RESEARCH ARTICLE

Development and Validation of a High Throughput Lc-Ms/Ms Method for Quantitation of Ipilimumab in Human Plasma

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ABSTRACT

For the analytical determination of ipilimumab employing cetuximab as a reference standard, a simple, fast, accurate, robust, and repeatable LC-MS/MS methodology was devised. This study investigated current developments in analytical LC-MS/MS methodologies. The Waters symmetry C_{18} column (150 x 4.6 mm, 3.5) was employed at ambient temperature under isocratic elution. Acetonitrile (ACN) and 0.1% aqueous solution of formic acid were utilized as mobile phase at 1.0-mL per min flow rate. The injection volume was 10 µL, and the operation period was 7 minutes. The overall methodology duration was 7 minutes, with a retention time being 3.124 minutes for Ipilimumab. Validated across a static linearity range of 12.50–100 ng/mL for Ipilimumab with a correlation value of 0.99961. Outcomes for precision, matrix effect, recovery, accuracy, and stability fell within acceptable parameters.

Keywords: Analysis, Cetuximab, Human plasma, Ipilimumab, LC-MS/MS, USFDA guidelines,

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Conflict of interest: None

INTRODUCTION

Ipilimumab (IPB) is employed as an adjunctive treatment for individuals who have undergone surgery to eradicate cutaneous and lymph vascular invasion melanoma.[1] IPB is prescribed for adults and children over 12 if the lump could not be resected or has progressed (migrated to other organs).[2-5] The protein chemical formula is $C_{6572}H_{10126}N_{1734}O_{2080}S_{40.}$ Protein average Weight is approximately 148000.0 Da[6-7] The literature survey revealed that no work had been done.

EXPERIMENTAL

Materials

Chemicals and Reagents

Ipilimumab and cetuximab samples from Biocon, Bangalore. LCMS grade acetonitrile, LCMS grade formic acid, and other chemicals were procured from Merck chemical division, Mumbai. HPLC-grade water supplied by the Milli-Q water purification system was utilized during the experiment.

Equipment

HPLC system (model number waters alliance e2695) was coupled with a mass spectroscopy QTRAP 5500 triple quadrupole device (sciex). Utilizing the Empower 2.0 software, operations were carried out under chromatographic settings.

Methodology

Chromatographic Conditions

An isocratic elution-based separation with Waters symmetric C18 (150 x 4.6 mm, 3.5 microns) columns was performed at ambient temperature.Acetonitrile(ACN) and 0.1% aqueous formic acid were utilized as mobile phases at a rate of 1.0 milliliters per minute. A volume of 10 µL was injected in each run, and the running duration was 7 minutes.

Preparation of standard and internal control (IC) samples

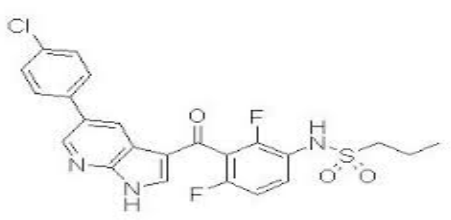

Figure 1: Structure of Ipilimumab

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Ipilimumab Standard Stock Solution Preparation

IPB working standard (5 mg) was poured in a 10 mL flask and the volume was raised up to the mark by the addition of dilutants. Again diluted 0.1 to 10 mL with dilutant. From this solution, take 0.4 mL and transfer it into a 10 mL flask. This is the Ipilimumab stock solution.

Cetuximab (CTX) Stock Solution Preparation

Cetuximab (CTX) working standard (5 mg) was poured in a 10 mL flask and the volume was increased upto the mark by adding dilutant. Again diluted 0.1 to 10 mL with dilutant. From this solution, take 0.4 mL and transfer it into a 10 mL flask. This is the cetuximab stock solution.

Preparation of Standard Solution (50 ng/mL of Ipilimumab)

Transferred 500 µL of the standard stock solution into a 2 mL centrifugation tube. 500 µL of dilutant,200 µL of plasma, 300 µL of ACN, and 500 µL of CTX were mixedwith this solution. Centrifuge it to 20 minutes. Filter the supernatant liquid and transfer it into an HPLC vial.

Ipilimumab Sample Stock Solution Preparation

IPB sample (1-mL) was poured in a 10 mL flask and the volume was raised up to the mark by addition of dilutants. Further diluted 0.1 to 10 mL with diluent. From this solution, take 0.4 mL and transfer it into a 10 mL flask. This is the Ipilimumab stock solution.

Extraction procedure

The centrifuged plasma samples were designated accordingly to corresponding time frames. 200 µL of plasma was mixed thoroughly with 500 µL of dilutant. Further, add 300 µL of Acetonitrile, which causes the precipitation of proteins, and combine using a vortex mixture. For 15–20 minutes, it was centrifuged at 4000 rpm. The supernatant liquid was collected in an autosampler vial.

Extracted Sample Preparation

An appropriate lot of plasma samples were taken out of the freezer and left to defrost at ambient temp. and vortex the tubes.

Table 1: Optimized chromatographic conditions

Parameter	*Description*
Column	Water Symmetry C_{18}, 150 mm x 4.6 mm, 3.5 µm
Mobile phase	ACN: 0.1% Formic acid (50:50)
Flow rate	1.0 millilitres per minute
Injection volume	10 µL
Retention time	Ipilimumab 3.124 minutes
Run time	7 minutes
Rinsing volume	100 µL
Detector	PDA
Autosampler temperature	Ambient
Column oven temperature	Ambient

Arrange the pre-labeled empty tubes as per the batch sequence, and aliquot 200.000 µL of plasma, then vortex 300.000 µL of ACN for 15 minutes add 500.000 µL of STD Stock, then add 500.000 µL of IS STD Stock, vortex for 15 minutes and finally 500.000 µL of dilutant.The samples were agitated for around 5 minutes at 2500 rotations per minute, followed by centrifugation at four thousand rotations per minute for next five minutes. About 1.000 mL of supernatant was obtained.

Unextracted Sample Preparation

Take 500.000 µL of STD Stock solution into pre-labeled tubes. Add 500.000 µL of ISTD working solution and vortex to mix. Add 1000.000 µL of mobile phase and combine by agitation. Pour the required proportion to pre-labelled HPLC vials and injects 10.00 µL into LC-MS/MS.

Optimized Bio-analytical Conditions

Optimized chromatographic conditions are summarized in Table 1.

Bioanalytical Method Validation

The technique's selectivity, sensitivity, linearity, recovery, precision, and accuracy, matrix effect, repeatability of reinjection, as well as stability were established.

Selectivity

Selectivity was determined by testing 6 distinct plasma samples and examining interference at their respective retention times (RTs).

Matrix Effect

The height-is-to-area percentage of 6 distinct pure plasma samples were compared to establish the matrix state for IPB. The tests were duplicated using six separate plasma batches with a precision below 15% for MQC values.

Precision and Accuracy

Repetitive assessments of untreated samples at the high-, low-, and medium-quality control (HQC, LQC and MQC) levels as well as lower LoD, allowed for their analysis. An overall CV must be below 15%, and the accuracy must be below 15%, excluding LLOQ, which must be under 20%.

Recovery

Ipilimab is extracted from 6 samples to repeat the assay at every control sample concentration. By comparing the height-is-to-areas of extracted samples with those of unextracted samples, %recovery was then determined.

Carryover

Carryover is the occurrence of a modest analyte trace after the insertion of a blank after the insertion of a sample that generates a prominent peak of the exact analyte.

Dilution Integrity

The integrity of dilution must be demonstrated by combining the matrix and analyte at a concentration more than the ULOQC and then diluting the sample using a blank matrix.

Stability

By contrasting the stability of the stock solution to the sample from the freshly-prepared stock sample, stability is determined. By contrasting the stability of the stock solution to the sample from the freshly-prepared stock sample, stability is determined. Six repetitions were used to evaluate plasma samples' stability at LQC and HQC levels. In accordance with US FDA standards, the sample was deemed stable when the shift was below 15%. The perfection of injected plasma maintained at ambient temperature for 24 hours was tested. The stability of loaded plasma samples maintained in an auto-sampler at ambient temperature for 24 hours periodwas evaluated. By analyzing extracted samples, which were inserted instantly as well as those that were pumped back after being stored for 12 hours and 18 hours at 2–8°C, the stability of auto-sampler (HQC, LQC, and MQC) was investigated. By analyzing extracted samples inserted instantly as well as those reinjected after preservation for 12 hours and 18 hours at -20°C ± 3°C, the repeatability of technique was investigated. By juxtaposing previously inserted IC samples with stability samples that had previously been stored at -31°C and then left to defrost three times, the freeze-thaw stability was ascertained. For the examination of protracted stability, the amounts after 24 hours were contrasted to the starting amount.

The solvent extraction technique exhibited excellent recoveries and selectivity. The enhanced detection settings, chromatography settings, and extraction method enabled a decrease in assay time and a precise and accurate determination of Ipilimumab in plasma samples. The parent and production mass spectrums of ipilimumab and cetuximab are shown in Figures 2 and 3.

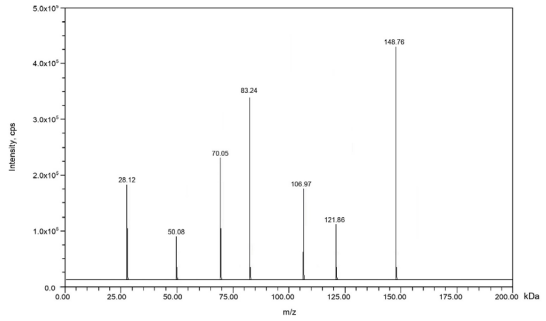

Figure 2: Mass spectrum of fragmentation pattern for Ipilimumab (148.7683.24)

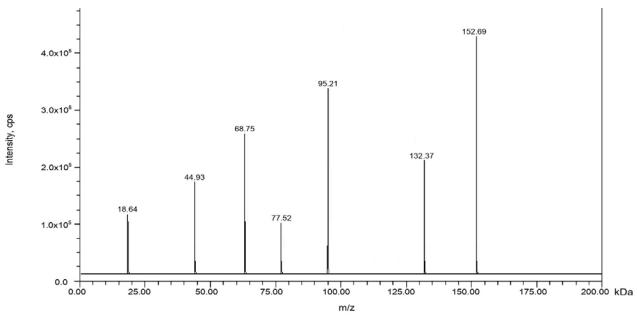

Figure 3: Mass fragmentation pattern of Cetuximab (152.69→95.21)

RESULTS AND DISCUSSION

Specificity

Figures 4 and 5 depict the chromatograms obtained for blank plasma samples and standard plasma samples, respectively. It was noticed that the chromatograms of both blank plasma and standard included no interfering peaks.

Matrix Effect

The percentage RSD of ion silencing/upregulation within the signal was reported to be 1% for Ipilimumab LCMS/MS, indicating that the matrix influence on sample ionizing is within appropriate limits under such conditions. IPB's matrix impact LQC and HQC, correspondingly, were reported to be 99.52 and 101.81%. The CV of Ipilimumab equaled 0.45 at the LQC level but 0.25 at the HQC level. It demonstrates that the matrix influence on the ionizing of the sample does not exceed the permissible range.

Linearity

The calibration curves showed linear for the concentrations of Ipilimumab varying from 12.50 to 100 ng/mL. The average coefficient of correlation equals 0.999. The ratios of the analyte peak area: IS peak area was used to quantify samples. Peak area ratios versus plasma concentrations were graphically represented. Linearity findings of Ipilimumab are summarized in Table 2, and respective calibration graphs are depicted in Figure 6.

Precision and Accuracy

Multiple IC samples were integrated with every test's data to calculate precision and accuracy. It was evident from the presented statistics that the technique was accurate and

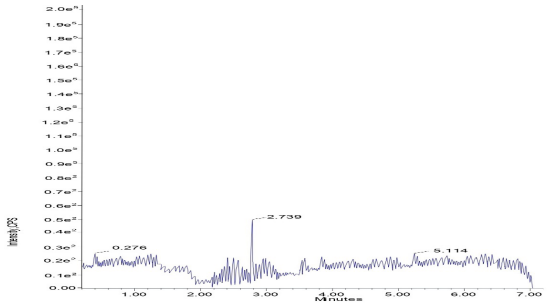

Figure 4: Chromatogram of human Blank plasma

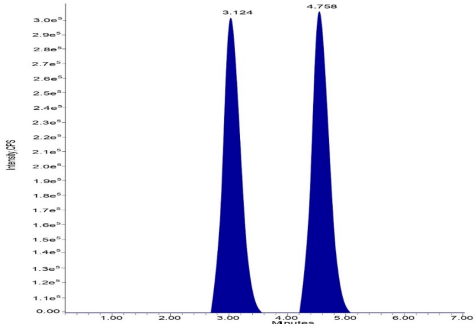

Figure 5: Chromatogram of standard human Plasma

Table 2: Linearity results of ipilimumab

Final conc. in ng/mL	*RES*	*Area response ratio*
0	0	0.0
12.50	0.885	0.255
25.00	1.743	0.503
37.50	2.493	0.724
50.00	3.241	0.937
62.50	4.056	1.169
75.00	4.862	1.400
100.00	6.467	1.881
Slope	0.0184	
Intercept	0.02763	
R^2 value	0.99961	

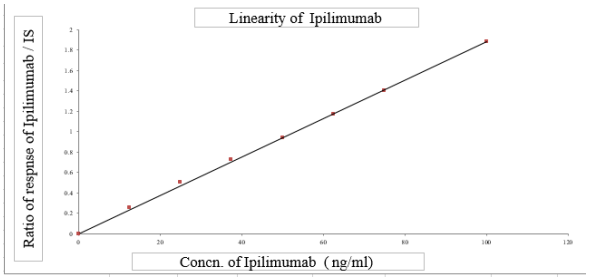

Figure 6: Calibration plot for concentration v/s area ratio of ipilimumab

effective. Table 3 displays the accuracy and precision values for Ipilimumab.

Recovery

Reported recovery values for Ipilimumab at the LQC, HQC, and MQC levels indicated that the proposed approach had excellent extraction yield. Ipilimumab's recoveries were reported to be 98.85%–101.49% at LQC, HQC, and MQC levels, and %CV varied between 0.30 and 4.50. The findings indicate that the proposed technique had a high extraction yield.

Ruggedness

At HQC, MQC, LLQC, and LQC levels, both the % recovery as well as %CV of Ipilimumab assessed by two separate analyzers and on two separate columns were well within a permissible range. The findings demonstrated the technique's durability. Ipilimumab had recoveries ranging between 97.48 and 101.91 percent. The %CV for Ipilimumab varied around 0.03–0.67. The finding shows that the technique is rugged.

Autosampler Carryover

Following sequential injections of both LLQC and ULQC levels at the RTs of Ipilimumab, the peak area responsiveness of Ipilimumab was not detected with the blank plasma samples. Consequently, this approach lacks auto-sampler carryover.

Table 3: Precision and accuracy results of ipilimumab

Acquisition Batch ID	Date	HQC	MQC	LQC	LLQC
		Nominal Concentration (ng/mL)			
		75.0	50.0	25.0	6.0
		Analyte peak area			
		$4.853x10^5$	$3.249x10^5$	$1.733x10^5$	$0.388x10^5$
		$4.868x10^5$	$3.260x10^5$	$1.749x10^5$	$0.365x10^5$
		$4.854x10^5$	$3.236x10^5$	$1.727x10^5$	$0.374x10^5$
		$4.843x10^5$	$3.228x10^5$	$1.740x10^5$	$0.381x10^5$
		$4.865x10^5$	$3.263x10^5$	$1.722x10^5$	$0.366x10^5$
		$4.833x10^5$	$3.236x10^5$	$1.737x10^5$	$0.341x10^5$
n		6	6	6	6
Mean		$4.852x10^5$	$3.245x10^5$	$1.734x10^5$	$0.369x10^5$
SD		0.012	0.013	0.008	0.0014
% CV		0.25	0.44	0.51	4.05
% Mean Accuracy		100.85%	102.64%	98.54%	99.90%

Table 4: Stability results of ipilimumab

Stability experiment spiked plasma	*Concentration*	*Analyte peak area*	*% CV*
Benchtop Stability	LOQ-25.0	$1.788x10^5$	0.25
	MQC-50.0	$3.236x10^5$	0.18
	HQC-75.0	$4.850x10^5$	0.38
Freeze-Thaw stability	LOQ-25.0	$1.800x10^5$	0.89
	MQC-50.0	$3.350 x10^5$	1.15
	HQC-75.0	$4.700x10^5$	0.70
Short term stability	LOQ-25.0	$1.600x10^5$	1.87
	MQC-50.0	$3.589 x10^5$	2.37
	HQC-75.0	$4.879x10^5$	3.47

Stability

For solution stability testing, IPB preparations were formulated using dilutants and stored under refrigeration at 2–8°C. Newly prepared stock solutions were compared to those prepared 24 hours previously. The benchtop stability as well as autosampler were consistent lasting for 24 hours, and also for 24 hours in the auto-sampler at 20°C. Storage stability revealed that Ipilimumab was stable at a tempreture range of -30°C for as long as 24 hours. The cumulative stability findings of Ipilimumab are shown in Table 4.

CONCLUSION

The present study was conducted to establish a convenient, economical, robust, and accurate technique for assessing Ipilimumab in LC-MS/MS utilizing cetuximab as a reference standard. The overall chromatographic operating time was 7 minutes, with a retention time of 3.143 minutes for ipilimumab. The approach is verified across a dynamically linear range between 12.50 and 100 ng/mL for ipilimumab having a coefficient of correlation (r2) of 0.999. The intra-batch, as well as inter-batch accuracy (%CV), was below 15% for all five levels (LLOQ, HQC, MQC, ULOQ, and LQC). This may be confirmed based on USFDA regulations.

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