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Development and Validation of UV-Visible Spectrophotometric Method for the Analysis of Methotrexate in Pharmaceutical Formulations

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Abstract: ***A simple, sensitive, selective rapid spectrophotometric method has been developed for the determination of post synaptic α_1- Adrino receptor antagonist Methotrexate in pure form and pharmaceutical formulations based on the comparative method of analysis and recovery studies like reductive coupling reaction and diazotization with1,10-PT,2,2-BP, FC reagent, $NaNO_2$/HCl and $k_3FeCN_6/FeCl_3$ at P^H–4.0 which is extractable at 430 nm,480nm,680nm,440nm & 680nm. Beer's law is obeyed in the concentration ranges 0.5 -3 µg/ml,1-6 µg/ml,0.4-2 µg/ml1-4 µg/ml and 0.1 -0.5 µg/ml . The developed method was applied directly and easily for the analysis of the Pharmaceutical formulations and R.S.D was found to be 0.46%,1.5%,0.6%,0.88% and 0.89% and Recovery 99.92 ± 0.12 , 99.19 ± 0.12, 99.81 ± 0.12 100.03 ± 0.72 and 99.95 ± 0.15 respectively. The method was completely validated and proven to be rugged. The interferences of the other ingredients and excipients were not observed. The repeatability and the performance of the proved method were established by point and internal hypothesis and through recovery studies.***

Keywords: Spectrophotometry, Methotrexate,

I. INTRODUCTION

Methotrexate-{(2S)-2-[(4-{[(2,-diaminopteridin-6-1(methyl)](methyl) amino} phenyl) form amide]} is a Antineoplastic anti-metabolite. Methotrexate anti-tumor activity is a result of the inhibition of folic acid reductase, leading to inhibition of DNA synthesis and inhibition of cellular replication. It is structurally related to prazosin (Figure1)

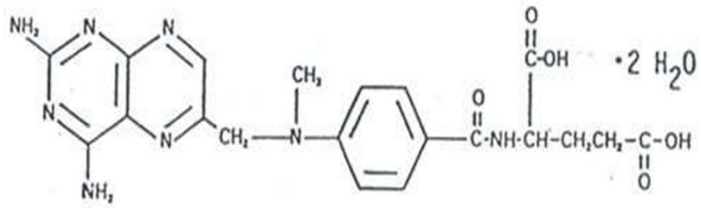

Figure.1

There is however no reported UV- Visible spectrophotometric method for the comparative colored complex method formational analysis of Methotrexate in its technical grade and formulations. UV- visible spectrophotometric method for the quantitative determination of Methotrexate the Functional group used for color development of Methotrexate was primary amine group. Early analysis of methotrexate in Human plasma by HPLC with fluorescence detection , HPLC determination of methotrexate polyglutamates after Low-Dose methotrexate therapy in patients with Rheumatoid arthritis31,Quality control of methotrexate by HPLC46 and Polarographic and voltammetric methods26-29 for the quantitation of MTX in pharmaceuticals and plasma samples have been published. In the present study an attempt has been made to develop simple UV-Visible spectrophotometric method for quantitative estimation of Methotrexate in its technical grade and formulations. The result obtain in this method was based on complex and diazotization reactions formation reaction of Methotrexate with 1,10-PT,2,2-BP,FC reagent $NaNO_2$/HCl , $k_3FeCN_6/FeCl_3$

An attempt has been made to develop and validate to ensure their accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in various gradients.

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II. MATERIALS AND METHODS

A. Preparation of Standard drug Stock and Working Solutions

The stock solution (1mg/ml) of Methotrexate was prepared by dissolving 100mg of the drug in 20 ml Dimethyle Sulfoxide and made up to 100mi with Dimethyle Sulfoxide to get a clear solution. A portions of this stock solution diluted step wise to get the working standard solutions of concentrations 100 µg ml^{-1}

B. Preparation of reagents

All the chemicals and reagents used were of analytical grade solutions were prepared in double distilled water.

- 1,10-Phenothraline(0.01M)
- Ferric chloride (0.003M)
- Ortho phosphoric acid (0.2M,0.02M)
- 2,2-Bipyridiene (0.01M)
- $K_3Fe(CN_6)$ (0.2%)
- HCl(1N)
- Folin-Ciocaltaeu 1:2 diluted
- Sodium Carbonates (20%)
- Sodium Nitrite(0.1%)

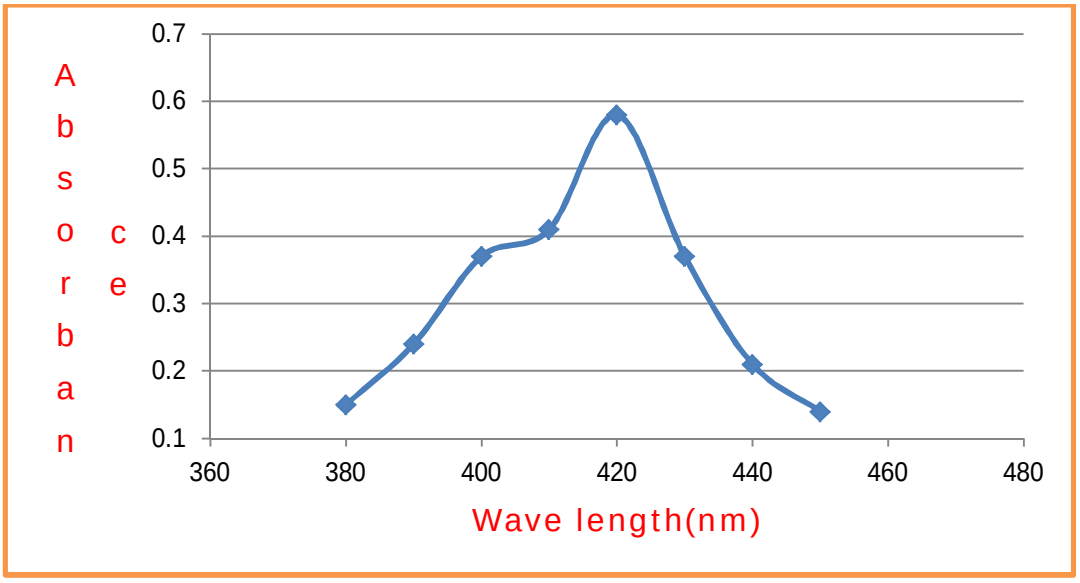
C. Develop and used procedures

The following new procedures were developed on the basis of reactions of the functional group present in Methotrexate and recommended then for the determination of Methotrexate in bulk, dosage and pharmaceutical formulations.

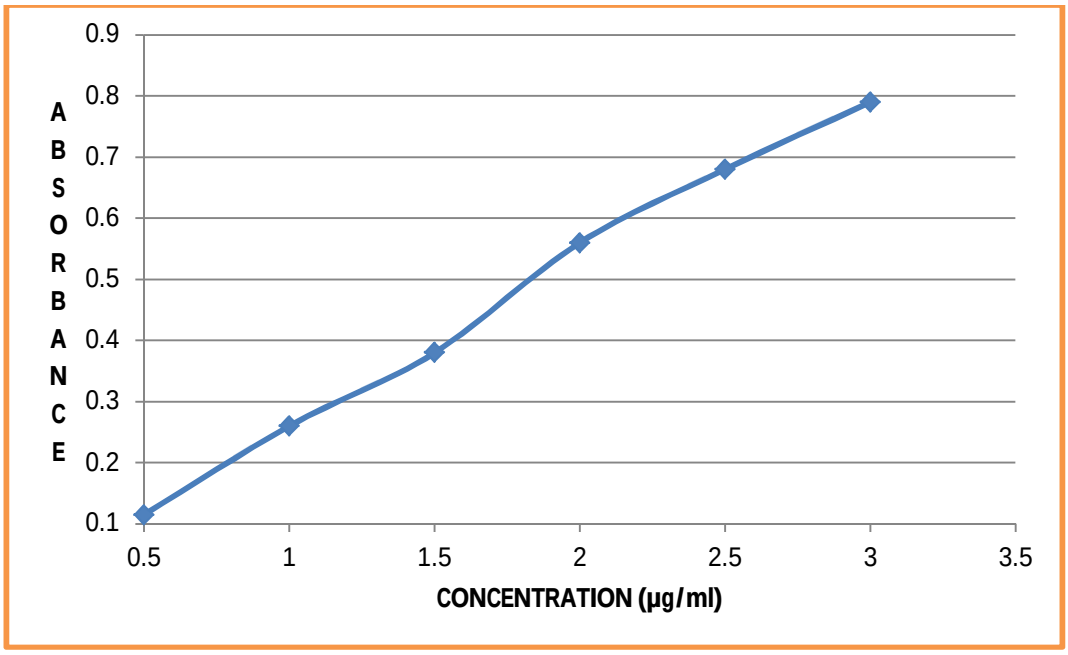
1) Method-1

Aliquots of standard drug solutions of MTX ranging from 0.5 to 3.0 ml (1ml=100 µg) were transferred to a series of 10ml graduated tubes. To each tube1ml of 1,10-PT ligand solution was added followed by 1ml Ferric Chloride solution and the resulting solution was heated for 15min at 100^0 C and finally 2ml of ortho phosphoric acid was added. The volume was made up to 10ml with distilled water and the absorbance of the orange red colored chromogen was measured at 430 nm against the corresponding reagent bank. The amount of MTX was computed from the Beer-Lambert's plot

Absorption spectrum of Methotrexate with 1,10-phenanthroline.



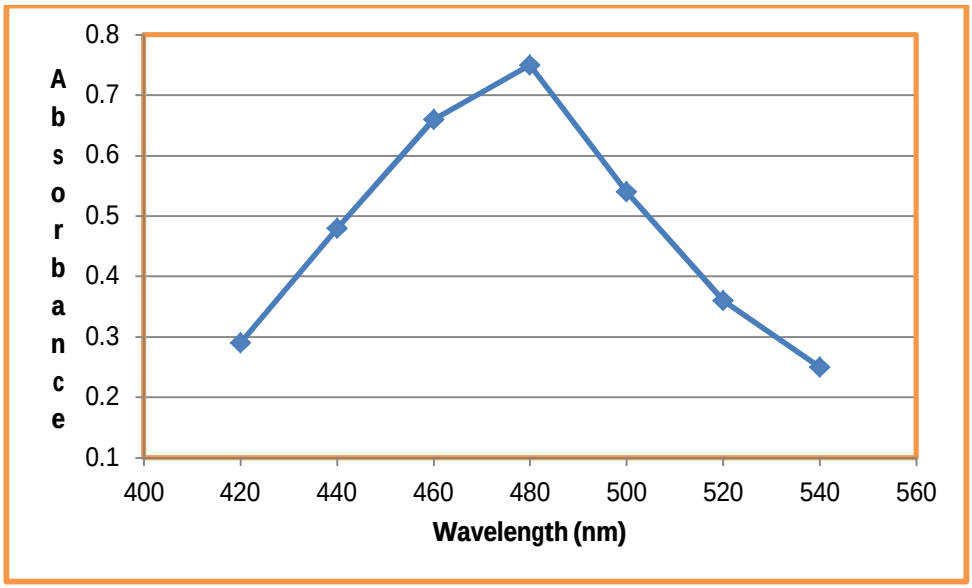
Beers law plot of Methotrexate with 1,10-phenanthroline



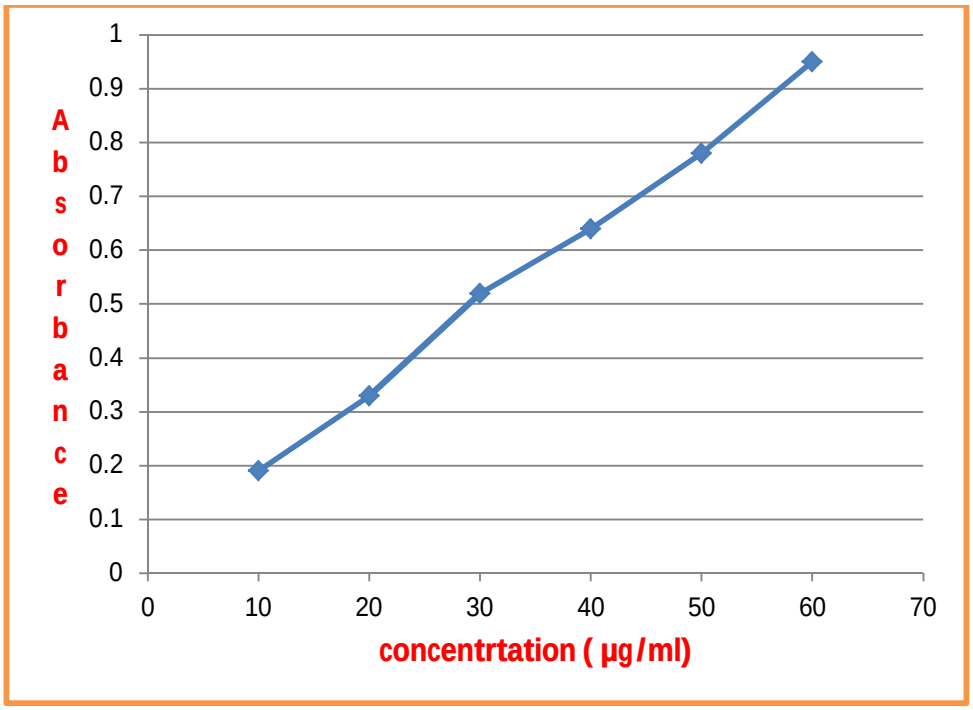
2) Method:2

The Methotrexate solutions ranging from 1ml to 6 ml (1ml=100 µg) were taken into series of 10ml graduated test tubes. 1ml of 2,2 Bypyridine solution was added followed by 1ml of ferric chloride solution heated for 15min at 100^0C and 2ml of ortho phosphoric acid added. The volume was made up to 10ml with distilled water. The absorbance of the orange colored chromogen was measured at 480nm against corresponding reagent blank. The amount of methotrexate was computed following previously described procedure.

Absorption spectrum of Methotrexate with 2,2-Bipyridine.



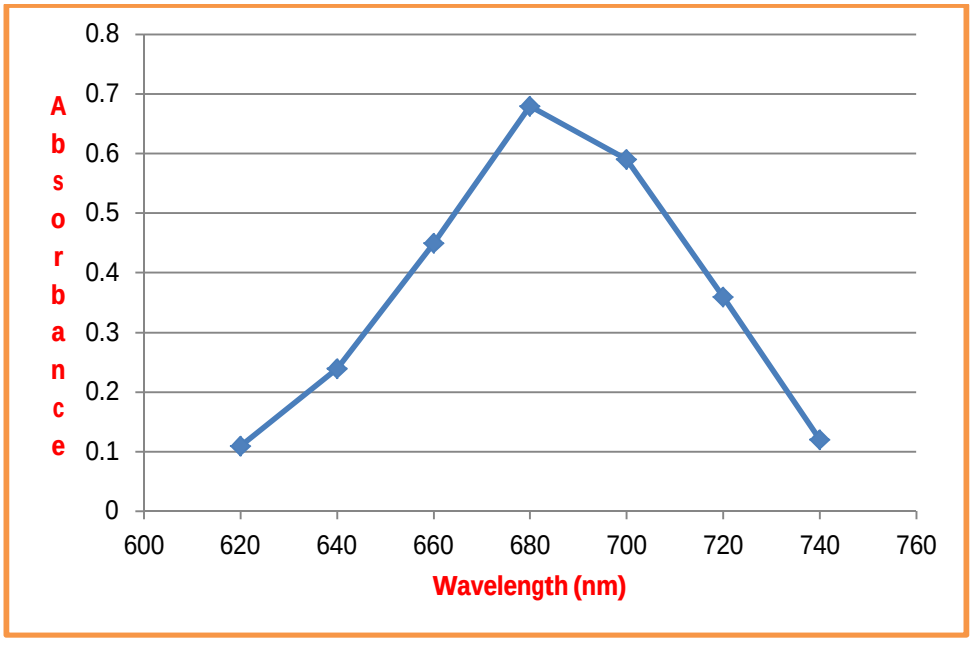
Beers law plot of Methotrexate with 2,2-Bipyridine



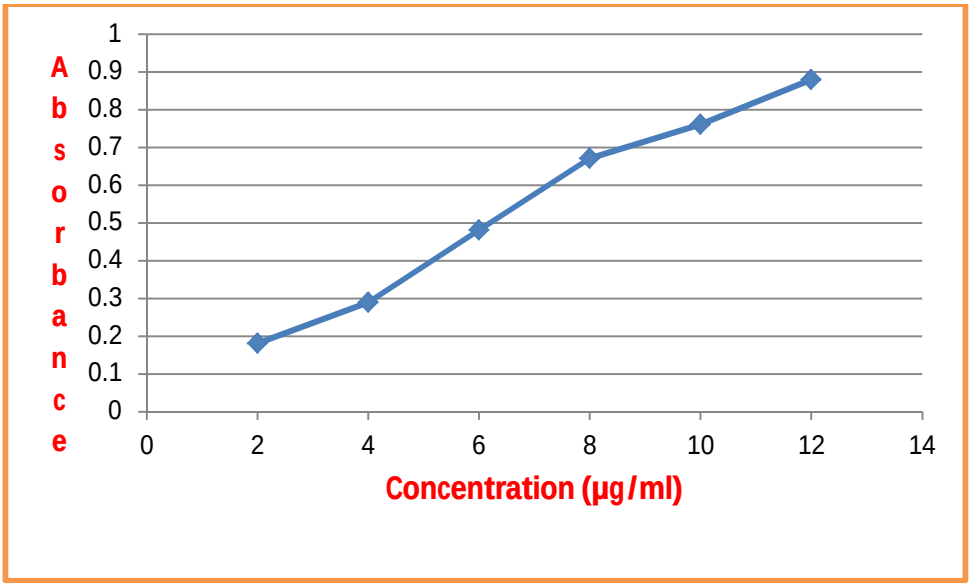
3) Method:3

The Methotrexate solutions ranging from 0.4 to 2ml (1ml=100 µg) were taken into series of 10ml graduated test tubes. 2ml of sodium carbonate and 2ml Folin-Ciocltaeu reagent were added and kept aside for 20min. The volume was made up to 10ml with distilled water. The absorbance of the blue colored chromogen was measured at 680 nm against corresponding reagent blank. The amount of methotrexate was computed following previously described procedure.

Absorption spectrum of Methotrexate with FC Reagent /Na_2 CO_3



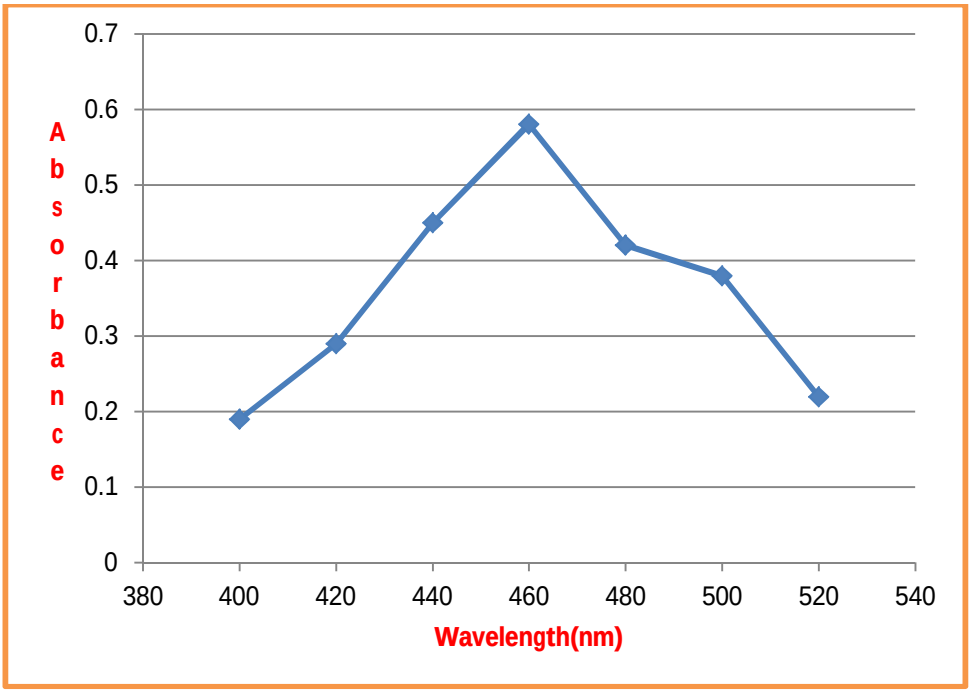
Beers law plot of Methotrexate with FC Reagent /Na_2 CO_3



4) Method: 4

Solution of concentrated HCl (5N,1ml) and sodium nitrite (0.1%,1ml) were added to the drug solutions of methotrexate taking from 1-4ml (1ml =500g/ml) in a series of 10ml volumetric flasks and kept aside for 5 min. The contents in each volumetric flask were made up to 10ml with distilled water. . The absorbance of the Yellow colored chromogen was measured at 440 nm against corresponding reagent blank. The amount of methotrexate was computed following previously described procedure

Absorption spectrum of Methotrexate with $NaNO_2$/HCl



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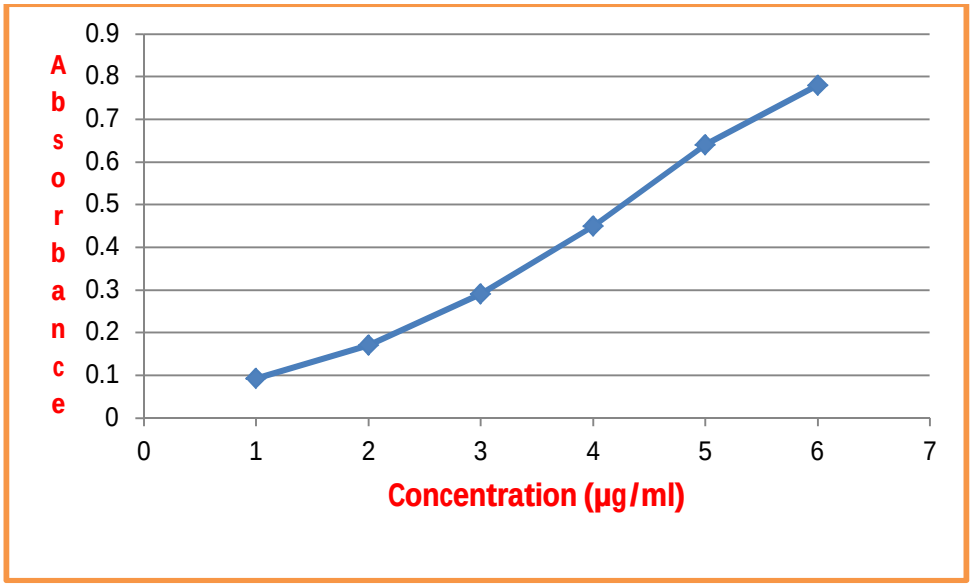
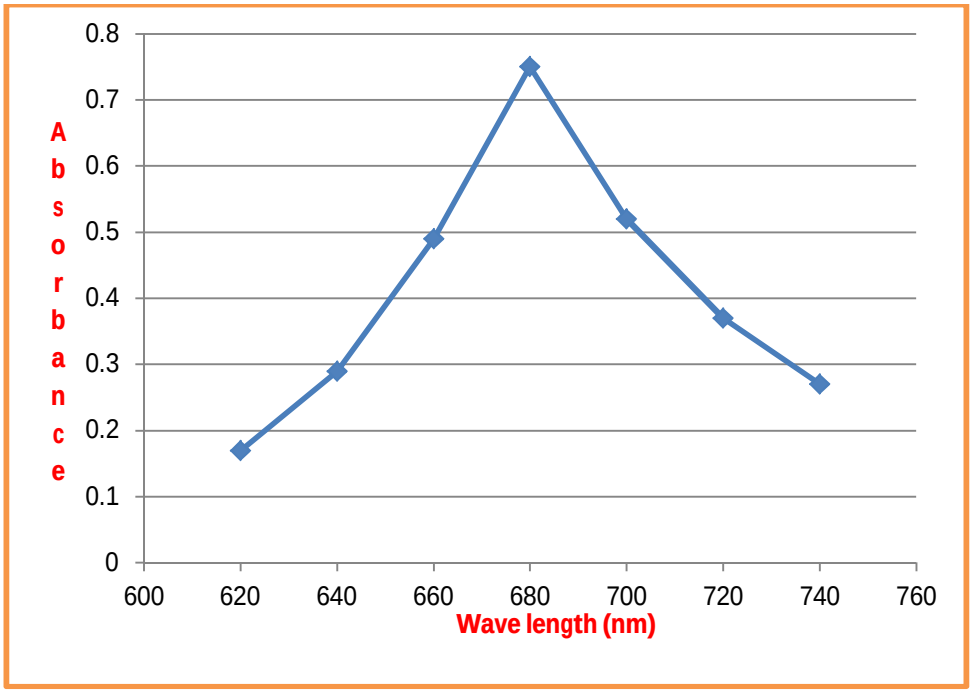
Beers law plot of Methotrexate with $NaNO_2$/HCl



5) Method: 5

Ferric chloride (0.5%,1ml) Potassium ferric cyanide (0.2%,2ml)were taken in a series of 10ml graduated test tubes and methotrexate solutions from 0.1 to 0.5 ml (1ml=100g) were introduced to these. They were kept for 10min and HCL (1N1ml)m was added to each one of them. The bluish green colored chromogen absorbance was measured at 680nm against reagent blank. The amount of methotrexate was determined as done previously.

Absorption spectrum of Methotrexate with $K_3Fe(CN)_6/FeCl_3$



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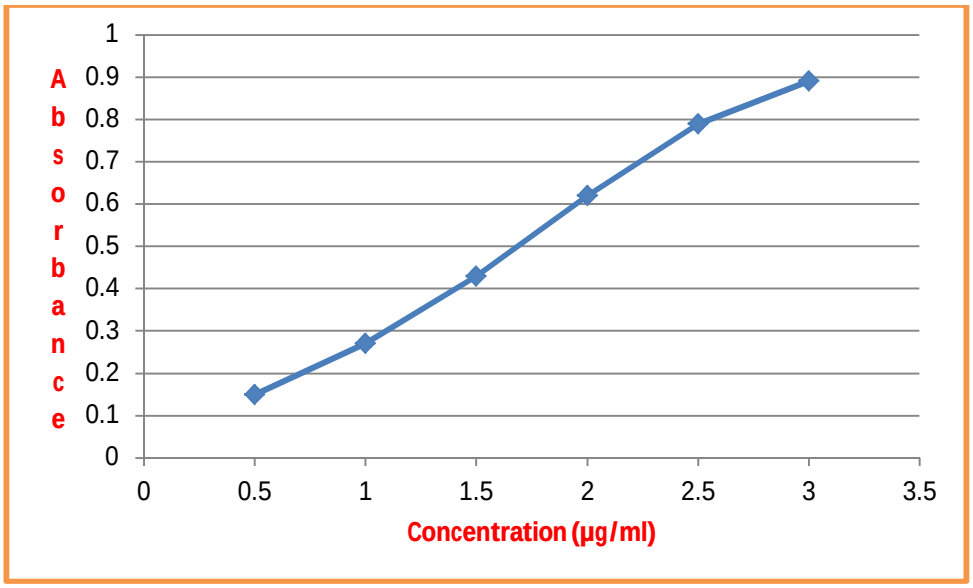
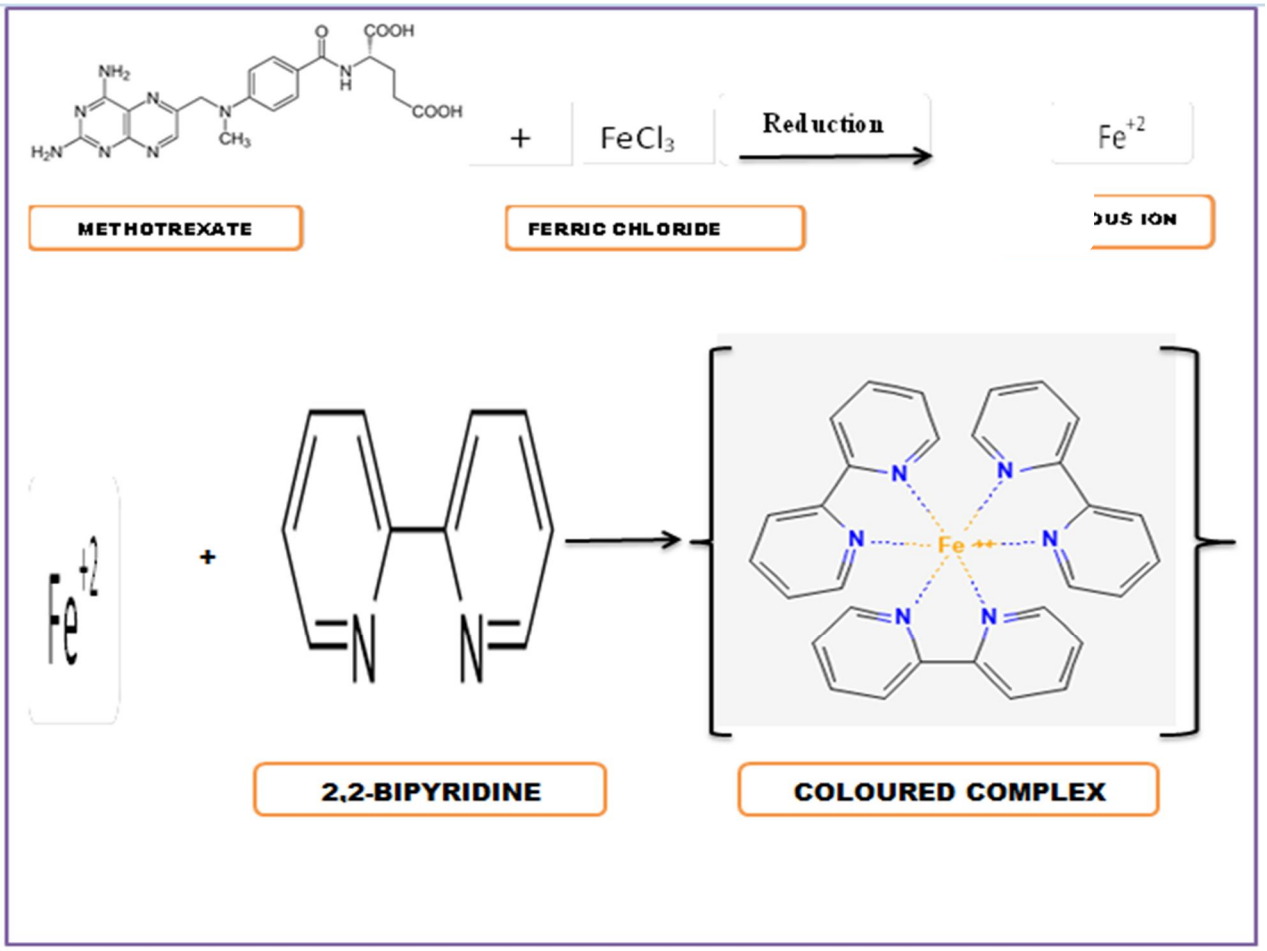
Beers law plot of Methotrexate with $K_3Fe(CN)_6/FeCl_3$



Method M-1&2: In these methods depends on reduction of Fe^{+3} to Fe^{+2} Subsequent orange colored complex formation of the resulting Fe^{+2} ion with 1,10 – Phenanthroline and 2,2,-Bipyridine.



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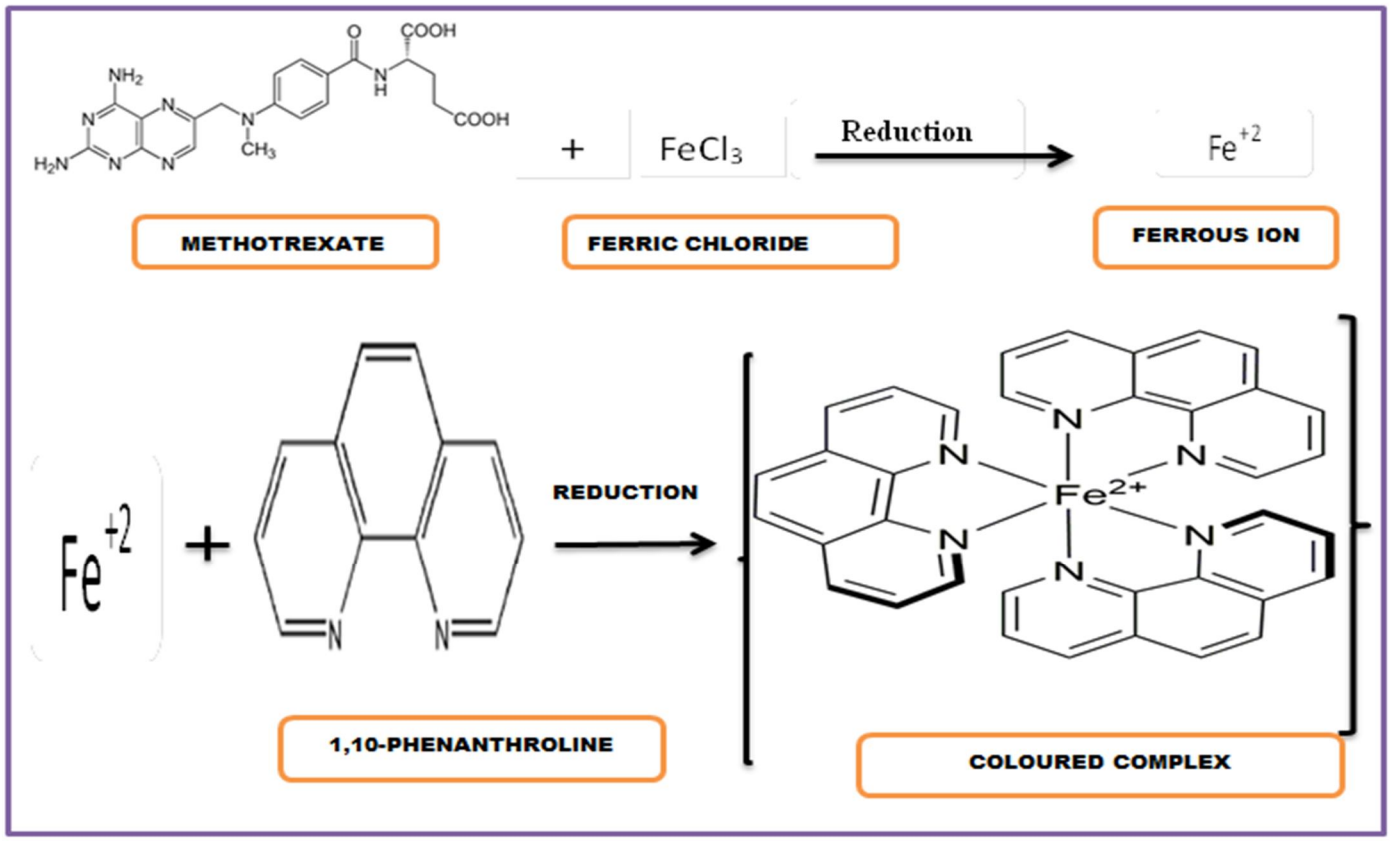
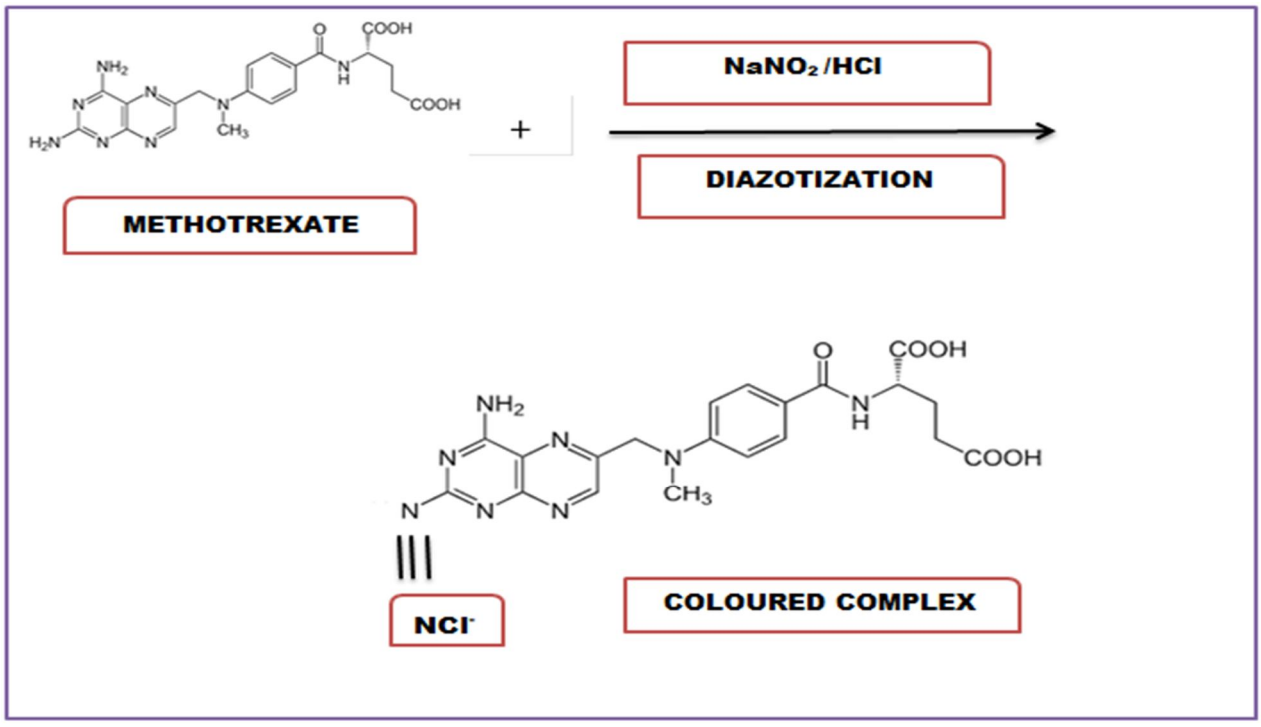


Method-3: The drug Methotrexate is subjected to a simple diazotization reaction to produce a colored complex.



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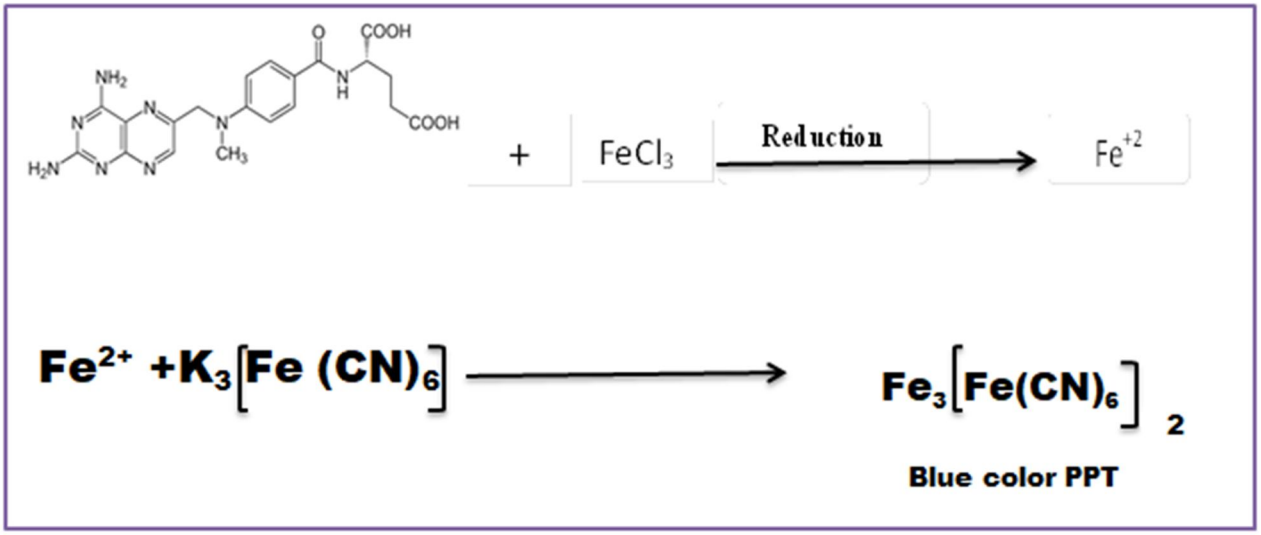
Method-4: In this method Methotrexate reduces Fe^{3+} to Fe^{2+} which subsequently reacts with potassium ferri cyanide to produce blue coloured chromogen.

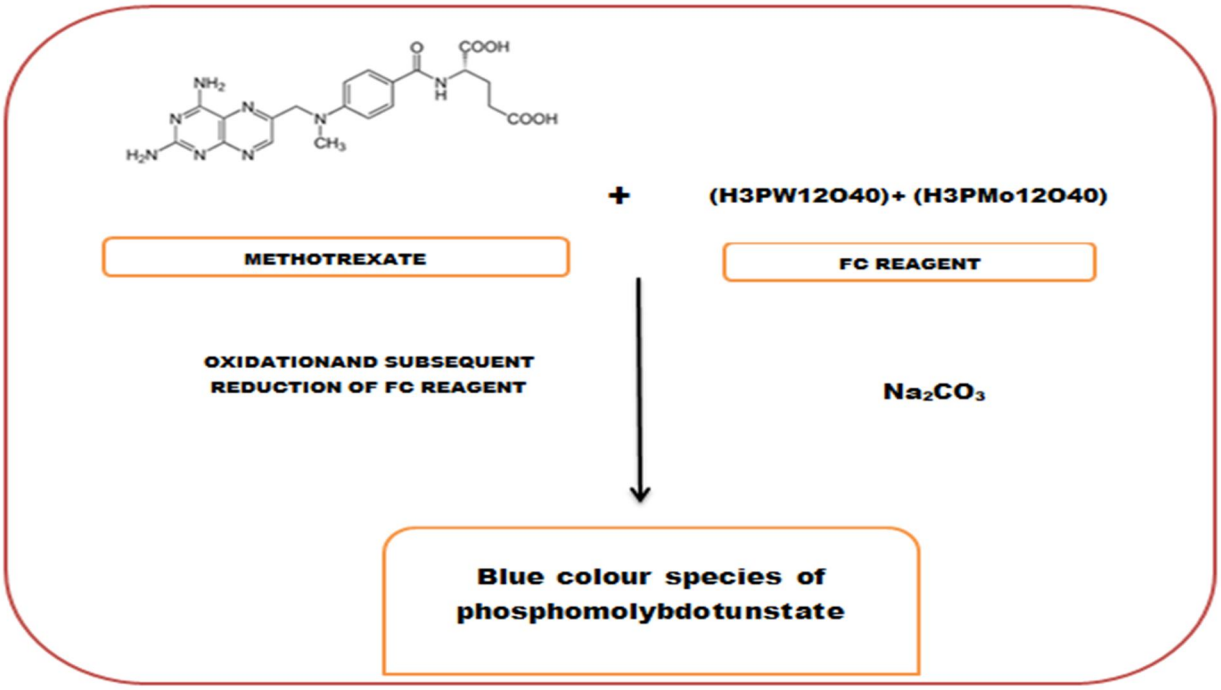
When ferrous ions (Fe^{2+}) react with potassium ferricyanide ($K_3[Fe(CN)_6]$), they form a blue precipitate called Turnbull's blue of Ferrous ferricyanide $Fe_3 (Fe(CN_6)_2$



Method-5: The colour formation of FC reagent with Methotrexate may be due to the formation of reduced blue colour species of phosphomolybdotunstate derived from FC reagent.

The Folin-Ciocalteu (F-C) reagent is a complex mixture, not a single compound with a defined molecular structure. It is primarily composed of phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMo12O40), which are yellow in colour.,

The reaction between a Folin-Ciocalteu (F-C) reagent, an amine, and sodium carbonate involves the oxidation of the amine in an alkaline medium (provided by sodium carbonate) and the subsequent reduction of the F-C reagent. This reduction of the F-C reagent produces a colored product



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III. RESULTS AND DISCUSSION

Optical And Regression Chatracteristics Precision And Accuracy Of The Used Methods For Methotrexate

PARAMETER	M -1	M-2	M-3	M-4	M-5
λ_{Max} (nm)	430	480	680	680	440
Beer's law limits (g/ml)	1 to 5	10 to 50	2 to 10	2to10	1 to 5
Molar absorptivity (1mole/cm)	9.3X103	7..28X103	7.4X103	6.26X103	7.57X103
Sandell's sensitivity (g/cm/0.001Absorbance unit)	0.019362	0.001517	0.001632	0.013	0.0202
Regression equation (Y=a+bc)					
Slope (b)	0.0027	0.0263	0.0125	0.0241	0.015
Intercept(a)	0.0175	0.1364	0.0488	0.2132	0.2046
Correlation coefficient®	0.9963	0.9947	0.9975	0.991	0.9728
Standard deviation	0.0029	0.0051	0.0045	0.0041	0.004
% Relative standard deviation	0.46	1.5	0.68	0.88	0.89
% range of errors (confidence limits)					
0.05 level	±0.5921	±1.3654	±0.4224	±0.5625	±0.6235
0.01level	±0.593	±1.8986	±0.5624	±1.3236	±1.0122
%error in bulk samples					

Assay And Recovery Of Methotrexate In Dosage Forms

Method	Pharmaceutical formulation	labelled amount (mg)	proposed method			Found by reference method ±S.D	%recovery by proposed methods ±S.D
			Amount found	t	f		
M-1	Tablet-I	500mg	499.96±0.089	0.854	2.502	500.02 ±0.082	99.92 ±0.23
	Tablet-II	500mg	500.64±0.014	0.614	1.362	498 ±0.041	100.2±0.45
	Tablet-III	500mg	499.14± 0.031	0.714	2.365	500.91±0.013	99.12±0.43
M-2	Tablet-I	500mg	500.81 ± 0.013	0.782	1.117	500.18±0.031	99.19±012
	Tablet-II	500mg	500.64 ±0.014	0.614	1.362	499.20 ± 0.041	99.91±0.62
	Tablet-III	500mg	499.8 ± 0.031	0.174	1.989	500.98 ± 0.013	100.44±0.68
M-3	Tablet-I	500mg	500.55 ±0.064	0.936	2.718	500.04 ±0.046	99.88±0.55
	Tablet-II	500mg	499.79 ± 0.070	0.537	1.665	500.05± 0.014	99.81±0.12
	Tablet-III	500mg	500.92 ± 0.021	1.012	1.383	500.02 ± 0.021	100.34±0.41
M-4	Tablet-I	500mg	499.79 ±0.014	0.316	1.632	500.05± 0.056	100.03±0.72
	Tablet-II	500mg	500.21 ±0.062	1.121	1.916	500.06 ±0.082	100.6±0.84
	Tablet-III	500mg	499.88 ±0.031	0.174	1.989	500.98± 0.013	100.44±0.68
M-5	Tablet-I	500mg	499.94 ±0.047	0.474	1.9864	500.14 ± 0.074	100.4±0.37
	Tablet-II	500mg	500.47 ±0.053	0.617	2.154	499.94 ±0.036	100.21±71
	Tablet-III	500mg	500.34 ±0.021	0.326	1.485	499.02 ±0.026	99.98±0.15

Average ± standard deviations of eight determinations the t and f values refer to comparison of the used method. Theoretical value at 95%confidance limits t=2.365 and f=4.88

IV. CONCLUSIONS

In the present study, an analytical method was developed for quantitative analysis of drug in its pure and formulations.The experimental results demonstrated a properly conducted validation study of UV-Vis spectrophotometric method for analytical

determination of MTX .The suggested methodologies showed high specificity, linearity, precision, accuracy, robustness with low limit of detection and quantification, which demonstrates the reliability required for recovery of the MTX in these drug delivery systems. The analytical methods were sufficiently simple, rapid, and suitable and may be safely used in the quantitative analysis of MTX .

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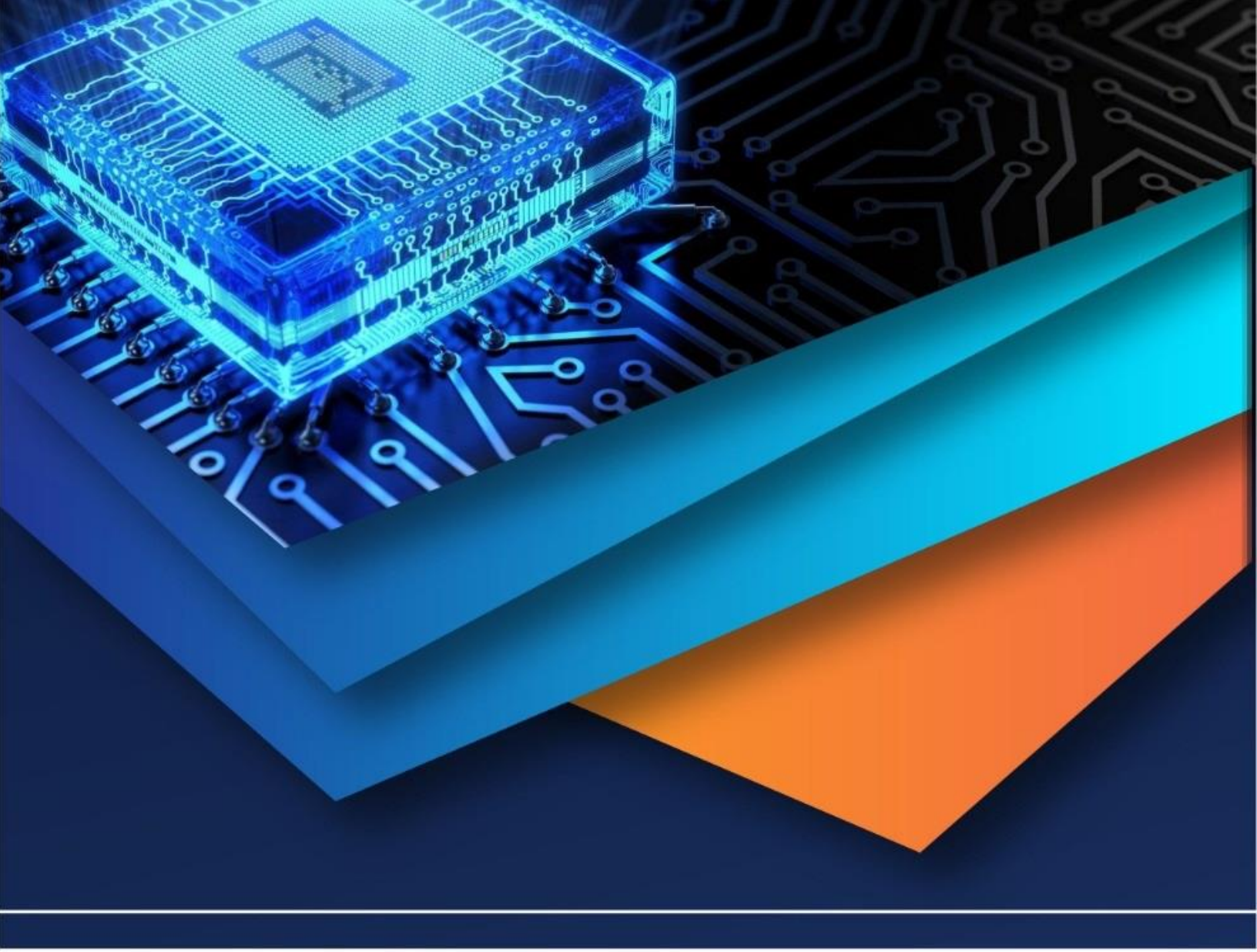

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