Original Article

Simultaneous Determination of Linezolid and Levamisole Hydrochloride in a Fixed Dose Combination

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ABSTRACT

Background: The combination of antibiotics with an immune-stimulant can be used for the prevention of antimicrobial resistance. Multilayer tablets of linezolid and levamisole hydrochloride can be a solution of this problem. **Objective:** Aim of the present study was to High Performance Liquid Chromatography method development and validation as per the International Council for Harmonisation guidelines for analysis of both the drugs simultaneously in a single unit dosage form. **Methods:** Ammonium acetate and acetonitrile in the ratio 65:35 (%v/v) was used as mobile phase. Ultra violet detection was carried out at 236 nm and flow rate was kept as 0.9 mL/min. **Results:** Linezolid and levamisole hydrochloride were found at 4.61 min and 6.45 min retention times, respectively. **Conclusion:** The study confirmed that the present method can be used for routine simultaneous analysis of linezolid and levamisole hydrochloride.

Key words: HPLC, Levamisole hydrochloride, Linezolid, Antibacterial resistance, Antimicrobial resistance.

INTRODUCTION

Antimicrobial resistance (AMR) has become a serious problem for a public health and it is a growing concern around the world.[1] Progressively, governments around the world have started to pay attention to a serious problem that it threatens the successes of contemporary medicine.[2] Antibacterial resistance (ABR) is an undisputable circumstance and will continue to grow in future. ABR is a main problem among various types of antimicrobial resistance.[2] It causes economic and social implications which increases the cost of treatment, longer hospitalization and leads to high rate of hospitalization.[1]

Failure to tackle drug resistant infections will lead to at least 10 million extra deaths per year and cost the global economy up to $100tn (£64tn) by 2050.[3] As per Former Goldman Sachs chief economist Jim O'Neill, in a short term scenario AMR signifies a highly confident threatening in comparison of the climate change.[3]

Indian consumes the highest amount of antibiotics in the world, but Indians consume (at 10.7 units per candidate) less than half of the amount per candidate as in the US (at 22.0 units per candidate). China is the second-largest consumer of antibiotics (at 7.5 units per candidates).[4] It has been reported that due to AMR the most populous counties such as India and China, may face 2 million and 1 million deaths a year, respectively by 2050.[3] Past guidelines are partially effective for dealing with resistance issues. Therefore, novel approaches are required to solve the issues associated with antimicrobial resistance.

To avoid the antimicrobial resistance prescriber should have optimum knowledge of general medicines, genetic host factors, microbial virulence, immune-stimulants, physicochemical properties of drugs, etc.[5] As the immune-boosting elements are used as alternatives to the antibiotics to reduce

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the resistance,[6] it is expected that the combination of antibiotics and immune-stimulant can overcome the problems of AMR. Hence in the present study we have formulated a fixed dose combination of an antibiotic (i.e., linezolid) and an immune-stimulant (i.e., levamisole hydrochloride) for the prevention of AMR.

Linezolid is an antibiotic and belongs to oxazolidinone class. The *in vitro* and *in vivo* spectrum of activity of linezolid is primarily against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), streptococci, vancomycin-resistant enterococci (VRE), penicillin-resistant *Staphylococcus pneumoniae* (PRSP), etc. It is most commonly given in the skin/soft tissue infections and pneumonia. It may be used in a variety of other infections.[7-14] But, the main problem associated with the drug is resistance.

Levamisole is one of the ideal candidates in this fixed dose combination, because of its immunomodulatory effect.[15] It has been used as an immune-stimulator to stimulate the immune system either alone or in combination with other substances in the treatment of cancer, HIV, tuberculosis, etc. and to improve weight gain also.[16,17]

In the view of above facts, we have formulated multilayer tablets of linezolid and levamisole hydrochloride. But, no method has been reported till date for the estimation of linezolid and levamisole hydrochloride simultaneously in a same combination. Aim of present study was to develop and validate an HPLC method for the simultaneously estimation of both the drugs in multilayer tablets.

MATERIALS AND METHODS

Reagents and Chemicals

Linezolid was received as a gift sample from Glenmark Generics Ltd. (Mumbai, India) and levamisole hydrochloride was also obtained as a gift sample from Aurochem Pharmaceuticals Pvt. Ltd. (Mumbai, India). Avicel PH 101, sodium starch glycolate and glacial acetic acid were purchased from SD Fine Chem Ltd. (Mumbai, India). Acetonitrile (HPLC grade) was procured from Merck Specialities Pvt. Ltd. (Mumbai, India). HPMC E5 was procured from Lobachemie Pvt. Ltd. (Mumbai, India). Croscarmellose sodium was received as gift sample from Lupin Research Park (Pune, India). Aerosil was procured from Evonik Degussa India Pvt. Ltd. (Mumbai, India). Lactose was procured from Sisco Research Laboratories (Mumbai, India). Magnesium stearate was purchased from GS Chemical (New Delhi, India). Sodium lauryl sulphate was purchased from Nice Chemicals Pvt. Ltd. (Kerala, India). Ammonium acetate was purchased from Spectrochem Pvt. Ltd. (Mumbai, India). Hydrochloric acid was procured from Suvidhinath Lab (Vadodara, India). Methanol was procured from Finar Ltd. (Ahmedabad, India). Milli Q (ultra-pure) water (more than 18.0 MΩ cm resistivity) was produced in the Pharmaceutics department research laboratory.

Instrumentation and Apparatus

Analysis was performed using HPLC (LC-2010CHT, Shimadzu Corporation, Kyoto, Japan) connected with dual wavelength UV detector, quaternary low pressure gradient pumps, high-throughput auto-sampler and column oven was used for the present study. For data acquisition, monitoring and processing output chromatographs LC software solution 5.57 release was used. The mobile phase and stock solutions were prepared with Milli Q (ultra-pure) water, which was prepared in department research laboratory using Millipore water purification system (Direct-Q® 3, Millipore Corporation, Billerica, MA, USA). For weighing of the drug and standard chemicals sensitive analytical balance (Sartorius CPA 225D, Sartorius AG, Germany) was used. The prepared buffer was passed through 0.22 μ membrane filter (Pall Pvt. Ltd., Bangalore, India) using a glass vacuum filtration assembly (Merck Millipore, Merck Life Science Pvt. Ltd., Mumbai, India) connected with rotary vacuum pump (Turbo Blower Manufacturer, Mumbai, India). The pH of buffer was measured with a pH meter (Eutech Instruments pH 510) using a glass electrode (Van London, Co., USA). Then the buffer was degassed using ultrasonic bath (Ultrasonic Cleaner-15L, Equitron – Medica Instrument Mfg. Co., Mumbai, India) to reduce the noise in baseline.

Preparation of Multilayer Tablets

As shown in Table 1, the required quantity of linezolid blend was weighed and half of its amount was filled in die cavity and pressed slightly. Then weighed amount of levamisole hydrochloride blend was filled and above it remaining half amount of linezolid blend was filled and then it was punched using manually operated tablet punching machine. Multilayer tablet was prepared to prevent separation of layers.

Preparation of Solutions

Preparation of Mobile Phase

Mobile phase used for the present study was consist of ammonium acetate (pH 4.0) and acetonitrile in the ratio 65:35 (%v/v). The pH of buffer was selected on the basis of high elution which was achieved after few trials. Ammonium acetate was used in a very low concentration (20 mM) to prevent the precipitation and

obstructions of HPLC system. The low concentration of buffer was chosen for column safety.[18,19]

Preparation of Stock Solution of Linezolid

Linezolid (10 mg) was weighed and dissolved in 1 mL of methanol and volume was made up to 10 mL with Milli Q water to obtain 1000 µg/mL of drug. The solution (2.0 mL) was taken and diluted with Milli Q water to get 80 µg/mL of drug.

Preparation of Stock Solution of Levamisole Hydrochloride

Levamisole hydrochloride (11.78 mg, equivalent to 10 mg of levamisole) was weighed and dissolved in few milli litre of Milli Q water. Then the volume was made up to 10 mL with Milli Q water to produce 1000 µg/mL of drug. The 250 µL of solution was taken and diluted with Milli Q water to produce 10 µg/mL of drug.

Preparation of Standard Solution

The 1.5 mL solution of linezolid (80 µg/mL) was mixed with 1.2 mL solution of levamisole hydrochloride (10 µg/mL) and further diluted to 10 mL with mobile phase (buffer: acetonitrile 65:35% v/v) to produce 12 µg/mL and 1.2 µg/mL for linezolid and levamisole, respectively. The mixture was injected (20 µL) into HPLC system and peak area was calculated.

Development of HPLC Method

A simultaneous method for estimation of linezolid and levamisole hydrochloride in fixed dose combination by HPLC was developed adopting following conditions such as Genesis® C_{18} column (250 mm × 4.6 mm id, 4 µm, BDS 120 Å) as a stationary phase, mobile phase as ammonium acetate (pH 4.0) and acetonitrile in the ratio 65:35 (%v/v), flow rate (0.8 mL/min), UV detector with wavelength (at isosbestic point) 236 nm, column oven temperature at 25ºC, injection volume 20 µL and run time 8 min.

Method Validation

Method is validated as per the ICH Q2(R1) guidelines.[19] The following typical parameters considered were considered for method validation.[18,20-24]

Specificity

Interference from placebo was determined at retention time of linezolid and levamisole hydrochloride using HPLC.

Linearity

The seven concentrations of each drug i.e. between 2 to 24 µg/mL for linezolid and between 0.2 to 2.4 µg/mL for levamisole were prepared. Each concentration was analysed thrice as independent samples by injecting 20 µL and the mean peak area for each concentration was calculated. Calibration curve was constructed by using observed peak area versus concentration of analyte. The coefficient of determinant (r^2) and linear regression were determined from the constructed calibration curve.[18,24]

Precision

Precision was performed as intraday and interday precision.[18,24]

i. Intraday precision

Peak area obtained from mixture of linezolid and levamisole standard solution was analysed two times in a day as six independent samples analysis at a time. Then the peak area and percent relative standard deviation (% RSD) was calculated.[18,24]

ii. Interday precision

In this study, the mixture of linezolid and levamisole standard solution was analysed in two different days as six independent samples analysis at a time. Then the peak area and percent relative standard deviation (%RSD) was calculated.[18,24]

Robustness[21,22]

i. Effect of pH of buffer

The effect of pH of buffer was assessed with variations in pH by ± 0.2 units. The standard mixture of both the drugs was analysed using the above developed HPLC method as three independent samples.

ii. Effect of flow rate

The effect of flow rate was determined with variations in flow rate by ± 0.1 mL/min. The standard mixture of both the drugs was analysed using the above developed HPLC method as three independent samples.

iii. Effect of wavelength

The effect of wavelength was assessed with variations in wavelength by ± 2.0 nm. The standard mixture of both the drugs was analysed using the above developed HPLC method as three independent samples.

Limit of detection and Limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were determined using the reported methods[18,21-24] and formulae for determination of same are as given below:[18,22,24]

$$LOD = 3.3 \times \frac{SD}{slope}$$

$$LOQ = 10 \times \frac{SD}{Slope}$$

Where, 'SD' is related to the least standard deviation value obtained in response and 'slope' is obtained from the linearity.

Application of method in estimation of linezolid and levamisole hydrochloride in fixed dose combination

Drug content in multilayer tablet was performed by the developed and validated HPLC method. Three tablets were crushed to powder and 230 mg of powder was taken in 100 mL volumetric flask. Methanol-water (1:1) mixture was added in to the flask to dissolve the powder and was sonicated and the volume was made up to 100 mL with same solvent mixture. Solution was filtered using Whatmann filter paper (no 1). The 1.0 mL of filtrate was taken and further diluted with mobile phase to produce 10 mL. The resultant solution was filtered through membrane filter with pore size 0.22 µ. This solution was injected (20 µL) in HPLC system. Peak area was recorded for both the drugs and the drug content was calculated. The drug content was calculated using the below given formula:

$$\text{Drug content} = 100 \times \frac{\text{Obtained Concentration}}{\text{Theoretical Concentration}}$$

RESULTS AND DISCUSSION

Selection of Wavelength

Wavelength of drugs was selected on the basis of isosbestic point of their wavelengths. At 236 nm both drugs showed the same absorbance (isosbestic point), hence 236 nm was selected as detection wavelength for HPLC.

Method Validation

Specificity

The developed method was used for estimation of linezolid and levamisole hydrochloride in fixed dose multilayer tablets. As shown in Figure 1, the retention times of linezolid and levamisole hydrochloride were found to be 4.61 min and 6.45 min, respectively. It confirms the method is fast and also confirms the absence of interference of one drug on the estimation of another drug.

Linearity

The calibration curves were constructed for both the drugs by plotting the obtained HPLC peak area against concentration to get the coefficient values and linear regression equations. The correlation values obtained for linezolid and levamisole hydrochloride are 0.999 and 0.998, respectively. The coefficient of determinant values are the evidence of strong relationship between the peak area and concentrations. Similarly, the linear regression equations obtained for the linezolid and levamisole hydrochloride are 27149x + 1028.5 and 43910x + 6003.4, respectively.

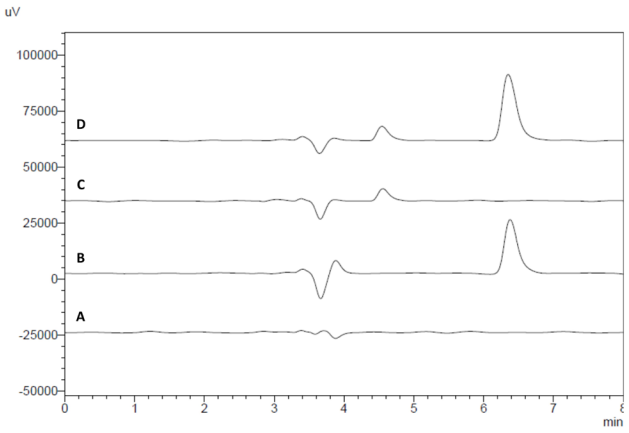

Figure 1: Chromatogram of placebo (A), levamisole hydrochloride (B), linezolid (C) and levamisole hydrochloride and linezolid in combination (D).

Precision

The percent relative standard deviation from intraday precision of linezolid and levamisole hydrochloride was found to be 0.91% and 0.94%, respectively. Similarly, percent relative standard deviation from interday precision of linezolid and levamisole hydrochloride was found to be 1.14% and 1.85%, respectively. The obtained relative standard deviation values are found to be within the range. Since as per the acceptance criteria of ICH, the relative standard deviation values for intraday and interday precisions should be less than 1% and less than 2%, respectively. The obtained results confirmed the method is precise.

Robustness

In all the deliberately altered chromatographic conditions (pH, wavelength and flow rate) the elution of drugs was remained same without any interference of impurities. The obtained percentage relative standard deviation (peak area) of both the drugs with the deliberately alterations in chromatographic conditions was found to be less than 2%. The obtained results of the percent relative standard deviations of peak area are reported in Table 2.

Limit of detection and Limit of quantitation

LOD and LOQ were determined using reported methods on signal to noise ratio.[18,21,22,24,25] LOD values of linezolid and levamizole hydrochloride were found to be 0.635 µg/mL and 0.051 µg/mL, respectively. Similarly, LOQ

Table 1: Composition of multilayer tablet		
Ingredients	**Linezolid layer (mg/tablet)**	**Levamisole hydrochloride layer (mg/tablet)**
Linezolid	600	-
Levamisole hydrochloride	-	88
Avicel PH 101	-	10
HPMC E5	-	35
Lactose	100	-
Sodium starch glycolate	30	-
Croscarmellose sodium	-	4.8
Sodium lauryl sulphate	7.3	-
Magnesium stearate	7.3	-
Aerosil	1.46	0.27

Table 2: Effect of variable parameters on mean peak area of linezolid and levamisole hydrochloride

Variable Parameters	Linezolid		Levamisole hydrochloride	
	Peak area*	%RSD	Peak area*	%RSD
pH 3.8	378579.3 ± 3722.39	0.98	81294.7 ± 612.45	0.75
pH 4.2	381862.3 ± 4821.19	1.26	68086.7 ± 612.49	0.90
Flow rate 0.7 mL/min	436734.7 ± 5222.01	1.19	82619.3 ± 1618.06	1.95
Flow rate 0.9 mL/min	354807.0 ± 1531.87	0.43	56501.0 ± 728.38	1.28
234 nm	322414.3 ± 4902.13	1.52	73293.7 ± 1425.45	1.94
238 nm	456560.7 ± 2251.2	0.49%	53167.7 ± 773.72	1.45

*Data is represented as Mean ± SD, n =3.

values of linezolid and levamizole hydrochloride were found to be 1.925 μg/mL and 0.157 μg/mL, respectively. The results evidence that the present method is sensitivity.

Application of method in estimation of linezolid and levamisole hydrochloride in fixed dose combination

The present HPLC developed method was successfully applied for the estimation of drugs present in the multilayer tablets. Samples were analysed as three independent analysis and the drug content was calculated. The drug content of linezolid and levamizole hydrochloride were found to be 103.66 ± 0.59% and 108.21 ± 1.22%, respectively. The drug content of levamisole hydrochloride was found to be more than the specifications for a finished product (i.e. ± 5%). This may be due to manual error or weight/content variations in the manufactured tablets.

CONCLUSION

Due to increase in cases of antibiotic resistance (resistance towards linezolid and vancomycin) and immune evasion by MRSA, it is postulated that combining an antibiotic and immune stimulant as a single unit dosage form to treat MRSA infection can become a novel therapeutic approach. To formulate a fixed dose combination of antibiotic and immunostimulant, linezolid was selected as antibiotic and levamisole hydrochloride was selected as immunostimulant. Chromatographic method was developed and validated for analysis of drugs in multilayer tablet. The validation results of developed HPLC method confirms that the present method is simple, rapid, specific, precise and robust. The study also concludes that HPLC method can be used for routine quality control analysis of linezolid and levamisole hydrochloride in pharmaceutical dosage forms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED

ABR: Antibacterial resistance; **AMR:** Antimicrobial resistance; **BDS:** Base Deactivated Silica; **HIV:** Human Immunodeficiency Virus; **HPLC:** High Performance Liquid Chromatography; **HPMC:** Hydroxy propyl methylcellulose; **ICH:** International Council For Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **MRSA:** Methicillin-resistant *Staphylococcus aureus*; **PRSP:** Penicillin-resistant *Staphylococcus pneumoniae;* **RSD:** Relative Standard Deviation; **SD:** Standard Deviation; **UV:** Ultra violet; **VRE:** Vancomycin-resistant enterococci.

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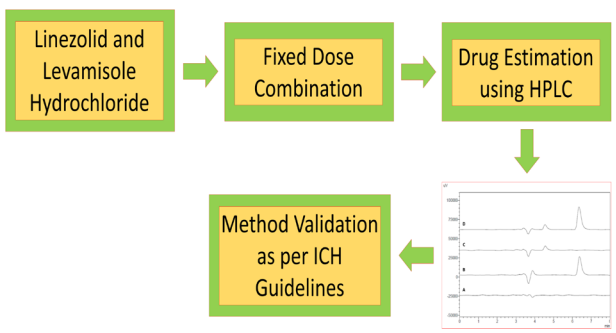
PICTORIAL ABSTRACT



SUMMARY

- Preparation of bilayer tablets of linezolid and levamisole hydrochloride.
- An HPLC method was developed and validated for estimation of linezolid and levamisole hydrochloride in fixed dose combination.
- HPLC analytical method was developed as per ICH Q2 R1 guidelines.
- Ammonium acetate and acetonitrile in the ratio 65:35 (%v/v) was used as mobile phase.
- Drugs detection was carried out at 236 nm and flow rate was kept as 0.9 mL/min.
- Linezolid and levamisole hydrochloride were found at 4.61 min and 6.45 min, respectively.
- All validation parameters were found to be within the acceptable criteria.
- The obtained results confirmed that the present method can be used for routine analysis of linezolid and levamisole hydrochloride in bulk dosage forms.

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