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Research Article

A New Highly Effective Development and Validation of Trastuzumab and Hyaluronidase-Oysk in Bulk and Pharmaceutical Dosage form by Using RP-HPLC Method

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Abstract

A simple, rapid, precise, sensitive, and reproducible Reverse phase High-performance liquid chromatography (RP-HPLC) method has been developed for the quantitative analysis of Trastuzumab and Hyaluronidase-OYSK in the pharmaceutical dosage form. Chromatographic separation of Trastuzumab and Hyaluronidase-OYSK was achieved on Waters Alliance-e2695by using Agilent Eclipse XDB (250x 4.6mm, 5µ) column and the mobile phase containing Acetonitrile: Ammonium formate pH-3.0/OPA in the ratio of 30:70% v/v. The flow rate was 1.0 ml/min; detection was carried out by absorption at 228nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Trastuzumab and Hyaluronidase-OYSK were NLT 2000 and should not be more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Trastuzumab and Hyaluronidase-OYSK study of its stability.

Introduction

Trastuzumab is a biologic agent primarily used in the treatment of HER2-positive breast cancer. It may be used as adjuvant therapy for localized disease or as first-line therapy for metastatic disease. Trastuzumab is a recombinant humanized IgG1 monoclonal antibody against the HER-2 receptor, a member of the epidermal growth factor receptors which is a photo-oncogene. Over-expressed in breast tumour cells, HER-2 overamplifies the signal provided by other receptors of the HER family by forming heterodimers [1-7]. It can be used alone or in

combination with other chemotherapeutic drugs.

Trastuzumab and hyaluronidase-risk injection is also used during and after treatment with other medications to decrease the chance that a certain type of breast cancer will return. Trastuzumab and hyaluronidase-oysk injection is in a class of medications called monoclonal antibodies. Trastuzumab and hyaluronidase-oysk injection is in a class of medications called monoclonal antibodies. It works by stopping the growth of cancer cells. Trastuzumab and hyaluronidase-oysk combination prevents the growth of some tumors that produce extra amounts of a certain substance known as the HER2 protein [8]. It should only be used in patients whose tumors have been shown to produce extra amounts of this protein (HER2 overexpression).

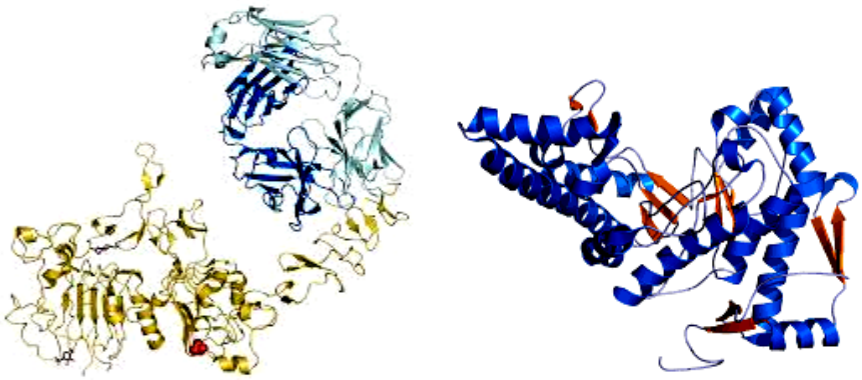

Fig 1. Structure of Trastuzumab and hyaluronidase-oysk

A literature survey reported that, few methods are available for simultaneous estimation of Trastuzumab and hyaluronidase-oysk and a few articles reported spectrophotometric techniques for estimation of Trastuzumabalone and with other drugs such as, LC-MS/MS, and RP-HPLC[9-12].This study aims to create a simple, precise, accurate, relatively sensitive, and fast RP-HPLC technique for estimating Trastuzumab and hyaluronidase-oyskin bulk and tablet formulations. The developed method was validated as per ICH guidelines and can be applied successfully to quality control determinations[13-23].

Materials and Methods

Equipment: HPLC- ALLIANCE, UV/VIS spectrophotometer- UV-1700, Pipettes, beakers and Burettes- Borosil, Ultra sonicator- UCA 701- Unichrome, Pump-Isocratic model.

Reagents & Chemicals: Acetonitrile HPLC grade received from Rankem, Water (Milli Q), Ammonium Formate and Formic Acid of HPLC grade received from analytical reagents.

Preparation of standard solution

Accurately weigh and transfer 600 mg of Trastuzumab, 5 mg of Hyaluronidase-oysk working standard into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent and again pipette 0.5ml of the above Hyaluronidase-Oysk solution into 10ml volumetric flask and dilute up to the mark with diluent(Stock solution)

Further pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. (6000ppm

of Trastuzumab, 2.5ppm of Hyaluronidase-oysk)

Sample Solution Preparation:

Accurately weighed and transfer 0.5ml of Trastuzumab and Hyaluronidase-oysksampleinto a 10mL clean dry volumetric flask add Diluent and sonicate it up to 30 mins to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (6000ppm ofTrastuzumab, 2.5ppm of Hyaluronidase-oysk)

Chromatographic condition:

Use suitable High Performance Liquid Chromatographic equipped with PDA detector.

Column: Agilent eclipse XBD (250x 4.6mm, 5µ)

Mobile phase ratio: Acetonitrile: Ammonium formate PH-3.0 /OPA (30:70)

Detection wavelength: 228 nm

Flow rate: 1ml/min

Injection volume:10µl

Run time: 8min

Preparation of Diluent: Acetonitrile is used as a diluent.

Procedure:

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Trastuzumab and Hyaluronidase-oyskpeaks and calculate the %Assay by using the formulae.

METHOD VALIDATION:

Specificity:

Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drugs was specific**.**

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 600 mg of Trastuzumab, 5 mg of Hyaluronidase-oyskworking standard into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent and again pipette 0.5ml of the above Hyaluronidase-Oysk solution into 10ml volumetric flask and dilute up to the mark with diluent. (Stock solution).

Preparation of Level – I (1500ppm of Trastuzumab, 0.63ppm of Hyaluronidase-oysk):

0.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (3000ppm of Trastuzumab, 1.25ppm of Hyaluronidase-oysk):

0.50 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (4500ppm of Trastuzumab, 1.88ppm of Hyaluronidase-oysk):

0.75 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – IV (6000ppm of Trastuzumab, 2.50ppm of Hyaluronidase-oysk)

1.0 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent**.**

Preparation of Level – V (7500ppm of Trastuzumab, 3.13ppm of Hyaluronidase-oysk)

1.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent**.**

Preparation of Level – VI (9000ppm of Trastuzumab, 3.75ppm of Hyaluronidase-oysk)

1.5 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent**.**

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Preparation Accuracy Sample solutions:

For preparation of 50% solution:

Accurately take and transfer 0.25 ml of Trastuzumab and Hyaluronidase-oysk sample into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.(3000ppm of Trastuzumab, 1.25ppm of Hyaluronidase-oysk)

For preparation of 100% solution:

Accurately take and transfer 0.50ml of Trastuzumab and Hyaluronidase-oysk sample into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (6000ppm of Trastuzumab, 2.50ppm of Hyaluronidase-oysk)

For preparation of 150% solution:

Accurately weigh and transfer 0.75 ml of Trastuzumab and Hyaluronidase-oysk sample into a 10 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (9000ppm of Trastuzumab, 3.75ppm of Hyaluronidase-oysk)

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Precision

Precision is the degree of repeatability of an analytical method under normal operation conditions.

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD.

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 6000ppm of Trastuzumab, 2.5ppm of Hyaluronidase-oysk).

Limit of detection (LOD) and limit of quantification (LOQ):

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma / S$
$LOQ = 10 \times \sigma / S$

Results and Discussion

The Trastuzumab peak was observed at 2.092 min with peak area 3854216, tailing factor 1.05Hyaluronidase-oysk peak was observed at 3.469min, with peak area 99653, tailing factor 1.03 and resolution 8.35.

Table 1: Results for (Optimized trail)

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Trastuzumab	2.092	3854216	12493	1.05	
2	Hyaluronidase-oysk	3.469	99653	15370	1.03	8.35

Table 2: Optimized chromatographic conditions

PARAMETERS	OBSERVATION
Instrument used	Waters HPLC with auto sampler and PDA detector.
Injection volume	10µl
Mobile Phase	Acetonitrile: Ammonium formate pH-3.0/OPA (30:70)
Column	Agilent Eclipse XDB (250x4.6 mm, 5 µ)
Detection Wavelength	228nm
Flow Rate	1 mL/min
Runtime	8min
Temperature	Ambient (25° C)
Mode of separation	Isocratic mode

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table 3: System suitability parameters for Trastuzumab & Hyaluronidase-oysk

S.No.	Parameter	Trastuzumab	Hyaluronidase-oysk
1	Retention time	2.092	3.469
2	Plate count	12493	15370
3	Tailing factor	1.05	1.03
4	Resolution	----	8.35
5	%RSD	0.36	0.19

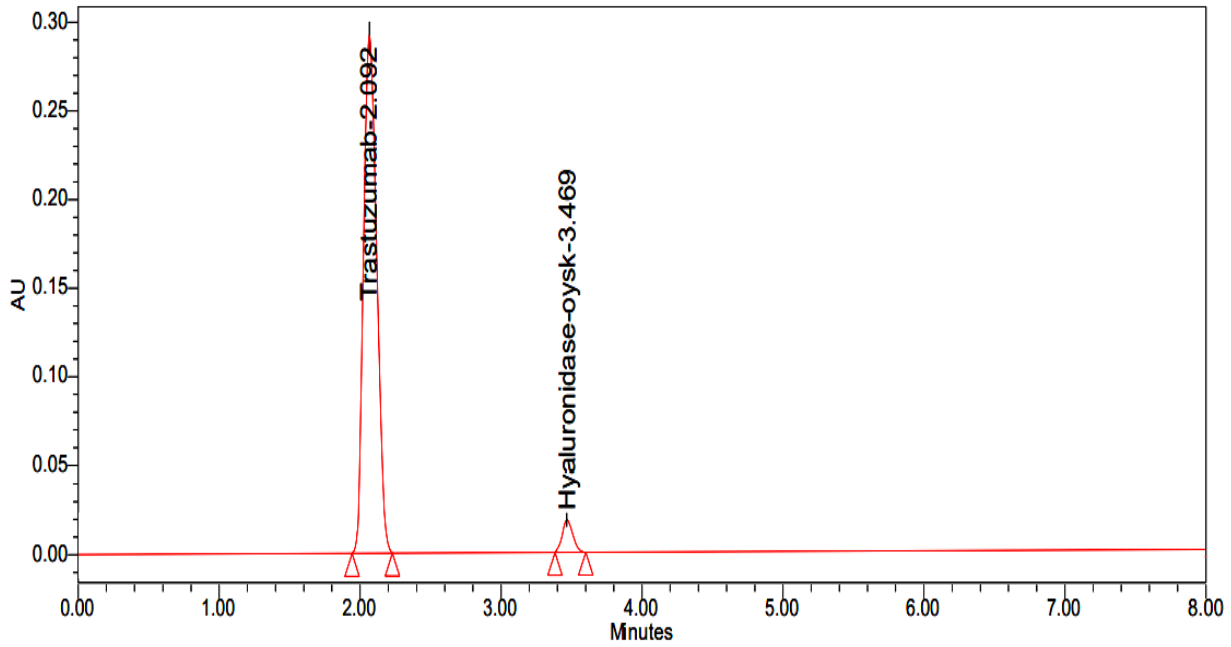

Fig. 2: Chromatogram of standard

Acceptance Criteria: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Analytical Method Validation

The method was validated for its linearity range, accuracy, precision, and specificity. Method validation was carried out as per ICH guidelines.

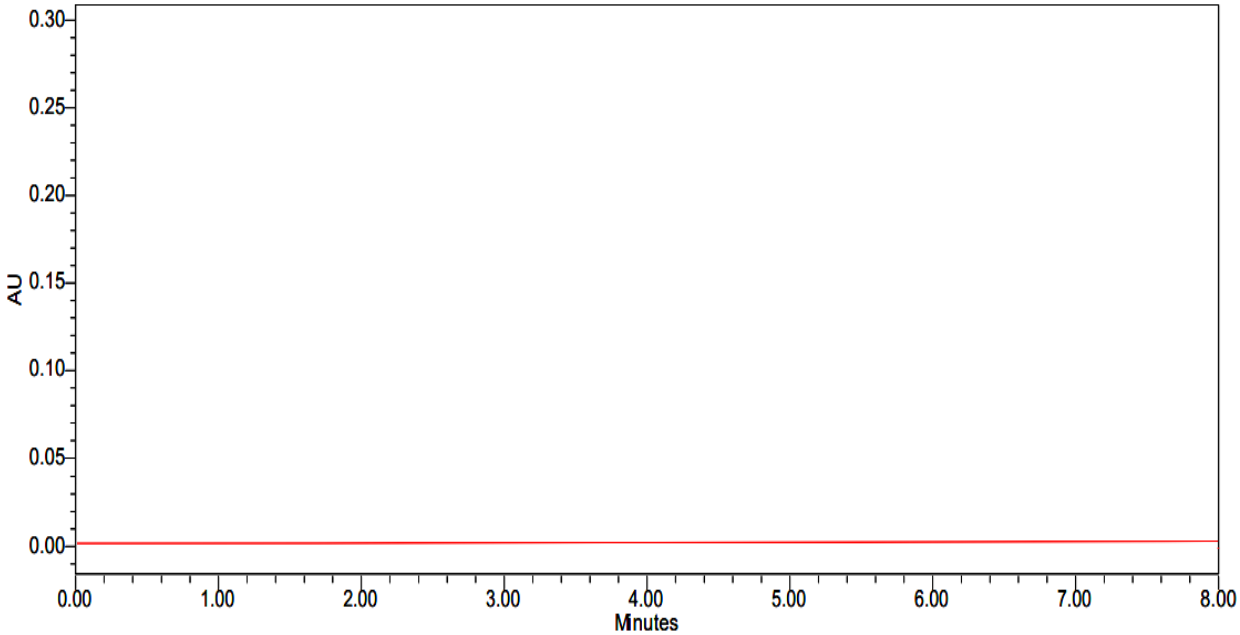

Fig. 3: Chromatogram of blank

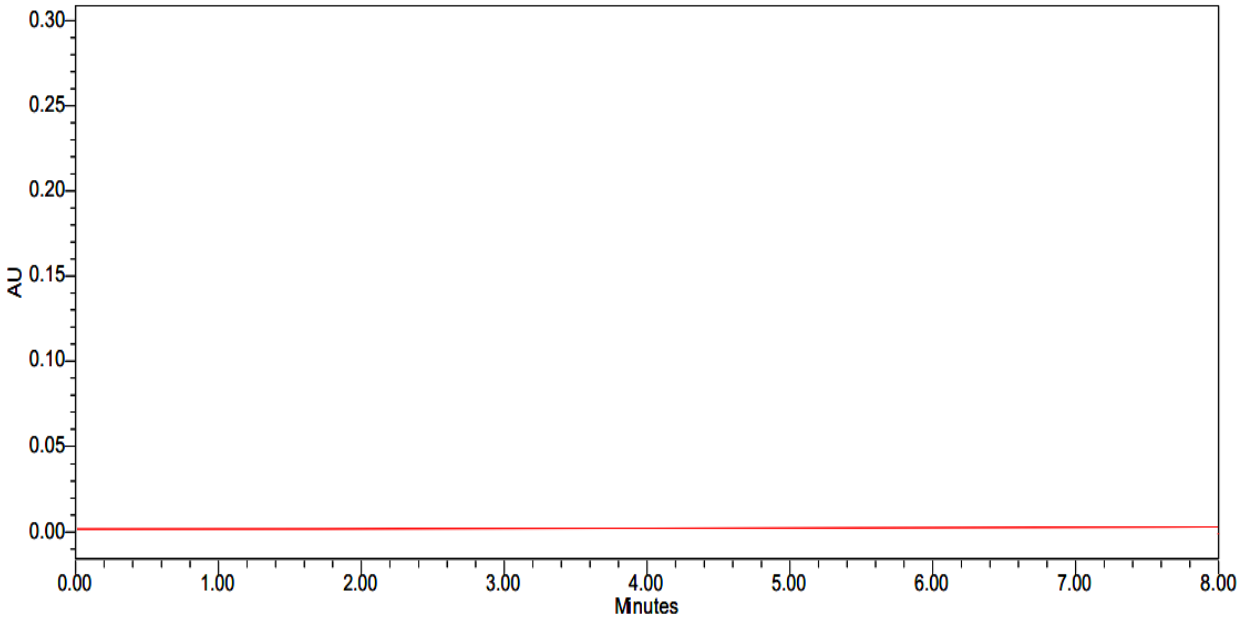

Fig. 4: Chromatogram of placebo

System Precision:

Table 4: System precision table of Trastuzumab & Hyaluronidase-oysk

S. No	Concentration Trastuzumab(µg/ml)	Area of Trastuzumab	Concentration of Hyaluronidase-oysk (µg/ml)	Area of Hyaluronidase-oysk
1.	6000	3854216	2.5	99553
2.	6000	3876933	2.5	99628
3.	6000	3889548	2.5	99826
4.	6000	3871639	2.5	99741
5.	6000	3867871	2.5	99848
6.	6000	3885520	2.5	99882
Mean	3874288		99746.33	
S.D	12783.6		131.52	
%RSD	0.32		0.13	

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.32% and 0.13% respectively for Trastuzumab and Hyaluronidase-oysk. As the limit of Precision was less than "2" the system precision was passed in this method.

Linearity:

Table 5: Results of linearity for Trastuzumab & Hyaluronidase-oysk

S.NO	Trastuzumab		Hyaluronidase-oysk	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	1500.00	1084369	0.63	24757
2	3000.00	1954782	1.25	49682
3	4500.00	3012467	1.88	74235
4	6000.00	3864271	2.50	99524
5	7500.00	4883659	3.13	125469
6	9000.00	6033548	3.75	147522
Regression equation	y = 657.35x +18080.18		y =39647.54x +116.43	
Slope	657.35		39647.54	
Intercept	18080.18		116.43	
R^2	0.99940		0.99989	

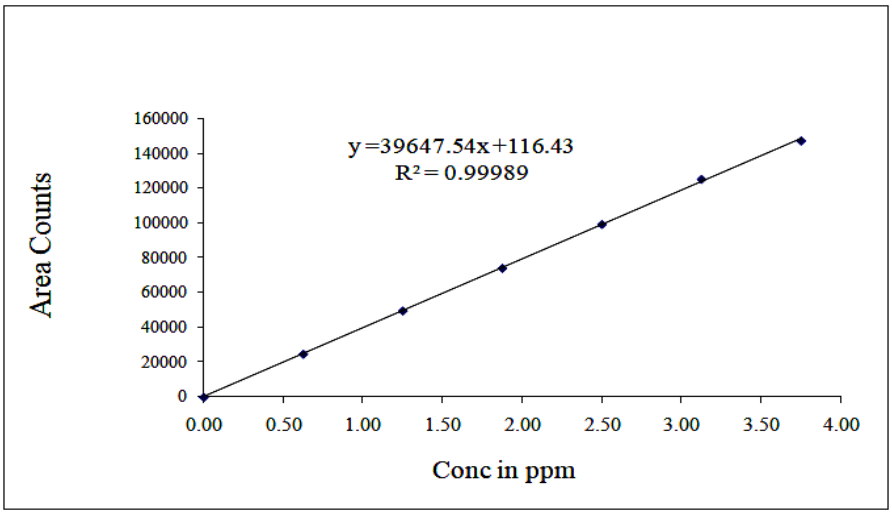

Fig. 5: Calibration curve for Hyaluronidase-oysk

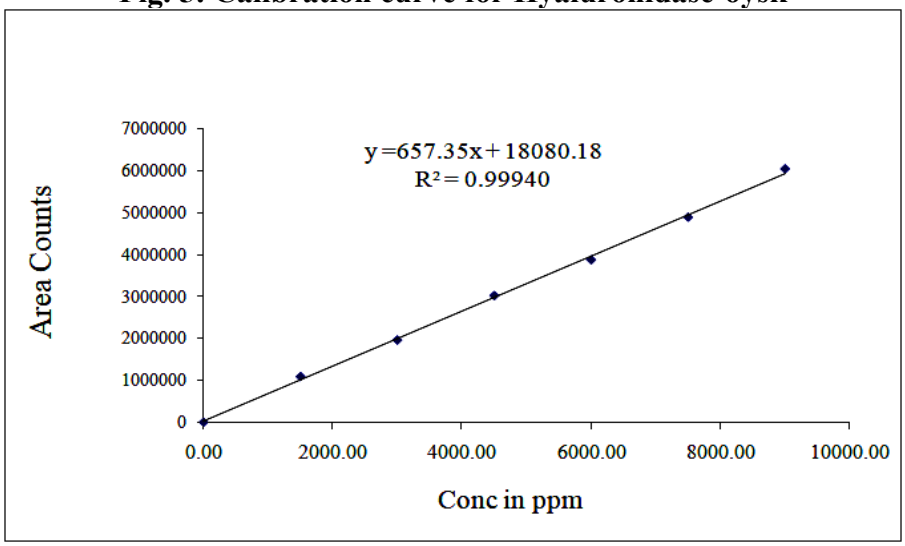

Fig. 6: Calibration curve for Trastuzumab

Assay

Table No.6: Assay of Trastuzumab & Hyaluronidase-oysk

Brand	Drug	Area	Avg sample area (n=2)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Std purity	Amount found (µg/ml)	% assay
Herceptin-Hylecta	Trastuzumab	3854126	3866052	6000	6000	120	99.9	59.850	99.8
		3877978							
	Hyaluronidase-oysk	99452	99483	2.5	2.5	0.05	99.8	0.025	100.0
		99514							

Intermediate precision (Day_ Day Precision):

Table 7: Intermediate Precision (Day variation) for Trastuzumab and Hyaluronidase-oysk by RP-HPLC method

S. No.	Area for Trastuzumab		Area for Hyaluronidase-oysk	
	Day-1	Day-2	Day-1	Day-2
1	3852103	3872160	99456	99636
2	3866396	3859321	99252	99012
3	3840137	3844206	99413	99423
4	3844106	3880137	99789	99897
5	3896354	3843214	99528	99431
6	3874136	3871305	99989	99894
Average	3862205	3861724	99571	99549
Standard Deviation	21178.708	15459.748	269.832	336.412
%RSD	0.55	0.40	0.27	0.34

The % RSD for the area of six standard injections results should not be more than 2%.

Accuracy:

Table 8: Accuracy results of Trastuzumab by RP-HPLC method

% Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1935238	30.00	29.96	99.9	100.4
	1945426	30.00	30.12	100.4	
	1955214	30.00	30.27	100.9	
100%	3851632	60.00	59.62	99.4	100.0
	3884127	60.00	60.13	100.2	
	3895260	60.00	60.3	100.5	
150%	5771236	90.00	89.34	99.3	99.5
	5784582	90.00	89.55	99.5	
	5792628	90.00	89.67	99.6	

Table 9: Accuracy results for Hyaluronidase-oysk by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	50856	0.013	0.013	100.0	100.0
	50386	0.013	0.013	100.0	
	52638	0.013	0.013	100.0	
100%	98686	0.025	0.025	100.0	100.0
	98837	0.025	0.025	100.0	
	99578	0.025	0.025	100.0	
150%	153434	0.038	0.038	100.0	100.0
	150236	0.038	0.038	100.0	
	152715	0.038	0.038	100.0	

Discussion:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.0% and 100.0% for Trastuzumab and Hyaluronidase-oysk respectively.

LOD and LOQ (µg/ml):

Table 10: Sensitivity parameters (LOD & LOQ) by RP-HPLC

Name of drug	LOD(µg/ml)	s/n	LOQ(µg/ml)	s/n
Trastuzumab	0.600	3	1.800	10
Hyaluronidase-oysk	0.075	3	0.250	10

Conclusion

The work concludes that the developed method was simple, precise, accurate and sensitive for the simultaneous assessment of the Trastuzumab and Hyaluronidase-oysk in drug product. Retention time of Trastuzumab and Hyaluronidase-oysk were found to be 2.092 and 3.469 minutes. Three levels of Accuracy samples (50%, 100% 150%) were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.5% and 100% for Trastuzumab and Hyaluronidase-oysk respectively. LOD, LOQ values for Trastuzumab and Hyaluronidase-oysk were 0.6, 0.075 µg/ml and 1.8, 0.25 µg/ml respectively.

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