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Original Article

Rapid, Simple and Low-Cost Reverse-Phase High-Performance Liquid Chromatography Tool for Estimation of Methotrexate: Testing Application in Standard Laboratory Bulk, Marketed Dosage Form, and Release kinetics in Nanoformulation

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ABSTRACT

Background: Methotrexate (MTX) is one of the utmost commonly prescribed drugs, which is official in the IP, BP, and USP. MTX is a potent drug that can cause serious side effects if not used in the correct quantity. Assaying MTX is vital to guarantee that the drug exists in the required concentration in the sample. Developing a simple, rapid, and cost-effective analytical tool for its estimation has always been a thrust area of research. In this line, this investigation reports a simple, rapid, and low-cost Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method with an ultraviolet detection channel for estimating MTX in bulk samples. **Materials and Methods:** The technique employs an isocratic mobile phase comprising a readily available solvent system having 50 mM sodium acetate buffer (pH-3.6) and acetonitrile in 90:10, v/v, which flows through the column at a constant flow rate of 1.0 mL/min. A Thermo C-18 Column (5-micron, 250 mmx4.60 mm) was used as the stationary phase for separation. Considering the optimized chromatography parameters, sensitivity, and selectivity of a method for drugs, 307 nm was selected as the detection wavelength for the UV-visible detector. The validation studies were performed by fulfilling the requirements of ICH guidelines. **Results and Conclusion:** The method was found to be specific, linear (including intra- and inter-day precision), accurate, and precise for routine testing of MTX. The developed RP-HPLC method has been successfully applied to determine MTX in standard Laboratory Bulk samples and commercial Dosage Formulations, as well as to monitor the release kinetics of MTX from an in-house developed Nanoformulation. The developed method represents a valuable tool in the laboratory analysis and monitoring of MTX during routine quality control testing and toxicity studies.

Keywords: Methotrexate, Routine analysis, Reverse-phase high-performance liquid chromatography, Estimation, Validation, Application, Standard Laboratory Bulk, Nanoformulation, Marketed Formulation.

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INTRODUCTION

Methotrexate (MTX; $C_{20}H_{22}N_8O_5$; Mol. wt: 454.4 da; Figure 1) is one of the most commonly prescribed drugs, which is official in the IP, BP, and USP.[1] It is an antifolate antimetabolite, which is employed as frontline therapeutics for the chemotherapy of carious cancers.[2-4] It also acts as an immunosuppressant that suppresses the growth of certain cells, especially cancer cells, bone marrow cells, and skin cells.[5] MTX is also a commonly prescribed medicine for treating rheumatoid arthritis and other severe psoriasis by destroying cells in the ailing skin that grow too swiftly.[2]

MTX is a potent drug that can elicits grave side effects if not used in the correct quantity and concentration.[6,7] Therefore, it is essential to monitor MTX concentration to ensure that it is within the prescribed therapeutic range to avoid harm to the patient. Quality control is an essential aspect of any analytical method.[8] Assaying MTX is vital to guarantee that the drug exists in the exact concentration in the sample.[9] Hence, developing a simple, rapid, improved, and cost-effective analytical tool for its estimation has always been a thrust area of research.

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In this line, the ARK MTX Assay is a homogeneous enzyme immunoassay is available commercially. It is intended for quantitatively determining MTX in samples to help ensure appropriate therapy. However, an antibody-based detection assay is sometimes a costlier and time-consuming affair.[10] Another strategy was developed for detecting MTX *employing* the quenching behavior of MTX on the fluorescent strength of dendritic silica nanoparticles with terbium-doping. The method was found to be sensitive, but the procedure requires additional steps to prepare terbium-doped dendritic silica particles.[11] In a similar line, Goksel *et al.* also developed a method for the detection of MTX in samples at clinically applicable levels engaging electro-chemical Surface-enhanced Raman spectroscopy on a customized benchtop Raman spectrometer. Such methods were also found to be valid for detecting MTX at the point of need but require a specialized Raman device.[12]

Furthermore, Chen *et al.,* tried to develop Protein-decorated gold nanocomposite as fluorescent labelled probe for detecting MTX. However, the reported methodology cannot be routinely engaged for MTX determination due to the complexity and additional requirements of protein-templated gold nanoparticles.[13] Immunoassays are yet another analytical technique that has been used to estimate MTX. However, as per the latest case report by Sharma *et al.*, it may be noted that the immunoassay tools are prone to show interference by moieties with same type of structure that of MTX, including folinic acid (leucovorin).[14]

Literature reports several spectrophotometric approaches for MTX analysis in pharmaceutical and bulk preparations; however, the application scope of these analytical methods is limited due to their low sensitivity and accuracy.[15] Furthermore, other analytical protocols using electrophoretic capillary method,[16] isotachophoresis,[17] and voltametric techniques have also been reported.[18] Notably, this analytical approach bears drawback such as long analysis time, additional need of derivatizing the molecule, as well as inferior purity estimation in pharmaceutical formulations.

Sophisticated analytical methods such as Volumetric Absorptive Micro Sampling (VAMS), Solid Phase Extraction (SPE), Liquid Chromatography-Mass Spectrometry (LC-MS),[19] Liquid chromatography-mass spectrometry[20] Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)[21] were also developed with enthusiasm for MTX analysis. Though these techniques represent the sensitive, accurate, and convenient approach of MTX analysis, but these methods demand exceptionally costlier instrumentation, laborious maintenance, and laboratory support systems for their routine analytical application.

In this line, this investigation rightly reports development and validation of a new, rapid, convenient, and economical Reverse Phase High Performance Liquid Chromatography (RP-HPLC) technique with an ultraviolet detection channel for estimating MTX in bulk samples. The developed RP-HPLC method has also been successfully applied to estimate MTX in standard Laboratory Bulk Stock samples, Commercial Dosage Formulations, as well as to study the release kinetics of MTX from plain and from an in-house developed Nanoformulation. The method employs an isocratic mobile phase comprising a readily available solvent system having 50 milli mole sodium acetate buffer (pH-3.6) and acetonitrile in 90:10, v/v (constant solvent flow rate: 1.0 mL/ min). Each time, the mobile phase was degassed 30 min before usage and then its filtration was done using nylon membrane filters (Millex® Syringe Filters; Pore diameter: 0.22 µm). A Thermo C18-Column with dimension of 4.60 mmx250 mm, and 5 µm particle size was employed for the separation as a stationary phase. Taking lead from the chromatographic conditions, observed method selectivity and sensitivity for the drug, 307.0 nm was designated as the selected wavelength for the UV detection.

The validation was performed by duly complying the requirements of ICH guidelines. The method was found to be simple, specific, and linear with intra- and inter-day precision for routine testing of MTX. The developed method represents a valuable tool in the laboratory analysis and monitoring of MTX during routine quality control testing and toxicity studies of anticancer chemotherapy. As detailed account of experimental procedures, results, and discussions are presented in the following section of the manuscript.

MATERIALS AND METHODS

Materials and reagents

MTX nanoformulation and standard laboratory samples were developed in lab. Bovine Serum Albumin was purchased from HiMedia Laboratories). MTX, Dialysis tubing (Molecular weight cut-off of 12K Dalton; Sigma, USA), and membrane filter (Millex® Syringe Filters; Pore size 0.45 µm/0.22 µm) were purchased from Fisher Scientific Ltd., Sodium phosphate, sodium chloride, and sodium acetate were obtained from Sigma Aldrich. All reagents engaged in this study were of HPLC/analytical grade, namely: sodium acetate buffer, acetonitrile Methanol and acetic acid (Merck) while HPLC-grade water from Qualigens, Mumbai (India). Each time, the mobile phase was degassed 30 min before usage and then filtration was attained using a Millex® Syringe Filters of 0.22 µm pore size.

Chromatographic Instrument

MTX Separation protocol was performed using a HPLC system (Waters™; Model: 784) fitted with a injector in manual mode and a binary pump Waters™; Model: 515). The system was equipped with a Thermo C18 column (5-micron, 4.60 mmx250 mm) with constant flow and constant pressure delivery, and UV-visible detector connected to software N 2000 for controlling the instrument as well as data processing. The column was

equilibrated for at least 30 min with the solvent flowing through the system with a flow rate of 1 mL/min at column temperature pre-set at 25°C.

Chromatographic conditions

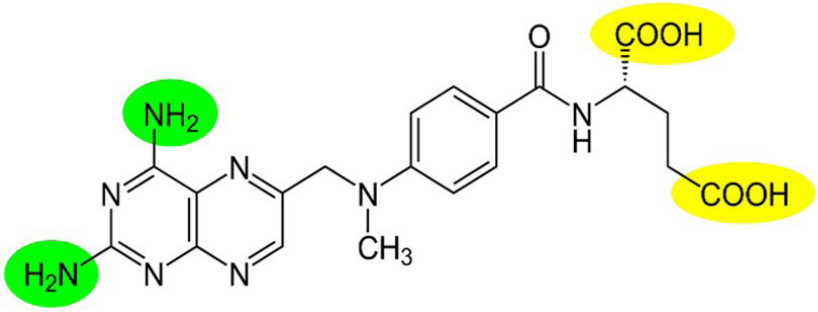
An isocratic mobile phase consisted of 50 mM sodium acetate buffer (pH 3.6): acetonitrile (90:10, v:v), which is flowing through the Thermo C18-Column with dimension of 4.60 mmx250 mm, and 5 µm particle size (Flow rate: 1.0 mL/min). Each time, the filtration of mobile phase was accomplished through Millex® Syringe Filters of 0.22 µm pore size and was degassed 30 min before use. The mobile phase was filtered through a Millex® Syringe Filters of 0.22 µm pore size and degassed with helium purge for 15 min. The mobile phase components were pumped from the reservoir to the column at a flow rate of 1.0 mL/min. By considering the chromatographic parameter, sensitivity, and selectivity of the method for drugs, 307 nm was selected as the detection wavelength for UV-Visible detector. Tables 1 and 2 and Figure 2 briefs the system suitability parameters as obtained during the analytical method optimization of MTX.

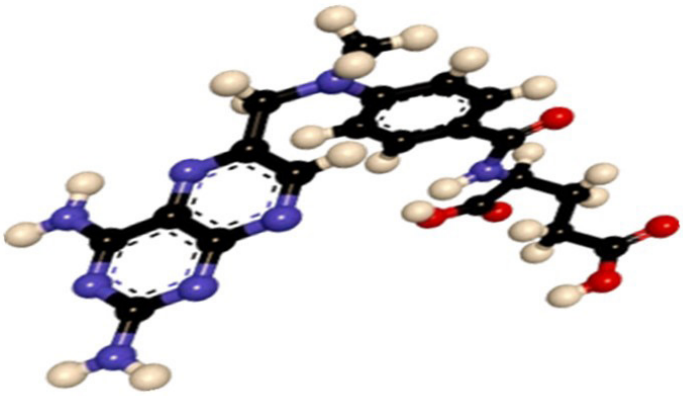
Standard stock solution preparation

Accurately weighed 10.0 mg of reference standard methotrexate was transferred into a 10 mL volumetric flask, dissolved in 5 mL of acetonitrile, and volume was made up to 10 mL with acetonitrile to get the concentration of solution 1000 g/mL (Stock-A). From this, 5 mL of stock-A was taken and diluted up to 50 mL with acetonitrile to get a concentration of 100 g/mL (Stock solution-B). (Corresponding to the linearity range stated in Table 3).

Working standard solution

An accurately weighed quantity of MTX (10.0 mg reference standard) was transferred into a 100 mL of volumetric flask and solubilized in a 0.015 N solution of sodium hydroxide (NaOH). Resultant solution in the volumetric flask was vigorously shaken for 10 min, and then the volume of the solution was made up





$CF: C_{20}H_{22}N_8O_5$

Mol. Wt: 454.439 Da

Chemical name:

(2S)-2-[[4-[(2,4-diaminopteridin-6-yl)methyl-methylamino]benzoyl]amino]pentanedioic acid

Disease	Min. Dose	Max. Dose
Rheumatoid arthritis	7.5 mg	30 mg
Crohn's disease (O)	12.5 mg	25 mg
Lupus (O)	7.5 mg	20 mg
Psoriasis	10 mg	30 mg

Figure 1: Chemical structure and key pharmaceutical details of Methotrexate.

to the 100 mL mark to acquire the standard MTX solution of (stock solution: 100 µg/mL). This stock solution was aliquoted and serially diluted with the respective volume of mobile phase solvent to get the working solutions of desired strength (perse the linearity and regression parameters as presented in Table 3). Following this, 20 µL volume of these standard solutions were injected in triplicate were made and chromatographed under the conditions as mentioned in Table 1. Then, the stock concentration of each standard solution were plotted against the area-under-the-curve (AUC) obtained from each corresponding run to obtain the standard calibration curve plot (Table 4).

Application of developed RP-HPLC Method for the analysis of MTX in standard Laboratory Bulk Stock samples

To test the applicability of the developed analytical method, three standard quality control laboratory MTX samples (650, 12500, and 2500 ng/mL; *n*=3) obtained from the Quality Control department as blended and blinded samples. Twenty microlitre injections from each sample were made and chromatographed under the conditions as mentioned above in Table 5. The obtained area under the curve was extrapolated using the validated equation and linearity graph to determine the Amount of MTX present in the formulation. Then the Amount analyzed (%) employing the developed analytical method was then determined.

Application of developed RP-HPLC Method determine MTX in Commercial Marketed Dosage Formulations

The applicability and suitability of the developed analytical method were tested using Methotex™ Gatwell Pvt. Ltd., Biotrexate®, Care First, Methocel™ Celon, and Plastomet-50, K Laboratories. Briefly, the sample was diluted suitably with solvent system and then chromatography was performed precisely to carry out the assay of MTX, as presented in Table 1. The obtained area under the curve was extrapolated using the validated equation and linearity graph to determine the Amount of MTX present in the formulation. Then the Amount determined (%) employing the developed analytical method.

Application of developed RP-HPLC Method to monitor release kinetics of MTX from an in-house developed Nanoformulation

Furthermore, the applicability and suitability of the developed analytical method in studying the release kinetic behavior of MTX from MTX-albumin nanoparticle preparation was also tested using extensive dialysis techniques. Briefly, nanoformulation of MTX and plain MTX were placed into a dialysis bag (MWCO: 12 KDa) and placed inside a beaker containing 500 mL of dissolution media as PBS pH 7.4 with 0.5 % v/v Sodium Lauryl Sulfate (SLS). The entire drug release assembly was maintained at 37±2°C with continuous magnetic stirring at speed of 300 rpm. At predetermined time (0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 48 hr), 1 mL sample were collected and diluted in an suitable fashion with the solvent system [(Mobile phase; 50 mM sodium acetate buffer (pH 3.6): acetonitrile (90:10, v:v)] and the chromatography of MTX was performed to carry out the MTX-assay as presented in the Table 1. Notably, each time, the release media was replaced with same quantity of fresh dissolution medium to preserve the sink condition in the study strictly. The obtained area under the curve was extrapolated using the validated equation and linearity graph to determine the strength of MTX present in the formulation. Then, the amount was estimated (%) employing the developed analytical method, and the release pattern was graphed by plotting time-interval on the x axis and the percent MTX release on the y-axis.

Table 1: Chromatographic Instrument and Optimized Chromatographic conditions.

System profile Stationary phase: Pump design	Mobile phase; Flow rate (mL/ min)	Gross Run time (min)	Column Temp (0°C)	Volume of injection loop (µl)	Detection wavelength (nm)	Sample Retention time (min)
Waters model no 784;Thermo (C-18) (5 µm, 250 mmx4.60 mm); water 515 binary pump	Isocratic System; 50 mM sodium acetate buffer (pH-3.6): acetonitrile (90:10, v:v); 1.0 mL/ min	11 min	25°C	20 µL fixed loop	307 nm	5.86 min

Table 2: System suitability parameters as obtained during the analytical method optimization of MTX.

Compound	t	n	R	T
MTX	5.86	3587	0.997	0.386

MTX: Methotrexate; R: Retention factor; T: Tailing factor; t: Retention time; n: No. of theoretical plates.

Table 3: Performance, detection parameters, linear regression, and statistical properties of the proposed method.

Linearity range* (g/mL)	Regression data			S^{φ}	S^{Ψ}	LOD^{ω} (g/mL)	LOQ^{Ω} (g/mL)
	a^{α}	b^{β}	r^{γ}				
0.5-3.5 g/mL	14736	225080	0.9996	2738	1487	0.041374	0.12241

*Results are represented as average of three observations. Here, α: Intercept; β: Slope; γ: Correlation coefficient; φ: Standard deviation of intercept; ψ: Standard deviation of slope; ω: Limit of detection; Ω: Limit of quantitation.

Table 4: Precision, accuracy and variation parameters of analysed MTX as obtained by developed method.

QC Sample strength (ng/mL)*	Measured conc. (ng/mL)	% Recovery	SD $^{\gamma}$	COV $^{\Psi}$
Intra-day (n=4)				
750	734.93	97.99	13.72	0.01866
1500	1454.04	96.94	17.64	0.01213
2000	1968.15	98.41	7.36	0.00374
3000	2943.62	98.12	25.96	0.00882
Inter day (n=4)				
750	748.50	99.80	19.86	0.02653
1500	1450.90	96.73	19.46	0.01342
2000	1960.43	98.02	22.55	0.01150
3000	2936.98	97.90	38.09	0.01297

*All experiments were performed in triplicate (n=3); γ: Standard Deviation; ψ: Coefficient of Variation (COV).

RESULTS AND DISCUSSION

MTX is a potent drug that can precipitate stern adverse effects if not used in the correct quantity and concentration. Assaying MTX is vital to guarantee that the drug exists in the exact concentration in the sample. Developing a simple, rapid, improved, and cost-effective analytical tool for its estimation has always been a thrust area of research. In this line, this investigation reports a new, rapid, simple, and low-cost RP-HPLC analytical tool with an UV detection channel for estimating MTX in bulk samples.

Optimization of chromatographic conditions

The MTX Separation protocol was developed on a reverse phase Liquid chromatography system (Waters™; Model: 784) fitted with an injector in manual mode and a binary pump Waters™; Model: 515). The system was equipped with a Thermo C_{18} column (5 micron, 4.60 mm x 250 mm) with constant flow rate and constant pressure delivery, and UV-Visible detector connected to the software system N 2000. For the optimization of the HPLC assay parameters, the influence of the composition of acetonitrile as well as the pH levels in the mobile phase were also evaluated. First, the column was equilibrated for 30 min (Constant Flow rate: 1.0 mL/min) while the column bed was pre-set at 25°C. The method employs an isocratic mobile phase comprising a readily available solvent system having 50 mM sodium acetate buffer (pH-3.6) and acetonitrile in 90:10, v:v, which flows through the column at a constant flow rate of 1.0 mL/ min (Table 1 and Figure 2).

Effect of acetonitrile levels in the mobile phase

The outcome of this investigation shows generation of a suitable peak attributes using the mobile phase system comprising of 10 % v/v acetonitrile. Varying proportion of acetonitrile in the mobile phase system was investigated as a function of MTX retention time as well as symmetry of MTX peak. This investigation concluded that 10%v/v acetonitrile offered optimal resolution condition that results the peak with symmetric and clearly-defined peak. The low acetonitrile concentration below 10% v/v, elicited the peak with prominent tailing and prolonged retention times. Conclusively, an increasing the proportion of acetonitrile beyond 10 %v/v yielded the peak with unsuitable peak shape and attributes.

Influence of buffer pH

The influence of pH of the buffer (aqueous) of the mobile phase system was investigated at various pH values between 3-7, which was adjusted employing required volumes of acetic acid or NaOH. The buffers of varying pH were used with 10%v/v of acetonitrile as the mobile phase system. The pH has a notable influence on the MTX retention as well as peak symmetry of MTX. The outcome of this study inferred that the chromatography mobile phase condition with pH 3.6 buffer offered the most optimum chromatography and resolution.

Concentration ranges and calibration graphs

Under the optimal chromatography conditions, observed sensitivity, and selectivity property of the method for MTX, the detection wavelength of 307.0 nm was set for the UV detector system. Under this chromatographic condition, MTX showed a retention time of 5.86 min following a 20 µL fixed loop injection of standard. Under this condition, the system-suitability parameters, including the theoretical plates, retention factor, and the tailing factor, are 3587, 0.997, and 0.386, respectively (Table 2).

Furthermore, under the optimized chromatography conditions, the linearity was obtained when a plot was developed between the standard concentration of drug and the peak area under curve (Table 3). The intercept, slope, and the correlation coefficient were determined and calculated under the guideline range. The higher values of the correlation coefficient (*r*, 0.9996) with insignificant value of intercept suggested acceptable linearity of the obtained standard calibration curve. For this assessment, the relative standard deviation and the standard deviations were also calculated and were found to be well under control and within standard range.

The linearity range under optimized chromatographic condition was found to be 0.5-3.5 g/mL following the straight-line equation of y=223633x-11819 and average regression data values as intercept: 14736; Slope: 225080, and Correlation coefficient to be 9996. This corresponds to the Standard deviation of intercept and Standard deviation of slope to be 2738 and 1487, respectively.

Detection and quantitation limits

The Limits of Detection (LOD) and the Limits of Quantitation (LOQ) of the developed analytical method were determined from the linear regression equation. Notably, limit of detection, and limit of quantitation were found to be 0.041374 g/mL and 0.12241 g/mL which shown in Table 3. The validation studies were performed by duly fulfilling the requirements of ICH guidelines. The developed analytical method was found to be accurate, selective, linear, precise robust with both inter-and-intra-day precision for routine testing of MTX.

Precision and accuracy

The investigate was performed in triplicate onto the samples of different proportions to determine the precision, coefficient of

Table 5: Application of developed RP-HPLC Method to determine MTX in standard Laboratory Bulk samples.

Sample ID	Standard concentration of QL Lab sample	Observed Amount (ng/mL)	Determined Amount (%)	SD ɣ	COV ψ
Blinded QC Sample-A	650 ng/mL	639.02	98.31	8.56	0.01339
Blinded QC Sample-B	1250 ng/mL	1218.45	97.48	6.51	0.00534
Blinded QC Sample-C	2500 ng/mL	2409.97	96.4	58.88	0.02425

*All experiments were performed in triplicate (*n*=3); ɣ: Standard Deviation; ψ: Coefficient of Variation (COV).

Table 6: Application of developed RP-HPLC method for the detection of MTX in commercial formulation.

Commercially marketed preparation	Reference Claim	Reference Claim Concentration (ng/mL)	Observed Amount (ng/mL)	Determined Amount (%)	SD ɣ	COV ψ
Methotex™ Gatwell Pvt. Ltd.	Methotrexate Injection Ip 500 mg/20 mL	2500 ng/mL	2453.269	98.13	48.33	0.0197
Biotrexate®, Care First	Methotrexate Injection Ip 50 mg/2 mL	2500 ng/mL	2479.293	99.17	21.02	0.0085
Methocel ™ Celon	Methotrexate Injection iv/Ip 50 mg/2mL	2500 ng/mL	2454.973	98.20	28.14	0.0115
Plastomet-50, K Laboratories	Methotrexate Injection Ip 50 mg/2 mL	2500 ng/mL	2451.723	98.07	34.61	0.0141

*All experiments were performed in triplicate (*n*=3); ɣ: Standard Deviation; ψ: Coefficient of Variation (COV).

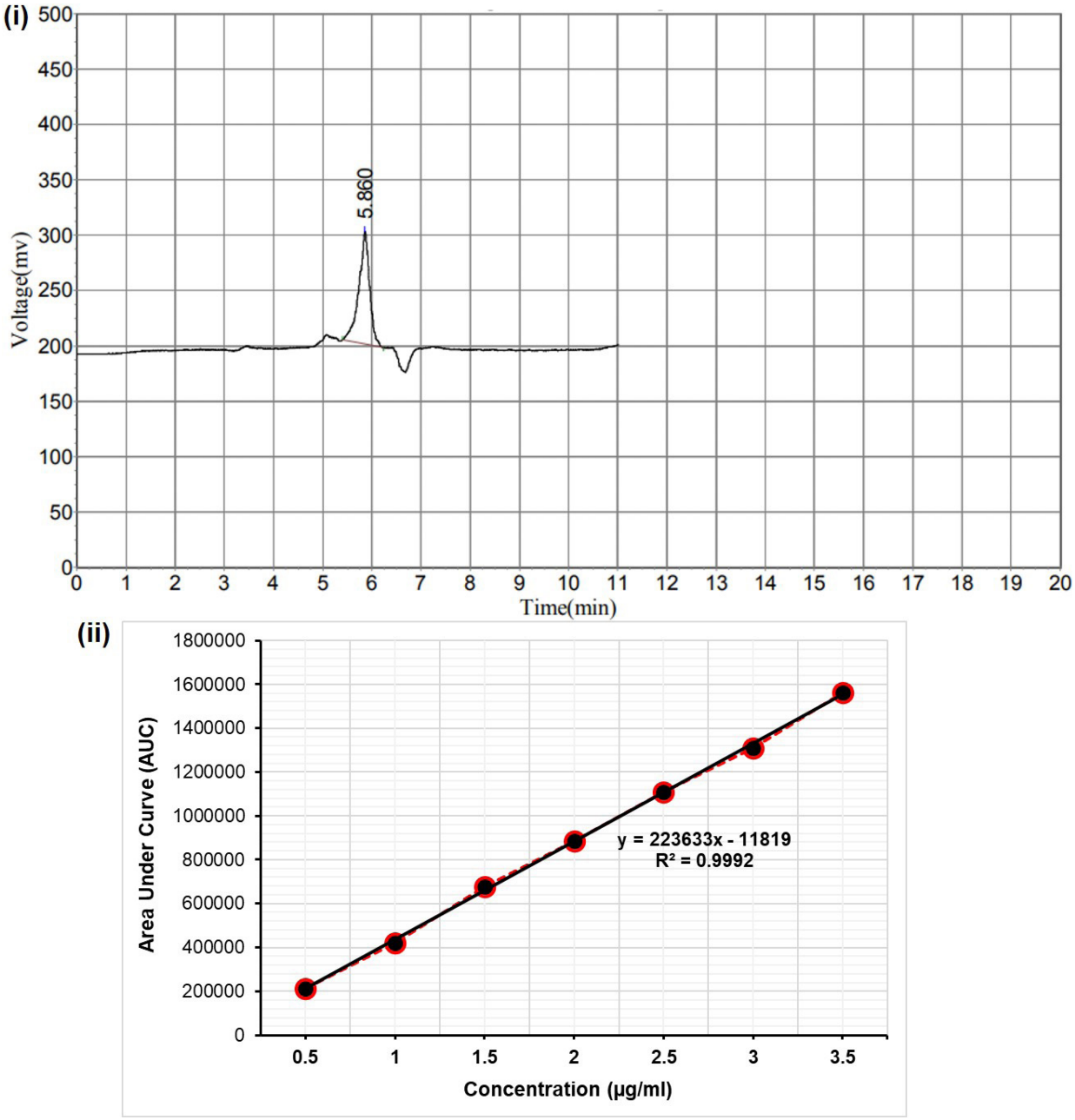


Figure 2: (i) MTX chromatogram and (ii) Linearity of MTX obtained following the injection of MTX (Injection volume: 20 µL) under optimized chromatographic conditions.

variation; and the accuracy of the developed HPLC method. The outcome of this investigation as depicted in Table 4 infers acceptable accuracy and precision linked to the developed analytical method. The analysis of the standard samples of MTX by the developed method showed acceptable precision and accuracy with significant recovery of MTX with more than 97% estimation of the labelled claim in the standard QC samples. The precision and accuracy showed acceptable results on both intra-day and inter-day analysis

Application of developed RP-HPLC method for the detection of MTX in standard Laboratory Bulk Stock samples, an in-house developed Nanoformulation, and Commercial Dosage Formulations

The developed RP-HPLC method has also been successfully applied to estimate MTX in standard Laboratory Bulk Stock samples, an in-house developed Nanoformulation, and Commercial Dosage Formulations. Briefly, the sample were

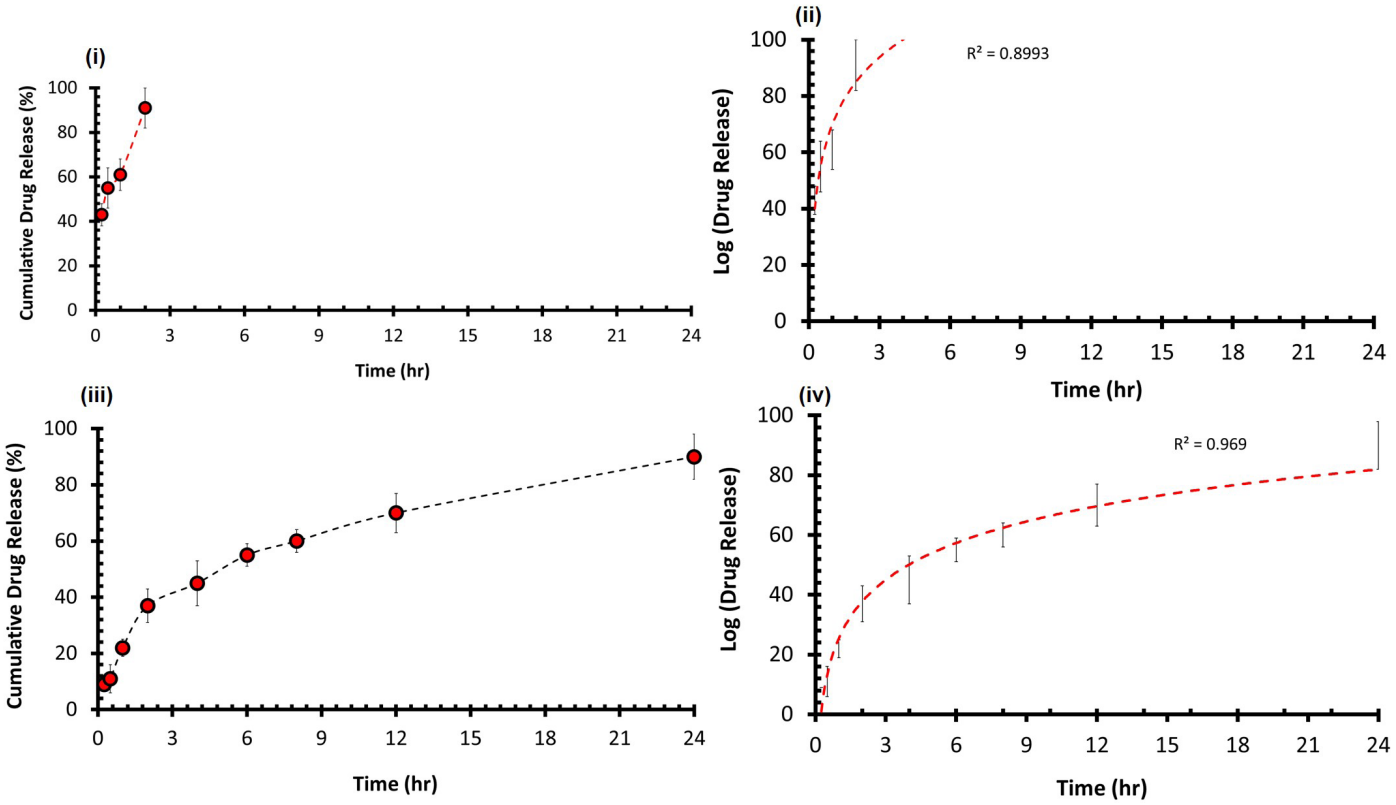


Figure 3: Application of developed RP-HPLC method to study the release kinetics of MTX from plain and in MTX-Nanoformulation.

suitably diluted using solvent mobile phase and chromatography was performed to do MTX assay (Table 1). The obtained area under curve was extrapolated using the validated equation and linearity graph to determine the Amount of MTX present in the formulation. For this, three standard blinded QC samples viz 650 ng/mL, 1250 ng/mL and 2500 ng/mL were procured from QC department (Table 5). In case of Standard concentration of QC Lab sample of concentration 650 ng/mL, and average of 639.02±8.56, with a coefficient of variation value of 0.01339. In similar lines, in case of standard of QC Lab sample of 1250 ng/mL and 2500 ng/mL concentrations, an average of 1218.45± and 2409.97±58.88 Amount was recovered using the developed method with a coefficient of variation value of 0.00534 and 0.02425, respectively.

Furthermore, the applicability and suitability of the developed analytical method was tested using Methotex™ Gatwell Pvt. Ltd., Biotrexate®, Care First, Methocel™ Celon, and Plastomet-50, K Laboratories (Table 6). Notably, in the case of Methotex™ Gatwell Pvt. Ltd., with a reference label claim of Methotrexate Injection Ip 500 mg/20 mL (2500 ng/mL) showed an appreciable amount of MTX, equivalent to 2453.269±48.33 ng/mL, was determined using the developed method. This recovery is approximately equal to 98.13% with a Coefficient of Variation of 0.0197. Another marketed preparation, Biotrexate®, Care First with label claim of Methotrexate Injection Ip 50 mg/2 mL (2500 ng/mL) determined 2479.293±21.02 equals to 99.17 recoveries with a Coefficient of Variation of 0.0085.

Similarly, Methocel ™ Celon (Methotrexate Injection iv/Ip 50 mg/2 mL) with a label claim of 2500 ng/mL showed 2454.973±28.14 (Coefficient of Variation: 0.0115). The marketed product Plastomet-50 from K Laboratories exists in the market with a label claim of Methotrexate Injection Ip 50 mg/2mL (2500 ng/mL) has been recovered to a level of 2451.723±34.61 with recovery of 98.07% at Coefficient of Variation of 0.0141. The acceptable level of determination deciphers the developed method to be capable of their engagement for the determination of MTX in the marketed preparations.

The method confirmed the rapid release of MTX from the conventional MTX formulation with 91.29±5.86% MTX release from the preparations at 2 hr time point. This release behavior as in agreement with the reported release pattern of MTX from its conventional formulations.[22] This is the reason, a sustained MTX releasing formulation most vocally in the form of nanoformulation has been largely attempted and advocated.[23] In this contour, the developed HPLC analytical tool was engaged for the investigation of the release behavior of MTX from its nanoformulation. Interestingly, the development of MTX in its nanoformulation form significantly improves its release behaviour with mere 37±2.95% MTX release at 2 hr time point as against to approximately 91.29±5.86 % in case of plain

MTX ($p<0.05$; Figure 3). The MTX-nanoformulation, offered a sustained release pattern of MTX till 24 hr, which was confirmed by the developed method wherein 90% MTX was released from its nanoformulation after 24 hr.

CONCLUSION

In conclusion, this investigation reports the development, validation and application of a RP-HPLC analytical methodology can be conveniently and routinely applied for MTX determination in standard Laboratory Bulk Stock samples, an in-house developed Nanoformulation, and Commercial Dosage Formulations. The analytical method as developed, validated and as applied in this research is easily translatable in regards to its cost-effectiveness, engagement of simple and easily available solvents that can be highly adaptable to the academic research, industrial QC testing as well as for regulatory confirmatory analytical investigations. The developed analytical method is significantly selective with no interference with the additives in the sample. The developed analytical tool is advocated for routine MTX monitoring as a valuable aid for the convenient analytical and laboratory sampling concerning standardization as well as for toxicity monitoring of antitumor therapy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SPE: Solid phase extraction; **VAMS:** Volumetric absorptive micro sampling; **MTX:** Methotrexate; **MWCO:** Molecular weight cut-off; **UPLC-MS/MS:** Ultra-performance liquid chromatography-tandem mass spectrometry; **LC-MS:** Liquid chromatography-mass spectrometry; **COV:** Coefficient of Variation; **ICH:** International Council for Harmonisation; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **n:** No. of theoretical plates; **QC:** Quality control; **R:** Retention factor**; RP-HPLC:** Reverse-phase high-performance liquid chromatography; **RT:** Retention time; **SD:** Standard deviation of intercept; **SLS:** Sodium lauryl sulfate; **t:** Retention time**; T:** Tailing factor.

SUMMARY

This investigation reports the development, validation and application of a RP-HPLC analytical methodology can be conveniently and routinely applied for MTX determination in standard Laboratory Bulk Stock samples, an in-house developed Nanoformulation, and Commercial Dosage Formulations. The developed analytical method can be easily translatable in regards to its cost-effectiveness, engagement of simple and easily available solvents that can be highly adaptable to the academic research, industrial QC testing as well as for regulatory confirmatory analytical investigations. The developed analytical method is significantly selective with no interference with the additives in the sample. The developed method represents a valuable tool in the laboratory analysis and monitoring of MTX during routine quality control testing and toxicity studies.

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