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BIOANALYTICAL METHOD DEVELOMENT AND VALIDATION OF CAPECITABINE IN PLASMA BY RP-HPLC

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ABSTRACT

Bio analysis employed for the quantitative determination of drugs and their metabolites in biological fluids. Studies involving measurement of the drugs or metabolites in the biological fluids such as blood plasma, serum, urine, bile, CSF, etc. Capecitabine is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a prodrug, that is enzymatically converted to fluorouracil (antimetabolite) in the tumor, where it inhibits DNA synthesis and slows growth of tumor tissue. An Extensive literature survey revealed that there are bioanalytical methods for estimation of Capecitabine in plasma but comparatively the retention times were more. Hence an attempt was made in order to develop an bioanalytical method for estimation of Capacitabine in Plasma by RP-HPLC with less retention times using structurally similar 5-Bromo Uracil as Internal Standard. The method was developed using Mixed Phosphate buffer and Acetonitrile (P^H-5) in 55:45 ratio as mobile phase using Chomosil C_{18} column with flowrate of 1ml/min.The retention times were 2.8ins for plasma,3.85mins for Internal Standard and 5.02mins for Capecitabine.The proposed method was Simple, Accurate, Precise and Robust. The regression coefficient obtained was 0.9993.Hence the method can be used for estimation of Capecitabine in biological fluids.

INTRODUCTION:

Bio analysis employed for the quantitative determination of drugs and their metabolites in biological fluids. Studies involving measurement of the drugs or metabolites in the biological fluids such as blood plasma, serum, urine, bile, CSF, etc. requires the selective, sensitive, well characterized bio analytical method to yield reliable results which can be satisfactorily interpreted as well as to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte[1]. It is therefore paramount that the applied bioanalytical methods used are well characterised, fully validated and documented to a satisfactory standard in

order to yield reliable results. The validation of bioanalytical methods and the analysis of study samples for clinical trials in humans should be performed following the principles of Good Clinical Practice (GCP).[2] Capecitabine is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a prodrug, that is enzymatically converted to fluorouracil (antimetabolite) in the tumor, where it inhibits DNA synthesis and slows growth of tumor tissue.[3] Capecitabine is a prodrug that is selectively tumour-activated to its cytotoxic moiety, fluorouracil, by thymidine phosphorylase, an enzyme found in higher concentrations in many tumors compared to normal tissues or plasma.[4] Extensive literature survey reveals that there are few methods available to bioanalyse the drug in human or rat plasma by Rp-hplc with uv detection[5] but most of the methods were reported either without internal standard or with internal standard which were of higher cost[6-12],by LC-MS for clinical and preclinical sstudies[13],by LC-MS/MS method in human plasma[14], by LC-MS and CE-MS method for drug monitoring[15],The reported methods revealed that the retention time for capacitabine was also more. Hence an attempt is made in order to develop cost effective analytical method to bioanalyse capacitabine in human plasma using 5-Bromo Uracil as an internal standard.

2. EXPERIMENTAL SECTION:

In the present study, A economical, Simple and Accurate method was developed to estimate Capacetabine in human plasma.Solubility studies were performed using different solvents and the results are in Table No-1

2.1 SELECTION OF PRECIPITATING AGENT:

A Precipitating agent is selected based on solubility of drug in the precipitating agent and protein precipitation property of the agent.Since Capacetabine is freely soluble in Acetonitrile and Acetonitrile having good precipitation property, in the proposed method Acetonitrile has been used as precipitating agent.

2.2 SELECTION OF MOBILE PHASE:

Initially to estimate Capacitabine, numbers of mobile phase in different ratio were tried taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was mixed phosphate buffer and Acetonitrile in 55:45 ratio and the results are represented in Table No-2

2.3 SELECTION OF INTERNAL STANDARD:

The main aim of the work was to develop a cost effective and simple method. Hence structurally similar (5-Bromo Uracil) internal standard has been selected by which selection of mobile phase was made easier and which is of low cost too.

2.4 PREPARATION OF MOBILE PHASE:

Weigh accuretly about 1.625 gm of potassium dihydrogen phosphate and 0.3 gms of dipotassium hydrogen phosphate were dissolved in 1000ml of mineralized water.A mixture of 55 volumes of mixed phosphate buffer, 45 volumes of acetonitrile was prepared Before delivering into the system, mobbile phase was filtered through 0.45µm polytetrafuloroethylene(PTFE) filter and sonicated for 5 min .Through out the analysis,the temperature maintained was isocratic.The flow rate maintained for the proposed analysis was 1ml/min.The chromatograms recorded were at 293 nm using photo diode array as detector.

2.5 OPTIMISED CHROMATOGRAPHIC CONDITIONS:

After performing the preliminary studies and selecting the mobile phase, stationary phase and internal standard,the optimized chromatographic conditions are represented in Table No-3

2.6 PREPARATION OF WORKING STANDARDS:

Preparation of stock solutions: 10mg of CAP and IS were dissolved in 10ml of mixed phosphate buffer which gives 1000µg/ml.

Preparation of working solutions:

1ml of stock solution was added to 10ml of blank plasma and 1ml of stock solution of Capecitabine was added to 100ml of mixed phosphate buffer which gives 100µg/ml concentration.

Preparation of QC samples:

To eshtablish linearity of analytical method,a series of dilutions ranging from 50 to 150ng were prepared by taking 0.5ml,0.7ml,0.9ml,1.3ml and 1.5ml from stock solution separately in 100ml volumetric flasks and was made to 100ml with mobile phase. This gives solution of 50ng, 70ng, 90ng, 130ng and 150ng/ml of drug.All the solutions were vortexed for 1min, then 0.5ml aliquots were transferred to 1.5ml microsringe tubes and stored at -20ºC until used. A caliberation curve was plotted between mean peak area and respective concentrations and is shown in Fig -02

Preparation of sample:

0.5ml of plasma from central auricular artery of rabbit was collected, calibration standards were placed in 1.5ml microcentrifuge tubes.0.2ml of ISworking standards were added in each tube.The solutions were vortexed for 30s, sonicated for 1min and centrifuged at 3000rpm for 30mins.100µl of ultrafiltrate was injected into chromatographic systems using an autosampler.

2.7 RP-HPLC DATA OF CAPACITABINE:

A sample chromatogram of Capacitabine in plasma is shown in Fig-01 and Retention time for capacitabine was found to be 5.047mins,for internal standard 3.510mins and for plasma 2.8mins.

3. VALIDATION OF PROPOSED METHOD:

In order to improve specificity and to minimize the interferences due to plasma and solvent system that may occur at lower wavelengths, we performed analysis at 293nm. Under described conditions, Capacitabine and IS were resolved with resolution factor greater than 3.0 with runtime 8mins.

3.1 LINEARITY:

Linear regression analysis was used to calculate slope, intercept and correlation coefficient (r^2).The linearity over the range of was found quite satisfactory and was reproducible over the time. From the mean of AUC observed and response concentration values, the response ratio was calculated by dividing AUC with respective concentrations. The Standard deviation and RSD were within the limits. The results are tabulated in Table No-4

3.2 ACCURACY AND PRECISION:

Interday and Intraday recision and accuracy of assay was determined at concentrations of 50 to 150ng/ml over three days. Intraday precision was less than 2.75% and interday was less than 4.70%.The intraday and interday accuracy were in the range of 99-107% and the results are represented in TableNo-5

3.3 REPRODUCIBILITY:

Reproducibility was performed by chemical to chemical(use of rankem in place of merck) for five calibration standards and %RSD was less than two and the results are tabulated in TableNo-6

3.4 ROBUSTNESS:

As per ICH guidelines,small variations were made to check the method capacity to remain unchanged.No significant chage were observed in tailing factor and retention times with small changes in flowrate and wavelength.Results are tabulated in TableNo-7

3.5 LOD and LOQ:

LOD and LOQ of the method was calculated based on standard deviation of response and slope of linearity curve and the results are in Table-8.

Table 1: Solubility of drug in different solvents:

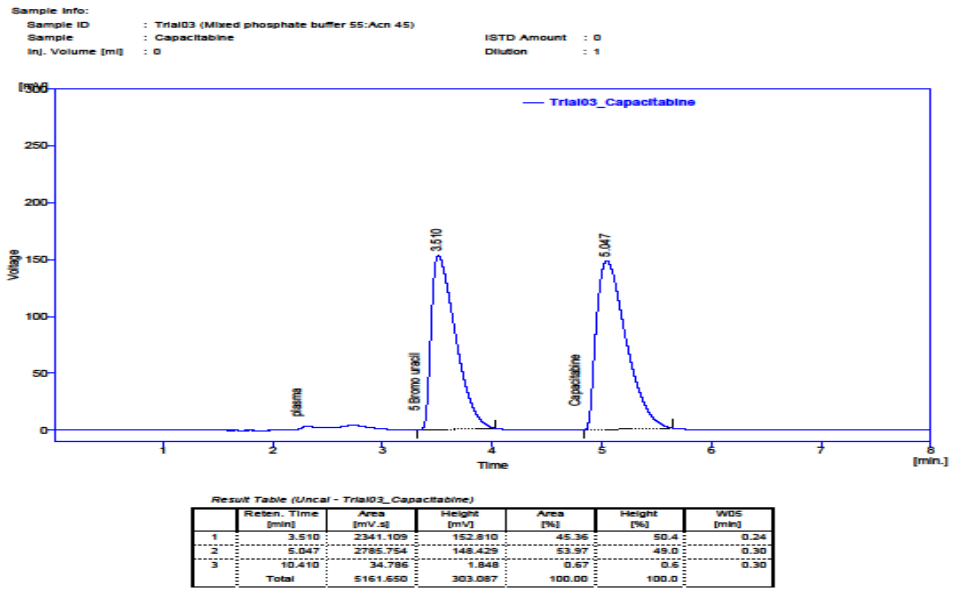
SOLVENT	SOLUBILITY
Water	Freely soluble
0.1N HCL	Insoluble
0.1N NAOH	Insoluble
Methanol	Freely soluble
Acetonitrile	soluble
Acetate Buffer	soluble
Phosphate buffer	soluble

Table 2: Mobile phase selection

MOBILE PHASE	RATIO	FLOWRATE	RESPONSE
Methanol:water	50:50	1ml/min	peak not found
Acetonitrile:water	50:50	1ml/min	peak not found
Methanol: Acetonitrile	50:50	1ml/min	peak not found
KH2PO4:Acetoitrile(PH-3.5)	20:80 v/v	1ml/min	Poor resolution
KH2PO4:Acetonitrile(PH-4)	30:70 v/v	1ml/min	Poor resolution
KH2PO4+K2HPO4:Acetonitrile(PH-5)	55:45 v/v	Iml /min	Satisfactory result

Table 3: Optimized chromatographic conditions:

Column	Chromosil C18
Mobile Phase	Mixed Phosphate Buffer:Acetonitrile(55:45)
Flow Rate	1ml/min
Tempearture	25 degree
Sample Size	100µl
Detection Wavelength	293nm
Retention Time	2.8mins for plama,3.85mins for IS&5.02mins for Capacitabine
Internal Standard	5-Bromo Uracil



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	WDS [min]
1	3.510	2341.109	152.810	45.36	50.4	0.24
2	5.047	2785.754	148.429	53.97	49.0	0.30
3	10.410	34.786	1.848	0.67	0.6	0.30
	Total	5161.650	303.087	100.00	100.0	

Fig-1: RP-HPLC Chromatogram of plasma, 5-bromo uracil (is) and capacitabine

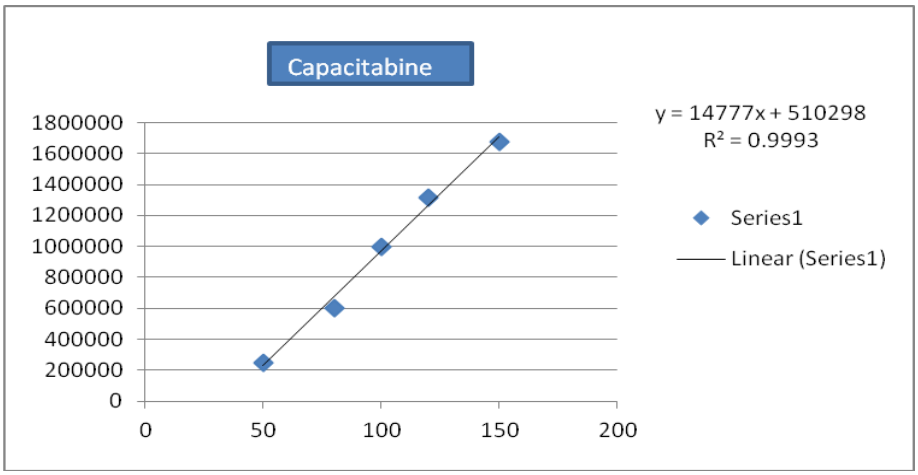


Fig 2: Linearity curve of capacitabine

Table 4: Linearity data

Replicates	Concentration in ng	Mean AUC	Response Ratio
1	50	1886.042	37.7208
2	70	2274.595	32.4942
3	90	2785.784	30.9531
4	130	3212.346	24.7103
5	150	3765.963	25.1064

Table 5: Accuracy and precision data: intra-day (n=10)

Nominal conc(ng/ml)	Conc found(ng/ml)	precision	Accuracy
50	50.03	1.45	100.06
70	69.54	2.23	99.34
90	90.29	2.75	100.32
130	131.81	2.64	101.39
150	152.12	1.58	101.41

Interday (n=20)

Nominal conc(ng/ml)	Conc found(ng/ml)	precision	Accuracy
50	50.07	4.69	100.14
70	69.75	3.32	99.64
90	90.23	2.94	100.25
130	131.08	2.24	100.83
150	152.59	2.36	101.72

Table 6: Reproducibility of method:

Replicate	Concentration found				
	50	70	90	130	150
R-1	49.1	69.2	89.4	128.9	149.6
R-2	48.7	69.4	87.9	129.3	148.9
R-3	49.4	69.3	88.7	128.3	147.8
R-4	48.5	66.7	89.1	128.9	146.9
R-5	49.7	68.6	88.9	129.9	147.9
Mean	49.08	68.64	88.80	129.06	148.22
%RSD	0.052	0.117	0.058	0.060	0.107

Table 7: Results of robustness

Parameter	Capacitabine	Internal Standard	
	Retention time(min)		Tailing factor
Flow Rate			
0.8 ml/min	5.012	3.512	1.452
1.2 ml/min	5.044	3.514	1.333
Wavelength			
292nm	5.056	3.515	1.400
294nm	5.002	3.511	1.400

Table 8: LOD and LOQ of Capecitabine

Drug	LOD(ng/ml)±SD	LOQ(ng/ml)±SD
CAP	5.3±0.005	6.1±0.003

CONCLUSION:

An attempt was made to develop cost effective bio analytical method for estimation of capacitabine in rabbit plasma with less retention time using structurally similar Internal Standard 5-Bromo Uracil. In summary the proposed method is Rapid, Sesitive, Specific, Accurate, Precise and Reproducible. The chromatogram developed has well resolved peak without any interferences and hence the method can be used for estimation of Capecitabine in biological fluids.

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