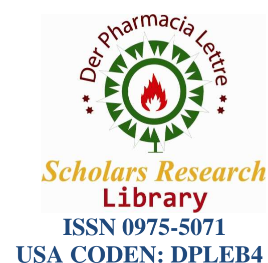
Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2013, 5 (3):249-260
(http://scholarsresearchlibrary.com/archive.html)



ISSN 0975-5071
USA CODEN: DPLEB4

An improved RP-HPLC method for the quantitative determination of capecitabine in bulk and pharmaceutical tablet dosage form

P. Ravisankar[*1, 2], G. Devala Rao[3], M. Naveen Kumar[1], M. Krishna Chaitanya[1]

[1]*Department of Pharmaceutical Analysis and Quality Assurance, Vignan Pharmacy College, Vadlamudi, Guntur, A.P., India*
[2]*Faculty of Science, Sri Chandrasekharendra Saraswathi Viswa MahaVidyalaya (SCSVMV University), Enathur, Kanchipuram, T.N., India*
[3]*Department of Pharmaceutical Analysis, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, A.P., India*

ABSTRACT

A simple, specific, accurate, and precise reverse phase high performance liquid chromatographic method was developed and validated for the estimation of Capecitabine in tablet dosage forms. A Welchrom C_{18}column having 250 × 4.6 mm i.d., 5 µm particle size in isocratic mode, with mobile phase containing Methanol:Acetonitrile:Water (50:30:20,v/v, pH adjusted to 4.6 using Triethylamine) was used. The flow rate was 1.0 ml/min and effluents are monitored at 245 nm. The retention time of Capecitabine is 4.123 min. The method was validated for specificity, linearity, accuracy, precision, limit of quantification, limit of detection, robustness in accordance with ICH guidelines. Limit of detection and limit of quantification for estimation of Capecitabine found to be 0.169247 µg/mL and 0.512872 µg/mL. Recovery of Capecitabine in tablet formulation was found to be 99.88 %. Proposed method was successfully applied for the quantitative determination of Capecitabine in commercially available tablet dosage forms.

Keywords: Capecitabine, RP-HPLC, Determination, Validation, Capiibine.

INTRODUCTION

Capecitabine (CAP) is an orally administered chemotherapeutic agent used in the treatment of colorectal cancer [1-2], metastatic breast cancer, stomach, pancreas, liver, gullet (esophagus) and skin cancers. It is chemically pentyl[1-(3,4-dihydroxy-5-methyltetrahydrofuran-2-yl)-5-fluoro-2-oxo-1H-pyrimidin-4-yl]carbamate (Fig. 1). CAP is aprodrug, which is converted to 5-fluorouracil (5-FU) [3]. Oral CAP undergoes sequential hydrolysis and deamination reactions in the liver to produce 5'-deoxy-5-fluorouridine. This is converted to 5-FU by thymidine phosphorylase (also known as platelet-derived growth factor). As this enzyme is abundant in tumor tissue there is some tumor specificity in the patient's exposure to 5-FU. 5-FU inturn inhibits the thymidylatesynthetase, blocking the methylation of deoxyuridylic acid to thymidylic acid, interfering with DNA, and to a lesser degree, RNA synthesis and decreases growth of tumor tissue. Since it lacks selectivity towards tumor cells, 5-FU also exhibits significant toxicity. Prodrugs of 5-FU have been developed to improve efficacy and to reduce side effect toxicity5-FU was until recently the only drug used extensively for advanced colorectal cancer. There is now some evidence to suggest that 5-FU is most active when given by prolonged intravenous infusion. This is not very convenient for patients because it requires protracted venous access and infusion devices. Oral treatmentis not a viable alternative because the absorption of 5-FU from the Gastro-Intestinal Tract is low and unpredictable. This problem has led to the development of orally bio-available 5-FU prodrug such as CAP.

Literature survey reveals that various methods have been reported for estimation of CAP in biological matrices such as plasma, which includes the use of High Performance Liquid Chromatography (RP-HPLC) with UV detection [4-13], Diode Array Detection (DAD) [14], Liquid Chromatography-Mass Spectrometry (LC-MS)[15-22]. But the reported methods have either longer chromatographic run time, less sensitivity and peak symmetry. The present study involves development of a validated HPLC method for the estimation of CAP in tablet dosage form, which is fast, simple, and sensitive with less run time and good peak symmetry. This method also provides rapid estimation, excellent peak shape, use of smaller sample volumes and buffer volumes, providing cost savings. Finally, the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines [23].

MATERIALS AND METHODS

Chemicals and Reagents:
The reference sample of Capecitabine standard was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Acetonitrile (HPLC grade) and triethylamine (HPLC grade) were purchased from Merck Pharmaceuticals Private Ltd., Mumbai, India. Methanol and water used were of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial capsules of Cefdinir formulation was procured from local market. CAPIIBINE tablets containing 500mg of CAP are manufactured by Dr.Reddy's Laboratories Pvt. Ltd., Hyderabad, India.

Instruments and Chromatographic conditions:
Chromatographic separations were achieved by using Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was injected into the HPLC system. The HPLC system was equipped with "Spinchrom" data acquisition software.Separations were performed on the reversed phase column using a mixture of methanol, acetonitrile and water (pH adjusted to 4.6 using o-phosphoric acid) in ratio of 50:30:20, v/v as mobile phase. Triethylamine was used as column modifier. The mobile phase was delivered at a flow rate of 1 mL/min. Eluate was monitored at 245 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

Preparation of Reagents and Standards
a. Mobile phase:
The mobile phase was prepared by mixing of methanol, acetonitrile and water (all of HPLC grade) in the ratio of 50:30:20, v/v. Then pH is adjusted to 4.6 with 0.1N *o*-phosphoric acid and 0.5ml triethylamine is added as column modifier. It is filtered through 0.45 µm nylon membrane filter and then sonicated for degassing.
b. Stock and Working Standard Solutions:
Accurately weigh and transfer about 100 mg of CAP, dissolve in a 100ml volumetric flask with mobile phase. This is stock standard solution of CAP with concentration of 1000 µg/mL. Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with mobile phase. The concentrations of CAP are 2.0, 4.0, 6.0, 8.0, 10.0 µg/mL, respectively.

Tablet Sample preparation:
Weigh accurately not less than 20 tablets and determine average weight. Crush the tablets of CAP (CAPIIBINE) into fine powder. Weigh equivalent to 100 mg of CAP into 100 mL volumetric flask. Add 70 mL mobile phase and sonicate until dissolution is complete. Make up the volume to 100 mL. Pipette out 1.0 mL of solution into a 100 mL volumetric flask and dilute with mobile phase upto the mark. Mix well.The resulting solution was filtered using 0.2 µm filter and degassed by sonication.

Selection of detection wavelength:
The UV spectrum of diluted solutions of various concentrations of CAP in mobile phase was recorded using UV spectrophotometer. The wavelengthof maximum absorbance was observed at 245nm. This wavelength was used for detection of CAP.

Calibration curve for Capecitabine:
Replicates of each calibration standard solutions (2,4,6,8,10 µg/mL) were injected using a 20µl fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of CAP on X-axis and peak areas of standard CAP on Y-axis and regression equations were computed for CAP.

VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

System suitability:

Set up the chromatographic system, allow the HPLC system to stabilize for 40 min.Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of CAP standard (%RSD NMT 2.0). If system suitability parameters are met, then inject sample (CAPIIBINE) preparation in duplicate and record the chromatograms.

Specificity:

The specificity of the proposed methodwas demonstrated by studying the effect of variousexcipients and other additives usually present in the formulations of CAP in the determinations under optimum conditions. The blank, standard, placebo, placebo spiked with analyte and test preparations were analyzed as per the method to examine the interference of blank and placebo with CAP peaks. The common excipients such as lactose anhydrous, microcrystalline cellulose, purified talc and magnesium stearate have been added to the placebo solution and injected and tested.Furthermore the well-shaped peaks also indicate the specificity of the method. The chromatogram for placebo indicating the specificity of developed method is presented in Fig. 2.

Linearity:

Linearity for CAP was determined by preparing standard solutions at different concentrations from 50% to 150% of the test concentration. The linearity graphs for the proposed assay methods were obtained over the concentration range of 2-10 µg/ml of CAP. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed.

Precision:

Intra-day and inter-day precision of the procedure were determined by performing six determinations at the same concentration (10µg/mL) of CAP during the same day, under the same experimental conditions and on a different day respectively. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

Accuracy/Recovery:

The accuracy of the method was evaluated in triplicate at 3 different concentrations equivalent to 80%, 100% and 120% of the active ingredient, by adding a known amount of CAP standard to a sample with pre-determined amount of CAP. The recovered amount of CAP, %RSD of recovery, % recovery of each concentration is calculated to determine the accuracy.

Robustness:

The Robustness of developed analytical method was established by the analysis of CAP under different experimental conditions such as making deliberate changes in chromatographic conditions like flow rate (± 0.2 ml/min), detection wavelength (±5 nm) and Mobile phase composition (±5%).

LOD and LOQ:

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve.

RESULTS AND DISCUSSION

The present study was aimed to develop a rapid, accurate and precise HPLC method for the determination of CAP in pharmaceutical dosage forms in order to set up analysis of the component peaks under isocratic conditions, mixtures of methanol, acetonitrile and HPLC grade water in different combinations were tested as mobile phase on a C_{18} stationary phase. A combination of methanol, acetonitrile and HPLC grade water in a ratio of 50:30:20, v/v, with pH adjusted to 4.6 using o-phosphoric acid and triethylamine as column modifier at a flow rate of 1mL/min was proved to be the most suitable of all combinations of mobile phase tried since the chromatographic peak obtained was well

shape symmetrical peak. The retention time for CAP was found to be4.123 min. UV spectra of CAP showed that the drug absorbed maximum at 245 nm, so this wavelength was selected as the detection wavelength. System suitability parameters and optimized chromatographic conditions are shown in Table 1. The calibration curve for CAP was found to be linear over the range of 2-10 µg/mL. The data of regression analysis of the calibration curve is shown in Table 2 and Table 3. A good linear relationship (R^2=0.9997) was observed between the concentrations of CAP and the corresponding peak areas. The regression equation of the calibration curve was found to beY= 4.713 + 108.73X where Y is the peak area and X is the concentration of CAP. The developed method was applied to the assay of CAP tablets. The experimental results are given in Table 4. The results were very close to labeled value of commercial tablets. The representative standard and sample chromatograms of CAP are shown in Fig. 3 and Fig. 4 respectively. The representative chromatograms of the standard CAP concentrations are shown in Fig. 5 to Fig. 9. The linearity graph is shown in Fig. 10. The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo (Fig. 2) with sample peak. They do not disturb the elution or quantification of CAP; furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 5. Precision was studied to find out intra-day and inter-day variations in the test methods of CAP for the three times on the same day and different day. The %RSD for intra-day and inter-day precision variations studied at 10µg/mL obtained were 0.6929 and 1.0206respectively showed a low Coefficient of Variation. This reveals that the proposed method is quite precise and reproducible and the precision results for intra-day and inter-day are shown in Table 6 and Table 7 respectively.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS AND SYSTEM SUITABILITY PARAMETERS OF PROPOSED RP-HPLC METHOD FOR CAPECITABINE

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 mm i.d. X 250mm, 5µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Mobile phase	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1mL/min.
Detection wave length	UV at 245 nm.
Run time	6 minutes
Column back pressure	156kgf
Temperature	Ambient temperature(25ºC)
Volume of injection loop	20µL
Retention time (t_R)	4.123 min
Theoretical plates[th.pl] (Efficiency)	10,080
Theoretical plates per meter[t.p/m]	201,597
Tailing factor (asymmetry factor)	1.069

TABLE 2: LINEAR REGRESSION DATA OF THE PROPOSED HPLC METHOD OF CAPECITABINE

Parameter	Method
Detection wavelength(λ_{max})	UV at 245 nm
Linearity range (µg/mL)	2-10µg/mL
Regression equation (Y = a + bX)	Y=4.713 + 108.73X
Slope(b)	108.73
Intercept(a)	4.713
Standard error of slope (S_b)	0.920965
Standard error of intercept (S_a)	5.576724
Standard error of estimation (S_e)	7.705352
Regression coefficient (R^2)	0.9997
% Relative standard deviation* i.e., Coefficient of variation(CV)	1.220368
Percentage range of errors* (Confidence limits)	
0.005significance level	1.447048
0.001 significance level	2.269799

**Average of 6 determinations; acceptance criteria < 2.0.*

The % recoveries of the drug solutions were studied at 3 different concentration levels. The % individual recovery and the % RSD values at each level were within the acceptance limits. The results are presented in Table 8. Generally the mean percentage recovery of CAP at each level was not less than 99% and not more than 101%. Robustness was done by deliberate changes in the chromatographic conditions like mobile phase flow rate, temperature, mobile phase composition etc.,deliberate changes in developed method had not much affected the peak tailing, theoretical peaks and % assay which indicates that the present method is robust. As a matter of fact, the

robustness results are presented in Table 9. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) was 0.169247 µg/mL and the limit of quantitation (LOQ) was 0.512872 µg/mL which shows that this method is very sensitive. The results are presented in Table 10.

TABLE 3: CALIBRATION DATA OF THE PROPOSED HPLC METHOD FOR ESTIMATION OF CAPECITABINE

S.No	Concentration, µg/mL.	Retention time, (t_R)min.	Peak area, mV.s.
1.	0	-	0
2.	2	4.123	219.489
3.	4	4.123	447.109
4.	6	4.113	659.469
5.	8	4.113	881.887
6.	10	4.123	1082.382
Slope		108.73	
Intercept		4.713	
Correlation Coefficient [CC] (r)		0.999856	
Squared CC (R^2)		0.999713	
Residual sum of squares		237.489	

TABLE 4: ASSAY RESULTS OF CAPECITABINE FORMULATION

S. No	Formulations	Labelled amount	Amount found	% Assay ±SD*
1	CAPIIBINE tablets (Dr.Reddy's laboratoriesLtd.,Hyderabad)	500mg/tablet	502.59mg/tablet	100.518±0.689%

**Average of 6 determinations; SD is standard deviation.*

TABLE 5: SPECIFICITY STUDY FOR CAPECITABINE

Name of the solution	Retention time, (t_R)min.
Mobile phase	No peaks
Placebo	No peaks
Capecitabine, 10 µg/mL	4.123 min.

TABLE 6: RESULTS OF PRECISION STUDY (INTRA-DAY) FOR CAPECITABINE

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD#
Capecitabine	10	1	1084.86	0.6929
		2	1082.52	
		3	1094.92	
		4	1079.643	
		5	1080.405	
		6	1096.864	

#Acceptance criteria < 2.0.

TABLE 7: RESULTS OF PRECISION STUDY (INTER-DAY) FOR CAPECITABINE

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD#
Capecitabine	10	1	1082.31	1.0206
		2	1097.408	
		3	1099.086	
		4	1106.074	
		5	1076.819	
		6	1088.227	

#Acceptance criteria < 2.0.

TABLE 8: RECOVERY DATA OF THE PROPOSED RP-HPLC METHOD FOR CAPECITABINE

Recovery level	Amount added (mg)	Total amount (mg)	Amount found (mg)	Amount recovered (mg)	% recovery	Mean % Recovery ± SD	%RSD#
80%	79.82	179.82	179.71	79.71	99.86	99.92±0.11	0.1123
	79.76	179.76	179.64	79.64	99.84		
	79.92	179.92	179.96	79.96	100.05		
100%	99.91	199.91	199.82	99.82	99.90	99.90±0.02	0.0200
	99.87	199.87	199.76	99.76	99.88		
	99.89	199.89	199.82	99.82	99.92		
120%	119.84	219.84	219.75	119.75	99.92	99.98±0.10	0.1059
	119.90	219.90	219.81	119.81	99.92		
	119.83	219.83	219.96	119.96	100.10		

#acceptance criteria < 2.0.

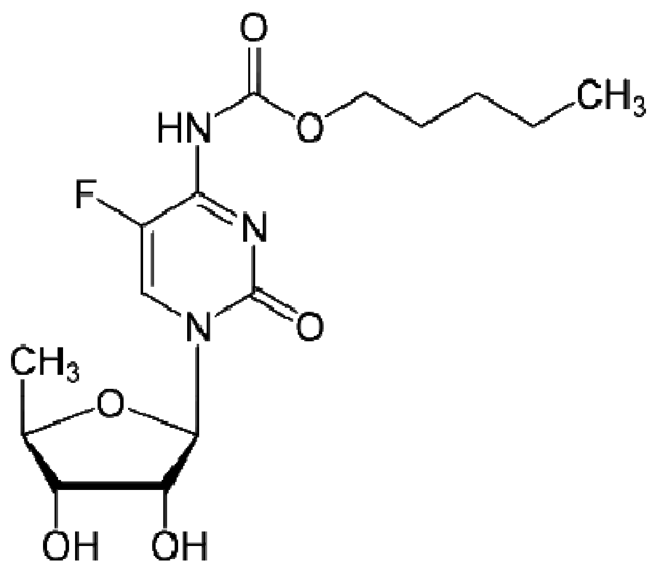


Fig. 1: Structure of Capecitabine

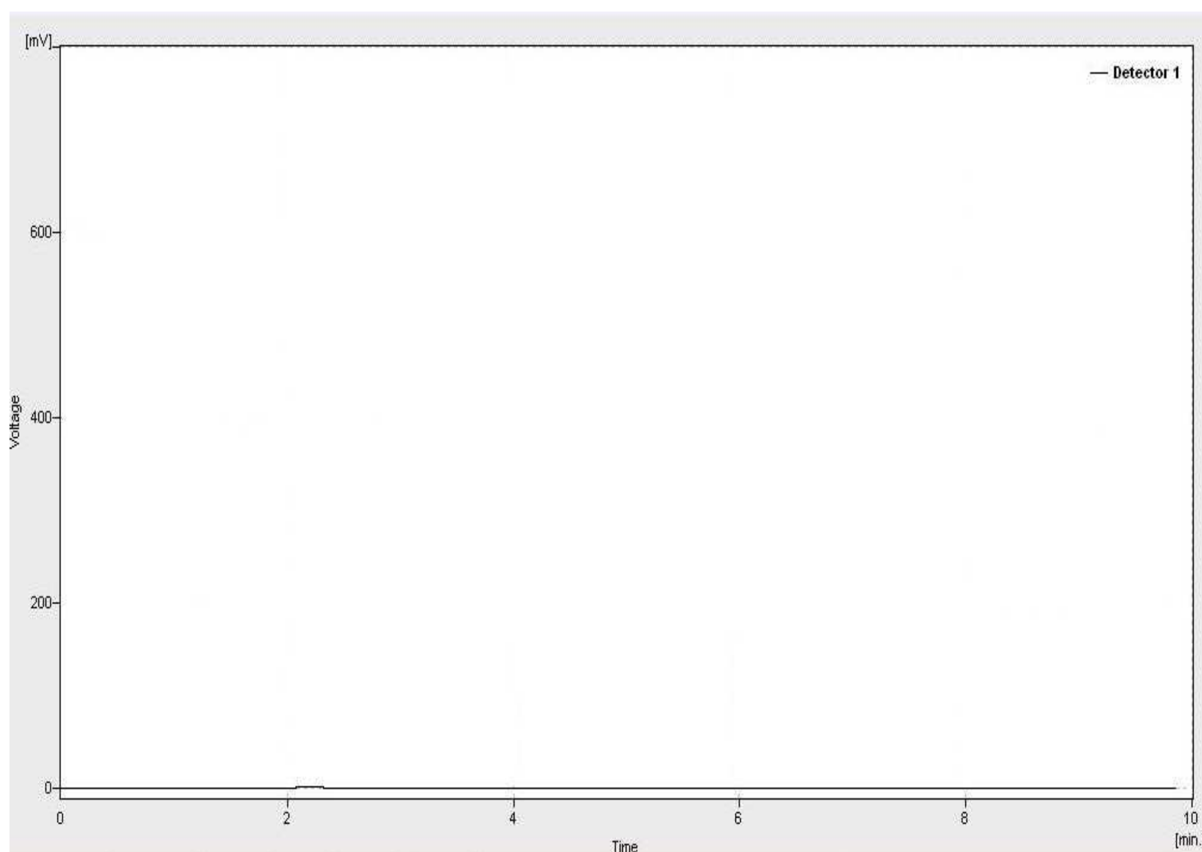


Fig 2: Chromatogram of placebo

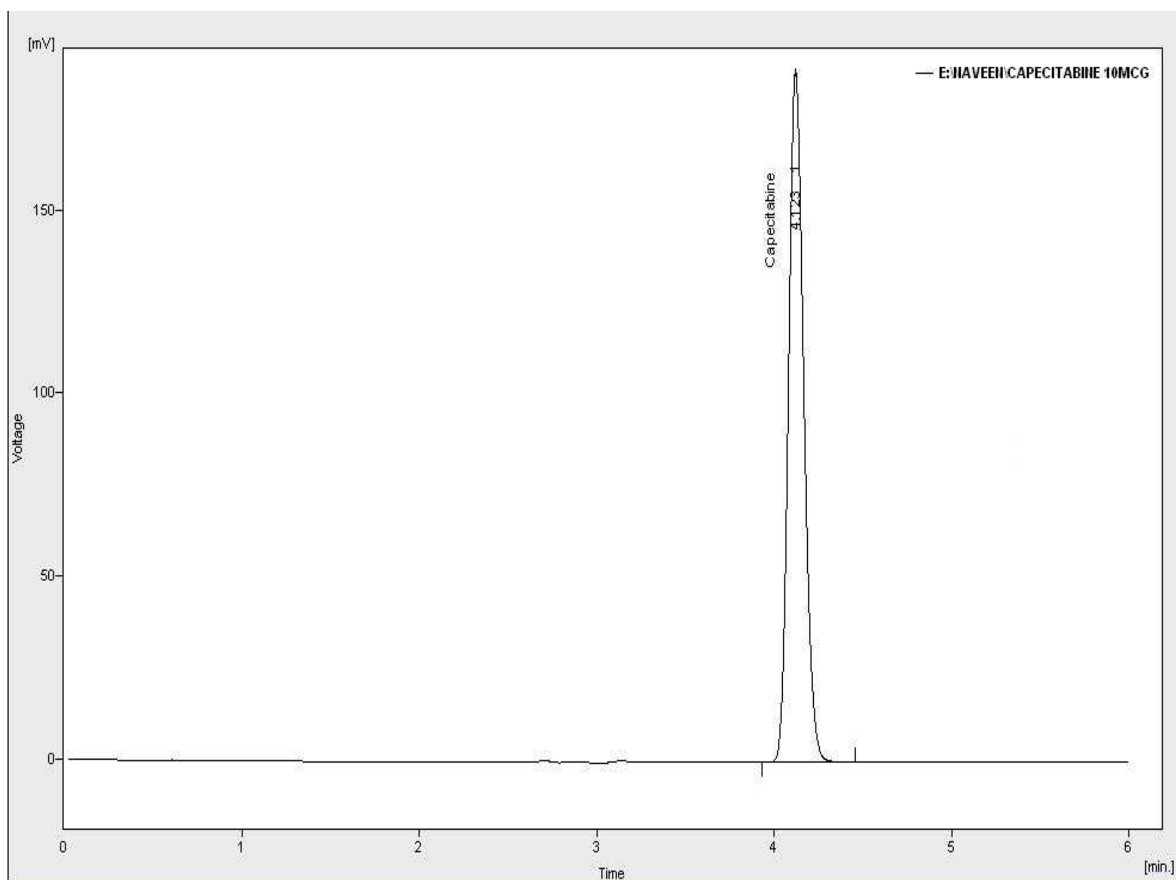


Fig. 3: A typical chromatogram of Capecitabine standard

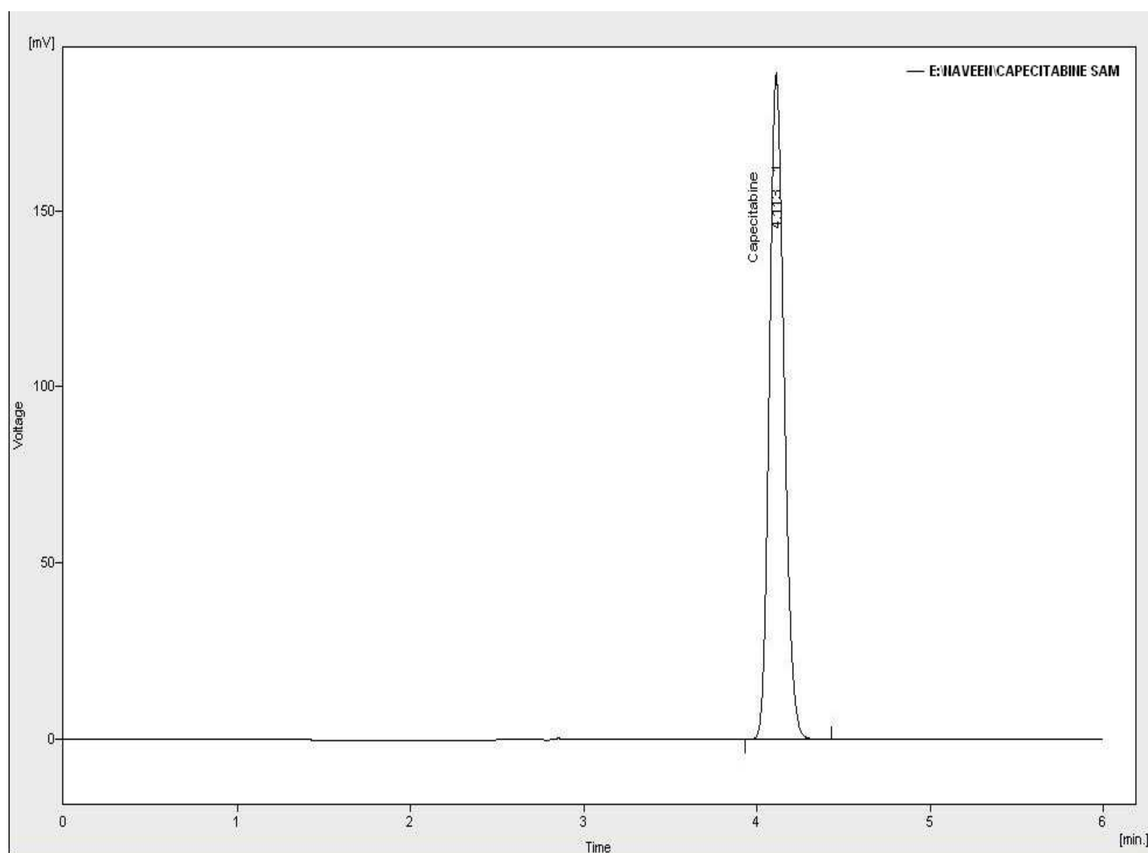


Fig. 4: Chromatogram of market formulation (CAPIIBINE 500 mg tablets) of Capecitabine

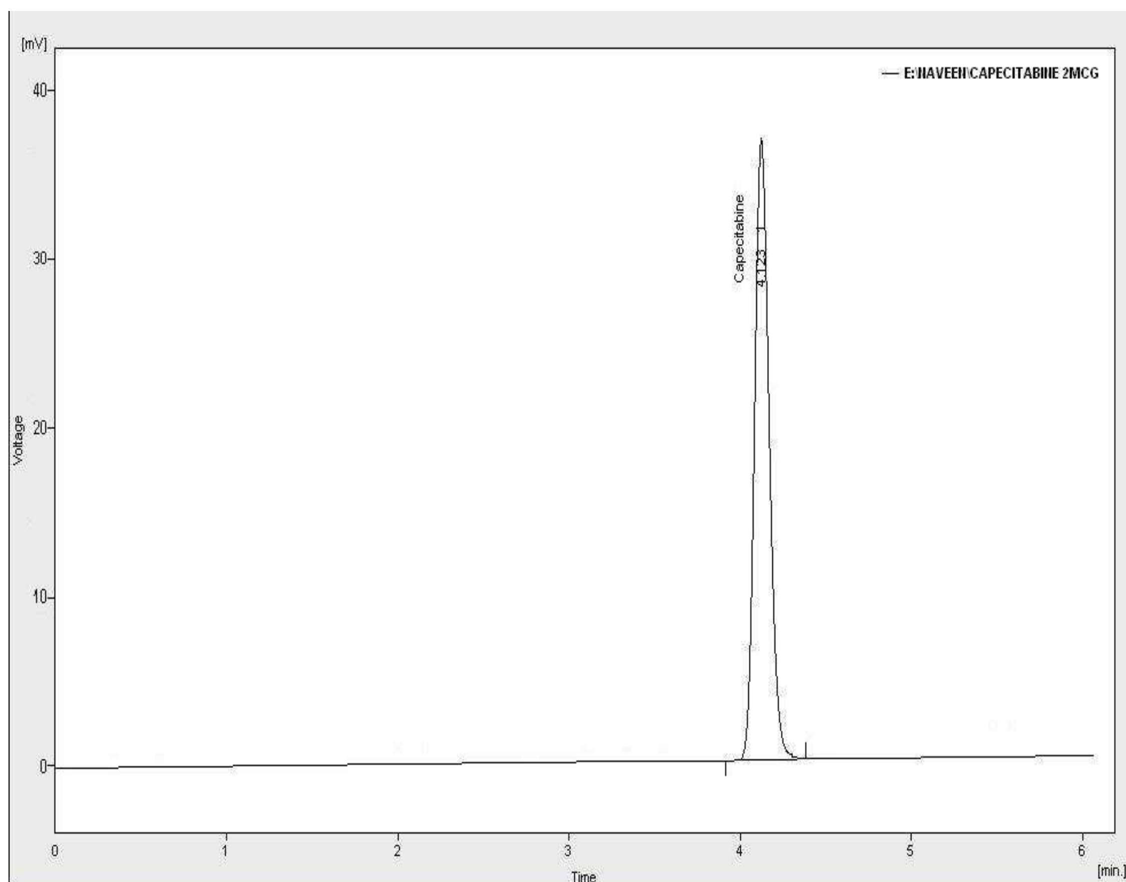


Fig. 5: Standard chromatogram of Capecitabine (2 µg/ml)

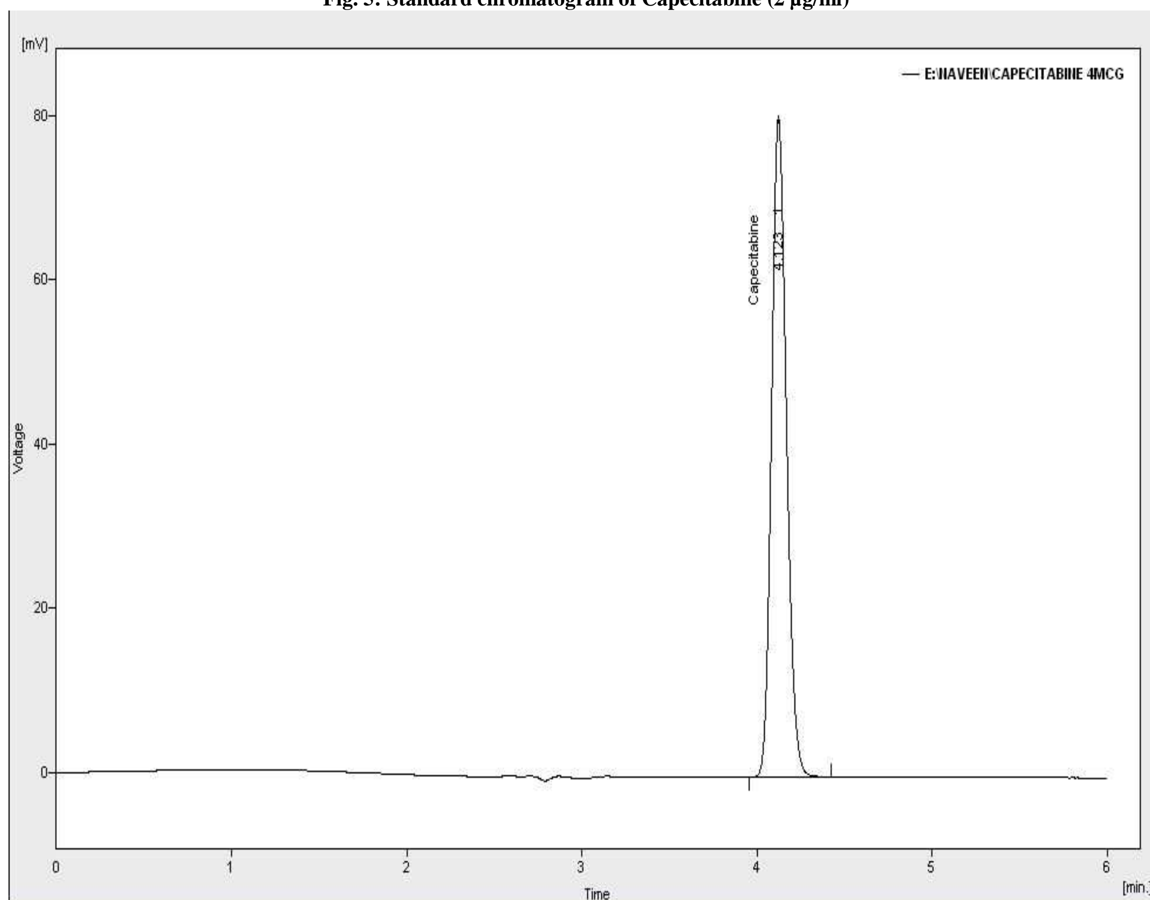


Fig. 6: Standard chromatogram of Capecitabine (4µg/ml)

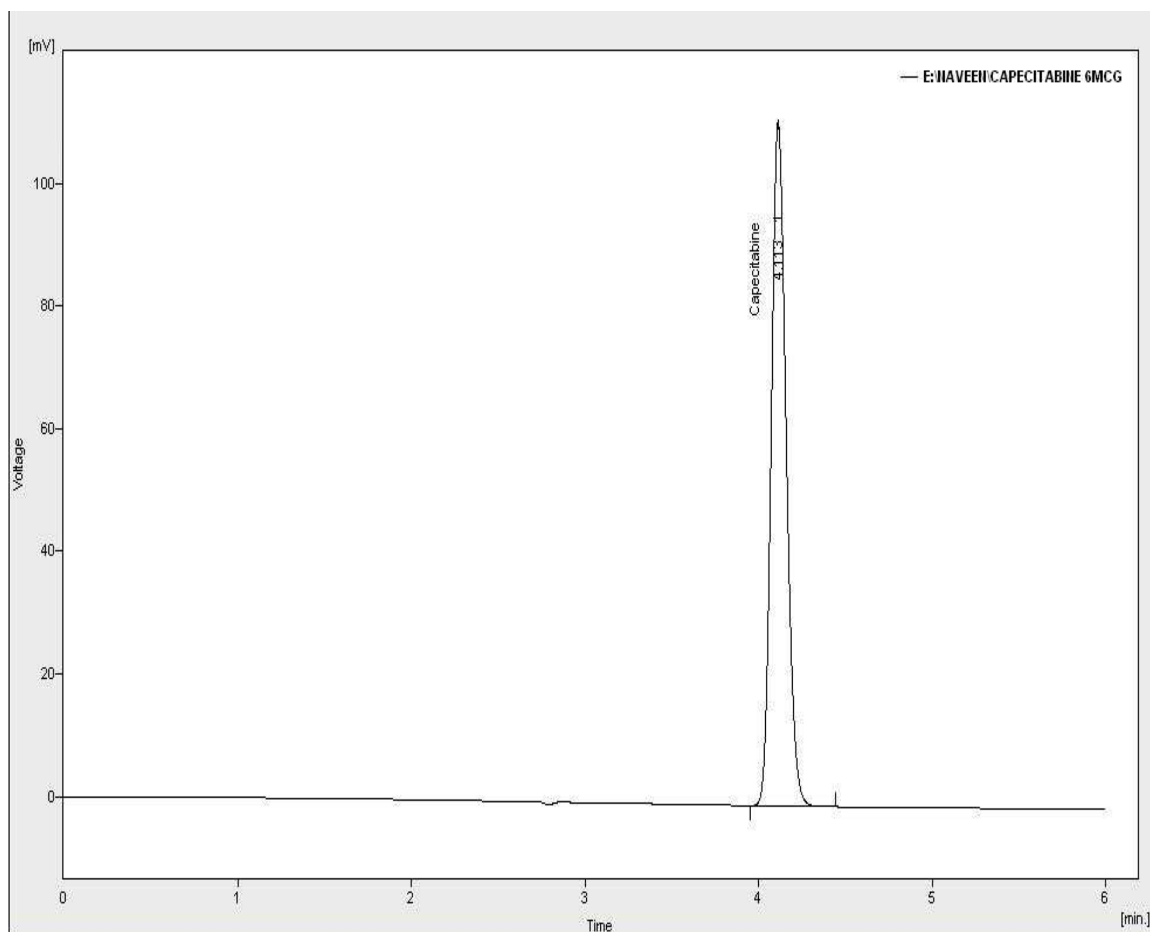


Fig. 7: Standard chromatogram of Capecitabine (6 µg/ml)

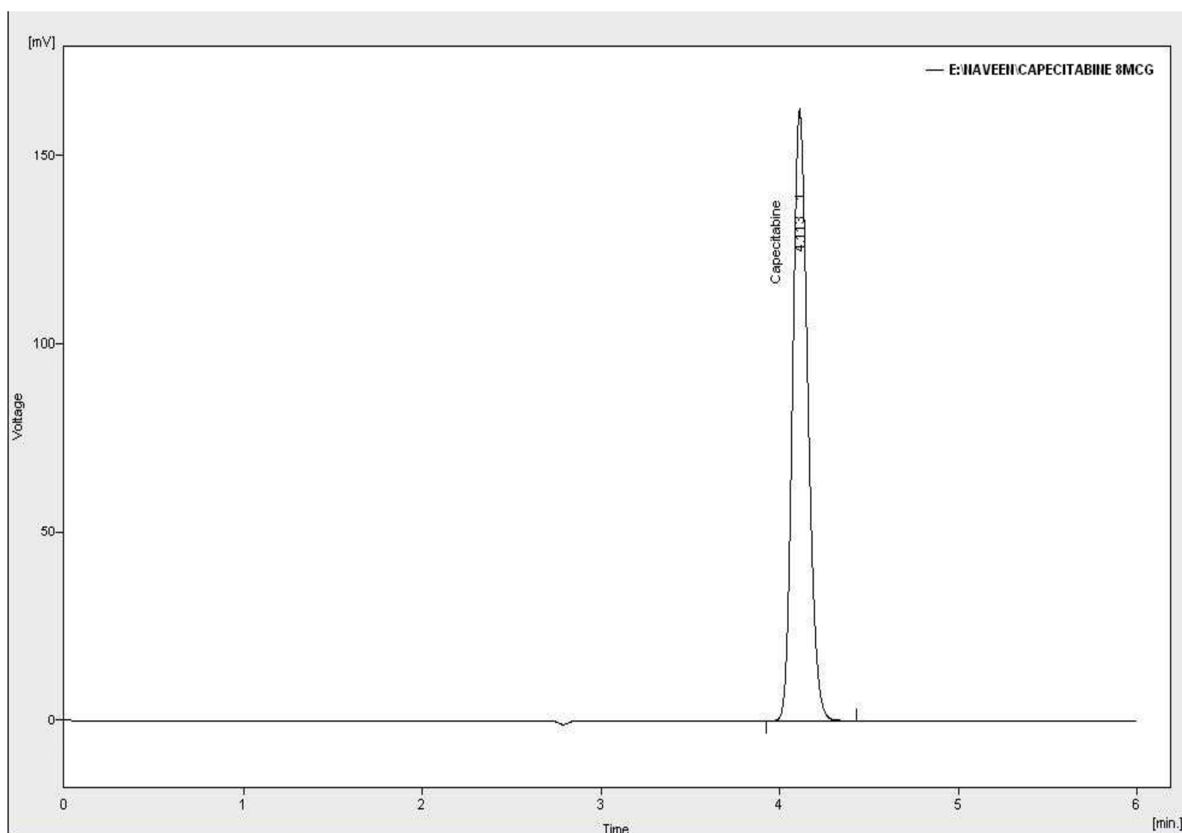


Fig. 8: Standard chromatogram of Capecitabine (8 µg/ml)

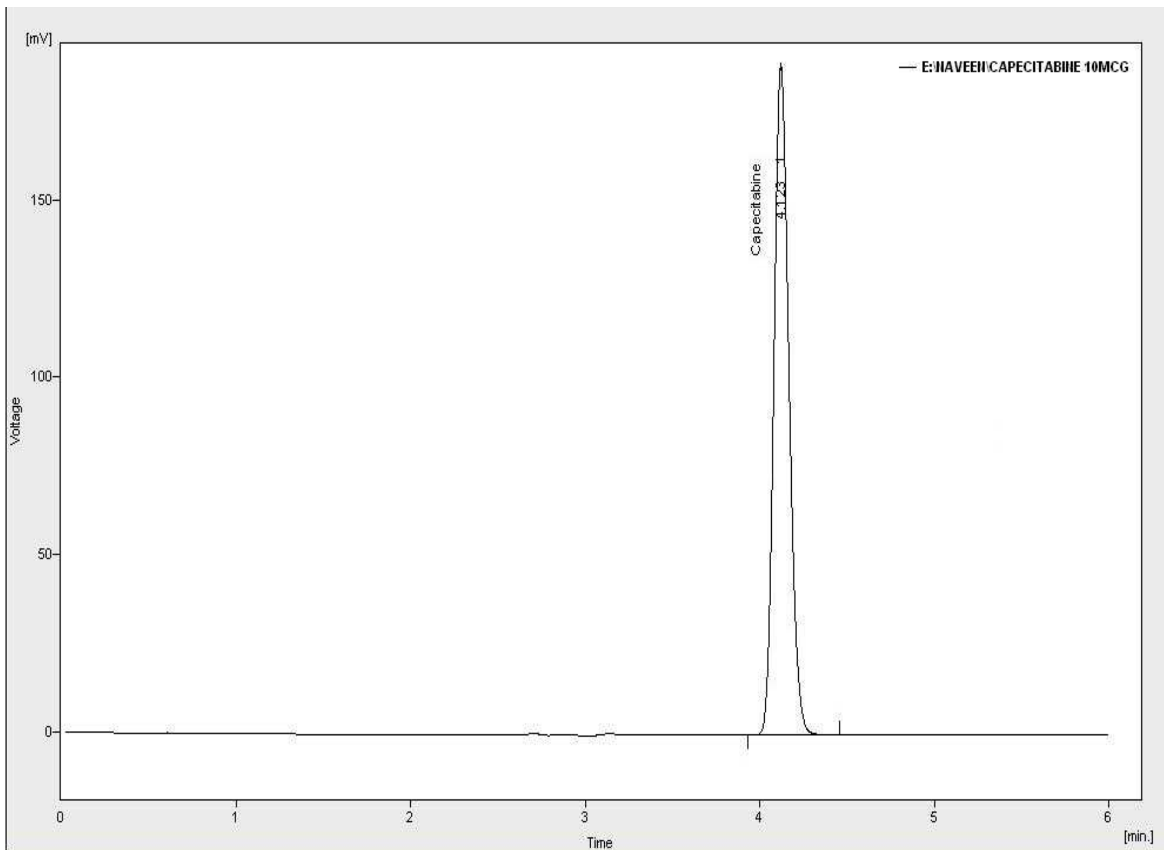


Fig. 9: Standard chromatogram of Capecitabine (10 µg/ml)

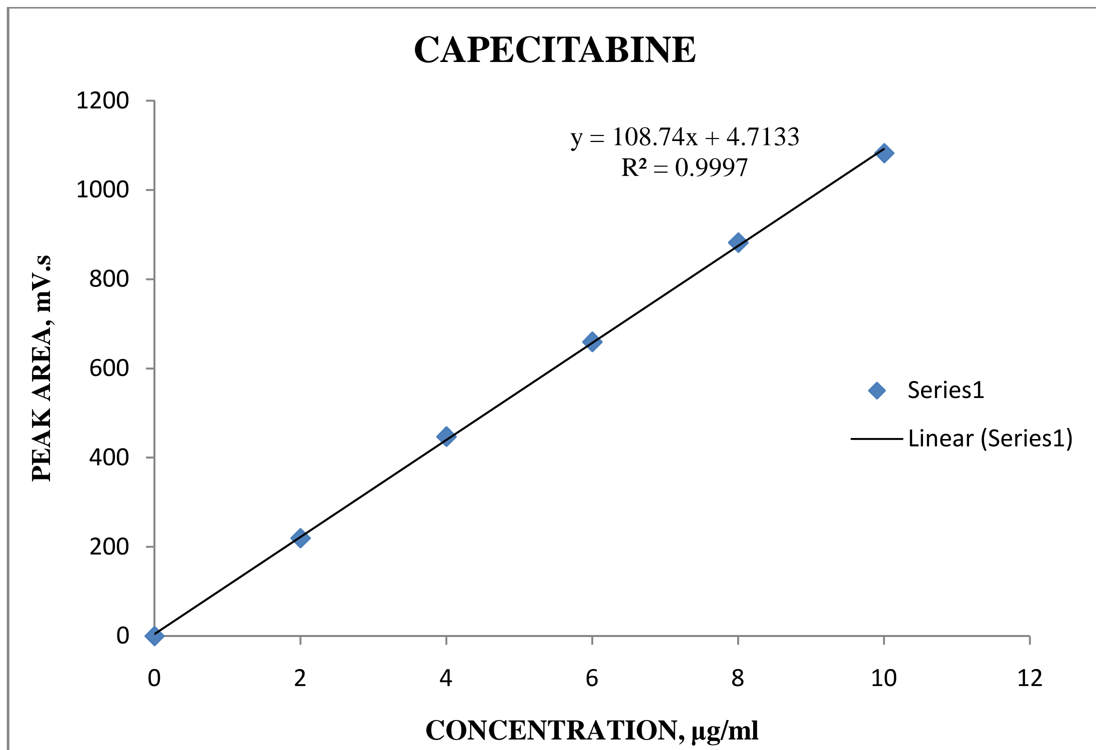


Fig. 10: Calibration plot of Capecitabine

TABLE 9: ROBUSTNESS RESULTS OF CAPECITABINE

S. no	Parameter[a]	Optimized	Used	Retention time (t_R), min	Plate count[$]	Peak asymmetry[#]	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	4.462	10426	1.076	*Robust
			1.2 mL/min	3.886	9724	1.066	*Robust
2.	Detection wavelength (±5nm)	245 nm	240nm	4.123	10080	1.069	Robust
			250nm	4.113	10032	1.066	Robust
3.	Mobile phase composition (±5 %)	50:50, v/v	55:45, v/v	4.354	10360	1.072	*Robust
			45:55, v/v	3.916	9856	1.070	*Robust

Acceptance criteria (Limits):
[#]Peak Asymmetry < 1.5, [$] Plate count> 3000
**Significant change in Retention time*

TABLE 10: LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

Limit of Detection(LOD)	0.169247 µg/mL
Limit of Quantitation(LOQ)	0.512872 µg/mL

CONCLUSION

A New validated RP-HPLC method has been developed for the quantitative determination of CAP in bulk and pharmaceutical tablet dosage forms. Statistical analysis of the results shows that the proposed method had good precision and accuracy. The method was completely validated shows satisfactory results for all the method validation parameters tested and method was free from interference of the other active ingredients and additives used in the formulation. The drug solutions employed in the study were stable upto 48 hours. The tailing factor, numbers of theoretical plates are within the acceptable limits. In fact, results of the study indicate that the developed method was found to be simple, reliable, accurate, linear, sensitive, economical, and reproducible and have short run time which makes the method rapid. Hence it can be concluded that this method may be employed for the routine quality control analysis of CAP in active pharmaceutical ingredient (API) and pharmaceutical capsule preparations.

Acknowledgements
The authors would like to thank Hetero Labs for providing the samples of Capecitabine. We are highly grateful to Dr.L.Rathaiah, Honorable Chairman, Vignan group of institutions, Vadlamudi, Guntur, for providing the necessary facilities to carry out this research work.

REFERENCES

[1] Esmaeilzadeh Mahdi and KazemzadehFariba, *Annals of Biological Research*, **2012**, 3 (1):622-627.
[2] Laila Fatima Ali Asghar, Chetan B. Chure, SajeevChandran, *Der Pharmacia Lettre*, **2011**, 3(1): 425-441.
[3] Sanjay Kumar Lanjhiyana, Jawahar Singh Dang, DeapriyaGarabadu, SweetyLanjhiyana, Priyanka Sharma Garabadu, Amitabh Arya, *Der Pharmacia Lettre*, **2010**, 2(4): 255-273.
[4] Zufıa, L., A. Aldaz, and J. Giraldez, *Journal of chromatography B*, **2004**, 809(1):51-58.
[5] Dhananjeyan MR, Liu J, Bykowski C, Trendel JA, Sarver JG, Ando H, Erhardt PW, *Journal of chromatography A*, **2007**, 1138(1-2):101-108.
[6] Jayaseelan S, Bajivali SK, Ramesh U, Sekar V, Perumal P, *Int J Chem tech Res*, **2010**, 2(4):2086-2090.
[7] Farkouh A, Ettlinger D, Schueller J, Georgopoulos A, Scheithauer W, Czejka M, *Anticancer research*, **2010**, 30(12):5207-5211.
[8] Rajesh V, Anupama B, Jagathi V, Praveen PS, *Journal of Chemistry*, **2011**, 8(3):1212-1217.
[9] Prakash KV, Rao JV, Raju NA, (2008). *Oriental Journal of Chemistry*, **2008**, 24(1):335-338.
[10] Kumar KR, Rao CMP, Rao CB, Chandra KB,*Int J Chem Tech Res*, **2010**, 2(1):307-311.
[11] Ye M, Fu Q, Zhu Z, *Chinese J of Hospital Pharm*, **2004**, 24(11):678-680.
[12] Qureshi MS, Munir A, Ahmad M, Mahmood A,*African J of Pharmacy and Pharmacology*, **2011**, 5(7):915-922.
[13] Devanaboyina N, Kishore YS, Pushpalatha P, Mamatha N, Venkatesh P, *Int J Sci Inventions Today*, **2013**, 2(1), 21-30.
[14] Di Paolo A, Danesi R, Ciofi L, Vannozzi F, Bocci G, Lastella M, Del Tacca M, *Therapeutic drug monitoring*, **2005**, 27(3):362-368.
[15] Guichard SM., Iain Mayer, Duncan IJ, *Journal of chromatography B*, **2005**, 826(1):232-237.
[16] Siethoff C, Orth M, Ortling A, Brendel E, Wagner-Redeker W, *Journal of mass spectrometry*, **2004**, 39(8):884-889.
[17] Licea-Perez, Hermes, Sherry Wang, Chester Bowen, *Journal of chromatography B,* **2009**, 877(11):1040-1046.
[18] Vainchtein LD, Rosing H, Schellens JH, Beijnen JH, *Biomedical Chromatography*, **2010**, 24(4):374-386.

[19] Montange D, Bérard M, Demarchi M, Muret P, Piédoux S, Kantelip JP, Royer B, *Journal of Mass Spectrometry*, **2010**, 45(6):670-677.
[20] Salvador A, Millerioux L, Renou A, *Chromatographia*, **2006**, 63(11-12):609-615.
[21] Švobaitė R, Solassol I, Pinguet F, Mazard T, Ivanauskas L, Ychou M, Bressolle FMM, *Journal of Liquid Chromatography & Related Technologies*, **2010**, 33(19):1705-1719.
[22] Deenen MJ, Rosing H, Hillebrand MJ, Schellens JH, Beijnen JH, *Journal of Chromatography B*, **2012**, 913–914:30–40.
[23] ICH, Q2B, Harmonized Tripartite Guideline, Validation of Analytical Procedure: Methodology, IFPMA, in: Proceedings of the International Conference on Harmonization, Geneva, March **1996**.