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METHOD DEVELOPMENT AND VALIDATION OF TRASTUZUMAB AND FULVESTRANT IN TABLET DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

Objective: To develop a new simple, precise, accurate and reproducible RP-HPLC method for simultaneous estimation of bulk and pharmaceutical formulations.

Methods: Separation of Trastuzumab and Fulvestrant was successfully achieved on a column: Inertsil C18 250×4.6mm, 5µm equivalent in an isocratic mode utilizing 0.1% KH2PO4: Methanol (70:30) at a flow rate of 1 ml/min and elute was monitored at 233nm.

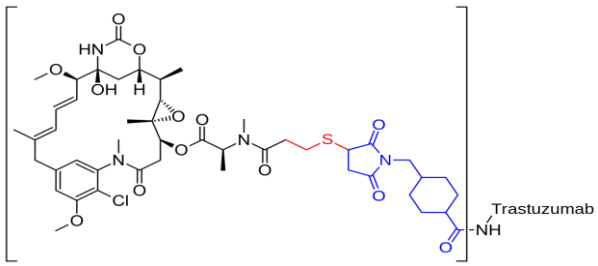
Results: The retention time [Rt] of Trastuzumab and Fulvestrant was 3.05±0.01 and 3.57±0.01min respectively. The precision was found with<1.5% of %RSD. The method was validated and the response was found to be linear in drug concentration of 50-150 µg/ml for Trastuzumab and Fulvestrant. The values of the correlation coefficient were found to 0.999 for Trastuzumab and Fulvestrant respectively. The percentage recovery was found to be within the specified range i.e., 98-102 % for three drugs. The LOD and LOQ for Trastuzumab were found to be 1.172 and 3.908 respectively. The LOD and LOQ for Fulvestrant were found to be 0.6181 and 2.0604 respectively.

Conclusion: The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Accuracy, Precession, Specificity and Robustness.

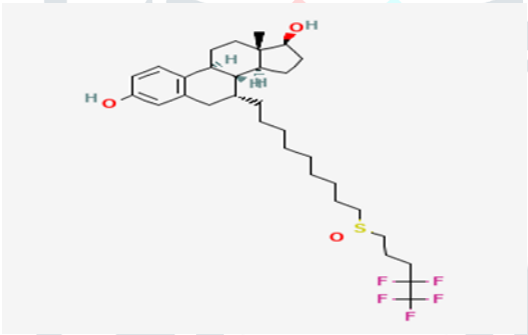
Keywords: Trastuzumab and Fulvestrant, Validation, RP-HPLC.

INTRODUCTION

Trastuzumab contains the active substance (anti-p185, rhuMab HER2), which is a humanized monoclonal antibody that binds to the HER2 protein. HER-2 is a transmembrane-spanning receptor-like protein, which is structurally related to the epidermal growth factor receptor and has been shown to inhibit the proliferation of human tumor cells that overexpress HER2 both in vitro and in vivo. trastuzumab is presented as a white to pale yellow lyophilized powder for concentrate for solution for infusion. Herceptin is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress HER2:



CHEMICAL FORMULA : $C_{6448}H_{9948}N_{1720}O_{2012}S_{44}·(C_{47}H_{62}ClN_4O_{13}S)$



13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol

Molecular Weight: 606.8 g/mol

FULVESTRAN is indicated, for the treatment of estrogen receptor positive, locally advanced or metastatic breast cancer in postmenopausal women: not previously treated with endocrine therapy, or, with disease relapse on or after adjuvant antiestrogen therapy, or disease progression on antiestrogen therapy.in combination

Antineoplastic agents that are used to treat hormone-sensitive tumors. Hormone-sensitive tumors may be hormone-dependent, hormone-responsive, or both. A hormone-dependent tumor regresses on removal of the hormonal stimulus, by surgery or pharmacological block. Hormone-responsive tumors may regress when pharmacologic amounts of hormones are administered regardless of whether previous signs of hormone sensitivity were observed. The major hormone-responsive cancers include carcinomas of the breast, prostate, and endometrium; lymphomas; and certain leukemias.

Trastuzumab and fulvestrant combination therapy is one of the treatment options for patients with hormone receptor- and human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer; however, there are limited studies evaluating the efficacy of this combination therapy.

MATERIALS AND METHODS

Instrumentation

WATERS HPLC, Model: e2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. C18 (4.6 x 250 mm, 5 µm, Make: Sunsil) column was used for separations. ASCOSET company ER200A model Electronic balance was used. ENERTECH company model SE60US Ultra-Sonicator was used for Preparation of standard samples.

Chemicals and Reagents

The standard samples **trastuzumab and fulvestrant** are procured from the K K PHARMA SOLUTIONS, Hyderabad. M formulation (AF-kit, Madras Pharmaceuticals, and Chennai) was procured from the local market. HPLC grade methanol were obtained from Merck Life Sciences, Mumbai, India. Analytical grade solvents and other chemicals were acquired from SD Fine Chemicals, Mumbai, India. HPLC Grade Water was obtained from UV Scientifics Hyderabad, filtered through 0.45 µ nylon membrane for the HPLC experiments.

Preparation of buffer

Phosphate buffer was prepared by dissolving 13.6 g of potassium dihydrogen *ortho*-phosphate in 1000 ml of water (HPLC grade) and pH was adjusted to 4.8 with *ortho*-phosphoric acid and solution was filtered through 0.45 µ Millipore nylon filter.

Preparation of mobile phase

Methanol: 0.1 % KH_2PO_4 in the ratio of 70:30 % v/v was prepared and it was filtered through 0.45 µ Millipore nylon filter. They are mixed and sonicated for 20min. The resultant solution was used as the mobile phase.

Chromatographic conditions

The method was developed by using column: Inertsil C18 250×4.6mm, 5µm equivalent in an isocratic mode utilizing mobile phase 0.1% potassium dihydrogen *ortho*-phosphate buffer (pH-4.8 adjusted with *ortho*-phosphoric acid): Methanol (70:30). The sample injection volume was 10 µl. The mobile phase was filtered through 0.45 µ Millipore nylon filter under vacuum filtration. Flow rate of the mobile phase is 1 ml/min and eluted compounds were monitored at 233nm.

Preparation of standard mixture of trastuzumab and fulvestrant : Accurately weigh and transfer 150mg of trastuzumab, 250mg of fulvestrant into 100ml of volumetric flask and add 20ml of Methanol and sonicate 10min (or) shake 5min and make with Methanol. Transfer 1ml of the above solution into 10ml volumetric flask dilute to volume with water. The resulted solution (10 µl) was injected into the HPLC system by employing optimized chromatographic conditions.

Preparation of sample mixture of trastuzumab and fulvestrant

Commercially available 20 tablets were weighed and the average weight of each tablet was determined individually. These Tablets were crushed into a fine powder and the equivalent weight (500mg, 150mg) of trastuzumab and fulvestrant of active ingredients were transfer into a 100ml of volumetric flask and add 20ml of Methanol and sonicate 20min (or) shake 10min and makeup with Methanol. Transfers above solution 1ml into 10ml of the volumetric flask dilute the volume with water. And the solution was filtered through 0.45µm filter before injecting into HPLC system. The resulted solution (10 µl) was injected into the HPLC system by employing optimized chromatographic conditions.

Method Validation

Method validation was performed using standard and sample solutions of analyte as per ICH guidelines for proposed method [22-23]. The following validation parameters performed such as specificity, linearity, precision, accuracy, robustness, LOD and LOQ etc.

RESULTS AND DISCUSSION

1. SYSTEM SUITABILITY:

Table 9: System suitability data of Trastuzumab and Fulvestrant

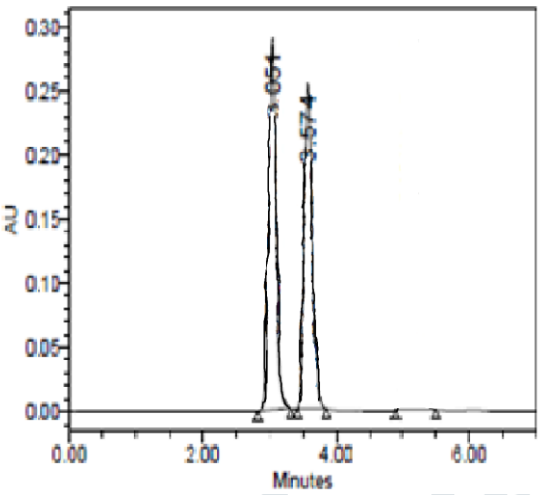
parameter	Trastuzumab	Fulvestrant	Acceptance criteria
Retention time	3.050	3.575	±10
Theoretical plates	3625	4191	>2500
Tailing factor	0.99	1.20	<2.00
% RSD	0.6	0.5	<2.00

Standard Results of TRASTUZUMAB

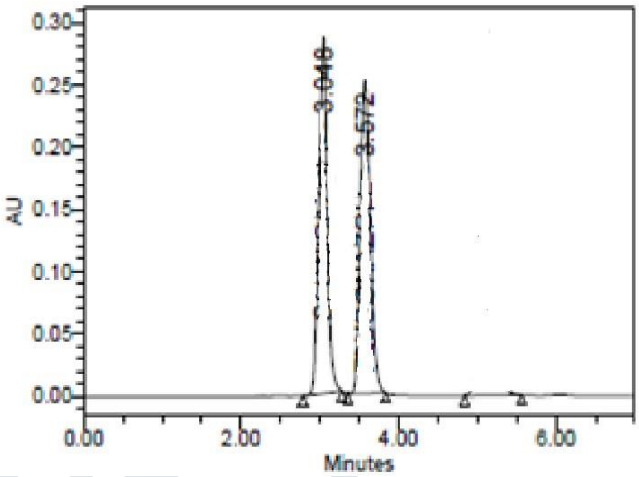
S.no	Sampl name	RT	Area	USP plate count	USP tailing
1.	Injection1	3.051	2174332	3720	0.99
2.	Injection 2	3.051	2161386	3744	0.98
3.	Injection 3	3.048	2152914	3900	0.99

Standard Results of FULVESTRANT

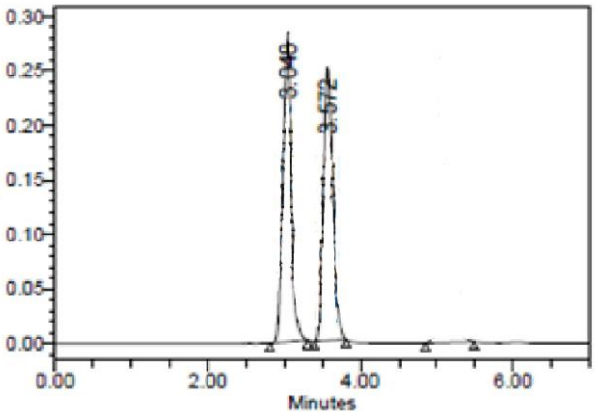
S.no	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection 1	3.574	2134038	4200	1.20
2.	Injection 2	3.575	2157373	4116	1.21
3.	Injection 3	3.572	2136622	4143	1.21



Typical Chromatogram of Standard-2; Injection-1



Typical Chromatogram of Standard-2; Injection-2



Typical Chromatogram of Standard-2; Injection-3

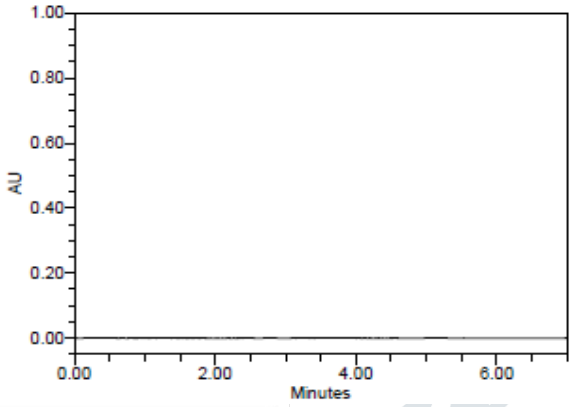
RESULT

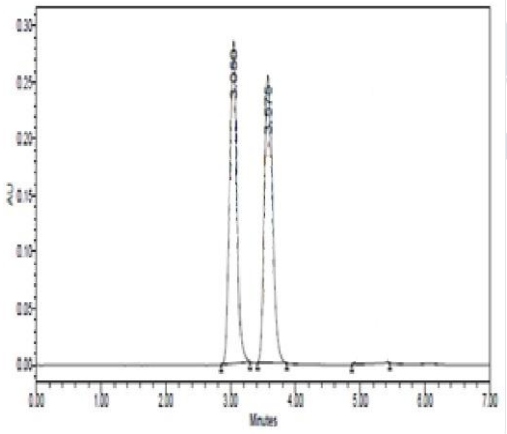
Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

2. SPECIFICITY:

Table 10: Specificity data for TRASTUZUMAB AND FULVESTRANT

S no	Sample name	TRASTUZUMAB area	Rt	FULVESTRANT area	Rt	Rt
1	Standard	2165086	3.050	2137258	3.575	5.121
2	Sample	2160575	3.047	2145650	3.568	5.101
3	Blank	-			-	-
4	Placebo	-			-	-





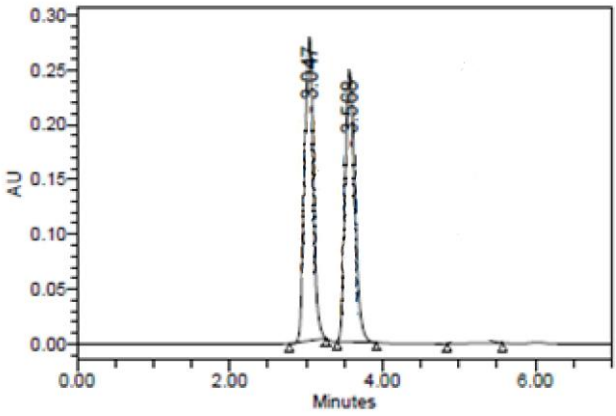


Fig 14: chromatogram representing specificity of standard **Fig 15: chromatogram representing specificity of sample**

RESULT

The forced degradation study showed the method was highly specific, the chromatographic peaks does not interfere with any other impurities**.** This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

3. ACCURACY:

Table 14: Accuracy (%recovery) results of TRASTUZUMAB

S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	680.00	250.000	250.72	100	100
		2	680.00	250.000	249.40	100	
		3	680.00	250.000	248.69	99	
2	100%	1	1360.00	500.000	499.00	100	100
		2	1360.00	500.000	497.67	100	
		3	1360.00	500.000	498.57	100	
3	150%	1	2040.00	750.000	748.20	100	100
		2	2040.00	750.000	747.23	100	
		3	2040.00	750.000	747.87	100	

Table 16: Accuracy (%recovery) results of FULVESTRANT

S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	680.00	74.250	74.37	100	100
		2	680.00	74.250	74.43	100	
		3	680.00	74.250	74.73	101	
2	100%	1	1360.00	148.500	149.08	100	100
		2	1360.00	148.500	149.29	100	
		3	1360.00	148.500	149.38	100	
3	150%	1	2040.00	222.750	223.49	100	100
		2	2040.00	222.750	223.25	100	
		3	2040.00	222.750	223.57	100	

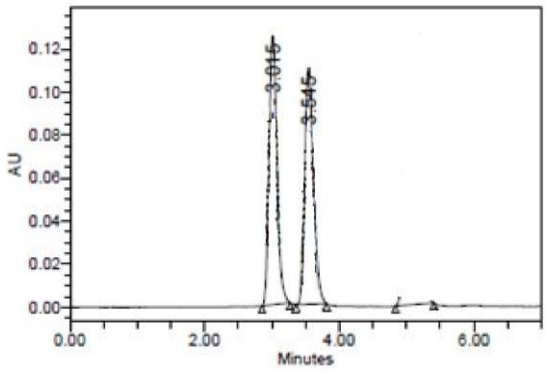


Fig 21: Typical chromatogram for Accuracy 50 %

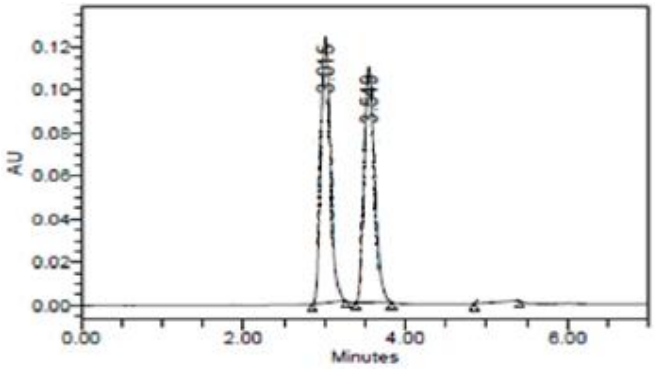


Fig 22: Typical chromatogram for Accuracy 100 %

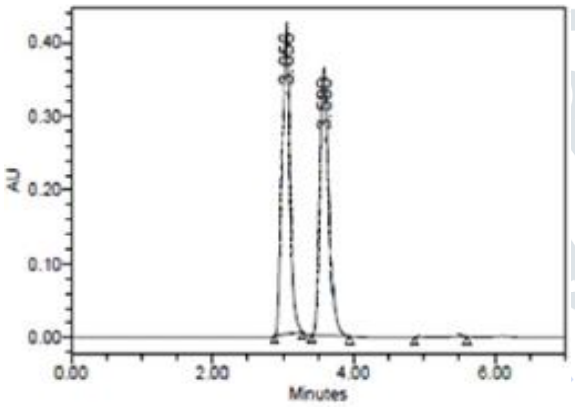


Fig 23: Typical chromatogram for Accuracy 150 %

RESULT

% Recovery was 100.00% for **TRASTUZUMAB** and 100.00% for **FULVESTRANT**. All the results indicates that the method is highly accurate.

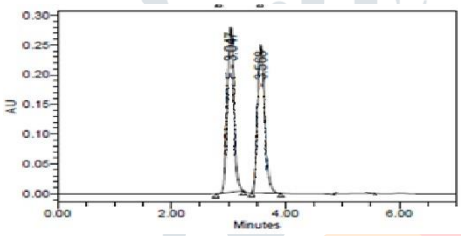
4. PRECISION:

Table17: Precision data for TRASTUZUMAB

S.no	RT	Area	%Assay
injection1	3.047	2160575	99
injection2	3.044	2160799	99
injection3	3.046	2164280	100
injection4	3.043	2162402	100
injection5	3.045	2160485	99
injection6	3.041	2166296	100
Mean			100
Std. Dev.			0.11
% RSD			0.11

Table 18: Precision data for FULVESTRANT

S.no	RT	Area	%Assay
injection1	3.568	2145650	99
injection 2	3.564	2142729	99
injection 3	3.562	2145818	99
injection 4	3.558	2149646	100
Mean			99
Std. Dev.			0.11
%RSD			0.11



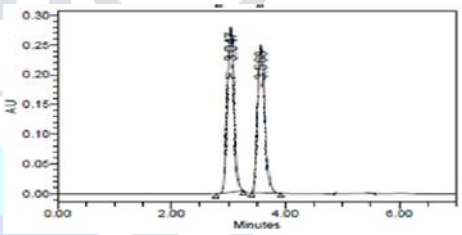
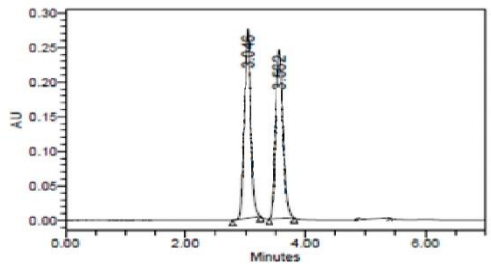


Fig 24: Chromatogram for precision injection 1 **Fig 25: Chromatogram for precision injection 2**



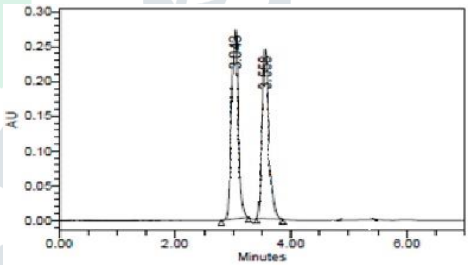


Fig 26: Chromatogram for precision injection 3 **Fig 27: Chromatogram for precision injection 4**

RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise**.**

5. LINEARITY:

Table 19: Linearity data for TRASTUZUMAB

s.no	Conc(µg/ml)	RT	Area
1.	50	3.017	1086149
2.	75	3.032	1620133
3.	100	3.042	2176798
4.	125	3.049	2701273
5.	150	3.056	3245695
(r^2)			0.999

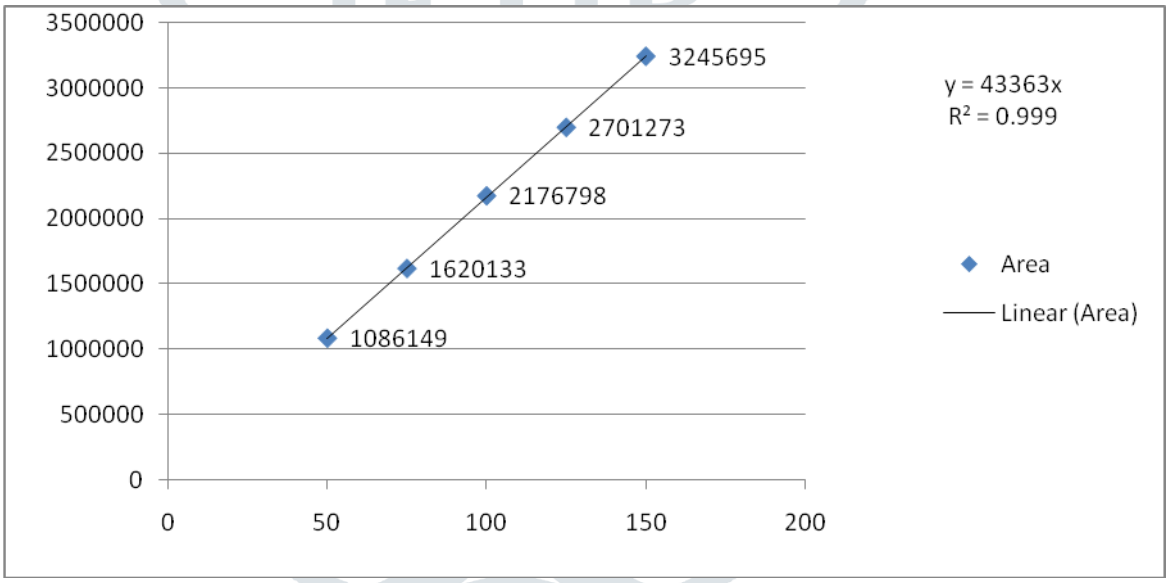


Fig 30: Linearity plot of TRASTUZUMAB

Table 20: Linearity data for FULVESTRANT

s.no	Conc(µg/ml)	RT	Area
1.	50	3.565	1076304
2.	75	3.568	1605787
3.	100	3.570	2142647
4.	125	3.571	26755400
5.	150	3.572	3217208
(r^2)			0.999

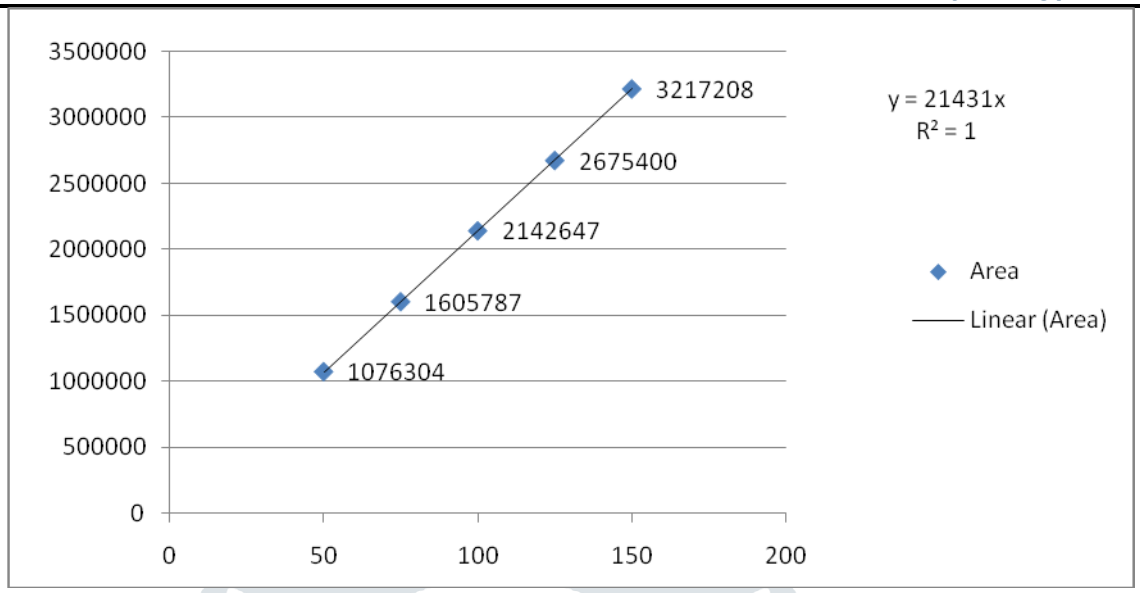


Fig 31: Linearity plot of FULVESTRANT

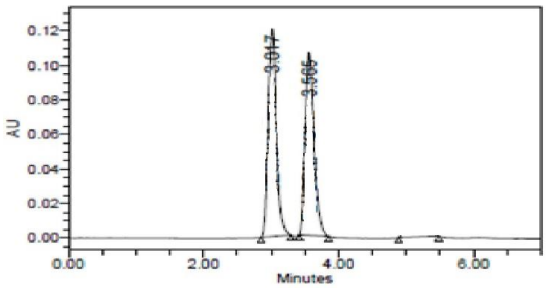


Fig32: Chromatogram representing linearity 1

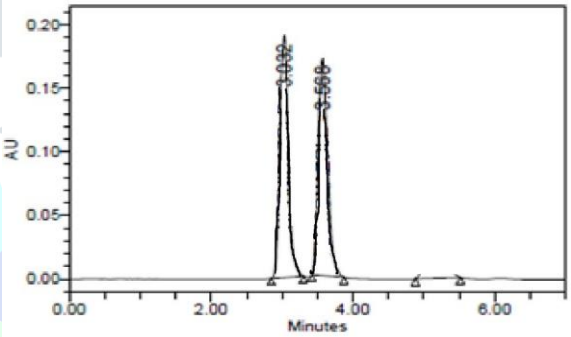


Fig 33: Chromatogram representing linearity 2

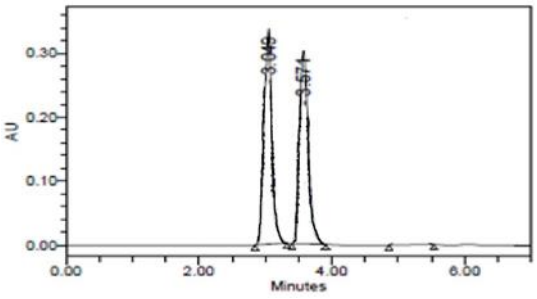


Fig 34: Chromatogram representing linearity 3

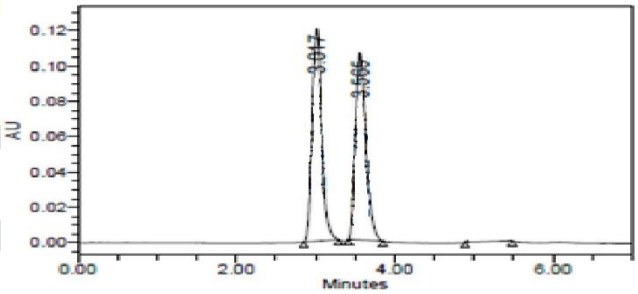


Fig 35: Chromatogram representing linearity 4

RESULT

A linear relationship between peak areas versus concentrations was observed for **TRASTUZUMAB, FULVESTRANT** in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.999 for both **TRASTUZUMAB, FULVESTRANT** which prove that the method is linear in the range of 50% to 150%.

6. ROBUSTNESS:

Table 21: Robustness data for TRASTUZUMAB

Parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.8ml/min)	3.795	3197	1.06
Increased flow rate(1.2ml/min)	2.525	3218	1.04
Decreased temperature(20^0c)	3.793	3088	1.06
Increased temperature(30^0c)	2.527	3202	1.05

Table 22 : Robustness data for FULVESTRANT

parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate (0.8ml/min)	4.409	3984	1.35
Increased flow rate (1.2ml/min)	2.957	3437	1.34
Decreased temperature(20^0c)	4.403	3955	1.36
Increased temperature(30^0c)	2.958	3305	1.34

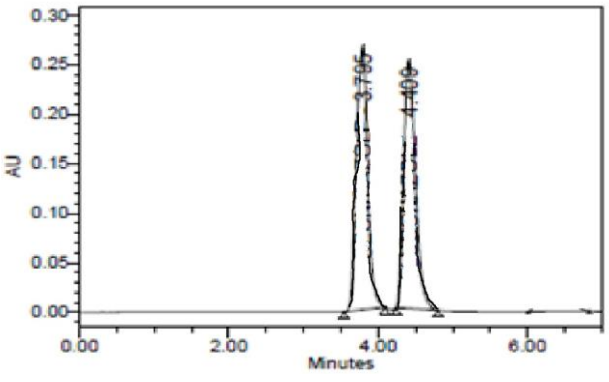


Fig 37: Chromatogram for decreased flow rate

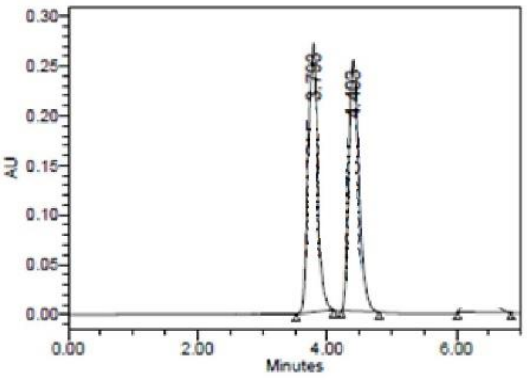
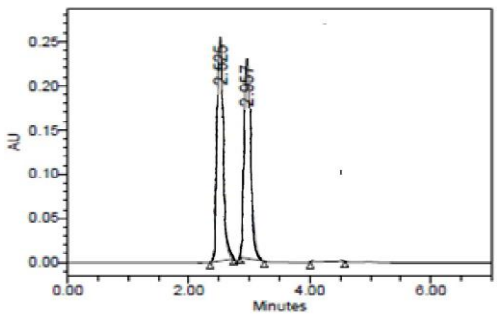


Fig 38: Chromatogram for increased flow rate



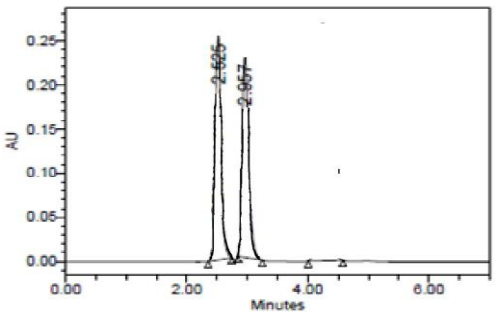


Fig 39: Chromatogram for decreased temperature Fig 40: Chromatogram for increased temperature

RESULT

The results of Robustness of the present method had shown that changes made in the Flow and Temperature did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.

7. LIMIT OF DETCTION:

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

$$LOD = 3.3* \sigma/S$$

LOD for Trastuzumab = 1.172

LOD for Fulvestrant =0.6181

LOD data for TRASTUZUMAB, FULVESTRANT

s.no	Sample name	RT	Area
1	**TRASTUZUMAB**	2.986	6668
2	**FULVESTRANT**	3.550	4668

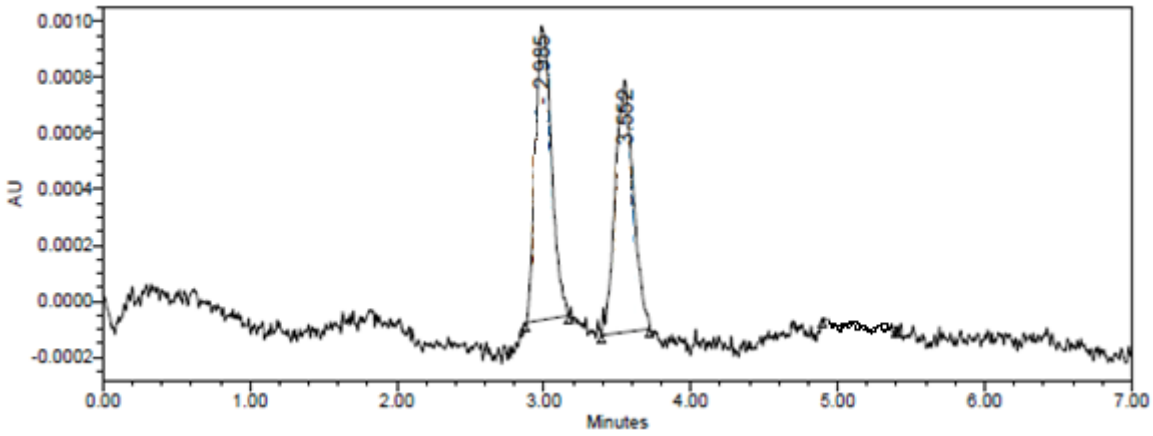


Fig 41: Chromatrogram for LOD

8. LIMIT OF QUANTIFICATION:

Minimum concentration of standard component in which the peak of the standard gets detected and quantification

$$LOQ = 10*\sigma/S$$

LOQ for **TRASTUZUMAB** =0.782

LOQ for **FULVESTRANT** =0.5494

LOQ data for TRASTUZUMAB, FULVESTRANT

S.no	Sample name	RT	Area
1	Trastuzumab	2.985	7968
2	Fulvestrant	3.552	7698

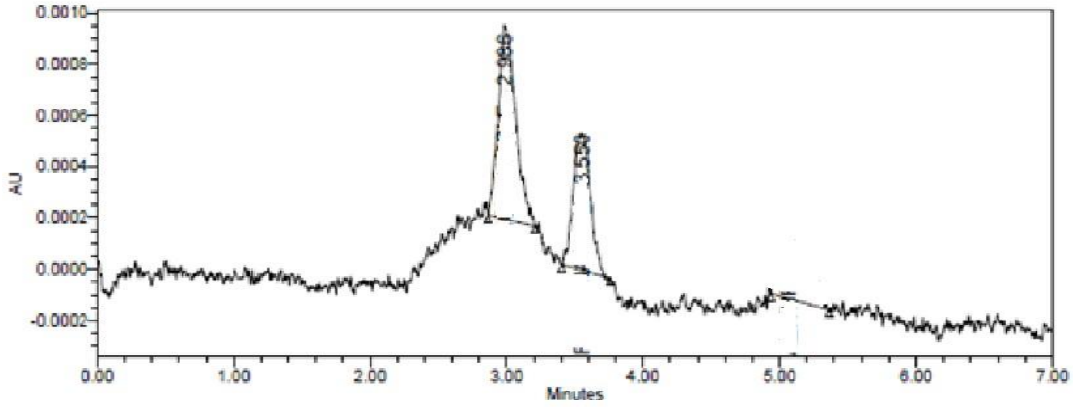


Fig 42: Chromatogram for LOQ

CONCLUSION

The study is focused to develop and validate an accurate and reliable reverse phase HPLC method was proposed for separation and simultaneous estimation of Trastuzumab, Fulvestrant in tablet dosage form

The buffer choice and mobile phase composition, column selection were found to be crucial in the separation these drugs.

Based on the results obtained it is found that the proposed method is accurate, precise, reproducible, rapid and economical and can be used in the routine analysis of combination kit product containing Trastuzumab, Fulvestrant dosage forms.

References

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