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Original Article

BIO ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF CAPECITABINE AND DOCETAXEL AND ITS APPLICATION TO PHARMACOKINETIC STUDIES USING LC-MS/MS

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ABSTRACT

Objective: An easy, quick, precise, active and reproducible Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) technique was developed for the bio-analytical method of Capecitabine and Docetaxel using D_9-Capecitabine and D_9-Docetaxel as Internal Standards (IS).

Methods: This article summarizes the recent progress on bioanalytical LC-MS/MS methods using Symmetry C_{18} column (150x4.6 mm, 3.5µ) and an organic mobile phase of 0.1% formic acid and Acetonitrile in 80:20 v/v.

Results: Analysis was carried out within 5 min over a good linear concentration range from 37.5ng/ml to 300ng/ml (r^2= 0.9999±0.008) for Capecitabine and 10ng/ml to 80ng/ml (r^2=0.9993±0.005) for Docetaxel. Accuracy, precision, recovery, matrix effect and stability results were found to be within suitable limits.

Conclusion: The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with United States Food and Drug Administration (USFDA) guidelines and applied effectively for the investigation of pharmacokinetic studies in rats.

Keywords: Capecitabine, Docetaxel, LC-MS/MS, USFDA guidelines, Rat plasma

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INTRODUCTION

Capecitabine is an orally administered chemotherapeutic agent used in the treatment of metastatic breast [1, 2] and colorectal cancers [3, 4]. Capecitabine is a prodrug that is enzymatically converted to fluorouracil (antimetabolite [5, 6]) in the tumor [7], where it inhibits DNA (Deoxyribo Nucleic Acid) synthesis [8, 9] and slows growth of tumor tissue. Common side effects include abdominal pain [10], vomiting, diarrhea [11, 12], weakness, and rashes. Other severe side effects include blood clotting problems [13, 14], allergic reactions [15], heart problems such as cardiomyopathy [16, 17], and low blood cell counts. Use during pregnancy may result in harm to the fetus. Capecitabine, inside the body, is converted to 5-fluorouracil (5-FU) through which it acts. It belongs to the class of medications known as fluoropyrimidines, which also includes 5-FU and tegafur [18].

Docetaxel (DTX or DXL), sold under the brand name Taxotere among others, is a chemotherapy medication used to treat a number of types of cancer. This includes breast cancer, head and neck cancer [19, 20], stomach cancer [21], prostate cancer and non-small-cell lung cancer [22, 23].

Common side effects include hair loss, cytopenia (low blood cell counts), numbness, shortness of breath [24], nausea, vomiting, and muscle pains. Other severe side effects include allergic reactions and future cancers. Docetaxel-induced pneumotoxicity is also a well recognized adverse effect which has to be identified timely and treated after with holding the drug. Side effects are more common in people with liver problems [25]. Use during pregnancy may harm the baby. Docetaxel is in the taxane family of medications. It works by disrupting the normal function of microtubules [26, 27] and thereby stopping cell division.

Till date, no method is available for bio-analysis of Capecitabine and Docetaxel in any type of biological matrix. The aim of the study was to develop a new rapid and sensitive LC-MS/MS method for the simultaneous estimation of Capecitabine and Docetaxel in rat plasma using D_9-Capecitabine and D_9-Docetaxel as internal standards.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and Formic acid, water (HPLC grade) were purchased from Merck (India) Ltd, Worli, Mumbai, India. All APIs of Capecitabine and Docetaxel as reference standards were procured from Zydus Cadila Healthcare Ltd, Ahmedabad.

Equipment

An HPLC (High-Performance Liquid Chromatography) system (Waters Alliance e2695 model) connected with the mass spectrometer QTRAP 5500 triple quadrupole instrument was used. By the ABSCIEX software operation was performed [28-30].

Pharmacokinetic study

Selection of animals

In vivo pharmacokinetic studies, 6 healthy white New Zealand rats (app. 250 g) were obtained from Biological E Limited, Hyderabad, India. The protocol of animal study was approved by the institute of animal ethics committee (Reg. No: 1074/PO/Re/S/20/CPCSEA).

Chromatographic conditions

Chromatographic separation, using Symmetry C_{18} (150 x 4.6 mm, 3.5 micron) columns, was administered in isocratic mode at room temperature. A mobile phase mixture of 0.1 percent Formic acid and acetonitrile at 80:20 v/v with a flow of 1.0 ml/min was used. 10 µl was the injection rate and the run time was 5 min.

Preparation of standard and internal control samples

Preparation of capecitabine parent stock solution

Take 6 mg of the Capecitabine working standard into a 100 ml volumetric flask and 70 ml of diluents and sonicate for 10 min to dissolve the contents completely and makeup to the mark with diluent. Further dilution by taking 1 ml into 10 ml volumetric flask.

Preparation of docetaxel parent stock solution

Take 5 mg of the Docetaxel working standard into a 100 ml volumetric flask and 70 ml of diluents and sonicate for 10 min to dissolve the contents completely and makeup to the mark with diluent. Further dilution by taking 0.32 ml into 10 ml volumetric flask.

Preparation of standard stock solution

1 ml of Capecitabine parent stock solution and 1 ml of Docetaxel parent stock solution were taken into 10 ml volumetric flask and made up to the mark with diluents.

In the same way, internal standard stock solutions also.

Preparation of standard solution

For standard preparation 200 µl of plasma was taken and 300 µl of Acetonitrile (ACN) into a 2 ml centrifuge tube and 500 µl of standard stock solutions and 500 µl of IS and 500 µl of diluents were added and vortexed for 10 min. These samples were further subjected for centrifuge at 4000rpm for 20 min. Collect the solution and filter through 0.45µ nylon syringe filter and the clear solution was transferred into a vial and injected into a system.

Bio-analytical method validation

The method was validated [31-39] in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

Selectivity

By analyzing the six different rat's plasma samples and to check interference at the retention time, selectivity was conducted.

Matrix effect

By comparing the height area ratio from the six various drug free plasma samples for Capecitabine and Docetaxel to get a matrix effect. Experiments were performed at MQC levels in triplicate with six different plasma lots with the suitable precision of ≤ 15 %.

Precision and accuracy

It was determined by replicate analysis of internal control samples at a Lower Limit of Quality Control (LLOQC), Low Quality Control (LQC), Medium Quality Control (MQC), High Quality Control (HQC) levels. The % CV (Coefficient Variance) should be less than 15 % and accuracy should be within 15% except LLOQ where 20%.

Recovery

The analysis of six samples reproduced at each internal control concentration is by extracting the Capecitabine and Docetaxel. By comparing the height areas of extracted standards to the height areas of unextracted standards, recovery is evaluated [40].

Carryover

Carryover [41, 42] deals with the analyte retained by the chromatographic system during the matrix with an analyte concentration Upper Limit of Quality Control (ULOQC) and above the diluting this sample with blank matrix.

Dilution integrity

By spiking the matrix with an analyte concentration above the ULOQC and diluting this sample with a blank matrix, the dilution integrity [43] should be explained.

Stability

By comparing the act of stock solution stability [44] under the stability sample with the sample from the fresh stock sample preparation. Sample 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 a smaller than 15 % as per US FDA guidelines [45]. The perfectness of spiked rat plasma stored at room temperature was evaluated for 24 h. The stability of spiked rat plasma stored at RT in an auto sampler was evaluated for 24 h. The autosampler stability (LQC, MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8 °C 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 dry extract stability at room temperature after 12 h and 18 h at-20 °±3 °C the freeze-thaw stability was conducted by comparing the steadiness samples that had been frozen at-31 °C and thawed 3 times, with freshly spiked internal control samples. The short-term stability was conducted 7 d at 7 °C. For long-term stability evaluation the concentrations obtained after 24 h were compared with initial concentration.

Pharmacokinetic study

Before experimentation, all animals are starved overnight and had water ad-libitum. Topical anesthetic procedure was used. Pharmacokinetic evaluation was performed for Capecitabine and Docetaxel formulations. The samples were administered to each rat under fasting conditions. After oral administration of Capecitabine and Docetaxel, blood samples were collected from rat marginal ear vein using a 25-gauge, 5/8 in needle by clipping the marginal ear vein with a paper clip shown in fig. 1 with the volume of 0.3 ml at 0.5, 1, 1.5, 2, 2.5 and 3 h. The blood was collected in Eppendorf containing 10% EDTA (Ethylene Diamine Tetra Acetic acid) solution. Blood was centrifuged at 4000 rpm for 20 min at 2-8 °C temperature. The clear supernatant plasma was collected and stored at-30 °C till its analysis. The plasma samples were treated for liquid-liquid phase extraction and analyzed for drug content with a developed analytical methods. After the study, the animals were returned to the animal house for rehabilitation.

The pharmacokinetic parameters for Capecitabine and Docetaxel oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC (Area Under the Curve), C_{max} (Maximum Concentration), T_{max} (Time to reach peak concentration) the time at which C_{max} occurred, Data was measured by the trapezoidal rule method from time zero to infinity of the concentration-time curve. C_{max} and T_{max} were obtained from the graph. All values are expressed in mean±SD. (SD–Standard Deviation).

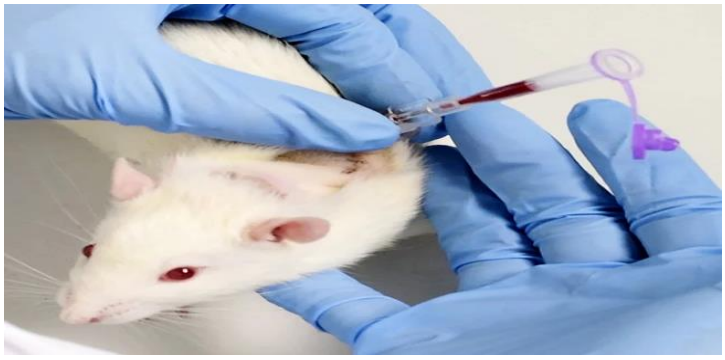


Fig. 1: Sampling of rat

RESULTS AND DISCUSSION

The maximum response on air pressure chemical ionization mode selected in this method is by having the electrospray ionization. The mobile phase flow of 10 µl/min Capecitabine and Docetaxel are highly responsive in the positive ion mode to offer sensitivity and signal stability with continuous flow to electro spray ions.

Specificity

The specificity of the method to research Capecitabine and Docetaxel simultaneously is proved. The chromatograms of blank and standard as shown in fig. 2, 3. The chromatograms of blank rat plasma and standard having no interference peaks were observed.

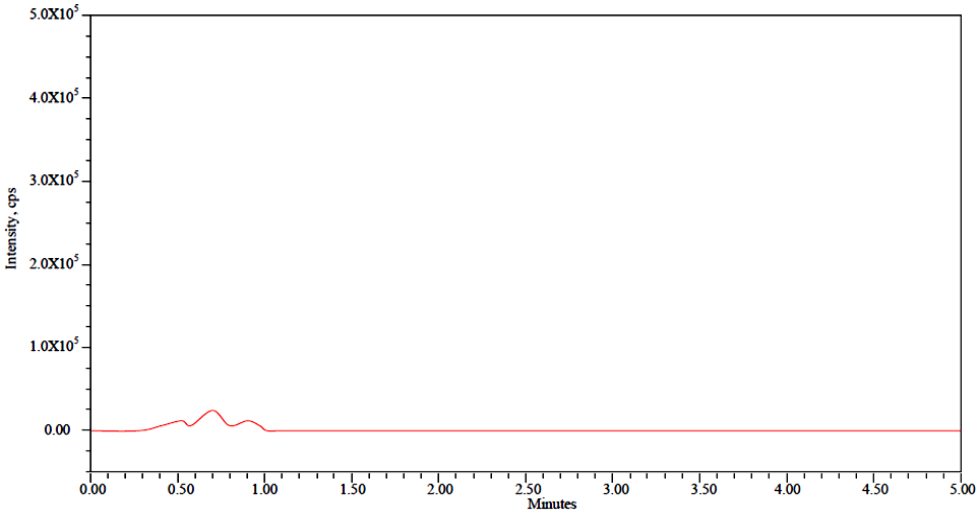


Fig. 2: **Chromatogram of blank**

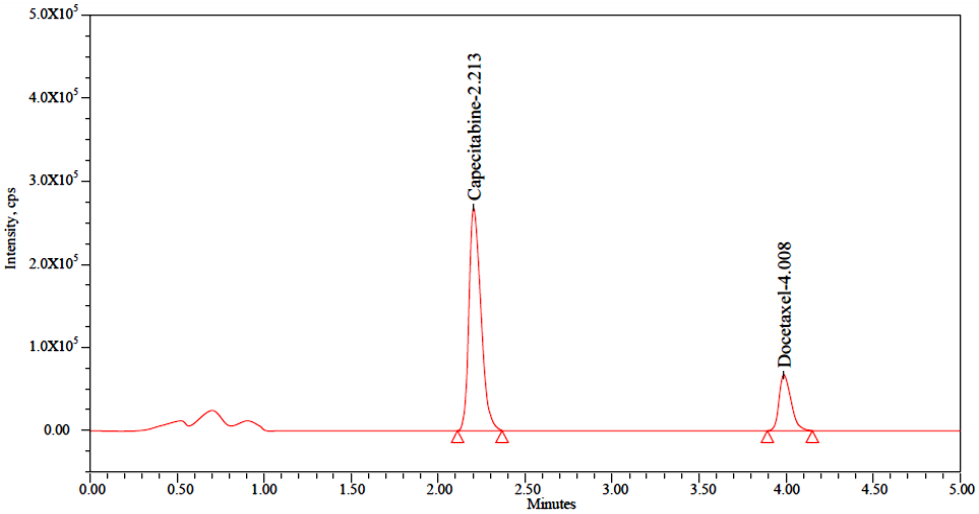


Fig. 3: Chromatogram of standard

Matrix effect

Percent RSD (Relative Standard Deviation) for within the signal, ion suppression/enhancement was observed as 1.0 percent for Capecitabine and Docetaxel in LC-MS/MS, suggesting that under these circumstances, the matrix effect [46] on analyte ionization is within an acceptable range of ionization. In matrix effect, LQC and HQC of Capecitabine were 96.12 and 97.90 and Docetaxel were 96.93, 97.71%. %CV of both drugs at LQC level were 0.73, 4.00 and HQC level is 0.22, 0.28 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

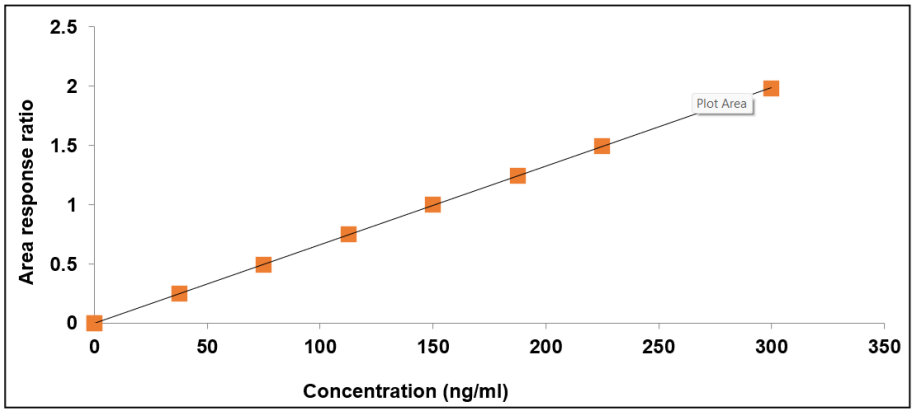
The peak area ratio of calibration standards was proportional to the concentration. The concentration range of Capecitabine is 15-300 ng/ml and Docetaxel is 4-80 ng/ml. Linearity results of Capecitabine and Docetaxel were shown in following table 1 and their calibration plots were shown in fig. 4 [47]. The calibration curves appeared linear and the coefficient of correlation was found to be 0.999 for Capecitabine and Docetaxel.

Precision and accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision [48] were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of Capecitabine and Docetaxel were shown in table 2, 3. Capecitabine accuracy results in quality control samples 95.10-98.81 and Docetaxel accuracy results in quality control samples 91.97-98.88. Capecitabine and Docetaxel CV is<5% of total internal control samples.

Table 1: Results of linearity

Linearity	Capecitabine		Docetaxel	
	Conc (ng/ml)	Area response ratio	Conc (ng/ml)	Area response ratio
1	37.50	0.252	10.00	0.289
2	75.00	0.497	20.00	0.553
3	112.50	0.752	30.00	0.822
4	150.00	0.999	40.00	1.067
5	187.50	1.244	50.00	1.395
6	225.00	1.496	60.00	1.629
7	300.00	1.984	80.00	2.180
Slope		0.0066	Slope	0.0269
Intercept		0.00390	Intercept	0.01858
CC		0.99997	CC	0.99932



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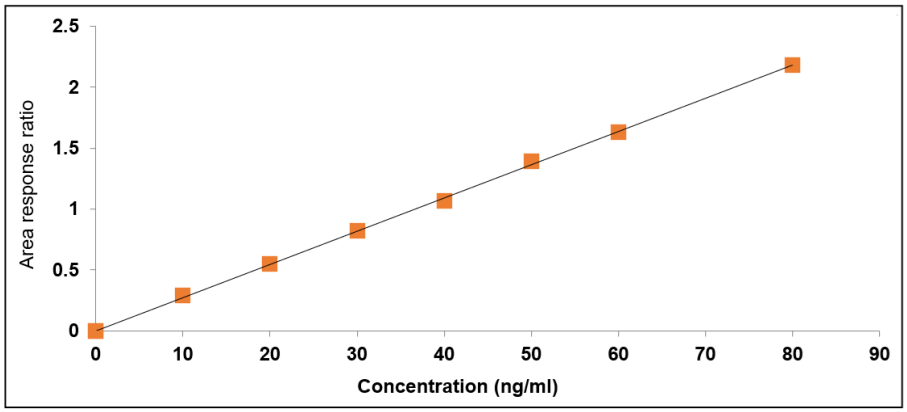

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Fig. 4: Calibration plots of (A) Capecitabine and (B) Docetaxel

Table 2: Precision and accuracy of capecitabine

S. No.	HQC	MQC	LQC	LLQC
	Nominal concentration (ng/ml)			
	225	150	75	15
	Analyte peak area			
1	$4.052x10^5$	$2.713x10^5$	$1.335x10^5$	$0.254x10^5$
2	$4.063x10^5$	$2.709x10^5$	$1.326x10^5$	$0.267x10^5$
3	$4.042x10^5$	$2.689x10^5$	$1.318x10^5$	$0.261x10^5$
4	$4.051x10^5$	$2.702x10^5$	$1.325x10^5$	$0.254x10^5$
5	$4.065x10^5$	$2.705x10^5$	$1.319x10^5$	$0.271x10^5$
6	$4.041x10^5$	$2.684x10^5$	$1.321x10^5$	$0.253x10^5$
n	6	6	6	6
Mean	$4.052x10^5$	$2.700x10^5$	$1.324x10^5$	$0.260x10^5$
SD	0.01011	0. 01145	0.00626	0.00764
% CV	0.25	0.42	0.47	2.94
% Accuracy	98.81%	98.76%	96.85%	95.10%

(n=6). High-Quality Control (HQC), Medium Quality Control (MQC), Low-Quality Control (LQC), Lower Limit of Quality Control (LLOQC).

Table 3: Precision and accuracy of docetaxel

S. No.	HQC	MQC	LQC	LLQC
	Nominal Concentration (ng/ml)			
	60	40	20	4
	Analyte peak area			
1	$1.023x10^5$	$0.675x10^5$	$0.321x10^5$	$0.064x10^5$
2	$1.013x10^5$	$0.668x10^5$	$0.337x10^5$	$0.068x10^5$
3	$1.022x10^5$	$0.677x10^5$	$0.326x10^5$	$0.054x10^5$
4	$1.005x10^5$	$0.662x10^5$	$0.321x10^5$	$0.063x10^5$
5	$1.013x10^5$	$0.664x10^5$	$0.339x10^5$	$0.061x10^5$
6	$1.018x10^5$	$0.681x10^5$	$0.325x10^5$	$0.065x10^5$
n	6	6	6	6
Mean	$1.016x10^5$	$0.671x10^5$	$0.328x10^5$	$0.063x10^5$
SD	0.00674	0.00763	0.00791	0.00476
% CV	0.66	1.14	2.41	7.62
% Accuracy	98.88%	97.96%	95.77%	91.97%

(n=6). High Quality Control (HQC), Medium Quality Control (MQC), Low Quality Control (LQC), Lower Limit of Quality Control (LLOQC).

Recovery

The recoveries for Capecitabine and Docetaxel at LQC, MQC and HQC levels demonstrated that the bioanalytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for Capecitabine (95.39%-98.24%) and Docetaxel (98.00%-94.86%) at LQC, MQC and HQC levels and % CV ranged from 0.13-1.72 for Capecitabine and 0.39-1.79 for Docetaxel. The results demonstrated that the bioanalytical method had good extraction efficiency.

Ruggedness

The percent recoveries and percent CV of Capecitabine and Docetaxel determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The results proved the method is ruggedness. The percent recoveries ranged from 96.89–97.81% for Capecitabine and 96.06%-97.96% for Docetaxel. The %CV values ranged from 0.08-1.78 for Capecitabine and 0.29–2.02 for Docetaxel. The results proved the method is ruggedness.

Autosampler carryover

Peak area response of Capecitabine and Docetaxel, wasn't observed within the blank rat plasma samples after successive injections of LLQC and ULQC at the retention times of Capecitabine and Docetaxel. In autosampler carryover, this method doesn't exhibit autosampler carryover.

Stability

Capecitabine and Docetaxel solutions were prepared with diluents for solution stability analysis and placed in a refrigerator at 2-8 °C. Fresh stock solutions were associated with stock solutions that were prepared 24 h earlier. The plasma stability of the bench top and autosampler was stable for 24 h, and 24 h at 20 °C in the autosampler. It became apparent from future stability that Capecitabine and Docetaxel were stable at a storage temperature of-30 °C for up to 24 h. The overall stability results of Capecitabine and docetaxel have been stated in the below table 4, 5.

***In vivo* pharmacokinetic evaluation**

The plasma concentration time profiles of Capecitabine and Docetaxel in rat are shown in fig. 5. The graph indicated bell shaped curves in both the cases of experimental formulation. Capecitabine and Docetaxel could be traced to be present in the blood for 2.5 h and 2 h after oral and intravenous administration, which indicates the effectiveness of drug release from the formulation.

The pharmacokinetic parameters C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t}, $AUC_{0-\infty}$, were calculated and the data is shown in table 6. The C_{max} for Capecitabine and Docetaxel were found to be 140.069 ng/ml and 37.946 ng/ml, respectively. The T_{max} for Capecitabine and Docetaxel were found to be 1 h and 0.5 h, respectively. The t½ values were 2.5 h and 2 h, respectively for Capecitabine and Docetaxel. The AUC0-t for Capecitabine and Docetaxel were found to be 243 and 33 ng-hr/ml, respectively. The pharmacokinetic parameters were shown in table 6.

Table 4: Stability results of capecitabine

Stability experiment spiked plasma		Mean area±SD	% CV	% Recovery
Benchtop stability	LQC	$1.322x10^5$±0.00631	0.48	96.71
	MQC	$2.664x10^5$±0.00816	0.31	97.44
	HQC	$4.020x10^5$±0.00569	0.14	98.02
Autosampler stability	LQC	$1.325x10^5$±0.00790	0.60	96.93
	MQC	$2.659x10^5$±0.00481	0.18	97.26
	HQC	$4.047x10^5$±0.01431	0.35	98.68
Long term (Day28) stability	LQC	$1.146x10^5$±0.00283	0.25	83.83
	MQC	$2.335x10^5$±0.00350	0.15	85.41
	HQC	$3.499x10^5$±0.00909	0.26	85.32
Wet extract 18 H stability	LQC	$1.305x10^5$±0.00335	0.25	95.46
	MQC	$2.644x10^5$±0.01431	0.54	96.71
	HQC	$3.975x10^5$±0.00274	0.07	96.93
Dry extract 18 H stability	LQC	$1.305x10^5$±0.00327	0.25	95.46
	MQC	$2.640x10^5$±0.01382	0.52	96.56
	HQC	$3.974x10^5$±0.00216	0.05	96.90
Freeze thaw stability	LQC	$1.315x10^5$±0.05716	4.35	96.20
	MQC	$2.692x10^5$±0.01296	0.47	98.46
	HQC	$4.011x10^5$±0.00631	0.16	97.81
Short term stability	LQC	$1.282x10^5$±0.00591	0.46	93.78
	MQC	$2.605x10^5$±0.00286	0.11	95.28
	HQC	$3.905x10^5$±0.00288	0.07	95.22

n=6. High-Quality Control (HQC), Medium Quality Control (MQC), Low-Quality Control (LQC), Lower Limit of Quality Control (LLOQC).

Table 5: Stability results of docetaxel

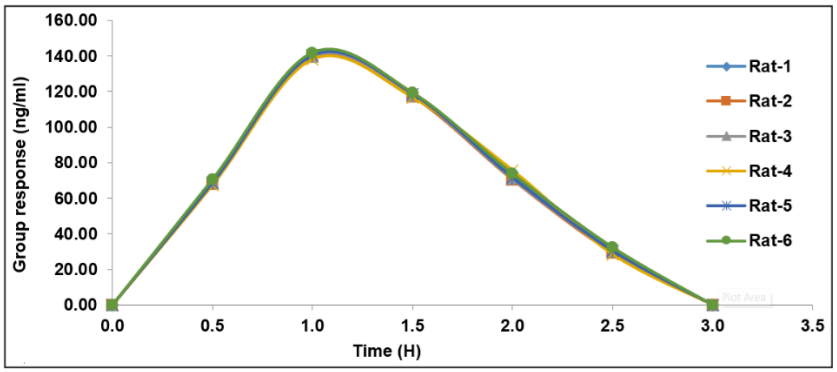
Stability experiment spiked plasma		Mean area±SD	% CV	%Recovery
Bench top stability	LQC	0.333x10^5±0.00787	2.36	97.23
	MQC	0.667x10^5±0.00402	0.60	97.37
	HQC	1.006x10^5±0.00916	0.91	97.91
Autosampler stability	LQC	0.323x10^5±0.00465	1.44	94.31
	MQC	0.670x10^5±0.00549	0.82	97.81
	HQC	1.009x10^5±0.00579	0.57	98.20
Long term	LQC	0.282x10^5±0.00628	2.23	82.34
(Day 28) stability	MQC	0.579x10^5±0.00956	1.65	84.53
	HQC	0.889x10^5±0.00467	0.52	86.52
Wet extract 18 H stability	LQC	0.328x10^5±0.00643	1.96	95.77
	MQC	0.659x10^5±0.00612	0.93	96.20
	HQC	0.995x10^5±0.00286	0.29	96.84
Dry extract 18 h stability	LQC	0.324x10^5±0.00258	0.80	94.60
	MQC	0.655x10^5±0.00258	0.39	95.62
	HQC	0.996x10^5±0.00242	0.24	96.93
Freeze thaw stability	LQC	0.329x10^5±0.00643	1.96	96.06
	MQC	0.669x10^5±0.00519	0.78	97.66
	HQC	1.014x10^5±0.00216	0.21	98.69
Short term stability	LQC	0.320x10^5±0.00692	2.16	93.43
	MQC	0.646x10^5±0.00280	0.43	94.31
	HQC	0.985x10^5±0.00280	0.28	95.86

n=6. High-Quality Control (HQC), Medium Quality Control (MQC), Low-Quality Control (LQC), Lower Limit of Quality Control (LLOQC).

Table 6: Pharmacokinetic parameters of capecitabine and docetaxel

Pharmacokinetic parameters	Capecitabine	Docetaxel
AUC_{0-t}	243 ng-h/ml	33 ng-h/ml
C_{max}	140.069 ng/ml	37.946 ng/ml
$AUC_{0-\infty}$	243 ng-h/ml	33 ng-h/ml
t_{max}	1 h	0.5 h
$T_{1/2}$	2.5 h	2 h

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, AUC_{0-t}: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration



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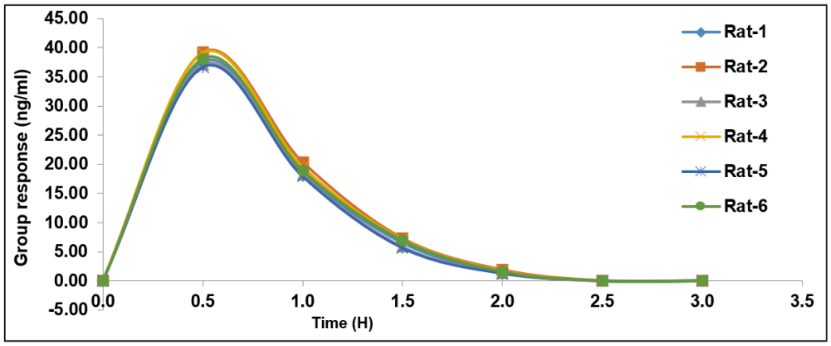

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Fig. 5: Recovery plot (A) Capecitabine and (B) Docetaxel

CONCLUSION

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of Capecitabine and Docetaxel in rat plasma. Here the described method is a rugged, fast, reproducible bio-analytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

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AUTHORS CONTRIBUTIONS

Nagul shareef has carried out the research samples and prepared the manuscript. Padmavathi has collected the literature and information about the drug. Aravind supported solution preparation in analysis. Mannam Subbarao check the data and reviewed the article.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest.

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