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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RIBAVIRIN IN RAT PLASMA BY RP-HPLC METHOD

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ABSTRACT

A simple, rapid, selective, sensitive, accurate and precise High Performance Liquid Chromatography (HPLC) with UV detection method has been developed and validated for determination of Ribavirin in rat plasma. C18 (250x4.6mm) column was used with the mobile phase containing a mixture of Acetonitrile: Water (60:40v/v). The flow rate was 1ml/min and drug was monitored at 218 nm. Plasma samples were processed using acetonitrile as precipitating agent to extract drug. The linearity for Tolperisone hydrochloride was found to be 0.5 to 12 µg/ml with regression coefficient (r^2) 0.9990. The recovery was found to be 88.09%.

KEYWORDS- Ribavirin, Reverse phases HPLC, Accuracy, Precision, Robustness, LOD, LOQ, and Specificity.

INTRODUCTION

Ribavirin also known as Tribavirin. Ribavirin is antiviral drug. Chemically, it is described as 1-beta-D-Ribofuranosyl-1, 2, 4-triazole-3-carboxamide.The molecular formula is C8H12N4O5 which corresponds to molecular weight 244.2. Ribavarin is extremely water soluble. It is more stable in acidic medium. Ribavirin is used to treat preliminary hepatitis[1,2,3] Several studies like application and mechanism in hepatitis C,[4] efficacy and safety for chronic. The aim of this work was to develop a simple, accurate, reproducible and sensitive method for determination of Ribavirin in rat plasma using rapid, convenient and simple reverse phase HPLC method.

MATERIALS AND METHODS

1. Reagents & chemicals used

a. Acetronitrile (HPLC Grade)

b. Methanol (HPLC Grade)

c. Double distilled water

d. Ortho phosphoric acid (AR grade).

2. Preparation of mobile phase

The mobile phase Acetonitrile: Water (60:40 v/v) was prepared and filtered through 0.45 µm membrane filter & sonicated on ultrasonic bath for 15 min.

3. Preparation of standard stock solution

Ribavirin standard stock solution was prepared by transferring 10 mg of Ribavirin working standard into a 100ml volumetric flask, approximately 30 ml of distilled water was added and sonicated for 20 min. The volume was made up to 100 ml with HPLC grade distilled water to get the concentration of 100 µg/ml. This solution was filtered through a 0.45µm pore size Nylon 66 membrane filter.

4. Separation of Plasma from Rat blood

The blood was removed from the healthy rat by retro orbital method of blood collection. Blood will be collected into purple top EDTA tubes and centrifuged (3000 rpm) at 4^0 C for 20 minutes. After centrifugation using clean pipette technique place 1.0 ml of plasma into 1.5ml eppendorf tube labeled with tracking number and “plasma”.

5. Preparation of sample solution

Sample solution was prepared by taking 0.90 ml of rat plasma and 100 µl of working standard solution of 10 µg/ml and 1 ml of precipitating agent (acetonitrile) to precipitate plasma protein, were added and mixed.

Table 1: Spiking Ribavirin in plasma.

Concentration (µg/ml)	Vol. of Spiking (µl)	Vol. of plasma (ml)	Final vol. (ml)	Final conc. (µg/ml)
5	100	0.9	1	0.5
10	100	0.9	1	1
20	100	0.9	1	2
40	100	0.9	1	4
60	100	0.9	1	6

80	100	0.9	1	8
100	100	0.9	1	10
120	100	0.9	1	12

Method Development[7, 8]

The fundamental parameters for Bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The measurements for each analyte in the biological matrix should be validated. Typical method development and establishment for Bioanalytical method include determination of

(1) Selectivity,

(2) Accuracy, precision, recovery,

(3) Calibration curve and

(4) Stability of analyte in spiked samples.

1. Selectivity

Analysis of blank sample of the appropriate biological matrix (plasma) should be obtained from at least six sources were tested for interference, & no interference at reported retention time was found.

Calibration curve

The concentration range over, which the linearity was found to be 0.5-12 µg/ml. The results are shown in Table 2.

Preparation of quality control standards

The quality control standard solution 1.5µg/ml, 7.5µg/ml, 12µg/ml were prepared.

Accuracy & Precision

Accuracy was measured using three determinations of LQC (1.5 µg/ml.), MQC (7.5 µg/ml.) and HQC (12 µg/ml.).

The precision was carried out by within batch intraday& inter batch precision.

Accuracy & Precision within batch

The within batch accuracy & precision was performed in single day by taking three different concentrations, & each concentration has three determination.(Table 4).

Inter batch Accuracy & Precision

The inter batch accuracy & precision was performed in different days by taking three different concentrations, & each concentration has three determination.(Table 5).

1. Recovery

Recovery experiment should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with unextracted standards that represent 100% recovery.

2. Stability

a. Freeze & Thaw stability

The freeze–thaw cycle was repeated two more times, and then analyzed on the third cycle.

b. Short term temperature stability

The short term stability was performed by three aliquots of each of the low and high concentrations were tested at room temperature and kept at this temperature from 8 hours and analysed.

c. Long term stability

Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions for 15 days.[9,10,11,12,13]

RESULT AND SUMMERY

Table 2: Linearity of Ribavirin.

Sr. No	Concentration (µg/ml)	Peak Area* (mAU)
1	0.5	58260
2	1	105520
3	2	212040
4	4	436150
5	6	699120
6	8	932160
7	10	1165200
8	12	1398240

***Average of Three determinations**

Table 3: Calibration Curve of Ribavirin in Rat Plasma.

Sr. No	Con. (µg/ml)	Peak Area* (mAU)
1	0.5	59288
2	1	118576
3	2	237152
4	4	464304
5	6	711456
6	8	948608
7	10	1185760
8	12	1422912

Table 4: Within-Batch Accuracy & Precision.

Quality control sample	Amt.Added (µg/ml)	Peak Area*	Amt. found (µg/ml)	%Accuracy	% C.V
LQC	1.5	167864	1.49	97.95	1.1358
	1.5	165864	1.48	95.83	
	1.5	166864	1.49	96.39	
MQC	7.5	879320	7.49	96.95	0.4282
	7.5	860320	7.50	97.06	
	7.5	877320	7.47	97.72	
HQC	12	1222912	11.99	96.95	1.0163
	12	1262912	12.50	97.6	
	12	1262912	12.37	98.72	

***Average of six determination**

Table 5: Inter Batch Accuracy and Precision.

Quality control sample	Amt. Added (µg/ml)	Peak Area*	Amt found (µg/ml)	% Accuracy	% C.V
LQC	1.5	150864	1.52	97.64	1.1348
	1.5	159864	1.51	96.07	
	1.5	161864	1.53	98.20	
MQC	7.5	899320	7.66	96.20	0.5813
	7.5	900320	7.67	97.31	
	7.5	897320	7.39	96.60	
HQC	12	1242912	12.16	96.35	0.8309
	12	1322912	11.99	97.95	
	12	1265912	12.35	96.97	

Table 6: Recovery Study.

Conc. (µg/ml)	Peak Area* (Extracted)	Peak Area* (Un-extracted)	% Recovery
1.5	182872	212869	85.90
7.5	890327	910298	90.80
12	1412919	1612922	87.59

***Average of six determinatio**

Table 7: Freeze and Thaw Stability.

Conc.	Peak Area *	Conc. Found*	% Purity*	S.D *	% C.V *
1.5 µg/ml	174205	1.46 µg/ml	97.89	0.8554	0.87
12µg/ml	1389572	11.71 µg/ml	97.61	0.4041	0.41

***Average of six determination**

Table 8: Short Term Stability.

Conc.(µg/ml)	Peak Area *	Conc. Found (µg/ml)*	% Purity*	S.D*	%C.V.*
1.5	177009	1.49	99.47	0.8764	0.8811
12	1392916	11.74	97.84	0.7050	0.7050

***Average of three determinations**

Table 9: Long term Stability.

Conc.	Peak Area*	Conc. Found (µg/ml)*	%Purity*	S.D*	% C.V*
1.5 µg/ml	166342	1.48	97.09	0.5559	0.5559
12 µg/ml	1309582	11.03	91.99	0.4041	0.4393

***Average of three determinations**

Table 10: Results and Summary.

% Recovery	L.Q.C (1.5 µg/ml)	M.Q.C (7.5µg/ml)	H.Q.C (12 µg/ml)
1	85.90	90.80	87.59
Stability (% C.V)	**L.Q.C**	**H.Q.C**	
1	Freeze & thaw	1.46	11.71
2	Short term	1.49	11.74
3	Long term	1.48	11.03

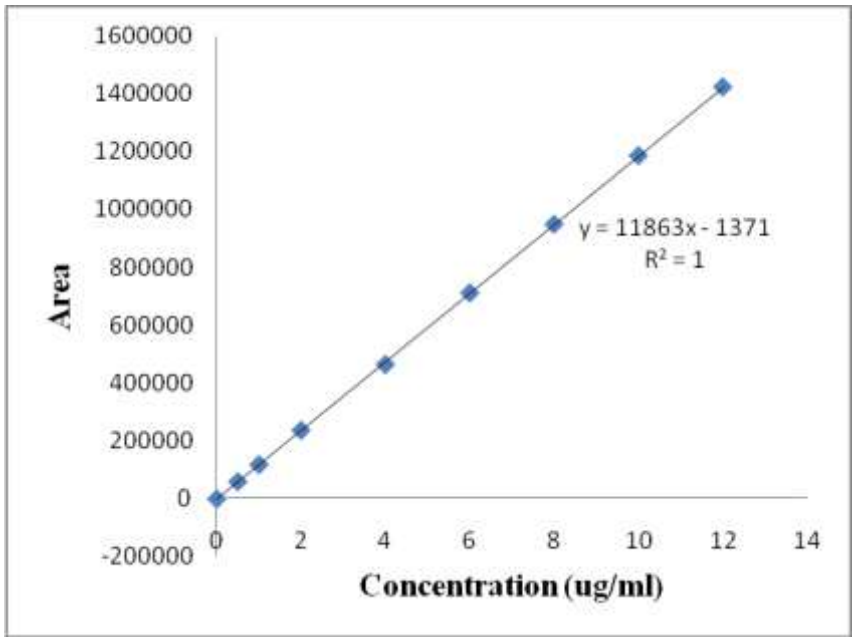

Fig. 1: Calibration Curve of Ribavirin in Rat Plasma.

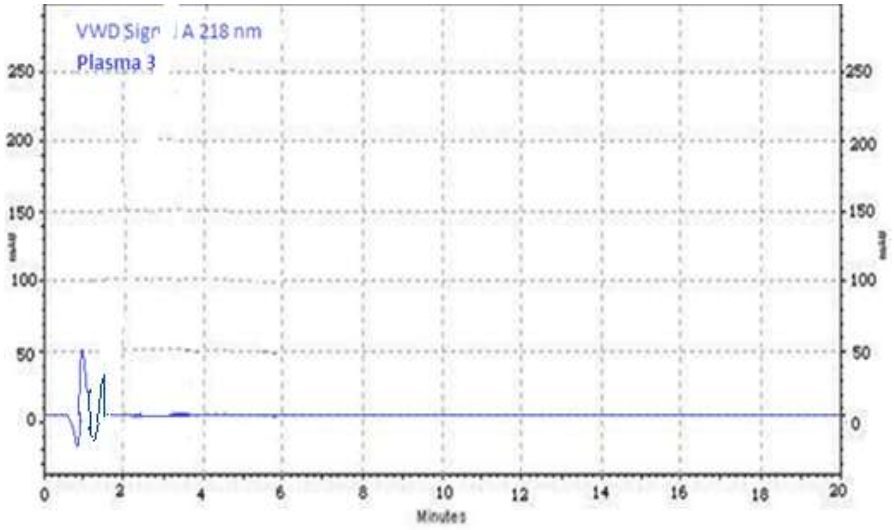

Fig. 2: Chromatogram of Blank Plasma.

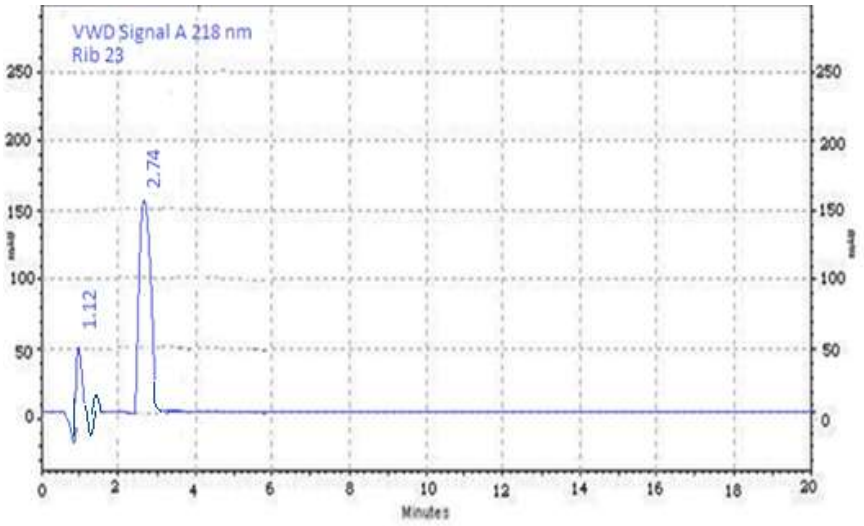

Fig. 3: Chromatogram of Extracted Sample.

CONCLUSION

Bioanalytical method for Ribavirin has been developed and method was validated as per US-FDA guideline. The proposed methods were found to be simple, accurate, precise and reproducible and can be applied for analysis of drug in rat plasma. The proposed method was also applied for the estimation of bioavailability, bioequivalence, pharmacokinetic & toxicokinetic data of Tablet formualtion.

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