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# Development and Validation of Novel RP-HPLC Method for Determination of Assay and Related Substances of Flucytosine in Drug Product

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A novel, rapid, simple and cost-effective RP-HPLC method was developed for the assay of flucytosine and its related substances in drug product (tablets). The developed method was subjected to validation challenges as per the ICH guidelines and the method found to follow all the validation challenges successfully. Method employed uses a column with L1 end-capped stationary phase having dimensions of 250 mm × 4.6 mm, 5 µm. The mobile phase comprised of 10 mM phosphate buffer and methanol in gradient mode. The detection was performed at 210 nm. The flucytosine peak eluted at about 5.5 min making the method cost-effective. In this method, the related substances were also quantified. Owing to short run time, simple chromatographic conditions and sample preparation, the method is claimed to be ideal for the routine high throughput analysis leading to low cost of testing.

**Keywords: Flucytosine, RP-HPLC, Related substances.**

## INTRODUCTION

Fungal infections are one of the opportunistic infections that affect HIV patients and considered difficult to treat diseases as the treatment involves prolonged medication and requires high level of patient compliance. Fungal infections may be local or systemic and treatment regimen depends on the severity of disease and type of infections [1]. Systemic fungal infections that affect HIV/AIDS patients are mainly *Pneumocystis jirovecii* (pneumocystosis), *Cryptococcus neoformans* (cryptococcosis), *Histoplasma capsulatum* (histoplasmosis) and *Talaromyces (Penicillium) marneffei* (talaromycosis). Affordable, rapid point-of-care diagnostic tests (as have been developed for cryptococcosis) are urgently needed for pneumocystosis, talaromycosis and histoplasmosis. Additionally, easy access to antifungal drugs, including amphotericin B, liposomal amphotericin B and flucytosine, need to be much more widely available to tackle this healthcare challenge. Such measures, together with continued international efforts in education and training in the management of fungal disease, have the potential to improve patient outcomes substantially [2].

To enhance access and make the drugs more affordable to patients, decrease in production cost of antifungal drugs is an urgent unmet need. Since evolution of high-tech analytical instruments, the cost of testing has increased significantly due to enhanced cost of spares, requirement of ultra-high purity reagents/chemicals and specialized instrument specific requirements. The regulatory requirements, which are mandatory to comply in order to receive marketing authorization have also evolved over a period of time and further adds to the cost of production. Development of short, convenient, robust and cost-effective methods play a significant role in containing the cost of testing and thus helps in enhancing access of critical medicines to vulnerable population. Facts suggest that there is an unmet need for a simple cost effective novel HPLC method that could estimate assay and related substances of flucytosine in the same chromatographic conditions [3-5].

Flucytosine is a synthetic antimycotic compound, which was synthesized first time in 1957. The susceptible fungal cells convert flucytosine into 5-fluorouracil (5-FU), which is further converted into metabolites that inhibit fungal RNA and DNA synthesis. Interest in 5-fluorouracil has been renewed because of two recent developments: (i) it is now increasingly used in combination with a number of azole antifungal agents, such as ketoconazole, fluconazole and itraconazole; (ii) it plays an important role in a new therapeutic approach in the treatment of certain tumors, especially colorectal carcinoma [6].

Although few investigators developed some analytical methods for assay and related substances of flucytosine in drug products [5,7-9], however these methods lack the ability to estimate the assay and related substances in same chromatographic conditions. The method developed by Paladugu *et al.* requires the use of internal standard and also lacks the capability to estimate the related substances of flucytosine [5]. Bhatt *et al.* [7] also reported a method, but found unsuitable for tablets formulation as the method is isocratic and developed for the simultaneous estimation of flucytosine and its related impurities in the injectable pharmaceuticals. Since tablet formulation contains complex mixture of excipients, interference was observed in this method and was found unsuitable for the selected tablet formulation. An assay method developed by Ubale *et al.* [8], which has long run time of 70 min and also lacks the ability to quantify the related substances. Similarly, Murugesan *et al.* [9] utilizes ultra-fast liquid chromatography (UFLC) method for the assay of flucytosine, which is costlier than conventional HPLC and the method also lacks an ability to separate the impurities. Few other methods are also available for the quantification of flucytosine in biological fluids, but they require sophisticated and costly instruments like liquid chromatographic mass spectrometry (LC-MS) and also have similar inherent limitations for the estimation of flucytosine and its related substances [10-13].

Considering the unmet need, in an endeavor to enhance access of flucytosine (5-FC), a sensitive, cost effective and rapid HPLC method was developed in this study. This method is capable to assay and the related substances of flucytosine in flucytosine tablets using RP-HPLC in the same chromatographic conditions.

## EXPERIMENTAL

The chemical such as HPLC grade $KH_2PO_4$, orthophosphoric acid, HPLC grade $H_2O$, HPLC grade methanol and Millipore 0.45 µm filter were procured from Merck, India. The study involved use of waters Quaternary pump HPLC equipped with PDA detector and C18, 250 × 4.6 mm, 5 µm analytical column of Waters Sunfire®.

**Method development and validation:** Flucytosine is an amino compound containing electron withdrawing fluorine and oxygen atoms. The molecular structure indicates that the compound is hydrophobic in nature and hence reversed phase chromatographic method would be suitable. Flucytosine is a basic compound with not so bulky structure hence highly acidic pH of mobile phase may lead to early elution of flucytosine and its impurities (known and unknown). Since, the main purpose of this study is to develop a common method for assay and its related substances, hence phosphate buffer of pH 5.5 was selected in order to attain the reasonable retention of flucytosine to enable its separation from impurities. The method is cost effective and based upon the solvent selectivity studies, methanol was selected as the organic modifier of the mobile phase. Based on the above considerations, trials were undertaken and a simple, rapid and cost-effective RP-HPLC method was developed for the assay of flucytosine tablets and its related substances method (stability indicating). The developed method was subjected to validation challenges as per ICH guidelines [14].

The developed method consists of the following chromatographic conditions: mobile phase A: 10 mM potassium dihydrogen phosphate pH 5.5, mobile phase B: methanol, column: Waters Sunfire® $C_{18}$, 250 mm × 4.6 mm, 5 µm, flow rate: 1.0 mL/min, wavelength: 210 nm, column oven temperature: 30 ± 2 ºC, injection volume: 10 µL, diluent: water and methanol in the ratio of 95:5 v/v, respectively. Sample solution concentration for assay: 0.05 mg/mL and 1.0 mg/mL for the related substances tests. The flow program conditions and representative chromatogram are shown in Table-1 and Fig. 1, respectively. The retention time of flucytosine and 5-fluorouracil was found to be 5.8 and 6.9 min, respectively.

TABLE-1
FLOW PROGRAM CONDITIONS

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 8 | 95 | 5 |
| 12 | 30 | 70 |
| 15 | 30 | 70 |
| 16 | 95 | 5 |
| 20 | 95 | 5 |

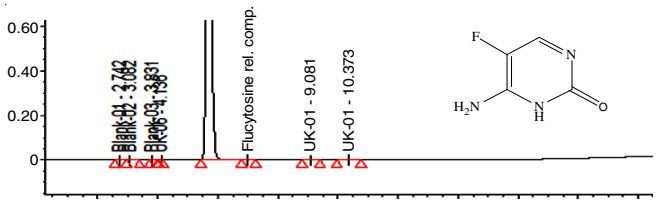


Fig. 1. RP-HPLC chromatogram of flucytosine

## RESULTS AND DISCUSSION

The method for the assay of flucytosine and its related substances was developed and further validated as per ICH guidelines [14]. The system suitability data for the developed method for determination of assay of flucytosine and related substances is given in Table-2. The obtained results met the predefined acceptance criteria.

TABLE-2
SYSTEM SUITABILITY DATA FOR DETERMINATION OF ASSAY OF FLUCYTOSINE AND RELATED SUBSTANCES

| Parameters | Assay (% RSD of replicate standard injection) | Related substances (% RSD of replicate standard injection) |
|---|---|---|
| Forced degradation | 0.4 | 1.2 |
| Repeatability | 0.3 | 0.8 |
| Linearity | 0.5 | 2.1 |
| Accuracy | 0.4 | 1.5 |
| Ruggedness | 0.6 | 0.9 |
| Robustness | 0.3 | 2.4 |
| Limit | NMT 2.0% | NMT 5.0% |

**Linearity:** The linearity of the test method was established from 50% to 150% of the target concentration for assay and LOQ (0.05% level) to 150% of limit concentration (0.5% for

flucytosine and 0.2% for 5-fluorouracil) for related substances. Flucytosine exhibited linear behaviour in the specified range for assay. Similarly, flucytosine and 5-fluorouracil exhibited linear response for related substances analysis. The linearity data and corresponding linearity plots are presented in Table-3 and Fig. 2.

**Accuracy:** Accuracy was performed in triplicate at three different levels *i.e.* 50%, 100% and 150%. The individual and mean accuracy at each level for flucytosine was found to be between 98.0 to 102.0%. The accuracy results met the predefined acceptance criteria.

**Accuracy for the related substances:** The study was performed at 50%, 100% and 150% (triplicate preparations at each level) of limit concentration (0.2%) for 5-fluorouracil. The individual and mean accuracy at each level for 5-fluorouracil was found to be between 80.0 to 120.0%. The accuracy data is presented in Table-4.

**Specificity:** Specificity of the method was established by spiking 5-fluorouracil (impurity of flucytosine) in placebo (prepared by mixing commonly used excipients, as the sample was market sample hence exact placebo composition was unknown) and all the peaks were observed to be well separated from the analyte peaks. The purity of flucytosine peak was assessed, purity angle was found less than auto purity threshold indicating that the flucytosine peak was pure.

For related substances, specificity of the method was established by spiking 5-fluorouracil (impurity of flucytosine) in sample. The method was found to be specific. Peak purity was evaluated, purity angle for flucytosine peak was found to be less than auto purity threshold in the spiked sample. The method met the validation challenge of specificity. Forced degradation studies were also performed, where prominent degradation was observed during acid, alkali and thermal degradation. The purity angle was found less than purity threshold for flucytosine peak in all degradation conditions, the method met the criteria for it to be stability indicating. The results from the forced degradation study are tabulated in Table-5.

**Precision:** Precision study was performed by preparing six sample each for method precision and intermediate precision study. For related substances, 5-fluorouracil was spiked at 0.2% level. The %RSD for assay results of flucytosine was found to be below 1.0% and results of 5-fluorouracil and total impurities was found to be below 5.0% for both method precision and intermediate precision studies.

**Limit of quantitation (LOQ):** Precision at LOQ was performed for 5-fluorouracil and flucytosine active pharmaceutical ingredient at LOQ level (0.05% level as per ICH guideline) in the placebo and injected six times. The %RSD values for six replicate injections and signal-to-noise ratio are presented in Table-6.

TABLE-3
LINEARITY DATA FOR DEVELOPED ANALYTICAL METHOD

| Assay | | | Related substances (flucytosine) | | | Related substances (5-fluorouracil) | | |
|---|---|---|---|---|---|---|---|---|
| Linearity level (%) | Conc. (ppm) | Area counts | Linearity level (%) | Conc. (ppm) | Area counts | Linearity level (%) | Conc. (ppm) | Area counts |
| 50 | 25.1 | 1552366 | LOQ | 1.02 | 62284 | LOQ | 0.51 | 31287 |
| 80 | 40.2 | 2485919 | 50% | 5.10 | 305924 | 60% | 1.22 | 73471 |
| 100 | 50.2 | 3071630 | 75% | 7.65 | 472898 | 80% | 1.64 | 97812 |
| 120 | 60.2 | 3697234 | 100% | 10.20 | 614326 | 100% | 2.04 | 120834 |
| 150 | 75.3 | 4685275 | 125% | 12.75 | 773430 | 125% | 2.56 | 152984 |
| | | | 150% | 15.30 | 936522 | 150% | 3.06 | 184023 |
| Correlation coefficient: 0.99978 | | | Correlation coefficient: 0.999855<br>Slope: 61024 | | | Correlation coefficient: 0.99989<br>Slope: 59715 | | |

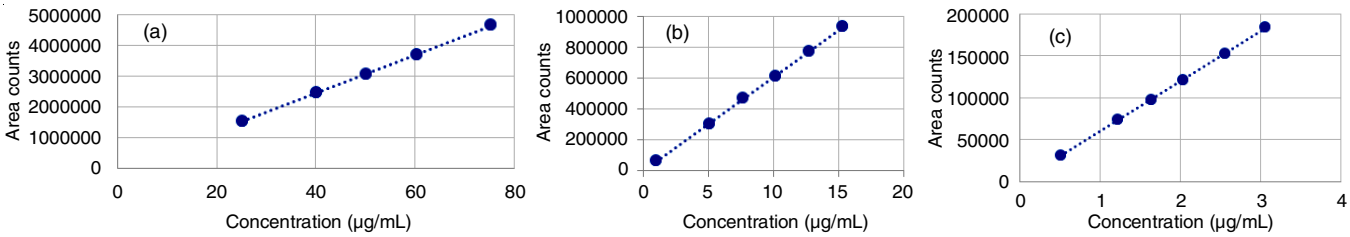


Fig. 2. Linearity plots of (a) flucytosine, (b) flucytosine related substances and (c) 5-fluorouracil

TABLE-4
ACCURACY DATA OF DEVELOPED ANALYTICAL METHOD

| Assay (flucytosine) | | | Related substances (5-fluorouracil) | | |
|---|---|---|---|---|---|
| Criteria | Accuracy level | Average % recovery | Criteria | Accuracy level | Average % recovery |
| Average % recovery | 50% | 99.2 | Average % recovery | 50% | 92.3 |
| | 100% | 100.3 | | 100% | 96.7 |
| | 150% | 99.6 | | 150% | 98.3 |
| RSD (% recovery) | 50% | 2.2 | RSD (% recovery) | 50% | 2.8 |
| | 100% | 3.1 | | 100% | 1.9 |
| | 150% | 1.4 | | 150% | 3.1 |

TABLE-5
FORCED DEGRADATION DATA

| Control sample (no treatment) | | Flucytosine peak purity | |
|---|---|---|---|
| | | Purity angle | Purity threshold |
| | | 0.232 | 0.392 |
| Forced degradation study | | | |
| Samples | Condition | % Degradation (5-fluorouracil) | Flucytosine peak purity |
| | | | Purity angle / Purity threshold |
| Acid degradation | 5 N HCl at 80 °C for 3 h | 0.864 | 0.068 / 0.316 |
| Alkali degradation | 5 N NaOH at 80 °C for 3 h | 0.324 | 0.073 / 0.243 |
| Peroxide degradation | 30% $H_2O_2$ for 6 h at room temperature | 0.124 | 0.196 / 0.467 |
| Thermal degradation | 80 °C for 3 h | 0.052 | 0.328 / 0.723 |
| Humidity degradation | 25 °C/90%RH/72 h | 0.042 | 0.164 / 0.323 |
| Photo stability | Exposed to UV light at 200-watt h/Sq.mt and white light for about 1.2 million lux hours | 0.082 | 0.153 / 0.298 |

TABLE-6
LIMIT OF QUANTITATION (LOQ)

| Parameter | Criteria | 5-Fluorouracil | Flucytosine |
|---|---|---|---|
| Precision at LOQ | % RSD | 1.1 | 2.1 |
| | Signal to noise ratio | 11 | 11 |

**Robustness:** The robustness study was performed by evaluating the impact of below mentioned changes on system suitability parameters *i.e.* tailing factor and %RSD for replicate injections: (i) flow rate: ±10%, (ii) column oven temperature: ± 5 ºC and (iii) wavelength: ± 5 nm. The robustness study encompassed evaluation of impact of flow rate, column oven temperature and wavelength. All the system suitability criteria were met in robustness study, and thereby confirmed that the method is robust.

## Conclusion

A simple, rapid and cost-effective RP-HPLC method was successfully developed for the simultaneous assay of flucytosine and its related substances in flucytosine tablets. The method covers all the validation challenges as per ICH recommendations. Present study recommends that developed method can be applied for high throughput and to reduce cost of production at affordable cost.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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