Egypt. J. Chem. **Vol. 67**, No. 10 pp. 559- 571(2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Chromatographic and UV−Spectrophotometric Methods for Simultaneous Determination of Ketoprofen along with Tilmicosin or Tylosin in Poultry Plasma and Veterinary Formulations

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Abstract

Two sensitive and accurate methods UPLC−MS and UV−spectrophotometry were adopted for the determination of ketoprofen simultaneously in two binary mixtures with the phosphate salts of either tilmicosin or tylosin. UPLC−MS method was applied to conduct efficient separation of the drugs. For ketoprofen, R^2=0.9999 was obtained over the concentration range of 0.5−5.0 ng mL^{-1}. While the range of 1.0−10.0 ng mL^{-1} for each of tilmicosin and tylosin showed correlation coefficients of 0.9999 and 0.9998, respectively. Four UV−spectrophotometric methods; dual wavelength, mean centering, ratio difference and ratio derivative methods were adopted for the determination of these drugs in propylene glycol. For the UV−spectrophotometric methods, the estimated linearity ranges for ketoprofen (2.0−30.0 µg mL^{-1}) and both tilmicosin and tylosin (5.0−100.0 µg mL^{-1}) showed excellent correlation coefficients. UPLC−MS and UV−spectrophotometric methods were validated following ICH guidelines. It is noteworthy that the two techniques are the first to be developed for the simultaneous quantification of Ketoprofen along with tilmicosin, or tylosin, in both bulk and two new veterinary formulations Ketotilmizide ® and Painless Plus ®. Furthermore, the proposed UPLC−MS method was utilized efficiently for the simultaneous estimation of a binary mixture of ketoprofen with either tilmicosin or tylosin in spiked biological poultry plasma samples.

Keywords: Ketoprofen; Tilmicosin; Tylosin; UPLC; Plasma; UV−Spectrophotometry.

1. Introduction

Macrolide antibiotics are protein synthesis inhibitors that act by attaching to the 50S component of the bacterial ribosome [1]. The studied macrolide; tilmicosin and tylosin were universally used for the treatment of various disorders. Tilmicosin is the best choice for respiratory infections in broiler chickens [2]. Tylosin has an anti−Gram−positive bacteria effect with a wide range of safety [3]. Additionally, tylosin is considered as a growth promoter in animal feeds [4]. Bacterial infections are usually accompanied by inflammatory manifestations, including fever, redness, local pain, and soreness [5]. Thus, it is essential to use nonsteroidal anti−inflammatory drugs to alleviate these symptoms. This created a demand for an anti−inflammatory antibiotic combination. Therefore, a new veterinary formulation combining ketoprofen as an analgesic and antipyretic with macrolides antibiotics, tilmicosin or tylosin for the treatment of infectious disorders is of significant importance.

Scientists have documented multiple analytical methods for detecting ketoprofen, tilmicosin, and tylosin in their pure forms, veterinary medications, and biological samples. The techniques employed in the previous studies encompass potentiometry [6,7], IR-spectroscopy [8,9], UV−Spectroscopy [10,11] and gas chromatography [12,13]. Multiple approaches have been outlined for the quantitative estimation of the investigated drugs using HPLC combined with mass spectrometry or a diode array ultraviolet detector [14-21]. It was found that a potentiometric sensor was developed to estimate the concentration of ketoprofen and to detect tilmicosin and tylosin residues [6,7]. However, potentiometric methods have inherent limitations such as instability

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Receive Date: 15 July, 2024, Revise Date: 04 September 2024, Accept Date: 11 September 2024
DOI: 10.21608/ejchem.2024.304663.10024

and calibration difficulties.

IR-spectroscopy was employed to detect ketoprofen with a high level of sensitivity ranging from 1000 to 4000 µg mL^{-1} [8]. Also, non-destructive and cost-effective mid-infrared spectroscopy combined with chemometrics was utilized to determine tylosin residues in milk [9]. Furthermore, Hassan A.A. et al [10] used $NaHCO_3$ as a diluent to analyze ketoprofen spectrophotometrically within the concentration range of 2.5-15 µg mL^{-1}. While the derivative spectrophotometry was employed for measuring tylosin and enrofloxacin simultaneously in various samples of chicken muscle, liver, and kidney [11]. Although the IR and UV-spectrophotometric techniques are simple and cost-effective, no method has been reported for the quantification of the proposed drugs simultaneously.

Gurupadayya B.M. et al [12] developed a gas chromatographic method to quantify ketoprofen in its pharmaceutical formulations within the concentration range of 20–100 µg mL^{-1}. In addition, GC-tandem mass spectrometry was applied to quantify tilmicosin residues in poultry muscle and pork following precolumn derivatization [13]. However, it is well-known that GC is a costly method with complex procedures for sample preparation.

Shishov, A [14] developed HPLC-MS method for the determination of ketoprofen in beef liver samples. The separation was carried out using methanol and 0.05% aqueous solution of formic acid as a mobile phase. While Andraws, G., and Trefi, S. [15] suggested an ion-pair HPLC method to detect ketoprofen using a mobile phase consisting of cetrimide and acetonitrile mixture. Although this approach has the advantage of no use of buffers in the mobile phase, the column could be damaged.as some drug ions tend to form strong associations with the stationary phase. Nevertheless, once the initial column characteristics are modified, the column will not be restored to its previous state, even after thorough column flushing. Also, several RP-HPLC methods have been reported for the analysis of ketoprofen, tilmicosin and tylosin that apply UV detection [16-21].

However these methods were lacking sensitivity and selectivity for the determination of the target analytes. It was obvious from the thorough analysis of the literature that the three active components we investigated were never simultaneously quantified using a singular analytical method. Due to the high level of sensitivity, specificity and versatility of UPLC-MS, it is the technique of choice in our study. Moreover, UPLC-MS provides rapid separation along with crucial data on the molecular ion, which is valuable for confirming the chemical's identity.

This work introduces effective and precise UPLC−MS and UV−spectrophotometric methods for measuring the amounts of ketoprofen and tilmicosin, or ketoprofen and tylosin, in two recently created veterinary formulations. The approaches are validated in compliance with ICH requirements. Furthermore, our objective is to optimize the UPLC-MS parameters and assess the method's efficacy in analyzing the three medications in chicken plasma samples that have been artificially spiked with these drug substances.

2. Materials and methods

1.1. Chemical and reagents

Pure ketoprofen; B. N. KPO−2102001, tilmicosin phosphate; B. N. K81170304/ WS and tylosin phosphate; B. N. L210303017. The drugs were kindly supplied by Hubei Xunda Pharmaceutical Co., Ltd, NINGIXIA TAIRUI Pharmaceutical Co., Ltd, NINGIXIA TAIYICIN BITECH Co. pharmaceutical industries company; Ltd, respectively. The purity is 98.8%.

Ketotilmizide® injectable solution; B. N. RDS−201, the product of Delta Pharma for Amoun vet company, is labelled to contain 90.0 mg mL^{-1} of ketoprofen and 300.0 mg mL^{-1} of tilmicosin. Painless Plus® injectable solution; B. N. PPS−011, the product of Arab company for gelatin and pharmaceutical products (Arab caps) for Vetopharm Nerhadou International, is labeled to contain 60.0 mg mL^{-1} of ketoprofen and 200.0 mg mL^{-1} of tylosin. Both products were obtained from the local market.

Acetonitrile, (Sigma Aldrich, Germany). Methanol, (Fisher, England). Formic acid pure analytical grade Merck (Darmstadt, Germany). Propylene glycol (Adwic, Cairo, Egypt).

1.2. Instrumentation

The UPLC (Waters, USA) connected to H−Class system, Electrospray ionization and a tandem mass spectrometer in addition to C_{18} column (50.0 mm × 2.1 mm × 1.7 µm). A 3.5 kV was the value of capillary voltage with 20 V cone voltage and the voltage applied was 2.5 V, 150◦C was the temperature of the source while was desolvation gas temperature. The flow rate was adjusted to 0.4 $mL.min^{-1}$, with nitrogen being employed as both the desolvation and cone gas. In addition, Spectrophotometer (UV−Vis, Shimadzu 1601, Japan) and pH meter (Hanna, Romania) were used.

1.3. Standards and QC samples Preparation

1.3.1. UPLC−MS

Stock solutions of 100.0 µg mL^{-1} of each standard drug were dissolved in aqueous methanolic solution. Dilution of the working standard solution was done in methanol at appropriate low concentrations to prepare 50.0 ng mL^{-1} of ketoprofen and 100.0 ng mL^{-1} of tilmicosin and tylosin.

1.3.2. UV−spectrophotometry

Propylene glycol was used to make stock solutions with a concentration of 1000.0 µg mL^{-1}. Then, three separate working standard solutions of 100.0 µg mL^{-1} were prepared by proper dilution.

3. Procedures for method validation

The suggested analytical method was validated based on linearity, accuracy, precision, and selectivity.

3.1. UPLC−MS Linearity

Accurately measured aliquots of working standard 50.0 ng mL^{-1} equivalent to 5.0−50.0 ng of ketoprofen were diluted one−tenth using a mixture of methanol and water in a 1:1 ratio (v/v). Similarly, one−tenth dilution was made for 100.0 ng mL^{-1} tilmicosin and tylosin working standard solutions to obtain solutions equivalent to 10.0−100.0 ng. Finally, using the selected conditions of chromatography; **Table 1**, 10 µL of each prepared dilution were injected in triplicate to obtain the calibration curve regression equation. Relating the peak area to the drug concentration to determine the regression parameters.

3.2. UV−spectrophotometry Linearity

Propylene glycol was used to dissolve accurately measured aliquots of either the stock or the working standard solutions in 10 mL volumetric flasks. Serial dilutions equivalent to a range of concentration of 2.0−30.0 µg mL^{-1} for ketoprofen, and a range of 5.0−100.0 µg mL^{-1} for tilmicosin and tylosin were prepared. The scan of the produced solutions was from 200.0 to 400.0 nm to get zero−order spectra.

3.2.1. Dual wavelength method

Direct measurement of the three drugs was conducted where ketoprofen was estimated in the presence of either tilmicosin or tylosin at 255−317 nm and 257−311 nm, respectively. The two wavelengths 239 and 274 nm were the best for the determination of each of tilmicosin or tylosin with ketoprofen. The absorbance differences were plotted against the respective drug concentration to create the calibration curves, and then precise equations of regression were computed accurately.

3.2.2. Mean centering method.

Ketoprofen spectra were divided by 30 µg mL^{-1} tilmicosin or tylosin spectrum followed by mean centering of the obtained spectra. The amplitude of the observed peaks was found at 246 nm. Two calibration curves were constructed correlating the mean centered peak amplitudes to the corresponding ketoprofen concentrations to construct two calibration curves to be used for calculating the regression equations. The same procedure was adopted for the quantification of tilmicosin or tylosin using a spectrum of 30 µg mL^{-1} ketoprofen as a divisor. The calibration curves were constructed by relating the peak amplitudes at 315 nm for tilmicosin and 314 nm for tylosin to their respective concentrations. Regression equations were derived from these data.

3.2.3. Ratio difference method

The amplitude difference of ratio spectra at 245−270 nm, 300−270 nm and 304−270 nm for ketoprofen, tilmicosin and tylosin were plotted versus the corresponding concentration to construct the calibration curves, and the regression equation of each drug was determined from these curves.

3.2.4. Derivative ratio method

The first derivative of the ratio spectra (^{1}DR) of the cited drugs were calculated using Δλ=4 nm and a scaling factor of 1. Ketoprofen had first derivative signals at 218, 238 and 259 nm, whereas tilmicosin and tylosin ^{1}DR amplitudes were at 285 and 320 nm. Then, the signal values were plotted versus the corresponding drug concentration, then regression equations were derived.

3.3. Assay of laboratory binary mixtures

3.3.1. UPLC MS/MS method

In 10 mL volumetric flasks, aliquots of the standard ketoprofen solution (50.0 ng mL^{-1}) and the tilmicosin standard solution (100.0 ng mL^{-1}) were combined. Volumes were diluted with methanol: water (1:1, v/v). Inject 10 µL of the resulting mixes as shown in **Table 1**. The binary mixtures of ketoprofen and tylosin were prepared in the same manner. The drugs concentrations were calculated using the respective regression equations obtained following the methods described under "3.1."

3.3.2. UV−spectrophotometric method

Different aliquots of the working solutions equivalent to 20.0–300.0 µg for ketoprofen and 50.0–1000.0 µg for either tilmicosin or tylosin; were delivered into 10 mL volumetric flasks. Then, propylene glycol was used as a diluent. The spectra of the obtained solutions were collected. The

calculation of the drug concentration was done using the respective regression equation obtained following the methods described under "3.2."

3.4. Application to Veterinary Formulations

3.4.1. UPLC MS method

Five Ketotilmizide® injectable solution contents were mixed. A solution labelled to contain 9.0 ng mL^{-1} ketoprofen and 30.0 ng mL^{-1} tilmicosin was prepared using methanol: water (1:1, v/v). The Content of five Painless plus® injectable solutions was well mixed. A solution claimed to contain 6.0 ng mL^{-1} ketoprofen and 30.0 ng mL^{-1} tylosin was obtained using the same diluent.

3.4.2. UV−spectrophotometric methods

Ketotilmizide® and Painless plus® were dissolved in propylene glycol to obtain two clear solutions. Ketotilmizide® solution is assumed to contain 45.0 µg mL^{-1} ketoprofen and 150.0 µg mL^{-1} tilmicosin, whereas Painless plus® is claimed to contain 30.0 µg mL^{-1} ketoprofen and 100.0 µg mL^{-1} tylosin. Analysis of the prepared solutions was achieved by UPLC−MS and UV−spectrophotometric methods as detailed under "3.1." and each drug concentration was found using the respective equation.

3.5. Application to Poultry Plasma

Into two sets of centrifuge tubes, one mL of the chicken plasma was added and spiked with various aliquots of the standard solutions of 50.0 ng mL^{-1} ketoprofen equivalent to 0.5−5.0 ng of ketoprofen. The first set of flasks were loaded with 100.0 ng mL^{-1} tilmicosin in the range of 1.0−10.0 ng. Similarly, the other set was treated with 100.0 ng mL^{-1} tylosin equivalent to 1.0−10.0 ng.

The spiked samples were mixed with three mL of acetonitrile to promote protein precipitation. Then, the mixture was subjected to vortex mixing for 1 min., followed by centrifugation at 5000 rpm for 20 min. After filtration through syringe filters (0.22 µm), the supernatant was passed into 10-mL volumetric flasks to be diluted to the mark with 50% aqueous methanol.

In addition, a blank plasma sample unspiked with the two drug mixes was also prepared using the above procedure to ensure the absence of the cited drugs in the poultry chicken plasma. Ten µL of each solution was analyzed by UPLC−MS method as the procedure described under "3.1." The average peak areas were plotted versus the corresponding drug concentration. The regression equations were derived from which the recovery % was calculated.

The blood samples were withdrawn from the brachial wing vein of an adult chicken [22, 23]. Sample withdrawal was conducted following a protocol approved by the Ethics Committee, Faculty of Pharmacy (Girls), Al-Azhar University, Egypt (NUB-439–2023).

4. Results and Discussion

4.1. UPLC−MS method

We developed a novel optimized UPLC method for quantification of the three cited drugs in the two binary mixtures. The stationary phase utilized was Acquity BEH C_{18} with dimensions of 100.0 × 2.1 mm. Multiple mobile phases were employed, including aqueous acetonitrile, buffered acetonitrile (pH 6.5) and 0.1% acidified acetonitrile. Effective gradient separation was conducted using mobile phase mixes as eluent. The composition of mobile phase A is aqueous 0.1% formic acid, while mobile phase B consists of 0.1% formic acid in acetonitrile. The conditions of gradient elution are illustrated in **Table 1**

Various wavelengths ranging from 200.0 to 400.0 nm and flow rates ranging from 0.30 to 1.50 $mL.min^{-1}$ were studied. It was proved that the wavelength with the highest detector response was 254 nm, and the ideal flow rate was found to be 0.40 $mL.min^{-1}$, as indicated in **Table 1**.

Table 1: Chromatographic conditions of the UPLC−MS method

Column	Acquity BEH C_{18} column (50 mm × 2.1 mm × 1.7 µm) (waters, Ireland)
Mobile phase	A: 0.1% formic acid in H_2O. B: 0.1% formic acid in acetonitrile
Gradient elution	Different ratios of A and B at each time interval : 0 min.: 95 % A+5 % B, 0.5 min.: 95% A+5 % B, 4.5 min.: 0 % A+100 % B, 6 min.: 0 % A+100 % B, 7 min.: 95 % A+5 % B, 7.5 min.: 95 % A+5 % B.
Flow rate	0.4 mL min.
Inj. Volume	0.4 µL
Temp	Ambient
Detector	MS−SIM

In summary, the most optimal result was obtained with a short analysis, where a distinct and well−defined peak was found at a retention time of 4.04±0.14 min. for ketoprofen, due to m/z=255.2. Tilmicosin peak appeared at Rt 3.20±0.11 min., due to a peak at m/z=869.3, whereas tylosin showed a peak at Rt 3.44±0.13 min., due to a peak at m/z=917.2. All m/z appeared at [M+H]+ because of the positive ionization mode of the ESI; **Fig. 1.**

Unfortunately, the peaks of tilmicosin and tylosin could not be efficiently separated, so ketoprofen was estimated in two binary mixtures, i.e., in the presence of either tilmicosin or tylosin. On the other hand, the suggested UPLC method was effectively utilized to determine the medicines under investigation in poultry plasma without any interference. Well−resolved peaks of the targeted drugs ions appeared at the obtained retention time without any interfering substances Also, the representative chromatograms of plasma samples spiked with ketoprofen and tilmicosin; or ketoprofen and tylosin were compared with the chromatogram of blank plasma sample. It was obvious that neither interfering nor coeluting components appeared, confirming method selectivity.

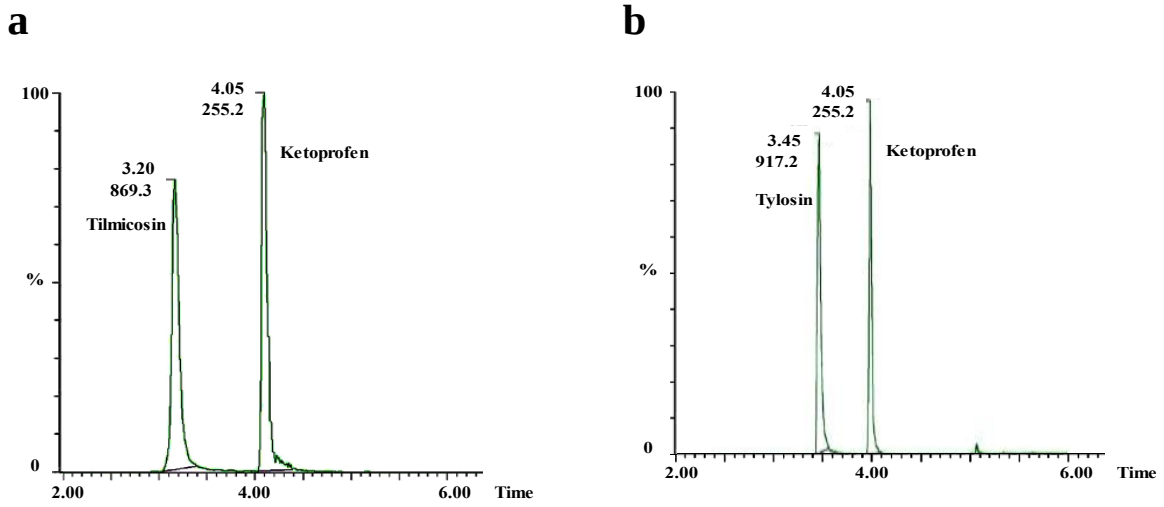

Fig. 1. Single ion chromatogram of a) ketoprofen (0.1 µg mL^{-1}) and tilmicosin (0.3 µg mL^{-1}) and b) ketoprofen (0.1 µg mL^{-1}) and tylosin (0.3 µg mL^{-1}).

4.2. UV− spectrophotometric methods

Ketoprofen absorption band strongly overlaps with that of tilmicosin and tylosin; **Fig.2**. The presence of propylene glycol (a common solvent usually used in the commercially available veterinary formulations) interferes significantly with the spectrophotometric estimation of the target analytes [24]. This resulted in difficulty in the spectrophotometric QC analysis of ketoprofen due to the presence of either tilmicosin or tylosin. As a result, three methods of manipulating ratio spectra were selected for the analysis of the cited drugs in their pure forms, binary mixture, and veterinary formulations. The methods are the mean centering method, ratio spectra and first derivative ratio method using propylene glycol as the solvent to cancel its interfering effect. An efficient selection of a divisor concentration is a crucial determinant of sensitivity and noise [25]. A fourth method was the conventional dual wavelength that affords good resolution of the interfering spectra with minimal data manipulation.

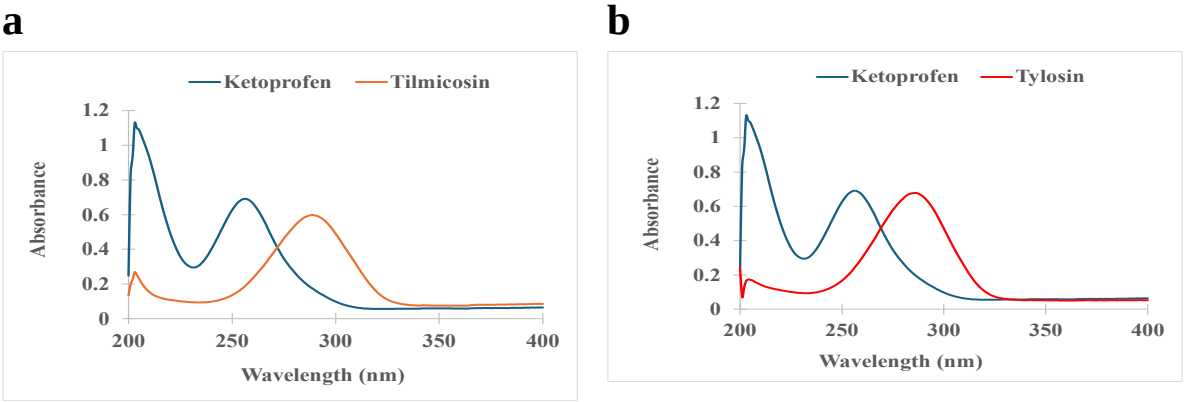

Fig. 2. Zero−order absorption spectra of a) ketoprofen 10 µg mL^{-1} and tilmicosin 30 µg mL^{-1} b) ketoprofen 10 µg mL^{-1} and tylosin 30 µg mL^{-1}

4.2.1. Dual wavelength method

Dual wavelength is known to offer an efficient tool for a better resolution when signal overlap exists [26]. This approach is based on the principle that the concentration of the component of interest is directly proportional to the absorbance difference between two points on the mixture spectra. From the overlain spectra shown in **Fig. 2**, the wavelength couples selected for the estimation of ketoprofen in the presence of either tilmicosin or tylosin were 255−317 nm and 257−311 nm, respectively. While the two wavelengths 239−274 nm were selected for determination of each of tilmicosin or tylosin with ketoprofen.

4.2.2. Mean centering method.

This approach relies on the technique of mean centering of ratio spectra [24]. Upon determination of ketoprofen, 30 µg mL^{-1} tilmicosin or tylosin were chosen as a divisor. The calculation of the mean centering of the ratio spectra was in the wavelength range of 210−300 nm and reasonable linearity was obtained at 246 nm; **Fig. 3 a & b**. For tilmicosin or tylosin estimation, the selected divisor was 30 µg mL^{-1} ketoprofen over 200−350 nm and the determination was done at 315 and 314, respectively; **Fig. 3 c & d**.

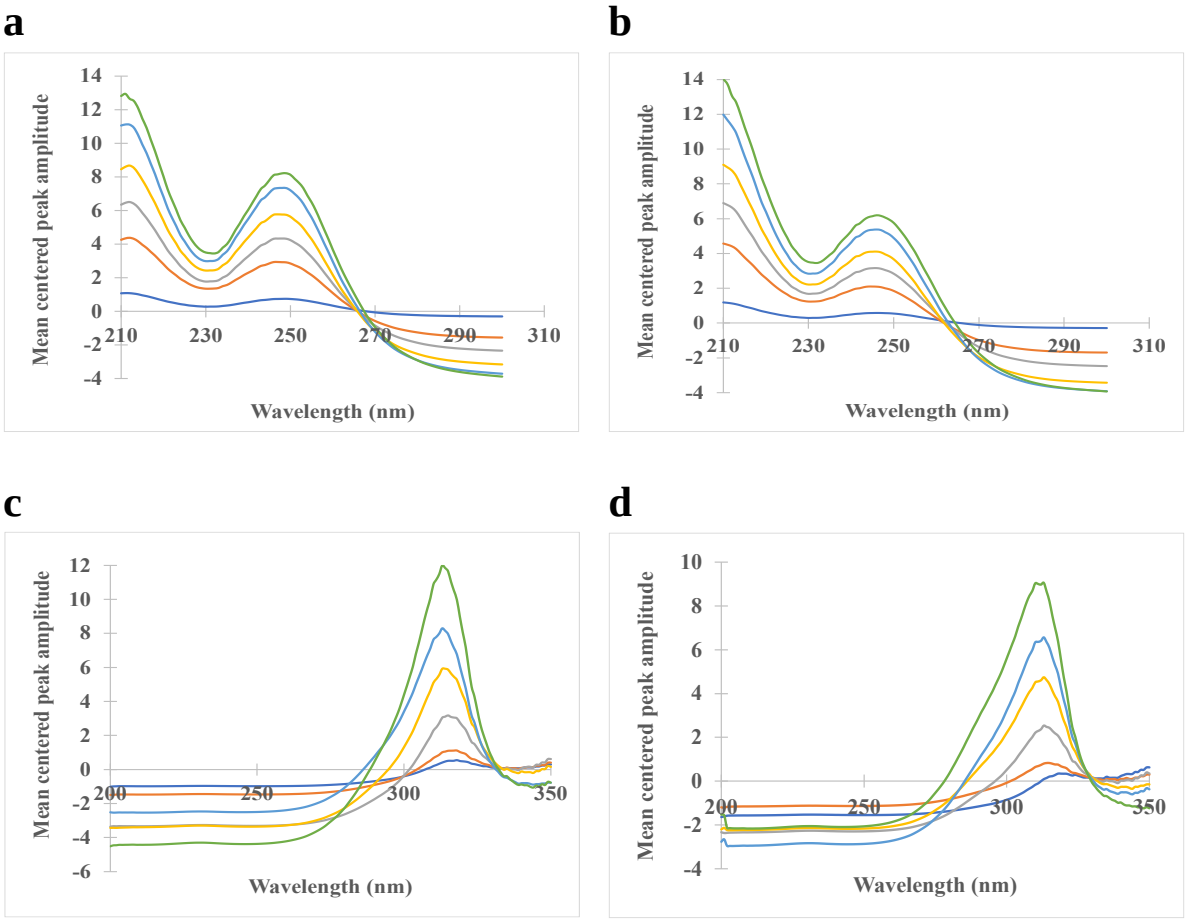

Fig. 3. Mean centered ratio spectra of ketoprofen (2−30 µg mL^{-1}) using a) tilmicosin 30 µg mL^{-1} as a divisor or b) tylosin 30 µg mL^{-1} as a divisor; and Mean centered ratio spectra of c) tilmicosin and d) tylosin; (5−100 µg mL^{-1}) using ketoprofen 30 µg mL^{-1} as a divisor.

4.2.3. Ratio difference method

This method is based on finding a good linearity between the difference in absorbance at two specific wavelengths in the ratio spectra and the corresponding drug concentration [25,26]. Ketoprofen was determined using a divisor of 30 µg mL^{-1} tilmicosin or tylosin where the chosen amplitudes were at 245 and 270 nm; **Fig. 4 a & b**. Likewise, the wavelengths chosen for estimating tilmicosin or tylosin using standard ketoprofen (30 µg mL^{-1}) as a divisor were 300−270 nm and 304−270 nm, respectively, as shown in **Fig. 4 c & d**.

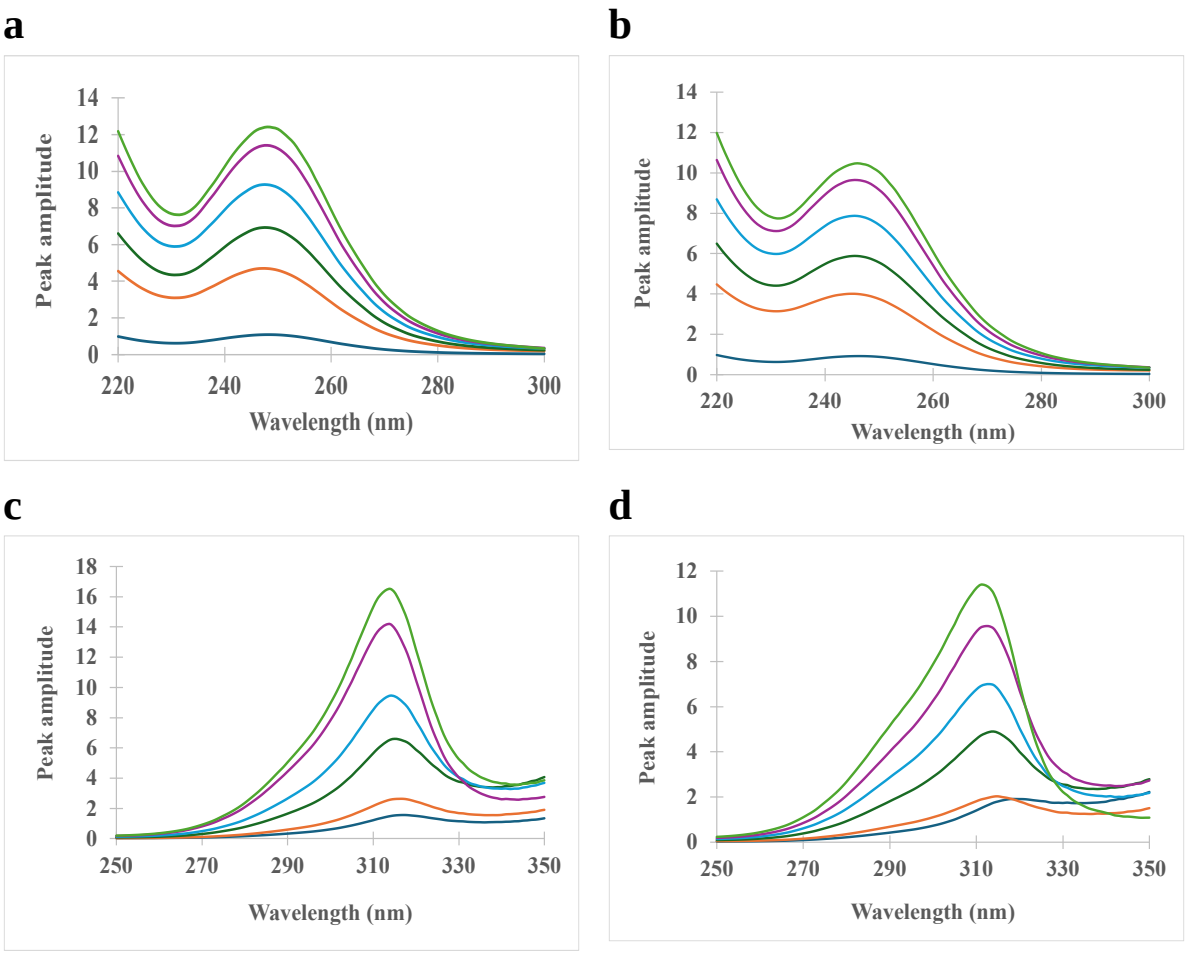

Fig. 4. Ratio spectra of ketoprofen (2−30 µg mL^{-1}) using a) tilmicosin 30 µg mL^{-1} as a divisor or b) tylosin 30 µg mL^{-1} as a divisor; and Ratio spectra of c) tilmicosin and d) tylosin; (5−100 µg mL^{-1}) using ketoprofen 30 µg mL^{-1} as a divisor.

4.2.4. Derivative ratio method

This method relies on the derivatization of the ratio spectrum, which is calculated after recording the zero−order spectrum [27]. Initially, ketoprofen ratio spectra were deduced using 30 µg mL^{-1} tilmicosin or tylosin as a divisor. Meanwhile, the ratio spectra of tilmicosin or tylosin were divided by 30 µg mL^{-1} ketoprofen. Secondly, the influence of two parameters, namely, delta lambda and scaling factor were investigated. Calculating the first derivative of the ratio spectra of the cited drugs using Δλ=4 nm and scaling factor of 1 showed reliable results. Finally, ketoprofen was estimated at 218, 238 and 259 nm in the presence of either tilmicosin or tylosin; **Fig. 5 a & b**. The two wavelengths 285 and 320 nm were the amplitudes at which tilmicosin or tylosin were determined; **Fig. 5 c & d**.

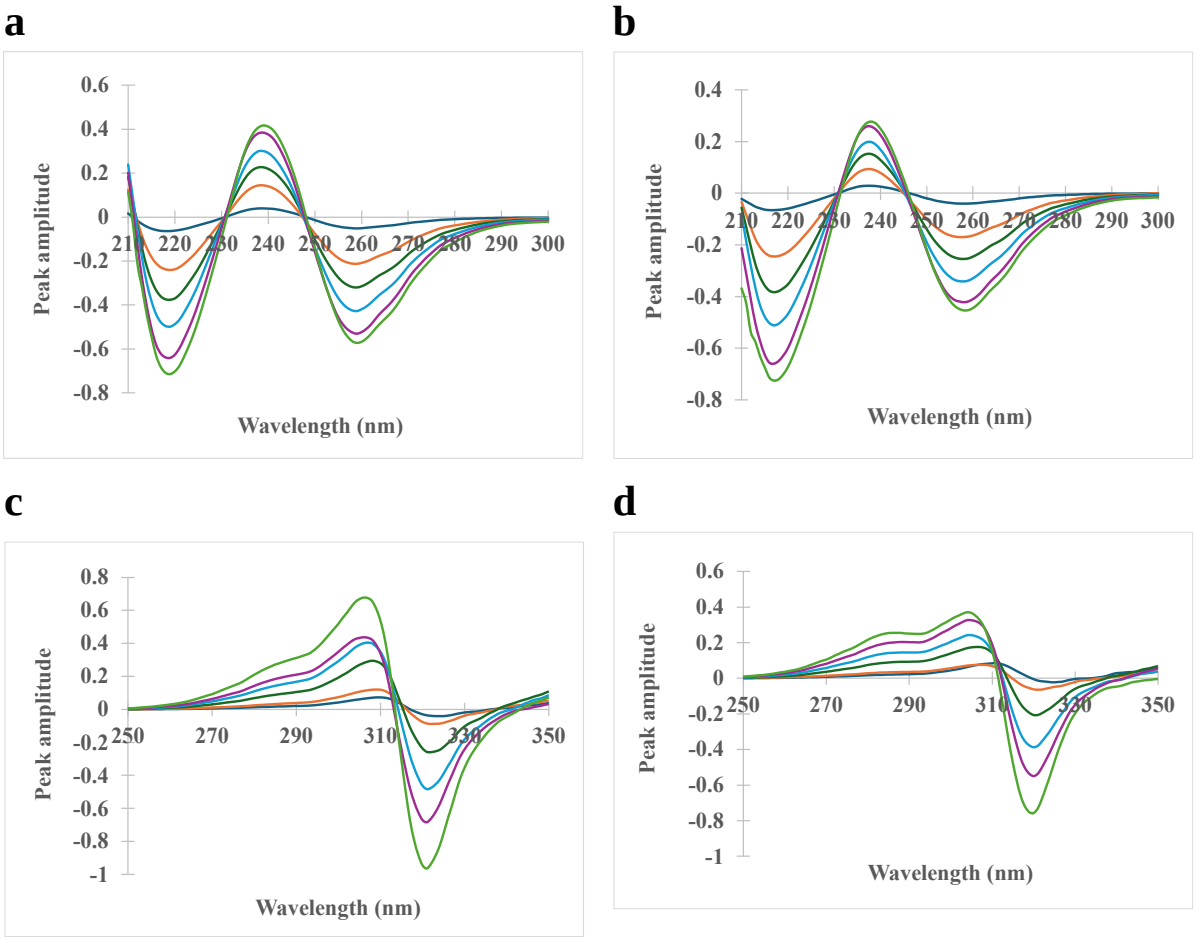

Fig. 5. First derivative of the ratio spectra of ketoprofen (2−30 µg mL^{-1}) using a) tilmicosin 30 µg mL^{-1} as a divisor or b) tylosin 30 µg mL^{-1} as a divisor; and First derivative of the ratio spectra of c) tilmicosin and d) tylosin; (5−100 µg mL^{-1}) using ketoprofen 30 µg mL^{-1} as a divisor.

4.3. Method validation

The validation of the proposed methods was achieved according to ICH guidelines [28].

4.3.1. Linearity

The response was linear in the range of 0.5−5.0 ng mL^{-1} for ketoprofen and 1.0−10.0 ng mL^{-1} for both tilmicosin and tylosin using the developed UPLC−MS method: **Table 2a**. Moreover, the spiked plasma samples showed a linear relationship over the mentioned concentration range of the studied drug with R^2 greater than 0.999; **Table 2a**.

While the spectrophotometric methods showed a linear relationship within 2.0−30.0 µg mL^{-1} for ketoprofen and 5.0−100.0 µg mL^{-1} for each of tilmicosin and tylosin. The parameters of regression were presented in **Tables 2b and 2**

Table 2a: Parameters of regression for the determination of ketoprofen together with either tilmicosin or tylosin by the suggested UPLC−MS method

	Regression equations	R^2	Ranges (ng mL^{-1})
UPLC−MS / Methanol	y = 12974x - 1025.9 a	0.9999 a	0.5−5.0 a
	y = 19973x + 4019.8 b	0.9999 b	1.0−10.0 b
	y = 15880x - 3621.3 c	0.9998 c	1.0−10.0 c
UPLC−MS / Poultry plasma	y = 14327x + 158.85 a	0.9999 a	
	y = 15408x + 5900.5 b	0.9998 b	
	y = 16533x + 4232.3 c	0.9999 c	

The letters a, b and c represent the regression data of ketoprofen, tilmicosin and tylosin respectively.

Table 2b: Parameters of regression for the determination of ketoprofen and tilmicosin by the suggested UV−spectrophotometric methods

UV−spectrophotometric methods	Regression equations	R^2	Ranges (μg mL^{-1})
Dual λ	y = 0.0614x + 0.043 a	0.9999 a	
	y = 0.0114x - 0.0003 b	0.9998 b	
Mean centering	y = 0.2588x + 0.3715 a	0.9999 a	
	y = 0.1223x - 0.3962 b	0.9999 b	
Ratio Difference	y = 0.1561x + 0.1023 a	0.9999 a	
	y = 0.0781x + 0.1972 b	0.9999 b	2–30 a
Ratio derivative			5−100 b
218 nm a	y = 0.0251x - 0.0039 a	0.9999 a	
238 nm a	y = 0.0143x + 0.0139 a	0.9999 a	
259 nm a	y = 0.0207x + 0.0088 a	0.9999 a	
285 nm b	y = 0.0027x + 0.0069 b	0.9999 b	
320 nm b	y = 0.0097x - 0.0159 b	0.9999 b	

The letters a and b represent the regression data of ketoprofen and tilmicosin respectively.

Table 2c: Parameters of regression for the determination of ketoprofen and tylosin by the suggested UV−spectrophotometric methods

UV−spectrophotometric methods	Regression equations	R^2	Ranges (μg mL^{-1})
Dual λ	y = 0.0626x + 0.0283 a	0.9998 a	
	y = 0.015x + 0.0133 c	0.9999 c	
Mean centering	y = 0.2052x + 0.0575 a	0.9999 a	
	y = 0.0985x - 0.3421 c	0.9999 c	
Ratio Difference	y = 0.0857x + 0.1268 a	0.9997 a	
	y = 0.0946x + 0.2553 c	0.9998 c	2–30 a
Ratio derivative			5−100 c
218 nm a	y = 0.0248x + 0.0034 a	0.9999 a	
238 nm a	y = 0.0147x + 0.004 a	0.9997 a	
259 nm a	y = 0.0206x + 0.0093 a	0.9999 a	
285 nm c	y = 0.0027x + 0.0057 c	0.9999 c	
320 nm c	y = 0.0086x - 0.0423 c	0.9999 c	

The letters a and c represent the regression data of ketoprofen and tylosin respectively.

4.3.2. Accuracy

It was evaluated in triplicates through the range of linearity for each drug. For the UPLC−MS method, it was found to be 100.15%±1.87 for ketoprofen. The accuracy was found to be 100.48%±0.08 and 100.01%±1.51 for tilmicosin and tylosin, respectively.

The accuracy of ketoprofen and tilmicosin were found to be 100.81%±0.34 and 99.99%±1.67 for the dual wavelength method, 101.11%±0.30 and 99.78%±0.75 for the mean centering method, 99.48%±0.74 and 100.12%±0.35 for ratio difference method. While the derivative ratio method accuracies were 99.74%±0.31, 99.79%±0.66 and 100.44%±0.71 for ketoprofen at 218 nm, 238 nm, and 259 nm, respectively, and 100.59%±1.48 and 99.38%±1.31 for tilmicosin at 285 nm and 320 nm, respectively.

The accuracy of ketoprofen and tylosin were found to be 100.62%±0.44 and 99.88%±1.13 for the dual wavelength method, 100.15%±0.88 and 99.96%±1.11 for the mean centering method, 100.15%±0.33, 101.17%±0.52 for ratio difference method. While the derivative ratio accuracies were 99.09% ±0.15, 100.25% ±1.18 and 100.46% ±0.54 for ketoprofen at 218 nm, 238 nm and 259nm, respectively, and 100.95 %±0.35 and 99.02 %±0.93 for tylosin at 285 nm and 320 nm, respectively

4.3.3. Precision

The evaluation of precision was achieved by the calculation of the intraday and interday RSD%. The established UPLC method showed that the RSD% of intraday range was 0.07−1.25%, while that of interday was between 0.12 and 0.43%. The UV−spectrophotometric methods were confirmed to be repeatable and reproducible acquiring RSD% < 2.

4.3.4. Selectivity

Laboratory combinations of the studied drugs were produced for analysis using the established procedures. Four proportions of ketoprofen and tilmicosin or ketoprofen and tylosin were mixed; taking into consideration the ratio of the commercial dosage administration form (Ketoprofen, together with tilmicosin or tylosin 1:3). The UPLC−MS technique showed recovery percentages (%R) of ketoprofen to be 99.92±0.36 and tilmicosin %R was determined to be 100.39±0.47, whereas that of ketoprofen and tylosin was discovered to be

98.89±0.83 and 99.98±0.44, respectively.

The dual wavelength approach revealed mean recoveries of 99.72%±1.40 for ketoprofen and 99.49%±0.68 for tilmicosin. The mean centering method resulted in %R of 100.30±1.86 for ketoprofen and 100.01±1.48 for tilmicosin. The ratio difference method produced %R of 99.45±0.92 for ketoprofen and 100.15±0.96 for tilmicosin. The mean recoveries of the derivative ratio technique for ketoprofen were 100.72%±0.34, 99.01%±0.41, and 99.42%±1.40 at wavelengths 218 nm, 238 nm, and 259 nm, respectively. For tilmicosin, the %R were 100.23±1.06 and 100.10±0.60 at wavelengths 285 nm and 320 nm, respectively.

Also, the dual wavelength approach yielded a recovery percentage of 99.28±0.49 for ketoprofen and 101.22±0.72 for tylosin. The mean centering method resulted in %R of 100.51±0.94 for ketoprofen and 100.33±0.79 for tylosin. The ratio difference method showed mean recoveries of 100.49±0.60 for ketoprofen and 100.19±0.83 for tylosin. The %R of the derivative ratio technique for ketoprofen at wavelengths 218 nm, 238 nm, and 259 nm were 99.05±0.77, 98.73±0.43 and 99.26±0.77, respectively. For tylosin, the recovery % at wavelengths 285 nm and 320 nm were 99.65±1.05 and 100.10±0.66, respectively.

4.3.5. Analysis of Veterinary formulations

Furthermore, the proposed methods were effectively applied for ketoprofen and tilmicosin estimation in the Ketotilmizide® injectable solution, as well as ketoprofen with tylosin in the Painless Plus® injectable solution. The results revealed that excipients did not interfere, indicating the specificity of the methods; **Tables 3a and 3b**.

The standard addition approach was used to verify the proposed method's validity. The standard solutions of the two binary combinations of the proposed drugs were combined with specific aliquots of the formulations, with three different concentrations for each solution. Acceptable mean recoveries were found in the ranges of.101.27-99.98 for the UPLC−MS method and 101.44-98.16 for UV−spectrophotometry.

The suggested and previously published approaches [10,18,19] were compared using two statistical parameters, namely the t-test and F-test. These values fell within the allowed theoretical bounds, indicating that there was no significant difference between the suggested and reported approaches. The proposed methods were effectively utilized to concurrently determine ketoprofen with tilmicosin or tylosin, in contrast to the previously documented methods.

4.3.6. Analysis of spiked plasma samples

The UPLC approach offered a high level of sensitivity to allow the quantification of the two combinations of ketoprofen and tilmicosin or ketoprofen with tylosin in chicken plasma samples that were intentionally spiked. The results were calculated using the regression equation in **Table 2a**. The analysis results showed that the assay % was for ketoprofen 100.44±1.26, 98.79±1.11 for tilmicosin, and 99.68±0.59 for tylosin. The selectivity of the UPLC technique was assessed by injecting a plasma sample devoid of any substances of interest. The data obtained from the blank plasma sample were examined for interference at the retention time of the analyte by comparing them with those data obtained from spiked plasma samples. The data indicate that the presented approach accurately estimates the target analytes in the spiked plasma without being affected by the natural components of the plasma matrix.

4.3.7. Comparison of the developed UPLC method and other HPLC methods

Multiple analytical techniques have been developed to determine the presence of the investigated substances in biological samples, liquid samples and pharmaceuticals using High Performance Liquid Chromatography; **Table 3** Based on a comparative analysis of the developed and disclosed HPLC procedures in the last ten years, drug substances evaluated using the proposed method exhibited a shorter analysis time compared to earlier methods [15,18,20] that also were unable to achieve simultaneous separation. In terms of range, the proposed method exhibits greater sensitivity compared to all of the mentioned published methods [14-21]. This study is noteworthy for being the first to establish a reliable and efficient UPLC-MS method for simultaneously quantifying three medicines, namely Ketoprofen, tilmicosin, and tylosin, in both bulk and in their combined dose forms. Undoubtedly, it can be inferred the UPLC method provided a significant degree of sensitivity and specificity, enabling the measurement of two combinations of ketoprofen and tilmicosin or ketoprofen with tylosin, in plasma samples in the quality control laboratory.

Table 3: Comparison between the previously published HPLC investigations and the current study.

Target analyte	Matrix	Mobile Phase	Elution technique	Detection & wavelength	Retention time (min)	Linearity range	Ref. No.
Ketoprofen	Beef liver	Formic acid and water (3:1, v/v)	Isocratic	MS/MS	-	0.3–500 µg kg^{-1}	14
	Capsules, tablets & ampules	Cetrimide and acetonitrile (50:50, v/v)	Isocratic	UV at 254	9.41	0.031–0.500 mg mL^{-1}	15
	Human plasma	Acetonitrile and trifluoroacetic acid in water (55:45, v/v)	Isocratic	UV at 257	1.7	153.2–19155 ng mL^{-1}	16
	Nano-emulsion gel	Acetonitrile and KH_2PO_4 buffer pH 3, (40:60)	Isocratic	UV at 254	-	0.5–60 µg mL^{-1}	17
Tilmicosin	Muscle, kidney and liver of chicken	Trifluoroacetic acid and acetonitrile	Gradient	UV at 287	6.86	0.05–5 µg mL^{-1}	18
Tylosin	Injectable solutions	100% methanol	Isocratic	UV at 280	0.774	0.5–20 µg mL^{-1}	19
	Parenteral Forms	water (60%) and methanol (40%)	Isocratic	PDA at 260	2.21	55-1100 µg mL^{-1}	20
	Wastewater effluents	Acetonitrile and 0.1M H_3PO_4 (60:40, v/v).	Isocratic	PDA at 290	5.4	5-100 µg mL^{-1}	21
• Ketoprofen • Tilmicosin • Tylosin	Oral solutions	Aqueous formic acid and acetous formic acid	Gradient	MS	• 4.05 • 3.20 • 3.45	• 0.5−5 • 1.0−10 • 1.0−10 ng mL^{-1}	Current Study

4.4. Solutions stability

The stability of the solutions containing three drugs in aqueous methanol or propylene glycol was assessed by the UPLC−MS and the UV−Spectrophotometric methods, respectively. These solutions demonstrated stability throughout two weeks, whether stored at room temperature or in a refrigerator.

5. Conclusion

Two efficient and rapid techniques were utilized to separate and quantify ketoprofen alongside tilmicosin in the Ketotilmizide® injectable solution, and with tylosin in the Painless Plus® injectable solution. Both UPLC−MS and UV−spectrophotometry effectively evaluated the purity of the three medicines. The three drugs in their binary combined formulations were initially analyzed using these methods. Moreover, the UPLC−MS approach showed exceptional sensitivity and specificity in quantifying the mentioned medications in poultry plasma. The proposed methodologies were validated in accordance with the requirements set by the International Council for Harmonisation (ICH), confirming their accuracy, precision, and robustness.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

The authors express their gratitude to the Animal Health Research Institute for offering the chicken plasma samples. Also, the authors are grateful to the supplier Pharmaceutical Industries company; Hubei Xunda Pharmaceutical Co., Ltd, and NINGIXIA TAIRUI Pharmaceutical Co., Ltd.

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