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Original Article

Quantification and Spectral Interference Studies of Selected Weak Chromophore Molecules Using ATR-FTIR Spectroscopy with Regression Tools

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ABSTRACT

Background: Weak chromophore molecules present challenges in analytical method development for routine quality control testing. This study focuses on three such molecules: favipiravir and molnupiravir, antiviral drugs repurposed for COVID-19 treatment, and etidronate disodium, used for osteoporosis and multiple myeloma. Existing analytical methods face limitations due to their non-chromophore structures. **Objectives:** To address this, we developed three non-destructive ATR-FTIR (Attenuated Total Reflectance Fourier Transform Infrared) spectroscopic methods for purity assessment. **Materials and Methods:** Infrared spectra were scanned in the full IR region (4000-667 cm^{-1}), with calibration curves generated via TQ Analyst Pro software using partial least squares regression. The fingerprint regions (1393-1062 cm^{-1}) for favipiravir; Near-IR regions (1700-667 cm^{-1}) for molnupiravir and etidronate disodium) demonstrated excellent linearity ($r^2 \approx 0.9998$-1.000). **Results:** The residual mean standard errors of calibration was <0.0025 for three active molecules, and intra-day/inter-day precision had relative standard deviations less than 5% after validation as per ICH guidelines. **Conclusion:** Interference studies confirmed that no spectral overlaps with inactive ingredients after comparing both placebos with and without all three above drugs, allowing potential application of this method for routine analysis of the formulation. These methods offer a sensitive, specific, and accurate and alternative method for quantitative analysis of weak chromophore molecules.

Keywords: Attenuated total reflectance infrared spectroscopy, Etidronate, Favipiravir, Molnupiravir, Quantification, Validation.

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INTRODUCTION

Chromophore molecules

A chromophore is a structural part of a molecule responsible for absorbing Ultraviolet-visible (UV-vis) light. Upon excitation, this region undergoes significant changes in geometry or electron density. In contrast, non-chromophore molecules lack such structural units, and their residual parts do not participate effectively in the absorption process. As a result, these molecules either do not absorb or only weakly absorb UV-visible light. This is due to the lack of effective electronic transitions between the excitation centre and the rest of the molecule, as seen in compounds such as molnupiravir, favipiravir, and etidronate disodium. When two chromophores are present in proximity within a molecule, they can influence both the position and intensity of the absorption band. If their interaction is weak, the overall absorption remains limited, and the chromophores are considered weak contributors. In complex molecules with multiple chromophore regions, numerous localized transitions occur, complicating the absorption pattern. These types of molecules present significant challenges for analytical detection using High Performance Liquid Chromatography (HPLC) with UV detection, especially when they are non-fluorescent. To enable effective analysis, chemical derivatization is often required to introduce chromophore groups. However, this process increases the complexity, time, and cost of the analytical method, and often suffers from poor reproducibility.[1]

Molnupiravir

Molnupiravir (MLN) is an antiviral drug indicated for the treatment of mild to moderate COVID-19 infections. MLN was formerly studied for management of influenza but acquired emergency use authorization by Food and Drug Administration

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(FDA) on 2021 after the emergence of COVID-19 pandemic.[2] The drug plays unique mechanism of inhibition termed as viral error catastrophe which cause excessive mutation of the viral genome until it becomes lethal to the virus.[3] The chemical structure of MLN is shown Figure 1 comprising of ester, hydroxyl group and amine which are attached to the pyrimidine ring with their strong stretching vibrations in their infrared spectrum, especially Specific peaks were identified as C=O bond from ketone at 1684 cm^{-1}, N-H stretching at 1638 cm^{-1} and C-N stretching at 1190 cm^{-1}.

MLN is soluble in organic solvent such as Dimethyl Sulfoxide (DMSO) and Dimethyl Formamide (DMF) with solubility of approximately 30 mg/mL. It is also slightly soluble in methanol and ethyl acetate. The molecular weight of MLN is 329.31g/mole with the melting point range between 156-159ºC.[4] MLN is stable both chemically and physically under long term and accelerated and light conditions following ICH guidelines.[5]

Several quantitative analyses of MLN using various methods had been reported and validated. This includes the classic approaches such as RP-HPLC, LC/MS-MS, UV-Visible spectroscopy, and fluorescence spectroscopy.[6-9] However, these methods were associated with many limitations such as optimization of many validation parameters and method complexity, expensive, use of multiple toxic solvents, longer analysis times and deal with skillful and dedicated technicians to handle and obtain reproducible and accurate results.

Favipiravir

Favipiravir (FVP) is an antiviral drug which is initially licensed for the treatment of emerging influenza viral infections.[10] It is marketed under the brand name Avigan Tablets 200mg. Its pure form appears as white to light-yellow fine powder and is odourless. FVP is slightly soluble in water and ethanol, and sparingly soluble in methanol and acetonitrile.[11] The chemical structure of FVP is shown in Figure 2 with functional groups such as alkyl Fluoride (C-F), amide (O=C-N-H), pyrazine ring, amine (N-H) and Hydroxyl (O-H). Being a prodrug, it undergoes intracellular phosphoribosylation to turn into its active form before exerting its antiviral effect. Then, it works by selectively inhibiting the Ribonucleic Acid (RNA) polymerase of RNA viruses, hence leading to termination of viral protein synthesis which in turn suppresses the viral reproduction.[12,13]

Due to its antiviral activities against a wide range of RNA viruses, including SARS-CoV-2, Favipiravir (FVP) is now repurposed to be used in the treatment of COVID-19.[14] The main reason for drug repurposing is to accelerate the new drug discovery process to halt the progression of widespread disease.[15] Additionally, it is reported that FVP has demonstrated its antiviral activity against SARS-CoV-2 in both *in vitro* and *in vivo* studies with the promising results from the clinical studies.[16] FVP has been approved in the clinical practice of COVID-19 treatment in a few countries such as Russia, Saudi Arabia, and Thailand. There are also ongoing clinical trials in other countries such as China and Japan to explore the efficacy of FVP over other antiviral drugs in COVID-19 patients.[17] The increasing demand of FVP in the current COVID-19 pandemic has made it imperative to establish a reliable and fast analytical method to examine the purity of FVP.[18] With the latest advancement of analytical instruments, development and validation of new analytical methods is gaining attention widely.[19] In general, analytical method plays an essential role in discovery, development, evaluation, and quantification of new medicines in the pharmaceutical industry.[20] Before developing a new analytical method, it is important to predefine the purpose of analytical method, whether qualitative or quantitative, so that proper analytical instruments and methodology are adopted. To begin method development, the methodology must be modified from the established literature and the procedures should be performed accordingly. A well-developed analytical method serves as a basis to acquire high-quality analytical results which in turn important and support its routine use in quality control and quality assurance of drugs.[21] Hence, the method validation should come after method development to demonstrate the quality and applicability of the method for its intended purpose.[22]

Etidronate Disodium

Etidronate disodium (ET) belongs to the class of Bisphosphonate drugs, which inhibits osteoclast action and the resorption of bone. It is useful in the treatment of certain bone diseases (hypercalcemia of malignancy, Paget's disease, and osteoporosis). It is chemically classified as non-nitrogenous bisphosphonates. It is highly polar compound with no chromophore groups and cannot absorb in the UV-Region and exhibit non-fluorescence. The chemical structure of ET is as shown below Figure 3.

Attenuated Total Reflectance Fourier Transform Infrared (ATRFTIR) Spectroscopy study of the vibrational spectra of three biphosphonic acids [Etidronic acid (ED), nitrilotris (Methylenephosphonic Acid) (NTMP) and N,N-bis(2-hydroxyethyl)Amino Methyl Phosphonic acid (BHAMP) in aqueous solution] was studied in the range of buffers (pH 5-9), and bands assignments were given in the range of 2000-890 cm^{-1} wavenumber. Phosphonates have shown characteristic spectral bands due to the P-O stretching vibration at 1200-900 cm^{-1} wavenumber. The study was focused on midinfrared region, which shows important changes with change in pH, specially the ν(P-OH) at ~925 cm^{-1} and ν(PO_3^{2-}) at ~970 cm^{-1} vibrations. It was also investigated and reported that FTIR analysis revealed the evidence for the zwitterionic nature of BHAMP and NTMP in solution with a strong indication that the zwitterion in both compounds remains intact throughout the pH range investigated.[23]

A reversed-phase HPLC method with indirect UV detection was reported for the determination of etidronate disodium in bulk and

in tablet dosage forms. The method was used sodium salicylate as a probe component to the mobile phase. Etidornate was detected monitoring the decrease in the elution with background signal at 210 nm. The linearity of the method was studied over the concentration range of 50-300 µg/mL. Calibration plots of the area of negative analyte signals plotted against the concentration showed a high value of the correlation coefficient (0.999), indicating a good linearity of the proposed method. The method was also successfully extended for the analysis of alendronate sodium trihydrate and clodronate disodium tetrahydrate.[24]

A direct, and stability-indicating method for analysis of etidronate was reported without a UV chromophore. A mixed-mode column was selected to elute the etidronate from its impurities in an 8-min gradient method using a Charged Aerosol Detector (CAD) for detection. The method can be used for release and stability testing of etidronate and has applicability to other similar bisphosphonate compounds.[25]

Hence, the interest has now shifted to Fourier Transform Infrared (FTIR) spectroscopy which possess the capability and feasibility for quantitative analysis comparable with the methods. Recent advancement in FTIR featuring Attenuated Total Reflectance (ATR) had added with many favourable advantages such as simple operation, economical and non-destructive property.[26] These are achieved through the exclusion of novel procedure, Potassium Bromide (KBr) disc preparation requiring careful technique to obtain clear discs which will be reflected as perfect spectra.[27] General ATR-FTIR procedure allows for small sample portion from drug and KBr mixture to be directly measured to gain accurate quantification of the analyte in addition to the information regarding the chemical structure. The simplified method has shortened the overall analysis time while eliminating the use of press machines for KBr discs. It also reduces the use of toxic chemical reagents which are harmful to both human health and environment thus considered as green analytical method.[28]

Partial Least Squares (PLS) regression tool is one of the most common multivariate calibration methods for data analysis and developing quantitative models. PLS algorithm advances in capturing the maximum variances among both predictor and predicted variables while providing maximum correlation among them. In the context of spectroscopy, PLS algorithm is useful to correlate between spectral intensity and concentrations of analyte in establishing the best calibration curve. Hence, this study aims to develop new analytical method for MLN using ATR-FTIR spectroscopy with PLS algorithm which is rapid and simple to assist the analysis of MLN in conjunction to other methods.[29]

ATR-FTIR coupled with chemometrics tools became an alternative solution for qualitative and quantitative analysis of MLN, FVP, and ET. The ATR-FTIR sampling technique is fast, simple and produces no waste products. The present work describes a simple, rapid, inexpensive, and non-destructive ATR-FTIR-PLS method for the routine analysis of MLN, FVP and ET.

MATERIALS AND METHODS

Materials

Molnupiravir standard (≥ 99.0%), Favipiravir standard (99.0%), and Etidronate Disodium (99.0%) were obtained Hetero Drugs Group (India). Infrared (IR) grade potassium bromide (99.999%) and ethanol (95%) were purchased from Merck Company (Darmstadt, Germany).

Methods

Measurement of ATR-FTIR Spectra

Spectra were recorded with Nicolet™ iS5 FTIR spectrometer controlled by OMNIC software for spectra collection and TQ Analyst software 7.2 (Thermo Scientific, Madison, Wisconsin, USA) for data processing. The instrument is equipped with an iD5 ATR accessory featuring a top plate diamond crystal with a fixed angle of incidence of 42°. ATR-FTIR spectra were obtained in the 4000-600 cm^{-1} spectral region. All spectra were recorded at 4 cm^{-1} resolution with an average of 20 scans per spectrum. Ethanol was used to clean the diamond crystal of the ATR accessory before the application of each sample. The background spectrum of air was collected before the measurement of the spectrum of each sample. All measurements were taken at ambient temperature and samples were kept in an amber glass jar in a desiccator to protect them from absorbing moisture.

ATR-FTIR Calibration and optimization

Calibration standards of MLN, FVP, and ET ranging from 10-100% w/w were prepared by accurately weighing different amounts separately and mixing it with weighed amounts of Infrared (IR) grade KBr to get a total weight of 30 mg of each working standard. The standards and KBr were mixed in mortar till homogenization and vortex. All standards of MLN, FVP and ET were scanned in the spectral region of 4000-667 cm^{-1}. All spectra of calibration standards were processed using the TQ analyst software following the PLS regression. The baselines of the Full spectrum calibrations were corrected from 4000-667 cm^{-1}. The validity of the PLS model was based on the Root Mean Square Error of Calibration (RMSEC) and the Correlation Coefficient (r^2) values.

Validation and Accuracy of the ATR-FTIR Method

ATR-FTIR method was validated based on International Conference on Harmonization (ICH) guidelines on validation of analytical procedures.[30] The validation parameters include linearity, precision, the limit of detection, quantification, and accuracy. The linearity, LOD, and LOQ were determined as described.[31] The precision of the developed ATR-FTIR method by inter- and intra-day variations was evaluated by using the 10%

w/w and 60% w/w calibration standards. Intra-day (repeatability) was determined by four measurements of each concentration on the same day. Inter-day (reproducibility) precision was determined by the measurement of each concentration once a day for four consecutive days. The precision was expressed as the Relative Standard Deviation (RSD). The accuracy was defined as percentage Relative Error (%RE) content in preformulating samples of MLN and FVP were determined by directly applying it on the ATR accessory to get the corresponding FTIR spectrum.

RESULTS

Wavenumber Selection

ATR-FTIR is simple, fast, and non-destructive and efficient tool for the determination of active pharmaceutical ingredients.[32,33] It is useful for the determination of chemical products of natural origin for the detection of quality and adulteration of botanical products.[34-37] and quantification of bioproducts.[38,39] The quantification could be carried out in a simple manner taking advantage of the functional groups present in the chemical structure of the analyte of interest. This is done by selecting a band at which the molar absorptivity is high and there is no interference or overlapping between the selected band and other bands that could be found in the spectrum. This allows rapid determination of active pharmaceutical ingredients without the need for complex sample preparation by excluding the need for the extraction of the analyte in question.[40-42] To achieve this, there should be some specific chemical groups that result in unique bands and there is no need for the presence of a double bond conjugation system (chromophore) which is required for ultraviolet-visible spectroscopic analysis. However, less chromophore molecules like MLN, FVP and ET possess specific functional groups (C-NH-OHMLN; C-F, CO-NHFVP; C-OH, C-CH_3ET) where they absorb at unique stretching vibration that could be used to achieve higher absorptivity bands to carry out quantitative determination by this non-destructive technique.

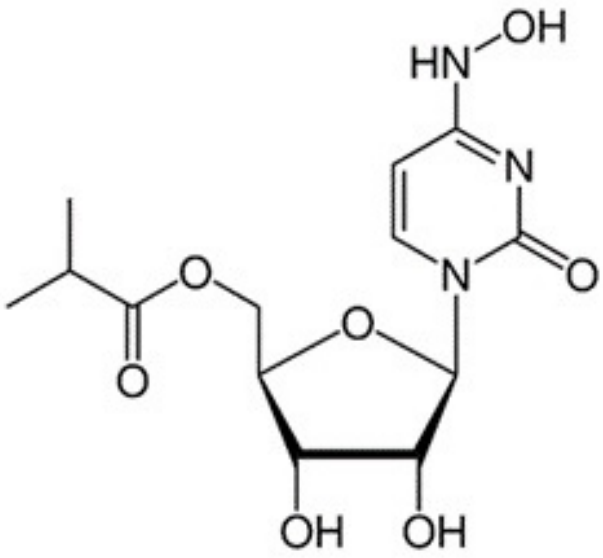

Figure 1: Molnupiravir chemical structure.

Molnupiravir (Mln)

Optimization of linearity for MLN

The FTIR spectra of MLN showed focusing on the 1700-667 cm^{-1} region, the identification of MLN was made as the significant peaks being analysed. Specific peaks were identified as C=O bond from ketone at 1684 cm^{-1}, N-H stretching at 1638 cm^{-1} and C-N stretching at 1190 cm^{-1} in Figure 4a. The overlay spectrum of different calibration standards of MLN was shown in Figure 4b.

The calibration curve of molnupiravir was established between 10-70% w/w. The linear regression coefficient (r^2) was as close to 1.00 as shown in Figure 4c below. The perfect linear fit was established at sensitive concentrations in the spectral range especially at 1684 cm^{-1} for carbonyl (C=O) stretching vibrations.

Favipiravir (FVP)

Establishment of Calibration curve for FVP

The FTIR spectra of FVP is showed focusing on the 1700-667 cm^{-1} region, the identification of FVP was made as the significant peaks being analysed. Specific peaks were identified as O-H bond from hydroxyl at 3200 cm^{-1}, C=O stretching at 1700-1630 cm^{-1} and C-F stretching at 1400-1000 cm^{-1} and strong amide bending frequency 980-960 cm^{-1}. The overlay spectrum of different calibration standards of FVP was shown in Figure 5a.

The serial concentrations in the range of 10-80% w/w of FVP were obtained using Potassium Bromide (KBr) as a diluent. A standard calibration curve, with the r^2 value of 0.9998 was obtained as

Table 1: Results of validation parameters for infrared method for Molnupiravir (MLN), Favipiravir (FVP), Etidronate disodium (ET) established. Results were shown significant and sensitive.

Drugs	MLN	FVP	ET
Spectral region	1700-1000 cm^{-1}	1700-667 cm^{-1}	1160-900 cm^{-1}
Number of Factors	5	5	5
RMSEC	0.00035	0.0013	0.00235
Calibration range (%w/w)	10-70	10-80	10-90
R^2 Value	1.0000	0.9998	1.0000
Limit of Detection (%w/w)	0.500	0.500	0.500
Limit of Quantification (%w/w)	2.000	1.500	1.000

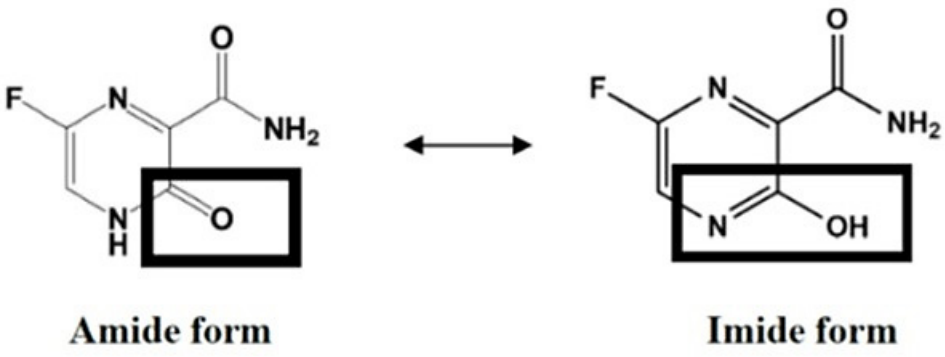

Figure 2: Chemical Structure of Favipiravir and its amide-imide tautomerism.

Table 2: Intraday and Interday precision results for Molnupiravir (MLN), Favipiravir (FVP), Etidronate disodium (ET).

Concentration (%w/w)		Precision (%RSD)
Intra-day (*n*=3) 10 60	MLN	3.45 0.78
Intra-day (*n*=3) 10 60	FVP	4.53 3.53
Intra-day (*n*=3) 10 60	ET	2.06 1.34
Intra-day (*n*=3) 10 60	MLN	6.40 4.37
Intra-day (*n*=3) 10 60	FVP	7.23 6.78
Intra-day (*n*=3) 10 60	ET	3.41 2.34

shown in Figure 5b. The corresponding linear regression equation was:

y=0.9998x + 0.0133 s

Shown in Figure 5c. In this case, if the r^2 value is approached to 1.00, reflecting a perfect linear relationship between the concentrations and the absorbance, indicates that the variation of analytical signal as a function of its concentration is sensitive.[43]

Etidronate disodium (Ed)

Linearity of ED standards

Different concentrations of ED from 10-90% w/w were prepared using Potassium Bromide (KBr) as a diluent and scanned all samples using ATR-FTIR as direct measurement. All spectra are processed at specific bands of functional groups C-OH, C-CH_3 particularly O-H stretching band at 1160 cm^{-1}, bending vibration in the range of 930-900 cm^{-1} and C-O stretching vibration at wave number at 1050 cm^{-1}. The overlay infrared spectra of ET (10-90% w/w) were overlapped to show the linearity with increased absorbance as shown in Figure 6a. The linear regression r^2 value was obtained 1.00 and the linear concentration range was established between 10-90% w/w shown in Figure 6b.

DISCUSSION

The development of the ATR-FTIR method for the determination of MLN, FVP and ET was based on partial least squares regression. This statistical tool can assess the spectral purity of individual spectra to quantify active pharmaceutical ingredients in a pharmaceutical formulation with complex excipients components. The initial step in this regression method was to select the spectral region that could provide the best prediction between actual values of MLN, FVP and ET with interpreted values by using the PLS regression calibration model followed by the prediction step to determine the concentrations of sample components spectra from the calibration results.[44,45]

The results were shown the best calibration results in the fingerprint regions (1393-1062 cm^{-1}) for favipiravir; near-IR regions (1700-667 cm^{-1}) for molnupiravir and etidronate disodium and they were demonstrated excellent linearity (r^2 ≈ 0.9998-1.000) in both regions for three weak chromophore molecules like MLN, FVP and ET. The calibration plots for three molecules were indicated an excellent agreement between the absorbance in the selected spectral region and concentration of the MLN, FVP and ET separately. The Residual Means Standard Error of Calibration (RMSEC) values were 0.00035, 0.0013 and 0.00235 respectively (less than 0.0025) for the comparison of the actual and computed concentration for the standards which demonstrated the accuracy and precision of the calibration at the specific spectral region. Low RMSEC values in this PLS algorithm values indicated better accuracy and excellent performance of this model.[46-47] This was considered as validated method and sensitive enough to carry out quantitative analysis of MLN, FVP and ET which lack double bond conjugations system making it unsuitable for direct UV detection. The calibration results of the ATR-FTIR method were shown in Table 1.

The calculated LOD and LOQ were computed in Table 2 with the validation results of the ATR-FTIR method. The precision was expressed in percent relative standard deviation (%RSD). The %RSD values for intra-day and inter-day precision were less than 8% indicating good repeatability of the proposed method.

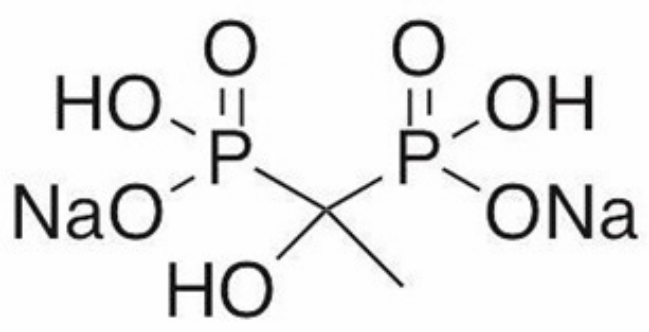

Figure 3: Chemical Structure of Etidronate disodium.

The accuracy was defined as percentage relative error (RE). The percentage relative error values ranging from 0.52-5.60% were obtained which indicate accuracy of the proposed method AOAC.[48]

INTERFERENCE AND QUNATIFICATION STUDIES OF FAVIPIRAVIR AND MOLNUPIRAVIR IN PREFORMULATING SAMPLES

Favipiravir

The infrared spectrum of excipient mixture was shown in Figure 7a. This spectrum was compared with the IR spectrum of 10% w/w favipiravir added with excipients as shown in Figure 7b to investigate the possibility of spectra interference due to presence of excipients in the sample. It was shown that most significant peaks remained present and not overlapped. Hence, the method

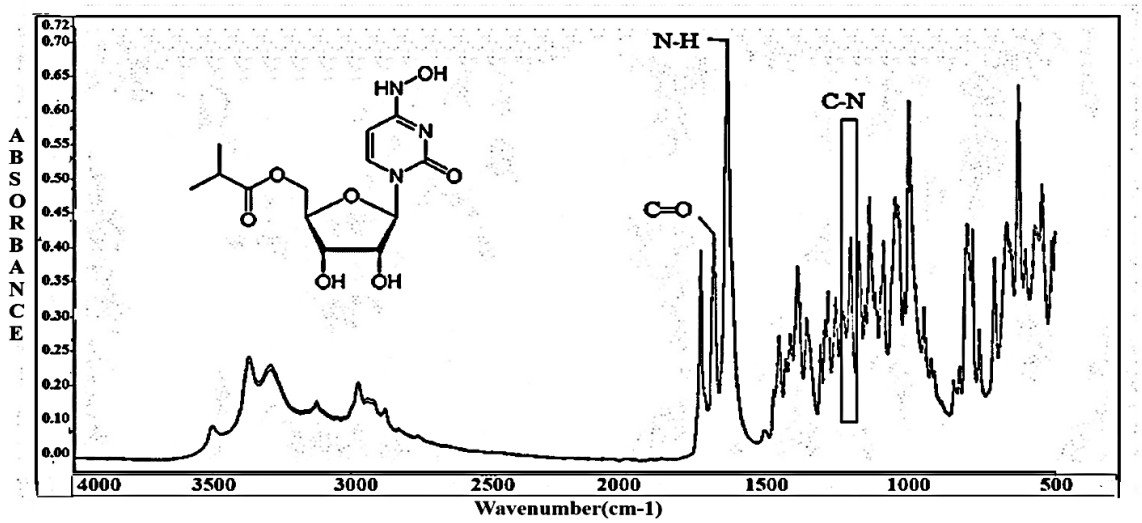

Figure 4a: ATR-FTIR Spectrum of molnupiravir.

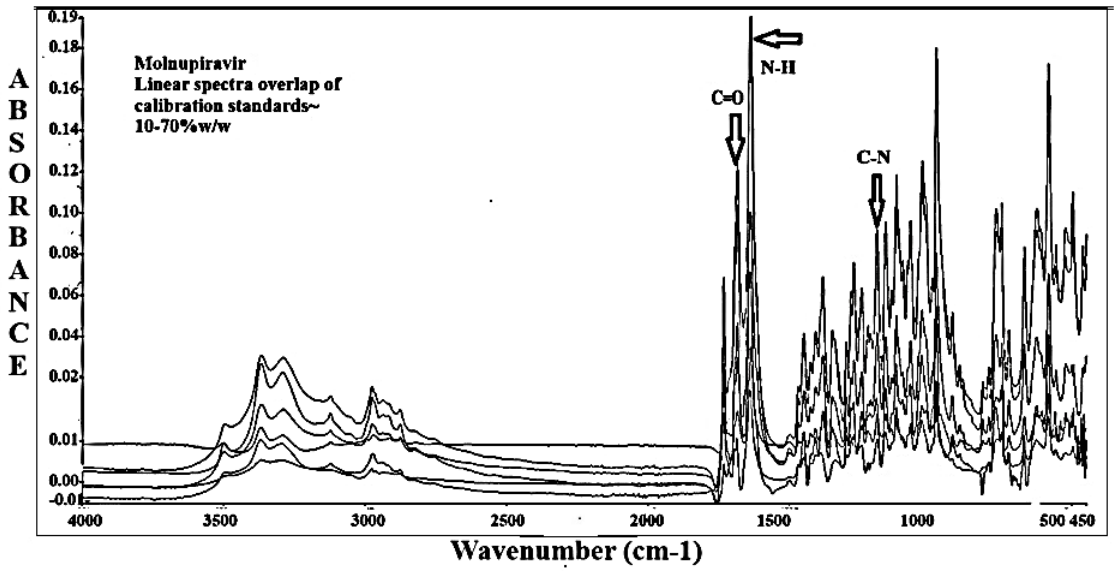

Figure 4b: Overlapped spectra of molnupiravir calibration standards (10-70%w/w) in the spectral region 1700-667 cm-1.

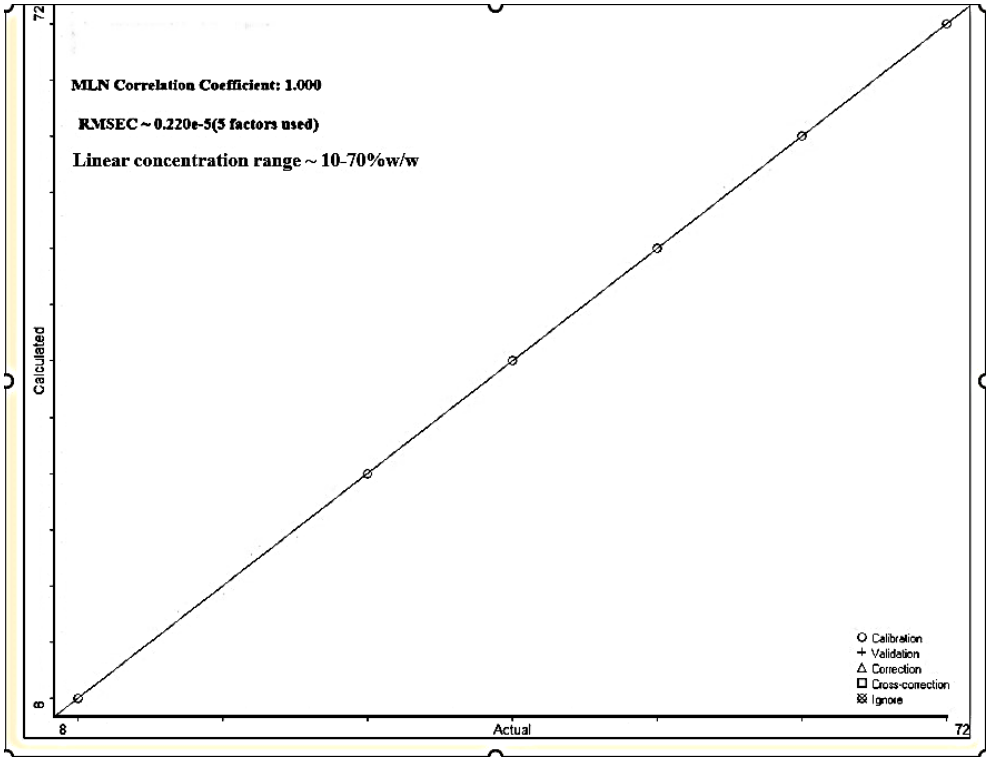

Figure 4c: PLS regression curve for molnupiravir linearity studies (10-70%w/w).

Table 3: Characteristic IR Peaks of Etidronate Disodium.

Functional Group	Expected Wavenumber (cm^{-1})	Functional group
O-H stretching (broad)	3200-3500	Hydroxyl groups (drug + excipients)
P-O stretching	1150-1100	Phosphonate group
P-O stretching	1050-950	Phosphonate linkage
P-O-H bending	900-850	Phosphonic acid group
C-H bending	1450-1350	Alkyl group

was claimed to be specific for this molecule because the presence of excipients or other impurities did not interfere with the pure spectral peaks of favipiravir.

It was clear that both spectra did not overlap with each other, indicating that FVP content could be detected and quantified even in the presence of other impurities as shown in Figure 7a. Thus, the method was suitable for quantifying FVP and as well as presence of impurities, but also applicable for quantification of FVP tablets in quality control. IR spectrum of 10% w/w favipiravir added with 3 excipients in test formulations was clearly seen there is no spectral overlap and interference of favipiravir detected at 1400-1000 cm^{-1}).

To further prove the method specificity, the IR spectra data for the 10% w/w working sample was incorporated into the established standard curve to correlate form the linear curve. It was revealed that r^2 value was 1.0000, with confidence interval of 99.7% indicating a very good specificity and the result was significant within limits ($p<0.05$) as shown in Figure 7b.

Molnupiravir

Qualitative method specificity was used for the spectral analysis, which was focused on the fingerprint region (1500-667 cm^{-1}) only to determine the specificity of this method towards molnupiravir (MLN). According to the overlapped spectra of placebo and MLN plus placebo as shown in Figure 8a, some peaks were seen to overlap with each other 1500- 450 cm^{-1} which indicates there is no significant interference by the placebo, especially in the specific area of the MLN spectral peak(1700 cm^{-1}) estimated in the linearity of MLN raw material, the spectral peaks remained uninterrupted which is useful information discovered for this method to extend for the quantification of formulations of MLN.

Fingerprint region from IR spectra of starch (A), lactose (B) and CMC (C) in transmittance vs wavenumbers (cm^{-1}) spectra.[49] Multiple peaks were present in the fingerprint region of individual spectrum, which directly contributed to the peaks in the placebo spectrum thus causing overlapped. Hence, no peak interference was seen in the wavenumber range of 1600-1000 cm^{-1} as highlighted in the box as shown in the Figure 8b. None of the excipients in the placebo chemically consists of either C-N or N-H bond. Moreover, the C-H bond peak at wavenumber of 981 cm^{-1}, which was shown not overlapping with the placebo. The interference was most likely due to the presence of C-O bonds in the placebo supported with the individual infrared spectrum of the excipients. Hence, this method was proven and specific to the

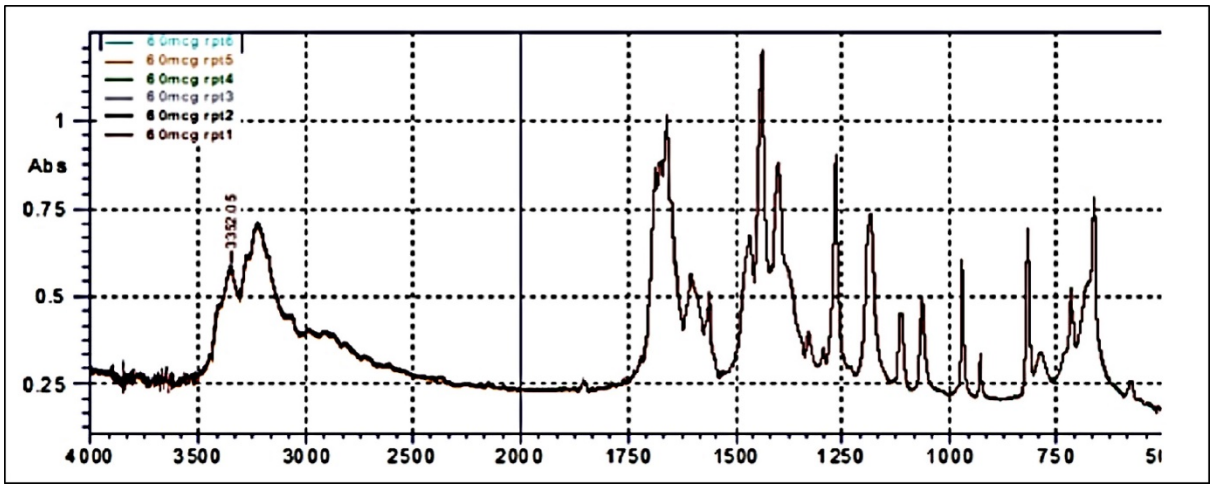

Figure 5a: Infrared spectrum of pure favipiravir powder.

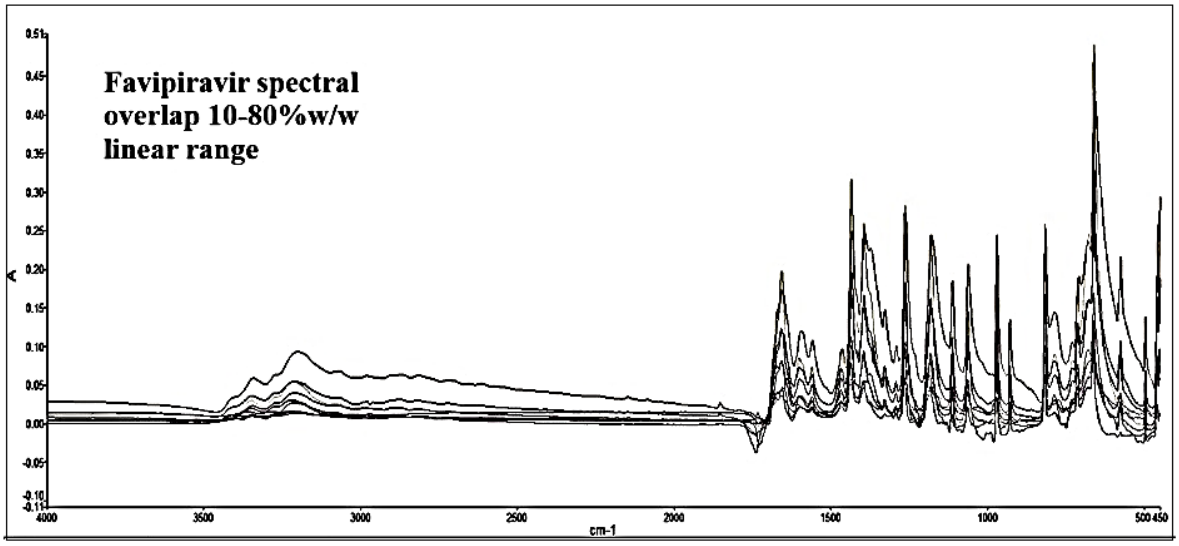

Figure 5b: Overlay infrared spectra of Favipiravir in the linear range of 10-80% w/w.

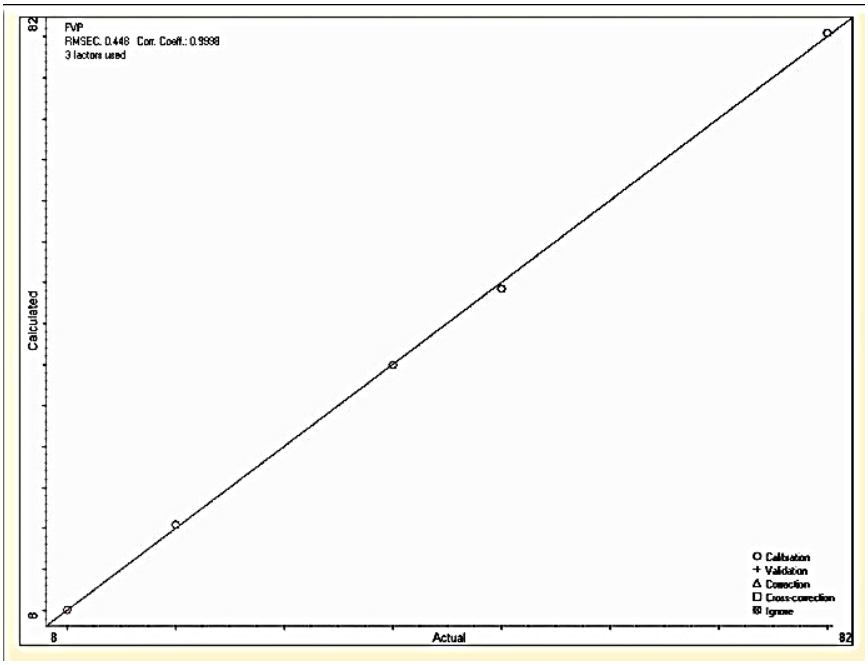

Figure 5c: PLS regression curve for favipiravir linearity studies (10-80%w/w).

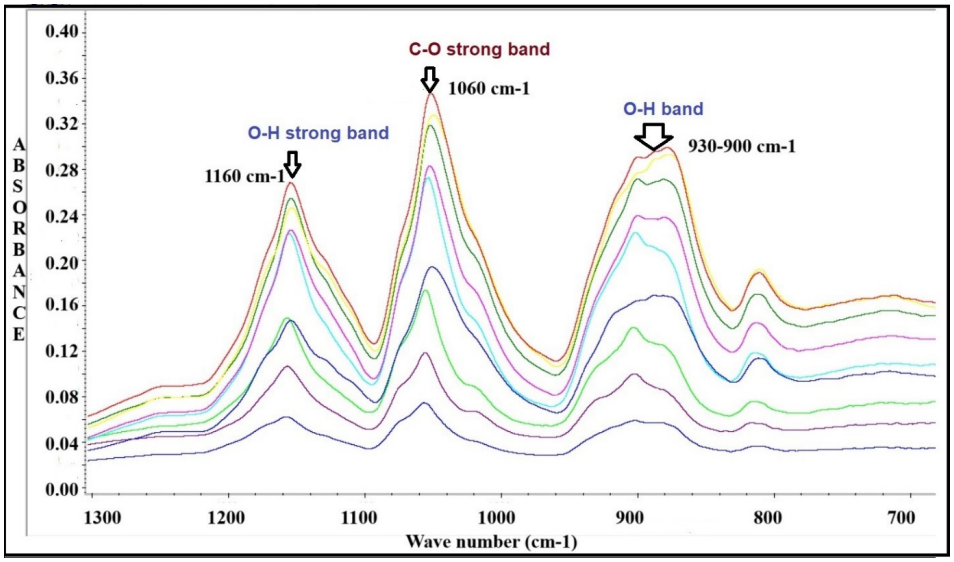

Figure 6a: Overlay spectra of different calibration standards of ET.

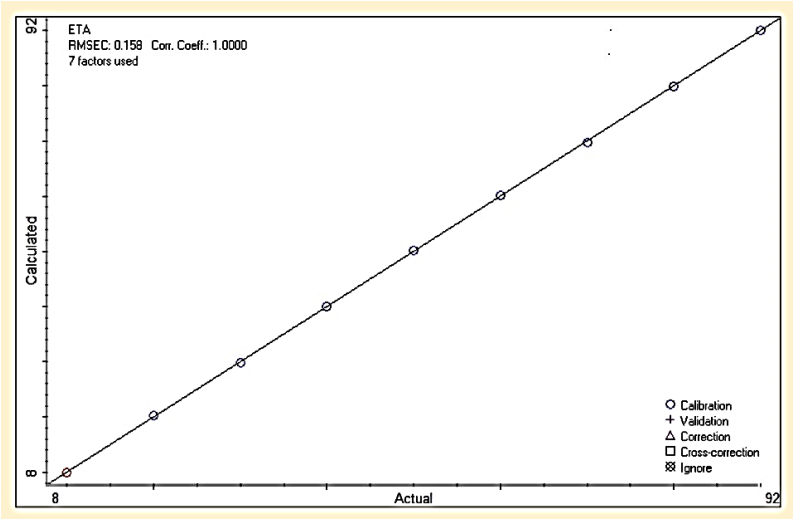

Figure 6b: PLS regression curve for favipiravir linearity studies (10-90%w/w).

MLN at the unique functional moieties such as N-H and C-N, which were detected with no interference from the excipients added in the placebo.

Etidronate disodium

The infrared (IR) spectral analysis of etidronate disodium was conducted to evaluate potential physicochemical interactions with commonly used excipients, including lactose, sodium Carboxymethyl Cellulose (sodium CMC), and starch. Etidronate disodium exhibited characteristic absorption bands corresponding to its functional groups, particularly strong *p*=O stretching vibrations in the region of 1150-1100 cm^{-1}, P-O stretching around 1050-950 cm^{-1}, and broad O-H stretching bands between 3200-3500 cm^{-1} (See Figure 6a). Lactose and starch showed prominent O-H stretching vibrations in the 3200-3600 cm^{-1} region and C-O stretching bands in the 1200-1000 cm^{-1} region, while CMC demonstrated Characteristic Carboxylate (-COO^-) asymmetric and symmetric stretching

Table 4: Characteristic IR Peaks of Excipients of lactose, starch, and Carboxy Methyl Cellulose (CMC).

Lactose	
Wavenumber (cm^{-1})	Functional group
3400-3200	O-H stretching
2920	C-H stretching
1150-1000	C-O stretching
Starch	
3400-3300	O-H stretching
2930	C-H stretching
1155-1020	C-O and C-O-C stretching
Carboxymethyl Cellulose (CMC)	
3400-3200	O-H stretching
~1600	COO^- asymmetric stretching
~1420	COO^- symmetric stretching
1100-1000	C-O stretching

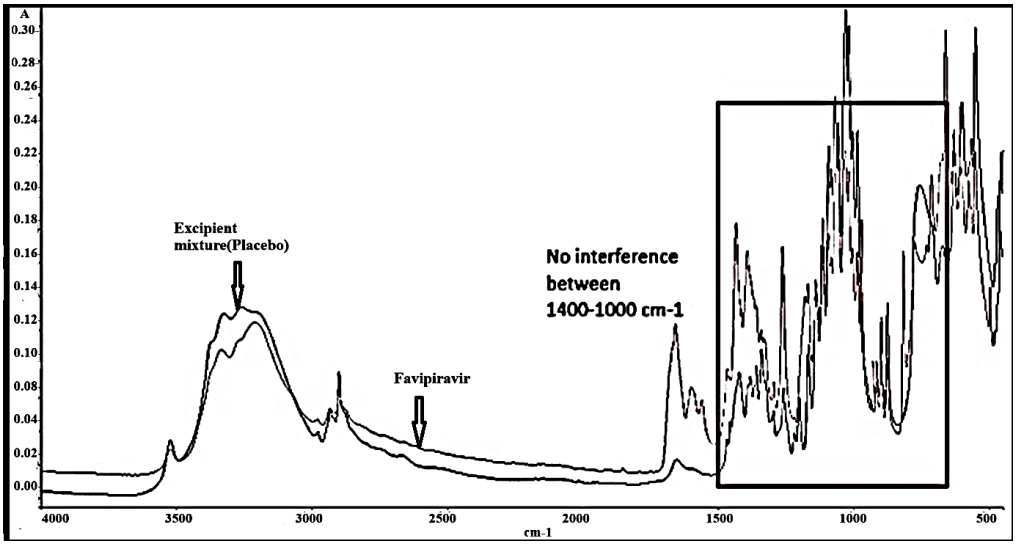

Figure 7a: The overlap spectra of excipient mixture and 10%w/w FVP added with excipient mixture.

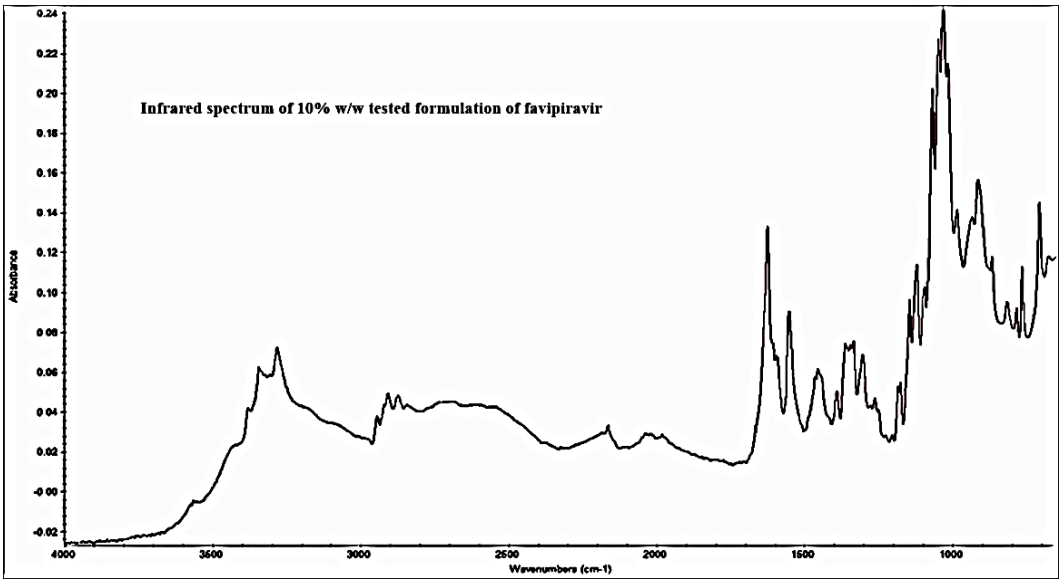

Figure 7b: Infrared spectrum of 10% w/w tested formulation of favipiravir where there are no peaks seen of other excipients present in the formulation.

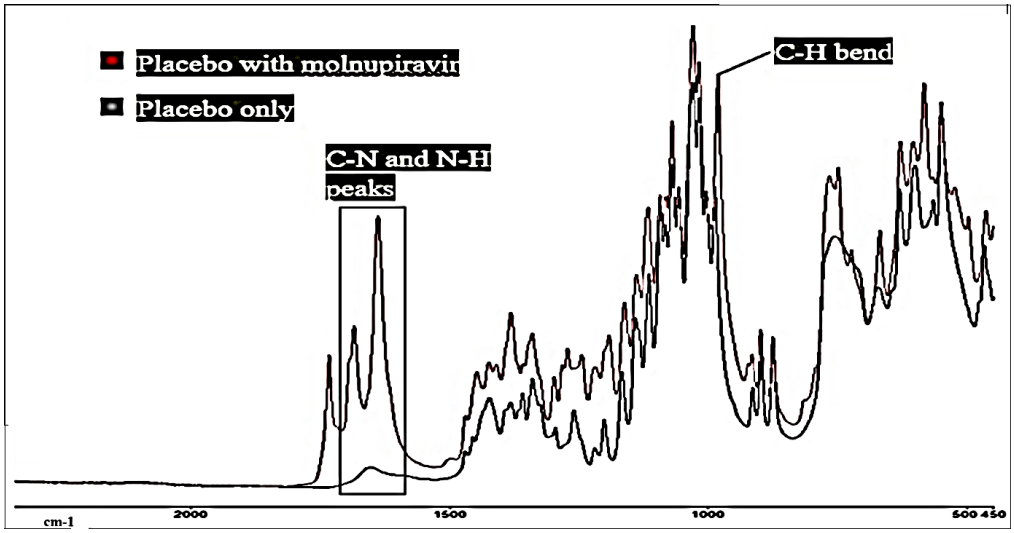

Figure 8a: Overlapped spectra of placebo and placebo with molnupiravir. Specifically noticed that there is no spectral overlap.

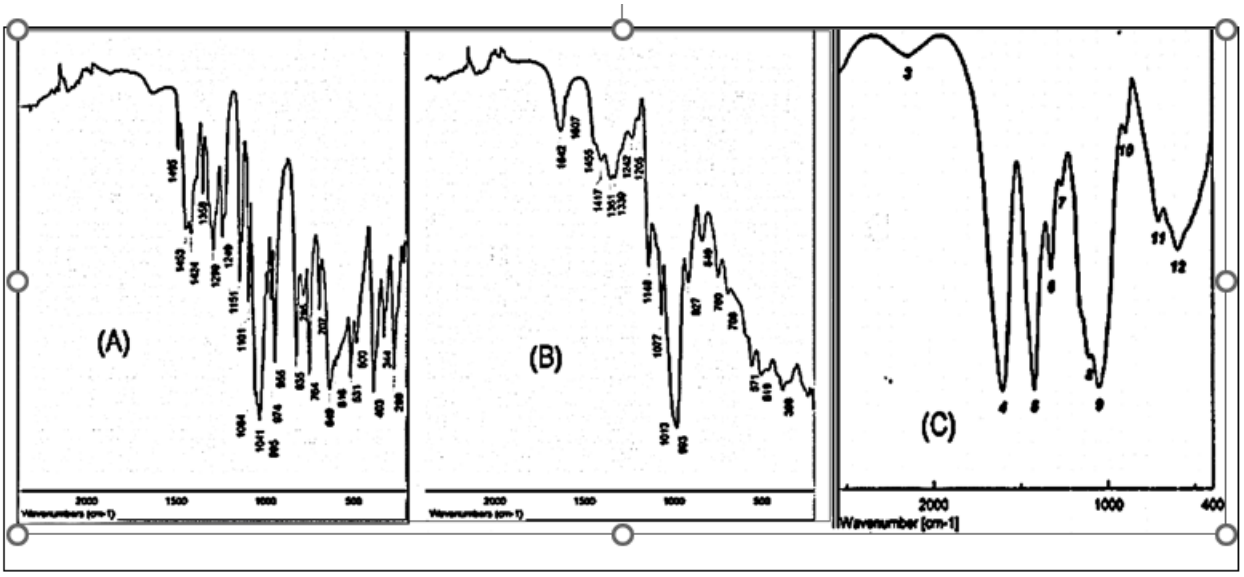

Figure 8b: Fingerprint region from IR spectra of starch (A), Lactose (B) and Carboxymethyl Cellulose (C).

near 1600 cm^{-1} and 1420 cm^{-1}, respectively. Due to the presence of overlapping hydroxyl and C-O functional groups among these compounds, partial spectral overlap was observed, particularly within the 1200-1000 cm^{-1} region.

However, comparative evaluation of the spectra revealed that the principal characteristic peaks of etidronate disodium remained intact without significant shifts, disappearance, or formation of new bands in the physical mixtures. The observed overlaps were attributed to coincidental superimposition of similar functional group vibrations rather than chemical interaction. No marked changes in peak intensity or position were detected that would indicate incompatibility between etidronate disodium and the selected excipients. These findings suggest that lactose, CMC, and starch are compatible with etidronate disodium under the studied conditions, supporting their suitability for formulation development.

Most of the reported methods used the normal transmission mode, which requires mechanical preparation of pellets of the sample using KBr which is not suitable for quantitative analysis of thick samples. ATR has penetration depth of about 200 nm depending on the ATR crystal material hence it is ideal for thick samples. The type of ATR crystal material used in quantitative analysis plays a vital role to ensure the quality and accuracy of the data. Theoretically, the crystal refractive index should be greater than the analyte refractive index to obtain a high-quality spectrum. Diamond crystal was used in this study. Diamond crystal has refractive index of 2.4, which is greater than the refractive indices of molnupiravir, favipiravir and etidronate disodium. In addition, ATR-FTIR sampling technique can measure a broad range of solid, liquid, and gas samples directly without requiring complex sample preparations. Though the mid-IR spectra were recorded without any sample pre-treatment, a continuous flow system requires a skilled analyst, and it is a time-consuming process compared to ATR-FTIR sampling technique. The continuous flow systems have constraints, including changes in flow rate (maintaining a constant pulse-free flow rate) and introduction of a precise volume of sample solution without disturbing the flow. ATR-FTIR coupled with PLS provides simple but accurate determination of pharmaceutical ingredients which lack a chromophore.

The representative IR spectral profile of drug placebo made with etidronate disodium formulated with starch, sodium CMC, and lactose as excipients. The exact spectrum might show slight variation in wavenumbers depending on the quality excipient used and also on the instrument resolution, and formulation ratios. The following data was obtained the expected combined spectral characteristics based on functional groups present in each component as shown in Tables 3 and 4.

Expected IR Spectrum of the placebo blend with etidronate and excipients

When etidronate disodium is formulated with starch, lactose, and sodium CMC, the IR spectrum typically showed A broad O-H stretching band (3200-3500 cm^{-1}) due to cumulative hydroxyl groups from drug and excipients. Strong overlapping peaks in the 1150-1000 cm^{-1} region, attributed to: (1) *p*=O and P-O stretching of etidronate; (2) C-O stretching from lactose and starch (3) C-O vibrations from sodium CMC; (4) Carboxylate peaks near 1600 and 1420 cm^{-1} from sodium CMC. The retention of characteristic phosphonate peaks of etidronate without significant shift. It is interpreted in a compatible placebo formulation, it was clearly observed that no disappearance of the etidronate's characteristic phosphonate peaks with no significant shift (>10-20 cm^{-1}) in *p*=O or P-O stretching frequencies and no new peaks indicating chemical interaction. The overlap was observed in the 1200-1000 cm^{-1} region is expected due to similar C-O and P-O functional groups and does not necessarily indicate incompatibility.

CONCLUSION

Molnupiravir (MLN), Favipiravir (FVP) and Etidonate disodium (ET) may contain impurities during the synthesis process. Hence, qualitative, and quantitative analysis should be done prior to marketing to ensure the drug is safe for use. Direct HPLC analysis of these three molecules, which cannot be carried out due to the lack of UV chromophore or double bond conjugation system in their structures and hence tedious derivatization procedures need to be done prior to their quantification and measurement for routine analysis is not feasible in industry, not reproducible and not cost effective. Current proposed methods developed by ATR-FTIR Spectroscopy combined with PLS are suitable for the quantification of MLN, FVP and ET in MIR region at 1700-667 cm^{-1}. These validated methods provide an alternative methodology for the quantification of MLN, FVP and ET in pharmaceutical formulations. The quantitative data is reproducible, reliable, cost-efficient, and reduces the usage of hazardous chemical reagents and solvents compared to liquid chromatography or mass spectrometry quantitative methods. The method can be applied for routine quality control analysis of MLN, FVP and ET.

NOVELTY AND SUMMARY OF THE RESEARCH

Non chromophore molecules are typical non-absorbing molecule under UV-visible light and non-fluorescing materials. Analytical R and D laboratories face challenges to develop new methods using sophisticated instruments like HPLC with UV detection. These molecules need derivatisation and complex analytical methods reported in the literature. Molnupiravir (MLN), Favipiravir (FVP) and Etidronate disodium (ET) are polar and very weak chromophore molecules. ATR-FTIR with PLS algorithm is an excellent analytical technique to estimate these molecules quantitatively using chemometric tools. In this research, three analytical methods developed and validated using ATR-FTIR spectroscopy with Partial Least Squares (PLS) algorithms. Three methods are exhibited with excellent linearity with simple faster, economical, non-destructive, and green analysis for the above molecules. Hence, these methods can be utilised for the routine analysis of MLN, FVP and ET in bulk drug and pharmaceutical dosage forms, which are precise, selective, and accurate.

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ABBREVIATIONS

ATR-FTIR: Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy; **AMK:** Amikacin; **HPLC:** High-Performance Liquid Chromatography; **UV:** Ultra-violet; **ATR:** Attenuated Total Reflectance; **IR:** Infrared; **KBr:** Potassium bromide; **PLS:** Partial Least Squares; **RMSEC:** Residual means standard error of calibration; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **FTIR:** Fourier Transform Infrared; **RSD:** Relative Standard deviation; **RE:** Relative error; **ICH:** International Conference on Harmonisation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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