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METHOD DEVELOPMENT AND VALIDATION REPORT FOR THE SIMULTANEOUS ESTIMATION OF GEMCITABINE AND CAPECITABINE USING LCMS

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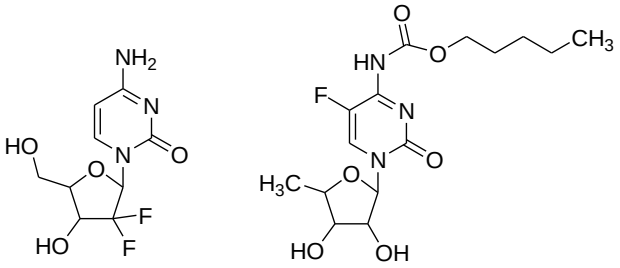
Abstract: A highly responsive and simple LC-MS/MS assay was developed and witnessed for the gradation of Gemcitabine and Capecitabine in rat plasma. Gemcitabine and Capecitabine were isolated from rat plasma using acetonitrile. The linearity curves are linear in a range of 10% to 200% of rat plasma for each analyte and its regression coefficient is 0.999. Quantitative recovery was observed in extracted in plasma and unextracted in without plasma of Gemcitabine and Capecitabine. Gemcitabine and Capecitabine are stable at conditions like wet extract, Bench top, Freeze thaw. This method is good in terms of accuracy, precision, recovery and stability.

Keywords: Gemcitabine, Capecitabine, LCMS, Method Development, Accuracy.

Introduction

Gemcitabine and Capecitabine are generally used as anti-curing reagents.[i] Mainly Gemcitabine cures the various cancers namely, breast cancer,[ii] ovarian cancer,[iii] non-small cell lung cancer,[iv] pancreatic cancer[v] and bladder cancer.[vi] Especially, pancreatic cancer, metastatic bladder cancer and metastatic non-small lung cancer are cured by the combination of Gemcitabine and cisplatin. In addition, the mixture of Gemcitabine and paclitaxel is used for breast cancer,[vii] cholangiocarcinoma[viii] and biliary tract cancers.[ix] Similarly, Capecitabine is also used curing for cancers like gastric cancer[x] and colorectal cancer.[xi] For breast cancer it is often used together with docetaxel, similarly, Ovarian cancer is cured by together with Capecitabine. However, they give common side effects include bone marrow suppression,[xii] liver and kidney problems,[xiii] nausea,[xiv] fever, rash, shortness of breath, mouth sores, diarrhea,[xv] neuropathy,[xvi] and hair loss. In addition, they also give other negative impacts such as abdominal pain,[xvii] vomiting, diarrhea, weakness, rashes and other severe side effects include blood clotting problems,[xviii] allergic reactions,[xix] heart problems such as cardiomyopathy,[xx] and low blood cell

counts. This medicine is not recommended to use during pregnancy, if they use, it harms to the baby. It is very harmful to kidney diseases people.



Gemcitabine **Capecitabine**

Fig 1: Structures of Gemcitabine and Capecitabine

In recent times, drug development and validation method has been established using bioanalysis.[xxi-xxx] It is the area of analytical chemistry in quantitative measurement of xenobiotics and biotics in biological systems. In this regard, here in, we would like to describe the bio analytical method based on liquid- liquid extraction and validated for quantification of Gemcitabine and Capecitabine in rat plasma.

Experimental

Materials and Methods

The APIs of Gemcitabine and Capecitabine were procured from Glenmark pharmaceuticals, Mumbai. LCMS grade of acetonitrile was get from JT Baker. Ortho Phosphoric acid of HPLC grade was procure from Merck in Mumbai and the rat plasma was get from Bharat Biotech Hyderabad.

Results and Discussion

Mass Spectrometry detection parameters optimization

Electrospray ionization (ESI) having maximum response over atmospheric pressure chemical ionization (APCI) mode selected in this method. The optimization of instrument to give sensitivity and signal stability during infusen of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at flow rate of 10µl/min. Gemcitabine and Capecitabine give more response in positive ion mode when compared to negative ion mode. The predominant peaks in the primary ESI spectra of Gemcitabine of MH^+ ions at m/z 263.2 and 321.7 and Capecitabine of MH^+ions at m/z 359.2 and 411.5 respectively. Internal standard D_6 Gemcitabine MH^+ ions at 263.8 to 321.5 and D_6 Capecitabine MH^+ ions at 359.6 to 411.9 respectively.

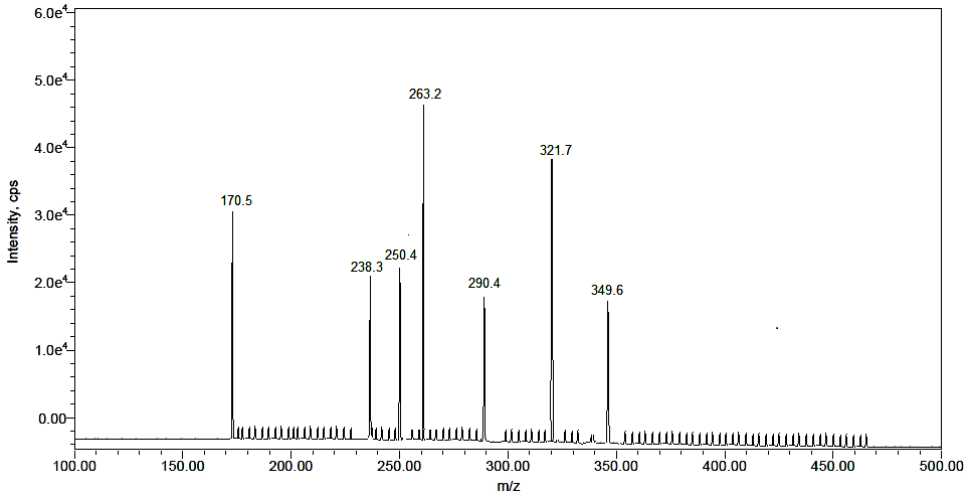


Fig 2 Mass Spectra of Gemcitabine

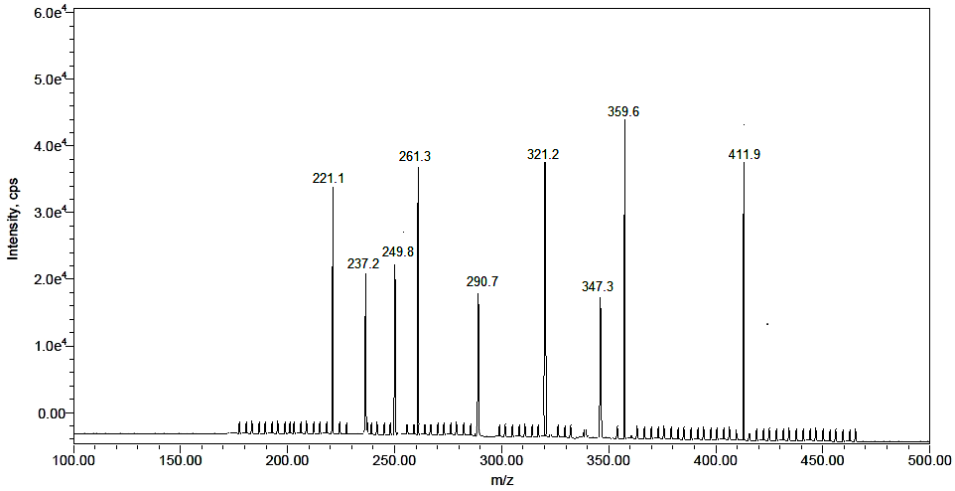


Fig 3 Mass Spectra of Capecitabine

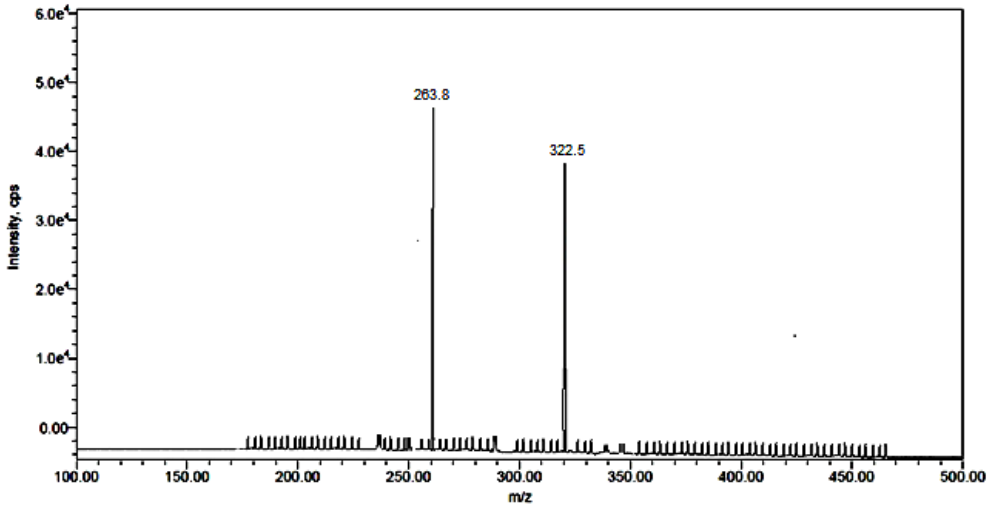


Fig 4 Mass spectra of D_6- Gemcitabine

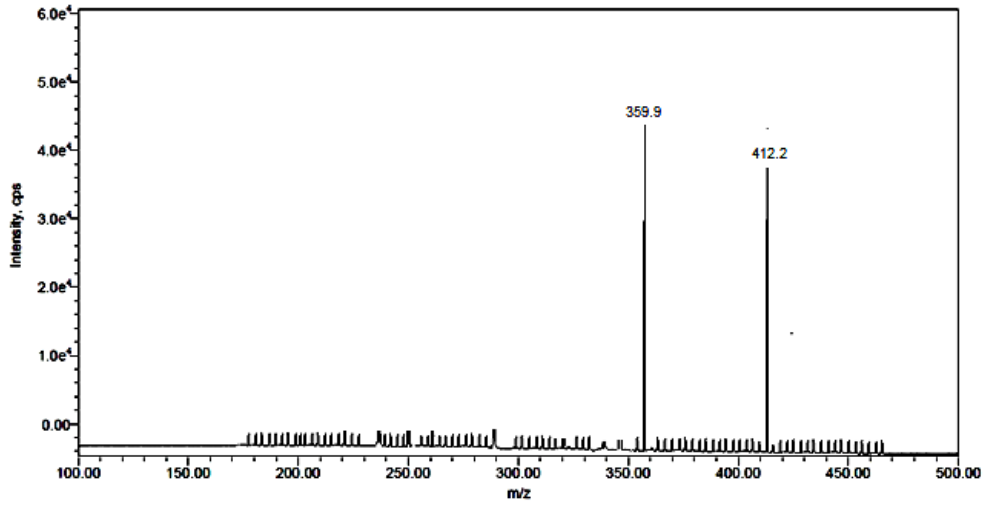


Fig 5 Mass spectra of D_6- Capecitabine

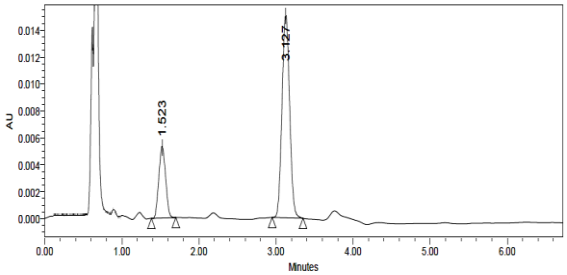
Chromatography Optimization

Different types of mobile phase are tried to develop the method they are included in below table-1. The pKa value of Gemcitabine and Capecitabine are 8.69 and 8.77 indicates it is strongest acidic. In acidic buffers very

Table 1 Method development Trials

S. No.	Mobile Phase (v/v)	Column	Observation
Trial-1	0.1% OPA: ACN (20:80)	Inertsil ODS (150x 4.6mm, 3.5µ)	Peak retention time is very low
Trial-2	0.1% OPA: ACN (30:70)	Inertsil ODS (150x4.6mm, 3.5µ)	Baseline is not sufficient
Trial-3	Water: ACN(30:70)	Luna Phenyl Hexyl (250x4.6mm, 3µ)	Resolution is low
Trial-4	Water: ACN (40:60)	Luna Phenyl Hexyl (250x4.6mm, 3µ)	Resolution is very low
Trial-5	Water: ACN (50:50)	Luna Phenyl Hexyl (250x4.6mm, 3µ)	All the parameters are Within the limit

sharp peaks are obtained. In all the trials we performed only acidic buffers are used. Finally we optimized the mobile phase of water and Acetonitrile in isocratic mode. Plate count and taliling were not within the limit when we use the mobile phase of water and acetonitrile in (50+50) v/v ratio. After that a mobile phase of water and Acetonitrile with changing combination was tried. An improvement in peak shape signal for Gemcitabine and Capecitabine and internal standard was observed using mobile phase as water and acetonitrile in 50:50 v/v. A column of Luna phenyl Hexyl (250 x 4.6mm, 3µm), flow rate of 1.0 ml/min was used. The drug and IS were eluted within 10min.



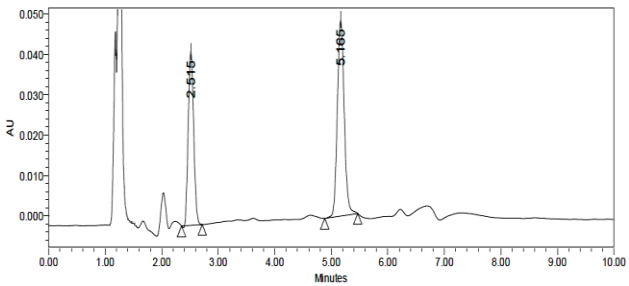


Fig 4 Chromatogram of trial-1

Fig 5 Chromatogram of trial-2

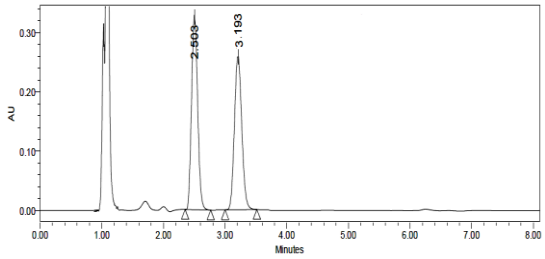


Fig 6 Chromatogram of trial-3

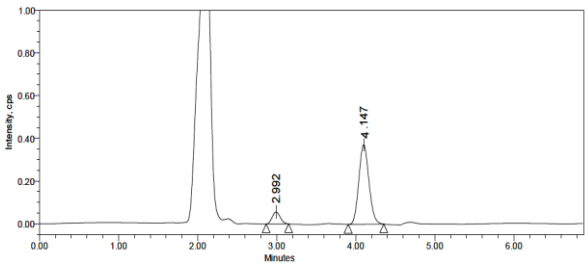


Fig 7 Chromatogram of trial-4

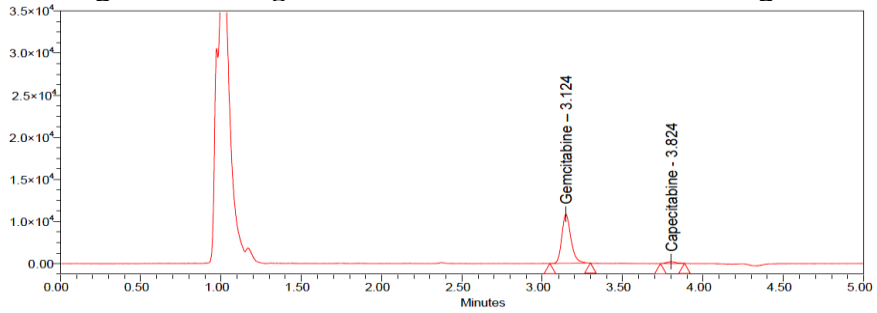


Fig 8 Chromatogram of trial-5

Extraction optimization

Sample was co-extracted proteins are removed from the prepared solution. Initially we tested with different extraction procedure protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE). In solid phase extraction stationary phase comes in the form of a packed syringe-shaped catridge, a 96 well plate, a 47mm flat disk packed with sorbent material in liquid handling syringe. Suppression effect in protein precipitation method for drug and internal standard is founded. Also we performed solid phase extraction and liquid-liquid extraction. In all extractions liquid liquid extraction suitable for extraction of the drug and internal standard. Several organic solvents (ethyl acetate, acetonitrile, chloroform, n-hexane, dichloromethane and methyl tertialry butyl ether) individuall as well as with combination in LLE to extract analyte from the plasma sample. Acetonitrile is good extraction solvent. D_6 - Gemcitabine and D_6- Capecitabine is good internal standard for this analysis. There is no significant effect in IS on analyte recovery, sensitivity or ion suppression. In liquid-liquid extraction method high recovery is observed.

Due to all optimized detection parameters, Chromatographic conditions and extraction procedure resulted in reduced in time of analysis with accurate and precise detection of Gemcitabine and Capecitabine in rat plasma.

Method Validation

Method validation of Gemcitabine and Capecitabine in rat plasma was done by as per US FDA guidelines. This method is validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, reproducibility and stability.

Selectivity and Sensitivity

Blank plasma and spiked plasma with a lower limit of quantification (LOQ) sample is shown in Fig. 3 and 4 for Gemcitabine and Capecitabine. The % interference of retention time of analytes between six different lots of rat plasma, including hemolyzed and lipedemic plasma containing K_2EDTA as an anti-coagulant was 0.00% for Gemcitabine and Capecitabine respectively, it is within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of Gemcitabine

and Capecitabine was prepared and analyzed. The %CV of the area ratios of these six replicates of samples was 1.06% and 1.17% for Gemcitabine and Capecitabine.

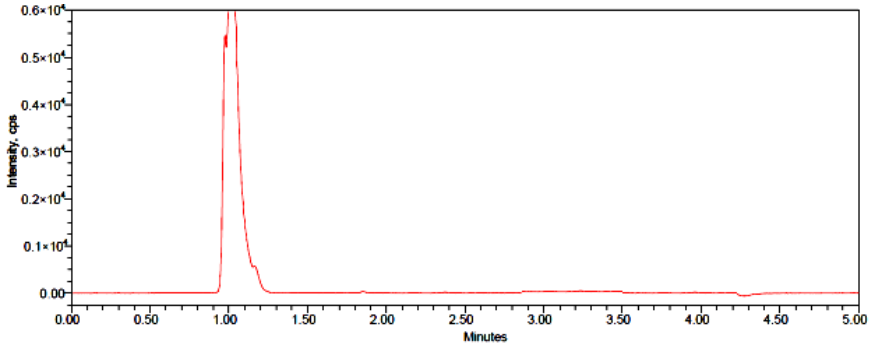


Fig 9 Blank plasma chromatogram of rat plasma

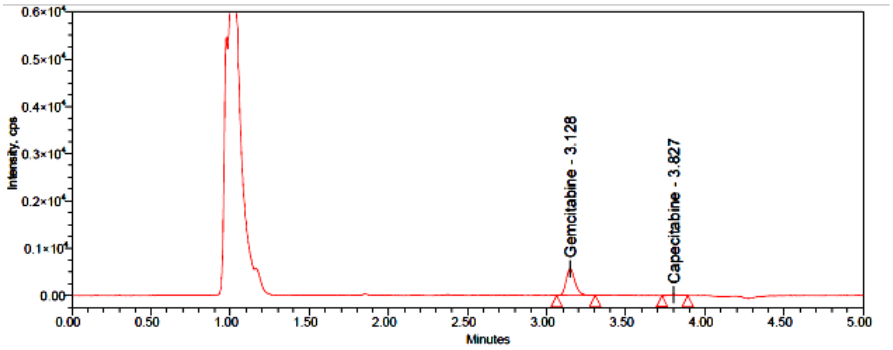


Fig 10 LLOQ chromatogram for rat plasma

Matrix effect

The %CV of ion suppression/enhancement in the signal was found to be 1.0% at MQC level for Gemcitabine and Capecitabine indicating that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

Linearity

The peak area ratios of calibration standards were proportional to the concentration of Gemcitabine and Capecitabine in each assay over the nominal concentration range of 2.5-75ng/ml of Gemcitabine and 1.25-37.5ng/ml of Capecitabine. The calibration curves appeared linear and were well described by least squares linear regression lines in Fig 5. The correlation coefficient was ≥0.999 for each drug.

Table 2 Linearity results of Gemcitabine

Linearity	Gemcitabine Conc (ng/ml)	Gemcitabine Peak response	IS peak response	Area ratio
Linearity-1	5	$0.145x10^4$	$1.562\ x10^4$	0.093
Linearity-2	12.5	$0.386\ x10^4$	$1.567\ x10^4$	0.246
Linearity-3	25	$0.762\ x10^4$	$1.545\ x10^4$	0.493
Linearity-4	37.5	$1.232\ x10^4$	$1.528\ x10^4$	0.806
Linearity-5	50	$1.542\ x10^4$	$1.534\ x10^4$	1.005
Linearity-6	62.5	$1.967\ x10^4$	$1.564\ x10^4$	1.258
Linearity-7	75	$2.393\ x10^4$	$1.537\ x10^4$	1.557
Linearity-8	100	$3.124\ x10^4$	$1.542\ x10^4$	2.026
Slope	0.0206			

Intercept	0.00998
CC	0.99902

Table 3 Linearity results of Capecitabine

Linearity	Capecitabine conc. (ng/ml)	Capecitabine peak response	IS peak response	Area ratio
Linearity-1	2.50	0.026	1.562	0.017
Linearity-2	6.25	0.075	1.567	0.048
Linearity-3	12.50	0.139	1.545	0.090
Linearity-4	18.75	0.201	1.528	0.132
Linearity-5	25.00	0.256	1.534	0.167
Linearity-6	31.25	0.342	1.564	0.219
Linearity-7	37.50	0.386	1.537	0.251
Linearity-8	50.00	0.529	1.542	0.343
Slope	0.0070			
Intercept	0.00034			
CC	0.99907			

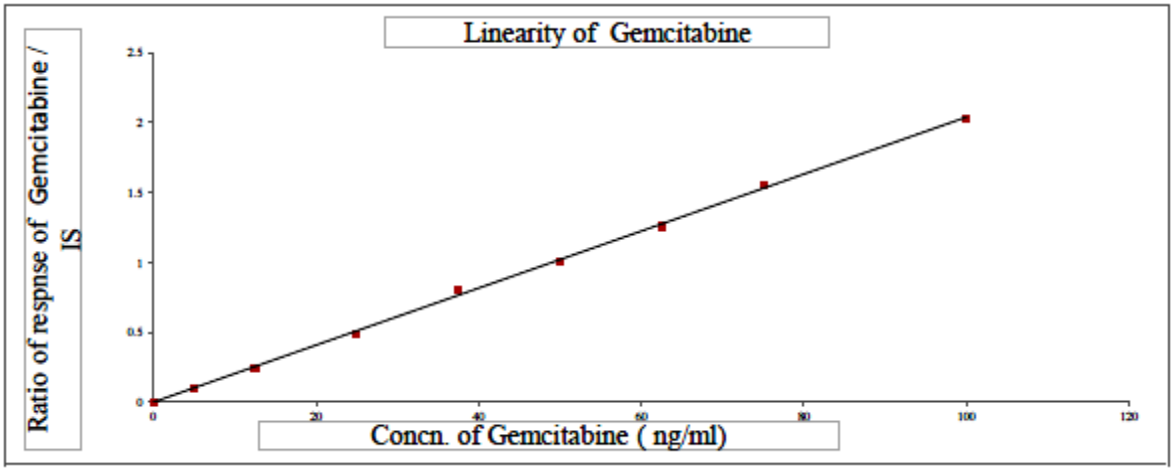


Fig 11 Calibration plot for Gemcitabine

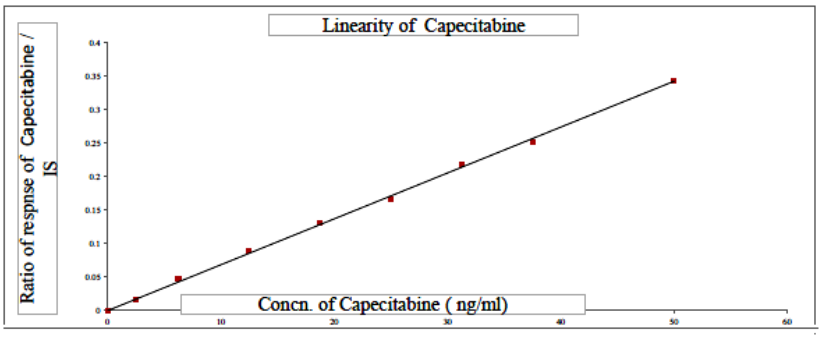


Fig 12 Calibration plot for Capecitabine

Precision and accuracy

The inter-run and accuracy were determined by pooling all individual assay results of replicate (n=6) quality control over five separate batch runs analyzed on four different days. The inter-run precision (%CV) was < 5% and inter-run accuracy was in between 98-101 for Gemcitabine

and Capecitabine. All these data presented in below table indicate that the method is precise and accurate.

Table 4 Comparision of within run and between run precision and accuracy for Gemcitabine

Nominal conc.(ng/ml)	Within run			Between run		
	Mean	Precision (%CV)	Accuracy	Mean	Precision (%CV)	Accuracy
2.5	2.486	0.62	99.89	2.561	0.64	99.75
25	25.241	0.81	99.53	25.275	0.86	100.21
50	50.362	0.94	98.09	50.344	0.99	100.05
75	75.367	1.15	99.41	75.314	1.21	99.81

Table 5 Comparision of within run and between run precision and accuracy for Capecitabine

Nominal conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (bg/ml)	Precision (%CV)	Accuracy
1.25	1.195	0.54	99.25	1.203	0.58	98.56
12.5	12.526	0.92	99.62	12.536	0.95	99.25
25	25.621	1.24	98.47	25.527	1.21	99.82
37.5	37.502	0.37	99.26	37.495	0.46	100.54

Recovery

We have prepared six aqueous spiked sample solutions of Gemcitabine and Capecitabine with low, average and high quality for examination of recovery testing. Very interestingly, extracted samples have provided the areas with same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Gemcitabine and Capecitabine recovery was 98.5%, 99.6% with a precision of 1.2% and 0.8%. This indicates that the extraction efficiency for Gemcitabine and Capecitabine was consistent and reproducible.

Reinjection and Reproducibility

In addition, we have also tested reinjection and reproducibility of the sample and they give good decent results. The change was less than 2.0 at LQC and HQC concentration levels hence batch can be reinjected in the case of instrument failure during real subject sample analysis. Furthermore, sample were prepared to be reinjected after 24 h, which shows % change less than 2.0% at LQC and HQC concentration levels; hence batch can be reinjected after 24 h in the case of instrument failure during real subject sample analysis.

Stabilities

In solution stability Gemcitabine and Capecitabine in solutions are prepared in diluent and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 24hours. The % change for Gemcitabine and Capecitabine was 1.02% respectively which indicates that stock solutions were stable at least for 24hours. Bench top and auto sampler stability for Gemcitabine and Capecitabine was investigated at LQC and HQC levels. Gemcitabine and Capecitabine was stable in plasma for at least 24h at room temperature, and 24h in an auto sampler at 20°C. It was confirmed that repeated freezing and thawing of plasma samples spiked with Gemcitabine and Capecitabine at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Gemcitabine and Capecitabine were stable in a matrix up to 24hours at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in table 4.

Table 6 Stability of the Gemcitabine

Stability Experiments		Spiked plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV (n=6)
Bench Top stability	LQC	25	25.3612	1.03
	HQC	75	75.4628	0.86
Auto sampler stability	LQC	25	25.6389	1.57
	HQC	75	75.4125	0.94
Long term stability	LQC	25	25.6956	1.51
	HQC	75	75.4201	1.06
Freeze thaw stability	LQC	25	25.9687	1.87
	HQC	75	75.5925	0.76

Table 7 Stability of the Capecitabine

Stability experiments		Spiked plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV (n=6)
Bench Top stability	LQC	12.5	12.5632	0.68
	HQC	37.5	37.5692	0.97
Auto sampler stability	LQC	12.5	12.5896	0.62
	HQC	37.5	37.5985	0.86
Long term stability	LQC	12.5	12.5635	0.99
	HQC	37.5	37.5965	1.13
Freeze thaw stability	LQC	12.5	12.6452	1.01
	HQC	37.5	37.4598	0.93

Application:

The validated method has been success fully to quantify Gemcitabine and Capecitabine in three groups of rats, under fasting conditions after administrations of 500mg tablet containing Capecitabine and 1 g/vial injection of gemcitabine as an oral dose. Drug sample was injected into rat body collected samples at different time intervals like 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0hr. After that samples are prepared as per test method injected into chromatographic system recorded the values. The pharmacokinetics parameters evaluated were C_{max} (maximum observed drug concentration during the study), AUC0-12 (area under the plasma concentration –time curve measured 2.0h for capecitabine and 1.5h for gemcitabine, using the trapezoidal rule) t_{max} (time to observed maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and $t_{1/2}$ (terminal half-life as determined by the quotient $0.693/K_{el}$).

The test/reference ratios for C_{max}, AUC0-12, and AUC of gemcitabine and capecitabine were 88.24, 92.56 respectively, and they were within the acceptance range of 80%-125% demonstrating the bio equivalence of the formulation of Gemcitabine and Capecitabine. The mean concentration versus time profile of Gemcitabine and Capecitabine in rat plasma as test and reference is shown.

Table 8 Mean pharmacokinetic parameters of Gemcitabine

Pharmacokinetic Parameters	Gemcitabine	Capecitabine
AUC_{0-t} (ng h/ml)	3.0	3.0
C_{max} (ng/ml)	1.547	0.236
$AUC_{0-\infty}$ (ng h/ml)	0-1.59	0-0.24
K_{el}	0.462	0.347
$t_{1/2}$	1.5	2.0
t_{max} (h)	1.5	2.0

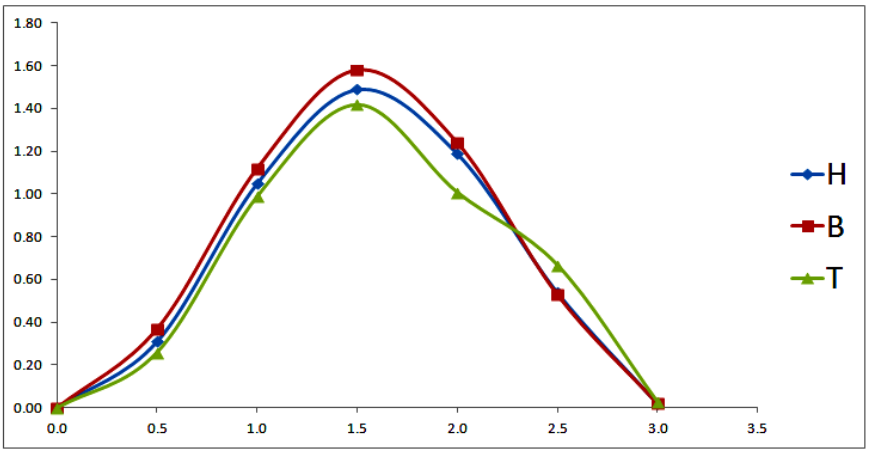


Fig 13 Recovery plot for Gemcitabine

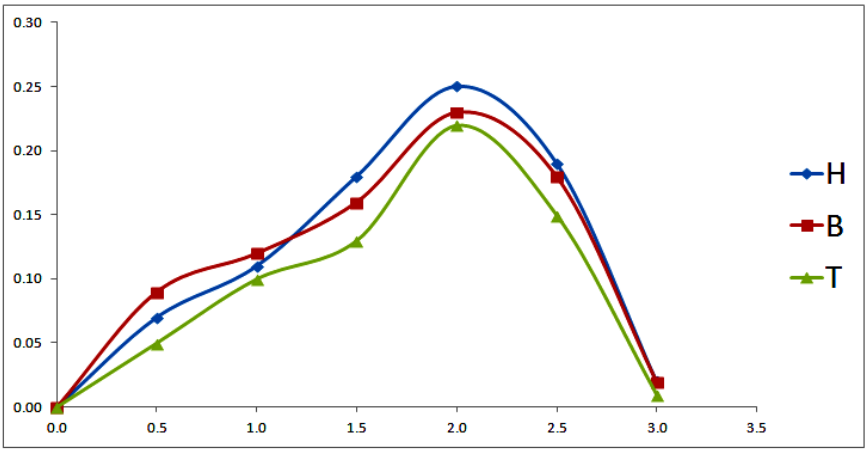


Fig 14 Recovery plot for Capecitabine

Conclusion

The proposed method was higher sensitive HPLC-ESI-MS/MS method for the determination of Gemcitabine and Capecitabine in rat plasma has been developed and validated for the first time. The method describes here is fast, rugged, reproducible bio analytical method. The developed method is simple and efficient and can be used in pharmacokinetics studies as well as in the monitoring of the investigated analyte in body fluids.

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