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A review of the bioanalytical methods for the quantitative determination of capecitabine and its metabolites in biological matrices

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Abstract

The bioanalysis of the oral anticancer drug capecitabine and its metabolites has been investigated extensively over the past years. This paper reviews methods for the bioanalysis of capecitabine and its metabolites. The focus of this review will be on sample pre-treatment, chromatography and detection. Furthermore, the choice of standards and analytical problems encountered during analysis of capecitabine and its metabolites in biological matrices will be discussed. The major challenges in the bioanalysis of capecitabine and its metabolites are the simultaneous extraction and analysis due to the differences in polarity of the analytes. Furthermore we evaluate currently described methods for the quantification of capecitabine and its metabolites. Future wishes and perspectives are stated that could serve as an inspiration for further development of assays for the quantification of capecitabine and its metabolites.

KEYWORDS

Fluoropyrimidines, capecitabine, bioanalysis, chromatography, mass spectrometry

1 | INTRODUCTION

Capecitabine (Xeloda ®) is an oral fluoropyrimidine-based chemotherapeutic agent indicated for the treatment of several malignancies including colon cancer, colorectal cancer and breast cancer ("Xeloda [SmPC],", 2017). Additionally, capecitabine is used for the treatment of gastric, pancreatic and head and neck cancer (Derissen et al., 2016). Capecitabine is an inactive prodrug that is rapidly absorbed from the gastrointestinal tract and enzymatically converted into the active agent 5-fluorouracil (5-FU) through three metabolic steps (Deng et al., 2015; Derissen et al., 2016; Licea-Perez, Wang, & Bowen, 2009). Thereupon, 5-FU is inactivated by dihydropyrimidine dehydrogenase (DPD) mainly in the liver to 5,6-dihydro-5-fluorouracil (5-FUH_2) which is further metabolized into α-fluoro-β-ureidopropionic (FUPA) and α-fluoro-β-alanine (FBAL) . Eventually, three metabolites are formed intracellularly which are ultimately responsible for the anticancer effect of capecitabine (complete metabolism is depicted in Figure 1). Pharmacokinetic parameters of capecitabine and its metabolites are characterized by substantial inter-individual variability which is likely caused by the variability in activity of enzymes involved in the metabolism of capecitabine (Deng et al., 2015; Reigner, Blesch, & Weidekamm, 2001). Therefore, bioanalytical assays for the quantitative determination and therapeutic drug monitoring of capecitabine and its metabolites are imperative and could improve the safety and efficacy of treatment with capecitabine and are essential in support of clinical pharmacological studies with capecitabine and 5-FU (Deenen, Rosing, Hillebrand, Schellens, & Beijnen, 2013; Deng et al., 2015). The simultaneous analysis of capecitabine and its metabolites can be

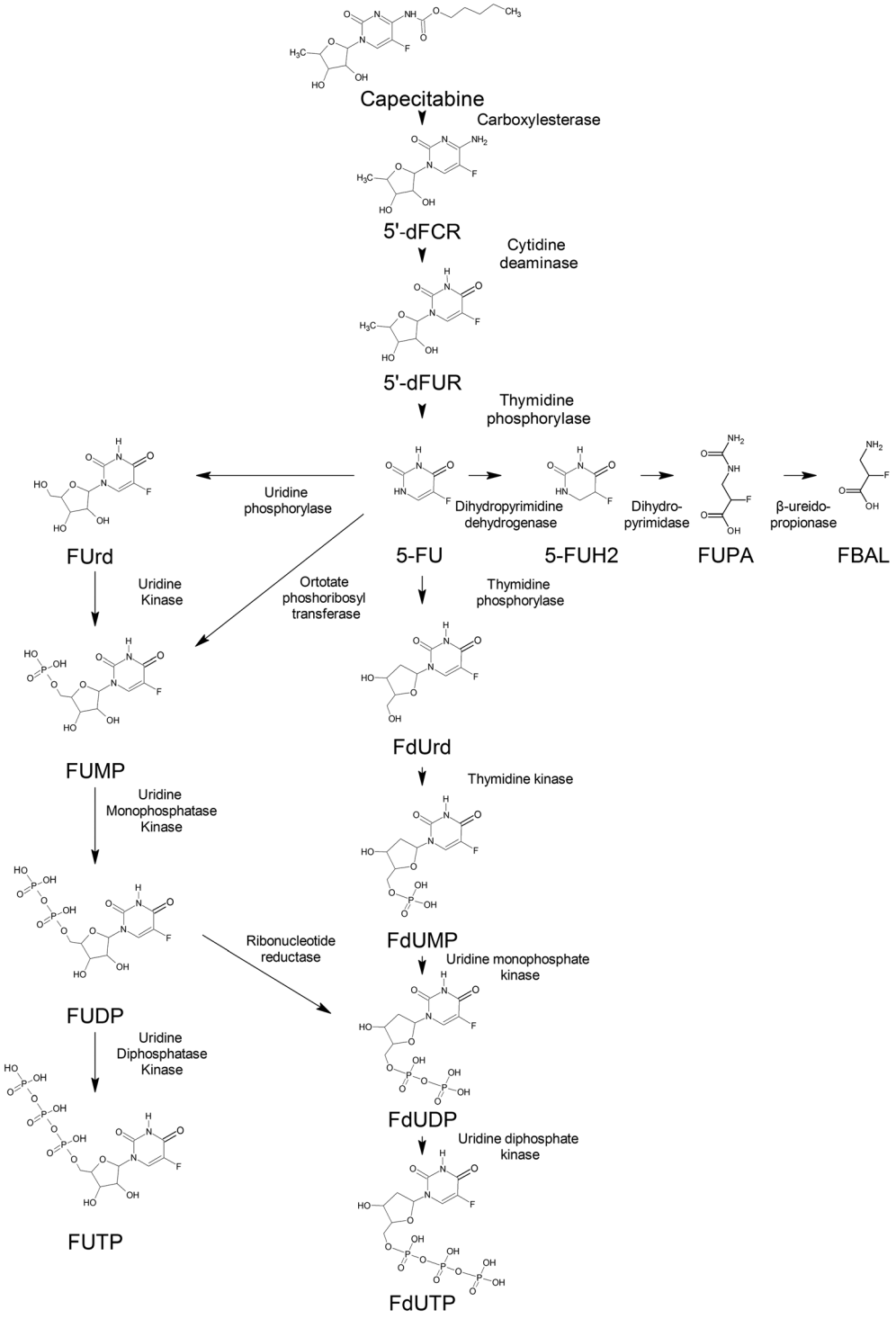


FIGURE 1 Metabolism of capecitabine. Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; 5-FUH2, 5,6-dihydro-5-fluorouracil; FBAL, α-fluoro-β-alanine; FdUDP, 5-Fluoro-2'-deoxyuridine 5'-diphosphate; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; FdUrd, 5- fluoro-2'-deoxyuridine; FdUTP, 5-fluoro-2'-deoxyuridine 5-'triphosphate; FUDP, 5-fluorouridine 5'- diphosphate; FUMP, 5-fluorouridine 5'-monophosphate; FUPA, α-fluoro-β-ureidopropionic; FUrd, 5-fluorouridine; FUTP, 5-fluorouridine 5'-triphosphate

challenging due to broad concentration ranges and varying polarity. Capecitabine has a long carbon chain and shows lipophilicity. During biotransformation the polarity of the metabolites gradually increase and become more hydrophilic (Wang et al., 2019). Plasma concentrations of the metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR) and 5'-deoxy-5-fluorouridine (5'-dFUR) are similar to the plasma concentration of capecitabine. However, plasma concentrations of 5-FU and its converted products are significantly lower (Figure 2) (Henricks et al., 2018). Large differences in retention time can be expected due the differences in polarity between capecitabine and its metabolites, which could pose a challenge for simultaneous analysis particularly using liquid chromatography (LC) (Deng et al., 2015; Montange et al., 2010). Furthermore, high selectivity is pivotal using mass spectrometry (MS) detection as the molecular weights of 5'-dFCR and 5'-dFUR differ by only one mass unit and therefore should be separated during chromatography. In addition the high hydrophilicity of 5-FU, 5-FUH_2, FUPA and FBAL hampers its isolation from a biological matrix. Several assays have been developed for the quantitative determination of capecitabine and its metabolites. This has shown to be troublesome due to the aforementioned challenges. Thus the aim of this review is

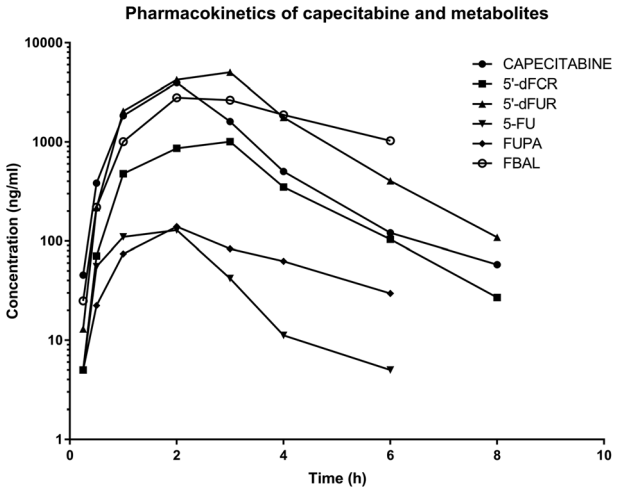


FIGURE 2 Representative of plasma concentration-time curves of capecitabine 5'-dFCR, 5'-dFUR, 5-FU, FUPA and FBAL in a patient with colorectal cancer treated with 1500 mg capecitabine..
Abbreviations: 5'-dFCR, 5'- deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine; FUPA, α-fluoro-β-ureidopropionic

to give an overview and discuss published methods for the bioanalysis of capecitabine and its metabolites. Bioanalytical assays for the determination of capecitabine and its metabolites are based on a chromatographic separation method combined with either MS or ultraviolet (UV) detection, preceded by a sample pretreatment procedure. In this review the three components of the bioanalytical method (sample pre-treatment, separation and detection) will be discussed separately. Moreover, the strengths and weaknesses of the different methods will be discussed. Table 1 gives an overview of the published bioanalytical methods for the determination of capecitabine and its metabolites.

2 | SAMPLE PRE-TREATMENT

Generally, biological samples (e.g. plasma, urine and tissues) cannot be analyzed directly and sample pre-treatment is required to remove endogenous compounds such as lipids, proteins and salts, which usually interfere with chromatographic separation of the analytes. Furthermore, using MS detection these compounds can pollute the system and influence the ionization efficiency of the mass spectrometer and therefore the sensitivity of the method. Hence, sample pre-treatment is an important step in the quantitative determination of capecitabine and its metabolites. The most applied techniques to remove interfering endogenous substrates are liquid-liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PP).

2.1 | Liquid-liquid extraction

LLE is a sample preparation technique based on the solubility of a compound in 2 different immiscible solvents. LLE is cheap and easily executed compared to SPE, but a disadvantage is the difficulty of automatizing LLE, which is readily possible for SPE. LLE is best suited for lipophilic analytes that are extracted from an aqueous matrix to an apolar organic matrix. LLE as sample pre-treatment for capecitabine and its metabolites is possible, however is complicated due to the differences in physiochemical properties between analytes. Which can lead to the extraction of endogenous compounds. Zufía, Aldaz, and Giráldez (2004) reported the use of a mixture of ethyl acetate and acetonitrile (4:1, v/v) after sample acidification with orthophosphoric acid to simultaneously extract capecitabine, 5'-dFUR, 5-FU and 5-FUH_2 from plasma. Whereas the pKa (amine-group) of these compounds lie close to 8, which is the pKa of 5-FU (Escoriaza, Aldaz, Calvo, & Giráldez, 1999) .To ensure that the analytes exist in neutral form during extraction, given that they are weak organic acids, sample acidification was applied. A mean recovery of 90.34% ± 9.48, 90.47% ± 8.95, 95.24% ± 3.96 and 91.5% ± 3.31 were obtained for capecitabine, 5'-dFUR, 5-FU and 5-FUH_2, respectively (Zufía, 2004). Interestingly, in Piórkowska et al. (2014) a similar extraction method is reported using a mixture of ethyl acetate and acetonitrile (4:1, v/v) but without sample acidification. Acidification was not deemed necessary because the assay was developed to only determine capecitabine concentrations (no metabolites) and could therefore be simplified. This allowed for a simplification (leaving out the acidification) of the extraction and a reduction of the extraction solvent volume (Piórkowska et al., 2014). A drawback is that the obtained recovery is lower compared to the method including acidification (67.6 – 71.2% vs. 90.3%). In Licea-Perez et al. (2009) a method was reported in which capecitabine and 5-FU were extracted and separated from FBAL by LLE using a mixture ethyl acetate and acetonitrile (8:3, v/v). The significant differences in polarity between capecitabine, 5-FU and FBAL lead to difficulties in simultaneous extraction of all analytes and poor retention of 5-FU and FBAL in reversed phase chromatography. A derivatization step with dansyl chloride was introduced to overcome these difficulties (Licea-Perez et al., 2009). After extraction the organic phase, containing 5-FU and capecitabine, were transferred to a 96-well plate and dansyl-chloride in acetonitrile and sodium bicarbonate were added and vortex mixed for 3 minutes and incubated for 30 min. Further purification of capecitabine and 5-FU was achieved by a second extraction with tertiary-methyl-butyl ether (tBME). The aqueous phase, containing FBAL, was incubated with dansyl-chloride in acetone and sodium bicarbonate. Further purification was achieved by utilizing SPE. A clear drawback of this method is that an extra step is introduced which complicates the sample pre-treatment and prolongs the duration of sample pretreatment (Licea-Perez et al., 2009). Wang et al. (2019) reported that they tested several separation methods (SPE and PP) and extraction fluids, but that the use of a one-step liquid-liquid extraction of capecitabine, 5-'dFCR, 5'-dFUR, 5-fluoro-2'-deoxyuridine (FdUrd), 5-FU and 5-FUH_2 by ethyl acetate and isopropanol (19:1, v/v) gave optimal extraction recovery (59.3% to 90.2%) (Wang et al., 2019).

TABLE 1 Overview of bioanalytical methods for capecitabine and its metabolites

Analyte	Matrix/Tissue	Species	Sample (µl or mg)	Sample preparation	Detection method	(Internal) Standard	Runtime (min)	Reference
Capecitabine 5'-dFCR 5'-dFUR	Plasma	Human	500	On-line SPE	MS	5'-CUDR	24	(Xu & Grem, 2003)
Capecitabine 5-FU	Plasma	Human	250	LLE	MS	Fluvastatin $[^{15}N_2]$-5-FU	8	(Siethoff et al., 2004)
Capecitabine 5'-dFUR 5-FU 5-FUH_2	Plasma	Human	500	LLE	UV	Tegafur Tegafur 5-BU 5-BU	30	(Zufía et al., 2004)
Capecitabine 5'-dFCR 5'-dFUR 5-FU	P + L + T P + L + T P + L + T P + L + T	H or M H or M H or M H or M	50 50 50 50	PP	MS	Ex: Capecitabine Ex: 5'-dFCR Ex: 5'-dFUR Ex: 5-FU	12	(Guichard et al., 2005)
Capecitabine 5'-dFCR 5'-dFUR 5-FU	P + S + B P + S + B P + S + B P + S + B	M or R M or R M or R M or R	-	PP	UV	Ex: Capecitabine Ex: 5'-dFCR Ex: 5'-dFUR Ex: 5-FU	10	(Dhananjeyan et al., 2007)
Capecitabine 5'-dFCR 5'-dFUR 5-FU	Plasma	Human	500	SPE	MS	$[^2H_8]$-capecitabine 3-Methyl-uridine 3-Methyl-uridine $[^{15}N_2]$-5-FU	14	(Salvador et al., 2006)
Capecitabine 5-FU FBAL	Plasma	Human	50	LLE LLE SPE	MS	$[^2H_{11}]$-capecitabine $[^{13}C_1,^{15}N_2]$-5-FU $[^{13}C_3]$-FBAL	4,5	(Licea-Perez et al., 2009)
Capecitabine	Plasma	Human	1000	SPE	UV	Ex: Capecitabine	6,5	(Farkouh et al., 2010)
Capecitabine 5'-dFCR 5'-dFUR 5-FU	Plasma	Human	500	LLE	MS	Carbamazapine 5-CldUrd 5-CldUrd $[^{13}C_1,^{15}N_2]$-5-FU	15	(Montange et al., 2010)
Capecitabine 5'-dFCR 5'-dFUR 5-FU 5-FUH_2	Plasma	Human	200	PP	MS	Fludarabine 5-chlorouracil 5-chlorouracil 5-chlorouracil 5-chlorouracil	15	(Vainchtein et al., 2010)
5'-dFCR 5'-dFUR	Plasma	Human	1000	SPE	UV	Ex: 5'-dFCR Ex: 5'-dFUR	30	(Buchner et al., 2013)
Capecitabine	Plasma	Human	100	PP	MS	$[^2H_{11}]$-capecitabine	9	

(Continues)

TABLE 1 (Continued)

Analyte	Matrix/Tissue	Species	Sample (µl or mg)	Sample preparation	Detection method	(Internal) Standard	Runtime (min)	Reference
5'-dFCR 5'-dFUR						[$^{13}C_1$,$^{15}N_2$]-5'-dFCR [$^{13}C_1$,$^{15}N_2$]-5'-dFUR		(Deenen et al., 2013)
5-FU 5-FUH_2 FUPA FBAL	Plasma	Human	100	PP	MS	[$^{13}C_1$,$^{15}N_2$]-5-FU [$^{13}C_1$,$^{15}N_2$]-FUH_2 [$^{13}C_3$]-FUPA [$^{13}C_3$]-FBAL	5	(Deenen et al., 2013)
Capecitabine	Plasma	Human	1000	LLE	UV	Voriconazole	8	(Piórkowska et al., 2014)
Capecitabine 5'-dFCR 5'-dFUR 5-FU	Plasma	Human	100	PP	MS	[$^{2}H_{11}$]-capecitabine [$^{13}C_1$,$^{15}N_2$]-5'-dFCR [$^{13}C_1$,$^{15}N_2$]-5'-dFUR [$^{13}C_1$,$^{15}N_2$]-5-FU	10,5	(Deng et al., 2015)
Capecitabine	Serum	Human	100	PP	UV	Gemcitabine	-	(Thorat et al., 2018)
Capecitabine 5'-dFCR 5'-dFUR 5-FU 5-FdUrd 5-FUH_2	Plasma	Human	100	LLE	MS	Fludarabine Fludarabine Fludarabine 5-Chlorouracil Fludarabine 5-Chlorouracil	5	(Z. Wang et al., 2019)

Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-BU, 5-Bromouracil; 5-CldUrd, 5-Chloro- 2-deoxyUridine; 5-CUDR, 5-chloro-2'-deoxyuridine; 5-FdUrd, 5-fluoro-2'-deoxyuridin;5-FU, 5-Fluorouracil; 5-FUH2, 5,6-dihydro-5-fluorouracil; B, bile; Ex, external; FBAL, α-fluoro-β-alanine; FUPA, α-fluoro- β-ureidopropionic; H, human; L, liver; LLE, liquid-liquid extraction; M, mouse; MS, mass spectrometry; PP, protein precipitation; R, rabbit; S, serum; SPE, solid phase extraction; T, tumor; UV, ultra violet.

2.2 | Solid-phase extraction

SPE is a commonly used sample preparation technique by which analytes that are dissolved in a liquid matrix are separated from other compounds based on chemical and physical characteristics. Usually the analyte is retained on a SPE cartridge, after which the interfering compounds are removed by a washing step. Thereafter, the analyte is eluted from the solid phase cartridge using an elution solvent. SPE can be applied in a wide range of analyses, due to the availability of many different types of cartridges and solvents. Nevertheless, the differences in polarity between capecitabine and its metabolites make the choice of cartridge and eluent challenging (Stokvis, Rosing, & Beijnen, 2005). Salvador, Millerioux, and Renou (2006) describes a SPE method using an Atoll XWP extraction cartridge which allowed for simultaneous extraction of capecitabine and metabolites (5'-dFCR, 5'-dFUR and 5-FU) with a high recovery (>90%), but not the smaller more polar metabolites of 5-FU. The Atoll XWP cartridge consists out of polystyrene divinylbenzene, which shows similar chemistry to C18 columns (Salvador et al., 2006). Licea-Perez et al. (2009) reported a SPE method with the use of an Oasis MAX 96 column for the extraction of FBAL out of the aqueous phase after an initial LLE step (Licea-Perez et al., 2009). Buchner et al. (2013) and Farkouh et al. (2010) applied Oasis HLB cartridges for the separation of the analytes from the matrix (Buchner et al., 2013; Farkouh et al., 2010). The Oasis HLB is an all-purpose polymeric reversed-phased column. A practical advantage of this cartridge over others is that these columns maintain their interaction capacities, without the need to keep the phases moisturized. Furthermore, the Oasis HLB column also can retain polar analytes while having both hydrophilic and lipophilic properties. A disadvantage of SPE is that it is labor-intensive and costly compared to LLE and PP. This is unfavorable for assays intended for use in a routine clinical setting (e.g. therapeutic drug monitoring). A way to reduce the workload of SPE is by utilizing on-line SPE, which has been described by Xu and Grem (2003) as a sample pre-treatment method

(Xu and Grem, 2003). On-line sampling brings sample handling back to a minimum, improving the sample throughput and reproducibility. The major advantage of SPE is that it often results in a clean final extract compared to LLE and PP, due to the SPE cartridges being very efficient for the removal of interfering endogenous substances.

2.3 | Protein precipitation

Due to its simplicity and good recovery rates PP is frequently chosen as sample pre-treatment in bioanalytical procedures. It involves the addition of a protein precipitating solvent, subsequent homogenizing and centrifugation, after which, the clear supernatant with analytes can be used further for analysis. Generally, methanol, acetonitrile or a mixture with either of these organic solvents is used for PP. For this review mixtures of methanol and water, trichloroacetic acid (TCA) or mixtures containing acetonitrile were found for PP. Dhananjeyan et al. (2007), Deng et al. (2015) and Thorat, Chikhale, and Tajne (2018) used methanol as the precipitating solvent (Deng et al., 2015; Dhananjeyan et al., 2007; Thorat et al., 2018). Dhananjeyan et al. (2007) reported a single-step PP method using a mixture of methanol-water (50:50, v/v), by which recoveries of >85% were obtained for capecitabine, 5'-dFCR, 5'-dFUR and 5-FU from biological matrices which include mouse plasma, mouse serum and rabbit bile, without having to make concessions on the assays selectivity or specificity (Dhananjeyan et al., 2007). Guichard, Mayer, and Jodrell (2005) reported two separate single step PP methods for the extraction of capecitabine, 5-'dFCR, 5'-FUR and 5-FU from human and mouse plasma, human tumor tissue and mouse liver tissue. Acetonitrile was used to extract the analytes from plasma. It was shown that mouse plasma contains high concentrations of carboxylesterase, which converts capecitabine to 5'-dFCR. To prevent this conversion samples were thawed on ice. A mixture of ammonium acetate: acetonitrile (1:3, v/v) was used to extract the analytes from the tissues (Guichard et al., 2005). Thorat *et al*. (2018) observed a higher efficiency of extraction with methanol then with acetonitrile or a mixture of acetonitrile and methanol (Thorat et al., 2018). Vainchtein, Rosing, Schellens, and Beijnen (2010) tested several sample pre-treatment methods including LLE and PP but high degradation of FUH_2 was found when evaporating the supernatant. To prevent a evaporation step and to efficiently precipitate the plasma proteins, 10% (v/v) trichloroacetic acid (TCA) in water was finally used (Vainchtein et al., 2010). Deenen et al. (2013) developed two separate assays, one assay for the quantitative determination of capecitabine, 5'-dFCR and 5'-dFUR and another assay for 5-FU, 5-FUH2, FUPA and FBAL (Deenen et al., 2013). For sample pre-treatment the same PP procedure as described by Vainchtein, Rosing, Schellens, and Beijnen (2010) was considered and tested, but not deemed suitable due to the acid environment catalyzing the conversion of $^{2}H_{11}$-capecitabine (stable isotope) into 5'-dFUR. Instead, a pH-neutral solution of methanol and acetonitrile (1:1, v/v) was selected as precipitating solvent. For the 5-FU assay, proteins were precipitated with acetonitrile in an acetonitrile-plasma ratio of 4:1 (v/v) (Deenen et al., 2013; Vainchtein et al., 2010). During pre-validation experiments of 5-FU assay it was seen that the sensitivity of 5-FUH_2 significantly increased using a HybridSPE-phospholipid technology (PPT) cartridge filter, which removes phospholipids from the extract. This increased the 5-FUH_2 signal significantly, which led to a gain of sensitivity of factor 50. Therefore, 5-FUH_2 was quantified after filtration, whereas 5-FU, FUPA and FBAL were quantified without filtration (Deenen et al., 2013). A disadvantage of PP is that it yields less clean samples compared to SPE and LLE, which can cause matrix effect (ionization of co-eluting components in the matrix) during detection with MS. This was shown by Wang *et al*. whom have tested both LLE and PP and reported that LLE could remove interfering endogenous components at the greatest extent and subsequently showed less of a matrix effect compared to PP (Wang et al., 2019). Nevertheless, PP can still be an excellent option for sample pre-treatment of capecitabine and its metabolites. If a very sensitive assay and low lower limit of quantification is required, clean extracts and therefore SPE or LLE are preferred. On the contrary, if adequate sensitivity can be reached by using PP as sample pre-treatment, PP is preferred due to easy applicability and low cost.

2.4 | Microextractions

Although LLE, SPE are widely used, these methods can be laborious or consume a great amount of organic solvent. Currently, more attention is being paid to the development of sample pre-treatment methods that are more efficient and environmentally friendly (Wu et al., 2010). A novel sample pre-treatment method that has gained popularity is microextraction. Extraction techniques are classified as a microextraction if the volume of the extracting phase is very small in relation to the volume of the sample. (He & Concheiro-Guisan, 2019). Based on the extraction phase a microextraction method can be classified as solid-phase microextraction (SPME) or liquid-phase microextraction (LPME). The extraction phase in SPME can be either a tube design or a fiber design. The tube or the fiber are exposed to the sample and the analytes are removed from matrix. The difference between SPE is that the objective of SPME is never that of exhaustive extraction, in contrary to SPE (Pawliszyn & Lord, 2000). LPME usually makes use of a hollow fiber (HF) which is dipped into a water immiscible solvent, such as 1-octanol. This solvent fills the pores of the HF, after which the inner lumen of the hollow fiber is filled with an acceptor solution. The HF is placed in the sample and extracts the analytes from the sample (Gjelstad & Pedersen-Bjergaard, 2013; He & Concheiro-Guisan, 2019). Recently, it was shown that by modifying HF with sorbents that they can be used for solid-phase microextraction, thereby combining SPME and LPME which is named solid-liquid-phase microextraction (SLPME) (Yang, Chen, & Shi, 2015). Forough et al. (2017) reported the development of a HF-SLPME method for the simultaneous extraction of capecitabine and 5-FU. In this method HFs of which the lumen was embedded with silver nanoparticles were introduced to strengthen the absorption capacity and provide and extra way for solute transport of the LPME. Therefore combining both LPME and SPME. In this case mobil composition of

matter No. 41 (MCM-41) was used due to its mesoporous characteristics and anchored to silver nano-particles due to the their specific interaction with selected organic functional groups. By using this method both capecitabine and 5-FU could be extracted from the plasma with a high recovery and therefore could be considered as sample pretreatment for capecitabine and its metabolites (Forough et al., 2017).

3 | CHROMATOGRAPHY

Liquid chromatography (LC) is the main separation method utilized for the bio-analysis of capecitabine and its metabolites. Separation of analytes from structurally related endogenous compounds is an important step since these can interfere in analyte detection. (Stokvis et al., 2005). Most of the assays discussed in this review use high pressure liquid chromatography (HPLC), which is the most common type of chromatography. Licea-Perez *et al*. were the only ones to report the use of ultra-high pressure liquid chromatography (UHPLC). UHPLC utilizes smaller particles in column packing and a higher pressure which reduces the run time and improves the resolution. A disadvantage of the higher pressure utilized in UHPLC is the reduced column life due to the higher pressure (Gumustas, Kurbanoglu, Uslu, & Ozkan, 2013). An important difference between assays utilizing UV detection and MS detection is that the MS assays use the chromatography step primarily to separate the analytes from any matrix components. Whereas most LC-UV assays use chromatography to separate the analytes, internal standards (ISs) or potential metabolites. Due to the selectivity of the MS, co-eluting peaks do not necessarily cause interference with the detection of the analytes (Stokvis et al., 2005). Yet, co-eluting endogenous components in the matrix can affect the quantitation of the analytes due to matrix effect. Therefore if matrix effect occurs chromatographic conditions or run time can be adjusted to enhance the chromatographic separation between the component causing the matrix effect and the analyte (Jemal, 2000; Srinivas, 2009). While capecitabine is a lipophilic compound (Log P = 0.84, as calculated by Benet, Broccatelli, and Oprea (2011) using the method of Leo (Benet et al., 2011; Leo, 1993)), the metabolites are more hydrophilic. These compounds elute rapidly from reversed-phase columns even with mobile phases containing a low percentage of organic content, which complicates the development of a chromatographic method to simultaneously analyze these compounds (Salvador et al., 2006; Xu & Grem, 2003). Normal phase or ion-exchange chromatography are not easy applicable with MS due to incompatible organic solvents and modifiers being used here. Moreover, additives to the mobile phase such as phosphates and strong acids as trifluoroacetic acid (TFA) are undesirable because they contaminate and reduce the MS signal significantly (Stokvis et al., 2005). Furthermore, to ensure stable retention times on a HPLC column the pH of the mobile phase should preferably be approximately 2 units above or below the pKa of the analytes to assure that they are >99% unionized. The most common components of the mobile phase for capecitabine and its metabolites are acetonitrile, methanol, formic acid and ammonium acetate (see Table 1). Moreover, the majority of the chromatographic runs were carried out in a gradient mode allowing for simultaneous analysis of capecitabine and its more polar metabolites. Several stationary phases have been exploited in the assays described in this review (see Table 1). Most assays employed a reversed phase column with C18 modified material. Siethoff, Orth, Ortling, Brendel, and Wagner-Redeker (2004) describe the use of a column switching method. Two different columns, possessing different polarities were used to analyze capecitabine and 5-FU simultaneously (Siethoff, Orth, Ortling, Brendel, & Wagner-Redeker, 2004). A Hypercarb (porous grafitic carbon) column to simultaneously quantify capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and 5-FUH2 was used by Vainchtein et al. (2010). This column showed unique separation properties and can retain a wide range of small polar and non-polar compounds, allowing for the simultaneous analysis of capecitabine and subsequently formed more polar metabolites (Vainchtein et al., 2010). But over time and upon frequent application of the assay it was seen, however, that the signal intensity decreased, which led to significant loss of sensitivity. Therefore a new assay was developed utilizing a different type of chromatography. Two different assays were developed due to differences in polarity of capecitabine, 5'-dFCR and 5'-dFUR on the one hand compared to 5-FU and its more polar metabolites on the other hand. Standard reversed phase chromatography was used for capecitabine, 5'-dFCR and 5'-dFUR. Hydrophilic interaction liquid chromatography was utilized for 5-FU. 5-FUH_2, FUPA and FBAL due to the more polar nature of these analytes. (Deenen et al., 2013; Vainchtein et al., 2010). Run times of the described assays vary between approximately 5 and 30 min, depending on the analytes being analyzed. Several assays described in this review have shown that run time higher than 10 min are not necessary (See Table 1). Which allows for high-throughput analysis of capecitabine in a routine clinical setting.

3.1 | Thin layer chromatography

Interestingly, Thorat *et al*. (2010) reported the use of high-performance thin layer chromatography (HPTLC) using a TLC plate pre-coated with silica gel to rapidly and easily determine capecitabine concentrations (Thorat et al., 2018). The TLC plates were analyzed using a densitrometric scanner and the lower limit of quantification was 250 ng/ml. The accuracy ranged from 96.0% to 102.7% and intra-day precision and inter-day precision were 12.6% and 13.9% or less at each quality control level, respectively. The method was developed and applied for therapeutic drug monitoring purposes in serum for capecitabine alone and in combination with oxaliplatin. It was shown that developed HPTLC method was sufficiently sensitive and accurate for this purpose. TLC has the advantage of being cheaper, easier and quicker, due to being able to analyze multiple samples simultaneously compared to other established analytical methods (Thorat et al., 2018). A major disadvantage of TLC is that it cannot be coupled easily with other techniques such as mass spectrometry which leads to a higher detection limit compared to other assays utilizing other techniques. Furthermore, TLC plates have a limited length which limits the length of separation, TLC is an open system which can be influenced by exogenous factors such as temperature and humidity. It can be concluded that LC is the preferred separation method compared with TLC. Yet, the described HPTLC method by Thorat *et al*. can still be a

viable option if proper equipment for LC is not available or a simple and fast method for the quantification of capecitabine is needed.

4 | DETECTION

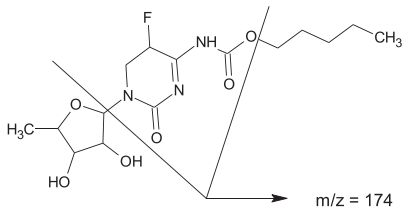
MS is a sensitive and selective detector which is suitable for detection of a large range of compounds. Triple quadrupole (TQ) MS is the most applied and first choice in the quantitative analysis of capecitabine and its metabolites (Stokvis et al., 2005). To be able to detect a compound with MS the analyte needs to be, negatively or positively ionized. The most applied technique for the ionization of capecitabine and its metabolites is by electrospray ionization (ESI). In Licea-Perez et al. (2009) a variant of ESI is described in which heated gas is used for desolvation of the eluent, by a turbo ion spray (TIS) (Licea-Perez et al., 2009). Another form of ionization is atmospheric pressure chemical ionization (APCI) which is described by Montagne *et al*., using both positive and negative modes (Montange et al., 2010). Both ESI and APCI were considered but best signals were acquired with APCI, which also showed less of a matrix effect and decreased chemical background noise (Montange et al., 2010). Furthermore, it was shown by Deenen *et al*. that 5-FUH_2 is influenced by the presence of phospholipids in the matrix, which reduced the sensitivity of 5-FUH_2 significantly (Deenen et al., 2013). It is known that phospholipids may influence the signal by causing matrix effect by either signal enhancement or suppression (Xia & Jemal, 2009; Yang, Chen, & Shi, 2015). Therefore, the presence of phospholipids should be kept to a minimum or separated from the influenced analytes during chromatography. After ionization the ions enter the mass spectrometer and ion selection and detection take place. Detection can be executed by means of selecting the molecular ion in selective ion monitoring (SIM) or multiple reaction monitoring (MRM) modes. Operating in MRM allows for the identification and detection of the analyte by means of both the parent ion and typical fragment product ion for TQ platforms. A higher sensitivity and selectivity is reached by MRM in comparison to SIM (Stokvis et al., 2005). Fragmentation of capecitabine and its metabolites was described extensively by Deenen et al. (2013) and Vainchtein *et al*. (Deenen et al., 2013; Vainchtein et al., 2010) (depicted in Table 2). The most abundant product ion of capecitabine is a product ion with a *m/z* of 174 in Vainchtein et al. (2010) and 130 in Deenen et al. (2013). This difference in *m/z* of 44 can be explained by the loss of a sugar moiety and a pentane chain as described in Vainchtein et al. (2010) instead of the pentanoic acid described in Deenen et al. (2013). The reported fragmentation of 5'-dFUR, 5-FU and 5-FUH_2 was similar in both articles. The *m/z* of the product ion of 5'-dFUR was 108, corresponding with the loss of a sugar moiety and the fluorine atom. The product ions of 5-FU and 5-FUH_2 had a *m/z* of 42 and 83 which corresponds with the loss of a formamide moiety or fluoroethane moiety. (Deenen et al., 2013; Vainchtein et al., 2010) Additionally, the fragmentation of FUPA and FBAL were described by Deenen et al. (2013). The most abundant product ion of FUPA and FBAL had a *m/z* of 106 and 86, respectively. This corresponds with the loss of a formamide moiety for FUPA and a fluoroethane moiety for FBAL. Deng et al. (2015) reported the use of a polarity switching method between ESI- and ESI+ in a single run (Deng et al., 2015). This method was chosen due to the chemical noise found when analyzing 5'-dFCR, 5'-dFUR and 5-FU in ESI+ mode compared to ESI-. Capecitabine was analyzed under ESI+ (Deng et al., 2015). Several papers referred to in this review have applied UV detection as detection method. Due to different absorption maxima of capecitabine and its metabolites the setting of the wavelength must be well considered. Capecitabine exhibits UV absorption maxima at approximately 214, 241 and 305 nm. While the exhibited UV absorption maxima of 5'-dFCR and 5-FUH2 is at 285 nm and 205 nm, respectively. 5-FU and 5'-dFUR exhibit a maximum at approximately 205 nm and 266 nm. Zufía et al. (2004) described a method in which multiple wavelengths were monitored to detect capecitabine, 5'-dFUR, 5-FU and 5-FUH_2. Dhananjeyan et al. (2007) detected capecitabine, 5'-dFCR, 5'-dFUR and 5-FU at a wavelength of 254 nm, at this wavelength capecitabine and the measured metabolites showed near equal absorption. Piórkowska et al. (2014) and Farkouh *et al*. (2014) both described an assay which only quantifies capecitabine and set the wavelength at 305 nm (Dhananjeyan et al., 2007; Farkouh et al., 2010; Piórkowska et al., 2014; Zufía et al., 2004). Drawbacks of the described assays utilizing UV detection are the relatively low sensitivity for pharmacokinetic studies in humans (Dhananjeyan et al., 2007; Farkouh et al., 2010) and the long runtime seen Zufía et al. (2004). In contrary to the mentioned assays, the assay developed by Piórkowska et al. (2014) has satisfactory sensitivity for application in pharmacokinetic studies in humans and has an acceptable runtime (8 min), but only quantifies capecitabine (Piórkowska et al., 2014). UV detection is thus a viable option for the quantification of capecitabine, but if quantification of the metabolites is needed MS detection is to be preferred due to its higher sensitivity.

5 | INTERNAL STANDARDS

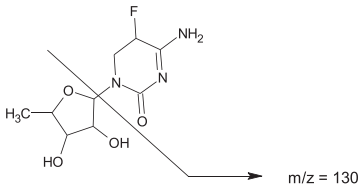
Differences in sample pre-treatment, instrumental related parameters or experimental conditions can cause variations in concentrations detected. The variations can be corrected by using either an external standard (ExS) or internal standard (IS) (Stokvis et al., 2005). This is especially important with MS detection were the signal can be variable and differ due to matrix effect, whereas the signal in UV detection is more stable. Three assays described in this review used an ExS to correct for the variations (Buchner et al., 2013; Dhananjeyan et al., 2007; Guichard et al., 2005). Although it is well known that ISs reduce the effect of interfering matrix components, minimizes sample processing errors and the variability of detection, ISs are not always required (Imre et al., 2019). ISs standards are preferred due to being more accurate. Most of the described assays use ISs that are structurally related to the analytes. Examples of these are 5-bromouracil (5-BU) and 5-chlorouracil (5-CU). The use of 5-BU in LC-MS/MS was described by Vainchtein et al. (2010) but showed a reasonably high ion suppression, due to elution along with other endogenous plasma components. For that reason 5-CU was chosen over 5-BU (Vainchtein et al., 2010). Furthermore, prescribed drugs such as fluvastatin,

TABLE 2 Fragmentation of capecitabine and a subsequent metabolites as described by Vainchtein et al. and Deenen *et al.* (A) = Capecitabine, (B) = 5'-dFCR, (C) = 5'-dFUR, (D) = 5-FU, (E) = 5-FUH2, (F) = FUPA, (G) = FBAL

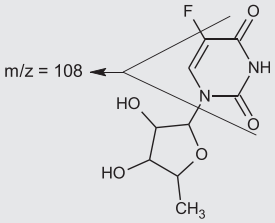
A



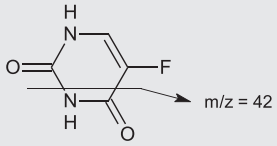
B



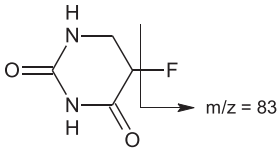
C



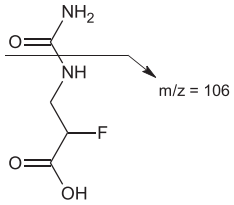
(D)



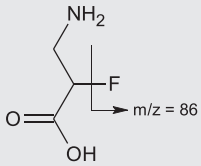
(E)



(F)



(G)



carbamazepine, voriconazole, gemcitabine and fludarabine have been used as IS ((Montange et al., 2010; Piórkowska et al., 2014; Siethoff et al., 2004; Thorat et al., 2018; Wang et al., 2019) It can be questioned if using prescribed drugs, especially commonly prescribed drugs such as fluvastatin, carbamazepine and voriconazole should be used as an IS, due to the limitation in the applicability of the assay. Depending on the use of the assay prescribed drugs can be used as an IS in for example pharamacology studies in healthy volunteers, but are not suited for clinical use such as therapeutic drug monitoring (Srinivas, 2016). The most ideal IS for MS is a stable isotopically labeled identical isomer, due to its identical behavior to the analyte in terms of matrix effect (Jemal, 2000; Wang, Cyronak, & Yang, 2007). Yet until recently stable isotopically labeled ISs of capecitabine and its metabolites were unavailable. Salvador et al. (2006) were the first to describe the use of stable isotopically labeled $^{2}H_{8}$-capecitabine as IS (Salvador et al., 2006). In the years thereafter Deenen et al. (2013) and Deng et al. (2015) both described methods using $^{2}H_{11}$-capecitabine as IS (Deenen et al., 2013; Deng et al., 2015). A disadvantage reported by Deenen et al. (2013) is the rapid conversion of $^{2}H_{11}$-capecitabine into 5'-dFUR in an acid environment. Storage of plasma samples at 2-8 °C that were processed with TCA for a couple of hours resulted in an unacceptable increase in 5'-dFUR of more than 25%, whereas the concentration of capecitabine decreased with a similar amount. A pH-neutral solution of methanol and acetonitrile (1:1, v/v) was used in which stability of $^{2}H_{11}$-capecitabine was sufficient (Deenen et al., 2013). Regarding stable isotopically labeled ISs, ^{13}C or ^{15}N are the preferred labeled ISs. The retention time of deuterium (^{2}H) labeled isotopes can differ slightly compared to analyte due to the deuterium isotope effect. Deuterated ISs can elute slightly earlier on a reverse phase chromatography system due to being less lipophilic than the analyte, which can lead to an inadequate correction of the variations (Wang et al., 2007). As far as we know this problem has not been reported for ^{13}C and ^{15}N labeled ISs which have the same retention time as the analyte.

6 | INTRACELLULAR METABOLITES—FDUMP, FUTP AND FDUTP

Capecitabine is ultimately metabolized into and 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), 5-fluorouridine 5'-triphosphate (FUTP), and 5-fluoro-2'-deoxyuridine 5'triphosphate (FdUTP) (5-FU nucleotides) (Deenen et al., 2013; Derissen et al., 2016). FdUMP inhibits thymidylate synthase (TS), which leads to accumulation of deoxyuridine triphosphate (dUTP) and exhaustion of deoxythymidine triphosphate (dTTP) which leads to an intracellular metabolic imbalance. This interferes with DNA synthesis and repair, ultimately leading to apoptosis. FUTP and FdUTP are incorporated in RNA and DNA, which disrupts normal RNA processing and function and leads to DNA damage (Derissen et al., 2016; Grem, 1997; Wang et al., 2019). These nucleotides are not included in assays for the quantification of capecitabine or 5-FU as they are formed intracellularly and are not emerging in plasma. However, since these nucleotides are held responsible for the anticancer activity and toxicity, quantification of intracellular concentrations could be interesting. Through the years several assays for the quantification for FdUMP, FUTP and FdUTP have been described. Nonetheless, most assays were not applicable for clinical use for various reasons (e.g. the use of radioactive-labeled nucleotides or lack of sensitivity) (Procházková, Liu, Friess, Aebi, & Thormann, 2001; Weckbecker, 1991). To date and to our knowledge only Derissen, Hillebrand, Rosing, Schellens, and Beijnen (2015) have reported the development of an LC-MS/MS assay that can quantify FdUMP, FUTP and FdUTP intracellularly, in a clinical setting (Derissen et al., 2015). Quantification of FdUMP, FUTP and FdUTP in peripheral blood mononuclear cells (PBMCs) by cell lysis and subsequent extraction with methanol was described. A common obstacle in the quantification of nucleotides is the interference by other structurally related endogenous nucleotides. Sufficient separation of analytes and endogenous compounds during chromatography was achieved by using a Biobasic anion exchange column and a gradient in which the amount of ammonium acetate and the pH were gradually decreased and increased, respectively. This LC-MS/MS assay showed to be applicable for the measurement of intracellular FUTP concentrations after administration of capecitabine to patients. For the measurement of FdUTP and FdUMP a higher sensitivity is needed (Derissen et al., 2015).

7 | ANALYTICAL CHALLENGES AND FUTURE PERSPECTIVES

Analysis of capecitabine and its metabolites has some difficulties. The major challenge is the simultaneous extraction, chromatographic separation and detection of capecitabine and its metabolites due to the large differences in polarity. To date no assay has been reported which can measure capecitabine, its metabolites and those of 5-FU simultaneously. Vainchtein et al. (2010) has reported an assay which was capable of quantifying capecitabine and its metabolites, 5-FU and 5-FUH2. But it was not robust enough and upon frequent application of this assay signal intensity decreased, which led to a significant loss of sensitivity (Vainchtein et al., 2010). Deenen et al. (2013) developed a method, which consisted of two separate assays, to quantify capecitabine and its 'large' metabolites and 5-FU and its subsequent 'small' metabolites. Furthermore, attention should be paid to sample pre-treatment due to the influence of phospholipids on the signal intensity of 5-FUH_2. Another challenge faced in the bioanalysis of capecitabine and its metabolites is the quantification of these analytes from tumor tissue. Tumor tissue analysis is challenging compared to liquid matrices such as plasma due to difficulty of tissue sampling and the heterogeneous nature of tumor tissue. Therefore, aspects such as sample collection, tissue homogenization, extractability from the tissue and stability should be carefully considered (Xue et al., 2012). It was shown by Guichard *et al*. that quantification of capecitabine and it metabolites in tumor tissue is possible with a sensitivity comparable to previous published work (Guichard, 2005; Reigner et al., 2003; Siethoff et al., 2004). Another interesting issue is that quality controls which are prepared by spiking the control matrix with the test analyte do not always mimic the activity of the analyte in samples from treated patients. Samples from treated patients can differ from the control matrix due to various reasons including incurred instability, matrix effect and sample inhomogeneity (Vazvaei, 2018). To ensure reproducibility of the measured concentrations, repeated measurement of analytes within a selected sample set of treated patients in separate runs on different days is advised. This is also known as incurred sample reanalysis (ISR) and is mostly applied during drug discovery stage (Giri, Patel, Joshi, Giri, & Srinivas, 2019; Vazvaei, 2018). Most assays described in this review did not perform any kind of ISR strategy. Therefore, reproducibility cannot be certainly be assured and should be considered during the development stage of the assay. Improvement in the quantification of capecitabine and metabolites could be the development of an integrated assay which can also quantify 5-FU and all its metabolites simultaneously. Furthermore, more efficient and environmentally friendly sample pre-treatment methods such as microextractions should be considered. In addition, future research should consider the possibility of simultaneously quantifying capecitabine and its metabolites and concomitant drugs which could stimulate the use of the assays for therapeutic drug monitoring of capecitabine. As was shown by Thorat *et al*., whom have quantified capecitabine and oxaliplatin using one assay (Thorat et al., 2018). Moreover, development of an assay able to quantify all cytotoxic intracellular 5-FU nucleotides should be considered which could provide valuable information concerning the pharmacokinetics of the active metabolites. This information could potentially be used to assess the exposure to treatment with fluoropyrimidines and to assess the risk of developing severe fluoropyrimidine-related toxicity in patients. Subsequently, it should be kept in mind that this method is heavily dependent on the amount of metabolites being released from the cell matrix, which can differ and can be difficult to assess.

8 | CONCLUSION

HPLC methods coupled to UV detection have been successfully applied for the quantification of capecitabine and its metabolites in biological

matrices. However, LC coupled to tandem MS is now the preferred method for determining capecitabine in biological samples due to improved selectivity and sensitivity. This allows for better assessment of the pharmacokinetic parameters of capecitabine and metabolites. LLE, SPE and PP are described as sample pre-treatment. PP was the most reported technique due to being easy and fast to perform. LLE and SPE were described but are labor intensive and costly, but yield cleaner extracts compared to PP. Differences in polarity between capecitabine and metabolites raise problems in the simultaneous bioanalysis of these compounds. Due to these differences sample pre-treatment and chromatographic separation are complicated. An 'all-in-one' system fulfilling all bioanalytical validation requirements, remains to be developed. Furthermore, the measurement of the active metabolites of capecitabine is complicated by their intracellular formation. The intracellular metabolites are present in the cells of patients in very low concentrations, therefore high sensitivity is required.

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