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Development and validation of a rapid reverse-phase HPLC method for the determination of methotrexate from nanostructured liquid crystalline systems

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A reversed-phase liquid chromatography (RP-LC) method was successfully developed and validated for the determination of methotrexate in nanostructured liquid crystalline systems composed by polyether functional siloxane and silicone polyether copolymer. The LC method was performed on RP C18-ODS column, Agilent Zorbax® (4.6 x 250 mm, 5 µm), maintained at room temperature, with a mobile phase constituted by a mixture of 50 mM ammonium acetate buffer (pH 6.0) and methanol (77:23,v/v) with a flow rate of 1.0 mL/min, using ultraviolet detection at 313 nm. The parameters used in the validation process were linearity, specificity, intra and inter-day precision, accuracy, robustness. The quantitation and detection limits yielded good results. The calibration plot assumed linear behavior from 5.0-150.0 µg. mL^{-1} (r^2= 0.9999). The methotrexate was subjected to oxidation, acid, base and neutral degradation, photolysis and heat as stress conditions. There were no interfering peaks at or near the retention time of methotrexate. The nanostructured liquid crystalline systems did not interfere with the analysis and the recovery was quantitative. The intra and inter-day assay relative standard deviation were less than 0.20 %. The method developed proved to be simple, sensitive, accurate, precise, reproducible and therefore adequate for routine analysis of methotrexate in nanostructured liquid crystalline systems.

1. Introduction

Methotrexate (Fig. 1) is an antimetabolite structurally similar to folic acid, that inhibits in a competitive manner the activity of dihydrofolate-reductase enzyme, and is considered a specific chemotherapeutic for phase S (synthesis) of the cellular cycle (Bournerias and Chosidow 1994; Olsen 1991). It is also effective for the treatment of psoriasis when administered by the oral or parenteral route over long periods of time. (Naldi 2004).

The systemic use of methotrexate may cause numerous side effects, notably hepatotoxic effects (Bookbinder et al. 1984; Dooren-Greebe et al. 1994). It is routinely used in the treatment of acute lymphoblastic leukemia, choriocarcinoma and related trophoblastic tumours (Calabresi and Parks 1975). To reduce such effects, it would clearly be preferable to administer methotrexate topically (Hwang et al. 1995). The most important problem in topical administration of methotrexate is that the drug is hydrosoluble, mostly dissociated at physiological pH and its capacity for passive diffusion is thus limited.

One of the possibilities for increasing the penetration of drugs through the skin is the use of nanostructured systems such as lamellar liquid crystals. Liquid crystals are associated with states of matter intermediate between the almost perfect long-range positional and orientational order of solid crystals and the long-range disorder found in isotropic liquids (Gray and Winsor 1974). They have wide ranges of applications in material science, household products, chemical reaction media, electrochemical biosensors, protein crystallization, gene therapy and pharmaceutical vehicles (Landau et al. 1997; Mezzenga et al. 2005). The use of vehicles having a liquid crystalline structure to carry drugs for topical use has been employed. It allows an easier diffusion of biological active substances through the skin besides having a considerable solubilizing capacity for both oil and water soluble compounds (Farkas et al. 2000; Nesseem 2001). Furthermore, liquid crystals are thermodynamically stable and can be stored for long periods of time without phase separation (Makai et al. 2003).

Thus, the purpose of the presented study was to develop and validate a simple and fast reversed-phase liquid chromatography (RP-LC) method for the quantitative analysis of methotrexate in nanostructured liquid crystalline systems of lamellar phase composed by polyether functional siloxane and silicone polyether copolymer to the topical treatment of psoriasis. This method was validated following the ICH guidelines and resolution 899 of Brazilian National Health Surveillance Agency (ANVISA), assuring the therapeutic efficacy and contributing to improvement of the quality control.

2. Investigations and results

A reversed-phase HPLC method for the assessment of methotrexate associated with nanostructured liquid crystalline system has been proposed. The analytical parameters of specificity, linearity, LOD, LOQ, precision, accuracy and robustness were evaluated to validate the method, according to ICH recommendation and resolution 899 of Brazilian National Health Surveillance Agency (ANVISA). Forced-degradation studies were performed to evaluate the specificity and the amount of methotrexate recovered

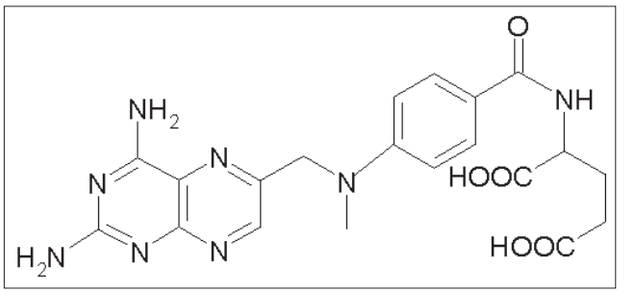

Fig. 1: Chemical structure of methotrexate.

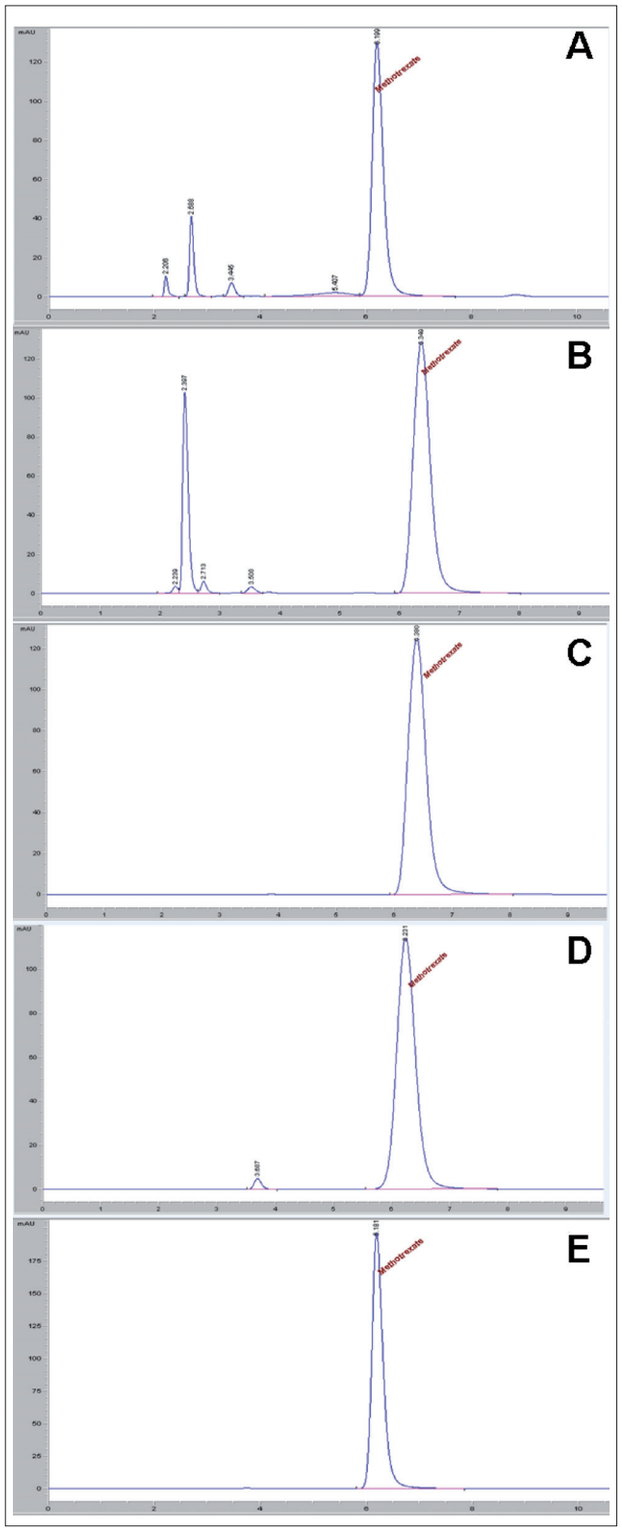

Fig. 2: Chromatograms obtained from 50 µg.mL^{-1}methotrexate after forced degradation under different conditions: a. exposure to UV radiation at 254 nm for 6 h, b. oxidation with 3% hydrogen peroxide for 6 h at room temperature, c. acidic conditions at 60 ºC for 8 h, d. basic conditions at 60 ºC for 8 h and e. neutral condition at 60 ºC for 8 h.

in each test. The purpose of the specificity test was to determine whether new degradation products were produced during forced degradation of methotrexate drug substance and whether any degradation products were resolved from the chromatographic peak obtained for methotrexate (Fig. 2). The photolytic condition

Table 1: Method accuracy results for methotrexate in nanostructured liquid crystalline systems

	Added concentration (µg.mL^{-1})	Concentration found (µg.mL^{-1})	Recovery (%)	RSD (%) n = 3
R1	5	4.91	98.20	0.15
R2	50	49.78	99.60	0.11
R3	150	149.07	99.40	0.19

Table 2: Effects of the analytical parameters on content, during robustness testing of the chromatographic method for methotrexate quantitation

Youden's Test Effect	Content* (%)
Column model (A = Zorbax®ODS 18; a = Zorbax®XDB 18)	101.06 – 100.26 = 0.80
Mobile phase pH (B = 6,0 ; b = 6,5)	100.81 – 100.51 = 0.30
Mobile phase flow rate (mL/min) (C = 1,0 ; c = 0,8)	100.45 – 100.86 = 0.41
Proportion of mobile phase (D = 77:23 ; d = 80:20)	100.50 – 100.82 = 0.32
Wavelength (nm) (E = 303 ; e = 308)	100.39 – 100.93 = 0.54
Injection volume (µL) (F = 20 ; f = 25)	100.43 – 100.88 = 0.45
Column temperature (°C) (G = 25 ; g = 30)	100.85 – 100.47 = 0.38

Mean of content obtained in normal conditions – Mean of content obtained in modified conditions

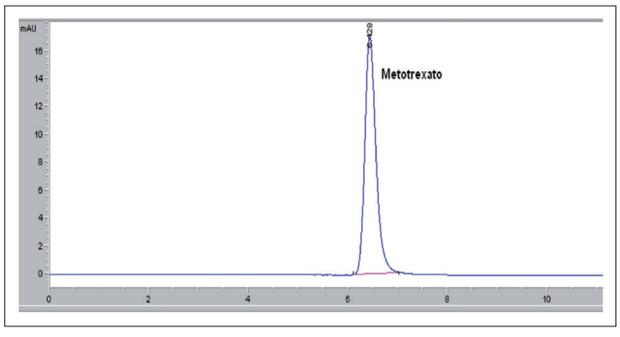

Fig. 3: Representative RP-LC chromatogram of methotrexate in nanostructured liquid crystalline systems of lamellar phase (50 µg.mL^{-1}). Chromatographic conditions: 4.6 x 250 mm i.d., 5 µm particle size, RP C18-ODS column, Agilent Zorbax® with 77:23 (v/v) 50 mM ammonium acetate buffer (pH adjusted to 6.0 with acetic acid) and methanol as mobile phase with a flow rate of 1.0 mL/ min; detection wavelength 313 nm.

exhibited a significant decrease of methotrexate peak's area with three visible degradation peaks and also observed an additional peak close to that of methotrexate that confirms that the drug is photosensible. The oxidative condition also exhibited a slightly decrease of the area, with four well visible degradation peaks, confirming the reactivity of the methotrexate with oxygen. The neutral and acid conditions did not exhibit a significant decrease of methotrexate's area and no degradation peak was observed. Under basic conditions only one degradation peak was observed. This method is able to separate the peaks of the degradation products from methotrexate's peak and the chromatograms showed that the method is specific, and there is no interference or overlaps of the compounds of nanostructured liquid crystalline systems with

Table 3: Results of the system suitability test

	Retention time	Tailing factor	Theoretical plates	Peak area	Capacity factor
Mean (n = 6)	6.43	0.68	4530	2833	2.1
RSD (%)	0.32	0.10	1.00	0.16	0.91

RSD relative standard deviation (%)

is rugged, as shown in Table 2. The system-suitability test is an important part of an analytical method indicating that the system is suitable for the intended analysis. The criteria used for system-suitability tests at each stage of method development will vary with the requirements of the method and its intended application.
System-suitability studies were conducted as specified in United States Pharmacopoeia Convention. The characteristics measured were retention time, tailing factor, column efficiency, peak area,

Table 4: Analytical parameters, values and experimental conditions during robustness evaluation of the chromatographic method

Analytical Parameter	Value (X/x)	Experimental condition							
		1	2	3	4	5	6	7	8
Column model	A/a (Agilent Zorbax®ODS 18/ Agilent Zorbax-®XDB 18)	A	A	A	A	a	a	a	a
Mobile phase pH	B/b (6.0 / 6.5)	B	B	b	b	B	B	b	b
Mobile phase flow rate (mL/ min)	C/c (1.0 / 0.8)	C	c	C	c	C	c	C	c
Proportion of mobile phase (%)	D/d (77:23 / 80:20)	D	D	d	d	d	d	D	D
Wavelength (nm)	E/e (303 / 308)	E	e	E	e	e	E	e	E
Injection volume	F/f (20 µL / 25 µL)	F	f	f	F	F	f	f	F
Column temperature	G/g (25°C / 30°C)	G	g	g	G	g	G	G	g
Result		***s***	***t***	***u***	***v***	***w***	***x***	***y***	***z***

lamellar arrangement with the methotrexate response at 313 nm detection wavelength. So it can be used in stability studies.
The calibration plot for methotrexate was constructed by plotting peak area against eight concentration. It was found to be linear in the range 5-150 µg.mL^{-1} with a correlation coefficient of 0.9999, a representative linear regression equation was y =57,9768x - 2,0175 (r^2 = 0.9999). These data were validated by analysis of variance (ANOVA), which indicated significant linear regression and no significant deviation from linearity ($P < 0.05$). The repeatability (intra-day precision) of the analytical method was calculated as the RSD of assays of nine samples of methotrexate covering the specified range for the procedure, on the same day, under the same experimental conditions.
The obtained RSD value was 0.13 %. The intermediate precision (inter-day precision) was assessed by analyzing two samples on two days by two analysts. The mean values obtained were 99,80% (RSD = 0.20%) on the first day and 99.63% (RSD = 0.19%) on the second day, respectively, confirming that the method is sufficiently precise. The accuracy of the LC method was confirmed by determining the average recoveries from the samples by applying the standard addition method. Mean recoveries from these three different levels (lower, medium and upper concentration) corresponding to 5, 50 and 150 µg.mL^{-1} respectively, are shown in Table 1. The results indicated a good accuracy of the proposed method. For calculating of the LOD and LOQ, a calibration equation, y = 57,9768 x - 2,0175, was generated by using the mean values of the three independent analytical curves. The LOD and LOQ calculated were 0.16 µg. mL^{-1} and 0.47 µg. mL^{-1}, respectively. The LOQ evaluated in an experimental assay, with a precision lower than 5% and accuracy within ± 5%, was found to be 0,05 µg.mL^{-1}. The difference (X – x), the mean values, standard deviations and the criterion ($s\sqrt{2}$), were calculated and used to evaluate the results. The calculated criterion was 0.968. The results meet the acceptance criterion and no significant changes in chromatographic behavior were observed when the experimental conditions were changed slightly, demonstrating that the method and capacity factor. The values obtained are shown in Table 3. No interference from the sample solvent or from impurities was observed at the detection wavelength.
In conclusion, a simple, rapid and sensitive RP-HPLC method for the determination of methotrexate in nanostructured liquid crystalline systems of lamellar phase composed by polyether functional siloxane and silicone polyether copolymer was developed and validated. The proposed method was demonstrated to be linear, precise, accurate and specific based on method validation, uses simple reagents and minimum sample-preparation procedures. The results from forced degradation revealed no interference with the methotrexate peak that proved to be stability-indicating and therefore useful in routine analysis of methotrexate in these kinds of formulations.

3. Discussion

More than 70 papers describing chromatographic assays for methotrexate and its metabolites have been published. A wide array of experimental conditions for sample preparation, analyte separation and detection have been employed (Rubino 2001). Choice of an analytical method depends on factors such as the nature of the drug, the complexity of the sample, and the intended use. The aim of the study was to develop a HPLC assay for analysis of methotrexate in nanostructured liquid crystalline systems of lamellar phase composed by polyether functional siloxane and silicone polyether copolymer. The results demonstrated that the method uses a simple mobile phase and has good reproducibility and accuracy, and be regarded as can therefore useful in routine analysis. The retention time of methotrexate (6.4 min) allows a fast determination of the drug, free from any coeluting peak as shown in Fig. 3.

4. Experimental

4.1. Chemical and reagents

Methotrexate was supplied from Sigma-Aldrich (USA) with a 99.5% purity certificated. Nanostructured liquid crystalline systems of lamellar phase composed by polyether functional siloxane and silicone polyether copolymer was kindly obtained by

Dow Corning®, São Paulo, Brazil. All chemicals used were pharmaceutical or special analytical grade. Analytical reagent grade ammonium acetate buffer was purchased from Merck, HPLC grade methanol from Tedia (USA) and acetic acid from Quimis. For all of the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

4.2. Chromatographic conditions

The RP-LC method was performed on a Agilent Technologies LC system, model 1100 UV–visible detector. The detector was set to 313 nm and peak areas were integrated automatically by use a computer using the HPchem software program. A RP C18-ODS column, Agilent Zorbax® (4.6 x 250 mm, 5 µm) was employed with a mobile phase constituted by a mixture of 50 m*M* ammonium acetate buffer (pH 6.0 adjusted by addition of acetic acid) and methanol (77:23,v/v), in an isocratic mode with a flow rate of 1.0 mL/min. The HPLC system was performed at room temperature (25±1°C). The injection volume was 20 µL for both the reference substance and the samples. The mobile phase was filtered with a 0.45 µm membrane filter and degassed with helium for 15 min before use.

4.3. Preparation of reference substance solution

The stock solution was prepared by accurately weighing 10 mg methotrexate reference substance, and transferring it to a 50 mL volumetric flask. Mobile phase buffer was added to volume, to give a final concentration of 200 $\mu g.mL^{-1}$ of methotrexate. The stock solution was prepared daily, diluted to an appropriate concentration in mobile phase buffer and filtered with a 0.45 µm membrane filter (Millipore).

4.4. Preparation of sample solutions

Nanostructured liquid crystalline systems of lamellar arrangement were prepared by mixing the polyether functional siloxane as surfactant, with silicone polyether copolymer as oily phase and phosphate buffer 0,01*M* pH 7.4 as aqueous phase and 1.25 % of methotrexate. An amount of liquid crystal phases with 1.25% of methotrexate equivalent to 100 mg was accurately weighed and transferred to 25 mL volumetric flask. Mobile phase buffer was added to volume, to give a final concentration of 50 $\mu g.mL^{-1}$. The samples was prepared daily, diluted with mobile phase buffer, filtered through a 0.45 µm membrane filter and injected. The amount of the drug was calculated against the reference substance. Blank liquid crystal phases were prepared according to the procedure previously described, omitting the drug. All samples were prepared in triplicate.

4.5. RP-HPLC method assessment

Method was performed following ICH guidelines and resolution 899 of Brazilian National Health Surveillance Agency (ANVISA) for specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness and system suitability test and their acceptance criteria (ICH 2005; Brazil 2003).

4.5.1. Specificity and selectivity

A stability-indicating method is defined as an analytical method that accurately quantifies the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities (Alsante et al. 2007). The stability-indicating capability of the method was determined by subjecting a reference sample solution (50.0 $\mu g.mL^{-1}$) to accelerated degradation under acidic, basic, neutral, oxidative, and photolytic conditions to evaluate the interference in the quantitation of methotrexate. Degraded samples were prepared by treating 50 $\mu g.mL^{-1}$ of reference substance solutions with acid (0.1 *M* hydrochloric acid) and base (0.1 *M* sodium hydroxide) in a hot water bath at 60 ºC for 5 h. The solutions were cooled at room temperature and neutralized. For the study under neutral conditions, the drug was dissolved in mobile phase buffer and heated at 60 ºC for 6 h. Oxidative degradation was induced by storing the sample solutions in 3% hydrogen peroxide, at room temperature for 4 h, protected from light. Photodegradation was induced by exposing the samples in a photostability chamber to 200 W h/m² of near ultraviolet light (254 nm) for 6 h and the UV lamp was positioned 15 cm from the samples. After the procedure, the samples were diluted with the mobile phase buffer to a final concentration of 50 $\mu g.mL^{-1}$. The interference of the excipients of the pharmaceutical formulation was determined by the injection of a sample containing only placebo (nanostructured liquid crystalline systems with lamellar arrangement were prepared by mixing the polyether functional siloxane as surfactant with silicone polyether copolymer as oily phase and phosphate buffer 0.01*M* pH 7.4 as aqueous phase without methotrexate). Then, the stability-indicating capability of the method was established by the acceptable separation of degradation products peaks from methotrexate peak.

4.5.2. Linearity and range

The linearity was evaluated by preparing three independent analytical curves, each one with eight reference substance concentrations of methotrexate, in the range of 5-150 $\mu g.mL^{-1}$, prepared in mobile phase buffer. Before injection of the solutions, the column was equilibrated for at least 20 min with the mobile phase flowing through the system. Three injections of 20 µL each of standard solutions were prepared to check the repeatability of the detector response at each concentration. Peak areas for methotrexate were plotted as a function of concentration to obtain the calibration plot. The eight concentrations of the reference substance solution were subjected to regression analysis in order to calculate the calibration equation and correlation coefficient.

4.5.3. Precision

The precision of the analytical procedure was evaluated by determination of the repeatability of the method by assaying nine samples of methotrexate covering the specified range for the procedure, with three concentrations with three replicates each, on the same day, under the same experimental conditions. The response factors of the drug peaks and the mean and percentage relative standard deviations (RSD) of the response factor of the peaks were calculated. The intermediate precision of the method was assessed by carrying out the analysis on two days (inter-day), with two analysts, using the same LC instrument, in the same laboratory.

4.5.4. Accuracy

The accuracy was evaluated as follows: Nanostructured liquid crystalline systems with lamellar arrangement were spiked with known quantities of methotrexate, at three different levels (lower, medium and upper concentration) corresponding to 5, 50 and 150 $\mu g.mL^{-1}$. The accuracy was calculated as the percentage of the drug recovered from the sample and expressed as relative percentage (%) in accordance with the recommendations of the ICH guideline and resolution 899 of Brazilian National Health Surveillance Agency (ANVISA)

4.5.5. Limit of detection and quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated, as defined by ICH guideline and resolution 899 of Brazilian National Health Surveillance Agency (ANVISA), using the mean values of three independent analytical curves, determined by a linear-regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respectively, were multiplied by the ratio from the standard deviation of the intercept and the slope. The LOQ was also evaluated in an experimental assay. The analytical curves were constructed in the range 5– 150 $\mu g.mL^{-1}$.

4.5.6. Robustness

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 the routine analysis. The robustness evaluation of the chromatographic method for the methotreate quantitation was performed using the method proposed by Youden and Steiner (1975). It was determined by analyzing the same samples (50 $\mu g.mL^{-1}$) under variations of seven analytical parameters of the method including different columns. Two Youden`s tests were carried out and the seven analytical parameters employed, as well as the introduced variations are demonstrated in Table 4. The analytical conditions at the nominal values are represented by capital letters and the conditions with the small variation are represented by lowercase letters. The results obtained of each experiment were represented by the letters s, t, u, v, w, x, y and z, respectively. From these results it is possible to estimate the effect of each variable by obtaining the difference of the four analyses that have the nominal value (capital letter) and the four analyses with the alternative value (lowercase letter). For example, to evaluate the effect of the column supplier in the final result of the analyses, the following equation was employed: $[(s + t + u + v)/ 4 – (w + x + y + z)/ 4]$ (César and Pianetti 2009). Considering the mean and the standard deviation of the eight results, the following criterion was applied: If the value of the difference (A-a … G - g > s$\sqrt{}$ 2), the variable has a significant effect and the method is sensitive to changes in the variable concerned (Bedregal et al.2008).

4.5.7. System suitability test

The system suitability test was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed, using seven replicates injections of a reference solution containing 50 $\mu g.mL^{-1}$of methotrexate. The parameters measured were peak area, retention time , number of theoretical plates, capacity factor, tailing factor and peak symmetry.

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Conflicts of interest: None declared.

References

Alsante KM, Ando A, Brown R, Ensing J, Hatajik TD, Kong W, Tsuda Y (2007) The role of degradant profiling in active pharmaceutical ingredients and drug products. Adv Drug Deliv Rev 59: 29-37.

Bedregal P, Torres B, Ubillús M, Mendonza P, Montoya E (2008) Robustness in NAA evaluated by the Youden and Steiner test. J Rad Nuc Chem 278: 801-806.

Bookbinder SA, Espinoza LR, Fensk NA, Germain BF, Vasey FB (1984) Methotrexate: its use in the rheumatic diseases. Clin Exp Rheum 2:185–193.

Bournerias I, Chosidow O (1994) Méthotrexate et psoriasis Pharmacologie et prise en charge thérapeutique. Ann Dermatol Venereol 121:69-74.

Brazil (2003) Guide for validation of analytical and bioanalytical methods Resolution 899 of Brazilian National Health Surveillance Agency (ANVISA).

Calabresi, P, Parks R E Jr. (1975) The Pharmacological Basis of Therapeutics. In: Goodman L S Gilman A (ed) 5th ed., New York, p. 1248-1307.

César IDC, Pianetti GA (2009) Robustness evaluation of the chromatographic method for the quantitation of lumefantrine using Youden`s test. Braz J Pharm Sci 45: 235-240.

Dooren-Greebe RJ, Kuijpers ALA, Mulder J, Boo T, Kerkhof PCM (1994) Methotrexate revisited: effects of long-term treatment in psoriasis. Br J Derm 130: 204–210.

Farkas E, Zelkó R, Németh Z, Pálinkás J, Marton S, Rácz I (2000) The effect of liquid crystalline structure on chlorhexidine diacetate release. Int J Pharm 193: 239–245.
Gray GW, Winsor PA (1974) Liquid Crystals & Plastic Crystal. Ellis Horwood, Chichester, vol.1, p.139
Hwang GC, Lin AY, Chen W, Sharpe RJ (1995) Development and optimization of a methotrexate topical formulation. Drug Dev Ind Pharm 21: 1941–1952.
ICH (2003) International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Stability Testing of New Drugs Substance and Products Q1A(R2).
ICH (2005) International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Validation of analytical procedures: text and methodology Q2(R1).
Landau EM, Rummel G, Cowan-Jacob SW, Rosenbuch JP (1997) Crystallization of a polar protein and small molecules from the aqueous compartment of lipidic cubic phases. J Phys Chem B 101: 1935– 1937.
Makai M, Csányi E, Németh Z, Pálinkás J, Erós I (2003) Structure and drug release of lamellar liquid crystals containing glycerol. Int J Pharm 256: 95–107.
Mezzenga R, Meyer C, Servais C, Romoscanu AI, Sagalowicz L, Hayward RC (2005) Shear rheology of lyotropic liquid crystals: a case study Langmuir 21: 3322–3333.
Naldi L (2004) Epidemiology of psoriasis. Curr Drug Targets Inflamm Allergy 3: 121-128.
Nesseem DI (2001) Formulation and evaluation of itraconazole by liquid crystal for topical delivery system. J Pharm Biomed Anal 26: 387–399.
Olsen EA (1991) The pharmacology of methotrexate. J Am Acad Dermatol 25: 306-318.
Rubino FM (2001) Separation methods for methotrexate, its structural analogues and metabolites.J Chromatogr B: Biom Sci Appl 764: 217-254.
United States Pharmacopoeia Convention (2010) United States pharmacopeia, 33th edn. USP, Rockville.
Youden W J (1975) Statistical manual of the association of official analytical chemists (No. LC-0111). Aoac International.