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A Reversed-phase High Performance Liquid Chromatography (HPLC) method for bio-analysis of Methotrexate

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Abstract

Methotrexate (MTX) is a chemotherapeutic agent used in the treatment of many disorders, including autoimmune diseases and cancers. The availability of a reliable analysis method for drug assay in biological fluids of interest is a prerequisite for all pharmacokinetic studies in humans or animal models. Considering the complex matrices of the biological specimens, as well as the low concentrations of the majority of the drugs in biological fluids, the development of an available yet sensitive method for the bioanalytical studies is often a challenging issue. For drug assay in aqueous, plasma, animal brain, and liver tissue environments in a concentration range of 25-600 ng/ml, a reverse phase high performance liquid chromatography (RP-HPLC) was developed. System suitability tests indicated an acceptable analytic separation efficiency, and peak shapes prove high selectivity of the method. Limit of detection (LOD) and limit of quantification (LOQ) were 10 ng/ml and 25ng/ml, which reflects method sensitivity. Regression analysis showed a linear correlation between area under the curve (AUC) of peaks and corresponding MTX concentrations. The within-day and between-day precision and accuracy were both in acceptable ranges. Recovery index of the method for median concentration (200 ng/ml) was about 74%. The developed method is in accordance with the acceptable criteria of analytical method validation. The sensitivity of the method in all the tested matrices makes the method suitable in terms of detection and quantitation of low concentration samples throughout the study. Moreover, the assay method has a fairly short run-time and lacks any significant interference.

Keywords: Bioanalysis, HPLC, Methotrexate.

1. Introduction

Methotrexate (MTX) [L-(+)-(P-[((2,4-diamino-6-pteridinyl) methyl) methylamino] benzoyl) glutamic acid] is an antineoplasm agent with a yellowish appearance. The ranges of decomposition and melting point temperatures of MTX are 185-204 °C and 182-189 °C, respectively. MTX, is an anti-metabolite of folic acid used as a chemotherapeutic agent (1). MTX stops thymine production in the cells thus interferes in DNA replication. In fact, MTX affects DNA replication the same as 5-fluorouracil (5-FU). But, unlike 5-FU, which reduces the action of thymidylate synthetase, MTX attaches tightly to dihydrofolate reductase (DHFR) and leads to depletion of the associated folate cofactors (2). Moreover, MTX is widely used as an alternative treatment for psoriasis and rheumatoid arthritis, when they are hard to treat by the conventional therapies.

MTX is absorbed rapidly after oral ad-

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ministration. The maximum plasma concentration level is attained in about 1-2 hrs. It then undergoes hepatic and intracellular metabolism and converts to an active metabolite. The elimination half-life of MTX is about 3-10 hrs for doses less than or equal to 30 mg/m^2. In higher doses, its half-life expands to 8-15 hrs. Renal excretion is the primary route of elimination, with 50-90% of the administered dose excreted intact in the urine within 24 hrs. Methotrexate is widely utilized in the treatment of a variety of malignancies, including leukemias and solid tumors. Treatment of solid tumors may require high doses of MTX, which are typically followed by the administration of calcium folinate to reduce MTX toxicity (3). Since calcium folinate accumulation is a transporter mediated process, it penetrates into normal cells in higher amounts compared to malignant cells; therefore, it serves as an ‘‘assistant agent’’ for normal cells in the MTX toxicity conditions. MTX is also utilized in breast carcinomas as an adjuvant therapy in several combination drug regimens. Other indications of methotrexate are in the treatment of psoriasis and rheumatoid arthritis. The resistance to MTX can happen in different ways, such as increased concentrations of DHFR, decreased transport of MTX into target cells, decreased bioactivation of MTX, and decreased thymidylate synthase activity (4).

The availability of a reliable analysis method for drug assay in biological fluids of interest is a prerequisite for all pharmacokinetic studies in humans or animal models (5-7). Considering the complex matrices of the biological specimens, as well as the low concentrations of most drugs in the biological fluids, development of an available while sensitive method for the bioanalytical studies is often a challenging issue.

To conduct further pharmacokinetic studies about the drug, a simple and selective bioanalytical high performance liquid chromatography with ultraviolet detector (HPLC-UV) assay for MTX in the PBS, plasma, brain homogenate, and liver homogenate of rats is developed and validated in this study. The bioanalytical method that is used here is similar to the methods used for other biological samples. It is in accordance with the acceptable criteria of analytical method validation, in addition to having a fairly short run-time and lack of any significant interference.

2. Materials and Methods

2.1. Materials

Methotrexate was kindly donated by Loghman Pharmaceutical Co. (Tehran, Iran).
All other chemicals, solvents, and reagents were of chemical or analytical grade and were purchased locally.

2.2. Instrument setup

The MTX concentrations in the samples throughout the study were determined by a reversed-phase high performance liquid chromatography (HPLC) method. The chromatographic system consisted of a C18 column (Eurosphere 100-5 C18, 150×4.6 mm, Germany) as the stationary phase and a mixture of phosphate buffer (0.01 M, pH 3.9) and acetonitrile (85:15) as the mobile phase. A pump-controller unit (Knauer, Wellchrom®, model k-1001, Berlin, Germany) and a Rheodyne injector (Rheodyne, Model 7725, USA) equipped with a 20 µl loop were used for solvent delivery (flow rate 1 ml/min) and sample injection, respectively. The analyte detection was carried out at a wavelength of 307 nm by a UV-detector (Knauer, model k-2600, Berlin, Germany). The chromatograms were analyzed using compatible software (EZChrom Elite®, Germany). A complete series of analytical method validation tests were carried out via the developed HPLC method (7, 8).

2.3. Sample preparation

Tissues were weighed, then homogenized in distilled water using a tissue homogenizer (IKA, model T25 Ultra Turrax, Germany). The amount of the distilled water was twice the tissue weight. To 225 µl of aqueous solution of plasma, brain, or liver homogenate, 25 µl of para-amino acetophenone (PAAP) (2 µg/ml) as the internal standard, and 40 µl of trichloro-acetic acid (2N in ethanol) were added; then the mixture was shaken for 30 min and centrifuged at 12,000 g for 15 min. Finally, 50 µl of the clear supernatant was injected to the chromatographic system.

2.4. Standard preparation

Stock solutions of 10 µg/ml of methotrex-

ate in distilled water were prepared. The standards were prepared using pooled PBS, human free plasma, and also rat drug free brain and liver obtained from healthy and untreated rats, as diluent. Finally, the drug concentrations of 25, 50, 100, 200, 300, 400, 500, and 600ng/ml were prepared by 1:10 dilution of the corresponding solutions with brain and liver homogenate, plasma, and PBS.

2.5. System suitability tests

The following parameters were calculated as system suitability indices of the developed method:

Number of theoretical plates (N)=$5.54(Rt/W_{h/2})^2$
Peak symmetry=W/2f
Retainability (k')=(Rt/ta)-1

Where, "Rt" is the peak retention time, "W" is the peak width at 0.05 peak height, "$W_{h/2}$"is the peak width at 0.5 peak height, "f" is the front half-width of the peak at 0.05 peak height, and "ta" is the retention time of non-retained peak (solvent front) (9.10).

2.6. Analysis validation tests

The developed method was validated by evaluation of selectivity, specificity, linearity, the limit of quantification (LOQ), precision, accuracy, and extraction recovery according to the US Food and Drug Administration (FDA) guidelines (11).

2.6.1. Selectivity

The selectivity of the method was explored by analyzing six individual human and rat blank plasma samples and also rat blank brain and liver samples to discover if the endogenous plasma, brain and liver compounds may interfere with the analytes and internal standard.

2.6.2. Limits of detection and quantitation

Limit of quantitation (LOQ) of the method was determined as the lowest methotrexate concentration capable of being quantitated with an acceptable accuracy (*i.e.* 80-120%) and precision (*i.e.* 20%). Limit of detection (LOD) was determined as the methotrexate concentration that produced a signal/noise ratio of 3.

2.6.3. Linearity

Serial samples, with the drug concentrations indicated in section 2.4 and the described preparation method, were injected to the chromatograph in three separate runs. In each case, the linear regression analysis was carried out on the known added concentrations of methotrexate against the corresponding peak AUC. Then the regression coefficient (r), slope, and intercept of the resulting calibration curves were determined.

2.6.4. Precision

2.6.4.1. Within-day variations

Three samples with methotrexate concentrations of 25, 100, 400, and 600 ng/ml (from low, middle, and high region of the standard curve) were prepared in triplicate and analyzed by the developed HPLC method, in the same day. The coefficient of variations (CV%) of the corresponding determined concentrations were calculated in each case.

2.6.4.2. Between-day variations

In three different days, three samples from upper, intermediate, and lower concentration regions used for the construction of standard curve (the same concentrations used in within-day variations test) were prepared and analyzed by the HPLC method. Then, the corresponding CV% was calculated in each case.

2.6.5. Absolute recovery

For each sample tested for within- and between-day variations, the absolute recovery of the method was determined as the ratio of the measured concentration (based on the standard curve) to the corresponding added (nominal) concentration (9).

2.6.6. Relative recovery

The percent ratio of the peak areas of the drug samples in each biological matrix (plasma, brain, and liver) to the corresponding peak areas after direct injection of aqueous solution of the analyte was determined as the index of the relative recovery of methotrexate from the mentioned matrices.

3. Results and Discussion

3.1. Drug assay

Considering the need for the assay of methotrexate throughout the experiments, developing an available, reliable, and easy-to-use reversed phase HPLC method was one of our primary objectives in this study. We adopted and validated the method for the drug assay in PBS, as well as brain and liver homogenates of rats.

3.1.1. System suitability tests

The outcomes of these series of tests were indicative of a proper separation efficacy and peak shape. However, there was some tailing, which was constant and size-proportional, therefore minimally harmful to results, if the peak area is considered as the response.

The number of theoretical plates (N), peak symmetry, and retentability (k') of the method were 2250, 1.03 and 4.98, respectively.

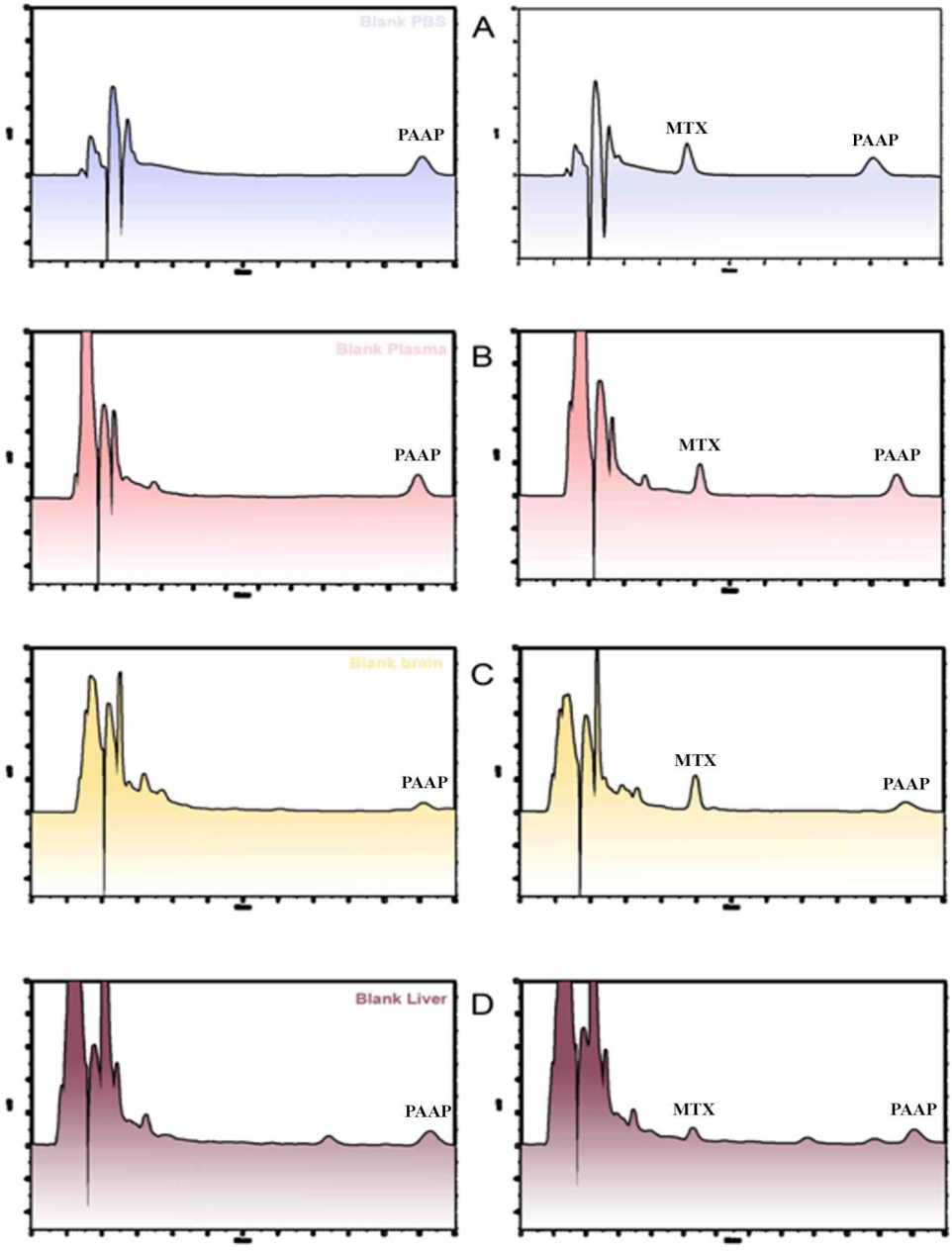

Figure 1. Typical chromatograms of the used HPLC method: Matrices were spiked by 600ng/ml MTX and 2µg/ml Para-amino acetophenone(PAAP) as an internal standard; PBS (A), Plasma (B), Brain (C) and Liver (D) compared with the relevant blank.

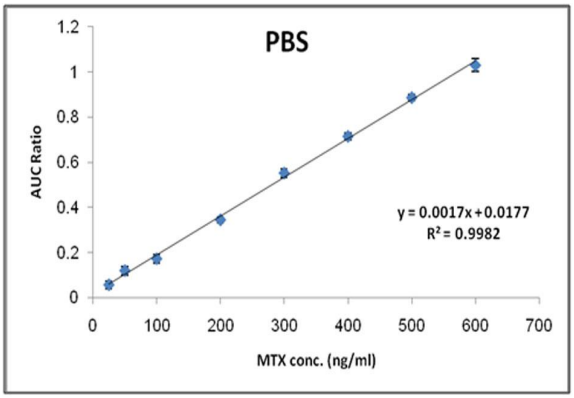

Figure 2. Calibration curves of MTX in PBS. (n=3).

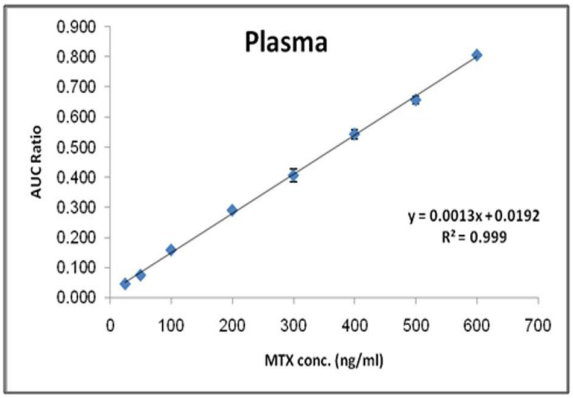

Figure 3. Calibration curves of MTX in plasma. (n=3).

3.1.2. Analysis validation tests

Validation of an analysis method is the process by which the method is tested by the developer or user for its linearity, range of applicability, accuracy, and precision for the intended purpose. The results of the linearity tests showed that the method produced linear response throughout the methotrexate concentration ranges of 25-600 ng/ml. The results of the within- and between-day variations indicated acceptable degrees of repeatability and reproducibility for the method. In addition the data reveals that the method is accurate, which ensures obtaining reliable results by the method. The values of LOD and LOQ reflect that the method is highly sensitive and can be used reliably.

3.1.2.1. Specificity and selectivity

Selectivity of the used HPLC method was evaluated in PBS, plasma, brain, and liver matrices and the separation achieved using the present analysis method for methotrexate is presented in Fig. 1. As indicated, no interfering endogenous components are evident with the retention times of all analytes in blank plasma, brain, and liver matrices extracts. Also, an acceptable resolution is seen in these data between the methotrexate and para-amino acetophenone (PAAP) as the internal standard. In other words, at wavelengths of 307 nm, endogenous plasma, rat brain, and liver components have negligible responses.

3.1.2.2. Limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) of the method were 10ng/ml and 25ng/ml, respectively.

3.1.2.3. Linearity

The linearity of the relationship between peak area ratios and corresponding concentrations were demonstrated by the regression analysis in each case. The method produced linear responses throughout the wide methotrexate concentration range of 25-600 ng/ml for PBS, plasma, brain, and liver. The results are summarized in Table 1 and are also shown in detail in Figs. 2 to 5.

3.1.2.4. Precision

The within- and between-day variations of the method are shown in Table 2.

3.1.2.5. Absolute recovery (Accuracy)

The within- and between-day absolute recovery values of the method are shown in Table 2.

Table 1. Calibration curve characteristics of methotrexate in PBS, plasma, brain and liver (n=3).

Samples	R square	Adjusted R square	Standard Error	Slope	F-value of Regression	Significance of F-value
PBS	0.9981	0.9978	0.0170	0.0017	3292.25	1.88E-09
Plasma	0.9990	0.9989	0.0096	0.0013	5916.74	3.25E-10
Brain	0.9989	0.9988	0.0200	0.0027	5621.41	3.79E-10
Liver	0.9985	0.9983	0.0086	0.0010	4105.06	9.72E-10

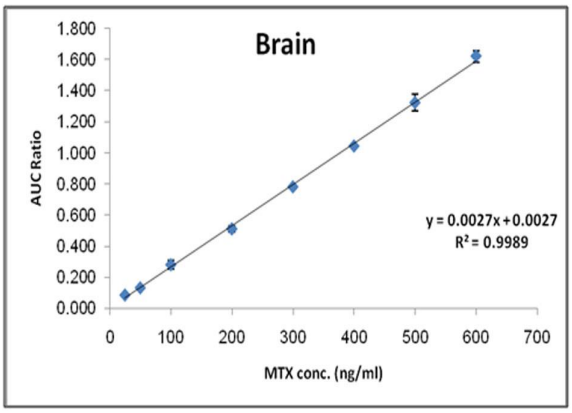

Figure 4. Calibration curves of MTX in brain homogenate. (n=3).

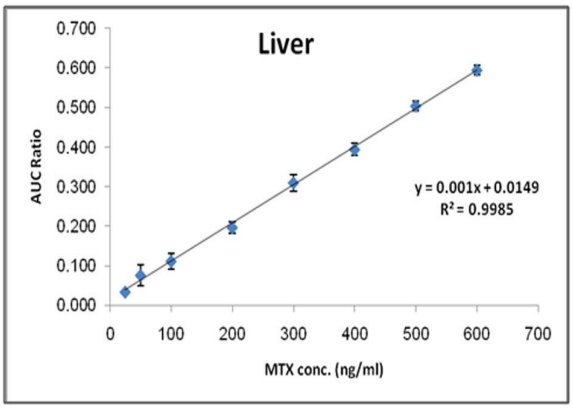

Figure 5. Calibration curves of MTX in liver homogenate. (n=3).

3.1.2.6. Relative recovery

The relative recoveries of the developed HPLC method in plasma, brain, and liver homogenates are shown in Table 2.

4. Conclusion

The reversed-phase HPLC methods for the determination of methotrexate in PBS, rat plasma, brain homogenate, and liver homogenate were developed and validated. The validation study supported the selected conditions by confirming that the assays were specific, accurate, linear, and precise. This method has a very short run time. It determines MTX very selectively with a baseline resolution, and it could be used in pharmacokinetic studies.

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Conflict of Interest

None declared.

Table 2. Within and between day variations of the assay method for quantitation of MTX (n=3).

MTX conc. (ng/ml)		Between day		Within day		Recovery
		CV%	Accuracy%	CV%	Accuracy%	Mean±SD
PBS	25	6.59	93.62	7.37	91.66	-
	100	2.85	89.61	5.26	90.92	-
	400	7.20	94.12	1.80	86.38	-
	600	4.81	98.83	4.15	93.43	-
Plasma	25	10.25	102.81	3.46	91.09	86.24±2.70
	100	9.17	97.39	4.78	107.50	92.52±8.13
	400	2.72	98.04	2.88	100.85	89.89±4.06
	600	3.39	98.98	0.81	100.86	83.11±3.05
Brain	25	9.61	96.95	2.85	107.53	75.35±5.68
	100	3.52	94.63	2.24	97.08	65.07±3.19
	400	4.03	96.31	5.95	91.85	60.95±2.98
	600	7.54	99.23	6.24	91.75	65.45±6.48
Liver	25	8.56	102.19	3.56	94.09	67.96±4.41
	100	8.58	99.42	3.28	99.93	66.82±5.09
	400	2.85	97.72	4.11	94.83	65.13±1.46
	600	3.33	99.44	2.22	96.64	61.37±3.69

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