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Rapid and simplified HPLC-UV method with on-line wavelengths switching for determination of capecitabine in human plasma

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Received October 27, 2013, accepted January 13, 2014

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Pharmazie 69: 500–505 (2014) *doi: 10.1691/ph.2014.3223*

Capecitabine is an important oral fluoropyrimidine anticancer drug. The purpose of this study was to overcome limitations of previously reported methods and propose an optimized and widely available tool for analysis of capecitabine in human plasma. The simplification of the liquid-liquid extraction procedure involved elimination of unnecessary addition of phosphoric acid and reduction of the extraction solvent volume. The use of voriconazole as the internal standard, combined with gradient elution and on-line wavelength switching, assured very high within- and between-run precision of results (relative standard deviation < 7.1% for lower limit of quantification) and enabled the reduction of the total chromatographic run time to 8 min. The calibration curve was linear within the range of 0.05–10.00 μg/mL and the method selectivity was confirmed in the presence of capecitabine metabolites. All validation parameters met the acceptance criteria set by international regulatory guidances, which proves that the method leads to reliable results. The method may be applied in human pharmacokinetic studies, for the regulatory purposes and therapeutic drug monitoring.

1. Introduction

Over the last decade, several new oral fluoropyrimidines have been developed and studied for the treatment of many types of cancers (Adjei 1999; Bunnell and Winer 1998; Diasio 1999; Hoff et al. 1999; MacDonald 1999; Meropol 1998). The oral fluorinated pyrimidines are compounds that may emulate the effect of the continuous infusion of the tumor-selective cytotoxic moiety 5-fluorouracil (5-FU). Capecitabine (N^4-pentoxycarbonyl-5'-deoxy-5-fluorocytidine) which is an orally administered, non-cytotoxic prodrug was developed to reduce adverse effects of 5-FU. After almost complete absorption from the gastrointestinal tract capecitabine is easily converted to its metabolites: 5'-deoxy-5-fluorocytidine (5'-DFCR), 5'-deoxy-5-fluorouridine (5'-DFUR), 5-FU, 5-fluorodihydropyrimidine-2,4-dione (FUH_2), N-carbamoyl-2-fluoro-β-alanine (FUPA), 3-amino-2-fluoropropionic acid (FBAL), respectively (Fig. 1). After oral administration the peak plasma concentration for the drug and its metabolites occurs shortly ($t_{max} = 2.0$ h) and its elimination half-life is approximately 1 h (Reigner et al. 2001). LC-MS methods permit the simultaneous measurement of plasma parent drug and its metabolites, however the reported LC-MS methods used for the determination of capecitabine in human plasma allowed to quantify the compound mostly in a range of 0.001−1.00 μg/mL (Guichard et al. 2005; Licea-Perez et al. 2009; Montange et al. 2010; Salvador et al. 2006; Siethoff et al. 2004, Švobaitė et al. 2009; Vainchtein et al. 2010), with exception of two methods with linearity range over 6 μg/mL (Deenen et al. 2013; Xu and Grem 2003). According to our knowledge, so far only three HPLC-UV methods for the analysis of capecitabine in human plasma were reported (Farkouh et al. 2010; Reigner et al. 1998, Zufia et al. 2004). For the most sensitive HPLC-UV method a lower limit of quantification (LLOQ) of 0.025 μg/mL was reported (Zufia et al. 2004) and the linearity range aimed 20.00 μg/mL (Farkouh et al. 2010; Zufia et al. 2004). Except one method was used for therapeutic drug monitoring (Farkouh et al. 2010), other methods allow concomitant determination of metabolites. A method for the determination of capecitabine in mouse plasma was also reported, but its linearity range of 1−500 μg/mL seems to be not suitable for human pharmacokinetic studies (Dhananjeyan et al. 2007).

Among the reported methods one can hardly find a cost-effective tool based on simple equipment for determination of capecitabine as only analyte in a large number of samples, for example during bioequivalence studies or therapeutic drug monitoring (Farkouh et al. 2010). Bioequivalence studies based on the analysis of parent drug and quantitation of metabolites is not required by regulatory agencies. The developed method was designed for the application in bioequivalence studies conducted in patients receiving capecitabine in a single oral dose of 1500 mg during the course of chemotherapy treatment. After analysis of available pharmacokinetic data the linearity range was specified as 0.05−10.00 μg/mL. Therefore, one can surprisingly conclude that the linearity ranges for reported HPLC-UV methods are more suitable than those of LC-MS methods in this case.

Because capecitabine was the only analyte of interest, the aim of the method development in this study was to avoid the impact of metabolites on the determination of parent drug during the successive steps of the analysis. At our best knowledge there is no publication yet, which presents a method for the determination of capecitabine, developed according to the recent European

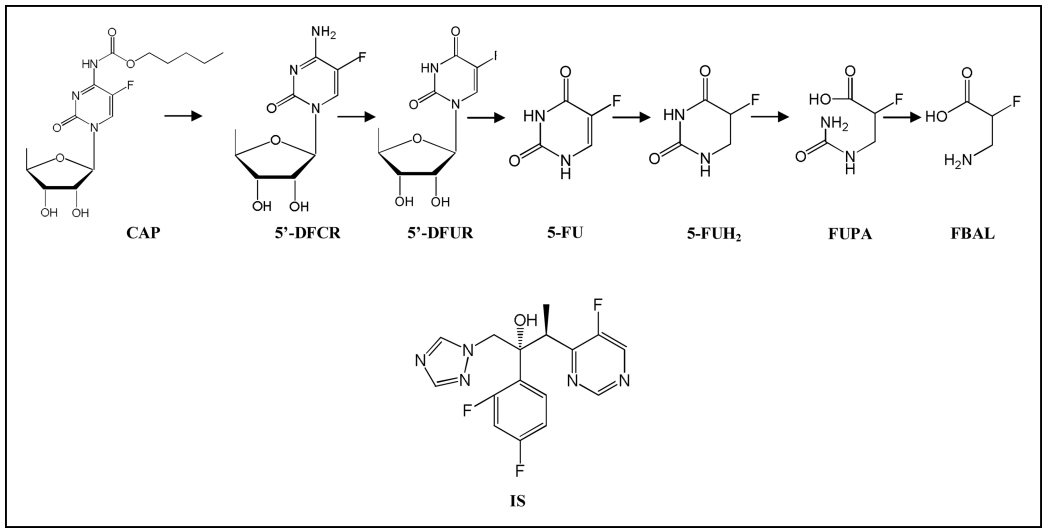

Fig. 1: Chemical structures of capecitabine (CAP) and its major metabolites: 5'-deoxy-5-fluorocytidine (5'-DFCR), 5-fluoro-5'deoxyuridine (5'-DFUR), 5-fluorouracil (5-FU), 5-fluorodihydropyrimidine-2,4-dione (FUH_2), N-carbamoyl-2-fluoro-β-alanine (FUPA), 3-amino-2-fluoropropionic acid (FBAL) and the internal standard voriconazole (IS).

Medicines Agency guideline on bioanalytical validation (EMA 2011). Moreover, each of the reported HPLC-UV methods has some limitations, i.e. insufficient sensitivity and lack of internal standard (Dhananjeyan et al. 2007; Farkouh et al. 2010), extensive sample pretreatment (Farkouh et al. 2010; Reigner et al. 1998) or long chromatographic run time (Zufia et al. 2004). Summing up, there is lack of a cost-effective tool designed for application in bioequivalence studies after single, oral administration of capecitabine in standard doses.

This paper intends to fill this niche and describe a simple, rapid and sensitive HPLC-UV method with optimized sample preparation based on liquid-liquid extraction of capecitabine from human plasma suitable for application in pharmacokinetic studies. Taking into account the importance of the drug, the need for an optimized and cost-effective tool for analysis of capecitabine seems to be unambiguous.

2. Investigations and results

2.1. Method development and optimization

The initial phase of the study was performed in the Department of Drug Chemistry, Medical University of Warsaw, and then the method was transferred for further optimization and validation to the Pharmacology Department of Pharmaceutical Research Institute.

Capecitabine exhibits UV absorption maxima at 214, 241 and 305 nm (Fig. 2), while the maximum absorption for the internal standard is observed at 260 nm. The interferences from endogenous and exogenous substances are presented in human plasma at lower wavelengths. The wavelength of 305 nm was selected as optimal for the determination of capecitabine, but it was not suitable for the internal standard. Therefore, on-line wavelength switching from 305 to 265 nm was applied during the analysis (Fig. 3). The internal standard peak appeared in the retention time of the chromatogram, which is almost free from the interfering peaks derived from plasma. The use of voriconazole as internal standard in combination with on-line wavelength switching of the detector is one of key novelties in the described method. It enabled the simultaneous determination of capecitabine and internal standard and reduced the total chromatographic run time to 8 min. Such a short run time, similar to that achieved by Reigner et al. (1998), is very important to facilitate the analysis of a large number of samples. Other HPLC-UV methods had a longer chromatographic run time of 10 min (Dhananjeyan et al. 2007) and 30 min (Zufia et al. 2004), which is associated with the concomitant determination of capecitabine metabolites. To enable such a short run time numerous compounds were screened to find an internal standard with retention time similar to capecitabine under the developed chromatographic conditions. Although voriconazole is a weak base and capecitabine is a weak acid, the extraction recoveries of both drugs met the acceptance criteria demanded by the FDA guideline (2001). The extraction recovery of capecitabine for studied concentrations 0.15, 4.00 and 8.00 ng/mL were consistent: 54.9%, 61.5% and 61.8%, respectively. The extraction recovery of voriconazole (one concentration in all capecitabine plasma samples) was similar to that of capecitabine and ranged 67.6–71.2%. What was even more important, the extraction recovery of the internal standard was not affected by the capecitabine concentration and assured very high precision of the results (Table 1). Therefore, voriconazole was selected as an appropriate internal standard.

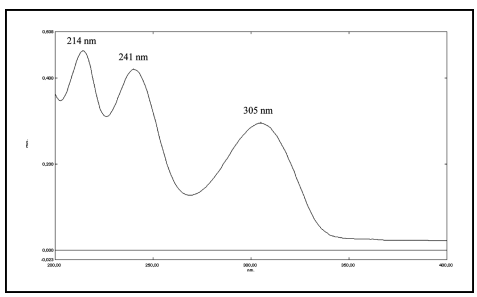

Fig. 2: The UV spectrum of capecitabine.

The mobile phase components were based on the assay by Zufia et al. (2004). To separate capecitabine from interfering compounds extracted from plasma and to fasten the method run-time, the gradient elution with 0.1% formic acid and methanol was applied. Moreover, the formic acid content was decreased from the previously reported 1% (Zufia et al. 2004) to 0.1%, possibly extending the life of an HPLC column. The appropriate selection of chromatographic column, including lower diameter of particles, was an important factor in achieving good chromatographic peak shape and resolution as well as a short run time.

It turned out that the liquid-liquid extraction procedure described by Zufia et al. (2004) did not allow proper selectivity in our hands, and therefore was further optimized and simplified. The best results were achieved with 3 mL of ethyl acetate/acetonitrile (4:1, v/v) without ortophosphoric acid, therefore one step of the sample preparation was eliminated. Moreover, the volume of the organic solvent used was 40% reduced in comparison to the previous report (Zufia et al. 2004) and therefore the method may

Table 1: Accuracy and precision (n = 6)

Nominal concentration (μg/mL)	Concentration found (mean, μg/mL)	Accuracy (mean, %)	Precision (RSD, %)
	Day 1		
0.05	0.05	105.3	7.07
0.15	0.16	107.0	1.71
4.00	3.83	95.7	3.70
8.00	7.75	96.9	1.66
	Day 2		
0.05	0.06	116.7	1.77
0.15	0.16	106.4	2.37
4.00	3.71	92.7	4.33
8.00	7.01	87.6	1.56
	Day 3		
0.05	0.05	104.7	2.31
0.15	0.15	102.0	2.36
4.00	4.13	103.2	1.43
8.00	7.68	96.0	5.12

be considered to be more environmental friendly. As in case of assay reported by Fakough et al. (2010), to achieve appropriate sensitivity rather high plasma sample volume of 1 mL was necessary, what may be considered in certain cases as a limitation of the method. The extraction recovery of capecitabine was sufficient to quantify the lowest concentration of capecitabine reliably with the acceptable accuracy and precision.

2.2. Method validation

The validation of the bioanalytical method was performed according to international guidelines (EMA 2011, FDA 2001), in compliance with the principles of Good Laboratory Practice (GLP). The main characteristics essential to ensure the reliability of analytical results were: selectivity, lower limit of quantification, linearity, accuracy, precision, dilution integrity, stability of capecitabine in the biological matrix, stability of capecitabine and the internal standard in the stock and working solutions as well as in the extracts.

2.2.1. Selectivity

Possible interferences from the human plasma were evaluated by analyzing blank human plasma samples derived from six sources, including haemolysed and hyperlipidaemic plasma.

One of the most important tasks during bioanalytical method development and validation is to minimize the impact of the major or unstable metabolites on the determination of a parent drug. The evaluation of the interference caused by capecitabine metabolites was performed by assaying six plasma samples containing the metabolites at concentrations corresponding to the maxima expected in the real samples: 4.0 μg/mL for 5'DFCR, 5'DFUR, and FBAL as well as 0.5 μg/mL for 5-FU, FUH_2, and FUPA (Deenen et al. 2013). The aim of the method development in this case was only to avoid the impact of metabolites on determination of parent drug, therefore, metabolite concentration did not have to be quantified. According to guideline (EMA 2011), the absence of interfering components is accepted if the response (peak height or area) is less than 20% of LLOQ and 5% for the internal standard. The evaluation of the selectivity in the absence and presence of capecitabine metabolites indicated lack of interferences at the retention time of capecitabine and the internal standard (Fig. 4) and therefore this was the best possible result to be achieved.

2.2.2. Limit of quantification

The lower limit of quantification was determined by analyzing minimum five plasma samples at 0.05 μg/mL. (Fig. 5, Table 1). The 90% confidence intervals for the accuracy within one day and within three days were within the ranges of 99.2–111.5% and 99.2–118.4%, respectively. The 90% confidence intervals for within one day and within three days' precision were within the ranges of 5.48–13.12% and 1.52–13.12%, respectively. For each chromatogram the signal to noise ratio exceeded 5.

2.2.3. Linearity

The calibration curve, constructed by plotting the peak area ratios of capecitabine to the internal standard against the nominal concentrations of capecitabine, was linear within the range of 0.05–10.00 μg/mL. The calibration curves were obtained by a linear regression analysis with a weighting factor of $1/x^2$ selected according to the minimum sum of percentage relative errors (Almeida et al. 2002), when weighting was not applied the linear range was limited to 0.15–10.00 μg/mL. The values of regression parameters for the curve, described by the equation: $y = ax + b$, were: $a = 0.2468$, $b = -0.0007$ and $r = 0.9986$ (n = 6). All regression parameters were statistically significant ($\alpha = 0.05$, $df = n - 2$). During the validation 14 calibration curves were prepared and all of them met the acceptance criteria. It should be emphasized that only 2 of 112 calibration standards did not meet acceptance criteria and were rejected during the whole study (Fig. 6).

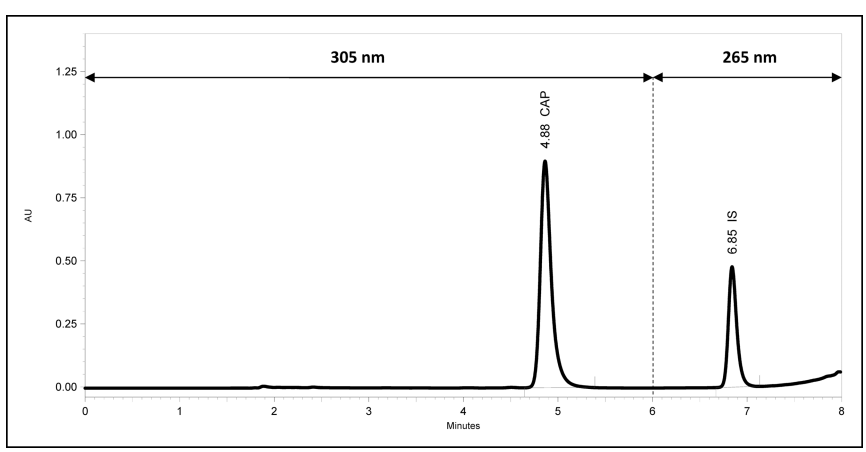

Fig. 3: Chromatogram of the extracted plasma sample containing capecitabine at 10.00 μg/mL (CAP 4.88 min) and the working concentration of the internal standard voriconazole (IS 6.85 min); with on-line wavelength switching from 305 nm to 265 nm at 6.0 min.

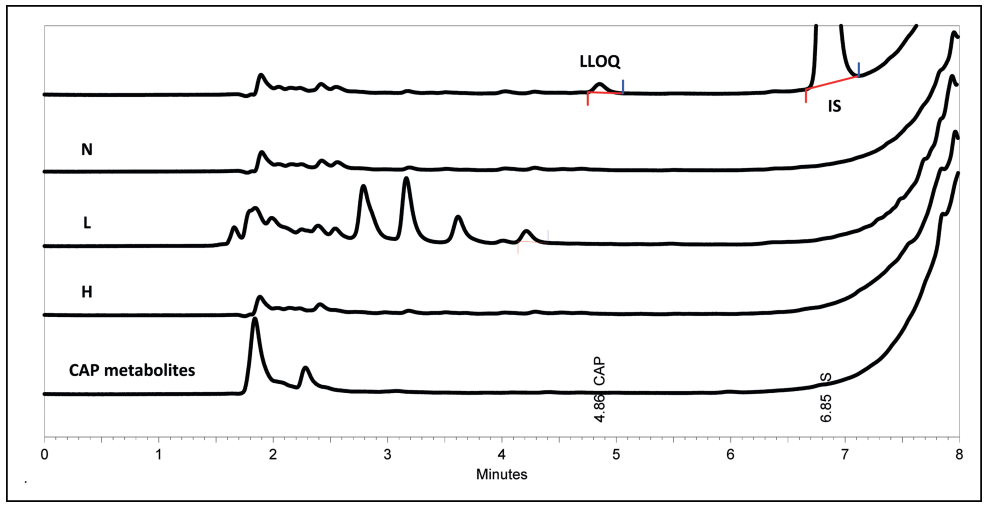

Fig. 4: Chromatograms of extracted blank plasma samples (N - standard plasma, L - hyperlipidaemic plasma, H - haemolysed plasma), the extracted plasma sample containing capecitabine metabolites and lower limit of quantification (LLOQ, capecitabine at 0.05 μg/mL and working concentration of internal standard).

2.2.4. *Accuracy, precision and dilution integrity*

Precision and accuracy of the method were determined within one day and within three days using QC samples, each in six replicates (Table 1). The 90% confidence intervals for within one day and within three days' accuracy were within the ranges of 92.8–108.5% and 86.4–108.5%, respectively. The 90% confidence intervals for within one day and within three days' precision were within the ranges of 0.82–6.23% and 0.82–10.92%, respectively. The precision and accuracy met the acceptance criteria 85–115%.

The possibility of plasma samples dilution 1:1 (v/v) with blank human plasma prior to the sample processing, e.g. in the case of concentration exceeding the calibration range, was studied for the QC samples at 16.00 μg/mL. The obtained accuracy of 104.8% and precision of 2.55% confirmed the dilution integrity and indicated the possibility of the further extension of the linearity range. The experiments performed during the method optimization suggested that the calibration range may be extended up to 16.00 μg/mL, if necessary.

2.2.5. *Stability*

The stability studies of capecitabine in human plasma were preceded by the detailed stability studies of the active pharmaceutical substance (Łaszcz et al. 2011). The method of stability evaluation described in this paper based on the calculation of C.I. could be considered superior to the standard approach, because it includes uncertainty of the measurement.

Table 2: The stability of capecitabine in human plasma (n = 6)

Stability test	Storage period	Temperature	Stability (90% confidence interval,%)	
			0.15 μg/mL	8.00 μg/mL
Short-term	4 hours	room temperature	86.3–99.2	94.4–98.6
Freeze and thaw	3 cycles	≤ − 14 °C	93.5–97.4	95.1–99.2
Long-term	21 days	≤ − 14 °C	96.1–102.2	87.8–98.0

Therefore, the application of confidence intervals during bioanalytical method validation may be considered as the improvement of the quality of data obtained during pharmacokinetic studies. No effect of 3 freeze-thaw cycles at ≤ − 14 °C on the stability of capecitabine was observed. Capecitabine was found to be stable in human plasma for at least 4 h at room temperature and after storage at ≤ − 14 °C for at least 21 days. For each test and each concentration 90% confidence interval for the mean stability fell within the acceptance criteria of 85–115% (Table 2). Also the test and reference samples fell within 85–115% of their nominal concentration.

Surprisingly, we could not find any information in the literature about the capecitabine stability in the stock and working solutions used in bioanalytical methods. Moreover, the solvents used for the preparation of the solutions were rarely reported.

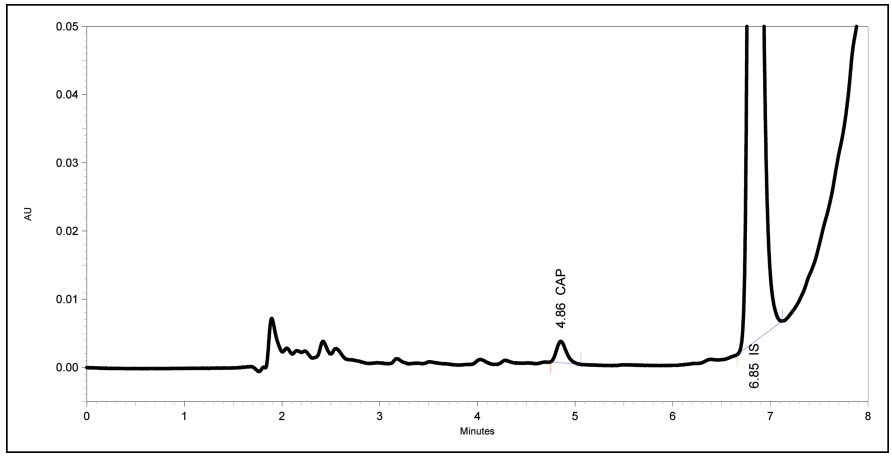

Fig. 5: Chromatogram of the extracted plasma sample containing capecitabine (CAP) at 0.05 μg/mL (lower limit of quantification) and the working concentration of the internal standard (IS).

Table 3: Standard stock and working solution stability (n = 6)

	Analyte	Concentration (μg/mL)	Storage period	Storage condition	Stability (90% confidence interval, %)
Stock solution	capecitabine	1000.0	20 days	freezer $\leq -6\,^{\circ}C$	104.7−105.2
	internal standard	1000.0	28 days		93.6−94.2
	capecitabine	1000.0	24 hours	room temperature	101.8−102.7
	internal standard	1000.0			94.9−95.6
Working solution	capecitabine	500.0	17 days	refrigerator $\leq 12\,^{\circ}C$	108.1−108.8
	capecitabine	1.00	8 days		100.1−100.8
	internal standard	120.0	9 days		108.7−109.4
	capecitabine	500.0	24 hours	room temperature	96.6−97.2
	capecitabine	1.00			102.8−103.5
	internal standard	120.0			96.0−96.8

This paper is the first report on the capecitabine stability in the stock and working solutions prepared in MeOH and 50% MeOH, respectively. For all compounds the 90% confidence intervals for the mean stability in each storage condition fell within the acceptance criteria of 90–110% (Table 3).

2.2.6. System suitability test

The chromatographic performance of the method was monitored by five injections of the system suitability test solution in each sequence. The RSD for the peak area did not exceed 0.5% and 1.3% for capecitabine and the internal standard, respectively. The theoretical plates were over 6000 and 10000 for capecitabine and the internal standard. For both compounds the asymmetry at 10% of the peak height was below 1.5. All those results confirmed constant characteristic of the method during the validation.

3. Discussion

Although capecitabine determination in human plasma seems to be widely described, each of the previously reported HPLC-UV methods has significant limitations. Briefly, two methods lack sufficient sensitivity for pharmacokinetic studies in humans and do not use an internal standard, what raises serious questions on the results reliability in routine analysis (Dhananjeyan et al. 2007; Farkouh et al. 2010). Two other methods can be characterized by complicated sample preparation by combined protein precipitation and solid phase extraction (Reigner et al. 1998) or long analytical run time of 30 min (Zufia et al. 2004), which makes them unsuitable for application in studies with a large number of samples.

Although the method presented in this paper is hardly a breakthrough novelty, major improvements in the determination of capecitabine are reported. The optimization and simplification of liquid-liquid extraction procedure involved elimination of unnecessary addition of phosphoric acid and reduction of the extraction solvent volume. The use of voriconazole as the internal standard, combined with gradient elution and on-line wavelength switching, assured very high precision of results (relative standard deviation < 7.1% for LLOQ) and enabled the reduction of the total chromatographic run time to 8 min. This is equal to the shortest run time among HPLC-UV methods for the determination of capecitabine (Reigner et al. 1998). Contrary to assay reported by Farkouh et al. (2010), the method selectivity was confirmed in the presence of capecitabine metabolites.

The results of capecitabine stability study in the stock and working solutions may also prove to be valuable as, according to our knowledge, they have never been cited before. All validation parameters, defined according to the international bioanalytical guidances (EMA 2011, FDA 2001), met the acceptance criteria, which proves that the method leads to reliable results.

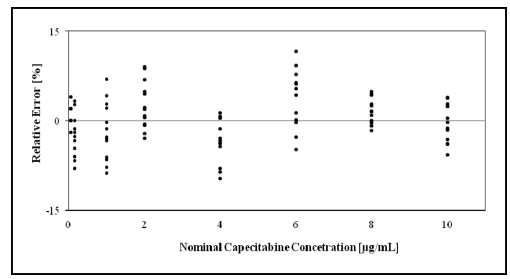

Fig. 6: Percentage relative error plotted against capecitabine nominal concentration; weighting factor $w = 1/x^2$ (n = 14).

A rapid, reproducible and validated HPLC-UV method for the determination of a socially important anticancer drug capecitabine is reported. The presented method is an optimized tool for the cost-efficient analysis of capecitabine in plasma samples, based on a widely available instrumentation. The assay employs a simplified liquid-liquid extraction procedure with rapid chromatographic elution leading to accurate and precise results in the linearity range of 0.05–10.00 μg/mL. Thus, it is well suited for the pharmacokinetic studies in humans, where metabolites determination is not necessary and a large number of samples should be analyzed in a short time. The method may be applied in human pharmacokinetic studies, including the regulatory purposes and therapeutic drug monitoring.

4. Experimental

4.1. Materials

Capecitabine was synthesized in the Pharmaceutical Research Institute (Warsaw, Poland). Voriconazole (internal standard) was supplied by Sigma-Aldrich (Saint Louis, USA). The capecitabine metabolites: 5'-DFCR, 5'-DFUR, 5-FU, FUH_2, FUPA, FBAL were obtained from CCN Industries Ltd (Xiamen, China), bioKEMIX (Hillsborough, NJ, U.S.A), Sigma-Aldrich (Saint Louis, USA), Santa Cruz Biotechnology, Inc. (Dallas, Texas, U.S.A.), Toronto Research Chemicals Inc. (Toronto, Ontario, Canada), Sigma-Aldrich (Saint Louis, USA), respectively. Methanol (MeOH), and acetonitrile, both of HPLC grade, were supplied by Merck (Darmstadt, Germany) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Ethyl acetate 99.8%, formic acid 100% and dimethylsulfoxide were purchased from POCH, (Gliwice, Poland).

Blank plasma was obtained from the Regional Center of Blood Donation and Treatment (Warsaw, Poland). Sodium citrate was used as an anticoagulant.

4.2. Instrumentation

The HPLC system consisted of a system controller SCL-10A VP, two pumps LC-10AD VP connected to an autosampler SIL-10A, an UV-Vis detector SPD-10A VP (Shimadzu, Duisburg, Germany) and a column oven of Decade II detector (Antec Leyden, NV Zoeterwoude, Netherlands). The data processing software was Class-VP v. 6.13 (Shimadzu, Duisburg, Germany). The separation was performed on an Atlantis dC18 analytical column (150 x 4.6 mm, 3.5 μm), which was preceded by a guard column SecurityGuard C18 (both Waters, Milford, MA, USA).

4.3. Chromatographic conditions

The method used gradient elution with a total run time of 8 min. The mobile phase consisted of 0.1% formic acid (A) and MeOH (B). The flow-rate of the mobile phase was 1.1 mL/min. The starting eluent was 46% A and 54% B. The proportion of the eluent B was increased linearly to 90% in 4 min and then returned to the initial composition in 6 min. The last 2 min were used to re-equilibrate the column. The sample volume of 70 μL was injected into the column whose temperature was maintained at 40 ± 2 °C. The retention times of capecitabine and the internal standard were around 4.9 min and 6.9 min, respectively. Capecitabine was monitored at a wavelength of 305 nm and online wavelength switching was set at 6 min to monitor the internal standard at 265 nm (Fig. 3).

4.4. Standard stock and working solutions

The standard stock solutions of capecitabine and the internal standard were prepared in MeOH (1000 μg/mL) and stored in a freezer at ≤ - 6 °C. The standard stock solutions of capecitabine metabolites (5'-DFCR, 5'-DFUR, 5-FU, FUH_2, FUPA) were prepared immediately before analysis in MeOH, except the FBAL stock solution which was prepared in dimethylsulfoxide. The standard working solutions of capecitabine and the internal standard were prepared in 50% MeOH and were stored in a refrigerator at ≤ 12 °C.

4.5. Calibration standards and quality control plasma samples

The calibration standards were prepared at following capecitabine concentration levels: 0.05, 0.15, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 μg/mL. Quality control (QC) samples contained capecitabine at three concentration levels: 0.15, 4.00 and 8.00 μg/mL. The calibration standards and QC samples were prepared by spiking blank human plasma with the capecitabine working solution. The calibration standards were prepared immediately before analysis. The QC samples were prepared in a larger volume, frozen at ≤ -14 °C and thawed gradually during the study.

4.6. Sample preparation

A 1 mL aliquot of a human plasma sample was transferred to the extraction tube. Following the addition of 50 μL of the internal standard solution (120.0 μg/mL), the sample was vortex mixed. Then 3 mL of ethyl acetate/acetonitrile (4:1, v/v) was added and mixed. The sample was centrifuged at 3500 rpm and frozen. Then the organic layer was transferred to a glass tube and evaporated under the stream of nitrogen. The dry residue was dissolved in 200 μL of 50% MeOH, the solution was centrifuged at 3500 rpm and transferred to an autosampler vial. A 70 μL aliquot of reconstituted extract was injected into the HPLC system.

4.7. Statistical analysis

The statistical analysis of stability, based on the application of confidence intervals, included the comparison of two experimental data sets (Timm et al. 1985; Rudzki and Leś 2008). Briefly, the possible outliers in each of result sets (concentrations in stability and reference samples) were identified. Then the logarithmic transformation of data was applied to correct the possible skew of data distribution. Following the homoscedascity examination with an *F*-test applied at a significance level $\alpha = 0.01$, the lower and upper limits of two-sided 90% confidence interval were calculated either using pooled variance (in the case of equal variances) or individual variances of the studied and reference samples. The outliers were detected with a two-sided Dixon Q-test at a significance level $\alpha = 0.10$ and discarded from the calculations of stability and the calibration curve parameters.

Acknowledgements: The authors wish to thank the National Centre for Research and Development of the Republic of Poland for their financial support under the project No. KB/151/13364/IT1-B/U/08. The authors gratefully acknowledge the technical assistance of Mrs. Magdalena Troć and Mrs. Krystyna Serafin-Byczak.

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