In vitro studies for BCS classification of an antiviral agent, favipiravir

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ABSTRACT: Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a pyrazine carboxamide derivative, which is a purine nucleic acid analog, which is an antiviral agent used in the treatment of influenza. Since the recent outbreak caused by 2019-novel coronavirus (nCoV), there has been a search for effective antiviral agents to be used in the treatment of coronavirus disease 2019 (COVID-19), and favipiravir has been one of the options which provides a broad-spectrum therapy. Herein, we studied the aqueous solubility and in vitro permeability characteristics of favipiravir in order to shed light on the BCS classification of this antiviral agent used in COVID-19 therapy. The in vitro solubility was assessed using saturated solution of favipiravir in four different aqueous media and the solubility values were evaluated during 72 h at 37 °C. The solubility of favipiravir was between 4.48 to 8.5 mg/ml, which is 5.85 to 10.63 times of calculated solubility limit. Caco-2 cell monolayers were utilized for the permeability assessment, and the drug solutions in three different concentrations including the highest dose required for bioequivalence exemption of the immediate release dosage form were applied. The effect of efflux transporters on the permeability of favipiravir was also determined using a P-gp inhibitor, Verapamil HCl. According to the data obtained from the in vitro studies, favipiravir can be considered as a representative of class I compound.

KEYWORDS: Favipiravir; antiviral therapy; BCS classification; solubility; permeability; Caco-2 cell monolayer.

1. INTRODUCTION

Favipiravir (6-fluoro-3-hydroxypyrazine-2-carboxamide) is a pyrazine carboxamide derivative, with a molecular weight of 157.1 g/mol and pKa value of 5.1, and it is an antiviral agent used in the treatment of influenza [1-3]. Since the recent outbreak caused by 2019-novel coronavirus (nCoV), there has been a search for effective antiviral agents for the treatment of coronavirus disease 2019 (COVID-19), and favipiravir has been one of the emerging options, which is approved in the treatment and has still been enrolled in several clinical trials including USA, and UK [2, 4, 5].

Favipiravir, also known as T-705, was discovered by Toyama Chemical Co., Ltd. during the assessment of a group of approximately 30000 compounds against influenza [6]. It is a synthetic prodrug which is designed as a derivative of the lead compound T-1105 [4]. Favipiravir turns into its active form, favipiravir-RTP (favipiravir ribofuranosyl-5B-triphosphate), within the tissue after phosphoribosylation [4, 7]. After phosphoribosylation, it selectively and potently inhibit RNA dependent RNA polymerase (RdRp) [7]. Its affinity to RdRp enzyme to inhibit the viral protein synthesis causes its broad-spectrum antiviral effect including the resistant strains, since various types of RNA viruses have RdRp domain. In addition, it has antiviral activities against RNA viruses other than influenza [7]. Since the RdRp enzyme has a pivotal role in the replication of coronavirus, favipiravir is promising to address the unmet medical needs. So, the repurposing
of the existing nucleoside analogs such as favipiravir and small molecule drugs would have an impact in the treatment of COVID-19 patients [5].

Favipiravir is approved for the treatment of influenza in Japan, before the recent COVID-19 pandemic, in 2014 [1, 6, 7]. It has recently received emergency use approval in Italy, and it has been in use in countries including Japan, Russia, Saudi Arabia, Ukraine and Turkey for the treatment of SARS-CoV-2 [4]. There still ongoing clinical trials on the subject of SARS-CoV-2 all over the world including USA, Turkey, Germany, UK, Hungary, Egypt and China [2].

Biopharmaceutics Classification System (BCS) divides drug molecules into four classes based on their aqueous solubility and intestinal permeability. It is a scientific framework, and the scope is explained in detail with the guidelines provided by regulatory authorities. BCS is crucial, since the waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms are regulated based on this classification. According to the related guidelines, the drugs exhibiting high solubility could be the subject of BCS-based biowaiver concept, whether or not they show high permeability.

There are four BCS classes as follows; Class I: High Solubility – High Permeability, Class II: Low Solubility – High Permeability, Class III: High Solubility – Low Permeability, Class IV: Low Solubility – Low Permeability.

FDA guideline published in 2000 was the first one describing the regulations for waiving bioavailability and bioequivalence studies for high solubility and high permeability drugs. Later on FDA, EMA and WHO published guidance for biowaiver of high solubility drugs, Class I and Class III [8], and recently final adoption of a multidisciplinary guideline for the harmonization of current regional guidelines, ICH M9 on biopharmaceutics classification system based biowaivers, came into effect in 2020 [9].

The guidelines suggest experimental methods for the evaluation of aqueous solubility and permeability. According to the ICH M9 guideline, the solubility of a drug substance is considered as high in case the highest single therapeutic dose is completely soluble in 250 ml or less of aqueous media over the pH range of 1.2–6.8 at 37±1°C [9, 10]. In order to evaluate the solubility of the test drug, an extensive solubility study including at least three different pHs in the range of 1–6.8 should be conducted. The selected pH values should cover pH 1.2, 4.5 and 6.8, and the sampling time points should be chosen reasonably to include the expected duration of absorption as well as to ensure reaching the equilibrium. The number of pH conditions could be determined according to the pKa value of the drug, and the class of the drug is determined according to the lowest solubility data obtained in the aforementioned pH range. Each time point should at least have three samples to determine the solubility with a validated method. In addition, the stability of the drug substance with not more than 10 % degradation during the time period of the solubility studies should be demonstrated [9, 10].

For the evaluation of the permeability, the guidelines suggest human pharmacokinetic studies. Furthermore, the permeability of the drug substance can also be assessed by validated and standardized systems capable of predicting the extent of drug absorption such as in vitro Caco-2 cell culture methods [9, 10].

In this study, the solubility and permeability of favipiravir was assessed according to the related guidelines and the BCS classification was conducted using in vitro methods.

2. RESULTS

2.1. Solubility studies

The solubility studies were conducted during 72 hours and the results are given in Figure 1. The solubility of favipiravir increased with increasing pH showing a maximum solubility at pH 6.8. The highest solubility value recorded was 8.5 ± 0.078 mg/ml at 4 h for pH 6.8 buffer, and the lowest solubility was 4.68 ± 0.045 mg/ml at 4 h for distilled water. The lowest measured solubility was 5.85 times higher than the solubility limit for favipiravir. The summary of the solubility data is given in Table 1.

The BCS classification limit for favipiravir was calculated as 0.8 mg/ml, according to the highest dose required for bioequivalence exemption of the immediate release dosage form, which is 200 mg for favipiravir. For the determination of high solubility, the dissolution of the highest dose is required in a volume of 250 ml or less at studied pH conditions.
Figure 1. Results of solubility studies of favipiravir for distilled water, 0.1 N HCl, pH 4.5, pH 6.8 (n=3).

Table 1. The summary of solubility studies of favipiravir in distilled water, 0.1 N HCl, pH 4.5 and pH 6.8 (n=3).

<table>
<thead>
<tr>
<th>Favipiravir</th>
<th>Distilled water</th>
<th>0.1 N HCl</th>
<th>pH 4.5</th>
<th>pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum solubility (mg/ml)</td>
<td>4.68</td>
<td>5.30</td>
<td>5.76</td>
<td>8.50</td>
</tr>
<tr>
<td>SD</td>
<td>0.045</td>
<td>0.080</td>
<td>0.255</td>
<td>0.078</td>
</tr>
<tr>
<td>RSD %</td>
<td>1.0</td>
<td>1.5</td>
<td>4.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

In addition, the evaluation of stability during solubility studies are given in Table 2. The data has shown no sign of decomposition during 72 h period of solubility studies. After 72 h, the change in the amount of drug was found between 0.08 to 1.73 %, showing the stability of the drug substance in four different aqueous media used for solubility evaluation. The sampling points for solubility studies were determined accordingly as 0, 2, 4, 8, 12, 24, 48 and 72 h based on stability data.

Table 2. The results of stability evaluation conducted during solubility studies of favipiravir (n=3).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Distilled water</th>
<th>0.1 N HCl</th>
<th>pH 4.5</th>
<th>pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change %</td>
<td>RSD %</td>
<td>Change %</td>
<td>RSD %</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1.35</td>
<td>0.26</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>0.73</td>
<td>0.77</td>
<td>0.14</td>
<td>0.35</td>
</tr>
<tr>
<td>8</td>
<td>0.17</td>
<td>0.1</td>
<td>0.20</td>
<td>0.39</td>
</tr>
<tr>
<td>12</td>
<td>0.52</td>
<td>0.06</td>
<td>0.83</td>
<td>1.33</td>
</tr>
<tr>
<td>16</td>
<td>1.25</td>
<td>0.42</td>
<td>1.00</td>
<td>0.04</td>
</tr>
<tr>
<td>20</td>
<td>1.54</td>
<td>0.11</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>24</td>
<td>1.26</td>
<td>0.58</td>
<td>1.42</td>
<td>0.66</td>
</tr>
<tr>
<td>32</td>
<td>1.24</td>
<td>0.4</td>
<td>1.07</td>
<td>0.17</td>
</tr>
<tr>
<td>48</td>
<td>0.91</td>
<td>0.76</td>
<td>1.63</td>
<td>0.15</td>
</tr>
<tr>
<td>72</td>
<td>1.26</td>
<td>0.7</td>
<td>1.73</td>
<td>0.2</td>
</tr>
</tbody>
</table>

2.2. Permeability studies

The effect of initial drug concentration on the measured in vitro permeability of favipiravir was assessed using three different concentrations starting from the highest strength dissolved in 250 ml, and additional two concentrations. The concentrations used in the study were 0.8 mg/ml, 0.4 mg/ml and 0.2 mg/ml.
The apparent permeabilities of favipiravir were calculated as $1.35 \times 10^{-5} \pm 0.0171 \times 10^{-5}$ cm/s, $1.18 \times 10^{-5} \pm 0.0093 \times 10^{-5}$ cm/s, and $1.09 \times 10^{-5} \pm 0.0458 \times 10^{-5}$ cm/s for the doses of 0.8, 0.4 and 0.2 mg/ml, respectively (Figure 2).

Figure 2. Comparison of the apparent permeabilities of the test drug in different concentrations and the reference metoprolol (0.4 mg/ml). Verapamil HCl was applied to assess the influence of a specific P-gp inhibitor on the permeability of the test compound. The in vitro permeability of favipiravir was found to be dependent on the concentration applied as the apparent permeability decreased with the decreasing concentration ($n=3$). ns: not significant, VP: Verapamil HCl-50 µM, * $p<0.05$.

Metoprolol tartrate (0.4 mg/ml) was used as model drug for permeability assay method for its high permeability characteristics ($f_a \geq 85\%$). The apparent permeability of metoprolol tartrate was found as $0.856 \times 10^{-5} \pm 0.0428 \times 10^{-5}$ cm/s. According to the apparent permeability data, the permeability ratio of favipiravir/metoprolol was calculated, and the permeability ratio of test/metoprolol was greater than 1. The results are shown in Table 3.

The dependence of in vitro permeability of favipiravir on efflux transporters was assessed using Verapamil HCl (50 µM) as a P-gp inhibitor.

The apparent permeability of favipiravir in the presence of Verapamil was $1.38 \times 10^{-5} \pm 0.0183 \times 10^{-5}$ cm/s, $1.27 \times 10^{-5} \pm 0.0541 \times 10^{-5}$ cm/s, and $1.17 \times 10^{-5} \pm 0.0607 \times 10^{-5}$ cm/s for the doses of 0.8, 0.4 and 0.2 mg/ml, respectively. For favipiravir doses of 0.8 and 0.2 mg/ml, no significant effect was observed ($p>0.05$) in the presence of P-gp inhibitor. On the other hand, a significant increase in permeability was observed for 0.4 mg/ml without P-gp inhibitor ($p<0.05$).

Table 3. The permeability ratio of test compound. Test compound (favipiravir) was applied in three different concentrations as 0.8, 0.4 and 0.2 mg/ml. The reference compound was metoprolol tartrate ($n=3$).

<table>
<thead>
<tr>
<th>Favipiravir Concentration</th>
<th>Test / Reference Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 mg/ml</td>
<td>1.57 ± 0.019</td>
</tr>
<tr>
<td>0.4 mg/ml</td>
<td>1.38 ± 0.011</td>
</tr>
<tr>
<td>0.2 mg/ml</td>
<td>1.27 ± 0.054</td>
</tr>
<tr>
<td>0.8 mg/ml + VP</td>
<td>1.62 ± 0.021</td>
</tr>
<tr>
<td>0.4 mg/ml + VP</td>
<td>1.49 ± 0.063</td>
</tr>
<tr>
<td>0.2 mg/ml + VP</td>
<td>1.37 ± 0.071</td>
</tr>
</tbody>
</table>

a: VP; Verapamil HCl (50 µM)

The ratio of apparent permeability ($P_{app}$) of the apical-to-basolateral direction between the verapamil-treated and non-treated groups were determined. The efflux ratio of favipiravir was calculated as equation 1 [11]:

$$Efflux\ ratio = P_{app AP\to BL} with\ Verapamil / P_{app AP\to BL}$$ (Eq. 1)
The efflux ratio of favipiravir for 0.8, 0.4 and 0.2 mg/ml doses were found to be <2 for the selected drug concentrations (1.03, 1.08 and 1.07, respectively), showing the lack of dependence on the measured in vitro permeability on efflux transporters.

The results have shown that in vitro permeability of favipiravir was dependent on the initial drug concentration (Figure 2). A significant difference was observed between the doses of 0.8 and 0.4 mg/ml, 0.8 and 0.2 mg/ml as well as between 0.4 and 0.2 mg/ml (p=0.0002, p<0.0001 and p=0.0308, respectively). The difference was also statistically significant for the groups treated with Verapamil (p=0.0108, p<0.0001 and p=0.0147, respectively).

In addition, the integrity of cell monolayers during study was determined by TEER measurement. The electrical resistance of the cells was found to be above 500 Ω.cm² after the 2 hours of incubation confirming the integrity and tolerability of Caco-2 cells to the applied drug solutions.

3. DISCUSSION

BCS classifies drug substances based on their aqueous solubility and intestinal permeability. The dissolution, solubility, and intestinal permeability are three major factors effecting the rate and extent of drug absorption from IR solid oral dosage forms according to the FDA guideline [10].

The drug is defined as high soluble or low soluble according to the limit concentration. The limit concentration is relative to the highest dose required for bioequivalence exemption of the immediate release dosage form. Depending on the defined highest dose, the drug active substance is considered to be 'highly soluble' when dissolved in a volume of 250 ml or less in the pH range of pH 1.0 – pH 6.8 [10]. According to the ICH guideline, the classification of the drug substance should be determined depending on the lowest measured solubility over the studied pH range [9].

In this study, BCS class of favipiravir was determined utilizing the solubility data of favipiravir in the pH range of pH 1.0 – pH 6.8, and the results were evaluated according to the classification limit of favipiravir, which is 0.8 mg/ml. The solubility of favipiravir was found to be approximately 5.85 to 10.63 times higher than the high solubility classification limit, implying the high soluble nature of the drug. The stability of the drug substance was also shown using four different media used in the solubility studies and no sign of significant degradation above 5 % was observed during the period of 72 h.

Caco-2 cell line is one of the most preferred cell lines to mimic the monolayer of intestinal epithelium. It has been a model for in vitro prediction of drug absorption over 40 years since its establishment from colon carcinoma [12, 13]. Although there are studies for alternative in vitro methods for permeability evaluation in comparison to Caco-2 cell line such as different cell line models utilizing HT29-MTX or MDCK cells as well as lipid-based membrane models, the popularity of Caco-2 cell model still continues [14, 15]. In addition, the use of organic solvents for membrane models like parallel artificial membrane permeability assays (PAMPA) are still under investigation for solvent-free alternatives [15-17].

Caco-2 cell line forms monolayer structure after 14 to 21 days following confluency, and it expresses several morphological and functional characteristics of the mature enterocytes like tight junctions and brush border on the apical side. The passage number of the cell line is reported to affect the proliferation rate and formation of the monolayer structure; thus, the culturing conditions and the passaging information should be taken into consideration when it comes to the comparison between studies conducted by different laboratories [12, 13, 18].

Although there some problems coming with the use of Caco-2 cell line such as stability and reproducibility, inter-laboratory culturing differences or challenges of using living systems, Caco-2 cell line still has opportunities like offering a cellular model incorporating a mucous barrier, which is especially important for poorly soluble and lipophilic drugs [14]. Furthermore, there are number of transporters described in different origins of Caco-2 cells such as peptide transporters like PepT1 and HPT, sugar transporters like SGLTI and GLUT5, and P-glycoprotein (P-gp) transporters [18].

In this study, the effect of efflux transporter on the permeability of the drug was also evaluated using Caco-2 cell monolayers. Caco-2 cell line, which is reported to have increased mRNA levels of P-gp transporters between the culture days of 7-21 [18, 19], were used for the studies. According to the guidelines, the efflux
ratio of more than 2 for the selected drug concentrations suggests involvement of an efflux transporter, and the efflux ratio less than 0.5 could be the indication of an active transporter [9, 10, 20]. Verapamil HCl was used as P-gp inhibitor to evaluate this involvement of efflux transporters in the transport of favipiravir [21]. The results have shown that the transport of favipiravir is not affected from P-gp, since the efflux ratios for three different concentrations were found to be approximately 1.

The permeability of a drug in Caco-2 monolayer could be used for the evaluation of the absorption after oral administration. The studies showed the correlation between high permeability in Caco-2 cell line and the complete oral absorption from human intestine. In case when the drug is completely absorbed from human intestine, the permeability is also high, showing apparent permeability above $1 \times 10^{-6}$ cm/s [13, 22]. For the permeability determination of favipiravir, metoprolol was used as reference permeability standard, since it has fraction absorbed (FA) over 85 % [9, 11, 23]. It is reported that if a drug has permeability above that of metoprolol, it would be considered highly permeable [23, 24]. The permeability of metoprolol tartrate through Caco-2 monolayers was $8.56 \times 10^{-6}$ cm/s, which is in accordance with the literature [11]. The permeability of favipiravir was found to be higher than that of metoprolol, and the permeability ratio of favipiravir was found between 1.27 to 1.62 for three different concentrations with or without P-gp inhibitor. Thus, the favipiravir could be considered highly permeable.

4. CONCLUSION

The presented paper shows the data of in vitro methods for biopharmaceutical drug classification of an antiviral drug, favipiravir. The BCS classification of favipiravir was conducted utilizing the solubility data of favipiravir in the pH range of pH 1.0 – pH 6.8, and the permeability was assessed with Caco-2 cell monolayer.

The lowest solubility value of favipiravir was approximately five-times higher than the solubility limit and the permeability was higher than the reference standard. Thus, it can be concluded that favipiravir can be considered as a representative of class I compound according to the BCS due to its high solubility and high permeability characteristics.

5. MATERIALS AND METHODS

5.1. Materials

Favipiravir was purchased from Biophore, India. Hydrochloric acid, Acetic acid, Potassium dihydrogen phosphate, Sodium hydroxide, Trifluoroacetic acid and Sodium acetate trihydrate were from Merck, Germany. Dulbecco’s Modified Eagle Medium (DMEM), Trypsin-EDTA, Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were purchased from Biochrom, Germany. Caco-2 cell was purchased from ATCC (HTB-37™). All other chemicals were analytical grade, unless otherwise stated.

5.2. Solubility studies

Solubility studies were conducted in accordance with ICH M9 guideline to determine the solubility of favipiravir in different buffer systems [9]. Three pH conditions were selected among physiological pH conditions in order to simulate the gastrointestinal tract. Supersaturated solutions of favipiravir were prepared using distilled water, 0.1 N HCl, pH 4.5 Acetate buffer and pH 6.8 Phosphate buffer. Briefly, the supersaturated solutions of drug were prepared by adding 5 grams of active ingredient to a flask containing 100 ml of buffers. The solutions were re-saturated by addition of favipiravir if the active substance was dissolved during the analysis. The solutions were placed in a shaker at a speed of 600 rpm at 37 °C. The samples are taken at specified time points and filtered through 0.45 µm regenerated cellulose filter. The samples were diluted (1/50) before analysis, and the amount of favipiravir was determined using a validated HPLC method. The experiments were carried out in triplicate; average values and accompanied RSD values (expressed in %) are presented. The results of the solubility studies are in Table 1.
In order to determine the stability of the active substance in experimental conditions, the standard solution at 100 % concentration was kept in distilled water, 0.1 N HCl, pH 4.5 and pH 6.8 for 72 hours and analyzed using the aforementioned validated HPLC method. The details of the HPLC method is given under analytical methods. The results of the stability studies are summarized in Table 2.

5.2.1. Preparation of buffer solutions

0.1 N HCl: Purified water was added into volumetric flask, and 8.3 ml of 37 % HCl was added and mixed. Sufficient purified water was added to produce 1000 ml.

pH 4.5 Acetate Buffer: Purified water was added into volumetric flask, and 2.99 g Sodium acetate trihydrate (C₂H₃NaO₂·3H₂O) and 14.0 ml 2 N Acetic acid were mixed until dissolved. Sufficient purified water was added to produce 1000 ml, and pH was checked.

pH: 6.8 Phosphate Buffer: Purified water was added into volumetric flask, and 6.8 g Potassium dihydrogen phosphate (KH₂PO₄) and 0.9 g Sodium hydroxide (NaOH) were mixed until dissolved. Sufficient purified water was added to produce 1000 ml, and pH is checked.

5.3. Permeability studies

Permeability studies were performed with Caco-2 cell line (ATCC® HTB-37™, passage number between 10 to 15), to mimic the monolayer structure of intestinal cells. DMEM medium was prepared to contain 4 mM L-Glutamine and Sodium Pyruvate, 10 % FBS, 0.5% Penicillin (10,000 units) and streptomycin sulfate (10,000 μg / mL). Caco-2 cells were seeded into 75 cm² flasks and incubated at 37 °C in an environment containing 5 % CO₂. The cells were removed and resuspended with trypsin-EDTA solution at a concentration of 0.025% after reaching 80 % confluency, and then stained with trypan blue to determine cell numbers using a hemocytometer.

For the permeability study, the cells were seeded into the inserts (ThinCert™, 12 wells, 1 μM pore diameter, transparent) at a concentration of 12 x 10⁴ cells/mL. 0.5 mL of cell suspension was added to the apical section and 1 mL of medium to the basolateral section. The plates were incubated at 37 °C in an environment containing 5 % CO₂. The culture medium was changed every other day and permeability studies were performed after 21 days when the cell monolayer reached confluency.

The integrity of the cell monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) before and after the drug application. The TEER values of Caco-2 cell monolayers were measured by epithelial voltohmmeter (Millicell – ERS, Merck), and the cell monolayers with an electrical resistance of 500 Ω.cm² and above were used for evaluation.

At the end of 21 days, the medium in the apical and basolateral compartments was discarded and the cells were washed with transport medium. Briefly, 0.5 mL of HBSS containing 10 mM HEPES (without phenol red) was applied to the apical side and 1 mL to the basolateral side, and the cells were left with transport medium to ensure that they have reached equilibrium. Favipiravir solutions with three different concentrations (0.8 mg/ml, 0.4 mg/ml and 0.2 mg/ml) and metoprolol tartrate solution (0.4 mg/ml) were applied to the apical compartment in transport medium and kept at 37 °C in water bath at 60 rpm (n=4). Verapamil HCl (50 µM) was also used to determine the effect of P-gp efflux on the transport mechanism of favipiravir.

After 2 hours of incubation, the medium in the basolateral compartment was collected and stored at −20 °C for HPLC analysis. After the HPLC analysis the apparent permeability coefficients (P_app) were calculated [11]. The cell integrity was confirmed by measuring the electrical resistance of the cell monolayers after 2 hours of incubation.

The statistical analysis was performed using Prism 7.0 software. The difference between the groups were analyzed using 2-way ANOVA test. The groups, which exhibited statistical difference, were further evaluated by Tukey’s multiple comparison test. All results were expressed as mean ± SD, unless indicated otherwise.
5.4. Analytical methods

The amount of favipiravir samples obtained during solubility and permeability studies were analyzed using a validated HPLC method. An isocratic analytical method was developed using reverse phase C18 column (Phenomenex InertClone™ 5 µm ODS (2) 150Å, 250 x 4.6 mm). The mobile phase was distilled water with 0.05 % TFA and Acetonitrile (80:20, v/v) at a flow rate of 1 ml/min. The detection of favipiravir was conducted using UV detector at 323 nm.

For the quantification of metoprolol tartrate in the samples obtained from basolateral compartment after permeability studies, an isocratic HPLC method was conducted using Agilent 1200 series equipped with reverse phase C18 column (Phenomenex InertClone™ 5 µm ODS (2) 150Å, 250 x 4.6 mm). The mobile phase was prepared using pH 3 Phosphate buffer and Acetonitrile (30:70) at a flow rate of 1 ml/min. 10 μl of samples were injected and the absorbance was obtained at 232 nm [25].

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