

## DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF METHOTREXATE INCORPORATED INTO POLYMERIC IMPLANTS: APPLICATION TO QUALITY CONTROL ANALYSES

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### ABSTRACT

This study reports the development and validation of an analytical UV spectrophotometric method for determination of methotrexate incorporated into implants based on poly( $\epsilon$ -caprolactone) and released from them. The detection of the drug was carried out in 0.1 ml/L hydrochloric acid at 307 nm. It provided specificity for the methotrexate in direct contact with the poly( $\epsilon$ -caprolactone). The method was linear in the range of between 4 and 14  $\mu$ g/mL presenting a good correlation coefficient ( $r = 0.999$ ). The method was robust; the average accuracies of three concentrations ranged from 80 to 120%, and precision showed low relative standard deviation ( $< 2.00\%$ ).

**Keywords:** UV spectrophotometric method, Validation, Methotrexate-loaded poly( $\epsilon$ -caprolactone) implants.

### INTRODUCTION

Methotrexate is an antineoplastic drug (Figure 1), which acts as an antimetabolite of folic acid and interferes with the formation of DNA, RNA, and protein<sup>1</sup>. Its main target is the dihydrofolate reductase, which inhibits the synthesis of new DNA by restricting the supply of deoxythymidine triphosphate and of purine nucleotides, leading to the misincorporation of the uracil into DNA<sup>2,3</sup>.

This drug is widely used in the treatment of human malignancies including childhood acute lymphocytic leukemia, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, breast cancer, psoriasis, choriocarcinoma and related trophoblastic tumors<sup>4</sup>.

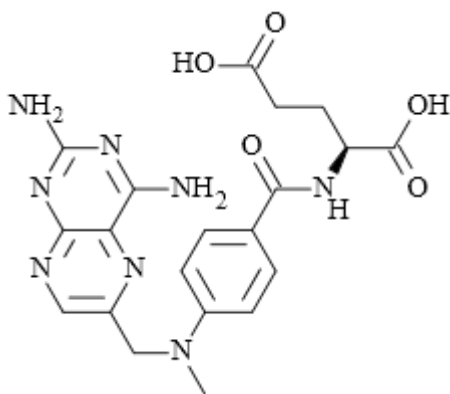


Fig. 1: Chemical structure of methotrexate

Despite of the methotrexate is quite effective for the treatment of human cancer; undesirable side effects following the drug administration have been reported such as toxicity to normal cells, drug resistance, nephrotoxicity, bone marrow suppression, acute and chronic hepatotoxicity, interstitial pneumonitis and chronic interstitial obstructive pulmonary disease<sup>4,5</sup>. In order to minimize the side effects induced by the methotrexate and to increase delivery efficiency to tumors, delivery systems containing this drug have been developed. For example, Li and coworkers<sup>6</sup> reported the development of micelles based on methotrexate and poly(ethylene glycol)-block poly(2-hydroxyethyl L-aspartamide). Yang and coworkers<sup>7</sup> described the synthesis of nanoparticles composed by methoxy poly(ethyleneglycol)-grafted chitosan that encapsulated the methotrexate. In this study, methotrexate loaded poly( $\epsilon$ -caprolactone) implants were developed to provide the controlled release of the drug direct in the target site.

The therapeutic importance of the methotrexate has prompted the development of many analytical methods for quantifying the drug in different samples. In the literature, high performance chromatographic (HPLC) methods with UV detection for assaying methotrexate entrapped in polymeric nanoparticles were described<sup>8,9</sup>. The official pharmacopeias<sup>10,11</sup> also report that the quantitation of methotrexate raw material, tablets and injections is based on an isocratic HPLC method with UV detection. Additionally, some reports have described HPLC methods coupled with mass spectrometry for determination of methotrexate and other active principles isolated from biological samples<sup>12,13,14</sup>. Methotrexate and its major metabolites in biological fluids by HPLC coupled with spectrofluorometry were also determined and reported by different authors<sup>15,16</sup>. Although these methods provide the selective measurement of the drug and its by-products, these techniques do not represent viable analytical methods to quantify the methotrexate in routine analyses of quality control.

In this study, a simple and reliable UV spectrophotometric method was developed and validated to quantify the methotrexate incorporated into poly( $\epsilon$ -caprolactone) implants. This method was also applied for determination of the methotrexate released from the polymeric implants for prolonged period. The method was validated according to the International Conference on Harmonization guidelines<sup>17</sup> regarding the following parameters: specificity, linearity, precision, accuracy and robustness. The proposed UV spectrophotometric method could be widely used in routine analysis of quality control laboratories.

### MATERIALS AND METHODS

#### Materials

Methotrexate reference standard was purchased from Sigma Chemical Co (USA). Poly( $\epsilon$ -caprolactone) was purchased from Sigma Chemical Co (USA) (PCL; MW  $\sim 14,000$ ; density = 1.145 g/ml at 25  $^{\circ}$ C). Acetonitrile and hydrochloric acid analytical grade were obtained from Vetec (Brazil) and J. T. Baker. The water was distilled and freshly used.

#### Instrumentation and analytical conditions

A Shimadzu model 1700 (Japan) double beam UV/Visible spectrophotometer with spectral width of 2 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matched quartz cell was used to measure absorbance of all solutions. Spectra were automatically obtained by UV-Probe system software. The detection of the drug was carried out in 0.1 mol/L hydrochloric acid at 307 nm. A Shimadzu analytical balance model AUW220D (Japan) and an ultrasonic bath Quimis (Brazil) were also used.

### Preparation of solutions

**Methotrexate standard solution:** approximately 5 mg of methotrexate reference compound were dissolved in a 50 mL volumetric flask. Approximately 35 mL of 0.1 mol/L hydrochloric acid was added, the solution was sonicated for 5 minutes and the volume was adjusted to 50 mL with 0.1 mol/L hydrochloric acid. An aliquot (1 mL) was transferred to a 10 mL volumetric flask and the volume was completed with 0.1 mol/L hydrochloric acid, to obtain a solution at 10 µg/mL.

**Methotrexate sample solution:** one implant was transferred to a 50 mL volumetric flask and the 0.1 mol/L hydrochloric acid (35 mL) was added. The solution was sonicated for 5 minutes and the volume was adjusted to 50 mL with the same solvent. The solution was filtered. An aliquot (1 mL) was transferred to a 10 mL volumetric flask and the volume was completed with 0.1 mol/L hydrochloric acid, to obtain a solution at 10 µg/mL.

**Placebo solution:** one implant containing poly( $\epsilon$ -caprolactone) (without drug) was transferred to a 50 mL volumetric flask and the 0.1 mol/L hydrochloric acid (35 mL) was added. The solution was sonicated for 5 minutes and the volume was adjusted to 50 mL with the same solvent. The solution was filtered. An aliquot (1 mL) was transferred to a 10 mL volumetric flask and the volume was completed with 0.1 mol/L hydrochloric acid.

### Method validation

The method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures<sup>17</sup>.

### Specificity

Placebo and methotrexate standard solutions were prepared as previously described. The UV spectra were recorded in the range of 200 to 400 nm. Specificity was evaluated comparing the UV spectra of methotrexate reference compound and that obtained for placebo represented by solubilize poly( $\epsilon$ -caprolactone). To achieve the specificity of the method, no interferences or overlaps with methotrexate response at 307 nm were allowed. Further, selectivity was evaluated by comparing the average concentration of methotrexate ( $n = 6$ ) of the two groups (sample and standard solution) through the Student's *t* test ( $p < 0.05$ ).

### Linearity

Aliquots of the methotrexate standard solution were diluted in 0.1 mol/L hydrochloric acid to six different concentrations (4, 6, 8, 10, 12 and 14 µg/mL). A calibration curve for concentration versus absorbance was plotted and the obtained data were subjected to linear regression analysis using the least square method. The correlation coefficient was also calculated.

### Precision

The intra-day precision (repeatability) was evaluated by analyzing eight replicates of methotrexate sample solutions ( $n = 8$ ), at test concentration (10 µg/mL). Similarly, the inter-day precision (reproducibility) was evaluated in two consecutive days ( $n = 16$ ). The concentration of methotrexate into the implants was determined and the relative standard deviation (RSD) was calculated.

### Accuracy

Methotrexate standard solutions, at three different concentration levels (8, 10 and 12 µg/mL), were added to placebo solutions. At each level, solutions were prepared in triplicate and the recovery percentage was calculated. The mean percentage recovery of methotrexate at each level between 98 and 102% indicated the accuracy of the UV method<sup>18</sup>.

### Robustness

Six sample solutions were prepared and analyzed using the established conditions and by variation of the following analytical parameters: two hydrochloric acid and acetonitrile suppliers.

Methotrexate contents and RSD were determined for each condition. The obtained data were submitted to statistical analysis (Student-*t* test) at 0.05 significance level<sup>19</sup>.

### Preparation of the implants containing poly( $\epsilon$ -caprolactone) and methotrexate

The implants were prepared by fully blending methotrexate particles with melting poly( $\epsilon$ -caprolactone) and then molding the blends into spherical implants using a metallic mold. Briefly, poly( $\epsilon$ -caprolactone) was heated until it was completely melted. Afterward methotrexate was added slowly into the melting poly( $\epsilon$ -caprolactone) and mixed at approximately 60 °C for 20 min at a screw speed of 50 rpm. The resultant blend was collected and further molded into spherical implants (6 mm in diameter) using a metallic mold at approximately 60 °C. The methotrexate-loaded poly( $\epsilon$ -caprolactone) implants contained approximately 25.0% (w/w) of the drug corresponding to 5 mg of methotrexate. Implants without drug were also prepared.

### Determination of methotrexate content in the implants

For the determination of content uniformity of methotrexate in the polymeric implants, the procedure stated in the general chapter <905> uniformity of dosage units of the United States Pharmacopeia<sup>10</sup> was followed. Ten implants were selected and weighted. The methotrexate sample solutions were prepared as previously described. The amount of the drug in the implant was determined by applying the validated UV spectrophotometric method. The obtained amount of methotrexate in each implant (mg) was calculated and the results were expressed as the percent of the pre-indicated value (approximately 5 mg). The relative standard deviation was also calculated.

### In vitro release of methotrexate from the implants

The *in vitro* release of methotrexate was carried out during 90 days. The methotrexate-loaded poly( $\epsilon$ -caprolactone) implants were placed in different tubes containing 3 mL of phosphate buffer solution (PBS pH = 7.4) ( $n = 6$ ). These tubes were placed inside an incubator set at 37°C and 30 rpm. At predetermined intervals, 3 mL of the PBS was sampled and the same volume of fresh PBS was added to each tube. The amount of methotrexate released from each implant was assayed by the validated UV spectrophotometric method, and expressed as the cumulative percentage of the drug released in the medium. The average of the obtained measurements was calculated and used to plot the release profile curve.

## RESULTS AND DISCUSSION

In this study, an UV spectrophotometric method was developed and validated for determination of methotrexate content incorporated into poly( $\epsilon$ -caprolactone) implants and released from these implantable devices. The objective of this work was to develop a simple, rapid and less environmental toxic method for quantitation of the methotrexate. For this reason, the 0.1 mol/L hydrochloric acid was selected as diluting solvent and a minimum amount of acetonitrile was used to solubilize the poly( $\epsilon$ -caprolactone). Environmental concerns are also related to the use of organic solvents for the sample preparation and in the application of analytical methods. However, the developed UV method overcomes this type of problem by replacing more toxic solvents with hydrochloric acid solution. Additionally, the 0.1 mol/L hydrochloric acid solution was used as solvent for the methotrexate once it shows a pH-dependent aqueous solubility. Therefore, in the lower pH range, methotrexate increases its aqueous solubility<sup>20</sup>.

Initially, an UV spectroscopic scanning run of the methotrexate standard and sample solutions provided an intense absorption band with maximum wavelength at 307 nm. The UV spectra of the placebo solution was also recorded and it was verified the absence of interferences or overlaps with the methotrexate response at 307 nm, indicating that the specificity of the method under the described conditions. In addition, there was no significant difference ( $p < 0.05$ ) between the average concentrations of the methotrexate standard solutions ( $99.56 \pm 0.48\%$ ) and sample solutions (containing implants) ( $100.67 \pm 0.87\%$ ) determined by the UV method.

Considering the previous results, the developed method presented adequate selectivity for the determination of methotrexate in polymeric implants.

The linearity is determined by the ability of the method to obtain test results, which are directly proportional to the concentration of the compounds of interest in the sample<sup>21</sup>. The standard calibration curve was constructed by plotting methotrexate concentration versus absorbance values obtained at 307 nm. The calibration curve was linear over the range of 4 to 14 µg/mL and the representative linear equation was  $y = 0.0035x + 0.2342$ , calculated by the least squares method. The correlation coefficient was over 0.999 indicating highly significant correlation between concentration and absorbance value<sup>22</sup>. The significance of the intercept obtained in the calibration curve was tested and this parameter was not statistically significant ( $p > 0.05$ ), consequently, it can be considered that the curve passes through the origin.

In the intra-day precision (repeatability) ( $n = 8$ ), the mean content of methotrexate in the implants was 99.77% (RSD = 1.62%). For the inter-day precision (reproducibility) ( $n = 16$ ), the obtained mean was 100.37% (RSD = 1.99%). The RSD values were below 5% for the concentration tested, thus indicating appropriate intra and inter-assay precision of the UV spectrophotometric method<sup>23</sup>.

The accuracy of the method was expressed as the percent recovery of methotrexate, at three concentration levels, added to placebo solutions. The obtained results ranged between 99.0 and 101.0% for the concentrations applied (Table 1). All the percent recovery indicated the accuracy of the UV method and, consequentially, an agreement between the theoretical value and the real value of concentration<sup>24</sup>.

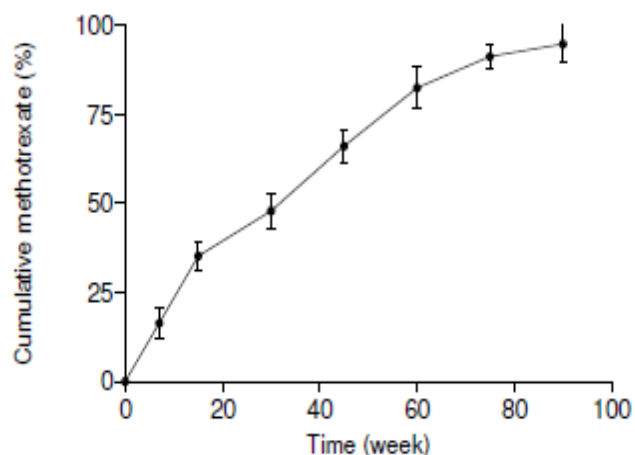
**Table 1: Accuracy percent recovery of methotrexate added to placebo solutions**

Methotrexate concentration (µg/mL)	Recovery (%)	RSD (%)
8	99.71	0.25
10	98.28	0.38
12	100.14	1.39

The reliability of the proposed method was also evaluated by means of the robustness test. Statistical analysis showed no significant difference between results obtained employing analytical conditions previously established for the method and for experiments in which variations were introduced ( $p < 0.05$ ). Therefore, the method demonstrated to be robust for different suppliers of hydrochloric acid and acetonitrile.

The validated UV spectrophotometric method was applied to determine the methotrexate content in the polymeric implants. The result of uniformity content revealed that the methotrexate presented a uniform distribution throughout the polymeric matrix. No units were outside the range of 85-115% of the pre-indicated amount of the drug [25% (w/w), corresponding to approximately 5mg of the drug per implant]. The relative standard deviation obtained was 3.4%. Therefore, the obtained results showed that the processing technique used in this study is reproducible and resulted in a uniform distribution of the drug in the poly(ε-caprolactone) matrix.

This method was also used to quantify the methotrexate released from the polymeric implants. Figure 2 demonstrated the *in vitro* cumulative release profile of the drug from the implantable devices for 90 days. The drug was leaked slowly from the implants controlled by diffusion. According to Merkli and coworkers (1998)<sup>25</sup> the poly(ε-caprolactone) is characterized by a very low hydrolysis rate, which can vary from months to years. Since it exhibits slow biodegradation rate via bulk hydrolyzation of the ester bonds<sup>26</sup>, the release mechanism of the methotrexate was predominantly by diffusion through polymeric matrix or the voids left by the depleted drug.



**Fig. 2: *In vitro* cumulative methotrexate released from poly(ε-caprolactone) implants. Results represent mean ± standard deviation (n = 6)**

## CONCLUSION

The proposed UV spectrophotometric method for determination of methotrexate incorporated into poly(ε-caprolactone) implants was validated and showed to be specific, linear, precise and accurate. This method was successfully applied to determine the methotrexate content in the polymeric implants and released from them for 90 days. The analytical method could be widely used in routine of quality control laboratories, since it showed to be simple and fast. Additionally, it allowed decreasing of the use of organic solvents during the sample preparation, which represents an advantage of the proposed method with regard to the environmental preservation. Finally, the UV spectrophotometric method could be used in routine quality control analyses.

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