ISSN: 2320 - 4230

Journal of Drug Discovery and Therapeutics

Available Online at www.jddt.in

CODEN: - JDDTBP (Source: - American Chemical Society)

Volume 5, Issue 3, March: 2017, 09-20

Research Article

RP-HPLC ESTIMATION OF METHOTREXATE AND TRETINOIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS.

Ashish Agrawal* [1], Manoj Sharma [2]

[1] **Research Scholar, Bhagwant University, Ajmer, Rajasthan, India.**

[2] **School of Studies in Pharmaceutical Sciences, Jiwaji University, Gwalior, India.**

Received 30 January 2017; Accepted 15 March. 2017

ABSTRACT

A simple, sensitive and selective RP-HPLC method with for the estimation of Methotrexate and tretinoin in pharmaceutical formulation and in spiked plasma developed and validate in present work. Chromatographic separation of drug is performed with a 250X4.6 mm, 5µm diameter particles RP C-18 column and the mobile phase consisted of a mixture of ACN: Buffer (85:15, v/v). Isocratic elution at a flow rate of 1 ml/min with UV 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: RP-HPLC, Leukemia, Methotrexate (MTX) and Tretinoin, Linearity and Calibration curve, malignant disorder

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 1/3rd of all cancers in children, acute myelogenous leukemia (AML) being the most common type. Leukemia therapy relies generally on combination chemotherapy utilizing a number of different anticancer drugs[25].

A drug-combination of interest commonly engaged in treating leukemia includes Methotrexate (MTX) and Tretinoin (all-*trans* retinoic acid, ATRA).[28] MTX is an antimetabolite and antifolate drug used in 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 has been reported. But 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. So it goes very important to develop a simple, precise, and accurate, analytical method for estimation of MTX and Tretinoin individual as well as simultaneous in bulk and pharmaceutical dosage forms using HPLC.

MATERIAL & 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 Authentification of both the procured drugs was done by UV and FT-IR spectroscopy.

Determination of solubility of both drugs in different solvents.

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

RP-HPLC method for the Simultaneous estimation of MTX and ATRA

Selection of Chromatographic Method

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

Selection of Suitable Analytical Wavelength

10 mg of reference standard MTX in was dissolved in 100 mL of 0.1N NaOH to yield stock solution of 100µg/mL. This solution was scanned in spectrum mode over the entire UV range between 400 to 200 nm using UV spectrophotometer (Thermospectronic, Merk). Similarly the UV spectra of stock solution of ATRA (10 µg/mL) was scanned and then both spectra were overlap to each other which show a point (isobestic point) at higher absorbance of both drugs occurs. The UV overlap spectra so obtained showed the wavelength of maximum absorbance (λmax) at 340 nm, which was selected as working wavelength for the analysis.

Selection of Mobile Phase

The pure solution of MTX and ATRA (2 µg/mL; 20 µL) prepared in 0.01N NaOH was injected into RP-HPLC system and run in different mobile phases viz: 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 were tried in different proportions to obtain ideal mobile phase for effective separation of both drugs.

Preparation of Mobile Phase

ACN (85ml) was mixed with freshly prepared 40 mM buffer solution (15ml), then finally acetic acid was added in to 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 MTX-ATRA

Reference standard of MTX and ATRA were accurately weighed (10mg 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 micron whatman filter paper. The working mixed standard solution of MTX-ATRA was prepared from suitable aliquots of stock solution were pipette out and volumes were made up to the mark with mobile phase.

Linearity and Calibration curve

From MTX standard stock solution, aliquots are made with diluents to obtain concentration of 1-6µg/mL of MTX, in the same way ATRA dilutions are prepared with diluents to obtain concentration of 1-16µg/mL of ATRA. The solution of (20 µL) was injected into column with the help of Hamilton syringe. All measurements were repeated six times for each concentration. The calibration curves of the area under curve Vs concentration were recorded for both drugs. ***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 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.

Method Validation[49-51]

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

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.

a) 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.

b) Interday and Intraday Precision

Variations of results within the same (Intra) day,

variation of results between days (Interday) 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.

Limit of Detection (LOD) and Limit of Quantization (LOQ)

The LOD and LOQ were separately determined based on the calibration curves. The Standard Deviation 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 Hrs. The Standard deviation, Coefficient of variation and Relative standard deviation was 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 were studied. The mixed standard solution containing, MTX (2 µg/mL) and ATRA (4 µg/mL) was injected into sample injector of RP- HPLC three times under the varied conditions.

System Suitability Parameters:

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

RESULTS

Determination of solubility of both drugs in different solvents

Table 1: Result of Solubility of Drugs in Different Solvents

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

Keywords: (+++), freely Soluble; (++), Soluble; (+), sparingly soluble; (-), insoluble

Selection of Analytical Wavelength[52-53]

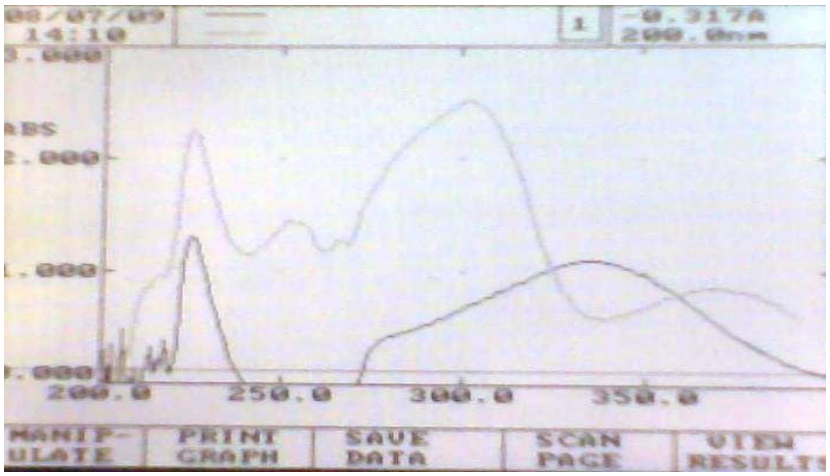

Fig. 1: Overlay UV spectra of MTX-ATRA pure

Selection of Mobile Phase

Fig. 2: MTX- ATRA in ACN: buffer (75:25)

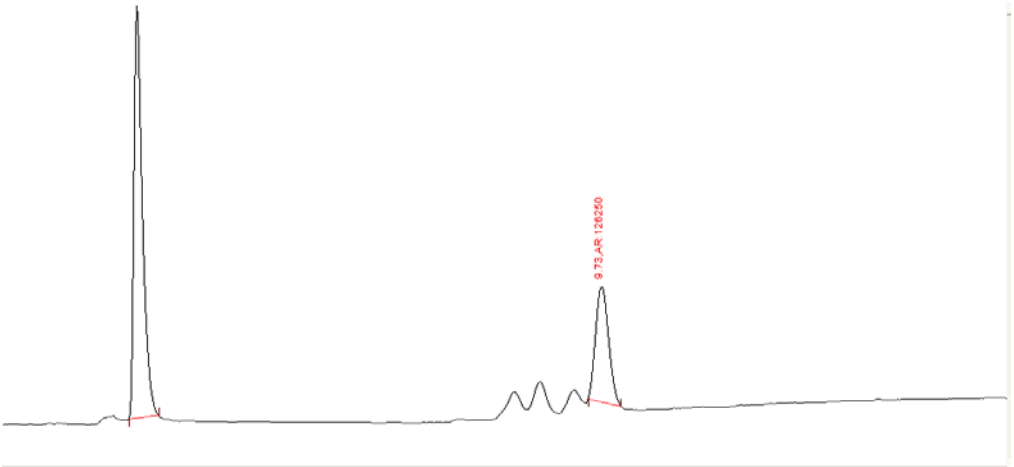

Fig. 3: MTX- ATRA in ACN: buffer (80:20)

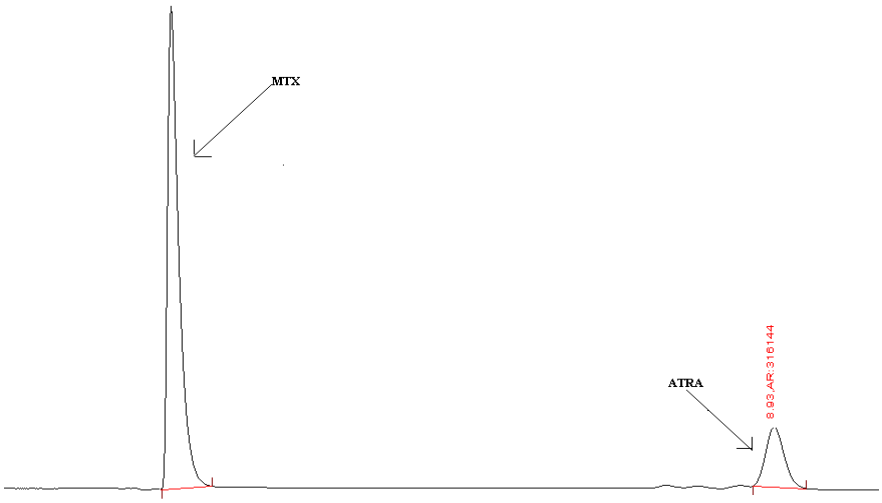

Fig.4: MTX- ATRA in ACN: buffer (85:15)

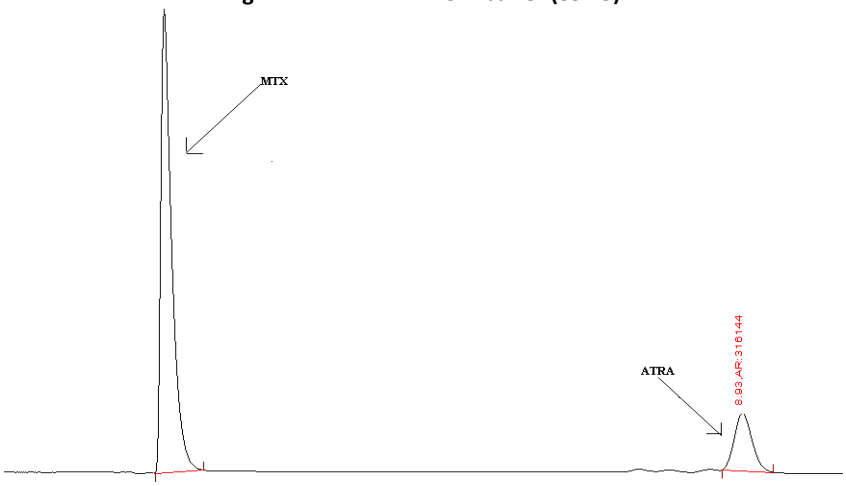

Fig. 5:- Typical Chromatogram of MTX and ATRA in Mixed Standards

Linearity and Calibration curve

Table 2: Linear Regression Data for linearity of MTX and ATRA

Drug	Linearity range * (µg/ml)	Slope*	Intercept *	Regression Coefficient(r^2)* ± S.D
MTX	1-6	598822	596740	0.9989±0.0001
ATRA	1 - 16	127525	10134	0.9998±0.0001

*Denotes average of three determinations

Analysis of binary mixed standard solution

Table 3: Result of analysis of binary mixtures of drugs

Conc. Of mixed std. (µg/ml)		AUC of MTX	AUC of ATRA	Expected Conc. µg/ml		Conc. Found µg/ml		% Found	
MTX	**ATRA**			**MTX**	**ATRA**	**MTX**	**ATRA**	**MTX**	**ATRA**
1	2	657109	226745	1	2	0.99	1.99	99.80	99.90
2	4	1156973	452465	2	4	1.98	3.98	99.76	99.50
3	6	1808860	653872	3	6	2.97	5.98	99.70	99.85
4	8	2421707	913367	4	8	3.99	7.97	99.80	99.60
5	10	2990267	109654	5	10	4.99	9.98	99.68	99.89

MTX- methotrexate , ATRA- tretinoin

Analysis of Commercial Formulation

Table 4: Results of analysis of marketed formulation

Sr. No.	Expected Conc.		Conc. 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					98.446	99.10
SD					0.8057	0.7362
RSD					0.024	0.023

Formulation:- Tablet: Newtrexate (Emil pharmaceutical, Tarapur),

Gel: Tretinoin gel 0.1%(Alpharma ,USPD)

Method Validation

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

1. 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%.

Table No. 5 Results of recovery study of MTX-ATRA marketed formulation

Replicate	Amount taken (µg/ml)		Amount added at (µg/ml)			% Recovery	
	MTX	ATRA	%	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						99.79	99.87
S.D						0.431	0.307
RSD						0.004	0.003
COVAR						0.432	0.308

Precision

Method Repeatability

Table 6: Data of Method Repeatability of MTX and ATRA Formulation

Drug	Label claim	Amount Found* (%)	Standard Deviation*	% Co-efficient 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

Interday and Intraday Precision

Table 7: Intraday & Inter day precision of MTX and ATRA Formulation

Intraday precision			**Inter day precision**		
	% Label Claim			**% Label Claim**	
	MTX	**ATRA**		**MTX**	**ATRA**
After 1hr	99.50	100.50	First day	99.10	100.10
After2hr	99.63	99.97	Second day	98.20	99.90
After3hr	99.50	99.95	Third day	97.35	99.70
After4hr	99.20	99.10			
After5hr	98.50	99..00			
Mean	99.17	99.42	**Mean**	98.22	99.90
SD	0.204	0.172	**SD**	0.340	0.126
% COV	0.205	0.172	**% COV**	0.252	0.126

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Sample solution was subjected to Limit of Detection (LOD) and Limit of Quantitation (LOQ) studies, results are given in Table No.6.11.

Table 8: LOD and LOQ of MTX and ATRA Formulation

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

System Suitability / Repeatability

Table 9: HPLC System suitability of MTX and ATRA Formulation

Injection No	**Standard Response**	
	MTX 2(µg/mL)	**ATRA 4(µg/mL)**
1.	1115973	452765
2.	1115750	452701
3.	1115130	452720
4.	1115127	452750
5.	1115247	452738
Average	1115342	452736
SD	420.9301	27
%RSD	0.25	0.012

Robustness

The results of robustness are given in Table No. 10.

Table 10: HPLC Robustness of MTX-ATRA Formulation

Effect on AUC

S. No	Sys 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	5957	2761	5959	2750	5947	2766	5956	2755	5953	2759
SD	13.1	11.5	12.3	10.5	10.4	14.5	9.5	9.2	15.4	12.6

Effect on Retention time

S. No	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	1.94	8.98	1.93	8.96	1.93	8.93	1.94	8.96	1.94	8.96
SD	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.02	0.01

System Suitability Parameters

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
Therotical plate Number (N)	2562	1535
Tailing Factor (T)	0.58	0.85
Resolution (R)	6.8	

Discussion:

Methotrexate 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 Methotrexate and Tretinoin in bulk and in pharmaceutical dosage form.

Estimation of Methotrexate–Tretinoin combination by RP-HPLC method was achieved by LachroCART RP C_{18} 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 340nm.The retention time of Methotrexate and Tretinoin were found to be 1.95 and 8.98 min respectively. Linearity range was 1-6µg/ml for Methotrexate and 1-16 µg/ml for Tretinoin, regression coefficient values 0.9989 and 0.9997 respectively.

On the basis of the fixed parameters, the method of estimation was validated, for following parameters. Precision studies were carried out using parameters like repeatability, interday and intraday precision for MTX and ATRA. Results showed that the % C.V was 0.0311, 0.0472 and SD for interday was 0.654, 0.745 and SD for intraday was 0.594, 0.799 respectively. This shows that SD, %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 % 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 parameter. Results of robustness showed that no significant change in Retention time and Area under curve by small variation in method parameter.
System suitability test were carried out as per USP-24 and all suitability parameter of both method comes 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 Methotrexate and Tretinoin.

REFERNCES

1. M. Cociglio, D. Hillaire-Buys, C.Alric; Determination of MTX and 7-hydroxyMTX by LC for routine monitoring of plasma levels; Journal of Chromatography B, 674 (1995), 101-110.
2. E.A. McCrudden, S.E. Tett; Improved HPLC determination of MTX and its major metabolite in plasma using a poly (styrene-divinybenzene) column; Journal of Chromatography B, 721 (1999), 87-92.
3. V. Ioffe, T. Rubinstein, R. Zabetski; RP-HPLC of polar lipids II.Procedure for a phospholipid derivative of MTX; Journal of Chromatography A, 1031 (2004), 249-258.
4. D. Abd El-Hady, N. Abo El-Maali, R. Gotti; MTX determination in pharmaceutical by enantioselective HPLC; Journal of Pharmaceutical and Biomedical Analysis, 37 (2005), 919-925.
5. A. Zarghi, Giovanni Puglisi; HPLC determination of stability of Tretinoin in tretinoin-minoxidil solution; Pharmaceutica Acta helvetiae, 73 (1998), 163-165.
6. Cutaneous Drug Reaction Case Reports; Am J Clin Dermatol ,142 (2000), 316-7.
7. M. Brisaert, M. Gabriels, V. Matthijis; Liposomes with Tretinoin: a physical and chemical evaluation; Journal of Pharmaceutical and Biomedical Analysis, 26 (2001), 909-917.
8. Tretinoin treatment before carbon-dioxide laser resurfacing: A clinical and biochemical analysis; American Academy of Dertatology, Inc. 2004.
9. D. Abd El-Hady, N. Abo El-Maali, V. Matthijis ; A rapid HPLC method for determination of stability of Tretinoin in tretinoin isotretinoin dermatological formulations; Journal of Chromatography A ,103 (2005), 183-193.
10. M. Bassam, Tashtoush, Elaine L. Jacobson; A rapid HPLC method for simultaneous determination of Tretinoin and isotretinoin in dermatological formulations; Journal of Pharmaceutical and Biomedical Analysis, 43 (2007), 859-864.

11. Jorge E. Cortes, Hagop Kantarjian; All-Trans Retinoic Acid followed by chemotherapy for salvage of refractory or relapsed Acute Promyelocytic Leukemia; University of Texas, M.D. Anderson Cancer Center, Houston. 1994.

12. Rodney R. Lrson, M. B. Khazaeli, H. Kenneth Dillon; Development of an HPLC method for simultaneous analysis of five Antineoplastic agents; Applied Occupational and Enviromental Hygiene, 18 (2003), 109-119.

13. Ossama T. Fahmy, Mohamed A. Korany, Hadir M. Maher; HPLC determination of some co-administered anticancer drugs in pharmaceutical preparations and in spiked human plasma; Journal of Pharmaceutical and Biomedical Analysis, 34 (2004), 1099-1107.

14. Yuerong Rose Ye, Edin Bektic, Barry Hunt; Simultaneous determination of Tretinoin and clindamycin phosphate and their degradation products in topical formulation by RP-HPLC; Journal of Separation Science, 27 (2004), 71-77

15. Laura Sabatini, Anna Barbieri, Marco Tosi; A new HPLC/ eletrospray ionization tandem mass spectrometric method for simultaneous determination of cyclophosphamide, MTX and 5-fluorouracil as markers of surface contamination for occupational exposure monitoring; Journal of Mass Spectrometry , 40 (2005), 669-674.

16. Lubor Urbanek, Lenka Krcmova, Dagmar Solichova, Petr Solich; Development and validation of a liquid chromatography method for simultaneous determination of α-tocopherol, retinol and retinyl ester in human serum using a monolithic column for monitoring of anticancer therapy side effects; Journal of Separation Science, 29 2006,2485-2493.

17. Meade, J. B., Karp, J. E., Guo, C., Adoo, C. B. S., et al; Timed sequential therapy of acute myelogenous leukemia in adults: a phase II study of retinoids in combination with the sequential administration of cytosine arabinoside, idarubicin and etoposide. Leukemia Research 27, 313–321 (2003).

18. Bauchez, A. S., Lunardi, J., Pernin, C., Marti-Battle, D., Fagret, D.; Detection of chemoresistance profile of cell lines K562, KB, GLC4, and HL60 through characterisation of the hmdr1, mrp, and Irp transcripts. In Vivo 15, 101-104 (2001).

19. Fenaux, P., Chastang, C., Chevret, S., Sanz, M., Dombret, H., et al,; A Randomized Comparison of All Transretinoic Acid (TRETINOIN) Followed by Chemotherapy and TRETINOIN Plus Chemotherapy and the Role of Maintenance Therapy in Newly Diagnosed Acute Promyelocytic Leukemia. Blood 94, 1192-1200 (1999).

20. Douer, D., Koeffler, H. P.; Retinoic acid enhances colony stimulating factor induced clonal growth of normal myeloid progenitor cells in vitro. Experimental cell research 138, 193-198 (1982).

21. Cassinat, B., Chevret, S., Zassadowski, F., Balitrand, N., Guillemot, I. et al., In vitro all-trans retinoic acid sensitivity of acute promyelocytic leukemia blasts: a novel indicator of poor patient outcome, Blood 98, 2862-2864 (2001).

22. Sanz, M. A., Martin, G., Cocco, F. L.; Choice of chemotherapy in induction, consolidation and maintenance in acute prommyelocytic leukemia, Best Practice and Research Clinical Haematology. 16, 433-451 (2003).

23. Camitta, B., Leventhal, B., Lauer, S., Shuster, J. J., Adair, S., Casper, J., Civin, C., Graham, M., Mahoney, D., Munoz, L.; Intermediate dose intravenous MTX and mercaptopurine therapy for non-T, non-B acute lymphocytic leukemia of childhood: a paediatric oncology group study. J. Clinical Oncology 7, 1539-1544 (1989).

24. Koh, L. P., Goh, Y. T., Teoh, G., Tan, P.; Treatment of Acute Promyelocytic Leukemia Using a Combination of All-trans Retinoic Acid and Chemotherapy. Ann. Acad. Med. Singapore 30, 401-408 (2001).

25. Snyder L.R., Kirkland J.J, Practical HPLC Method Development, 2nd Edition, John Wiley and Sons Inc, 2000,2(1), 38-42.

26. Lindsay Sandy, HPLC By Open Learning, Johan Wiley and Sons, London, 1991, 30-45.

27. Quality Assurance Guide, Organization of Pharmaceutical Producer of India; Section 7.2, 1-20.

28. Validation of Analytical Procedures: Methodology, ICH Harmonized Tripartite Guidelines, 1996, 1-8.

29. Quality Assuarance of Pharmaceutical – A Compendium of guidelines and Related materials, WHO, Geneva, 1997, I; 119-124.

30. Validation of analytical Procedures Text and Methodology; U.S.F.D.A Technical Review Guide: 2005.

31. Ingle, J. D;Crouch, S.R; Spectrochemical Analysis; Printince-Hall; New Jersy, (1988), 338, 478-480

Beckett, A. H, Stenlake J. B, Practical Pharmaceutical Chemistry, 4th Edition, II, CBS Publishers and distributers, 1997, 1-8, 162-167, 275-337