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Stability Indicating RP-HPLC Method for Simultaneous Determination of L- Methyl folate and Escitalopram in Bulk and Pharmaceutical Formulation

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ABSTRACT

Stabiliy indicating Reverse phase -HPLC Method been described for the simultaneous estimation of L-Methyl folate and Escitalopram in combined tablet formulation form. Chromatographic separation was carried out by using reversed phase HPLC and the method was achieved on a ODS column with UV detection. The mobile phase was optimized with Acetonitrile : 0.01% H_3PO_4 in water 35:65 (%V/V) Flow rate of 1.0ml/min and The wavelength was selected at 212nm. The drug was stressed by acidic, alkaline, oxidative, thermal and photolytic conditions and the degradation samples were analyzed by the proposed method. Degradation studies showed that all the two drugs were degraded under oxidative, acidic, alkaline and thermal conditions, Minor degradation observed under photolytic and hydrolysis condition. Analytical Method validation parameters such as specificity, linearity, accuracy, precision, Ruggedness and Robustness were determined and System suitability of all the parameters was passed. Hence this method was stability indicating method, It can be used for the routine and Stability analysis of L-Methyl folate and Escitalopram in pharmaceutical dosage forms.

Keywords: RP-HPLC, L-Methyl folate and Escitalopram.

INTRODUCTION

Levomefolic acid(L-Methyl folate)

Levomefolic acid (fig- 1) was primary biologically active form of folic acid used at the cellular level for DNA reproduction. A-vitamin (B9) essential to human health and function. One of its most notable functions is its role in creating key neurotransmitters or brain chemicals that regulate human mood, cognitive ability and arousal. The three primary brain chemicals are dopamine, norepinephrine and serotonin. Chemically, these

brain chemicals are referred to as monoamines. Abnormal amounts (either too much or too little) of these chemicals can cause various forms of mental illness and disease including depression, schizophrenia and attention deficit disorder.

Our body does not create its own supply of folic acid (also known as folate), it must be acquired from the diet by eating foods or taking supplements containing this b-vitamin. Your body then takes the dietary folic acid and uses the enzyme MTHFR (methylenetetrahydrofolate reductase) to transform it into levomefolic acid[1]. Your body can then use it to carry out a specific range of reactions and function. Levomefolic acid is used to produce the neurotransmitter nor epinephrine Levomefolic acid (and folic acid in turn) has been proposed for treatment of cardiovascular disease[2,3] and advanced cancers such as breast and colorectal cancers[citation needed] A lack (deficiency) of folate in the human body can be caused by certain diseases, by taking certain medications, or by not getting enough folate in your diet. Folate deficiency can lead to decreased red blood cells, or anemia. Folate deficiency can also cause high levels of a certain amino acid in the blood, a condition called hyperhomocysteinemia (HYE-per-HOE-moe-sis-tin-EE-mee-a).L-methylfolate is not an antidepressant or anti-psychotic medication. However, l-methylfolate may enhance the effects of antidepressant medications. Literature survey reveals that there were less number of analytical methods for L-methylfolate or in combination with other drugs including spectro scopic and chromatographic methods are reported

Escitalopram

Escitalopram (trade names Lexapro, Cipralex) (Fig 2) is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class. It is approved by the U.S. Food and Drug Administration (FDA) for the treatment of major depressive disorder and generalized anxiety disorder in adults; other indications include social anxiety disorder, panic disorder and obsessive-compulsive disorder. Escitalopram is the S-stereoisomer (enantiomer) of the earlier Lundbeck drug citalopram (Celexa), hence the name escitalopram. Escitalopram is noted for its high selectivity of serotonin reuptake inhibition and, as a result has fewer side effects not related to its serotonergic activity. According to a meta-analysis of 12 new-generation antidepressants, escitalopram and sertraline (Zoloft) are the best in terms of efficacy and acceptability in the acute-phase treatment of adults with major depression; however, sertraline may be a better choice because of the lower cost. escitalopram exhibits superior therapeutic properties to citalopram or merely represents an example of "ever greening" is controversial

The utility of antidepressant drugs in the treatment of mild-to-moderate depression is itself controversial. In those with very severe depression there is a large benefit[4]. The most recent Reviews concluded (with caveats in some cases) that escitalopram is modestly superior to citalopram in efficacy and/or tolerability[5]. Literature survey reveals that there were many analytical methods for escitalopram or in combination with other drugs including spectro scopic and chromatographic methods are reported Simultaneous estimation[6] of escitalopram and single estimation[7] of escitalopram has been done previously in UV and also in Colourometry[8]

Simultaneous determination of escitalopram oxalate and clonazepam in combined tablets by HPTLC[9] and RP HPL has also been performed. but there is no method established for the stability indicating RP-HPLC under stress for this combination. The present work describes the development of stability indicating RP-HPLC method, which can quantify these components simultaneously from a combined dosage form The present RP-HPLC method was validated[10,11] and applied under stressed conditions according to International conference on harmonization (ICH) guideline There stability indicating assay methods helps in establishing the inherent stability of the drug which provides assurance on detection changes in identity, purity and potency of the product on exposure to various conditions.In the present study, Levomefolic acid, Escitalopram is exposed to a variety of stress like acidic, basic, thermal, photolytic and oxidative stress conditions[12]. According to ICH guidelines the stress testing of the drug substances helps in identifying the likely degradation products. The aim of the present work was to develop stability indicating method for determination of all the two drugs in presence of its degradation products.

MATERIALS AND METHODS

Chemicals and reagents

L-Methyl folate and Escitalopram standard was obtained from reputed companies, HPLC grade Methanol, Water, Ortho phosphoric acid and Acetonitrile were purchased from Merck Pvt limited, Mumbai. 0.45 µm nylon membrane filter papers were purchased from Pall Life Sciences, Mumbai. A combined dosage tablet lefodap plus was purchased from Med plus Pharmacy.

Instrumentation

In the present study Performed with Waters 2998 PDA detector and Waters Empower2 software was used Shimadzu double beam UV-Visible spectrophotometer was used for spectral analysis and the data was generated and recorded by UV probe software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were and weighed by using Denver electronic analytical balance andsartorious analytical balanace (SI-234).

Determination of maximum absorbance

L-Methyl folate and Escitalopram standard solution was scanned in the range of 200-400 nm against mobile phase as blank. L-Methyl folate and Escitalopram shows maximum absorbance at 212nm. The wave length selected for the determination of L-Methyl folat and Escitalopram is 212nm.

Diluent

Water: Methanol (50:50)

Preparation of standard stock solution

6mg of L- Methyl folate and 8mg Escitalopram weighed in to 10ml volumetric flasks separately. Add 3/4ml of diluents, sonicated to up to dissolve the drugs and make up to the mark with diluents, finally 600ìg/ml of L- Methyl folate and 800ìg/ml of Escitalopram Standard stock solutions of concentration were prepared using diluent. Fromtake 1ml of standard stock solution, in to a 10ml Volumetric flask and and added dilent mixed well dilueted to get 60µg/ml of L- Methyl folate and 80ìg/ml of Escitalopram with diluent.

Preparation of Tablet formulation

Taken weight of 20 tablets of lefodap plus and calculate the average weight then crush the tablets and weighted the equivalent to 1 tablet taken into a 25ml volumetric flask, added the 10ml of diluents and sonicated for 30 min, further dilueted up volume with diluent and filtered through 0.45µ filter. Then taken 2ml of above filter solution into a 10 ml volumetric flask and made upto volume with diluent. The finally sample solution concentrations of 60µg /ml of L-Methyl folate and 80µg/ml of Escitalopram was obtained.

Chromatographic conditions

The present study explains the development and validation for the estimation of L-Methyl folate and Escitalopram in Bulk and tablet forms using the most commonly employed ODS 250mm x 4.6 mm, 5m.column with UV detection. The mobile phase was optimized with Acetonitrile : 0.01% H_3PO_4in water 35:65 (%V/V). The wavelength was detected and selected at 212nm, Iso-absorptive point for both the drugs. Good resolution was carried out at 212nm and both drugs showed good absorbance at this wavelength with minimum interference of the other drug. Analysis was carried out at column temperature 30°C temperature. Compounds were separated with a mobile phase consisting of

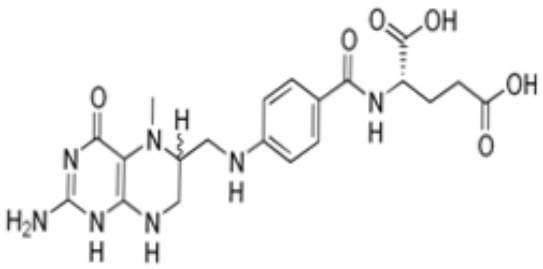

Fig. 1: Structure of Levomefolic acid

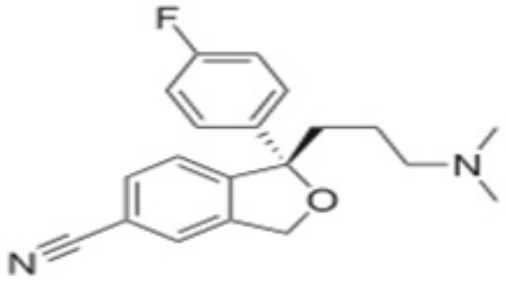

Fig. 2: Structure of Escitalopram

Acetonitrile : 0.01% H_3PO_4in water 35:65 (%V/V). Flow rate of 1.0 ml/min and detection of wavelength at 212nm. The mobile phase was filtered by using 0.45µm membrane filter paper and solicited and Degassed in Ultrasonicator for 10min.Optimized chromatographic conditions were shown in table-1 Standard and blank and formulation chromatograms were shown in figure no.3, 4 and 5. All parameters of method was validated as per the USFD and ICH guidelines.

Analytical Validation of the method

Assay Analytical method Validation of L-Methyl folate and Escitalopram by HPLC was carried out with respect to the following parameters.

System suitability

System suitability test was performed before each validation parameter. system suitability result shown in table-2. In the system suitability

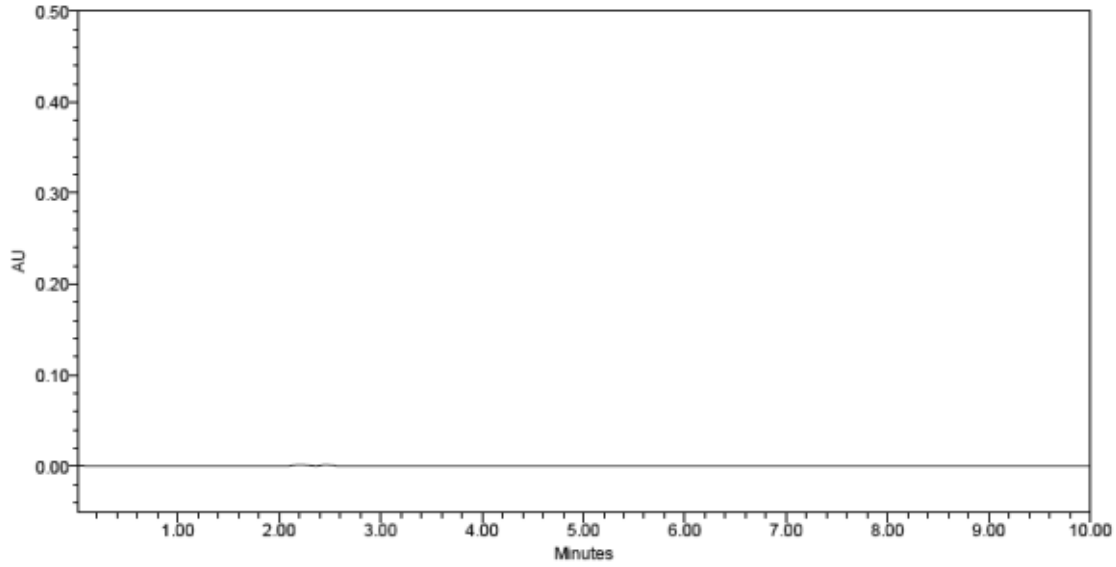

Fig. 3: Blank chromatogram of L-Methyl folate and Escitalopram

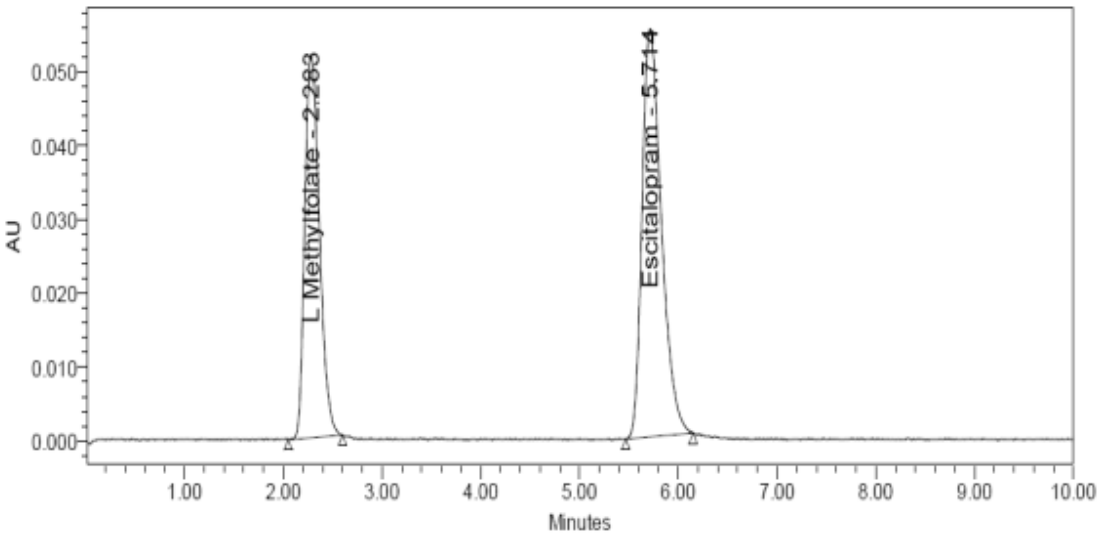

Fig. 4: Standard chromatogram of L-Methyl folate and Escitalopram

test L-Methyl folate and Escitalopram peaks were separated with good resolution, such as tailing factors, theoretical plates, and repeatability against the specifications set for the method. The tailing factor was within ≤ 2, theoretical plate number of ≥ 2000, which satisfied the acceptance criteria.

Table 1: Optimized chromatographic conditions for L-Methyl folate and Escitalopram

S. No.	Parameter	Results
1	Mobile Phase	Acetonitrile : 0.01% H_3PO_4in water 35:65 (%V/V)
2	Wavelength	212nm
3	Column	ODS 250mm x 4.6 mm, 5m.
4	Injection volume	10mL
5	Flow Rate	1.0ml/min
6	Pump Mode	Isocratic
7	Run time	10 min

Linearity and range

Linearity of L-Methyl folate and Escitalopram detector response of assay method was found by injecting seven standard solutions with concentration ranging from 15µg/ml to 90µg/ml for L-Methyl folate and 20µg/ml to 120µg/ml for Escitalopram of the test concentration and a graph was plotted for concentration versus peak area. Good linear relation was observed within the concentrations and the study. Regression equation was found to be y= 8862.x + 204.1 (r^2=1.000) for L-Methyl folate and y= 9273.x + 1391 (r^2=0.999) for Escitalopram

Y=slope, m=intercept, c=correlation coefficient. The results were shown in Table-3, Table-4, fig-6 and fig -7

Precision

Repeatability of results called as Precision. In this parameter injecting 6 eplicate injections of the solution 60ìg/ml L-Methyl folate and 80ìg/ml of Escitalopram respectively.The %RSD was below 2.0% there found to be 0.87% and 0.79. Variability of the method was Checked by analyzing the solution on the same day (intra-day precision) and on other

Table 2: System suitability Results for L-Methylfolate and Escitalopram

S.No	Parameter	Results
1	Api Concentration	L- Methyl folate – 60 µg/mlEscitalopram - 80 µg/ml
2	RT	L- Methyl folate – 2.280min Escitalopram - 5.697 min
3	Resolution	L- Methyl folate – ……..Escitalopram -10.81
4	Area	L- Methyl folate – 517514Escitalopram - 730351
5	Theoretical Plates	L- Methyl folate – 1161Escitalopram - 4374
6	Tailing Factor	L- Methyl folate – 1.32Escitalopram -1.41

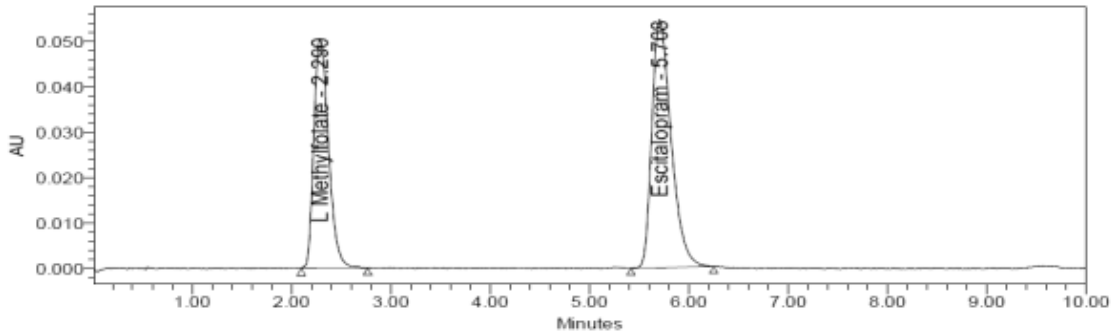

Fig. 5: Formulation chromatogram of L-Methyl folate and Escitalopram

different day (inter- day precision). The results were obtained for intra-day precision %RSDs were below 2.0% they were 0.87 % and 0.79 % respectively. The inter-day precisions %RSDs were 0.66% and 0.36 %, respectively it is also below 2.0%. The results were shown in table-5.

Accuracy

Accuracy of the method was Studied by internal standard addition method. A known concentration amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent of recovery was calculated by comparing the area before and after the addition of the standard drug. As per ICH Guidelines Accuracy parameter performed at least 3 levels. The Recovery of method was performed at 50%, 100%and 150% levels of standard concentration for both L-Methyl folate and Escitalopram drugs. We were prepared above given 3 levels solutions in triplicate and injected as per the proposed method. % recovery was calculated for each level a found to be within the acceptance criteria of 98-102% for both drugs as per guidelines. This Results showed that the recoveries of L-Methyl folate and Escitalopram by the proposed methods are satisfactory. %RSD accepted within the limit of 2.0%. The results are shown in table-6 and table-7

Ruggedness

Ruggedness was performed by the analyst to analyst variability, % RSD) was calculated. The %RSD was below 2.0%, Cumulative %RSD both analysts also found below 2.0%. The results are shown in table-5and table-8.

Robustness

To perform the robustness of the method, Experimental Chromatographic conditions such as

Table 3: Results for linearity of L-Methyl folate

No	Concentration (µg/ml)	Peak area
1	0	0
2	15	132701
3	30	264945
4	45	400021
5	60	534130
6	75	665262
7	90	795920

Table 4: Results for linearity of Escitalopram

S. No	Concentration (µg/ml)	Peak area
1	0	0
2	20	183208
3	40	372861
4	60	562544
5	80	745938
6	100	933617
7	120	1106405

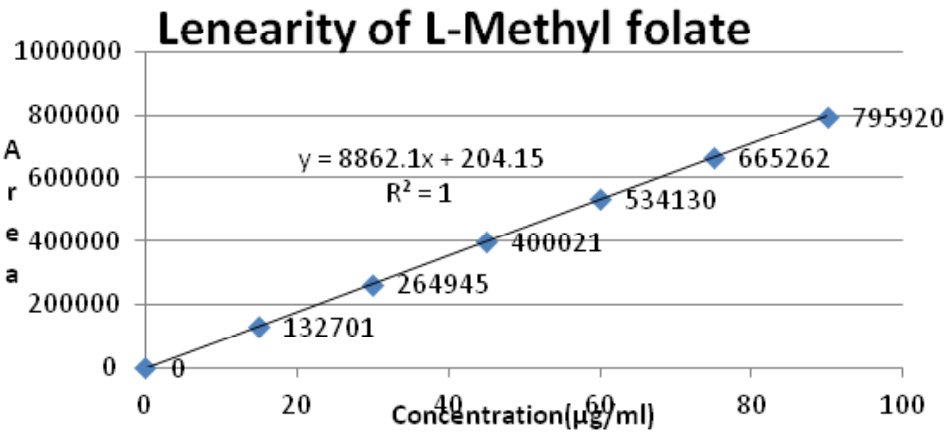

Fig. 6: Linearity of L-Methyl folate

the Flow rate ,temperature and composition of the mobile phase. The small changes were tested The results are obtained small variations which do not effected on the results It indicates that the Analytical method was robust. The results were shown on the Table-9

Limit of Detection and Quantification Limit

Determination of the Limit of Detection and Limit of Quantification was performed by standard deviation method. Standard with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the

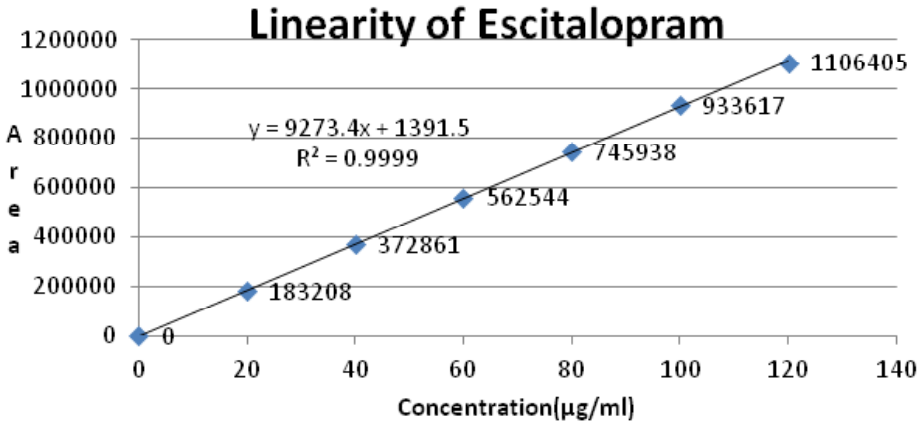

Fig. 7: Linearity of Escitalopram

Table 5: Precision Results for of L-Methyl folate and Escitalopram

S.No	L-Methyl folate	Escitalopram	L-Methyl folate	Escitalopram
	Intraday precision		Inter day precision	
1	519055	730022	510325	726226
2	513781	724488	509391	722452
3	513863	725274	506107	722991
4	516973	720769	502404	719327
5	506829	734154	511588	725318
6	518129	735207	509145	721058
RSD	0.87	0.79	0.66	0.36

Table 6: Results for Accuracy of L-Methyl folate

S. No	% Recovery	Concentration in µg/ml Target	Spiked	Total	Amount Found	% recovery	% RSD
1	50%	60	30	90	30.08	100.27	0.38
2		60	30	90	29.90	99.68	
3		60	30	90	30.12	100.39	
4	100%	60	60	120	59.71	99.52	0.94
5		60	60	120	60.17	100.29	
6		60	60	120	59.06	98.43	
7	150%	60	90	150	89.88	99.87	1.02
8		60	90	150	90.93	101.03	
9		60	90	150	89.09	98.99	

analyte can be reliably detected. Limit of Detection is generally considered acceptable 3.3*ó/slop for estimating the detection limit. The quantification limit 10.0*ó/slop is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. The results are shown on the table-10

Formulation

We were prepared assay sample solution injected into the HPLC. The assay results were calculated against standard peak areas.the calculated % assay was found to be 99.41% for L-Methyl folate and 99.91 % for Escitalopram. Hence the method can use for the simultaneous estimation of L-Methyl folate and Escitalopram in pharmaceutical formulation and stability sample analysis. Results of the formulation analysis were shown in table -11.

Solution stability

The standard and samples solutions of L-Methyl folate and Escitalopram does not change their concentration up to 24hrs. So standard and samples solution of L-Methyl folate and Escitalopram Stable up to 24 hours. The results were shown in table-12

Force Degradation Studies

Forced degradation study undertaken to demonstrate the specificity of method, when developing the stability- indicating methods, particularly when little information is available about potential degradation of products. These studies also provide information about the degradation pathways and degradation products that could form during storage period in different conditions. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development manufacturing and packaging in which knowledge of chemical behavior can be used improve a drug product

Table 7: Results for Accuracy of Escitalopram

S. No	% Recovery	Concentration in µg/ml Target	Spiked	Total	Amount Found	% recovery	% RSD
1	50%	80	40	120	39.40	98.50	1.49
2		80	40	120	40.55	101.38	
3		80	40	120	39.71	99.28	
4	100%	80	80	160	79.70	99.63	1.14
5		80	80	160	79.16	98.96	
6		80	80	160	80.94	101.18	
7	150%	80	120	200	119.22	99.35	0.90
8		80	120	200	120.17	100.14	
9		80	120	200	118.03	98.36	

Table 8: Result for Ruggedness of L-Methyl folate and Escitalopram

S. No	L-Methyl folate	Escitalopram
1	510325	726226
2	509391	722452
3	506107	722991
4	502404	719327
5	511588	725318
6	509145	721058
RSD	0.66	0.36

Oxidative Stress

To 1 ml of sample stock solution of L-Methyl folate and Escitalopram and 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 2 hours at 60ºc. For HPLC Analysis , The Resultant solution was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system Recorded the chromatograms to assess the stability of sample.

Acid Degradation Studies

To 1 ml of stock s solution L-Methyl folate and Escitalopram, 1ml of 2N Hydrochloric acid was added and refluxed for 30min at 60ºc. The Resultant solution was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system. Recorded the chromatograms to assess the stability of sample

Base Degradation Studies

To 1 ml of stock solution L-Methyl folate and Escitalopram, 1ml of 2N sodium hydroxide was added and refluxed for 30mins at 60ºc. The Resultant solution was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system. Recorded the chromatograms to assess the stability of sample.

Thermal Degradation Studies

The thermal degradation carried out by sample solution was kept in oven at 105 ºc for 6 hours. For HPLC analysis, The Resultant solution was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system Recorded the chromatograms to assess the stability of sample.

Photolytic degradation studies

The photochemical degradation was also studied by exposing the tablet powder to UV Light by kept the beaker cointaining tablet powder in UV Chamber for 7days or 200 Watt hours/min in photo stability chamber. For HPLC Analysis, The Resultant solution was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system. Recorded the chromatograms to assess the stability of sample.

Hydrolysis Degradation Studies

Hydrolysis Degradation Study carried out by refluxing the drug in water for 6hrs at a temperature of 60°C for HPLC analysis, The Resultant solution

Table 9: Results for robustness of the L-Methyl folate and Escitalopram

S. No	Condition	Change	L-Methyl folate		Escitalopram	
			Mean Area	%RSD	Mean Area	%RSD
1	Standard	……..	514471	0.9	730082	0.8
2	MP 1	40:60(%V/V)	513547	1.1	734666	1.1
3	MP 2	30:70(%V/V)	502632	0.9	725802	1.4
4	Flow-1	0.9 mL/min	589325	1.1	834351	1.4
5	Flow-2	1.1 mL/min	471802	0.9	675146	1.5
6	Oven Temp-1	25°C	496975	1.2	709669	1.2
7	Oven Temp-2	35°C	499556	0.7	713144	1.2

Table 10: Results for LOD and LOQ

Parameter	L-Methyl folate (µg/ml)	Escitalopram (µg/ml)
LOD	0.06	0.26
LOQ	0.18	0.79

Table 11: Result for formulation of L-Methyl folate and Escitalopram

S. No	Drug	Brand	Dosage	Amount Prepared	Amount Found	% Assay
1	L-Methyl folate	Lefodep Plus	7.5mg	60	59.65	99.41
2	Escitalopram		10.0mg	80	79.93	99.91

Table 12: Results for stability

Time in hrs	Peak Area	
	L-Methyl folate	Escitalopram
0	502700	717606
24	508145	722895

was diluted to Required concentration 60µg/ml of L-Methyl folate & 80µg/ml of Escitalopram solution and injected into the HPLC system recorded the chromatograms to assess the stability of sample. The Degradation results shown in table-13

Table 13: Result for Degrdation of L-Methyl folate and Escitalopram

Degradation	Assay of L-Methyl folate	Assay of Escitalopram
water	98.89	99.00
UV	98.66	97.43
Thermal	96.09	96.08
Acid	94.08	94.09
Base	93.14	93.35
Peroxide	91.97	91.97

RESULTS AND DISCUSSION

The present experiment was aimed to develop and validated stability indicating RP- HPLC method for simultaneous estimation of L-Methyl folate and Escitalopram. for selection of mobile phase ,After several Experiment trails with different mobile phase compositions, a Acetonitrile : 0.01% H_3PO_4in water 35:65 (%V/V) was found to be desired composition. This ratio was optimized for the simultaneous estimation of all the two drugs.

The analytical data obtained from the linearity parameter ,linear regression analysis showed a linear relationship in peak areas and concentration in the range of 15µg/ml to 90µg/ml for L-Methyl folate and 20µg/ml to 120µg/ml for Escitalopram of the test concentration. The method was precise, repeatable results were obtained. The %RSD values for intra-day and inter-day precision less than 2.0%. The developed method was accurate recovery obtained at each levelas per guide lines

The limit of detection of L-Methyl folate and Escitalopram was found to be 0.06ìg/mL and 0.26ìg/mL ,limit of quantification was 0.18ìg/mL and0.79ìg/mL . While studying the robustness, the system suitability results of all the two drugs were within acceptance criteria,

The established method was robust system suitability parameter passed affected after varying the parameters like flow rate and Temparature. The assay of commercial tablets was established with present chromatographic condition developed and it was found to be more accurate and reliable. The percentage label claim present in tablet formulation was found to be 99.41, 99.91 for L-Methyl folate and Escitalopram respectively.

The drugs were exposed to acidic, base, oxidative, thermal and photolytic conditions and the stressed samples were analyzed by the proposed method. Degradation studies showed that all the two drugs were degraded under, thermal, Acid, Base and peroxide conditions. The drug peak areas decreased sufficiently with drastic change in the Rt values.

Thus the developed RP-HPLC Stability inicating method was found to be simple, rapid, sensitive, accurate, precise and specific for the simultaneous estimation of two drugs in bulk and pharmaceutical dosage forms and for routine analysis in stability studies of these drugs.

CONCLUSION

The Stability indicating RP-HPLC method was developed and validated simultaneous estmation and quantitative determination of L-Methyl folate and Escitalopram from in its formulation. All the validation parameters were found to be within the limits according to the ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and

the method was found to be suitable for the routine and stability analysis of the marketed formulation.

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