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Stability Indicating HPTLC Method of Molnupiravir and Comparative Study of Degradant with Marketed Molnupiravir Impurity- A

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Abstract
The purpose of this study was to create, optimise, and validate a high-performance thin layer chromatographic (HPTLC) method for identifying Molnupiravir (MOL) and its impurity (IMP-MOL). (3aR,4R,6R,6aR) -6-(4-(hydroxyamino) -2-oxopyrimidin-1(2H)-yl) Molnupiravir A is 2,2 dimethyltetrahydrofuro [3,4-d][1,3]dioxol-4-yl)methyl isobutyrate (MOL IMP) Over concentration ranges of 0.1 µg/band to 0.6 µg/band and 0.02 to 0.6 µg/band, the proposed technique was employed to analyse Molnupiravir and its impurity, with mean percentage recovery of 99.92% ±1.521 and 99.28% ±2.296, respectively. This method has been done with the separation of two components and ends with the densitometric measurement of the separated peaks at 276 nm. The separation was done on silica gel HPTLC F254 plates with a Toluene: n-Butanol: Methanol: Water developing system (5:3:1.5:0.5, by volume). The MOL was kept under conditions like oxidative, hydrolytic, thermal stress, and photolytic tests that the International Conference on Harmonization (ICH) requires. In acid, alkali, and oxidative hydrolysis, the MOL was unstable, but it was not affected by acidic, heat or UV light. The alkaline degradation of Molnupiravir was studied using the proposed HPTLC approach. The degradant are separated using HPTLC method, and their structures are confirmed using IR, MS, and NMR spectrum data.

Keywords
Molnupiravir: Impurity: HPTLC: IR: NMR: Mass Spectrometry.

1. INTRODUCTION

The coronavirus disease 2019 (Covid-19) pandemic, which was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in about 270 million confirmed cases and over 5.2 million documented deaths around the world.(1) A significant number of patients with Covid-19, mostly elderly adults and those with prior illnesses, require hospitalisation (e.g., obesity, diabetes mellitus, and serious cardiac conditions). (2-4) Several vaccinations that are highly successful in lowering hospitalisation and death have been approved, but immunisation coverage is still insufficient.

Molnupiravir (MOL) is a small-molecule N-hydroxycytidine (NHC) ribonucleoside prodrug with action against SARS-CoV-2 and other RNA viruses, as well as a high resistance barrier.(5-12) NHC is phosphorylated intracellularly to NHC triphosphate after molnupiravir is taken orally.

Molnupiravir (also known as EIDD-2801/MK-4482) is a prodrug of the active antiviral ribonucleoside analogue b-D-N4-hydroxycytidine (NHC; EIDD-1931) (13) (Fig 1). In this paper we discussed the Impurity profile of Molnupiravir – A,(MOL IMP) chemically the impurity A is 3AR,4R,6R,6AR)-6-((E)-4-(hydroxiamino)-2-oxo-3,4-dihydropyrimidine-1(2H)-YL)-2,2-dimethyltetrahydrofuoro[3,4-D][1,3]dioxol-4-yl)methyl having an molecular weight of 369.40 g/mol and molecular formula is $C_{16}H_{23}N_3O_7$. (Fig 2)

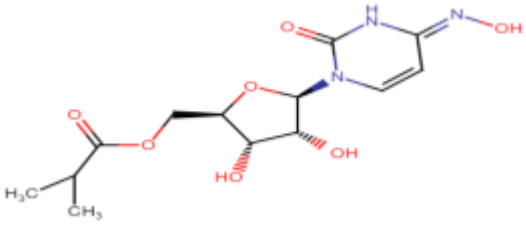

Fig. 1. Structure of Molnupiravir

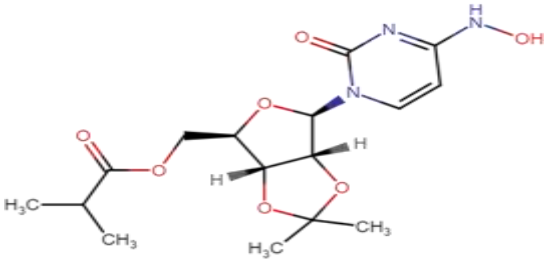

Fig. 2. Structure of Mol. Impurity

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There is no report in the literature concerning the separation and identification of molnupiravir impurities that we are aware of. The amount of permissible level for a known and unknown related component (impurity) should be less than 0.15 and 0.10 percent, respectively, according to the general parameters given by ICH (14) QA3 to qualify the drug material. The impurities present in the MOL material must be identified and characterised in order to meet the severe regulatory standards. There has also been no research onto the force degradation of MOL.

2. EXPERIMENTAL

2.1. Instrumentation

2.1.1. High performance thin layer chromatography

For HPTLC-densitometric determinations, Camag TLC Scanner 3 and Camag TLC sampler Linomat IV (Camag, Muttenz, Switzerland) are provided with a 100 µl syringe. The following criteria are taken into account: data resolution: 100 mm/step; band width: 6 mm; result output: chromatogram and integrated peak area; slit dimensions: 6×0.3 mm; scanning speed: 20 mm/s; spraying rate: 10 µl/s; data resolution: 100 µm/step; band width: 6 mm; result output: chromatogram and integrated peak area 0.25 mm silica gel 60 F254 was deposited on HPTLC aluminium plates (20x20 cm) (Merck, Germany).

2.1.2. Mass spectrometry

A triple quadruple mass spectrometer, the PE Sciex type API 3000, was used for the turbo spray and MS–MS experiments. By varying the capillary voltage between +5000 and 4500 V, positive and negative electrospray MS data were acquired. The collision energy was ramped from 30 to 60 V in the nitrogen atmosphere to create the MS–MS data.

2.1.3. NMR spectroscopy

The 1H NMR were recorded on Varian Mercury plus 400 MHz, Gemini 200 MHz, using DMSO-d6, and a mixture of DMSO-d6 and CDCl3 as solvents and trimethylsilane (TMS) as the internal standard.

2.1.4. FT-IR spectroscopy

The IR spectra were acquired using a Perkin Elmer 1600 series FT-IR spectrophotometer in the solid state as KBr dispersion medium.

2.1.5. Material and reagents

Pure standard MOL pure sample was kindly provided by Simson Pharma Limited Dahisar East Mumbai (India). MOL IMP was purchased from Simson Pharma Limited Dahisar East Mumbai (India) with a purity of 99.87% based on the company certificated.

2.2. Method development and validation

To achieve the best separation between MOL and its MOL IMP, different developing systems with different compositions and ratios were tried, including Toluene: n-Butanol: Methanol: Water (5:3:1:1, by volume), Toluene: n-Butanol: Methanol: Water (5:3:1.5:0.5, by volume), Toluene: n-Butanol: Methanol: DMSO (5:3:1:1, by volume). Toluene: n-Butanol: Methanol: Water was discovered to be the optimum developing system (5:3:1.5:0.5, by volume).

The proposed method's validation was carried out in accordance with the International Conference on Harmonization (ICH) guidelines. (14) shown in Table 1

Table 1. Regression and Analytical Parameters of the Proposed Method for Determination of MOL and MOL IMP

Parameter	HPTLC	
	Molnupiravir	Molnupiravir Impurity A
Linearity	0.1 to 0.6 µg/band	0.02 to 0.6 µg/band
Slope	361.08	425.13
Intercept	511.74	17.04
Correlation coefficient($r^{2)}$	0.998	0.997
Accuracy	99.21% ± 1.125	99.35% ± 2.235
Precision: Interday (RSD%)	5.44	0.5404
Precision: Intraday (RSD%)	1.4263	0.5153
Robustness (RSD%) a)Water (1±0.5 ml)	2.106	2.8534
b)Saturation Time (25±5 min)	1.12	5.16
LOD	0.044 µg/band	0.021 µg/band
LOQ	0.13 µg/band	0.064 µg/band

2.3. Forced degradation

Stress tests were performed according to ICH guidelines Q1A (R2). (15) The MOL was put through a variety of stress tests, including hydrolysis, oxidation, dry heat, and photolysis. Result shown in Table 2.

2.3.1. Hydrolytic degradation

Separate aliquots of MOL were tested under acidic and alkaline conditions. 1 mL of 100 mg/mL MOL, heated for 4.0 hours at 80º C with 1 mL each of HCL (0.1N) and NaOH (0.1N) for hydrolytic breakdown, after the requisite exposure, acid-treated samples were neutralised with an equal

strength of base, and vice versa. After that, the samples were run through HPTLC to see if any degradation products were present as shown in Fig. 3 and 4.

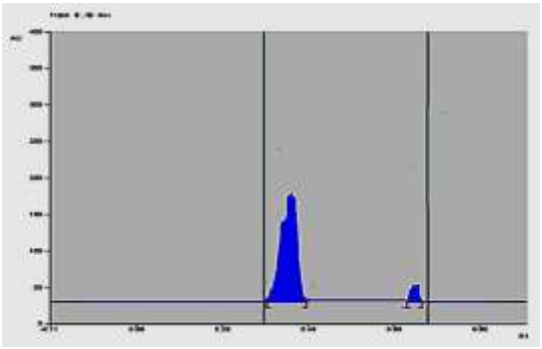

Fig. 3. Chromatogram showing acid degradation of MOL

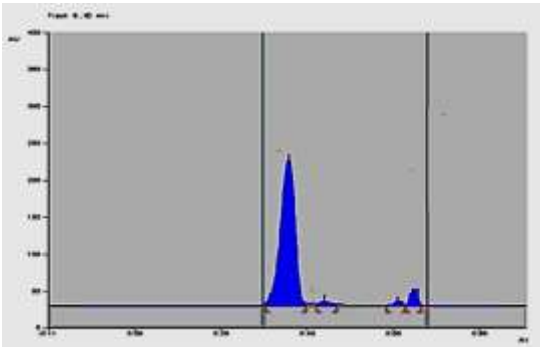

Fig. 4 Chromatogram showing alkali degradation of MOL

2.3.2. Oxidative degradation

1 ml of 100 g mL-1 MOL solution was treated with 1 ml 6 % H_2O_2 at room temperature for 4.0 hours to assess oxidative degradation as shown in Fig. 5

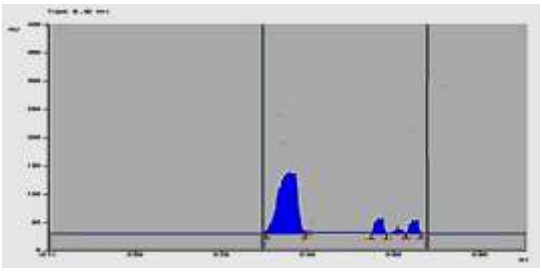

Fig. 5 Chromatogram showing oxidative degradation of MOL

2.3.3. Thermal degradation

An aliquot of MOL was employed in the solid form, which was degraded in the oven for 12 hours at 80° C in a sealed glass ampoule. The powder was dissolved in methanol to a final concentration at the end of the incubation period. After that, the samples were run through HPTLC to see if any degradation products were present as shown in Fig. 6

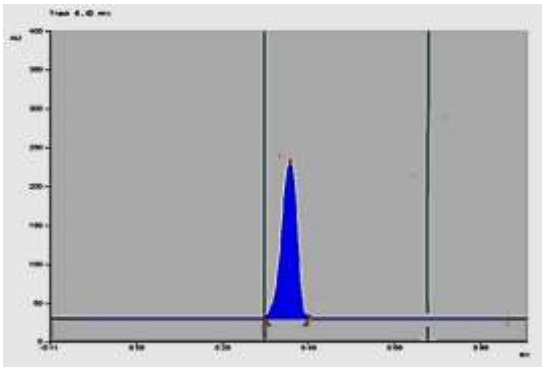

Fig 6 Chromatogram showing thermal degradation of MOL

2.3.4. Photo degradation

In a photo stability chamber, solid MOL and MOL solutions made in methanol (100 g/mL) were subjected to 12 hours of fluorescent light and 200 Whm"2 of UV light as shown in Fig.7.

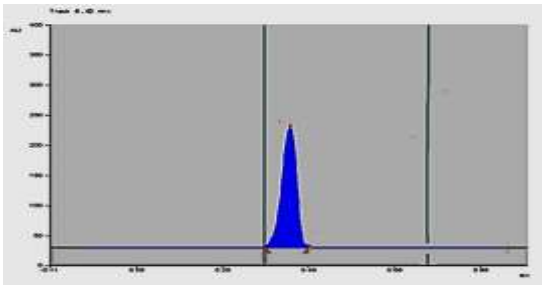

Fig 7. Chromatogram showing Photo degradation of MOL

Table 2. Result of degradation study

Sr. No	Condition	% Degradation of MOL
1	0.1 N NaOH condition	97.21 %
2	0.1 N HCL condition	92.14 %
3	6 % H_2O2 condition	78.33 %
4	UV light (12 hrs)	No degradation

2.3.5. Extensive forced degradation

In volumetric flasks, 100 mg of MOL were accurately weighed and dissolved in 25 mL methanol. After that, 25 mL 1 N NaOH was added, and the flask was heated for 24 hours in a temperature-controlled oven at 80º C. The solutions were concentrated nearly dry under vacuum, cooled to room temperature (25º C), and then quantitatively transferred into a 100 mL volumetric flask, where they were neutralised with 1N HCL and the volume was made with methanol. The proposed HPTLC method confirmed complete alkaline degradation of the studied MOL, where no peaks corresponding to intact MOL were detected in degraded samples and the concentrations of the MOL were calculated from the corresponding regression equations where no peaks

corresponding to intact MOL were detected as shown in Fig 8.

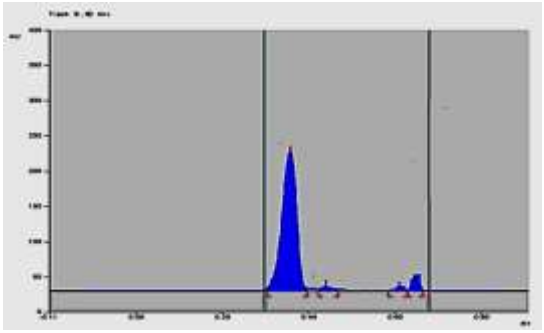

Fig 8. Chromatogram showing Extensive degradation in Alkali of MOL

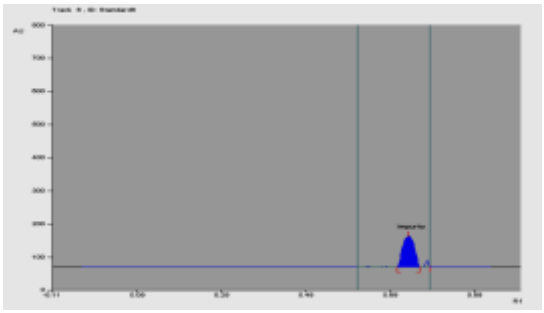

Fig 9. Chromatogram showing Marketed MOL-IMP

3. RESULT AND DISCUSSION

3.1. Structure elucidation of degradant

The IR spectrum (KBr) of DP was characterized by the absorption frequency of aromatic ring C-H (strch) 2960.85 cm^{-1} and C-N (strch) 1302.40 cm^{-1}, C=C (strch) 1589.83 cm^{-1}, –NH band (strch) at 3382.81cm^{-1}, and C=O at 1621.97cm^{-1}, C-O at 1252.31cm^{-1} and O-H 3031.42 cm^{-1}. The mass spectrum of DP was characterized by the appearance of the molecular ion peaks at 113.0 m/z and 184.0 m/z and 279.1 m/z (M & M+2) which confirm the molecular weight of the suggested degradation product & compared with MOL degradant in Table 3. The 1H NMR spectrum (DMSO-d6) showed an additional signal at δ 3.405 ppm indicating the presence of another methoxy group in the degradant. 1H NMR spectrum is remarkable, protons at δ 7.063 ppm, corresponding to –NH and at δ 7.392 ppm corresponding to –OH shown in Table 3.

3.2. Characterization

We compared our degradant result to the marketed impurity i.e Molnupiravir A (MOL IMP) we found that our values are closer to marketed Impurity. The IR spectrum (KBr) of marketed impurity was characterized by the absorption frequency of aromatic ring C-H (strch) 2934.57 cm^{-1} and C-N (strch) 1498.26 cm^{-1} , C=C(strch) 1689.55cm^{-1}, –NH band (def) at 3276.99 cm^{-1}, and C=O at 1749.53 cm^{-1}, C-O at 1247.14 cm^{-1} and O-H 2990.41 cm^{-1}. The mass spectrum of MOL IMP was characterized by the appearance of the molecular ion peaks at 370.0 m/z which confirm the molecular weight of the marketed impurity. Compared with MOL degradant in Table 3 .The 1H NMR spectrum (DMSO-d6) showed an additional signal at δ 2.577 ppm indicating the presence of another methoxy group in the impurity. 1H NMR spectrum is remarkable, protons at δ 6.896 ppm, corresponding to –NH and at δ 6.876 ppm corresponding to –OH is shown in Table 3.

3.3. Compare with the marketed Known Molnupiravir Impurity A

We compare the degradant of Mol with marketed known Impurity i.e, 2,2 dimethyltetrahydrofuro [3,4-d][1,3]dioxol-4-yl)methyl isobutyrate (Molnupiravir A) and from the characterization of degradant of MOL and MOL IMP by IR, MS, NMR spectroscopy we found that our degradant is comparatively similar to the marketed known Impurity up to 86-87 % is shown in Table 3.

The purpose of this study was to create, optimise, and validate a high-performance thin layer chromatographic (HPTLC) method for identifying Molnupiravir (MOL) and its impurity (IMP-MOL). The HPTLC method offers the advantages of being selective, sensitive, accurate, and speedy analytical approach, as well as reducing sample preparations, laboratory usage, and material costs. It offers the advantage of being both cost-effective and time-saving in comparison to the HPLC approach

Table 3. FT-IR, MS and NMR of Degradant of MOL and Impurity

Sr.No	Compound	IR	MS	NMR
1.	Degradant of MOL	C-H (strch) 2960.85, C-N (strch) 1302.40, C=C (strch) 1589.83, NH (strch) 3382.81, C=O 1621.97, C-O 1252.31 and O-H 3031.42	m/z Measured EI-MS in Acidic 113.0 m/z, 184.0 m/z and 279.1 m/z	1H NMR δ 3.405, NH δ 7.063 and OH δ 7.392
2.	Marketed Impurity	C-H (strch) 2934.57, C-N (strch) 1498.26, C=C (strch) 1689.55 NH (def) 3276.99, C=O 1749.53, C-O 1247.14 and O-H 2990.41	m/z Measured EI-MS in Acidic	1H NMR δ 2.577, NH δ 6.896 and OH δ 6.876

The MOL was kept under conditions like oxidative, hydrolytic, thermal stress, and photolytic tests that the International Conference on Harmonization (ICH) requires. In acid, alkali, and oxidative hydrolysis, the MOL was unstable, but it was not affected by acidic, heat or UV light. The alkaline degradation of Molnupiravir was studied using the proposed HPTLC approach. The degradant are separated using HPTLC method, and their structures are confirmed using IR, MS, and NMR spectrum data.

4. CONCLUSION

HPTLC (analytical and preparative), IR, mass spectrometry, as well as N MR (1H and 13C NMR) techniques were utilized for the determination of the process related impurity in Molnupiravir drug substance. The extraction, purification, isolation, and identification of the impurity were achieved by using HPTLC, IR, and mass spectrometry. The ICH guidelines were followed in the development, optimization, and validation of a high performance thin layer chromatography method. According to ICH guidelines, developed method proved to be sensitive, precise, specific, and efficient. Compared synthesized degradants of molnupiravir with marketed impurities of molnupiravir, the impurities are comparatively similar upto 86-87% to the synthesized degradants. In future we can isolate and characterised a number of other impurities from Molnupiravir bulk and formulations and the proposed analytical methods will be useful for estimating these impurities.

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REFERENCES

[1] WHO coronavirus (COVID-19) dashboard; Geneva: World Health Organization, 2021 (https://covid19.who.int).

[2] Stokes, E.K., Zambrano, L.D., Anderson, K.N., et al.; Coronavirus disease 2019 case surveillance — United States, January 22–May 30, 2020; MMWR Morb Mortal Wkly Rep 2020;69:759-65.

[3] Ko, J.Y., Danielson, M.L., Town, M., et al.; Risk factors for coronavirus disease 2019 (COVID-19)–associated hospitalization: COVID-19–associated hospitalization surveillance network and behavioral risk factor surveillance system; Clin Infect Dis 2021;72(11):e695-e703.

[4] Kompaniyets, L., Goodman, A.B., Belay, B., et al.; Body mass index and risk for COVID-19-related hospitalization, intensive care unit admission, invasive mechanical ventilation, and death — United States, March–December 2020; MMWR Morb Mortal Wkly Rep 2021;70:355-61

[5] Yoon, J.J., Toots, M., Lee, S., et al.; Orally efficacious broad-spectrum ribonucleoside analog inhibitor of influenza and respiratory syncytial viruses; Antimicrob Agents Chemother 2018;62(8):e00766-18.

[6] Cox, R.M., Wolf, J.D., Plemper, R.K.; Therapeutically administered ribonucleoside analogue MK-4482/EIDD-2801 blocks SARS-CoV-2 transmission in ferrets; Nat Microbiol 2021;6:11-8.

[7] Sheahan, T.P., Sims, A.C., Zhou, S., et al.; An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice; Sci Transl Med 2020;12(541):eabb5883.

[8] Wahl, A., Gralinski, L.E., Johnson, C.E., et al.; SARS-CoV-2 infection is effectively treated and prevented by EIDD-2801; Nature 2021;591:451-7.

[9] Abdelnabi, R., Foo, C.S., De-Jonghe, S., Maes, P., Weynand, B., Neyts, J.; Molnupiravir inhibits the replication of the emerging SARS-CoV-2 variants of concern (VoCs) in a hamster infection model; J Infect Dis 2021;224:749-53.

[10] Agostini, M.L., Pruijssers, A.J., Chappell, J.D., et al.; Small-molecule antiviral betad-N4 -hydroxycytidine inhibits a proofreading-intact coronavirus with a high genetic barrier to resistance; J Virol 2019; 93(24):e01348-19.

[11] Urakova, N., Kuznetsova, V., Crossman, D.K., et al.; β-d-N4 -hydroxycytidine is a potent anti-alphavirus compound that induces a high level of mutations in the viral genome; J Virol 2018;92(3):e01965-e17.

[12] Grobler, J., Strizki, J., Murgolo, N., et al.; Molnupiravir maintains antiviral activity against SARS-CoV-2 variants in vitro and in early clinical studies. In: Proceedings and abstracts of IDWeek 2021, September 29–October 3, 2021; Arlington, VA: Infectious Diseases Society of America, 2021

[13] Painter, G.R., Bowen, R.A., Bluemling, G.R., DeBergh, J., Edpuganti, V., Gruddanti, P.R., Guthrie, D.B., Hager, M., Kuiper, D.L., Lockwood, M.A., Mitchell, D.G., Natchus,

M.G., Sticher, Z.M., Kolykhalov, A.A.; The prophylactic and therapeutic activity of a broadly active ribonucleoside analog in a murine model of intranasal Venezuelan equine encephalitis virus infection. 2019; Antiviral Res 171:104597; https://doi.org/10.1016/j.antiviral.2019.104597.

[14] ICH Guideline Q3A (R); Impurities in New Drug Substances; February 7, 2002.

[15] ICH Guideline Q1A (R2); Stability Testing of New Drug Substances and Product; February, 2003.

روش HPTLC تشخیص پایداری مولناپیراویل و مطالعه مقایسه‌ای تخریب با ناخالصی مولناپیراویل نشان‌دار شده A

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تاریخ دریافت: ۳۱ تیر ۱۴۰۱ تاریخ پذیرش: ۰۱ مهر ۱۴۰۱

چکیده

هدف از این مطالعه عبارت بود از ایجاد، بهینه‌سازی و اعتبارسنجی یک روش کروماتوگرافی لایه نازک با کارآیی بالا (HPTLC) برای تشخیص مولناپیراویل (MOL) و ناخالصی آن (IMP-MOL). تکنیک پیشنهادشده برای اندازه‌گیری گستره‌های غلظتی ۰٫۶–۰٫۱ µg/band و ۰٫۶–۰٫۰۲ µg/band برای MOL و ناخالصی‌اش با درصد بازیابی‌های به ترتیب ۱٫۵۲۱±۹۹٫۹۲٪ و ۲٫۲۹۶±۹۹٫۲۸٪ بکار رفت. این روش با جداسازی اجزا و پایانه‌ها و از طریق اندازه‌گیری دانسیتومتری پیک‌های جداشده در طول موج ۲۷۶ نانومتر انجام گرفت. جداسازی بر روی پلیت‌های HPTLC از نوع سیلیکاژل F254 و با سیستم توسعه‌یافته شامل آب، متانل، n–بوتانل و تولوئن با نسبت حجمی به ترتیب ۰٫۵، ۱٫۵، ۳ و ۵ انجام شد. MOL تحت شرایط مشابه آنچه در سمینار بین المللی هماهنگ‌سازی (ICH) ملزم کرده است، اعم از شرایط اکسیداسیون، هیدرولیتیکی، فشارهای دمایی و تست های فتولیتیکی قرارداده شد. در شرایط هیدرولیز اسیدی، قلیایی و اکسیدی MOL ناپایدار بود، اما تحت شرایط اسیدی، گرما یا نور UV تحت تاثیر قرار نمی‌گرفت. تخریب قلیایی MOL با استفاده از نگرش HPTLC ذکر شده، مطالعه شد. تخریب شده‌ها با روش HPTLC جداسازی شدند و ساختارشان با داده‌های طیفی IR، MS و NMR مورد تایید قرار گرفت.

واژه‌های کلیدی

مولناپیراویل؛ ناخالصی؛ HPTLC؛ IR؛ NMR؛ اسپکترومتری جرمی.