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Development and Validation of Spectrophotometric method for determination of Methotrexate Incorporated into PLGA Implants

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Abstract

A simple and reliable spectrophotometric method was developed and validated for the determination of methotrexate incorporated into PLGA implants 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 PLGA. The method was linear in the range of between 4 and 14 µg/mL presenting a good correlation coefficient (r = 0.99). The average accuracies of three concentrations ranged from 98.64 to 100.76 % and precision was close to 2%. The method showed robustness, remaining unaffected by deliberate variations in relevant parameters.The validated method can be successfully applied to determine methotrexate loaded PLGA implants.

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INTRODUCTION

Methotrexate, chemically known as 4-amino-10-methylfolic acid or 4-amino-4-deoxy-10-

methylpteroyl-L-glutamic acid, is an antineoplastic which acts as an inhibitor of dihydrofolateredutase (DHFR), an essential enzyme in the biosynthesis of thymidylate, required for DNA replication[1,2](Figure 1).The methotrexate is widely used in the treatment of 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[3,4].
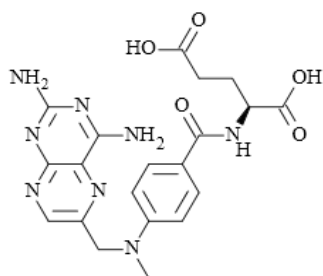

Fig. 1: Chemical structure of the methotrexate

Many analytical methods for methotrexate analysis, including fluorescence polarization immunoassay[5], radioimmunoassay [6] and enzyme immunoassay[7], have been reported to determine the plasma methotrexate level[8]. These methods are at least to some degree prone to interference from drug metabolites, endogenous factors, hemolysis of the sample and various patient disease states [8-10].

Different HPLC methods have been described for simultaneous determination of methotrexate and its metabolites using a variety of stationary and mobile phases, and ultraviolet and mass spectrometricdetections. Turci and co-workers [11] developed a HPLC method tandem mass spectrometry to quantify in wipe and air samples from the hospital environment. Each step of this method was firstly developed and optimized using ultraviolet detection, and afterwards tandem mass spectrometry was applied to obtain a lower limit of quantitation when the expected methotrexate level was less than the analytical ultraviolet detection

limit. Fang and co-workers [12] reported the development and validation of a HPLC method coupled with ultraviolet detection for simultaneous determination of three process- related impurities in cell-based bioproduction of many active biopharmaceuticals: yeastolates, triton X-100 and methotrexate. The suitability of this method for analyses of recombinant human hyaluronidase in-process (viral inactivation) and final manufacturing materials was demonstrated, and trace levels of yeastolates, triton X-100 and methotrexate were reliably measured. Finally, Alcântara and co-workers [13] developed a HPLC coupled with ultraviolet detection for simultaneous determination of 5-fluorouracil, methotrexate, doxorubicin, cyclophosphamide and ifosfamide. This method was considered useful to quantify multiple drugs in exposure assessments. Therefore, there are several analytical methods for determination of methotrexate in biological samples; however they are not appropriate to quantify the drug in routine quality control analyses of implantable devices.

In this study, a spectrophotometric method was developed and validated for determination of methotrexate incorporated into PLGA implants. The proposed method offered the advantages of being simple, reliable, rapid and effective. Therefore, it was hypothesized that the spectrophotometric method could be applied in quality control analyses of implants containing methotrexate in direct contact with PLGA matrix.

MATERIALS AND METHODS
Materials
Methotrexate reference standard was purchased from Sigma Chemical Co., USA. Poly(lactic-co-glycolic acid)was purchased from Boehringer Ingelheim Pharma GmbH & Co., Germany (PLGA 75:25, inherent viscosity (i.v.) = 0.50 - 0.70 dL/g - Resomer® RG 755 S). Acetonitrile and hydrochloric acid analytical grade were obtained from Vetec

(Brazil) and J. T. Baker, respectively. 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 10 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 5 mL of acetonitrile and 35 mL of 0.1 mol/L hydrochloric acid was added. The solution was sonicated for 10 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 PLGA (without drug) was transferred to a 50 mL volumetric flask and 5 mL of acetonitrile and 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 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 [14].

Specificity

Placebo solution and methotrexate standard solution were prepared as previously described. The spectra were recorded in the range of 200 to 400 nm. Specificity was evaluated by comparing the spectra of methotrexate reference compound and that obtained for placebo represented by solubilizingPLGA. 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

The calibration curve was obtained using five standard solutions in different concentrations of methotrexate (4, 6, 8, 10, 12 and 14 µg/mL) in 3 independent replicates run in random order. These assays were performed on 2 different days. The constructed calibration curves were assessed using residue analysis (homoscedascity, normality, and independence of residues) and linear regression analysis was done by the ordinal least squares method [15].

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 PLGA

implants was determined and the relative standard deviation (RSD) was calculated.

Accuracy

Methotrexate standard solutions, at three different concentrations 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 analytical method[16].

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 calculated for each condition by calibration plot equation. All data were evaluated by analysis of variance (ANOVA) in a significance level ($p < 0.05$)[17].

Preparation of the methotrexate-loaded PLGA implants

The implants were prepared by fully blending methotrexate particles with melting PLGA and then molding the blends into cylindrical implants using a metallic mold. Briefly, PLGA was heated until it was completely melted. Afterward methotrexate was added slowly into the melting PLGA and mixed at approximately 120 ᵒC for 20 min at a screw speed of 50 rpm. The resultant blend was collected and further molded into cylindrical implants using a metallic mold at approximately 120 ᵒC.The methotrexate-loaded PLGA implants contained approximately 25.0% (w/w) of the drug corresponding to 1.5 mg of methotrexate (MTX PLGA implants). Implants without drug were also prepared (PLGA implants).

Determination of methotrexate content in the PLGAimplants

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[18] 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 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 1.5 mg). The relative standard deviation was also calculated.

In vitro release of methotrexate from the PLGA implants

The *in vitro* release of methotrexate was carried out during 30 days. The methotrexate-loaded PLGA implants were placed in different tubes containing 3 mL of phosphate buffer solution (PBS pH = 7.4) (n = 5). These tubes were placed inside an incubator set at 37ᵒC 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 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
Method validation

The spectra of methotrexate standard solution and placebo solution were recorded. It was verified the absence of interferences or overlaps with the methotrexate response at 307 nm, demonstrating the specificity of the spectrophotometric method. Moreover, the statistical analysis (t-Student test)

revealed that there was no significant difference (p < 0.05) between the average concentration of the methotrexate standard solutions (99.78 %) and sample solutions (containing implants) (99.45 %) determined by the method. Therefore, this analytical method showed selectivity to quantify unequivocally methotrexate in the presence of PLGA.

The linearity of the method was tested by plotting a calibration curve over the range of 4 to 14µg/mL of methotrexate, which was subjected to regression analysis by the least square method. The representative linear equation was y = 0.0004x + 0.0199, where y and x were absorbance and concentration (µg/mL), respectively. 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[19].The correlation coefficient (r) was higher than 0.99, showing highly significant correlation between concentration and peak area[20].The coefficient of determination (r^2) of the calibration curve was 0.9912, implying that 99.12% of the total variance of the absorbances was explained by the varying methotrexate concentration. Finally, the linear model proved to be adequate since the residues followed a normal distribution pattern and were independent, the homoscedasticity could be observed and the lack of fit was not significant[16].

In the intra-day precision (repeatability) (n = 8), the mean content of methotrexate in the PLGA implants was 101.82% (RSD = 1.98%). For the inter-day precision (reproducibility) (n = 16), the obtained mean was 99.80% (RSD = 0.48%). The RSD values were below 5% for the concentration tested, thus indicating appropriate intra and inter-assay precision of the spectrophotometric method[20].

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 99.03 and 101.54% for the concentrations applied with acceptable RSD limits, indicating that the method was accurate(Table 1).

Table 1: Percent of recovery of methotrexate spiked in the placebo solution

Methotrexateconcentration (µg/mL)	Recovery (%)	RSD (%)
8	100.76	0.96
10	98.64	0.87
12	99.23	0.18

Robustness statistical analysis (Table 2) showed no significant difference (p < 0.05) between standardized (nominal) analytical conditions and deliberate variations. The variations in hydrochloric acid and acetonitrile suppliers did not strongly affect the determination of methotrexate incorporated into PLGA implants.

Table 2: Spectrophotometric results of robustness for methotrexate-loaded PLGA implants.

Reagents	Methotrexate assay (%)	RSD (%)
Hydrochloric acid (supplier 1)	99.67	0.79
Hydrochloric acid (supplier 2)	99.21	0.88
Acetonitrile (supplier 1)	100.45	1.34
Acetonitrile (supplier 2)	100.89	1.02

Determination of methotrexate content in the PLGA implants

The validated spectrophotometric method described was applied to determine themethotrexatecontent incorporated into PLGA implants. The results of content uniformity showed that methotrexate presented a uniform distribution in the PLGA implants. No unit was outside the range of 85.0–115.0% of the pre-indicated amount of methotrexate (approximately 1.5 mg of the drug per implant). The relative standard deviation for replicates was 5.49%.

***In vitro* release of methotrexate from PLGA implants**

The drug leached from the implantable devices was quantified by the validated spectrophotometric method. Figure 2 shows the cumulative release curve of methotrexate from PLGA implants over a period of 30 days. The implants led to a controlled release of the drug, since approximately 24% of the methotrexate was leached from the implantable devices during the follow-up period.
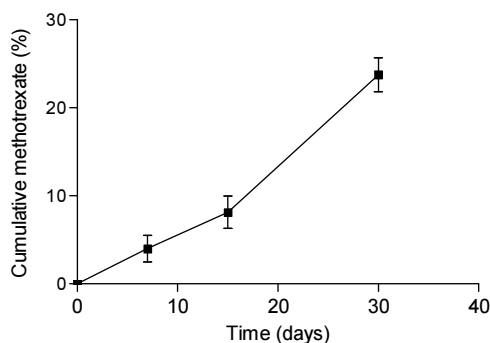

Fig. 2: *In vitro* methotrexate release from PLGA implants. Results represent mean ± standard deviation (n = 5).

CONCLUSION

In this study, it was developed a spectrophotometric method for the determination of methotrexate incorporated into PLGA implants. This analytical method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures; and showed to be selective, linear, precise, accurate and robust.

Acknowledgments

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