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A stability-illustrating HPLC-DAD method for assessment of two veterinary anti-parasitic drugs: appraisal of the method's greenness and blueness

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Abstract

This paper represents an effective and reliable high-performance liquid chromatography-diode array detector (HPLC-DAD) method for the regular assay of Clorsulon (CLR) and Moxidectin (MOX) anti-parasitic drugs in injection solution and pure powder without derivatization processes. The mobile phase was composed of acetonitrile: methanol: water: acetic acid (56.0: 36.0: 7.5: 0.5 by volume). Besides, a Supelcosil C18 (4.60 mm ×15.0 cm, 5.0 µm) column was selected for completing the separation and quantitation of the two aforementioned veterinary drugs at a wavelength of 254 nm. The flow rate was set at 2.0 mL min^{-1} at the isocratic approach. We have conducted the degradation experiments using the HPLC-DAD instrument, adhering to the guidelines of the International Conference of Harmonization (ICH), subjecting CLR and MOX to light, heat, basic, acidic, and oxidative stressful conditions to figure out the ideal storage conditions and the possible medications that can be co-administered with them. CLR and MOX were quantified linearly from 400.0 to 1200.0 and from 40.0 to 120.0 µg mL^{-1}, respectively. The maximum recorded degradation results were in acidic, basic, and oxidative conditions. Therefore, strong basic or acidic medications and oxidants shouldn't be combined with CLR and MOX in a co-formulated medication. Greenness, carbon footprint, and blueness assessments for the novel method were conducted to verify the sustainability and functionality. The thirteen subdivisions in the GAPI pictogram, which were categorized as either green or yellow, refer to moderate green aspects. The final AGREE score of 0.56 and the majority of its subdivisions, ranging from dark green to yellow, indicated a relatively moderate level of greenness. This was primarily due to the significant acetonitrile content (56%) in the mobile phase. Using the HEXAGON method, the ultimate score is 0 out of 5 since the total calculated carbon footprint is less than 0.10. An eco-friendly method is one with a reduced carbon footprint score. The innovative HPLC method's functioning and utility are indicated via its overall BAGI score of 80.0. Generally, the outcomes of the AGREE and GAPI pictograms indicate that the HPLC-DAD has a greenness feature, despite its moderate sensitivity.

Keywords HPLC-DAD, Clorsulon, Moxidectin, BAGI, AGREE, GAPI, Green chemistry

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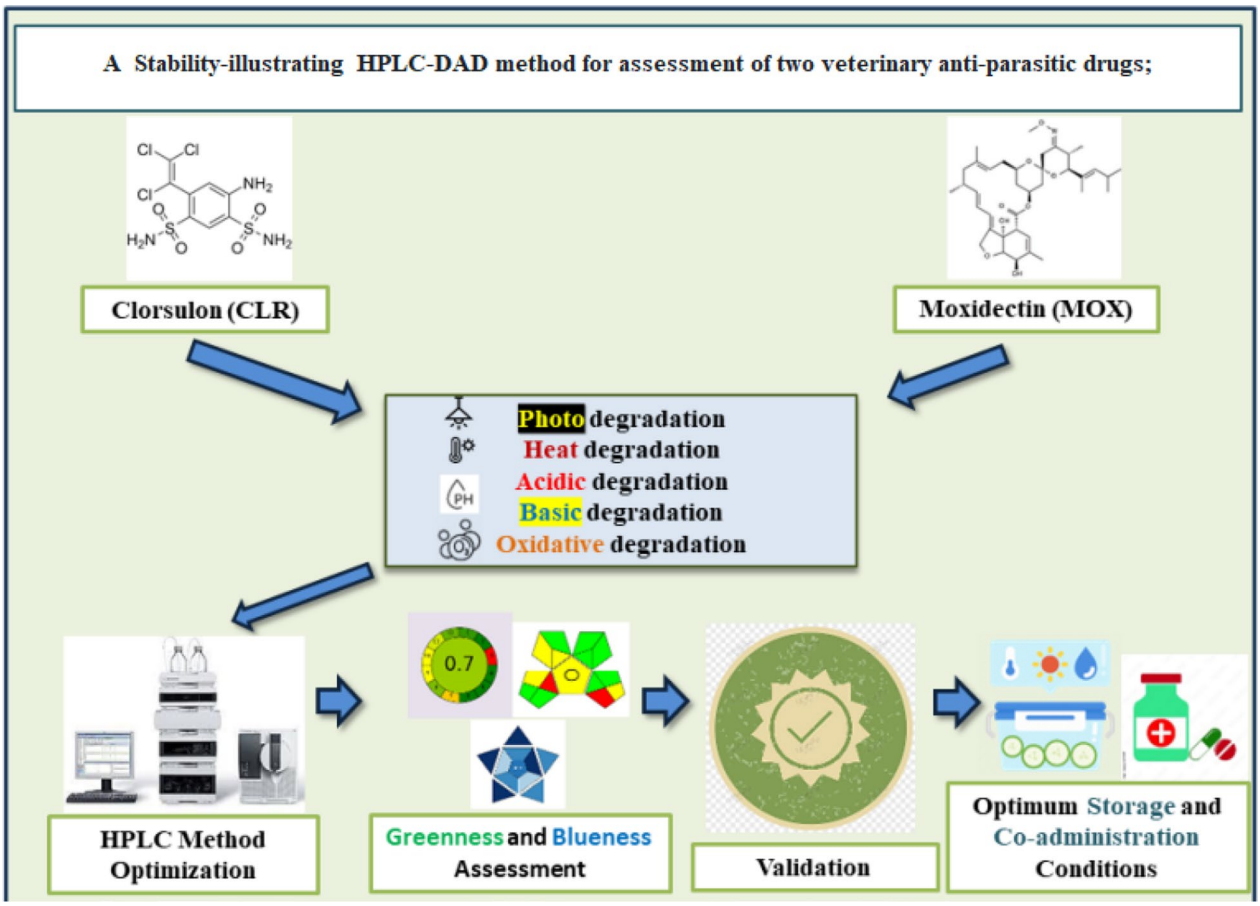
Graphical Abstract



Introduction

For precise drug quantification free from interference from any contaminants and/or degradations, an effective stability-indicating method must be developed [1–3]. Implementing an environmentally friendly and sustainable analytical technique is also important for maintaining a clean environment for our world and the health of scientists [4–7]. These days, there is a lot of interest in sustainability and green analytical chemistry. Techniques based on sustainable chromatography are frequently employed in the management of contaminants found in water, air, and soil [8, 9]. Applications for sustainability are also expanded to include the potential of extracting solvents being reused before chromatographic analysis and the decrease of extracting solvents [10–13]. It is greatly welcomed when sustainability principles are applied to chromatography-based procedures to guarantee both operator safety and environmental preservation. These environmentally friendly guidelines must be followed in their entirety [6]. Thus, the analyst should bear in mind that the quality and applicability of the developed method will be enhanced by the use of minimum hazardous chemicals. Green chemistry principles started to be required for pharmaceutical quality control on a daily basis [14–16].

Regarding this investigation, a recently studied veterinary benzenedisulfonamide derivative with strong fasciolicidal activity is called Clorsulon (CLR). Through specific antagonistic interactions with fluke phosphoglycerate mutase and kinase, it disrupts the energy metabolism. Cattle internal and external parasites are commonly treated and controlled with the use of CLR injectable medication as a parasiticide [17]. Fig. S1 supplementary data shows CLR, molecular weight 380.66 g mol^{-1}, chemical structure, and formula [$C_8H_8Cl_3N_3O_4S_2$]. By fermenting Streptomyces cyano-griseus, Moxidectin (MOX), a semisynthetic derivative of nemadectin, is produced. MOX's molecular weight is 639.819 g mol^{-1} and

its formula is [$C_{37}H_{53}NO_8$], revealed in Fig. S1 supplementary data. MOX is an anthelmintic medication used to prevent and treat intestinal and heartworms. It works by killing parasitic worms, or helminths [18]. It is present in medications administered for sheep, cattle, horses, dogs, and cats. Depending on the treatment, there are several ways to apply moxidectin: oral, topical, and injectable preparations. Moxisulon Solution for Injection is the brand name used to distribute the CLR and MOX injectable solution. Fig. S2 supplementary data shows the UV spectrum for both CLR and MOX employing a spectrophotometric detector.

Recently, quite a few HPLC studies for the CLR assay in their dosage form [17, 19–21]. Two HPLC techniques, one for CLR assay results in milk [22], the second in the kidney of cattle [23], and the UPLC method for CLR assay results in commercial bulk drug substance batches [24]. A UV-visible spectrophotometric method was used for the assay of CLR in its dosage form [25]. MOX was determined in human plasma using HPLC study [18], while in serum [26], in cattle tissues [27] using LC-MS, and in cattle hair by LC–MS/MS [28]. UV-spectrophotometric method used for the assay of MOX [29], spectrofluorimetric method [30], and matrix solid-phase dispersion technique for the determination of MOX in bovine tissues [31]. The LC-MS instrument's high cost and high energy consumption prevented it from being used on a daily basis for CLR and MOX tests in pharmaceutical companies' quality control [32]. Actually, as of the writing of this text, no stability-indicating HPLC-DAD procedures have been found that have been published for the analysis of CLR and MOX as a mixture simultaneously, either with or without chemical derivatization.

Because of its applicability and effectiveness, HPLC is the best practical method from chromatography in analyzing medications when contaminants and degradates coexist in dosage forms [33]. The main goal of this study is to use green chemistry procedures to deliver the first straightforward, environmentally friendly HPLC-DAD for CLR and MOX as a therapeutically effective mixture [34] together with their degradation byproducts. Furthermore, the objective was extended to suggest optimal storage settings for CLR and MOX mixtures in veterinary pharmacies by utilizing the stability-representative method's results. Finally, the two automated and current greenness assessment tools, AGREE and GAPI, have been used to evaluate the method's effectiveness in terms of environmentally friendly aspects.

Experimental

Reagents, parenteral formulation, and pure chemicals

The Drug Authority in Egypt (El-Maadi, Egypt) provided friendly supplies of Clorsulon (CLR), Moxidectin (MOX), a genuine, pristine impurity proportion less than 1.98%, and MOXISULON Solution for Injection (Merial S.A.S., France). A sterilized injectable solution that is prepared for usage containing CLR and MOX is called MOXISULON Solution for Injection. A milliliter (mL) of MOXISULON solution contains 400 mg of formal glycerol, 300 mg of propylene glycol, 100 mg of CLR, and 10 mg of MOX.

The chromatographic analysis employed gradient-grade acetonitrile and methanol content of at least 99.9% for each, which were purchased from the German company Sigma Aldrich Chemie GmbH in Steinheim. Acetic acid was purchased from El Nasr Pharm. (Abu-Zabal, Egypt). Internal preparation of deionized water was accomplished by using the (arium®) mini Ultrapure Water System (Geottingen, Germany). Prior to chromatographic analysis, all liquid solutions underwent filtering and degassing. In the filtration process, (Tisch®) nylon membrane filters with a pore size of 5.0 µm, 47.0 mm, were employed.

Instrumentation

1200 Series Agilent with G1316A TCC Thermostatted Column Compartment, G1367C HiP-ALS SL autosampler, online degasser (G1322A), quadruple gradient pump (G1312A), and temperature controller (G1316A) were used. For chemical detection and quantification, the UV detector (G1315B)'s chem station software was programmed. Additionally, Memmert Co.'s (Schwabach, Germany) equipment was used for the sonication procedure.

Procedure and chromatographic conditions

The optimization of the HPLC-DAD method was attempted multiple times. One variable at a time optimization was used to change a variety of factors, including composition, speed of the mobile phase, detection wavelength as shown in Fig. 1 and S3. The ideal parameters for producing the best chromatograms with regard to ultimate peak area, lowest backdrop noise, and sharpness for the main peaks for CLR and MOX are shown by Table 1.

Approaches for preparing standard and pharmaceutical solutions

A. Preparation of standard solutions

The primary standard solution was prepared by adding 70 mL of liquid mobile solution to 100 mL glass flasks after 200 and 20 mg of pure powder CLR and MOX were carefully added, one at a time, respectively. Each glass flask was then sonicated for thirty minutes. The glass flasks were then filled with the liquid mobile solution until the final volume reached 100 mL, giving concentrations of 2000.00 and 200.00 µg mL^{-1} for

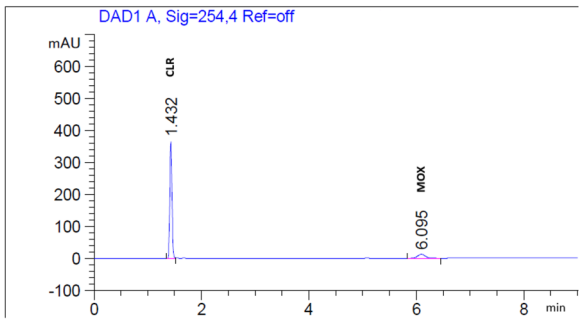


Fig. 1 RP-HPLC chromatogram of CLR (800 µg mL^{-1}) and MOX (80 µg mL^{-1}) throughout the best analysis subsets; employing the stationary phase of C-18, mobile phase of acetonitrile: methanol: water: acetic acid (56: 36: 7.5: 0.50 by volume), and flow rate 2 mL min^{-1} at 254 nm

CLR and MOX, respectively. The secondary standard solution was prepared by transferring 10 mL from the primary standard solution to a 50 mL volumetric flask and then diluted to volume using the mobile phase, giving concentrations of 400.00 and 40.00 µg mL^{-1} for CLR and MOX, respectively.

B. MOXISULON prepared liquid for injection

One milliliter of the injectable MOXISULON solution, which contained 100 mg of CLR and 10 mg of MOX, was carefully added to a 50 mL flask to prepare the primary standard solution, then 30 mL of liquid mobile solution was added and the glass flask was then sonicated for thirty minutes. The glass flasks were then filled with the liquid mobile solution until the final volume reached 50 mL giving concentration (2000.00, and 200.00 µg mL^{-1}) for CLR and MOX, respectively. 10 mL from (the primary standard solution) was transferred to 50 mL volumetric flask, then diluted to volume using the mobile phase giving concentration (400.00, and 40.00 µg mL^{-1}) for CLR and MOX, respectively.

Procedures for the analysis of the CLR and MOX stability study

In accordance with ICH guidelines, methodologies for HPLC degrading stabilization [35] were carried out to analyze CLR and MOX in the MOXISULON formula in various stressed settings. Table 2 provided complete descriptions of the various deterioration environments. Additionally, the blank solution, composed of all formulation components except the drugs under investigation, was examined for comparative purposes. It was set up by adding all of the previously listed inactive additives, expressed as a percentage, to the MOXISULON solution without CLR or MOX.

In terms of light treatment, from the previously prepared solutions in Sect. 2.4.A, concentrations of 800.00 and 80.00 µg mL^{-1} for CLR and MOX, respectively, were exposed to either UV radiation for 12 h or direct sunshine for two days before being filtered via a syringe filter. The studied drug liquids underwent an eight-hour thermally regulated water bath at 80 °C as part of the heat-based procedure. The studied drug solution was then quickly filtered through sterile syringe filters after cooling.

Using a 50 mL volumetric flask, 200 mg of CLR and 20 mg of MOX were mixed with 5 mL of 1 N HCl and NaOH, each one at a time for the acid and basic assessments. After that, the bottle was heated for 60 min in a thermally controlled water bath set at 80 °C, and 1 N HCl or NaOH was used to maintain the solution's pH at 7.0 ± 0.1. The final size was then achieved using the mobile phase and sonicating it for thirty minutes, giving a primary solution with concentrations of 4000.00 and 400.00 µg mL^{-1} for CLR and MOX, respectively. 10 mL from the primary solution was transferred to a 50 mL volumetric flask, then diluted to volume using the mobile phase, giving a secondary solution with concentrations of 800.00 and 80.00 µg mL^{-1} for CLR and MOX, respectively. Additionally, in H_2O_2 treatment, a 50 mL volumetric bottle containing 200 mg of CLR, 20 mg of MOX, and

Table 1 Chromatographic parameters for CLR and MOX analysis using the RP-HPLC chromatographic method

Stationary phase	Supelcosil C18 (4.6-mm×15-cm, 5µ)	
Mobile phase	Acetonitrile: Methanol: Water: Acetic acid (56:36:7.5:0.50, by volume)	
Detector	UV (254 nm)	
Pumping sytem	Isocratic	
Temperature	Room temperature at 25 ^{0}C	
The Injected volume	10 µL	
Flow rate	2.0 mL min^{-1}	
Total run time	9 min	
Retention times	CLR: 1.43 min	MOX: 6.05 min
	Acidic degradation products (3.50 min) Basic and oxidative degradation products (3.10 min)	

Table 2 The ratio of recovery to degradation for CLR (800 µg mL^{-1}) and MOX (80 µg mL^{-1}); the samples were subjected to (a) photodegradation, (b) thermal degradation, (c) acidic degradation, (d) basic degradation, and (e) oxidative degradation

Mode of degradation	The number of samples used (*n*)	An explanation of the conditions	Area		Assay %		Degradation %	
			CLR	MOX	CLR	MOX	CLR	MOX
Photo	3	Light for (48 h)/ UV for (12 h.)	1059.405	122.333	100.91	98.90	11.58	1.10
Standard deviation (SD)	–		0.50	0.10	–			
Thermal	3	At 80 °C for (8 h.)	928.292	120.852	88.42	97.70	0.91	2.30
Standard deviation (SD)			1.00	0.50	–			
Acidic	3	1 N HCl at 80 °C for (1 h)	836.835	78.359	79.71	63.35	**23.00**	**36.65**
Standard deviation (SD)			1.50	1.00	–			
Basic	3	1 N NaOH at 80 °C for (1 h)	808.385	82.268	77.00	66.51	20.29	**33.49**
Standard deviation (SD)			0.50	0.50	–			
Oxidative	3	0.50% H_2O_2 at 80 °C for (1 h)	760.502	83.749	72.44	67.71	**27.56**	32.29
Standard deviation (SD)	–		2.00	0.50	–			

Bold underlined values indicate the two highest degradation conditions for each drug

5 mL of H_2O_2 (0.50%) was placed in a thermally adjusted water bath set to 80 °C for 60 min. After that, the pH of the solution was adjusted to 7.0 ± 0.1 using 1 N NaOH, and 35 mL of the liquid system was added. The bottle was then shaken for thirty minutes using a sonicator. Finally, the liquid solution was diluted like in the previous manner to reach final concentrations (800.00 and 80.00 µg mL^{-1}) for CLR and MOX, respectively.

Evaluation of the validity of the HPLC approach

The International Conference of Harmonization (ICH) guidelines for endorsement of verification of techniques are considered regarding many parameters, including efficient linear manner, precision, specificity, accuracy, robustness, ruggedness, limits of detection, and limit of quantitation [36].

Evaluation of the expected HPLC method's environmental benefits using the AGREE and GAPI tools

The automated software AGREE [37] and GAPI [38] were mostly utilized for method greenness assessments in order to verify the risks to the environment and analysts. We looked at the created pictograms for evaluation and methodologies, whereas subdivisions in green stand for totally safe analytical processes. Numerous publications have proven the effectiveness and dependability of the aforementioned tools [39].

Results and discussions

Even though there aren't many published LC-MS and LC-MS/MS approaches for the CLR and MOX tests in pharmacokinetics investigations, high-performance liquid chromatography is the recommended apparatus for analyzing medicines in authentic shape, together with medicinal products, as the introduction describes due to concerns about energy and cost consumption [32]. Furthermore, the HPLC method's speed and environmental friendliness significantly increase its dependability and suitability for everyday drug assays [40]. The daily quality control in pharmaceutical firms greatly depends on the improvement of a straightforward and eco-friendly stability-proving technique for a combination of CLR and MOX.

Outcomes of accelerated degradation studies

Figure 2 showed the collective chromatograms for the CLR and MOX mixture in various intensive degradation conditions, illustrating how well the approach separates the two compounds from potential degradates. Besides, the chromatograms for degradation studies were also displayed in their original state in supplementary file; whereas figure S3 illustrates the chromatogram of pure standard of the studied drugs, figure S4 illustrates a blank sample, figure S5 illustrates the studied drugs under photo degradation, figure S6 illustrates the studied drugs under thermal degradation, figure S7 illustrates the studied drugs under acidic degradation, figure S8 illustrates the studied drugs under basic degradation, and figure S9 illustrates the studied drugs under oxidative degradation. In conclusion, there was a clear separation between the peaks of CLR and MOX from those of other degradates. Table 1 lists the minor degradation products for the different demanding conditions. The minor peaks had retention durations of 3.10 and 3.50 min, but the CLR and

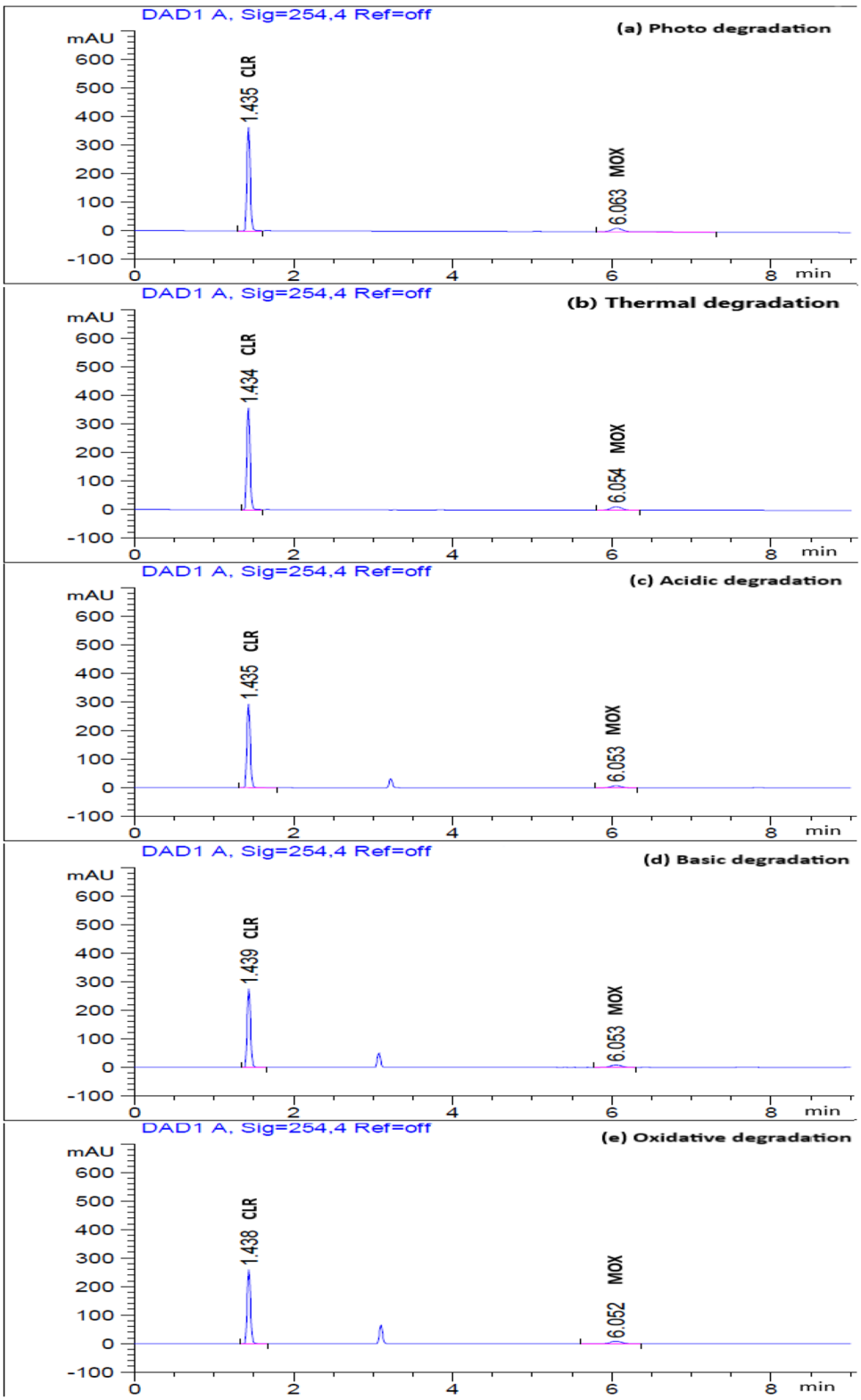


Fig. 2 HPLC chromatograms of the stability results of CLR (800 µg mL^{-1}) and MOX (80 µg mL^{-1}); the samples were subjected to (**a**) photodegradation, (**b**) thermal degradation, (**c**) acidic degradation, (**d**) basic degradation, and (**e**) oxidative degradation; the stationary phase was C-18, mobile phase of acetonitrile: methanol: water: acetic acid (56: 36: 7.5: 0.50 by volume), and flow rate 2 mL min^{-1} at 254 nm

MOX peaks had retention durations of 1.43 and 6.05 min. Table 2 displays the ratios of degradations. For CLR, the oxidative environment showed the highest degradation [about 27.56%], while the light degradation setting showed the lowest degradation [about 0.91%]. Likewise, for MOX, the acid environment showed the highest degradation [about 36.65%], while the light degradation setting showed the lowest degradation [about 1.10%]. One little peak emerged at 3.50 min as a result of the acidic degradations of the materials. The acidic degradations caused one small peak to appear at 3.50 min. The degradates in the basic and oxidative degradations were identical, giving only one peak that appeared at 3.10 min, and. There were no peaks for either photodegradation or heat-induced deterioration. The maximum observed retention times of 1.43 and 6.05 were measured over the whole 9-minute run to ensure that no additional small peaks were eluted from the column. The chromatographic conditions stated above, as reported in Table 1, enable rapid and efficient separation. The innovative study offers recommendations for CLR and MOX storage, highlighting how crucial it is to shield it from light and heat and the significance of using other drugs with caution, especially those that are oxidative, basic, or acidic.

Examining optimization for the HPLC technique

The C8 and cyanogen columns were eliminated from the selection process for the stationary phase. Conversely, as shown in Fig. 1, Supelcosil C18 (4.6 mm x 15 cm, 5 µ) produced acceptable resolution. Since CLR and MOX are basic in nature (pKa = 9.61 and 12.80 at 25 °C, respectively), the cyano column was disregarded. Furthermore, the stability and chromatographic behaviour of CLR and MOX may be impacted by the abundance of polar functional groups in MOX, such as secondary and tertiary amine groups, and the abundance of sulfoxide and hydroxyl groups in CLR. These functional groups may interact or establish hydrogen bonds on a cyano column, which could affect retention and separation. When the C8 column was compared to the C18 column, a wider peak was produced. As a result, C18 was chosen based on its peak form and was anticipated to have good degradation product resolution.

Our first preference for the mobile phase was acetonitrile, methanol, and water from an environmentally friendly standpoint. For the watery portion, a tiny amount of acetic acid was added. Because CLR and MOX are basic, adding acetic acid (pH = 4 ± 0.05) to the mobile system was also necessary to prevent peak tailing. The best mobile system components were also tested with several buffer types, and acetic acid alone was suggested as the pH adjuster. Furthermore, in order to improve peak uniformity, and resolution parameters an aqueous-free solvents, such as methanol or acetonitrile, were required especially for MOX which has high molecular weight and low solubility in water (0.50 mg/L) make its separation a challenging point [18]. Many non-aqueous solvents were tested, with ethanol being the most environmentally friendly option compared to acetonitrile and methanol. The chromatogram that was created for the ethanol case was insufficient. Furthermore, the researchers did their best to increase the ratio of water to organic solvents. However, inconvenient results were achieved in terms of peak symmetry and resolution. Using acetonitrile, methanol, water, and acetic acid (56: 36: 7.5: 0.50 by volume) as a mobile system, the best chromatogram was finally obtained. The wavelength of 254 nm was chosen for the DAD detector setup because it increases the sensitivity of the approach by coincidentally matching the λ_{max} for both MOX and CLR.

To produce the best chromatogram in the shortest amount of time, numerous attempts were undertaken to optimize the flow rate. The retention periods for CLR and MOX were comparatively lengthy when operating at 0.5 mL min^{-1}, free from degradation product interference. However, when operating at 1.0 mL min^{-1}, the resolution was insufficient. The best results were obtained when the flow rate was set at 2.0 mL min^{-1}. There were no other adjustments made to the ambient temperature.

Examining HPLC-DAD validation implications

To guarantee that the novel HPLC has a suitable linear scope and quantitation limit in addition to being robust, accurate, precise, specific, rugged, and reliable, the International Conference on Harmonization (ICH) practices were implemented [36]. Table 3 compiles all of the validation factor data, which were all satisfactory and in line with ICH principles. The supplemental PDF file S1 contains demonstrations of the comprehensive chromatograms for validation items. Figure S3 represented the HPLC chromatogram of pure standards for CLR and MOX using the aforementioned liquid system at 254 nm, while Figure S4 referred to the HPLC chromatogram of the blank sample. Therefore, the method specificity was concluded.

As shown in Table 4, five concentration values of each of the pure standards for CLR and MOX were chosen in order to examine linearity and create the regression equation. For CLR, the estimated linearity varied from 400.00 to 1200.00 µg mL^{-1}, while for MOX, it was between 40.00 and 120.00 µg mL^{-1}. Concerning the linear equation adaptations that were produced for each of CLR and MOX, the correlation coefficient value was close to unity. Though the CLR's calibrating equation was [Y = 1.3047 X + 2.0172], $r = 0.99988$, it was [Y = 1.5447 X + 0.6618] for MOX and $r = 0.99984$. A complete positive relation

Table 3 Validation data for the new stability-illustrating RP-HPLC method for CLR and MOX include linear range, robustness, detection and quantification limits, precision, accuracy, specificity, ruggedness, and robustness

Items for validation	Quantified values		Approved standards consistent with the ICH protocol [38]
	CLR	MOX	
Linearity and range	400.0 to 1200.0 µg mL^{-1} $r = 0.99988$	40.0 to 120.0 µg mL^{-1} $r = 0.99984$	$r \geq 0.99$
Precision (Calculated using 6 replicates)	0.15	0.37	RSD ≤ 2%
Accuracy (recovery ± SD)	99.82% ±0.440	99.58% ±1.379	100 ± 2%
Specificity/ Selectivity	Finely resolved peak for CLR from the other peaks for degradation products	Finely resolved peak for MOX from the other peaks for degradation products	Interference was not detected
LOD	18.67 µg mL^{-1}	2.17 µg mL^{-1}	Applying the equation: 3.3 x σ / S (σ = the standard deviation of the response, S = the slope of the calibration curve)
LOQ	56.58 µg mL^{-1}	6.58 µg mL^{-1}	Applying the equation: 10 x σ / S
Ruggedness (RSD of peak areas for 3 replicates)	1.38 (various days) 0.26 (various analysts)	1.64 (various days) 0.66 (various analysts)	For every modification, Pooled RSD should be ≤ 2%
Robustness (Slightly manipulates components of the mobile phase)	1.68	1.31	For every modification, Pooled RSD should be ≤ 2%

Table 4 The anticipated linear range from (400.00: 1200.00 µg mL^{-1} for CLR) and (40.00: 120.00 µg mL^{-1} for MOX), utilizing the best chromatographic conditions revealed stability-indicating RP-HPLC method

Conc. (µg mL^{-1})		Peak area		Mean	
CLR	MOX	CLR	MOX	CLR	MOX
400.00	40.00	525.394	61.740	527.4	61.5
		527.956	61.261		
		528.833	61.508		
600.00	60.00	782.792	91.971	783.6	92.1
		783.083	92.170		
		784.927	92.055		
800.00	80.00	1044.618	121.937	1045.3	122.8
		1045.958	123.585		
		1045.289	122.743		
1000.00	100.00	1298.089	152.808	1297.2	152.5
		1295.592	152.107		
		1297.778	152.492		
1200.00	120.00	1574.006	185.974	1575.3	185.8
		1576.079	185.765		
		1575.739	185.566		

$r = 0.99988$ for CLR
$r = 0.99984$ for MOX

between the variables CLR and MOX concentrations and their observed peak areas is indicated by correlation coefficient r values, which are close to unity. Additionally, as previously mentioned, 100 mg of CLR and 10 mg of MOX are included in each mL of the MOXISULON veterinary solution. With the use of the liquid mobile phase and successive dilutions, these can be measured effectively.

In order to test the accuracy of the procedure, Standard CLR and MOX are added to a blank sample that is made up of every component of the formulation, at predetermined amounts to spike the samples. The accuracy was assessed using a total of three tests at an identical quantity, and the concentrations' recovery percentages were computed. The assessment is done at 50%, 100%, and 150% of the middle concentration. Different concentrations are measured: for CLR, the middle concentration is 800.00 µg mL^{-1}, it is comparable to (400.00, 800.00, and 1200.00 µg mL^{-1}), correspondingly. Similarly, for MOX, the middle concentration is 80.00 µg mL^{-1}, it is comparable to (40.00, 80.00, and 120.00 µg mL^{-1}), correspondingly. Predicted on the estimated recovery rates for specified concentration ± SD, the accuracy was evaluated; 100% ± 2 should be the accepted threshold. The correctness of the expected HPLC-DAD method was demonstrated by the recovery percentages (99.99%, 99.32%, and 100.12%) for CLR and (98.93%, 98.64%, and 101.16%) for MOX that were shown in Table 5. The inclusion of the three various concentrations stated above demonstrates that the procedure is inclusive of accuracy at each locus in the range of linearity.

To evaluate technique precision, six measurements were performed again for the same concentrations of CLR (800.00 µg mL^{-1}) and MOX (80.00 µg mL^{-1}) on the same day. The statistical test of relative standard

Table 5 The accuracy of CLR and MOX at different concentrations (50, 100, and 150% of the intermediate concentration; 800.00 and 80.00 µg mL^{-1}, respectively) as indicated by the proposed stability-indicating RP-HPLC method

Concentration (%)	CLR			MOX		
	Peak area	Recovery %	Average recovery (%)	Peak area	Recovery %	Average recovery (%)
50% (400.00 µg mL^{-1} for CLR) (40.00 µg mL^{-1} for MOX)	526.011	100.21	99.99	60.901	98.48	98.93
	525.531	100.12		61.919	100.12	
	523.154	99.67		60.728	98.20	
100% (800.00 µg mL^{-1} for CLR) (80.00 µg mL^{-1} for MOX)	1043.824	99.42	99.32	120.619	97.51	98.64
	1042.882	99.33		122.501	99.03	
	1041.555	99.21		122.929	99.38	
150% (1200.00 µg mL^{-1} for CLR) (120.00 µg mL^{-1} for MOX)	1577.745	100.19	100.15	188.170	100.42	101.16
	1571.860	99.81		187.364	99.99	
	1581.684	100.44		187.539	100.08	
Maximum recovery	99.32%			98.64%		
Minimum recovery	100.15%			101.16%		
Mean	99.82%			99.58%		

deviation was used to evaluate the precision. The repeatability of the HPLC-DAD method was determined by RSD using Microsoft Excel computations, and the results were 0.15% and 0.37% for CLR and MOX, respectively. These results are shown in Table 6. A smaller RSD generally denotes a more precise approach.

By contrasting the retention durations and peak areas for the CLR and MOX standards, injection solutions, and placebo, the expected HPLC method's specificity was considered. The predicted HPLC-DAD's specificity is confirmed by Table 7's comparable retention periods and peak area values. Supplemental PDF file S1 contained illustrations of the HPLC chromatograms for the injectable fluids, placebo, and CLR and MOX standards. Additionally, as can be shown in Fig. 2, HPLC chromatograms for CLR and MOX in various stressful settings demonstrated an excellent resolution for CLR and MOX peaks without interfering with the peaks of degradates. This validated the new RP-HPLC approach's efficacy and selectivity.

Three replicates with different days and analysts were used to test the HPLC-DAD's ruggedness, utilizing the same concentration (800.00 µg mL^{-1}) for CLR and (80.00 µg mL^{-1}) for MOX. The ruggedness of the expected HPLC-DAD approach was evaluated using the pooled relative standard deviation (SD) values (1.38 and 0.26%) for CLR and (1.64 and 0.66%) for MOX, which are listed in Tables 8 and 9. Pooled RSD is a statistical method that calculates the combined variability from different data sets. A lower pooled RSD value indicates

Table 6 Using the RSD calculation, the precision of the estimated stability-indicating RP-HPLC method was assessed for six measurements at the same concentrations of CLR (800.00 µg mL^{-1}) and MOX (80.00 µg mL^{-1})

Number of measurements	Peak area		Recovery percent		Guidelines for acceptance [38]
	CLR	MOX	CLR	MOX	
Measurement no. 1	1049.399	123.912	100.35	100.81	
Measurement no. 2	1047.867	123.050	100.20	100.11	
Measurement no. 3	1048.650	123.153	100.28	100.19	
Measurement no. 4	1050.972	123.941	100.50	100.83	
Measurement no. 5	1050.216	124.058	100.43	100.93	
Measurement no. 6	1052.002	124.042	100.60	100.91	
Mean of peak area	1049.9	123.7	100.39	100.63	
SD	1.5	0.5	0.15	0.37	
RSD	0.15%	0.37%	0.15%	0.37%	RSD ≤ 2%

Table 7 Data for the retention time and peak area for the concentration levels of CLR (800.00 µg mL^{-1}) and MOX (80.00 µg mL^{-1}) in pure standard, test pharmaceutical solution, and placebo to assess the specificity of the innovative stability RP-HPLC approach

Test Title	CLR			MOX		
	Conc (µg mL^{-1})	Peak RT	Peak Area	Conc (µg mL^{-1})	Peak RT	Peak Area
Standard	800.0	1.432	1033.663	80.0	6.009	121.763
Test	800.0	1.424	1031.853	80.0	6.029	122.087
Placebo	0	No Peak was found	No Peak was found	0	No Peak was found	No Peak was found

Table 8 The detailed information for the proposed RP-HPLC method's ruggedness for CLR and MOX (interday) at the same concentration (800.00 µg mL^{-1}) for CLR and (80.00 µg mL^{-1}) for MOX

Replicate	CLR		MOX		Guidelines for acceptance [38]
	First day	Second day	First day	Second day	
No. 1 peak area (mAU)	1086.274	1060.398	125.984	123.748	
No. 2 peak area (mAU)	1086.831	1060.356	128.019	123.448	
No. 3 peak area (mAU)	1090.219	1061.799	128.180	124.765	
Average per day and recovery percent	1087.78 104.02	1060.85 101.44	127.39 103.62	123.99 100.78	
Pooled Mean	1074.3		125.7		
Pooled SD	14.8		2.1		
Pooled RSD	1.38		1.64		≤ 2%

Table 9 The detailed information for the proposed RP-HPLC method's ruggedness for CLR and MOX (analyst to analyst) at the same concentration (800.00 µg mL^{-1}) for CLR and (80.00 µg mL^{-1}) for MOX

Replicate	CLR		MOX		Guidelines for acceptance [38]
	First analyst	Second analyst	First analyst	Second analyst	
No. 1 peak area (mAU)	1055.214	1049.000	122.456	124.302	
No. 2 peak area (mAU)	1054.257	1049.863	122.951	122.951	
No. 3 peak area (mAU)	1052.875	1048.960	124.094	122.433	
Average per analyst and recovery percent	1054.12 100.80	1049.27 100.33	127.17 103.44	123.23 100.26	
Pooled Mean	1051.7		123.2		
Pooled SD	2.80		0.80		
Pooled RSD	0.26		0.66		≤ 2%

better precision and ruggedness of the novel stability-illustrating method.

In order to verify the robustness of the approach, triple replicates at the same concentration (800.00 µg mL^{-1} for CLR and 80.00 µg mL^{-1} for MOX) were evaluated in two mobile systems with marginally different water/methyl alcohol ratios. Based on the pooled relative SD values (1.68%) for CLR and (1.31%) for MOX shown in Table 10, it was determined that the predicted HPLC-DAD method was robust.

Table 3 displays the values and formulae used in the computation of the limits of detection and quantitation. For CLR, the limits of detection (LOD) and limits of quantitation (LOQ) were 18.67 µg mL^{-1} and 56.58 µg mL^{-1}, while for MOX, they were 2.17 µg mL^{-1} and 6.58 µg mL^{-1}, respectively. The formulas [3.3 x σ / S] and [10 x σ / S], respectively [36], were used to calculate the values for LOD and LOQ. S denotes the calibration curve's slope, while σ denotes the standard deviation of the responses of analytes.

Table 10 The detailed information for the robustness of the proposed RP-HPLC method for CLR and MOX at a concentration of (800.00 µg mL^{-1}) for CLR and (80.00 µg mL^{-1}) for MOX with mild variations in the composition of the mobile phase

Replicate	CLR		MOX		Guidelines for acceptance [38]
	1st condition*	2nd condition**	1st condition*	2nd condition**	
1	1096.252	1052.571	126.995	123.768	
2	1058.558	1047.004	124.586	122.535	
3	1057.908	1052.877	123.030	123.153	
Pooled Mean	1060.9		124.0		
Pooled SD	17.8		1.6		
Pooled RSD	1.68		1.31		**≤ 2%**

* Acetonitrile: methanol: water: acetic acid (55: 37: 7.5: 0.50 by volume)

** Acetonitrile: methanol: water: acetic acid (57: 35: 7.5: 0.50 by volume)

Table 11 The results of the suitable parameters for a novel RP-HPLC method for the CLR and MOX assay, including tailing factor, retention time, capacity factor, and number of theoretical plates

Guidelines for System Suitability	Recorded data for the Expected approach		Reference values [38]
	CLR	MOX	
Retention Time (Rt) ± SD	1.43 ± 0.02	6.05 ± 0.03	> 1
Capacity Factor (K)	Not calculated as the diluting liquid was the same mobile phase (no peak for pure mobile phase was recorded)		1–10 acceptable
Theoretical Plate (N)	4965	8346	The efficiency increases with its value
HETP = height equivalent to theoretical plate (cm/plate).	0.0030	0.0018	Column efficiency rises as the HETP value decreases. Column efficiency rises as the HETP value decreases
Tailing Factor (T)	0.93	0.94	≤ 2
Resolution factor (Rs)	26.54		≥ 1.5
Selectivity factor (α)	7.33		> 1

The percentage of back to front width at a 10% signal height was used to calculate the tailing factor. N = length of column (L)/ height for the theoretical plate (HETP). Rt was noted automatically. $K=(Rt- Rt_{(0)})/ Rt_{(0)}$, $Rs=2(Rt_b-Rt_a)/(W_b+W_a)$, $\alpha=K_b/K_a$

To ascertain whether or not the innovative LC approach was appropriate to the analysis purpose, its suitability was further evaluated, achieving satisfactory results [41], shown in Table 11.

Review for the sustainability of the novel LC analytical approach

The reliability of the HPLC approach was assessed using 2 computational green meter methodologies: AGREE [37] and GAPI [38]. In the GAPI pictogram (Fig. 3), there were only three red subsets identified: stats 1, 7, and 15 show that offline testing techniques were used, acetonitrile was used as one component in the mobile phase, and that there was no waste management for the reagents used. The remaining thirteen parts, which were categorized as either green or yellow, accepted the established HPLC-DAD stability method. Moreover, the proposed stability method's eco-friendly appraisal is approved by the AGREE pictogram (Fig. 4). Due to the position of the offline analytical device, only red subsection 3 was

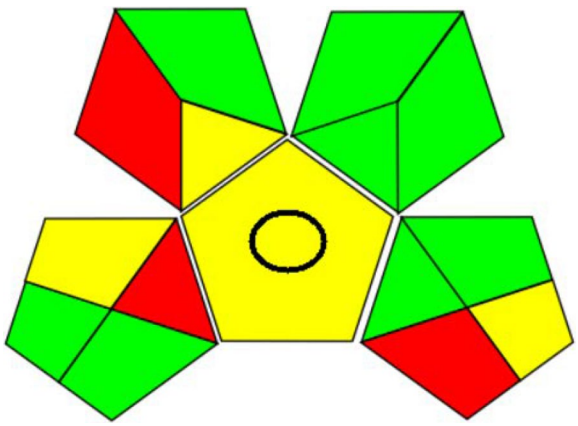


Fig. 3 Pictogram representing the Green Analytic Procedure Index (GAPI) for the HPLC-DAD method's expected stability for CLR and MOX

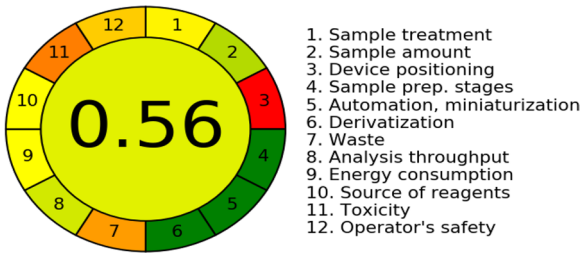


Fig. 4 Green analytical procedure Index (AGREE) pictogram for HPLC-DAD method for CLR and MOX, which indicates expected stability

observed. Subdivision 7 exhibited an orange color due to a mobile liquid waste volume of approximately 18 mL per run, which is considered a moderate amount. Besides, subdivision 11 was also orange in color because of acetonitrile toxicity (56% of the mobile phase composition). With a combined score of 0.56, the other subdivisions, which varied from dark green to yellow due to a high percentage of acetonitrile in the mobile liquid phase, also demonstrated the relative greenness merit.

The amount of waste generated can be computed by multiplying the flow rate by 9 mL of solvent (a combination of acetonitrile, methanol, water, and acetic acid) for the entire run period. Section 7 of the AGREE tool classifies this volume as moderate. As a result, a light orange hue is generated, signifying a moderate amount of waste. For instance, the greenest solvents are methanol and water. We made every effort to stay away from acetonitrile. However, employing acetonitrile without it produced inaccurate chromatograms. Methanol mixed with acetonitrile is a compostable and (non-perfluoroalkyl, non-polyfluoroalkyl) fluid. It is crucial to remember that methanol and acetonitrile are dangerous substances even though they are not PFAS chemicals.

Furthermore, carbon footprint (kg CO_2) is a crucial environmental and sustainable indicator. It might be determined via using the HEXAGON method and expressed as kg CO_2 equivalent, as per the 2019 study by Ballester-Caudet and colleagues [42]. Supplementary data (page 3, assessment of carbon footprint environmental impact) contains an equation for the calculation and findings. The total amount of carbon dioxide equivalent left behind was 0.00741 kg. Using the HEXAGON method, the ultimate score is 0 out of 5 since the total calculated carbon footprint is less than 0.10. An eco-friendly method is one with a reduced carbon footprint score [15].

Without a doubt, the new HPLC-DAD stability method and its application to the MOXISULON injectable solution, when compared to the previously reported LC-MS bioanalytical methods, have the advantages of being greener, more economical, consuming less energy, and being simpler to implement, as shown in Table 12.

The innovative HPLC Approach's benefits for the Pharmaceutical Sector

Considering the apparatus for HPLC is more affordable than that for LC-MS, the novel HPLC approach frequently serves as the initial cost-saving technique for the measurement of MOX and CLR. Furthermore, when the compounds are isolated from the related degradation products, the HPLC method—which is easier to use than the LC-MS method—is recommended for verifying efficacy and medication safety in frequent quality assurance

Table 12 A comparison between a few published HPLC, LC-MS, and LC-Ms/MS methods for the CLR and MOX assay and the new HPLC-DAD method's matrix, merit, total analysis time, cost-effectiveness, ease of implementation, and quantitation limits

Techniques	Merits and the matrix	Quantitative confines	Simplicity of use and cost-effectiveness	Total time spent on analysis	References
HPLC	Ivermectin and Clorsulon in Ivercam injection	25 µg mL^{-1}	Costly extraction techniques are used, assay CLR only without MOX	12 min	[19]
HPLC	Assay of Lvermectin and Clorsulon in Combined Pharmaceutical Dosage Form	100 µg mL^{-1}	Costly extraction techniques are used, assay CLR only without MOX	8 min	[20]
HPLC	Clorsulon and Ivermectin in pharmaceutical formulation	4 µg mL^{-1}	Costly extraction techniques are used, assay CLR only without MOX	25 min	[21]
HPLC	Ivermectin and Clorsulon in an injectable finished product	1000 µg mL^{-1}	Costly extraction techniques are used with high temperature, assay CLR only without MOX	45 min	[17]
HPLC-DAD	Comparatively green in pure powder and injection solution stability method	400 µg mL^{-1} for CLR 40 µg mL^{-1} for MOX	Economical and doesn't require any extraction procedures	9 min	Present approach

Graphical Abstract

analyses of CLR and MOX. Chromatography-grade liquids are generally easier to get, less expensive, and more widely available on the market than LC-MS-grade ones. Additional purification procedures are performed on LC-MS-grade solvents to reduce contaminants that may interfere with mass spectrometry analysis. In addition, the HPLC equipment uses about ten times less energy than the LC-MS one.

Suggestions regarding CLR and MOX Storage

Degradation ratio results shown in Table 2 indicate that strong basic or acidic medications and oxidants shouldn't be combined with CLR and MOX in a co-formulated medication. It is best to keep CLR and MOX out of direct sunlight and heat.

Applicability and functionality of the method using the Blue Applicability Grade Index (BAGI) method

Manousi and her team [43] published the BAGI tool lately in 2023. In order to provide a pictogram and a score that demonstrate the applicability and efficiency of an analytical approach, BAGI takes 10 elements into account. To make the analytical process considered practical, it is recommended that the final score be higher than 60. The innovative HPLC method's functioning and utility are indicated by its final score of 80.0 in Fig. 5.

Fig. 5 Evaluation of the novel method's blueness using the BAGI methodology

Restrictions and potential futures

Since our main objective was to assess both CLR and MOX in sterile pharmaceutical solutions utilising an accessible and easy-to-use chromatographic apparatus, animal tissues and fluids were not used in the testing of the HPLC-DAD approach. Furthermore, because the LC-MS approach is able to identify at the nanogram scale, it may be the best method for evaluating CLR and MOX in animal tissues and fluids. While doing this research, our laboratory did not have access to LC-MS or GC-MS, which are suggested methods for determining the molecular weight of each degradate. Furthermore, more investigation is needed to clarify the mechanism of CLR and MOX degradation. In addition, other detectors like the refractive index, corona spray, and evaporative light scattering detector (ELSD) could be explored in comparative stability experiments to obtain a comprehensive view of their degradation profiles.

Conclusions

For the first time, the straightforward, reliable, sustainable, and consistency-suggestive LC approach was improved to evaluate Clorsulon and Moxidectin in pure and injectable solutions. Furthermore, a ratio of 0.91% in the light stimulus to 27.56% in the oxidative environment for CLR and 1.10% in the enticement of light and 36.65% within the acidic conditions for MOX was observed for the degradation of Clorsulon and Moxidectin in all severe conditions. In less than nine minutes, the peaks for Clorsulon and Moxidectin were clearly separated from the peaks for all other degradation products. Refrain from exposing Clorsulon and Moxidectin to heat or light, and use caution when co-formulated with basic, acidic, or oxidative medications. Finally, the new approach worked well for repeatable tests for Moxidectin and Clorsulon in pharmaceutical companies' quality control laboratories.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13065-025-01401-9.

Supplementary Material 1

Author contributions

"M. A.:. Investigation, Data curation, resources, writing-original draft, writing-review, and editing. H.S. and F. K.: Methodology, funding acquisition, project administration, and editing. A. N.: Writing-review and editing. L. M.: Methodology, investigation, editing and reviewing. M. G.: Data curation, writing-review and editing, and project administration. All authors reviewed the manuscript."

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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