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Determination of Capecitabine and Its Metabolites in Plasma of Egyptian Colorectal Cancer Patients

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Abstract: The incidence of colorectal cancer (CRC) is increasing worldwide. It has variable signs and symptoms starting from changes in bowel habit to nausea and vomiting. Chemotherapeutic agents are often prescribed in CRC such as Capecitabine (CCB) and 5-Fluorouracil (FU). CCB is the prodrug of FU in oral dosage form, which makes it preferable by physicians, since no hospitalization is needed for drug administration. CCB is activated to FU in a three-step reaction producing 5′-deoxy-5-fluorocytidine (DFCR) (by carboxylesterase (CES) enzyme), then 5′-deoxy-5-fluorouridine (DFUR) (by cytidine deaminase (CDD) enzyme) and finally FU (by thymidine phosphorylase (TP) enzyme), the active form, which is later deactivated to give 5,6-dihydro-5-fluorouracil (DHFU). Different patients exhibit variable drug responses and adverse in response to CCB therapy, despite being treated by the same dose, which could be attributed to the occurrence of different possible enzyme single nucleotide polymorphisms (SNPs) along the activation and deactivation pathways of CCB. The most commonly occurring toxicities in CCB therapy are hand-foot syndrome and diarrhea. This study aims at developing and validating a new method for the simultaneous determination of CCB and its metabolites by HPLC-UV, followed by a correlation study with the toxicities occurring during therapy, where predictions of toxicity could be based on metabolites' levels instead of the tedious process of genotyping. A new superior analytical method was optimized by a quality-by-design approach using DryLab® 2000 software achieving a baseline resolution of the six analytes within the least possible gradient time of 10 min. The method also showed linearity (in a range from 1 to 500 µg/mL), accuracy, precision and robustness upon validation: The LOD was found to be 3.0 ng/mL for DHFU and CCB, and 0.3 ng/mL for DFUR, DFCR and FU. The LOQ was found to be 10.0 ng/mL for DHFU and CCB, and 1.0 ng/mL for DFUR, DFCR and FU. The clinical results showed a positive correlation between the concentration of DFCR and mucositis and between the concentration of DFUR and hand-foot syndrome, confirming that this technique could be used for predicting such toxicities.

Keywords: quality by design; colorectal cancer; Capecitabine; metabolites; HPLC; cancer

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1. Introduction

Capecitabine (CCB) (Pentyl [1-(3, 4-dihydroxy-5-methyltetrahydrofuran-2-yl)-5-fluoro-2-oxo-1H-pyrimidin-4-yl] carbamate, $C_{15}H_{22}FN_3O_6$, FM: 359.99 [1]) is an oral chemotherapeutic agent (Figure 1). CCB belongs to the fluoropyrimidine carbamate family, primarily employed in the treatment of colorectal cancer as a neo-adjuvant therapy with radiation, adjuvant therapy or for metastatic cases. It is marketed as a pro-drug where it is activated to the well-known anti-neoplastic agent 5-Fluorouracil (FU) with the unique advantage of being orally administered in contrast to FU [2]. CCB oral administration makes it much

more convenient for patients compared to intravenous 5-FU, providing better patient compliance, eliminating the need for frequent hospitalization and intravenous infusions. This convenience can lead to better patient compliance and improved quality of life.

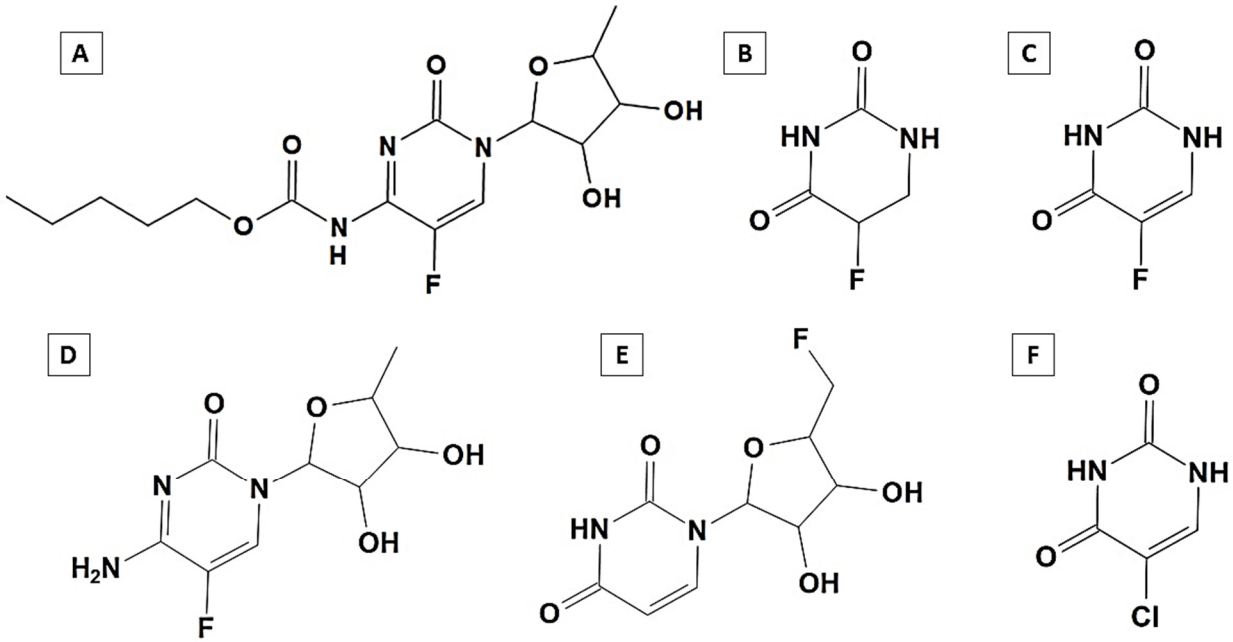

Figure 1. The chemical structures of the six analytes CCB, DHFU, FU, DFCR, DFUR and CLU are labeled (**A**–**F**).

The first step in CCB metabolism occurs by hepatic Carboxylesterase (CES) 1 and 2 giving 5′-deoxy-5-fluorocytidine (DFCR) which is then converted to 5′-deoxy-5-fluorouridine (DFUR) by Cytidine Deaminase (CDD) present in liver and tumor tissues. The activation of DFUR occurs by Thymidine Phosphorylase (TP) producing FU. Since the enzymes involved in catalytic activation are found in higher levels in tumor tissues than in normal tissues, the selective activation of CCB to FU in the tumor tissue occurs. Following oral administration of CCB, the average ratio of FU concentration in tumor-to-plasma is 21.4, whereas the average ratio in healthy tissues-to-plasma is 8.9. Prior to excretion, FU is deactivated by Dihydropyrimidine Dehydrogenase (DPD) to 5,6-dihydro-5-fluorouracil (DHFU) where DPD activity is the rate-limiting step, followed by Dihydropyrimidinase cleavage of the pyrimidine ring to yield 5-fluoro-ureidopropionic acid (FUPA). Finally, β-Ureido-Propionase cleaves FUPA to give α-fluoro-β-alanine (FBAL) excreted in the urine. CCB undergoes rapid oral absorption, followed by extensive metabolism to DFCR and DFUR (Figure 2). At the dose of 1250 mg/m^2 on Day 14, the time taken for the concentration to peak in plasma (t_{max} in hours) was 1.50, 2.00, 2.00 and 2.00 for CCB, DFCR, DFUR and FU, respectively. The AUC values (μg·h/mL) were 7.75, 7.24, 24.6 and 2.03 [1,3,4]. Figure 1 shows the chemical structure of CCB and its major metabolites DFCR, DFUR, FU and DHFU.

In various studies, the quantification of drug metabolite concentrations has served as a crucial indicator of the activity of metabolizing enzymes. For instance, several investigations have linked the presence of DPD SNPs to diminished DPD activity, leading to reduced FU clearance and elevated FU plasma levels, along with notably diminished DHFU plasma levels [5–7]. Concerning the metabolites in the CCB activation pathway, a correlation was found between the DFUR AUC in plasma and toxicity following the oral administration of CCB in monkeys, mice and rats [8]. Clinical studies examining drug concentrations on different days have revealed associations between FU AUC and Grade 3–4 hyperbilirubinemia, FU AUC and time to disease progression, as well as DFUR C_{max} and survival [9]. Notably, both 5′DFUR AUC and FU AUC exhibited significant but

quantitatively moderate and reversible accumulation throughout the treatment course, with comparable AUC values on Day 2 of Cycle 1 and Day 2 of Cycle 2 [10].

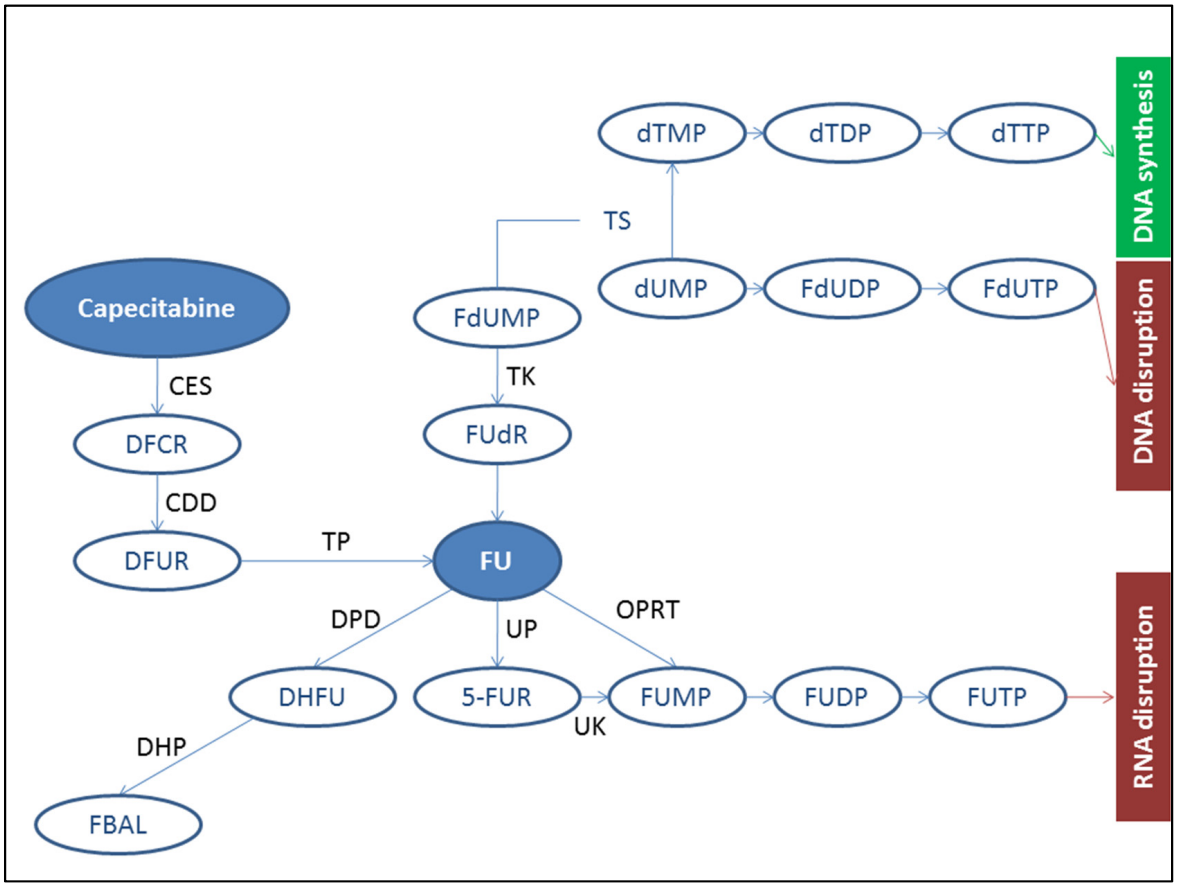

Figure 2. Metabolism of CCB [5].

Various analytical methods have been developed for the qualitative and quantitative analysis of CCB and its diverse metabolites. An LC-MS/MS assay has been established for quantifying CCB, DFCR, DFUR, FU, and DHFU in human plasma. This assay, employing 200 µL human plasma aliquots, offers quantification within a range of 10–1000 ng/mL for CCB, 10–5000 ng/mL for DFCR and DFUR and 50–5000 ng/mL for FU and DHFU, utilizing a mixture of fludarabine and 5-chlorouracil as an internal standard (IS). Separation was achieved using a 30 × 2.1 mm Hypercarb (porous graphitic carbon) column with a liquid chromatography (LC) runtime of 15 min. The retention times for CCB, fludarabine, DFUR, 5-chlorouracil, DFCR, FU and DHFU were 11.5, 8.5, 6.5, 6.2, 6.0, 5.5 and 2.8 min, respectively. This method was applied to evaluate the pharmacokinetic profile of CCB and its metabolites in plasma from treated cancer patients [11].

An LC/MS method was reported to separate CCB, DFUR, DFCR and FU using 50 µL plasma. CCB and DFUR, DFCR and FU curves were linear over a range of 5–1000 ng/mL, 10–2000 ng/mL and 50 to 10,000 ng/mL, respectively. Compounds were separated on a Develosil ODS-UG-3 column (4.6 mm × 150 mm, 3 µm with the mobile phase consisting of acidified water and acetonitrile. The method used gradient elution with a total runtime of 12 min and the column was maintained at 30 °C [12].

Another LC/MS method has been reported for the separation of CCB, DFUR, DFCR and FU using just 50 µL of plasma. Calibration curves for CCB, DFUR, DFCR and FU demonstrated linearity within the ranges of 5–1000 ng/mL, 10–2000 ng/mL, and 50–10,000 ng/mL, respectively. Separation was performed on a Develosil ODS-UG-3 column (4.6 mm × 150 mm, 3 µm) with a mobile phase consisting of acidified water and

acetonitrile. The method employed gradient elution with a total runtime of 12 min and the column temperature maintained at 30 °C [13].

The previously reported HPLC-UV method conducted was an assay for the determination of plasma capecitabine, DFUR, FU and DHFU. The limit of detection was 0.01 µg/mL for CCB, and its nucleoside metabolites and the limit of quantification was 0.025 µg/mL where the linear range was from 0.025 to 10,000 µg/mL. The retention times of DHFU, FU, DFUR and CCB were 3.6, 4.4, 11.4 and 20.4 min, respectively, and the internal standard retention times were 8.7 and 12.2 min for 5-bromouracil (BU) and 5-Fluoro-1-(tetrahydro-2-furyl) uracil, respectively. The column used was an Atlantis dC18 and gradient elution was performed using a mobile phase consisting of acidified water and methanol. The extraction was performed using 500 µL of plasma, and the detection wavelength was at 205 nm, 266 nm and 310 nm simultaneously [14].

CCB and its metabolites primarily circulate in the blood bound to albumin. Therefore, their concentrations in plasma are typically measured after extraction and liberation from protein binding. While some studies have employed the "Liquid-Liquid Extraction" (LLE) method for analyte extraction [12,14], others have adopted a simpler approach, involving protein precipitation [11,13]. The latter method was chosen for its simplicity, speed and high recovery percentages.

The aim of this study is the use of systematic HPLC method development aided with DryLab® to design a quantitative analytical method with a defined two-dimensional design space for the routine analysis of CCB and its major metabolites DFCR, DFUR, FU and DHFU in human plasma. The main target in this development is to obtain a short runtime, with the best possible resolution, with exact determination of areas with major robustness to provide enough motivation for professionals to adopt it in every hospital equipped with the necessary instruments for drug monitoring purposes. Finally, method validation must be performed to ensure its reliability. 5-Chlorouracil (CLU) was chosen as the internal standard because it is not naturally present in the sample, it is structurally closely related to the analytes, and it has acceptable retention time and recovery from plasma (Figure 1).

The study aims to correlate the patient's clinical data with the metabolites' levels quantified by the developed method to assess their relationship with CCB toxicity, to predict drug response and toxicity in individual patients in order to permit dose tailoring and optimization of the effect with the fewest possible toxicity symptoms.

2. Materials and Methods

2.1. HPLC Method Development for the Determination of Capecitabine and Its Metabolites Using HPLC-UV

2.1.1. Chemicals and Reagents

CCB together with its major metabolites DFCR, DFUR, FU and DHFU were purchased from Santa Cruz Biotechnology (USA) and have been stored in portions at low temperatures (<−20 °C) and continuously protected from light and humidity. The absence of degradation products was confirmed via chromatography. 5-Chlorouracil (CLU), used as an internal standard, and Trifluoroacetic Acid (TFA) were bought from Sigma-Aldrich (Hamburg, Germany).

Methanol (HPLC grade), Phosphoric acid, Ammonium Acetate and Tetrabutyl Ammonium Hydrogen Sulfate were also purchased from Sigma-Aldrich (Hamburg, Germany). Ultra-pure water was always freshly prepared throughout the analyses using Purelab UHQ water (ELGA, Woodridge, IL 60517, USA).

2.1.2. Equipment and Software

Chromatographic separations were performed on a Thermo Finnigan Spectrasystem® (Hertfordshire, UK) coupled to a Spectrasystem UV3000 detector. Three columns were used throughout this study and their performance compared (Table 1). A Phenomenex Fusion-RP AJ0-7557-S (dimensions (mm): 3 × 4 particle size (µm): 4) guard column was also used throughout the study. The HPLC method development was performed using

DryLab®2000 and PeakMatch® software (Molnár Institute for Applied Chromatography, Berlin, Germany).

Table 1. Columns used throughout the study and their characteristics.

Column Type	Source	Particle Size	Dimensions (Mm)	Features
HYPERSIL GOLD C18	Thermo Scientific, Waltham, MA, USA	3 µm	150 × 4.6	C18 (Commonly used in pharmaceutical and biomedical applications)
HYPERSIL GOLD C8	Thermo Scientific	5 µm	250 × 4.6	C8
SYNERGI FUSION-RP	Phenomenex, Torrance, CA, USA	4 µm	150 × 4.6	Polar embedded ligand and hydrophobic ligand, Tetramethylsilane (TMS) end capping. Stable within a pH range of 1.5–10.0 [15]

2.1.3. The Choice of Column and Method Development Strategy

In order to achieve the best resolution in the least retention time of CCB and its metabolites, three different columns were tested in this study. The first column used was the Hypersil Gold C18; next, the Hypersil Gold C8 column was tested; followed by the Synergi Fusion-RP Column.

In an attempt to enhance the retention of the most polar metabolites, ion-pair chromatography was employed. Ion-pairing reagents, such as tetraalkylammonium salts, were added to the mobile phase to facilitate the retention of acidic or basic analytes [16]. For acidic analytes, tetraalkylammonium salts were used to temporarily form a complex with the analyte's polar groups, while allowing the hydrophobic groups to interact with the RP column [17]. Two different runs were conducted with different aqueous mobile phases (A) utilizing the gradient time (t_G) versus temperature (T) approach (Table 2).

The initial conditions for gradient elution were 5% B (methanol) and phosphate buffer pH 2.6 (A) with a runtime of 20 min. These conditions were tested on the Synergi Fusion-RP column, and while a good resolution and reasonable retention times were achieved for most analytes, CCB exhibited a high retention time of 16.2 min. To optimize the method, four different runs were performed using DryLab® software (Table 2). The run with the highest T (45 °C) and the shortest t_G exhibited the shortest retention times due to the temperature-increased kinetic energy.

The choice of the gradient time versus temperature approach for method development was based on two main factors: (i) the retention of the analytes and the resolution of their separation are highly dependent on column temperature. By varying the temperature, the kinetic energy of the molecules can be modulated, influencing their retention and separation [18]. (ii) The water content of the mobile phase is a critical factor affecting the elution of analytes from a C18 column [19]. By varying the mobile phase composition and gradient elution method, using acidified water of pH 2.6 as the aqueous mobile phase (A) and methanol as the organic mobile phase (B), the elution properties and resolution of the analytes can be optimized.

2.2. Validation of the Newly Developed HPLC Method

According to the ICH guidelines [20], the newly developed HPLC method was validated to ensure its linearity, precision and accuracy of analytical results. To validate the method linearity, calibration standards were prepared within the following concentration ranges: DHFU and CCB: 10 to 2 × 10^4 ng/mL, DFUR and DFCR: 1 to 2 × 10^4 ng/mL and FU: 1 to 1 × 10^5 ng/mL) (Table 3). Five calibration curves were constructed for each metabolite on three different days and the slope, intercept and the coefficient of correlation were calculated. The concentrations of quality control samples were determined in duplicate on different days, and precision was expressed as the relative standard deviation or coefficient of variation (C.V.%), calculated as C.V.% = (standard deviation)/mean × 100.

Table 2. Chromatographic conditions for experimental runs initially performed using different types of column: "run1 and run2", different ion-pairing reagents to enhance the retention of polar metabolites: "run2 and run3", 4 basic drylab: "drylab1, drylab2, drylab3, drylab4" and the run using optimum conditions generated by drylab: "optimum".

Run	Column	T_g (Min)	T (°C)	Mobile Phase A	Mobile Phase B	%B Range	Detection	Flow Rate	Injection Volume
RUN 1 (Figure 3C)	Hypersil Gold C18	20	25	Acidified water with phosphoric acid, pH 2.6	Methanol	5–100% B	205, 266, 310 (nm)	1 (mL/min)	25 (µL)
RUN 2 (Figure 3D)	Hypersil Gold C8	20	25	Acidified water with phosphoric acid, pH 2.6					
RUN 3 (Figure 3E)		20	25	20 mM ammonium acetate at pH 4.0					
RUN 4 (Figure 3F)		20	25	5.0 mM tetrabutylammonium hydrogen sulfate at pH 8.0					
DRYLAB1 (Figure 4A)	synergi fusion RP	20	25	Acidified water with phosphoric acid, pH 2.6					
DRYLAB2 (Figure 4B)		60	25						
DRYLAB3 (Figure 4C)		60	45						
DRYLAB4 (Figure 4D)		20	45						
OPTIMUM (Figure 4E)		27	45			0–7 min (5–75% B), 7–20 min (75–100% B)			

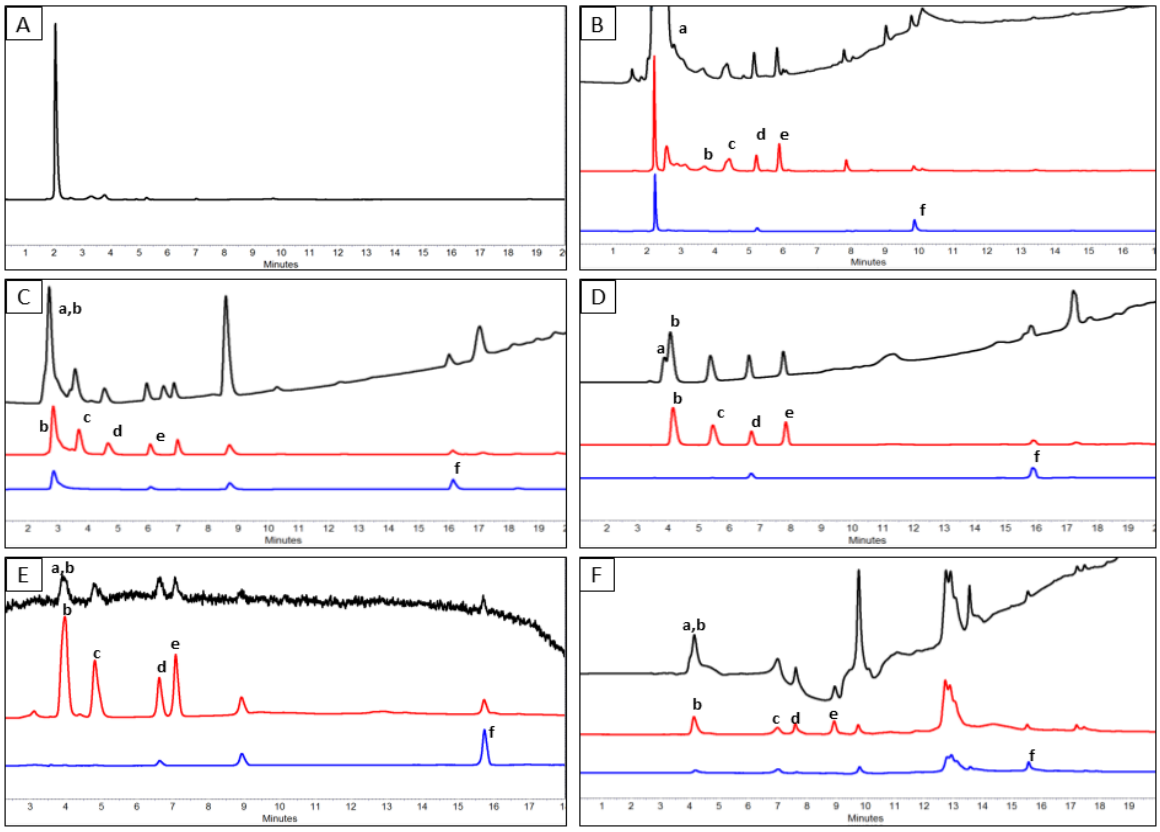

Figure 3. (**A**): Extracted unspiked plasma sample (detector: 266 nm), (**B**): Experimental chromatogram of extracted plasma sample spiked with the six analytes of interest, (**C**): Experimental chromatogram

of a standard mixture of six analytes of interest using a Hypersil Gold C18 column (K′ of 1st peak is 0.33), (**D**): Experimental chromatogram of a standard mixture of six analytes of interest using a Hypersil Gold C8 column (K′ of 1st peak is 0.44), (**E**): Experimental chromatogram of a standard mixture of six analytes of interest using a Hypersil Gold C8 column with mobile phase (A: 20 mM ammonium acetate at pH 4.0, pH 2.6, B: Methanol), (**F**): Experimental chromatogram of a standard mixture of six analytes of interest using a Hypersil Gold C8 column with mobile phase (A: 5 mM tetrabutylammonium hydrogen sulfate at pH 8.0, pH 2.6, B: Methanol). Experimental Conditions (common for Figure 3B–F): analytes (DHFU, FU, CLU, DFCR, DFUR and CCB) are labeled as a, b, c, d, e and f, respectively. Internal standard: CLU at a concentration of 104 ng/mL. Detection wavelengths: 205 nm (black), 266 nm (red) and 310 nm (blue) simultaneously. Gradient elution: 5–100% B, stepwise gradient time: 20 min (0–7 min: 5–75% B, 7–20 min: 75–100% B), runtime: 27 min, temperature: 25 °C, flow rate: 1 mL/min, injected volume: 25 µL.

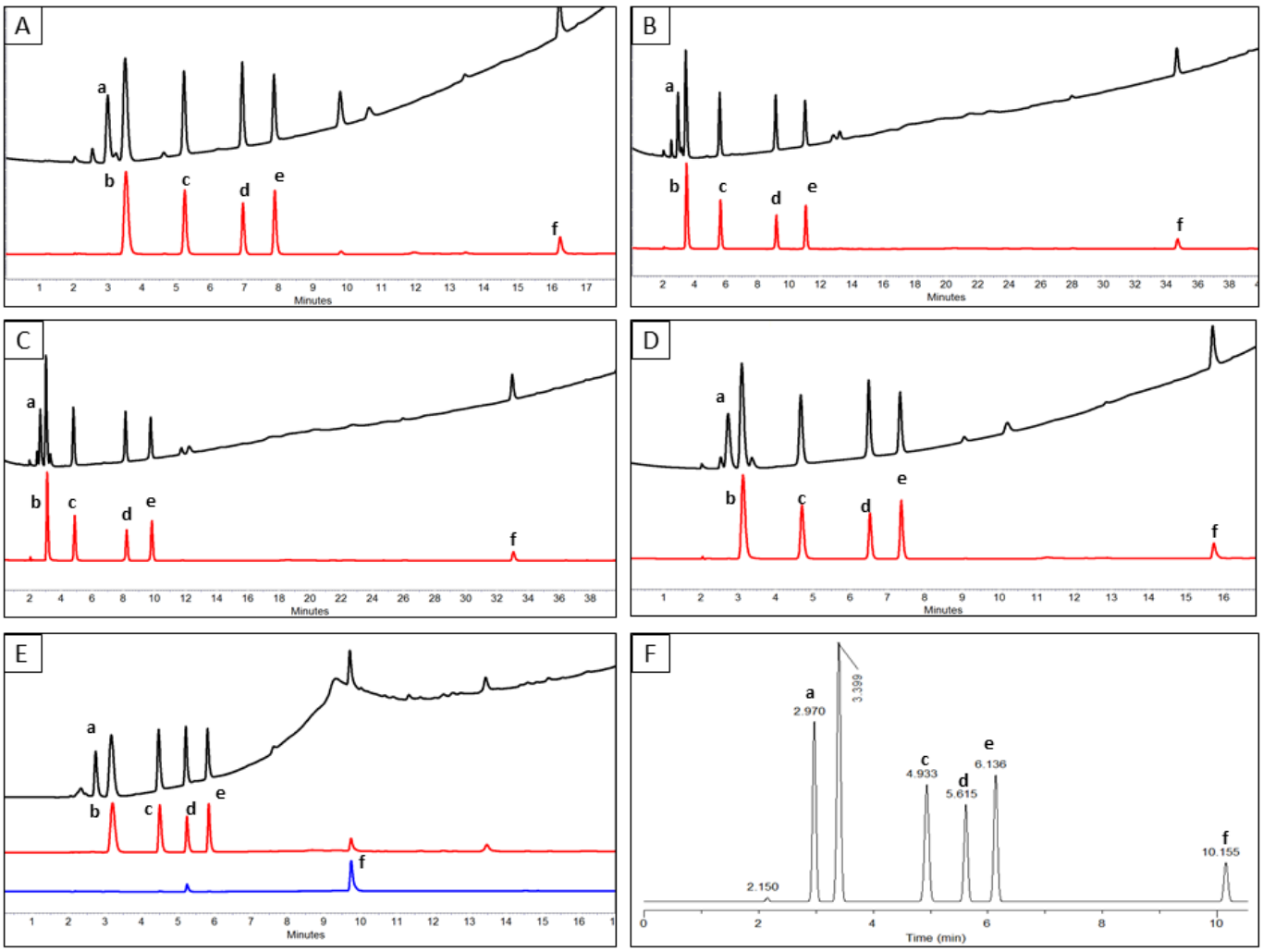

Figure 4. (**A**–**D**) Chromatograms of the 4 basic "synergi fusion-RP column" runs of altering temperatures and gradient (aqueous: t_G = 20 min, T = 25 °C; B: t_G = 60 min, T = 25 °C; C: t_G = 60 min, T = 45 °C; D: t_G = 20 min, T = 45 °C). (**E**) Experimental chromatogram of a standard mixture of the six analytes of interest on a Synergi Fusion-RP column using the optimum chromatographic conditions predicted from the 2D resolution map (mobile phase A: acidified water with phosphoric acid, pH 2.6, mobile phase B: methanol; 0–7 min (5–75% B), 7–20 min (75–100% B), t_G: 27 min, T = 45 °C, flow rate: 1 mL/min, injected volume: 25 µL). (**F**) Predicted chromatogram proposed by DryLab®. The six analytes of interest DHFU, FU, CLU, DFCR, DFUR and CCB are labeled a, b, c, d, e and f, respectively. The detection λ is 205 nm (black), 266 nm (red) and 310 nm (blue) simultaneously.

Table 3. Stock concentration of analytes used in the HPLC method validation.

Analyte	DHFU	FU	DFCR	DFUR	CCB
Conc 1 (ng/mL)	10	1	1	1	10
Conc 2 (ng/mL)	$1 * 10^2$	$1 * 10^2$	$1 * 10^2$	$1 * 10^2$	$1 * 10^2$
Conc 3 (ng/mL)	$1 * 10^3$	$1 * 10^3$	$1 * 10^3$	$1 * 10^3$	$1 * 10^3$
Conc 4 (ng/mL)	$1 * 10^4$	$1 * 10^4$	$1 * 10^4$	$1 * 10^4$	$1 * 10^4$
Conc 5 (ng/mL)	$2 * 10^4$	$2 * 10^4$	$1 * 10^5$	$2 * 10^4$	$2 * 10^4$

The accuracy of the developed method was validated using quality control samples with different concentrations of the 5 analytes, shown in Table 3, as spiked plasma samples to assess accuracy. The %Bias was calculated as %Bias = [(measured value − true value)/true value] × 100.

The limits of detection (LOD) and quantification (LOQ) were determined for each analyte, with LOD defined as the lowest detectable concentration at a signal-to-noise ratio of 3 and LOQ as the lowest concentration with a signal-to-noise ratio of 10.

Selectivity was also proven by comparison between the chromatogram of unspiked plasma of a healthy donor with the chromatogram of plasma of a healthy donor spiked with the internal standard and the 5 analytes of interest to prove that the response is only the result of the compounds of interest.

Validation during routine analysis was also performed by calculating the slope, the intercept of the regression line and the coefficient of correlation. During routine analysis, only FU, DFCR and DFUR (prepared in the 3 concentrations: $1 * 10^3$ ng/mL, $1 * 10^4$ ng/mL and $2 * 10^4$ ng/mL) were considered in the quality control samples, as the application of the current study focuses on the quantification of the activation pathway metabolites of CCB.

2.3. Analytes Extraction from Plasma and Assay Application to Patient Plasma Samples

The sample population of this study consisted of 20 CRC patients. Samples of 2–3 mL venous blood were obtained in EDTA tubes in triplicate from each patient. The sampling points were set at Day 1 of Cycle 1, Day 14 of Cycle 1 and Day 1 of Cycle 2, two hours after CCB oral ingestion. These sampling times were chosen to coincide with the patients' visits to the NCI so as to facilitate the process of sample collection, especially since it was noted that there is no unified protocol used for sample collection when monitoring CCB's concentration levels [3,9,10,21]. For certain patients, a sample on Day 7 of Cycle 1 was interchanged with the sample on Day 14 of Cycle 1 (both being in the drug's steady state). The sampling points were set to be during Cycle 1 so as to fulfill the aim of this study, which is using the plasma concentrations of CCB and its metabolites as an indicator to predict the toxicities that might occur later during the 8 cycles of CCB therapy.

Periodic follow-ups with the patients were continuously performed in order to trace the patients' status, ensure their compliance and most importantly to investigate signs of toxicity and possible reasons for therapy stoppage. Before samples were taken, the purpose and plan of the study were fully explained to the patient, and written consent was obtained. This was performed after approval was obtained from the Ethics Committee of the German University in Cairo, in addition to an IRB approval from the NCI's board. Information regarding the prescribed dose of CCB was obtained, together with the evaluation of toxicity symptoms and grades for each patient over the first 4 to 6 cycles of treatment.

The adopted extraction procedure was based on that developed by Vainchtein et al. [11] being rapid, simple and exhibits high percent recovery. Analytes' extraction was conducted using a simple protein precipitation step as follows: 20 µL internal standard (CLU, 100 µg/mL) was added to a 160 µL plasma sample followed by vortexing for 10 s. Plasma proteins were then precipitated with 20 µL 99% (*v*/*v*) TFA in water, followed by vortexing for 1 min at 1400 rpm. Samples were centrifuged for 20 min at 14,000 rpm at 4 °C. Finally, the clear supernatant was transferred to a glass auto sampler vial with an insert [11].

3. Results

3.1. Analytical Method Development

3.1.1. Preliminary Trials for the Choice of a Suitable Analytical Column

The Hypersil Gold C18 column, initially used to resolve the five analytes of interest, resulted in unretained FU and DHFU being highly polar with retention factors (K′) of 0.33 and 0.4 for DHFU and FU, respectively (Figure 3C). Since the retention factor is smaller than 0.5, the method could not be accepted as per the recommendations of the ICH [20]. The Hypersil Gold C8 column resulted in a similar selectivity with lower retention times for the analytes of interest (Figure 3D). Changing the aqueous mobile phase (A) to 20 mM ammonium acetate at pH 4.0 (Figure 3E) or 5 mM tetrabutylammonium hydrogen sulfate at pH 8.0 (Figure 3F) did not solve the FU-DHFU separation problem as the retention factors values of the least retained peak remained less than 0.5. Finally, the Synergi Fusion-RP column successfully achieved retention and the full separation of DHFU and FU, with K′ of DHFU = 0.6, K′ of FU = 0.83 and the separation factor (α) = 1.4 (Figure 4). While good resolution and reasonable retention times were achieved for most analytes, CCB exhibited a high retention time of 16.2 min.

3.1.2. Computer-Assisted Analytical Method Optimization

The results from 4 "Synergi Fusion-RP column" basic runs Figure 4A–D) were fed into DryLab Software in order to generate a 2D resolution map correlating the resolution (R_s) of the critical peak peaks (DHFU and FU) to the studied factors (t_G and T) (Figure 5). Using the resolution map (Figure 5), the best resolution was found to be a stepwise gradient, with the following chromatographic conditions: Mobile phase (A: Acidified water with phosphoric acid, pH 2.6, B: Methanol), gradient: 5–100% B, stepwise gradient time: 20 min; 0–7 min (5–75% B), 7–20 min (75–100% B), runtime: 27 min, temperature: 25 °C, detection: 205, 266 and 310 nm simultaneously, flow rate: 1 mL/min and injected volume: 25 µL. This stepwise gradient was designed to decrease the retention time of CCB from 16.2 min to 9.9 min, together with enhancing the peaks resolution of the critical peak pair (DHFU and FU). This predicted method was practically implemented and showed an excellent match between the experimental chromatogram (Figure 4E) and the predicted one proposed by DryLab® (Figure 4F), achieving the best resolution in the shortest possible t_G. Peak comparisons were made based on the retention factor and the % error in prediction ranged from 2.6% to 10.8%.

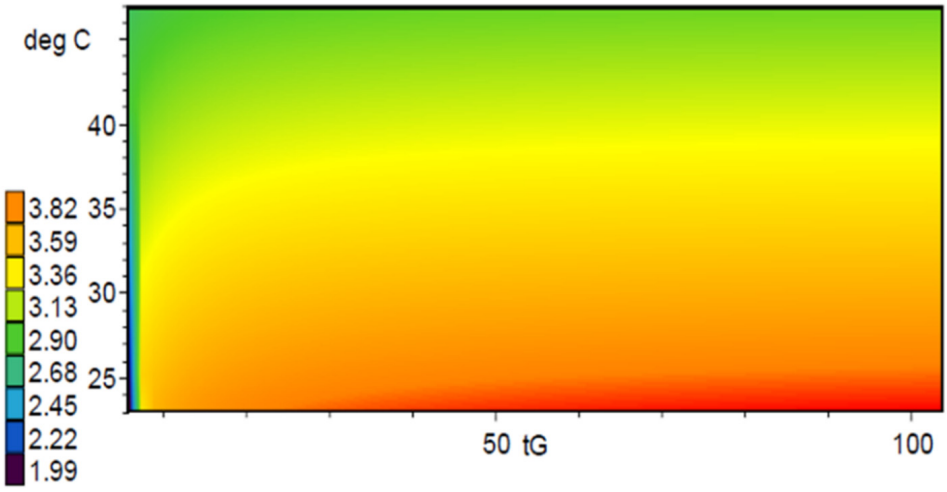

Figure 5. The 2D resolution map of T versus t_G generated by DryLab®; The best resolution of the critical peak pair is achieved by implementing the conditions offered by the red area of the map, while the blue areas offer the worst resolutions. Colors in between offer various resolution options.

3.2. Validation of the Developed HPLC Method

The developed method in this study proved to have an excellent resolution, selectivity and robustness, attaining validity as set by the ICH guidelines [20]. The method proved to be selective to CCB and its metabolites, DFCR, DFUR, FU and DHFU, in the presence of endogenous matrix components. It was found that the spiked plasma (Figure 3B) demonstrated the appearance of six significant peaks that were not present in the unspiked plasma chromatogram (Figure 3A). The comparison showed baseline separation of the six compounds at retention times 2.8, 3.2, 4.4, 5.2, 5.8 and 9.9 min for DHFU, FU, CLU, DFCR, DFUR and CCB, respectively, eluting all compounds in less than 10 min.

Moreover, the method was linear over a range of 10 ng/mL to 2×10^4 ng/mL for DHFU and CCB, 1 ng/mL to 2×10^4 ng/mL for DFUR and DFCR and 1 ng/mL to 1×10^5 ng/mL for FU, with a correlation coefficient >0.98. In addition, all results for the intra- and inter-day precision and accuracy were within the acceptable limits [22]. The slope, the intercept of the regression line and the coefficient of correlation calculated during routine analysis indicated no deviation from the pre-study results. Linearity ranges and correlation coefficients for the calibration curves along with the precision, LOD and LOQ values for the analytes are shown in Table 4. The adopted extraction procedure was based on that developed by Vainchtein et al. [11], being rapid, simple and exhibiting higher recoveries compared to other methods. The selectivity of the newly developed method was proved by comparison between the chromatogram of unspiked plasma of a healthy donor with the chromatogram of plasma of a healthy donor spiked with the internal standard and the five analytes of interest to prove that the response is only the result of the compounds of interest.

Table 4. Selected assay validation parameters.

Parameter	DHFU	FU	DFCR	DFUR	CCB
LINEARITY RANGE (NG/ML)	From 10 to 2×10^4	From 1 to 2×10^4	From 1 to 1×10^5	From 1 to 2×10^4	From 10 to 2×10^4
CORRELATION COEFFICIENT (R)	>0.98	>0.98	>0.98	>0.98	>0.98
INTRA-DAY PRECISION (CV%)	<15%	<15%	<15%	<15%	<15%
INTER-DAY PRECISION (CV%)	<10%	<10%	<10%	<10%	<10%
PRE-STUDY CALIBRATION CURVE CV%	1.1–4.9%	1.6–7.9%	1.2–4.7%	1.7–6.7%	1.6–8.4%
ROUTINE QC SAMPLE CV%	2.79–5.36%	2.69–9.14%	1.5–10.6%	2.69–9.14%	1.5–10.6%
PRE-STUDY CALIBRATION CURVE %BIAS	−4.7% to 5.5%	−3.6% to 6.8%	1.5% to 6.6%	1.2% to 3.2%	−3.8% to 3.5%
ROUTINE QC SAMPLE %BIAS	−7.2% to 8.9%	1.7% to 4.2%	−1.27% to 5.7%	1.7% to 4.2%	−1.27% to 5.7%
LOD (NG/ML)	3 ng/mL	0.3 ng/mL	0.3 ng/mL	0.3 ng/mL	3 ng/mL
LOQ (NG/ML)	10 ng/mL	1 ng/mL	1 ng/mL	1 ng/mL	10 ng/mL

3.3. A Prospective Study on Egyptian Colorectal Cancer Patients

In this study, 20 patients newly diagnosed with CRC, aged from 31 to 73 years, were recruited. The recruitment process was conducted over the period of six months starting from May 2014 until October 2014 in the National Cancer Institute (NCI) of Egypt. Patients included in the study were treated with CCB as single agent or combined therapy with Oxaliplatin (CAPOX). Patients having impaired hepatic or renal functions were excluded, due to its high influence on the efficiency of metabolism and hence the levels of CCB and its metabolites in plasma. Among the most reported toxicities of Oxaliplatin is peripheral neuropathy, which is not among the main toxicities caused by CCB. The possibility of Oxaliplatin increasing toxic effects specifically caused by CCB was neglected in the scope of this study, especially since CAPOX is usually the treatment of choice for CRC, and CCB is less often prescribed as a single agent. After running all the patients' samples using the newly developed and validated HPLC method, the toxicities they experienced over their treatment cycles were followed up (Table 5). Figure 6 illustrates the metabolite profiles

in plasma patient samples (Patients 2 and 19), providing a visual representation of the concentration levels of FU, DFCR and DFUR. These 2 patients depicted in the figure are presented as representatives of the larger population of 20 patients included in our study.

Table 5. Plasma concentrations of FU, DFCR and DFUR of the recruited patients at the selected sampling times. Patient information and data about the grades of CCB-induced toxicities are included.

P#	Sex	Age	Regimen	Conc. of FU ($\times 10^3$ ng/mL)			Conc. of DFCR ($\times 10^3$ ng/mL)			Conc. of DFUR ($\times 10^3$ ng/mL)			Toxicity Grade						
				D1 C1	D14 C1	D1 C2	D1 C1	D14 C1	D1 C2	D1 C1	D14 C1	D1 C2	A	N	V	D	C	F	M	H
1	M	46	CCB	43	37	33	2	6	0	4	11	1	0	1	0	2	1	0	1	3
2	M	44	CAPOX	55	52	31	14	1	2	7	1	1	1	1	0	3	1	1	1	2
3	M	63	CAPOX	58	98	0	3	1	0	3	2	0	2	1	2	1	0	0	0	1
4	F	55	CAPOX	44	26	0	0	2	0	13	8	0	0	0	0	0	1	0	1	1
5	F	60	CCB	122	50	41	1	2	514	3	2	1	1	1	0	1	0	0	0	0
6	M	31	CAPOX	70	85	132	2	4	0	1	3	2	0	1	1	0	1	1	0	2
7	M	33	CCB	50	80	0	12	5	0	4	5	0	0	0	0	0	0	0	0	0
8	F	36	CAPOX	52	0	70	5	0	6	10	0	3	1	0	2	0	0	0	0	0
9	F	51	CAPOX	28	81	37	1	698	1	1	3	2	1	1	2	2	2	0	0	1
10	M	70	CAPOX	50	77	62	8	1	6	5	1	3	2	0	0	0	3	0	2	0
11	M	32	CAPOX	24	27	28	0	2	3	2	2	3	3	0	3	2	2	0	1	0
12	M	64	CCB	78	47	0	21	0	0	3	1	0	0	1	2	0	1	1	1	3
13	F	62	CAPOX	102	48	31	2	2	0	2	3	1	1	1	0	1	1	1	0	3
14	F	47	CAPOX	35	38	18	1	2	1	3	3	4	2	2	2	3	1	1	2	3
15	M	37	CAPOX	28	39	45	3	1	1	5	2	1	1	0	0	0	0	0	0	0
16	M	48	CAPOX	31	86	146	5	9	1	4	8	24	0	1	0	1	2	0	1	3
17	M	58	CAPOX	13	16	18	5	1	1	5	1	1	2	1	0	3	1	1	1	0
18	M	73	CAPOX	54	0	0	1	0	0	1	0	0	0	0	0	3	0	0	0	0
19	F	54	CAPOX	40	49	29	2	1	1	1	0	1	1	1	1	2	1	1	0	0
20	F	68	CAPOX	58	0	0	1	0	0	6	0	0	2	1	1	0	1	0	0	3

The population of patients examined consisted of 60% males and 40% females. According to age groups, the 30s age group was found to form 25% of the population, the same as the 60s age group. The 40s age group was found to form 20% of the population, the same as the 50s age group, and finally 10% of the patients were in their 70s.

In order to evaluate the prevalence of the toxicity grades for the studied toxicities, the percentage of occurrence of each toxicity grade was calculated for all toxic adverse events, giving the results presented in Figure 7.

3.4. Correlations between Plasma Concentration of FU, DFCR, DFUR and Toxicity

A newly developed method was employed in this study to measure the levels of CCB and its metabolites in 20 Egyptian colorectal cancer patients recruited from the NCI in Egypt. Since CCB patients are outpatients, samples were taken depending on the accessibility of the patient and his/her general condition. For 20 patients at three different sampling times, a total of 51 samples were successfully collected.

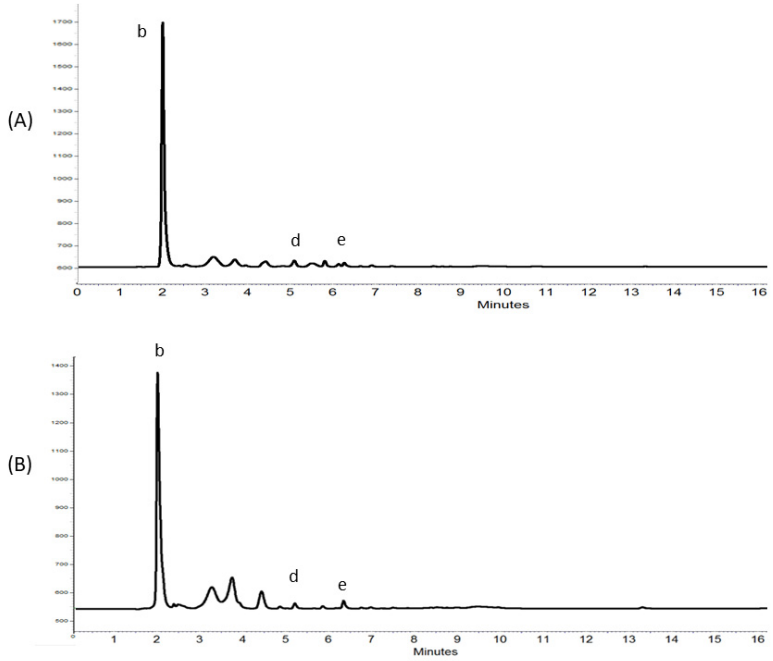

Figure 6. Chromatogram representing the extracted patients' plasma after CCB ingestion (Day 1) for Patient 2 (**A**) and Patient 19 (**B**). The metabolites FU, DFCR and DFUR are labeled as b, d and e, respectively.

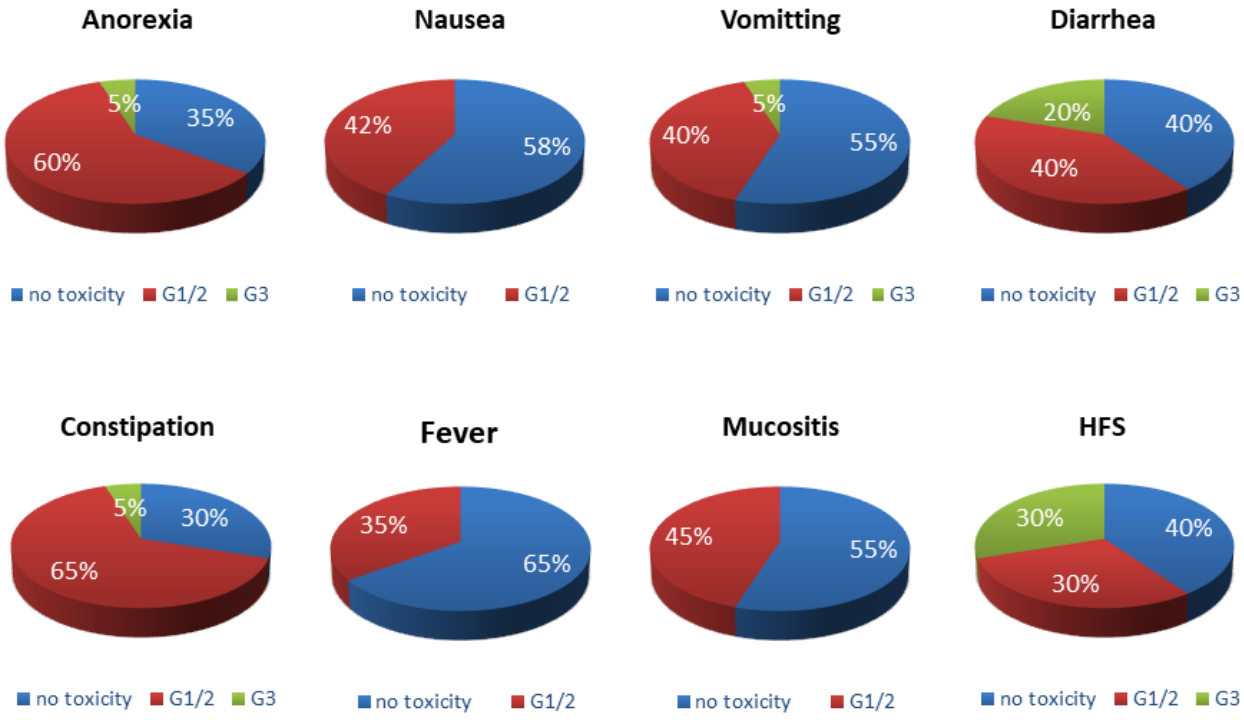

Figure 7. Occurrence percentage of each toxicity grade for anorexia, nausea, vomiting, diarrhea, constipation, fever, mucositis and HFS.

A significant positive correlation was found between the level of DFUR and FU with a Pearson correlation (r) of 0.664 (p = 0.000) (Figure 8A), in agreement with the previous discovery of the significant increase in the AUC of DFUR and FU during the treatment course [10].

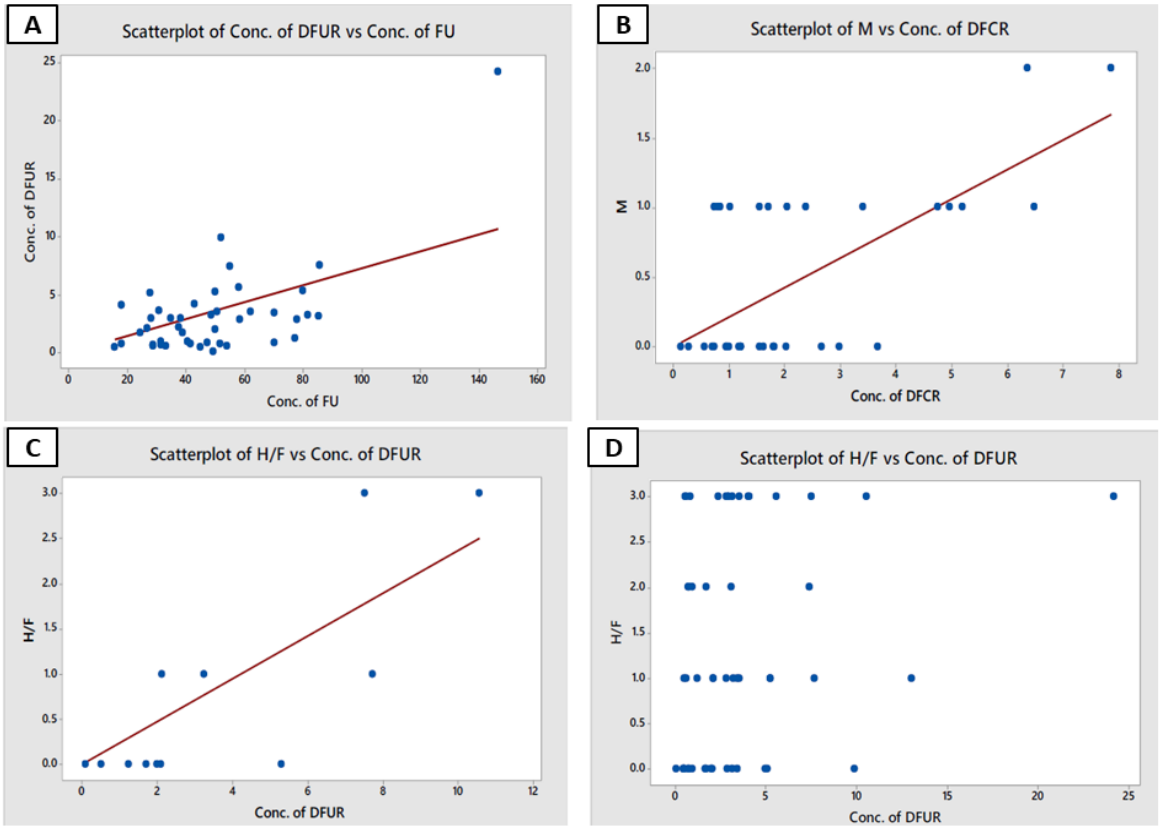

Figure 8. (**A**) Scatterplot showing the relation between the concentration of DFUR and the concentration of FU. Pearson correlation = 0.664, *p*-value = 0.000. (**B**) Scatterplot showing the relation between the concentration of DFCR and mucositis toxicity grades. Pearson correlation = 0.647, *p*-value = 0.000. (**C**) Scatterplot showing the relation between the concentration of DFUR (in steady state) and hand-foot syndrome toxicity grades. Pearson correlation = 0.822, *p*-value = 0.001. (**D**) Scatterplot showing the relation between the concentration of DFUR (in steady state) and hand-foot syndrome toxicity grades. Pearson correlation = 0.822, *p*-value = 0.001.

A positive correlation was found between the concentration of DFCR and mucositis/stomatitis, with a Pearson correlation (r) of 0.647 (p = 0.000) (Figure 8B). This important finding is reported for the first time in this study. Although SNPs in the gene of the CES enzyme were correlated repeatedly with the occurrence of diarrhea, no significant correlation was found between the concentration of DFCR and the occurrence of such toxicity. This could be attributed to the following reasons: the small sample size and the possible occurrence of diarrhea due to Oxaliplatin intake.

Another positive correlation was found between the concentration of DFUR (in steady state) and HFS, with a Pearson correlation (r) of 0.822 (p = 0.001) (Figure 8C). This finding in particular was remarkably interesting because previous studies have related SNPs in the gene of the CDD enzyme (leading to enzyme hyperactivity) with the occurrence of HFS [23,24]. This finding proves that the concentration of DFUR could be linked to CDD hyperactivity, and possibly to HFS toxicity prediction as an alternative to the expensive genotyping techniques adopted in clinical laboratories. It was observed that the concentration of DFUR only showed a significant positive correlation with hand-foot syndrome when measured in the steady state (samples taken on Day 14 or Day 7 of Cycle 1), compared to the concentration of DFUR measured on Day 1 of Cycle 1 and Day 1 of Cycle 2 which showed a very weak correlation and no statistical significance (Figure 8D).

After establishing a significant positive correlation between the concentration of DFUR in the steady state and HFS toxicity grades, the idea of exploring the DFUR concentration ranges in the steady state where such toxicity occurs emerged. Over the DFUR concentra-

tion range of 0.11–5.31 ($\times 10^3$ ng/mL), no HFS toxicity was observed. Along the DFUR concentration. range of 0.74–7.72 ($\times 10^3$ ng/mL), G1/2 HFS toxicity was noted. During the DFUR concentration range of 0.87–10.58 ($\times 10^3$ ng/mL), G3 HFS toxicity occurred (Figure 9). This means that above the DFUR concentration of 5.31 ($\times 10^3$ ng/mL), HFS toxicity is expected to occur in general, and above the concentration of 7.72 ($\times 10^3$ ng/mL) G3 HFS specifically is expected to happen.

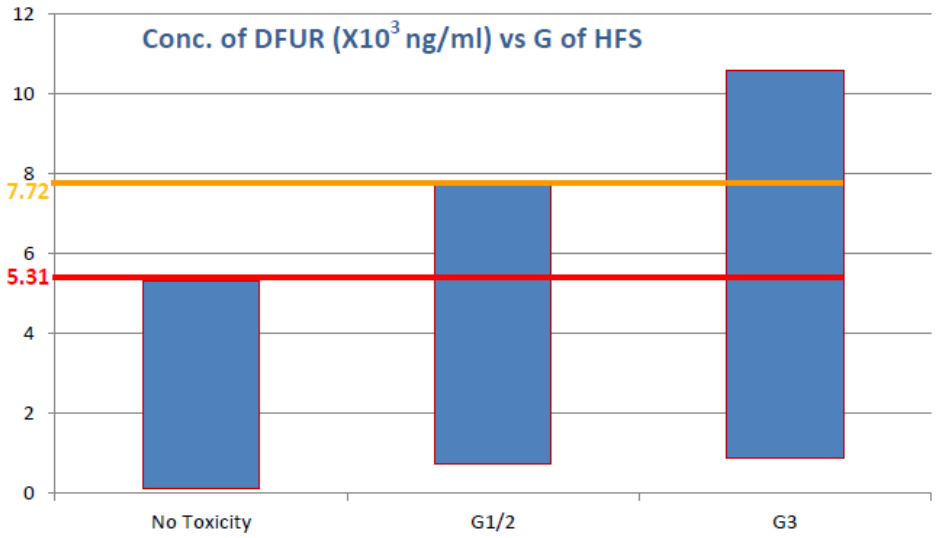

Figure 9. Concentration of DFUR ($\times 10^3$ ng/mL) vs. HFS toxicity grade.

In this study, no correlation was found between the concentration of FU and any of the toxicities and none was found between the concentration of DFCR or DFUR and the rest of the toxicities. Although there was a previously established correlation between two SNPs found in the CDD enzyme and Grade 2–4 diarrhea [25], this study showed no correlation between the concentration of DFUR and diarrhea. This inconsistency could be attributed to the small sample size, since the population size in the Loganayagam et al. study was 244 patients on CCB treatment. Also, the low incidence of G3/4 diarrhea in the population of this study could be the reason for the inability to correlate the toxicity to the DFUR plasma level, and hence, to the SNPs found in the CDD enzyme.

As a conclusion from these correlations, it was found that 45% of the sample population exhibited mucositis toxicity and 65% of the population showed HFS toxicity, where the incidence of such toxicities was positively correlated with the levels of DFCR and DFUR in plasma, respectively. This means that by measuring the plasma levels of these metabolites in Cycle 1 of treatment, the prediction of such toxicities is possible and accordingly, dose optimization can be performed from the beginning of the treatment. Also, this reflects the possibility of SNPs' occurrence in the CES and CDD enzymes in the Egyptian population, which needs further investigation by a comprehensive genotyping study.

4. Discussion

The decision to develop an HPLC method using a UV detector instead of an MS detector for the analysis of CCB and its metabolites was driven by several factors, including the ease of operation, excellent linearity, precision, accuracy and sensitivity at picogram levels, which rivals MS sensitivity. While MS detectors offer superior selectivity, HPLC-UV instruments are more prevalent and accessible in clinical settings, particularly in developing countries with a high incidence of colorectal cancer. Existing methods typically measure CCB alongside only a subset of its metabolites, necessitating the development of a comprehensive method for enhanced pharmacokinetics monitoring and toxicity prediction. Moreover, the aim was to minimize the required plasma volume for analysis, reduce runtimes and establish a linear method across a wider concentration range while

using a cost-effective mobile phase. Comparing the new method to the Vainchtein et al. approach [11] revealed its cost-efficiency, employing a more affordable UV detector and a simpler mobile phase (methanol instead of acetonitrile, tetrahydrofuran and 2-propranolol). This approach is particularly convenient for application in countries like Egypt, where HPLC-UV instruments are more readily available in research and clinical facilities.

Regarding recovery percentages, the protein precipitation method closely aligns with Vainchtein et al.'s study, differing primarily in the use of TCA as the precipitating agent instead of TFA. The ease of the procedures employed in this method and low costs requirements make it a good candidate for routine clinical use and research studies.

The choice of CLU as the internal standard was guided by several important considerations. First, CLU does not naturally occur in the sample, making it a suitable reference compound. Second, its structural similarity to the analytes makes it a suitable candidate for internal standardization. Additionally, CLU exhibits a favorable retention time and recovery characteristics from plasma, as evidenced in Figure 1.

The Synergi Fusion-RP column emerged as the most practical option for separating CCB, an extremely hydrophobic compound, and its metabolites, which include FU and DHFU, both of which are comparatively more hydrophilic. A comparison between the chromatograms obtained from the Synergi Fusion-RP column (Figure 4A) and a traditional C18 column (Figure 4C), utilizing identical mobile phase conditions for the same sample mixture, revealed that the Synergi Fusion-RP column provides enhanced retention for polar compounds due to the presence of polar embedded groups in the stationary phase. Simultaneously, it offers reduced retention for hydrophobic compounds, resulting in shorter analysis times.

Regarding the linearity range, the newly developed method surpasses the Vainchtein et al. study [11] by offering a broader linear range. In terms of method duration, the newly developed method outperforms the Vainchtein method, with the last peak eluted at 9.9 min in the new method compared to 11.5 min in the Vainchtein method. The volume of plasma required for analysis is comparable between the two methods, with the Vainchtein method needing 200 µL of plasma, while the new method requires only 160 µL.

The newly developed method enables accurate and reproducible measurements of CCB and its metabolites (DFCR, DFUR, FU and DHFU) in plasma. This capability is vital for assessing metabolite profiles and understanding drug metabolism variations among colorectal cancer patients. Precise quantification of metabolite concentrations can facilitate the calculation of optimal dosages to achieve therapeutic efficacy while minimizing toxicity, resulting in cost-effective treatment and reduced adverse events. Notably, no G4 toxicity occurred in the study population. Regarding symptoms like nausea, mucositis and fever, no patients experienced G3 toxicity. However, 30% of patients exhibited G3 hand-foot syndrome (HFS), 20% had G3 diarrhea, while 5% of patients each suffered from G3 anorexia, vomiting and constipation.

Despite the promising advantages of the newly developed method, it is important to acknowledge certain limitations inherent to this study. Firstly, the evaluation of this method was conducted in a specific clinical setting, and its applicability to broader patient populations and diverse clinical scenarios may require further validation. Given the low number of samples analyzed in this study and their inherent heterogeneity, it is imperative to approach the reported correlations between metabolite concentrations and observed adverse events with caution. These correlations should be considered putative, requiring further investigation in larger and more diverse patient cohorts. Moreover, it is noteworthy that several variables, such as the type and stage of tumor progression, body weight and complete therapeutic protocols, were not considered during the analysis. Therefore, the conclusions drawn from this study must be corroborated through more comprehensive and in-depth investigations.

5. Conclusions

In conclusion, the aims of this study were completely fulfilled, where a new superior method for the simultaneous determination of plasma levels of CCB, DHFU, FU, DFUR, DFCR and CLU was developed using DryLab® and validated according to the ICH guidelines. Moreover, the study reports for the first time a significant positive correlation between the concentration of DFCR and mucositis/stomatitis, and a significant positive correlation between the concentration of DFUR and hand-foot syndrome, confirming that measuring the plasma concentration of DFCR and DFUR in the steady state during Cycle 1 of CCB therapy can be an indicator for the CES and CDD enzyme activity, and possibly a predictor for the common toxicities associated with CCB which are mucositis and hand-foot syndrome. Drug dosage could be adjusted based on metabolites' levels to avoid the emergence of these two types of toxicities.

6. Recommendations

As demonstrated, HPLC determination of CCB and its metabolites, together with their correlation with different CCB-induced toxicities, seems to be a very promising area of research. Therefore, in order to obtain more generalized and comprehensive results, examining a larger sample representing the Egyptian population is recommended. Also, future investigation of the deactivation pathway of CCB would be very beneficial, since the DPD enzyme is considered the rate-limiting step of the metabolism, making DHFU a very interesting metabolite to investigate in the Egyptian population. Finally, it is recommended to perform a correlation study at the end of the eight cycles of treatment between the metabolites' levels and efficacy of CCB.

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Abbreviations

CCB	Capecitabine
CDD	cytidine deaminase
CLU	5-Chlorouracil
DFCR	5′-deoxy-5-fluorocytidine
CES	carboxylesterase
DFUR	5′-deoxy-5-fluorouridine
DHFU	5,6-dihydro-5-fluorouracil
DPD	Dihydropyrimidine Dehydrogenase
HPLC	high-pressure liquid chromatography
FBAL	α-fluoro-β-alanine
FU	5-Fluorouracil

FUPA	5-fluoro-ureidopropionic acid
IS	internal standard
LC	liquid chromatography
SNPs	single nucleotide polymorphisms
TP	thymidine phosphorylase

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