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RESEARCH ARTICLE

Reverse-phase High-performance Liquid Chromatography Estimation of Methotrexate and Tretinoin in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

A simple, sensitive, and selective reverse-phase high-performance liquid chromatography (RP-HPLC) method with for the estimation of methotrexate (MTX) and tretinoin in pharmaceutical formulation and in spiked plasma developed and validate in the present work. Chromatographic separation of drug is performed with a 250 mm × 4.6 mm, 5 µm diameter particles RP C-18 column, and the mobile phase consisted of a mixture of Acetonitrile (ACN):buffer (85:15, v/v). Isocratic elution at a flow rate of 1 ml/min with ultraviolet detection at 340 nm at ambient temperature is used in this method. The proposed RP-HPLC method is successfully applied for the determination of MTX and tretinoin in pharmaceutical preparation and spiked plasma samples. The validation studies are carried out and it's fulfilling ICH requirements. The method is found to be specific, linear, precise (including both intra- and inter-day precision), accurate, and robust. This proposed method may represent a valuable aid in the laboratory monitoring of the toxicity of anticancer chemotherapy.

Keywords: Leukemia, linearity and calibration curve, malignant disorder, methotrexate and tretinoin, reverse-phase high-performance liquid chromatography.

INTRODUCTION

Leukemia is a malignant disorder of bone marrow and blood and is the most widespread cancer in children and teenagers. It accounts for about one-third of all cancers in children, acute myelogenous leukemia being the most common type. Leukemia therapy relies generally on combination chemotherapy utilizing a number of different anticancer drugs.[17-24] A drug combination of interest commonly engaged in treating leukemia includes methotrexate (MTX) and tretinoin (all-transretinoic acid [ATRA]).[28] MTX is an antimetabolite and antifolate drug used in the treatment of cancer and autoimmune diseases. MTX interferes with the growth of certain cells of the body, especially cells that reproduce quickly, such as cancer cells, bone marrow cells, and skin cells.

Extensive literature survey reveals that very few spectrophotometric and chromatographic methods have been reported. However, still, no method has been reported for the individual as well as simultaneous estimation of these drugs. Almost all the analytical methods reported are bioanalytical methods. Hence, it goes very important to develop a simple, precise, and accurate analytical method for the estimation of MTX and tretinoin individual as well as simultaneous in bulk and pharmaceutical dosage forms using high-performance liquid chromatography (HPLC).[1-16, 26-27]

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade acetonitrile, methanol, and acetic acid were purchased from Merck, while HPLC grade water was purchased from Qualigens, Mumbai, India. The drugs ATRA and MTX were received as gift samples from Shalaks Pharmaceuticals (P) Ltd., New Delhi, India, and M/s Dabur Research Foundation, Hyderabad, India, respectively.

Identification of drugs

The identification as well as authentication of both the procured drugs was done by ultraviolet (UV) and FT-IR spectroscopy.

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Determination of solubility of both drugs in different solvents

Solubility of MTX and ATRA was observed by dissolving them in different solvents and the observed results are shown in Table 1.

RP-HPLC method for the simultaneous estimation of methotrexate and all-transretinoic acid

Selection of chromatographic method

Proper selection of the chromatographic method depends on the nature of the sample (ionic/ionizable/neutral), its molecular weight, and solubility. Herewith, reverse-phase-HPLC (RP-HPLC) (Lachrom Merk, Series 7100) was selected for the separations as well as estimation of MTX-ATRA combination due to simplicity and suitability associated with the method.

Selection of suitable analytical wavelength

About 10 mg of reference standard MTX was dissolved in 100 mL of 0.1 N NaOH to yield stock solution of 100 µg/mL. This solution was scanned in spectrum mode over the entire UV range between 400 and 200 nm using UV spectrophotometer (Thermo Spectronic, Merk). Similarly, the UV spectra of stock solution of ATRA (10 µg/mL) were scanned, and then, both spectra were overlap to each other which show a point (isosbestic point) at higher absorbance of both drugs occur. The UV overlap spectra so obtained showed the wavelength of maximum absorbance (λ_{max}) at 340 nm, which was selected as working wavelength for the analysis. Overlay Spectra Shows in Figure 1.

Table 1: Result of the solubility of drugs in different solvents

Solvents	Solubility	
	MTX	ATRA
0.1 N NaOH	+++	+++
Water	+	++
Acetonitrile	++	+++
Methanol	++	++
Chloroform	-	+
Ether	-	+

+++: Freely soluble, ++: Soluble, +: Sparingly soluble, -: Insoluble.
MTX: Methotrexate, ATRA: All-transretinoic acid

Table 2: Linear regression data for linearity of methotrexate and all-transretinoic acid

Drug	Linearity range* (µg/ml)	Slope*	Intercept*	Regression coefficient (r^2)*±SD
MTX	1–6	598822	596740	0.9989±0.0001
ATRA	1–16	127525	10134	0.9998±0.0001

*Denotes average of three determinations. MTX: Methotrexate, ATRA: All-transretinoic acid, SD: Standard deviation

Selection of mobile phase

The pure solution of MTX and ATRA (2 µg/mL; 20 µL) prepared in 0.01 N NaOH was injected into RP-HPLC system and run in different mobile phases, namely methanol:water, methanol:phosphate buffer, ACN:methanol:water, ACN:methanol:water (with acetic acid 0.1%–1.3% v/v), and ACN:buffer (85:15) and was tried in different proportions to obtain ideal mobile phase for effective separation of both drugs. All chromatogram of different mobile phase obtained shown in Figure 1-4.

Preparation of mobile phase

ACN (85 ml) was mixed with freshly prepared 40 mM buffer solution (15 ml); then, finally, acetic acid was added into mixture; pH of resultant solution was 3.0. Final mobile phase was ultrasonicated for 20 min and then filtered through 0.45 µm Whatman filter paper.

Preparation of mixed standard solution of methotrexate-all-transretinoic acid

Reference standard of MTX and ATRA was accurately weighed (10 mg each), transferred to 100 mL volumetric flask, and dissolved in 0.01 N NaOH. The flask was vigorously shaken for 10 min and the volume was made up to the mark with the same solvent to obtain standard stock solution of MTX-ATRA (100 µg/mL; stock solution). This resultant stock solution was filtered through a 0.45 µ Whatman filter paper. The working mixed standard solution of MTX-ATRA was prepared from suitable aliquots of stock solution where pipette out and volumes were made up to the mark with mobile phase and the observed results are shown in Table 3. The chromatogram of mixed standards of both drugs shown in Figure 5.

Linearity and calibration curve

From MTX standard stock solution, aliquots are made with diluents to obtain concentration

of 1–6 µg/mL of MTX; in the same way, ATRA dilutions are prepared with diluents to obtain concentration of 1–16 µg/mL of ATRA. The solution of 20 µL was injected into column with the help of Hamilton syringe. All measurements were repeated 6 times for each concentration. The calibration curves of the area under curve versus concentration were recorded for both drugs and the observed results are shown in Table 2.

Preparation of sample solution for MTX and ATRA in mixed standards

From standard stock solutions of both standards drugs, the working mixed standard solution of MTX-ATRA having final concentration of 2 and 4 µg/mL, respectively, was prepared by diluting with mobile phase. The final solution of 20 µL was injected into column with the help of syringe and area was recorded and the observed results are shown in Table 4.

Method validation[29-32]

On the basis of fixed parameters, the method of estimation was validated for the following parameters.

Accuracy (recovery studies)

To check the degree of accuracy of the developed method, recovery studies were performed at 80%, 100%, and 120% of the label claim. The solutions were analyzed by RP-HPLC method as described above. At each level, three determinations were performe and the observed results are shown in Table 5.

Precision

The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple sample of homogenous sample.

Method repeatability

Degree of repeatability of the method and suitable statistical evaluation was carried out. Six samples of pharmaceutical formulation and prepared mixed standard solution of both drugs were analyzed. The percentage mean content, its S. D, C. V, and S. E. were calculated.

Interday and intraday precision

Variations of results within the same (intra) day and variation of results between days (interday)

Table 3: Result of the analysis of binary mixtures of drugs

Concentration of mixed standard (mg/ml)		AUC of MTX	AUC of ATRA	Expected concentration µg/ml		Concentration found µg/ml		Found (%)	
MTX	ATRA			MTX	ATRA	MTX	ATRA	MTX	ATRA
1	2	657,109	226,745	1	2	0.99	1.99	99.80	99.90
2	4	1,156,973	452,465	2	4	1.98	3.98	99.76	99.50
3	6	1,808,860	653,872	3	6	2.97	5.98	99.70	99.85
4	8	2,421,707	913,367	4	8	3.99	7.97	99.80	99.60
5	10	2,990,267	109,654	5	10	4.99	9.98	99.68	99.89

MTX: Methotrexate, ATRA: All-transretinoic acid, AUC: Area under the curve

Table 4: Results of the analysis of marketed formulation

Serial number	Expected concentration		Concentration found		Drug found (%)	
	MTX (mg/tab)	ATRA (%/gel)	MTX (mg/tab)	ATRA (%/gel)	MTX	ATRA
1	7.5	0.1	7.49	0.99	99.75	99.70
2	7.5	0.1	7.42	0.98	98.00	98.08
3	7.5	0.1	7.48	0.99	99.00	99.10
4	7.5	0.1	7.49	0.97	98.75	99.60
5	7.5	0.1	7.48	0.98	98.90	98.33
6	7.5	0.1	7.49	0.99	99.75	99.25
Mean±SD					98.446±0.8057	99.10±.7362
RSD					0.024	0.023

SD: Standard deviation, RSD: Relative standard deviation, MTX: Methotrexate, ATRA: All-transretinoic acid

Table 5: Results of the recovery study of methotrexate-all-transretinoic acid marketed formulation

Replicate	Amount taken (μg/ml)		Amount added at (μg/ml)			Recovery (%)	
	MTX	ATRA	Percentage	MTX	ATRA	MTX	ATRA
1	4	8	80	3.2	6.4	99.50	99.80
2	4	8		3.2	6.4	100.10	100.40
3	4	8		3.2	6.4	100.00	99.60
1	4	8	100	4	8	99.50	99.70
2	4	8		4	8	100.20	99.90
3	4	8		4	8	100.30	99.70
1	4	8	120	4.8	9.6	99.00	100.30
2	4	8		4.8	9.6	100.00	100.00
3	4	8		4.8	9.6	99.50	99.50
Mean±SD						99.79±0.431	99.87±0.307
RSD						0.004	0.003
Covar.						0.432	0.308

SD: Standard deviation, RSD: Relative standard deviation, MTX: Methotrexate, ATRA: All-transretinoic acid

were analyzed. Intraday precision was determined by analyzing sample solutions at different time intervals on the same day and on different day for interday precision and the observed results are shown in Table 7.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were separately determined based on the calibration curves and the observed results are shown in Table 8. The standard deviation (SD) of the y-intercepts and slope of the regression lines were used.

Solution stability

Solution stability was performed by taking chromatogram after a fixed time interval. The solution of MTX and ATRA was found to be stable for at list 24 h. The SD, coefficient of variation, and relative SD (RSD) were calculated.

Robustness

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of change in pH of mobile phase, flow rate, mobile phase ratio on the retention time, theoretical plates, area under curve, and percentage content of MTX and ATRA was studied. The mixed standard solution containing, MTX (2 μg/mL) and ATRA (4 μg/mL), was injected into sample injector of RP-HPLC 3 times under the varied conditions.

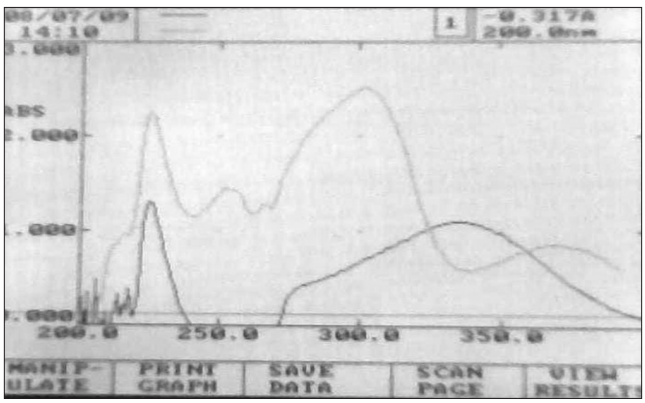

Figure 1: Overlay ultraviolet spectra of methotrexate-all-transretinoic pure Figure 1: Overlay ultraviolet spectra of methotrexate-all-transretinoic pure

System suitability parameters

As per USP-24, system suitability tests were carried out on freshly prepared standard stock solution of MTX and ATRA. 2 μL of MTX and 4 μg/mL of ATRA solution were injected under optimized chromatographic condition and the following parameters were studied to evaluate the suitability of the system Observed results are shown in Table 9.

RESULTS

Determination of the solubility of both drugs in different solvents

Determination of solubility of both drugs in was performing in different solvents. Maximum

solubility was found in Acetonitrile and methanol. Obtained results are shown in Table 1.

Selection of analytical wavelength[25]

Selection of suitable analytical wavelength done by using UV spectrophotometer (Thermo Spectronic, Merk). The UV overlap spectra so obtained showed the wavelength of maximum absorbance (λmax) at 340 nm. Obtained results shown in Figure 1.

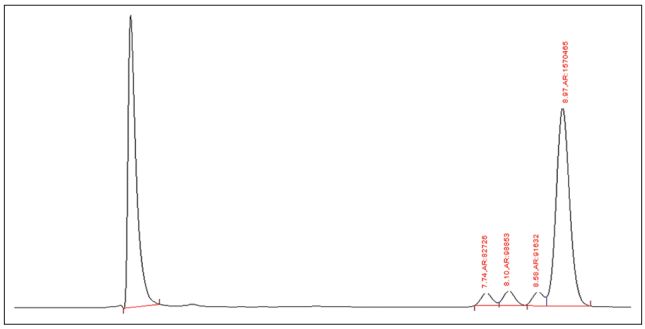

Figure 2: Methotrexate-all-transretinoic acid in ACN:buffer (75:25)

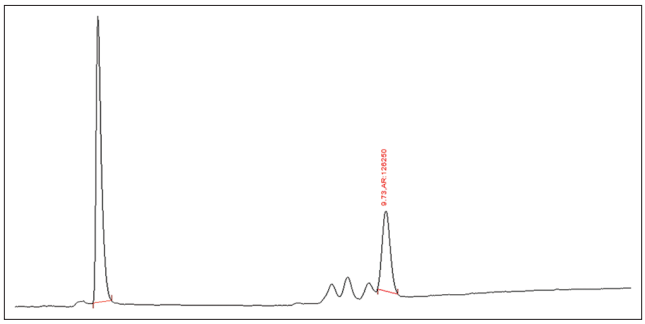

Figure 3: Methotrexate-all-transretinoic acid in ACN:buffer (80:20)

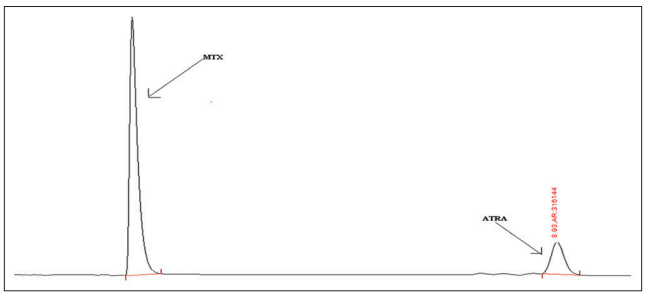

Figure 4: Methotrexate-all-transretinoic acid in ACN:buffer (85:15)

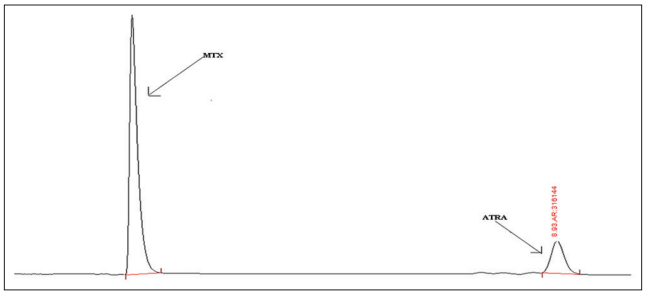

Figure 5: Typical chromatogram of methotrexate and all-transretinoic acid in mixed standards

Selection of mobile phase

For the best selection of mobile phase pure solution of MTX and ATRA (2 µg/mL; 20 µL) was injected into RP-HPLC system and run. We found ACN: buffer (85:15, v/v) was ideal mobile phase for effective separation of both drugs.

Linearity and calibration curve

At predetermined chromatographic conditions the linearity range was found 1–6 µg/ml for MTX and 1–16 µg/ml for Tretinoin with regression

Table 6: Data of method repeatability of methotrexate and all-transretinoic acid formulation

Drug	Label claim	Amount found* (%)	Standard deviation*	Coefficient of variation (%)*	RSD*
MTX (mg/tab)	7.5	99.64	0.0787	0.0311	0.019
ATRA (%gel)	0.1	98.12	0.098	0.0477	0.041

*Denotes average of six determinations. RSD: Relative standard deviation, MTX: Methotrexate, ATRA: All-transretinoic acid

Table 7: Intraday and interday precision of methotrexate and all-transretinoic acid formulation

Time Interval	Label claim (%)	
	MTX	ATRA
Intraday precision		
After 1 h	99.50	100.50
After 2 h	99.63	99.97
After 3 h	99.50	99.95
After 4 h	99.20	99.10
After 5 h	98.50	99.00
Mean	99.17	99.42
SD	0.204	0.172
COV (%)	0.205	0.172
Interday precision		
1st day	99.10	100.10
2nd day	98.20	99.90
3rd day	97.35	99.70
Mean	98.22	99.90
SD	0.340	0.126
COV (%)	0.252	0.126

MTX: Methotrexate, ATRA: All-transretinoic acid, SD: Standard deviation

coefficient values were 0.9989 and 0.9997 respectively. Results shown in Table 2.

Analysis of binary mixed standard solution

The working mixed standard solution of MTX-ATRA having final concentration of 2 and 4 µg/Ml were injected and obtained results shown in Table 3.

Analysis of commercial formulation

Analysis of marketed formulation done at predetermined chromatographic conditions and obtained results shown in Table 4.

Table 8: LOD and LOQ of methotrexate and all-transretinoic acid formulation

Drug	LOD (µg/mL)	LOQ (µg/mL)
MTX	0.6	0.8
ATRA	0.5	0.7

MTX: Methotrexate, ATRA: All-transretinoic acid, LOD: Limit of detection

Table 9: High-performance liquid chromatographic system suitability of methotrexate and all-transretinoic acid formulation

Injection number	Standard response	
	MTX 2 (µg/mL)	ATRA 4 (µg/mL)
1	1,115,973	452,765
2	1,115,750	452,701
3	1,115,130	452,720
4	1,115,127	452,750
5	1,115,247	452,738
Average	1,115,342	452,736
SD	420.9301	27
%RSD	0.25	0.012

SD: Standard deviation, RSD: Relative standard deviation, MTX: Methotrexate, ATRA: All-transretinoic acid

Method validation

On the basis of fixed parameters, the method of estimation was validated for the following parameters.

Accuracy (recovery studies)

Recovery studies were carried out at 80%, 100%, and 120% level. The results for recovery studies showed that results were within acceptable limits, above 99% and below 101%.

Precision

Method repeatability

The percentage means content, its S. D, C. V, and S. E. were calculated. All results come under limit as per guideline.

Interday and intraday precision

Obtained Results shown in table 5 and table 6. Results shows method is precise.

LOD and LOQ

Sample solution was subjected to LOD and LOQ studies; results are giWven in Tables 6 and 11.

System suitability/repeatability

The results of system suitability where shown in Table 9.

Table 10: High-performance liquid chromatographic robustness of methotrexate-all-transretinoic acid formulation

Effect on AUC										
Serial number	System suitability		Flow (−10%)		Flow (+10%)		pH=2.97		pH=3.03	
	MTX	ATRA	MTX	ATRA	MTX	ATRA	MTX	ATRA	MTX	ATRA
1	5973	2765	5965	2760	5950	2768	5971	2765	5978	2767
2	5945	2770	5955	2740	5945	2779	5949	2754	5940	2770
3	5956	2748	5950	2750	5940	2741	5950	2749	5965	2742
Mean±SD	5957±13.1	2761±11.5	5959±12.3	2750±10.5	5947±10.4	2766±14.5	5956±9.5	2755±9.2	5953±15.4	2759±12.6
Effect on RT										
Serial number	RT		Flow (−10%)		Flow (+10%)		pH=2.97		pH=3.03	
	MTX	ATRA	MTX	ATRA	MTX	ATRA	MTX	ATRA	MTX	ATRA
1	1.95	8.98	1.93	8.97	1.94	8.95	1.96	8.99	1.94	8.95
2	1.93	8.97	1.95	8.95	1.92	8.92	1.94	8.95	1.92	8.96
3	1.94	8.98	1.92	8.96	1.94	8.93	1.92	8.94	1.96	8.97
Mean±SD	1.94±0.01	8.98±0.02	1.93±0.01	8.96±0.01	1.93±0.02	8.93±0.02	1.94±0.02	8.96±0.01	1.94±0.02	8.96±0.01

SD: Standard deviation, RT: Retention time, MTX: Methotrexate, ATRA: All-transretinoic acid, AUC: Area under the curve

Table 11: System suitability test parameters

System suitability parameters	Proposed method	
	MTX	ATRA
Retention time (t_R)	1.95	8.98
Capacity factor (k)	0.95	7.98
Theoretical plate number (*n*)	2562	1535
Tailing factor (T)	0.58	0.85
Resolution (R)	6.8	

MTX: Methotrexate, ATRA: All-transretinoic acid

Robustness

The results of robustness are given in Table 10.

System suitability parameters

Various parameters were calculate and obtained results shown in Table 11.

DISCUSSION

MTX and tretinoin both drugs are most commonly used drugs. Literature survey reveals that tretinoin and MTX are given in combination for effective management of leukemia. The combination has shown great success rates in clinical practice.

A simple, rapid, accurate, and precise reverse-phase high-performance liquid chromatographic and UV methods were developed and validated for simultaneous estimation of MTX and tretinoin in bulk and in pharmaceutical dosage form.

Estimation of MTX-tretinoin combination by RP-HPLC method was achieved by LiChroCART RP C18 column and ACN:buffer (85:15 v/v)) of resultant pH 3.0, as mobile phase, at a flow rate of 1.2 ml/min and measured at 340 nm. The retention time of MTX and tretinoin was found to be 1.95 and 8.98 min, respectively. Linearity range was 1–6 μg/ml for MTX and 1–16 μg/ml for tretinoin; regression coefficient values were 0.9989 and 0.9997, respectively.

On the basis of the fixed parameters, the method of estimation was validated, for the following parameters. Precision studies were carried out using parameters such as repeatability, interday, and intraday precision for MTX and ATRA. Results showed that the percentage C. V was 0.0311 and 0.0472 and SD for interday was 0.654 and 0.745 and SD for intraday was 0.594 and 0.799, respectively. This shows that SD and %RSD of RP-HPLC method was under limit which shows that method was precise. For accuracy studied three replicate injections, each of three different test concentrations in the range of 80, 100, and 120% of labeled claim of formulation under study has percentage recovery. The results for recovery studies showed that results were within acceptable limits, above 99% and below 101%. Robustness studies were carried out using different analyst parameters. Results of robustness showed that no significant change in retention time and area under curve by small variation in method parameter.

System suitability test was carried out as per USP-24 and all suitability parameters of both methods come under acceptable limit.

CONCLUSION

Validation of the developed analytical methods shows good regression values at their respective wavelengths and the results of recovery study revealed that any small change in the drug concentration in the solution could be accurately determined by the proposed method. Hence, proposed methods are new, simple, cost effective, accurate, sensitive, and precise and could be adopted for routine quality control analysis of MTX and tretinoin.

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