate of 1 ml/min. The chromatographic analysis was performed in isocratic mode at 280 nm detection wavelength with a total run time of 7 min and a retention time of 6.1-6.3 min. The protein precipitation method was employed to prepare rat plasma samples for chromatographic analysis. Linearity, sensitivity, accuracy, precision, selectivity, and stability of the developed method were determined in accordance with the USFDA bioanalytical method validation guidelines. The method showed linearity over 100–5000 ng/ml concentration range with 60 ng/ml as limit of detection and 100 ng/ml as limit of quantitation. The accuracy was within a relative error of 6% of the nominal drug concentrations and precision was under 7% coefficient of variance for intra-day as well as inter-day analyses. Stability of plasma samples was also acceptable as per USFDA guidelines. The selectivity of the method for risperidone was confirmed by the absence of any interfering peaks at the retention time of the drug.

KEYWORDS: Reverse phase-HPLC method, PDA detector, risperidone, rat plasma, validation.**INTRODUCTION**

Risperidone is an atypical antipsychotic agent of benzisoxazole class having a high affinity for D2 and 5-HT_{2A} receptors.^[1] It is widely used in the treatment of schizophrenia as it effectively manages positive as well as negative symptoms of schizophrenia. Unlike typical antipsychotics it has a very less propensity to produce extrapyramidal symptoms associated with long term drug therapy.^[2]

For quantification of risperidone from biological samples, several LC-MS methods have been reported mainly for simultaneous determination with other drug substances.^[3,4,5,6,7,8] Recently a UPLC-Tandem MS method has also been reported for its estimation in rat plasma.^[9] As compared to widely used HPLC methods, the LCMS methods are associated with limitation of expensive instrumental setup which restricts its availability in the academic bioanalytical research laboratories. Although some HPLC methods have been reported for determination of risperidone in biological samples using UV detector^[10,11,12,13] but no HPLC method using a photo-diode array (PDA) detector is developed for its estimation in rat plasma for biostudies.

There are many advantages of PDA detector in HPLC drug estimation like faster estimation simultaneously at multiple wavelengths, high wavelength precision, sensitivity, and ruggedness as compared to conventional UV detector.^[14]

This study reports a validated reverse phase HPLC method employing a PDA detector for the determination of risperidone in rat plasma.

MATERIALS AND METHODS

Risperidone was supplied as gift sample by M/S RPG Life Sciences, Mumbai, India. HPLC grade acetonitrile was purchased from Merck, Mumbai. HPLC grade water was obtained from the water purification system (Millipore, India) of our lab. Membrane filter of 0.45 µm pore size was purchased from Millipore, India. All other chemicals and reagents were of analytical grade and purchased from the local market.

Preparation of samples for HPLC analysis

The blood sample collected from albino rats in centrifuge tubes containing anticoagulant solution was centrifuged for 10 min at 5000 rpm at 4 °C to separate the plasma. A

stock solution of 1 mg/ml risperidone was prepared in acetonitrile to prepare standard risperidone solutions of different concentrations by diluting with acetonitrile. Standard risperidone solution in rat plasma was prepared by spiking 100 μ l of the rat plasma with 100 μ l of risperidone standard solution, adding 200 μ l of acetonitrile in it and then mixing on a vortex mixer for 10 min to completely precipitate the plasma proteins present. The mixture was then centrifuged for 10 min at 5000 rpm at 4 °C and the supernatant was separated and subjected to chromatographic analysis.

HPLC conditions

The HPLC analysis was done on a chromatographic system (Waters Corporation, USA) equipped with a photodiode array detector (Waters 2996) and Empower software. The column used was a 250 mm $\times$ 4.6 mm, 5 μ m Hypersil BDS Phenyl column (Thermo Fisher Scientific, USA). A mixture of 0.01 M sodium dihydrogen phosphate buffer (pH 6) and acetonitrile (40:60 ratio) was used as the mobile phase with a flow rate of 1 ml/min. The injection volume was 20 μ l with retention time of 6.1-6.3 min and total run time of 7 min. The detector wavelength was 280 nm.

Method validation

The developed analytical method was validated according to the bioanalytical method validation guidelines of USFDA.^[15]

Linearity

The standard solutions of risperidone in rat plasma were prepared in 100-5000 ng/ml concentration range (n=6), and analyzed starting from lower to higher concentration on the HPLC system equipped with a PDA detector. The calibration curve was prepared by plotting chromatographic peak areas against drug concentrations and its linearity was confirmed statistically.

Sensitivity

The sensitivity is the minimum measurable analyte concentration and is calculated using signal to baseline noise (S/N) ratio. Serially diluted standard risperidone solutions were spiked into rat plasma, analyzed and S/N ratio of each was calculated (n=6). The limit of detection (LOD) and lower limit of quantification (LLOQ) were the concentrations that yielded the S/N ratios of 3:1 and 5:1 respectively.

Accuracy

Accuracy of the developed reverse phase-HPLC method was determined by the recovery method.^[16] Three nominal concentrations of standard risperidone solution in rat plasma (100 ng/ml, 1000 ng/ml, and 5000 ng/ml) were estimated (n=6) and the average recovery was calculated. Accuracy of the method was calculated as the percent relative error of the recovered amount.

Precision

Precision of the developed method was determined by analyzing the nominal drug concentrations (100 ng/ml, 1000 ng/ml, and 5000 ng/ml) of standard risperidone solution in rat plasma (n=6) and recording peak areas of the respective chromatograms. The within-run precision was determined by analyzing the nominal drug concentrations in the same run on the same day and the between-run precision was determined by analyzing them on three different days. The precision was reported as the percent coefficient of variation.

Selectivity

The selectivity of the method was established by analyzing blank plasma samples obtained from six different rats separately (n=6) and comparing the resulting chromatograms with those obtained from the analysis of standard risperidone solutions in rat plasma at LLOQ to detect any possible interference caused by endogenous plasma substances at the retention time.

Stability

The bench top stability, long-term stability, and freeze/thaw stability of standard risperidone solutions in rat plasma was determined (n=3) by estimating their concentrations after keeping at room temperature for twenty four hours, at -20 °C for two months, and after three freeze/thaw cycles. The stability analysis was done at a lower concentration (100 ng/ml) and a higher concentration (5000 ng/ml).

RESULTS AND DISCUSSION

Selection and optimization of HPLC conditions

The aim of optimization of HPLC conditions was to achieve an efficient and selective separation of risperidone in rat plasma with a good peak shape and least tailing. Three types of columns were tried namely Thermo Hypersil BDS C-18 column, Waters C-8 Column, and Thermo Hypersil BDS Phenyl column. The mixtures of acetonitrile and phosphate buffer in different ratios as the mobile phase were used to get a sharp and symmetric peak with minimum retention time.

The Hypersil BDS Phenyl column (4.6 mm $\times$ 250 mm, 5 μ m) was chosen for further study as it produced most selective separation, sharpest peak shapes, maximum signal to noise ratio, and minimum retention time and peak tailing. The mixture of acetonitrile and 0.01 M sodium dihydrogen phosphate buffer (pH 6) in 60:40 ratio was selected as the mobile phase because it provided the sharpest and most resolved peaks. The detection wavelength of 280 nm resulted in retention time of 6.1-6.3 min and total run time of 7 min.

Risperidone was extracted from plasma samples by protein precipitation method using acetonitrile as extraction solvent since it completely precipitates plasma proteins and risperidone is sufficiently soluble in it.^[17]

Method validation

Linearity

The calibration curve constructed between the chromatographic peak area of risperidone against its concentration was linear over the concentration range of 100–5000 ng/ml (Fig. 1) with correlation coefficient (r^2) of 0.9989. This confirmed the linearity of the developed method.

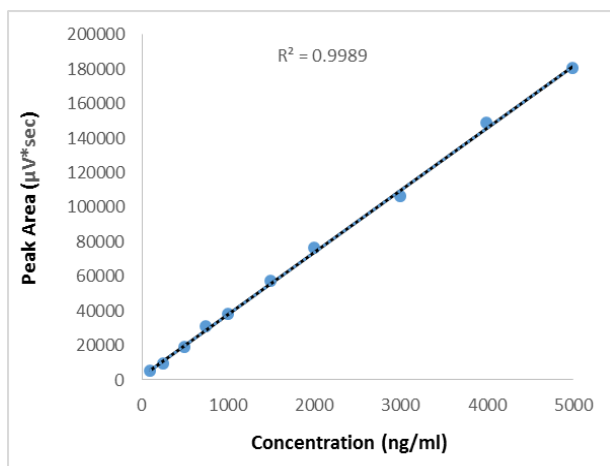


Fig. 1: Calibration curve of risperidone in rat plasma at 280 nm.

Sensitivity

The least concentration of analyte which could be measured accurately and precisely from the standard risperidone solution in rat plasma with an S/N ratio of

5:1 was found to be 100 ng/ml and the least quantity of analyte giving an S/N ratio of 3:1 between peak area and baseline noise, i.e., limit of detection was 60 ng/ml.

Accuracy

The accuracy of the developed method was expressed as percent relative error of the recovered amount from risperidone standard solutions in rat plasma and was found to be -4.25%, 2.97%, and 3.13% intra-day and -5.07%, 2.15%, and -2.40% inter-day respectively for 100 ng/ml, 1000 ng/ml, and 5000 ng/ml concentrations (table 1). The recovery of drug was under $\pm 6\%$ limits of nominal drug concentration that is well below the USFDA prescribed limits of $\pm 15\%$.^[15] This established the accuracy of developed method.

Precision

Precision of the developed method was expressed as % coefficient of variation (CV) and determined by analyzing three concentrations of standard risperidone solutions in rat plasma, i.e., 100 ng/ml, 1000 ng/ml, and 5000 ng/ml respectively. The % coefficient of variation for intra-day precision was between 1.91% and 6.12% and that for inter-day precision on three different days was between 1.42% and 5.98% (table 1). Since both intra-day and inter-day precisions were under 7% CV which is well within the USFDA prescribed limit of 15% CV^[15] and therefore established the precision of developed method.

Table 1: Accuracy and precision results.

Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy RE (%)	Precision CV (%)
Intra-day			
100	104.64	-4.25	6.12
1000	377.33	2.97	1.91
5000	1036.81	3.13	3.39
Inter-day			
100	105.80	-5.07	5.98
1000	411.84	2.15	3.34
5000	1042.02	-2.40	1.42

Selectivity

The chromatogram obtained from blank plasma showed few peaks between 2 min and 4 min of detection time and no signals afterwards (Fig. 2A), while the chromatogram of spiked plasma samples showed sharp peak of risperidone at its retention time (Fig. 2B). Since there was no visible peak at the retention time of risperidone, it can be concluded that rat plasma did not cause any interference in spiked rat plasma samples and therefore the developed method is selective for analysis of risperidone in rat plasma.

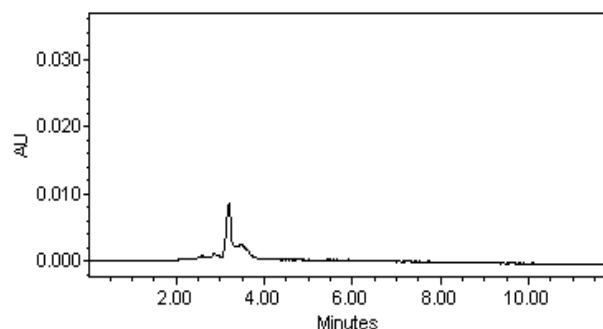


Fig. 2: A. HPLC chromatogram of blank plasma.

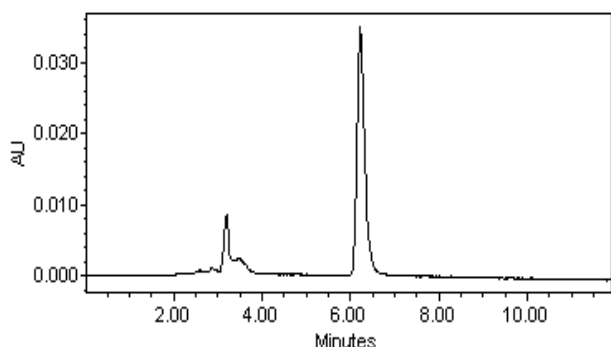


Fig. 2: B. HPLC chromatogram of plasma spiked with risperidone.

Table 2: Stability study results.

Stability study type	Stability study test condition	Nominal drug concentration (ng/ml)	Concentration after study* (ng/ml)	RE (%)	% Nominal concentration analyzed	CV (%)
Bench-top	24 hours at RT	100	97.35	-2.65	97.35	5.11
		5000	5049.23	0.98	100.98	2.35
Long-term	2 months at -20 °C	100	96.20	-3.80	96.20	5.93
		5000	4848.13	-3.04	96.96	2.02
Freeze-thaw	3 cycles	100	100.51	0.507	100.51	5.74
		5000	5124.60	2.49	102.49	1.28

*Mean (n = 3).

CONCLUSION

A reverse phase liquid chromatographic method for the estimation of risperidone in rat plasma was developed using PDA detector and validated as per USFDA bioanalytical method validation guidelines. The retention time of risperidone was 6.1-6.3 min and the total run time was 7 min. The method has good accuracy and precision with linearity range of 100-5000 ng/ml drug concentration ($r^2 = 0.9989$), and LLOQ and LOD of 100 ng/ml and 60 ng/ml respectively. The plasma samples remained stable under all the storage conditions studied.

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