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Research Article

A novel isocratic RP-HPLC method development and validation for estimation of 5HMF in Levofloxacin Hemihydrate intravenous infusion

Narasimha Swamy Lakka*[1], Nishant Goswami[2]

Analytical Research and Development Dr.Reddy's Laboratories Ltd. IPDO, Bachupally, Hyderabad-500072, Andhra Pradesh, India

ABSTRACT

A RP-HPLC method was developed for estimation of 5-Hydroxy methyl furfural content in Levofloxacin Hemihydrate intravenous infusion 5mg/ml. It involved a 150mm x 4.6mm, 5µm LichroSphere C-18 column with 40°C temperature. The separation was achieved on simple isocratic method. A mixture of pH 3.0 buffer (0.04M, ortho phosphoric acid buffer, adjusted pH 3.0 with triethylamine) - acetonitrile (87:13, *v/v*) was used as the mobile phase. The flow rate was 1.0ml/min, injection volume was 5µl and the detection wavelength was 284nm. The retention time of 5HMF was 2.7min. The total runtime was 12min within which active compound and degradation products were separated. The developed method successfully applied to the determination of 5HMF content in pharmaceutical preparations. The developed RP-HPLC method was validated with respect to linearity, accuracy, specificity, limit of detection, limit of quantification, precision, robustness and ruggedness.

Keywords: 5HMF; Method development; Method validation; RP HPLC.

1. INTRODUCTION

Hydroxymethylfurfural (HMF), also 5-(Hydroxymethyl) furfural, is an organic compound derived from dehydration of sugars. IUPAC name of this compound is 5-(hydroxymethyl)-2-furaldehyde, its molecular formula and molecular weight are C6H6O3 and 126.11 g/mol; respectively. This colourless solid is highly water-soluble. The molecule is a derivative of furan, containing both aldehyde and alcohol functional groups. HMF has been identified in a wide variety of heat-processed foods including milk, fruit juices, spirits, honey, etc. HMF, which is derived from cellulose without use of fermentation, is a potential "carbon-neutral" feedstock for fuels and chemicals.

Anhydrous Glucose (anhydrous dextrose) is one of the ingredient in the formulation of the Levofloxacin Hemihydrate intravenous infusion. During the stability of infusion, glucose is converting into 5HMF. Hence, a new RP HPLC method has been developed and validated for 5HMF estimation. [Haibo Zhao, et al, 2007; ICH-Q2 (R1); ICH- Q3A (R2); ICH- Q3B (R2)].

Toxicological studies have revealed significant adverse effects of 5HMF on human blood cells and tumerogenic potential in association with colon cancer, although there have been several contradictory reports on the mutagenic and /or anti-mutagenic effects of 5HMF. The potential toxic, mutagenic, and carcinogenic effects of 5HMF have been determined and amounts of the compound in food are limited by regulation (Hayriye M et al, 2009).

Levofloxacin hydrochloride, a cyclic amine, is a synthetic antiviral drug and a derivate of adamantane, like a similar drug amantadine. Levofloxacin hydrochloride is inhibitory to the in vitro replication of influenza A virus isolates from each of the three antigenic subtypes (H1N1, H2H2 and H3N2) that have been isolated from man. Levofloxacin hydrochloride has little or no activity against influenza B virus. Levofloxacin hydrochloride does not appear to interfere with the immunogenicity of inactivated influenza A vaccine.

Accordingly, the aim of the present study was to estimate the 5HMF content in the Levofloxacin intravenous infusion 5mg/ml under a variety of ICH recommended test conditions and to develop a stability-indicating estimation method for 5HMF [ICH-Q1A; ICH-Q1B] (Fig 1).

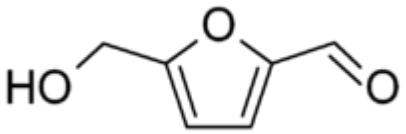

Figure 1: Chemical structure of 5HMF

In the literature there are limited methods have been reported [Roger m et al, 2003; Lenka Vorlová et al, 2006; Siqin Hu et al, 2009;

* Corresponding Author
Email: nslakka@gmail.com; narasimhasl@drreddys.com
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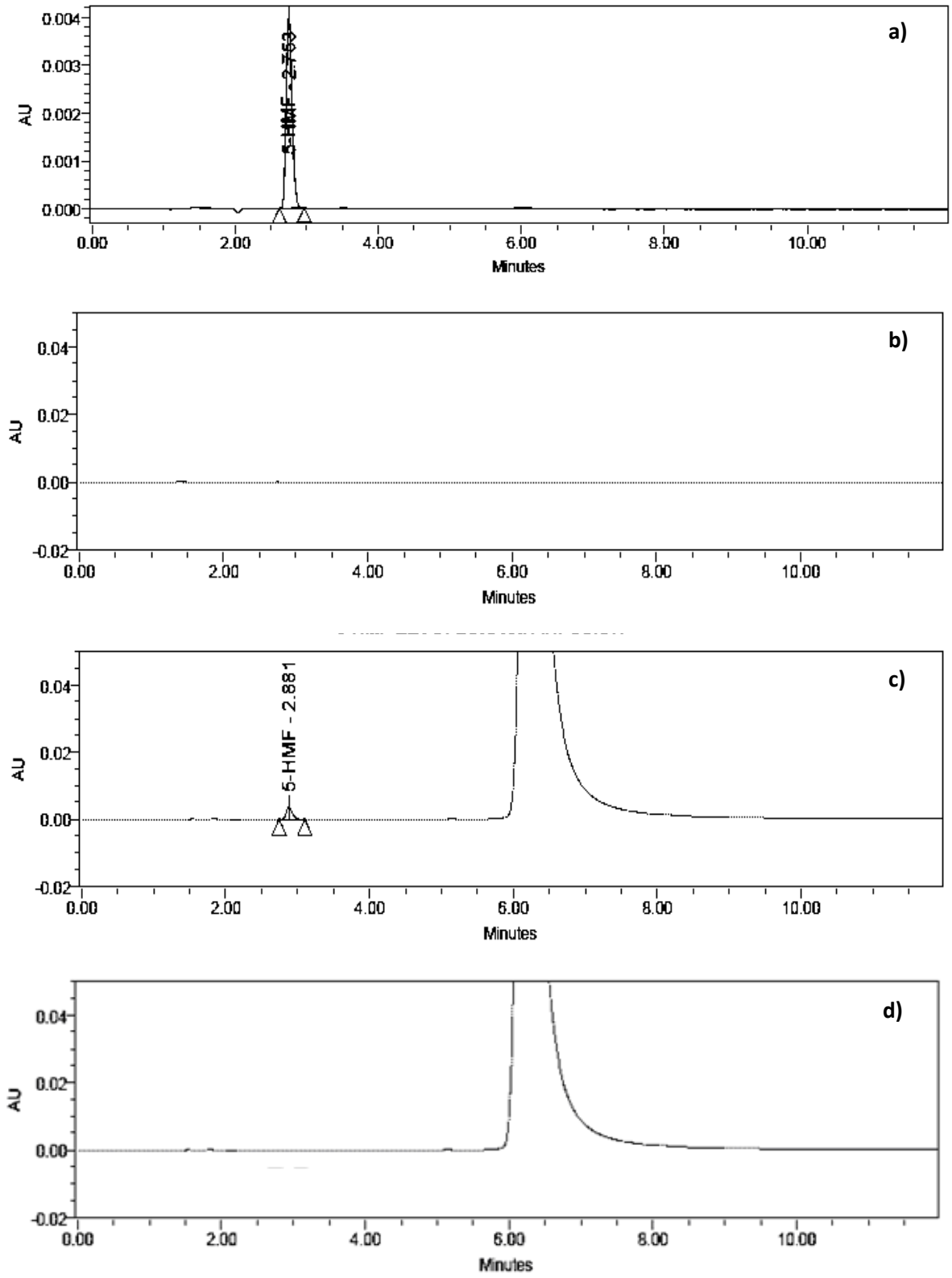

Figure 2: A typical chromatogram of a) 5HMF, b) blank, c) finished product, d) placebo of finished product

Chen Lei et al, 2009; I.G.Zenkevich et al, 2001; Hyoung s. Lee et al, 1986; Spano N et al, 2006]. A LC method has been reported for the determination of 5HMF in combination with other drugs. A HPTLC method has been reported for Turkish fruits and vinegars [Hayriye M et al, 2009; K. Kukurova et al, 2005], and one more method has been reported for honey and other Sugar-containing materials [Charles H et al, 2006]. A specific quantitative chromatographic assay method has been reported for autoclaved d-glucose infusion fluids [C. T. Hung et al, 2008]. A reversed-phase HPLC method has been reported for the strawberry tree honey samples [Nadia Spano et al, 2005]. No method was found with

complete method development and validation in intravenous infusions in pharmaceutical preparations.

Reverse phase high performance liquid chromatography (RPHPLC) is a one of the technique in liquid chromatography. A novel isocratic reproducible reverse phase high performance liquid chromatography method was developed for estimation of 5HMF in Levofloxacin Hemihydrate intravenous infusion 5mg/ml.

2. EXPERIMENTAL

2.1. Chemicals

HMF Standard was supplied by Sigmaaldrich. Commercially available Levofloxacin Hemihydrate intravenous infusions 5mg/ml were manufactured by Dr. Reddy's laboratories limited, Hyderabad, India. The HPLC grade acetonitrile, analytical grade triethylamine and ortho phosphoric acid were purchased from Merck, Darmstadt, Germany; water was prepared by using Millipore Milli Q Plus water purification system

2.2. Equipment

The Agilent 1200 series HPLC system we used consists of a binary solvent manager, a sample manager and a UV detector. The output signal was monitored and processed using Empower software.

2.3. Chromatographic Conditions

The chromatographic column used was a LichroSphere C-18, 150mm x 4.6mm. i.d with 5µm particles. A mixture of pH 3.0 buffer (0.04M, ortho phosphoric acid buffer, adjusted pH 3.0 with triethylamine) - acetonitrile (87:13, v/v) was used as the mobile phase. The flow rate of mobile phase was 1.0 ml/min and the detection was monitored at a wavelength 284nm. The column temperature was maintained at 40˚C and injection volume was 5µl (Fig 2).

2.4. Preparation of Stock Solutions

A stock solution of 5HMF standard (0.0125mg/ml of 5HMF) was prepared by dissolving an appropriate amount in mobile phase. Final working standard solution concentration was s 0.00125 mg/ml of 5HMF was prepared from above stock solution (0.2% of target concentration) in mobile phase for limit test determination of 5HMF.

2.5. Preparation of Sample Solution

Transferred 5ml (each 1ml contains 5mg of Levofloxacin Hemihydrate) of Levofloxacin Hemihydrate intravenous infusion sample in to 20ml of volumetric flask dissolved and diluted up to volume with mobile phase (1.250 mg/ml of Levofloxacin Hemihydrate). Pipette out 5 ml of above test stock solution in to 10 ml volumetric flask and diluted up to the volume with mobile phase (0.625mg/ml of Levofloxacin Hemihydrate). This solution was filtered using 0.45 µm (Nylon 66- membrane) filter.

2.6. System Suitability Solution Criteria

The system suitability was assessed by six replicate analyses of the drugs at concentrations of 0.00125 mg/ml of 5HMF. The acceptance criteria were not more than 10.0% for the RSD. The tailing factor should be not more than 2.0. The column efficiency should not be less than 3000 theoretical plates for 5HMF peak.

2.7. Method Validation

Method validation was performed as per ICH guidance for determination of 5HMF in the formulations. The following validation characteristics were addressed: system suitability, system precision, linearity, detection limit, quantification limit, precision, accuracy, robustness, ruggedness and specificity.

2.7.1. System Suitability

The system suitability test solution was injected and the chromatographic parameters like USP tailing factor and, plate counts for six replicate injections of 5HMF were evaluated for proving the system suitability.

2.7.2. **System precision**

The system precision parameter was evaluated by injecting six replicate injections standard preparation of 5HMF and reported the %RSD of six injections.

2.7.3. Precision of test method

The precision of the estimation method of 5HMF was evaluated by carrying out six independent assays of (0.00125 mg/ml of 5HMF) test samples by spiking 5HMF. The percentage of RSD of six assay values was calculated. Different analyst from the same laboratory evaluated the intermediate precision of the test method.

2.7.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for 5HMF were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentration.

2.7.5. Linearity

Linearity solutions were prepared from stock solution at five concentration levels from LOQ level to 200% of analyte concentrations (0.033µg/ml to 2.500µg/ml). The slope, Y-intercept and correlation coefficient were calculated.

2.7.6. Accuracy

The accuracy of the method was evaluated in triplicate at five concentration levels, i.e. 50%, 75%, 100%, 125% and 150% of target test concentration (0.625 mg/ml of Levofloxacin Hemihydrate) in infusions. The percentage of recoveries of 5HMF was calculated.

2.7.7. Specificity

The specificity of the method was established by injecting duplicate sample preparations of placebo of Levof-

Table 1: Precision results of 5-Hydroxy Methyl Furfural

Intra-day precision	Active Name	Pre-1 % Assay	Pre-2 % Assay	Pre-3 % Assay	Pre-4 % Assay	Pre-5 % Assay	Pre-6 % Assay	% Mean	% RSD
	5HMF	101.7	99.6	98.1	98.2	102.0	99.3	99.8	1.7
Inter-day precision	5HMF	98.6	98.8	101.2	101.1	100.7	101.4	100.4	1.2

Table 2: Linearity of 5-Hydroxy Methyl Furfural

S. No.	Concentration level (in)		Response of Detector
1.	0.033µg/ml	0.005% (LOQ)	645
2.	0.3125µg/ml	25%	5129
3.	0.6250µg/ml	50%	10377
4.	1.2500µg/ml	100%	20821
5.	1.5625µg/ml	125%	25661
6.	1.8750µg/ml	150%	29188
7.	2.500µg/ml	200%	41243
Coefficient of Correlation (r)			0.999
Slope (a)			0.00006
Intercept (b)			0.005

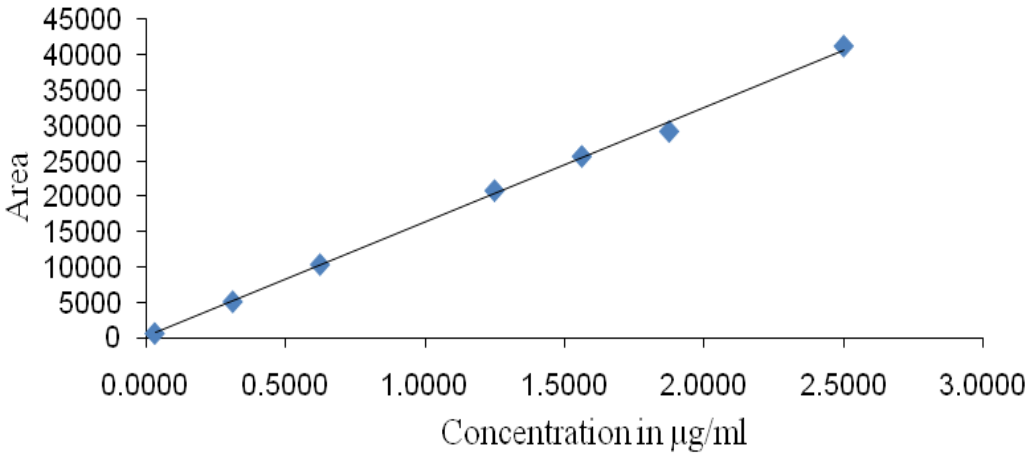

Figure 3: Linearity graph of 5-Hydroxy Methyl Furfural

loxacin Hemihydrate without glucose content in the infusion. No, interference found due to ingredients which are used in the formulation.

2.7.8. Solution Stability and Mobile Phase Stability

The solution stability of 5HMF was carried out by leaving the test solution in tightly capped volumetric flask at room temperature and as well as refrigerator for 48 hours. The mobile phase stability was also carried out by assaying the freshly prepared standard solution for 12 hours interval up to 48 hours. The mobile phase preparation was kept constant during the study period. The percentage of RSD of assay of 5HMF was calculated for the study period during mobile phase and solution stability experiments.

2.7.9. Robustness

The robustness of a method is its capacity to remain unaffected by small changes in conditions. To determine the robustness of the method the experimental conditions were deliberately altered and assay, USP tailing factor, plate count and % R.S.D. results for assay were evaluated.

The mobile phase flow rate was 1.0mL min^{-1}. This was changed by 0.2 units to 0.8 and 1.2mL min^{-1} and the effect was studied. Similarly, the effect of column temperature was studied at 35°C and 45°C instead of 40°C. The effect of mobile phase composition was changed by 10% organic phase in mobile phase to 90% (11.7) and 110% (14.3) instead of buffer (pH 3.0) - acetonitrile 87:13 (*v*/*v*). The effect of mobile phase buffer pH was studied at pH 2.8 and pH 3.2 instead of pH 3.0.

3. RESULTS AND DISCUSSION

3.1. Method Development and optimization of assay method

The method was optimized by using RP-HLC UV detector. The main target of the chromatographic method is to get the content of 5HMF in Levofloxacin Hemihy-

Table 3: Percentage Recovery of 5-Hydroxy Methyl Furfural

Recovery level	µg/ml added	µg/ml found	Recovery (%) (*n* = 3)
	5HMF	5HMF	5HMF
50%	0.6217	0.6040	97.2
75%	0.9326	0.9224	98.9
100%	1.2434	1.2044	96.9
125%	1.5543	1.5437	99.3
150%	1.8652	1.8282	98.0

n = 3 determinations

Table 4: Robustness study results

Condition	USP		% R.S.D of results
	Tailing	Plate counts	
Flow rate (±0.2 ml/min of the optimum flow)			
0.8ml min^{-1}	1.2	6674	0.8
1.2ml min^{-1}	1.2	5254	0.7
Mobile phase composition (±10% of optimum organic modifier concentration)			
11.7	1.2	5784	0.2
14.3	1.2	5687	0.2
Temperature (±5°C of optimum temperature)			
35 °C	1.2	5764	1.2
45 °C	1.2	5750	0.2
pH (±0.2 of optimum pH)			
2.8	1.2	5757	0.2
3.2	1.2	5857	0.1

drate infusion. The samples were run using different stationary phases like C18, C8, Cyano and Mobile phases containing buffer like phosphate with different pH (2-7) and using organic modifiers like acetonitrile and methanol in the mobile phase. But the separation was satisfactory in the adopted chromatographic conditions only. It indicated that the isocratic with 13% acetonitrile as organic modifier in mobile phase was successful in separating 5HMF in infusion.

3.2. Validation of Developed Stability-Indicating method

3.2.1. System Suitability

The system suitability test solution was injected and the chromatographic parameters like USP tailing factor and plate counts were evaluated**.** The USP tailing factor and plate counts were 1.2 and 5757.

3.2.2. System precision

The system suitability test solution was injected and the chromatographic parameters like relative standard deviation for six replicate injections of 5HMF was evaluated and found to be within the limit. The relative standard deviation for replicate injections was 0.2%.

3.2.3. Precision (inter-day precision and intra-day precision)

The percentage RSD values for the precision study were 1.7%, 1.2% (inter-day precision). This is confirming good precision of the method. The complete results are shown in Table 1.

3.2.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection and limit of quantification of 5HMF were 0.01µg/ml and 0.033µg/ml for 5µl injection volume.

3.2.5. Linearity

Linear calibration plot for this method was obtained over the calibration ranges tested, i.e. from 0.033µg /ml to 2.500µg/ml for 5HMF and the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the peak area and concentration of the analyte. The mean regressions equations were found as A = 0.000062 × C - 0.005 (r^2 = 0.9981, *n* = 7). *A = a C + b*, where A is the peak area ratio of the 5HMF, *a is* the slope, *b* is the intercept and *C* is concentration of the measured solution in µg mL^{-1}. The results show that an excellent correlation existed between the peak area and concentration of the analyte. The complete results are shown in Table 2 and Fig 3.

3.2.6. Accuracy

The percentage recovery of 5HMF in Levofloxacin Hemihydrate intravenous infusion ranged from 96.9% to 99.3%. Excellent recoveries were made at each added concentration. The complete results are shown in Table 3.

3.2.7. *Solution Stability and Mobile Phase Stability*

The solution stability and mobile phase stability experiment data confirms that sample solutions and mobile phase used during the assay were stable up to 48 hours.

3.2.8. *Specificity*

Fig 2.(d) shows that the method was sufficiently specific to the drug. The specificity of the method was established by injecting duplicate sample preparations of placebo of Levofloxacin Hemihydrate without glucose content in the infusion. No, interference found due to ingredients which are used in the formulation, it indicating that the method remains specific under tested conditions.

3.3.8. *Robustness*

For robustness study, for all changes of conditions the sample was assayed in triplicate. When the effect of altering one set of conditions was tested, the other conditions were held constant at the optimum values. The USP tailing factor, plate counts and %RSD were calculated for each condition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust. The complete results are shown in Table 4.

4. CONCLUSIONS

A novel isocratic RP-HPLC method prove to be simple, linear, precise, accurate, robust, rugged and specific. The total runtime was 12min within which the drugs and their formulation products were separated. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method can be used for quantitative determination of the 5HMF in Levofloxacin Hemihydrate intravenous infusion 5mg/ml in the presence of finished products in stability by the industry.

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