Original Article

DEVELOPMENT AND VALIDATION OF A HIGHLY SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR THE ESTIMATION OF METHOTREXATE (MTX) PURE DRUG AND MARKETED FORMULATION IN SPIKED RAT PLASMA

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ABSTRACT

Objective: Methotrexate (MTX) is used as an anti-tumor agent, and its clinical pharmacokinetics requires an accurate method for estimation of its plasma concentration. We describe a rapid, selective and sensitive HPLC method coupled with UV detection for determination of MTX in rat plasma.

Methods: Internal standard (IS; caffeine) was added to plasma aliquots prior to protein precipitation with acetonitrile. MTX and IS were eluted on a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) protected by a guard column (4 mm × 3 mm ×5 μm) (Torrance, CA, USA). The mobile phase comprised a mixture of aqueous 0.01 M phosphate buffer (pH 3.9)/acetonitrile (89/11%, v/v). To further improve the applicability of the method, a marketed formulation of high dose MTX (Folitrax 10 mg, IPCA) was spiked in rat plasma and the developed method was applied for the detection of MTX.

Results: Adequate specificity, precision, and accuracy of the proposed method were demonstrated over the concentration range of 10 to 1000 ng/ml.

Conclusion: This developed method was more sensitive and specific than any reported methods for the estimation of MTX and hence was successfully applied to measure plasma MTX concentrations in rat plasma which can be useful to elucidate the pharmacokinetic data of MTX.

Keywords: Methotrexate, Folitrax, Protein precipitation, Rat plasma, Caffeine

INTRODUCTION

MTX is a conventional anti-metabolite drug and forms a major part of many cancer treatment regimens [1]. It acts by inhibiting folic acid, which is essential for DNA synthesis and hence is therapeutically effective on cancer cells expressing folate receptors on their surfaces [2]. MTX is prominently employed as a chemotherapeutic agent for human malignancies such as acute lymphoblastic leukemia, malignant lymphoma, osteosarcoma, breast cancer and head and neck cancer [3]. MTX is also used as an immunosuppressive medication for the therapeutic management of inflammatory diseases such as rheumatoid arthritis, acute psoriasis and uveitis [4]. MTX can be taken orally or administered by injection (subcutaneous, intramuscular, intravenous or intrathecal) [5].

However, the usefulness of MTX is greatly hindered by the toxicities associated with the drug due to which monitoring the plasma concentrations would help to quantify the drug and be valuable to predict and prevent the probable toxicity of drug [4].

Literature survey revealed that numerous methods for estimation of methotrexate have been reported which include HPLC analysis with ultraviolet detection and fluorescence detection, radioimmunoassay, dihydrofolate reductase inhibition assay, enzyme multiplied immunoassay, fluorescence polarization immunoassay, enzyme immunoassay and capillary zone electrophoresis with laser–induced fluorescence detection [4]. For our purposes, HPLC approaches provide the greatest compromise between sensitivity, selectivity, assay time and assay expense as the aim of the research was also to improve the industrial or commercial applicability of the method.

Further, there are several HPLC assays, some which are suitable for use after high doses such as those used for cancer treatment [5-10]. However, these methods have not been established in an animal model such as rat plasma.

Joseph P. Balthasar *et al.* has also described a high-performance liquid chromatographic assay for MTX in the presence and absence of anti-methotrexate antibody fragments in rat and mouse plasma. However, the method involves the pre-column derivatization of MTX by sodium hydrosulfite and detection by fluorescence detector which becomes multiple steps, time-consuming and less economical [11].

In our survey to find a sensitive HPLC assay, we found that the assay reported by Eftihia Asprodini et al. appeared most useful. This HPLC involved monitoring of MTX in Osteosarcoma Patients. The method reported a Limit of Quantitation (LOQ) of 0.01 μM and the Limit of Detection (LOD) was 0.003 μM [12]. However when we applied the method to the analysis of MTX in rat plasma we observed lower sensitivity. Further, we also tested the suitability of the method for industrial adaptation by spiking the rat plasma with the samples of a marketed formulation Folitrax tab 10 mg (IPCA laboratories). We have, however, successfully modified the method of Eftihia Asprodini et al. for our purposes.

Hence, in this paper, we describe a simple, economical and convenient HPLC procedure with UV detection and simple sample preparation for determination of MTX in rat plasma samples which has not been effectively tried out. The method is also applied to a marketed formulation to improve the industrial applicability of the method. The other objective was to validate the assay as per the International Conference on Harmonization (ICH) guidelines [Q2 (R1)].

MATERIALS AND METHODS

Materials

MTX was obtained as a kind gift sample from Machen Product India Pvt. Ltd. (Mumbai). Caffeine was supplied by Himedia (Mumbai, India). The purity of both the compounds was found to be more than 99%. HPLC grade acetonitrile was purchased from SD fine-chem limited (Mumbai, India). Other chemicals used were HPLC and analytical grade. Water used for the preparation of aqueous mobile phase in all experiments was processed through a Milli-Q water purification system supplied by Millipore (Bangalore, Karnataka, India) filtered through a 0.22 μm filter. Rat blood was collected from healthy male Wistar rats (200–250 g).

Experimental

Equipment

A Shimadzu (Japan) HPLC LC-2010 HT equipped with a Serial dual plunger, microvolume (10μL on the primary side, 5μL on secondary)

and autosampler was used for the chromatographic separation. Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) protected by a guard column (4 mm × 3 mm ×5 μm) (Torrance, CA, USA) was employed for the analysis. The detection was carried out with the UV-Visible SPDM20A detector. LC solutions software was used for the interpretation of the results.

Separation of plasma from rat blood

Rat plasma was collected from healthy male Wistar rats (200–250 g), which were fasted overnight before blood collection with free access to water. The animals were subjected to acclimatization to laboratory conditions one week before experiments and fed with standard rat diet. The animals were kept under controlled conditions of a 12: 12 h light: dark cycle, with a temperature of 22±3ºC and a relative humidity of 50±5%RH. Rat blood was collected from the retro-orbital plexus by micro capillary technique under light ether anesthesia and taken into 3 ml coated vacutainer tubes and centrifuged at 10, 000 rpm for 10 min using cold centrifuge (Remi Model TC 650 D) to extract the plasma. The plasma was stored in deep freeze at–4 °C until further analysis.

Chromatography

Chromatographic separations were performed on Shimadzu (Japan) HPLC LC-2010 HT PhenomenexC18 column (250 mm × 4.6 mm, 5 μm) maintained at 30 ◦C, using a thermostatically controlled column heater. The mobile phase comprised a mixture of aqueous 0.01 M phosphate buffer (pH 3.9)/acetonitrile (89/11%, v/v) and the pH was adjusted to 3.9 with glacial acetic acid (HPLC grade). The flow rate was set at 1.0 mL/min. The mobile phase was degassed by ultrasonication prior to use. The UV detection wavelength was set at 303 nm.

Preparation of standard solutions

A standard stock solution (1 mg/ml) of MTX was prepared in 0.05M sodium hydroxide (NaOH). Primary working standard solution (10 μg/ml) we prepared by serial dilution of the standard stock solution with the mobile phase. A stock solution (1 mg/ml) of IS was prepared by accurately weighing an appropriate amount of caffeine and dissolving it in the mobile phase. Working standard solutions (10 μg/ml) were prepared by making appropriate serial dilutions of the IS stock solution in the mobile phase. The standard solutions were stored at −15 ◦C to −25 ◦C, protected from light and were used within seven days.

Plasma sample extraction and processing

150μl of drug-free plasma was spiked with MTX and IS by adding 150μl of the caffeine solution and 150μl of the MTX solution to the plasma. The final concentration of IS was set to 1.0 μg/ml. After the addition of 150μl of acetonitrile as precipitating agent to the drug-spiked plasma, the solutions were vortex-mixed for 1 minute followed by centrifugation at 15,000 rpm for 15 min using a cold centrifuge Remi Model TC 650 D. The supernatant organic layer (200 μl) was separated and 50 μl aliquot was injected onto the chromatographic system for analysis [12].

Preparation of calibration standards and quality control samples

Calibration curve (CC) was prepared by serial dilution of MTX stock solution (10μg/ml) in the range of 10, 20, 30, 40, 50, 100, 200, 400, 600, 800 and 1000ng/ml. Similarly, five standard solutions were prepared by serial dilution of MTX stock solution (10 μg/ml) in the range of 1, 2, 3, 4 and 5ng/ml in order to determine the LOD and LOQ. Calibration standards were also prepared by spiking 150μL of blank plasma with 150μL of the appropriate working solution resulting in concentrations of 10, 20, 30, 40, 50, 100, 200, 400, 600, 800 and 1000ng/ml and 1, 2, 3, 4 and 5ng/ml of MTX. Stock solution (1μg/ml) of I. S was prepared in mobile phase and stored at −20°C. The solutions were stable for seven days when stored at room temperature (20–25°C). All solutions were used within one week. Quality control (QC) samples (low quality control (LQC),100 ng/ml; medium quality control (MQC), 1000 ng/ml; high quality control (HQC), 2500ng/ml; limit of quantification (LOQ), 5 ng/ml were prepared by spiking 0.15 mL aliquot of blank plasma with 150 μL of spiking solution of drug as well

as the IS. All solutions were stored in the refrigerator at 4.0±2.0◦C. The bulk spiked CC and QC samples were stored at −20◦C and brought to room temperature before use [12].

Validation of the developed method

All the validation studies were carried out as per ICH guidelines [Q2 (R1)] by six consecutive replicate injections of the standard and sample solutions. Selectivity is a tool to determine the ability of the analytical method to differentiate and quantify the analyte in the presence of some components expected in the sample. Sensitivity was determined by analyzing control rat plasma in replicates (n = 6) spiked with the analyte at the lowest level of the calibration standard, that is 0.010 μg/ml. Accuracy and precision of the quality control (QC) samples were assessed using the calibration curve. Assay precision was quantified by expressing the standard deviation of the measurements as a percentage of the average value. The accuracy was determined for each spiked control by comparing the nominal.

Extraction recovery

The recovery of MTX and IS were determined. The recovery of MTX was determined at a concentration of 10, 100 and 1000 ng/ml and for IS was determined at a concentration of 1000ng/ml. Six replicates at each concentration level with peak area response from non-extracted control samples prepared at the same concentration level were prepared and injected into the HPLC system.

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing MTX at three different levels, i.e., 10, 100 and 1000 ng/ml. The inter-assay precision was determined by analyzing the three levels on six different runs. The criteria for acceptability of the data included accuracy within±15% Deviation (DEV) from the nominal values and precision within 15% Relative Standard Deviation (RSD). For intra-day, accuracy and precision at each concentration were assayed on the same day. The inter-day accuracy and precision were evaluated for three subsequent days [14, 15].

LOD and LOQ

The LOD and LOQ were determined at 3.3 and 10 times the baseline noise, respectively [13].

Ruggedness

From the stock solution, sample solutions of MTX (10ng/ml, 100ng/ml, 500ng/ml and 1000ng/ml) were equipped and analyzed by two different analysts employing analogous operational and environmental surroundings. The peak area was calculated for identical concentration solutions six times.

Estimation of MTX marketed formulation spiked in rat plasma

To further improve the applicability of the method, a marketed formulation of high dose MTX (Folitrax 10 mg, IPCA) was spiked in rat plasma and the developed method was applied for the detection of MTX. Standard stock solution (1 mg/ml) of Folitrax was prepared in 0.05 M NaOH solution and subsequent dilutions with mobile phase were carried out to obtain a concentration (100ng/ml). The final sample was prepared as described in the previous section.

RESULTS AND DISCUSSION

High-performance liquid chromatography method development and optimization

Column chemistry, solvent type, solvent strength, detection wavelength, and flow rate were varied to determine the chromatographic conditions with an aim of greater sensitivity and separation. The mobile phase conditions were optimized for the purpose of minimizing any background noise. After trying different columns, the selection was narrowed down to the reversed phase Phenomenex C18 column of stationary phase giving satisfactory sensitivity, resolution and retention time. Mobile phase and flow rate selection was based on peak parameters *viz.*, area, height, tailing, theoretical plates, capacity factor and resolution.

Method validation

Validation

Short elution time, good separation between MTX and IS, and baselines with low background noise were achieved by using a reversed phase Phenomenex C18 column with low carbon load. The highlight of the research was the selection of the pH of phosphate buffer and alteration in the ratio of organic to aqueous phase which gave favorable retention times of MTX and IS. MTX gave a retention time of 7.5 min and IS showed a good sharp peak at 10.25 min by UV detection at 303 nm.

The proposed method is suitable for quantification of MTX and IS in rat plasma samples. It showed specificity since I. S. and MTX were well resolved and no interfering peaks from endogenous components of rat plasma were observed, as can be seen from fig. 1 and fig. 2.
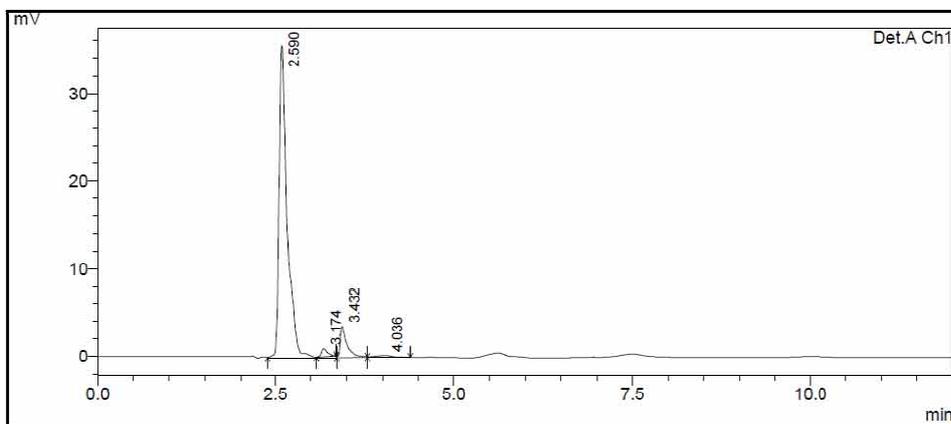

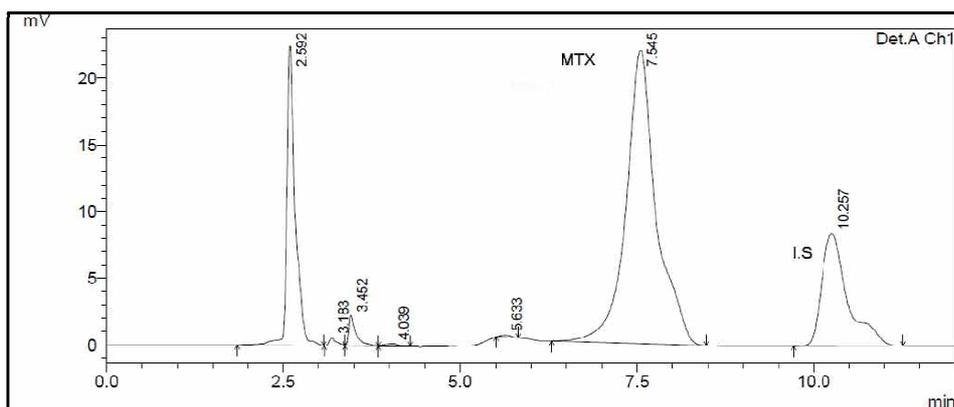**Fig. 1: HPLC chromatogram of blank rat plasma**

Fig. 2: HPLC chromatogram of rat plasma spiked with MTX pure drug and I. S

Linearity, LOD, and LOQ

The peak area ratios of drug to IS for the calibration standards were proportional to the concentration of each drug in plasma over the range which was tested. The calibration curves were linear over the range of 10 ng to 1000 ng/ml resulted in the regression equation $y = 157.1x + 774.3$ ($r > 0.998$) (fig. 3). The LOD and LOQ of the calibration graph were 4 ng/ml and 5 ng/ml
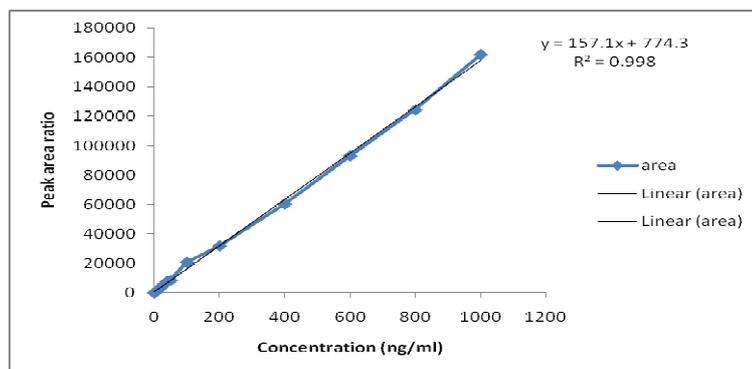

Fig. 3: Linear standard curve determination of methotrexate in serum (concentration range 10-1000 ng/ml)

Extraction recovery

The absolute recovery of MTX from plasma was calculated by comparing the peak area obtained from extracts of spiked plasma samples with the peak area obtained from the direct injection of known amounts of standard solutions of MTX. The overall extraction yields of 10, 100 and 1000 ng/ml MTX in plasma were 93 to 96% [table 1]. The outcome of the study illustrated that at least 15 min of centrifugation at 15,000 rpm was needed for protein denaturation completely when 150μl acetonitrile was added to 150 μl plasma sample.

Table 1: Recovery of MTX and IS from spiked rat samples

*Plasma concentration of MTX (ng/ml)	Recovery of extraction (%) (mean+SD., n=6)	Plasma concentration of I. S (μg/ml)	Recovery of extraction (%) (mean+SD, n=6)
10	93.02+1.006	1	92.95+1.15
100	96.93+0.407		
1000	96.39+1.028		

*(n=6)

Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in table 2. Intra-day and inter-day precision (% R. SD) of the methods were lower than 14% and were within the acceptable limits to be in concurrence with the guidelines for United States Pharmacopeial (USP) norms method validation which is considered to be within 15% (RSD).[15-17] The accuracy of both the methods was also good with the deviation between the nominal concentration and calculated concentration for MTX well below the limits of±15%. Precision and accuracy data indicated that the methods to extract MTX from plasma and tissues are highly reproducible and robust. The values accuracy and precision were acceptable in view of the international recommendation that the precision and accuracy should not exceed 15% [15, 16].

Ruggedness

Two different analysts performed the same analysis under the same experimental conditions. The contents of the drug were not greatly affected by these changes as obvious from the low values of % RSD (2%), indicating ruggedness of the method as shown in table 3. %RSD values of less than 2% were acquired for repetitive measurements and operators.

Table 2: Intra-and inter-day precision and accuracy determination of MTX concentration in spiked rat plasma samples

*Spiked concentration (ng/ml)	Measured concentration (mean+SD)		Precision (%RSD)		Accuracy (Relative error, %)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
10	7.14+0.46	7.07+0.41	6.46	5.86	-28.56	-29.28
100	112.83+4.49	113+5.51	3.97	4.87	12.83	13
1000	1068.6+6.21	1062.5+6.25	0.58	1.03	6.86	6.25

*(n = 6 at each concentration for intra-day and n = 6 d for interday precision).

Table 3: Values of ruggedness studies for the developed method

Drug	*Concentration ng/ml	Analyst 1 amount found%+SD	%RSD	Analyst 2 amount found%+SD	%RSD
MTX	10	7.07+0.41	5.86	7.10+0.20	2.89
	100	113+5.51	4.87	113+5.89	5.22
	500	512.5+3.61	0.7	512.16+4.62	0.9
	1000	1062.5+9.19	1.03	1066.33+7.71	0.72

*(n=6)

Detection of methotrexate in plasma samples

The developed method was found suitable for the detection of MTX marketed formulation (Folitrax 10 mg) in the plasma sample. MTX gave a sharp peak at 7.04 min with a good resolution. The outcome of the study was that no interfering peaks of any of the excipients were observed as shown in fig. 4. Hence, the presently developed method has the potential of detecting MTX in rat plasma samples which is an important animal model for preclinical studies. Further, the method also has applicability to quantify the drug in a marketed formulation in the presence of rat plasma which makes the method more applicable to study the pharmacokinetics of drug using a rat as an animal model which has not been described in the previously described HPLC methods.
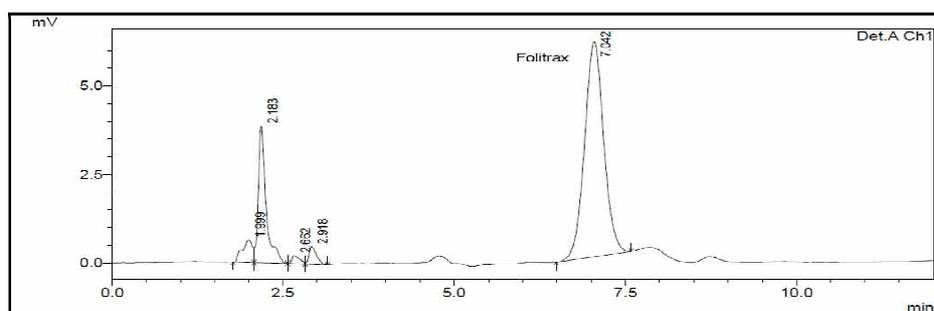

Fig. 4: HPLC chromatogram of rat plasma spiked with MTX marketed formulation (Folitrax 10 mg)

CONCLUSION

A simple and sensitive method for the determination of MTX, a chemotherapeutic agent, in spiked rat plasma by HPLC was developed and validated. Protein precipitation method was employed for sample preparation followed by chromatographic separation and UV detection. No interfering peaks were observed at the elution times of MTX and IS. Adequate specificity, precision, and accuracy of the proposed method were demonstrated over the concentration range of 10 to 1000 ng/ml. The method was accurate, reproducible, specific, and provided excellent separation and enable the quantification of MTX in rat plasma. The study was also extrapolated for determination of MTX in tablet formulation which would improve the industrial applicability as well as detection of MTX levels after administering the chemotherapeutic dose of the drug.

CONFLICT OF INTERESTS

Declared none

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