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A Stability-Indicating HPLC Method for Favipiravir and its Related Substances

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Abstract

Background and Aims: This study aimed to improve an HPLC method for the quantification of favipiravir (FVP) and its impurities and apply it to a marketed pharmaceutical product.

Methods: Chromatographic separations were achieved on a C18 column. The mobile phase comprised 10 mM potassium dihydrogen phosphate buffer (pH 4.0):acetonitrile (90:10 v/v) and acetonitrile in the gradient mode. The peaks were detected at 238 nm and the flow rate was set at 1.5 mL/min. The optimised method has been validated as per the International Conference on Harmonisation (ICH) guidelines Q2 (R1). A sharp peak of FVP was obtained at 5.5 min with no interfering peaks. In addition, the degradation study was conducted under acidic, basic, oxidative, photolytic, and thermal stress conditions.

Results: In the calibration curve experiments, the linearity was between 0.5 – 3 µg/mL and r2=0.9985. Recovery ranging from 97.5% to 102.2% for the drug and impurities. The limit of detection (LOD) and limit of quantification (LOQ) concentrations of FVP and its impurities were 0.07 µg/mL and 0.2 µg/mL using s/n:3, respectively. The precision studies were carried out and the relative standard deviation (RSD) values were found to be between 1.01 and 1.65% and 0.67-1.75% for intra-day precision and inter-day precision; respectively.

No degradation peak appeared in the acidic hydrolysis, base hydrolysis, and photolysis stress studies. When the drug was subjected to thermal degradation, impurity B was observed. The degradation of the drug substances was obtained at 0.67% and 1.87% by thermal degradation and oxidation; respectively.

Conclusion: The stability-indicating chromatographic methods for the determination of FVP and related components were developed and applied in tablets.

Keywords Favipiravir · Pharmaceutical preparation · Stability-indicating · Validation

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INTRODUCTION

Coronavirus Disease 2019 (COVID-2019) outbreak in Wuhan City in December 2019 that became a pandemic rapidly. Coronovirus is a group of viruses categorised under alpha and beta coronavirus (Agrahari et al., 2021). Many treatment approaches and drugs have been investigated against COVID-19. Antiviral drugs are used in clinical trials, including lopinavir/ritonavir, FVP, remdesivir, oseltamivir, ASC09/ritonavir, (ASC09F), umifenovir (arbidol, abidol), ribavirin, darunavir, and cobicistat (Babaei et al., 2021).

FVP (6-Fluoro-3-hydroxypyzanine-2-carboxamide) is an antiviral pharmaceutical agent that was developed against the influenza virus by Toyama Chemical Co. Ltd (Toyama Chemicals. Summary of the Product Characteristics of Avigan). FVP inhibits the RNA-dependent RNA polymerase (RdRp) of RNA viruses (Furuta et al., 2013). FVP has an extremely good bioavailability (~94%) and a short half-life (2.5-5 h.) (Agraval et al., 2020).

According to the literature search, several analytical techniques, including HPLC-UV and spectrofluorometric methods, have been widely described and used for the analysis of pharmaceutical preparations. (Bulduk., 2020; Megahed et al., 2020, Gülşen & Ertürk Toker, 2024, Sangani & Patel, 2024). In this study, a novel HPLC method was developed and thoroughly validated for the simultaneous analysis of FVP and its degradation impurities. The method was optimised to ensure accurate and precise quantification of both the active pharmaceutical ingredient and its degradation products, which are crucial for assessing the stability and quality of the drug.

Literature review reveals that there is no identified impurity for FVP. Two basic impurities were determined after the investigation of the synthesis steps of the active pharmaceutical ingredient (API). The chemical structures are presented in Figure 1.

The aim of this work was to develop and validate an HPLC method according to ICH parameters. This developed method can be applied for FVP and related substances in a Favimol 200 mg Film Coated Tablet.

MATERIALS AND METHODS

Chemicals

FVP and its impurities were supplied by Honour Lab. Limited (India). The FVP pharmaceutical preparation was provided by a local drugstore. HPLC analytical grade chemicals and reagents were purchased from Merck, E. Merck A.G. Darmstadt (Germany).

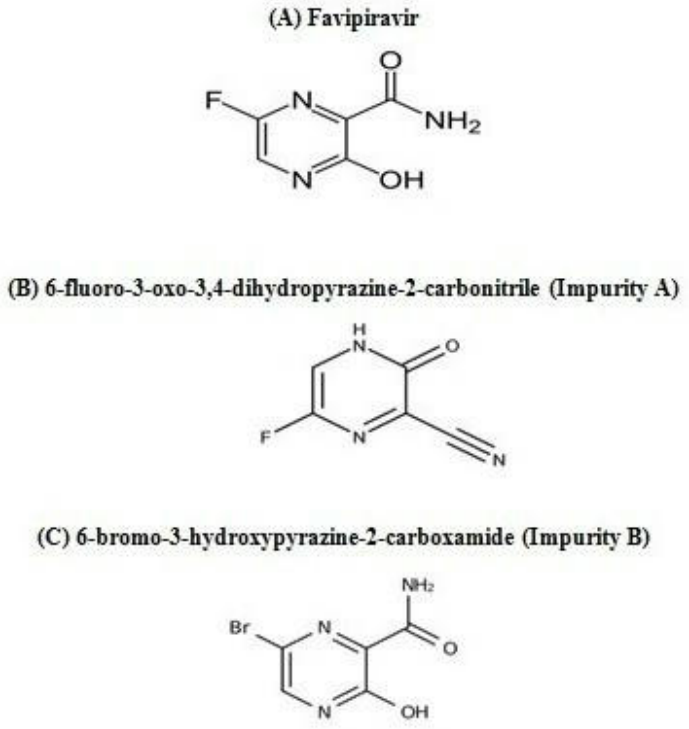

Figure 1. Chemical structures of favipiravir and its related substances (PubChem)

The Chromatographic Conditions

The HPLC system consisted of a Shimadzu (Japan) equipped with an autosampler system model SIL-20ACHT, a pump model LC-20AT, a column oven model CTO-10ASP, and a detector model SPD-M20A. A PDA detector was used for the wavelength monitoring of the standard solution FVP and its impurities.

Analysis and separation was performed on a column of InertSustainSwift C18 (250 mm x 4,6 mm, 5 µm) using a gradient combination of 10 mM potassium dihydrogen phosphate buffer (pH 4.0):acetonitrile (90:10 v/v) and acetonitrile at an isocratic flow rate of 1.5mL/min. The HPLC gradient program is presented in Table 1. The column temperature was 25˚C. The eluent was monitored at 238 nm.

Table 1. The HPLC gradient program

Time (minutes)	*B% concentration*
0	2
1.5	2
2	10
7	10
8	2
15	2

B: Phase B (Acetonitrile concentration)

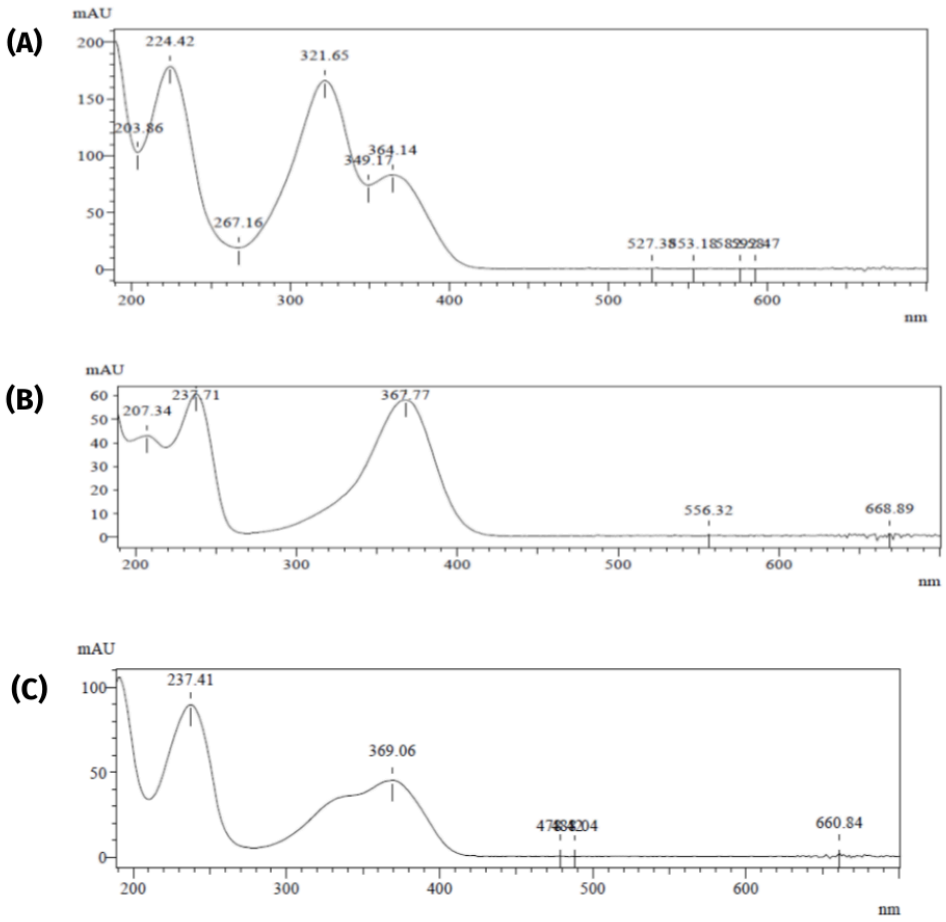

Figure 2. Spectrum of favipiravir (A), impurity A (B), and impurity B (C).

Preparation of The Standard Solutions

Two milligrams of FVP, 2 mg impurity A and 2 mg impurity B were weighed and dissolved in approximately 20 mL mobile phase A and diluted to 50 mL with the same solvent. This working standard solution was obtained 0.002 mg/mL concentration for all compounds.

Portions of 0.002 mg/mL FVP solution, 0.002 mg/mL impurity A solution, 0.002 mg/mL impurity B solution, and 0.002 mg/mL working standard solution were injected into the chromatographic system.

Sample Preparation

The amount of pharmaceutical preparation (Favimol 200 mg Film Coated Tablet) equivalent to 50 mg FVP was weighed and added in about 10 mL mobile phase A and sonicated for 30 minutes. The final concentration was obtained at 2 mg/mL after dilution to volume.

Selection Of The Working Wavelength

The wavelength for monitoring was selected by scanning the standard solution of FVP and its impurities using a PDA detector. The optimum monitoring condition was observed at 238 nm (Figure 2) for the developed method. No significant interference peaks were observed, and the chromatographic peaks of both FVP and its impurities were clearly identified.

Method Validation

The optimised method was validated according to the ICH Q2 Guideline (2023) for evaluating system linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and robustness.

Linearity

Five different standard solutions of FVP and its impurities were prepared within the concentration range of 0.5-3 µg/mL. The regression line was assessed using statistical methods and the calculated coefficient of correlation, slope and intercept.

LOD and LOQ

The standard solution was diluted several times and injected these solutions sequentially. Signal/noise (S/N) values were measured for calculating the LOD and LOQ by using the standard method. LOD and LOQ values determined using these equations:

$$LOD = 3 \times S/N \qquad LOQ = 10 \times S/N$$

Accuracy

The standard stock solution and sample solution were used to prepare for 1 µg/mL, 2 µg/mL and 3 µg/mL. Standard solutions were spiked with the sample solutions and injected for the accuracy studies. The area was calculated for the recovery percentage.

Precision

The intra-day precision, also known as repeatability, was assessed by analysing the calibration curves of the five standard solutions. The inter-day precision was determined over three days. The precision was expressed as the relative standard deviation (RSD%).

Robustness

The robustness of the analytical method was determined by evaluating the effect of small variations in the method parameters such as the pH of the mobile phase, column temperature, and detection wavelength. pH of mobile phase was changed ±0.1. The column temperature was changed ± 2^{0}C. Detection wavelength was changed from 235 nm to 240 nm. The solution stability of the standard solution was determined at ambient conditions for 48 h. The robustness of the method was performed as per the standard guidelines.

Forced Degradation Studies

The stress degradation studies were carried out under various stress conditions in accordance with the ICH guidelines Q1A (R2). Forced degradation was performed under the following conditions: acidic (0.1 N HCl at room temperature for 1 h), basic (0.1 N NaOH at room temperature for 1 h), thermal (at 80^{0}C for 1 h), oxidation (0.3% v/v H_2O_2 at room temperature for 1 h), and photolytic (at 360 nm for 12 h). Degradation samples were analysed using this developed method. A peak purity test was performed for all degradation samples using a PDA detector.

RESULTS

Chromatographic Conditions

Many trial-and-error methods were tried for the optimisation of chromatographic conditions. Methanol and acetonitrile were tried with the buffer solution for the selection of the mobile phase. The obtained peaks were tailed by using methanol with the buffer solution. A binary mixture of 10 mM potassium dihydrogen phosphate pH 4.0 : acetonitrile (mobile phase A) (90:10 v/v) and acetonitrile (mobile phase B) was used at a flow rate of 1.5 mL/min. Literature search and some experimental trials showed that FVP was slightly soluble in water. Mobile phase A was tried as the solution. Mobile phase A was more soluble than water for FVP and its impurities. Chromatographic separation was performed using a C18 column with a separation gradient program (Table 1). In the optimisation of the chromatographic method, system suitability parameters such as resolution (R), theoretical plates (N), the tailing factor, and the retention time (tR) were evaluated. Results are presented in Table 2.

Table 2. System suitability results

Compound	Retention time (minutes)	Number of theoretical plates	Tailing factor	Resolution
Favipiravir	5.531	7828	1.132	-
impurity A	5.902	3157	1.138	2.094
impurity B	6.854	3003	1.034	4.171

Within the determined analysis conditions, the retention times of FVP, impurity A, and impurity B were determined as 5.5 min, 5.9 min, and 6.8 min, respectively. Superior resolution and sharp peaks were obtained, and all compounds were detected. The PDA detector was used for wavelength optimisation, and 238 nm was selected as the optimal wavelength. Typical chromatograms obtained using this developed method are shown in Figure 3.

Linearity

The regression lines were plotted using peak area versus concentration data through the method of least squares analysis. The concentration range study was conducted between 0.5 and 3 µg/mL, with correlation coefficient values of 0.9985 for FVP and its related impurities, as shown in Table 3.

LOD and LOQ

The LOD and LOQ concentrations of FVP and its impurities were 0.07 µg/mL and 0.2 µg/mL using S/N:3, respectively.

Precision

The precision studies were carried out for three consecutive days to analyse the quantities of FVP and its impurities. The RSD values were determined to be between 1.01% and 1.65% for intraday precision and between 0.67% and 1.75% for inter-day precision. Detailed precision data are given in Table 3.

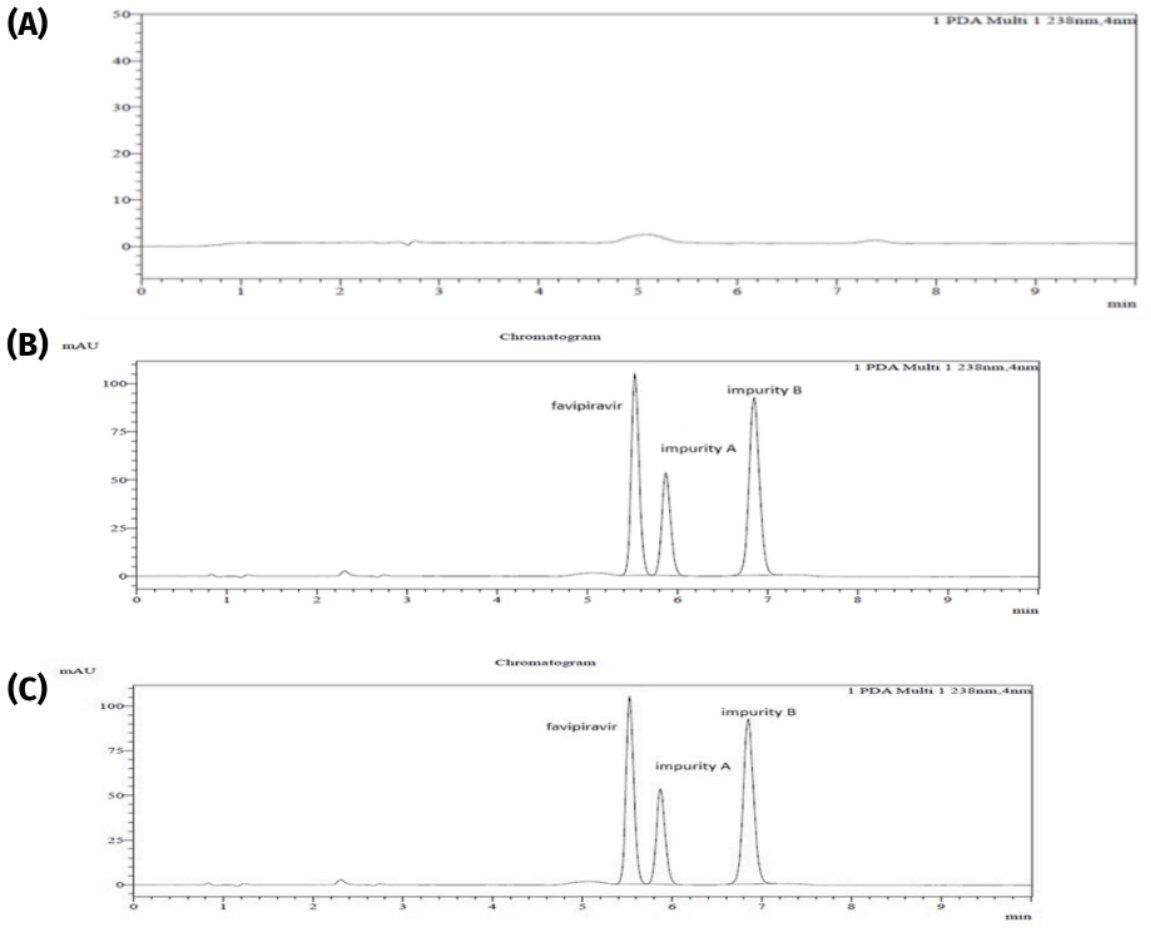

Figure 3. Representative chromatograms of (A) diluent , (B) placebo, and (C) favipiravir standard

Table 3. Results of linearity, precision, LOD, and LOQ.

Parameter	Favipiravir	Impurity A	Impurity B
Slope	297538874	175127282	342277564
Intercept	23244	14623	29424
Correlation coefficient	0.9985	0.9985	0.9985
Linearity range (µg/mL)	0.5-3	0.5-3	0.5-3
LOD	0.07	0.07	0.07
LOQ	0.2	0.2	0.2
Intraday precision (SD)[a]	0.04	0.02	0.02
Intraday precision (RSD)[a]	1.65	1.02	1.01
Interday precision [a] (SD)[b]	0.01	0.04	0.04
Interday precision [a] (RSD)[b]	0.67	1.75	1.73

[a]n=5, [b]Results of three different days

Accuracy

The accuracy of the procedure for FVP and its impurities was determined using the standard addition technique. The recovery was calculated by comparing the theoretical concentration with the experimental concentration. The percentage recoveries for the drug and impurities ranged from 97.5% to 102.2%. The relative standard deviations were calculated for each level. Table 4 shows the recoveries of FVP and its impurities.

Table 4. Recovery results for favipiravir and its impurities

Compound	Total amount found [a](µg/mL)	Recovery (%)	SD	RSD[b] (%)
Favipiravir	1.13	100.6	1.53	1.52
	2.08	100.2	1.15	1.15
	3.14	101.4	0.54	0.53
Impurity A	1.04	100.3	1.55	1.54
	2.15	99.3	1.00	1.00
	3.09	101.5	0.41	0.41
Impurity C	1.03	98.5	0.80	0.81
	2.15	98.6	1.38	1.40
	3.18	101.5	0.30	0.29

[a]n=6, [b]Relative standard deviation

Robustness

Predetermined variations were performed under the experimental conditions to assess their robustness. Changes were pH (±0.1), column oven temperature (±2°C) and in the detected wavelength (235nm and 240 nm). Any of the modified

chromatographic conditions had not a significant effect on the results. The obtained results are given in Table 5.

Table 5. Results of the robustness study

Conditions	Impurity A (%)	Impurity B (%)	SD
Validation conditions	0.095	0.105	0.11
Mobile phase (pH 3.9)	0.101	0.108	0.15
Mobile phase (pH 4.1)	0.106	0.110	0.22
Column temperature (23ºC)	0.098	0.115	0.26
Column temperature (27ºC)	0.103	0.104	0.24
Wavelength (235 nm)	0.094	0.105	0.19
Wavelength (240 nm)	0.092	0.104	0.20

Stability

The standard solution was stored at room temperature (25°C) for 48 h and tested using the developed method periodically. All of the solution stability studies demonstrated that the standard solution was stable in mobile phase A for 48 h at room temperature. The obtained results are given in Table 6.

Table 6. Table of standard solution stability (room condition for 48 h)

Time (h)	Favipiravir (µg/mL)	Impurity A (µg/mL)	Impurity B (µg/mL)
0	2.27	2.11	2.06
1	2.28	2.11	2.05
3	2.26	2.11	2.05
9	2.27	2.12	2.06
24	2.27	2.11	2.05
48	2.26	2.13	2.04
SD	0.01	0.01	0.01
% RSD	0.33	0.40	0.37

Analysis of the commercial drugs

The method was applied in a commercial tablet, and the obtained results are given in Table 7.

Table 7. Favipiravir assay on commercial pharmaceutical products

Active ingredient	Recovery[a] (%)	SD	RSD (%)
Favipiravir	99.58	0.47	0.31

[a]n=5

Forced Degradation Studies

Forced degradation samples were analysed under the conditions described above, and FVP and its impurities were determined using PDA detector. No significant degradation was observed in the acidic hydrolysis, base hydrolysis, and photolysis stress studies. When the drug was subjected to thermal degradation, impurity B was observed. The degradation of the drug substances was obtained at 0.67% and 1.87% by thermal degradation and oxidation; respectively (Table 8).

Table 8. Forced degradation results

Stress Condition	Degradation (%)	Purity Threshold
Acid degradation	-	1.000
Base degradation	-	1.000
Oxidation	0.67	1.000
Thermal stress	1.87	1.000
Day light degradation	-	1.000

DISCUSSION

In this study, a highly specific, precise, accurate, and robust analytical method was developed and validated for the determination of favipiravir and its related impurities in Favimol 200 mg Film Coated Tablets. The method was designed to ensure that both the active pharmaceutical ingredient (API) and its potential degradation products could be effectively and efficiently quantified in the tablet formulation. The mobile phase compositions and other analytical conditions were meticulously determined based on an extensive review of the relevant literature and systematic experimental trials. (Bulduk., 2020; Megahed et al., 2020, Gülşen & Ertürk Toker, 2024, Sangani & Patel, 2024). These conditions were fine-tuned to optimise the separation and detection of Favipiravir and its impurities while minimising interference from other components in the tablet matrix.

A key feature of the developed method is its simplicity and rapidity. The analysis is conducted within a short run time, which not only improves efficiency but also facilitates quick comparisons with other published HPLC methods for related substance analysis, including those outlined in China patents CN104914185A and CN104914185B (Guangling et al., 2015 and Guangk ing et al., 2016).

Furthermore, extensive degradation studies were carried out to evaluate the stability of favipiravir under various stress conditions, including alkaline, acidic, oxidative, and thermal environments. These studies are crucial for understanding the stability profile of the drug and for ensuring that any degradation products are adequately accounted for in the analytical process. The results demonstrated that all degradation products formed under these stress conditions, as well as any process-related impurities, were successfully separated from the drug substance. This is particularly important for ensuring the accuracy of the analysis and for guaranteeing that any potential impurities do not interfere with the measurement of the active ingredient.

CONCLUSION

This method offers a comprehensive solution for the analysis of favipiravir and its impurities in pharmaceutical formulations, and it can be effectively used not only for routine quality control but also for stability studies required by regulatory authorities. The simplicity, speed, and robustness of the method make it a promising tool for pharmaceutical manufacturers and researchers involved in the development and production of favipiravir-based therapies.

Peer Review Externally peer-reviewed.
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Conflict of Interest The authors have no conflict of interest to declare.
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Toyama Chemicals. Summary of Product Characteristics of Avigan.

Favipiravir | C5H4FN3O2 | CID 492405 - PubChem

6-Fluoro-3-oxo-3,4-dihydropyrazine-2-carbonitrile | C5H2FN3O | CID 22662785 - PubChem

6-Bromo-3-hydroxypyrazine-2-carboxamide | C5H4BrN3O2 | CID 9794418 - PubChem