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Article

A small-scale method of sample preparation suitable for simultaneous HPLC-UV assay of capecitabine and its 5′-DFCR metabolite in mouse blood plasma

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The objective of the study was to develop an easy, cheap, effective, and safe, small-scale method for sample preparation suitable for the simultaneous high-performance liquid chromatography (HPLC)-ultraviolet (UV) assay of capecitabine and its 5′-deoxy-5-fluorocytidine (5′-DFCR) metabolite in mouse blood plasma. The suitability of the proposed method of sample preparation was verified by the optimal effectiveness and efficiency achieved in the overall analytical workflow. The chromatographic separation of capecitabine and its first metabolite was performed on a Hypersil GOLD aQ column with a mobile phase consisting of 1% formic acid, methanol, and water, and run in a gradient elution mode. The absence of interfering endogenous components at the retention times of each analyte was confirmed by the chromatographic analysis of blank matrices and matrices spiked with the corresponding standards. The absence of any tactile matrix effect was also recorded. For the first time, the effect of the vacutainer's anticoagulant on the extraction efficiency of both analytes was evaluated. The method was found to be accurate, precise, and specific. The estimated mean "extraction" efficiencies were ≥90% for each analyte. The lower limit of quantitation for both capecitabine and 5′-DFCR was 0.05 μg/mL.

Keywords: Capecitabine. 5′-deoxy-5-fluorocytidine. Mouse blood plasma. Sample preparation. HPLC.

INTRODUCTION

Capecitabine is an orally administered antineoplastic prodrug, a member of the fluoropyrimidine class, and an ally in the therapy of a number of different types of benign and malignant neoplasias (Murphy, Middleton, 2012). Inside the organism, the prodrug in question is easily converted into its pharmacologically active metabolite, 5-fluorouracil (Scheme 1) (Avendaño, Menéndez, 2015). The metabolic activation of capecitabine requires the consecutive participation of three different and highly specialized enzymes: carboxylesterase (CES), cytidine deaminase (CDD), and thymidine phosphorylase (TP) (Avendaño, Menéndez, 2015).

Individual differences in the expression of each of these three prodrug-activating enzymes may, to some extent, serve as prerequisites for the lower functional (antineoplastic) potential of anticancer therapeutic drugs. That is why, from a therapeutic point of view, the actual concentration of capecitabine and its metabolites should be quantified *in vivo*.

In modern pharmaceutical and biomedical analysis, sample preparation is an integral part of the analytic process and has been identified as "the bottleneck of

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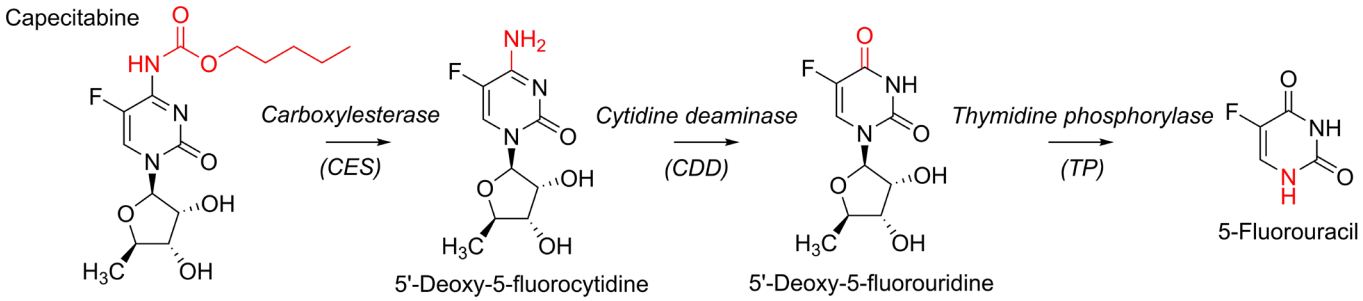
analytical methodology" (Abdulra'uf, Sirhan, Huat Tan, 2012). In fact, the importance of sample preparation in this case cannot be neglected. Ensuring that the "right choice" is made when selecting a proper sample preparation method (or technique) is considered a must for obtaining reliable and meaningful analytical results (Moldoveanu, David, 2015; Moldoveanu, 2004).



Scheme 1 – A simplified scheme of capecitabine metabolism.

Use of protein precipitation has become more commonplace as a sample preparation strategy due to its abilities to:

(i) work reasonably and remarkably well with small-scale (microliter) samples;
(ii) ensure excellent assay performance and specificity, even at lower analyte concentrations;
(iii) function just enough to meet all analytical needs; and,
(iv) be conducted using simple and conventional equipment, and with inexpensive and innocuous reagents.

Actually, the effectiveness of the protein precipitation process has been proven in numerous studies (Wang *et al.*, 2012; Alshammari *et al.*, 2015). The aim of the present study was to further verify the implementation of the protein precipitation method using the antineoplastic agent capecitabine and its first metabolite, 5′-deoxy-5-fluorocytidine (5′-DFCR). In this work, the qualitative and quantitative composition of each biological specimen was accurately assessed using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Thorat, Chikhale, Tajne, 2019; Vijaya Jyothi *et al.*, 2018; Hassanlou *et al.*, 2016; Piórkowska *et al.*, 2014; Komarov *et al.*, 2014; Farkouh *et al.*, 2010; Zufía, Aldaz, Giráldez, 2004).

We have thus focused our attention to the development of a suitable sample preparation method for the simultaneous HPLC-UV assay of capecitabine and its 5′-DFCR metabolite in mouse blood plasma.

MATERIAL AND METHODS

Chemicals

Capecitabine (Pharmaceutical Secondary Standard, CRM; ≥99%) and 5′-deoxy-5-fluorocytidine (5′-DFCR: 95%) were purchased from Sigma-Aldrich Co. (USA) and Fluorochem Ltd. (UK), respectively. Formic acid (99%–100%) was supplied by Chem-Lab (Belgium). Zinc sulfate monohydrate (99%) was purchased from Acros Organics (Belgium). The solvents used (methanol, absolute ethanol, propan-1-ol, and propan-2-ol) for the protein precipitation procedures were of HPLC-grade purity (Fisher Chemicals, UK). Water was purified using Water Still for double distillation (Gesellschaft für labortechnik mbH, Germany). Bi-distilled and de-aerated water was used for all experiments.

Standard stock and working solutions

The standard stock solutions of capecitabine (1,000.0 µg/mL) and 5′-DFCR (1,000.0 µg/mL) were prepared by dissolving 20.0 mg of the respective standards in 20.0

mL of water. The obtained solutions were serially diluted with water to produce working standard concentrations of 5.0, 4.0, 3.0, 2.0, 1.0, 0.5, 0.1, and 0.05 μg/mL for each analyte. To construct the calibration curves, the same series of standard solutions were HPLC assayed; excellent linearity was demonstrated for both analytes (R^2 >0.998).

Animals and experimental design

ICR albino mice were provided from the Institute of Experimental Morphology, Pathology, and Anthropology with Museum (Bulgarian Academy of Sciences, Sofia, Bulgaria). The mice were housed in cages containing up to six mice each; the mice were acclimated for 1 week under controlled conditions, including temperatures of 20°C±2°C, humidity of 55%±10%, and 12-hour light–dark cycles (where the lights remained on from 7:00 a.m. to 7:00 p.m.). For all experiments, the mice were housed under specific pathogen-free conditions and were allowed free access to food and water. Age-matched males (7–9 weeks old, weighing 25–30 g) were used for all experiments. The experiments were performed in accordance with the Institutional Guideline No. РД-09-27/21.05.2019 for Animal Experiments from the IEMPAM – BAS and the Bulgarian Food Safety Agency (permission No. 96).

The experimental animals were divided into eight groups with six individuals each. The first group served as the control group, whereas groups 2–7 were used for analytical profiling. The individuals from the last (8th) group received a single oral, nominal dose of 370.0 mg capecitabine per kg of body mass (the utilized dose was deemed to be appropriate and equal to the recommended human therapeutic dose). Further, the tested drug was administered in the form of an aqueous suspension. For the purpose of this study, 74.0 mg of capecitabine was suspended in 2.0 mL of water at 37°C for 5 min. The resulting suspension was administered orally (via disposable mouse feeding needles [20 G, 1.9 mm tip, 38 mm]) at a volume of 0.25 mL/animal.

Upon capecitabine administration, blood samples were taken from each specimen after 30 minutes – the time needed to achieve the optimal bioavailability of both the prodrug and its first metabolite (Onodera *et al.*, 2000). The collected blood samples were separately transferred in EDTA-coated (Boen Healthcare Co., Ltd.) and heparinized vacutainers (Thermo Fisher Scientific). The samples were immediately centrifuged (6,000 rpm for 5 min at room temperature) and the plasma was separated, quick frozen (at approximately –80°C), and immediately transported in a dry-ice box. Before the HPLC analysis, the plasma samples were allowed to thaw at room temperature and were thoroughly homogenized.

Sample preparation

For protein precipitation (Figure 1), aliquots of 100 μL of plasma were placed into sterile 10.0 mL screw-capped glass tubes. The samples were vortexed in the presence of 30 μL of 1% formic acid and 100 μL of 3% $ZnSO_4xH_2O$ at 5,000 rpm for 2 min. The obtained homogenates were treated with equal volumes (800 μL) of ethanol and were vortexed for 2 min at 5,000 rpm once again. The formed coagulates were solidified by centrifugation at 6,000 rpm for 5 min. The liquid residues were thoroughly collected and quantitatively transferred into new glass tubes of the same capacity. The solid remnants were resuspended in 300 μL of ethanol, vortexed, and centrifuged again. The resulting supernatants were combined with the initial ones, and evaporated to dryness under a gentle stream of N_2 at 40°C, using an SBH CONC/1 concentrator equipped with a SBH130D/3 block heater (Cole-Palmer Ltd., UK).

The sample preparation was conducted using a nearly identical procedure where, instead of ethanol, propan-1-ol and propan-2-ol was used.

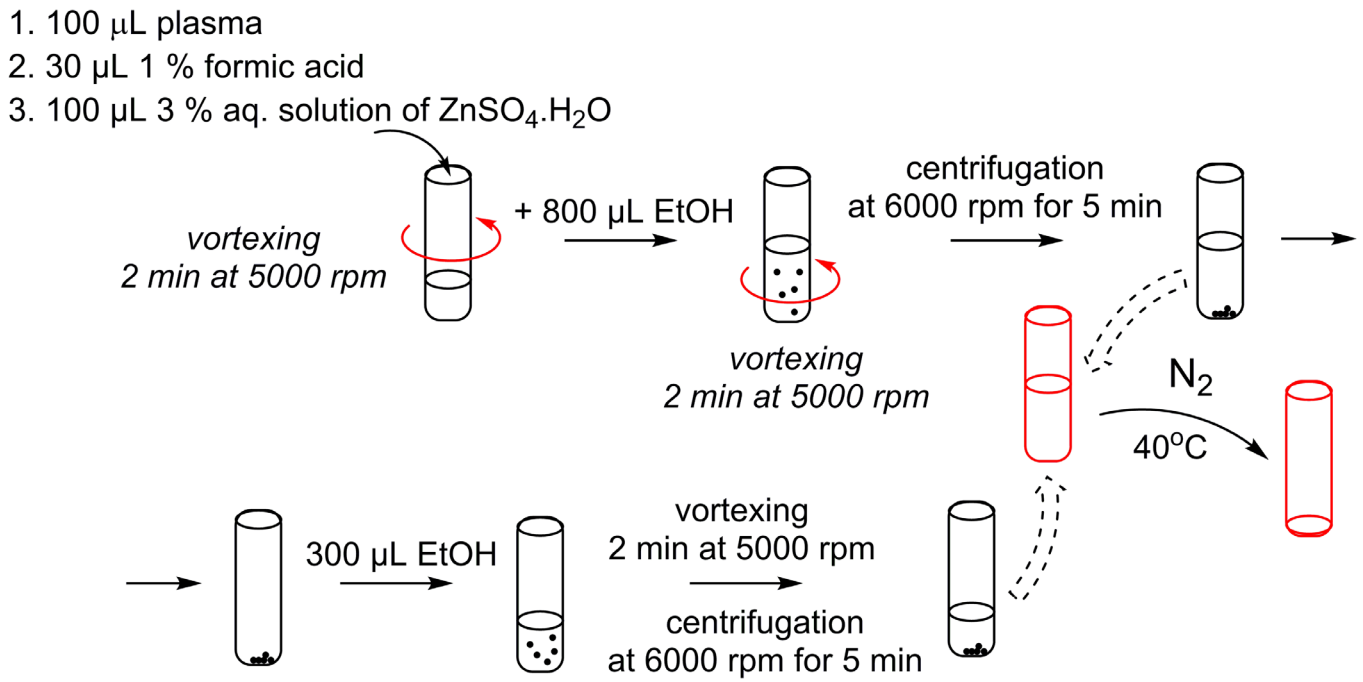


FIGURE 1 – Schematic of the sample preparation method.

The dried residues were reconstituted in 200 µL of water and thoroughly homogenized by vortexing at 5,000 rpm for 2 min. The samples were additionally centrifuged at 6,000 rpm for 5 min, and the obtained clear aqueous fractions were transferred to 1.5 mL septum-capped vials containing 200 µL glass inserts (02-NV, Thermo Fisher Scientific). The autosampler was programmed to inject 20 µL of each sample into the chromatographic system.

Plasma spiking

The spiked plasma samples were obtained by mixing mouse plasma and standard aqueous solutions of capecitabine and 5′-DFCR. Equal volumes (100 µL) of each working standard (capecitabine or 5′-DFCR) solution were added separately to aliquots of 100 µL of mouse plasma in 10 mL glass tubes. All samples were thoroughly mixed at 6,000 rpm for 2 min; a series of samples with varying analyte concentrations (5.0, 3.0, and 1.0 µg/mL) were obtained.

The spiked plasma samples were treated according to the aforementioned protocol for protein precipitation; the total content of each vial (200 µL) was thoroughly withdrawn and subject to analysis.

Instrument, conditions, and software

Chromatographic analysis was performed with a high-performance liquid chromatographer (Thermo Scientific UltiMate 3000 Analytical LC System) equipped with a quaternary pump (Dionex UltiMate 3000 LPG-3400SD Quaternary Pump), an automatic injector (Dionex UltiMate 3000 Autosampler), a variable wavelength detector (Dionex UltiMate 3000 VWD), and a diode array detector (Dionex UltiMate 3000 DAD-3000 Diode Array Detector; Thermo Fisher Scientific).

The analytes underwent HPLC analysis under reversed-phase conditions on a Hypersil GOLD aQ C18 analytical column (150×4.6 mm, 5 µm; Thermo Fisher Scientific), fitted with a pre-column from the same stationary phase (Hypersil GOLD aQ C18; 10×4.0 mm, 5 µm; Thermo Fisher Scientific). The column temperature was maintained at 30°C, and the autosampler was kept at 10°C. The eluent was monitored using a PDA detector at

280 nm (for 5′-DFCR) and 306 nm (for capecitabine). The total duration of each run was 30 minutes. Twenty µL of each sample was injected into HPLC system.

Chromatographic separation was achieved in gradient mode with a mobile phase consisting of water, methanol, and 1% formic acid. A flow rate of 0.8 mL/min was found to be appropriate (Table I).

Table I - Gradient elution profile for HPLC analysis.

Time (min)	Water (v/v %)	Methanol (v/v/ %)	1% formic acid (v/v %)	Flow rate (mL/min)
0.00	90	0	10	0.8
20.0	20	70	10	0.8
30.0	90	0	10	0.8

The system's instrumental control, as well as the data acquisition and analysis processes were performed using Chromeleon software, version 7.2 (Thermo Fisher Scientific).

Validation of the HPLC method

The validation of the HPLC method was performed according to the criteria proposed in the Eurachem Guide (2020), and in compliance with the principles of Good Laboratory Practice (GLP).

Limit of quantification (LOQ)

LOQ was determined by analyzing a series of standard solutions featuring known concentrations of the tested analytes. A signal-to-noise ratio of 10 was considered acceptable for estimating the quantification limit.

Accuracy and precision

Within-day precision of the method did not exceed 0.1%. Moreover, during the validation process, the calibration curves were reconstructed and all met the acceptance criteria of accuracy (RSD) of ≤0.5%.

Linearity

The calibration curves were constructed by plotting the peak area ratios of capecitabine and 5′-DFCR, respectively, against their nominal concentrations. Linearity, in both the cases, was evaluated by linear regression analysis based on the least square regression method, were seven working standard solutions were measured at a concentration range of 0.1–5.0 µg/mL.

Quantification of capecitabine and 5′-DFCR in plasma samples

The presence of capecitabine and 5′-DFCR in all biological samples was quantified using an external standardization method. The spike recovery test was performed according to the methodology described by Dhananjeyan *et al.* (2007). In this study, each sample was analyzed in triplicate. Furthermore, the recoveries of capecitabine and 5′-DFCR were evaluated in triplicate at three different concentration levels (1.0, 3.0, and 5.0 µg/mL). The results for each recovery (Re) are expressed in percentages.

Stability

All measurements with capecitabine and 5′-DFCR were performed, wherever possible, at low temperatures (up to no more than 10°C) and at shorter periods of time.

Specificity and sensitivity

The specificity of the HPLC method was evaluated by comparing the obtained chromatograms for the blank and real biological samples (Figure 4). The sensitivity of the method was evaluated according to the described validation protocol, where LOQ responses of each analyte were compared with those of the interference in the blank matrices at the same retention times. For each chromatogram, the analyte response exceeded 10 times that of the blank one.

RESULTS AND DISCUSSION

Optimization of the sample preparation method

This work reports the chromatographic analysis of *in* and *ex vivo*-loaded mouse blood samples spiked with capecitabine and its first metabolite, 5'-DFCR. For the purpose of the chromatographic separation process, the analytic gradient reverse-phase HPLC procedure developed by Zufía, Aldaz, and Giráldez (2004) was successfully employed. With the imposed modifications to the standard protocol for sample preparation, the need to use routine and expensive filtration materials, time-consuming strategies, and toxic solvents was eliminated. The effectiveness of the protein precipitation method described here was found to be a suitable technique for "insulating" target organic analytes from mouse blood and plasma samples, and was assessed during several experimental tests using small amounts of different eco-friendly, organic solvents – alcohols.

In this research, three different precipitating solvents (aliphatic alcohols) were utilized: ethanol, propan-1-ol, and propan-2-ol. The selected solvents were chosen not only because of their availability and safety, but also because of their inertness towards the testing analytes. Furthermore, the solubility of both analytes in aquo–alcoholic mixtures should exceed their protein–plasmatic affinity.

Actually, the accomplished and analytically evaluated average percentage recoveries for each concentration level of spiked specimens (achieved by using each of the selected alcohols) were more than passable (Table II).

Table II - Results from recovery studies of capecitabine and 5′-DFCR.

Precipitating solvent	Recovery of capecitabine ± RSD (%)			Recovery of 5′-DFCR ± RSD (%)		
	C_{spike} [§]			C_{spike}		
	1.0 µg/mL	3.0 µg/mL	5.0 µg/mL	1.0 µg/mL	3.0 µg/mL	5.0 µg/mL
Ethanol	98.9 ± 0.17	97.8 ± 0.06	95 ± 0.0	99.7 ± 1.53	95.7 ± 1.16	95.0 ± 1.0
Propan-1-ol	96.26 ± 0.2	94.6 ± 0.06	92.8 ± 0.06	102.5 ± 0.5	96.6 ± 1.06	96.5 ± 1.06
Propan-2-ol	95.8 ± 0.06	92.6 ± 0.0	91.8 ± 0.0	95.1 ± 0.78	98.8 ± 1.0	99.0 ± 1.3

[§] *The concentration of the spiked analyte.*

For all concentrations, the evaluated recovery values of capecitabine and 5′-DFCR were found to be not less than 90% (with repeatability up to ±0.17 % for capecitabine, and no more than 1.53% for 5′-DFCR-spiked specimens). Moreover, the obtained recovery values (Re) for both analytes did not cross the upper limit of 103%.

Overall, the present method yielded good results, not only for the tested prodrug, but for its first metabolite, 5′-DFCR, as well. This result was not achieved in the study by Zufía, Aldaz, and Giráldez (2004).

Despite the advantages associated with the current method, several arguments could, admittedly, be made against it, particularly in terms of its continuance and complicacy. However, for all the reasons given above, none of these limitations should be considered as a relevant factor.

Given the high recovery values for both analytes, one should not overlook the fact that the high levels of extracted capecitabine and 5′-DFCR were most likely due to the combined effect of the used salting out and settling agents (formic acid and $ZnSO_4$), as well as to the imposed re-extraction step (as noted in Figure 1).

However, when assaying the recovery of 5′-DFCR, there was unacceptable variability in the quality of the obtained results (as high as 1.54%), which fell outside of the standard range (Table II). One potential reason underlying the higher deviations in the repeatability of the applied method was the lower purity of the analyzed

5′-DFCR. On the other hand, the errors made when weighting 5′-DFCR may have also occurred given the high hygroscopicity of the metabolite in question – mistakes that cannot be clearly defined or subjected to statistical analysis at this point.

However, the 5′-DFCR extraction procedure could have been completed with higher analytical accuracy and precision, *ab ovo*, if carried out properly in compliance with all of the aforementioned requirements, and if carried out with an additional number of scientifically justified animals. Overall, we found that there were no interfering, endogenous matrix constituents eluted during the course of the retention times for both analytes (Figure 2); this held true when each of the six different blank plasma samples.

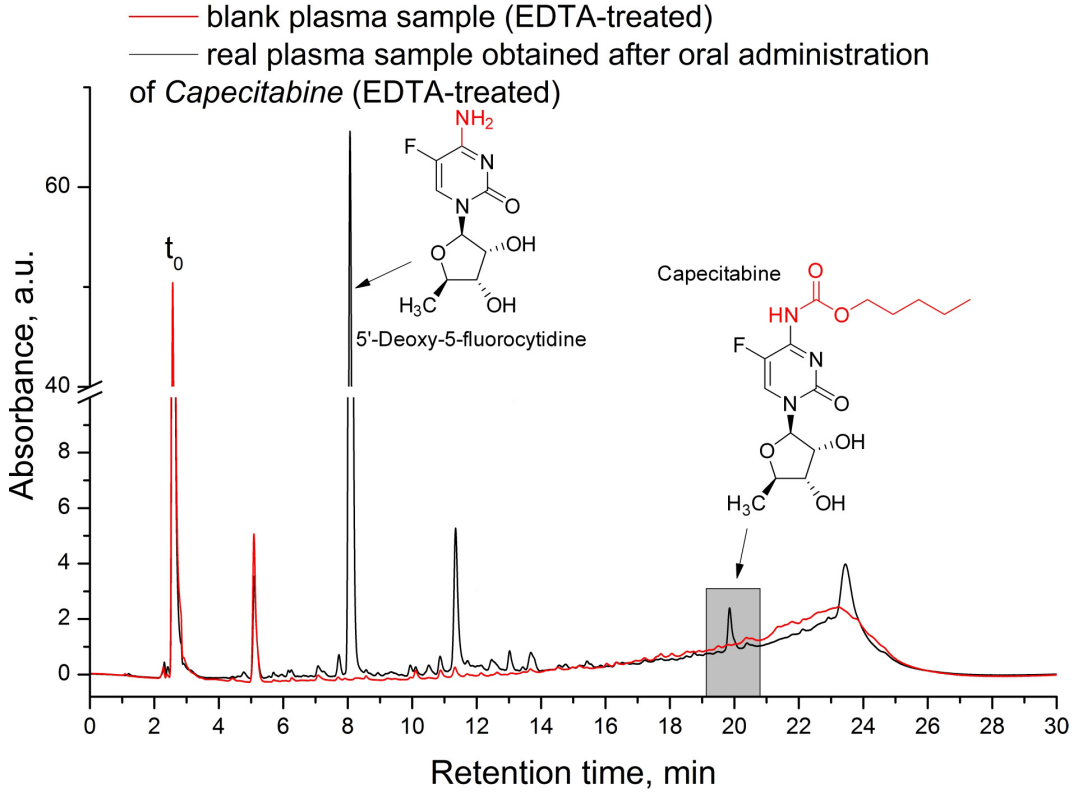


FIGURE 2 – Representative chromatograms of a blank plasma sample and of a real plasma sample obtained following the oral administration of capecitabine; the black arrows indicate the elution positions of capecitabine and its first metabolite.

The "good" repeatability of the method applied in this work may also be regarded as an analytical measure to ensure the absence of interfering effects. The suitableness of the sample preparation method was also reflected both in the sharp and symmetrical chromatographic peaks that were obtained with minimal tailing (Table III), and in the relatively high instrumental sensitivity that was achieved (Figures 3 and 4). In this regard, several other basic analytical characteristics of the proposed method are also presented in Table III.

Table III - Chromatographic parameters for the assayed analytes

Parameters	Results
Selectivity	Pass
Accuracy	≤ 0.5 %
Precision	≤ 0.1 %
Recovery	Pass
Bench top stability	6 h
Coolant stability	> 24 h
LOQ (capecitabine)	0.05 µg/mL
LOQ (5′-DFCR)	0.05 µg/mL
Linearity (capecitabine)	0.99981
Linearity (5′-DFCR)	0.99861
Theoretical plates (capecitabine)	117881
Theoretical plates (5′-DFCR)	35574
Resolution	49.04
Tailing factor (capecitabine)	1.48
Tailing factor (5′-DFCR)	1.47
Capacity factor (capecitabine)	15.49
Capacity factor (5′-DFCR)	5.46

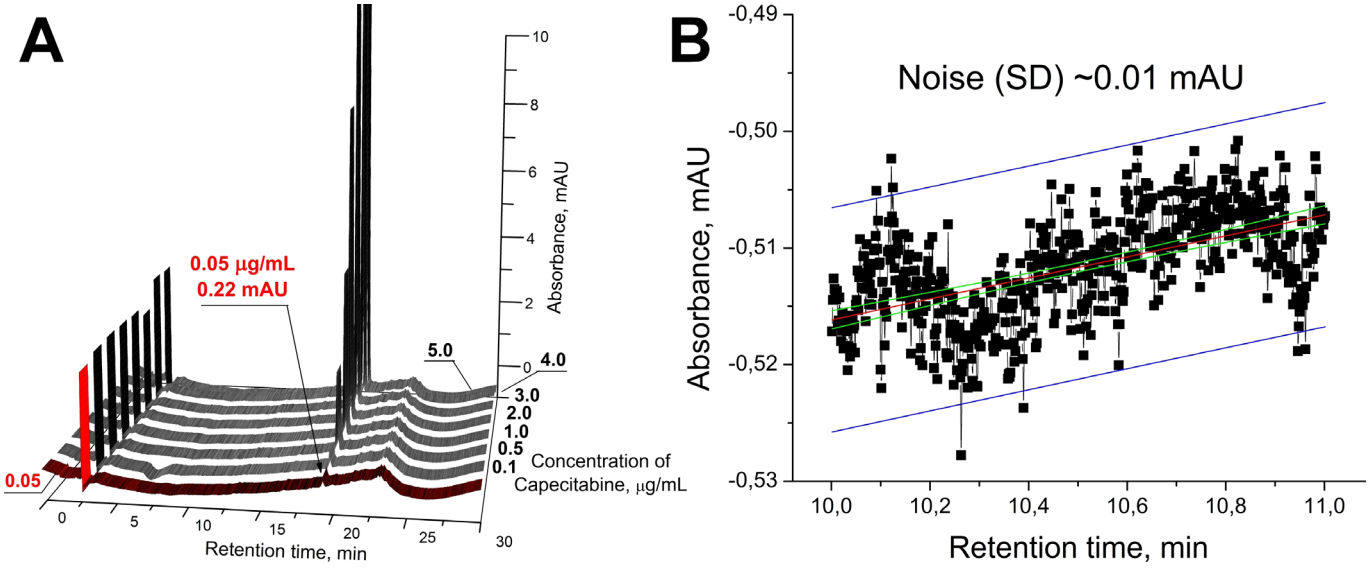


FIGURE 3 – a) Representative chromatograms of capecitabine at different concentrations. b) The estimated SD (noise) of the input chromatographic signal (baseline) of the obtained first chromatogram (at 0.05 µg/mL).

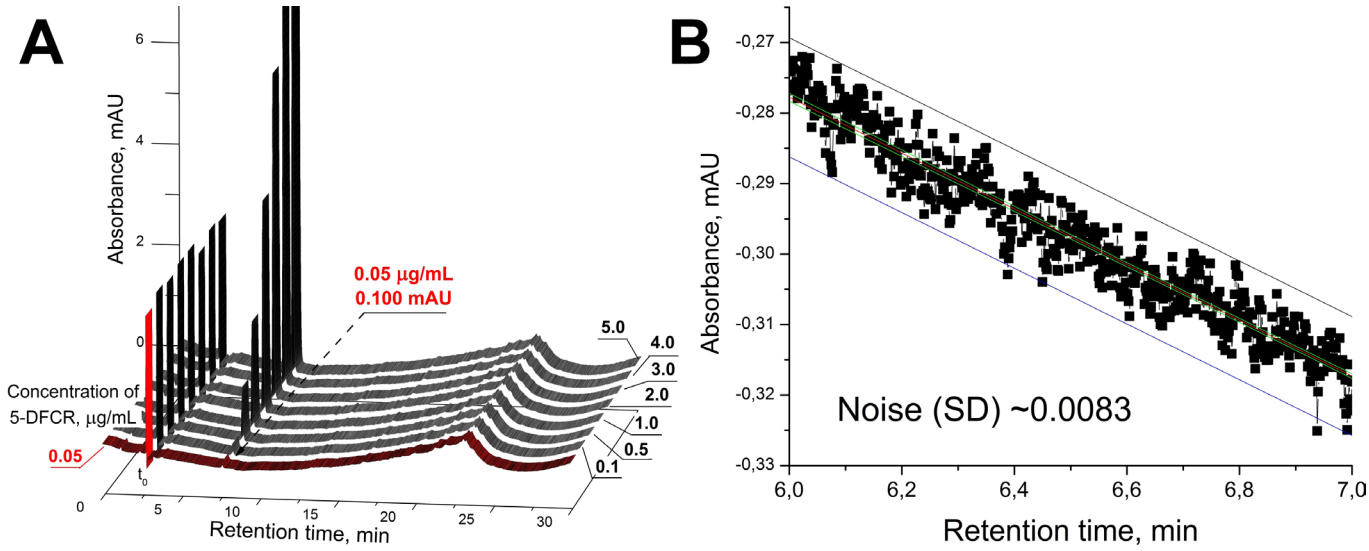


FIGURE 4 – a) Representative chromatograms of 5′-DFCR at different concentrations. b) The estimated SD (noise) of the input chromatographic signal (baseline) of the first chromatogram obtained (at 0.05 µg/mL).

Further, given the appropriate choice of chromatographic column used, the greatest advantages of the adapted HPLC method were maintained *in toto* (and even enhanced to a certain extent, if the extractability of 5′-DFCR is taken into account) (Zufía, Aldaz, Giráldez, 2004). However, the relatively long run time of the HPLC analysis allowed us to adequately separate the examined analytes from co-extracting matrix interferences and the hypothetical presence of other, artificial compounds (even with CES-modulating activity). This finding will be subjected to further pharmacological studies.

Thus, the findings from the analysis indicate that the sample preparation method applied in this study may be perceived as reliable (Table III), and no less valuable than the previously reported methodologies (Table IV).

Table IV - Comparative summary of different HPLC methods developed for analysis of *Capecitabine* and its first metabolite

Method of sample preparation	Used solvents	Applied calib. method	Plasma (µL)	Analyzed analyte/s	Recovery (%)	Source
LLE†	acetonitrile / ethyl acetate	IS‡	500	capecitabine	83.3÷101.1	(Zufía, Aldaz, Giráldez, 2004)
LLE	acetonitrile / ethyl acetate	IS	1000	capecitabine	95.7÷107	(Piórkowska *et al*., 2014)
PP†	ethanol	ES‡	400	capecitabine	95.98÷102.5	(Hassanlou *et al*., 2016)
PP	methanol	ES	100	capecitabine 5′-DFCR	83.3÷103.5 86.6÷107	(Dhananjeyan *et al*., 2007)
PP	methanol	IS	1000	capecitabine	97.22	(Jayaseelan *et al*., 2017)
PP SPE†	acetonitrile / acetic acid methanol	IS	500	capecitabine 5′-DFCR	not presented	(Budman *et al*., 1998)

Table IV - Comparative summary of different HPLC methods developed for analysis of *Capecitabine* and its first metabolite

Method of sample preparation	Used solvents	Applied calib. method	Plasma (µL)	Analyzed analyte/s	Recovery (%)	Source
SPE	methanol / water	ES	1000	capecitabine	97.8÷106.6	(Farkouh *et al*., 2010)
LLE	ethyl acetate / isopropanol	IS	100	capecitabine 5-DFCR	85.26±3.69 80.89±3.04	(Wang *et al*., 2019)
LLE	ethyl acetate / methanol	IS	500	capecitabine 5-DFCR	95.7÷104 100÷105	(Švobaitė *et al*., 2010)

† *LLE - liquid-liquid extraction; PP - protein precipitation; SPE - solid-phase extraction.;* ‡ *IS - Internal standard; ES - External standard.*

The present method is advantageous over the previously reported: (i) it is capable of detecting and quantifying much lower concentrations of the target analytes in real plasma samples (within and beyond the accepted therapeutic range); (ii) it can overcome some of the limitations associated with using smaller sample volumes (from 50–100 µL); (iii) it can enable the user to analyze up to three specimens per mouse and with smaller sample injection volumes of 20 µL; and (iv) it is eco-friendly and cost-effective. For the analysis, however, the choice to use precipitating solvents was limited to ethanol given that this solvent performed better when used with the limited number of test specimens reported in this study.

Effect of vacutainer anticoagulants on the extractability of capecitabine and 5′-DFCR

From a clinical and analytical standpoint, it is essential to identify a "reliable" blood collection tube (vacutainer) that can ensure each directly withdrawn blood sample is usable for its intended purposes (Stargel *et al.*, 1979; Shi, Rossum, Bowen, 2012). It is well known that the use of commercial blood sampling tubes may affect the blood composition in such a way that it alters the plasma fraction (Toennes, Kauert, 2001; Mena-Bravo, Priego-Capote, Luque de Castro, 2015).

To select the most appropriate vacutainer, we initiated a set of parallel measures (under strictly controlled experimental conditions) in which the effect of including vacutainer additives (anticoagulants) was evaluated. The obtained experimental data are presented in Figure 5.

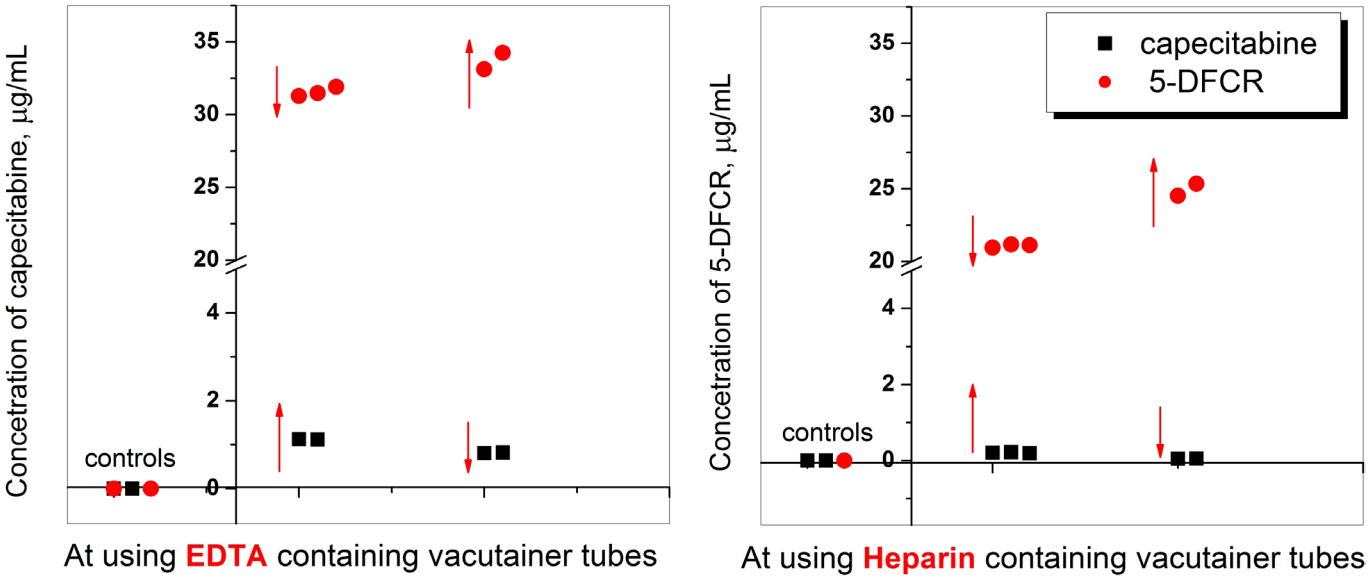


FIGURE 5 – Comparison of total capecitabine and 5′-DCFR yields in plasma samples separated using EDTA-treated (a) and heparinized tubes (b).

As seen from the figure, the comparison between plasma samples treated with each of the tested anticoagulants (EDTA and heparin) reveals a fairly high level of inter-animal variability in response to the examined prodrug. It is reasonable to suppose that all of these differences are caused by the expected individual fractions in the level of CES and CDD expression in the small cohort of animals treated with the antineoplastic agent (Scheme 1). This fact was further supported by the registered and expected counter-directional variations in the concentrations of the examined analytes (as shown in Figure 5, with red arrows).

As for the effect of the vacutainer anticoagulants on the extractability of both analytes, there is irrefutable evidence to support that the extent of capecitabine and 5′-DFCR extractability is dramatically enhanced in cases where EDTA-containing vacutainers were used. The data in Figure 5 illustrate the superiority of EDTA-based tubes over the heparinized ones. This finding was clearly demonstrated by the marked increase in the total extractability of both analytes. The most important benefit noted in this case, however, was the increasing degree of capecitabine separation from real biological samples – from detection to the necessary LOQ range. Moreover, the current detection value enabled us to determine the bioavailability of the prodrug in question after its *per oral* administration.

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, a validated protein precipitation protocol was developed for the simultaneous extraction of capecitabine and 5′-DFCR from mouse blood plasma. This protocol also resulted in a more precise and accurate HPLC assay of both analytes. This study further evaluated the influence of two different anticoagulants (vacutainers) on the reliability of the overall analytical method. This analytical method was able to assay the concentration of the analyte in a real pharmacokinetic situation, as demonstrated by improvements in the detection limit of capecitabine after selecting an appropriate blood collection vacutainer and implementing a suitable sample preparation technique.

Additional studies to support this analytical protocol are currently in progress. Most of these studies have been designed to demonstrate whether the proposed analytical method is suitable for detecting and monitoring differences in the bioavailability of both analytes in the presence of CES-modulating agents.

DECLARATION OF COMPETING INTEREST

All authors declare no conflicts of interest.

ETHICS STATEMENT

All biological tests were performed in compliance with regulations for biological testing on animals.

ACKNOWLEDGMENTS

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