Development And Validation Of Stability Indicating Method for Immunotherapy (Ctla-4) Agent By Reverse Phase Ultra Performance Liquid Chromatography

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ABSTRACT

The current investigation was pointed at developing and progressively validating novel, simple, robust, accurate, rapid, reproducible, and stability-indicating reverse phase- liquid chromatography method has been established for the assay of Ipilimumab in bulk and pharmaceutical dosage forms. ipilimumab was eluted from an Agilent C18 (50 x 2.6mm, 1.7µm) column at 15°C. Isocratic elution was performed using the mobile phase having mixture of Phosphate buffer : Methanol in the proportion of (10:90v/v). The Rate flow was maintained at 0.3 ml/min. and the maximum wavelength for column effluents was detected at 225nm using a PDA detector. The developed method has shown elution at 3 min. The whole analytical method validation was done satisfactorily as per ICH regulations. The recovery studies were performed at levels of 50%, 100% and 150%. The % recovery results were in the range of 99.91 to 100.81%, . The current method's regression co-efficient of 0.9989 showed that it was linear at concentrations between 20 and 120 µg/ml. The % relative standard deviation results were seen to be < 2%. ipilimumab was subjected to several stress conditions such as acid, neutral, base, peroxide, photolytic, and thermal for the stability testing. More recovery and minimal SD were observed. This approach was simple and affordable making it suitable for application in industries as a standard method of QC department.

Key words: RP-UPLC, PDA detector, Ipilimumab stability indicating studies and ICH Guidelines.

INTRODUCTION

Ipilimumab (IPB) is used as an additional therapy for patients who had surgery for cutaneous and lymphatic vascular invasion melanoma[1]. Cytotoxic T-lymphocyte antigen - 4 (CTLA - 4) is conjugate by the immunotherapy drug ipilimumab (brand name: Yervoy®), which also stops CTLA-4 from interacting with its ligands, CD80/CD86. It has been discovered that blocking CTLA-4 signalling increases T-cell activation and proliferation and may ameliorate T-cell responsiveness. Immune cells called T-cells are able to recognise and combat cancerous cells[2]. If the lump was undetectable or had advanced (migrated to other organs), IPB is recommended for adults and children over the age of 12[3]. The atomic structure of a protein is $C_{6572}H_{10126}N_{1734}O_{2080}S_{40}$. The average protein weight is around 148000.0 da.[4-6]. The literature review indicated that no work had been done on UPLC.

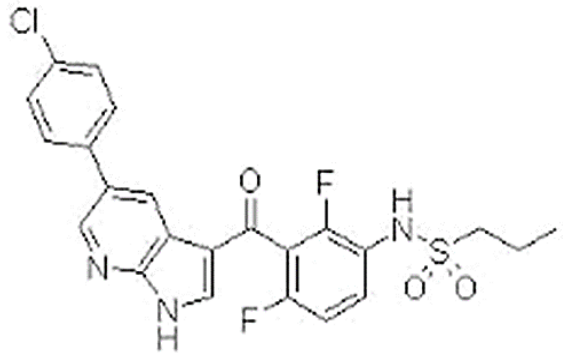

Figure 1: Structure of Ipilimumab.

MATERIALS AND METHODS

Instrumentation

Shimadzu (LC 20 AT VP) UPLC scheme on Inertsil C18, ODS (50x2.6 mm ID, 1.7µm particle size) column was utilized to create a UPLC approach for ipilimumab. The device has a PDA detector and an auto sampler. The samples were placed into a 4µl rheodyne injector port. Employing the programme Spin Chrome, data was analyzed. For measuring pH, a Global digital pH meter was applied.

Chemicals and solvents

Ipilimumab were offered as free samples by Chandra Labs in Hyderabad, India. From E.Merck (India) Ltd. in Mumbai, India, UPLC grade water, sodium hydroxide, methanol, and phosphate buffer were achieved.

Chromatographic conditions

It was found that phosphate buffer and methanol (10 : 90, v/v) built the optimum mobile phase for an ideal chromatographic separation for assessing of ipilimumab. The solvent combination was ultrasonically addressed and passed through a 0.45µm membrane filter before use. It passed through the column at a flow rate of 0.3ml/min. Injection volume was 4µl, and the column was held at room temperature. By pumping the mobile phase through the column for at least 15 min, the column was made equilateral before the drug solution was added. The drug detection was seen at 225nm. The run was permitted for 3 minutes.

Phosphate buffer Preparation [buffer pH 3]

By pairing 6.8gr of phosphate buffer with 1000 ml of water, phosphate buffer solution may be produced. Use sodium hydroxide to bring the pH of this solution to 3.

Mobile phase Preparation

Mix 900 ml of Methanol UPLC (90%) with 100 ml of phosphate buffer (10%), and then degas the mixture in an ultrasonic bath for 5 minutes. Filter finished a 4.5µ filter while using a vacuum.

Standard & Sample Solution Preparation

Standard Solution

Measure and transfer carefully. An void, dry volumetric flask of 25 ml has been stuffed with 25 mg of ipilimumab. Diluent is added, entirely dissolved, and the flask is sterilized to raise the volume to the desired level. Using Stock Solution.

Ipilimumab stock solution indicated above, 0.8 ml, poured into a 10 ml volumetric flask, diluted to the appropriate concentration.

Sample Solution

A precise weight is used to determine the amount of Ipilimumab and it is then put into a dry, clean volumetric flask weighing 25 ml of the sample. The sample is completely dispersed in diluents before being sonicated to enhance the volume to the required amount. (Stock solution)

The remaining 0.8 ml of ipilimumab from the stock solution mentioned earlier must be poured into a 10 ml volumetric flask and diluted to the required concentration.

Wavelength selection

The UV spectrum is 4 µg/ml. Scannable wavelength between 200-400nm were used to record ipilimumab in methanol. The wavelength of 225nm was chosen from the UV spectrum. Drug has strong absorption at this wavelength.

Procedure

The column's temperature was held stable at that level. The run was tentatively set for 3 minutes. The column was made equal by pumping the mobile phase through it for at least 15 min before the delivery of the drug solutions. Six times, 4 µl of each of the standard and sample solutions were injected into the chromatographic device at a flow rate of 0.3 ml/min to produce the appropriate chromatograms. From these chromatograms, the average area alongside the peak of each dilution was computed. (Figure 3).

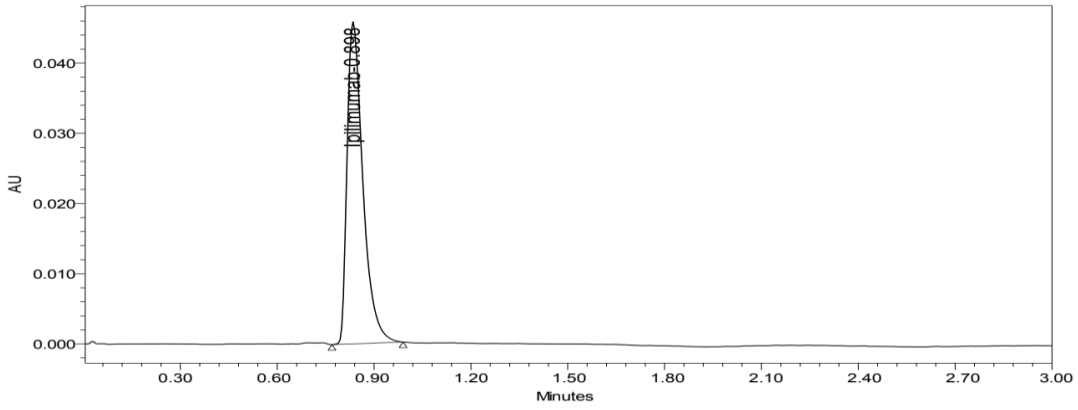

Figure 2: Ipilimumab standard chromatogram.

METHOD VALIDATION

Linearity

Ipilimumab standard solutions were divided into several aliquots and placed into six distinct 10 ml volumetric flasks. The flasks were then occupied to the mark with diluent, resulting in final concentrations of Ipilimumab that ranged from 20 to 120µg/ml. The UPLC system was filled with the aforementioned solutions while sustaining a uniform injection volume. The peak point for all samples were obtained with a UV detector at 225nm. Plotting medication concentration vs peak regions allowed for the creation of the linearity curves. This curve's regression equation was calculated. Later, the amount of pharmaceutical in tablet dosage forms was estimated using this regression equation (Figure.3).

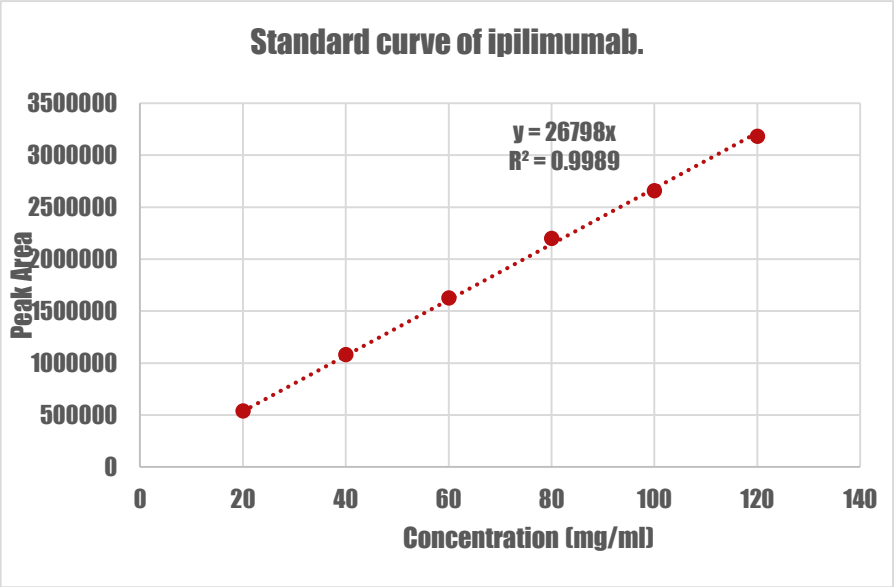

Figure 3: Calibration Plot of Ipilimumab.

Precision

Ipilimumab precision was used throughout and between days to determine its potency. A total of three shots were aimed at each sample. The peak area measurements were given as %RSD.

Accuracy

Recovery tests of ipilimumab at three concentrations i.e. 50%, 100% and 150%. were used to evaluate the method's accuracy. To determine Ipilimumab % Recovery, a fixed amount was added to the pre analysed sample three times at each level.

System suitability

Ipilimumab was investigated in six duplicate probes, and system suitability traits such as tailing factor, theoretical plates, and retention time frame were assessed and compared to bench mark values. The theoretical plate numbers (N) of at least 2000 for each peak, a %RSD of peak areas of not more than 2%, and tailing factors of not more than 2.0 are necessary to permit ipilimumab.

Limit of detection and quantification

The Limit of Detection (LOD) and Quantification (LOQ) of the method were decided by injecting progressively decreasing amounts of the Ipilimumab standard solutions. LOD and LOQ were attained by using the signal to noise ratio. The LOD and LOQ were found using the 3.3 σ/S and 10 σ/S formulae to use. S is the corresponding calibration curve's slope, and is the peak of the areas' standard deviation.

Robustness

By deliberately changing small aspects of the process, such as rate of flow, ambulant phase ratio, and temperature, the robustness of the approach was assessed.

RESULTS AND DISCUSSION

By optimizing the UPLC process, a method for synchronous estimation of ipilimumab in lozenge dosage form using a UPLC system on an Inertsil C18 (50x2.6mm, 1.7µm particle size) column in isocratic manner with flexible phase composition was created[7-11]. Using phosphate buffer and methanol (10:90, v/v), a peak with maximal separation, nice development, and resolution was achieved. The study concentrated on flow rates of 0.3 ml/min. 3.0 min. were found to be the retention times for ipilimumab, and a flow rate of 0.3 ml/min offered the finest signal to noise ratio and decent separation time. The run time lasted for three minutes. The components of the medication were detected using the UV detector at 225nm. Table 1, displayed the outcomes of improved chromatographic conditions. The chromatograms show no interference from normal excipients and chemicals found in tablet formulations when they were examined for the appearance of any new peaks under perfect conditions. The peak regions were also compared to the norm and found to be within limits. As shown in the chromatogram, symmetrical peaks are produced as two analytes are eluted. The typical chromatogram of the Praziquantal standard is shown in Figure 1. Ipilimumab's linearity was discovered to be between 20 and 120 ng/ml. The correlation coefficient (r^2) for ipilimumab was found to be 0.9989. It was shown that the regression equation of the linearity plot of Ipilimumab concentration over its peak area is y=2679.8x+53596, where x is the concentration of Ipilimumab (g/ml) and y is the corresponding peak area. The findings demonstrate that, within the recommended concentration range, there is a substantial correlation between peak area and drug concentration. Tables 2 and Figures 3 provide the linearity findings and calibration curves, respectively. The approach is precise since the % RSD for the scheme, method and half way precision for ipilimumab was determined to be within the limitations (limit % RSD 2.0%). Ipilimumab's precision information was provided in Table 3. The medicine Ipilimumab had a recovery rate of 99.91 to 100.81%, and this high recovery rate demonstrates the great accuracy of the suggested approach. Table 4 shows the findings of Ipilimumab accuracy trials. The robustness studies showed that the drug's determination was not significantly impacted. As a result, the test procedure is reliable for determining the drug's quantity. The % RSD for replicate Ipilimumab injections were determined to be within the permitted range in all deliberately altered scenarios. Table 5 displays the findings of the Ipilimumab robustness investigations.

Throughout the validation, the stability's subject area changes in the test material's area responses will be tracked. Stress testing must be done in order to clarify the inherent stability properties of the active ingredient as part of the authorized stability assessment of new pharmacological substances and products.Table 6 displayed the findings of the stability studies.

Table 1: Optimized chromatographic situation

S.No	Parameters	conditions
1	Mobile Phase	Phosphate buffer: Methanol (10:90) ratio
2	Column	Waters BEH C18(50x2.6mm, 1.7µm) UPLC COLUMN
3	Column Temperature	Ambient
4	Wavelength	225 nm.
5	Shot Value	4µl
6	Rate flow	0.3 mL per min
7	Run time	3 min

Table 2: Linearity data of Ipilimumab

Injection	Standard Area
1	541386
2	1082736
3	1627143
4	2199868
5	2658905
6	3181776
Mean	188.1969
SD	989450.36

Table 3: Precision of Ipilimumab

S. No		Area
System Precision	1	550667
	2	551067
	3	563139
	4	553794
	5	561645
	6	568453
	Mean	558127.5
	SD	7326.6
	%RSD	1.3
	1	570667
	2	571067

Method Precision	3	563139
	4	573794
	5	561645
	6	568453
	Mean	568127.5
	SD	4779.4
	%RSD	0.8
Intermediate precision	1	556273
	2	551993
	3	558740
	4	549512
	5	541747
	6	548239
	Mean	551084.0
	SD	6068.1
	%RSD	1.1

Table 4: Accuracy studies of Ipilimumab

Concentration (At Specification Level)	**Area**	**Amount Added (Mg)**	**Amount Found (Mg)**	**% Recovery**	**Mean Recovery**
50%	273183	12.5	12.49	99.91	100.38
100%	549201	25	25.11	100.43	
150%	826898	37.5	37.80	100.81	

Table 5: Robustness of Ipilimumab

S. No.	**Conditions**	**%RSD**
1	Rate Flow (-)	1.69
2	Flow rate (+)	1.65
3	Mobile phase (-)	1.59
4	Mobile phase (+)	1.60
5	Temperature (-)	1.65
6	Temperature (+)	1.66

STABILITY STUDIES

Stress testing must be done in order to clarify the inherent stability properties of the active chemical, according to the ICH regulation entitled stability testing of novel pharmaceuticals and products. The purpose of this work was to use the proposed methodology to carry out stress degradation investigations on the Ipilimumab[12].

Acidic degradation.

Add 3 ml of 0.1N HCl to a 10 ml volumetric flask after adding 0.8 ml of the preceding solution by the pipetting process. The volumetric flask was neutralized with 0.1 N NaOH and diluted to a final volume of 10 ml after being kept at 60 °C for 24 hours. Use 0.44µ syringe filters to filter the solution prior filling the vials.

Alkaline degradation.

After pipetting 0.8 ml of the preceding solution into the flask, add 3 ml of 0.1N NaOH. Later on, the volumetric flask was kept at 60 °C for 24 hours before being neutralized with 0.1 N HCl and dilute to a final amount of 10 ml. Utilizing syringe filters with a pore size of 0.44µ, pour the filtered solution into vials.

Thermal degradation.

The material was collected in a petridish and maintained in a hot air oven for three hours at 110^0C. The sample was then captured, diluted with diluents, and sent through a UPLC to be examined.

Oxidative degradation.

Pipette A 10 ml volumetric flask was filled with 0.8 ml of the stock solution, 1 ml of 30% hydrogen peroxide, and the volume was then diluted to the necessary concentration. The volumetric flask was then kept at room temperature for 15 minutes. Using 0.45µ syringe filters, put the filtered solution in vials.

Photo degradation.

Pipette 0.8 ml of the stock solution into a volumetric flask with a 10 ml capacity. After subjecting the flask to sunlight for 24 hours, dilute with diluent to the desired volume is carried out. Using 0.45µ syringe filters, put the filtered solution in vials.

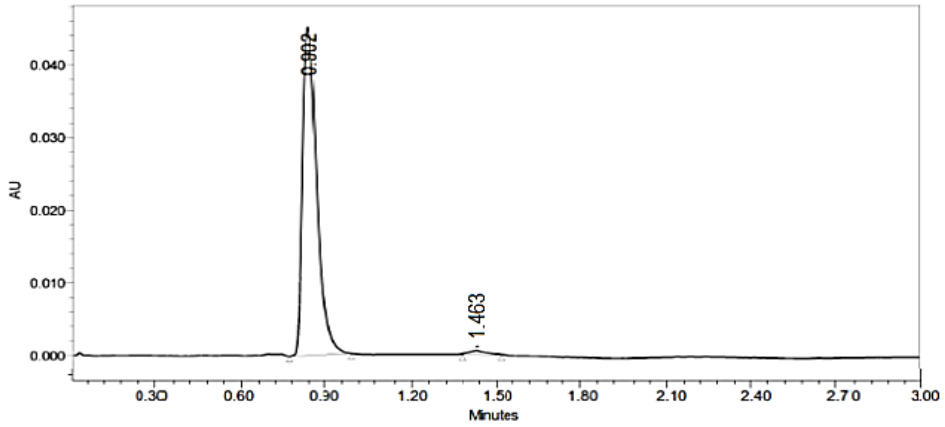

Figure 4: Peak of acid degradation.

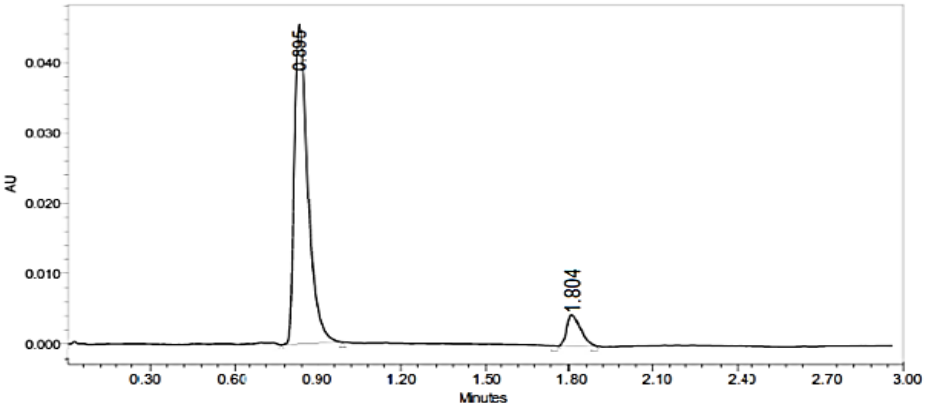

Figure 5: Peak of Basic degradation.

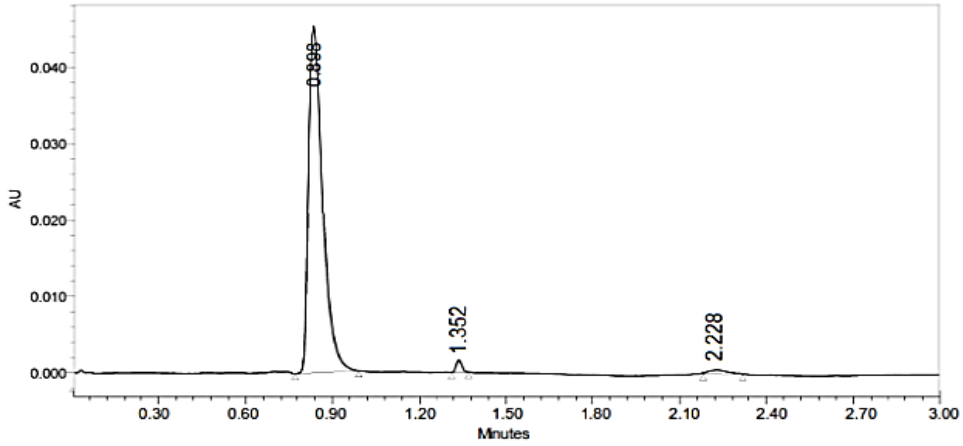

Figure 6: Peak of Thermal Degradation.

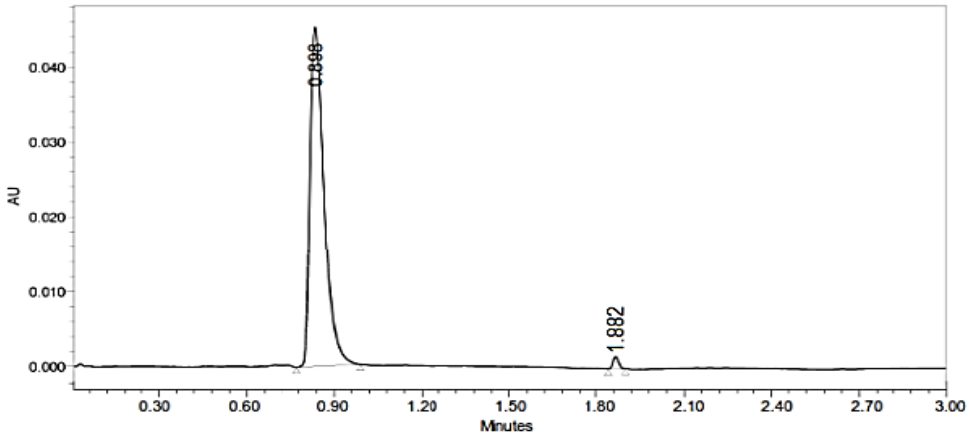

Figure 7: Peak of Oxidative Degradation.

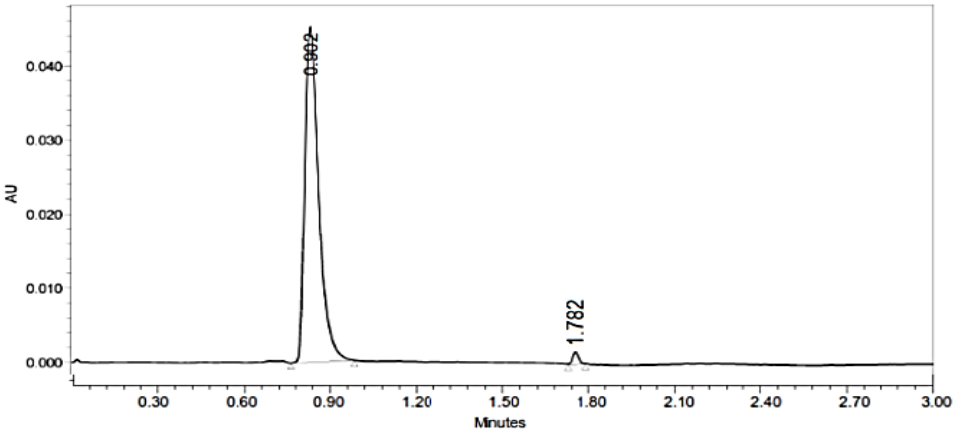

Figure 8: Peak of Photo Degradation.

Table 6: Degradation studies data

Sample Name	Ipilimumab	
	Area	% Degraded
Standard	0	0
Acid	510362	6.49
Base	529039	5.06
Peroxide	533748	5.20
Thermal	516206	5.41
Photo	506476	7.20

Acceptance criteria: For all degradation study, the % Degradation should be between 5-30%.

CONCLUSION

We present in this article simple, selective, validated and well-defined stability that shows gradient RP-UPLC methodology for the quantitative estimation of Ipilimumab. the product of degradation formed during the stress conditions and the related active pharma ingredients are well separated and peaks were well resolved from each other and separate with an appropriate retention time indicating that the proposed method to be fast, simple, feasible and affordable in RS condition.

Therefore the developed method during stability tests, it can be used for routine analysis of production samples and to verify the quality of drug sample during stability studies.

Compliance and Ethical Standards

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Author's Contribution

Each author contributed to the conception, design and execution of the study and agreed to submit the current journal.

REFERENCES

1. D. B. Johnson,C. Peng, R. G. Abramson, F. Ye, S. Zhao, J. D. Wolchok, J. A. Sosman, R. D. Carvajal, C. E.Ariyan: Oncologist, 20(2015), 648-52.
2. Chu, E., & DeVita, V. T: Physicians' cancer chemotherapy drug manual, 2015.
3. J. R. Thumar, H. M. Kluger, Oncology, 24(2010), 1280-1288.
4. V. A. Trinh, B. Hagen, Journal of Oncology Pharmacy Practice, 19(2013), 195-201.
5. C. Fellner: Pharmacy and Therapeutics, 37(2012), 503.
6. FDA-Approved Drug Products: Yervoy (Ipilimumab) for intravenous injection, May-(2022).
7. Reddy KTK, Haque MA, et al., Development and Validation of a High Throughput LC - MS/MS Method for Quantitation of Ipilimumab in Human Plasma, International Journal of Pharmaceutical Quality Assurance, 13(2022), 303-307.
8. Sharmila Alladil, Ravi Kumar Konda and Mallikarjuna B, et al., Stability Indicating HPLC Method for Simultaneous Determination of Drugs Nivolumab and Ipilimumab, Journal of Drug and Alcohol Research, 11(2022), 1-5.
9. Sravya D, Kuber BR, et al., A stability-indicating reverse phase HPLC method development and validation for newly approved drug, Belzutifan in bulk and pharmaceutical dosage forms, J Appl Pharm Sci, 13(2023), 233–240.
10. Ram babu K, Ramadevi P, et al., Determination of Umbralisib using reverse phase ultra performance liquid chromatography in bulk and pharmaceutical dosage form, J Appl Pharm Sci, 12(2022), 172–178.
11. Tandrima M, Shiva Kumar G, et al., Stability Indicating RP UPLC Method Development & Validation for Quantitation of Doravirine in Tablet Formulation, J Pharm Negative Results, 13(2022), 1776-1782.
12. A .Venkata Suresh Babu and Hemant K.Sharma, Stability Indicative and Cost Effective Analytical Method Development and Validation Of Favipiravir and Oseltamivir in Bulk And Pharmaceutical Dosage Form By Using Rp-Hplc, Journal of Drug and Alcohol Research, 11(2022), 1-7.