Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 919–925
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Methotrexate determination in pharmaceuticals by enantioselective HPLC

D. Abd El-Hady[b], N. Abo El-Maali[b], R. Gotti[a], C. Bertucci[a], F. Mancini[a], V. Andrisano[a,*]

[a] *Dipartimento di Scienze Farmaceutiche, Via Belmeloro 6, Universita di Bologna, 40126 Bologna, Italy*
[b] *Department of Chemistry, Faculty of Science, Assiut University, 71516 Assiut, Egypt*

Received 20 May 2004; received in revised form 13 July 2004; accepted 16 July 2004
Available online 18 November 2004

Abstract

A simple, sensitive and selective high performance liquid chromatographic method with UV detection for the chiral separation of racemic methotrexate (*rac*-Mtx) and enantiomeric purity of L-methotrexate in pharmaceutical formulations was developed and validated. The chiral separation was optimized studying both the nature of the stationary phase by using Chirobiotic T^{TM}, Chiracel OJ and human serum albumin columns and the effect of the mobile phase composition. The best results in terms of enantioresolution and enantioselectivity were achieved with a polar organic mobile phase on Chirobiotic T^{TM} stationary phase. Essential steps in method validation such as precision, accuracy, suitability and stability were studied according to ICH guidelines. At wavelength 303 nm, the limit of detection (S/N = 3) was found to be 0.9 μg/ml for *rac*-Mtx. The separation of D-Mtx at 0.2% (w/w) level (as limit of quantitation) from the main drug L-Mtx was successfully obtained with 1.72 enantioresolution value. Enantiomeric purity of L-Mtx was determined in pharmaceutical formulations (tablets and injections) with inter- and intra-days relative standard deviation ≤ 1.6%. Under the validated stereoselective HPLC conditions for methotrexate, folic acid was also analysed.

Keywords: Methotrexate; Enantioselective HPLC; Chirobiotic T^{TM} stationary phase; Pharmaceutical formulations

1. Introduction

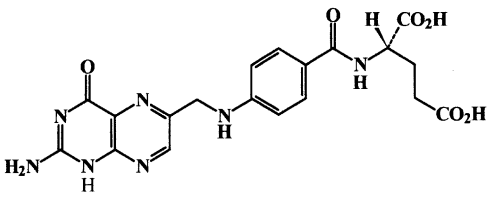
L-Methotrexate (L-Mtx) is a drug included into the antineoplastic and antireumatic therapeutic categories. It belongs to antifolates which produced the first striking, although temporary remission in leukemia and the first cure of a solid tumor, choriocarcinoma [1–5]. D-Mtx can be present as impurity from raeemization of the synthetic L-Mtx product or during the shelf life of the pharmaceutical product, due to improper storage conditions [6,7]. It was found that the pharmacokinetics of D-Mtx is different from that of L-Mtx. Although D-Mtx exhibits similar dihydrofolate reductase (DHFR) inhibitory effect, yet D-Mtx is much less toxic (40-fold lower LD_{50}) and its antitumor effect is proportionately reduced [6]. Many analytical methods have been reported to analyze Mtx in pharmaceutical formulations and in biological fluids by using LC [8–14], capillary zone electrophoresis [15,16], spectrophotometric [17,18] and voltammetric techniques [19–23]. However, there are few reported analytical methods which discriminate between L- and D-enantiomers of Mtx [15,24,25]. These methods suffer from long time analysis, pre-step derivatization or not satisfactory resolution for enantiomeric purity estimation in pharmaceutical formulations.

The aim of this work was to develop and validate a stereoselective HPLC method with UV detection based on the use of a chiral stationary phase for the direct enantioselective analysis of Mtx in pharmaceutical formulations such as tablets and injections. Concerning the optimization of the stereoselective chromatographic resolution, some chiral stationary phases were tested: Chirobiotic T^{TM}, Chiracel OJ and human serum albumin (HSA) columns under several chromatographic conditions in direct and reversed phase. Under the optimised chromatographic conditions, the method validation was performed and the two Mtx enantiomers

* Corresponding author. Tel.: +39 051 2099742; fax: +39 051 2099734.
E-mail address: vincenza.andrisano@unibo.it (V. Andrisano).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.07.046

Folic acid



L-Methotrexate

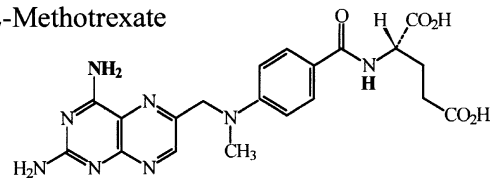


Fig. 1. Structures of the drugs investigated.

identified and determined in pharmaceutical formulations such as tablets and injections.

Moreover, as there is no reports in literature for the simultaneous quantitation of Mtx and folic acid (FA), which is often administer together with Mtx, the method was also validated for FA. In Fig. 1 the structures of Mtx and FA are shown. Low levels of FA may play a role in cancer development, particularly cancers of the cervix, lung, and colon [2]. It was found that patients who took folic acid supplements along with the arthritis drug methotrexate were less likely to have a malfunctioning liver and less side effects than those taking just methotrexate [3–5].

2. Experimental

2.1. Materials

DL-Amethopterin hydrate (*rac*-Mtx), L-Amethopterin (L-Mtx) and D-Amethopterin (D-Mtx) were purchased from Aldrich (Milano, Italy). *N*-[4-{[(2,4-diamino-6-pteridinyl)-methyl]methyl amine}benzoyl] L-glutamic acid (folic acid) was obtained from Fluka. Methanol (ultrapure for HPLC), acetonitrile 200 (for UV), *n*-hexane (95%), isopropanol (ultrapure for HPLC) and sodium hydroxide were obtained from Carlo Erba (Milan, Italy). Triethylamine (99%) was purchased from Sigma and acetic acid (99.7%) was obtained from Aldrich (Germany). The pharmaceutical formulations (tablets and injections, Wyeth Lederle S.P.A.) were locally purchased from pharmacies (Bologna, Italy). All the other chemicals were of analytical reagent grade (Carlo Erba Reagenti, Milano, Italy) and were used without further purification. Water used for the preparations of solutions and mobile phases was purified by a Milli-Rx apparatus (Millipore, Milford, MA, USA). All organic solvents were used without further purifications.

2.2. Apparatus

Separations on Chirobiotic T^{TM} (teicoplanin) HPLC stationary phase (250 mm × 4.6 mm i.d., Astec, Whippany, NJ, USA) and Chiralcel OJ [250 mm × 4.6 mm i.d., Daicel Chemical Industries, Ltd., column, cellulose tris(4-methylbenzoate)] were carried out with a Beckman 110 B solvent delivery system, a Rehodyne injection valve and a Beckman System Gold 166 Detector. Data were collected with Gold Nouveau Chromatography Data System, Version 1.6 (Beckman Instruments Inc.). A constant mobile phase flow rate (0.8 ml/min) was provided and UV detection was carried out at 303 nm. Samples were injected using loop injections (5 μl) and chromatographic analyses were performed at room temperature (20 ± 2.0 °C).

The chromatographic analyses related to the use of HSA chiral stationary phase (150 mm × 4.6 mm i.d.) (Shandon Scientific, Runcorn, UK) containing HSA immobilized on a 7 μm diol silica support, were carried out using the solvent delivery system (Jasco PU-980 Intelligent HPLC pump) equipped with Rehodyne Model 7125 injector with a 20 μl sample loop. The eluents were monitored by Jasco MD 910 Multi-wavelength Detector (DAD) connected to a computer station. For routine analysis the detector wavelength was set at 303 nm. The temperature was maintained constant at 30 °C using a column heater/chiller (Model 7955, Jones Chromatography Ltd., UK).

The pH values of the mobile phases were adjusted by using a pH-meter (basic 20, Crison) at 20 ± 2.0 °C. This instrument was calibrated by using standard universal buffer solutions at different pHs.

2.3. Chromatographic conditions

The mobile phases for Chirobiotic T^{TM} HPLC column were composed of different percentages (0.1, 0.2, 1.0, and 2.0%) of acetic acid and triethylamine in the presence of 100% methanol (polar organic phase). Under the reversed phase mode, the mobile phases contained either water or 1.0% triethylamine acetate (TEAA) buffer with different amounts of methanol or acetonitrile as organic modifiers. The buffer was prepared by dissolving 2.0 ml triethylamine in water; glacial acetic acid was added to adjust the appropriate pH value before bringing the volume to 200 ml in a volumetric flask.

The mobile phases used for Chiralcel OJ column consisted of isopropanol and *n*-hexane (80:20) (v/v) as normal phase mode and acetonitrile in combination with water in reversed phase mode. All mobile phases were filtered through a 0.45 μm membrane filter and degassed before using for HPLC analysis.

The chromatographic conditions for chiral separation of *rac*-Mtx. on HSA chiral stationary phase consisted of phosphate buffer (50 mM, pH 7.4):*n*-propanol (97.5:2.5 or 99:1) (v/v). Sample solution at 0.1 mg/ml concentration were injected.

2.4. Calibration curves

2.4.1. Standard stock solutions

Standard stock solutions of *rac*-, L-, D-Mtx and folic acid (1.0 mg/ml) were dissolved in 0.1 M sodium hydroxide and were stored at 4 °C. Daily required volumes of each analyte stock solutions were diluted in the same solvent to prepare the standard working solutions.

2.4.2. rac-Mtx and folic acid calibration curves

Working solutions were prepared by diluting the stock solution with the same solvent to obtain concentrations ranged within 3–250 μg/ml of both *rac*-Mtx and folic acid. Each solution was stirred for about 30 s, sonicated for about 5.0 min and then filtered before injection into the HPLC system. Duplicate injections were performed for each solution. Optimized chromatographic conditions involved the use of Chirobiotic T^{TM} column with a mobile phase composed of 0.2% acetic acid and 0.1% triethylamine in methanol (polar organic phase), flow rate 0.8 ml/min at 20 °C with UV detection at 303 nm. For obtaining the calibration graphs, the analyte peak heights were plotted against the correspondent analyte concentrations (μg/ml).

2.4.3. Standard addition of D-Mtx

Standard stock solutions of D-Mtx (1000 μg/ml) and L-Mtx (2625 μg/ml) were prepared in 0.1 M sodium hydroxide.

Five calibration solutions were prepared by mixing 200 μl of L-Mtx solution with 10 μl of diluted working solutions of D-Mtx to give D-Mtx concentrations ranged from 5 to 62.5 μg/ml thus corresponding to 0.2–2.5% (D-/L-Mtx) (w/w) in the presence of a constant amount of L-Mtx (525 μg). The solution containing 0% of D-Mtx was also prepared by mixing 200 μl of L-Mtx solution with 10 μl of 0.1 M sodium hydroxide. Each solution was stirred for about 30 s, sonicated for about 5 min and then filtered before injection into the HPLC system. Chromatograms were acquired by using the HPLC chromatographic conditions as reported in Section 2.4.2. The standard addition calibration graph was obtained by plotting the peak height of D-Mtx against the percentage of D-Mtx.

2.5. Stereoselective analysis of pharmaceutical formulations

The developed method was applied to the analysis of commercially available formulations (tablets and injections). For the analysis of the distomer D-Mtx, solutions from tablets were prepared by grinding 13 tablets in a mortar; aliquots of 100 mg corresponding to the average weight of all the 13 tablets were accurately weighed and dissolved in 0.1 M sodium hydroxide solution by stirring and sonicating for 5 min at room temperature, in order to obtain final sample solutions which contained about 2500 μg/ml of *rac*-Mtx as declared by the manufacturer. Injection solutions were prepared by mixing 200 μl of the commercial formulations with 400 μl of 0.1 M sodium hydroxide, stirring, sonicating and filtrating; similarly to the samples extracted from the tablets, the final concentration of *rac*-Mtx was about 2500 μg/ml.

The analysis of L-Mtx in both tablets and injections was carried out on the same sample solutions previously obtained after a 30 times dilution with 0.1 M sodium hydroxide.

The content of each enantiomer was determined by using external standard working solutions from pure reference compounds.

3. Results and discussion

A large number of drugs with one stereogenic center has been resolved using macrocyclic antibiotic and polysaccharide based stationary phases [31,34–37]. Macrocyclic antibiotics represent a relatively new class of chiral selectors, which have been introduced by Armstrong et al. [26–28]. One of the recent selectors is Chirobiotic T^{TM} (teicoplanin) phase. This column is packed with a chromatographic stationary phase produced by chemically bonding the macrocyclic glycopeptide teicoplanin to a 5 μm silica matrix through a spacer arm, which yields several ether linkages that are hydrolytically stable. Teicoplanin is considerably surface active having three sugar moieties surrounding four cavities and a methyl dodecyl side chain. All of the defined mechanisms for chiral recognition of this kind of stationary phase are contained within this structure. These include complexation, hydrogen bonding, dipole stacking and steric interaction as well as the inclusion mechanism typical of cyclodextrins.

Another type of chiral selector used in search of optimal enantioselective chromatographic separation is Chiracel OJ, one of the cellulose derivative-based phases [39]. These phases introduced by Ichida et al. [29] and Okamoto et al. [30] found application to the chiral separation of a broad range of compounds with carboxylic groups [31–33]. Therefore, the study of the chiral recognition of cellulose tris(4-methyl benzoate) (Chiracel OJ), for the separation of Mtx enantiomers and for the analysis of folic acid was thought to be promising. For these reasons, the enantiomeric separation of *rac*-Mtx was investigated on Chirobiotic T^{TM} and Chiralcel OJ columns. Finally a HSA based chiral stationary phase was employed because the enantioselective separation of *rac*-Mtx was reported to occur by using bovine serum albumin [25]. Further this column was employed in the view of performing binding studies on human serum albumin (HSA), which plays a major role in the plasma binding of neutral or acidic drugs. This binding is often stereoselective with respect to chiral molecules and can affect the pharmacokinetic and pharmacodynamic profiles of each enantiomer. The Mtx enantioselective binding to HSA will allow further drug–drug interaction studies.

Table 1
The effect of acid–base ratio in a new polar organic mode on the separation of *rac*-Mtx and folic acid on a Chirobiotic T^{TM} stationary phase

Acetic acid/TEA	Methotrexate		Folic acid		
	k_1	k_2	α	R_s	k
1/2	4.8	4.8	–	–	0.6
0.1/0.2	2.9	2.9	–	–	0.7
1/1	4.1	4.7	1.1	2.3	0.4
0.1/0.1	1.7	2.2	1.3	2.5	0.6
2/1	2.0	2.5	1.2	3.0	0.2
0.2/0.1	2.2	3.6	1.6	4.1	0.2

k_1: capacity factor of first eluting enantiomer; k_2: capacity factor of second eluting enantiomer; α: enantioselectivity value, R_s: enantioresolution value; t_r: retention time [$k = t_r - t_0/t_0$, $\alpha = k_2/k_1$, $R_s = 2(t_2 - t_1)/(W_1 + W_2)$].

3.1. Stereoselective rac-Mtx separation

3.1.1. Stereoselective separation of rac-Mtx on the teicoplanin-based chiral stationary phase (Chirobiotic T^{TM})

The stereoselective separation of *rac*-Mtx and FA was first approached on teicoplanin-based stationary phase by using a polar organic mode and the reversed phase mode. The polar organic mode was originally developed as a novel way to obtain difficult enantioselective separations on cyclodextrin-based phases, while suppressing inclusion complex formation [38]. Therefore this mode was approached due to the presence of strong polar groups in the macrocyclic peptides and the presence of more than one functional group in Mtx and FA capable of interacting with the chiral selector (some of these groups are near or on the stereogenic center). The key factor in obtaining complete separation with the polar organic phase was found to be the acid/base ratio. The concentrations of acid and base were varied from 2.0 to 0.1% in methanol. In fact, above 2% the mobile phase becomes too polar and the system assumes typical reversed phase behaviour, whereas below 0.1% it behaves as in normal phase chromatography [38]. No separation of *rac*-Mtx enantiomers in the presence of 1/2 acid/base ratio occurred, while increasing the acid percentage, enantioselectivity and enantioresolution of *rac*-Mtx increased. Table 1 shows the influence of percentage variations of both acetic acid and triethylamine on the enantioselectivity (α) and enantioresolution (R_s) of *rac*-Mtx and for the separation of FA at room temperature with a constant flow rate of 0.8 ml/min. It was found that the best selectivity with good resolution and acceptable retention times were achieved with 0.2% acetic acid and 0.1% triethylamine in MeOH as mobile phase.

In Fig. 2A and B the reported *rac*-Mtx chromatographic enantioseparations were pursued by using two different acid/base ratios in the mobile phase. A better resolution due to increased efficiency was achieved by using as mobile phase a mixture consisting of 0.2% acetic acid/0.1% TEA in methanol, the two enantiomers being eluted with retention times of 15.4 and 22.4 min, respectively (Fig. 2B). In the same conditions, the simultaneous separation of folic acid was performed: its retention time decreased from 7.1 min

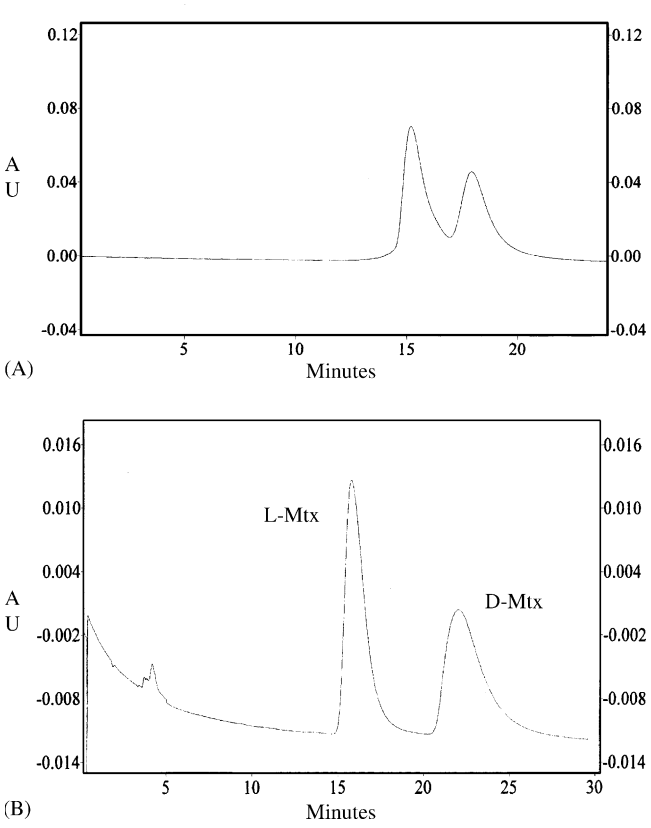


Fig. 2. (A) Chiral separation of *rac*-Mtx. Chromatographic conditions: Chirobiotic T^{TM} (250 mm × 4.6 mm i.d.) stationary phase, with a mobile phase consisting of acetic acid, TEA, methanol (0.1:0.1:100) (v/v), 5 μl injection, flow rate 0.8 ml/min at 20 °C with UV detection at 303 nm. (B) Chiral separation of *rac*-Mtx. Chromatographic conditions: Chirobiotic T^{TM} (250 mm × 4.6 mm i.d.) stationary phase, with a mobile phase consisting of acetic acid, TEA, methanol (0.2:0.1:100) (v/v), 5 μl injection, flow rate 0.8 ml/min at 20 °C with UV detection at 303 nm.

(1/2 acid/base ratio) to 5.0 min at 2/1 acid/base ratio. Therefore, a satisfactory separation between FA and the Mtx enantiomers was achieved for the simultaneous determination of both drugs under the above conditions.

By using a reversed phase mode on antibiotic stationary phases, it was reported that the nature of organic modifiers affects the selectivity of separation and the presence of buffers increases the efficiency and the resolution [38]. Therefore, the enantioseparation of *rac*-Mtx and FA with mixtures consisting of 20% methanol and 10% acetonitrile and water or TEA buffer as mobile phases was investigated.

The observed enantioselectivity and enantioseparation values obtained by using a mixture consisting of methanol and water (20:80) (v/v) as a mobile phase at room temperature at a flow rate of 0.8 ml/min. (R_t = 14.4 and 19.8 for L- and D-Mtx, respectively), were found less satisfactory than the values under the optimized new polar organic phase conditions. Replacing methanol with acetonitrile, the enantioselectivity of Mtx enantiomers was reduced with a concomitant loss of retention. The influence of TEA buffer (instead of water), pH values as well as type and content of organic modifier

Table 2
The effect of mobile phase composition in a reversed phase mode on the separation of *rac*-Mtx and folic acid on a Chirobiotic T^{TM} stationary phase

Composition	Methotrexate		Folic acid		
	k_1	k_2	α	R_s	k
20% MeOH + 80% water	2.3	3.7	1.6	2.6	1.7
20% MeOH + 80% TEA buffer (pH 4.0)	More than 50 min	–	–	–	0.4
10% AcCN + 90% water	0.2	0.3	1.7	1.1	–
10% AcCN + 90%TEAA buffer (pH 4.0)	More than 50 min	–	–	–	–

(methanol or acetonitrile) on *rac*-Mtx separation were also tested. It was found that the separation of enantiomers was not achieved till 50 min at pH 4.0, using methanol or acetonitrile as organic modifier. By increasing pH values to pH 7.0, the retention times were reduced to 35 min with bad enantioselectivity and enantioseparation values. The effect of mobile phase composition on the enantioseparation of Mtx and FA is reported in Table 2.

3.1.2. Stereoselective separation of rac-Mtx on the cellulose-based chiral stationary phase

Chiral separation of Mtx was performed on a cellulose tris(4-methylbenzoate) phase (Chiralcel OJ) in normal phase and reversed phase modes. The separation of folic acid was also studied using the two chromatographic modes. Mtx and FA were not resolved up to a retention time of 50 min by using mobile phases consisting of mixtures of isopropanol and *n*-hexane in various relative percentages. Under the reversed mode conditions, mixtures of acetonitrile in combination with water were employed, but Mtx enantiomers were poorly separated with long retention time (above 45 min), with folic acid being eluted at about 35 min. Therefore, the enantioselective separation of Mtx was not satisfactorily obtained on Chiracel OJ when compared with Chirobiotic T^{TM} phase.

3.1.3. Stereoselective separation of rac-Mtx on HSA chiral stationary phase

The stereoselective separation of *rac*-Mtx and FA on HSA stationary phase was obtained by using mixtures containing *n*-propanol and phosphate buffer, pH 7.4 at 30 °C. The capacity factors values at 1.0% *n*-propanol were found to be $k_1 = 2.9$, $k_2 = 3.8$ ($\alpha = 1.3$) and the values at 2.5% *n*-propanol were $k_1 = 2.4$, $k_2 = 2.6$ ($\alpha = 1.1$) for L- and D-Mtx, respectively. However, the enantioresolution was found lower than that obtained with the Chirobiotic T^{TM} stationary phase. The elution order of methotrexate enantiomers was established by the injection of each pure enantiomer under the same conditions and it was found that L-Mtx was less retained than D-Mtx.

3.2. Method validation

From preliminary trials to optimize the conditions for *rac*-Mtx and FA separation upon teicoplanin-based column, it was found that the polar organic mode using a mixture consisting of acetic acid:triethylamine:methanol (0.2:0.1:100) (v/v), at a flow rate of 0.8 ml/min at 20 °C gave the best enantioselectivity and enantioseparation values with suitable short retention times. Therefore, under these optimal conditions the method was validated and applied to the enantioselective analysis of D- and L-Mtx in tablets and injections commercially available samples.

3.2.1. Elution order of Mtx enantiomers

The elution order of Mtx enantiomers was determined by injecting L- and D-Mtx standard solutions; D-Mtx was retained strongly than L-Mtx, which indicates that the diastereomeric D-Mtx-teicoplanin complex is more stable than the L-Mtx one. Under the optimized conditions the enantioresolution evaluated in a non-racemic mixture containing D-Mtx at 0.2% (w/w) level was about 1.8.

3.2.2. Linearity

The linearity of response was studied on six different standard solutions of both *rac*-Mtx and FA in the concentration range of 3–250 and 6–300 μg/ml, respectively; these concentration ranges resulted suitable to test the linearity at the levels normally observed for Mtx and folic acid in pharmaceutical formulations and biological matrices.

The calibration graphs were constructed by plotting the normalized peak-heights (*Y*, AU) versus the correspondent analyte concentrations (*X*, μg/ml); the obtained relationships are shown in Table 3. The reported statistical data represent the average correlation coefficient, slope and intercept for three separated standard curves obtained in three different days. Good linearity was therefore obtained even in the absence of internal standard.

3.2.2.1. Standard addition of D-Mtx. As a further confirmation of the method linearity, the response of D-Mtx as a function of its concentration was evaluated in the presence of a high and constant amount of the drug L-Mtx. Due to the presence of the distomer D-Mtx in the pharmaceutical preparations of L-Mtx (tablets and injections) and standard reference compound, these experiments were carried out by means of the standard addition method.

Precisely, five working solutions with a fixed L-Mtx amount (2500 μg/ml) and D-Mtx concentration ranged from 5 to 62.5 μg/ml, thus correspondent to 0.2–2.5% (D-/L-Mtx) (w/w), were subjected to enantioselective HPLC analysis as described in Section 2.4.2. The average peak heights (*Y*, AU)

Table 3
Linearity data obtained for Mtx and folic acid ($n = 6$)

Analyte	Concentration range (∝g/ml)	Slope	Slope S.D.	Intercept	Intercept S.D.	S.E.	R^2
L-Mtx	1.5–125	37.5763	0.1252	−0.0172	0.0152	0.0274	0.9999
D-Mtx	1.5–125	12.5478	0.0641	−0.0004	0.0077	0.0303	0.9999
Folic acid	6–300	11.0034	0.0259	0.0025	0.0112	0.0156	0.9998

obtained as difference from each calibration point and the unspiked reference sample were plotted against the correspondent percentage concentrations of D-Mtx (X%) (w/w); the results of the linear regression analysis ($n = 5$) indicate that the standard deviation (S.D. ≈ 0.0017) with correlation coefficient ($r^2 \approx 0.9986$) between Y and X is attainable over the studied range (slope = 0.14364 ± 0.00338; Y intercept = −0.00499 ± 0.00471)

3.2.3. Sensitivity

The method sensitivity was evaluated by progressive dilution of a standard mixture of *rac*-Mtx and folic acid and detecting the signal at the wavelength of 303 nm. *Rac*-Mtx and FA solutions (0.9 and 3.8 μg/ml, respectively) provided a signal-to-noise ratio of approximately 3 (limit of detection; LOD). Due to the importance of evaluating the presence of D-Mtx as chiral impurity of L-Mtx, the limit of quantitation of the distomer (D-Mtx) was evaluated by analysing a mixture of L-/D-Mtx 2500/5.0 μg/ml thus correspondent to 0.2% (w/w) of the distomer. The R.S.D.% of the peak height obtained by 10 replicated injections resulted to be 3.15%; this value of D-Mtx concentration can be considered as an adequately low limit of quantitation.

3.2.4. Retention time and peak height repeatability

Multiple injections from a single sample solution were performed to demonstrate the repeatability of the migration time and the peak height under constant conditions of solvent composition, temperature and flow rate. Ten replicate runs of a standard mixture L-/D-Mtx (2500/5.0 μg/ml) (LOQ) were carried out in three different days. The obtained retention times and peak heights R.S.D.s were found to be 1.01 and 1.08% for L-Mtx and 1.85 and 3.15% for D-Mtx, respectively, indicating an adequate instrumental precision at impurity level.

3.2.5. Accuracy, precision and applications

As the applied HPLC separation can be considered adequately selective, the accuracy of the whole analysis is substantially related to the extraction procedure efficiency.

This procedure was actually very simple, involving only a dilution step (analysis of injections) or liquid–solid extraction with sodium hydroxide in ultrasonic bath (analysis of tablets). A typical chromatogram for the analysis of Mtx in tablets is shown in Fig. 3.

The accuracy of the method was therefore evaluated for L- and D-Mtx taking as references highly pure standard compounds and by comparing their responses with those obtained

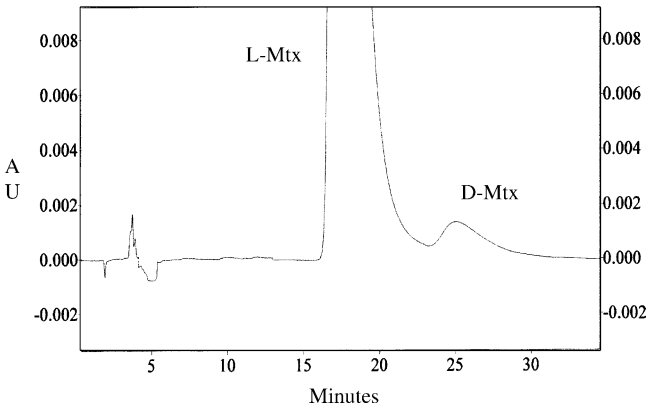


Fig. 3. Enantioselective HPLC chromatogram of a MTX sample solution obtained from commercial tablets. Peaks: (1) L-Mtx; (2) D-Mtx. See Fig. 2B for chromatographic conditions.

with different bulks supplied (tablets and injections). The sum of the found amounts of both D- and L-Mtx in each analysed sample was compared with the content of *rac*-Mtx declared by the manufacturer to estimate the recovery of the method; values in the ranges 99.5–101.2 and 99.8–101.6% for tablets and injections respectively were obtained. All the analytical values fall within labeled amount of 90–110% required by USPXXV [40].

In Table 4 the accuracy and precision data evaluated by reporting the percentage amount of the single enantiomers on the declared *rac*-Mtx are reported.

A further confirmation of the accuracy of the determination was obtained by evaluating the level of D-Mtx in the analysed pharmaceuticals, by means of interpolation with the standard addition curve. The found data were in agreement with those obtained with the single point calibration method as no significant differences were observed by applying the F- and t-test at the 95% confidence level.

Table 4
Data for the determination of L- and D-Mtx in pharmaceutical formulations

L-Mtx	Found[a] (%)	R.S.D.% (intraday) ($n = 3$)	R.S.D.% (interday) ($n = 9$)
Tablets	98.55	0.42	0.71
Injections	98.30	0.68	0.69
D-Mtx	Found[a] (%)	R.S.D.% (intraday) ($n = 3$)	R.S.D.% (interday) ($n = 9$)
Tablets	1.45	1.25	1.53
Injections	1.70	0.44	0.47

[a]Calculated as percentage of the declared content.

4. Conclusion

A simple, specific, sensitive, stable and relatively rapid HPLC method has been successfully developed for the stereoselective determination of L- and D-Mtx and for the simultaneous determination of folic acid. Both the optimization and validation of the method were performed and the obtained results demonstrated that the proposed stereoselective HPLC method is useful for the application to L-Mtx pharmaceutical formulations quality control. Further, the preliminary results obtained for the investigation of Mtx enantiomers and folic acid interactions with human serum albumin (HSA) will be followed by more specific studies to elucidate the Mtx enantiomer protein affinity, binding sites and drug interactions.

Acknowledgment

This work was supported by a grant from MURST (Cofin. 2002, Rome, Italy). Thanks are due to CIRB, University of Bologna, for the availability of the Beckman liquid chromatograph.

References

[1] J.R. Bertino, J. Clin. Oncol. 11 (1993) 5–14.
[2] Cancers, Nutrition and Food. World Cancer Research Fund/American Institute for Cancer Research, Washington, DC, 1997.
[3] G.K. Endresen, G. Husby, Tidsskr Nor Laegeforen 119 (1999) 534–537.
[4] Z. Ortiz, B. Shea, M.E. Suarez-Almazor, D. Moher, G.A. Wells, P. Tugwell, J. Rheumatol. 25 (1998) 36–43.
[5] O.K. Jensen, C. Rasmussen, F. Mollerup, P.B. Christensen, H. Hansen, S. Ekelund, A.M. Thulstrup, J. Rheumatol. 29 (2002) 1615–1618.
[6] S.M. Cramer, J.H. Schornagel, K.K. Kalghatgi, J.R. Bertino, C. Horvath, Cancer Res. 44 (1984) 1843–1846.
[7] J. Hendel, H. Brodthagen, Eur. J. Clin. Pharmacol. 26 (1984) 103–107.
[8] G. Rule, M. Chappie, J. Henion, Anal. Chem. 73 (2001) 439–443.
[9] J. Chaldek, L. Sispera, J. Martinkova, J. Chromatogr. B: Biomed. Sci. 744 (2000) 307–313.
[10] E.D. Lobo, J.P. Balthasar, J. Chromatogr. B: Biomed. Sci. 736 (1999) 191–199.
[11] R. Turci, G. Micoli, C. Minoia, Rapid Commun. Mass Spectrom. 14 (2000) 685–691.
[12] C. Wu, Z. Yang, G. Lin, T. Lu, Zhongguo Yiyuan Yaoxue Zazhi 19 (1999) 272–274.
[13] L. Skibinska, J. Gregorczyk, Chemia Analityczna 46 (2001) 329–336.
[14] R.S. Myers, P.T. Paton, A. Dastgah, J.R. Martell, C.E. Berkman, Anal. Biochem. 275 (1999) 187–191.
[15] C.Y. Kuo, H.L. Wu, S.M. Wu, Anal. Chim. Acta 471 (2002) 211–217.
[16] F. Sczesny, G. Hempel, J. Boos, G. Blaschke, J. Chromatogr. B: Biomed. Sci. 718 (1998) 177–185.
[17] X.-N. Chen, C.-X. Zhang, J.-R. Lu, Fenxi. Ceshi. Xueba. 19 (2000) 47–54.
[18] C.S. Sastry, J.S.V.M. Rao, Anal. Lett. 29 (1996) 1763–1778.
[19] Z.-J. Sun, J.-B. Hu, Y.-G. Qian, Y.-Q. Lu, Q.-L. Li, GaodengXuex. Huax. Xueb. 23 (2002) 541–545.
[20] J. Wang, P. Tuzhi, S. Meng, T. Tapia, Talanta 33 (1986) 707–712.
[21] M.A. Malone, A.C. Garcia, P.T. Blanco, M.R. Smyth, Anal. Meth. Instrum. 1 (1993) 164–171.
[22] A. Temizer, A.N. Onar, Talanta 35 (1988) 805–806.
[23] T.R.I. Cataldi, A. Guerrieri, F. Palmisano, P.G. Zambonin, Analyst 113 (1988) 869–873.
[24] R.-I. Stefan, R.G. Bokretsion, J.F. Van Staden, H.Y. Aboul-Enein, Talanta 60 (2003) 983–990.
[25] E. Brandsteterova, K. Marcincinova, J. Lehotay, A. Zbojova, J. Halko, Neoplasma 40 (1993) 241–245.
[26] D.W. Armstrong, Y.B. Tang, S.S. Chen, Y.W. Zhou, C. Bagwill, J.R. Chen, Anal. Chem. 66 (1994) 1473–1484.
[27] D.W. Armstrong, Y. Liu, K.H. Ekborg-Ott, Chirality 7 (1995) 474–497.
[28] T.J. Ward, A.B. Farris III, J. Chromatogr. A 906 (2001) 73–89.
[29] A. Ichida, T. Shibata, I. Okamoto, Y. Yuki, H. Namikoshi, Y. Toga, Chromatographia 19 (1984) 280–284.
[30] Y. Okamoto, K. Hatada, M. Kawashima, J. Am. Chem. Soc. 106 (1984) 5357–5359.
[31] E. Yashima, Y. Okamoto, Bull. Chem. Soc. Jpn. 68 (1995) 3289–3307.
[32] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020–1043.
[33] E. Yashima, J. Chromatogr. A 906 (2001) 105–125.
[34] E. Yashima, P. Sahavattanapong, Y. Okamoto, Chirality 8 (1996) 446–451.
[35] E. Yashima, H. Fukaya, Y. Okamoto, J. Chromatogr. A 677 (1994) 11–19.
[36] H.Y. Aboul-Enein, M.R. Islam, Biomed. Chromatogr. 5 (1991) 74–77.
[37] H.Y. Aboul-Enein, S.A. Bakr, Chirality 9 (1997) 10–12.
[38] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 13 (1999) 520–524.
[39] H.Y. Aboul-Enein, J. Chromatogr. A. 906 (2001) 185–193.
[40] US Pharmacopeia 25-National Formulary 20, Asian Ed., 2002, pp. 1111–1112.