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LC-MS/MS Method Development and Validation for the Determination of Favipiravir in Pure and Tablet Dosage Forms

Short Title: LC-MS/MS Determination of Favipiravir

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ABSTRACT
Objectives: The analytical method development and validation for the determination of Favipiravir (FVPR) in pure and tablet dosage forms by LC/MS-MS Technique.
Materials and methods: A simple LC-MS/MS method was developed for the determination of a new antiviral drug, FVPR in pharmaceutical formulations. The stationary phase employed was Shim pack GISS, C18 (100 mm × 2.1 mm, 1.9 µm) column and mobile phase used in pump A was 10.0 mM ammonium acetate and in pump B methanol was used. The gradient program was used with a fixed mobile phase flow rate at 0.4 $mLmin^{-1}$. The total run time was 5.0 minutes. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines. The established method found better outcomes.
Results: The linearity graph was found in the range of 50-200 $ngmL^{-1}$, and the correlation coefficient value (R^2) obtained was found to be 1.0. The limit of detection and limit of quantification were 4.044 $ngmL^{-1}$ and 12.253 $ngmL^{-1}$, respectively. Tremendous recovery outcomes were observed and found to be 101%, 99.0% and 99.5% for the FVPR at 150% upper, 100% middle and 50% lower concentrations, respectively.
Conclusion: All obtained outcomes were complying with the ICH guidelines. The developed method was simple, unique, accurate, robust, precise, and reproducible for the determination of FVPR in tablet formulation. The method is novel and could be adopted in the formulation industry.
KEYWORDS: Favipiravir, LC-MS/MS, Method development, Method validation, Quantification.

INTRODUCTION

Favipiravir (FVPR) is an antiviral drug used for the treatment of all three types of influenza A, B and C. [1] The IUPAC name of FVPR is 6-fluoro-3-hydroxy-2-pyrazine carboxamide with molecular formula $C_5H_4FN_3O_2$ (Figure 1). This is a pyrazine carboxamide derivative. Melting point is about 187 ^{0}C to 193 ^{0}C. It is sparingly soluble in water but completely soluble in organic solvents such as ethanol, DMSO and DMF. FVPR is a prodrug that goes through intracellular ribosylation and phosphorylation into a dynamic form of favipiravir ribofuranosyl-5'-triphosphate (favipiravir RTP), inhibits viral RdRp (RNA-dependent RNA polymerase) and, can bind, transcript and replicate the viral genome and thereby inhibit the viral RNA polymerase. [2-4] Despite being vital against influenza, it was also revealed that the FVPR exhibits antiviral activity against alpha-, filo-, bunya-, arena-, flavi-, noroviruses and currently COVID-19. [5, 6]

The literature survey revealed that few analytical methods are reported for the determination of FVPR. Chromatographic separation method using HPLC with a runtime of 60 minutes was reported. [7, 8] A LC-MS/MS method reported for the bio analysis of the antiviral drug FVPR in human plasma. [9] The quantification of FVPR in pharmaceutical formulations by HPLC-UV method in which the total run time showed 15 minutes and limit of detection (LOD) and limit of quantification (LOQ) concentrations were in mgmL^{-1}. [10] HPLC and spectrofluorimetric methods were developed by Ibrahim et. al.[11] for the determination of FVPR. In HPLC method, the FVPR peak was eluted at 4.0 minutes and total run time shown was 10.0 minutes [11]. A pharmacokinetics method was developed by Thi, et. al., for the estimation of FVPR in Ebola-Infected patients.[12] The spectrofluorimetric method has been developed by Megahed, et. al., for determination of FVPR and quantified in human plasma. [13] A liquid chromatrography-tandem mass spectrometric method was reported for the determination of multiple antiviral drugs.[14] Another LC-MS/MS method was reported for the quantification of FVPR in human plasma. [15, 16] A RP-HPLC method was reported for the determination of FVPR in spiked human plasma.[17] The LOQ of the reported method was 0.72 µgmL^{-1} and the linearity range was 0.2 µgmL^{-1} to 3.2 µgmL^{-1}. In the proposed method, the results were obtained in µgmL^{-1}. Another LC-MS method has been developed for the assay of FVPR in human plasma, the LOQ of the method was found to be at 80 ngmL^{-1} and the linear range reported was between 80 ngmL^{-1} to 30000 ngmL^{-1}. [18] The sensitivity of this method is quite more than the proposed method. A LC/LC–MS method[19] was reported in the literature for the determination of FVPR. This method is quite different form the proposed method. This method studied the route of degradation mechanism and degradation impurities are determined. The LOD and LOQ of the method were 0.09 µgmL^{-1} and 0.027 µgmL^{-1}, respectively. The results of the reported methods are tabulated in **Table 1**.

Keeping the drawbacks of the reported LC-MS methods in mind, we developed and validated the LC/MS-MS method for the determination of FVPR in pure and tablet dosage forms. In the proposed LC-MS/MS method, the total run time was 5.0 minutes and FVPR peak was eluted at 1.9 minutes. The LOD and LOQ concentrations were found in ngmL^{-1} concentrations. Hence the proposed method is sensitive than other reported methods. These outcomes clearly indicated that the established method is simple, accurate, reproducible and robust than the reported methods.

MATERIALS AND METHODS

Instruments

The Shimadzu prominence HPLC and LCMS-8045 instruments were used for the proposed method development and validation for the determination of FVPR. The HPLC instrument consists of a deuterium lamp as the source of light, an UV detector, quaternary pump and an auto-injector. The MS/MS system used was Shimadzu LCMS-8045 which achieves both high sensitivity and ultra-high-speed detection, outfitted by a heated ESI probe. The LCMS-8045 has the maximum sensitivity in its category which is designed to maximize sensitivity and minimize contamination by high-temperature heating block, heated ESI probe, drying gas, heated desolvation line. The Lab Solutions software was used for the analysis and interpretation of data. These all factors provide the robust instrumentation for the determination of FVPR.

Chemicals and reagents

More than 98% purity of FVPR pure drug was provided by Karnataka Antibiotics and Pharmaceuticals Ltd., Bengaluru, India, as a gift sample. FVPR tablets (label claim, 200 mg, commercial name-Avigan-200mg, manufacturer-Dr. Reddy's Lab Ltd., INDIA) were commercially purchased from local medical shops. The HPLC grade methanol and ammonium acetate were procured from Merck Ltd. India. HPLC grade ultra-purified water by Millipore purifier instrument was employed for the study. The stationary phase used was Shim-pack GISS, column (C18, 2.1x100 mm, ID.144 cm, and 1.9µm) was obtained from Shimadzu Ltd. Japan.

Mobile phase preparation, standard stock solution, and dilutions

The mobile phase consists of 0.1 M ammonium acetate buffer of pH 6.5 in pump A and methanol in pump B. The standard stock solution of FVPR was prepared by dissolving accurately weighed 100 mg of FVPR into a 100 mL standard volumetric flask and made up to the mark with mobile phase. The prepared standard stock solution was of the concentration of 1000 μgmL^{-1}. From the above stock solution, 1.0 mL was pipetted out into another 1000 mL standard volumetric flask and made up to the mark with mobile phase. The concentration of the resulting working standard solution was 10 $ngmL^{-1}$. Similarly, the working standard solutions of different concentration of FVPR were prepared from least to maximum dilutions to examine the parameters of interest such as linearity, accuracy, recovery, LOD and LOQ of the proposed method. For the assay analysis, the test sample weights were taken according to the standard equivalent and the following formula was used for the determination of test sample weights:

$$\frac{\text{Standard weight x average weights of 20 tablets}}{\text{Label claim of 1 tablet}}$$

Chromatographic conditions

In method development, the chromatographic conditions play an important role. The mobile phase consists of 10 mM ammonium acetate buffer of pH 6.5 in pump A and methanol in pump B, followed by gradient program as shown in **Table 2**. The stationary phase used was Shim-pack GISS column. The flow rate of the mobile phase was fixed at 0.4 $mLmin^{-1}$. The column oven temperature was kept at 40 ^0C and the wavelength of detection was fixed at 323 nm throughout the method development and validation. The sample injection volume was fixed at 10 µL. With these chromatographic conditions, the FVPR sharp peak was eluted at 1.9 minutes. The total run time was fixed at 5.0 minutes.

Mass Spectroscopy conditions

The MS/MS system used was Shimadzu LCMS-8045 consisting of heated ESI Probe High-temperature gas supplements the nebulizer gas, which improves the desolvation efficiency. This facilitates the ionization of a wide range of compounds. High-Voltage Power Supply for Polarity Switching, which assists fork ultrafast scan speed (30,000 u/s) and polarity switching time of 5 ms was maintained. The high-speed acquisition benefits the laboratory by reducing run times for increased throughput, and also shortens method development time. The system is designed to be robust. The heated desolvation line, high-temperature heating block, heated ESI probe, drying gas and center optics all proceed to minimize contamination and maximize sensitivity. Lab Solutions software was used to analyze the complete method development and validation for the determination of FVPR and offers the latest features designed to streamline workflows and allow analysis to be started lacking long hours of method establishment.

The mass spectrometer with electrospray ionization (ESI) probe operated in positive polarity, the data acquisition and processing were performed by using the Lab Solutions software. The distinctive working conditions were as follows: nebulization gas flow was fixed at 3 $Lmin^{-1}$, heating gas flow was kept constant at 10 $Lmin^{-1}$, interference temperature was fixed at 150 ^0C, heat block temperature was kept constant at 300 ^0C and drying gas flow was fixed at 10 $Lmin^{-1}$. These conditions were maintained for mass spectrometer throughout method development and validation process.

RESULTS AND DISCUSSION

Method development

The two-phases i.e., mobile and stationary phase play an important role in the proposed method development and validation for the determination of FVPR. The mobile phase was balanced by analyzing different trials with a various mixture of solution A (Pump A) and solution B (Pump B) followed by gradient time programs. The FVPR sharp peak was eluted after various trials in mobile phase ratios with a gradient time program. Expected peak was not eluted suitably after analyzing different ratios of pump A and B mobile phase. Hence, the pump A mobile phase was replaced by 10 mM ammonium acetate of pH 6.5 and methanol in pump B. Wavelength of detection was fixed at 323 nm. The gradient time program was fixed as shown in **Table 2**. With these conditions, the FVPR sharp peak was eluted with good baseline in chromatograms as shown in **Figure 2**. In mass spectrum, three peaks were observed which are, m/z=84.95, m/z=113.05, and m/z=141.1 shown in **Figure 3**. Hence for the whole method development and validation pump A was used for 10 mM ammonium acetate of pH 6.5 and pump B for methanol followed by the gradient time program as mentioned in Table 1. With these various trials, good peaks were observed in both chromatogram as well as in mass spectrum and approximate fragment structures (ionized ion fragments) found and revealed in **Figure 4**. All the parameters of the proposed method were in compliance with the ICH guidelines. [20]

Method validation

Linearity

In the proposed LC-MS/MS method, five different concentrations between 50 to 200 *n*gmL^{-1} standard solutions of FVPR were injected and examined. The regression significance was found suitable (R^2=1.0). Y=bX+C equation was used for the determination of the R^2 value. The linearity graph of FVPR was designed by different areas against the different concentrations of the FVPR solutions. The resultant graph revealed a straight line for the FVPR as shown in **Figure 5**. The outcomes indicated that the method could be analyzed at a wide range of concentrations. Hence developed LC-MS/MS method is supposed to be validated. The results are shown in **Table 3**.

Precision

In the proposed method precision data were found to be excellent and in accordance to ICH guidelines. The outcomes were found to be precise and well within the range. On the same day and on different days, the six separately spiked standard solutions and test solution were analyzed repetitively for the precision parameter. The intra-day and inter-day performance were examined and outcomes revealed that there were not many deviations in the obtained results. The percentage of relative standard deviation (RSD%) of test solution of six individual assay outcomes were found less than 2.0 %.Therefore it can be concluded that the developed LC-MS/MS method is precise. The results are revealed in **Table 3.**

Limit of detection (LOD) and limit of quantification (LOQ)

Several methods for determining the detection and quantification limits are described in the ICH guideline.[20] These include visual assessment, signal-to-noise ratio calculations, response standard deviation calculations, and calibration curve slope calculations. The LOD and LOQ in the current study were determined using the third approach and were based on the 3.3 x (σ/m) and 10 x (σ/m) criteria, respectively. σ stands for the standard deviation of the y-intercept of the regression line and m for the slope of the calibration curve. The LOD and LOQ of the proposed method were found to be 4.044 *n*gmL^{-1} and 12.253 *n*gmL^{-1}, respectively. These results indicated that the method was very sensitive for the determination of FVPR. The results are tabulated in **Table 3**.

Recovery

In the recovery parameter, data was accomplished by three different concentration solutions of FVPR; lower, middle, upper, and blank was spiked at 50%, 100%, and 150% against the standard solution. The results obtained were fulfilling ICH guidelines. Hence the established method was excellent. The standard formula was used to calculate outcomes. The data of the recovery parameter was found satisfactory as shown in **Table 4**. The limit of the recovery range accepted is 98-102%. The obtained outcomes were well within the range for all three different concentrations. Therefore, the recovery parameter indicated that the proposed method can be used in the industry.

Specificity

The standard procedures were used for the assay of FVPR. The clear and separated peak was found in liquid chromatography and in the case of a spectrometer, there were three peaks eluted at m/z-84.95, m/z-113.05, and m/z-141.1 respectively. When injected these solutions separately, the consistent retention time and m/z obtained for both standard (working standard) as well as test solution (formulation), the outcomes were found between 98.0 % to 102.0 %. Hence assay data were complying with ICH guidelines. It was also observed that there was no probable excipients peaks interference for the determination of FVPR. The following excipients were used for the specificity parameter: microcrystalline cellulose, starch, magnesium stearate, lactose monohydrate, micro powder silica gel, and magnesium sulfate. These excipients did not interfere during the assay of FVPR by LC-MS/MS method. Therefore the proposed method revealed specificity for FVPR assay. The results of the assay were found to be satisfactory and are shown in **Table 4**.

Robustness studies

The robustness parameter contains the deliberate changes in the developed method. The known concentration of the standard solution of FVPR was injected at different conditions, i.e., column oven temperature was changed from 40 ^{0}C to 35 ^{0}C and 45 ^{0}C and flow rate variation in the mobile phase ranging from 0.3 mLmin^{-1} to 0.5 mLmin^{-1}. The results are shown in **Table 5.** The acquired outcomes were satisfactory and comply with the ICH guidelines. There were no many deviations in the overall results. Hence the established method can be used under varying conditions. Thus, the established LC-MS/MS method is robust.

Solution stability

Solution stability of FVPR was studied up to 48 h by keeping the solutions at 8 ^{0}C. To study this parameter, the standard (50 ngmL^{-1}) as well as the test solutions from 0 h to 48 h were injected. The obtained assay results were found to be 101.5%, 101.1%, 99.8% and 99.0% for the 0, 12, 24 and 48 h, respectively. On observing these data, it can be concluded that there was no much deviation in the area and the calculated assay values, up to 0 h to 48.0 h. Since the FVPR solution stability results were found stable up to 48 h, the developed LC-MS/MS is stable for the determination of FVPR.

DISCUSSION

The critical literature survey (**Table 1**) exposed that there were not many analytical methods on LC-MS/MS for the estimation of FVPR in bulk and formulations. Most of the LC-MS/MS methods report the analysis FVPR in bio matrix like plasma and body fluids. Further, some of the liquid chromatographic methods used different stationary phase and mobile phases for the determination of FVPR in bulk and formulation forms. The reported analytical methods for the determination of FVPR were less sensitive, takes more time for the analysis. Hence it was planned to develop the highly sensitive, simple, reproducible, rugged and robust analytical method for the determination of FVPR in pure and pharmaceutical formulations. In the proposed method, the LOD and LOQ values were found to be 4.044 ngmL^{-1} and 12.253 ngmL^{-1}, respectively and linearity range was found between 50 ngmL^{-1} to 200 ngmL^{-1} for the five different concentrations (R^2=1.0). The results of the solution stability studies were found well within the limit. The recovery and assay data were found to be acceptable and better than literature methods. The developed method was highly sensitive, simple, accurate, rugged, reproducible and robust. The proposed method is novel and exclusive which can be employed in the industries for the routine analysis of FVPR. The proposed method overcomes most of the limitations of the reported methods. This proposed method is cost effective. The total run time of the method was very less. Hence the method is reliable for the rapid analysis of FVPR and can reproduce the accurate and precise results for the formulation samples also.

CONCLUSIONS

The majority of the formulations of an antiviral drug have the analytical methods for their determination such as LC-MS/MS, HPLC, UPLC, and UV-spectroscopic methods. FVPR is an antiviral drug used to prevent COVID-19 and other influenza. We developed an analytical method and validated the method by the LC-MS/MS instrument. The established method was highly sensitive, reproducible and rugged. Above all, all the parameters outcomes were complying with ICH guidelines. Thus, the proposed LC-MS/MS method has exposed the determination of FVPR in bulk and formulations.

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21.

TABLE 1. Comparison of the statistical data of the reported methods and proposed methods

Ref. No.	Analytical method	Results	Remarks
7.	HPLC	LOD- 0.2 $mgmL^{-1}$ LOQ- *NA	Analyzed related substances of FVPR
8.	HPLC	LOD- 0.2 $mgmL^{-1}$ LOQ- NA	Analyzed related substances of FVPR
9.	LC-MS/MS	LOD- NA LOQ- 100 $ngmL^{-1}$	FVPR determined in human plasma
10.	HPLC-UV	LOD- 1.20 μgmL^{-1} LOQ- 3.60 μgmL^{-1}	Different mobile phase used, mixture of 50 mM potassium dihydrogen phosphate (pH 2.3) and acetonitrile (90:10, v/v)
11.	HPLC and spectrofluorimetric	LOD- 0.985 LOQ- 2.986	FVPR determined in human plasma samples. Mobile phase used as 0.02 M Brij-35, 0.15 M Sodium dodecyl sulfate, and 0.02 M disodium hydrogen phosphate, pH 5.0
12.	Parmacokinetics	LOD- NA LOQ- NA	Other than HPLC-UV method developed
13.	Spectrofluorimetric	LOD- 9.44 $ngmL^{-1}$ LOQ- 28.60 $ngmL^{-1}$	Other than HPLC-UV method developed
14.	LC-MS/MS	LOD- 25990 μgmL^{-1} LOQ- NA	FVPR determined in human serum
15.	LC-MS/MS	LOD- NA LOQ- 60 $ngmL^{-1}$	FVPR identified in human plasma

16	LC-MS/MS	LOD- NA LOQ- 0.062 µg/mL	FVPR estimated in human serum
17	LC-MS/MS	LOD- NA LOQ-0.72 μgmL^{-1}	FVPR spiked in human plasma
18	LC-MS/MS	LOD- NA LOQ- 80 $ngmL^{-1}$	FVPR determined in human plasma
19	LC-MS/MS	LOD- 0.09 μgmL^{-1} LOQ- 0.027μgmL^{-1}	Determine Impurity of FVPR and degradation rout mechanism

*NA= Not available

Table 2. Mobile phase gradient program

Time in minutes	Pump A (10 mM ammonium acetate of pH-6.5)	Pump B (Methanol)
0.01	90	10
2.00	40	60
3.10	90	10
5.00	90	10

Table 3. Precision, LOD, LOQ and Linearity outcomes,

Parameters	Results	LIMIT
Precision		
Intra day	0.09 %RSD	NMT-2.0 %
Inter day	0.05 %RSD	NMT-2.0 %
LOD	4.044 $ngmL^{-1}$	-
LOQ	12.253 $ngmL^{-1}$	-
Linearity		
Range	50-200 $ngmL^{-1}$	-
Slope (b)	10122	-
Intercept (c)	42721	-
Correlation coefficient (R^2)	1.000	R^2- above 0.995
Standard Error of the Intercept	5063.3606	-
Standard Deviation of the Intercept	12402.650	-

Table 4. Recovery and assay results

Parameter	Brand and Label claim/tablet	Amount found mg/tablet	Concentrations in %	Assay, %	Recovery, %	*RSD %	Limit
Assay (Spiking FVPR)	Avigan 200 mg	201.0	100	100.5	-	0.02	
			50	-	100.1	0.51	98.0-102.0%
Recovery	-	-	100	-	101.5	0.72	
			150	-	101.2	0.64	

*RSD- Relative standard deviation

Table 5. Robustness data of FVPR

Parameters	**Actual**	**Low**	**High**
Flow variation	0.4 $mLmin^{-1}$	0.3 $mLmin^{-1}$	0.5 $mLmin^{-1}$
Column temperature (^0C)	40	38	42
RSD%	0.9	1.1	1.3

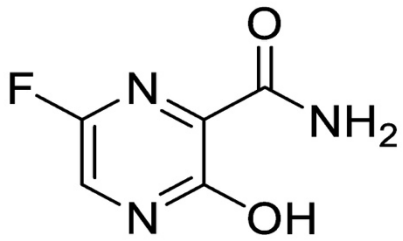


Figure 1. Structure of FVPR.

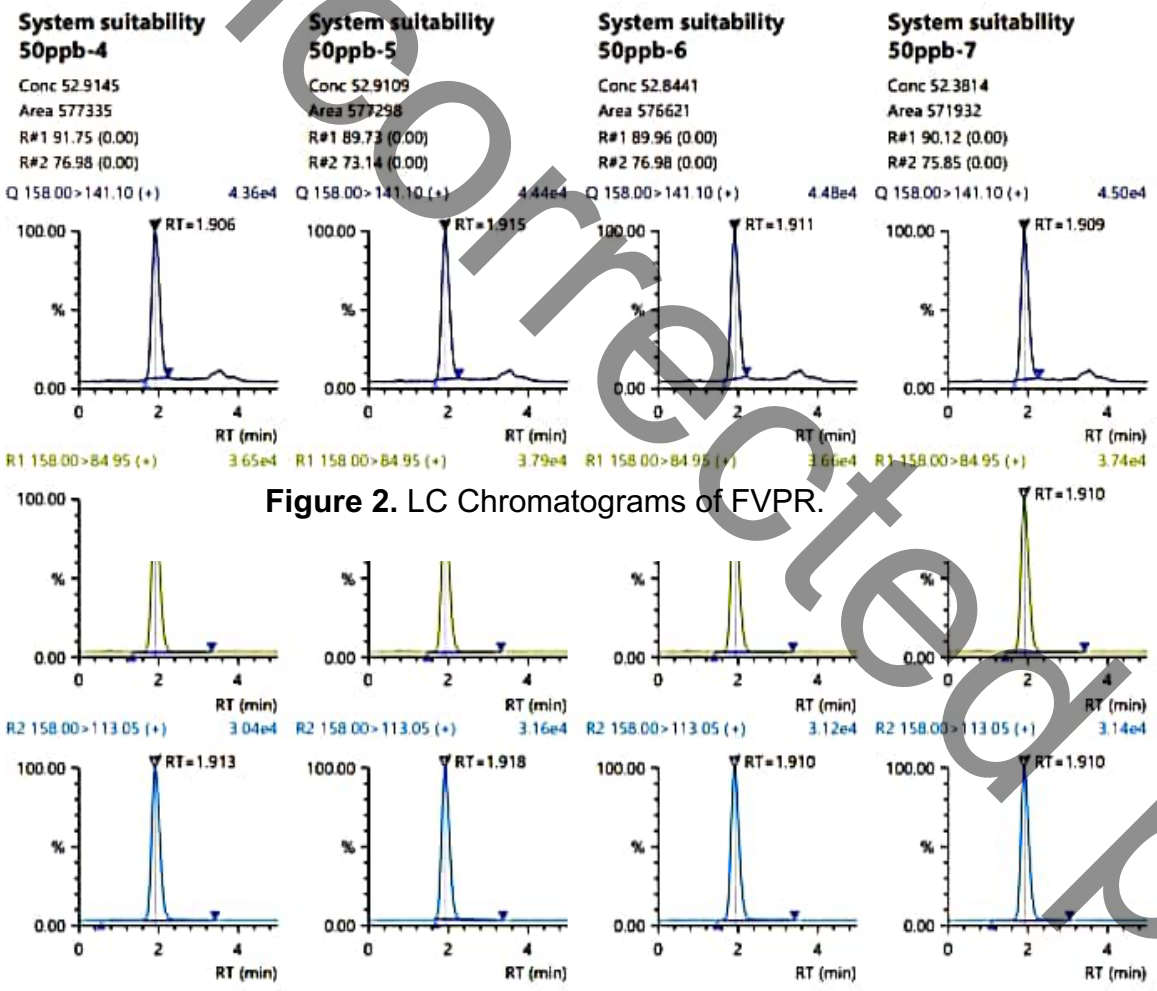
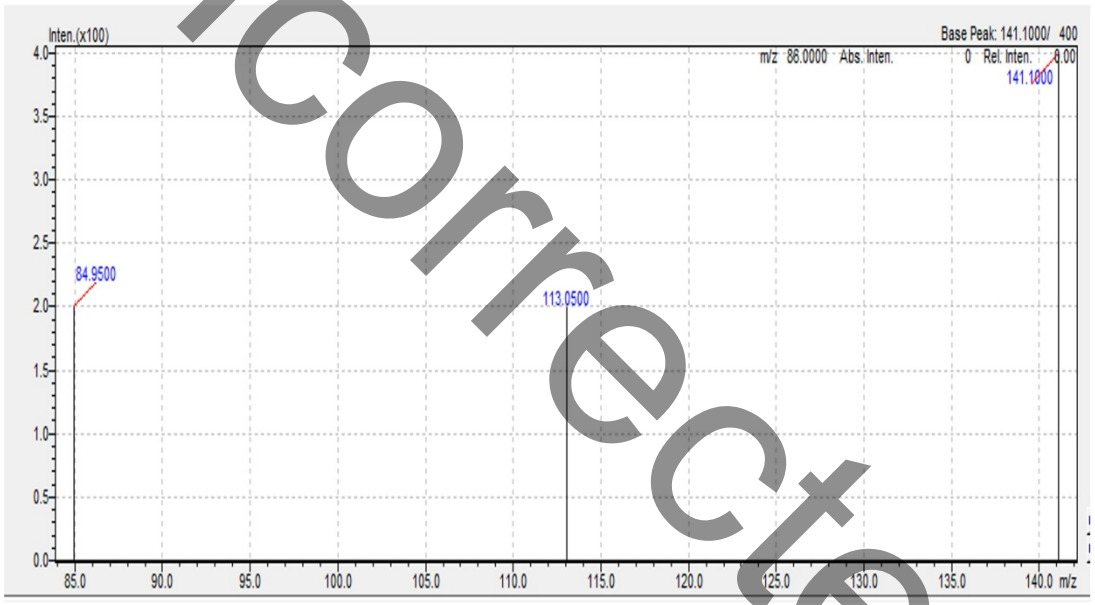
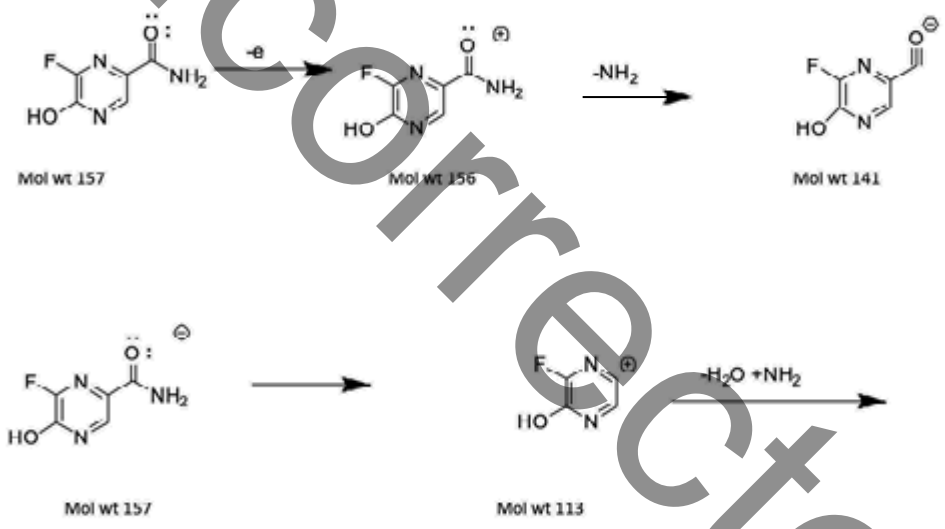


Figure 2. LC Chromatograms of FVPR.





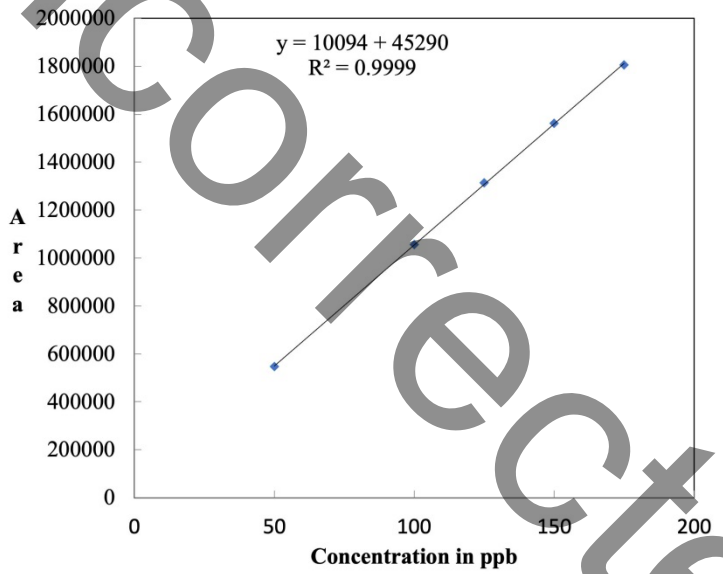


Figure 5. Linearity graph of FVPR.