Egypt. J. Chem. **Vol. 64,** No. 3 pp. 1601 - 1614 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Facile validated HPLC method using photodiode array detector for the combined analysis of etodolac and 5-FU in bulk and tablet dosage form

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Abstract

The aim of the investigation was to develop and validate a reversed-phase high pressure liquid chromatography (RP-HPLC) method for the simultaneous estimation of etodolac (ETD) and 5-FU in bulk and coated tablet dosage form. The method was validated in terms of linearity, precision, accuracy, and specificity, LOD, and LOQ according to ICH guidelines. The standard instrument parameters were optimized using C18 reverse phase Inertsil® ODS (250 × 4.6 mm, 5µ particle size) column with system equipped with photodiode array (PDA) detector. An optimized ratio of mobile phase in RP-HPLC under low pressure gradient mode comprising of 60:40 % v/v of acetonitrile and di-potassium hydrogen phosphate buffer (pH 6.4; 25 mM), provided sharp peaks (having adequate molar absorptivity at λmax of 280 nm) with short retention time of 4.3+/-0.2 and 11.5+/-0.3 min for 5-FU and etodolac, respectively. Results indicated the method was linear, precise, accurate, rugged and robust with RSD values <2%. The percentage recoveries of both the drugs were within the limits of 90-110% at various spiked concentrations. The proposed method was found to be highly effective and could be successfully employed for the quantification of 5-FU and ETD in bulk drug and tablet formulations for routine analysis.

Keywords: RP-HPLC, 5-FU, ETD, validation, tablets, photodiode array (PDA)

Introduction

For more than five decades, 5-FU has been employed in oncological practice as an individualized therapeutic agent as well as in combination regimens in various forms of cancer [1-2]. Pharmacokinetic based dose adjustment of 5-FU as well as its combinations with suitable agents at sub-optimal dose ranges has significantly improved clinical outcomes by reducing toxicities and improving efficacy [3-4].

Several topical combination formulations of 5-FU with NSAIDs such as salicylic acid, especially COX-2 specific inhibitors such as diclofenac sodium (i.e. topical creams, or solutions) are currently being marketed for the treatment of skin cancers (i.e. actinic or solar keratosis and superficial basal cell carcinomas). Such formulations include *Fluorac*® having composition of fluorouracil 5 g/100g plus diclofenac sodium-1g/100g by Burke Therapeutics, LLC, USA; *Verrunex*® a generic topical cream available as kit containing fluorouracil-0.5 g/0.5g plus salicylic acid-1.2g/1.2g by Accumix Pharmaceuticals, in USA; and *Actikerall*® (a combination of fluorouracil-0.5% plus salicylic acid-10%) launched by Cipher Pharmaceuticals Inc., Canada in 2016 [5].

Although, combination of 5-FU with etodolac has not been clinically approved yet, however literature investigations have clearly demonstrated the combined synergistic potential of 5-FU in combination with ETD in various preclinical and clinical studies in diverse types of cancers viz. ovarian cancer, pancreatic cancer, colorectal cancer and head

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Receive Date: 06 October 2020, Revise Date: 12 November 2020, Accept Date: 07 December 2020

DOI: 10.21608/EJCHEM.2020.45336.2922

& neck cancer respectively [6-9]. The combination of specific COX-2 agents (e.g. ETD, diclofenac, sulindac etc.) with 5-FU at sub therapeutic doses have been employed by the clinical researchers globally to reduce the drug associated toxicity potential of 5-FU. **Figure 1** shows the structural formulae of 5-FU and ETD, respectively. Recent investigations have reported the modulation of pharmacokinetic properties of 5-FU (owing to its incomplete oral absorption, short half-life and peak-valley phenomenon) via pharmaceutical approaches such as co-crystallization [10]. Preclinical studies have revealed the ability of NSAIDs (e.g. indomethacin, NS-398) to interact with 5-FU and modulate its dihydropyrimidine dehydrogenase mRNA expression or enzyme activity. Literature reports have also stated that metabolism of etodolac gets reduced in the presence of 5-FU [11]. Therefore, it becomes crucial to assess the pharmaceutical drug-drug interaction behavior in dry bulk form.

Further, various analytical reports have shown validated quantification methods for the determination of 5-FU alone in samples of biological matrices employing GC/MS [12], HPLC [13,14], hydrophilic interaction liquid chromatography-APCI-mass spectrometry [15], liquid chromatography tandem mass spectrometry (LC–MS/MS) [16-18] and in pharmaceutical dosage forms such as polymeric nanoparticles [19] or ETD alone [20-21]. Furthermore, **Table 1** summarizes the comparative data of analytical methods/chromatographic studies in chronological order carried out in the last decade on individual analytes 5-FU or ETD or in combination with the agents of other therapeutic class /analytes from the literature [22-38]. This comparative analysis offer distinct understanding of the sensitivity (in terms of LOD/LLOQ) of the analytical method using PDA detector conducted in the present study in comparison to other methods for the analysis of 5-FU or ETD or in combination with agents of other therapeutic class.

It is a matter of the fact that no *in* vitro study for the simultaneous estimation of 5-FU with ETD and its interaction analysis in bulk form as well as in samples of pharmaceutical dosage form has been reported. Moreover, due to high interference by pH responsive enteric polymers, no sensitive technique is presently available for the simultaneous estimation of 5-FU with ETD.

The major aim of the present work was to develop a facile HPLC method using PDA detector for the quantitative simultaneous estimation of 5-FU and ETD bulk as well as in enteric coated tablet dosage form for routine in vitro analytical estimations.

Materials and Methods

Chemicals and reagents

All chemicals were analytical grade and used as received. All solutions were prepared in Milli-Q® deionized water from a Millipore water purification system (Bedford, MA, USA). 5-Fluorouracil was obtained as gift sample from Getwell Pharmaceuticals Pvt. Ltd., (Gurgaon, India). Etodolac USP with stated purity of 99% (Batch no. 1266802) was obtained as gift sample from IPCA Laboratories Pvt. Ltd, Mumbai, India. Potassium dihydrogen phosphate (KH_2PO_4), HPLC grade acetonitrile and HPLC grade methanol was obtained from Merck Specialities Pvt. Ltd., (Mumbai, India) respectively. Mobile phase was filtered using 0.45µ nylon filters from Millipore Co. (MA, USA).

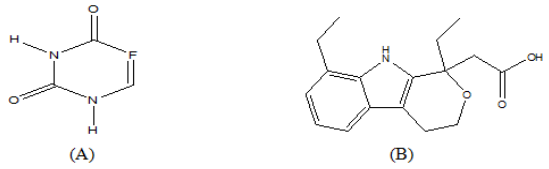

Figure 1: Structural formulae of 5-FU and ETD

Design of enteric coated tablets

In order to develop pH responsive enteric coated delivery system, core tablets of 5-FU and ETD (batch 5-FET) were designed alongwith other tablet excipients such as MCC PH 102, talc (2% w/w) and magnesium stearate (1% w/w). Briefly, 5-FU, ETD and MCC were screened together through sieve #60 mesh to obtain uniform powder blend. Then, the resulting blend was mixed in inflated polybag for ten minutes.

Subsequently, the powder blend was granulated using 5% w/v polyvinyl pyrrolidone (PVP). PVP dispersion was prepared by dissolving 2.5 g of PVP in 50 ml of distilled water and used as binder to form wet solid mass. The wet mass was passed through sieve #12 mesh to form granules which were dried in tray dryer at 50 ºC±2 ºC. The granules were later mixed with 2% w/w talc and 1% w/w magnesium stearate to obtain better flow properties. The final granulating blend was subjected to direct compression to obtain adjuvant core tablets.

Table 1: Comparative analytical methods carried out in the last decade on individual analytes 5-FU or ETD or in combination with the agents

Analytical Method/Sample	Mobile phase composition	Detector and wavelength	LOD/LLOQ (ng/mL)	Analytes as Single agent		Analytes in combination with other class of agents	References
				5-FU	ETD		
HPLC/ simultaneous determination in bulk drug and enteric coated tablets	Acetonitrile (ACN): 25mM (KH_2PO_4) at 60:40 % v/v dissolved in distilled water. pH-6.4 adjusted with orthophosphoric acid	PDA-280 nm	101 and 307 (5-FU); 57 and 173 (ETD)	----	----	5-FU+ ETD (As adjuvant therapy)	Present study
HPLC-MS/MS/plasma	A: water containing 0.5% acetic acid B: ACN containing 0.5% acetic acid	MS	5 and 10	5-FU	---	----	[22]
UV spectrometric and RP-HPLC (Stability Indicating Assay) in bulk and tablets	ACN: Methanol:65:35 %v/v	UV-226 nm	315800	----	ETD	----	[23]
Chemometrics-assisted spectrophotometry (UV Spectrometry) in commercial tablets	Methanol as solvent	240–440 nm	Not Applicable	----	----	ETD + thiocholchicoside (THC)	[24]
HPLC/ simultaneous determination in human plasma	water (pH 4.0): CH_3OH (98:2 v/v); and water (pH 4.0):methanol:ACN (70:13:17 v/v/v)	diode array detector (195-270 nm)	2 and 10 (5-FU); 30 and 150 (EP); 200 and 1000 (CP)	----	---	5-FU + epirubicin (EP) + cyclophosphamide (CP) and their metabolites	[25]
HPLC/Skin tissue	ACN and 0.1% phosphoric acid aqueous solution (4:96 or 2:98, v/v, (pH 2.6)	UV-265 nm	33-300	5-FU	---	----	[26]
HPLC/mobile phase and plasma	5 mmol/L potassium dihydrogen phosphate (pH 6.0) and methanol at a ratio of 96:4 v/v	UV- 254 nm	Not reported	5-FU	---	----	[27]

UPLC-MS/surface samples	0.1% FA (solution A) and ACN (solution B)	Tandem MS with ESI	0.5~ 5-FU; 1 ~ DOC/DOX	---	---	5-FU + Cyclophosphamide (CYP) + docetaxel (DOC) and doxorubicin (DOX)	[28]
HPLC/Stability Assay	HPLC grade water only	UV -269 nm	7.10 and 21.5	5-FU	---	----	[29]
HPLC-MS/MS	A: 0.1% acetic acid in ACN (v/v) B: 0.1% Acetic acid in water	MS	5	5-FU	---	----	[30]
HPLC/ human and rabbit plasma	de-ionized distilled water, pH adjusted at 3.2 with perchloric acid.	UV -260 nm	24.80/40 (human plasma) and ; 50.05/80 (rabbit plasma)	5-FU	---	----	[31]
UV Spectrometric simultaneous estimation in bulk and tablets	Methanol as solvent	λmax of ETD-259.4; λmax of THC-236 nm	88 (ETD) and 129 (THC)/ 291 (ETD and 949 (THC)	---	---	ETD and thiocholchicoside (THC)	[32]
HPLC-DAD in plasma	phosphate buffer (pH~4) : acetonitrile (45 : 55, v/v)	DAD detector-254 nm	33 (PTZ) and 918 (ETD) /98.8. (PTZ) and 2783 (ETD)	---	---	ETD + Pantazoprazole (PTZ)	[33]
HPLC in mobile phase and tablets	acetate buffer:ACN: 55:45% v/v	PDA-221 nm	130/400 for ETD; 90/280 for impurity H	---	---	ETD with impurity H	[20]
HPLC/ human plasma and pharmacokinetic study with marketed tablet	10 mM potassium phosphate buffer (adjusted to pH 7.5)–ACN (70 : 30, v/v)	UV-230 nm	100	---	---	ETD with paracetamol	[34]
HPLC/polymeric nanoparticles	ACN and water (10:90, v/v)	PDA- 265 nm	10.86 and 32.78	5-FU	---	----	[19]
HPLC/bulk drug, marketed injection, w/o	methanol:water (50:50 % v/v)	UV -254 nm	50 and 150	5-FU	---	----	[35]

and w/o/w nanoemulsions and stress studies							
HPLC-MS/MS/plasma	A: ACN B: 5mM ammonium acetate and 0.1% formic acid	MS	1	5-FU	---	----	[36]
HPLC/simultaneous determination in human plasma	0.05 M disodium hydrogen phosphate and ACN (65:35 v/v)	UV-variable wavelength detector (VWD) at 266, 254 and 198 nm	78 and 98 (5-FU); 96 and 150 (AD) 123 and 150 (CP)	----	---	5-FU, adriamycin (AD) and cyclophosphamide (CP)	[37]
HPLC/simultaneous determination in fixed dose combination tablets	phosphate buffer (KH2PO4) of pH 5.5 : Methanol : Acetonitrile : Tri-ethylamine (40 : 40 : 20 : 1.5)	PDA-257 nm	0.57/1700 ~ ETD; 160/510 ~ TOLP	----	---	ETD with tolperisone hydrochloride (TOLP)	[38]
HPLC/plasma	methanol and 20 mmol/L ammonium formate	UV -265 nm	5	5-FU	---	----	[13]

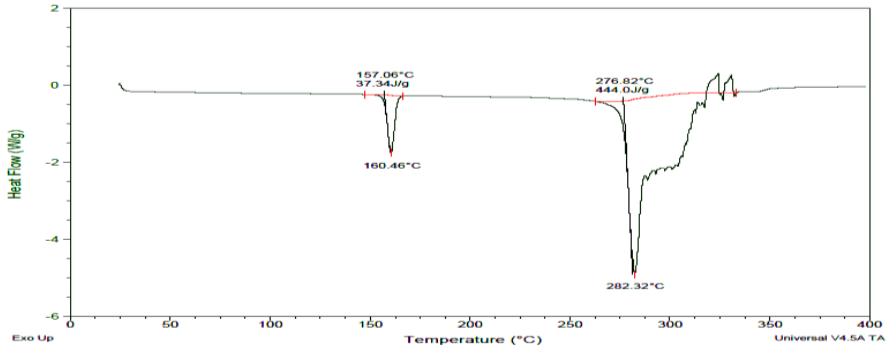

Figure 2: Drug interaction studies between the analytes, ETD and 5-FU

Table 2: Analysis of pH responsive enteric coated system

Formulation	Drug/ Amount added	Amount (mg) obtained Mean ± S.D.	%Assay	%RSD
5-FET (coated at 2.95% w/w)	5-FU (100 mg)	99.33± 3.56	99.33	3.593
	ETD (200 mg)	201.1±3.79	100.55	1.885

Preparation of coating solution

A mixed film coating solution consisting of three polymers, ethyl cellulose: Eudragit ®S-100: starch in a ratio of 2:1:1 was prepared by dissolving in a 3:1 ratio blend of IPA: ethanol. The total polymer concentration in the coating solution was 4 %w/v. 12.5 % v/v of dibutyl phthalate was also added drop wise to the coating solution with stirring at 2000 rpm for 30 minute to obtain clear solution. The resulting solution was used for the mixed film enteric coating of the adjuvant core tablets.

Coating of combined tablet formulation

Tablets containing 5-FU and ETD were coated using pan coating technique at 0.91-2.95 %w/w of coating. Tablets were coated with inlet air temperature of 40-50 ºC and coating process was continued till the desired level of coating thickness was achieved.

Drug interaction behaviour

The suitability and physiochemical compatibility of the drugs (5-FU and ETD) in solid state was evaluated using DSC (Q20 series, TA instruments, USA). Physical mixtures of 5-FU and ETD were prepared, in equal proportions (1:1) and were subjected to DSC analysis in order to analyze their interaction.

Analysis of drugs in pH responsive enteric coated system

Tablet powder equivalent to dose of drugs (100 mg and 200 mg each, respectively) was dissolved in 100 ml volume of phosphate buffer (pH 6.8) and the resultant solution was initially vortexed for ten minutes followed by sonication for five minutes. These, samples were filtered through a 0.45µ membrane filter after required dilution. The solutions were subjected to HPLC analysis and peak areas were measured. The analysis of enteric tablet powder was conducted in triplicate (**Table 2**).

Preparation of test solution

Twenty tablets were weighed and finely powdered in a mortar. Tablet powder containing 10 mg of each 5-FU and ETD was accurately weighed and transferred to a 100 mL calibrated flask. Around 50 mL of phosphate buffer solution (pH 6.8) was added, and the resultant solution was sonicated in an ultrasonicator bath for 10 minutes. The final volume was made up to the mark with the same solvent mixture. The solution was filtered through 0.45µ membrane filter. This solution obtained having concentration of 100 µg mL^{-1} each of 5-FU and ETD was used for the analytical investigation.

HPLC equipment and chromatographic conditions

Samples were analysed on HPLC system (Waters® Delta 600 HPLC) fortified with a Waters 600 controller pump, Waters® 2996 PDA detector and a degasser module from Waters, Milford, USA. The retention of the drug compounds was carried out on Inertsil® ODS-3 column (C-18, 250 × 4.6 mm, 5µ). The system was equipped with Waters Empower 2® software which was exploited for data acquisition and processing. The mobile phase was run using gradient system. The flow rate of the mobile phase was set at 1.0 mL min^{-1}. The Inertsil® ODS-3 column (C-18, 250 × 4.6 mm, 5µ) was maintained at 30°C and the measurements were made with 20 µL of injection volume.

Preparation of the mobile phase

The composition of mobile phase was acetonitrile: 25mM potassium dihydrogen phosphate (KH_2PO_4) (60:40) dissolved in distilled water. The pH of mobile phase was adjusted to 6.4 with diluted orthophosphoric acid.

Preparation of phosphate buffer

A weighed amount of sodium phosphate tribasic dodecahydrate (M.W. 380.12) was dissolved in distilled water in order to obtain 0.02 M solution. The resulting solution was uniformly mixed with 0.1N HCl (in a ratio of 1:3 respectively) to obtain phosphate buffer (pH 6.8). Further, the pH of the phosphate buffer was cross-validated with the help of a pH meter.

Preparation of standard stock solutions

The standard stock solution containing 1mg mL^{-1} each of 5-FU and ETD was prepared by

dissolving the both drugs together in phosphate buffer pH 6.8 and diluting with the same diluent. An aliquot of 0.5 mL from the above prepared stock solution (containing 5-FU and ETD) was transferred to 50 mL calibrated volumetric flask and the volume was made up to the mark with the same solvent mixture to in order to obtain a standard stock solution having a concentration of 100 µg mL^{-1} for both drugs. Calibration curves were plotted by diluting this standard stock solution to the appropriate volume with phosphate buffer pH 6.8.

Method validation

The developed method was validated according to ICH guidelines [39]. To check the system performance, the system suitability parameters were measured. System precision was determined on six replicate injections of standard preparations. Number of theoretical plates and asymmetry were measured.

Linearity and range

An analytical method is tested for its linearity in order to validate a proportional relationship of response versus analyte concentration over the working range. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is from 80-120% of the target concentration. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r^2) of >0.998 is generally considered as evidence of acceptable fit of the data to the regression line.

In the present study, linearity of the method was obtained by analysing three replicates of five concentrations of standard drug solution dissolved in phosphate buffer (pH 6.8). A stock solution of the drug dissolved in phosphate buffer pH 6.8 at a concentration of 100 µg/ml was prepared. Further serial dilutions were prepared with concentration ranging from 1 µg/ml to 100 µg/ml. The prepared concentrations were injected into the column and the respective area of the chromatogram obtained for each injection was determined. A graph was plotted between area of the chromatogram and the drug concentration and the correlation coefficient of the calibration curve was calculated.

Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. Precision of the analytical method was determined by system repeatability studies. For this, 10 µl of the drug solution of concentration 2, 4 and 5 µg/ml was injected six times in the column and the percent relative standard deviation (% RSD) was obtained for the respective drug peak areas.

Precision criterion for an assay method is that the instrument precision (RSD) should be ≤1%, and for the impurity assay, at the limit of quantitation, the instrument precision (repeatability) should be ≤ 2%.

Intra-day precision

The intra-day precision was carried out at three different concentrations (2, 4 and 5 µg/ml). Three injections were administered into the column in triplicate and analyzed. This protocol was repeated twice on the same say under similar conditions and % RSD was obtained.

Inter-day precision

The inter-day precision was determined at three different concentrations of (2, 4 and 5 µg/ml). Three injections were injected into the column in triplicate and were analyzed. This procedure was repeated was two consecutive days and % RSD was obtained.

Accuracy as recovery

Accuracy was determined by recovery studies using standard addition method. The pre-analyzed samples of both 5-FU and ETD were spiked with extra 10%, 20%, and 30% of the standard drugs and the mixtures were analyzed by the proposed method. The experiment was conducted in triplicate.

Robustness

The effect of slight variations in chromatographic conditions such as change in flow rate (±0.2 mL/min) and wavelength of detection (±2 nm), were investigated to determine the robustness of RP-HPLC-PDA method for the simultaneous estimation of 5-FU and ETD and their %RSD was determined.

Detection and quantitation limits

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using following equations:

$$LOD = 3.3 \times \frac{SD}{S} \quad \text{..... (1)}$$

and $$LOQ = 10 \times \frac{SD}{S} \quad \text{.....(2)}$$

where SD is standard deviation of response (area of the chromatogram) and S is the average of the slope of the calibration curve.

System suitability

System suitability tests are vital parameters of optimized RP-HPLC-PDA method which are utilized to authenticate the reproducibility of analytical method. To ascertain its effectiveness, certain system suitability test parameters were analyzed.

Table 3: Optimized chromatographic conditions

Parameter	Optimized Condition
Instrument	HPLC system (Waters Delta 600 HPLC)
Mobile Phase	Acetonitrile: 25mM potassium dihydrogen phosphate (KH_2PO_4) (60:40)
Column	Inertsil® ODS-3 column (C-18, 250 × 4.6 mm, 5µ)
Injection Volume	20µl
Flow rate	1.0 mL min^{-1}.
Detection Wavelength	275 nm
Run time	17 minutes
Temperature	30ºC

Results and discussion

The prime significance of application of PDA method is because of detection of entire spectrum simultaneously in three different dimensions i.e. light intensity, time and wavelength in comparison to simple UV-visible detection. The present method is quite useful in avoiding any interference of the drug with the excipients which are embedded in the polymeric network such as matrix and coated formulations. None of the analytical technique has been employed for the quantification of both drugs in formulations containing 5-FU and ETD using RP-HPLC-PDA method.

In the present study, both 5-FU and ETD have different physiochemical properties. 5-FU, a water soluble molecule, exhibits pH independent solubility whereas ETD, a hydrophobic molecule shows pH dependent solubility (in alkaline media at pH 6.5-7.5). The apposite wavelength for the detection of analytes was observed by scanning the UV spectrophotometry in the range of 200-400 nm for both 5-FU and ETD in alkaline media conditions of pH 6.8. The maximum wavelength of both analytes was observed in the range of 260-290 nm. Hence, the wavelength of the UV detector was kept at 280 nm.

The physicochemical interaction between the analytes in the solid state was also analyzed using DSC calorimetric analysis. The DSC thermogram of physical mixture (in ratio of 1:1) showed the characteristic endothermic peak of both the drugs (**Figure 2**). 5-FU showed a melting endothermic peak at 282.32 ºC with onset temperature of 276.82 ºC and heat of enthalpy of 444 J/g. In case of ETD, the drug showed its endotherm at 160.46 ºC with onset temperature of 157.06 ºC and heat of enthalpy of 37.34 J/g. The DSC studies did not reveal any type of physical or chemical state change for both 5-FU and ETD.

In order to develop suitable RP-HPLC-PDA method for the simultaneous estimation of 5-FU and ETD, different chromatographic conditions were employed and optimized chromatographic conditions were established. The chromatogram of the two drugs 5-FU and ETD indicated diverse solubilities and they eluted at different retention times (**Figure 3**). The optimized conditions developed for the simultaneous determination of both drugs are summarized in the **Table 3**. RP-HPLC-PDA analytical method for the simultaneous estimation of 5-FU and ETD was validated as per the ICH (Q2) guidelines.

Various validation characteristics (such as selectivity, linearity, precision, accuracy, robustness, LOD, LOQ and system suitability parameters) were investigated. The selectivity of the method was assessed with respect to interference due to the presence of any other excipients. It was observed that both the drugs 5-FU and ETD were successfully eluted from its excipients. Thus, the HPLC method presented in this study was found to be selective as depicted in **Figure 4.**

A linear relationship between area of chromatogram and concentration (µg/ml) was obtained in the range of 1-5 µg/ml for 5-FU as well as ETD. The data obtained was subjected to linear-regression analysis and calibration curves were obtained for 5-FU and ETD from which the linear regression equation was computed and found to be *y*=37675x-467.4, r^2=0.9973 for 5-FU and y=26799x+8183.7, r^2 = 0.9909 respectively (**Figures 5 and 6**).

Precision studies were carried out for both 5-FU as well as ETD. The summary of data for both 5-FU and ETD, indicating intra-day and inter-day precision is shown in **Table 4**. The developed method was found to be precise for both inter-day and intra-day in case of 5-FU and ETD with % RSD value <2%. From the above mentioned **Table 4**, it was evident that the developed method exhibited sufficient precision for ETD with corresponding % RSD values ranging from 1.18% to 1.78% for intra-day and 0.135% to 1.89% for inter-day precision studies. These values were found to be well within the permissible limits of 6.0%. Precision studies for 5-FU indicated that the analytical method was precise for determination of the drug. The corresponding % RSD values were found to be in the range of 0.968-1.28% for intraday whereas 1.09-1.32% for the inter-day precision.

Accuracy of the method was calculated by recovery studies at three levels by standard addition method. Recovery studies were performed for the both analytes (5-FU and ETD) and sufficient recovery of the drug analyte was obtained at each level of added concentration as summarized in **Table 5**. The percent mean recovery for the 5-FU was obtained in the range of 98.24% to 102.80% (n=3 for each level) with % RSD values of 0.136 to 0.418. Hence, the method showed acceptable limit of recovery of the spiked concentration of 5-FU.

Furthermore, ETD samples also showed good recovery when injected in a similar level of concentration pattern **(Table 5).** Mean percent recovery for the ETD was obtained in the range of 101.76 % to 103.56 % with % RSD values in the range of 0.227 to 0.980. The limit of detection as calculated by standard formula as given in ICH guidelines was found to be 0.101 µg/ml for 5-FU and 0.057 µg/ml for ETD. The RP-HPLC-PDA method for the simultaneous estimation of 5-FU and ETD was found to be robust with % RSD <2 and the results of relative retention time (R_t) at various flow rate and wavelength are showed in **Table 6.**

The limit of quantitation as calculated by standard formula as given in ICH guidelines was found to be 0.307 µg/ ml for 5-FU and 0.173 µg/ml for ETD. The system suitability parameters (such as retention time, symmetry factor, K-prime, theoretical plates, resolution, capacity factor, and tailing factor) have been compiled in **Table 7.**

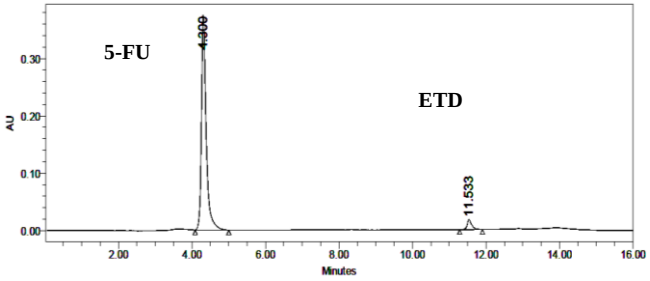

Figure 3: Chromatogram of 5-FU and ETD

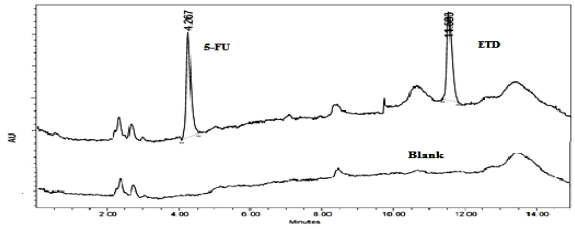

Figure 4: Overlay chromatograms of blank and sample preparation

Table 4: Precision data for 5-FU and ETD

Conc. of drug (µg/ml)	Precision studies for 5-FU (n=6)						Precision studies for ETD (n=6)					
	Intra-day precision			Inter-day precision			Intra-day precision			Inter-day precision		
	Mean amount (n=6)	% amount	% RSD	Mean amount (n=6)	% amount	% RSD	Mean amount (n=6)	% amount	% RSD	Mean amount (n=6)	% amount	% RSD
2	1.49	74.68	1.28	2.05	102.71	1.09	1.81	90.61	1.78	2.15	107.57	0.135
4	3.22	80.69	0.941	3.99	99.89	1.19	3.77	94.44	1.57	3.98	99.71	1.42
5	4.16	83.36	0.968	5.13	102.78	1.32	4.88	97.68	1.18	5.07	101.46	1.89

Table 5: Accuracy studies for 5-FU and ETD

Amount of each analyte (µg/ml)	Accuracy studies for 5-FU				Accuracy studies for ETD			
	Amount obtained (µg/ml)	% recovery	Mean % recovery	% RSD	Amount obtained (µg/ml)	% recovery	Mean % recovery	% RSD
10 + 10	19.592	97.96			20.541	102.70		
10 + 10	19.614	98.06	98.24	0.418	20.311	101.55	102.60	0.980
10 + 10	19.744	98.71			20.712	103.56		
10 + 20	29.313	97.71			37.157	100.89		
10 + 20	29.377	97.92	97.96	0.289	30.604	102.01	101.76	0.769
10 + 20	29.482	98.27			30.720	102.40		
10 + 30	41.059	102.64			41.345	103.36		
10 + 30	41.164	102.91	102.80	0.136	41.401	103.50	103.56	0.227
10 + 30	41.144	102.85			41.528	103.82		

Table 6: Data for Robustness

Parameters		Retention time	
Flow rate (±0.2 mL/min)	**Wavelength (±5nm)**	**5-FU (mean±%RSD)**	**ETD (mean±%RSD)**
0.8mL/min	-	4.22±0.21	11.49±0.17
1.0mL/min	-	4.31±0.22	11.72±0.14
-	275nm	4.3±0.395	11.70±0.084
-	280nm	4.24±0.59	11.89±0.083

Table 7: System suitability parameters

S. No.	Parameters	5-FU	ETD
1	Retention time	4.3	11.55
3	Symmetry Factor	0.82	1.45
4	K Prime	3.27	10.53
5	Theoretical Plates	6978.2	41585.45
6	Resolution	5.0	35.97
7	Capacity factor	0.94	4.11
8	Tailing factor	0.61	0.37

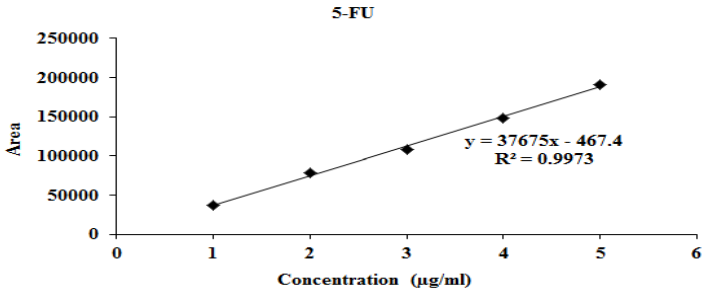

Figure 5: Linearity plot of 5-FU

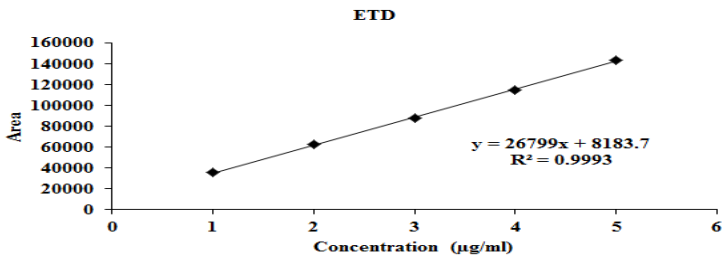

Figure 6: Linearity plot of ETD

Conclusion

The present RP-HPLC-PDA method for the simultaneous estimation of 5-FU and ETD offers various advantages in terms of simplicity in mobile phase, rapidity, and reproducibility. The validation data indicated good precision, accuracy, and reliability of the method for its intended use in simultaneous determination of 5-FU and ETD in bulk and tablet dosage form. Further, the validated method could be further exploited for the quantification of 5-FU and ETD necessary for the therapeutic drug monitoring in *in vitro*, pharmacokinetic studies, bioassays and clinical studies.

Competing interests

The authors confirm that this article content has no conflict of interest.

Acknowledgements

The authors express gratitude towards University Grants Commission, New Delhi for providing financial assistance to carry out this work. We are also thankful to Getwell Pharmaceuticals Pvt. Ltd., (Gurgaon, India) and IPCA Laboratories Pvt. Ltd, (Mumbai, India) for providing gift samples of 5-FU and ETD respectively.

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