Development and Validation of a Fast RP-HPLC Method for Determination of Methotrexate Entrapment Efficiency in Polymeric Nanocapsules

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Abstract

A rapid and effective isocratic chromatographic procedure is successfully developed to determinate methotrexate (MTX) entrapment efficiency (EE) in polymeric nanocapsules using reversed-phase high-performance liquid chromatography. The method employed a RP-C_{18} Shimadzu Shim-pack CLC-ODS (150 mm × 4.6 mm, 5 µm) column with mobile phase constituted by a mixture of water–acetonitrile–tetrahydrofuran (65:30:5 v/v/v; pH 3.0) at a flow rate of 0.8 mL/min. The eluate is monitored with a UV detector set at 313 nm. The parameters used in the validation process are: linearity, specificity, precision, accuracy, and limit of quantitation (LOQ). The linearity is evaluated by a calibration curve in the concentration range of 10–50 µg/mL and presented a correlation coefficient of 0.9998. The polymers (PLA or PLA-PEG), oil, and surfactants used in the nanocapsule formulation did not interfere with analysis and the recovery was quantitative. The intra and inter-day assay relative standard deviation were less than 0.72%. Results are satisfactory, and the method proved to be adequate for the determination of methotrexate in nanocpsules formulations.

Introduction

Methotrexate (MTX), 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 dihydrofolate redutase (DHFR), an essential enzyme in the biosynthesis of thymidylate, required for DNA replication (1). It is routinely used in the treatment of acute lymphoblastic leukemia, psoriasis, choriocarcinoma, and related trophoblastic tumours (2).

Conventional chemotherapy using MTX shows low specificity and selectivity of action. It is administered in relatively high doses, which often leads to drug resistance and causes nonspecific toxicities in normal proliferating cells, limiting the permissible dose of the drug (3–7).

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In the last few years, new strategies have been developed in order to decrease the toxicity of active molecules by driving them to the target site. In this context, the use of polymeric nanoparticles can be a useful approach to concentrate the loaded drug on the focus of the disease and reducing both the given dose and the side of effects (8).

Biocompatible and biodegradable polymers such as Poly (DL-lactic acid) (PLA) and poly(DL-lactic acid-co-glycolic acid) copolymer (PLGA) are clinically available as medical devices and drug carriers because of their biodegradability and biocompatibility (9,10). Much attention has been paid to the Poly(DL-lactic acid)-poly(ethylene glycol) block copolymer (PLA-PEG), which is a diblock copolymer with hydrophilic and hydrophobic blocks, because it allows the formation of a stable nanoparticulate suspension in an aqueous solvent, where PLA chains form the core and PEG chains are located outside (11,12). The PEG shell prevents the interaction of PLA core with biomolecules, cells and tissues (13,14).

The nanocapsules are systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, which is proposed to protect the drug from degradation, targeting site of action, and reduction of toxicity or side effects. Therefore, the determination of the drug entrapment efficiency (EE) is an essential parameter to guarantee a significant amount of nanocapsules loaded with drug. In this way, an efficient quantitative measurement of drug EE within nanoparticles needs to be established, considering its major importance to quality control of the colloidal systems (15,16). A number of techniques, including gel-filtration, dialysis, and ultracentrifugation are available for the study of drug EE (17).

MTX can be detected under a variety of chromatographic conditions (i.e., on different stationary phases) under a choice of mobile phase conditions (acidic or neutral, employing ion-pair or micellar chromatography), followed by several detection techniques (UV–vis spectrophotometry, pre- or post-columm oxidation and fluorimetry, electrochemistry, mass spectrometry).

Rubino (21) reported a review about the separation methods for methotrexate, describing the instrumental conditions and the performance of several published chromatographic methods employed to measure MTX, its metabolites, and some analogues

in clinical and biological research. Abd El-Hady et al. (19) presented a liquid chromatographic method with UV detection for the chiral separation of racemic methotrexate in pharmaceutical formulations. Gao and Jiang (20) developed an rapid-phase high-performance liquid chromatographic method with UV detection to quantify metotrexate in brain tissues. And Li and co-workers, (18) reported an HPLC method with online post-column electrochemical oxidation and fluorescence detection for the determination of plasma MTX level and whole-blood total MTX (MTX + MTXPG) level (18–21). Several reports have described methods for quantitating MTX, however, most of them have been focused on their separation in biological samples, a few assays employed for the quality control of MTX (21), and no one for the determination of MTX in nanoparticles. In fact, there is no official assay described in the pharmacopoeias for quantification of MTX in nanocapsules.

Since our research involves the development of PLA and PLA-PEG nanocapsules containing methotrexate, the objective of this paper was to develop and validate a rapid and effective chromatographic procedure for the determination of methotrexate EE in polymeric nanocapsules using RP-HPLC with UV detector.

Experimental

Materials and chemicals

Poly(DL-lactic acid) was supplied from Sigma-Aldrich (St. Louis, MO). Poly(DL-lactic acid)-poly(ethylene glycol) diblock copolymer (PLA-PEG 49KD, 20% PEG 5KD) was obtained from Alkermes (Cambridge, MA). Methotrexate sodium salt (state purity 100.1 %; batch nº 04076101) was provided by Biosintética (Biometrox, São Paulo, Brazil). Miglyol 812N and sorbitan monoolate (Span 80) were purchased from Sasol (Westlake, LA) and Bereca (Rio de Janeiro, Brazil), respectively. Polysorbate 80 (Tween 80) was obtained from Delaware (Porto Alegre, Brazil). Ultrapure water was provided by a Milli-Q purification system (Millipore, Milford, MA). Acetonitrile and tetrahydrofuran HPLC grade were purchased from Vetec (Rio de Janeiro, Brazil). Other solvents and reagents used were analytical grade.

Table I. The Linearity and Limit of Quantitation

Range of linearity (µg/mL)	Peak area (mean and RSD)*
10	959133.33 + 0.333
20	1874552.22 + 1.122
30	2758222.78 + 0.646
40	3682658.67 + 0.139
50	4642992.67 + 0.508
Equation	$y = 91758x + 30764$
r^2	0.9998
LOQ	2.61 µg/mL

* Mean + R.S.D ($n = 3$.)

Apparatus and chromatographic conditions

The HPLC analysis was performed on a Shimadzu LC-10A system (Kyoto, Japan) equipped with a LC-10AD pump, SPD-10AV variable-wavelength detector (set at 313 nm), SCL-10Avp controller unit and the sample injection performed via a Rheodyne 7125 valve with a 20 µL loop. A RP C_{18} Shimadzu Shim-pack CLC-ODS (150 mm × 4.6 mm, 5 µm) was employed with a mobile phase constituted by water, acetonitrile and tetrahydrofuran (65:30:5 v/v/v), adjusted to pH 3.0 with perchloric acid, in an isocratic system, at a flow rate of 0.8 mL/min. The column was operated at 40 ± 1°C. The injection volume was 20 µL for all standards and samples. Data acquisition was performed using CLASS-VP software by measurement of detected peak areas.

Preparation of nanocapsules

Nanocapsules were prepared according to nanoprecipitation method Fessi et al., 1989 (22). Briefly, 40 mg of polymer (PLA or PLA-PEG) and 40 mg Span 80 were dissolved in 15 mL of acetone containing 125 µL of Mygliol 812N and 100 µL MTX (2.5 mg). This organic solution was added dropwise into aqueous phase (30 mL). The aqueous phase was constituited by deionized water adjusted at pH 5.0 with perchloric acid, containing 45 mg of Tween 80. After stirring magnetically at room temperature for 10 min, the organic solvent was removed using a rotary evaporator (Quimis Q-344B2) and the final volume was adjusted to 10 mL.

Blank nanocapsules were prepared according to the procedure previously described, omitting obviously the drug. All samples were prepared in triplicate.

Standard and standard solutions

The stock standard solution of MTX was prepared in mobile phase at the concentration of 500 µg/mL. The standard solutions were prepared by diluting the stock solution to: 10, 20, 30, 40, and 50 µg/mL in mobile phase.

Method validation

The objective of validation of an analytical procedure is to demonstrate that it is adequate for its intended purpose. To meet current pharmaceutical regulatory guidelines ICH, USP, EP (23–25) a number of parameters must be investigated in order to validate analytical methods such as linearity, specificity, precision, accuracy, and limit of quantitation (LOQ).

Linearity

The linearity response was assessed in the range of 10–50 µg/mL. Appropriate amounts of the stock solution were diluted with mobile phase, yielding concentrations of 10, 20, 30, 40, and 50 µg/mL. Triplicate injections of each were carried out. The linear regression was used for data evaluation. Peak area ratios of standard compounds were plotted against theoretical concentrations of standards. Linearity was expressed as a correlation coefficient; the value must be > 0.9990.

Precision

The repeatability (intra-day) and intermediate (inter-day) precision of the method were demonstrated by analyzing six nanocapsules samples of PLA or PLA-PEG with the same con-

centration of MTX (30 µg/mL) during the same day and 3 different days under the same conditions. Precision was expressed as relative standard deviation (RSD); the results must be less than 2%.

Specificity

Blank PLA and PLA-PEG nanocapsules were prepared to evaluate method specificity. They were treated in the same manner as the samples and injected into the system to investigate the ingredient interference on the selectivity of the methotrexate separation. Triplicate injections of each were made.

Accuracy

The accuracy was evaluated by a recovery test. Blank nanocapsules of PLA or PLA-PEG were spiked with known amounts of MTX, at three different levels (lower, medium, and upper concentration) corresponding to 12.5, 25, and 37.5 µg/mL. The recovery of the added standard was determined in triplicate analysis and calculated by the formula:

$$R\% = \left(\frac{Snc - Bnc}{Ss}\right) \times 100$$

in which *R* is the recovery, *Snc* is the spiked nanocapsule, *Bnc* is the blank nanocapsule, and *Ss* is the standard solution.

Limit of quantitation

The limit of quantitation was based on the standard deviation of the response and the slope of the constructed calibration curve. The LOQ may be expressed as:

$$LOQ = \left(\frac{10\sigma}{S}\right)$$

where σ is the standard deviation of the response and *S* is the slope of calibration curve.

Drug entrapment efficiency

The entrapment efficiency (%) was estimated as being the difference between the total concentration of methotrexate found in the nanocapsule suspensions after the complete dissolution in acetonitrile and the concentration of drug in the supernatant obtained by suspension ultrafiltration/centrifugation procedure using Ultrafree-MC membranes (100000 NMWL; Millipore). The drug content was expressed in micrograms of methotrexate per milliliter of suspension.

Table II. Results of the Precision

Sample nanocapsule* (30 µg/mL)		Experimental Amount (µg/mL; Mean ± RSD)	Recovery (%)
PLA nanocapsules	Day 1	29.75 + 0.37	99.18
	Day 2	29.95 + 0.14	99.84
	Day 3	30.23 + 0.22	100.78
	Inter-day	29.92 + 0.72	99.75
PLA-PEG nanocapsules	Day 1	29.96 + 0.56	99.87
	Day 2	30.21+ 0.88	100.71
	Day 3	30.43 + 1.49	101.45
	Inter-day	30.20 + 0.79	100.67

* (n = 6)

Table III. Results From the Recovery Test

Sample	Level (µg/mL)	Mean recovery + RSD*
MTX-PLA nanocapsule	12.5	100.36 + 0.23
	25	99.54 + 0.13
	37.5	101.23 + 0.32
MTX-PLA-PEG nanocapsule	12.5	96.74 + 0.44
	25	98.11 + 0.10
	37.5	100.33 + 0.37

* Mean + RSD (= 3).

Results and Discussion

An isocratic RP-HPLC method with UV detection was proposed as a suitable method for quantitative determination of methotrexate entrapment efficiency in polymeric nanocapsules. The chromatographic conditions such as the portions of mobile phase were adjusted as a function of the drug physicochemical characteristics and did not interfere in the structure of nanocapsule. MTX can be eluted under isocratic conditions by employing mixtures of aqueous buffers at pH values ranging from 2.5 to 6.7. Therefore, the pH selected was 3.0 because under this pH conditions MTX can be found in its undissociated form (pK_a 3.8, 4.8, 5.6) (18). The main advantage of the proposed method is the short time analysis and simplified mobile phase. By not using a buffered system, column lifetime should be increased.

Under the experimental conditions, the linearity of the method was determined using five concentrations levels ranging from 10 to 50 µg/mL of methotrexate standard solution. The calibration curve was constructed by plotting concentration versus corresponding mean peak area. The method was linear, showing a correlation coefficient of 0.9998. All the results are shown in Table I. The representative linear equation was: $y = 91758x + 30764$. The linear equation obtained from the drug substance by dilution of a standard stock solution was used to quantify MTX in both PLA and PLA-PEG nanocapsules (20). The limit of quantitation calculated was 2.61 µg/mL.

The specificity of the method was evaluated by analyzing a blank PLA and PLA-PEG nanocapsules. The chromatograms (Figure 1) showed that the method is specific, and there is no interference or overlaps of the compounds of nanocapsules with the methotrexate response at 313 nm detection wavelength.

The repeatability and intermediate precision was determined by performing six replicate analyses of the same sample and evaluated by RSD of the peak area of methotrexate. The mean peak area and the relative standard deviation are reported in Table II. The obtained values indicate good precision.

The accuracy was determined using a recovery test by fortifying blank PLA and PLA-PEG nanocapsules with methotrexate

standard at 3 levels and assaying by the developed method. The mean values of the percent recoveries for each concentration of methotrexate are show in Table III ranging from 96% to 101%. The accuracy of the method was determined by applying the Student's *t*-test to each level (12.5, 25, and 37.5 µg/mL) and to PLA and PLA-PEG samples. The computed values for all samples were lower than the tabulated *t*-value of 2.78 ($P < 0.05$), indicating no significant difference between the added and the estimated quantities.

The proposed method was applied to determine MTX entrapment efficiency (EE) in nanocapsules. The amount of drug used to prepare nanocapsules was 2.5 mg and the mean EE values of PLA and PLA-PEG nanocapsules were 79.31% and 59.97%, respectively. All results demonstrated RSD of < 2.0% from triplicate analysis of each formulation. Considering water-soluble drugs usually have a poor encapsulation efficiency (26), the validated method had successfully been applied to the assay of EE of methotrexate-loaded nanocapsules.

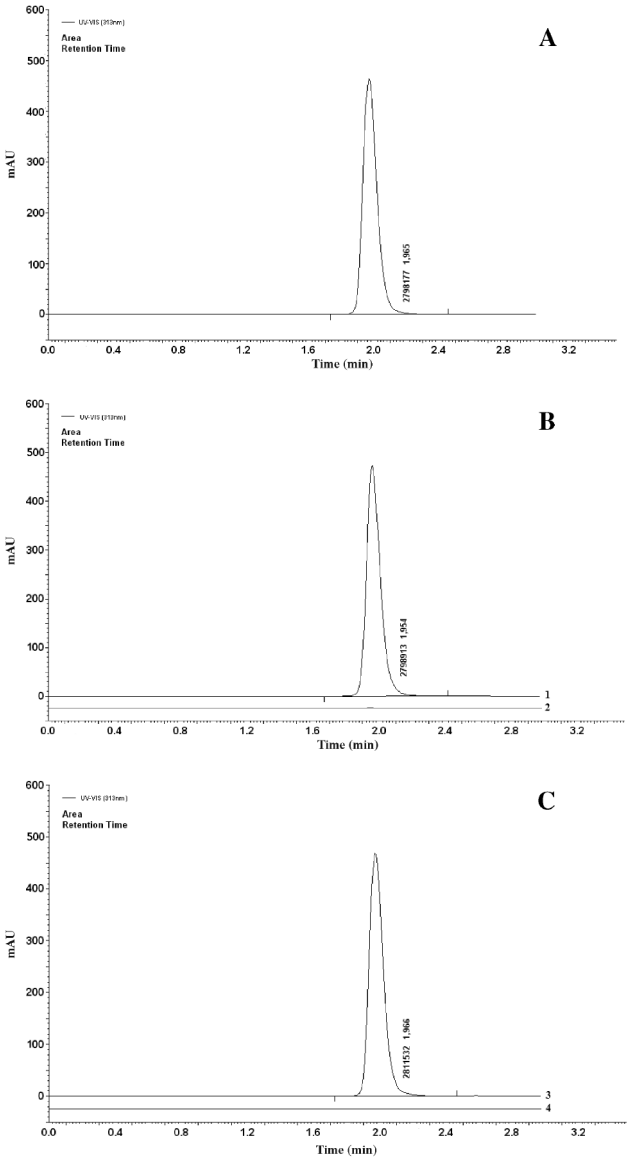

Figure 1. Typical chromatograms obtained under the experimental conditions. Standard solution of 30 µg/mL (A). 1: PLA-PEG nanocapsules of 30 µg/mL MTX, and 2: Blank PLA-PEG nanocapsules (B). 3: PLA nanocapsules of 30 µg/mL MTX, and 4: Blank PLA nanocapsules (C).

Conclusions

A reliable isocratic RP-HPLC method for the determination of methotrexate EE in PLA and PLA-PEG nanocapsules has been developed and validated. The proposed method proved to be simple, rapid and effective. The results confirm that the method can be a suitable technique to quantify methotrexate in nanocapsules formulations, encouraging its application for quality.

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