

## SIMULTANEOUS DETERMINATION OF LEVAMISOLE AND OXYCLOZANIDE IN THE PHARMACEUTICAL PREPARATION BY GC-MS

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### ABSTRACT

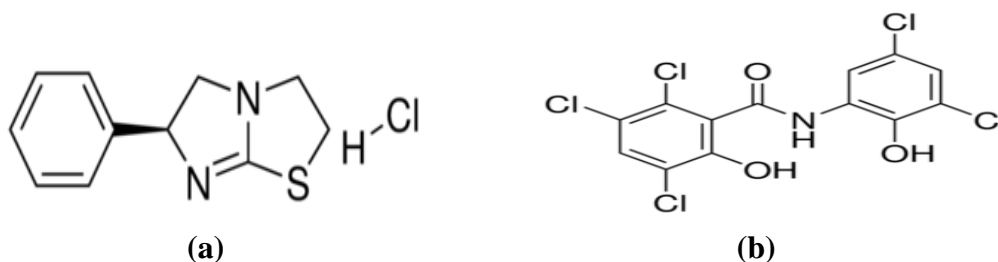
A sensitive, precise, and accurate gas chromatography-mass spectrometry method was developed for the simultaneous determination of levamisole and oxyclozanide in pure samples and pharmaceutical preparation. Gas chromatography was presented as a simple separation analytical method for the simultaneous analysis of the deliberated drugs within a shorter analytical run time. In this study, separation was achieved using a ZB5 column with (30 m × 0.53 mm, 1.50 μm), and helium as a carrier gas. The proposed method showed well separation between the drugs and each other and had good accuracy. The method showed to be linear ( $r^2 = 0.9998$ ), precise (RSD < 0.496%), accurate (recovery of 99.37% for levamisole and 100.14% for oxyclozanide), specific and robust. LOD and LOQ values were 0.935 ng mL<sup>-1</sup> and 3.085 ng mL<sup>-1</sup> respectively for levamisole and 0.884 ng mL<sup>-1</sup> and 2.917 ng mL<sup>-1</sup> respectively for oxyclozanide. The proposed method obtained well separation and had perfect accuracy. The method was validated according to ICH guidelines and carried out to determine the cited drugs in their pharmaceutical preparation.

**Keywords:** Levamisole, Oxyclozanide, Gas chromatography, Pharmaceutical preparation.

### INTRODUCTION

Levamisole, (6S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole, is an active levo-isomer of tetramisole, that is used in combination with oxyclozanide for the treatment of worm infestations (The British Pharmacopoeia 2011, Martindale 2009). Levamisole is soluble in methanol, and practically insoluble in water. It has a molecular formula of C<sub>11</sub>H<sub>12</sub>N<sub>2</sub>S, and molecular weight of 204.29 g/mol as shown in **Fig 1(a)** (The British Pharmacopoeia 2011). Levamisole is official in European Pharmacopoeia (The European Pharmacopoeia 2008), British Pharmacopoeia (The British Pharmacopoeia 2011), and United states Pharmacopoeia (The United States pharmacopoeia 2011). Oxyclozanide, 3,3',5,5',6-pentachloro-2'-hydroxy - salicylanilide, is an anthelmintic drug (The British Pharmacopoeia 2011). Oxyclozanide is freely soluble in acetone, methanol and ethanol, and practically insoluble in water. It has a molecular formula of

$C_{13}H_{6}Cl_5NO_3$ , and molecular weight of 401.45 g/mol as shown in **Fig 1(b)** (The British Pharmacopoeia 2011). Oxytoclozanide is official in British Pharmacopoeia (The British Pharmacopoeia 2011). Levamisole and oxytoclozanide combination therapy is formulated as an oral suspension, and indicated for the treatment of worm infestations in animals (Mohamed et al. 2014). Levozan<sup>®</sup> is a medication used to treat worm infestations in animals. It is approved for treatment and control of parasitic gastroenteritis, verminous bronchitis, and liver flukes infestation. There are different analytical techniques applied for the determination of levamisole in pharmaceutical formulation and in biological fluid such as titrimetric (Cao et al. 1992, Xu et al. 1997), spectrophotometric (Syed et al. 2020), HPLC (Tong et al. 2011, Cherlet et al. 2000), and GC methods (Trehy et al. 2011). There are different analytical techniques applied for the determination of oxytoclozanide in pharmaceutical preparation and in biological fluid such as titrimetric (The British Pharmacopoeia 2011), spectrophotometric (Mohamed et al. 2014, Dinc et al. 2002), HPLC (Khan et al. 2000), and GC methods (Bluethgen et al. 1982) and there are another available instrumental techniques for the determination of levamisole and oxytoclozanide as a combination in the pharmaceutical preparation, the available techniques include a spectrophotometric method, TLC, and HPLC methods (Whelan et al. 2010). The aim of the present work was to develop a validate new method for the determination of levamisole and oxytoclozanide in pure form and its pharmaceutical preparation. The developed method was validated in accordance with ICH guidelines Q2 (R1) (ICH Q2 (R1) 2005).



**Figure 1: structural formula of (a) levamisole and (b) oxytoclozanide.**

## 1. EXPERIMENTAL

### 2.1. Pure sample

Pure levamisole (Its purity was found to be  $99.48 \pm 1.061\%$  according to the official method (BP, 2011)) and oxytoclozanide (Its purity was found to be  $99.69 \pm 0.976\%$  according to the manufacturer's method) were obtained from Brihan Laboratories Pvt Ltd Bhosari, Pune, and kindly supplied by Egyptian International Center for Import (Cairo, Egypt).

### 2.2. Pharmaceutical preparation

Levozan<sup>®</sup> oral suspension (Power Vet Company, Batch number. V100503, and expire date April- 2023) imported by International Center for Import (Cairo, Egypt). Each 1ml is claimed to contain 30 mg of Levamisole and 60 mg of oxytoclozanide.

### 2.3. Chemicals and reagents

Methanol, HPLC grade (Sigma-Aldrich, Steinheim, Germany).

Double deionized Water.

Helium gas.

Phosphate buffer (25Mm, pH 7) prepared using monosodium phosphate and disodium phosphate as prescribed in USA pharmacopeia (Sigma-Aldrich, Steinheim, Germany).

Acetonitrile, HPLC grade (Sigma-Aldrich, Steinheim, Germany).

Ortho-phosphoric acid (Sigma-Aldrich, Steinheim, Germany).

N,O-Bis(trimethylsilyl)acetamide (BSA), for GC derivatization, LiChropur™, ≥ 98.5% (GC) (Sigma-Aldrich, Steinheim, Germany) ( made by the reaction of acetamide with a large excess of Chlorotrimethylsilane in the presence of Triethylamine).

Sodium decanesulfonate (30 mM) and triethyl amine were purchased from Pectrochem Pvt. Ltd., Mumbai.

### 2.4. Apparatus

The GC Perkin Elmer Clarus 500 model (Perkin Elmer Technologies, USA), Mass detector (Perkin Elmer Technologies, USA).The control of the GC system and data processing were performed using PerkinElmer TurboMass™ GC/MS software.

### 2.5. Standard solutions

Standard stock solution (200 µg mL<sup>-1</sup>) of each levamisole and oxyclozanide were prepared separately in methanol. Preparation of working solutions of levamisole and oxyclozanide in the required concentration range was adjusted by dilution of standard stock solutions with the same solvent and derivatization was performed using BSA. The stock solutions were prepared once a month, kept at 2–8 °C in a refrigerator and brought to room temperature before use.

### 2.6. Procedure

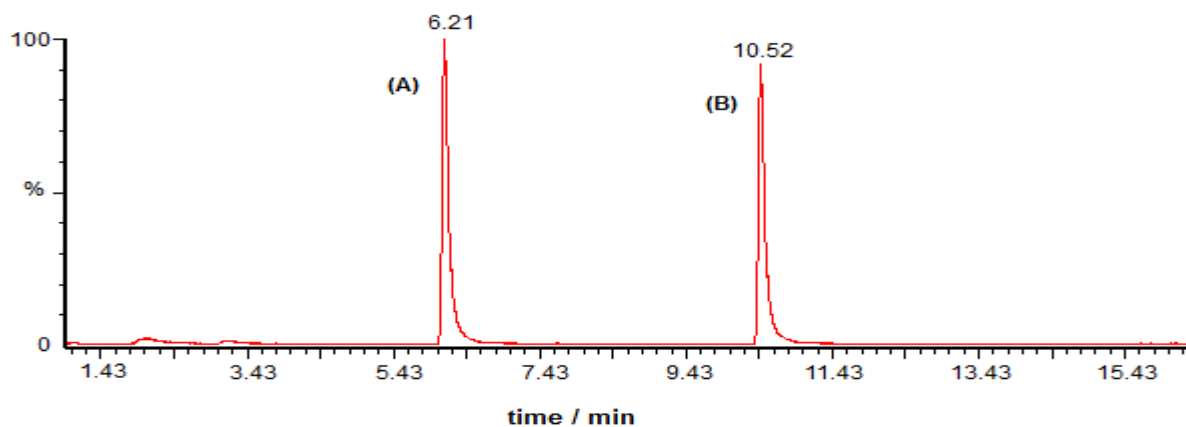
#### 2.6.1. Chromatographic condition

A Zebron ZB5 column (30m, 0.53 mm, 1.50 µm, Phenomenex, USA) was used for the chromatographic separation. Helium was applied as a carrier gas, with a flow rate of 1.5 mL min<sup>-1</sup> and an injection volume of 2 µL. The oven temperature program was as follows: initial temperature maintained at 70 °C for 1 min, raised to 270 °C at a rate of 20 °C /min, hold 4 min, then raised to 300 °C at a rate of 10 °C/min, hold 3 min, and the injector temperature was maintained at 280 °C. Methanol was used as a diluent. The mass spectrometry ionization modes were electron ionization (standard) positive/negative

chemical ionization (optional); compounds leaving the GC column are fragmented by electron impact. Selected ion monitoring (SIM) was used as an acquisition mode in order to increase the detector sensitivity of the measurement. The charged fragments are detected, and the subsequent spectrum obtained can be used for identifying the molecule. Fragmentation patterns are reproducible and can be used to produce quantitative measurements.

### 2.6.2. Construction of the calibration graph

Six different concentrations of standard solutions (4–120 ng mL<sup>-1</sup>) of both levamisole and oxyclozanide were injected separately into GC-MS system. The procedure was performed in triplicate for each concentration. The analyte response (Peak Area) obtained was plotted against the corresponding concentration of the analyte (expressed as ng mL<sup>-1</sup>).



**Figure 2. GC chromatogram of levamisole (A) (60 ng mL<sup>-1</sup>) and oxyclozanide (B) (60 ng mL<sup>-1</sup>).**

### Derivatization

Derivatization of standards and sample solutions were prepared from the dry residues (where the standard and samples solutions were diluted to obtain a solution containing 12 ng mL<sup>-1</sup> of levamisole and 24 ng mL<sup>-1</sup> of oxyclozanide, and then 250  $\mu$ L of the solution in microvial was evaporated to dryness using nitrogen) by reacting with 50  $\mu$ L BSA solution at 80 °C for 15 min in an airtight glass vial; the obtained solutions were cooled before being injected into GC.

### 2.6.3. Application to pharmaceutical preparation

Two mL of Levozan<sup>®</sup> oral suspension equivalent to 60 mg of levamisole and 120 mg of oxyclozanide, were quantitatively transferred to a 100 mL volumetric flask, and the volume was made up to 50 mL with methanol. The solution was shaken vigorously for 20 min and filtrated. The volume was completed with diluent to produce a stock solution labeled to contain 0.6 mg mL<sup>-1</sup> levamisole and 1.2 mg mL<sup>-1</sup> of oxyclozanide. This

stock solution was diluted to obtain a test sample solution containing 12 ng mL<sup>-1</sup> of levamisole and 24 ng mL<sup>-1</sup> of oxyclozanide, and then 250 µL of the test sample solution in microvial was evaporated to dryness using nitrogen followed by derivatization using BSA and then injected into GC-MS system.

## 2. RESULTS AND DISCUSSION

### 3.1. Method development

Optimization of mass spectrometry parameters and chromatographic conditions was achieved to develop and validate a selective and rapid assay method for the determination of levamisole and oxyclozanide.

The determination of levamisole and oxyclozanide was successful by applying the GC-MS method in pure samples and pharmaceutical preparations without any interference in the results or between the compounds and each other. Several mobile phases were used to identify the mentioned compounds as a mixture and the best choice was methanol. No obstacles were observed during the analysis, and it was great.

This method contributes to the determination of levamisole and oxyclozanide using a developed, accurate and rapid method, and all the results of the analyzes were within the permissible limits.

### Optimization of experimental conditions

The chromatographic separation was optimized after taking into account the resolution between the drugs and their degradation product. Helium was applied as a carrier gas. The column was performed by a flow rate of 1.5 mL min<sup>-1</sup> and an injection volume of 2 µL. Methanol was the best choice to separate the intact drugs from their degradation product. The chromatogram of the standard solution of levamisole and oxyclozanide was shown in Fig. 2.

### 3.2. Method validation

#### 3.2.1. Linearity and range

A linearity relationship was established by plotting the peak area values of the analyte versus the corresponding concentrations in ng mL<sup>-1</sup>. The regression data results were summarized in **Table 1**.

#### 3.2.2. Limit of detection and limit of quantification

LOD and LOQ were calculated according to ICH by comparing measured signals from samples with known low concentrations of the analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. The analytical parameters of the proposed methods are summarized in **Table 1**

### 3.2.3. Accuracy and precision.

Accuracy and precision were evaluated at three different concentration levels within the same day to obtain repeatability (intra-day precision) and over three different days to obtain intermediate precision (inter-day precision). The accuracy and precision were calculated and expressed in terms of percent recovery and standard deviation, respectively. All values were within the acceptance variability limits as shown in **Table1**.

### 3.2.4. Specificity

The specificity of the method was confirmed by determination of different laboratory prepared mixtures of levamisole and oxyclozanide in different ratios. The provided chromatograms revealed that levamisole and oxyclozanide were clearly completely separated from each other confirming the specificity and selectivity of the proposed method. Also the specificity was evaluated by observing possible interference from suspension excipients. This was achieved by the analysis of suspension where the recorded chromatograms did not show any additional peaks when compared to those of the synthetic mixture.

**Table 1: Regression and validation data for estimation of levamisole and oxyclozanide by the proposed method.**

Parameter	Levamisole	Oxyclozanide
Linearity range (ng mL <sup>-1</sup> )	4-120	4-120
LOD (ng mL <sup>-1</sup> )	0.935	0.884
LOQ (ng mL <sup>-1</sup> )	3.085	2.917
Regression parameter*	Y= a+ b C	Y= a+ b C
Correlation coefficient	0.9998	0.9998
Slope (b)	54189	3986.7
Intercept (a)	-29084	-1500.9
Precision (% RSD)		
Repeatability	0.288	0.296
Intermediate precision	0.374	0.383
Robustness (%RSD)		
Flow Rate (±0.1mL)	0.478	0.496
GC oven temperature (±1 °C)	0.347	0.384

\*Y= a + bC, where Y is the peak area and C is the concentration in ng mL<sup>-1</sup>.

**Table 2: Recovery study of levamisole and oxyclozanide by applying standard addition technique:**

Drug	Pharmaceutical taken (ng mL <sup>-1</sup> )	Pure added (ng mL <sup>-1</sup> )	Pure found (ng mL <sup>-1</sup> )	%Recovery
Levamisole	20	16	15.87	99.21
		20	19.92	99.58
		24	23.83	99.32
		Mean ± % RSD		99.37 ± 0.845
Oxyclozanide	20	16	16.04	100.24
		20	19.94	99.72
		24	24.11	100.46
		Mean ± % RSD		100.14 ± 0.646

**3.2.5. System suitability**

System suitability test was applied to verify that an analytical method was suitable for its intended purpose; the items measured were resolution, tailing factor, and theoretical plate and all results were observed within the acceptance range, as shown in **Table 3**.

**Table 3: System suitability test for levamisole and oxyclozanide**

Criteria	Results	
	Levamisole	Oxyclozanide
The %RSD for five replication injections of standard preparation for levamisole and oxyclozanide	0.294	0.314
Resolution	1.26	1.26
The Tailing factor	1.56	1.43
Theoretical Plates	2675	2968

**3.2.6. Robustness**

Robustness was evaluated by changing the flow rate ( $1.5 \pm 0.1$  ml min<sup>-1</sup>). The effect of GC oven temperature was studied on pure samples of the selection drugs at  $70 \pm 1^\circ\text{C}$  (optimized temperature was  $70^\circ\text{C}$ ). The measured response variances were the % RSDs as shown in **Table 1**. 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.

### 3.2.7. Application to the finished product

The proposed method was applied successfully for determination of levamisole and oxyclozanide in levozan<sup>®</sup> oral suspension. The obtained results showed absence of any interference from either excipients or additives. The results of the proposed method were compared with that of the reported method (Kemal. et al. 2020). The proposed method showed good accuracy and precision for assay of levamisole and oxyclozanide in Levozan<sup>®</sup> oral suspension and the values were listed in **Table 4**.

**Table 4: Results obtained after determination of levamisole and oxyclozanide in Levozan oral suspension and comparison with the reported method.**

Parameter	Proposed method		Reported method *	
	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide
<b>n<sup>a</sup></b>	5	5	5	5
<b>%R</b>	99.37	100.14	100.37	100.45
<b>%RSD</b>	0.845	0.646	0.848	0.800
<b>SD</b>	0.840	0.647	0.851	0.803
<b>Variance</b>	0.705	0.418	0.724	0.645
<b>Student's t-test</b>				
	1.877	0.658	—	—
<b>(2.306)<sup>b</sup></b>				
<b>F-value</b>	0.974	0.648	—	—
<b>(6.388)<sup>b</sup></b>				

<sup>a</sup>Experiments number.

<sup>b</sup>Tabulated values of “t “and “F” at (P = 0.05).

**\*Reported method: HPLC method using C18 column, mobile phase consisting of acetonitrile: methanol: 25mM phosphate buffer at pH 7.0 containing 30 mM sodium decanesulfonate and triethylamine (50:50:1 by volume) with pH adjusted to 7.0 using ortho-phosphoric acid [51:49 by volume], and using UV detector at, 220 nm (Kemal. et al. 2020).**

The proposed work shows better sensitivity than the reported method, as when using highly sensitive automated systems such as GC-MS we can get better and more accurate results (linear range, accuracy, precision, recovery, sample volume and sample preparation time) besides that it shortens time and effort.

### 3. CONCLUSION

This method is sensitive, selective and rapid, which can be applied for determination of levamisole and oxyclozanide in their pharmaceutical preparation by GC –MS. These methods have wider range with good accuracy and precision. They can be used for the routine analysis of both drugs in pharmaceutical preparation.



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### التحديد المتزامن لمادة الليفاميزول والأوكسيكلوزانيد في المستحضر الصيدلاني باستخدام

#### كروماتوجرافيا الغاز- قياس الطيف الكتلي

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تم تطوير طريقة قياس الطيف الكتلي الحساسة والدقيقة للغاز من أجل التحديد المتزامن للليفاميزول والأوكسيكلوزانيد في العينة النقية والمستحضرات الصيدلانية. تم تقديم كروماتوجرافيا الغاز كطريقة تحليلية بسيطة للفصل من أجل التحليل المتزامن للعقاقير المدروسة خلال وقت تشغيل تحليلي أقصر. في هذه الدراسة ، تم تحقيق الفصل باستخدام عمود ZB5 (30 م × 0.53 مم ، 1.50 ميكرومتر) ، والهليوم كغاز حامل. أظهرت الطريقة المقترحة فصل جيد بين الأدوية بعضها البعض بدقة جيدة. أظهرت الطريقة أنها خطية ( $r^2 = 0.9998$ ) ودقيقة ( $RSD > 0.496\%$ ) و (محكمة 99.37% للليفاميزول و 100.14% للأوكسيكلوزانيد) ومحددة وقوية. كانت قيم LOD و LOQ 0.935 نانوغرام / مل و 3.085 نانوغرام / مل على التوالي للليفاميزول و 0.884 نانوغرام / مل و 2.917 نانوغرام / مل على التوالي للأوكسيكلوزانيد. الطريقة المقترحة حصلت على فصل جيد ودقة تامة. تم التحقق من صحة الطريقة وفقاً لإرشادات ICH وتم تنفيذها لتحديد الأدوية المذكورة في مستحضراتها الصيدلانية.

**الكلمات المفتاحية :** ليفاميزول ، أوكسيكلوزانيد ، كروماتوجرافيا الغاز - قياس الطيف الكتلي، المستحضرات الصيدلانية.