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ORIGINAL ARTICLE

On-line coupling of derivatization with pre-concentration to determine trace levels of methotrexate

Samy Emara[a,*], Tsutomu Masujima[b], Walaa Zarad[a], Maha Kamal[c], Ramzia EL-Bagary[d]

[a]*Pharmaceutical Chemistry Department, Faculty of Pharmacy, Misr International University, Km 28 Ismailia Road, Cairo, Egypt*
[b]*Analytical Molecular Medicine and Devices Laboratory, Hiroshima University, Graduate School of Biomedical Sciences, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[c]*Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Modern Sciences and Arts University, 26 July Mehwar Road intersection with Wahat Road, 6 October City, Egypt*
[d]*Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El Aini St., Cairo 11562, Egypt*

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Fluorescence detection

Abstract A new simple, sensitive and precise green analytical procedure using an automated packed-reactor derivatization technique coupled with on-line solid-phase enrichment (SPEn) has been developed and evaluated to determine trace levels of methotrexate (MTX). The method was based on injection of MTX into a flowing stream of phosphate buffer (0.04 M, pH 3.4), carried through the packed oxidant reactor of Cerium (IV) trihydroxyhydroperoxide for oxidative cleavage of the drug into highly fluorescent product, 2,4-diaminopteridine-6-carboxylic acid, followed by SPEn on a head of short ODS column (10 mm × 4.6 mm i.d., 5 μm particle size). The flow rate was 0.25 mL/min and packed reactor temperature was 40 °C. The trapped product was back-flush eluted from the ODS column to the detector by column-switching with an environmentally friendly mobile phase consisting of ethanol and phosphate buffer (0.04 M, pH 3.4) in the ratio of 5:95 (v/v). The eluent was monitored at emission and excitation wavelengths of 460 and 360 nm, respectively. The calibration curve was linear over the concentration range of 1.25–50 ng/mL with a detection limit of 0.08 ng/mL.

*Corresponding author. Tel.: +20 2 01004052675; fax: +20 2 447715 66.
E-mail address: emara_miu@yahoo.com (S. Emara).
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The method was successfully applied to determine MTX in pharmaceutical formulations with mean percentage recovery ranging from 99.48 to 99.60.

1. Introduction

Methotrexate (MTX, 2,4-diamino-4-deoxy-N^{10}-methylpteroglutamic acid) is one of the most widely used antifolate (Scheme 1) drugs that show significant antitumor activity in acute leukemia and other neoplastic diseases [1].

Many methods have been developed for the determination of MTX, including spectrofluorimetry [2–4], immunoassay [5], capillary electrophoresis [6,7] and high-performance liquid chromatography (HPLC) with UV detection [8–13]. Due to its higher sensitivity and selectivity, the measurement of low MTX level was more precise in the HPLC with fluorescence detection [14–25]. MTX does not show native fluorescence but it can be derivatized into strongly fluorescent product after variety of successful analytical schemes. Several methodologies have been developed for the derivatization of MTX into a highly fluorescent product, 2,4-diaminopteridine-6-carboxylic acid. These methods were based on photo-oxidative irradiation [14,20], electrochemical oxidation [16,17], oxidation with permanganate [2,4,18,21,25] and hydrogen peroxide [19,22–24]. The United State Pharmacopoeia for the determination of MTX is based on HPLC with UV detection [26].

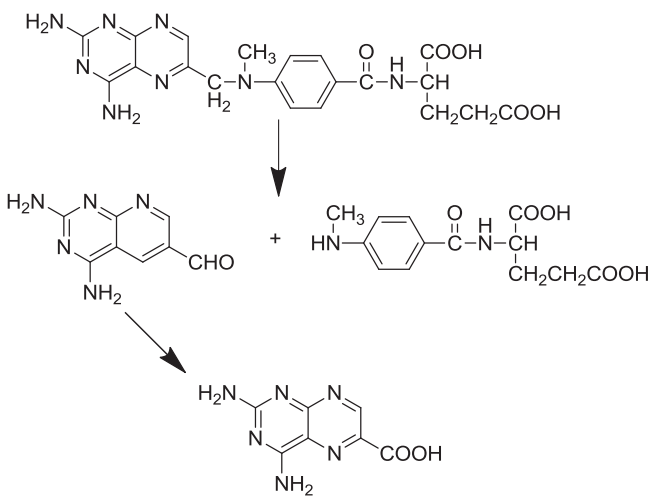
In recent years, more strict regulation related to the quality control of pharmaceuticals led to increasing demands on automation of the analytical assays carried out in appropriate control laboratories. The flow injection analysis (FIA) became a versatile instrumental tool that contributed substantially to the development of automation in pharmaceutical analysis due to its simplicity, low cost and relatively short analysis time. A survey of the literatures reveled that there are few methods for FIA determination of MTX in pharmaceutical formulations [27,28]. These methods were based on the oxidation of MTX into highly fluorescent product 2,4-diaminopteridine-6-carboxylic acid by on-line electrochemical oxidation and acidic potassium permanganate. A sequential injection analysis has also been reported for the determination of MTX using amperometric biosensor as detector [29]. Cerium (IV) trihydroxyhydroperoxide (CTH) has been introduced as a packed reactor in a flowing system for conversion of MTX into a highly fluorescent product, 2,4-diaminopteridine-6-carboxylic acid (Scheme 1) [30].

Determination of trace amount of drugs is an important issue in quality control of pharmaceuticals. Appropriate selection of pre-concentration methods for on-line sample processing and suitable detection allows the development of FIA methods for such analysis. Especially satisfactory for this purpose is the application of a measuring system combining FIA with on-line solid-phase enrichment (SPEn). In the present work, a single line FIA packed reactor procedure combined with on-line SPEn was developed to determine MTX. The method was based on oxidative cleavage of MTX into highly fluorescent product, 2,4-diaminopteridine-6-carboxylic acid, by means of CTH packed oxidant. At the same time the fluorescent product of MTX was pre-concentrated on a suitable short ODS column before detection. This approach presented interesting features such as: (i) high selectivity; (ii) high sensitivity; (iii) rapidity, simplicity and low cost, compared to well established FIA methods. In addition, packed reactor FIA used for the oxidative cleavage of MTX avoids the use of chemical reagents, which frequently are expensive and/or harmful; it is therefore an environmentally friendly analytical method. Moreover, the implementation of FIA-SPEn is a very attractive and fruitful research field, since this approach is appropriated as automated pre-concentration method, in order to determine MTX at trace levels (0.27 ng/mL). The proposed method was fully validated and proved to be adequate for quantification of MTX in pharmaceutical formulations.

2. Experimental

2.1. Instrumentation

A single line FIA-SPEn manifold, illustrated in Fig. 1, consisted of two solvent delivery pumps (Agilent 1100 Series Iso



Scheme 1. Structures of the investigated compounds.

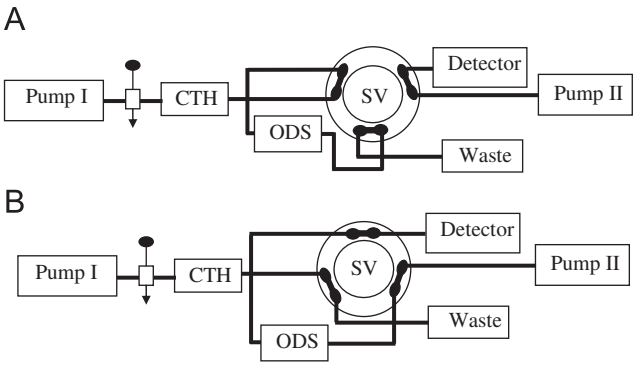


Fig. 1 Schematic diagram of on-line-SPEn coupled to packed reactor FIA for the trace analysis of MTX in pharmaceutical formulations. (A) Shows the system in initial position, ready for sample injection, derivatization and enrichment steps. (B) Displays the back-flush elution and detection step. SV, 6-port switching valve.

pump G1310A); one used to deliver the carrier solution at a flow rate of 0.25 mL/min and the other to deliver isocratic mobile phase at a flow rate of 1 mL/min. This system was equipped with two columns; one was a short (70 mm × 4.6 mm i.d.) CTH packed oxidant column for oxidative-cleavage of MTX into highly fluorescent product and the other was a small TSK gel ODS-80 TM column (10 mm × 4.6 mm i.d., 5 μm particle size) for pre-concentration of the fluorescent product. A model 7125 sample injection valve (400 μL) and a model 7010 flow direction switching valve were applied to load the sample onto the CTH packed oxidant and facilitate oxidative cleavage of MTX into highly fluorescent product and to elute the enriched analyte in a back-flush elution mode to the detector, (Rheodyne, Berkeley, CA, USA) respectively. A fluorescence detector (Agilent 1200 series, G1321A) monitored the eluent, set at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data acquisition was performed on an Agilent LC ChemStation software. The ODS column temperature was ambient, while that of the CTH packed reactor was 40 °C.

2.2. Reagents and chemicals

MTX (99.83%) was obtained from Kyowa Hakki (Tokyo, Japan). The present method was applied to the determination of MTX in its pharmaceutical formulations: (1) Methotrexate tablets (Batch No. 1368454) 2.5 mg of MTX and (2) Methotrexate vials (Batch No. 1236627) 50 mg of MTX (Orion Corporation, Finland). Ethanol used was HPLC grade (BDH, Poole, UK). Distilled water was used for the preparation of all reagents and solutions. Potassium dihydrogen phosphate, ortho-phosphoric, chloroform and isopropyl alcohol used were of analytical grades. TSK gel ODS-80 TM silica (5 μm) was from Tosoh Corporation (Tokyo, Japan).

2.3. Standard solutions

Stock standard solution of MTX (100 μg/mL) was prepared by dissolving an accurately weighed amount of MTX (25 mg) in 250 mL of distilled water. A known volume of the stock standard solution was diluted with the same solvent to obtain a concentration of 1 μg/mL MTX (solution A). The standard solutions for calibration were prepared daily by serial dilutions of appropriate volumes of solution A to produce MTX standard solutions in the final concentration range of 1.25–50 ng/mL. An aliquot of 400 μL was analyzed for MTX according to the proposed procedure. The standard solutions were stored frozen at −20 °C until used.

2.4. Tablets

A total of 20 tablets containing MTX as the active ingredient were weighed and finally powdered. A portion of the powder equivalent to 25 mg of MTX was accurately weighed and transferred to a 250 mL calibrated flask and dissolved in about 200 mL of distilled water using an ultra sonic bath. The solution was diluted to the volume with the same solvent and then filtered. The first portion of the filtrate was discarded and the remainder was used as a stock sample solution (solution A, 0.1 mg/mL). A known volume of solution A was diluted quantitatively with distilled water to obtain a concentration of 1 μg/mL MTX (solution B). A further dilution was carried out to obtain a final concentration of 20 ng/mL MTX. An aliquot of 400 μL was analyzed for MTX according to the proposed procedure.

2.5. Vials

An accurately volume equivalent to 25 mg MTX was transferred to a 250 mL calibrated flask and completed to the mark with distilled water. Serial dilutions as described under analysis of tablets were made to obtain a final concentration of 20 ng/mL. An aliquot of 400 μL was analyzed for MTX according to the proposed procedure.

2.6. Mobile phases

Two different mobile phases were employed in the assay procedure. One was phosphate buffer (0.04 M, pH 3.4) (**MI**), which was used to deliver sample to the CTH packed oxidant in the oxidation step. The other was an isocratic solvent system (**MII**) consisting of ethanol and phosphate buffer (0.04 M, pH 3.4) (5:95 v/v) which was used to elute the enriched fluorescent product in a back-flushed mode from the head of the small ODS column to the fluorescence detector. All mobile phases were freshly prepared on the day of use, filtered through 0.45 μm filters (Millipore, Billerica, MA), and degassed ultrasonically under vacuum.

2.7. Preparation of ODS and CTH columns

The ODS (TSK gel ODS-80 TM, 5 μm particle size) and CTH packing materials were suspended in chloroform and isopropyl alcohol, respectively. The suspended materials were degassed under vacuum with continuous stirring for 10 min. A stainless-steel cylinder (100 mm × 4.6 mm i.d.) was used as a reservoir for the packing materials. This reservoir was connected to a short column (10 mm × 4.6 mm i.d.) for ODS materials and another column (70 mm × 4.6 mm i.d.) for CTH oxidant. The suspended ODS and CTH supplied from the reservoir were packed into the corresponding columns with the aid of an HPLC pump at a flow-rate of 5 mL/min with ethanol

as a purge solvent (10 min). Pumping must continue until a constant pressure is reached. The cylinder was then disconnected and a mixture of ethanol and distilled water (1:1) was passed through the columns at a flow rate of 1 mL/min for further 10 min. The columns were then equilibrated with phosphate buffer (0.04 M, pH 3.4) at a flow rate of 1 mL/min for 30 min.

2.8. *General procedure*

A 400 μL aliquot of MTX sample was loaded into the injection valve and then injected into **MI** (position A, Fig. 1). The moving zone of MTX passed through the CTH packed reactor at a flow rate of 0.25 mL/min (pump I). The oxidative cleavage of MTX occurs during the flow of **MI** containing the drug through the CTH column. Pre-concentration was performed by means of the flow of the fluorescent product from the packed oxidant column to the short ODS column head. After 4 min the switching valve was switched into position B (Fig. 1). At this position, the **MII** could pass through the ODS column, where the fluorescent product was eluted in a back-flush mode to the detector. The flow rate was maintained at 1 mL/min and the fluorescence intensity of the eluting compound was monitored at emission and excitation wavelengths of 460 and 360 nm, respectively. At 5 min after injection, the switching valve was switched into position A to be ready for the next injection.

3. Results and discussion

The use of a packed reactor, in the described FIA-SPEn assembly, as an alternative to existing reagent solutions for the determination of MTX is dependent on optimization of the system to achieve maximum detector response. On the basis of experimental results, it can be stated that pH, concentration and flow rate of carrier stream as well as packed reactor temperature are the key parameters to improve derivatization step (position A, Fig. 1). The other parameters optimized were: pH and buffer concentration as well as the concentration of ethanol used as organic modifier in the elution step (position B, Fig. 1).

3.1. *Effect of pH and concentration of carrier stream*

Carrier stream pH is a very important chemical parameter that has to be investigated. The correct adjustment of this variable is necessary to improve the reaction completeness between the analyte and CTH packed reactor. The influence of this parameter on the analytical signal was studied in the pH range of 2.8–5 using buffer solutions from phosphate and acetate. Phosphate buffer gave the best performance as a carrier stream and was selected in all further experiments. The analytical signal reached its maximum value in the narrow range of pH 3.2–3.6 (Fig. 2). At pH < 3.0, a low signal response was observed which might suggest the decomposition of the peroxy groups of the CTH materials, whereas at pH > 3.8, the detector signals were decreased abruptly probably due to the low CTH reactor efficiency which reduces drastically the oxidative cleavage of MTX into highly fluorescent derivative. Accordingly, phosphate buffer solution of pH 3.4 was selected as the optimum carrier stream.

To find out the suitable salt concentration, phosphate buffers of concentrations varying from 0.02 to 0.1 M were

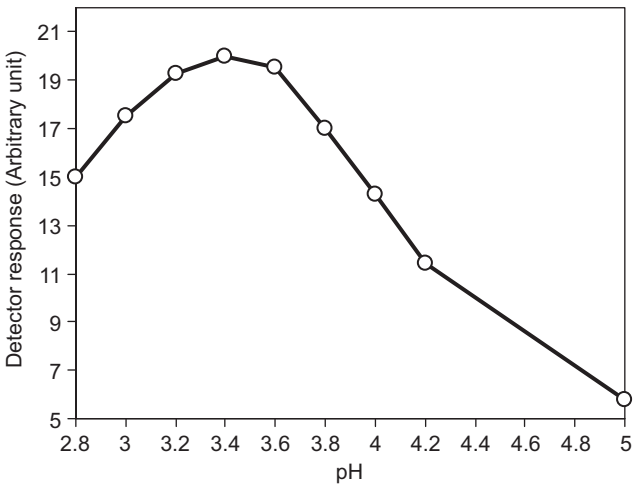


Fig. 2 Effect of phosphate buffer pH on the reaction efficiency of CTH packed reactor with MTX.

examined at the optimum pH. Best analytical signals were verified within the concentration range of 0.02–0.05 M (Fig. 3). Above this range, the peak area that corresponds to the analyte was significantly decreased probably due to greater quenching effect on the fluorescence signal intensity. Although, 0.02 M phosphate buffer showed slight improvement of signal intensity, 0.04 M was chosen as a compromise between detector response and precision.

3.2. *Effect of flow rate*

The contact time (residence time) between the sample zone containing MTX and the solid-phase reactor is very important for the reaction to proceed sufficiently and to achieve a substantial enhancement of the detector response. As the residence time depends on the flow rate of the carrier stream through the CTH packed reactor, a study of the flow rate was examined over the range of 0.25–2 mL/min (at 0.25 mL/min interval). The results showed an increase in the peak area at the lower flow rate down to 0.25 mL/min. It could be postulated that any decrease in the flow rate will increase the residence time between the moving zone of MTX and solid surface of CTH reactor, because of the increased diffusion of a sample zone over a larger area. The negative effect of the use of increasing flow rate on the reaction yield of MTX with CTH was also observed. Unlike previously reported assay using the conventional packed reactor FIA approach [30], a lower flow rate was justified in the present work for the determination of MTX because peak enrichment could be achieved on the top of the small ODS column. As far as the oxidation reaction proceeded, 2,4-diaminopteridine-6-carboxylic acid could be accumulated on the top of the ODS column with a zone width almost independent on the flow rate. A carrier flow rate of 0.25 mL/min was effective for the proposed FIA-SPEn method, achieving detection limit of 0.08 ng/mL for a sample size of 400 μL.

3.3. *Effect of temperature*

The oxidative cleavage of MTX into highly fluorescent product with CTH could be performed at room temperature while the highest detector response was achieved at elevated

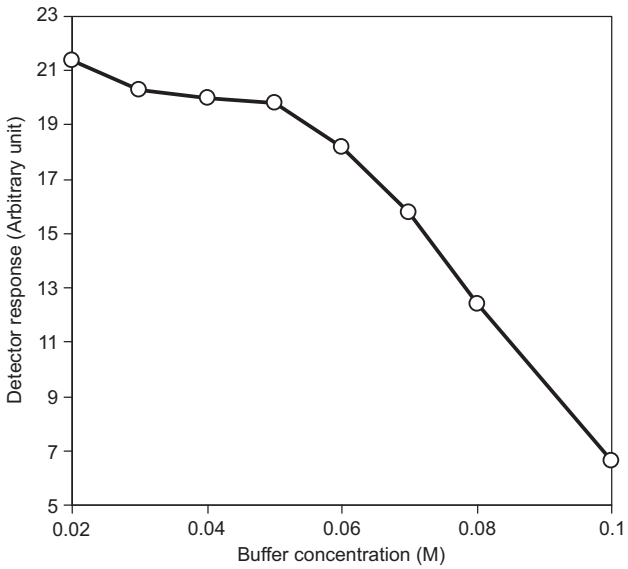


Fig. 3 Effect of phosphate buffer concentration on the reaction efficiency of CTH packed reactor with MTX.

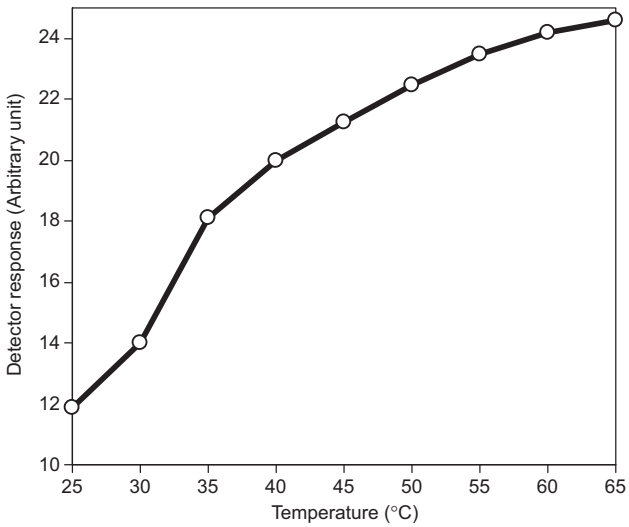


Fig. 4 Effect of temperature on the reaction efficiency of CTH packed reactor with MTX.

temperature. At the same time, experimental results showed a generally drastic effect of temperature on the CTH packed oxidant life span. Accordingly, the effect of temperature on the fluorescent intensity of MTX was investigated in the range of 25–65 °C by thermostating the packed oxidant reactor. It was found that if the reaction temperature was too low, the time of reaction needed was too long, and the oxidative cleavage could not be completed, while elevating the temperature within the range of 25–65 °C resulted in an increase in the reaction rate and peak area (Fig. 4). Owing to the packed oxidant limitations, 40 °C was chosen as the optimum value because under this condition, good sensitivity and reproducibility were achieved.

As a result, the optimal oxidation reaction efficiency of the CTH packed reactor could be achieved by using phosphate buffer (0.04 M, pH 3.4) at a flow rate of 0.25 mL/min and reactor temperature of 40 °C.

3.4. Sample volume

In an effort to push the concentration detection limit to a lower level, we attempted to use an on-line SPEn technique to pre-concentrate the fluorescent product before detection by loading a large sample volume of MTX. The design of a switching valve containing a small ODS column for on-line sample enrichment was described (Fig. 1, position A). Samples were loaded onto the CTH packed oxidant with a carrier mobile phase (**MI**) and pump I, while pre-concentration was performed by means of flowing of the fluorescent product from the packed reactor to the ODS column head. Different injection volumes (50–500 μL) were tested to introduce decreasing concentration of MTX. The efficiency of enrichment for fluorescent product was evaluated on the basis of the linearity of calibration curve constructed over sample volumes (50–400 μL, at 50 μL interval). It was found that, the CTH packed oxidant could tolerate large

volumes of MTX standard solution and the linear relationship (r^2 0.9995) between the peak area and the injected volumes was observed over the range of 50–400 μL sample volume. If too large sample volume is used (more than 400 μL), then the linearity of MTX between the peak area and concentration will be disturbed because a long residence time was found to be necessary to get reproducible results upon using large volume of MTX samples. Accordingly, sample volume of 400 μL MTX sample was selected as a compromise between the sensitivity and accuracy.

3.5. *Breakthrough study of ODS small column*

A small reversed-phase column was inserted in a switching valve between the solid CTH packed reactor and fluorescence detector as schematically shown in Fig. 1 (position **A**). For optimal on-line pre-concentration conditions, with respect to band broadening, the retention of the fluorescent product should be high at enrichment mode and low at elution mode, i.e., the reversed-phase column hydrophobicity should be as high as possible for complete trapping of the fluorescent product for enrichment. In the current study, TSK gel ODS-80 TM silica was chosen to be suitable packing material for the preparation of easily replaceable short column. A breakthrough study was performed by injecting 400 μL sample containing MTX (20 ng/mL) onto the ODS column, by means of **MI**. Increasing volume of **MI** was pumped through the column, before the switching valve was switched to position **B** (Fig. 1) and the trapped product was brought to the fluorescence detector with the analytical mobile phase (**MII**). With up to 10 mL of phosphate buffer (0.04 M, pH 3.4), pumped through the ODS column, the recovery of the analyte was above 99%. These results indicated complete trapping of MTX on the ODS column during the enrichment step.

3.6. *Optimization of the back-flush elution step*

Once the derivatization and enrichment step was terminated, the switching valve was switched to position **B** (Fig. 1) and the fluorescent product was transferred to the detector in a back-flush mode with **MII**. Thus, the time needed to elute the product from the ODS column and to transfer it to the detector with **MII** was then tested. The optimization procedure was continued by changing pH of the analytical mobile phase (**MII**) as well as changing the ratios of ethanol and phosphate buffer. Variation in the pH in the range of 2.0–6 weakly affected retention behavior of the fluorescent product. Variation of ethanol concentration was found to strongly affect retention behavior and band broadening of the fluorescent product. As a result, the optimal elution efficiency of the analytical mobile phase (**MII**) could be achieved by using a green solvent system of ethanol and phosphate buffer (0.04 M, pH 3.4) in the ratio 5:95 (v/v). Due to the elution strength of **MII**, a time period of less than 1 min was sufficient to perform complete elution of the fluorescent product from the head of ODS column (10 mm × 4.6 mm i.d.). By this means, much faster analysis was possible and the productivity of proposed FIA-SPEn method could be increased.

Table 1 Characteristic parameters for the regression equations of the proposed FIA method.

Parameters	MTX
Calibration range (ng/mL)	1.25–50
Detection limit (ng/mL)	0.08
Quantitation limit (ng/mL)	0.27
Slope (b)	1.4286
Standard error of the slope	0.0090
Intercept (*a*)	0.3030
Standard error of the intercept	0.2222
Correlation coefficient (r^2)	0.9997

$Y=a+bC$, where C is the concentration of MTX in ng/mL and Y is the peak area.

3.7. *Method validation*

3.7.1. *Linearity*

The calibration curve for MTX was constructed with seven concentrations (simultaneously prepared) ranging from 1.25 to 50 ng/mL. Calibration curve was constructed by plotting the measured peak area of 2,4-diaminopteridine-6-carboxylic acid versus concentration. Each concentration was repeated three times; this approach provided information on the variation in peak area values between samples of same concentration. The linearity of the calibration curve was validated by the high value of the correlation coefficient (0.9997). The coefficient for the linear equation $Y=a+bC$ was calculated using the linear regression least squares method, where Y is the peak area and C denotes the concentration in ng/mL of the tested compound. Characteristic parameters of the linear calibration curve are shown in Table 1.

3.7.2. *Limits of detection and quantification*

The limit of detection (LOD), defined as the lowest concentration of MTX that can be clearly detected above the base line signal, is estimated as three-times the signal-to-noise ratio. The LOD was determined ($n=3$) by injection of MTX in decreasing concentrations. The LOD was found to be 0.08 ng/mL (Table 1). The limit of quantification (LOQ) is often defined as 10 times the signal-to-noise ratio. The LOQ was determined ($n=3$) by injection of MTX in decreasing concentrations. The precision was calculated for each concentration. Then, the LOQ was calculated as the concentration, where the precision was less than or equal to 15% and was found to be 0.27 ng/mL (Table 1).

3.7.3. *Precision and accuracy*

The relative standard deviation (RSD %) and the relative error (RE %) of the mean measured concentration were served as measures of accuracy and precision for validation of the assay procedure. The intra- and inter-day assay precision and accuracy for MTX are summarized in Table 2. Within the examined range, the intra-day reproducibility and accuracy of the assay were excellent, with RSD % being in the range of 0.09–0.28 and with RE % ranging from 0.26 to 0.54. The inter-day RSD % were 0.11–0.32 and the mean RE % ranged from 0.34 to 0.65. Repeatability and reproducibility of MTX samples with high and low concentration levels were below 0.70%, indicating a reliable measurement using the proposed method (Table 2). RE

Table 2 Precision and accuracy validation of MTX.

Concentration (ng/mL)	Intra-assay[a]		Inter-assay[a]	
	Mean recovery (%)[a] ± RSD	Mean RE (%)	Mean recovery (%)[a] ± RSD	Mean RE (%)
5	99.62 ± 0.09	0.38	99.48 ± 0.11	0.52
10	99.74 ± 0.17	0.26	99.66 ± 0.19	0.34
20	99.65 ± 0.23	0.35	99.60 ± 0.27	0.40
40	99.46 ± 0.28	0.54	99.35 ± 0.32	0.65

[a]Average of five determinations.

was evaluated by back-calculation and expressed as the percent deviation between concentration added and concentration found according to the following:

$$RE\% = [(\text{conc. added} - \text{conc. found})/\text{conc. added}] \times 100\%$$

3.7.4. *Interference studies*

In order to examine the selectivity of the proposed method, the effect of common excipients normally used in pharmaceutical formulations was studied. Solutions containing MTX (20 ng/mL) in the presence of more than 100-folds of common additives such as maize starch, calcium hydrogen phosphate, magnesium stearate, mannitol, red ferric oxide, lactose, methylhydroxypropylcellulose, sodium stearyl fumarate, microcrystalline cellulose, hydrophobic colloidal silica and sucrose were prepared. The undissolved materials were filtered off before injection. No significant changes were observed on the results and recoveries in the range of 99.38–99.61% were obtained in all cases.

3.8. *Application*

The proposed FIA-SPEn method was applied for the determination of MTX in its commercial formulations together with the official USP method [26]. As indicated, the assay results, of pharmaceutical formulations, obtained by the proposed method were in accordance with those obtained by the official USP method (Table 3). The accuracy and precision of the developed method were further judged by applying *t*- and *F*-test at 95% confidence level. The experimental *t*- and *F*- values did not exceed the theoretical values, which support the similar accuracy and precision of the proposed and official USP methods (Table 3). The accuracy of the analytical method was also checked by standard addition method, which is applied by adding drug standard to previously analyzed tablets. The results showed that the derivatization procedure developed for the determination of MTX could be considered to be accurate within the concentration range investigated. Mean value is very close to the theoretical concentration, showing percentage recovery of 99.48 and 99.60 and RSD of 0.31 and 0.28 for MTX tablets and vials, respectively. These results indicate that the effects of the common additives and ingredients of the pharmaceutical formulations do not interfere with the determination of MTX.

High reagent consumption is the main disadvantage of all continuous FIA systems, which use the reagent continuously even when no sample is present in the apparatus [27–29,31]. In the present work, the packed reactor of CTH avoids this uneconomical approach in such a way that the sample zone meets the reactor in a controlled manner while the rest of the system is filled with phosphate buffer (0.04 M, pH 3.4). Also, the use of CTH reactor offers many significant improvements over the other FIA procedures for the determination of MTX. These improvements are simplification of manifold, saving reagents, no mixing problems, less pump noise and the reaction is very mild and the derivatization is achieved in a fairly short time. The use of phosphate buffer (0.04 M, pH 3.4) as a flowing stream and a mobile phase consisting of ethanol and phosphate buffer (0.04 M, pH 3.4) in the ratio of 5:95 (v/v) made this work as a contribution to environmentally friendly analytical chemistry. Moreover, the implementation of FIA-SPEn is a very attractive and fruitful research field, since this approach can achieve an improvement of sensitivity of around a 20-fold compared with the reported FIA method using CTH packed reactor [30].

Table 3 Determination of MTX in commercial formulations by the proposed and official methods.

Commercial formulation	Recovery (%) [a] ± RSD	
	Proposed method	Official method
Tablets (20 ng/mL)	99.56 ± 0.26	99.77 ± 0.21
Student's *t*-test	0.28 (2.30)[b]	
F-test	1.57 (6.38)[b]	
Vials (20 ng/mL)	99.68 ± 0.23	99.87 ± 0.20
Student's *t*-test	0.27 (2.30)[b]	
F-test	1.37 (6.38)[b]	

[a]Average of five determinations.
[b]Theoretical values at $p = 0.05$.

3.9. *Robustness*

To determine robustness of the proposed method, experimental conditions, namely flow rate, pH and concentration of the buffer solution used as a carrier stream, packed reactor temperature and organic content of the mobile phase were purposely altered and the detector responses were evaluated. Variation of each parameter by ±2% did not have a significant effect on the detector response.

4. Conclusion

The present study has demonstrated that MTX could be determined using an automated green analytical procedure based on the formation of fluorescent product, 2,4-diaminopteridine-6-carboxylic acid, on a CTH packed oxidant coupled with on-line SPEn on the head of a small ODS column for preconcentration before detection. The use of solid-phase reactor incorporated into the FIA manifold offers many significant improvements over the other FIA procedures for the determination of MTX. These improvements are simplicity of the procedure, no dangerous reagents used, very mild reaction conditions, no mixing problems and less pump noise. A high robustness degree of on-line derivatization-SPEn strategy, good reproducibility, and simplicity demonstrates the suitability of the method for routine analysis and quality control

of MTX in pharmaceutical formulations. Common excipients used as additives in pharmaceutical preparations did not interfere.

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