RESEARCH ARTICLE

Development and Validation of New LC-MS/MS Bioanalytical Method of Tremelimumab in Rat Plasma by Using Nivolumab as Internal Standard and Its Application with Pharmacokinetic Studies

Alapati Sahithi, Asha Deepti*

Department of Pharmaceutical Analysis, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India.

Received: 15th April, 2023; Revised: 01st May, 2024; Accepted: 19th May, 2024; Available Online: 25th June, 2024

ABSTRACT

Nivolumab was decided to be used as the IS. To separate the drugs, an isocratic mobile phase of acetonitrile (ACN): Ammonium formate containing formic acid (70:30) was adopted and delivered at 1-mL/min, along with a 150 x 4.6 mm x 3.5 µm Alliance, e2695 Luna Phenyl Hexyl column. Drug and IS, both of which display proton adducts approximately m/z 146.3685 to 120.0638 and m/z 143.7695 to 76.7964, might be detected simultaneously using MRM-positive modalities. Over a linearity level of 2.00 to 40.00 ng/mL, the method had a correlation value (r2) of 0.99977. The accuracy and precision of this method during the day ranged from 89.85 to 102.89% and 0.19 to 2.81%, respectively. Tremelimumab was reported to remain stable during three freeze-thaw cycles, benchtop tests, and postoperative stability studies. Cmax and Tmax values were acquired directly from experimental data. C_{max} and T_{max} averaged 18.024 ng/mL and 6 hours, respectively. At $t_{1/2}$ of 18 hours, plasma levels began to fall. The obtained AUC_{024} and AUC_0 values were 257 and 257 ng h/mL, respectively.

Keywords: Tremelimumab, Nivolumab, Pharmacokinetics, MRM, Ammonium formate, Formic acid.

International Journal of Drug Delivery Technology (2024); DOI: 10.25258/ijddt.14.2.20

How to cite this article: Sahithi A, Deepti A. Development and Validation of New LC-MS/MS Bioanalytical Method of Tremelimumab in Rat Plasma by Using Nivolumab as Internal Standard and Its Application with Pharmacokinetic Studies. International Journal of Drug Delivery Technology. 2024;14(2):727-731.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Tremelimumab (Figure 1) (TMB) is a completely humanized IgG monoclonal antibody that blocks the cancer-fighting T-cell suppressive receptor known as CTLA-4. Preliminary clinical studies of TMB's antineoplastic effects have been encouraging. Target amounts of antibodies in the blood required for a pharmacodynamic impact were anticipated by experimental research *in-vitro* and in large animal species. In line with the hypothesized mode of action, phase I human studies showed dose- or exposure-related responses.[2,3] The chemotherapeutic drug tremelimumab inhibits tumor development.[4] It interacts with human CTLA-4 having subnanomolar affinities and excellent selectivity.[5] Elevated IL-2 synthesis in *ex-vivo* blood stimulating experiments employing PBMCs from healthy individuals and cancer sufferers was dose-dependent, suggesting that tremelimumab enhanced T cell-induced cytotoxicity.[6] Tremelimumab additionally promoted the growth of effector T cells.[7-9]

MATERIALS AND METHODS

Chemicals and Reagents

- Pfizer Limited in Hyderabad, India, supplied both the tremelimumab (TMB) (99.98%) and the nivolumab (NVL) (99.95%).
- Merck (in Mumbai, India) supplied the acetonitrile: ammonium formate pH-2.5/formic acid 30:70 v/v.
- The K2EDTA-treated plasma of rats was collected (In-house: Bio needs, Bangalore).

The LC-MS/MS Instrument and its Parameters

An HPLC setup with a column oven, degasser, and autosampler was used for the test; the model was the Waters Alliance e-2695. The HPLC equipment was coupled to an ESI-compatible mass spectrometer (AB SCIEX QTRAP 5500) for analysis. The separation is achieved in the positive mode of turbo ion spray (ESI) and with unit resolution. TMB's and NVL's parent ion m/z measured 146.3685 and 143.7695, correspondingly,

*Author for Correspondence: salapati1@gitam.in

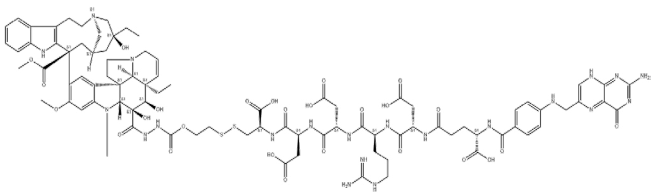

Figure 1: Chemical structure of tremelimumab

and their distinctive product ion m/z measured 120.0638 and 76.7964, correspondingly. These are the criteria for mass selection: Collision gas nitrogen, drying gas 120 to 250°C, 5 mL/min flow rate, and 550°C at the source. The TMB and NVL had the same entry, egress, and decluttering potentials of 10, 7, and 40 V, respectively, and the dwell time was 1 second.

Chromatographic Conditions

For this investigation, the 150 x 4.6 mm, 3.5 µm Luna Phenyl Hexyl Analytical Column was used. ACN and AmF were added to the mobile phase in a 70:30 (v/v) ratio. The mixture was then filtered using 0.45 µ filter paper. The mobile phase was added at a rate of 1-mL/min. During a 6-minute period, the median TMB retention time (RT) was 2.225 minutes.

Sample Preparation

TMB was isolated using the LLE method from rat plasma. This technique included adding plasma samples (80 µL, at the optimal dose) to already-labeled vials and letting them briefly reflux. It was then centrifuged at 4000 rpm at 20°C after being blended for approximately 10 minutes with 500 µL of standard stock and NVL stock. The supernate from each sample was placed in its own labeled tube and evaporated at 40°C until dry. The samples were diluted in 300 µL of ACN and 500 µL of diluents, then quickly vortexed, transferred to auto-sampler vials, and injected.

Method Validation

We assessed the specificity and sensitivity of the method by treating blank plasma, blank plasma spiked with TMB and NVL, and plasma samples (which included oral TMB administration) to various treatments and analyses.[10]

Linearity and sensitivity

By employing a linear least-squares regression analysis (1/X2 weighting), peak area ratios (Y) between the drug and IS doses (X) were plotted to generate a calibration graph .[11]

Precision and accuracy

LQC, MQC and HQC samples were run to evaluate the precision and accuracy on a single and different day. Measures of precision and accuracy had to match the basic guidelines. They were reported as the RSD and RE, respectively.[10] The relative standard deviation and the relative error should both be under 15%.[12]

Recovery and matrix effect

Analyte standards with and without matrix extracts were diluted using the mobile phase and compared for peak regions to determine the presence or absence of a matrix effect at three different concentrations (LQC, MQC, and HQC) (Zhang *et al*., 2014). Assessing the reaction of blank plasma samples taken before and after extraction allowed us to gauge the success of the recovery.[13]

Stability

The samples were tested for stability at low, medium, and high concentrations in rat plasma (n = 6 each). They were kept at 25°C for 24 hours to determine their short-term stability.[14] The effects of long-term storage at 28°C over 49 days were also investigated. Furthermore, 3 full freeze-thawing sessions (28–25°C) were performed to examine the freeze-thaw stability. Additionally, 30 days of storage at 4°C was used to assess the long-term stability of the stock solutions.[15] When the RE for a given analyte was less than or equal to 15%, we concluded that its stability had been maintained.[16,17]

Pharmacokinetic Evaluation of Tremelimumab in Plasma

The male Sprague-Dawley rats were obtained from Flair Labs in Surat, Gujarat. The rats were injected with TMB (25 mg/kg) once, and samples were collected at various time points ranging from 0 to 21 hours after treatment. Samples of blood were collected at regular intervals and stored in a K2 EDTA cuvette, each containing 5 mL. A blank sample (prior to drug administration) was also collected to ensure that no plasma interferences were visible. The plasma samples were carefully processed to separate the blood cells and subsequently stored at a very low temperature of -70°C. Before combining the samples with the QC samples, the plasma samples were introduced to four different amounts of the NVL. Analyzing the PK of TMB involved using the software WinNonlin (Version 5.2).

Research Statement

The guidelines and rules put out by the CPCSEA were adhered to scrupulously throughout this study. The Central Government of India established the CPCSEA to supervise the use of animals in scientific experiments. The Animal House Facility of Flair Labs (Surat, Gujarat, India) has been legally permitted to conduct educative experiments on small animals using the pharmacokinetics methodologies registered under 1250/PO/RcBi/S/27/CPCSEA. The protocol number CPCSEA/Flair Lab/PK/1250 covers all rat experiments included in this study, which were all carried out at the Flair Labs Animal House Facility. Without the use of anesthesia, but with all the necessary measures in place to prevent unnecessary pain and suffering for the animals involved, we perform our studies.

RESULTS AND DISCUSSION

Mass and Chromatographic Condition Optimization

For the purpose of determining the TMB and NVL, this investigation meticulously refined the chromatography and analytical variables. As per the findings, excellent chromatography behavior (especially peak uniformity, sensitivity, and brief run duration) and adequate ionization were achieved by chromatographic settings, notably the

constitution of the mobile phase. ACN and AmF, 30:70 v/v, eluted isocratically at 1-mL/min, produced an excellent peak shape and ionization. It was found that the responses of the TMB and NVL were detected in the positive ionization modality and that this was due to the fact that this method was used to produce the precursor and product ions under mass-condition-specific circumstances. TMB's and NVL's parent ion m/z measured 146.3685 and 143.7695, correspondingly, and their distinctive product ion m/z measured 120.0638 and, 76.7964, correspondingly. Figures 2 and 3 illustrate the mass spectra of TMB and NVL, respectively**.**

Method Validation

Specificity and selectivity

Figure 4 shows a basic chromatogram of blank plasma, Figure 5 shows a spiking of blank plasma with NVL, and Figure 6 shows a chromatogram of plasma samples collected when administered orally with TMB. Figure 6 shows that between the TMB retention period (3.652 minutes) and the IS, there was no substantial interference from indigenous constituents in the blank sample (2.225 minutes).

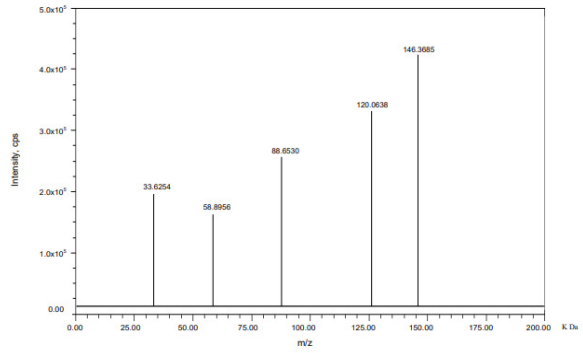

Figure 2: Tremelimumab parent and daughter ion mass spectra

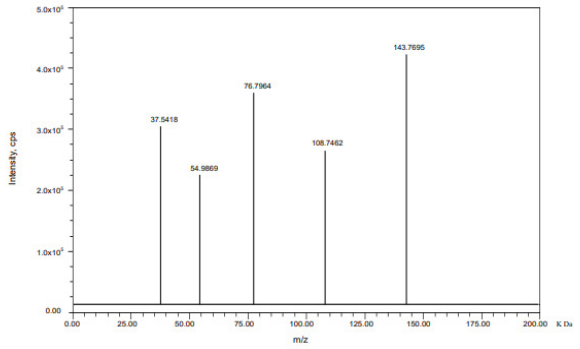

Figure 3: Nivolumab parent and daughter ion mass spectra

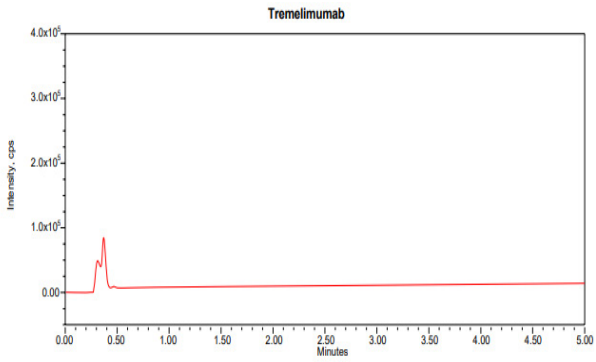

Figure 4: Blank plasma interference

Linearity

Based on the data, we can conclude that TMB content is linearly proportional to the response variable within a 2 to 40 ng/mL spectrum, with a LLoQ set at 2 ng/mL. The regression coefficient is y = 0.0480x + 0. 0008. Table 1 display the calibration curve results of tremelimumab

Precision and accuracy

Table 2 displays the precision and accuracy of TMB at low-, medium-, and high- concentrations, both within and between days, in rat plasma.

Stability

Using measurements taken from rat plasma before, during, and following three cycles of freeze/thaw (at -30°C and at room temperature), the drug's stability was established (Thulaseedhar *et al*., 2022; Kanchanamala *et al*., 2013). No detectable degradation of the medication was sensed after being stored in the autosampler for 6 hours, with the resultant amounts of TMB being about 105.89 to 112.54% of the calculated observations. Additionally, QC samples were stored at 30°C over 28 days to evaluate the medication's long-term

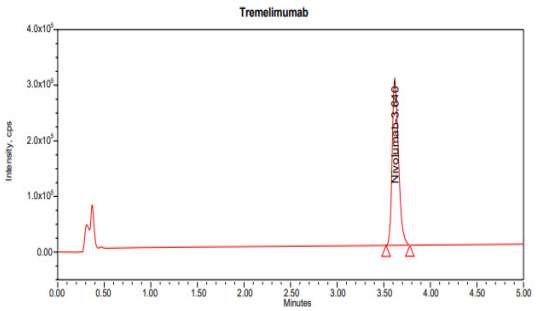

Figure 5: Blank plasma interference with IS

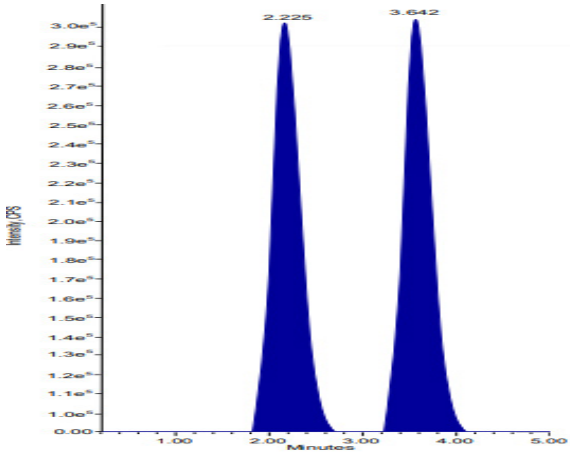

Figure 6: Standard chromatogram of tremelimumab and nivolumab

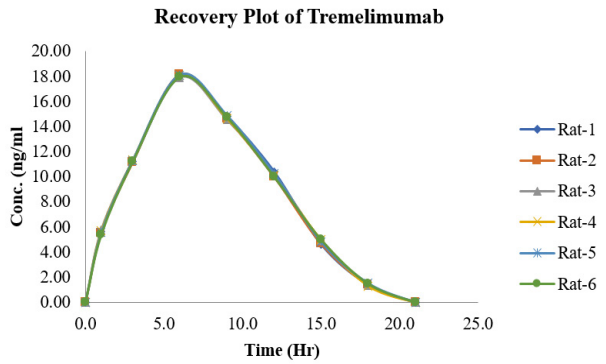

Figure 7: Plasma concentration-time profiles of TMB in rat plasma

Table 1: Calibration curve of tremelimumab

Concentration	*Peak area*		*Peak*
(ng/mL)	*Tremelimumab*	*Nivolumab*	*Ratio*
0	0	0	0
2	0.309	3.172	0.0974
5	0.753	3.181	0.2367
10	1.524	3.176	0.4798
15	2.318	3.175	0.7301
20	3.063	3.177	0.9641
25	3.835	3.184	1.2045
30	4.547	3.172	1.4335
40	6.121	3.180	1.9248
Slope	0.048018		
Intercept	0.000841		
R^2	0.99988		

Table 2: Tremelimumab precision and accuracy in rat plasma

Injection	*HQC*	*MQC*	*LQC*	*LLoQ*
	Nominal conc. (ng/mL)			
	30	*20*	*10*	*2*
	Drug peak area			
Mean	$4.528x10^5$	$3.057x10^5$	$1.527x10^5$	$0.302x10^5$
SD	0.009	0.009	0.043	0.001
%CV	0.19	0.29	2.81	0.33
%Mean Accuracy (%)	101.52	89.85	102.78	98.78

stability. 89.25 to 99.88% of the calculated observations were achieved in the concentration range (Table 3).

Recovery

TMB recovery was calculated to be 100.41, 95.78, and 101.19%, respectively, at three different concentrations of 10.0, 20.0, and 30.00 ng/mL. As a result, NVL retrieved 101.19% of its dosage, whereas TMB retrieved 95.78%.

Table 3: Tremelimumab stability in plasma samples

Stability conc. (10 ng/mL) (30 ng/mL)	*Mean response ± SD*	*RSD (%) (n = 6)*
Bench top stability	$1.515 x10^5 \pm 0.008$ $4.539 x10^5 \pm 0.002$	0.42 0.09
Auto sampler stability	$1.519 x10^5 \pm 0.012$ $4.548 x10^5 \pm 0.013$	0.63 0.53
Wet extract stability at 12 hours	$1.523x10^5 \pm 0.023$ $4.524 x10^5 \pm 0.019$	1.02 0.38
Wet extract stability at 18 hours	$1.525x10^5 \pm 0.012$ $4.532 x10^5 \pm 0.014$	0.78 0.13
Freeze-thaw	$1.518 x10^5 \pm 0.005$ $4.545 x10^5 \pm 0.024$	0.23 0.56
Short term	$1.532 x10^5 \pm 0.003$ $4.559 x10^5 \pm 0.010$	0.18 0.20
Long term (28 days)	$1.539 x10^5 \pm 0.008$ $4.539 x10^5 \pm 0.005$	0.15 0.19

Table 4: Pharmacokinetic parameters of tremelimumab

Pharmacokinetic parameters	*Tremelimumab*
AUC_{0-t}	257 ng-hr./mL
C_{max}	18.024 ng/mL
$AUC_{0-\infty}$	257 ng-hr./mL
t_{max}	6 hours
$T_{1/2}$	18 hours

Pharmacokinetics and statistical analysis

After administering 25 mg/kg of TMB intravenously to six rats, we effectively acquired a series of pharmacokinetic data using the newly designed Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) technology. It was discovered that the assay's sensitivity and specificity were enough for precisely describing the plasma pharmacokinetics of TMB in rats. The major pharmacokinetic characteristics of TMB after IV delivery were computed using a non-compartmental model and are shown in Table 4. Figure 7 illustrates the mean plasma concentration-time profiles of TMB following treatment. C_{max} and T_{max} values were acquired directly from experimental data. C_{max} and T_{max} averaged 18.024 ng/mL and 6 hours, respectively. At $t_{1/2}$ of 18 hours, plasma levels began to fall. The obtained AUC_{024} and AUC_0 values were 257 and 257 ng h/mL, respectively.

CONCLUSION

To determine Tremelimumab oral pharmacokinetics, we used a newly designed and established LC-MS/MS analytical approach for tremelimumab quantitation in rat plasma. TMB had a rapid time to C_{max} and exhibited subsequent uptake, as shown by pk characteristics. The acquired data might set the groundwork for preclinical and clinical research of TMB, as well as give benchmark and useful knowledge for the ongoing development of novel drugs and pharmacology pathways.

REFERENCES

1. Ezzeldin E, Iqbal M, Al-Salahi R, El-Nahhas T. Development and validation of a UPLC-MS/MS method for determination of motesanib in plasma: Application to metabolic stability and pharmacokinetic studies in rats. Journal of Pharmaceutical and Biomedical Analysis. 2019 Mar 20; 166:244-51.
2. Guo Z, Li B, Gu J, Zhu P, Su F, Bai R, Liang X, Xie Y. Simultaneous quantification and pharmacokinetic study of nine bioactive components of Orthosiphon stamineus Benth. Extract in Rat Plasma by UHPLC-MS/MS. Molecules. 2019 Aug 22;24(17):3057.
3. Hwang JR, Byeon Y, Kim D, Park SG: Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. Exp Mol Med. 2020 May;52(5):750-761.
4. Kanchanamala K, Nagiat T. H, Babu Rao C, Prakash K, Mukkanti K, Challa BR, Bhavyasri K. Bioanalytical method development and validation of milnacipran in rat plasma by LC–MS/MS detection and its application to a pharmacokinetic study. Journal of Pharmaceutical Analysis. 2013;3(6):481–488.
5. Li L, Feng R, Feng X, Chen Y, Liu X, Sun W, Zhang L. The development and validation of an HPLC-MS/MS method for the determination of eriocitrin in rat plasma and its application to a pharmacokinetic study. RSC advances. 2020;10(18):10552-8.
6. P. Venkata Rao, Ch., M. Ravi Kumar, Dantu Durga Rao. Simultaneous Determination Of Omeprazole And Domperidone In Capsules And *In-vitro* Dissolution Studies By Using Stability Indicating UPLC. Journal of liquid chromatography & related technologies. 2012 Oct 1;35(16):2322–32.
7. Niroja Vadagam, Sharath Babu Haridasyam, M. Venkatanarayana, Narasimha Swamy Lakka, Chinnakadoori SR. Separation and quantitative estimation of stereo-selective enantiomers of montelukast in pharmaceutical drug substance and tablets dosage forms by using stability-indicating normal phase-HPLC method. Chirality. 2023 Jul 17, 952-965.
8. Niroja Vadagam, Sharath Babu Haridasyam, Muvvala Venkatanarayana, Lakka NS, Chinnakadoori SR. Separation and quantitation of valacyclovir enantiomers using stability-indicating chiral liquid chromatography method with a chiral stationary phase of amylose tris-(3,5-dimethylphenylcarbamate). Separation science plus. 2023 Sep 21;6(12).
9. Lakka NS, Chandrasekar Kuppan, Niroja Vadagam, Poornima Ravinathan, Kalyani Chepuri, Chinnakadoori SR. Molecular docking, in-vitro anticancer evaluation and ADME profiling of 7-Oxo Midostaurin. Journal of molecular structure. 2023 Dec 1;1293:136159–9.
10. Niroja Vadagam, Sharath Babu Haridasyam, Muvvala Venkatanarayana, Lakka NS, Chinnakadoori SR. Separation and simultaneous estimation of enantiomers and Diastereomers of muscarinic receptor antagonist Solifenacin using stability-indicating Normal-phase HPLC technique with chiral stationary phase amylose tris-(3,5-dimethylphenylcarbamate). Chirality. 2023 Nov 23;36(2).
11. Mohan Pasham, Sharath Babu Haridasyam, Niroja Vadagam, N. V. V. D. Praveen Boppy, Chinnakadoori SR, Lakka NS. Separation and quantification of organic-related impurities of beta-adrenergic receptor blocking agent propranolol in pharmaceutical solid dosage forms: Impurity profiling using stability-indicating HPLC method. Separation science plus. 2023 Nov 13;7(1).
12. N. V. V. D. Praveen Boppy, Sharath Babu Haridasyam, Niroja Vadagam, Muvvala Venkatanarayana, Chinnakadoori SR, Lakka NS. Separation and quantification of organic-related impurities of anti-histamine drug hydroxyzine in pharmaceutical dosage forms using stability-indicating high-performance liquid chromatography, liquid chromatography-mass spectrometry, and high-resolution mass spectrometry techniques. Separation science plus. 2024 Feb 13;7(4).
13. Teja K, Reddy K, M Akiful Haque. Develop and validate a highly sensitive method for the estimation of Molnupiravir in rat plasma by high-performance liquid chromatography-tandem mass spectroscopy and its application to pharmacokinetic studies. 2022 Jan 1;13(S01).
14. A Critical Review On Bioanalytical Method Development and Validation of Few Oncology Drugs by Using LC-MS-MS. Journal of Pharmaceutical Negative Results. 2022 Jan 1;13.
15. Teja K, M. Akiful Haque. Bioanalytical method development and validation of atrasentan in human plasma using verapamil as internal standard by liquid chromatography coupled with tandem mass spectrometry. International Journal of Health Sciences (IJHS). 2022 Jul 7;625–38.
16. Konatham TK Reddy, Haque M. Molnupiravir Bioanalytical Method Development and Validation in Rat Plasma by LC-MS/MS Detection and Application to a Pharmacokinetic. International journal of pharmaceutical quality assurance. 2024 Mar 25;15(01):110–4.
17. Reddy KTK, Haque MA. Development and Validation of a High Throughput Lc-Ms/MS Method for Quantitation of Ipilimumab in Human Plasma. International Journal of Pharmaceutical Quality Assurance. 2022;13(3):303-307