Development and Validation of an Ecofriendly HPLC-UV Method for Determination of Capecitabine in Human Plasma: Application to Pharmacokinetic Studies

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ABSTRACT

A simple, rapid, cost-effective and green high-performance liquid chromatographic assay for determination of capecitabine in human plasma using a C_{18} reversed-phase analytical column was developed and validated. The separation was conducted by means of a mobile phase composed of formic acid solution (pH = 3): ethanol (55:45) running at a flow-rate of 1.0 mL min^{-1} with UV detection at 310 nm. The column temperature was set at 50 °C. Sample preparation involved protein precipitation by zinc sulfate–ethanol solution. This method is consistent with a high recovery of capecitabine in human plasma ranging from 95.98 to102.50 %.The calibration curves were linear over concentration range of 0.05–10.00 μg mL^{-1} ($r^2 > 0.9999$). Between- and within-day variability was less than 15 % and the bias was within ±15 %. This validated method was successfully applied to a pharmacokinetic study enrolling seven Iranian cancer patients after administration of a morning oral dose of 1500 mg.

KEYWORDS

Capecitabine, RP-HPLC method, ecofriendly, pharmacokinetics, human plasma.

1. Introduction

Colorectal and breast cancers, two of the most common types of neoplasms, are among the leading causes of death worldwide. These two diseases share some risk factors including smoking, exposure to environmental pollutants, physical inactivity, inadequate diet, excessive alcohol consumption and increased body-mass index from overweight to obese.[1–2] Thus, living a healthier lifestyle could be one of the strategies to prevent cancer. Beyond this, medical therapies are an essential part of cancer treatment.

Capecitabine is an oral 5-fluorouracil (5-FU) pro-drug commonly given to patients suffering from colorectal and breast cancer. It is rapidly and completely absorbed through the gastrointestinal wall as an intact molecule. This drug is first metabolized in the liver into 5′-deoxy-5-fluorocytidine (5′-DFCR) by human carboxyesterase isoenzyme-2 (hCES2). The metabolite is then converted into 5′-deoxy-5-fluorouridine (5′-DFUR) by cytidine deaminase in tumor cells and in the liver. Finally, 5′-DFUR is metabolized by thymidine phosphorylase into the cytotoxic agent 5-FU inside tumor cells.[3–5] The metabolism pathway of capecitabine is shown in Fig. 1.

During the past years, the healthcare costs especially for the anti-cancer drugs have progressively increased worldwide, and this has made the governments and authorities to consider regulations on drug pricing. It is therefore essential that high-quality, low-cost drug formulations are made available.[6] Conducting bioequivalence studies is of paramount importance to be sure about the efficacy and quality of such generic drugs. Regarding capecitabine, the Office of Generic Drugs (OGD) of the FDA

recommends a bioequivalence study to be carried out in cancer patients already receiving a stable dose (1250 mg/m2 twice daily) of this medicine. In this respect, both the FDA and the European Medicines Agency (EMA) bioequivalence guidelines[7–9] are on
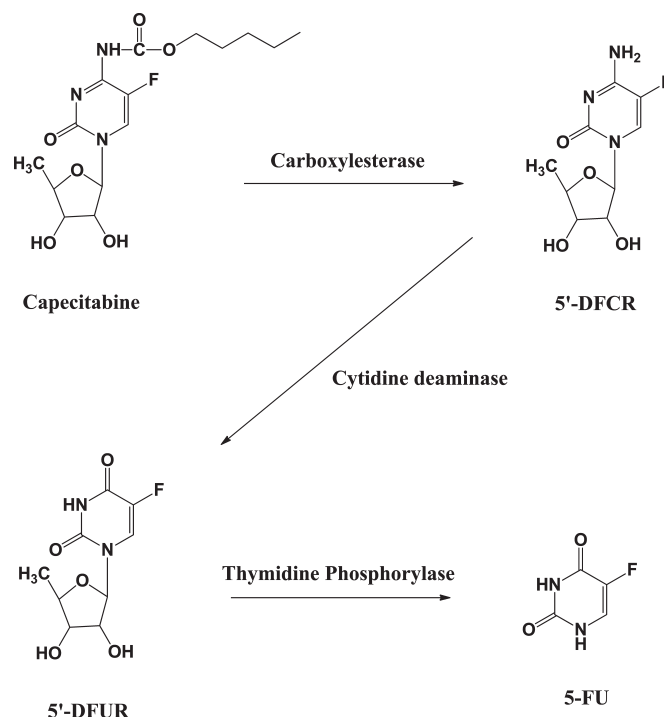

Figure 1 Metabolic pathway of capecitabine.

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the basis of comparison of capecitabine pharmacokinetic parameters obtained from the administration of the test and reference formulations. Therefore, simple chromatography methods for the bioanalysis of this drug should be available.

The most common methods to extract capecitabine from the plasma are liquid-liquid and solid-phase extraction, which are energy- and time-consuming sample-processing methods.[10–13] Moreover, in the most previously reported procedures, mass spectrometry has been used as a detection system.[13–18] These methods are highly specific and sensitive but involve expensive equipments which are not generally available in a clinical setting. The most important drawback of all previously published methods is the use of toxic and non-ecofriendly solvents such as acetonitrile in liquid chromatography or sample preparation steps. Because of inherent toxicity of such solvents,[19] safe detoxification of the waste is essential, which may lead to high to very high disposal costs. Otherwise, the analysis itself may be a source of environmental pollution. Therefore, it is reasonable to apply greener and more sustainable approaches in bioanalysis to avoid polluting the environment through such experiments.

This paper describes a rapid and sensitive and greener HPLC-UV method for analyzing capecitabine using direct protein precipitation. The method was successfully applied to assess pharmacokinetics of capecitabine following administration of a 1500 mg morning dose in seven cancer patients.

2. Experimental

2.1. Materials, Reagents and Chemicals

Qualified capecitabine standard (99.0 %) was kindly provided by Osveh Pharmaceuticals (Tehran, Iran). Absolute ethanol, gradient grade methanol and analytical grade reagents were purchased from Merck (Darmstadt, Germany). HPLC grade water was obtained through a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all the solutions.

2.2. Preparation of Standard Solutions

Standard stock solution (1.0 mg mL^{-1}) of capecitabine was prepared in methanol and stored in refrigerator at 4 ± 2 °C. This solution was found to be stable for at least one month at this temperature. Standard solutions were prepared daily from this solution by dilution with HPLC grade water.

2.3. Sample Preparation

An aliquot of 400 μL of human plasma was mixed with 300 μL of absolute ethanol and 30 μL of zinc sulfate solution (17 %) to precipitate the plasma proteins. The mixture was vortexed for 1 min and centrifuged at $15\,800 \times g$ in a 5415C Eppendorf centrifuge for 10 min. The clear supernatant was transferred into a clean tube and a volume of 100 μL was injected into HPLC column.

2.4. Apparatus and Chromatographic Conditions

The HPLC method was carried out on a Younglin (Hogye, South Korea), which was equipped with YL9104 Vacuum degasser, YL9110 Quaternary pump, YL9131 Column compartment, and YL9120 UV/VIS detector. The peak areas were integrated automatically by computer using an Autochro-3000 software program. A 100 μl volume of sample was introduced into a Rheodyne model 7725i injector. The elution was carried out on a C18 column (150 mm × 4.6 mm, 5 μm particle size) from Teknokroma (Barcelona, Spain) protected with a C18 guard column Teknokroma (Barcelona, Spain). All analyses were performed at the column temperature of 50 ± 1 °C under isocratic conditions with a mobile phase consisting of a solution of formic acid in water (pH = 3): ethanol (55:45) and a flow-rate of 1.0 mL min^{-1}, using UV detection at 310 nm. It is worth mentioning that the analysts were trained to manage the aqueous solutions containing this compound and also the potentially contaminated surfaces according to the previously published procedure.[20]

2.5. Data Evaluation and Method Validation

Method validation was carried out according to the *FDA Guidance for Industry Bioanalytical Method Validation*.[21] Data were evaluated using peak areas.

2.5.1. *Preparation of the Calibration Standards and Quality Control Samples*

In order to prepare quality control samples (QC) and calibration standards, firstly 100 μL of stock solution was vortex mixed with 900 μL of HPLC grade water. Then 100 μL of the prepared solution (100 μg mL^{-1}) was added into 900 μL of drug-free human plasma obtained from Iranian Blood Transfusion Organization (Tehran, Iran) and stepwise the final concentrations of 0.05, 0.25, 0.50, 2.50, 5.00, and 10.00 μg mL^{-1} for the calibration standards and 0, 0.05, 0.13, 1.00, 7.50 μg mL^{-1} for QC samples were prepared. The resulting mixtures were subjected to the sample pretreatment described in sample preparation section and injected into HPLC.

Five sets of such calibrators were prepared on five subsequent days. Each calibrator was injected in triplicate. Calibration data were acquired by plotting the peak area of capecitabine against the concentrations of the calibration standards, followed by a linear regression analysis.

2.5.2. *Precision, Accuracy, Limit of Quantification and Recovery*

Five replicate QC samples were injected between and within day. The assay precision was expressed as a percentage of RSD. Accuracy was calculated as the deviation of the mean from nominal concentration. The LOQ was defined as the lowest concentration of capecitabine at which the percentage deviation from the nominal concentration (accuracy) and the RSD were within ± 20 % and less than 20 %, respectively, considering at least ten times the response compared to that of the blank response. The recovery of capecitabine was determined by comparing the peak area of the processed QC samples with those obtained after the injection of the same amount of drug dissolved in HPLC grade water at different concentration levels.

2.5.3. *Specificity and Selectivity*

Control human plasma, obtained from 13 patients was assessed by aforementioned procedure and compared with respective spiked plasma samples to evaluate the selectivity of the method.

2.6. Application of the Method

Nine cancer patients (five males and four females) with histologically proven cancer who were treated with capecitabine (1500 mg twice daily) alone or together with other commonly used medicines in breast or colon cancer like cetuximab, dexamethasone, oxaliplatin were included in this study. The ages of patients were in the range of 54–79 years. Patients with a history of cardiovascular, renal or hepatic disorder were excluded from this study. The patients received 1500 mg capecitabine (Xeloda®; Hoffmann-La Roche Ltd., Basle, Switzerland) as a single oral dose after an overnight fast. The intake of food was delayed for 3 h post oral dose. Peripheral venous blood samples (5 mL each) were collected into heparinized tubes from each patient imme-

diately before and from 0.5 to 5 h after the morning dose. The blood samples were centrifuged immediately at 3000 × *g* for 10 min and plasma samples were stored at –20 °C until analysis.

This study was approved by Azad University of Pharmaceutical Sciences ethics committee (Reference number: 17697) and informed consents were obtained from each individual patient.

2.7. Calculation of the Pharmacokinetic Parameters

Plasma concentration–time curves of capecitabine were evaluated using non-compartmental analysis. The values of maximum plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were directly obtained from experimental observations. The elimination rate constant (k_e) was obtained from a log-linear regression analysis of the plasma concentration–time curves in the terminal phase. The terminal half-life ($t_{1/2}$) was calculated from k_e. The area under the concentration–time curve from 0 h to the last quantifiable concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. The area from the last measured concentration to infinity ($AUC_{t-\infty}$) was calculated by dividing the last measurable plasma concentration by the k_e. The clearance (CL) was calculated as fDose/$AUC_{0-\infty}$. Since the bioavailability of capecitabine was reported to be 100 %,[4] f was considered to be equal to 1.

The non-compartmental model analysis was performed on Microsoft Excel software version 2010. Data are expressed as mean ± standard deviation ($\overline{X}$ ± S.D.).

3. Results and Discussion

3.1. Selectivity, Specificity and Chromatography

The HPLC procedure was optimized with a view to develop a greener bioanalytical method. Green analytical techniques aim to minimize or eliminate the hazardous waste associated with analytical methods. In this context, it is reasonable to use ethanol-water as the mobile phase of choice for many RP-HPLC applications.[22] Therefore, ethanol was chosen as the organic modifier and different ratios of the ethanol and water were examined to obtain a symmetric peak of capecitabine with proper capacity factor. The best condition regarding retention time was achieved using a mobile phase consisting of ethanol:water (45:55, v/v), but the column pressure was too elevated at the flow rate of 1.0 mL min⁻¹. Therefore, the temperature was increased to 50 °C. Even so, the peak shape was not optimal under the aforementioned chromatographic condition. To solve this problem, water was replaced by a solution of formic acid in water (pH = 3). This change resulted in rather symmetric peaks with tailing factor of 1.31 ± 0.83 and theoretical plates of more than 2000. Figure 2 shows the chromatograms obtained from the method development.

According to these preliminary results, the detection wavelength of 310 nm, flow rate of 1.0 mL min⁻¹ and the mobile phase of formic acid solution (pH = 3):ethanol (55:45, v/v), were finalized. Under such chromatographic condition, capecitabine peak appeared at about 3.81 ± 0.06 min (*n* = 10) and no endogenous plasma component was eluted at this retention time, as evaluated by chromatograms of blank human plasma obtained from 13 patients and plasma spiked with capecitabine. A representative chromatogram from a plasma sample obtained 0.5 h after oral administration of capecitabine-based treatment from a patient, the blank plasma of the same patient and a chromatogram collected from human blank plasma spiked with
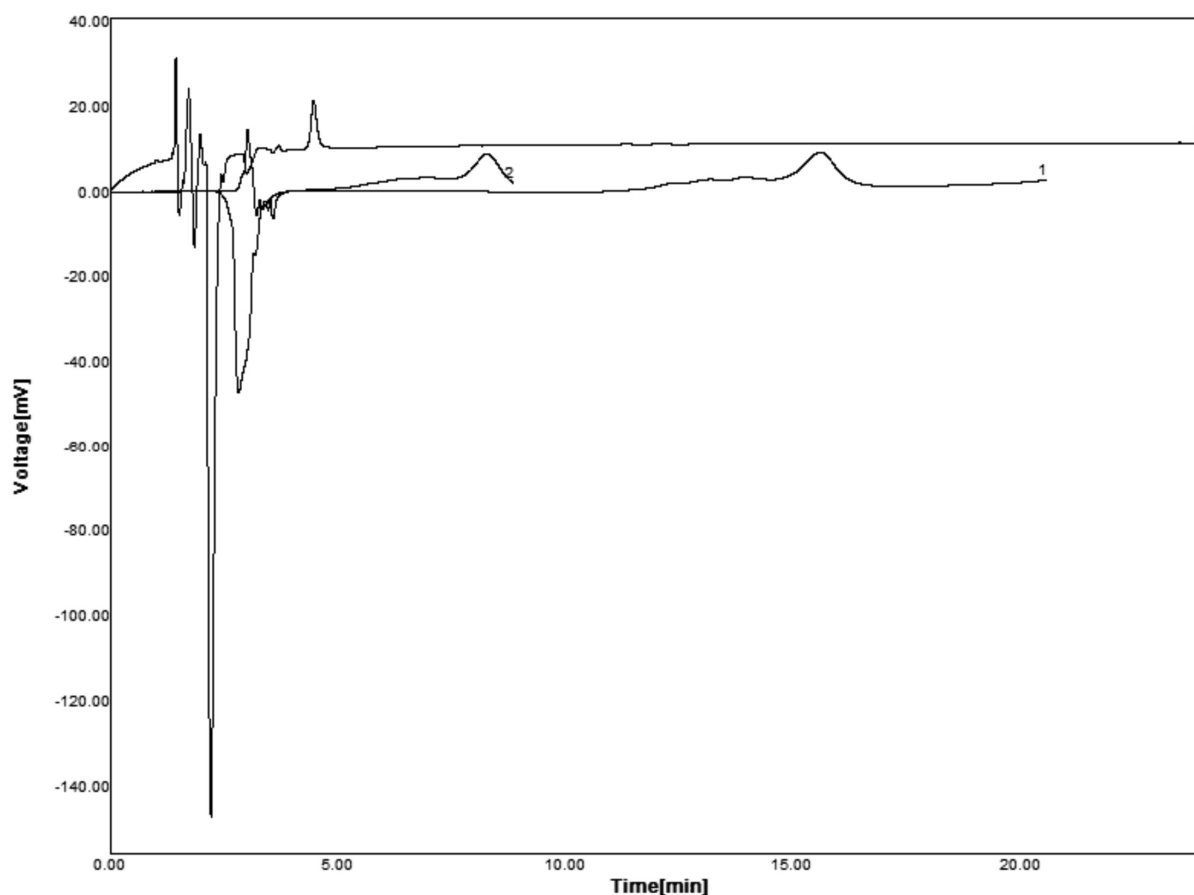

Figure 2 Chromatograms (1) and (2) were obtained from the injection of a standard solution of capecitabine in water using mobile phases consisting of 20 % and 40 % ethanol in HPLC grade water, respectively. Chromatogram 3 is the result of injection of the same standard solution using a mobile phase consisting of a solution of formic acid in water (pH = 3): ethanol (56:44). The column temperature was 50 °C ± 1 °C for the last chromatogram.

2.50 μg mL^{-1} capecitabine are shown in Fig. 3. It is worth mentioning that the major capecitabine metabolites like 5-flourouracil, 5′-DFCR and 5′-DFUR show their maximum absorbance at around 266 and 205 nm and do not have UV absorbance at 310 nm.[9–10] Therefore, they do not interfere with capecitabine assay at the chosen wavelength. Also, the co-administered drugs did not give any interfering peaks. No change in column efficacy and back pressure was observed over the entire study time.

3.2. Sample Preparation

The recovery of capecitabine was assessed using greener analytical solvents, ethanol and methanol. Although addition of three volumes ethanol to one volume of plasma produced complete precipitation of the plasma proteins, it was not satisfactory because of the higher LOQs. Lower LOQs and higher recoveries were achieved by adding zinc sulfate to ethanol. Interestingly, ethanol yielded sharper peaks and as a result lower LOQs compared to methanol. As shown in Table 1, the recoveries achieved in this study are in the range of 95.98–102.50 %.

3.3. Method Validation

Assay linearity was evaluated over the concentration range of 0.05–10.00 μg mL^{-1}, constructing six-point calibration curves. The equations for means of five standard curves is $y = (93.25x \pm 1.12) + (-2.10 \pm 0.26)$, ($r^2 = 0.9999$). Samples having concentration more than 10.00 μg mL^{-1} were first diluted with drug-free

plasma. LOQ, as previously defined, was 0.05 μg mL^{-1}, considering the mean accuracy value of 98.69 % and RSD value lower than 6.92 %, which seems to be proper to perform a bioequivalence and pharmacokinetic study.[6,24–27] This LOQ is lower than those of previously reported procedures[12,23] and comparable to that obtained using a HPLC-MS/MS method.[16] RSD values for between- and within-day determinations were found to be in the range of 5.58–6.92 % and 1.90–6.66 %, respectively (Table 1). These values document the high precision of the assay. They fulfill the validation criteria of an analytical method designed for pharmacokinetic and drug metabolism studies for which RSD values of less than 15 % are acceptable. Moreover, the accuracy was found to be in the range of 92.68–102.67 % (Table 1), that is acceptable for testing drug content in biological samples.

The stability of processed and non-processed quality control samples was assessed after four freeze–thaw cycles and after being kept for at least 5 h at ambient temperature ($n = 3$), respectively. The obtained accuracy and RSD values were in the range of 85–115 % and less than 15 %, respectively. These data confirm the stability of these samples.

3.4. Application of the Method

The proposed method was applied to determine the pharmacokinetic parameters of nine patients after receiving a 1500 mg (three 500 mg tablets) dose of capecitabine. In two out of nine patients, double peak phenomenon was observed in
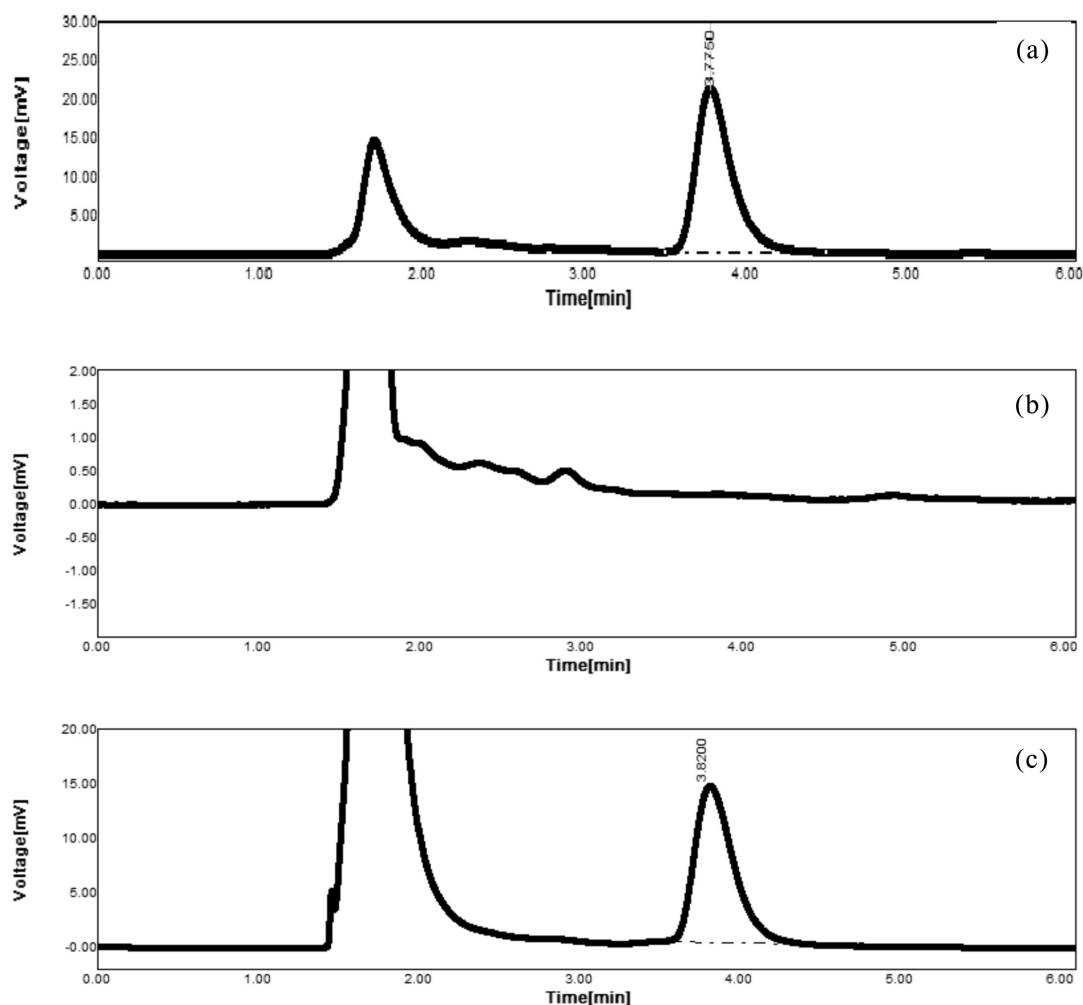

Figure 3 Chromatograms of (**a**) plasma of a patient 0.5 h after oral administration of a 1500 mg dose of capecitabine, (**b**) blank plasma of the same patient and (**c**) plasma spiked with 2.5 μg mL^{-1} capecitabine.

Table 1 Between- and within-day variability, accuracy and recovery for determination of capecitabine.

| Concentration /μg mL^{-1}	Between-day variability /$n = 5$		Within-day variability /$n = 5$		Recovery /$n = 5$	
	RSD/%	Accuracy/%	RSD/%	Accuracy/%	RSD/%	%
0.05	6.92	94.70	3.13	102.67	4.25	100.27
0.13	6.35	92.68	6.66	100.49	2.67	102.50
1.00	5.65	99.18	3.06	95.38	1.36	95.98
7.50	5.58	99.28	1.90	99.26	4.20	99.53

plasma concentration–time profiles, which could be due to disintegration of the tablets in gastrointestinal tract. The tablet that disintegrates slower may have delayed gastric emptying, resulting in a second absorption peak. Because there was not enough information in the terminal phase to calculate k_e, these two patients were excluded from the pharmacokinetic study. The C_{max}, T_{max}, AUC$_{0-\infty}$, $t_{1/2}$ and CL in the remaining seven patients were calculated to be 6.29 ± 5.06 μg mL^{-1}, 0.71 ± 0.27 h, 5.78 ± 3.22 μg h mL^{-1}, 0.53 ± 0.15 h and 345.31 ± 199.67 L h^{-1}, respectively. The typical plot of the capecitabine concentrations as a function of time in a patient is depicted in Fig. 4. The results of the main pharmacokinetic parameters are summarized in Table 2. Considering the fact that, capecitabine exhibits linear increases in C_{max} and AUC$_{0-\infty}$ when dosage increases,[4] it is possible to normalize the previously reported pharmacokinetic data to a dose of 1500 mg. Therefore, it can be concluded that the pharmacokinetic parameters obtained in this study were similar to the published pharmacokinetic studies[26,28] and it seems that there are no racial differences in capecitabine pharmacokinetics.

4. Conclusion

In this study a simple, economical and sensitive method for determination of capecitabine in human plasma was developed and fully validated on the basis of specificity, linearity, sensitivity, precision and accuracy, as well as recovery and stability. The method provided satisfactory sensitivity using a simple and fast protein precipitation procedure with total sample preparation time of about 15 min and did not involve any drying or reconstitution steps to achieve the desired sensitivity. This is suitable for the analysis of large numbers of biological samples, which normally exist in a bioequivalence or pharmacokinetic study and is in accordance with one of the principles of green analytical chemistry, which emphasizes on designing for energy efficiency.[29] The principal advantage of the method is the use of available environmentally friendly solvents for LC analyzing and sample preparation to follow the first principle of green chemistry which emphasizes on the waste prevention instead of remediation.[19] To the best of our knowledge this is the first method which has been introduced as 'green'. The assay was

Table 2 Individual pharmacokinetic data obtained from seven patients following oral administration of 1500 mg of capecitabine.

Patient	T_{max} /h	C_{max} /μg mL^{-1}	AUC$_{(0-\infty)}$ /μg h mL^{-1}	$t_{1/2}$ /h	CL /L h^{-1}
1	0.50	16.19	11.28	0.42	132.98
2	0.50	2.99	2.80	0.47	535.71
3	1.00	1.78	2.21	0.69	678.73
4	1.00	2.05	3.91	0.33	383.63
5	0.50	6.39	5.44	0.53	275.74
6	0.50	8.81	8.27	0.54	181.38
7	1.00	5.85	6.55	0.75	229.01
Mean	0.71	6.29	5.78	0.53	345.31
S.D.	0.27	5.06	3.22	0.15	199.67
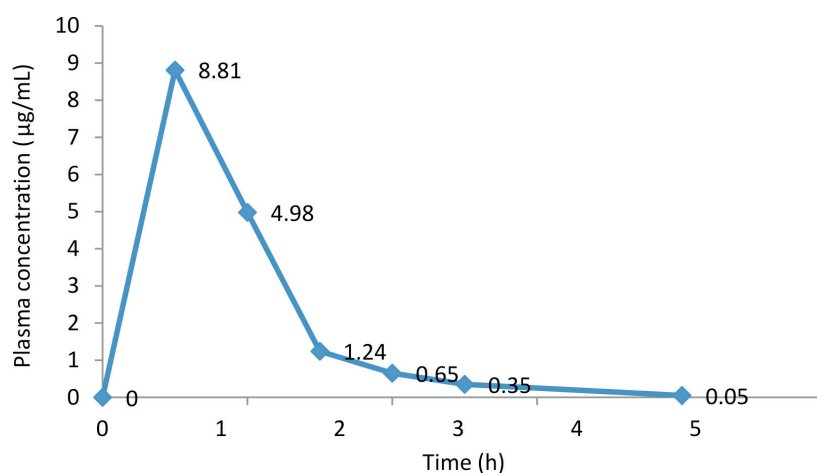
Figure 4 Plasma concentration–time profile of capecitabine after administration of a 1500 mg morning dose to a cancer patient.

successfully used in pharmacokinetic study of capecitabine after administration of a single oral dose of 1500 mg in seven Iranian cancer patients.

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