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SPECTROPHOTOMETRIC & RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN & ORNIDAZOLE

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Keywords:

Levofloxacin, Ornidazole, UV-Spectroscopy, Reverse phase Liquid Chromatography, Simultaneous equation method

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ABSTRACT: An accurate, precise and reproducible UV-spectro-photometric methods and liquid chromatographic assay method were developed and validated for the determination of Levofloxacin and Ornidazole in tablet dosage form. Spectrophotometric estimation was done by simultaneous equation method and 50% methanol as solvent. In this method λ_{max} for LEVO and OZ were selected at 293.5nm and 318nm. RP-HPLC analysis was carried out using Prontosil C-18 column (4.6 x 250mm, 5µ particle size) and mobile phase composed of Acetonitrile : 0.05% Ortho-phosphoric acid in water pH 3.0 (45:55% v/v)at a flow rate of 1.0 ml/min and chromatogram was recorded at 303 nm. Linearity was evaluated over the concentration range of 4 -20 µg/ml and 8-40 µg/mL for LEVO and OZ in both UV spectrophotometric and RP-HPLC method (the value of r^2= 0.999 found were by both the methods for LEVO and OZ). The developed methods were validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values therefore the both methods can be used for routine monitoring of LEVO and OZ in industry in the assay of bulk drug and tablets.

INTRODUCTION: Levofloxacin hemihydrates (LEVO) chemically (-)-(S)-9-fluoro2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4benzoxazine-6-carboxylic acid hemihydrate (**Fig. 1A**.), is a fluoroquinolone antimicrobials, is the active S-isomer isolated from the racemic ofloxacin [1-2]. Ornidazole (OZ) chemically; 1-chloro-3-(2-methyl-5-nitro-1H-irnidazole-1-yl) propane-2-ol (**Fig. 1B**.), is a 5-nitroimidazole derivative used as an anti-infective agent [1, 2].

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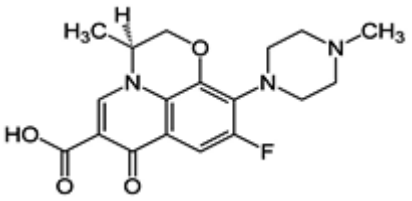
Levofloxacin hemihydrate is official in IP [3]. Numerous HPLC [4-10], UV [11] and HILIC/MS/MS [12] has been used to determine drugs in biological fluids.

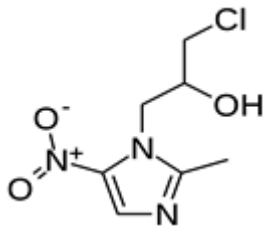
Ornidazole is official in IP [3] and USP [13]. The assay procedure mentioned in these pharmacopoeias uses non-aqueous titration for estimation of ornidazole. A literature survey reveals that ornidazole is estimated by glassy carbon electrode [14], UV [15] method, HPLC [16] method, LC-MS [17] method and calorimetry [18] method in solid dosage form and in biological fluids.

Some spectrophotometric [19, 20], HPLC [21] and HPTLC [22] methods have been reported for their simultaneous estimation with ofloxacin in the tablet dosage form.

However, to best of our knowledge, there is no reported UV-Spectrophotometric and RP-HPLC method available for simultaneous estimation of LEVO and OZ. The aim of the present work was to develop easy, economic, accurate, specific and precise spectrophotometric methods for simultaneous estimation of LEVO and OZ in bulk drugs and tablet dosage form.



(A)



(B)

FIG. 1: STRUCTURE OF (A) LEVOFLOXACIN (B) ORNIDAZOLE

EXPERIMENTAL:

Instrument: UV-spectrophotometric method utilizes a double beam double detector spectrophotometer, Shimadzu model-1700 having spectral bandwidth 3 nm and of wavelength accuracy ±1 nm, with 1cm quartz cells was used.

Liquid chromatographic system from Young Lin 9100 comprising of manual injector, YL 9111 quaternary pump for constant flow and constant pressure delivery and Photodiode array detector (YL 9160 detector) connected to software YL clarity for controlling the instrumentation as well as processing the data generated was used.

Reagents and chemicals:

Drugs: Pure sample of LEVO and OZ was obtained as gift sample from Intas Laboratories Pvt Ltd, and GSK Ltd. Mumbai, respectively.

Solvent: Acetonitrile (HPLC Grade), Methanol (AR Grade), ortho-phosphoric acid (AR Grade) obtained from Merck Chemical Division, Mumbai. Milli-Q was used to prepare water used in UV spectroscopy and RP-HPLC method.

Diluent: 50% Methanol used in UV Spectrophotometry and Acetonitrile: Water (50:50 % v/v) in RP-HPLC as diluents.

Linearity range and calibration graph:

1. **Preparation of Standard Stock Solutions of LEVO and OZ:** Standard stock solutions were prepared by dissolving separately 100 mg of each drug in diluents used in UV Spectrophotometry method (i.e. 50% Methanol) and RP-HPLC method (i.e. Acetonitrile :Water (50:50 % v/v)) and the flask was sonicated for about 10 min to solubilize the drugs (Stock-A).

2. **Preparation of Working Standard Solution for calibration curve:** For spectrophotometric method, further dilutions of aliquots of standard stock solution (1000 µg/ml) were carried out in ranging from 4-20µg/ml for LEVO and 8-40 µg/ml for OZ. UV Spectrum has been recorded in the range of 200-400nm **Fig. 2.** Calibration curve was plotted between concentrations versus absorbance at λmax for LEVO and OZ which are 293.5nm and 318nm respectively **Fig. 3, Fig. 4**. The result of their optical characteristics and linearity data of both drugs has been reported in the **Table 1**.

   For HPLC method, the standard solutions were prepared by dilution of aliquots of the standard stock solution (1000 µg/ml) with diluents to reach the linearity range of 4-20µg/ml for LEVO and 8-40 µg/ml for OZ. The chromatogram was recorded at 303 nm **Fig. 5A and 5B**.

   The peak areas were plotted against the corresponding concentrations to obtain the calibration graph **Fig. 6, Fig. 7**. The result of their optical characteristics and linearity data of both drugs has been reported in the **Table 1**.

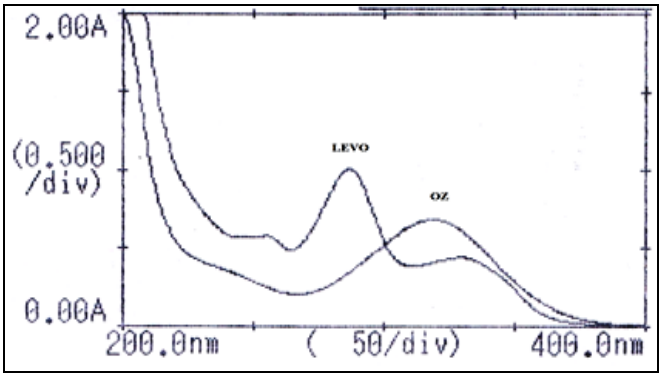


FIG. 2: OVERLAY SPECTRA OF LEVO AND OZ

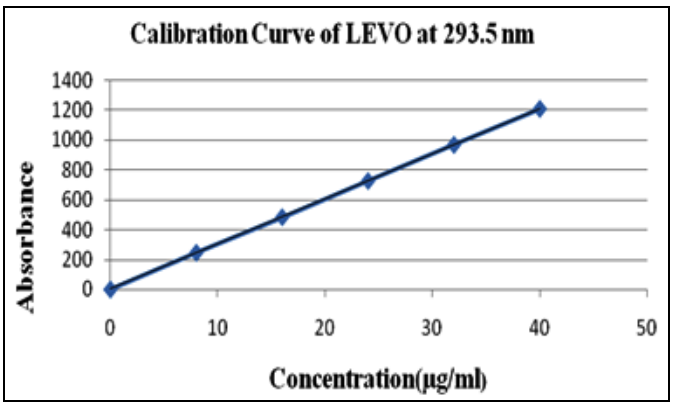


FIG. 3: CALIBRATION CURVE OF LEVO IN UV SPECTROPHOTOMETRIC METHOD

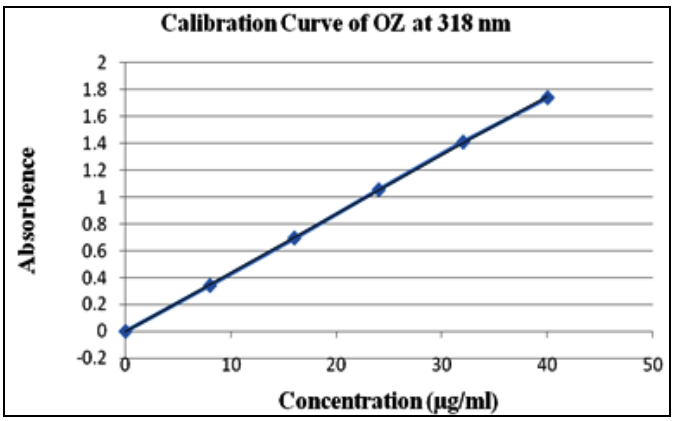
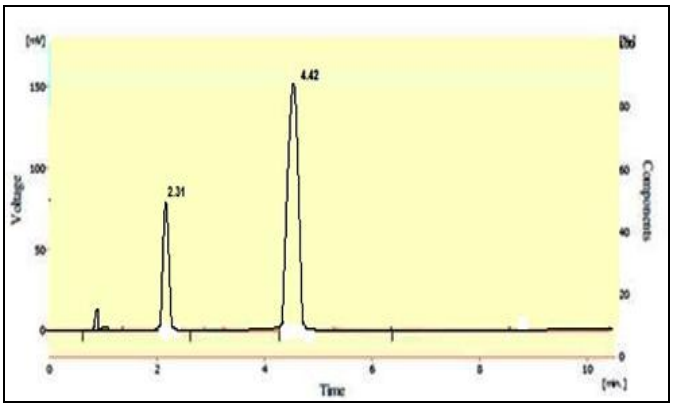
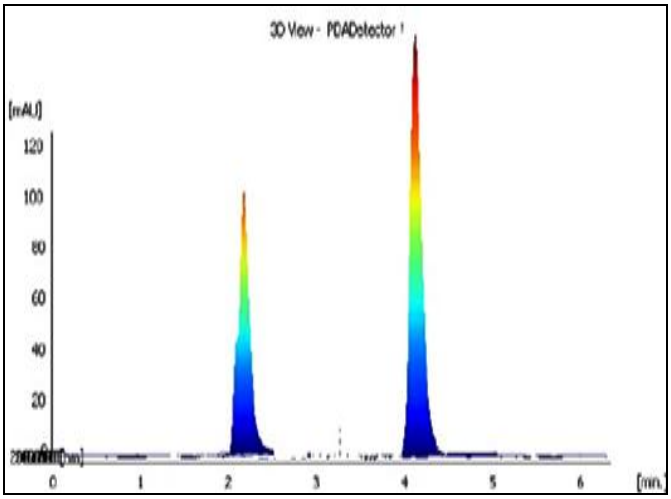


FIG. 4: CALIBRATION CURVE OF OZ IN UV SPECTROPHOTOMETRIC METHOD



(A)



(B)

FIG. 5: (A) CHROMATOGRAM AND (B) 3D VIEW OF LEVO AND OZ AT 303 nm

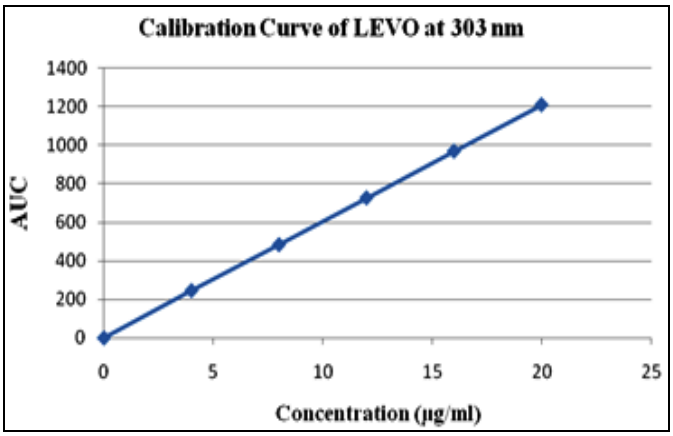


FIG. 6: CALIBRATION CURVE OF LEVO IN RP-HPLC METHOD

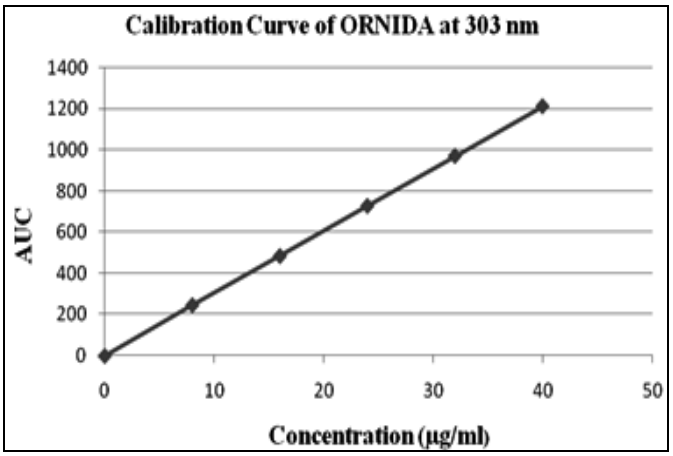


FIG. 7: CALIBRATION CURVE OF OZ IN RP-HPLC METHOD

TABLE 1: OPTICAL CHARACTERISTICS AND LINEARITY DATA OF LEVO AND OZ

Sr. No.	Parameters	UV Spectrophotometric Method		RP-HPLC Method	
		LEVO	OZ	LEVO	OZ
1	Working λ	293.5	318	303	303
2	Beer's law limit (µg/ml)	4-20	8-40	4-20	8-40
3	Correlation Coefficient (r^2)*	0.9997	0.9998	0.9998	0.9998
4	Slope (m)*	0.083	0.041	51.913	60.496
5	Intercept (c)*	-0.0325	-0.0178	-2.762	1.6097

*Average of five determination

UV Spectrophotometric Method:

Study of overlay spectra of drugs and selection of method: The spectra exhibit major absorbance maxima at 293.5 nm and 318 nm for LEVO and OZ respectively and isobestic point at 303 nm (**Fig. 2**). Due to difference in absorbance maxima and having no interference with each other so both drug can be simultaneously estimated by simultaneous equation method

Vierordt's simultaneous equation method (Method A): The wavelength 293.5 nm (λ_{max} of LEVO) and 318 nm (λ_{max} of OZ) was selected. The absorbencies of LEVO and OZ were measured at 293.5 nm and 318 nm. This method of analysis is based on the absorption of drugs X and Y at the wavelength maxima of the other. The quantification analysis of LEVO and OZ in a binary mixture was performed by using **Eqn-1** and **Eqn-2**. Where C_X and C_Y are the concentrations of LEVO and OZ respectively in the diluted sample, ax_1 and ax_2 are absorptivities of LEVO at λ_1 and λ_2, ay_1 and ay_2 are absorptivities of OZ at λ_1 and λ_2 respectively **Table 2.**

A_1 and A_2 are the absorbances of samples at the 293.5 and 318 nm respectively [23].

$$C_X = A_2ay_1 - A_1ay_2 / ax_2ay_1 - ax_1ay_2 \quad \textbf{Eqn.1}$$

$$C_Y = A_1ax_2 - A_2ax_1 / ax_2ay_1 - ax_1ay_2 \quad \textbf{Eqn.2}$$

TABLE 2: ABSORPTIVITIES OF LEVO (x) AND OZ (y) at λ_1 and λ_2

	Simultaneous Equation Method			
Drug	287 nm (λ_1)		320 nm (λ_2)	
LEVO	ax_1	0.0956	ax_2	0.0395
OZ	ay_1	00.0256	ay_2	0.0432

RP-HPLC Method:

System Suitability: The system suitability parameter was carried out to verify that the analytical system was working properly and could give accurate and precise result. The six replicates of reference standard, 16µg/ml were injected separately and chromatogram was recorded. The result of system suitability parameter is reported in the **Table 3.**

TABLE 3: RESULTS OF SYSTEM SUITSBILITY PARAMETERS

Sr. No.	PARAMETERS	LEVO (RT=2.74min)	OZ (RT=6.7min)
1	No. of Theoretical Plates	942.17 ± 14.22	873.2 ± 15.77
2	HETP	0.265 ± 0.00414	0.287 ± 0.0048
3	Tailing Factor	0.822 ± 0.136	0.867 ± 0.00599
4	Resolution	2.75	2.04

N=6

Analysis of Marketed Formulation: Twenty marketed tablets of LEVO and OZ (LOVOLKEM™-OZ, Alkem Lab.Ltd, Mumbai) were weighed and ground to a fine powder; amount equal to 25 mg of LEVO was taken in 100-ml volumetric flask. The OZ present in this amount of tablet powder was 50 mg; the ratio of all these drugs was 1:2. This was than dissolve in 25 ml of diluents by sonication for about 10 minutes. The volume is made up to the mark by diluents as per the UV Spectrophotometry method and RP-HPLC method and filtered by Whatmann filter paper (no.41) and the filtrate was used to prepare samples of different concentration. The statistical evaluation of tablet analysis by both methods has reported in **Table 4.**

TABLE 4: RESULTS AND STATISTICAL PARAMETERS FOR TABLET ANALYSIS (LOVOLKEM™-OZ**)**

S. No	Drug	Label Claim	Amount Found	MEAN*	S.D.*	%COV*	Std. Error*
UV Spectrophotometry Method	**LEVO**	250	249.00	99.6	0.426	0.427	0.174
	OZ	500	487.90	98.78	1.032	1.035	0.421
RP-HPLC	**LEVO**	250	246.41	98.56	0.699	0.701	0.286
	OZ	500	499.45	99.89	1.407	1.409	0.574

*Average of five determination

Validation Parameters: The optimized spectrophotometric and chromatographic methods were completely validated according to the procedure described in ICH guidelines Q2 (R1) for validation of analytical methods (Linearity, Accuracy, Precision and Robustness) [24].

Linearity: Linearity was studied by analyzing five standard solutions (n = 5) covering the range of 4-20 µg/ml and 8-40 µg/ml for LEVO and OZ respectively in both UV spectrophotometric and HPLC method. Calibration curves with concentration verses absorbance or peak area was plotted for each method and the obtained data were subjected to regression analysis using the least squares method. Linearity of LEVO and OZ was established by response ratios of drug. Response ratio of both drugs was calculated by dividing the absorbance or peak area with respective concentration **Table 5**.

TABLE 5: RESPONSE RATIOS OF LEVO AND OZ

S. No.	Concentration (µg/ml)		UV Spectrophotometric				RP-HPLC			
	LEVO	OZ	ABS	RR	ABS	RR	AUC	RR	AUC	RR
1.	4	8	0.381	0.095	0.338	0.042	204	51	240	30
2.	8	16	0.760	0.095	0.679	0.042	408	51	480	30
3.	12	24	1.145	0.095	1.002	0.042	612	51	720	30
4.	16	32	1.519	0.095	1.329	0.042	816	51	960	30
5.	**20**	40	1.899	0.095	1.668	0.042	1020	51	1200	30

Accuracy: The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100% and 120%. The recovery studies were carried out by adding known amount of standard solution of LEVO and OZ to reanalyzed tablet solutions. The resulting solutions were then re-analyzed by proposed methods.

In UV Spectrophotometric method, the value of mean recoveries was found to be in ranging from 98.32 to 99.28 for LEVO and 98.47 to 98.65 for OZ. The value of SD and %RSD less than 2 indicate the accuracy of method.

In RP-HPLC method, the value of mean recoveries was found to be in ranging from 98.21 to 99.93 for LEVO and 98.21 to 99.74 for OZ. Result of recovery study shown in Table 6.Total amount of drug found and percentage recovery was calculated. Result of recovery studies are reported in **Table 6.**

TABLE 6: RESULTS OF RECOVERY STUDIES ON MARKETED FORMULATIONS

Recovery Level %	% Recovery (Mean ± SD)*			
	UV Spectrophotometric method		RP-HPLC method	
	LEVO	OZ	LEVO	OZ
80	98.32 ±0.514	98.47±0.474	98.21 ± 0.89	98.21±0.409
100	99.28±0.231	98.61±0.3413	99.93±0.705	99.74±0.45
120	98.51±0.321	98.65±0.299	99.13±0.431	99.27± 0.36

*Average of five determination

Precision: Precision of the methods was studied at three levels as at repeatability, intermediate precision (Day to Day and analyst to analyst) and reproducibility **Table 7.**

Robustness: For the robustness of the analytical method we changed the ratio of hydrotropic solution. Instead the 50:50 ratios of sodium acetate and urea 60:40 sodium acetate and urea were used as solvent **Table 7**.

TABLE 7: RESULTS OF VALIDATION (MEAN±SD)

Parameter	UV Spectrophotometric method				RP-HPLC method			
Precision (Mean ± SD)*	LEVO	% RSD	OZ	%RSD	LEVO	%RSD	OZ	% RSD
Repeatability	98.15±0.092	0.094	98.91±0.172	0.175	98.87±0.231	0.234	99.1±0.40	0.48
Day to Day	98.26±0.264	0.096	98.19±0.109	0.111	98.9±0.168	0.171	98.2±0.35	0.38
Analyst to Analyst	97.99±0.115	0.115	98.26±0.134	0.136	99.24±0.136	0.140	99.73±0.24	0.25
Reproducibility	98.60±0.04	0.050	98.21±0.111	0.113	98.96±0.076	0.079	99.31±0.144	0.147
Robustness*	98.15±0.104	0.142	98.27±0.115	0.147	98.62±0.069	0.070	99.03±0.212	0.213

*Average of 5 replicate and 5 concentration.

LOD AND LOQ: Detection limit and Quantitation limit of described method were observed as 0.124µg/ml and 0.375µg/ml for LEVO and 0.122 µg/ml and 0.3702µg/ml for OZ Respectively in UV Spectrophotometric method and RP-HPLC method, based on the SD of response and slope, which meet the requirement of new method.

RESULTS AND DISCUSSIONS: The aim of the present work was to develop simple and reproducible UV-spectrophotometric and RP-HPLC method for the simultaneous determination of LEVO and OZ in solid pharmaceutical dosage forms. As the solubility of LEVO and OZ was sparingly soluble in water therefore mixture of methanol and water used (1:1, v/v) for spectrophotometry as solvent for preparation of all standard and sample solutions as easily available and cost effective, The mixture of acetonitrile and ortho-phosphoric acid in water pH 3.0 (45:55, v/v) used, which improved resolution and peak shape.

UV-Spectrophotometric Method: Based on the solubility and stability and spectral characteristics of the drugs, methanol and water used (1:1, v/v) for spectrophotometry as solvent. LEVO and OZ were show maximum absorbances at 293.5 and 318 nm respectively. LEVO and OZ follows the Beer's law in the concentration range of 4-20 µg/ml and 8-40 µg/ml respectively (r^2= 0.999 and 0.9998). Simultaneous equation method employs 293.5 and 318 nm as two analytical wavelengths, the optimized methods showed good reproducibility and recovery with ranging from 98.32 to 99.28 for LEVO and 98.47 to 98.65 for OZ. The mean percent label claims of tablet dosage by spectrophotometric method were found to be 99.6±0.426and 98.78±1.032 for LEVO and OZ respectively.

The standard deviation, coefficient of variance and standard error were obtained for LEVO and OZ was satisfactorily low. Result of precision at different level were found be within acceptable limits (RSD<2).

RP-HPLC Method: Several attempts were performed in order to get satisfactory resolution of LEVO and OZ in different mobile phases with various ratios mixture of organic phases and buffers by using C_{18} column. Initially the mobile phase used was mixture of water and methanol followed by water and acetonitrile in different ratios. The mobile phase used was acetonitrile-ammonium acetate buffer (pH 5.5) in the ratio (60:40, v/v) by isocratic elution could not give satisfactory resolution. Further acetonitrile and ortho-phosphoric acid in water (pH 3.0) in ratio of (45:55, v/v) mobile phase was used by isocratic elution shown satisfactory and good resolution at a flow rate of 1.0 mL/min.

The resolution was found reproducible and satisfactory. . LEVO and OZ follows the linearity in the concentration range of 4-20 µg/ml and 8-40 µg/ml respectively (r^2= 0.9998 and 0.9998) and chromatogram has recorded at 303nm. The value of mean recoveries was found to be in ranging from 98.21 to 99.93 for LEVO and 98.21 to 99.74 for OZ.

The mean percent label claims of tablet dosage by RP-HPLC method were found to be 98.56±0.699 and 99.89±1.407for LEVO and OZ Respectively. The standard deviation, coefficient of variance and standard error were obtained for LEVO and OZ was satisfactorily low. Result of precision at different level were found be within acceptable limits (RSD<2).

CONCLUSION: In conclusion, a RP- HPLC and simple reproducible UV-Spectrophotometric methods were developed and validated for the simultaneous determination of LEVO and OZ in solid dosage form. The advantage of UV method over HPLC method is that the proposed UV method does not require the elaborate treatment and procedures usually associated with chromatographic method. It is less time consuming and economical. A statistical comparison of the quantitative determination of LEVO and OZ shows that HPLC method as more accurate and precise than UV method.

The results indicate HPLC and UV Spectrophotometry methods are adequate methods to quantify LEVO and OZ in pure form and its dosage form. There was no interference by excipients in the tablets and the mobile phase is easy to prepare. Since these methods are simple, specific, rapid, precise and accurate, they may be successfully and conveniently adopted for routine quality control analysis of LEVO and OZ in bulk and pharmaceutical dosage form.

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