

Original Research Article

Stability-Indicating Methods for the Determination of Closantel Sodium by HPLC, Densitometry, Spectrofluorimetry and pH induced ΔA

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Abstract

Four stability-indicating methods were developed for the determination of closantel-Na in the presence of its alkaline degradation products. The first one was HPLC method in which efficient separation was achieved on a C₁₈ analytical column with isocratic elution and a mobile phase composed of two mixtures A and B in a ratio of (30:70, v/v). Mobile phase A of ammonium acetate buffer-acetonitrile- water (100:30:870, v/v/v) and mobile phase B of the same composition in a ratio (100:870:30, v/v/v). Bromohexine-HCl was used as an internal standard and linearity was obtained in the range of 0.5-10.0 $\mu\text{g/mL}$ with mean accuracy of 99.63% $\pm$ 0.49. The second method was a densitometric evaluation of thin-layer chromatograms of the drug using a mobile phase of ethyl acetate-methanol-ammonia (33%) (17:2:1, v/v/v) and scanned at 245 nm. Closantel-Na was separated from its three degradates with R_f 0.40, 0.05, 0.14 and 0.93, respectively; and determined in the range of 0.5-10.0 $\mu\text{g/spot}$ with mean accuracy of 100.11% $\pm$ 0.27. The third method was based on measuring the native fluorescence intensity of closantel-Na in ethanolic H₂SO₄ solution at 477 nm (λ_{ex} 337 nm) in the range of 0.5-5.0 $\mu\text{g/mL}$ with mean accuracy of 99.84% $\pm$ 0.52. The forth one was pH-induced ΔA spectrophotometry based on measuring the ΔA of closantel-Na in ethanolic HCl solution at 373.5 and 286.5 nm in the range of 6.0-100.0 $\mu\text{g/mL}$ with mean accuracy of 99.43% $\pm$ 0.61 and 99.73% $\pm$ 0.58; respectively. The four methods provided selective recovery of the intact

drug ($99.24\% \pm 0.61$, $100.21\% \pm 1.33$, $100.54\% \pm 1.06$ and $99.67\% \pm 0.96$ and $100.50\% \pm 1.14$; respectively.) in the presence of up to 90% its alkaline degradates by HPLC method and densitometric methods, 30% by the spectrofluorimetric method and 40% at λ max 373.5 nm and 15% at 286.5 nm, respectively; by the pH induced ΔA method. Successful application of the four methods for analyzing closantel-Na in Closa-Atak injectable solution was obtained and results were statistically analyzed and found to be in accordance with those given by a reported method.

Keywords: Stability-indicating HPLC, Densitometry, Spectrofluorimetry, ΔA , closantel sodium.

1. Introduction

Closantel- Na, N-[5-chloro-4-[(R, S)-(4-chlorophenyl) cyanomethyl]-2-methylphenyl]-2-hydroxy-3, 5-diiodobenzamide sodium salt dihydrate, is an anthelmintic. It acts by uncoupling oxidative phosphorylation and is effective against *Taenia pisiformis* in rabbits, *Ancylostoma caninum* and *Fasciola hepatica* and has prolonged effect on *Haemonchus* in sheep ⁽¹⁾. A survey of the literature revealed few analytical methods for the determination of closantel-Na, including spectrometric methods ^(2,3), densitometric method ⁽⁴⁾ and HPLC ⁽⁵⁻¹²⁾. The aim of the present study is to develop selective and accurate HPLC, densitometric and spectrofluorimetric methods to estimate closantel-Na in the presence of its alkaline degradation products and in its pharmaceutical formulation.

2. Experimental

2.1. Instruments

- HPLC (Agilent 1100 series, Waldbronn, Germany) consists of an Agilent pump, equipped with a variable wavelength detector and a 20 μ L volume injection loop, and Lichospher 60-S RP- column DOC (particle size of the packing material, 125 mm $\times$ 4 mm).
- Densitometer-Dual Wave Length Flying Spot CS-9301 (Shimadzu, Tokyo, Japan).
- Spectrofluotometer RF-1501 (Shimadzu, Tokyo, Japan).
- UV-Vis spectrophotometer (Shimadzu 1601, Japan).
- Thin layer chromatographic plates pre-coated with silica gel 60 F₂₅₄, 10x20 cm (Merck, Germany).
- Micro syringe 10 μ L capacity (Hamilton, Germany).

2.2. Materials and Methods

2.2.1. Materials

- Pure closantel-Na was supplied from Pharma Sweed, Egypt Co., Cairo, Egypt while pure bromohexine-HCl was supplied by Medmac Co., Jordan with purities of 99.75% and 99.84% as referred by the supplier, respectively.
- Closa-Atak –injectable solution, B.N.101920, labeled to contain 50 mg closantel-Na per mL, product of Bomac Laboratories Limited, Auckland, New Zealand; was purchased from a local market.
- Acetonitrile, HPLC grade (Scharlau chemie, Spain).
- Acetic acid, HPLC grade (Honil Limited, London, UK).
- Absolute Ethanol and Methanol, (Sigma Aldrich, Germany).
- Ethyl acetate and Ammonia Solution 33%, (Adwic. Cairo, Egypt).
- Ammonium acetate, (Adwic. Cairo, Egypt); 7.7 g/l solution prepared in water and adjusted to pH 4.3 with acetic acid.
- HCl and H₂SO₄, (Sigma Aldrich, Germany); 6 N and 0.1 N solutions prepared in ethanol; respectively.
- KOH pellets, (Nakamaruko Kawasaki, Japan); 6 N solution prepared in ethanol ⁽¹³⁾.

2.2.2. Standard solutions

Solutions of closantel-Na (0.05 mg mL⁻¹) and the internal standard (IS) bromohexine-HCl (0.1 mg mL⁻¹) were prepared separately in acetonitrile to be used in the HPLC method. A five mg mL⁻¹ methanolic solution of closantel-Na was used for the densitometric method. For the spectrofluorimetric method, standard stock solution of closantel-Na 0.1 mg mL⁻¹ was prepared in 0.1N ethanolic H₂SO₄ solution which is further diluted one tenth to obtain a working standard solution labeled to contain 10 µg mL⁻¹. A 0.2 mg mL⁻¹ standard solution of closantel-Na prepared in ethanol was utilized by the ΔA method.

2.2.3. Degraded solution

Accurately weighed 50 mg of closantel-Na was dissolved and refluxed with 50 mL of 6 N ethanolic KOH for 7 h. After cooling, 50 mL of 6 N ethanolic HCl was added and mixture evaporated to dryness under vacuum. The dry residue was extracted twice with 20 mL of acetonitrile and filtered into 50-mL volumetric flask and **made up** to volume with acetonitrile to obtain an alkali-hydrolyzed solution derived from one mg mL⁻¹ closantel-Na. This solution was further diluted with acetonitrile to obtain a working solution labeled to contain 0.05 mg mL⁻¹ degradates to be used in the HPLC method. For the densitometric method, the dry residue was extracted twice with 20 mL of methanol and filtered into 50-mL volumetric flask and **made up** to volume with methanol to obtain labeled to contain 1 mg mL⁻¹ degraded closantel-Na. Extraction for the spectrofluorimetric method was carried out twice with 20 mL

of 0.1 N ethanolic H₂SO₄ acid solution and filtered into 50-mL volumetric flask and made up to volume with the same solvent to obtain a solution of degradates derived from 1 mg mL⁻¹ drug which was then diluted by 100 folds to provide a solution labeled to contain 10 µg mL⁻¹ closantel-Na degradates. Extraction for the ΔA method was carried out twice with 20 mL of ethanol and filtered into 50-mL volumetric flask and made up to volume with the same solvent to obtain a solution of degradates derived from 0.5 mg mL⁻¹ drug which was then diluted by 5 folds to provide a solution labeled to contain 100 µg mL⁻¹ closantel-Na degradates.

2.2.4. Procedures

2.2.4.1. Linearity

HPLC method- Aliquots from the drug solution in acetonitrile (0.05 mg/ mL) equivalent to 0.005-0.100 mg closantel-Na were transferred to a series of 10-mL volumetric flasks to which 1.2 mL of IS solution was added and diluted to volume with acetonitrile. Twenty µL injections from each solution were chromatographed on the Lichospher 60-S RP-column using a mobile phase composed of two mixtures A and B (30:70, v/v). Mobile phase A of ammonium acetate buffer- acetonitrile- water (100:30:870, v/v/v) and mobile phase B of the same composition as A with a ratio of (100:870:30, v/v/v) at a flow rate of 0.5 mL/min and UV detection at 245 nm. The calculated peak area ratios of closantel-Na to IS were plotted with respect to the drug concentration and the regression parameters were deduced.

Densitometric method- Accurately measured aliquots containing 0.5-10.0 mg of closantel-Na from its standard solution (5 mg/mL) in methanol were introduced into 10-mL volumetric flasks and diluted to volume with methanol. Ten µL of each solution were applied to TLC plate pre-coated with silica gel 60 F₂₅₄ (10 × 20 cm; Merck, Germany) using a 10 µL microsyringe. The plates were developed in a mobile phase of ethyl acetate-methanol-ammonia 33% (17:2:1, v/v/v). Then allowed to dry in air and spots were scanned at 245 nm. The calibration curve representing the recorded area under the peak against drug concentration was plotted and the regression equation was computed.

Spectrofluorimetric method- Aliquots of working drug solution (10 µg /mL) in ethanolic H₂SO₄ equivalent to 5-50 µg closantel-Na were transferred to a series of 10-mL volumetric flasks and completed to the mark with the same solvent. Fluorescence intensity was recorded using 0.1 N ethanolic H₂SO₄ as a blank at λ_{em} 477 nm (λ_{ex} 337 nm). The calibration curve of

137 fluorescence intensity against drug concentration was plotted and the regression equation was
138 calculated.

139 **pH induced ΔA method-** Aliquots of standard solution of closantel-Na in ethanol (0.2
140 mg/mL) equivalent to 0.06 - 1.00 mg were transferred to two separate sets of 10-mL
141 volumetric flasks; one set was diluted to volume with ethanol and the second was adjusted to
142 volume with ethanolic HCl. The ΔA was determined at 373.5 and 286.5 nm using the
143 ethanolic solution in the sample beam and the acidic ethanol in the reference beam.
144 Calibration curves relating (ΔA) to drug concentration were constructed and the regression
145 equations were computed.

146

147 2.2.4.2. Assay of intact and degraded drug mixtures

148 **HPLC method-** Different volumes of standard drug solution (0.05 mg/mL) in acetonitrile
149 equivalent to 0.095-0.005 mg closantel-Na were transferred into a series of 10-mL volumetric
150 flasks containing 1.2 mL of IS and volumes of degraded solution equivalent to degradation
151 products derived from 0.005-0.095 mg. Flasks were made up to the mark with acetonitrile,
152 then 20 μ L of each solution was chromatographed on HPLC column following details under
153 “2.5.1.Linearity”.

154 **Densitometric method-** Volumes equivalent to 9.0-1.0 mg of closantel-Na from its standard
155 methanolic solution (5 mg/mL) were transferred to a series of 10-mL volumetric flasks, and
156 mixed with volumes from its degraded solution equivalent to degradation products derived
157 from 1.0-9.0 mg of the drug. Each flask was made up to the mark with methanol and then
158 analyzed by densitometric method as described under “2.5.1.Linearity”.

159 **Spectrofluorimetric method-** Aliquots equivalent to 49.0-1.0 μ g of closantel-Na from its
160 solution in 0.1 N ethanolic H₂SO₄ solution (10 μ g/mL) were transferred to a series of 10-mL
161 volumetric flasks. Different portions from its alkaline hydrolyzed solution equivalent to the
162 degradation products derived from 1.0-49.0 μ g of the drug. The volume was made up with
163 ethanolic H₂SO₄ solution and fluorescence intensity was recorded at λ_{em} 477 nm using λ_{ex} 337
164 nm as detailed under “2.5.1.Linearity”.

165 **pH induced ΔA method-** Aliquots equivalent to 98.0 - 2.0 μ g of closantel-Na from its
166 solution in ethanol (0.2 mg/mL) were transferred to two series of 10-mL
167 volumetric flasks. Different portions from its alkaline hydrolyzed solution equivalent to the
168 degradation products derived from 2.0 - 98.0 μ g of the drug. The volume was made up with

169 ethanol solution in one set and ethanolic HCl in the other set. The ΔA was determined at
170 373.5 and 286.5 nm as detailed under “2.5.1.Linearity”.

171 2.2.4.3. Application to pharmaceutical formulations

172 The contents of 5 Closa-Atak injectable solutions were thoroughly mixed and a volume
173 equivalent to 5 mg closantel-Na was pipetted into a 100-mL volumetric flask. Volume made
174 up with acetonitrile to obtain a solution labeled to 0.05 mg/mL closantel-Na to be used in the
175 HPLC method. For the densitometric method, volume equivalent to 250 mg closantel-Na was
176 pipetted into a 50-mL volumetric flask, completed to the mark with methanol to obtain a 5
177 mg/mL closantel-Na solution. For the spectrofluorimetric method, stock solution of closantel-
178 Na claimed to contain 0.1 mg mL⁻¹ was prepared in 0.1 N ethanolic H₂SO₄ solution which
179 was further diluted ten folds to obtain a 10 µg mL⁻¹ as a working solution. For the ΔA method,
180 volume equivalent to 20 mg closantel-Na was pipetted into a 100-mL volumetric flask, made
181 up to the mark with ethanol to obtain a 0.2 mg/mL closantel-Na solution. For each method,
182 the details under “2.5.1.Linearity” were followed and the concentration of the drug was
183 calculated from the corresponding regression equation.

184 3. Results and Discussion

185 3.1. Degradation of closantel sodium

186 Closantel contains cyanide and amide groups that are expected to be hydrolyzed.
187 Unfortunately closantel as sodium salt is insoluble in both aqueous and alcoholic acidic
188 medium as well as H₂O₂ solution. Thus, stressed alkaline hydrolytic degradation was studied
189 by refluxing the drug with 6 N ethanolic KOH for 7 hr. Then neutralization was carried out
190 with 6 N ethanolic HCl using universal pH paper followed by evaporation to dryness under
191 vacuum and extraction with methanol. The methanolic extract was tested by TLC using a
192 developing system of ethyl acetate-methanol-ammonia 33% (17:2:1, v/v/v). Three spots for
193 hydrolyzed solution were obtained at R_f 0.93, 0.14 and 0.05 whereas the spot related to intact
194 drug was at R_f 0.4.

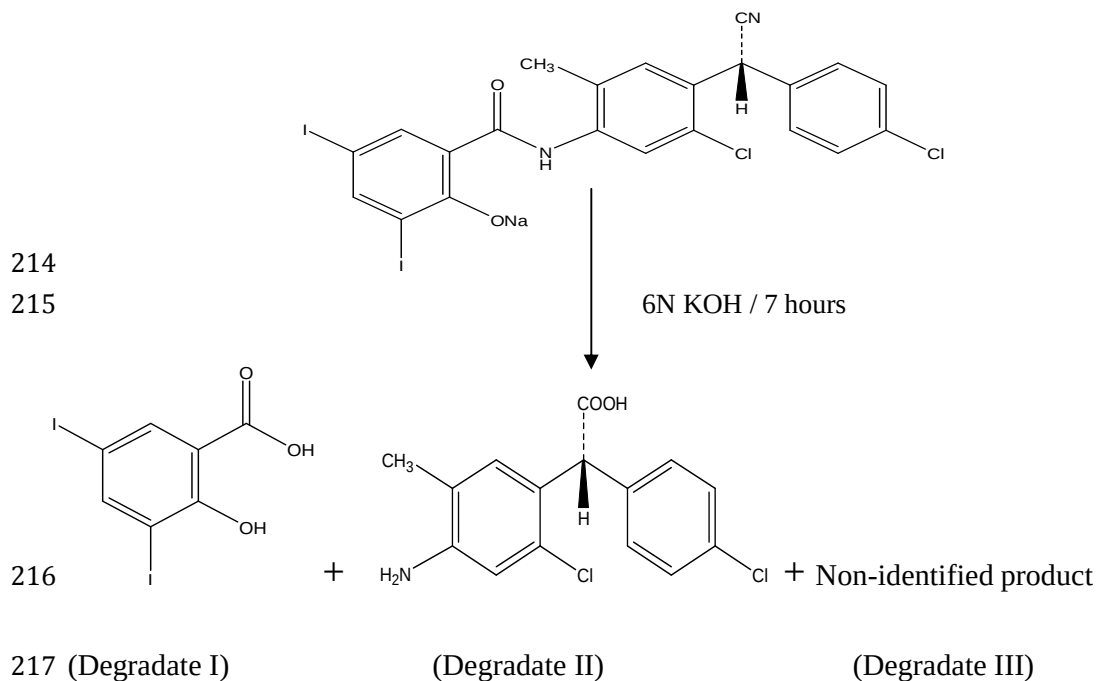
195 The methanolic extract of the spots of closantel-Na and its three alkaline degradates
196 were separated on TLC plates using the mentioned mobile phase and bands related to the
197 intact closantel-Na and each degradate were scratched, extracted with methanol and
198 evaporated to dryness. The residues were tested by both IR (using KBr disc) and MS where
199 the third degradate at R_f 0.05 could not be identified.

200 The IR spectrum of the intact drug showed characteristic absorption bands for cyanide
201 group and amidic carbonyl groups at 2268 cm⁻¹ and 1628 cm⁻¹, respectively. These bands

disappeared from the IR spectra of the alkaline degradate (I) and (II) and instead a new carbonyl of carboxylic acid functionality appeared at 1732 and 1736 cm^{-1} for two degradates, respectively; Figure (1). In addition, a broad band at 3398-3233 cm^{-1} appeared in the spectrum of degradate I which might be related two-OH of carboxylic and phenolic groups. Whereas the IR spectrum of degradate II showed a broad band at about 3395-3236 cm^{-1} which could be ascribed to carboxylic OH and amine NH_2 ; Fig 1.

Further confirmation was carried out by MS, where mass spectrum of intact closantel-Na was characterized by the molecular ion m/z at 663. While spectra of the two degradates (I) and (II) showed m/z at 390 and at 309, respectively; Fig 2.

This finding suggested the formation of salicylic acid moiety (degradate I) that was confirmed by positive FeCl_3 test and aminobiphenylacetic acid derivative (degradate II) as presented in the following scheme:



Scheme 1: Proposed alkaline degradation pathway of closantel-Na.

3.2. HPLC method

Chromatographic separation of closantel-Na and its degradation products was satisfactorily performed using a Lichospher 60-S RP-column DOC. Optimization of the mobile phase was carried out using different solvents with different ratios, acetonitrile-water, acetonitrile-methanol-water, acetonitrile-methanol-water- H_3PO_4 and acetonitrile-methanol-

ammonium acetate buffer. Best separation was achieved with isocratic elution with two mobile phases in ratios of (30:70, v/v). Mobile phase A: ammonium acetate buffer-acetonitrile-water (100:30:870, v/v/v) and Mobile phase B: ammonium acetate buffer-acetonitrile- water (100:870:30, v/v/v). Different flow rates (0.5-1.5 mL/ min) were studied, resolution of the intact drug from its degradates was achieved at a flow rate of 0.5 mL/min. More than one wavelength was tried and the most sensitive detector response was obtained at 245 nm. Under these optimum conditions, pure closantel-Na and IS exhibited two strong resolved peaks at R_t 12.47 ± 0.02 and 10.16 ± 0.06 min. where three peaks at 2.19 ± 0.01 , 3.22 ± 0.02 and 5.10 ± 0.01 min. were observed for closantel-Na alkaline degradates; Fig 3.

3.3. Densitometric method

The TLC densitometric method was used to determine closantel-Na in the presence of its degradation products using differences in their R_f values. Different developing systems such as toluene-methanol-chloroform-ammonia (33%) (5:3:6:0.1, by volume), chloroform-methanol- ammonia (13.5 M) (8.5:1.5:0.1, v/v/v)⁽⁴⁾, methanol-water-glacial acetic acid (5:5:0.1, v/v/v) and methanol-acetone (8:2, v/v) were attempted. Complete separation of the drug from its degradation products was achieved using a mobile phase of ethyl acetate-methanol-ammonia (33%) (17:2:1, v/v/v); **Fig 4**. The R_f value of the pure drug was 0.40, but the R_f value of its three alkaline degradates was 0.93, 0.14 and 0.05, respectively; using detection at 245 nm.

3.4. Spectrofluorimetric method

Closantel-Na was found to possess native characteristic fluorescence at 477 nm after excitation at 337 nm; Fig 5. Different media were adopted for studying fluorescence intensity as methanol, ethanol, acetone and aqueous, methanolic and ethanolic sulphuric. Maximum intensity was obtained in ethanolic H_2SO_4 so it was **used** throughout this work. The effect of sensitizing agents was also studied using cetrimid, triton, B-cyclodextrin, tween 20, sodium lauryl sulphat and gelatin. All were found to cause decrease in fluorescence intensity which may be attributed to the insolubility of the drug in water in which the sensitizing agent is solubilized. Thus none of them was used. The effect of time was also studied; it was found that fluorescence intensity was stable for more than one hour.

3.5. pH induced ΔA method

Closantel in its form as sodium salt exhibits maximum absorbance at 371.5 and 284 nm in ethanol. While in acidic-ethanol, a hypsochromic shift to 333.5 and 276.5 nm was observed due to trapping of phenate anion by hydrogen ions. The ΔA spectrum between ethanol (experiment) and ethanolic-HCl (reference) showed two bands at 373.5 nm and 286.5 nm which were adopted for spectrophotometric determination of closantel-Na; Fig 6. No interference from its alkaline hydrolyzed solution as it was found to exhibit maximum absorbance at 277 nm in ethanol and 299 nm in acidic ethanol; Fig 7.

3.5. Method validation

The four proposed methods were validated according to ICH guidelines⁽¹⁴⁾.

Linearity- A linear correlation was obtained between the peak areas ratio of closantel-Na to IS (bromohexine HCl), peak areas of the separated spots, fluorescence intensity or ΔA at $\lambda = 373.5$ and 286.5 nm and drug concentration in the range of 0.5 - 10.0 $\mu\text{g mL}^{-1}$, 0.5 - 10.0 $\mu\text{g/spot}$, 0.5 - 5.0 $\mu\text{g mL}^{-1}$ or 6.0 - 100.0 $\mu\text{g mL}^{-1}$ for HPLC, densitometric, spectrofluorimetric or pH induced ΔA method; respectively. The regression parameters were calculated and presented in Table (1) which indicated that densitometric method was the most sensitive one as indicated by the highest slope (688.79 ± 10.6).

Accuracy and precision- The accuracy of the proposed methods was tested using three concentrations of closantel-Na within the linearity range and found to range between 99.43 and 100.11%. Precision was also evaluated by calculating the intraday RSD%, which ranged between 0.17 and 1.88% and interday RSD%, 0.44-1.70% over a period of two months. This indicated the repeatability and reproducibility of the proposed methods; Table (1).

Selectivity- Laboratory prepared mixtures containing different ratios of the drug and its alkaline degradation products were analyzed. The methods were found to be valid at determining the pure drug in the presence of up to 90% of its degradation products by the HPLC and the densitometric methods while up to 30% for the spectrofluorimetric method and for the ΔA method up to 40% at λ_{max} 373.5 nm and 15% at 286.5 nm without any interference; as indicated by recoveries of 99.24-100.54% $\pm$ 0.61-1.33; Table (2). The selectivity of the proposed methods was further evaluated by successful analysis of the drug in its pharmaceutical formulation (Closa-Atak injectable solution) by HPLC, densitometric, spectrofluorometric and pH induced ΔA methods with mean percentage recoveries of 101.27 $\pm$ 1.43, 102.21 $\pm$ 1.37, 103.92 $\pm$ 0.95 and 100.71 $\pm$ 1.32 and 101.43 $\pm$ 1.58; respectively.

Validity of the proposed methods was further assessed by applying the standard addition technique; mean recovery of the added was 99.45% $\pm$ 0.96, 100.40% $\pm$ 1.16,

100.06% $\pm$ 1.64 and 100.77% $\pm$ 0.86 and 100.53% $\pm$ 1.12 for the four proposed methods, respectively; Table (3). These results ⁽¹⁴⁾ were compared with those obtained from the reported direct UV spectrophotometric method ⁽³⁾. As shown in Table (3), calculated *t*- and *F*-values were less than theoretical ones, indicating that there was no significant difference between the proposed and reported methods.

Stability of standard solution- The stability of standard closantel-Na solutions as 0.05 mg mL⁻¹ in acetonitrile, 5.00 mg mL⁻¹ in methanol, 0.10 mg mL⁻¹ in 0.10 N ethanolic H₂SO₄ solution and 0.20 mg mL⁻¹ in ethanol were evaluated by HPLC, densitometry, spectrofluorimetry and pH induced ΔA ; respectively. This was carried out through storing on laboratory bench and in the refrigerator at 4°C. The solutions were found to be stable for one week at room temperature and for two weeks in refrigerator.

4. Conclusion

The main criteria for developing successful stability-indicating HPLC, Densitometric, Spectrofluorimetric and ΔA methods to determine closantel-Na were established. The drug was susceptible to alkaline degradation; however, it was not subjected to acidic or oxidative stresses because of its insolubility in both solutions. Identification of alkaline degradates was accomplished and validation of the proposed methods was carried out according to ICH guidelines; the methods proved to be accurate, precise and rugged. The high sensitivity and selectivity of the methods can allow for using them in routine analysis.

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Table 1: Regression parameters and assay validation results for the determination of closantel-Na by the proposed methods

<i>Parameters</i>	<i>HPLC method</i>	<i>Densitometric method</i>	<i>Spectrofluorimetric method</i>	<i>Δ A method</i>	
linearity range	0.5-10.0 µg mL ⁻¹	0.5-10.0 µg/spot	0.5-5.0 µg mL ⁻¹	6.0-100.0 µg mL ⁻¹	
Regression Parameters				<u>(λ = 373.5nm)</u>	<u>(λ = 286.5nm)</u>
Slope ± S.D	12.11 ± 0.11	688.79 ± 10.59	33.92 ± 0.33	0.0147 ± 8.93486E-05	0.0147 ± 0.00018
Intercept ± S.D.	1.68 ± 0.64	67.69 ± 64.14	9.91 ± 0.99	0.0028 ± 0.0050	0.0039 ± 0.0104
S.D. of residual	0.854	85.482	1.309	0.0079	0.0163
Correlation coefficient	0.9997	0.9991	0.9996	0.9998	0.9992
Accuracy (R%± S.D.)	99.62 ± 0.49	100.11 ± 0.27	99.84 ± 0.52	99.43 ± 0.61	99.73 ± 0.58
Precision (RSD%, n=9)					
Intraday	0.52 – 1.23	0.89 – 1.45	1.06 – 1.88	0.48 – 1.53	0.17 – 1.15
Interday	1.13 – 1.29	0.67 – 1.47	0.60 – 1.70	0.48 – 1.07	0.44 – 1.58

354 **Table 2: Determination of closantel-Na in mixtures with its degradation products using the proposed methods**

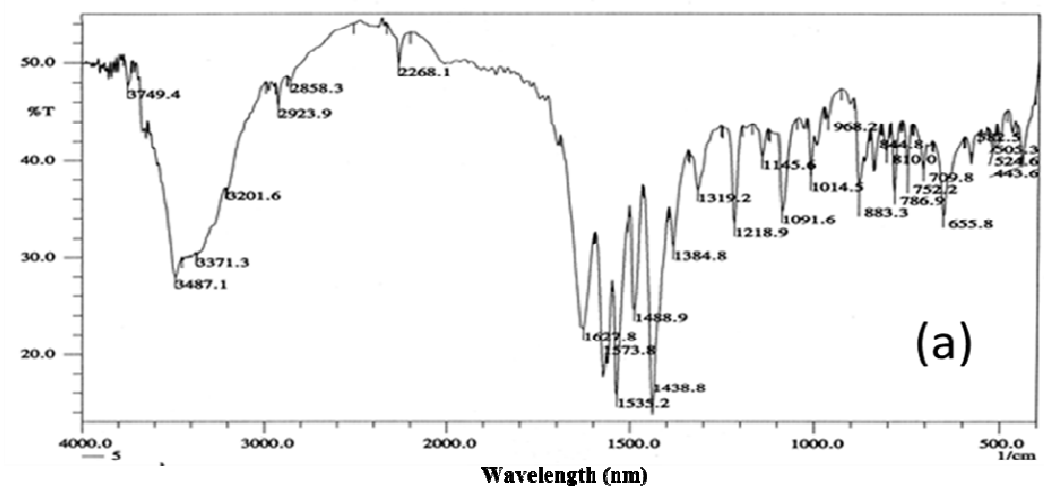
<u>HPLC method</u>			<u>Densitometric method</u>			<u>Spectrofluorimetric method</u>			<u>ΔA method</u>			
Intact ($\mu\text{g/mL}$)	Degradate ($\mu\text{g/mL}$)	R% of Intact	Intact ($\mu\text{g/spot}$)	Degradate ($\mu\text{g/spot}$)	R% of Intact	Intact ($\mu\text{g/mL}$)	Degradate ($\mu\text{g/mL}$)	R% of Intact	Intact ($\mu\text{g/mL}$)	Degradate ($\mu\text{g/mL}$)	R% of Intact At 373.5 nm	R% of Intact At 286.5 nm
9.5	0.5	100.08	9.0	1.0	100.05	4.8	0.2	100.29	98	2	98.45	101.35
9.0	1.0	99.21	8.0	2.0	101.01	4.5	0.5	99.26	95	5	98.55	99.90
8.0	2.0	98.80	6.0	4.0	101.70	4.3	0.7	100.83	93	7	99.28	98.46
6.0	4.0	98.42	2.0	8.0	100.16	4.0	1.0	98.72	90	10	100.39	101.14
5.0	5.0	99.19	1.0	9.0	98.15	3.8	1.2	101.66	88	12	100.05	100.73
3.0	7.0	99.38				3.5	1.5	101.70	85	15	100.06	101.41
1.0	9.0	98.71				3.0	2.0	105.73*	80	20	101.63	109.87*
						2.5	2.5	117.77*	75	25	99.97	108.49*
									70	30	99.44	
									60	40	98.89	
									50	50	96.63*	
									40	60	94.59*	
Mean $\pm$ S.D.	99.24 $\pm$ 0.61		100.21 $\pm$ 1.33			100.54 $\pm$ 1.06					99.67 $\pm$ 0.96	100.50 $\pm$ 1.14

355 *Rejected.

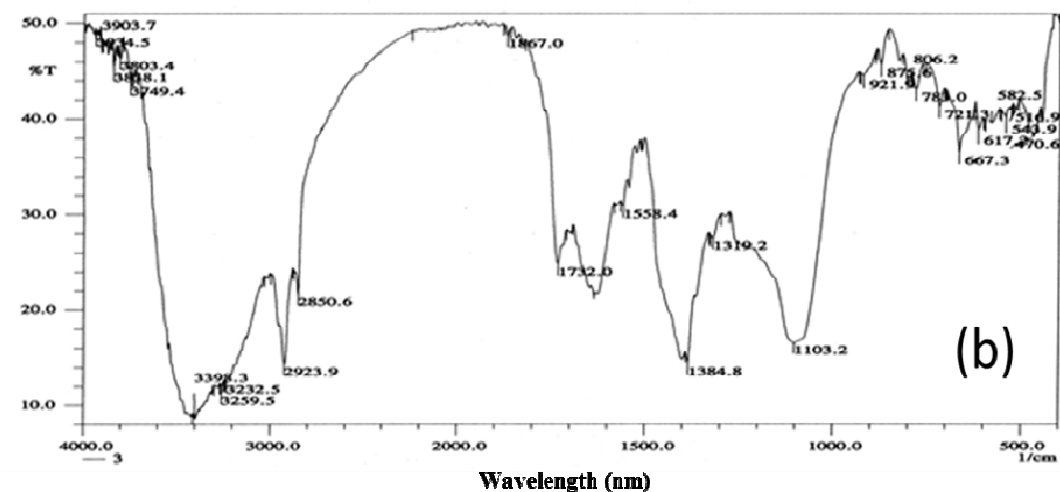
356 **Table 3: Statistical analysis of determination of closantel-Na in Closa-Atak injection by the proposed methods in**
357 **comparison with the reported method ⁽³⁾**

Parameters	<i>HPLC method</i>	<i>Densitometric method</i>	<i>Spectrofluorimetric method</i>	<i>Δ A method</i>	<i>Reported method ⁽³⁾</i>
Mean %	101.27	102.21	103.92	103.92	102.55
S.D.	1.43	1.37	0.95	0.955	1.207
Variance	2.04	1.89	0.91	0.92	1.46
N	5	5	5	5	5
t – test	1.534	0.413	1.990	1.990	-
F – test	1.401	1.296	1.602	1.602	-
Standard addition Mean% ± S.D.	99.45 ± 0.96	100.40 ± 1.16	100.06 ± 1.64	100.77 ± 0.86 at 373.5nm and 100.53 ± 1.12 at 286.5nm	-

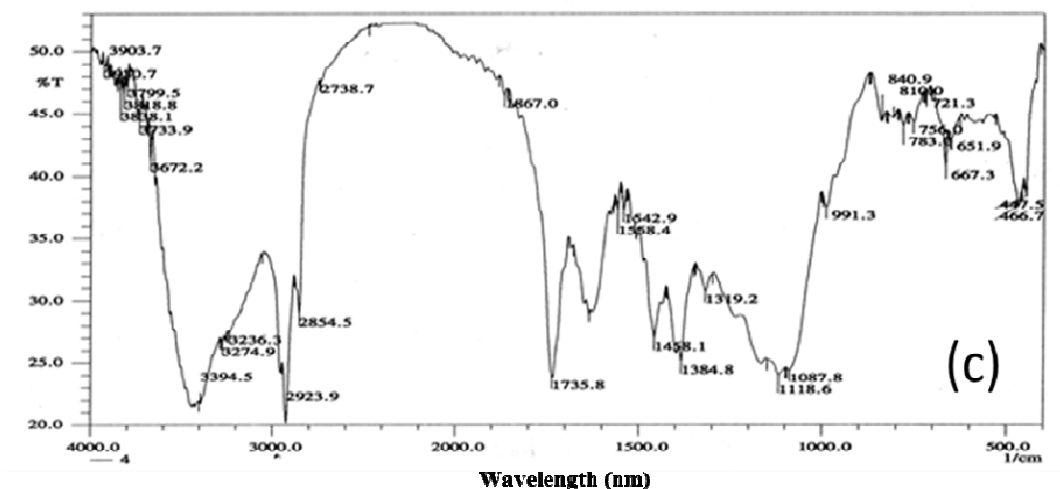
- 358 ➤ The theoretical *t*- and *F*- values at $p=0.05$ were 2.31 and 6.39; respectively.
- 359 ➤ The reported method ⁽³⁾ UV- measurements of drug at 284.8 nm in ethanol.



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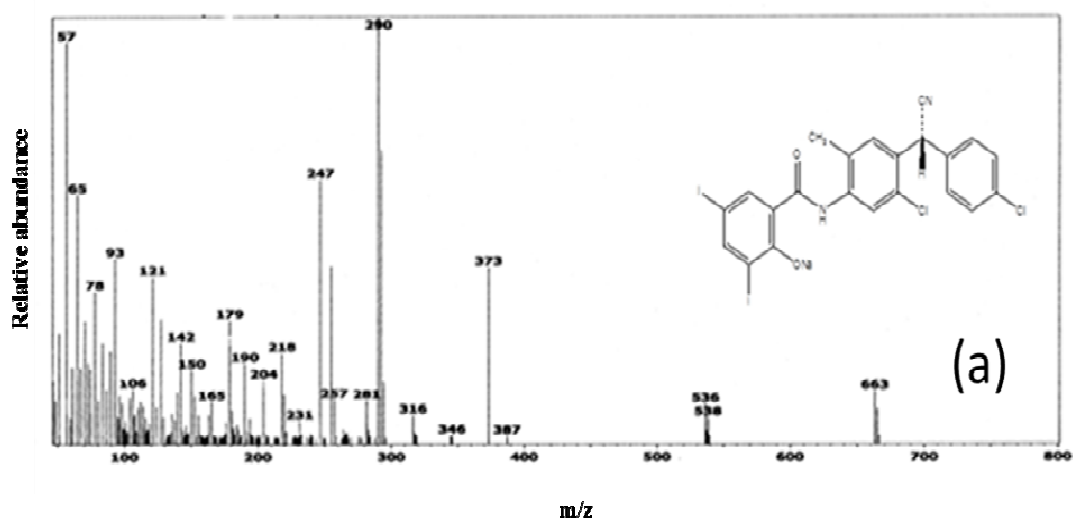


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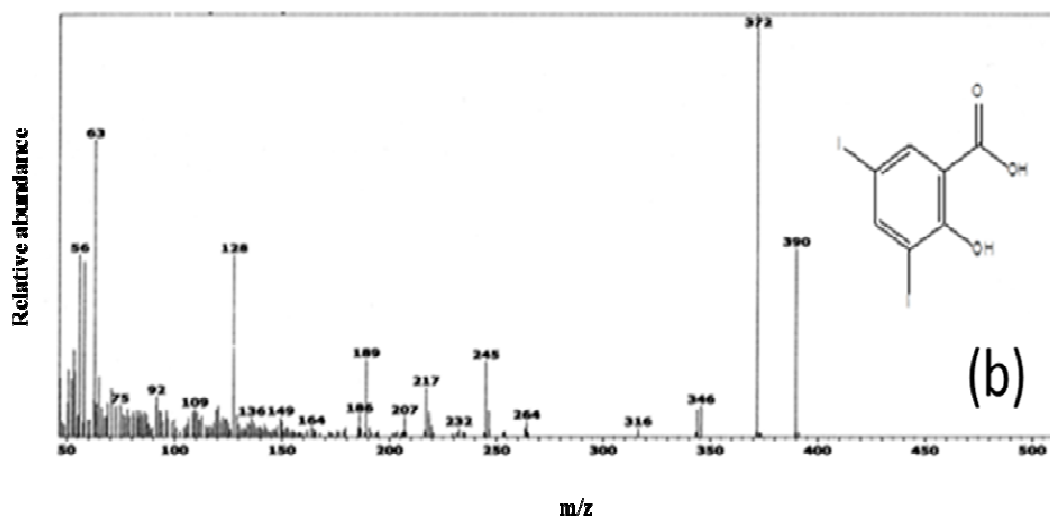


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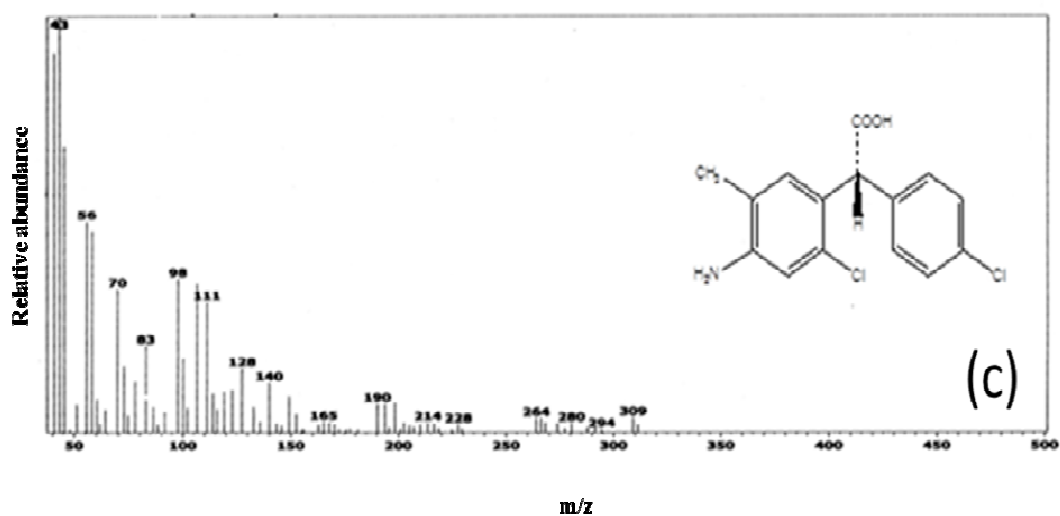
363 Fig1. : IR spectrum of (a) Intact closantel-Na. (b) Alkaline degradate I of
364 closantel-Na. (c) Alkaline degradate II of closantel-Na.



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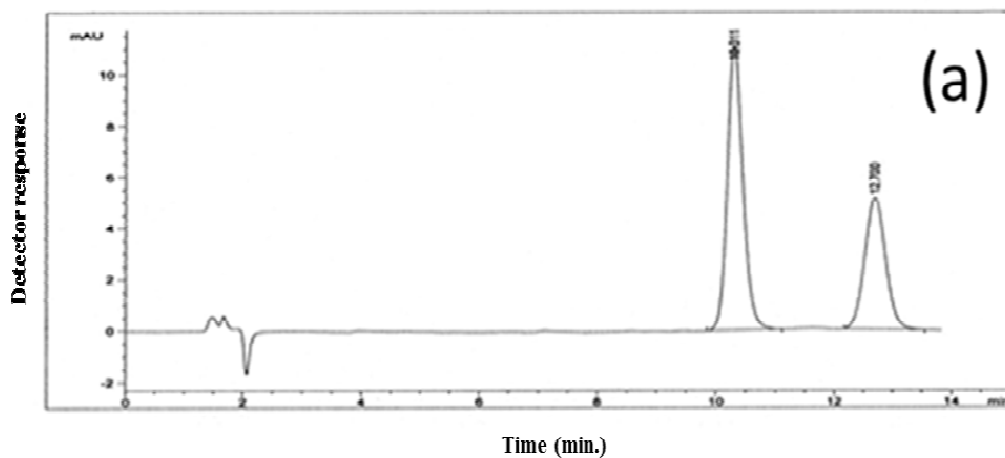


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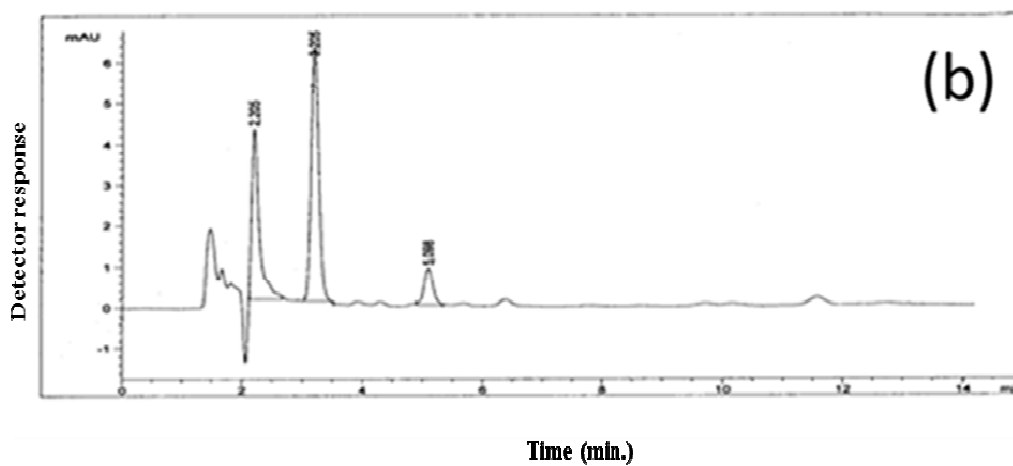


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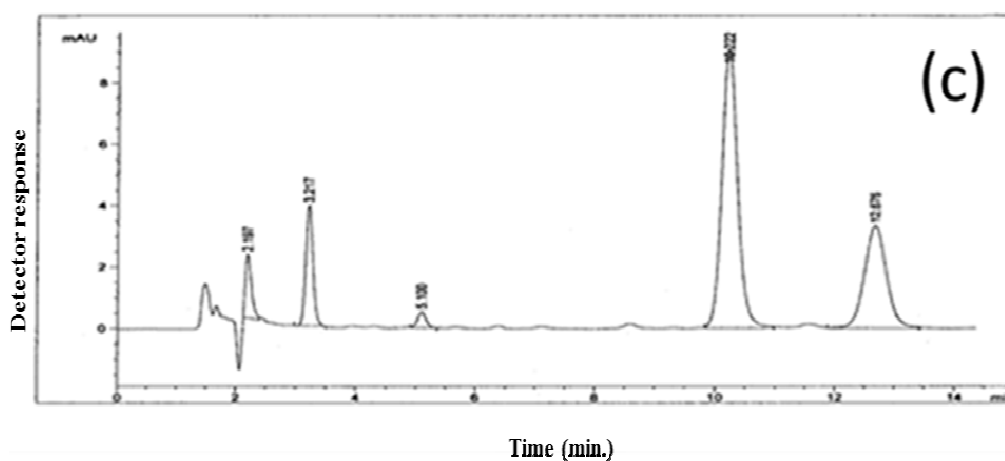
368 Fig 2 : Mass spectrum of (a) Intact closantel-Na. (b) Alkaline degradate I of
 369 closantel-Na. (c) Alkaline degradate II of closantel-Na.



370

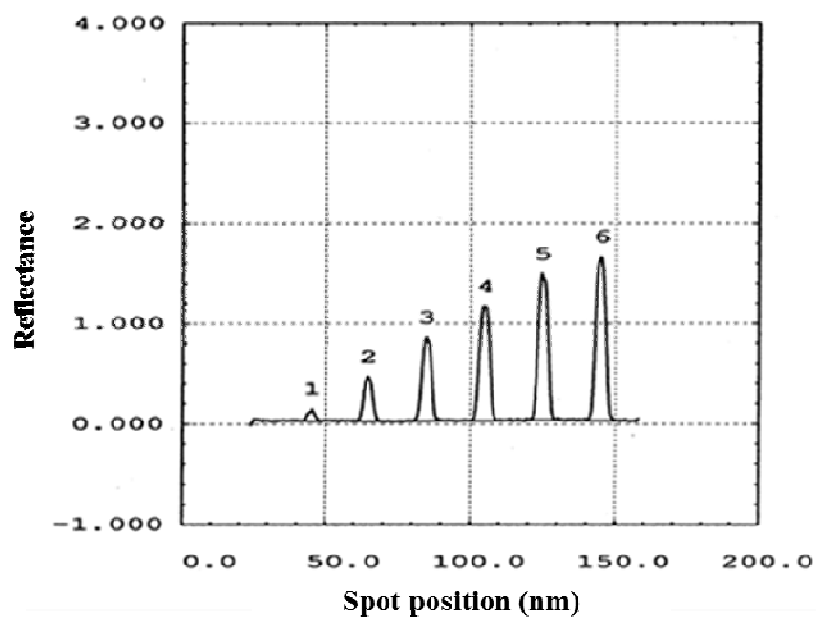


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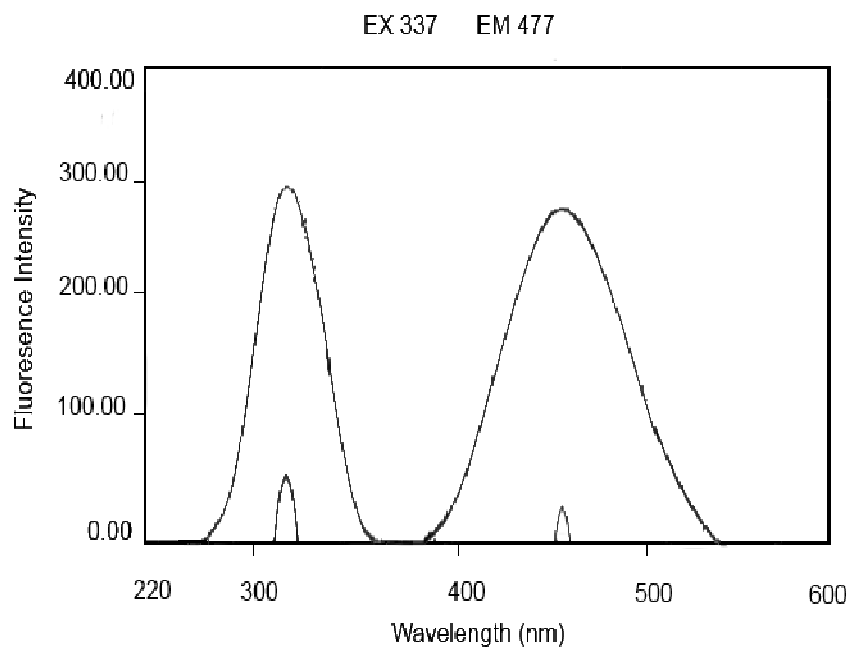


372

373 **Fig 3. : HPLC chromatogram at 245 nm for:**
 374 **(a) Closantel-Na ($5 \mu\text{g mL}^{-1}$) and bromohexine-HCl IS ($12 \mu\text{g mL}^{-1}$).**
 375 **(b) Alkaline degradates of closantel-Na ($5 \mu\text{g mL}^{-1}$).**
 376 **(c) Mixture of intact closantel-Na and its alkaline degradates ($3:7 \mu\text{g mL}^{-1}$)**
 377 **and bromohexine-HCl ($12 \mu\text{g mL}^{-1}$).**

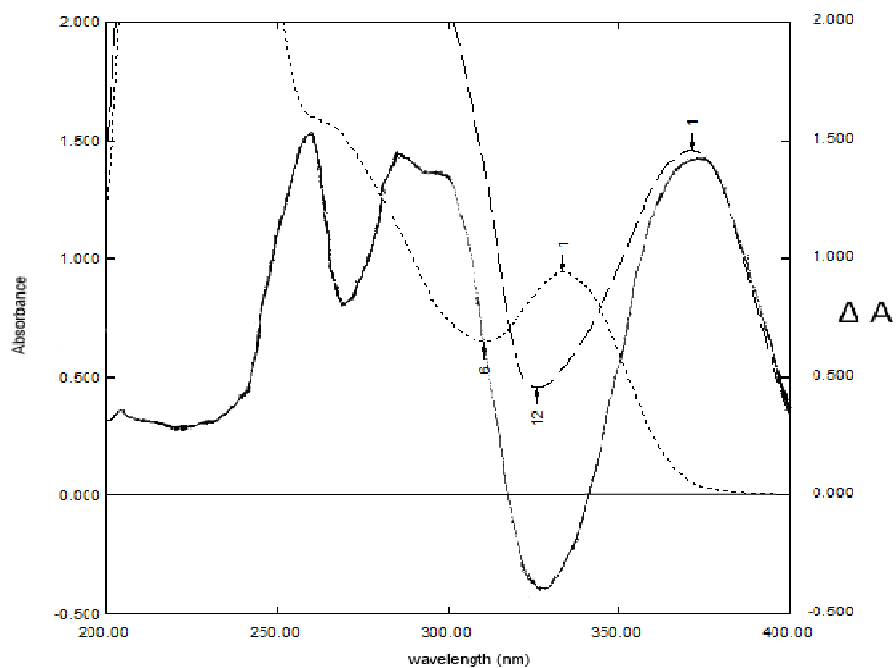


378
 379 **Fig4.:Densitometric chromatogram of closantel-Na (0.5-10 µg/spot)**
 380 **at 245 nm.**
 381

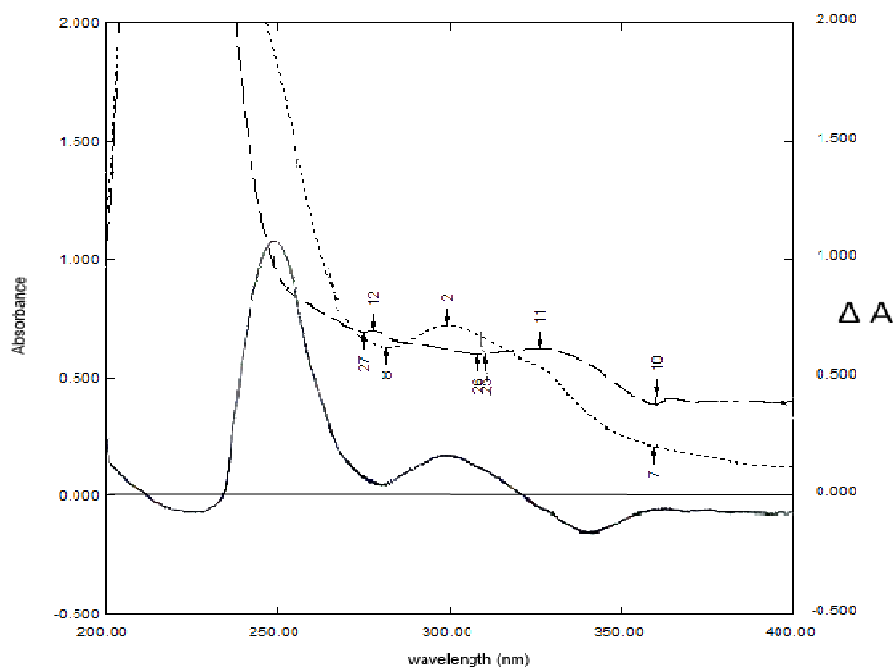


382
 383 **Fig 5.: Excitation and emission spectra of closantel-Na (3µg mL⁻¹) in**
 384 **ethanolic H₂SO₄ solution.**
 385
 386
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391 **Fig 6.: UV Spectra of closantel-Na ($100 \mu\text{g mL}^{-1}$) in ethanolic HCl (.....), in ethanol (---)**
392 **and their ΔA spectrum (—).**



393
394 **Fig 7.: UV Spectra of closantel-Na alkaline degradates ($100 \mu\text{g mL}^{-1}$) in ethanolic HCl**
395 **(.....), in ethanol (---) and their ΔA spectrum (—).**