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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF PROCESS IMPURITIES AND DEGRADATION PRODUCTS OF LEVOLEUCOVORIN CALCIUM FOR INJECTION

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Keywords:

RP-HPLC, Levoleucovorin Calcium, Process related impurities, Stability indicating method, Method validation

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ABSTRACT: Levoleucovorin calcium contains amide and aldehyde groups which makes it vulnerable to degradation. Stability indicating method for determination of all seven process-related impurities and degradation impurities in Fusilev® 50 mg/mL injection was developed with the help of RP-HPLC. A Forced degradation study supports method development to ensure stability indicating conditions. Solutions of the lyophilized injection were stress-tested in different conditions like Acidic, Basic, Oxidative, Thermal, Photolytic conditions, and the mass balance was found close to 90-110%. Validation has been performed according to ICH guidelines. The method was developed in ACE C18 column (150 × 4.6 mm, 3 µm) with binary gradient elution consisting of mobile phase A, which consists a mixture of Potassium Phosphate Triabasic and Tetra butyl ammonium hydrogen sulfate in water (pH 6.8 with diluted Ortho Phosphoric Acid, 0.05 M, 1000 ml) and mixed with Methanol in ratio of 90:10 % V/V and mobile phase B consist methanol at a flow rate of 0.5 mL/min and detection performed at 280 nm with column thermostat 40 °C, injection volume was 10 µl. The HPLC method allowed good resolution between peaks of Levoleucovorin Calcium, process impurities, and its degradation products with good linearity, precision, accuracy, specificity, LOD, LOQ. Regression analysis indicates the correlation coefficient value greater than 0.9900 for all process-related impurities and degradants. The LOD of all process impurities was at a level of 0.015%. The method has shown consistent recoveries for all process impurities (98.0%-102%). The method was found to be linear, accurate, precise, specific, sensitive, robust and stability-indicating.

INTRODUCTION: Levoleucovorin Calcium (LLC) is a salt form of calcium and chemically described as Calcium (2S)-2-[[4-[[[(6S)-2-amino-5-formyl-4-oxo-1, 4, 5, 6, 7, 8-hexahydropteridin-6-yl] methyl] amino] benzoyl] amino] pentanedioate pentahydrate **Fig. 1A** [1].

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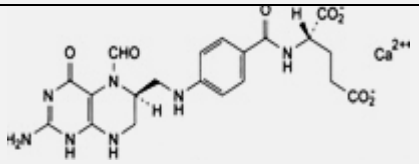
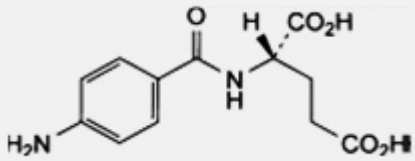
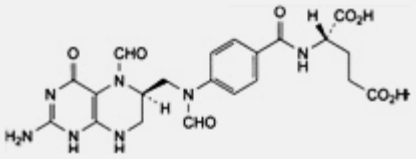
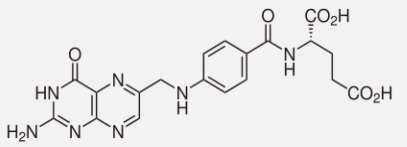
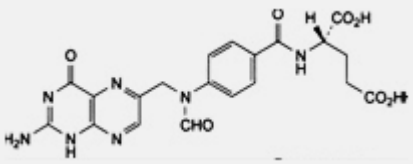
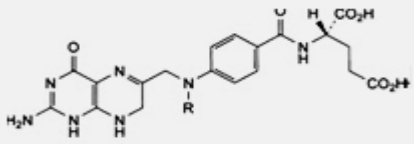
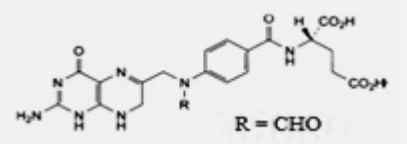
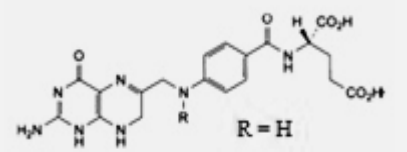
It is used to circumvent the inhibition of dihydrofolate reductase as a part of high dose methotrexate therapy and to potentiate fluorouracil in the treatment of colorectal cancer and an antidote to diminish the toxicity and counteract the effect of unintentional overdose of folic acid antagonists, such as trimethoprim and pyrimethamine [2].

LLC is stereochemically pure (6S, the 20S)-diastereomer and limits undesirable (6R, 2S)-isomer to the impurity of 0.5 % [1]. It is an active intermediate in the metabolism of folic acid, and only natural l-isomer is pharmacologically active [3]. Regulatory requirements for the identification,

qualification, and control of impurities in drug substance and their formulated products are now being explicitly defined and recommended by International Conference on Harmonization (ICH) that all routine impurities at or above the 0.1 % level should be identified through appropriate analytical methods [4-6]. An aqueous solution of LLC produces an insoluble degradation compound by oxidative degradation thereby all aqueous solutions are unstable and may form precipitate during handling and storage that have significant risk to patient [7]. LLC is known to contain seven process-related impurities, namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, and Imp-G **Table 1 (B-H)**. It is necessary to determine the impurities of LLC to ensure the quality, efficacy, and safety of the final pharmaceutical formulation and determining its degradation profile by the stability-indicating method.

TABLE 1: CHEMICAL NAME AND STRUCTURE FOR LEVOLEUCOVORIN CALCIUM AND ITS RELATED IMPURITIES

S. no.	Name	Structure	Chemical name
(A)	Levoleucovorin Calcium		Calcium (2*S*)-2-[[4-[[[(6*S*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl] methyl]amino]benzoyl]amino]pentanedioate pentahydrate
(B)	IMP-A		(2*S*)-2-[(4-aminobenzoyl)amino]pentanedioic acid,
(C)	5,10-diformyltetrahydrofolic acid (IMP-B)		(2*S*)-2-[[4-[[[(6*R*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl] nylamino]benzoyl]amino]pentanedioic acid
(D)	Folic acid (IMP-C)		(2S)-2-[[4-[(2-Amino-4-oxo-1H-pteridin-6-yl)methylamino]benzoyl]amino]pentanedioic acid
(E)	10-Formylfolic acid (IMP-D)		(2*S*)-2-[[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]formylamino]benzoyl]amino] pentanedioic acid
(F)	5-Formyltetrahydroptrroic acid (IMP-E)		4-[[[(6*S*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoic Acid
(G)	10-Formyldihydrofolic acid (IMP—F)		2*S*)-2-[[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]formylamino] benzoyl]amino]pentanedioic acid
(H)	Dihydrofolic acid (IMP-G)		H: (2*S*)-2-[[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino]benzoyl] amino]pentanedioic acid

Several analytical methods are available like capillary electrophoresis [3], LC-MS [8], HPLC [9,] and Compendial method in European Pharmacopoeia for determination of Imp-G and its dextroform [1], but all reported methods were not capable of determining LLC, its all seven process-related impurities as well as degradants.

Thus, the aim of the present research work is to develop a stability-indicating RP-HPLC method and validate the same for the determination and quantitative estimation of LLC, its known impurities along with degradation products in lyophilized injection formulation, according to the ICH guidelines.

MATERIALS AND METHODS:

Chemicals and Reagents: Qualified standards of Levoleucovorin Calcium (87.53%) and its process-related impurities Imp-A (100.5%), Imp-B (77.55%), Imp-C (92.2%), Imp-D (96.8%), Imp-E (72.0%), Imp-F (81.6%), and Imp-G (87.6%) were gifted by Zydus Cadila Healthcare Ltd. (Ahmedabad, India). Reagents were used Potassium Phosphate Triabasic (K_3PO_4), Tetrabutyal ammonium hydrogen sulfate (TBAHS), Tris Buffer (Hydroxymethyl, aminomethane) procured from Merck, United States. All the solvents were used HPLC-grade such as Methanol, Hydrochloric acid (HCl), and Ortho Phosphoric Acid (OPA) procured from Merck, United States. High purity water was prepared by using Milli Q plus water purification system (Millipore, Milford, MA, USA). Lyophilized form of Levoleucovorin Calcium for injection and Placebo (Mannitol) were formulated in the Department of Pharmaceutics, Zydus Cadila Healthcare Ltd.

Equipment: Dionex Ultimate 3000 equipped with an Autosampler, quaternary pump a thermostatted column compartment, and Photo Diode Array detector (Dionex Technologies) was used for method development, force degradation, and validation. The data were evaluated by Chromeleon software 6.8. Solutions were degassed by ultrasonication (Power sonic 420, Labtech, Korea). The pH of the mobile phase was adjusted as required by a pH meter (Thermo Orion model 420a, USA).

Chromatographic Condition: The method was developed using chromatographic column ACE C18 (150mm × 4.6mm × 3µm) by using Brownlee (15mm × 3mm × 7µm) as a guard column. The mobile phase was prepared in two solvent reservoirs A and B: Solvent reservoir A consists of a mixture of 1.15 g Potassium Phosphate Triabasic and 1.7 g Tetrabutyal ammonium hydrogen sulfate in water (pH adjusted to 6.8 with diluted orthophosphoric acid, 0.05 M, 1000 ml) used as a buffer and mixed with methanol in the ratio of (90:10) (%v/v). Solvent-B consists of Methanol. The flow rate used was 0.5 mL/min. The measurement was carried out at a wavelength of 280 nm for the analytes.

The column oven temperature was maintained at 40 °C; the injection volume was 10 µl. The diluent used as water for the stock solution of the drug, standard solution of drug and sample preparation, and 0.05 M Tris buffer (pH 8.1 with diluted HCl) for the preparation of impurities stock solutions. The HPLC step gradient method used the following runtime program: (Time in min/% Mobile Phase B (T/%B) - 0.0/15, 4/15, 10/20, 12/20, 30/35, 33/15, 40/15.

Preparation of Stock and Standard Solution: A stock solution of Levoleucovorin Calcium (0.64 mg/ml) was prepared by dissolving a specific amount of drug in a diluent, and further dilution was made to prepare a standard solution (0.032 mg/ml). Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, and Imp-G (0.1 mg/ml) were prepared individually and mixed by dissolving a specific amount in the diluent. A combined solution containing LLC (0.5 mg/ml) and spiked with a mixture of stock solution of impurities was prepared at the level of 0.05% was used as a system suitability standard.

Sample Preparation: The sample was prepared by reconstituting 1 vial of Levoleucovorin Calcium for injection equivalent to 50 mg with diluent and diluted to the appropriate volume to get a concentration of 0.5 mg/ml.

Method Validation: Developed method was validated as per ICH guideline Q2(R1) [10].

Specificity: Specificity is the ability to assess the analyte unequivocally in the presence of components that may be expected to be present. These studies were performed in two parts,

Specificity part A and Specificity part B [10]. In Specificity part, A, a spiked solution of Levo-leucovorin Calcium was used and observed the separation and resolution between Levoleucovorin Calcium and all process impurities, namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, and Imp-G and also peak purity index was also calculated for each analyte.

In specificity Part-B, forced degradation study was carried out on Lyophilized injection formulation under various stress conditions like acidic hydrolysis, basic hydrolysis, oxidative degradation, thermal degradation, photolytic degradation. For this purpose, Lyophilized injection formulation and control placebo sample were used. Taken 1 vial of Lyophilized injection formulation and reconstituted with 10 ml diluent and transferred into 100 mL volumetric flask. Three such sets were prepared for acidic hydrolysis, basic hydrolysis, oxidative degradation.

Acid Hydrolysis: Forced degradation studies in acidic media were performed by adding 1 mL of 0.1 N HCl in 10 ml of solution and in the placebo. The mixture was then heated at 100 °C for 1 h, after which it was cooled at room temperature and neutralized with 0.1 N NaOH. The resultant mixture was diluted up to 100 ml with diluent.

Base Hydrolysis: Forced degradation studies in basic media were performed by adding 1 mL of 0.1 N NaOH in 10 ml of solution and in the placebo. The mixture was then heated at 100 °C for 1 h, after which it was cooled at room temperature and neutralized with 0.1 N HCl. The resultant mixture was diluted up to 100 ml with diluent.

Oxidative Degradation: Forced degradation in oxidative media was performed by adding 1 mL of 1% H_2O_2 in 10 mL of solution and in the placebo. The mixture was then heated at 100 °C for 1 h, after which it was cooled at room temperature. The resultant mixture was diluted up to 100 ml with diluent.

Thermal Degradation: In thermal degradation, 1 vial of lyophilized injection formulation was put at 100 °C for 5 days in the oven, after which it was cooled at room temperature, reconstituted with 10 ml diluent, transferred into 100 mL volumetric flask, and diluted it up to 100 ml with diluent.

Photolytic Degradation: In photolytic degradation taken 1 vial of lyophilized injection formulation was put for 1 cycle in the photolytic chamber (U.V/Not less than 200-watt hour's square meters-1, VIS/ not less than 1.2 million Lux h). After completion of 1 cycle, reconstituted with 10 ml diluent, transferred into 100 mL volumetric flask, and diluted it up to 100 ml with diluent. Peak purity was carried out for Levoleucovorin Calcium peak by using a PDA detector in stress conditions. The mass balance studies were calculated for each type of stress study.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD is defined as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value and the LOQ was defined as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [10]. The LOD and LOQ for Levoleucovorin Calcium and its impurities were determined as an S/N ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in sample [10]. The linearity test solution was prepared from mixed impurities stock solution at six different concentration levels ranging from LOQ to 150% of specification limit (range was inclusive of concentration at LOQ, 50%, 80%, 100%, 120%, and 150%) and calculated the value of the coefficient of regression, slope, and Y-intercept by plotting the calibration curve of impurity area versus concentration individually.

Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between value which is accepted either as a true conventional value or an accepted reference value, and the value found [10]. The accuracy of the method was evaluated at three concentration levels (50%, 100%, and 150%) in triplicate in the dosage form.

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained

from multiple sampling of the same homogeneous sample under the prescribed conditions [10].

Precision may be considered at three levels: repeatability (same day), intermediate precision (Interday-Three different day, Intraday- different time interval on the same day), and reproducibility (different lab). Precision was carried out by injecting six replicates at the 100 % level. The RSD of the peak areas of each impurity was calculated.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage [10].

To determine the robustness of the developed method, the effect of small but deliberate variation in the chromatographic condition was evaluated. The condition was studied were flow rate (± 0.05 ml/min), column oven temperature (± 5° C), and mobile phase pH (± 0.1) and determine the resolution between all the peak.

RESULTS AND DISCUSSION:

Optimization of Chromatographic Condition: At the beginning of method development most important part was a screening of a column and selection of mobile phase, which has given good resolution with appropriate system suitability peak. LLC is an ion-forming substance, and it is very sensitive to pH; therefore, it was necessary to use the buffer as a mobile phase to maintain its buffer capacity and used an ion-pairing reagent for better separation. Based on pKa value of LLC 3.1, 4.8, and 10.4 [11] pH of mobile phase was selected 6.8. Preliminary studies were done and based on that select mobile phase - A, which consists a mixture of 1.15 g Potassium phosphates Triabasic and 1.7 g TBAHS as ion-pairing reagent (pH 6.8 by OPA, 0.05 M, 1000 ml) and mobile phase-B was Methanol.

But using this mobile phase composition, fungus growth was produced in Mobile phase - A due to the high concentration of buffer, which need to change mobile phase-A composition by adding methanol in a ratio of 990:10 %V/V. Initially, the trial took on Brownlee sphere ODS (100 × 4.1mm, 5µm) as a column, but the peak shape was not good due to the lower theoretical plate observed.

Then change the column YMC Ultra Hydrosphere C18 (100 × 3 mm, 2 µm) with isocratic mode, flow rate 0.05 ml/min, column oven temperature 50 °C, and detection wavelength 280 nm. When used, this isoocratic mode peaks to get merged, and peaks have poor resolution. Then change the isocratic mode to gradient mode (T/%B) - 0.0/20, 4/20, 25/27, 27/20, 30/20 with the same chromatographic condition, but peak shape was not good due to the lower theoretical plate observed.

Then change the column to ACE C18 (100 × 4.6 mm, 3 µm) used with guard column to increase the lifespan of columns and trial taken with a flow rate 0.5 ml/min, column oven temperature 40 °C, detection wavelength 280 nm, and gradient profile (T/%B) - 0.0/10, 4/10, 25/30, 27/30, 35/10. When used this gradient profile, one impurity gets merged between 10-20 min, thus change gradient profile (T/%B) - 0.0/10, 4/10, 10/12, 12/12, 27/30, 30/10, 40/10, and trail took, but there was observed co-eluting peak with the main peak at 30 min thus increase the buffer at 30 min which retains peak by using gradient profile (T/%B) - 0.0/10, 4/10, 10/12, 12/12, 30/26, 33/10, 40/10 which gave very long retention time because increase the buffer.

On the next trial, increase the organic modifier by using gradient profile (T/%B) - 0.0/15, 4/15, 10/20, 12/20, 30/35, 33/15, 40/15, which gave better resolution and theoretical plate, thus finalize these conditions and considered as an optimum chromatographic condition.

The optimum chromatographic condition was an ACE C18 column (150 × 4.6mm, 3µm) with binary gradient elution consisting of mobile phase A, which consists a mixture of 1.15 g M Potassium Phosphate Triabasic and 1.7 g Tetra butyl ammonium hydrogen sulphate in water (pH 6.8 adjusted with diluted OPA, 0.05 M, 1000 ml) and mixed with Methanol in ratio of 990:10 (%v/v) and mobile phase B consist methanol at a flow rate of 0.5 mL/min and detection performed at 280 nm with column thermostat 40 °C, injection volume was 10 µl.

Method Validation:

System Suitability: System suitability was used to verify the system is adequate for the analysis to be performed. System suitability parameters for the

LLC and impurities were evaluated, and the result is shown in following **Table 2**.

TABLE 2: SYSTEM SUITABILITY PARAMETERS FOR DRUG AND ITS PROCESS RELATED IMPURITIES AND KNOWN DEGRADATION IMPURITIES

Compound	Resolution	Asymmetry	Theoretical plates
Imp-E	0.00	0.99	2435
Imp-A	9.50	1.02	31910
Imp-F	5.36	1.05	42274
Imp-B	1.71	0.83	10129
LLC	2.33	0.69	19644
Imp-G	1.92	1.03	25364
Imp-D	1.57	0.69	82434
Imp-C	5.26	0.62	58623

Specificity: The resolution between all process-related impurities, degradants, and Levoleucovorin Calcium were evaluated **Fig. 1B**, Placebo Interference **Fig. 1A**, peak purity Chromatogram **Fig. 1C** and peak purity index for all analytes were found to be greater than >0.99. These both criteria give confirmation about the method specificity it means, the method was capable of determining drug in the presence of process-related impurities and degradation products **Fig. 2A-E** Mass balance results were calculated for all stresses samples and found to be 90-110% **Table 3**.

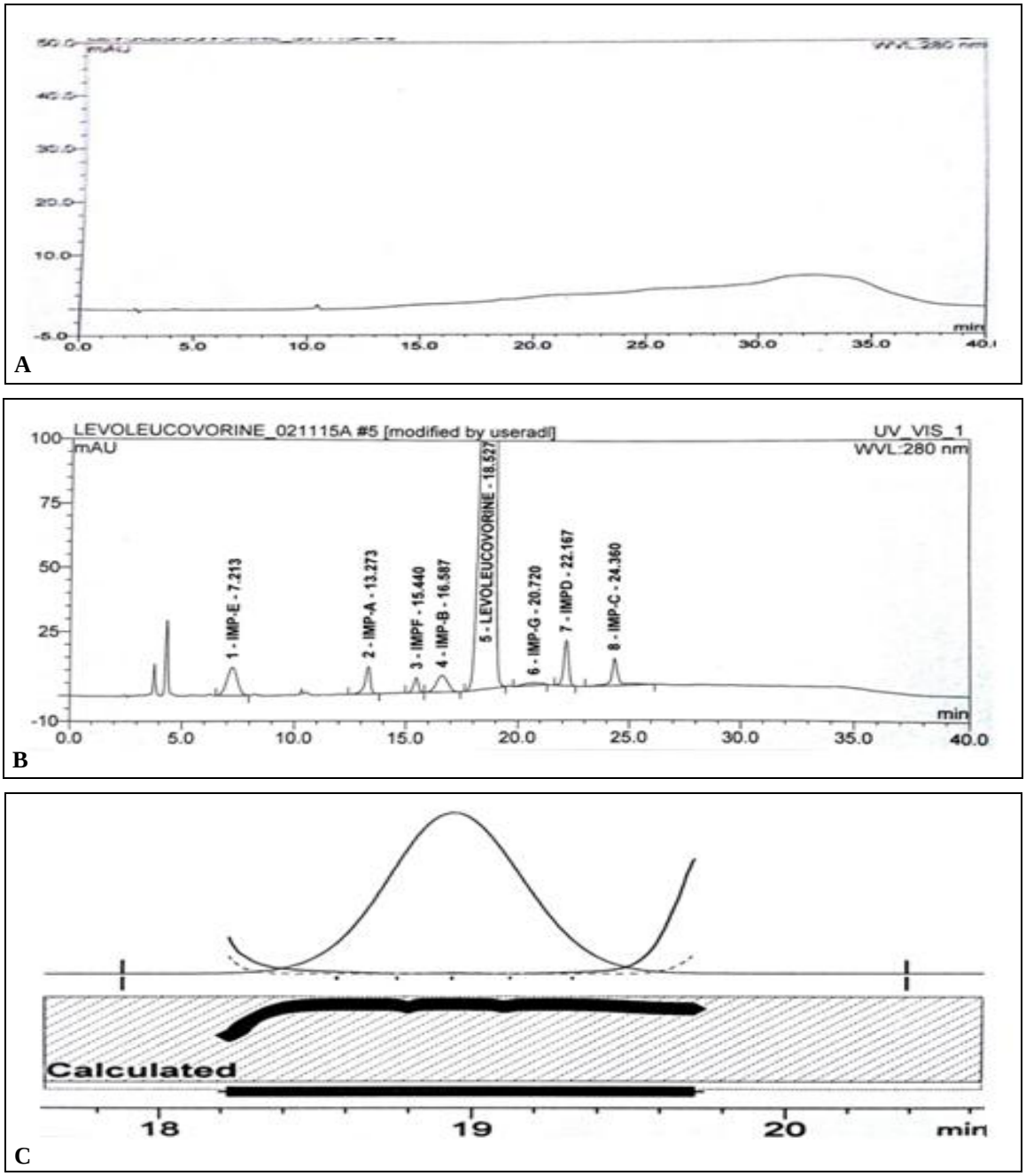
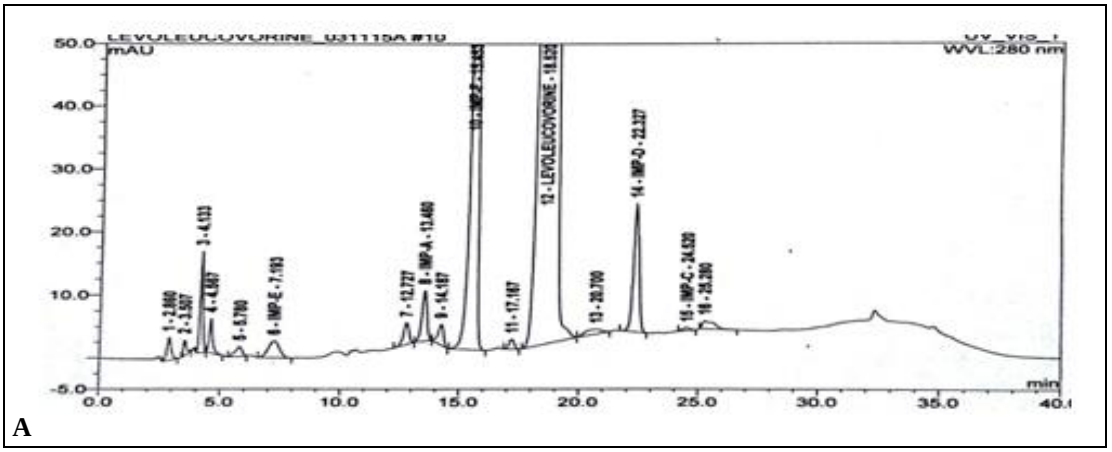


FIG. 1: TYPICAL CHROMATOGRAMS, (A) PLACEBO CHROMATOGRAM, (B) SAMPLE SPIKED CHROMATOGRAM (C) PEAK PURITY CHROMATOGRAM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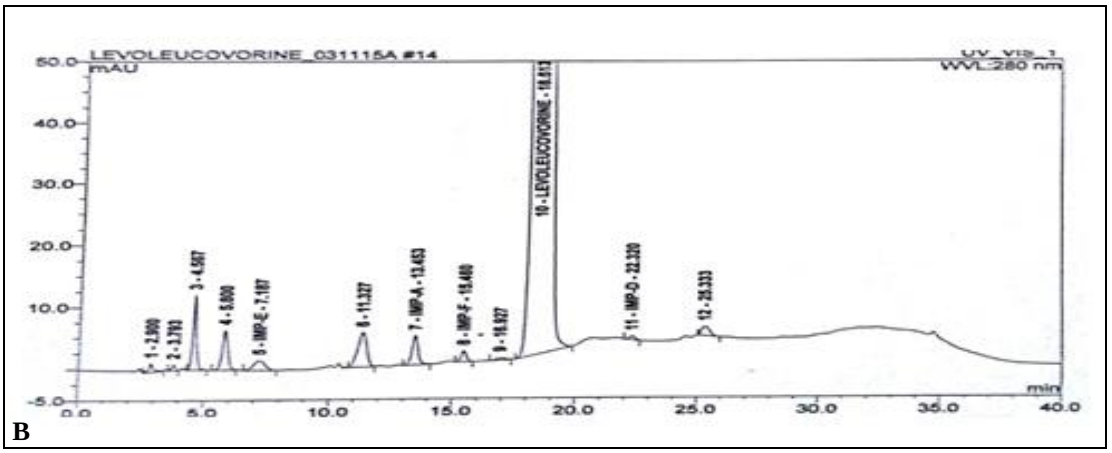
12-LEVOLEUCOVORINE
14-IMP-D
15-IMP-C
16-25.280

A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min
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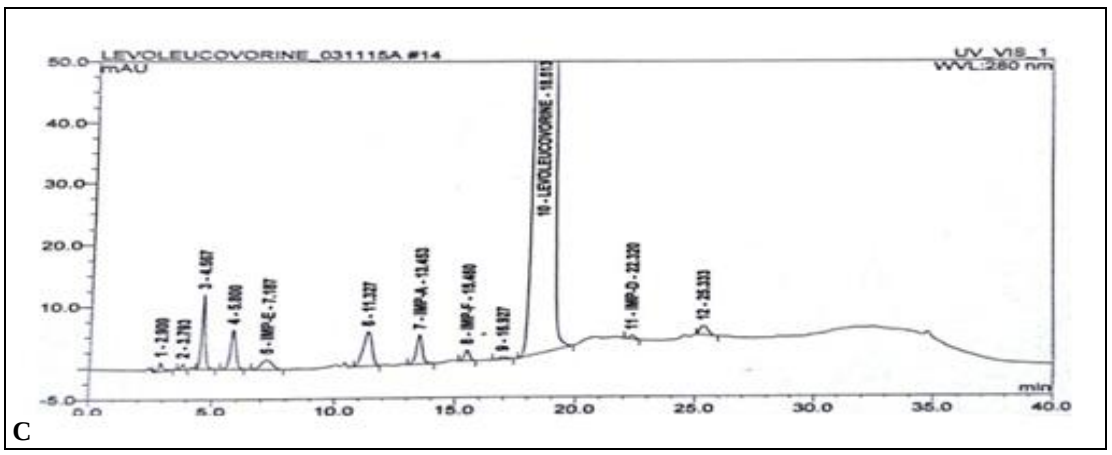
11-IMP-D
12-25.333

B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mAU
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min
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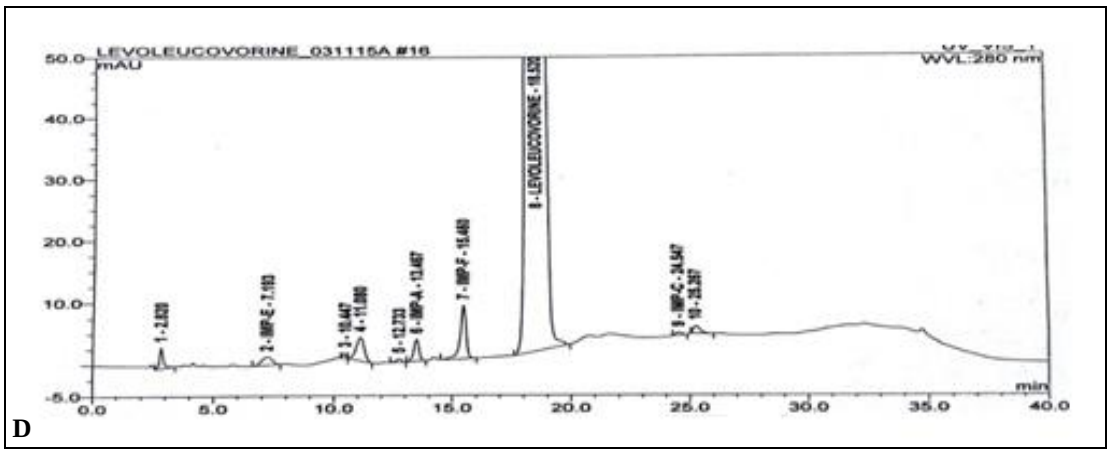
11-IMP-D
12-25.333

C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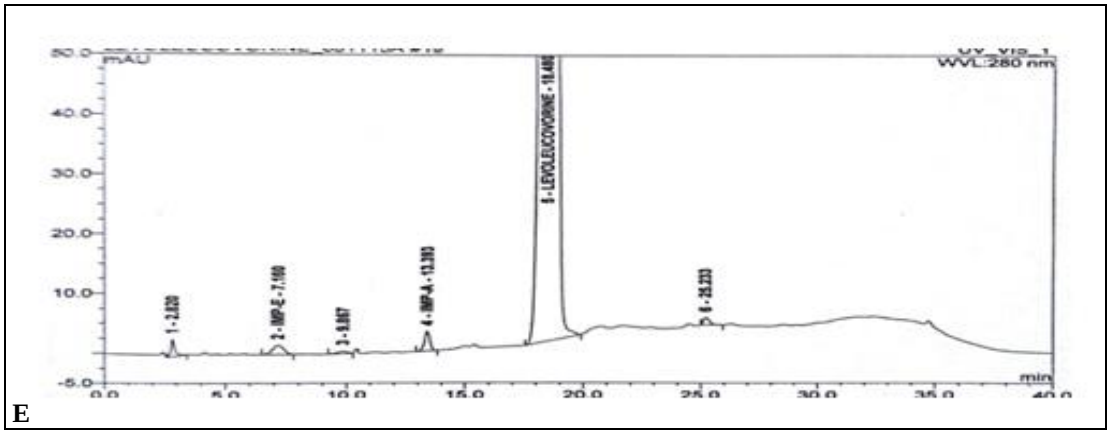
mAU
WVL:280 nm
min
8-LEVOLEUCOVORINE

D



E

FIG. 2: FORCED DEGRADATION CHROMATOGRAMS, (A) SAMPLE IN 0.1N HCl 1 mL 1 h 100 °C, (B) SAMPLE IN 0.1N NaOH 1 ML 1 HR 100 °C, (C) SAMPLE IN 1% H_2O_2 1 mL 1 h 100°C, (D) SAMPLE IN THERMAL DEGRADATION 5 DAY 100 °C, (E) SAMPLE IN PHOTOLYTIC DEGRADATION

TABLE 3: SPECIFICITY PART-B (STRESS) STUDIES REPRESENT DEGRADATION IN VARIOUS PARAMETERS

Degradation stage	Condition	Assay of Untreated Sample	%Assay Observed after Degradation	% Impurity	Mass Balance
Acidic Degradation	0.1 N HCl 1 mL 100 °C 1 h	103.2	82.13	16.84	95.90
Basic Degradation	0.1N NaOH 1 ml 100 °C 1 h	103.2	98.41	2.14	97.44
Oxidative Degradation	1% H_2O_2 100° C 1 h	103.2	100.24	1.66	97.57
Thermal Degradation	5 Day at 100 °C	103.2	100.59	1.67	99.09
Photolytic Degradation	1 cycle (not less than 1.2 million lux h)	103.2	102.89	0.45	101.80

Mass Balance = %Drug Impurity + % Assay Observed after Degradation / Initial Assay×100

Limit of Detection (LOD) and Limit of quantification (LOQ): LOD was obtained at the 0.015% level, whereas LOQ was obtained at the 0.05% level of all impurities. The proposed method was sensitive and capable of detecting and quantify the impurities at the lowest level.

Linearity: The linearity range of all impurities was found 0.5-1.5 µg/mL. The correlation coefficient was found to be >0.9900 for all the impurities **Table 4**. The result showed that an excellent correlation existed between the peak area and concentration of the analyte.

TABLE 4: LINEARITY DATA OF CALIBRATION CURVE FOR ALL PROCESS RELATED IMPURITIES

	Concentration (µg/mL)						R2
	LOQ	0.5	0.8	1	1.2	1.5	
Name	Area						
Imp-E	35455	92191	170011	221252	265705	309566	0.9910
Imp-A	12071	31234	50124	62468	74309	88769	0.9943
Imp-F	8495	21358	38381	49764	59942	77384	0.9997
Imp-B	10471	27542	49758	64115	78045	96596	0.9991
Imp-G	8152	30437	48299	60873	72445	90988	0.9948
Imp-D	14950	29567	52312	68254	82367	101733	0.9983
Imp-C	21228	38418	67916	88439	106126	138616	0.9965

Accuracy: Accuracy was determined by spiking the known impurities at 50%, 100%, and 150% of their specified limits in dosage forms. The % Recoveries of all the impurities were found to be in the range of 98 - 101% and RSD 0.8-1.5%, which indicates the accuracy of the proposed method in **Table 5**.

Precision: Repeatability was determined by analyzing six separate sample solutions spiked with all seven impurities. Reproducibility was determined by using two different labs, and Intermediate precision includes Interday precision and Intraday precision. Interday precision was performed by analyzing six samples on three different days, whereas Intraday was performed on the same day at a different time interval. The RSD was calculated from the result of obtaining observation. In all cases, the RSD was less than 2, which indicates the proposed method was precise **Table 6**.

TABLE 5: ACCURACY DATA FOR ALL PROCESS RELATED IMPURITIES

Name	% Level	Amount taken (mg)	Amount found (mg)	% Recovery	Mean % Recovery	RSD
Imp-A	50	0.503	0.494	99.890	100.454	
	100	1.005	1.005	100.580		0.955
	150	1.508	1.508	99.730		
Imp -B	50	0.504	0.490	99.178	98.840	0.954
	100	1.008	0.990	99.230		
	150	1.511	1.480	98.129		
Imp -C	50	0.507	0.490	99.630	99.110	0.863
	100	1.014	0.990	98.770		
	150	1.521	1.480	98.920		
Imp -D	50	0.557	0.496	99.175	99.484	1.026
	100	1.113	0.992	98.751		
	150	1.670	1.508	100.528		
IMP-E	50	0.504	0.496	99.245	99.571	0.823
	100	1.008	0.996	99.678		
	150	1.512	1.497	99.792		
IMP-F	50	0.494	0.500	100.130	99.881	0.899
	100	0.988	0.990	99.678		
	150	1.482	1.490	99.857		
Imp -G	50	0.510	0.493	98.661	99.590	1.491
	100	1.000	1.000	98.945		
	150	1.500	1.460	99.591		
	100	1.008	0.996	99.678		
	150	1.512	1.497	99.792		
IMP-F	50	0.494	0.500	100.130	99.881	0.899
	100	0.988	0.990	99.678		
	150	1.482	1.490	99.857		
Imp -G	50	0.510	0.493	98.661	99.590	1.491
	100	1.000	1.000	98.945		
	150	1.500	1.460	99.591		

TABLE 6: PRECISION DATA FOR ALL PROCESS RELATED IMPURITIES

Parameter		Conc. (µg/mL)	RSD						
		Name	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G
Repeatability		100	0.61	1.59	0.48	0.33	0.79	0.44	1.39
Reproducibility	Lab-1	100	1.30	1.24	0.36	1.39	1.13	0.39	1.23
	Lab-2		0.58	1.05	0.84	0.92	1.18	0.69	0.87
Intermediate precision	Intraday	100	0.76	0.89	0.69	1.28	0.24	0.26	1.02
	Interday		1.09	0.70	1.04	0.74	1.43	0.84	0.92

Robustness: In all deliberative varied chromatographic conditions (flow rate, column oven temperature, pH) all analytes were adequately resolved and elution order remained unchanged and determined the RRT of all process-related impurities with respect to the main peak, and it was the same on all the chromatographic condition **Table 7**.

TABLE 7: ROBUSTNESS DATA FOR ALL PROCESS RELATED IMPURITIES

Parameter	Value	Resolution							
		Imp -E	Imp-A	Imp-F	Imp -B	LLC	Imp -G	Imp-D	Imp -C
Flow rate	10% +	0.45	0.39	0.72	0.85	0.90	1.02	1.20	1.29
	10% -	0.55	0.38	0.71	0.84	0.91	1.02	1.19	1.30
Column temperature	10% +	35	0.39	0.72	0.87	0.91	1.05	1.20	1.29
	10% -	45	0.38	0.74	085	0.91	1.04	1.24	1.32
pH	10% +	6.6	0.38	0.72	0.85	0.92	1.04	1.22	1.30
	10% -	7.00	0.39	0.73	0.84	0.90	1.03	1.20	1.28

CONCLUSION: The developed stability indicating RP-HPLC method was simple, selective, rapid, specific, sensitive, linear, accurate, précis and robust. Therefore the method is found to be specific for the estimation of process-related impurities and degradation products with good resolution. It can be applied to the stability samples.

So the proposed method can be used in the pharmaceutical analysis for stability monitoring and routine quality control sample.

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