

## ELABORATION AND VALIDATION OF AN HPLC METHOD FOR THE QUANTITATIVE ASSAY OF 5-FLUOROURACIL

LAURA PALADE, IULIANA POPOVICI, GRATIELA POPA, ILEANA COJOCARU\*

*Pharmaceutical Technology Department, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iași  
16 University Street, 700115, Romania*

*\*corresponding author: mail.icojocaru@yahoo.com*

### Abstract

The study focused on elaborating and validating a quantitative assay method for 5-fluorouracil (5-FU) from vaginal bioadhesive tablets, using HPLC.

For the quantitative assessment of 5-FU from the bioadhesive tablets it was used the internal standard method. Pyridine 3-carbohydrazide (P3C) was chosen to be the internal standard used as its physical and chemical features were similar to those of 5-FU. The method relies on using HPLC for separating the two compounds, with UV detection at 270 nm.

Using the validation procedure we established the system's compatibility represented by the injection repeatability ( $RSD\%_{5FU} = 1.1006$ ;  $RSD\%_{P3C} = 1.9053$ ), the confidence interval at a safety level of 95%, repeatability of retention time ( $RSD\%_{5FU} = 1.8117$ ;  $RSD\%_{P3C} = 1.8694$ ), the separation degree of two consecutive peaks ( $Rs_{5FU/P3C} = 5.38$ ), the number of theoretical plates of the column ( $N_{5FU} = 3757$ ;  $N_{P3C} = 3110$ ). The method elaborated is accurate for the studied concentration range 0.0005 – 0.012mg/mL, with the average retrieval percentage of 103.4854 individual standard deviation 0.0148, relative standard deviation of 1.4127 and the maximum value of percent deviation of 4.16.

### Rezumat

Obiectivul lucrării a constat în elaborarea și validarea unei metode de determinare cantitativă a 5-fluorouracilului (5-FU) din comprimate bioadezive vaginale prin HPLC.

Pentru determinarea cantitativă a 5-FU din comprimate bioadezive s-a folosit metoda standardului intern. Ca standard intern s-a ales piridin 3-carbohidrazidă (P3C) pe baza caracteristicilor fizico-chimice similare cu 5-FU. Metoda se bazează pe separarea HPLC a celor doi compuși, cu detecție UV la 270 nm.

Prin procedura de validare am determinat compatibilitatea sistemului definită de repetabilitatea injectării ( $RSD\%_{5FU} = 1,1006$ ;  $RSD\%_{P3C} = 1,9053$ ), determinarea intervalului de încredere la un nivel de siguranță de 95%, repetabilitatea timpului de retenție ( $RSD\%_{5FU} = 1,8117$ ;  $RSD\%_{P3C} = 1,8694$ ), gradul de separarea a două picuri consecutive ( $Rs_{5FU/P3C} = 5,38$ ), numărul de talere teoretice ale coloanei ( $N_{5FU} = 3757$ ;  $N_{P3C} = 3110$ ). Metoda elaborată este exactă pe intervalul de concentrație cercetat 0,0005-0,012mg/mL, având procentul de regăsire mediu 103.4854, deviația standard individuală 0,0148, deviația standard relativă 1,4127, iar valoarea maximă a abaterilor procentuale este 4,16.

**Keywords:** HPLC dosage, 5-fluorouracil, validation method, bioadhesive tablets

## Introduction

Within our previous studies [1,2] there have been formulated, prepared and analyzed twelve original vaginal bioadhesive tablet formulae with 100 mg 5-fluorouracil (5-FU)/tablet. The pharmacotechnical control of vaginal bioadhesive tablets with 5-FU was performed by determining the specific quality characteristics [2].

For the quantitative determination of 5-FU in the bioadhesive tablets, a High Performance Liquid Chromatography (HPLC) method was developed which was afterwards validated according to the criteria existent in the literature [3, 4, 5].

## Materials and Methods

### Materials

***Chromatographic system HPLC Thermo Surveyor equipped with Surveyor LC –Pump, Autosampler Surveyor LC-Plus and UV-VIS detector***

***The column*** had as *stationary phase* octadecylsilane silica gel for chromatography (C<sub>18</sub>) (5µm) Thermo Fisher–Scientific Betasil C18 with the following dimensions  $l = 0.15$  m,  $\Phi = 4.6$  mm of stainless steel at a temperature of 37° C. The *mobile phase* was a mixture of ortophosphoric acid, water, tetrahydrofuran (V/V)(0.5: 97.5:2) and it was used a rate of 0.7mL/min.

***Detection*** was performed using a UV-VIS detector set at the wavelength of 270nm, band width 4; reference wavelength 450nm, band width 80nm.

The ***injected volume*** was 10 µL. ***The solution of internal standard (IS)*** consisted of 10 mg P3C (pyridine 3-carbohydrazide) dissolved in water and diluted with 10 mL of the same solvent ( $C_{SI} = 1.0$  mg/mL). ***The analyzed solution (AS)*** was obtained from a triturated vaginal bioadhesive tablet with sodium chloride and was transferred quantitatively into a vial of 100 mL. The resulting dispersion was subjected to ultrasounds for 10 minutes after which it was diluted with 100 mL of the same solvent. It was centrifugated at 3900 rpm for 10 minutes. To 1mL of supernatant it was added 1mL of internal standard solution and it was diluted with water to 100mL ( $C_{5FU} = 0.01$  mg/mL;  $C_{SI} = 0.01$ mg/mL). ***Reference solution (RS)*** was obtained by dissolving 100 mg 5-FU in water and it was diluted to 100 mL with the same solvent; 0.1 mL of this solution was quantitatively transferred in a 10 mL flask, 0.1 mL internal standard solution was added

and diluted at the sign with mobile phase ( $C_{MTP} = 0.01$  mg/mL;  $C_{SI} = 0.01$  mg/mL).

#### Method validation

To identify the 5-FU, P3C respectively, corresponding peaks, a solution containing the two compounds was injected and separately other solution with 5-FU alone.

For the *compatibility study* [3,5] the column was balanced with the mobile phase, flow of 0.7 mL/min, for 30 minutes and the reference solution *SR* was injected 10 times.

The 5-fluorouracil content was calculated based on the linear regression equation and reported to the internal standard concentration according to the formula:

$$\%5FU = \left( \frac{A_{st}}{A_{si}} - a \right) \times C_{si} \times F_{st} - \frac{b}{b}$$

where,  $A_{ST}$  = peak area corresponding to 5-fluorouracil in the solution analyzed;  $A_{SI}$  = peak area corresponding to the internal standard in the solution analyzed;  $C_{SI}$  = concentration of the internal standard in the solution analyzed;  $F_{ST}$  = the dilution factor corresponding to the solution analyzed;  $b$  = straight line calibration curve;  $a$  = intercept through the origin of the calibration curve.

For assessing the **linearity** [4] of the signal corresponding to 5-FU and 5FU/P3C respectively, the calibration curves were drawn on the concentration range 0.0005 and 0.012mg/mL 5-FU referring to a concentration of the internal standard of 10 µg/mL. Three independent series of this solution, for each concentration, were also performed.

The estimation of the peak area was performed using the linear regression equations:

**E1** Area = 216112946 x concentration - 42476.43 for 5-FU

**E2** Area = 2.1630 x concentration – 0.0419 for 5FU/P3C

For the study of **accuracy/exactness of the analysis method** [4] there have been prepared samples with quantities of almost 100 mg 5-FU, corresponding to the 100% percent compared to the theoretical content of 5-FU.

**Sample 1** consisting of 102.1 mg 5-fluorouracil, 60 mg carbomer 974 and 140 microcellulose crystalline was homogenized and shaped at a pressure of maximum 5t and from the resulting sample it was prepared the analyzed solution.

**Sample 2** consisting of 101.6 mg 5-fluorouracil, 60 mg carbomer 974 and 140 mg of microcellulose crystalline was homogenized and shaped at a pressure of maximum 5t. and from the resulting sample it was prepared the solution analyzed.

**Sample 3** consisting of 100.8 mg 5-fluorouracil, 60 mg carbomer 974 and 140 mg of microcellulose crystalline was homogenized and shaped at a pressure of maximum 5t. and from the resulting sample it was prepared the solution analyzed.

The retrieval concentration ( $C_{\text{retrieval}}$ ), the retrieval output (R%) of 5-FU from the reconstructed samples, the percent deviation (Xd%) and the experimental oblique were calculated.

## Results and Discussion

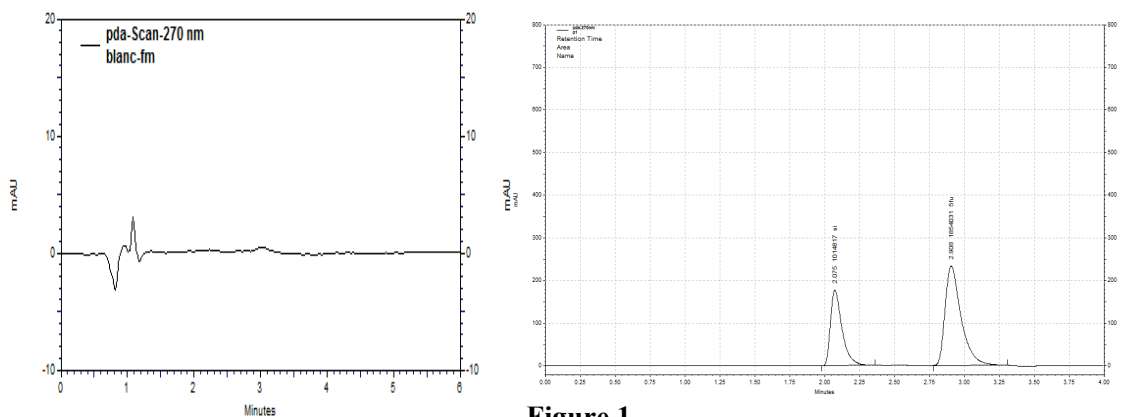
The results obtained at peak identification for 5-Fu and P3P are presented in table I:

**Table I**  
Peak identification for 5-FU and P3C

| Injected solution | Concentration of 5FU (mg/mL) | Concentration of P3C (mg/mL) | T <sub>r</sub> 5-FU (minutes) | T <sub>r</sub> P3C (minutes) | Area 5-FU | Area P3C |
|-------------------|------------------------------|------------------------------|-------------------------------|------------------------------|-----------|----------|
| SI1               | 0.01                         | 0.01                         | 2.90                          | 2.0                          | 2046079   | 977435   |
| SI2               | 0.01                         | -                            | 2.903                         |                              | 1998566   | -        |

Tr – retention time

It was noticed that the retention time of 5-FU was of almost 2.9 minutes and the retention time for P3C was of 2 minutes.



**Figure 1**

a. blank b. Peaks corresponding to the two samples  
5-FU mixed with P3C and the sample containing only 5-FU

In table II there are presented the results obtained at injection repeatability.

**Table II**  
Injection repeatability for the samples with 5-FU and P3C

| Sample  | 5FU 0.01 mg/mL |                       |                     | P3C 0.01 mg/mL |                       |                     |
|---------|----------------|-----------------------|---------------------|----------------|-----------------------|---------------------|
|         | Area           | Individual deviations | Relative deviations | Area           | Individual deviations | Relative deviations |
| SC1     | 2122582        | 9848.1                | 0.461825            | 904675         | 10601.3               | 1.158262            |
| SC2     | 2146008        | 13577.9               | 0.636734            | 902348         | 12928.3               | 1.412502            |
| SC3     | 2123555        | 8875.1                | 0.416197            | 902371         | 12905.3               | 1.40999             |
| SC4     | 2145236        | 12805.9               | 0.600531            | 908269         | 7007.3                | 0.765594            |
| SC5     | 2146256        | 13825.9               | 0.648364            | 936930         | 21653.7               | 2.36581             |
| SC6     | 2110569        | 21861.1               | 1.025173            | 885376         | 29900.3               | 3.266806            |
| SC7     | 2115623        | 16807.1               | 0.788167            | 925260         | 9983.7                | 1.090785            |
| SC8     | 2114256        | 18174.1               | 0.852272            | 933844         | 18567.7               | 2.028644            |
| SC9     | 2114562        | 17868.1               | 0.837922            | 935985         | 20708.7               | 2.262563            |
| SC10    | 2185654        | 53223.9               | 2.495927            | 917705         | 2428.7                | 0.265352            |
| Average | 2132430        |                       |                     | 915276.3       |                       |                     |
| SD      | 23471.01       |                       |                     | 17439.64       |                       |                     |
| RSD     | 1.10067        |                       |                     | 1.905397       |                       |                     |

From the results obtained it was noticed that injection repeatability (RSD%) was lower than 2 ( $RSD\%_{5FU} = 1.1006$ ,  $RSD\%_{P3C} = 1.9053$ ), the confidence interval for 5-FU was ranging between  $2132430 \pm 12474.71$  ( $2144904.71 - 2119955.29$ ), while for P3C ranged between  $915276.3 \pm 16788.98$  ( $932065.28 - 898487.32$ ).

The results obtained for retention time repeatability ( $RSD\%_{5FU} = 1.8117$ ,  $RSD\%_{P3C} = 1.8694$ ) correspond to the standards  $RSD\% \leq 2$ . The separation degree of the two consecutive peaks was of  $R_{S5-FU/P3C} = 5.38$ , within the standard values  $R_s > 2$ , the number of theoretical plates of the column which must be  $n > 2000$ , corresponds ( $N_{5FU} = 3757$ ,  $N_{P3C} = 3110$ ), and the stretching factor ( $T \leq 1.8$ ) also complies ( $T_{5-FU} = 1.62$ ;  $T_{P3C} = 1.63$ ). Taking into consideration the experimental values we can say that the system was compatible.

Further on there have been evaluated the linearity and the linearity interval for 5-FU. The results are presented in table III.

**Table III**  
The evaluation of the linearity and linearity interval for 5-FU

| $C_{5FU}(mg/mL)$<br>Area | 0.012   | 0.010   | 0.008   | 0.006   | 0.004  | 0.002    | 0.001    | 0.0005 |
|--------------------------|---------|---------|---------|---------|--------|----------|----------|--------|
| Series 1                 | 2554905 | 2122582 | 1687026 | 1242723 | 811793 | 364297   | 158536   | 102013 |
| Series 2                 | 2540549 | 2146008 | 1683366 | 1245170 | 820066 | 370410   | 161257   | 103253 |
| Series 3                 | 2542362 | 2136564 | 1692365 | 1246569 | 820125 | 368566   | 160236   | 102564 |
| Average                  | 2545939 | 2135051 | 1687586 | 1244821 | 817328 | 367757.7 | 160009.7 | 102610 |

For the 5-FU - P3C mixture, the results regarding linearity and the linearity interval evaluation are presented in table IV.

**Table IV**  
Linearity and linearity interval evaluation for 5-FU/P3C

| $C_{5FU}/C_{P3C}$<br>$A_{5FU}/A_{P3C}$ | 0.0012   | 0.010    | 0.008    | 0.006    | 0.004    | 0.002    | 0.001    | 0.0005   |
|----------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| Series 1                               | 2.545735 | 2.145416 | 1.650568 | 1.262882 | 0.815579 | 0.366604 | 0.163458 | 0.104156 |
| Series 2                               | 2.552328 | 2.165943 | 1.654744 | 1.266656 | 0.822122 | 0.369758 | 0.167029 | 0.105327 |
| Series 3                               | 2.548515 | 2.139555 | 1.692839 | 1.247445 | 0.807187 | 0.366817 | 0.158408 | 0.102747 |
| <b>Average</b>                         | 2.548859 | 2.150305 | 1.666050 | 1.258995 | 0.814963 | 0.367726 | 0.162965 | 0.104077 |

The corelograms obtained, point out a linear variation of the peak area depending of the sample's concentration, for both 5-FU and 5-FU/P3C. In the studied concentration range 0.012-0.0005mg/mL there is a linear dependence when using the internal standard method.

The last parameter assessed was the accuracy of HPLC method; the results obtained are presented in table V.

**Table IV**  
Evaluation of the accuracy of the HPLC method

| Samples | Ratio of theoretical concentrations -Xa | Ratio of area (5FU/P3C) | Ratio of retrieval concentrations - Xr | Retrieval percent % | Concentrations 5-FU (mg/mL) | Individual deviations | Percent deviations |
|---------|-----------------------------------------|-------------------------|----------------------------------------|---------------------|-----------------------------|-----------------------|--------------------|
| SP1     | 1.021                                   | 2.2513                  | 1.060194                               | 103.8388            | 0.010602                    | 0.039194              | 3.919417           |
| SP2     | 1.016                                   | 2.2459                  | 1.057698                               | 104.1041            | 0.010577                    | 0.041698              | 4.169764           |
| SP3     | 1.008                                   | 2.1932                  | 1.033333                               | 102.5132            | 0.010333                    | 0.025333              | 2.533333           |

The method is accurate for the studied concentration range of 0.0005-0.012mg/mL, with the mean retrieval percent of 103.4854%, individual standard deviation of 0.0148, relative standard deviation of 1.4127 and the maximum value of percent deviation of 4.16.

## Conclusions

An HPLC assay method was developed for the quantitative dosage of 5-fluorouracil from vaginal bioadhesive tablets, and was evaluated its compatibility, linearity, precision and accuracy.

The system's compatibility was defined by injection repeatability ( $RSD\%_{5FU} = 1.1006$ ,  $RSD\%_{P3C} = 1.9053$ ), determining the confidence interval at a safety level of 95%, retention time repeatability ( $RSD\%_{5FU} = 1.8117$ ;  $RSD\%_{P3C} = 1.8694$ ), separation level of two consecutive peaks ( $Rs_{5FU/P3C} = 5.38$ , number of theoretical plates of the column ( $N_{5FU} = 3757$ ;  $N_{P3C} = 3110$ )).

The elaborated method is accurate having the mean retrieval percent of 103.4854 and the maximum value of percent deviations of 4.16, below the acceptable limit – 5.

### References

1. Tomuță I., Iovanov R., Bodoki E., Leucuța S.E., Quantification of meloxicam and excipients on intact tablets by near infrared spectrometry and chemometry, *Farmacia*, 2010, 58(5), 559-571
2. Cojocaru I.C., Ochiuz L., Spac A., Popa G., Palade L., Popovici I., The validation of the UV spectrophotometric method for the assay of 5-fluorouracil, *Farmacia*, 2012, 60(3), 379-385
3. International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use- ICH-*Validation of Analytical Procedures: Text and Methodology* Q2(R1) Harmonised Tripartite Guideline, nov.London, 2005.
4. L. Roman, M. Bojiță, R. Săndulescu, D. L. Muntean *Validarea Metodelor de Analiză și Control- bazele teoretice și practice*, Editura Medicală, Bucuresti, 2007.
5. Rewier *Guidance-Validation of Chromatographic Methods*, Center for Drug Evaluation and Research, USA, nov.1994.
6. Talari R., Varshosaz J., Mostafavi A., Nokhodchi A., Development and validation of a novel RP-HPLC method for pharmacokinetic studies of gliclazide in rat, *Farmacia*, 2011, 59(3), 388-395

---

*Manuscript received: August 11<sup>th</sup> 2012*