

Determination of Methotrexate in pH-Sensitive Chitosan Nanoparticles by Validated RP-LC and UV Spectrophotometric Methods

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Abstract: Nanotechnology-based drug delivery systems are in constant development and, therefore, it is of great importance to have rapid, efficient and accurate analytical methodology to quantify the encapsulated drugs. Here, simple and fast methods, by reversed-phase liquid chromatography (RP-LC) and UV spectrophotometry, were developed and validated for the determination of methotrexate (MTX) in pH-sensitive chitosan nanoparticles (CS-NPs). NPs were prepared using a modified ionotropic complexation process, in which was included a surfactant derived from N^ε,N^ε-dioctanoyl lysine with an inorganic sodium counterion. The RP-LC method was carried out on a Waters XBridge™ C18 column (250 mm x 4.6 mm I.D., 5μm), with mobile phase consisted of potassium phosphate buffer (0.05 M, pH 3.2): acetonitrile (86:14, v/v), and UV detection set at 303 nm. The analyses of MTX content by the UV method were also accomplished at 303 nm, using 0.1 M sodium hydroxide as diluent. The measurements were linearly correlated with concentration for both methods in the 1 - 30 μg/mL range ($r > 0.9999$). The specificity tests showed that there was no interference of the NP components on the quantitative analyses. Precision (repeatability and intermediate precision) was demonstrated by a relative standard deviation lower than 1.5%, whereas the accuracy was assessed by the recovery of MTX from sample matrices, given mean value of ~99%. The proposed methods were applied for the analyses of MTX in different batches of NPs, and the results showed non-significant differences ($p > 0.05$) between the values obtained with both methodologies. Moreover, the RP-LC method was successfully used to determine the drug entrapment efficiency, and to quantify MTX during *in vitro* release assays and photolytic degradation studies. In conclusion, the validated methods are suitable to assay MTX in pH-sensitive CS-NPs without any interference from the polymer or surfactant.

Keywords: Chitosan nanoparticles, methotrexate, lysine-based surfactant, reversed-phase liquid chromatography, UV spectrophotometry, validation.

1. INTRODUCTION

Methotrexate (MTX, 2,4-diamino-N10-methyl-folic acid) is a dihydrofolate reductase (DHFR) inhibitor that is well established for the treatment of solid tumors and leukemias [1]. It is an antimetabolite drug that acts as an antagonist of folic acid, which is necessary for DNA synthesis [2]. Despite the current use of MTX to treat many types of cancer, its administration has the potential to cause severe side effects, including neurologic toxicity, renal failure and mucositis [3]. Therefore, it is of great importance the development of new approaches with higher specificity and effectiveness to deliver the chemotherapeutic agent to the tumor. Nanotechnology-based systems, with improved physicochemical properties, might appear as a promising tool to overcome the shortcomings

associated with conventional drug delivery strategies [4,5]. More specifically, polymeric nanoparticles (NPs) with pH-sensitive properties might achieve greater antitumor activity due to their specificity for the acidic extracellular space of tumors [6].

Chitosan (CS) is derived from natural sources and due to its biocompatibility, biodegradability and non-toxicity has been considered a harmless polymer to be used in the development of nanocarriers [7]. CS NPs have been investigated as a promising colloidal drug carrier for targeted delivery in cancer therapy [8-10]. CALVO et al. [11] firstly described the procedure for preparation of CS-based NPs by the ionotropic gelation method, where the natural polymer CS was dissolved in acetic acid (1% solution in water) and then a solution of the polyanion tripolyphosphate (TPP) was added dropwise under high stirring speed.

Several LC (Liquid chromatography) methods have been developed to determine MTX in different matrices

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and with various purposes, ranging from quality control to clinical pharmacokinetics and therapeutic drug monitoring [1,12,13]. Among these methods, a variety of chromatographic conditions have been described, including acidic or neutral mobile phase and UV, fluorimetry or mass spectrometry detection [1,14,15]. Likewise, standard analytical methodologies based on LC with UV detection are described in the main Pharmacopoeias for MTX as raw material [16-18] or injectable preparation [17,18]. However, these methods are suitable for pharmaceutical products without complex matrices, and not for formulations based on polymers and surfactants, as are the NPs. In the field of nanotechnology-based products, there is only one LC method reported in the literature, which comprises the determination of MTX entrapment efficiency in poly (DL-lactic acid) nanocapsules [19].

Therefore, the aim of the present work was to develop and validate a specific and sensitive RP-LC method, and also a simple and cost-effective UV spectrophotometric method, for the quantitative analysis of MTX in pH-sensitive CS-based NPs. Both methods were successfully applied to assess MTX content in different batches of NPs, while the LC procedure was also used to determine the drug entrapment efficiency, the MTX *in vitro* release profile and the photolytic degradation kinetics of the encapsulated drug.

2. EXPERIMENTAL

2.1. Chemical and Reagents

Methotrexate (MTX, state purity 100.1 %; batch no 1504478) was purchased from SM Empreendimentos Farmacêuticos Ltda. (São Paulo, SP, Brazil). Chitosan (CS) of low molecular weight (deacetylation degree, 75-85%; viscosity, 20-300 cP according to the manufacturer's data sheet), pentasodium tripolyphosphate (TPP) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Tedia (Fairfield, USA) and potassium phosphate from Merck (Darmstadt, Germany). All chemicals used were of pharmaceutical or special analytical grade. For all of the analyses, ultrapure water was purified using a Mega RO/UP - Mega Purity system.

2.2. Apparatus and Analytical Conditions

The RP-LC method was performed on a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with an

SCL-10A_{VP} system controller, a LC-10 AD_{VP} pump, a manual sample valve injector with a 20 μ L loop and an SPD-10A_{VP} ultraviolet (UV) detector. A Shimadzu Class VP V 6.14 software program was used for data acquisition and analysis of chromatograms. In addition, the evaluation of peak purity during the specificity test was performed on a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A photodiode array (PDA) detector. The analyses of MTX were carried out on a reversed-phase Waters (Milford, MA, USA) XBridge™ C18 column (250 mm x 4.6 mm I.D., with a particle size of 5 μ m and pore size of 110 Å). The LC system was operated isocratically, at room temperature, using a mobile phase consisted of potassium phosphate buffer (0.05 M, pH 3.2) and acetonitrile (86:14, v/v), which was filtered through a 0.45 μ m membrane filter (Millipore) and run at a flow rate of 1.0 mL/min. The UV detection was set at 303 nm and the injection volume was 20 μ L. Before injections, the column was equilibrated for at least 20 min with mobile phase flowing through the system, and each sample was filtered through a 0.45- μ m membrane.

The UV spectrophotometric experiments were performed on a double-beam UV-VIS spectrophotometer (Shimadzu, Japan), model UV-1800, with a fixed slit width (2 nm) and a 10 mm quartz cell was used to obtain spectrum and absorbance measurements. The wavelength was set at 303 nm and the diluent chose was 0.1 M sodium hydroxide (NaOH).

2.3. Preparation of Reference Substance Solution

The reference stock solution was prepared by dissolving MTX (20 mg) into a 20 mL volumetric flask with DMSO to give a final concentration of 1 mg/mL. The stock solution was stored at 2-8 °C, protected from light, and further diluted with mobile phase (for the LC analysis) or with 0.1 M NaOH (for the UV spectrophotometric measurements) to yield working solutions containing 1, 5, 10, 15, 20 and 30 μ g/mL. Finally, samples for LC analysis were filtered through a 0.45 μ m membrane filter (Millipore) before injection into the chromatographic system.

2.4. Preparation of Unloaded and MTX-Loaded Chitosan Nanoparticles

Chitosan-based NPs were prepared following the ionic gelation technique [11], which is based on the ionotropic complexation of CS with TPP anions. To the standard procedure, some modifications were included,

such as the incorporation of a biocompatible and pH-sensitive amino acid-based surfactant as a NP modifier [20]. The surfactant used is derived from N^α, N^ε-dioctanoyl lysine, has an inorganic sodium counterion (77KS) and was synthesized as previously described [21]. 77KS was included in the complexation process and the NPs were prepared with a selective CS:TPP:77KS ratio of 5:1:0.5 (w/w/w).

MTX-loaded CS NPs (MTX-CS-NPs) were prepared as follows: firstly, a MTX solution (at 0.1% w/v) was added to a premixed TPP and 77KS solution (both at 0.1%, w/v, with a TPP:77KS ratio equal to 1:0.5, w/w). MTX-NPs were then prepared by dropwise addition of this premixed solution (TPP:77KS:MTX, with a final ratio of 1:0.5:1, w/w/w) to CS (0.1%, w/v) previously dissolved in acetic acid solution (1%, v/v). The pH of the CS solution was adjusted to 5.5 with 1 M NaOH [22]. The NPs were produced at room temperature and dark conditions, under constant magnetic stirring (1000 rpm) for 20 min. Unloaded CS-NPs were prepared according to the procedure previously described, omitting the drug.

2.5. Validation of the RP-LC and UV Spectrophotometric Methods

The methods were validated by determinations of the following parameters: specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness, following the International Conference on Harmonisation (ICH) guideline [23].

Specificity was evaluated by analyzing unloaded CS-NPs, containing all the components of the formulation, except the drug. The samples were diluted with methanol and sonicated for 15 min in order to dissolve the NPs and extract any interference from them. The resulting solution was further diluted in mobile phase and injected into the LC system to investigate the ingredient interference on the selectivity of the MTX separation. Moreover, it was evaluated a sample of MTX-loaded NPs at a drug concentration of 15 µg/mL, following the same procedure described above. Then, the purity of MTX peak was determined using a PDA detector. For the UV method, samples with and without drug were diluted with 0.1 M NaOH after the extraction procedure, and absorption spectra were compared in order to verify the occurrence of any interference in wavelength of MTX maximum absorption.

The linearity was determined by constructing three independent analytical curves, each one with six concentrations of MTX, including the LOQ, in the range of 1 - 30 µg/mL, prepared in mobile phase or 0.1M NaOH for the LC or UV spectrophotometric method, respectively. Three replicate analyses of each sample were made to verify the repeatability of the responses. The peak areas of the chromatograms or the absorptions values were plotted against the respective concentrations of MTX to obtain the analytical curve. The results were subjected to regression analysis by a least-squares method to calculate calibration equation and correlation coefficient. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated, as defined by ICH [23] using the mean values of three independent analytical curves, where the factors 3.3 and 10 for the detection and quantification limits, respectively, were multiplied by the ratio from the standard deviation of the intercept and the slope.

The precision of the methods was determined by the repeatability and the intermediate precision. Repeatability was examined by analyzing six MTX-CS-NPs samples at the same concentration (15 µg/mL), on the same day, and under the same experimental conditions. Before analyses, samples were diluted and extracted as described for the specificity test. The intermediate precision was assessed by carrying out the analysis on three different days (inter-days), and also by other analysts performing the analysis in the same laboratory (between-analysts). Precision was expressed as relative standard deviation (RSD) and the results must be less than 2%.

Accuracy was evaluated assaying, in triplicate, samples of known concentrations of MTX-CS-NPs (10 µg/mL) spiked with three different concentrations of standard solution (2, 5 and 8 µg/mL) at three different levels (lower, medium, and upper concentration), giving sample solutions with concentrations of 12, 15 and 18 µg/mL, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively. The accuracy was calculated as the percentage of drug recovered from the sample (recovery %), which was calculated from differences between the responses obtained for spiked and unspiked solutions.

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis. Therefore, robustness was determined by analyzing MTX-CS-NPs samples (15 µg/mL) under a variety of

conditions with small variations of the method parameters. For LC method, the variations were performed in the flow rate, wavelength, buffer pH and proportion of acetonitrile, while for UV spectrophotometric method, changes were made in the wavelength and in the sonication time necessary to extract the drug from NPs.

The system suitability test was also carried out for the RP-LC method to evaluate the reproducibility of the system for the analysis to be performed, using six replicates injections of both reference and sample solutions containing 15 µg/mL of MTX. The parameters measured were peak area, retention time, theoretical plates and tailing factor (peak symmetry).

2.6. Analysis of MTX Content into the Formulations

For the quantitative analysis, the drug was extracted from the NPs with methanol, followed by sonication for 15 min. Then, the samples were diluted with mobile phase or 0.1 M NaOH for the LC or UV spectrophotometric measurements, respectively, to reach up a concentration of 15 µg/mL. Finally, the amount of drug was calculated against the reference substance. The results obtained were statistically compared by Student's *t* test and ANOVA in order to verify the similarity of the experimental values and, thus, to determine the equivalence of both methods for the assessment of MTX in CS-based NPs.

2.7. Drug Entrapment Efficiency

The entrapment efficiency (EE%) was estimated as being the difference between the total concentration of MTX found in the NP suspension after the complete dissolution in methanol and the concentration of drug in the ultrafiltrate after separation of the NPs by ultrafiltration/centrifugation technique using Amicon Ultra-0.5 Centrifugal Filters (10,000 Da MWCO, Millipore). EE% was calculated by the difference between the total and free MTX concentrations determined in the NPs (drug content) and in the ultrafiltrate, respectively, using the LC method.

2.8. *In Vitro* MTX Release Assay

In vitro release evaluations from pH-sensitive MTX-loaded CS-NPs were carried out for 8 h in phosphate buffer saline (PBS) at pH 7.4 and 5.4. An aliquot of NPs was placed in a dialysis bag (Sigma-Aldrich, 14000 MWCO) and suspended in 50 mL of PBS at 37°C under gentle magnetic stirring (100 rpm). At pre-determined intervals, 2 mL of medium was withdrawn and replaced with an equal volume of fresh medium.

The amount of MTX released was estimated by the RP-LC method. In order to assess the influence of the medium used, the method was co-validated for linearity and range using the release medium (PBS pH 7.4 or 5.4) as diluent.

2.9. UV Degradation Study

The system for irradiation of the samples consisted of a shortwave UV mercury lamp as UVC radiation source ($\lambda = 254$ nm), installed inside a photo stability chamber. Photodegradation was induced by exposing the samples (MTX-CS-NPs; CS:TPP:77KS, 5:1:0.5 w/w/w), in a covered transparent container, to the UV radiation for 6 h. Every hour, samples were taken from the chamber; the drug was extracted from NPs as previously described, and then analyzed by LC to determine the content of MTX.

The photolytic degradation kinetics of MTX-loaded NPs was calculated by the graphic method, through which it was possible to determine the degradation constant. Zero order, first order and second order graphics were drawn by plotting the drug residual content versus time, \ln of drug residual content versus time and $1/\text{drug residual content}$ versus time, respectively. The definition of kinetic order was performed by calculation of the correlation coefficient of each curve. The graph with the best fit was then considered to establish the kinetic order. Finally, half-life ($t_{1/2}$) and t_{90} value (time for 10% decomposition) were determined from the *k*-value [24].

2.10. Statistical Analyses

Statistical analyses were performed using Student's *t* test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Tukey's post-hoc test for multiple comparisons using SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Optimization of the Methods

Chromatographic and UV spectrophotometric methods have been widely used for many analytical purposes and, motivated by the growing development of nanotechnology, these analytical tools started to be applied for drug assay in nanomaterial-based products [25-27]. This innovative type of pharmaceuticals usually contains a complex matrix, including polymers and surfactants. In this way, the current analytical

methodology for the conventional pharmaceutical formulations may not be suitable for the analysis intended and, thus, specific analytical methods must be carefully developed and validated to demonstrate its suitability. For that reason, in this work, two analytical methods were proposed as important and appropriate tools to determine the antitumor drug MTX in pH-sensitive polymeric NPs.

The experimental conditions were adjusted in order to obtain efficient routine analysis. To achieve the best performance of the RP-LC method, the mobile phase was optimized to provide sufficient selectivity and sensitivity in a short separation time. Phosphate buffer resulted in high sensitivity and better separation in comparison with acidic aqueous solutions (ultrapure water acidified with phosphoric acid or glacial acetic acid). Even though acidic pH used to diminish the absorbance values of MTX [28], the pH selected was 3.2 because MTX (pK_a 3.8, 4.8 and 5.6) can be found mostly in its undissociated form under this condition [29]. The LC technique is very sensitive and, thus, a reduced absorbance is not critical for the good performance of the methodology. Moreover, the literature reports the quantification of MTX, in both pharmaceutical products and biological matrices, using mixtures of aqueous buffer at pH values ranging from 2.5 to 6.7 [28]. The acetonitrile was chosen as organic phase since it resulted in short analysis time, with improved peak symmetry (about 1.17). Different proportions of mobile phase were tested, and the one with 86:14, v/v (buffer-acetonitrile) was selected due to the fast elution of the analyte, achieved with acceptable chromatographic parameters. The main advantage of this method was that the total run time was set as only 8 min, allowing a fast determination of the drug, with low consumption of organic solvents.

Considering that simple methods are more affordable for the routine analysis of pharmaceuticals, a UV spectrophotometric method was also developed and further optimized for the detection wavelength and diluent. To choose the wavelength that allows the maximum absorption of MTX, scans were conducted in a range of 200–600 nm, and the set wavelength was the same of the LC method (303 nm). Likewise, the diluent selected (0.1 M NaOH) was simple, provide great sensitivity and did not show any interference or absorption within the drug. MTX strongly absorbs at UV-Vis range, due to the presence of the chromophore heteroaromatic pterine, and it was reported that the maximum absorbance is dependent on the pH. The highest molar extinction of MTX is obtained at neutral

pH, while acidic conditions use to reduce the absorbances [28]. This behavior justifies the selection of a NaOH solution as diluent, which gave good sensitivity to the analytical procedure. Finally, the possible precipitation of the sample after its redispersion in mobile phase or 0.1 M NaOH solution was also evaluated before analysis. No precipitation of any component of the formulation was observed, probably because of their low concentration in the final sample.

3.2. Validation of the Methods

Regarding the specificity evaluation, the chromatograms (Figure 1) and absorption spectra (Figure 2) showed that both LC and UV spectrophotometric methods, respectively, are specific, without any interference or overlaps of the components of NPs with the MTX response at the detection wavelength of 303 nm. In order to confirm this absence of interference in the LC method, a peak-purity evaluation using the PDA detector was carried out. These analyses showed that the MTX peak was free from any co-eluting peak (impurities and/or excipients), with values of peak purity index higher than 0.9999.

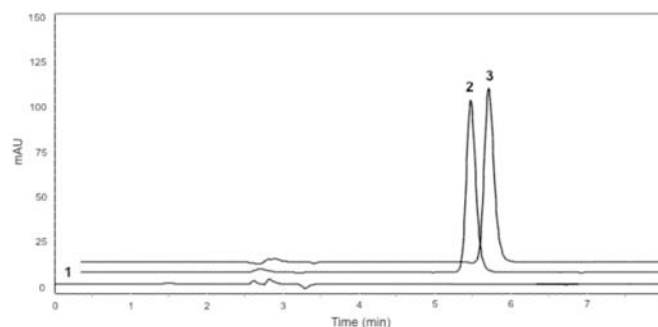


Figure 1: Representative RP-LC chromatograms obtained during the evaluation of the method specificity. Trace (1) represents the unloaded-NPs, (2) the reference solution of MTX (15 $\mu\text{g/mL}$) and (3) the MTX-CS-NPs (15 $\mu\text{g/mL}$).

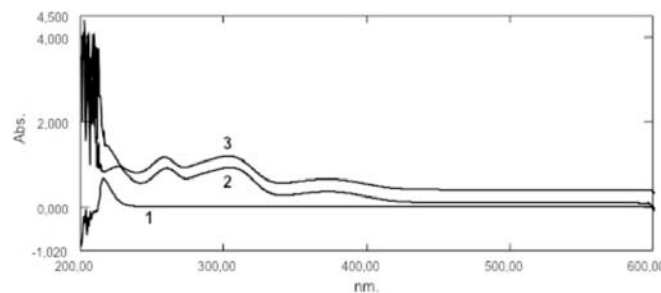


Figure 2: Absorption spectra obtained by UV spectrophotometric method during the evaluation of its specificity. Trace (1) represents the unloaded-NPs, (2) the MTX-CS-NPs (15 $\mu\text{g/mL}$) and (3) the reference solution of MTX (15 $\mu\text{g/mL}$).

Table 1: Repeatability[†] and Intermediate Precision[§] Data of RP-LC and UV Spectrophotometric Methods for MTX in Samples of Chitosan-Based NPs

	RP-LC		UV spectrophotometry	
	Recovery \pm SD (%)	RSD* (%)	Recovery \pm SD (%)	RSD* (%)
Intra-day (n = 6)	101.23 \pm 0.8402	0.83	103.61 \pm 1.68	1.62
Inter-day				
Day 1 (n = 3)	100.90 \pm 0.0048	0.48	100.34 \pm 0.0077	0.76
Day 2 (n = 3)	99.48 \pm 0.0148	1.49	102.19 \pm 0.01	0.98
Day 3 (n = 3)	102.43 \pm 0.0043	0.42	103.19 \pm 0.0163	1.58
Mean (n = 9)	100.94 \pm 0.0148	1.46	101.91 \pm 0.0145	1.42
Between-analysts				
Analyst 1 (n = 3)	100.67 \pm 0.0061	0.60	100.34 \pm 0.0077	0.76
Analyst 1 (n = 3)	102.14 \pm 0.0070	0.69	103.19 \pm 0.0163	1.58
Analyst 1 (n = 3)	101.35 \pm 0.045	0.45	104.14 \pm 0.0192	1.84
Mean (n = 9)	101.39 \pm 0.0074	0.73	102.56 \pm 0.0198	1.93

[†] intra-day[§] inter-day and between-analysts

* RSD = relative standard deviation

The analytical curves constructed for MTX were found to be linear for both methods in the 1 - 30 $\mu\text{g/mL}$ range. The linear regression equations obtained by the least-square method and the values of the correlation coefficient ($y = 60463.13x + 8207.2$, $r = 0.9999$, for RP-LC; and $y = 0.044205x + 0.001928$, $r = 0.9999$, for UV spectrophotometry; where x is concentration and y is the peak absolute area or absorbance) indicated the linearity of the analytical curves for both methods. Moreover, the validity of the assays was verified by analysis of variance (ANOVA). This revealed that the regression equations were linear ($F_{\text{calculated}} = 39,578.96 > F_{\text{critical}} = 4.75$, $p < 0.05$, for LC method; and $F_{\text{calculated}} = 17,325.12 > F_{\text{critical}} = 4.75$, $p < 0.05$, for UV spectrophotometric method) with no linearity deviation ($F_{\text{calculated}} = 0.31 < F_{\text{critical}} = 3.26$; $p > 0.05$, for LC method; and $F_{\text{calculated}} = 0.37 < F_{\text{critical}} = 3.26$; $p > 0.05$, for UV spectrophotometric method). The LOD and LOQ were calculated by using the mean of the slope and the standard deviation of intercept of three independent curves. The obtained values for LOD and LOQ were 0.37 and 1.25 $\mu\text{g/mL}$, respectively, for LC method, and 0.53 and 1.75 $\mu\text{g/mL}$, respectively, for UV spectrophotometric method. The LOQ evaluated in an experimental assay, with a precision lower than 5% and accuracy within $\pm 5\%$, was found to be 1 $\mu\text{g/mL}$ for both methods.

The precision, evaluated as repeatability of the methods, was studied by calculating the relative standard deviation (RSD) for six determinations of

MTX-CS-NPs samples, performed on the same day and under the same experimental conditions. The intermediate precision was assessed by analyzing two samples of MTX-loaded NPs on three different days (inter-day) and by three different analysts (between-analyst). The results are given in Table 1 and the obtained RSD values are lower than the acceptance criterion of 2%. Regarding the accuracy evaluation, good recoveries (98–102%) were obtained (Table 2), demonstrating that both methods are accurate within the desired range.

Table 2: Accuracy Data of RP-LC and UV Spectrophotometric Methods for MTX in Samples of Chitosan-Based NPs

Level ($\mu\text{g/mL}$)	RP-LC		UV spectrophotometry	
	Recovery (%)	RSD* (%)	Recovery (%)	RSD* (%)
12	99.36	0.47	100.59	0.55
15	99.35	0.12	98.83	0.80
18	99.47	0.01	98.02	0.60

* RSD = relative standard deviation.

The robustness was evaluated by making alterations on the experimental range of some of the most important parameters of the methods. It was studied the effects of performing variations on the wavelength (± 2 nm) for both methods, on the sonication time (± 3 min) for UV spectrophotometric method, and on the buffer solution pH (± 0.2), % of acetonitrile ($\pm 2\%$) and flow rate (± 0.1 mL/min), for LC

method. The analyses of reference and sample solutions containing 15 µg/mL of MTX were carried out in triplicate changing only one parameter at a time. The results of experimental range of variables evaluated were within the acceptable deviation ($RSD < 2\%$), which demonstrated that there were no significant changes in the chromatographic pattern and/or on assay data when small modifications were made in the experimental environment, thus showing the methods to be robust under the conditions tested.

Finally, a system suitability test was carried out to evaluate the adequacy and reproducibility of the LC system for the analysis to be performed. The RSD values calculated for the retention time, tailing factor and peak area were lower than 0.56, 0.62 and 1.41%, respectively. The number of theoretical plates was about 9405.65, with $RSD < 1.76\%$. The experimental results show that the parameters tested were within the acceptable range ($RSD < 2.0\%$), indicating that the system is suitable for the analysis intended.

3.3. Application of the Validated Methods

In order to demonstrate the applicability of both methods, pH-sensitive MTX-loaded NPs were assayed (six batches) using the conditions optimized and validated for each methodology (Table 3). The determination of drug content in sample solutions showed acceptable results (mean recovery of 105.29% and 105.62% for RP-LC and UV spectrophotometric methods, respectively). RSD values were lower than 2.0% from triplicate analysis of each sample, which indicates precise analytical methods. The experimental values of the two methods were compared statistically by the Student's *t* test and by ANOVA, showing non-significant differences (Calculated value < Critical value; $p > 0.05$).

It is of great importance the determination of the amount of drug that is really entrapped into the nanocarrier. Therefore, it is necessary to have a precise and accurate methodology to perform this assessment. In this line, the proposed RP-LC method was also applied to determine the EE% of MTX in NPs, and the mean value obtained was 38.37%.

In addition, the sensitivity of the LC method allowed the performance of *in vitro* release studies of MTX from the NPs as a function of pH. First of all, analytical curves were constructed using PBS pH 7.4 and 5.4 as diluents. It was observed that changing the sample diluent from mobile phase to PBS did not modify the

chromatographic performance and/or altered the quantification power of the validated LC method. Linear

Table 3: Comparison between the RP-LC and UV Spectrophotometric Methods Applied for the Analysis of MTX in Samples of Chitosan-Based NPs

Sample	RP-LC		UV spectrophotometry	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1	105.11	0.52	104.58	0.17
2	106.81	0.12	106.39	0.51
3	103.34	0.21	105.16	0.09
4	105.24	0.13	104.13	0.09
5	105.21	0.02	106.45	0.08
6	106.02	0.24	106.97	0.26
Mean	105.29	1.10	105.62	1.09
Statistical analysis	Critical value [§]		Calculated value	
Student's <i>t</i> test	2.23		0.63	
ANOVA	4.96		0.24	

* RSD = relative standard deviation.

[§]significance level $p = 0.05$

regression equations were obtained by the least-square method and the values of the correlation coefficient ($y = 59621.32x + 3766.19$ $r = 0.9998$, for PBS pH 7.4; and $y = 62270.37x + 1833.55$, $r = 0.9999$, for PBS pH 5.4; where x is concentration and y is the peak absolute area) indicated the linearity of both curves, independently of the pH. The cumulative amounts of MTX released from MTX-CS-NPs are shown in Figure 3. Both at physiological and acidic conditions, the drug showed an initial burst release, following by a sustained release up to 8 h. The amount of drug released at pH 5.4 was higher than at pH 7.4 in each schedule time. This behavior showed the pH-dependent properties of the formulation.

Finally, the LC method was successfully applied to assess the degradation profile of MTX entrapped in the NPs after exposure to UVC radiation. The chromatogram obtained after 6 h exposure of NPs to the radiation is presented in Figure 4. It was shown that ~90% of the drug degraded under the test conditions, and four detectable eluting degradation products were observed. The MTX peak purity index was determined, providing acceptable value. Altogether, these results indicated that the LC method might have a stability-indicating capability, which evidently has to be confirmed by additional forced degradation studies. On

the other hand, the graphics of zero order, first order and second order models were constructed for modeling the kinetics of MTX degradation in NPs after UVC exposure. The graphic with the best correlation coefficient was considered to establish the kinetic order and, thus, it was stated that the degradation profile of MTX in the nanostructure was according to zero order kinetics ($r = 0.9786$). The degradation constant was determined by the graphic equation and the value obtained was $0.0416 \mu\text{g mL}^{-1} \text{min}^{-1}$. The half-life ($t_{1/2}$) was determined from the k-value and the result obtained was very close to the experimental values. The calculated $t_{1/2}$ was 180.3 min, whereas ~60% of degradation occurs in 240 min. Moreover, the t_{90} value was estimated as 36.06 min under the tested conditions.

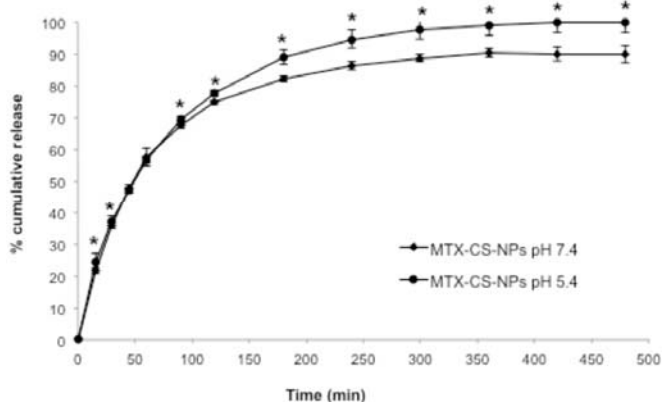


Figure 3: *In vitro* cumulative release profiles of MTX from CS-NPs in PBS pH 7.4 and 5.4. Results are expressed as mean \pm standard error of three independent experiments. Statistical analyses were performed by ANOVA followed by Tukey's multiple comparison test. *significantly different from PBS pH 7.4 ($p < 0.05$).

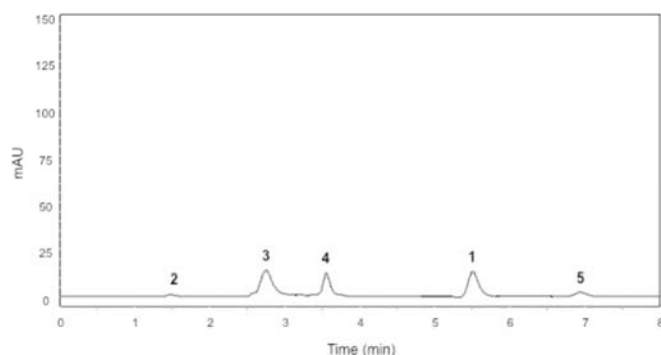


Figure 4: Representative RP-LC chromatogram of MTX-CS-NPs after 6 h exposure to UVC radiation. Peak (1) corresponds to MTX, and peaks (2), (3), (4) and (5) correspond to photodegradation products of MTX.

4. CONCLUSIONS

The results of the validation studies show that the RP-LC and UV spectrophotometric methods are specific, linear, precise and accurate. Both methodologies were successfully applied for assay MTX in pH-sensitive CS-based NPs, without any interference of their complex matrix composed of polymers and surfactants. Moreover, considering the efficiency and high-resolution of the chromatographic technique, the proposed RP-LC method was used to assess MTX entrapment efficiency, as well as to determine the *in vitro* MTX release profile and the photolytic degradation kinetics of the encapsulated drug. Altogether, the results proved that the validated methods could be suitable approaches to quantify MTX in complex matrices of nanotechnology-based polymeric formulations. Finally, it is worth mentioning that the availability of reliable analytical methodologies can contribute to improve the quality and to ensure the therapeutic efficacy of the new formulations that have been developed.

ACKNOWLEDGMENTS

This study was supported by Projects MAT2012-38047-C02-01 of the *Ministerio de Economía y Competitividad* (Spain), FEDER and 483264/2012-1 of the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq - Brazil). Daniele R. Nogueira holds a Postdoctoral fellowship from PNPd-CAPES (Brazil).

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Received on 05-11-2014

Accepted on 15-11-2014

Published on 28-01-2015

DOI: <http://dx.doi.org/10.14205/2309-4435.2014.02.02.3>© 2014 Nogueira *et al.*; Licensee Pharma Publisher.

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