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# Simultaneous Spectrophotometric Estimation of Methotrexate and All-*trans* Retinoic Acid in Mixture

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A novel, simple, accurate and precise UV spectrophotometric method has been developed for the simultaneous estimation of methotrexate and all-*trans* retinoic acid in mixture. Distinct solubility characteristics of both the drugs required the development of common solvent, which was found to be a combination containing 1.5 % v/v Tween-20 and 5.0 % methanol in PBS; pH 7.4 ($PBST_{20}$Met). Based on interaction studies, both the drugs were found to be imposing no effect on the absorption maxima of each other. In the proposed method, the signals were measured at 258 and 340 nm, respectively corresponding to the absorbance maxima of methotrexate and all-*trans* retinoic acid (ATRA). Both the drug obeys the Lambert-Beer's law in the concentration range of 2-12 µg/mL. Results were authenticated statistically as well as by recovery studies. The method has the advantage of high sensitivity, lower limit of detection and could find a niche application in on-going research for the development of methotrexate and all-*trans* retinoic acid based chemotherapeutic combination for the treatment of leukemia.

**Key Words: Methotrexate, All-*trans* retinoic acid, Simultaneous estimation.**

## INTRODUCTION

Leukemia accounts for about 1/3rd of all cancers in children less than 15 years of age and 1/4th of cancers occurring before the age 20 years. Effective maintenance of leukemia relies on chemotherapy utilizing a combination of different anticancer drugs[1]. All-*trans* retinoic acid (ATRA) is a derivative of vitamin-A and is chemically 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nona tetraenoic acid. Basically, it is not a chemotherapy drug, but reports suggest that the combination of ATRA with chemotherapy significantly improves the outcome of leukemia[2-4]. All-*trans* retinoic acid is also known to be able to inhibit AML blast cell proliferation[5]. Maintenance therapy may be useful in preventing relapses. Methotrexate (MTX) is (N-[4-{[(2,4-diamino-6-pteridinyl)methyl]methylamine}benzoyl]-L-glutamic acid. It is an antimetabolite and a drug of choice in inducing complete remission in leukemia[2,4].

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Literature survey reveals that ATRA and MTX are given in combination for effective management of leukaemia[5]. The combination has shown great success rates in clinical practice[3]. Methods have been reported for estimation of MTX by HPLC[6-8], liquid chromatography with fluorimetric detection[9], with tandem mass spectrometry detection[10] and for ATRA by gas chromatographic[11], gas-liquid chromatograpy[12], HPLC[13], LC/MS/MS[14]. A novel polymer based drug delivery system for the controlled and targeted delivery of MTX and ATRA in combination is currently under development in our laboratory. No analytical method has so far been reported for the simultaneous estimation of these drugs. A successful attempt is made herewith to estimate these two drugs simultaneously by spectrophotometric means.

## EXPERIMENTAL

All chemicals used were of analytical grade. Methanol was purchased from Qualigens Fine Chemicals (Mumbai, India), Tween 20 (CDH Laboratories, Mumbai, India), $Na_2HPO_4$ (Himedia Laboratories, Mumbai, India), NaCl (Ranbaxy Fine Chemicals Ltd., New Delhi, India), NaOH and $KH_2PO_4$ were purchased from E Merk (India) Ltd (Mumbai, India). Doubled distilled water was used throughout the study. ATRA and MTX were received as a gift samples from Shalaks pharmaceuticals (P) Ltd, New Delhi, India and m/s SunPharma, Vadodara, India respectively. All chemicals used were of analytical grade.

Spectral absorbance measurements were made on GBC Cintra-10, UV/ Visible spectrophotometer (Australia) at an scan speed of 1400 nm/min and data interval of 1.006 nm keeping the constant slit width of 2.0 nm.

**Methodology and solvent selection:** MTX is readily soluble in PBS 7.4 but ATRA is not, hence a combination containing 1.5 % v/v Tween-20 and 5.0 % methanol in PBS 7.4 ($PBST_{20}Met$) was selected. Methanol was used as a co-solvent while Tween-20 served as stabilizer to avoid precipitation of ATRA[15].

**Determination of absorption maxima ($\lambda_{max}$) and overlain spectra:** Stock solution of ATRA (100 µg/mL) was prepared by dissolving 10 mg of drug in 5 mL of methanol and diluted to 100 mL with $PBST_{20}Met$. For preparation of stock solution of MTX, 10 mg of drug was dissolved in 50 mL of $PBST_{20}Met$, 5 mL of methanol was added (so as to maintain the homogeneity) and the volume was made up to 100 mL with $PBST_{20}Met$. The working standard solutions were scanned in the entire UV range (between 200-400 nm) for obtaining the overlain spectra.

The absorption maxima ($\lambda_{max}$) of MTX and ATRA were found to be 258 nm ($\lambda_1$) and 340 nm ($\lambda_2$) respectively (Fig. 1). MTX and ATRA showed good linearity with absorbance in the concentration range of 2-12 µg/mL at their respective absorbance maxima and correlation coefficient were found to be < 1 in both the cases (Table-1). First of all equimolar solutions of both the drugs were mixed, continually stirred for 2 h in dark and then scanned over entire UV range (Fig. 2). Existence of individual peaks of both drugs at respective wavelengths and absence of occurrence of fresh peaks is representative of non-interacting nature of both the drugs.

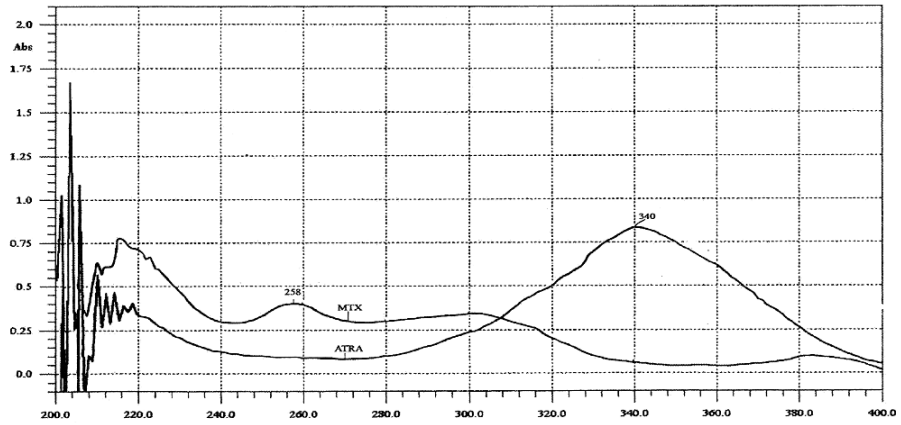


Fig. 1. Overlain spectra of methotrexate and ATRA in $PBST_{20}Met$

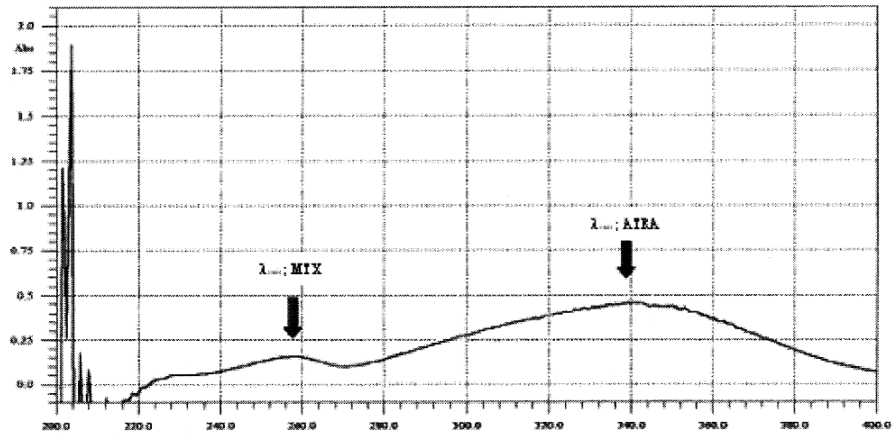


Fig. 2. UV scan of mixture containing both the drugs. Arrow represents the existence of respective peaks at wavelength, corresponding to $\lambda_{max}$ of both the drugs

**Development of simultaneous equation:** Six standard dilutions of both drugs having concentrations 2, 4, 6, 8, 10 and 12 μg/mL were prepared. The absorbance and absorptivity coefficients of these standard solutions in $PBST_{20}Met$ were determined at both selected wavelengths ($\lambda_1$ and $\lambda_2$). The optical properties and required values for calibration curve of MTX and ATRA are presented in Table-1, while Table-2 depicts the absorptivity values.

The quantitative estimation of drugs were carried out by solving simultaneous equations using Cramers rule and matrices, employing the mean absorptivity values (Table-2). A set of two simultaneous equations were framed (as presented below):

TABLE-1
REGRESSION AND OPTICAL CHARACTERISTICS OF MTX AND ATRA

| Parameters | MTX | | ATRA | |
|---|---|---|---|---|
| $\lambda_{max}$ in $PBST_{20}Met$ (nm) | 258 | | 340 | |
| Lambert-Beer's law range (µg/mL) | 2-12 | | 2-12 | |
| Regression values | 258 nm | 340 nm | 258 nm | 340 nm |
| Slope (m) | 0.04372 | 0.007836 | 0.0122 | 0.1146 |
| Intercept (c) | -0.05156 | -0.01 | -0.02351 | 0.06559 |
| Standard deviation | 0.1620 | 0.02956 | 0.04608 | 0.4293 |
| Standard error of mean | 0.06614 | 0.01204 | 0.01881 | 0.1752 |
| Correlation coefficient (r) | 0.9992 | 09937 | 0.9906 | 0.9991 |
| Coefficient of determination ($r^2$) | 0.9984 | 0.9875 | 0.999 | 0.9982 |
| Molar absorptivity (0.001 absorbance unit/mol cm/$dm^3$) | $14.87 \times 10^3$ | $2.63 \times 10^3$ | $2.21 \times 10^3$ | $38.44 \times 10^3$ |
| Sandell's sensitivity (µg/$cm^2$/0.001 absorbance unit) | 0.03054 | 0.17271 | 0.13532 | 0.00781 |

TABLE-2
ABSORPTIVITY VALUES FOR MTX AND ATRA

| Concentration (µg/mL) | Absorptvity | | | |
|---|---|---|---|---|
| | At 258 nm | | At 340 nm | |
| | MTX | ATRA | MTX | ATRA |
| 2 | 0.01749 | 0.00044 | 0.00283 | 0.14739 |
| 4 | 0.03038 | 0.00632 | 0.00533 | 0.13099 |
| 6 | 0.03467 | 0.00828 | 0.00616 | 0.12553 |
| 8 | 0.03683 | 0.00926 | 0.00658 | 0.12278 |
| 10 | 0.03811 | 0.00984 | 0.00683 | 0.12115 |
| 12 | 0.03897 | 0.01024 | 0.00700 | 0.12006 |
| Mean | 0.03274 ($a_{x1}$) | 0.00739 ($a_{y1}$) | 0.00579 ($a_{x2}$) | 0.12798 ($a_{y2}$) |

Values represent mean ± SD, (n = 3).

$$A_{258} = a_{x1}Cx + a_{y1}Cy \quad (1)$$

$$A_{340} = a_{x2}Cx + a_{y2}Cy \quad (2)$$

On putting values, we get:

$$A_{258} = 0.03274Cx + 0.00739Cy \quad (1')$$

$$A_{340} = 0.00579Cx + 0.12798Cy \quad (2')$$

where, $A_{258}$ and $A_{340}$ are the absorbance of diluted samples at 258 and 340 nm, respectively, while Cx and Cy represent the concentrations of MTX and ATRA, respectively in sample solution (µg/mL). By solving equation (1') and (2'), the values of Cx and Cy can be obtained as:

$$Cx = A_{258}0.12798 - A_{340}0.00739 / 0.004147 \quad (3)$$

$$Cy = A_{340}0.03274 - A_{258}0.00579 / 0.004147 \quad (4)$$

The method involves just the determination of absorbances of the sample mixture solution at 258 and 340 nm ($A_{258}$, $A_{340}$) and subsequent solving eqns. 3 and 4[16].

**Recovery studies:** Four standard laboratory samples of MTX and ATRA mixture (2:4, 4:8, 8:6 and 10:2; μg/mL: μg/mL) were randomly prepared in triplicate, its absorbance at both selected $\lambda_{max}$ were determined and the corresponding concentration was determined with the help of developed eqns. 3 and 4. Stastical analysis was performed with Graph Pad Instat Software (version 3.0, Graph Pad Software San Diego, California, USA) using one-way ANOVA followed by Tukey-Kramer multiple comparison test. Difference with $p > 0.05$ was considered statistically insignificant, whereas $p < 0.001$ was considered a very significant difference. The results of analysis as obtained in each instance were compared with theoretical value of 100 % and represented as ± SD.

## RESULTS AND DISCUSSION

MTX and ATRA are among the drug of choice administered in leukemia. Presently, development of a novel polymer based drug delivery system for the controlled and targeted delivery of MTX and ATRA combination is under progress in our Research Laboratory (PRL; India). This is the first ever-described analytical procedure for the simultaneous estimation MTX and ATRA in combination.

Distinct solubility characteristics of both the drugs required the development of common solvent, which was found to be a combination containing 1.5 % v/v Tween-20 and 5.0 % methanol in PBS 7.4, described as $PBST_{20}$Met in the paper. The fact that at $\lambda_{max}$ of one drug, the partner drug is showing the least absorbance was favourable towards the development of estimation method, based on simultaneous equation (Fig. 1). The determined optical properties and statistical parameters for MTX and ATRA are presented in Tables 1 and 2. The $\lambda_{max}$ of MTX and ATRA were found to be 258 nm ($\lambda_1$; $p > 0.05$) and 340 nm ($\lambda_2$; $p > 0.05$), respectively. Moreover, both the drugs were found to be non-interacting and impose no effect on the $\lambda_{max}$ of each other as is indicated from the UV scan of mixture containing both the drugs (Fig. 2).

The method requires only the determination of absorbance of sample solution containing both the drugs at the two selected working wavelengths, followed by simple mathematical calculations based on developed simultaneous eqauations[16]. This method was well validated by preliminary analysis of authentic laboratory samples and recovery studies. The recovery studies were performed on 5 standard mixtures containing 2:4, 4:8, 8:6, 10:2 and 2:10 proportions of MTX and ATRA, respectively. The results of analysis as obtained in each instance were compared with theoretical value of 100.0 % and represented as ± SD. Both the drugs were recovered to nearly 100 %, $p > 0.05$ (Table-3). The experiment was repeated 5 times in a day for intra-day and on 5 different days for inter-day precision. The outcome of the recovery studies revealed that the developed method is accurate, precise, rapid and reproducible for routine simultaneous detection of MTX and ATRA in mixtures. The method was found to be highly precise as is depicted from lower % RSD in both the cases.

TABLE-3
RECOVERY STUDIES OF VARIOUS MTX AND ATRA FROM STANDARD LABORATORY SAMPLES

| Sample code | Standard mixture solution (µg/mL) | | %MTX Recovered | %ATRA Recovered |
|---|---|---|---|---|
| | MTX | ATRA | | |
| $R_1$ | 2 | 4 | 100.32 ± 1.35 | 100.29 ± 1.15 |
| $R_2$ | 4 | 8 | 99.75 ± 1.00 | 99.84 ± 0.77 |
| $R_3$ | 8 | 6 | 100.11 ± 0.86 | 99.71 ± 0.34 |
| $R_4$ | 10 | 2 | 100.46 ± 1.07 | 99.43 ± 0.18 |
| $R_5$ | 2 | 10 | 99.71 ± 0.63 | 99.02 ± 0.85 |

Values represent mean ± SD, (n = 3); $R_1$, $R_2$, $R_3$, $R_4$ and $R_5$ signify the authentic laboratory samples.

This new analytical method is of particular use to product development scientists in on-going researches aiming at development of combined dosage formulation of MTX and ATRA for the effective treatment/maintenance of leukemia.

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