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Original Article

Optimized high performance liquid chromatography–ultraviolet detection method using core-shell particles for the therapeutic monitoring of methotrexate ☆

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ABSTRACT

Methotrexate (MTX) is an antineoplastic drug, and due to its high toxicity, the therapeutic drug monitoring is strictly conducted in the clinical practice. The chemometric optimization and validation of a high performance liquid chromatography (HPLC) method using core–shell particles is presented for the determination of MTX in plasma during therapeutic monitoring. Experimental design and response surface methodology (RSM) were applied for the optimization of the chromatographic system and the analyte extraction step. A Poroshell 120 EC-C18 (3.0 mm × 75 mm, 2.7 μm) column was used to obtain a fast and efficient separation in a complete run time of 4 min. The optimum conditions for the chromatographic system resulted in a mobile phase consisting of acetic acid/sodium acetate buffer solution (85.0 mM, pH=4.00) and 11.2% of acetonitrile at a flow rate of 0.4 mL/min. Selectivity, linearity, accuracy and precision were demonstrated in a range of 0.10–6.0 μM of MTX. The application of the optimized method required only 150 μL of patient plasma and a low consumption of solvent to provide rapid results.

1. Introduction

Methotrexate (MTX, 4-amino-N10-methylpteroylglutamic acid) is a cytotoxic drug used since the 1940s in the therapy of acute lymphoblastic leukemia (ALL), non-Hodking lymphoma and cerebral tumors [1].

The structure analogy between MTX and folic acid (Fig. 1) is the basis of its mechanism of action. Its antiproliferative action is developed by the competitive inhibition of dihydrofolate-reductase (DHFR), an enzyme involved in folic acid metabolism. At high doses, MTX follows different metabolic pathways of detoxification, producing two main metabolites, i.e., 7-hydroxy-MTX (7-OH-MTX) and 4-amino-4-deoxy-N10-methylpteroic acid (DAMPA) [2].

Since MTX inhibits a key cellular function, it is an important cytotoxic compound, especially on actively replicating cells, but also on other tissues [1–3]. Thus, MTX is one of the very few antineoplastic drugs for which therapeutic drug monitoring (TDM) is currently conducted in clinical practice, especially in high-dose protocols.

In order to allow the use of protocols with very high doses of MTX, a strategy called "leucovorin rescue therapy" has been devised. Administration of leucovorin at a scheduled time after the infusion of high-dose MTX is beneficial to healthy cells, and protects them from the cytotoxic action of MTX [1].

Several methods have been developed for the determination of MTX and its metabolites in human fluids. Firstly, bioanalytical methods using antibodies, such as radioimmunoassay [4,5], fluorescent polarization immunoassays (FPIA from Abbott) [6] and the enzyme multiplied immunoassay technique (EMIT from Behring Diagnostics) [7], have been used. These techniques have many advantages in simplicity, speed and cost. However, specificity can be compromised because antibodies can present cross-reactivity, leading to an overestimation of the actual MTX concentration. Recently, an electrochemical approach has been presented for the determination of MTX, which uses a poly (L-lysine) modified electrode in the presence of sodium dodecyl benzene sulfonate. This sensor provided satisfactory results for a wide linear concentration range, low detection limit, high selectivity and good stability. Its practical applicability has been proven by quantifying MTX in medicinal tablets, but it was not applied to detect the analyte in biological samples [8].

Among the separation methods, high-performance liquid chromatography (HPLC) using different detection modes, such as electrochemistry [9,10], fluorescence through pre- or post-column oxidation [11], UV [12,13] and mass spectrometry [14,15], is widely

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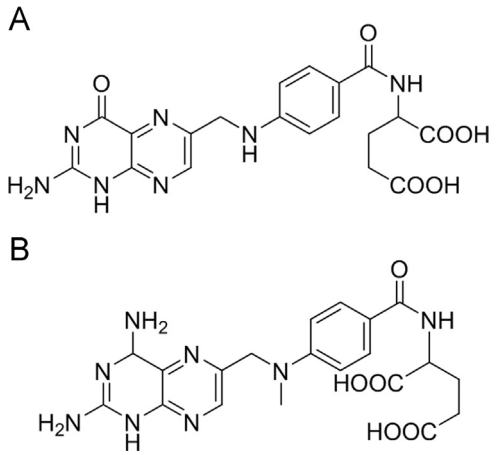

Fig. 1. Chemical structures of (A) folic acid and (B) methotrexate.

used for the determination of MTX in plasma for clinical purposes.

In the past few years, sub-3 μm core–shell particles columns for HPLC have been developed. The premise which drove their development is the reduced diffusion length for analytes inside the core–shell particles compared to their fully porous counterparts. As the thickness of the porous shell decreases, the faster mass transfer can lead to improved column efficiency and shorter elution time, reducing both total analysis time and organic solvent consumption [16,17].

Core–shell particles columns for biological samples have been reported for the determination of 25-hydroxymetabolites of vitamin D2 and D3 in serum [18], N-(ω)-hydroxy-nor-L-arginine, L-arginine and N-(ω)-ethyl-L-arginine in rat plasma [19] and boldine in rat plasma, urine and bile [20].

As for sample preparation, protein precipitation as the first step is highly recommended, as it is simple, fast and inexpensive. An extraction step can contribute to further clean-up, in which an organic solvent is employed to back-extract the solvent used for protein precipitation, leaving a smaller water volume that can be directly injected into the chromatographic system.

The development of a new analytical method may involve optimization approaches, for which experimental design, especially response surface analysis and Derringer's desirability function, are valuable tools [21,22].

Response surface methodology (RSM) is a statistical and mathematical technique used to model the experimental data and obtain the polynomial equation that best fits the response behavior [23]. When more than two responses are to be optimized simultaneously, the Derringer's desirability function is a useful strategy for finding the operative conditions that satisfy the optimization criteria for all the responses taken into account [24].

Once the method is developed and optimized, a full validation should be performed. The main characteristics of a bioanalytical method, which are essential for ensuring the acceptability of the performance and the reliability of analytical results, are selectivity, lower limit of quantification, response function and calibration range, accuracy, precision, matrix effects, stability of the analyte (s) in the biological matrix, and stability of the analyte(s) in the stock and working solutions [25].

In this study, a fast and efficient high performance liquid chromatography–ultraviolet (HPLC–UV) method was developed for the determination of MTX in human plasma applicable to the TDM. Both sample preparation and chromatographic separation were optimized and the method was validated according to the European Medicines Agency (EMA) guideline on bioanalytical method validation [25].

2. Materials and methods

2.1. Apparatus and software

The HPLC analyses were accomplished using an Agilent 1100 Series system, equipped with a quaternary pump, a membrane degasser, a thermostated column compartment, an autosampler and a diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). For data acquisition and processing, the Chemstation version B 0103 was used.

Experimental design, surface response modeling and desirability function calculations were performed using the Stat–Ease Design–Expert 8.0.0.

2.2. Chemicals, reagents and samples

Methotrexate sodium (Na_2MTX) freeze-dried preparation for injection was supplied by Microsules (Buenos Aires, Argentina). Acetonitrile (HPLC grade) was purchased from Aberkon Química (José León Suarez, Argentina). Glacial acetic acid (analytical grade) and chloroform (analytical grade) were purchased from Laboratorios Cicarelli (San Lorenzo, Argentina), while methylene chloride (analytical grade) and sodium acetate (analytical grade) were supplied by Anedra (Buenos Aires, Argentina). Purified HPLC grade water was obtained from a Milli-Q® system (Millipore, Milford, MA, USA).

Real unknown samples containing MTX were obtained from the remaining volume of plasma used in the laboratory for the TDM in patients hospitalized at "J.M. Iturraspe Hospital" in city of Santa Fe (Argentina). These samples were conserved at 4 °C for less than 6 h until analysis. Blank human plasmas were obtained from non-treated unidentified volunteers.

During the method development, pooled plasma samples prepared by mixing several samples from different hours post infusion were used to obtain an average concentration of MTX and 7-OH-MTX.

2.3. Calibration solutions and quality control (QC) samples

MTX stock standard solution at 1250 μM was prepared by weighing and dissolving an appropriate amount of Na_2MTX in 50.0 mL of purified water. Calibration solutions were prepared by adding appropriate volumes of stock standard solution to blank human plasma. The final MTX concentrations in the calibration solutions were 0.10, 0.50, 1.00, 1.50, 2.00, 4.00, and 6.00 μM.

QC samples were prepared by diluting the stock solution with blank plasma at 0.10, 0.30, 3.0 and 4.5 μM, to obtain low limit of quantification (QC-LLOQ, 0.10 μM), and low (QC-L, 0.30 μM), medium (QC-M, 3.0 μM) and high (QC-H, 4.5 μM) concentration levels, respectively.

2.4. Sample preparation

The procedure used for the extraction of the analyte from plasma samples consisted of two steps, i.e., protein precipitation using acetonitrile (ACN) with a ratio of 2:1 (ACN:plasma), and then the back-extraction of the solvent used for protein precipitation. For this purpose, chloroform and methylene chloride were evaluated. The performance of this procedure was optimized by experimental design and RSM.

2.5. Chromatographic separation

The separation was achieved using a Poroshell 120 EC-C18 (3.0 mm × 75 mm, 2.7 μm) column (Agilent Technologies) and monitored at 305 nm. The mobile phase consisted of acetic acid/

Table 1
Central composite design for chromatographic separation optimization.

Run	Block	Factors				Responses				
		SA[a]	pH	ACN (%)	Temp. (°C)	R_1	R_2	W_1	W_2	T (min)
1	1	62.5	5.50	16.25	35.0	0.94	1.17	–	–	1.01
2	1	87.5	4.00	8.75	25.0	10.89	10.99	0.300	0.330	10.2
3	1	87.5	4.00	16.25	35.0	2.55	2.59	0.078	0.081	1.53
4	1	87.5	5.50	8.75	35.0	6.76	3.03	0.390	0.350	5.40
5	1	75.0	4.75	12.50	30.0	1.92	0.00	–	–	1.57
6	1	87.5	5.50	16.25	25.0	2.01	0.00	–	–	1.08
7	1	62.5	5.50	8.75	25.0	4.62	1.07	0.530	0.620	4.20
8	1	62.5	4.00	8.75	35.0	3.04	1.39	0.840	1.68	6.17
9	1	62.5	4.00	16.25	25.0	2.95	3.28	0.069	0.077	1.62
10	1	75.0	4.75	12.50	30.0	5.63	5.79	0.120	0.120	3.02
11	2	75.0	4.75	12.50	30.0	3.09	1.68	0.230	0.250	2.34
12	2	62.5	4.00	16.25	35.0	2.48	2.45	0.077	0.084	1.50
13	2	75.0	4.75	12.50	30.0	3.73	2.34	0.190	0.200	2.47
14	2	87.5	5.50	8.75	25.0	6.76	2.35	0.420	0.470	5.41
15	2	87.5	5.50	16.25	35.0	2.03	0.00	–	–	1.04
16	2	62.5	4.00	8.75	25.0	11.61	12.33	0.260	0.350	11.5
17	2	62.5	5.50	8.75	35.0	7.92	4.14	0.310	0.260	5.45
18	2	62.5	5.50	16.25	25.0	2.03	0.00	–	–	1.04
19	2	87.5	4.00	8.75	35.0	9.83	9.22	0.220	0.240	7.01
20	2	87.5	4.00	16.25	25.0	2.88	3.32	0.067	0.073	1.61
21	3	75.0	4.75	20.00	30.0	1.76	0.00	–	–	1.02
22	3	100.0	4.75	12.50	30.0	3.41	1.83	0.190	0.200	2.21
23	3	75.0	4.75	12.50	30.0	3.39	1.73	0.190	0.220	2.19
24	3	75.0	3.25	12.50	30.0	3.34	5.94	0.076	0.076	2.13
25	3	75.0	6.25	12.50	30.0	5.07	8.79	0.087	0.099	3.04
26	3	75.0	4.75	12.50	30.0	2.43	0.66	0.210	0.300	1.72
27	3	50.0	4.75	12.50	30.0	3.34	1.66	0.210	0.240	2.29
28	3	75.0	4.75	12.50	20.0	4.67	2.98	0.160	0.190	2.67
29	3	75.0	4.75	5.00	30.0	19.1	–	0.350	–	–
30	3	75.0	4.75	12.50	30.0	4.63	2.61	0.120	0.140	2.17
31	3	75.0	4.75	12.50	40.0	6.16	4.00	0.060	0.064	1.96

[a] Sodium acetate concentration in mM.

sodium acetate buffer solution and acetonitrile, flowing at a rate of 0.4 mL/min, and the injection volume was 20 μL. The rest of the chromatographic parameters were optimized by experimental design and RSM.

2.6. Chromatographic optimization

In the first place, the responses to be optimized were selected in order to reach short analyses time and complete resolution between MTX and 7-OH-MTX peaks. It is worth noting that DAMPA was not considered for the optimization as it is a minor metabolite. A high-concentration solution of DAMPA was injected into the chromatographic system and several real samples were analyzed. It was confirmed that the level of DAMPA in plasma after infusion is undetectable by this method. Therefore, the five responses were resolution between MTX peak and the peak corresponding to endogenous plasma components (R_1), resolution between MTX and 7-OH-MTX peaks (R_2), complete runtime (T) and peak width of MTX (W_1) and 7-OH-MTX (W_2).

The analyzed factors, i.e., buffer concentration and pH, percentage of ACN in the mobile phase and oven temperature, were chosen from the literature as they have more influence on the responses under study.

The central composite design (CCD) consisted of 31 experiments, including the combinations of factors at different levels and seven central points. The ranges studied for the four factors were 50.0–100.0 mM for the buffer concentration, 3.25–6.25 for the buffer pH, 5.00%–20.00% of ACN in the mobile phase, and 20.0–40.0 °C for the oven temperature. The order of the experiments

Table 2
Full factorial design for extraction optimization.

Run	Factors			Responses	
	DPV[a]	ESV[b]	EST[c]	MTX area	EV[d]
1	200	200	C[e]	130.5	40
2	200	400	C[e]	127.5	40
3	400	600	C[e]	129.5	80
4	200	600	C[e]	130.7	40
5	400	400	C[e]	128.5	70
6	600	200	C[e]	40.1	70
7	400	400	C[e]	132.0	80
8	400	200	C[e]	55.5	100
9	600	400	C[e]	89.8	150
10	400	400	C[e]	123.0	100
11	600	600	C[e]	128.8	150
12	200	400	MC[f]	125.6	30
13	200	200	MC[f]	131.1	40
14	200	600	MC[f]	133.8	30
15	400	400	MC[f]	128.6	100
16	400	600	MC[f]	131.2	100
17	600	400	MC[f]	135.7	150
18	400	400	MC[f]	131.1	90
19	400	400	MC[f]	131.8	60
20	600	200	MC[f]	–	150
21	600	600	MC[f]	128.8	160
22	400	200	MC[f]	78.4	70

[a] Deproteinized plasma volume in μL.
[b] Extraction solvent volume in μL.
[c] Extraction solvent type.
[d] Extract volume in μL.
[e] Chloroform.
[f] Methylene chloride.

was randomized to minimize systematical error, and the experiments were divided into three blocks (Table 1). A pooled plasma sample containing both compounds, MTX and 7-OH-MTX, was employed in the optimization of experiments.

2.7. Extraction optimization

The selected factors for this optimization were volume of plasma after deproteinization and volume of extraction solvent. Chloroform and methylene chloride were evaluated for the extraction as categorical factors in the design. The optimized responses were MTX recovery, and the volume of the extract obtained after the extraction procedure.

A three-level full factorial design (FFD), consisting of 22 experiments, i.e., 11 for chloroform and 11 for methylene chloride, was built. The studied ranges were 200–600 µL for both factors (Table 2). A blank plasma sample spiked with MTX in a final concentration of 2.0 µM was employed for the optimization of experiments.

The experiments were performed in one block, and the order was randomized to minimize systematical errors.

2.8. Method validation

The method was validated following the EMA guideline on bioanalytical method validation. The following parameters were evaluated: selectivity, LLOQ, calibration curve regression model, accuracy, precision, dilution integrity and stability of the analyte in the stock solution under the entire period of storage and processing conditions.

3. Results and discussion

3.1. Chromatographic optimization

The responses obtained in the experimental design runs (Table 1) were fitted into suitable models obtained by the multiple regression technique and the least square method, in order to describe their behavior as a function of the analyzed factors. The second degree polynomial model used is shown in Eq. (1):

$$y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{1\le i\le j}^{k}\beta_{ij}x_i x_j+\sum_{i=1}^{k}\beta_{ii}x_i^2+\varepsilon \quad (1)$$

where y is the response to be optimized, x_{ij} are the factors, β_0 is the overall mean effect, β_i represents the effect of the factor x_i, β_{ij} is the effect of the ij interaction between the factors x_i and x_j, and ε is a random error component.

Some of the responses had to be transformed in order to achieve variance stabilization and normal distribution of residuals. The Box–Cox graphical strategy was used to find the appropriate transformation function [21]. Table 3 shows the transformations applied to the responses and the models adjusted by the analysis of variance (ANOVA) for each one. ANOVA is a statistical test used to analyze the differences between group means and their associated procedures. According to Eq. (1), the appropriate hypotheses for model evaluation are

$$H_0: \beta_i = b_{ij} = \ldots = b_k = 0$$
$$H_1: \beta_k \neq 0 \quad \text{for at least one } k \quad (2)$$

ANOVA estimates three variances: a total variance based on all the observation deviations from the grand mean; an error variance based on all the observation deviations from their appropriate treatment means, and a treatment variance. In this case, the treatment is the factor level. To determine the statistical significance of the model, the F-test is used. If the calculated F_0 exceeds $F_{\alpha,k-1,k(n-1)}$, the H_0 is rejected and there is at least one variable that contributes significantly to the model.

The desirability function was applied later for the simultaneous optimization of the responses. The optimization criteria (Table 4) were selected in order to reach short analysis time and good resolution between the peaks, and also to obtain symmetrical bands.

The levels for the factors that gave the maximum of the desirability function ($D=0.703$) were 85.0 mM and 4.00 for the buffer concentration and pH, respectively, 11.2% for ACN and 25 °C for the column oven. The response surface of the global desirability for pH and ACN percentage is shown in Fig. 2. These optimization results were experimentally checked and the experimental responses were compared with those predicted by the model. Predicted responses and experimental results are shown in Table 4. The chromatogram obtained at the optimal conditions is shown in Fig. 3. As can be seen, the peaks are completely resolved in a total run time of 4 min. In addition, the peaks for both compounds, i.e., MTX and 7-OH-MTX, show acceptable widths.

3.2. Extraction optimization

As it was done for the chromatographic optimization, each of the responses (Table 2) was fitted into a suitable model and ANOVA test was applied for model validations.

The models and significant terms are shown in Table 3. The models suggest that the volume of extract obtained was not influenced by the volume of extraction solvent employed, but it was affected by the volume of plasma. On the other hand, no factor was

Table 3
Models fitting.

Central composite design					Full factorial design				
Natural response	Transformation	Transformed response	Model	Significant terms[a]	Response	Chloroform		Methylene chloride	
						Model	Significant terms[a]	Model	Significant term[b]
R_1	Natural log	$y'=\ln R_1$	Linear	C	Area	Quadratic	A-B-AB-B^2	–	–
R_2	Square root	$y'=\sqrt{R_1+0.1233}$	2FI[c]	B-C-BD					
T	Inverse square root	$y'=\frac{1}{\sqrt{T}}$	Quadratic	B-C-C^2	Extract volume	Linear	A	Linear	A
W_1	–	–	Quadratic	C-D-AD-BC-BD-B^2-C^2-D^2					
W_2	–	–	Quadratic	C-D-AD-BC-BD-B^2-D^2					

[a] ANOVA test ($\alpha=0.05$): A=buffer concentration (mM), B=pH, C=ACN (%), D=temp. (°C).
[b] ANOVA test ($\alpha=0.05$): A=volume of deproteinized plasma (µL), B=volume of extraction solvent (µL).
[c] 2FI indicates model with linear terms and interaction.

Table 4
Criteria for the optimization of individual factors and responses.

Optimization	Factors	Response	Goal	Prediction	Experimental results
CCD		R_1	Maximize	6.43	7.40
		R_2	Maximize	7.19	9.40
		T	Minimize	5.51	4.70
		W_1	Minimize	0.058	0.142
		W_2	Minimize	0.054	0.148
FFD	DPV[a]		Minimize	–	–
	ESV[b]		Minimize	–	–
Chloroform		Area	Maximize	106.2	121.0
		EV[c]	Maximize	80	70
Methylene chloride		Area	Maximize	131.1	78.0
		EV	Maximize	88	50

CCD: central composite design. FFD: full factorial design.
[a] Deproteinized plasma volume.
[b] Extraction solvent volume.
[c] Extract volume in μL.

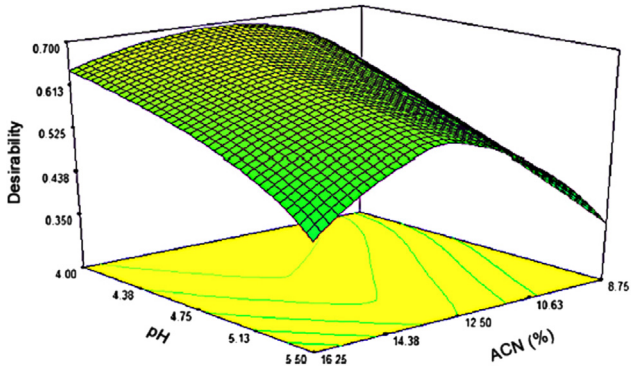

Fig. 2. Response surface of the global desirability as a function of pH and ACN. The other factors are at their optimum.

significant for the area of MTX when using methylene chloride as extraction solvent; therefore, no model could be built for this particular response. In the case of chloroform, the fitted model was quadratic and the response surface is shown in Fig. 4.

For the optimization of the responses, the desirability function was applied and the criteria were selected in order to achieve both higher MTX recovery and extract volume. In addition, the factors were also included in the global desirability function to minimize the organic solvent and the plasma volume required for the analysis.

The levels of the factors that maximized the desirability function were 350 μL of deproteinized plasma and 300 μL of chloroform, or 400 μL of deproteinized plasma and 200 μL of methylene chloride. The response surface of the global desirability for both factors and chloroform as extraction solvent is shown in Fig. 5. The optimization results were experimentally checked and the responses were compared with those predicted by the model. The optimization criteria and the results are shown in Table 4.

As can be seen in the experimental results, methylene chloride gives lower values than those predicted by the model, whereas chloroform shows better results according to the predictions, and presents much higher MTX recovery and more extract volume. Since chloroform achieves the goal of optimization, it was selected as extraction solvent and the optimized conditions were used.

Considering these results, the optimized extraction procedure

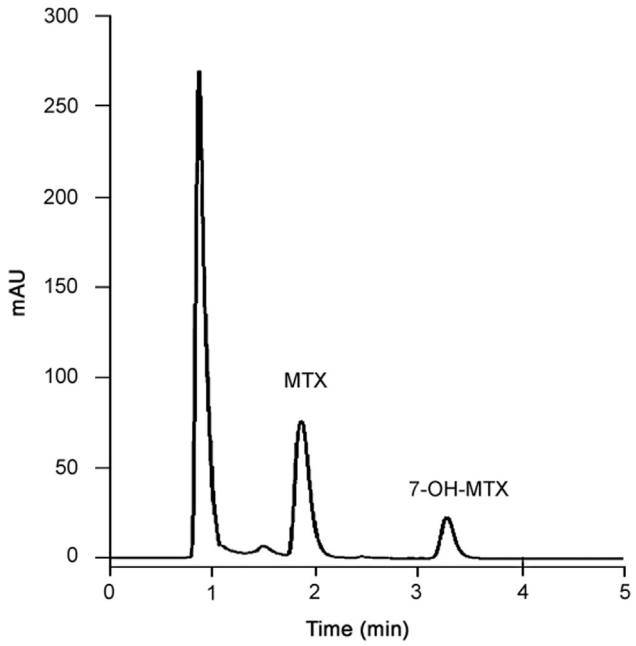

Fig. 3. Chromatogram of a pooled plasma sample obtained under the optimized conditions.

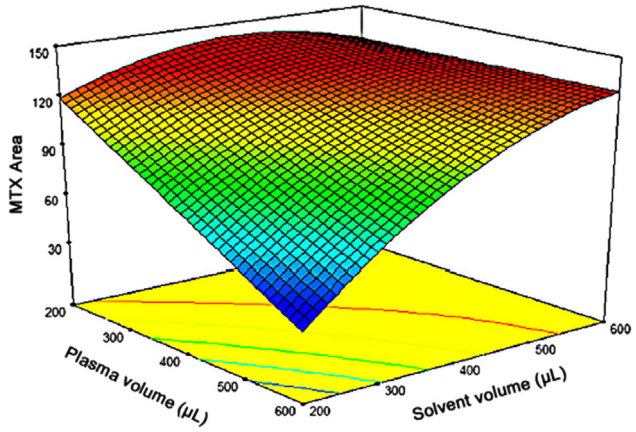

Fig. 4. Response surface for MTX area as a function of plasma volume and extraction solvent volume.

consisted in the addition of 300 μL of ACN to 150 μL of plasma for protein precipitation. Precipitated proteins were separated by centrifugation and 350 μL of deproteinized plasma were transferred to a new eppendorf tube. 300 μL of chloroform was added for back-extracting ACN. Finally, 50 μL of the aqueous extract was transferred to a vial for injection.

3.3. Method validation

3.3.1. Selectivity

The study of selectivity was carried out by evaluating four kinds of interferences, i.e., those produced by the blank matrix, metabolites, hemolysis and co-administrated medication. For the first case, six individual plasma samples from people who were not treated with MTX were tested. For metabolites and hemolysis studies, three plasma samples and three whole blood samples from people under MTX treatment were evaluated. The drugs selected as the co-administrated medication were folic acid, leucovorin, dexamethasone, mercaptopurine, ondansetron,

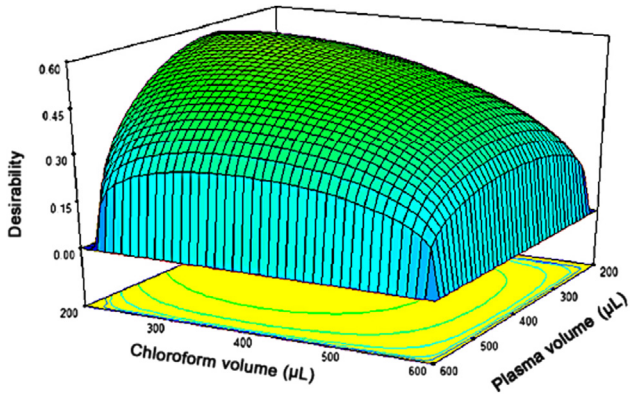

Fig. 5. Response surface of the global desirability as a function of solvent volume and plasma volume.

vancomycin and vincristine. These drugs are often simultaneously administered during the treatment with MTX and their interference needs to be tested.

The presence of any peak overlapped with the peak of MTX was studied. Except for folic acid, which has an absorption spectrum similar to that for MTX, the co-administered medication neither absorbs at the wavelength employed for MTX detection, nor produces strongly retained peaks, thus they do not interfere. The chromatographic run for the plasma spiked with folic acid showed its signal in the front peak, which is away from the retention time of MTX (Fig. 6).

For the rest of the tests, no peak with retention time similar to that expected for MTX was found. Therefore, it can be stated that the method is selective for the analyte under study.

3.3.2. LLOQ

The peak area for 0.05, 0.10 and 0.20 μM of MTX was obtained and the recovery and coefficient of variation (CV) were calculated for each one of them. The lowest concentration of MTX that satisfied the EMA specifications for precision and accuracy was 0.10 μM, for which a CV of 11% and a recovery of 89.6% were obtained. Therefore, this concentration was established as the LLOQ.

3.3.3. Calibration curve performance

The studied range for MTX concentration in plasma was from 0.10 μM to 6.0 μM at seven levels in triplicate, and the linear relationship between the concentration of MTX and the area of the peak was proven. Two calibration curves in the complete range on two different days, and a low level calibration curve in the range of 0.10–1.5 μM were performed. The back calculated concentrations of the calibration standards were within ± 15% of the nominal value, meeting the EMA criteria (Table 5).

3.3.4. Accuracy and precision

Accuracy and precision of the method were evaluated using five replicates of QC samples at LLOQ (QC-LLOQ), low (QC-L), middle (QC-M) and high (QC-H) concentration levels. The QC samples were analyzed against the calibration curve, and the obtained concentrations were compared with the nominal value. The CV was calculated for the replicates to evaluate precision. Both accuracy and precision were studied within a single run and between different runs; each of them was compared to the two calibrations previously performed. The results are shown in Table 6.

The obtained mean concentration was within 15% of the nominal values for the QC samples, except for the LLOQ which was within 20% of the nominal value. The CV values did not exceed 15% for the QC samples. These results met the EMA criteria; therefore, the accuracy and precision were demonstrated for the developed method.

3.3.5. Dilution integrity

Dilution integrity was demonstrated by spiking the blank matrix with an MTX concentration above the maximum concentration of the calibration curve and diluting this sample with blank matrix. For this purpose, a set of plasma samples were prepared containing 12.5, 25.0 and 62.5 μM of MTX. The dilution control samples were obtained by diluting 10 μL of these samples in

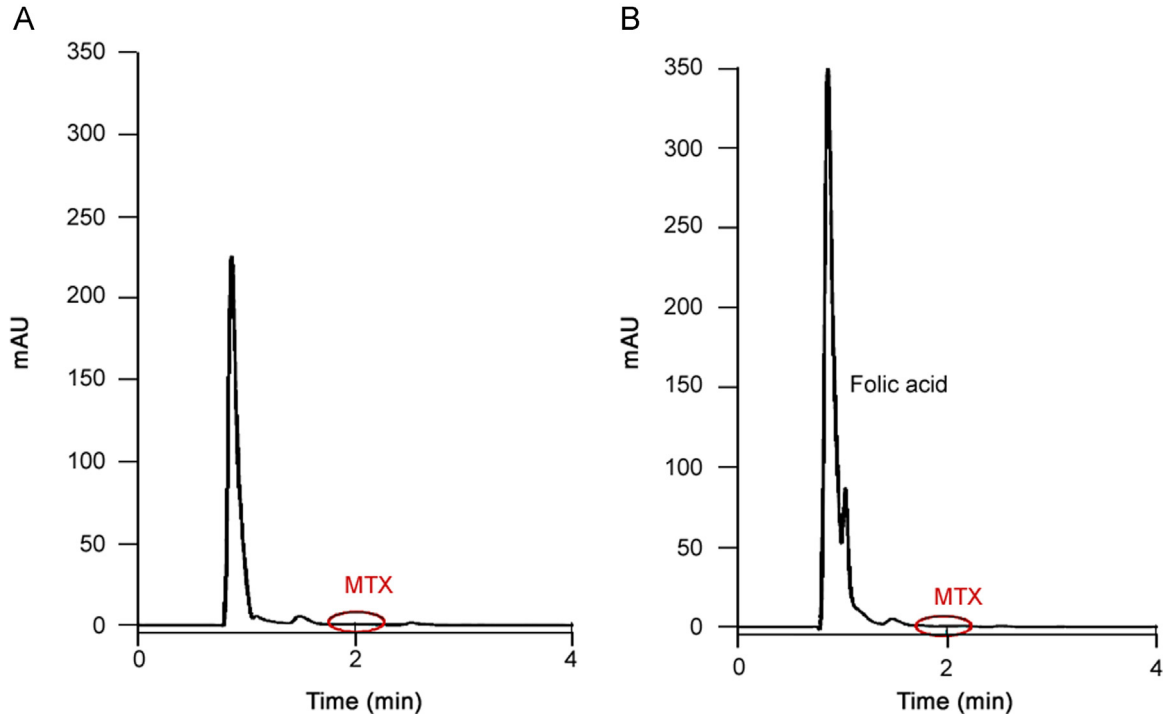

Fig. 6. Chromatograms obtained with the optimized conditions for (A) blank plasma and (B) blank plasma spiked with folic acid.

Table 5
Calibration curve performance.

Nominal[a]	Predicted[a]	Recovery (%)[b]	Predicted[a]	Recovery (%)[c]
0.102	0.109	106.9[d]	0.086	84.3[e]
	0.107	104.9[d]	0.091	89.2[e]
	0.103	101.0[d]	0.082	80.4[e]
0.510	0.503	98.6[d]	0.583	114.3[e]
	0.497	97.5[d]	0.540	105.9[e]
	0.482	94.5[d]	0.538	105.5[e]
1.021	1.018	99.7[d]	0.978	95.8[e]
	0.962	94.2[d]	0.957	93.7[e]
	0.932	91.3[d]	0.996	97.6[e]
1.531	1.522	99.4[d]	1.528	99.8[e]
	1.550	101.2[d]	1.528	99.8[e]
	1.531	100.0[d]	1.581	103.3[e]
2.042	2.021	99.0	2.166	106.1
	1.967	96.3	2.043	100.1
	1.934	94.7	1.967	96.3
4.083	4.081	100.0	4.287	105.0
	3.847	94.2	4.086	100.1
	3.736	91.5	4.239	103.8
6.125	6.077	99.2	5.495	89.7
	5.941	97.0	6.245	101.9
	6.026	98.4	6.424	104.9

[a] MTX concentration in μM.
[b] Calibration 1: slope=49.6; intercept=0.4.
[c] Calibration 2: slope=50.2; intercept=−0.9.
[d] Low level calibration 1: slope=52.9; intercept=−2.3.
[e] Low level calibration 2: slope=48.9; intercept=−0.75.

250 μL of blank plasma in order to obtain the final concentrations of 0.5, 1.0 and 2.5 μM. Five determinations per dilution factor were made. The obtained mean concentration was within 10% of the nominal values and the CV values did not exceed 1%.

These results show that when the expected MTX concentration exceeds the calibration range, a proper dilution can be made to obtain reliable results that are not affected by the sample treatment.

3.3.6. *Metabolism profiles in patients under treatment*

Several treatment protocols are currently used for high-dose MTX therapy including leucovorin administration to prevent the injury of normal cells. According to one of them, the dosage of MTX is performed routinely at 24, 42 and 48 h after starting the drug infusion.

Normograms guiding the duration and degree of rescue with leucovorin as a function of plasmatic concentration of MTX at different times after starting the drug administration are being used in ongoing clinical trials [26].

The levels of MTX as a function of the time after infusion were studied in different patients with the developed analytical method.

The pattern of MTX concentration vs. time depends on the patients, their renal function and their own metabolism. In Fig. 7, two profiles of MTX, especially distinct one from the other, are shown. Fig. 7A depicts three profiles from different patients. At 24 h after infusion, the levels of MTX in plasma were between 50 and 90 μM, decreasing the concentration below 0.25 μM after 48 h in all of them. Standard leucovorin rescue treatment was applied in these cases. Fig. 7B shows a completely different situation, in which the concentration of MTX after 48 h of infusion was 3.90 μM, which is 10 times higher than the value established by the protocol as acceptable. In this case, not only leucovorin rescue treatment was sustained, but also the dosage of MTX continued

Table 6
Accuracy and precision.

QC	Nominal[a]	Predicted[a]		Recovery (%)		CV (%)	
		Within-run	Between-run	Within-run	Between-run	Within-run	Between-run
QC-LLOQ	0.100	0.109	0.073	109.0	73.0	13.4	10.3
		0.101	0.081	101.0	81.0		
		0.088	0.086	88.0	86.0		
		0.099	0.090	99.0	90.0		
		0.103	0.082	103.0	82.0		
QC-L	0.299	0.310	0.258	103.7	86.3	9.6	6.9
		0.254	0.233	84.9	77.9		
		0.303	0.271	101.3	90.6		
		0.267	0.246	89.3	82.3		
		0.303	0.252	101.3	84.3		
QC-M	2.990	3.203	2.727	107.1	91.2	0.94	2.6
		3.122	2.892	104.4	96.7		
		3.185	2.741	106.5	91.7		
		3.170	2.787	106.0	93.2		
		3.168	2.856	109.2	95.5		
QC-H	4.486	4.736	4.608	105.6	102.7	0.72	2.2
		4.674	4.514	104.2	100.6		
		4.738	4.516	105.6	100.7		
		4.700	4.721	104.8	105.2		
		4.758	4.707	106.1	104.9		

[a] MTX concentration in μM.

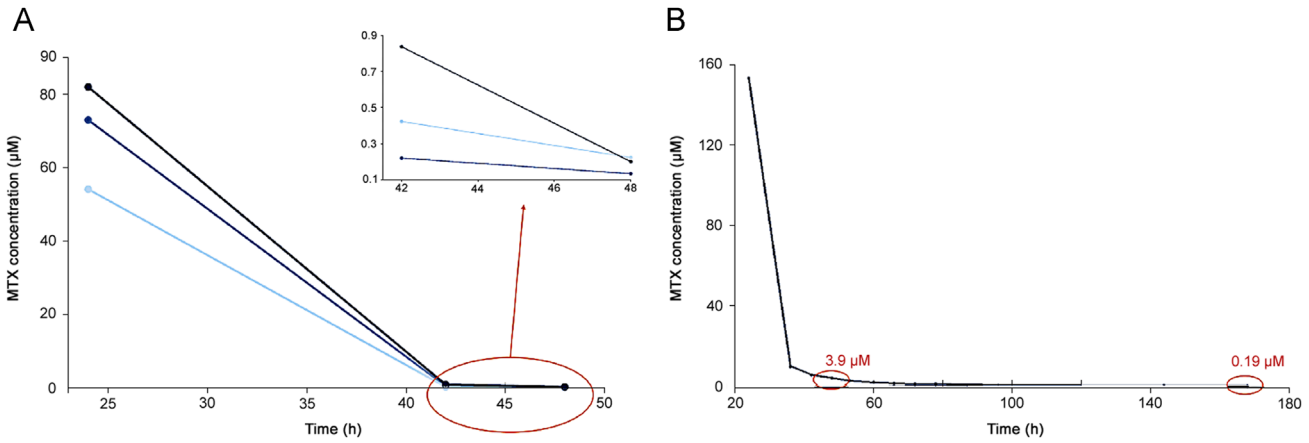

Fig. 7. Metabolism profiles of MTX.

until the concentration of MTX found in plasma was below 0.25 μM. This concentration, necessary to end the administration of leucovorine, was reached only at 168 h after the infusion, when 0.19 μM of MTX in plasma was detected.

The TDM gains importance in these kinds of situations, since the patient could have been exposed to high levels of MTX for a long period of time if the monitoring had not been performed, causing serious damage not only to neoplastic cells but also to other tissues. Furthermore, it is worth highlighting the importance of having an accurate method able to detect and quantify low levels of the drugs in a short time and give reliable results that allow making the proper medical decisions.

4. Conclusions

The developed method over this work for the determination of MTX in plasma by HPLC is fast, selective, accurate and precise, and allows us to obtain reliable results for the drug concentration in plasma samples.

The application of experimental design and multiple response optimization enabled the development of a chromatographic method with a complete run time of 4 min. In addition, the extraction step allows a simple and high analyte recovery from the sample, obtaining an aqueous extract free from proteins and lipids that could damage the chromatographic column. The small volume of plasma required for the analysis constitutes an additional advantage, especially when the samples are from pediatric patients.

The use of core–shell particles, instead of their fully porous counterparts, was an important additional tool for reducing both the analysis time and the volume of organic solvents employed during the runs.

This method was successfully applied to the routine therapeutic drug monitoring of MTX in patients with ALL, in order to prevent excessive toxicity. Furthermore, it could be applied to the TDM of new “targeted therapies” and in the development of innovative approaches to treatment individualization.

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