

# Simultaneous Determination of Oxyclozanide and Levamisole by Spectrophotometric and Chromatographic Methods

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## ABSTRACT

Three simple, accurate and sensitive methods were developed for simultaneous determination of oxyclozanide and levamisole. Method (A) was depending on zero-order absorption spectrophotometry for measuring oxyclozanide at 300 nm and derivative ratio spectrophotometry for levamisole using oxyclozanide as a divisor, then measuring the peak amplitude at 246 nm. Method (B) was TLC method, using silica gel 60 F<sub>254</sub> plates; the optimized mobile phase was ethyl acetate/ methanol/ ammonium hydroxide 33% (8:2:0.2 by volume). The spots were scanned densitometrically at 300 nm for oxyclozanide and 220 nm for levamisole. Method (C) was an HPLC method, performed on C18 column using acetonitrile/ methanol/ 0.05M potassium dihydrogen phosphate (60:20:20 by volume), the pH was adjusted to 3.5±0.2 with ortho-phosphoric acid as a mobile phase with a flow rate of 1 ml/min. Detection was performed at 220 nm. Linearity ranges were 5 – 40 µg/ml of oxyclozanide and levamisole for method (A), 1 – 6 µg/band of oxyclozanide and 2 - 10 µg/ band of levamisole for method (B) and 0.5 – 10 µg/ml of both drugs for method (C), the mean percentage recoveries were 100.21±0.844% for oxyclozanide and 99.53±0.920% for levamisole in case of method (A), 99.72±1.348% for oxyclozanide and 99.14±1.277% for levamisole in case of method (B) and 99.81±0.852% for oxyclozanide and 100.20±0.886% for levamisole in case of method (C). The proposed methods were found to be specific for both drugs in their binary mixture. Statistical comparison between the results obtained by these methods and the manufacturer's method for oxyclozanide and the official method for levamisole was done, and no significance difference was observed.

## INTRODUCTION

Oxyclozanide, is 3,3',5,5',6-pentachloro-2' hydroxy-salicylanilide, (Fig. 1). Oxyclozanide is a pale cream or cream colored powder, very slightly soluble in water, freely soluble in acetone; soluble in ethanol (96%); slightly soluble in chloroform (The British Pharmacopoeia, 2011). It is an anthelmintic drug used in veterinary medicine for the control of fascioliasis in cattle and sheep (Martindale "The Complete Drug Reference, 2009). Literature survey reveals few analytical methods for quantification of the drug in raw material, in pharmaceutical formulations and in biological fluids including titrimetric method (The British Pharmacopoeia, 2011), spectro-photometric methods (Dinc and Kanbur, 2002; Dinc and Onur, 1997; Lakshmi and Reddy, 1998),

HPLC methods (Khan *et al.*, 2000; Van-Tonder *et al.*, 1996) and gas chromatographic method (Bluethgen, 1982). Levamisole, is (6S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole, (Fig. 2).

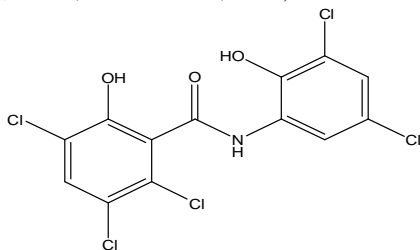
Levamisole is a white or almost white powder, slightly soluble in water, freely soluble in alcohol and in methanol (The British Pharmacopoeia, 2011). Levamisole is the active levo-isomer of tetramisole. It is used as an anthelmintic and as an adjuvant in malignant disease. It has also been tried in several conditions where its stimulant effect on the depressed immune response might be useful (Martindale "The Complete Drug Reference, 2009). The literature comprises several analytical methods for the determination of levamisole in raw material, in pharmaceutical formulations and in biological fluids including titrimetric methods (The British Pharmacopoeia, 2011; The United States pharmacopoeia, 2011; The European Pharmacopoeia, 2008; Xu, 1997; Cao *et al.*, 1992; Li, 1992; Billon *et al.*, 1985), spectrophotometric methods (Dinc *et al.*, 2009; Liang and Tao, 1992), HPLC methods (Tong *et al.*, 2011; Sari *et al.*, 2004), GC methods

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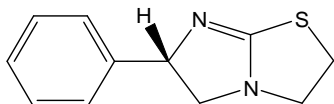
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(Schenck *et al.*, 1998; Loussouran *et al.*, 1995), electrophoretic method (Chankvetadze *et al.*, 2002) and electrochemical methods (Zhang *et al.*, 2002; Zhu and Gao, 1995).



**Fig. 1:** Oxyclozanide structure.



**Fig. 2:** Levamisole structure.

Investigation of the persistence of levamisole and oxyclozanide in milk and fate in cheese. Milk samples were analyzed by ultraperformance liquid chromatography coupled to tandem mass spectrometry (Whelan *et al.*, 2010).

This paper concerned with the development of a spectrophotometric and two chromatographic methods for the determination of oxyclozanide and levamisole in their pure powdered form, in their binary mixtures as well as in suspension forms.

The proposed methods are sensitive, fast and selective and can be easily applied for simultaneous determination of oxyclozanide and levamisole. Derivative spectrophotometric technique is of great utility for resolving some mixtures of compounds with overlapping spectra or determination of drugs in presence of their degradation products. TLC has become a routine analytical technique due to the possibility of application of several samples to be run simultaneously using a small amount of mobile phase, thus lowering the time and cost per analysis. HPLC has the advantages of its discriminating power to resolve the drug from its degradation product and its ability to operate at ambient temperature that would not contribute to the degradation of the drug.

## EXPERIMENTAL

### Equipments

HPLC system consists of an Agilent HPLC instrument, isocratic pump (Model G 1311 A pump, Agilent 1100 series), connected with an ultra violet detector (Model G1316 A, Agilent 1100 series). The injector was a manual Rheodyne injector (Model 7725/7725I, Rohnert Park, CA., USA) equipped with 20  $\mu$ l injector.

The instrument was connected to an IBM compatible personal computer (PC) and an HP disk jet 5652 printer. Densitometer CS-9301 PC dual wavelength flying spot scanning densitometer (Shimadzu). UV lamp with short wave length 254 nm (Desaga-Germany). UV-2400 PC Series Spectrophotometer

with two matched 1cm quartz cell (Shimadzu). TLC plates, 20  $\times$  20 cm aluminum plates precoated with 0.25 mm silica gel F254 (Fluka, Buchs, Switzerland).

### Reagents

Acetonitrile, methanol HPLC grade (s d fine-chem limited Mumbai, India). Ortho-phosphoric acid, disodium hydrogen phosphate, ethyl acetate, methanol, ammonium hydroxide and potassium dihydrogen phosphate (ADWIC Cairo, Egypt).

### Samples

#### Pure sample

##### Oxyclozanide

Pure sample was kindly supplied by Pharma Swede, 10<sup>th</sup> of Ramadan city, Egypt. Its purity was found to be 99.69 $\pm$ 0.976% according to the manufacturer's method

##### Levamisole

Pure sample was kindly supplied by Pharma Swede, 10<sup>th</sup> of Ramadan city, Egypt. Its purity was found to be 99.48 $\pm$ 1.061% according to the official method (BP, 2011).

### Pharmaceutical formulation

Zanide oral suspension-Batch number. V100502 imported by Middle East, Cairo, Egypt. Each 1 ml is claimed to contain 30 mg of oxyclozanide and 15 mg of Levamisole.

### Standard solutions

**Oxyclozanide stock standard solution:** (1 mg/ml) in methanol.

**Oxyclozanide working standard solution:** (0.1 mg/ml) in methanol.

**Levamisole stock standard solution:** (1 mg/ml) in methanol.

**Levamisole working standard solution:** (0.1 mg/ml) in methanol.

### Assessment of selectivity/specificity of the methods

Laboratory prepared mixtures containing different ratios of oxyclozanide and levamisole .

#### Method (A)

Aliquots of oxyclozanide and levamisole were accurately transferred from their corresponding working standard solutions (0.1 mg/ml) to prepare mixtures containing different ratios of the two drugs in methanol.

#### Method (B)

Aliquots of oxyclozanide and levamisole were accurately transferred from their corresponding stock standard solutions (1 mg/ml) to prepare mixtures containing different ratios of the two drugs in methanol.

**Method (C)**

Aliquots of oxyclozanide and levamisole were accurately transferred from their corresponding working standard solutions (0.1 mg/ml) to prepare mixtures containing different ratios of the two drugs in the mobile phase.

**Procedures****Method (A)***Linearity*

Linearity was performed by preparing solutions of concentration range (5-40 µg/ml) in methanol for both drugs from their working standard solutions (0.1 mg/ml). The spectra of the prepared solutions were scanned and stored in the computer. For oxyclozanide the zero-order absorption spectra were measured at 300 nm. A linear calibration curve was constructed relating the absorbance at 300 nm to the corresponding concentration of oxyclozanide and the corresponding regression equation was computed. While the determination of levamisole was performed by dividing the scanned spectra of the prepared solutions by the spectrum of 5 µg/ml of oxyclozanide then the first derivative of the ratio spectra <sup>1</sup>DD was obtained with  $\Delta \lambda=16$  and scaling factor 10. The peak amplitudes of the first derivative of the ratio spectra were measured at 246 nm (<sup>1</sup>DD<sub>246</sub>). A linear calibration curve was constructed relating the peak amplitude of the first derivative of the ratio spectra at 246 nm (<sup>1</sup>DD<sub>246</sub>) to the corresponding concentration of levamisole and the corresponding regression equation was computed.

*Accuracy*

The accuracy of the results was checked by applying the proposed method for the determination of different concentrations of pure oxyclozanide and levamisole within the linearity ranges. The concentrations were calculated from the corresponding regression equations. The mean recovery percentages and relative standard deviations were then calculated.

*Precision**Repeatability*

Three concentrations of oxyclozanide and levamisole (10, 20 and 30 µg/ml) were analyzed three times each, intra-day, using the previously mentioned procedures under section 2.6.1.1. The mean recovery percentages and relative standard deviations for both drugs were then calculated.

*Intermediate precision*

The above mentioned oxyclozanide and levamisole samples were analyzed on three successive days using the procedures stated under section 2.6.1.1. The mean recovery percentages and relative standard deviations for the studied drugs were then calculated.

*Assessment of selectivity/specificity of the method*

The absorption spectra of laboratory prepared mixtures containing different ratios of oxyclozanide and levamisole were

recorded. Then the procedures were completed as described in section 2.6.1.1. The concentrations were calculated using the regression equations.

*Application of the proposed method for the determination of oxyclozanide and levamisole in their pharmaceutical formulation*

The content of the Zanide oral-suspension bottle was shaken well, then 1 ml equivalent to 30 mg oxyclozanide and 15 mg levamisole was quantitatively transferred into a beaker, 50 ml methanol was added. The beaker was covered with watch glass and the solution was stirred for 30 minutes using a magnetic stirrer, filtered, the residue was washed three times each with 10 ml methanol and filtered, the collected filtrates were accurately transferred to prepare a solution of approx. 0.3 mg/ml of oxyclozanide and 0.15 mg/ml of levamisole in methanol. Suitable dilutions were made with the mobile phase to prepare a solution of approx. 30 µg/ml of oxyclozanide and 15 µg/ml of levamisole in methanol. Then the procedure was completed as described in section 2.6.1.1. The concentration of oxyclozanide and levamisole were calculated using the corresponding regression equations.

**Method (B)***Linearity*

Linearity was performed by preparing solutions of concentration range (100-600 µg/ml) for oxyclozanide and (200-1000 µg/ml) for levamisole in methanol from their stock standard solutions (1 mg/ml). Ten µl of the prepared solutions, using 10 µl Hamilton syringe, was applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase [ethyl acetate/ methanol/ 33% ammonium hydroxide (8:2:0.2 by volume) for one hour in ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the following conditions:

- Source of radiation: deuterium lamp.
- Photomode: Reflection.
- Scan mode: Zigzag.
- Result output: Chromatogram and area under the peak.
- Swing width: 10 mm.
- Wavelength: 300 nm for oxyclozanide and 220 nm for levamisole.

The scanning profiles for oxyclozanide and levamisole were obtained. The calibration curves relating the integrated area under the peak to the corresponding concentrations of the two drugs were constructed and the regression equations were computed.

*Accuracy*

The accuracy of the results was checked by applying the proposed method for the determination of different concentrations of pure oxyclozanide and levamisole within the linearity ranges. The concentrations were calculated from the corresponding regression equations. The mean recovery percentages and relative standard deviations were then calculated.

### Precision

#### Repeatability

Three concentrations of oxyclozanide and levamisole stock standard solutions (1, 3 and 5 µg/ band) and (4, 6, 8 µg /band) respectively were analyzed three times each, intra-day, using the previously mentioned procedure under section 2.6.2.1. The mean recovery percentages and relative standard deviations for both drugs were then calculated.

#### Intermediate precision

The above mentioned oxyclozanide and levamisole samples were analyzed on three successive days using the procedure stated under section 2.6.2.1. The mean recovery percentages and relative standard deviations for the studied drugs were then calculated.

#### Assessment of selectivity/specificity of the method

Ten µl from the prepared mixtures was spotted on TLC plates and the procedure was completed as described in section 2.6.2.1. The concentrations were calculated using the regression equations.

#### Application of the proposed method for the determination of oxyclozanide and levamisole in their pharmaceutical formulation

The content of the Zanide Oral-suspension bottle was shaken well, then 20 ml equivalent to 600 mg oxyclozanide and 300 mg levamisole was quantitatively transferred into a beaker, 50 ml methanol was added. The beaker was covered with watch glass and the solution was stirred for 30 minutes using a magnetic stirrer, filtered, the residue was washed three times each with 10 ml methanol and filtered, the collected filtrates were accurately transferred to prepare a solution of approx. 6 mg/ml of oxyclozanide and 3 mg/ml of levamisole in methanol. Suitable dilutions were made to prepare a solution of approx. 600 µg/ml of oxyclozanide and 300 µg/ml of levamisole in methanol. Then the procedure was completed as described in section 2.6.2.1. The concentration of oxyclozanide and levamisole were calculated using the corresponding regression equations.

### Method (C)

#### Linearity

Linearity was performed by preparing solutions of concentration range (0.5-10 µg/ml) for both drugs in the mobile phase from their working standard solutions (0.1 mg/ml). 20 µl of the previously prepared solutions was injected in triplicate using the following chromatographic conditions:

- Column: X Bridge, size 150×4.6 mm, C18, particle size 3.5µm. The column was equilibrated with the mobile phase until steady baseline obtained and column pressure was stabilized.
- Mobile phase: acetonitrile: methanol: 0.05M potassium dihydrogen phosphate in ratio (60:20:20 by volume) respectively, pH of the mobile phase was adjusted to

3.5±0.2 by ortho-phosphoric acid. The mobile phase was filtered using 0.45 µm Teflon membrane filters and degassed by ultrasonic vibrations for 30 min.

- Temperature: Ambient temperature.
- Flow rate: 1 ml/ min.
- Detector wavelength: 220 nm.
- Injection volume: 20 µl.

The chromatogram was obtained, the average peak area ratios obtained for each concentration of oxyclozanide and levamisole to that of external standard 10µg/ml were plotted versus the corresponding concentrations, and the regression equations was computed.

#### Accuracy

The accuracy of the results was checked by applying the previously mentioned procedure under linearity for different concentrations of pure oxyclozanide and levamisole within the linearity ranges. The concentrations of the drugs were calculated from the corresponding regression equations. The mean recovery percentages and relative standard deviations were then calculated.

### Precision

#### Repeatability

Three concentrations of oxyclozanide and levamisole (4, 6 and 8 µg/ml) were analyzed three times each, intra-day, using the previously mentioned procedures under section 2.6.3.1. The relative standard deviations for both drugs were then calculated.

#### Intermediate precision

The above mentioned oxyclozanide and levamisole samples were analyzed on three successive days using the procedures stated under section 2.6.3.1. The mean recovery percentages and relative standard deviations for the studied drugs were then calculated.

#### Assessment of selectivity/specificity of the method

20 µl from the prepared mixtures containing different ratios of oxyclozanide and levamisole was injected. Then the procedures were completed as described in section 2.6.3.1. The concentrations were calculated using the regression equations.

#### Application of the proposed method for the determination of oxyclozanide and levamisole in its pharmaceutical formulation

Two mls from the solution prepared (approx. 0.3 mg/ml of oxyclozanide and 0.15 mg/ml of levamisole) as described in section 2.6.1.5. were accurately transferred to prepare solution of approx. 6 µg/ml of oxyclozanide and 3 µg/ml of levamisole in the mobile phase.

Then the procedure was completed as described in section 2.6.3.1. The concentrations of oxyclozanide and levamisole were calculated using the corresponding regression equations.

### System suitability

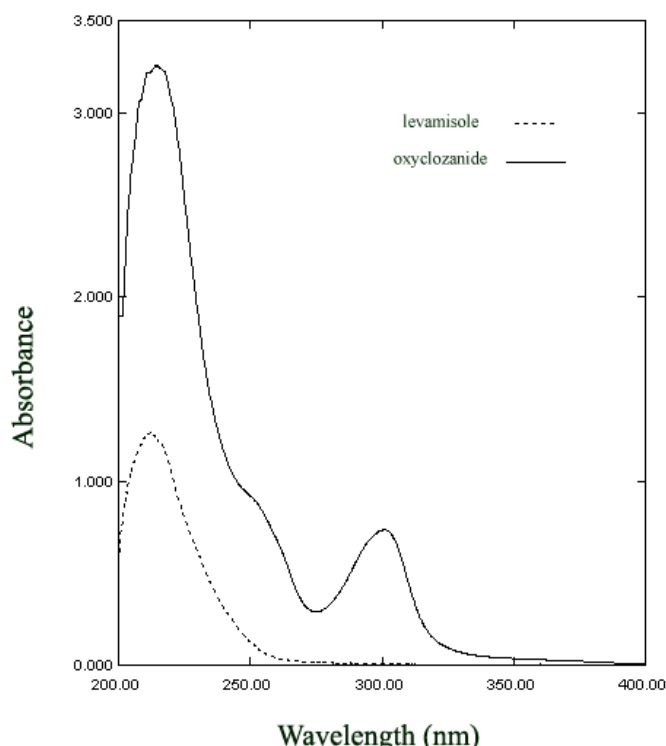
The tailing factor, the resolution factor, the selectivity factor, the theoretical plate count and the height equivalent to a theoretical plate (HETP) were calculated for each drug (USP 2011).

## RESULTS AND DISCUSSION

### Method (A)

This present work is concerned with the use of zero order spectrophotometry and first derivative of ratio <sup>1</sup>DD techniques for the quantitative determination of oxyclozanide and levamisole; respectively in the pure forms, in their binary mixtures as well as in pharmaceutical formulation.

The zero-order absorption spectra of oxyclozanide show no interference from levamisole, which allows the determination of oxyclozanide by measuring its zero-order spectra at 300nm, (Fig. 3).



**Figure 3:** Zero order spectra of oxyclozanide (30 µg/ml) and levamisole (15 µg/ml) in methanol.

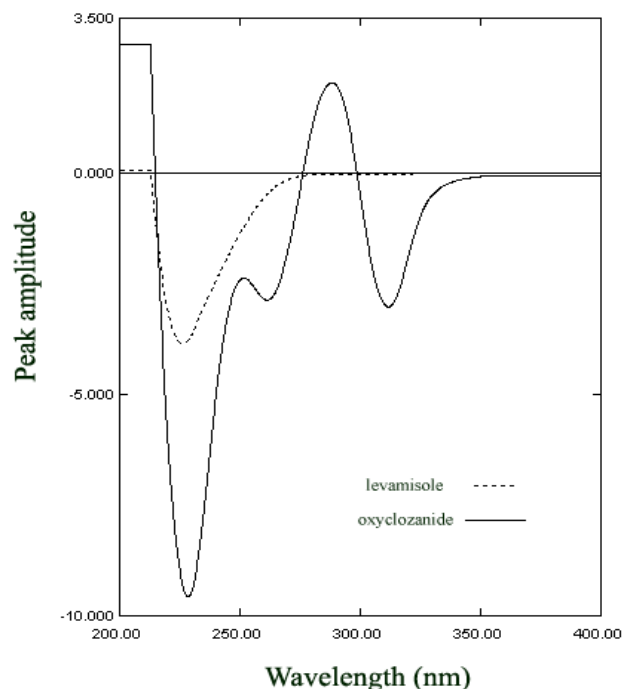
Linearity was studied and calibration curve was constructed relating the absorbance at 300 nm to the corresponding concentration of oxyclozanide. The proposed method was found to be valid in the range of (5-40 µg/ml) of oxyclozanide. The regression equation was computed and found to be

$$A = 0.0249 C + 0.0053 \quad r = 0.9995$$

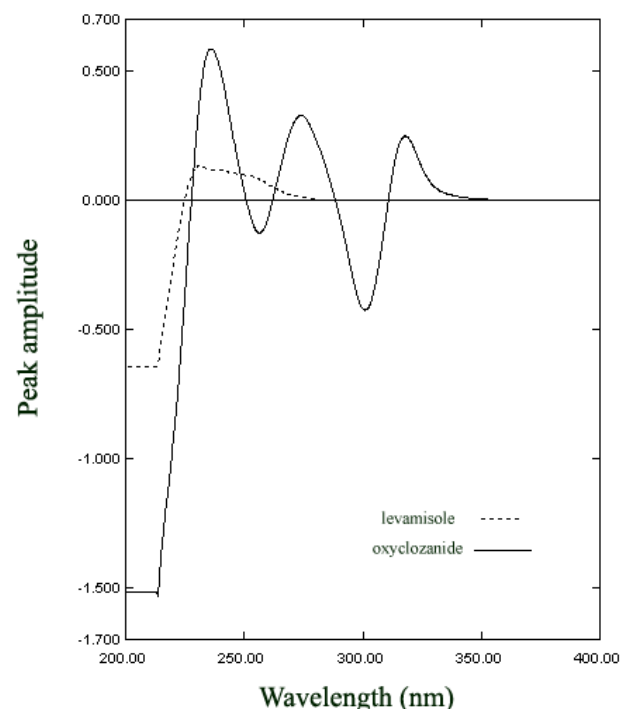
Where A is the absorbance, C is the concentration (µg/ml) and r is the correlation coefficient.

Levamisole shows great overlap from oxyclozanide that prevent the direct determination of levamisole, (Fig. 3). In an attempt to

resolve this overlap, derivative spectrophotometric methods were tried and it was found that first, second, third and fourth derivative failed to solve this overlapping, (Fig. 4-7). Derivative ratio method was applied; the spectra of levamisole were divided by the spectrum of (5 µg/ml) of oxyclozanide. Upon examining the first derivative of ratio spectra of levamisole, (Fig. 8), it is noticed that levamisole can be determined at 246 nm.



**Fig. 4:** First derivative spectra of oxyclozanide (30 µg/ml) and levamisole (15 µg/ml) in methanol.



**Fig. 5:** Second derivative spectra of oxyclozanide (30 µg/ml) and levamisole (15 µg/ml) in methanol.

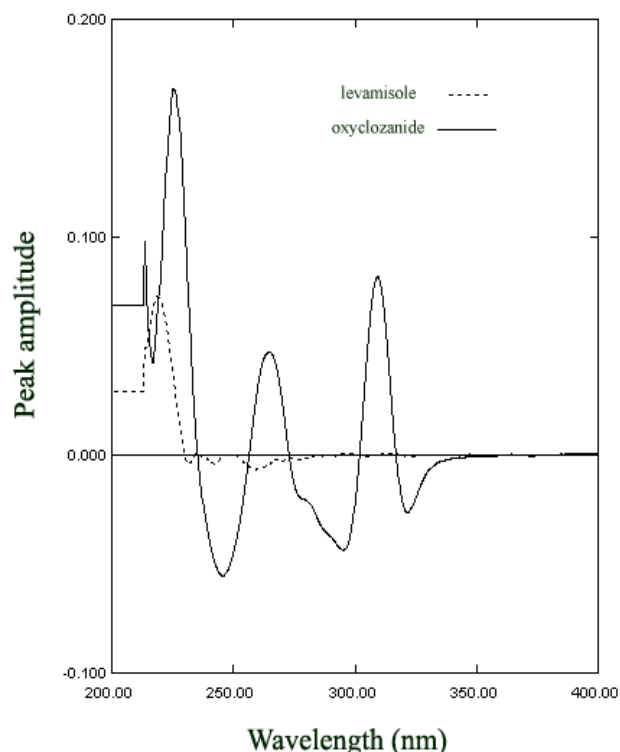


Fig. 6: Third derivative spectra of oxyclozanide (30 µg/ml) and levamisole (15 µg/ml) in methanol.

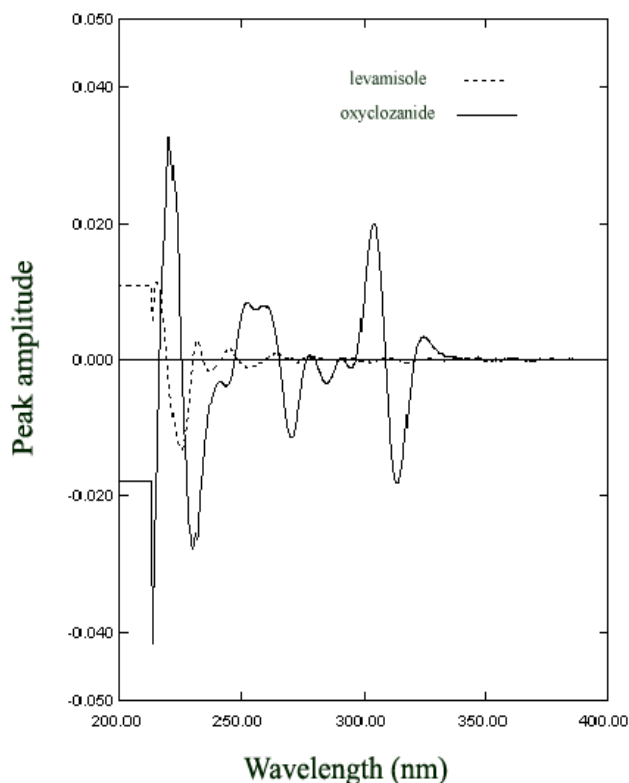


Fig. 7: Fourth derivative spectra of oxyclozanide (30 µg/ml) and levamisole (15 µg/ml) in methanol.

The main parameters that affect the shape of the derivative ratio spectra such as scanning speed and the wavelength increment over which the derivative is obtained ( $\Delta\lambda$ ) were studied

and it was found that fast scanning speed,  $\Delta\lambda = 16$  and scaling factor 10 gave best compromise in terms of signals to noise ratio, peak resolution and sensitivity throughout the determination. Careful choice of the concentration of the divisor was of great importance, so different concentrations of oxyclozanide were tried as a divisor. It was found that upon division by (5 µg/ml), best compromise in terms of sensitivity, repeatability and signals to noise ratio spectra were obtained.

Linearity was studied and the calibration curve was constructed relating the peak amplitude at 246 nm to the corresponding levamisole concentration. The proposed method was found to be valid in the range of (5-40 µg/ml). The regression equation was computed and found to be:

$${}^1DD_{246} = 0.0321 C + 0.0088 \quad r = 0.9997$$

Where  ${}^1DD_{246}$  is the peak amplitude of the first derivative of ratio spectra and C is the concentration (µg/ml) and r is the correlation coefficient.

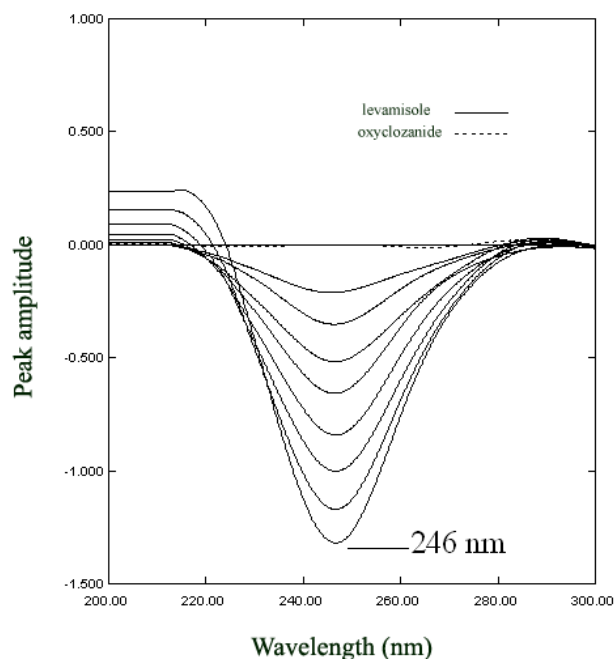


Fig. 8: First derivative of ratio spectra of levamisole (5-40 µg/ml), first derivative of ratio spectrum of oxyclozanide (15 µg/ml) using the spectrum of (5 µg/ml) of oxyclozanide as a divisor.

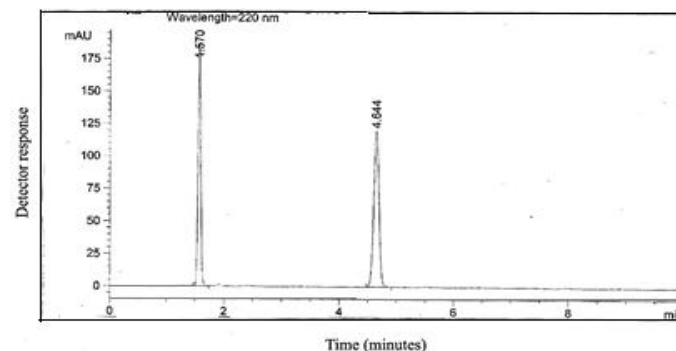


Fig. 9: HPLC chromatogram of a resolved mixture of oxyclozanide (6 µg/ml),  $R_t = 4.644$  and levamisole (6 µg/ml),  $R_t = 1.570$  using the specified chromatographic conditions.

The proposed method was successfully applied for the determination of the drugs in pure powder forms with mean percentage recovery of  $100.21 \pm 0.844\%$  for oxyclozanide and  $99.53 \pm 0.920\%$  for levamisole, (Table 1).

#### Method (B)

The proposed method is based on the difference in the  $R_f$  between the two drugs. Trials were carried out using different solvent systems to have a good separation of the two drugs, including methanol: n-butanol: 33% ammonium hydroxide (6:4:0.2 by volume) and methanol: toluene: 33% ammonium hydroxide (9:1:0.1 by volume), but no separation occurred. By using methanol: chloroform: 33% ammonium hydroxide (6:3:0.5 by volume) poor separation was observed. The use of ethyl acetate: methanol: 33% ammonium hydroxide solution (8:1: 0.2 by volume) resulted in low  $R_f$  value of oxyclozanide, while upon adjusting the ratio to (8:2: 0.2 by volume), good separation of the two drugs without tailing was obtained. The  $R_f$  values were found to be 0.43 for oxyclozanide and 0.73 for levamisole. The selected mobile phase allows the determination of the two drugs without interference from each other and without tailing of the separated spots providing better precision of the method.

Linear relationships were obtained between the integrated area under the peak and the corresponding concentration of oxyclozanide and levamisole in the range of 1-6  $\mu\text{g/spot}$  and 2-10  $\mu\text{g/spot}$ , respectively. The regression equations were computed and found to be:

$$A_1 = 0.0912 C_1 - 0.0338 \quad r = 0.9993 \quad \text{for oxyclozanide}$$

$$A_2 = 0.0275 C_2 + 0.0162 \quad r = 0.9993 \quad \text{for levamisole}$$

Where  $A_{1,2}$  are the integrated area under the peak for oxyclozanide and levamisole respectively,  $C_{1,2}$  are their corresponding concentrations ( $\mu\text{g/spot}$ ) and  $r$  is the correlation coefficient.

The proposed method was successfully applied for the determination of the two drugs in pure powder form with mean percentage recoveries of  $99.72 \pm 1.348\%$  and  $99.14 \pm 1.277\%$  for oxyclozanide and levamisole respectively, (Table 1).

#### Method (C)

High-performance liquid chromatographic method is described for the determination of oxyclozanide and levamisole; in the pure forms, in their binary mixture as well as in pharmaceutical formulation.

To optimize the proposed HPLC method, all the experimental conditions were investigated. Different mobile phases with different ratios were tried such as methanol: water in ratios (80:20 v/v) and (60:40 v/v) and also acetonitrile: methanol: 0.01M potassium dihydrogen phosphate in ratio (60:30:10 by volume), separation occurred but levamisole gave tailed peaks. Satisfactory separation was performed with a mobile phase consisting of acetonitrile: methanol: 0.05M potassium dihydrogen phosphate in ratio (60:20:20 by volume), pH was adjusted to  $3 \pm 0.2$  with ortho-phosphoric acid, with a flow rate of 1 ml/min and a retention time of  $4.644 \pm 0.03$  min for oxyclozanide and  $1.570 \pm$

0.03 min for levamisole, (Fig. 9). A linear relationships were obtained between the peak area ratio at the selected wavelength (220 nm) and the corresponding concentration of oxyclozanide and levamisole in the range of 0.5 – 10  $\mu\text{g/ml}$  for the two drugs. The regression equations were computed and found to be:

$$A_1 = 0.1005 C_1 - 0.0195 \quad r = 0.9995 \quad \text{for oxyclozanide}$$

$$A_2 = 0.0989 C_2 + 0.0036 \quad r = 0.9995 \quad \text{for levamisole}$$

Where  $A_{1,2}$  are the peak area ratio of oxyclozanide and levamisole respectively,  $C_{1,2}$  are their corresponding concentrations ( $\mu\text{g/ml}$ ) and  $r$  is the correlation coefficient.

The proposed method was successfully applied for the determination of the two drugs in pure powder forms with mean percentage recoveries of  $99.81 \pm 0.852\%$  and  $100.20 \pm 0.886\%$  for oxyclozanide and levamisole respectively, (Table 1).

System suitability test according to the United States Pharmacopoeia (The United States pharmacopeia, 2011) was used to verify that the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done. Accordingly, system suitability was checked by calculating the column efficiency (N), resolution (R), selectivity ( $\alpha$ ) and tailing factor (T), where the system was found to be suitable, (Table 2).

The specificity of the methods was proven by the analysis of laboratory prepared mixtures containing different ratios of both drugs (Table 3).

The usefulness of the proposed methods for the analysis of oxyclozanide and levamisole was studied by assaying Zanide oral suspension, (Table 4). Samples were also spiked in order to assess the validity of the proposed methods (Table 4).

Results obtained by the proposed method for the determination of pure samples of the drug were statistically compared to those obtained by the manufacturer's method (Middle East Company) for oxyclozanide and the official method (The British Pharmacopoeia, 2011) for levamisole and no significant differences were observed, (Table 5).

The accuracies were assessed by the determination of pure samples within the linearity ranges, the mean accuracies are given in Table (1).

The repeatability and interday precision were evaluated by assaying three freshly prepared solutions of both drugs in triplicate on the same day and on three successive days respectively at concentrations within the linearity ranges for each method. RSD% shows the precision of the methods, (Table 1).

Validation of the proposed methods was made by measuring range, accuracy, precision, repeatability, interday precision, linearity and specificity. Results obtained are depicted in Table (1).

This data render the applicability of the proposed method for the quality control of the drug formulation. The ICH guidelines (ICH guidelines, 2005) were followed throughout the study for methods validation.

**Table 1:** Results of validation parameters of the responses and the regression equations obtained by the proposed methods.

Parameters	Method (A)		Method (B)		Method (C)	
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
Slope <sup>a</sup>	0.0249	0.0321	0.0912	0.0275	0.1005	0.0989
S.E. of slope	0.000314	0.000335	0.00167	0.000612	0.001544	0.001527
Intercept <sup>a</sup>	0.0053	0.0088	-0.0338	0.0162	-0.0195	0.0036
S.E. of intercept	0.007939	0.008447	0.006503	0.004062	0.009357	0.009251
Correlation coefficient	0.9995	0.9997	0.9993	0.9993	0.9995	0.9995
Concentration range	5 – 40 µg/ml	5 – 40 µg/ml	1-6 µg/band	2-10 µg/band	0.5-10 µg/ml	0.5-10 µg/ml
Average accuracy (%)	100.21	99.53	99.72	99.14	99.81	100.20
S.D.	0.846	0.916	1.344	1.266	0.850	0.888
R.S.D. %	0.844	0.920	1.348	1.277	0.852	0.886
Specificity± R.S.D.	100.43±0.332	99.56±1.865	99.87±0.773	100.98±0.107	100.31±1.513	98.63±0.609
Repeatability <sup>b</sup> % ± R.S.D.	0.723	0.490	0.803	0.621	0.635	0.617
Intermediate precision <sup>c</sup> % ± R.S.D.	0.182	0.208	0.412	0.633	0.773	0.419

<sup>a</sup> Results of five determinations<sup>b</sup> n = 3×3<sup>c</sup> n = 3×3**Table 2:** Parameters of system suitability test of method (C).

Parameter	Obtained value
Relative retention time	Oxyclozanide 2.96 Levamisole 24.57
Resolution (R)	24.57
Capacity factor (K)	9.21
Tailing factor (T)	0.96
Column efficiency (N)	12680
HETP	0.0012 cm/plate

**Table 3:** Results of analysis of oxyclozanide and levamisole in laboratory prepared mixtures containing different ratios of both drugs in pure powder form by the proposed methods.

Ratio of oxyclozanide and levamisole	Method (A)				Method (B)				Method (C)			
	Concentration (µg/ml)		Recovery %		Concentration (µg/ band)		Recovery %		Concentration (µg/ml)		Recovery %	
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
2:1	30	15	100.05	98.16	4	2	99.47	101.06	6	3	98.56	99.32
1:1	30	30	100.58	101.67	4	4	100.76	100.86	6	6	101.07	98.20
1:2	15	30	100.55	98.86	2	4	99.38	101.01	3	6	101.30	98.38
Mean			100.43	99.56			99.87	100.98			100.31	98.63
S.D.			0.333	1.857			0.772	0.108			1.518	0.601
R.S.D. %			0.332	1.865			0.773	0.107			1.513	0.609

**Table 4:** Quantitative determination of oxyclozanide and levamisole in pharmaceutical formulation by the proposed methods and results of application of standard addition technique.

Zanide oral suspension B.N V100502	Method (A)							
	Found % <sup>a</sup>		Claimed amount taken (µg/ml)		Standard added (µg/ml)		Recovery % <sup>b</sup> of added	
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
	100.44	101.22	30	15	5	15	101.07	100.20
	99.61	101.02	30	15	7	20	99.55	99.69
	100.05	99.59	30	15		25	100.30	99.01
Mean	100.03	100.61					100.31	99.64
S.D.	0.415	0.890					0.758	0.597
R.S.D. %	0.415	0.884					0.756	0.599
Zanide oral suspension B.N V100502	Method (B)							
	Found % <sup>a</sup>		Claimed amount taken (µg/band)		Standard added (µg/band)		Recovery % <sup>b</sup> of added	
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
	99.50	100.40	3	3	1	3	98.80	98.50
	99.70	99.93	3	3	2	5	98.69	98.79
	99.56	98.26	3	3	3	7	100.39	100.38
Mean	99.25	99.53					99.29	99.22
S.D.	0.656	1.125					0.949	1.011
R.S.D. %	0.661	1.130					0.956	1.019



Table 4: continued .....

	Method (C)							
	Found % <sup>a</sup>		Claimed amount taken (µg/ml)		Standard added (µg/ml)		Recovery % <sup>b</sup> of added	
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
Zanide oral suspension B.N V100502	99.43	100.36	6	3	1	4	99.71	100.56
	98.12	99.84	6	3	2	5	100.79	99.79
	99.49	99.51	6	3	3	6	99.70	101.02
	99.15	99.24	6	3	4	7	98.26	100.51
Mean	99.05	99.74					99.62	100.47
S.D.	0.636	0.482					1.041	0.511
R.S.D. %	0.642	0.483					1.045	0.509

<sup>a</sup> Average of six determinations<sup>b</sup> Average of six determinationsTable 5: Statistical comparison of the results obtained by the proposed methods and the manufacturer's method<sup>[40]</sup> or the official method<sup>[11]</sup> for determination of oxyclozanide and levamisole respectively in pure powder forms.

Item	Method (A)		Method (B)		Method (C)		Manufacturer's method*	Official method**
	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole	Oxyclozanide	Levamisole
Mean	100.21	99.53	99.72	99.14	99.81	100.20	99.69	99.48
S.D.	0.846	0.916	1.344	1.266	0.850	0.888	0.973	1.055
R.S.D%	0.844	0.920	1.348	1.277	0.852	0.886	0.976	1.061
Variance	0.716	0.839	1.806	1.603	0.723	0.789	0.947	1.113
n	8	8	6	5	6	6	5	5
Student's t	1.020(2.201)	0.091(2.201)	0.041(2.262)	0.462(2.306)	0.218(2.262)	1.231(2.262)		
F test	1.323(4.88)	1.327(4.88)	1.907(5.05)	1.440(5.19)	1.310(5.19)	1.411(5.19)		

Figures in parentheses are the corresponding tabulated values at p = 0.05

\* HPLC method using C18 column, mobile phase methanol: water (80:20 v/v) and UV detection at 282 nm.

\*\* Non-aqueous titration method using 0.1 M perchloric acid as a titrant and naphtholbenzein as indicator.

## CONCLUSION

The proposed derivative ratio spectrophotometric, TLC and HPLC methods are simple, precise, accurate, and sensitive. These do not require any tedious extraction procedure. These methods have wider range with good accuracy and precision. They can be used for the routine analysis of both drugs in pharmaceutical formulations.

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