Development of Favipiravir Secondary Standard from Favipiravir Tablets

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Abstract

The aim of this study is to develop a secondary standard of favipiravir from favipiravir tablets for a replacement of costly reference standard. Firstly, favipiravir was isolated and purified from the powdered tablets by liquid-liquid extraction method using ethyl acetate and water. The total amount of extracted favipiravir yielded 31.25% and the extract was consequently identified using Fourier-transform infrared (FT-IR) spectroscopy. Secondly, quantity or purity of extracted favipiravir was determined using a validated ultraviolet (UV)-visible spectrophotometric method. The analysis result showed that the purity of favipiravir was 95.98% (as is) which the acceptance criteria has been limited within 98.0 – 102.0%. Unfortunately, although the purity of developed favipiravir secondary standard was nearly met the criteria, it could be preliminary used as a standard substance in some routine pharmaceutical laboratory procedures, such as identification, in-process quality control, including pharmacological studies.

Keywords : Favipiravir, tablets, secondary standard, UV-visible spectrophotometry

1. Introduction

Since the worldwide outbreak of coronavirus disease-2019 (COVID-19), one of potential drugs approved for the treatment of this infectious respiratory illness was favipiravir. In fact, favipiravir, also known as 6-fluoro-3-hydroxypyrazine-2-carboxamide (T-705), has been discovered since 2002 in Japan for the treatment of influenza through the mechanism of viral RNA-dependent RNA polymerase (RdRp) inhibition (Pavlova *et al.*, 2023). Recently, in Thailand, favipiravir still has been approved as one of recommended antivirals for the management of patients with mild symptomatic COVID-19 without pneumonia and no risk factors, including patients with mild to moderate pneumonia with risk factors (Department of Medical Services, 2023). Upon the success of favipiravir synthesis as an active pharmaceutical ingredient (API) by the National Science and Technology Development Agency (NSTDA), with the success of favipiravir tablets local production under the license of the Government Pharmaceutical Organization (GPO), the importation of favipiravir starting materials including finished products from overseas has decreased significantly (National Science and Technology Development Agency, 2021).

Not only the manufacturing process, but the quality control (QC) also plays an important role to assure the quality of any raw materials and pharmaceutical dosage forms before launching the products to the market. During the QC process, identification testing and assaying of APIs and finished products must be operated using reference standards. In general, pharmaceutical reference standards are categorized into 2 types. First, primary (compendial) standard or primary chemical reference substance is one that is widely acknowledged to have the appropriate qualities within a specified context, and whose assigned content when used as an assay standard is accepted without requiring comparison with another chemical substance (World Health Organization, 2007). Primary standard shown to have very high purity and may be obtained from the pharmacopoeias, such as the United States Pharmacopoeia (USP), European pharmacopoeia (EP), or Japanese Pharmacopoeia (JP) (USP Council of Experts, USP Reference Standards Committee, and Hauck, 2012). Whereas secondary (non-compendial)

standard or working standard is a substance whose characteristics are assigned and/or calibrated by comparison with a primary chemical reference substance to ensure its identity, strength, quality, purity, and potency (World Health Organization, 2007; USP Council of Experts, USP Reference Standards Committee, and Hauck, 2012). However, using secondary standards with assigned purity for routine analysis is preferable in many pharmaceutical laboratories due to the less expensive.

Focusing on the QC process of favipiravir tablets, including the case of analytical method validation, favipiravir reference standard and sample tablets are required for qualitative and quantitative analysis. Referred to the monograph, sampling of at least 20 favipiravir tablets is the first step of sample preparation for assaying (World Health Organization, 2021). After the completion of this step, all sampling tablets which were finely powdered cannot be used for any purposes. Hence, isolation of favipiravir from the remaining powdered tablets with suitable extraction and purification procedures could be advantageous. Moreover, purification, characterization, and purity estimation of extracted favipiravir by validated analytical methods were also conducted to certify the use of this substance as a secondary standard, and consequently replaced the use of an expensive reference standard.

2. Objectives

To develop a secondary standard of favipiravir from the powdered tablets and to determine the purity of extracted favipiravir (secondary standard) using a validated UV-visible spectrophotometric method.

3. Materials and methods

3.1 Materials

Favipiravir reference standard (assigned purity 99.48%) was purchased from the Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand). Powdered 200-mg favipiravir film-coated tablets (COVIVEL^{TM} 200, Strides Pharma Science Ltd., Bangalore, India) were obtained from the previous study (Poonsawat, Wongpayoon, and Thanarangsarit, 2023). All solvents and chemicals were analytical reagent (AR) grade. Ethyl acetate and NaCl were purchased from J.T. Baker (New Jersey, USA). Na_2SO_4 (anhydrous, 99%) was purchased from KemAus (New South Wales, Australia). Dichloromethane was purchased from Fluka (Buchs, Switzerland). Ethanol (absolute) was purchased from Merck (Darmstadt, Germany).

Rotary evaporator (Buchi Rotavapor R-205, Flawil, Switzerland) was used to remove the organic solvent after extraction process.

IR spectra were recorded using an FT-IR spectrometer (PerkinElmer Spectrum 100, Massachusetts, USA) equipped with attenuated total reflectance (ATR) accessory. Spectrum 6.1.0 software was used for data acquisition.

UV spectra and absorbance measurements were carried out using a double-beam UV-visible spectrophotometer (Jasco V-730, Tokyo, Japan) with a pair of identical 1-cm quartz cuvettes. Spectra Manager 2.5 software was used for data acquisition.

3.2 Methods

3.2.1 Extraction and purification of favipiravir from powdered tablets

Powdered tablets were weighed equivalent to 500 mg of favipiravir into a 250-mL separatory funnel, 50 mL of deionized (DI) water was then added, and mixed. The mixture was extracted with ethyl acetate (2 × 80 mL) and washed with 20 mL of 3 M NaCl (brine). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude extract was purified by precipitating in dichloromethane, filtered, and dried at room temperature to obtain a white to light yellow solid. Percentage yield (%yield) of favipiravir was also calculated from pooled purified extract (n = 2).

3.2.2 Identification of extracted favipiravir (secondary standard)

Favipiravir secondary standard was identified using spectrophotometric methods. For FT-IR spectroscopy, the sample was directly scanned in range of 650 – 4,000 cm^{-1} with ATR mode. The obtained FT-IR spectrum was compared with the reference standard under the same condition. In addition, for UV-visible spectrophotometry, UV spectrum of the sample solution was recorded in range of 200 – 400 nm and directly compared with the reference standard in terms of spectral characteristic and the wavelength of maximum absorbance (λ_{max}).

3.2.3 Determination of favipiravir secondary standard purity

A validated UV-visible spectrophotometric method from the previous study (Poonsawat, Wongpayoon, and Thanarangsarit, 2023) was conducted to estimate the content or purity of favipiravir secondary standard.

Preparation of calibration curve

A series of favipiravir reference standard solution at 2, 4, 6, 8, and 10 µg/mL were prepared by diluting of favipiravir stock standard solution (100 µg/mL) using ethanol : water (1:1, v/v) as a solvent. Absorbances of the standard solutions were measured at 227 nm (λ_{max}). Concentrations of favipiravir reference standard and measured absorbances were plotted to generate a calibration curve. Correlation coefficient (r) and regression equation were also determined and used for calculation of favipiravir purity.

Preparation of sample solution

Weighed accurately about 25 mg of favipiravir secondary standard into a 25-mL volumetric flask. Dissolved and adjusted to the volume with ethanol : water (1:1, v/v). Transferred 4.0 mL of the solution into a 25-mL volumetric flask and adjusted to the volume with the same solvent. Finally, 2.0 mL of the previous solution was pipetted and diluted to 50.0 mL with the same solvent to obtain the sample solution containing 6.4 µg/mL of favipiravir. The sample solutions were prepared in triplicate (n = 3) and then measured the absorbances at 227 nm. According to the International Meeting of World Pharmacopoeias (IMWP) criteria, favipiravir secondary standard contains not less than 98.0 and not more than 102.0% of favipiravir ($C_5H_4FN_3O_2$), calculated on the anhydrous basis (World Health Organization, 2021).

4. Results

4.1 Extraction and purification of favipiravir from powdered tablets

Starting from the extraction of powdered tablets containing 1,279.9 mg of favipiravir, 400 mg (31.25 %yield) of a white to light yellow powder was obtained after the purification process.

4.2 Identification of extracted favipiravir (secondary standard)

Spectral data of favipiravir secondary standard were obtained from 2 different spectrophotometric methods as mentioned above. The results showed that FT-IR spectrum of the sample complied to the reference standard (Figure 1). The N-H stretching peaks of amide at 3,346.54 and 3,212.83 cm^{-1}, C=O stretching peak of amide at 1,657.35 cm^{-1}, C-F stretching peak at 1,433.05 cm^{-1}, including C-N stretching peaks of aromatic amine at 1,393.25 and 1,262.55 cm^{-1} were noticed from the spectrum of sample. Meanwhile, the spectrum of favipiravir reference standard also exhibited the N-H stretching peaks of amide at 3,346.53 and 3,212.86 cm^{-1}, C=O stretching peak of amide at 1,655.42 cm^{-1}, C-F stretching peak at 1,432.57 cm^{-1}, as well as C-N stretching peaks of aromatic amine at 1,393.37 and 1,262.58 cm^{-1}.

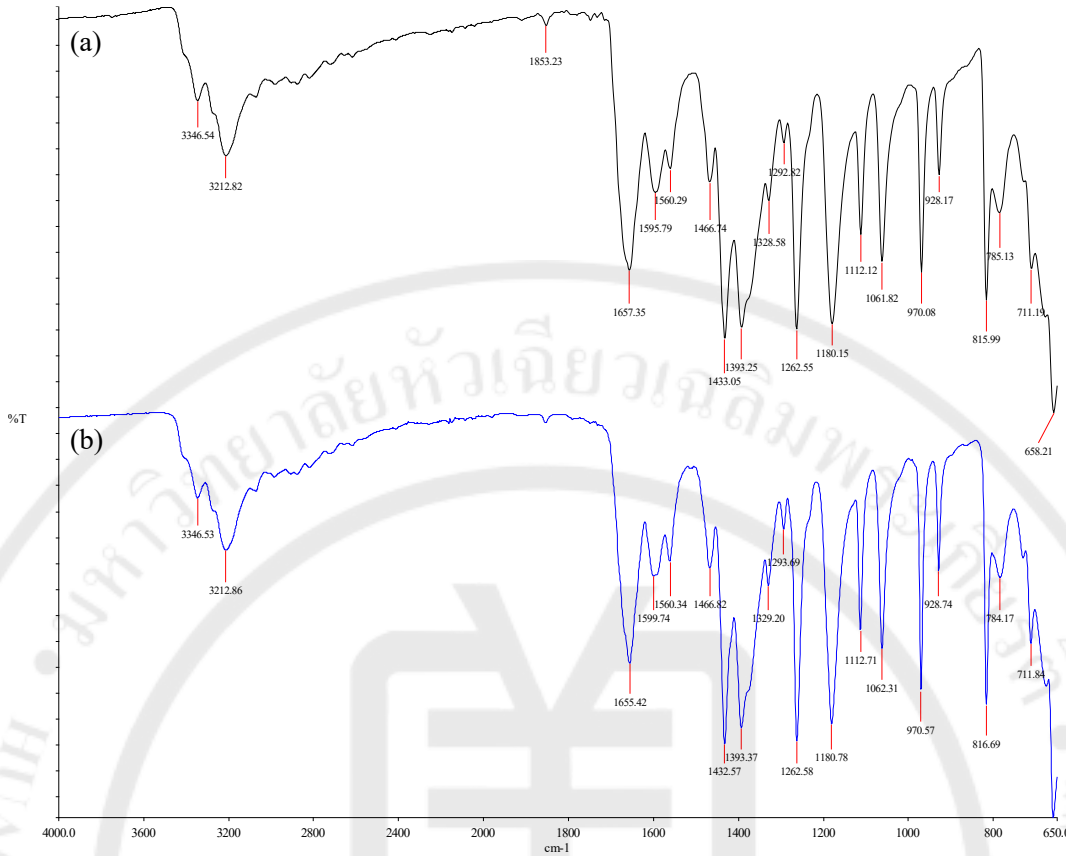


Figure 1 Stacked FT-IR spectra of favipiravir reference standard (a), and sample (b).

For UV-visible spectrophotometry, UV absorption spectra of the sample and reference standard were displayed in Figure 2. Spectral characteristic between 2 spectra was resembled and the λ_{max} was detected at 227 nm. These findings suggested that the extract from powdered tablets was truly identified as favipiravir.

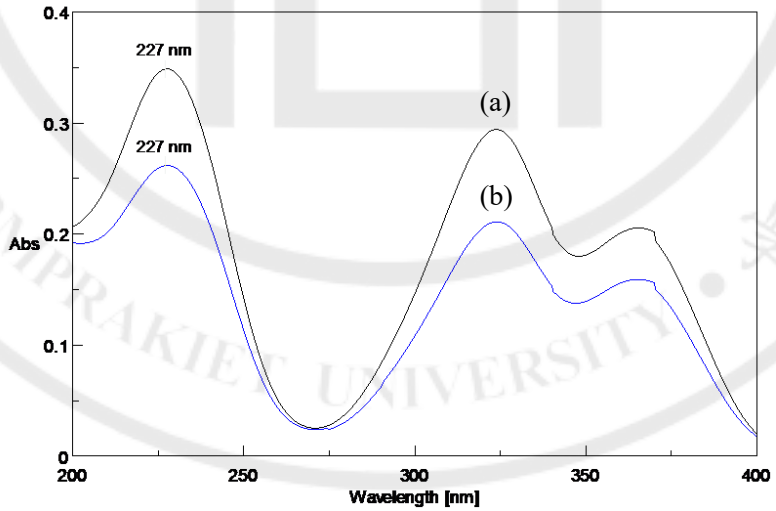


Figure 2 Overlay UV spectra of favipiravir reference standard at 8 µg/mL (a), and sample solution at 6.4 µg/mL (b) in ethanol : water (1:1, v/v).

4.3 Determination of favipiravir secondary standard purity

4.3.1 Calibration curve

The absorbances of 5 different concentrations of favipiravir reference standard solutions were measured at 227 nm. The results showed good linearity over the concentration

range of 2 – 10 µg/mL. From the regression analysis, the calibration curve of favipiravir was found to be linear with r-value of 0.9997 and regression equation of y = 0.0431x + 0.0154.

4.3.2 Purity of favipiravir secondary standard

Sample (favipiravir secondary standard) solutions at nominal concentration of 6.4 µg/mL were separately prepared in triplicate and measured for the absorbances at 227 nm. The content of favipiravir was then calculated by using the regression equation and the results were displayed in Table 1. The average amount or purity of favipiravir secondary standard was 95.98 %w/w (as is), which did not meet the IMWP acceptance criteria of favipiravir (98.0 – 102.0 %w/w, calculated on the anhydrous basis).

Table 1 Quantitative analysis results of favipiravir secondary standard (n = 3)

No.	Weight of sample (mg)	Absorbance at 227 nm	Concentration found (µg/mL)	Amount found (mg)	%w/w (as is)
1	26.0	0.2958	6.506	25.41	97.75
2	24.0	0.2690	5.884	22.98	95.77
3	25.4	0.2800	6.139	23.98	94.41
				Average	95.98
				SD	1.680

Additionally, total production costs for the development of favipiravir secondary standard were also estimated (Table 2). In this study, production of 400-mg secondary standard costed 314.62 Baht (0.79 Baht/mg), compared with 5,000 Baht (100 Baht/mg) of 50-mg reference standard used in this study (Bureau of Drug and Narcotic, 2021), and 18,380 Japanese Yen (approximately 4,438 Baht or 44.38 Baht/mg) of 100-mg marketed available IMWP reference standard (Pharmaceutical and Medical Device Regulatory Science Society of Japan, 2021). However, in fact, the presented cost of the developed favipiravir secondary standard in this study could not be directly compared with the commercial reference standard due to it was basically calculated using only the price of chemical and reagents (direct material). Direct and indirect labor costs, electricity, as well as total cost for QC processes must be included.

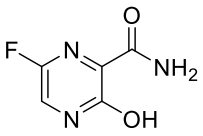
Table 2 Production cost (direct material) of favipiravir secondary standard (400 mg)

Chemical	Quantity per unit	Unit price (Baht)	Approximate amount used	Net price (Baht)
Ethyl acetate	2.5 L	1016.5	0.34 L	138.24
Na_2SO_4 anhydrous	1.0 kg	550	0.02 kg	11.00
Dichloromethane	2.5 L	590	0.05 L	11.80
Ethanol	2.5 L	963	0.29 L	110.74
NaCl	1.0 kg	250	0.18 kg	42.83
			Total (Baht)	314.62

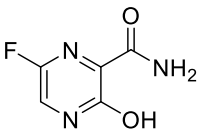
The results of qualitative and quantitative analysis for the developed favipiravir secondary standard were primarily summarized as a certificate of analysis (COA) and displayed in Table 3.

Table 3 Certificate of analysis (COA) of favipiravir secondary standard

Product name	Favipiravir
Molecular formula	$C_5H_4FN_3O_2$
Molecular weight	157.10
Chemical name	6-Fluoro-3-hydroxypyrazine-2-carboxamide
CAS number	259793-96-9



Test	Specification	Result
Appearance	White to light yellow powder	Conformed
Identification		
IR spectroscopy	The IR spectrum of sample corresponds to the standard	Conformed
UV-visible spectrophotometry	Absorption maxima at 227 nm (λ_{max}), the UV spectrum of sample corresponds to the standard	Conformed

Product name	Favipiravir	
Molecular formula	$C_5H_4FN_3O_2$	
Molecular weight	157.10	
Chemical name	6-Fluoro-3-hydroxypyrazine-2-carboxamide	
CAS number	259793-96-9	
Test	**Specification**	**Result**
Assay		
UV-visible spectrophotometry	98.0 – 102.0% (anhydrous basis)	95.98% (as is)
Water content	Not more than 0.6%	N/A
Packaging and storage	Preserve in tightly closed container and protected from light, preferably at the temperature about 5˚C	

Note: N/A = not applicable.

5. Discussion

According to the remaining of 200-mg favipiravir powdered tablets obtained after assaying from the previous study (Poonsawat, Wongpayoon, and Thanarangsarit, 2023), in this study, favipiravir was extracted and purified for the propose to use as a secondary or working standard. This value-added procedure started from liquid-liquid extraction technique using ethyl acetate and water. Due to poor solubility of favipiravir in many types of commonly used organic solvents or even water, selection of a suitable solvent for the partition was very important. Favipiravir is a weak acid with a pK_a of 5.1 at the phenolic group (Tuesuwan *et al.*, 2023) and it contains a primary amide (-$CONH_2$) in the structure, which is hydrolyzed under acidic or basic conditions. To prevent the hydrolysis, using aqueous acid (pH lower than 5.1) to convert favipiravir into a water-insoluble (unionized) form was not applied. For the extraction of favipiravir from the powdered tablets, ethyl acetate was chosen because it showed the highest solubility for favipiravir among laboratory available, water-immiscible organic solvents (Cui, Yan, and Zhang, 2023). After the extraction and purification processes, the amount of extracted favipiravir was very low (31.25 %yield), probably due to favipiravir is slightly soluble in water and may be lost during the step of partition. It was found that emulsion occurred while performing a separatory funnel technique because there was too high amount of the powdered tablets dispersed in aqueous layer. As aforementioned problem, the extraction and purification procedures must be modified to diminish the loss of favipiravir content. For example, either magnetic stirrer extraction or ultrasound-assisted extraction (UAE) using ethyl acetate without partitioning with water was preferred.

After the identity of favipiravir was confirmed using spectral information obtained from both FT-IR spectroscopy and UV-visible spectrophotometry, the content (%w/w) or %purity of favipiravir secondary standard was estimated using the validated UV-visible spectrophotometry, which was found to be a specific, linear, accurate, precise, and easy-to-operate method for assaying of favipiravir tablets (Poonsawat, Wongpayoon, and Thanarangsarit, 2023). To assure the linearity of analytical method, a calibration curve of favipiravir reference standard was reconstructed and the result showed that Beer's law obeyed at the λ_{max} of 227 nm with good linearity ($r \geq 0.995$). However, the calculated quantity of favipiravir secondary standard was lower than the IMWP acceptance criteria. In addition to the requirement stated that the calculated content of favipiravir based on the anhydrous basis was specified (World Health Organization, 2021). Thus, determination of water content in the sample using Karl-Fischer titration method should be done for reasonable conclusion.

Unfortunately, although the estimated purity of favipiravir did not meet the expected criteria and may not replace the reference standard, at least 90% purity of the substance was still accepted to use for the purity test in qualitative analysis, such as thin-layer chromatography (TLC). Furthermore, any known substances with greater than 95% purity were allowed to be used in qualitative and quantitative analysis, such as liquid chromatography (LC) or gas

chromatography (GC) (World Health Organization, 2007). Accordingly, various analytical methods used to determine the purity of chemical reference substances have been described in the guideline, UV spectrophotometric method is occasionally used for assaying due to limitations on the detection of impurities which mostly share similar structural properties to the standard. For this reason, high-performance liquid chromatography (HPLC), as an official assay method, seemed to be recommended for the determination of favipiravir secondary standard purity. Remarkably, there was no data of impurities testing specified in the monograph. Therefore, a validated stability-indicating method should be developed and applied for simultaneous determining impurities and assay of favipiravir.

6. Conclusion

The developed favipiravir secondary standard obtained from the extraction and purification of powdered tablets contained 95.98 %w/w of favipiravir, calculated on as is basis. Application of the validated UV-visible spectrophotometric method for the determination of favipiravir content was reliable and easy to operate. Even though the purity of favipiravir secondary standard did not meet the specified criteria, further usage of this substance with assigned purity in some pharmaceutical QC procedures was also acceptable as a working standard.

7. Acknowledgements

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