SIMULTANEOUS DETERMINATION OF LEVAMISOLE AND OXYCLOZANIDE IN THE PHARMACEUTICAL PREPARATION BY CAPILLARY ELECTROPHORESIS

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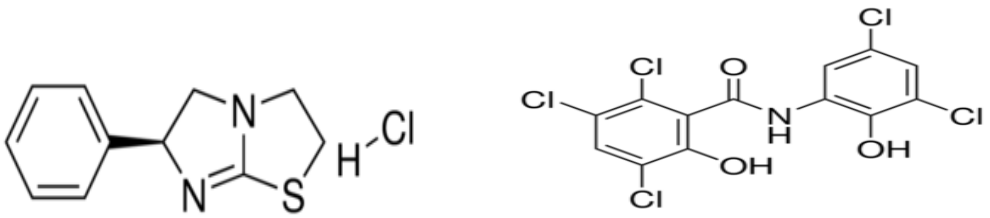
ABSTRACT

A sensitive, precise and accurate capillary electrophoresis method was developed for simultaneous determination of levamisole and oxyclozanide in pure sample and pharmaceutical preparation. Capillary electrophoresis 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 on fused silica capillary (30 cm - 50 µm internal diameter); background electrolyte solution consisted of phosphate buffer (40 mM, pH 7.9) and UV detection at 227 nm. The method showed to be linear (r^2 > 0.9998), precise (RSD <0.193%), accurate (recovery of 99.95% for levamisole and 100.12% for oxyclozanide), specific and robust. LOD and LOQ values were 0.099 µg mL^{-1} and 0.299 µg mL^{-1} respectively for levamisole and 0.075 µg mL^{-1} and 0.228 µg mL^{-1} respectively for oxyclozanide. The proposed method obtained well separation and had a perfect accuracy. The method was validated according to ICH guidelines and carried out for determination of the cited drugs in their pharmaceutical preparation.

KEYWORDS : Levamisole, Oxyclozanide, Capillary electrophoresis, Pharmaceutical preparation.

1. INTRODUCTION

Levamisole,(6S)-6-Phenyl-2,3,5,6-terahydroimidazo[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 $C_{11}H_{12}N_2S$, 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 pharmacopeia 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 $C_{13}H_6Cl_5NO_3$, and molecular weight of 401.45 g/mol as shown in **Fig 1(b)** (The British Pharmacopoeia 2011). Oxyclozanide is official in British Pharmacopoeia (The British Pharmacopoeia 2011). Levamisole and oxyclozanide combination therapy is formulated as an oral suspension, and indicated for the treatment of worm infestations in animals (Mohamed et al. 2014). Levozan® 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 oxyclozanide 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 oxyclozanide 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 and validate a new method for determination of levamisole and oxyclozanide in pure form and its pharmaceutical preparation. The developed method was validated in accordance with ICH guidelines Q2 (R1) (ICH Q2 (R1) 2005).



(a) **(b)**

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

2. EXPERIMENTAL

2.1. Pure sample

Pure levamisole (certified to contain 99.48%) and oxyclozanide (certified to contain 99.69%) were kindly supplied by Egyptian International Center for Import (Cairo, Egypt).

2.2. Pharmaceutical preparation

Levozan® oral suspension (Power Vet Company) imported by International Center for Import (Cairo, Egypt). Each 1ml is claimed to contain 30 mg of Levamisole and 60 mg of oxyclozanide.

2.3. Chemicals and reagents

Sodium hydroxide pellets (Sigma-Aldrich, Steinheim, Germany), prepared as 0.2N and 0.1N NaOH aqueous solutions.

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

Deionized Water.

Phosphate buffer (40 mM, PH 7.9) prepared using monosodium phosphate and disodium phosphate as prescribed in USA pharmacopeia.

2.4. Apparatus

Agilent Capillary Electrophoresis 7100 System (Agilent Technologies, Germany) equipped with UV-Visible diode-array detector (190–600 nm), and Agilent chem. station software was used for data analysis.

2.5. Standard solutions

Standard stock solution (100 µg mL^{-1}) of levamisole and (100 µg mL^{-1}) of oxyclozanide were prepared separately in a solvent containing methanol and phosphate buffer PH 7.9 (10:90 v/v). 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.

2.6. Procedure

2.6.1. Conditions for separation

- A bare fused silica capillary was obtained from Agilent Technologies and had the following dimensions 30 cm effective length, 50 µm internal diameter, and 365 µm outer diameter.
- The temperature of the capillary and the samples was maintained at 25 °C.

- The background electrolyte solution consisted of phosphate buffer (40 mM, pH 7.9).
- Samples were injected into the capillary by pressure at the anodic side at 10 m bar for 5 s.
- The electrophoresis was carried out by applying 25 kV to the capillary, with the cathode being at the detector end.
- Prior to its first use, the capillary was washed at 20 psi for 20 min. with 0.1 M sodium hydroxide solution, and then flushed with water for 5 min.
- The capillary was washed between runs with deionized water for 5 min then equilibrated with the running buffer for 5 min to ensure reproducibility of the assay.
- UV detection was carried out at 227 nm.

2.6.2. Construction of the calibration graph

Six different concentrations of standard solutions (4–40 µg mL^{-1}) of both levamisole and oxyclozanide were injected separately into the capillary electrophoresis 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 µg mL^{-1}).

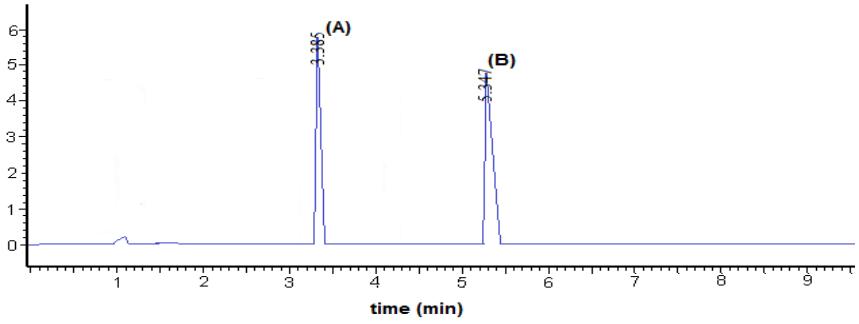


Figure 2. Electropherogram of levamisole (A) (24 µg mL^{-1}) and oxyclozanide (B) (24 µg mL^{-1}).

2.6.3. Application to pharmaceutical preparation

Two mL of Levozan® oral suspension equivalent to 60 mg of levamisole and 120 mg of oxyclozanide, quantitatively transferred to 100 mL volumetric flask and the volume was made up to 50 mL with methanol and phosphate buffer PH 7.9 (10:90 v/v). 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^{-1} levamisole and 1.2 mg mL^{-1} of oxyclozanide. This stock solution was diluted to obtain a test sample solution containing 12 µg mL^{-1} of levamisole and 24 µg $mL^{-1}$of oxyclozanide, and then injected into the capillary electrophoresis system.

3. RESULTS AND DISSCUSION

In the proposed method, capillary electrophoresis has been described for separation and simultaneous quantitation of levamisole and oxyclozanide. Capillary electrophoresis provides many advantages such as fast analysis times, minimum organic usage, and small quantities of solutions and thus overall lower consumable expenses can be attained.

3.1. Method development

Optimization of capillary electrophoresis system conditions was achieved to develop and validate a rapid and selective assay method for the determination of levamisole and oxyclozanide. Capillary electrophoresis technique separates ions according to their electrophoretic mobility using an applied voltage. The electrophoretic mobility depends on the radius of the atom, the viscosity, and the charge of the molecule. Capillary electrophoresis provides faster results and gives high resolution separation. It is a good technique compared to other technologies due to there is a large range of detection methods available. The capillary electrophoresis needs a careful selection of the buffer and PH to obtain the most accurate results and the best separation. Different concentrations of borate and phosphate buffers were tried at different pH values and concentrations. It was found that 40 mM of phosphate buffer pH 7.9 is optimum at which good separation and reproducible quantification of the studied compounds was successfully done. Phosphate buffer was tried at three different concentrations (20, 40, and 50mM) taking in account other factors constant. Phosphate buffer (40 mM) provides a good resolution with adequate analytical run time. The applied voltage is directly proportional to the resolution of compounds so it can affect the efficiency of analysis. The applied voltage was progressively increased from10 to 30 kV. The optimum resolution was obtained when applying a voltage of 25 kV. Finally, the electrophoretic separation of levamisole and oxyclozanide was carried out under the conditions described above and the migration times were 3.38 ± 0.02 and 5.34 ± 0.04 min for levamisole and oxyclozanide, respectively **Fig 2**.

3.2. Method validation

3.2.1. Linearity and range

The linearity of levamisole and oxyclozanide was evaluated by injecting six independent levels of calibration curve in the concentration range of 4-40 μgmL^{-1} for both levamisole and oxyclozanide in terms of slope, intercept and correlation coefficient values.

3.2.2. Precision

3.2.2.1. Repeatability (Intra-Day Precision)

The intra-day precision was calculated by analyzing three different concentrations (4, 24, 40 μgmL^{-1}) of levamisole and oxyclozanide, three times in the same day at interval of 1 hour, simultaneously and %RSD was calculated as shown in **Table 1**.

3.2.2.2. Intermediate Precision (Inter-Day Precision)

Inter-day precision was calculated daily by analyzing three different concentrations (4, 24, 40 μgmL^{-1}) of both levamisole and oxyclozanide, in three days and %RSD was calculated as shown in **Table 1**.

3.2.3. Accuracy (% Recovery)

Accuracy of the proposed method was confirmed by recovery study from marketed preparation at three levels (80%, 100% and 120%) of standard addition. Recovery percentage of levamisole and oxyclozanide were calculated and was found within accepted limits which achieve the accuracy of the method as shown in **Table 2**.

3.2.4. Limit of detection and limit of quantification

LOD and LOQ were determined by the mathematical equation according to ICH.

- **LOD = 3.3** x **σ/S**
- **LOQ = 10** x **σ/S**

Where, **σ** = Standard deviation of the response, and **S =** Slope.

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

Parameter	Levamisole	Oxyclozanide
Linearity range (μg mL^{-1})	4-40	4-40
LOD (μg mL^{-1})	0.099	0.075
LOQ (μg mL^{-1})	0.299	0.228
Regression parameter*	Y= a+ b C	Y= a+ b C
Correlation coefficient	0.9998	0.9999
Slope (b)	0.0177	0.0288
Intercept (a)	0.0021	0.0031
Precision (% RSD)		
Repeatability	0.117	0.176
Intermediate precision	0.161	0.193
Robustness (%RSD)		
	0.425 [a]	0.455 [a]
	0.284 [b]	0.301 [b]
	0.372 [c]	0.396 [c]
	0.558 [d]	0.493 [d]

*Y= a + bC, where Y is the peak area and C is the concentration in μg mL^{-1}.

[a] Values for three determinations with change in the pH of phosphate buffer (±0.2).

[b]Values for three determinations with change in the phosphate buffer concentration (±2 mM).

[c]Values for three determinations with change in the selected wavelength (±2 nm).

[d]Values for three determinations with change in the applied voltage (±0.2 KV).

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

Drug	Pharmaceutical taken (µg mL^{-1})	Pharmaceutical Found (µg mL^{-1})	Pure added (µg mL^{-1})	Pure found (µg mL^{-1})	%Recovery
Levamisole	10	9.91	8	8.01	100.13
			10	9.88	99.88
			12	11.98	99.83
		Mean ± % RSD			99.95 ± 0.161
Oxyclozanide	10	10.02	8	7.99	99.87
			10	10.04	100.4
			12	12.01	100.08
		Mean ± % RSD			100.12 ± 0.267

3.2.5. Specificity

The specificity of the method was confirmed by determination of different laboratory prepared mixtures of levamisole and oxyclozanide in different ratios. The provided electropherograms 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 electropherograms did not show any additional peaks when compared to those of the synthetic mixture.

3.2.6. 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	0.356	0.106
Resolution	1.17	
The Tailing factor	1.19	1.21
Theoretical Plates	2888	3013

3.2.7. Robustness

Robustness of the proposed method was evaluated by subjecting the method to small variations in method condition such as phosphate buffer concentration, PH of buffer, selected wavelength, and applied voltage. **Table 1** contains results of robustness in %RSD term.

3.2.8. Application to the finished product

The proposed method was applied successfully for determination of levamisole and oxyclozanide in levozan® 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(Mohamed et al. 2014). The proposed method showed good accuracy and precision for assay of levamisole and oxyclozanide in Levozan®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**
n[a]	5	5	5	5
%R	99.95	100.12	100.37	100.27
%RSD	0.356	0.106	0.801	0.461
SD	0.357	0.106	0.804	0.463
Variance	0.127	0.011	0.646	0.214
Student's t-test (2.306)[b]	0.636	0.852	____	------
F-value (6.388)[b]	0.197	0.052	____	-----

[a]Experiments number.

[b]Tabulated values of "t "and "F" at (P = 0.05).

*****Reported method: HPLC method using C18 column, mobile phase consisting of acetonitrile: methanol: 0.05M potassium dihydrogen phosphate (60: 20: 20 by volume) respectively, PH of the mobile phase was adjusted by ortho-phosphoric acid. and UV detector dual i.e, 210 and 303 nm (Mohamed. et al. 2014).

4. CONCLUSION

This method is sensitive, selective, and can be applied for determination of levamisole and oxyclozanide in their pharmaceutical preparation.

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التقدير المتزامن لليفاميزول وأوكسيكلوزانيد في المستحضر الصيدلاني عن طريق الرحلان الكهربائي الشعري

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تم تطوير طريقة الرحلان الكهربي الشعري الحساسة والدقيقة من أجل التقدير المتزامن لليفاميزول والأوكسيكلوزانيد في العينة النقية والتركيبة الصيدلانية. تم تقديم الرحلان الكهربي الشعري كأسلوب تحليلي بسيط للفصل من أجل التحليل المتزامن للعقاقير المحددة خلال وقت تشغيل تحليلي أقصر. في هذه الدراسة ، تم الفصل على شعيرات السيليكا المنصهرة (قطرها الداخلي 30 سم - 50 ميكرومتر) ؛ يتكون محلول المنحل بالكهرباء من محلول منظم الفوسفات (40 مل مول ، ودرجة الحموضة 7.9) وباستخدام الكشف بالأشعة فوق البنفسجية عند 227 نانومتر. أظهرت الطريقة أنها خطية (r^2> 0.9998) ودقيقة RSD <0.193 ٪ (ومحكمة 99.95٪ لليفاميزول و 100.12٪ للأوكسيكلوزانيد) ومحددة وقوية. كانت قيم LOD و LOQ 0.099 ميكروغرام / مل و 0.299 ميكروغرام/ مل على التوالى لليفاميزول و 0.075 ميكروغرام / مل و 0.228 ميكروغرام / مل على التوالى لأوكسيكلوزانيد. الطريقة المقترحة حصلت على فصل جيد ودقة تامة. تم التحقق من صحة الطريقة المقترحة وفقًا لإرشادات ICH وتم تنفيذها لتحديد الأدوية المذكورة في صيغتها الصيدلانية.

الكلمات المفتاحية : ليفاميزول ، أوكسيكلوزانيد ، الرحلان الكهربي ، المستحضرات الصيدلانية.