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Original Article

# DETERMINATION OF CAPECITABINE-AN ANTICANCER DRUG IN DRIED BLOOD SPOT BY LC-ESI-MS/MS

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**ABSTRACT**

**Objective:** Capecitabine (Cape), the first oral prodrug which belongs to the group of fluoro pyrimidines is the most frequently prescribed anticancer drug for the treatment of metastatic breast and colorectal cancers. The article describes a selective and robust method for determination of Cape in dried blood spots (DBS) by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Methods:** Cape fortified DBS was punched and extracted with ethyl acetate using capecitabine-d11 as the internal standard (IS). Chromatographic separation of Cape and IS from endogenous matrix was performed on Phenomenex Gemini C18 (150 × 4.6 mm, 5µm) column under isocratic condition using acetonitrile: 2 mmol ammonium formate (pH 3.0, adjusted with 0.1 % formic acid) (80:20, *v/v*) as the mobile phase. Detection and quantification were carried on a triple quadrupole mass spectrometer, using electro spray ionization technique in the positive ionization mode.

**Results:** The method was established over a concentration range of 10-10000 ng/ml. Accuracy, precision, selectivity, recovery, matrix effect and stability of the analyte were also estimated and the results were within the acceptance criteria. Further, precise results were obtained using an optimum spot volume of 10 µl with good spot homogeneity. Blood samples with hematocrit values varying from 24 % to 45 % gave acceptable results with good accuracy and precision.

**Conclusion:** The efficiency of dried blood spot sample preparation, short analysis time and high selectivity permits estimation of Cape in a small blood volume. The validation results suggest that the method is precise, accurate, and reproducible and can be useful in therapeutic drug monitoring of Cape.

**Keywords:** Capecitabine, Capecitabine-d11, Dried blood spot, Selective, LC-MS/MS, Post-column infusion.

## INTRODUCTION

Capecitabine (Cape, N4-pentyloxycarbonyl-5'-deoxy-5-fluoro-cytidine) is an orally administered fluoropyrimidine carbamate used in the treatment of metastatic breast and colorectal cancers [1]. The Food and Drug Administration (FDA) had approved Cape (Xeloda®; Hoffman-LaRoche, Nutley, NJ) in 2005 as an oral prodrug of 5-fluorouracil (5-FU) for use as monotherapy in the first line treatment of advanced colorectal cancer, adjuvant treatment of patients with stage III colon cancer and locally advanced or metastatic breast cancer [2]. Moreover, Cape is gradually replacing 5-FU in several indications, including gastric cancer. The principle mechanism of action of Cape is the inhibition of thymidylate synthase (TS) and incorporation into RNA and DNA. After oral administration and subsequent absorption across the digestive tract, it is converted to 5-FU through three sequential enzymatic reactions. It is first metabolized to 5-deoxy-5-fluorocytidine (5-DFCR) in the liver by the enzyme, carboxylesterase and then to 5-deoxy-5-fluorouridine (5-DFUR) in the liver and tumor tissue by cytidine deaminase. Finally, it is converted intracellularly to 5-FU by thymidine phosphorylase, an enzyme that is found in tumor tissue [3-5]. Cape is mainly eliminated as metabolites (>95 % of the dose) and the elimination half-life of the parent drug and its metabolites is around 1.0 h [5]. The bioavailability of Cape is nearly 100 % and its oral pharmacokinetics is linear, dependent on dose strength. The plasma protein binding (mainly to albumin) is 54% for Cape and about 10 %, 62 %, and 10 % for its metabolites 5-DFCR, 5-DFUR and 5-FU respectively [2].

In the last decade, dried blood spot (DBS) technique has proved to be a superior alternative micro sampling approach for quantitative bio analysis of drugs in pharmaceutical research and development. Blood micro-collection technique using filter paper has challenged the conventional, invasive blood sampling by venepuncture [6, 7]. DBS technique offers distinct benefits like reduced sample volume collection (usually 10–25 µl), simplified sample collection and processing procedures, lack of post-collection processing, lower costs of biological sample storage and transport, improved (bio)chemical drug stability compared with frozen samples, reduced biohazard risk with minimum facility for storage and shipment and many more [7-9]. Due to such wide range of advantages it has shown considerable promise for toxicokinetics and pharmacokinetic analysis and becomes one of the popular micro sampling techniques.

Several assays have been reported for the determination of Cape alone [10, 11], along with its active metabolites in different biological matrices such as mouse plasma [12, 13], mouse serum and rabbit bile [13] and human plasma [14-23]. In two other reports, Cape has been determined together with some multi-cytostatic compounds [24, 25]. Mainly, liquid chromatography with UV [10, 11, 13, 16] and mass spectrometry [12, 14, 15, 17-25] detection has been used for the quantification of Cape and/or its active metabolites in different matrices.

In order to derive the benefits of DBS, the analyses must be sensitive enough to quantify the target analyte concentration in a few microlitres of blood present in a punched DBS disk. Indeed, sensitivity may sometimes contribute as one of the major challenges for DBS analysis. This issue can be circumvented by using sensitive mass spectrometers such as triple quadropole mass spectrometers through which sufficient sensitivity and selectivity can be obtained with adequate confidence. A review of the literature revealed no DBS methods for the quantitation of Cape using LC-MS/MS and as such bioanalytical methods, illustrating the quantitative analysis of anticancer drugs in DBS are very limited. Thus, the aim of the present study was to develop and validate an LC–MS/MS method for the quantification of Cape in DBS. The method was fully validated based on the current regulatory guidelines [26]. The current method provides the simplicity and convenience inherent to the DBS technique, faster run time (2.5 min) and specificity through MS/MS detection.

## MATERIALS AND METHODS

### Standards and chemicals

The reference standard of capecitabine (purity, 99.8%) and capecitabine-d11 (IS, purity, 99.0 %) was procured from Toronto Research Chemicals Inc. (Ontario, Canada). HPLC grade methanol, acetonitrile and ammonium formate were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from a Milli-Q water purification system procured from Millipore (Bangalore, India). Sample collection cards (Whatman® FTA® DMPK-C) were purchased from GE Healthcare Europe GmbH (Diegem, Belgium). Harris Micro-Punch® and cutting mat were procured from Fisher Scientific. Fresh human blood with $K_3$EDTA as the anticoagulant was obtained from Supratech Micropath (Ahmedabad, India) and was stored at-20 °C until use.

### Liquid chromatography and mass spectrometry conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) was used for chromatographic separation of Cape and IS on Phenomenex Gemini C18 (150 mm × 4.6 mm, 5 µm) column, maintained at 40 °C in the column oven. The total chromatographic run time was 2.5 min. For isocratic elution the mobile phase consisting of acetonitrile and 2 mmol ammonium formate (pH 3.0, adjusted with 0.1 % formic acid) in water (80:20, *v/v*) and was delivered at a flow-rate of 1.0 mL/min. The total eluate from the column was split in 80:20 (*v/v*) ratio; flow directed to the electro spray interface was equivalent to 200 µl/min. The auto sampler temperature was maintained at 5 °C and the average pressure of the system was 1200 psi.

A triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with electro spray ionization and operating in positive ionization mode was used for detection of analyte and IS. For quantitation, multiple reaction monitoring (MRM) was used to monitor precursor→ production transitions for Cape and IS respectively. Optimized ion source, compound-dependent parameters and selected MRM transitions for Cape and IS are listed in table 1. Analyst classic software version 1.4.2 was used to control all parameters of HPLC and MS.

### Standard stock, calibration standards and quality control sample preparation

The standard stock solution of Cape (1000 µg/ml) was prepared by dissolving the requisite amount in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank blood (5 % of total volume of blank blood) with standard spiking solutions. A 10 µl fortified blood from each sample was spotted on filter paper and used as CS or QC samples. CSs were made at 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10000 ng/ml concentrations while QC samples were prepared at five concentration levels, 8000 ng/ml (high quality control, HQC), 4000/320 ng/ml (medium quality control, MQC-1/2), 30 ng/ml (low quality control, LQC) and 10 ng/ml (lower limit of quantification quality control, LLOQ QC). Stock solution (1000 µg/ml) of the IS was prepared by dissolving 2.0 mg of IS in 2.0 ml of methanol. An aliquot of 10 µl of this solution was further diluted to 20.0 ml in the same diluent to obtain a solution of 0.5 µg/ml.

**Table 1: Optimized mass parameters for capecitabine and capecitabine-d11**

| Parameter | Capecitabine | Capecitabine-d11 |
|---|---|---|
| ***Source dependent mass parameters*** | | |
| Gas 1 (Nebulizer gas) | 50 psig | |
| Ion spray voltage | 5800 V | |
| Heater gas | 60 psig | |
| Turbo heater temperature | 300 °C | |
| Entrance potential | 10 V | |
| Collision activation dissociation | 8 psig | |
| Curtain gas, nitrogen | 45 psig | |
| Quadrupole 1 and 3 | Unit mass resolution | |
| Dwell time | 200 ms | |
| ***Compound dependent mass parameters*** | | |
| Declustering potential | 50 V | |
| Cell exit potential | 10 V | |
| Collision energy | 30 eV | |
| MRM transition (*m/z*) | 360.1/244.4 | 371.3/255.1 |

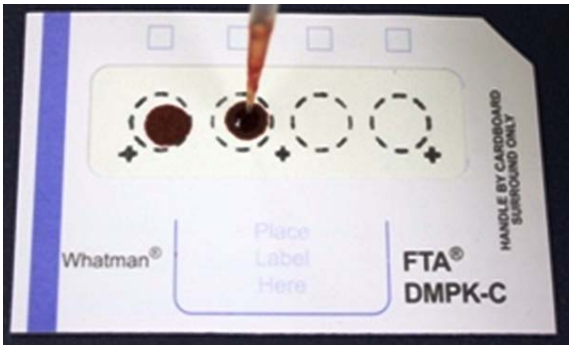


**Fig. 1: Representative snap of dried blood spot sampling**

### DBS sample preparation

Sample preparation was performed by spotting 10 µl fortified blood sample (CS or QC) onto the center of the printed circle and left to dry under ambient room temperature for at least 2 h (fig. 1). A 3-mm diameter disc was punched (Harris Micro-Punch®, 3-mm circle) out of the sample collection card and taken in eppendorf tubes, followed by the addition of 150 µl of 0.1 N HCl. The sample was vortexed and sonicated for about 5 min. An aliquot of 50 µl of IS working solution was added and vortexed for another 1.0 min. Thereafter the analyte and IS were extracted with 2.5 ml of ethyl acetate by vortex mixing for 5 min. Samples were centrifuged at 4000 rpm at 10 °C for 10 min. The supernatant was transferred into pre-labeled tubes and dried at 40 °C. The residue was reconstituted with 100 µl of mobile phase, briefly vortexed and 10 µl was injected into the LC-MS/MS system for analysis.

### Bioanalytical method validation study design

The method validation of Cape in DBS was done following the US FDA guidelines [26] and is similar to our previous work [27, 28].

A system suitability experiment was performed by injecting six consecutive injections using a standard aqueous mixture of Cape (4000 ng/ml) and IS (500 ng/ml) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. Carry-over effect of auto sampler was checked to verify any carryover of the analyte at the start and at the end of each batch. The

design of the experiment comprised of the following sequence of injections viz. extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample.

The selectivity of the method towards endogenous plasma matrix components was assessed in ten different batches of $K_3$EDTA whole blood. The samples were spiked with Cape at the LLOQ level and compared with double blank samples (without Cape and IS) for selectivity measurements. An aliquot of 10 µl of the samples was spotted on the sample collection card and processed. The selectivity of the method towards commonly used medications in human volunteers was done for acetaminophen, cetirizine, domperidone, ranitidine, diclofenac, ibuprofen, nicotine and caffeine in six different batches of blood having $K_3$EDTA as an anticoagulant. Their stock solutions were prepared by dissolving the requisite amount in methanol and water (50:50, *v/v*). Further, a mixed working solution of acetaminophen (1000 µg/ml), cetirizine (20 µg/ml), domperidone (1.0 µg/ml), ranitidine (27.5 µg/ml), diclofenac (100 µg/ml), ibuprofen (2250 µg/ml), nicotine (5.0 µg/ml) and caffeine (1000 µg/ml) were prepared in the same diluents, spiked in blood and analyzed under the same conditions at LQC and HQC levels in six replicates. These sets were processed along with freshly prepared CSs and qualifying QC samples in duplicate. As per the acceptance criteria, the % accuracy should be within 85 % to 115 %.

The linearity of the method was determined by analysis of five calibration curves containing ten non-zero concentrations. The area ratio response for analyte/IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was finalized during pre-method validation. A correlation coefficient ($r^2$) value>0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free blank blood. In addition, the analyte peak of LLOQ sample should be identifiable, discrete and reproducible with a precision (% CV) not greater than 20 % and accuracy within 80–120 %. The deviation of standards other than LLOQ from the nominal concentration should not be more than±15 %.

For determining the intra-batch accuracy and precision, replicate analysis of blood samples of Cape was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC-1/2 and HQC samples. Inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The deviation (% CV) at each concentration level from the nominal concentration was expected to be within±15 %, except LLOQ, for which it should be within±20 %. Similarly, the mean accuracy should not deviate by±15 %, except for the LLOQ where it can be±20 % of the nominal concentration.

The extraction recovery and matrix effect for the analyte and IS were calculated using the following expression as reported previously [29].

$$\text{Recovery} = \frac{\text{area response of processed sample}}{\text{area response of blank sample spiked with neat solution}} \times 100\ \%$$

$$\text{Matrix factor (MF)} = \frac{\text{area response of blank sample spiked with neat solution}}{\text{area response of neat solution}}$$

Ion suppression/enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing Cape (4000 ng/ml) was infused post column via a 'T' connector into the mobile phase at 10 µl/min employing an inbuilt infusion pump. Aliquots of 10 µl of extracted control blood were then injected into the column by the auto sampler and MRM LC-MS/MS chromatograms were acquired for Cape. Any dip in the baseline upon injection of extracted control blood (without IS) would indicate ion suppression while a peak at the retention time of Cape indicates ion enhancement.

All stability results were evaluated by measuring the concentration of stability samples against nominal concentration at LQC and HQC levels. Stock solutions of Cape and IS were checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from the nominal value was within±10.0 %. Bench top (at room temperature) and processed sample stability at 2-8 °C were performed at LQC and HQC ($n$ = 6 at each level). Long-term stability of dried blood spot samples stored at room temperature under ambient conditions was also studied at both these levels. The samples were considered stable if the deviation of mean calculated concentration of stability QC samples was within±15.0 %. Further, the blood stability of Cape on the cards incubated at 50 °C was assessed at LQC and HQC level.

To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by a different analyst while the second batch was studied on different equipment of the same make and model. The dilution integrity experiment was evaluated by preparing the spiked standard at concentration 20000 ng/ml for 1/4$^{th}$ dilution in the screened blood. The precision and accuracy for dilution integrity standards at 1/4$^{th}$ (5000 ng/ml) dilution was determined by analyzing the samples against calibration curve standards.

In addition to these procedures, other parameters which are DBS-specific like dried blood spot volume, spot spreadability, and effect of hematocrit were also studied in accordance with the EBF recommendations for DBS analysis [30]. The acceptance criteria for these experiments were same as those for stability assessments.

## RESULTS AND DISCUSSION

### Method development

In order to check optimum response for Cape and IS, both positive and negative ionization modes were tested. The signal intensities obtained were much higher in the positive mode than in the negative ion mode since Cape and IS have the ability to accept protons. This can be attributed to the basic nature of Cape which has a relatively high p*K*a value of 9.5 [23] and, as a result, gets readily protonated under the optimized acidic mobile phase conditions. The full scan Q1 MS spectra obtained by infusing 500 ng/ml solutions of Cape and IS contained abundant protonated precursor ions at *m/z* 360.1 and 371.3 respectively. The most abundant and characteristic productions were found at *m/z* 244.4 and 255.1 due to the loss of 2-methyltetrahydrofuran-3,4-diol moiety from the structure of Cape and Cape-d11 respectively as shown in fig. 2.

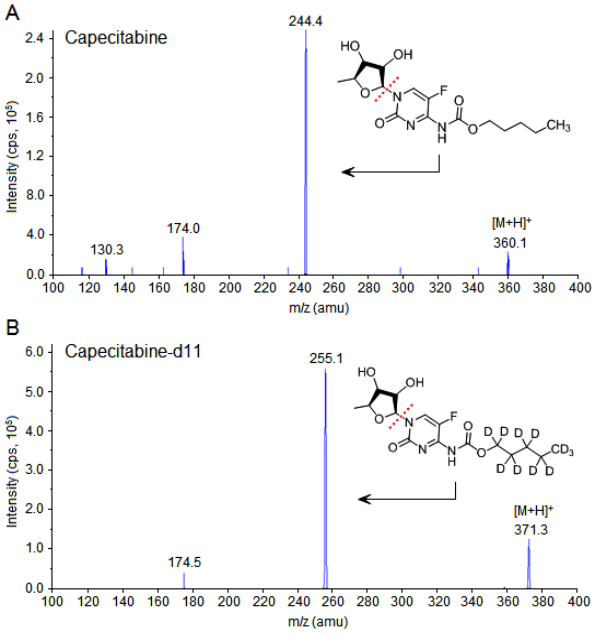


**Fig. 2: Production mass spectra of (A) capecitabine (*m/z* 360.1 → 244.4, scan range 100-400 amu) and (B) internal standard, capecitabine-d11 (*m/z* 371.3 → 255.1, scan range 100-400 amu) in the positive ionization mode**

During trials, the chromatography was tested on several columns like, ACE C18 (150 mm × 4.6 mm, 5.0 µm), Thermo Hypurity C18 (100 mm × 4.6 mm, 5.0 µm) and Phenomenex Gemini C18 (150 mm × 4.6 mm, 5.0 µm) to obtain an adequate response, a short run time, symmetric peak shapes, minimum matrix interference and solvent consumption. This was studied using different mobile phase solutions consisting of acidic buffers (acetic acid ammonium acetate, formic acid-ammonium format) and organic diluents like methanol/acetonitrile. The use of methanol in the mobile phase resulted in better selectivity, but the response was not adequate for Cape as well as IS, and also the peak shape was poor. Thus, acetonitrile was tested in varying proportions with acidic buffers. It was observed that the high content of acetonitrile in the mobile phase gave a sufficient response with adequate selectivity and assisted in eluting the analyte with a retention time of 1.812 min within a short run time of 2.5 min. It was found that 2.0 mmol ammonium formate (pH 3.0, adjusted with 0.1 % formic acid) in water: acetonitrile (20:80, *v/v*) was most suitable as mobile phase for analysis of Cape and IS on Gemini C18 (150 mm × 4.6 mm, 5.0 µm) column. Low pH buffer enhanced protonation and helped in eluting the analyte and IS completely without tailing. Although the other two columns afforded adequate retention and response, but the peak shape was comparatively poor and hence were not considered for further study. The reproducibility of retention time for Cape, expressed as % CV was ≤ 1 % for 100 injections on the same column.

The sensitivity achieved for Cape in the present work was 10.0 ng/ml. Representative MRM ion chromatograms in fig. 3 of extracted blank human plasma (without IS and analyte), blank plasma fortified with IS and Cape at LLOQ and ULOQ with IS demonstrate the selectivity of the method. The use of deuterated internal standard helped in the accurate quantification of analyte from DBS by maintaining good ionization efficiency of the analyte and thereby the accuracy of the data.

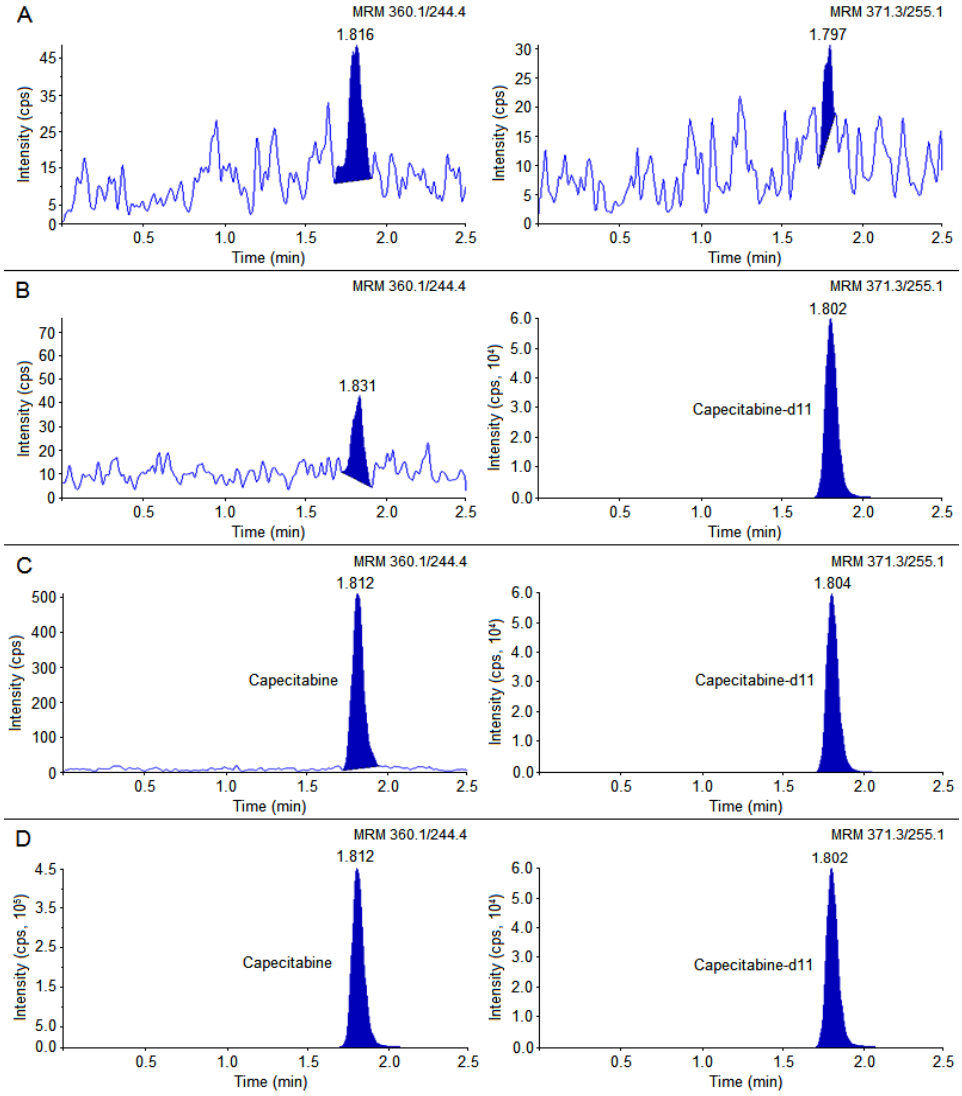


**Fig. 3: MRM ion chromatograms of (A) double blank plasma (without analyte and IS), (B) blank plasma and capecitabine-d11 (IS) (*m/z* 371.2 → 255.2), (C) capecitabine (*m/z* 360.2 → 244.2) at LLOQ and IS, (D) Capecitabine (*m/z* 360.2 → 244.2) at ULOQ and IS**

Cape being an anticancer drug and studied clinically on patients; it is crucial to minimize blood loss during subject sample analysis. Hence, alternate extraction procedures are necessary, which have a low sample requirement for processing without compromising the selectivity, sensitivity and efficiency of the developed method. A comparative study was done with three conventional extraction techniques to validate the potential of the optimized method using DBS. The DBS method was tested for % mean recovery and post-column infusion study for matrix effect with liquid-liquid extraction (LLE), solid phase extraction (SPE) and protein precipitation (PP) from plasma samples. The mean extraction recoveries obtained were 78 %, 85 %, 55 % and 87 % for LLE (with ethyl acetate), SPE (on Oasis extraction cartridge), PP (with acetonitrile) and DBS method respectively. The recovery obtained by DBS was comparable

with the SPE technique. The use of DBS permits a simple and straightforward approach for removing the analyte from the unwanted matrix components.

**Validation results**

**System suitability, system performance and carryover effect**

Precision (%CV) of the system suitability test was observed in the range of 0.02 to 0.15 % for the retention time and 0.28 to 1.02 % for the area ratio response of Cape/IS. The signal to noise ratio for system performance was ≥ 20 for Cape and IS. The carry-over evaluation was performed before and after each analytical run to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carryover≤ (0. 05 %) observed during auto sampler carryover experiment in extracted blank plasma (without IS and Cape) after subsequent injection of the highest CS at the retention time of Cape and IS.

**Linearity, accuracy and precision**

All five calibration curves were linear over the concentration range of 10-10000 ng/ml for Cape. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y$ = (0.0000833±0.0000017) $x$-(0.00000535 ±0.000018), where $y$ is the peak area ratio of the Cape/ IS and $x$ is the concentration of the Cape. The mean correlation coefficient ($r^2$) observed, was 0.9995. The accuracy and precision (% CV) for the calibration curve standards ranged from 97.3 to 102.4 % and 0.57 to 2.83 respectively for Cape. The lowest concentration (LLOQ, 10.0 ng/ml) in the standard curve was measured at a signal-to-noise ratio (S/N) ≥ 20. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at five QC levels (table 2). Precision (% CV) values for the intra-batch ranged from 1.3-4.6 and the accuracy were within 95.0 to 105.9 % for Cape. Similarly, for the inter-batch experiments, precision varied from 2.2–5.8 % and the accuracy was within 94.6 to 103.3 %.

**Extraction recovery and matrix effect**

The extraction recovery and MFs for the analyte and IS are presented in table 3. The extraction recovery for the analyte and IS varied from 85.2 to 89.4 % and 83.5 to 89.2 % respectively at all QC levels. Quantitative evaluation of matrix effect for the analyte and IS was carried out from the peak area response and expressed as MFs. The IS-normalized MF for Cape ranged from 1.02 to 1.05 across four QC levels.

**Table 2: Precision and accuracy data for capecitabine in dried blood spots**

| QC level (Nominal concentration, ng/ml) | Intra-batch ($n$ = 6) | | | Inter-batch ($n$ = 30, six replicates per batch) | | |
|---|---|---|---|---|---|---|
| | Mean conc. found (ng/ml) | % CV | % Accuracy | Mean conc. found (ng/ml) | % CV | % Accuracy |
| HQC (8000) | 7689 | 4.6 | 96.1 | 7810 | 5.8 | 97.6 |
| MQC-1 (4000) | 4235 | 2.8 | 105.9 | 4124 | 2.8 | 103.1 |
| MQC-2 (320) | 316 | 1.3 | 98.6 | 316 | 2.2 | 102.8 |
| LQC (30.0) | 28.5 | 2.1 | 95.0 | 28.5 | 2.5 | 103.3 |
| LLOQ QC (10.0) | 9.78 | 3.2 | 97.8 | 9.78 | 3.2 | 94.6 |

CV: Coefficient of variation; n: Number of replicates; LQC: low quality control; MQC: medium quality control; HQC: high quality control; LLOQ QC: lower limit of quantitation quality control

**Table 3: Extraction recovery and matrix effects for capecitabine and IS ($n$=6)**

| ***Extraction recovery*** | | | | | | |
|---|---|---|---|---|---|---|
| **QC level** | **Capecitabine** | | | **Capecitabine-d11 (IS)** | | |
| | **Area response** | | **Extraction recovery,** | **Area response** | | **Extraction recovery,** |
| | A | B | **% (A/B)** | A | B | **% (A/B)** |
| LQC | 10916 | 12813 | 85.2 | 450241 | 507600 | 88.7 |
| MQC-2 | 121091 | 136672 | 88.6 | 445086 | 498976 | 89.2 |
| MQC-1 | 1527309 | 1708400 | 89.4 | 437653 | 524136 | 83.5 |
| HQC | 2955532 | 3416800 | 86.5 | 445798 | 518974 | 85.9 |
| ***Matrix factors*** | | | | | | |
| **QC level** | **Capecitabine** | | | **Capecitabine-d11 (IS)** | | | |
| | **Area response** | | **Matrix factor** | **Area response** | | **Matrix factor** | **IS-normalized MF** |
| | **C** | **B** | **(B/C)** | **C** | **B** | **(B/C)** | |
| LQC | 14095 | 12813 | 0.909 | 580114 | 507600 | 0.875 | 1.04 |
| MQC-2 | 146173 | 136672 | 0.935 | 559874 | 498976 | 0.891 | 1.05 |
| MQC-1 | 1908826 | 1708400 | 0.895 | 596638 | 524136 | 0.878 | 1.02 |
| HQC | 3746491 | 3416800 | 0.912 | 588405 | 518974 | 0.882 | 1.03 |

*n*: Number of replicates; LQC: Low quality control; MQC: Medium quality control; HQC: High quality control; A: Mean area response of six replicates of processed samples (pre-fortified samples); B: Mean area response of six replicate samples prepared by spiking the blank sample with neat solution (post-fortified samples); C: Mean area response of six replicate samples prepared in mobile phase (neat samples)

Post-column analyte infusion is a useful tool for visual identification of interfering peaks that may cause matrix effects. fig. 4 shows the comparison of the profiles obtained by injection of extracted blank plasma using SPE, LLE, PP and DBS extraction technique after post-column infusion of Cape (4000 ng/ml). Any deviation (suppression/enhancement) caused by injection of these samples from the baseline indicates the existence of matrix effect. As evident from the results, except in the case of DBS, some matrix effect or signal suppression/enhancement was observed with the other extraction techniques between 1.1 and 1.9 min. Nevertheless, no such ion suppression or enhancement was found at the retention time of Cape with DBS, as evident from the flat baseline.

**Stability, dilution integrity and method ruggedness**

The stability of Cape and IS in DBS and stock solutions was examined under different storage conditions. Samples for short-term stability remained stable up to 8 h, while the stock solutions, for long term stability of Cape and IS were stable for a minimum of 7 d at a refrigerated temperature of 2-8 ℃. Cape in DBS at room temperature was stable for 8 h at 25 ℃ and under incubation at 50 ℃ up to 8 d. Processed sample stability was evaluated up to 69 h without significant loss of the analyte. DBS stored at ambient temperature for long term stability experiment was found stable for a minimum period of 60 d. The accuracy and precision of different stability experiments

in plasma at two QC levels are shown in table 4. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher Cape concentration above the upper limit of quantification (ULOQ). The precision and accuracy for dilution integrity of 1/4th dilution were 0.8-1.2 % and 101.2-106.0 % respectively. The precision and accuracy results for method ruggedness with different columns and analysts were within 0.8-4.0 % and 95.6-106.6 % for Cape respectively.

**Effect of spot volume, sample spread ability and impact of hematocrit**

To test the effect of DBS volume on the accuracy and precision of the method, different spot volumes (10, 15, 20, 25 and 30 µl) at LQC and HQC levels were analyzed in triplicate. It was found that at volumes ≥ 20 µl the results were less accurate compared to lower volumes (10 and 15 µl) as shown in table 5. In the present work 10 µl spot volume was selected based on better accuracy and precision data obtained.

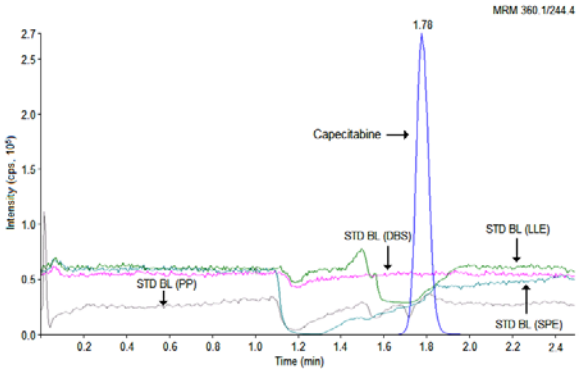


**Fig. 4: Comparison of extraction techniques (LLE, PP, SPE and DBS) for matrix effect by post-column infusion technique**

**Table 4: Stability of capecitabine in DBS under different conditions (*n* = 6)**

| Storage conditions | Nominal conc. (ng/ml) | Mean stability sample (ng/ml±SD) | Accuracy (%) | Precision (% CV) |
|---|---|---|---|---|
| Bench top stability, 8 h | 8000 | 7810±69.6 | 97.6 | 0.89 |
| | 30.0 | 28.1±0.63 | 93.3 | 2.24 |
| Processed sample stability, at 2-8 °C, 69 h | 8000 | 8150±83.4 | 94.5 | 1.02 |
| | 30.0 | 31.9±0.81 | 99.1 | 2.54 |
| Stability under incubation at 50 °C up to 8 d | 8000 | 7900±44.6 | 98.7 | 0.56 |
| | 30.0 | 29.3±0.40 | 96.7 | 1.36 |
| Long-term stability at ambient temperature, 60 d | 8000 | 8100±122.7 | 101.2 | 1.51 |
| | 30.0 | 31.0±0.94 | 103.3 | 3.03 |

*n*: Number of replicates; SD: Standard deviation; CV: coefficient of variation

**Table 5: Effect of DBS volumes on the quality control sample concentration (*n* = 3)**

| QC level | Spot volume (µl) | Concentration found (ng/ml) | Accuracy (%) | Precision (% CV) |
|---|---|---|---|---|
| Low quality control (30.0 ng/ml) | 10 | 30.4 | 101.3 | 3.2 |
| | 15 | 29.1 | 97.0 | 3.7 |
| | 20 | 28.5 | 95.1 | 2.7 |
| | 25 | 27.9 | 93.0 | 5.3 |
| | 30 | 26.7 | 89.1 | 4.2 |
| High quality control (8000 ng/ml) | 10 | 7946 | 99.3 | 2.1 |
| | 15 | 8262 | 103.3 | 1.7 |
| | 20 | 7643 | 95.5 | 3.4 |
| | 25 | 7427 | 92.8 | 2.4 |
| | 30 | 7144 | 89.3 | 2.8 |

*n*: Number of replicates; CV: coefficient of variation

**Table 6: Impact of hematocrit value on the quality control sample concentration (*n* = 3)**

| QC level | Hematocrit (%) | Concentration found (ng/ml) | Accuracy (%) | Precision (% CV) |
|---|---|---|---|---|
| Low quality control (30.0 ng/ml) | 24.5 | 30.7 | 102.3 | 4.5 |
| | 33.6 | 28.1 | 93.6 | 2.5 |
| | 44.2 | 27.8 | 92.7 | 3.6 |
| High quality control (8000 ng/ml) | 24.5 | 7856 | 98.2 | 1.2 |
| | 33.6 | 7806 | 97.6 | 1.9 |
| | 44.2 | 7764 | 97.1 | 3.8 |

The impact of sample spreadability was investigated by spotting 10 µl of spiked $K_3$EDTA whole blood at LQC and HQC levels in triplicate. A 3 mm disc punched and analyzed showed acceptable accuracy and precision results. The accuracy varied from 94.7 % to 102.3 % and the precision (% CV) was in the range of 1.5-5.2 % at the studied QC levels.

The effect of hematocrit, which defines the relative volume of red blood cells in whole blood greatly, influences the spot homogeneity. QC samples were prepared from whole blood having different hematocrit levels (24.5 %, 33.6 % and 44.2 %). The spiked samples at LQC and HQC levels (three replicates) were prepared using these whole blood samples with different hematocrit levels. The accuracy and precision of DBS samples ranged from 92.7-102.3 % and ≤ 4.5 % respectively (table 6). These results indicate no impact of hematocrit on the accuracy and precision of the developed method.

**Comparison of the DBS method with reported methods for capecitabine**

The developed method is the first attempt to determine Cape in DBS using any known analytical technique. Moreover, it employs the lowest sample volume for processing as compared to all previous methods [10, 11, 14-23]. Though the sensitivity is less compared to some of these methodologies [15, 17, 18] but the

present method requires only 2.5 min for chromatographic analysis, which is less than these methods. A comparative summary of existing methods and the salient features of the developed DBS method are shown in table 7.

**Table 7: Comparison of salient features of chromatographic methods developed for capecitabine in human plasma**

| S. No. | Detection technique | Sample volume (µl) | Linearity range (ng/ml) | Retention time (min); Run time (min) | Ref. |
|---|---|---|---|---|---|
| 1 | HPLC-UV | 1000 | 156–20000 | 6.223; 10.0 | [10] |
| 2 | HPLC-UV | 1000 | 50-10000 | 4.9; 8.0 | [11] |
| 3[a] | HPLC-MS | 500 | 50-10000 | 18.19; 24.0 | [14] |
| 4[a] | LC-MS/MS | 250 | 5.00-1000 | 1.43; 3.0 | [15] |
| 5[a] | HPLC-UV | 500 | 25-10000 | 20.4; 30 | [16] |
| 6[a] | LC-MS/MS | 500 | 1-5000 | 12.14; 14.0 | [17] |
| 7[a] | LC-MS/MS | 50 | 1-1000 | 2.47; 4.0 | [18] |
| 8[a] | LC-MS/MS | 500 | 150-3000 | 7.83; 15.0 | [19] |
| 9[a] | LC-MS/MS | 200 | 10-1000 | 11.5; 15.0 | [20] |
| 10[a] | LC-MS | 500 | 25-10000 | 9.0; 15.0 | [21] |
| 11[a] | LC-MS/MS | 100 | 50-6000 | 5.01; 9.0 | [22] |
| 12[a] | LC-MS/MS | 100 | 10-5000 | 8.2; 10.5 | [23] |
| 13[b] | LC-MS/MS | 10 (DBS) | 10-10000 | 1.81; 2.5 | PM |

*n*: Number of replicates; CV: coefficient of variation, [a]Along with metabolites; [b]In dried blood spots (DBS); PM: present method.

## CONCLUSION

A rugged and robust dried blood spots assay coupled with a LC–MS/MS was developed and validated for the extraction and analysis of Cape in human whole blood. The method offers significant advantages over those previously reported, in terms of lower sample requirements, the simplicity of extraction procedure and overall analysis time. The assay is able to quantify Cape over a dynamic concentration range of 10-10000 ng/ml in DBS and the results acquired were highly reproducible for 10 µl of spotting volume. The recovery obtained was quantitative and consistent across QC levels with no interference from endogenous or exogenous matrix components. The assay demonstrated the satisfactory long-term stability of Cape in DBS under ambient conditions. Further, blood samples having hematocrit values between 24 and 45 % demonstrated acceptable accuracy and precision in the quantitative measurements. This LC-MS/MS assay can be readily applied to the analysis of Cape in clinical laboratories for pharmacokinetics/ bioequivalence studies.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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