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Simultaneous Method Development and Validation of Trastuzumab and Hyaluronidase-Oysk and Its Pharmacokinetic studies with LC-MS/MS

Subba Rao Yarlagadda[1], Pavani Y[2], Subba Rao Mannam*

[1,2] *Research Scholars, Department of Chemistry, Acharya Nagarjuna Univeristy, Guntur, AP-522 510, India.*

Abstract:

A rapid, sensitive and selective bio-analytical method was developed and validated by Liquid chromatography – Mass spectrometry (LC-MS/MS) for the determination of Trastuzumab and Hyaluronidase-Oysk in rat plasma. An isocratic mode using the analytical column of waters symmetry C_{18} (150x4.6mm, 3.5 µm) and a mobile phase of 0.1% Formic acid (buffer) and Acetonitrile in the ratio of 70:30 was used and a positive mode of electrospray ionization detection was carried out using MS. The method was validated with a linearity range 1.2-24ng/ml of Trastuzumab and 0.06-1.2ng/ml of Hyaluronidase-oysk. The %CV values of intraday, interday precision and accuracy were found to be within the acceptance criteria. The %recovery of Trastuzumab was 99.5% and Hyaluronidase-oysk was 98.6% respectively. Using liquid liquid extraction, both the drugs were extracted from rat plasma. Stability of Verapamil and Trandolapril exist in all conditions like freeze thaw, auto sampler, bench top and long term stability studies as per US DFDA guidelines. This method was validated successfully in parameters like linearity, accuracy, recovery, stability and pharmacokinetic studies in rat plasma using LCMS/MS.

Keywords: Development, Hyaluronidase-oysk, LCMS/MS, Trastuzumab, Validation.

1. INTRODUCTION

Trastuzumab

Trastuzumab, sold under the brand name Herceptin among others, is a monoclonal antibody [1, 2] used to treat breast cancer [3, 4]. Specifically it is used for breast cancer that is HER2 receptor positive [5, 6]. It may be used by itself or together with other chemotherapy [7] medication. Trastuzumab is given by slow injection into a vein [8] and injection just under the skin. Common side effects include fever [9, 10], infection [11], cough [12, 13], head ache, trouble sleeping [14], and rash [15]. Other severe side effects include heart failure, allergic reactions [16], and lung disease [17]. Use during pregnancy may harm the baby. Trastuzumab works by binding to the HER2 receptor and slowing down cell duplication.

Hyaluronidase-Oysk

Hyaluronidases are a family of enzymes that catalyse the degradation of Hyaluronic acid (HA). Karl Meyer classified these enzymes in 1971 into three distinct groups, a scheme based on the enzyme reaction products [18]. The three main types of Hyaluronidases are two classes of eukaryotic endoglycosidase hydrolases and a prokaryotic lyase-type of glycosidase [19]. In humans, there are five functional hyaluronidases: HYAL1, HYAL2, HYAL3, HYAL4 and HYAL5 (also known as SPAM1) or PH-20): plus a pseudogene, HYAL6 (also known as HYALP1) [20, 21]. The genes for HYAL1-3 are clustered in chromosome 3 [22], while HYAL4-6 is clustered in chromosome 7 [23]. HYAL1 and HYAL2 are the major hyaluronidases in most tissues. GPI-anchored HYAL2 is responsible for cleaving high-molecular weight HA, which is mostly bound to the CD44 receptor. The resulting HA fragments of variable size are then further bydrolized by HYAL1 after being internalized into endo-lysosomes: this generates HA Oligosaccharides [24].

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Acetonitrile, formic acid and water (HPLC mark). Formic acid (HPLC mark) was purchased from Merck (India) Ltd., Worli, and Mumbai, India. All API's of Trastuzumab and Hyaluronidase-oysk as reference standards were procured from Spectrum Pharma Research Solutions pvt. Ltd., Hyderabad.

2.2 Equipment

An HPLC system (Waters Alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (Sciex) was used. Data was processed with Empower 2.0 software.

2.3 Chromatographic condition

An isocratic mode of separation with symmetry C_{18} (150x4.6mm, 3.5µ) column and a mixture of 0.1% Formic acid and Acetonitrile 70:30 v/v at a flow rate of 1.0ml/min was used as mobile phase. The injection volume was 10µl. The run time was 15min. Triple quadrupole mass spectrometer equipped with electro spray ionization and handled in positive ionization mode for tracking down and quantification of analytes and internal standards. The intensification of the source and compound parameters is Declustering potential: 40V, entrance potential: 10V, exit potential: 7, collision energy: 15V for Trastuzumab and 16V for Hyaluronidase-oysk. The source criteria were optimized as collision gas: 5, ion spray voltage: 5500V and temperature: 550°C.

2.4 Preparation of Standard and Quality Control Samples

The stock solution of Trastuzumab and Hyaluronidase-oysk was prepared in bulk for the calibration curve and quality control samples for the method validation exercise as well as subject sample analysis. The stock solution of Trastuzumab and Hyaluronidase-oysk was prepared in

mobile phase (diluents) concentration of 48ng/ml of Trastuzumab and 2.5ng/ml of Hyaluronidase-oysk. Primary dilutions and working standard solutions were used to prepare the calibration curve quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention time of Trastuzumab and Hyaluronidase-oysk. Eight pint standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Trastuzumab and Hyaluronidae-oysk. Calibration samples were made at concentrations of 1.2, 3, 6, 9, 12, 15, 18, 24 ng/ml of Trastuzumab and 0.06, 0.16, 0.31, 0.47, 0.63, 0.78, 0.94, 1.25 ng /ml of Hyaluronidase-oysk.

2.5 Standard Solution Preparation

For sample preparation, 200µl of plasma sample, 300µl of acetonitrile and 500µl of internal standard, 500µl of standard stock and 500µl of diluent to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 500rpm for 30min. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

2.6 Bio Analytical Method Validation

The method was validated according to US food and drug administration bio analytical method validation guidelines include system suitability, selectivity and specificity, LOD, LOQ, injector carry over, linearity, precision and accuracy, recovery, matrix effect, dilution integrity, re-injection reproducibility, ruggedness, sample stability studies were carried out to prove the capability of the proposed method.

2.6.1 Selectivity

Selectivity was performed by anlayzing the rat plasma samples from six different rats.

2.6.2 Matrix Effect

Matrix Effect for Trastuzumab and Hyaluronidase –oysk was evaluated by comparing the peak area ratio in the post extracted plasma sample from 6 different drug-free blank plasma samples and neat reconstitution samples. Experiments were performed at HQC, MQC and LQC levels in triplicate with six different plasma lots.

2.6.3 Dilution Integrity

Dilution integrity should be demonstrated by spiking the matrix with an anlayte concentration above the ULOQC and diluting this sample with blank matrix.

2.6.4 Precision and Accuracy

It was determined by replicate analysis of quality control samples (n=6) at a lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels.

2.6.5 Carry Over

The analyte retained by the chromatographic system during the injection of a sample that appears in subsequent blank or unknown samples.

2.6.6 Recovery

The extraction efficiencies of Trastuzumab and Hyaluronidase-oysk were determined by analysis of six replicates at each quality control concentration. The percentage recovery was evaluated by comparing the peak areas of extracted standards to the peak areas non extracted standards.

2.6.7 Stability

Stock solution stability was performed by comparing the area response of anlayte in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is less than 15% as per US FDA guidelines. The stability of spiked rat plasma samples stored at room temperature (bench top stability) was evaluated for 24h. The stability of spiked rat plasma stored at 2-8°C in auto sampler (auto sampler stability) was evaluated for 24h. The auto sampler stability was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were re injected after storing in the auto sampler at 2-8°C for 24h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately, with the samples that were re injected after storing in the auto sampler at 2-8°C for 24h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long-term stability evaluation the concentrations obtained after 24h were compared with initial concentration.

3. RESULTS AND DISCUSSION

Electro spray ionization (ESI) having maximum response over atmospheric pressure chemical ionization (APCI) mode selected in this method. The optimization of instrument to give sensitivity and signal stability during infusen of the analyte in the continuous flow of mobile phase to electro spray ion source operated at both polarities at flow rate of 10µl/min. Trastuzumab and Hyaluronidase-oysk give more response in positive ion mode when compared to negative ion mode.

To obtain the best chromatographic condition, different columns like C_{18}, C_8, and CN-propyl and mobile phases composed of 0.1% Formic acid and Acetonitrile were tested. The best chromatographic separation occurred on Waters Symmetry C_{18} column with a mobile phase of 0.1% Formic acid and Aceetonitrile in 70:30 ratio at a flow rate of 1ml/min.

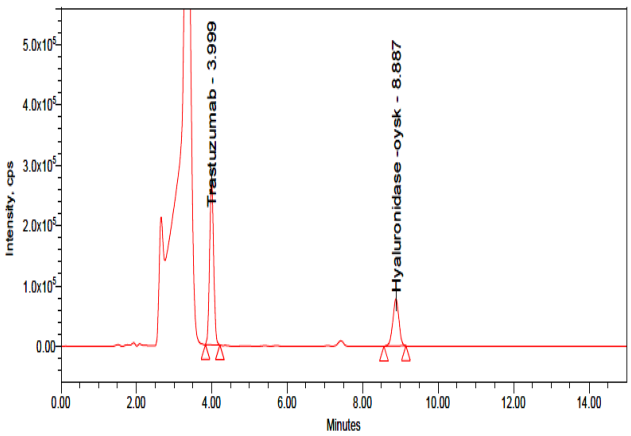


Fig. No. 1: Chromatogram of MQC

3.1 Bio analytical Method Validation

Matrix Effect

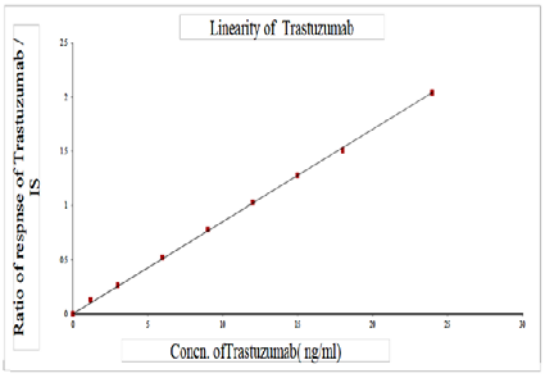
The % CV of ion suppression/enhancement in the signal was found to be 1.0% at MQC level for Trastuzumab and Hyaluronidase-oysk. It indicates that the matrix effect on the ionization of anlayte is within the acceptable limit.

Linearity

The peak area ratios of calibration standards were proportional to the concentration. The concentration range of Trastuzumab is 1.2-24 ng/ml and 0.06-1.2ng/ml of Hyaluronidase-oysk. The calibration curves were appeared linear and correlation coefficient was found to be 0.999 for Trastuzumab and Hyaluronidase-oysk. Linearity results of Trastuzumab and Hyaluronidase-oysk are shown in table 1.

Table 1: Linearity results of Trastuzumab and Hyaluronidase-Oysk

Linearity	Trastu -zumab conc. (ng/ml)	Trastu -zumab peak response	Hyaluronidase-Oysk conc. (ng/ml)	Hyaluronidase-oysk peak response
1	1.20	0.367	0.06	0.074
2	3.00	0.741	0.16	0.184
3	6.00	1.427	0.31	0.362
4	9.00	2.108	0.47	0.516
5	12.00	2.834	0.63	0.711
6	15.00	3.571	0.78	0.817
7	18.00	4.187	0.94	1.045
8	24.00	5.642	1.25	1.305
Slope	0.0822		1.6853	
Intercept	0.03281		0.00718	
CC	0.9999		0.9992	



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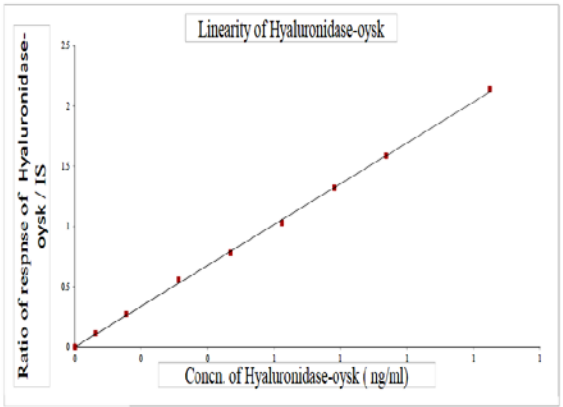

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Fig. No. 2: Calibration plot for (A) Tratuzumab and (B) Hyaluronidase-oysk

Precision and Accuracy

The inter-run and accuracy were determined by pooling all individual assay results of replicate quality control over five separate batch runs analyzed on four different days. The %CV of inter-run precision was <5% and the value of inter-run accuracy was in between 85 and 115 for Trastuzumab and Hyaluronidase-oysk. From the data given in the following table 2, 3 it was clear that the method is precise and accurate.

Recovery

For recovery determination of low, medium and high quality control concentration levels for Trastuzumab and Hyaluronidase-oysk were prepared, and the obtained areas for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery of Trastuzumab and Hyaluronidase-oysk was 98.5% and precision is 1.8%.This indicates that the extraction efficiency for Trastuzumab and Hyaluronidase-oysk.

Carry Over

Systematic error that may affect the measured value of the sample is called carryover. Sample carry over on a LC/MS system configured with Waters Alliance was evaluated using the following procedure. A 'system blank injection' of 10µl 0.1% Formic acid and Acetonitrile (70:30) was made onto a waters ZSpray triple quadrupole mass detector using flow injection analysis. From this we can say that it does not affect the accuracy and precision of the proposed method. Sample carry over was expressed as both %carryover and nL carryover. The sample carryover results are tabulated in table 4.

Reinjection Reproducibility

During real subject sample analysis, reinjection reproducibility was performed to check the instrument after hard ware deactivation due to any instrument failure. At LQC and HQC levels the change was less than 2.0, hence during real subject sample analysis the batch was re injected in the case of instrument failure. Samples were prepared and re injected after 24h, shows %change less than 2.0% at LQC and HQC levels, hence during real subject sample analysis in the case of instrument failure, batch can be re injected after 24h.

Stabilities

To check stablility of Trastuzumab and Hyaluronidase-oysk, stock solution was prepared and stored at 2-8°C in a refrigerator. Compare the stability of freshly prepared stock solution with stock solution prepared before 24h. From this we observed a % change of Trastuzumab and Hyaluronidase-oysk was 1.22% and 0.71% respectively, indicates that solutions are stable upto 24h. Bench top and auto sampler stabilities were observed at LQC and HQC levels.

At room temperature Trastuzumab and Hyaluronidase-oysk were stable in plasma for 24h, and 24h in auto sampler at 20°C. From this it was confirmed that, at LQC and HQC levels repeated freezing and thawing of plasma

samples spiked with Trastuzumab and Hyaluronidase-oysk did not affect their stability. From long-term stability it was clear that Trastuzumab and Hyaluronidase-oysk were stable upto 24h at a storage temperature of -30°C. The overall stablility results of Trastuzumab and Hyaluronidase-oysk are tabulated in table 5 and6.

Table 2: Within run and between run precision and accuracy of Trastuzumab

Nominal Conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision %CV)	Accuracy	Mean (ng/ml)	Precision %CV)	Accuracy
0.6	0.656	0.53	99.7	0.675	0.75	101.4
6	6.625	0.67	97.6	6.628	0.64	98.5
12	12.864	0.72	100.2	12.267	0.81	98.7
18	18.562	1.69	98.2	18.956	0.39	100.5

Table 3: Within run and between run precision and accuracy of Hyaluronidase-oysk

Nominal Conc (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision %CV)	Accuracy	Mean(ng/ml)	Precision(%CV)	Accuracy
0.03	0.032	0.95	100.5	0.038	0.87	102.6
0.3	0.328	0.65	100.7	0.357	0.79	100.8
0.6	0.637	0.52	99.8	0.651	0.52	99.4
0.9	0.995	0.46	99.5	0.924	0.49	97.4

Table 4: Results of Carryover

Concentration	% of Carryover	
	Trastuzumab	Hyaluronidase-oysk
Blank	0	0
LLQC	0.45	0.29
ULQC	0.78	0.67

Table 5: Stability results of Trastuzumab

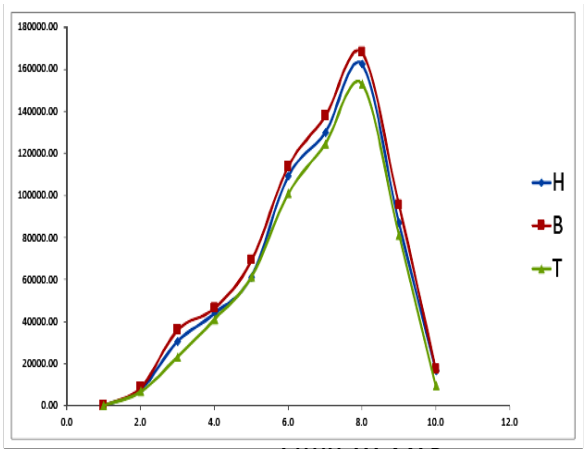
Stability experiments		Spiked plasma concentration (n=6, ng/ml)	Concentration Measured (n=6, ng/ml)	%CV
Bench top stability	LQC	6	6.254	0.26
	HQC	18	18.241	0.32
Auto sampler stability	LQC	6	6.239	0.22
	HQC	18	18.154	0.29
Long term stability	LQC	6	6.139	0.36
	HQC	18	18.267	0.21
Freeze thaw stability	LQC	6	6.255	0.48
	HQC	18	18.314	0.45
Wet Extract Stability	LQC	6	6.148	0.68
	HQC	18	18.247	0.54
Dry Extract Stability	LQC	6	6.152	0.59
	HQC	18	18.369	0.38
Short term stability	LQC	6	6.274	0.29
	HQC	18	18.325	0.47

Table 6: Stability results of Hyaluronidase-oysk

Stability experiments		Spiked plasma conc. (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV
Bench top stability	LQC	0.3	0.326	0.62
	HQC	0.9	0.985	0.54
Auto sampler stability	LQC	0.3	0.342	0.31
	HQC	0.9	0.958	0.29
Long term stability	LQC	0.3	0.316	0.72
	HQC	0.9	0.956	0.66
Freeze thaw stability	LQC	0.3	0.366	0.55
	HQC	0.9	0.974	0.89
Wet Extract Stability	LQC	0.3	0.324	0.26
	HQC	0.9	0.629	0.35
Dry Extract stability	LQC	0.3	0.335	0.35
	HQC	0.9	0.926	0.47
Short term Stability	LQC	0.3	0.337	0.39
	HQC	0.9	0.942	0.55

Table 7: Pharmacokinetic parameters of Trastuzumab and Hyaluronidase-Oysk

Pharmacokinetic parameters	Trastuzumab	Hyaluronidase-oysk
AUC_{0-t} (ng h/ml)	28 Days	2 Days
C_{max} (ng/ml)	8	0.4
$AUC_{0-\infty}$ (ng h/ml)	48 Days	21 Days
K_{el}	0.001	0.014
$T_{1/2}$	28 Days	2 D
T_{max} (h)	48 Days	21 D



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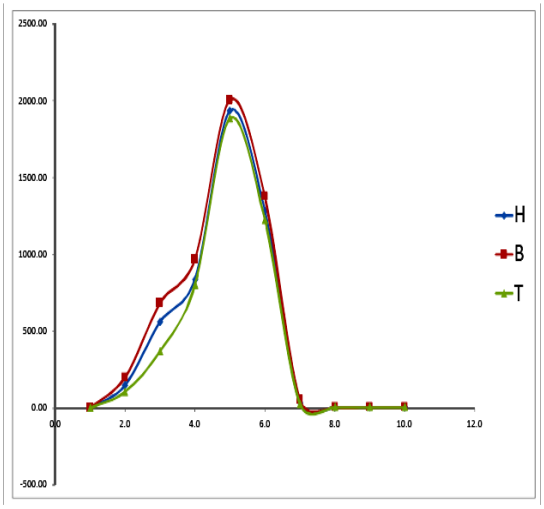

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Figure 3: Recovery plot for (3a) Trastuzumab and (3b) Hyaluronidase-oysk

Pharmacokinetic Study

The method has been validated successfully to quantify the concentration of Trastuzumab and Hyaluronidase-oysk in head, body and tail of rat, after administration of Trastuzumab and Hyaluronidase-oysk sample as an oral dose, under fasting condition. After injecting the drug samples into a rat body, collect the samples at different time intervals like 1 Hr, 0.5 D, 1 D, 2 D, 14 D, 21 D, 28 D, 35 D, 48 D hours from the rat body. After that as per test method sample is prepared and injected into the chromatographic system and record the values. The evaluated pharmacokinetic parameters were C_{max} (maximum observed drug concentration-time curve measured 48 D, using the trapezoidal rule), t_{max} (time to observed maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and $t_{1/2}$ (terminal half-life as determined by the quotient 0.693/K_{el}). The ratio of test/reference for C_{max}, AUC_{0-12}, and AUC were 86.27, 91.36 respectively and found to be within the acceptable limit of 85%-125%. **Table 7** shows the pharmacokinetic parameters of Trastuzumab and Hyaluronidase-oysk.

4. CONCLUSION

For the first time higher sensitive HPLC-ESI-MS/MS method was developed and validated for the determination of Trastuzumab and Hyaluronidase-oysk in rat plasma. Here the described method is fast, rugged, reproducible bio analytical method. Simple and efficient method was developed and can be used in pharmacokinetic studies and to check the investigated analyte in body fluids.

5. CONFLICT OF INTERESTS

Authors declared that there were no conflicts of interest.

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