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Research Article

DRUG FORMULATIONS

Smart Eco-Friendly Spectrophotometric Methods Resolving Highly Overlapping Spectra: Application to Veterinary Antibiotic Injections

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Abstract

Background: Over the last few years, mathematical manipulation has proved to be a very powerful means of successfully resolving severely overlapped spectra for various multicomponent mixtures.
Objective: Smart and environmentally friendly spectrophotometric determination approaches were used for two binary mixtures of fixed dose veterinary injections containing flunixin meglumine (FLU) combined with either florfenicol (FLR), or oxytetracycline HCl (OXY).
Methods: Regarding the first mixture, both FLU and FLR were determined by three successive resolution techniques, which were; constant multiplication coupled with spectrum subtraction (CM-SS), derivative ratio (DD^1), and ratio difference (RD), and two progressive resolution techniques which were absorbance subtraction (AS) and amplitude modulation (AM). Also, graphical representation of concentration of the two drugs through concentration value (CNV) method was also applied. Concerning the second mixture, both FLU and OXY showed severely overlapped spectra and a comparative study was conducted for the determination of each drug by constant center (CC), ratio subtraction via amplitude difference coupled with spectrum subtraction (RS/AD-SS), constant value via amplitude difference (CV-AD), and advanced concentration value (ACV) methods.
Results: Calibration graphs of the first mixture were linear over the range 5–40 μg/mL for FLU, and 3–40 μg/mL for FLR. The proposed methods overcame the problem of the overlapped spectra and the presence of a minor component in the mixture. Regarding the second mixture, calibration graphs were linear over the range 2.5–24 μg/mL for FLU and 4–28 μg/mL for OXY.
Conclusion: The proposed methods were successfully validated as per International Council for Harmonization (ICH) guidelines. The obtained results were statistically compared with the official or reported methods, showing no significant difference concerning accuracy and precision. The methods were evaluated for greenness by three different assessment tools: NEMI, analytical ecoscale, and GAPI.
Highlights: The methods were successfully applied for the simultaneous determination of the two combinations in synthetic mixtures and their marketed antibiotic veterinary injections: Megluflor® and Floxon®.

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Over the last few years, treatment of spectra through mathematical manipulation steps via the spectrophotometers' built-in software has proved to be a very powerful tool for resolving severely overlapped spectra for multicomponent mixtures (1). It has provided time- and cost-effective methods for quantitative analysis of drugs in fixed dose combinations with high selectivity, accuracy, and precision. Veterinary antibiotics are often mixed with analgesics and antipyretics in injections to be more convenient for administration to poultry or cattle suffering bacterial infectious diseases. The three drugs under investigation are flunexine meglumine (FLU), florfenicol (FLR) and oxytetracycline (OXY).

FLR, chemically designated as 2,2-dichloro-N-[(1R,2S)-3-fluoro-1-hydroxy-1–(4-methylsulfonylphenyl)propan-2-yl]acetamide (Figure 1a) is a fluorinated synthetic analog of thiamphenicol (2). It is a synthetic veterinary antibiotic used for bovine respiratory disease treament. FLR is licensed for use in the United States to control enteric septicemia in catfish.

FLU, chemically designated as (2R,3R,4R,5S)-6-(methylamino)hexane-1,2,3,4,5-pentol;2-[2-methyl-3-(trifluoromethyl)anilino]pyridine-3-carboxylic acid (Figure 1b), is a relatively potent non-narcotic, nonsteroidal analgesic with anti-inflammatory, anti-endotoxic, and anti-pyretic properties used in veterinary medicine for treatment of horses, cattle, and pigs (2). OXY, chemically designated as (4S,4aR,5S,5aR,6S,12aR)-4-(dimethylamino)-1,5,6,10,11,12a-hexahydroxy-6-methyl-3,12-dioxo-4,4a,5,5a-tetrahydrotetracene-2-carboxamide;hydrochloride (Figure 1c), is produced by Streptomyces rimosus exhibiting antimicrobial activity. Both FLU and OXY are official drugs stated in the United States Pharmacopeia (USP) (3). The literature revealed several analytical methods for quantitative determination of FLU, such as spectrophotometry (4), HPLC (5), and voltammetric methods (6). FLR was analyzed using spectrophotometry (7), LC (8), and GC (9). OXY hydrochloride, was determined using spectrophotometry (10), HPLC (11), and electrochemical methods (12). There was a reported method for simultaneous determination of OXY and FLR by spectrophotometry (13) and an HPLC method for the analysis of FLR and FLU mixtures (14), but there were no spectrophotometric methods for the latter mixture.

Multicomponent mixtures usually suffer from severely overlapped spectra which prompts the development of several resolution techniques to estimate the concentration of each component under investigation. The aim of this work was to develop green spectrophotometric methods in order to resolve the severe spectral overlap of binary mixtures of FLU with either FLR of OXY in synthetic mixtures in addition to their combined formulations, Floxon® (OXY and FLU) and Megluflor® (FLR and FLU) antibiotic veterinary injections, with high accuracy and precision via simple manipulation procedures. Along with that, we aimed to raise awareness of taking into consideration green chemistry, and to assess our work with greenness tools, NEMI, analytical ecoscale, and GAPI, to ensure very low hazard levels resulted throughout out analysis process (15–17).

Theoretical Background

Mixture A—FLU and FLR

(a) *Ratio difference (RD)*.—For a mixture of X and Y, whose zero spectra were overlapped severely, by using X as a divisor, the amplitude difference (P_1 –P_2) between two dissimilar wavelengths in the mixture's ratio spectra was directly proportional to the concentration of Y with no X interfering. Similarly, X can be also be determined, this time by using Y as a divisor.

(b) *Derivative ratio (DD^1)*.—For an X and Y mixture, having severely overlapped zero spectra, by using X as a divisor, the amplitude of derivative of the ratio spectra of mixture was directly proportional to the concentration of Y without any interference of X. Similarly, X could be determined, utilizing Y as a divisor this time.

(c) *Concentration value (CNV)*.—This method depends upon graphical representation of the spectra, in which the concentration value of the drug was obtained directly, representing the actual concentration without requiring substitution in the regression equation. It is applied by dividing the zero-order absorption spectrum of the mixture by zero-order absorption spectrum of the normalized spectrum of the more extended component, afterwards the constant obtained from the plateau region, which is parallel to the x-axis, directly represents the more extended component's concentration.

(d) *Absorbance subtraction (AS)*.—For two drugs X and Y with overlapping spectra intersecting at the isoabsorptive point, and Y is more extended than X, while X did not show any absorbance (A2) at another wavelength ($\lambda 2$), the isoabsorptive point, λ_{iso}, may be used for separate quantitative estimation of X and Y each in their mixture (X + Y). The absorbance values equal to X and Y at λ_{iso} were obtained using a response factor {$F = A_{iso}/A2$} which is constant for pure Y and is equal to the average of the ratio between the

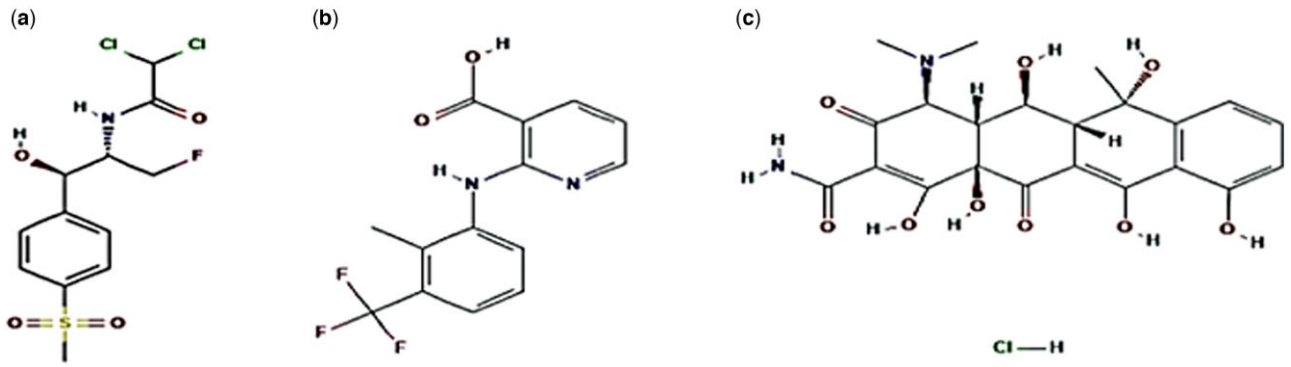

Figure 1. Chemical structures of (a) FLR, (b) FLU, and (c) OXY.

absorbance values of different concentrations of pure Y at λ_{iso} (A_{iso}) to those at λ2 (A2)

$$\text{Y absorbance in the mixture } \lambda_{iso} = F * A2$$
$$\text{X absorbance in the mixture } \lambda_{iso} = \lambda_{iso}\ (X + Y) - (F * A2)$$

The concentration of each X or Y individually is calculated by using the isoabsorptive point unified regression equation, that was obtained by recording the absorbance values of the zero-order curves of either X or Y at the λ_{iso} against their concentrations X or Y, respectively.

(e) *Amplitude modulation method (AM).*—For an X and Y mixture, where Y is extended over X and the zero-order absorption spectra of X and Y shows an isoabsorptive point. The ratio spectra using a normalized spectrum as a divisor, will retain the same isoabsorptive point. The constant obtained from the plateau parallel to the *x*-axis in the extended region represents PY at the isosbestic point. The peak amplitude of component X at the isosbestic point could be obtained by subtracting the PY from the total peak amplitude at the isosbestic point (PX = P Total—PY). The concentration of both components X and Y can be determined by substitution of PX and PY in the unified regression equation.

(f) *Constant multiplication coupled with spectrum subtraction (CM-SS).*—For a mixture of X and Y, where Y is more extended than X, dividing the spectrum of the laboratory mixture with a divisor of the extended component (Y') result in a plateau region parallel to the *x*-axis from which the constant Y/Y' is determined. This constant value is then multiplied with the divisor spectrum, which results in resolving the zero-order absorption spectrum of Y. Then spectrum subtraction is applied, where the resolved spectrum of Y is subtracted from the spectrum of the mixture (X + Y) to obtain the zero-order absorption spectrum of X.

Mixture B—FLU and OXY

(a) *Constant value via amplitude difference (CV-AD).*— This method is used in a mixture of two components (X and Y) showing severely overlapped spectra X. By using a divisor (X'), the division would result in a new curve which is

$$(X + Y)/X' = (P_X) + (P_Y)$$

where P_X is a constant. By subtracting the amplitude at two selected wavelengths λ_1 and λ_2 in the obtained ratio spectrum of the mixture, the constant (P_X) is thus cancelled to eliminate the contribution due to X, so then the difference would represent component Y only:

$$P_1 - P_2 = [(P_Y)_1 + \text{constant} - (P_Y)_2 + \text{constant})] \quad (1)$$

$$\Delta P = (P_Y)_1 - (P_Y)_2 \quad (2)$$

ΔP represents the difference of ratio spectra amplitudes at λ_1 and λ_2, $(P_Y)_1$ and $(P_Y)_2$ represent the amplitude of Y at λ_1 and λ_2 The regression equation which represents the linear relationship between ΔP of different concentration of pure Y at λ_1, and λ_2 using a certain concentration of X' as a divisor versus the corresponding ratio amplitude at one of the two selected wavelength λ_1 is:

$$\Delta P = \text{slope } (P_Y)_1 + \text{intercept} \quad (3)$$

Then, postulated $(P_Y)_1$ that is related to component Y only in the mixture X + Y is calculated by using the previous regression equation, using ΔP at the two selected wavelengths related to the mixture ratio spectrum. The constant value (P_X) may be obtained by subtracting the $P_{recorded}$ of the mixture at (λ_1), and its own $P_{postulated}$ at (λ_1) constant value = [$P_{Recorded}$]—[$P_{Postulated}$].The concentration of X was obtained by the regression equation that represents the correlation between amplitudes of the constant of ratio spectra, and the corresponding concentrations. Similarly, the concentration of Y in the mixture could be obtained by using Y as a divisor.

(b) *Constant center method (CC).*—Concerning a mixture of X and Y having a severely overlapped zero-order spectrum, upon utilizing X as a divisor (X'), the constant X/X' could be calculated via amplitude difference, and then multiplied by X' divisor spectrum. This step is known as constant multiplication which results in the zero-order absorption spectrum of X. The concentration of X could then be obtained using its corresponding regression equation which represents the linear relationship between the absorbance readings of zero-order curve of X at its λ_{max} versus its concentration. The concentration of Y in the mixture could then be determined by the same two previous steps.

(c) *Advanced concentration value (ACV).*—The advanced concentration value technique was applied using the same steps as CV-AD, by using the normalized Y spectrum which represents the absorptivity throughout the wavelengths, in which the measured constant value directly represents the concentration, while the recovery percentages will be calculated relative to a theoretical value.

(d) *Ratio subtraction via amplitude difference-coupled spectrum subtraction (RS/AD-SS).*—This method could be applied to be a step in a resolution technique; the constant obtained from the CV-AD is subtracted from a binary mixture (X+Y)/Y' – (Y/Y') to obtain X/Y' followed by divisor curve multiplication to obtain the resolved spectrum of X. Then the other drug could be calculated by subtracting the resolved spectrum of X from the mixture's spectrum.

Experimental

Apparatus and Software

Spectrophotometric measurements were carried out using a double-beam UV-Vis spectrophotometer model J-760 (Jasco, Japan) that is connected to a ACER-compatible computer, with 1.00 cm quartz cells. Scans were acquired in the range 200–400 nm at intervals of 0.1 nm. SpectraManager® software was used.

Chemicals and Reagents

(a) *Pure samples.*—Florfenicol with purity 101.08% ± 1.18 according to the reported method (7), Flunixin meglumine with purity 100.08 ± 1.26 according to the official method (3) and oxytetracycline hydrochloride with purity 99.48 ± 1.43 according to the official method (3) were kindly supplied by Pharmaswede Veterinary (Egypt).

(b) *Market samples.*—Floxon and Megluflor veterinary injections were kindly supplied by Pharmaswede Veterinary. Each 1 mL Floxon contains 108 mg OXY and 33 mg FLU, while each 1 mL Megluflur contains 16.5 mg FLU and 300 mg FLR.

(c) *Solvents*.—Hydrochloric acid (10%) was purchased from Sigma Aldrich (Germany).

Standard Solutions

(a) *FLU and FLR*.—
  (1) *FLU working solution*.—10 mg were dissolved in 50 mL distilled water in 100 mL volumetric flask, sonicated for 10 min, and completed till mark with distilled water.
  (2) *FLR working solution*.—10 mg were dissolved in 50 mL distilled water in 100 mL volumetric flask, sonicated for 10 min, and completed till mark with distilled water.

(b) *FLU and OXY*.—
  (1) *FLU working solution*.—10 mg were dissolved in 50 mL 0.1N HCl in 100 mL volumetric flask, sonicated for 10 min, and completed till mark with 0.1N HCl.
  (2) *OXY working solution*.—10 mg were dissolved in 50 mL 0.1N HCl in 100 mL volumetric flask, sonicated for 10 min, and completed till mark with 0.1N HCl.

Procedure

(a) *Spectral data for FLU, FLR, and OXY*.—Zero absorption spectra (D^0) of FLU, FLR, and OXY were scanned against blank at 200–400 nm.

(b) *Linearity and construction of calibration graphs*.—
  (1) *Mixture A (FLU and FLR)*.—Aliquots from the standard solution equivalent to 30-400 μg of FLR and 50-400 μg of FLU were carried into 10-mL volumetric flasks and completed till mark by distilled water.
  (2) *Mixture B, FLU and OXY*.—Aliquots from the working solution equivalent to: 40-280 μg of OXY and 25-240 μg of FLU, were transferred into 10-mL volumetric flasks and completed till mark with 0.1N HCl.

Mixture A, FLU and FLR.—*Regression equations axis*.—Regression equations which represents the linear relationships among the absorbance at λ_{max} of the scanned spectra of FLU and FLR at 280.1 or 225 nm, respectively; versus the corresponding FLU or FLR concentrations.

Ratio Difference (RD) and Derivative Ratio (DD^1)

(a) *FLU*.—The stored absorption spectra of FLU (5-40 μg/mL) were divided by the normalized FLR as a divisor, and the peak amplitudes at 207 nm and 224 nm were recorded. Then the first derivative was obtained and the peak at 213 nm was recorded.
  (1) *RD for FLU*.—The regression equation related the amplitudes difference (ΔP) at 207 and 224 nm of the ratio spectra to the corresponding FLU concentrations.
  (2) *DD^1 for FLU*.—The regression equation related peak amplitude at 213 nm of the first derivative of the ratio spectra to the corresponding FLU concentrations.

(b) *FLR*.—The stored absorption spectra of FLR (3-40 μg/mL) were divided by the normalized FLU as a divisor, then the peak amplitudes at 226 and 237 nm were recorded. Afterwards, the first derivative spectrum was obtained and the peak amplitude at 232.7 nm was recorded.
  (1) *RD for FLR*.—The regression equation relates amplitudes difference (ΔP) between 226 and 237 nm of the ratio spectra to the corresponding FLR concentrations.
  (2) *DD^1 for FLR*.—The regression equation relates amplitudes at 232.7 nm of the first derivative of the ratio spectra to the corresponding FLR concentrations.

Absorbance Subtraction Method (AS) for FLU and FLR

Standard solutions which contain 5–40 μg/mL of FLU and FLR were separately prepared in distilled water, and afterwards scanned against blank in the spectrophotometer.

Calculation of the Response Factor

The response factor is the ratio of the absorbance at the two wavelengths ($A_{216.2}/A_{333}$). The value of the response factor was found to be 2.9.

A unified regression equation,which represents the linear relationships between the absorbance at λ_{iso} of the scanned spectra of FLR or FLU at 216.2 nm versus the corresponding FLR or FLU concentrations, was constructed.

Amplitude Modulation (AM) for FLU and FLR

Ratio spectra of FLR or FLU using normalized FLU as divisor, were obtained. The calibration graph was done by plotting the amplitudes of the ratio spectra at 216.2 nm versus the corresponding concentrations in order to compute a unified regression equation.

Mixture B, FLU and OXY mixture.—*Regression equation axis*.—Regression equations which represents the linear relationships between the absorbance at λ_{max} of scanned spectra of FLU and OXY at 252 or 267.6 nm, respectively, versus the corresponding FLU and OXY concentration.

Amplitude Difference (AD)

A regression equation was constructed to relate the amplitude difference between the peak amplitudes 286 and 320 nm (ΔP) of the division spectra of OXY using a normalized spectrum of FLU as a divisor versus the peak amplitude at 286 nm.

A regression equation was constructed to relate the amplitude difference between 252 and 280 nm (ΔP) of the division spectra of FLU using a normalized spectrum of OXY as a divisor versus peak the amplitude at 252 nm.

Constant Value via Amplitude Difference (CV-AD)

(a) *CV-AD for FLU*.—The ratio spectra of FLU using normalized FLU as a divisor were obtained. The calibration graph was constructed by plotting the amplitude in the plateau region at 286 nm versus the corresponding concentration of FLU to calculate the regression equation.

(b) *CV-AD for OXY*.—The ratio spectra of OXY using normalized OXY as divisor were obtained. The calibration graph was then constructed by plotting the amplitude in the plateau region at 252 nm versus the corresponding concentration of OXY to calculate the regression equation.

Determination of FLU, FLR, and OXY in Synthetic Mixtures

Synthetic mixtures were fixed by mixing accurate aliquots of binary mixture FLU and FLR and binary mixture FLU and OXY from their previously prepared standard solutions. The final volume was reached by adding distilled water for the former mixture, while 0.1 N HCl for the latter one. The solutions were scanned over 200-400 nm.

(a) *Mixture A.*—

(1) *CM-SS.*—The lab mixture D^o spectrum was divided by the D^o spectrum of normalized FLU, then in the extended region (284–310 nm), the constant value was recorded and multiplied with the normalized FLU spectrum to obtain the resolved D^0 spectrum of FLU. The absorbance at 280.1 nm was substituted in the corresponding regression equation of FLU. The resolved FLU spectrum was subtracted from the total mixture spectrum, resulting in the zero-order absorption spectrum of FLR. The absorbance at 225 nm was substituted in the corresponding regression equation of FLR.

(2) *DR.*—The ratio spectrum of the laboratory mixture using FLU as divisor was obtained and the amplitude difference ΔP ($P_{226\,nm} - P_{237\,nm}$) was substituted in the corresponding regression equation to obtain FLR concentration, or using FLR as a divisor where the peak amplitude at ΔP ($P_{207\,nm} - P_{224\,nm}$) was substituted in the corresponding regression equation to obtain the FLU concentration

(3) *DD^1.*—The first derivatives of the previous spectra were obtained, where the amplitude at 213 nm of the first ratio spectrum was substituted in the corresponding regression equation to obtain the FLU concentration, and the amplitude at 232.7 nm of the second ratio spectrum was replaced in the corresponding regression equation to obtain the FLR concentration.

(4) *AS.*—The absorbance at 333 nm from the lab mixture spectrum was multiplied by the response factor ($A_{333} \times 2.9$), then substituted in the unified regression equation to obtain the FLU concentration. The recorded $A_{216.2}$ of the laboratory mixture was subtracted from the postulated FLU response to obtain the response due to FLR and then substituted in the unified regression equation to obtain the the FLR concentration.

(5) *AM.*—The first derivative of the ratio spectrum of the mixture using normalized FLU as a divisor was obtained. The response of FLU was recorded from the extended region (284—310nm), then substituted in the unified regression equation to obtain the FLU concentration. The response at 216.2 nm (λ_{iso}) was subtracted from the FLU response and substituted in the unified regression equation to obtain FLR concentrations.

(6) *CNV.*—The concentration value was obtained by dividing the zero-order absorption spectrum of FLU by the spectrum of normalized FLU; the reading obtained from the extended region (284–310 nm) represented directly the concentration of FLU. The recorded laboratory mixture peak amplitude at 216.2 nm was subtracted from the FLU concentration to obtain the FLR concentration.

(b) *Mixture B.*—The lab mixture spectrum (FLU + OXY) was divided by the spectrum of normalized FLU, the amplitude difference $\Delta P_{286\text{-}320}$ was substituted in the corresponding regression equation to obtain P_{286} due to OXY/FLU' which was then subtracted from recorded peak amplitude at P_{286} on the ratio spectrum of the laboratory mixture to obtain the constant representing FLU/FLU'. The lab mixture spectrum (OXY + FLU) was divided by the spectrum of normalized OXY, the amplitude difference $\Delta P_{252\text{-}280}$ was substituted in the corresponding regression equation to obtain P_{252} due to FLU/OXY' which was then subtracted from recorded peak amplitude at P_{252} on the ratio spectrum of the laboratory mixture to obtain the constant representing OXY/OXY'.

(1) *CC.*

(a) *FLU.*—The FLU/FLU' constant was then multiplied by the spectrum of normalized FLU to obtain the zero-order absorption spectrum of FLU in the mixture. The absorbance at its λ_{max} (252 nm) was substituted in the corresponding regression equation to obtain its concentration.

(b) *OXY.*—The OXY/OXY' constant was then multiplied by the spectrum of normalized OXY to obtain the zero-order absorption spectrum of OXY in the mixture. The absorbance at its λmax (267.6 nm) was substituted in the corresponding regression equation to obtain its concentration.

(2) *ADV.*—The constant values of both FLU/FLU' and OXY/OXY' were directly recorded representing concentration.

(3) *CV-AD.*—The constant values of FLU/FLU' and OXY/OXY' obtained were substituted in the corresponding regression equation to get the concentration of FLU or OXY.

(4) *RS/AD-SS.*

(a) *OXY.*—The constant FLU/FLU' obtained was subtracted from the binary mixture, followed by multiplication by the divisor spectrum, normalized FLU', to get the resolved spectrum of OXY.

(b) *FLU.*—The resolved spectrum of OXY obtained above was subtracted from the spectrum of the binary mixture to get the zero-order absorption spectrum of FLU, allowing measurement of the absorbance at its λ_{max} (252 nm).

Application to Veterinary Formulation

A 1 mL Megluflur injection, claimed to contain 300 mg FLR and 16.5 mg FLU, was transferred to a 100 mL volumetric flask and made up to the mark with distilled water. Then 1.25 mL was transferred into another 100 mL volumetric flask and spiked with a standard FLU aliquot equivalent to 3 μg/mL to get a working solution with final concentrations: 37.5 μg FLR and 5.06 μg FLU. To get the actual concentration of FLU present in the dosage form, the amount of standard added drug was subtracted from the total concentration of the drug found in the analyzed sample.

A 1 mL Floxon injection was transferred to a 100 mL volumetric flask and made up to the mark with 0.1 N HCl. Then 0.2 mL was transferred from this solution in a 100 mL volumetric flask and made up to the mark with 0.1 N HCl to obtain a working solution of concentration 21.6 μg/mL OXY and 6.6 μg/mL FLU.

The suggested methods were used for the analysis of the drugs under investigation. The drugs' concentrations were obtained from the corresponding regression equations, except for the advanced concentration value, and the concentration could be directly used for determination of the percentage recovery.

Results and Discussion

The large-animal industry is one of the largest and fastest growing industries in the world due to increased global demand; especially that it is relatively less cheaper and easier to handle than other industries. However, the large-animal industry often suffers from economic losses caused by diseases from various microorganisms, which makes it hard for the industry to

expand to meet the demands of consumers. The health of large animals is also very important for humans, as there are diseases that are directly transmitted to humans if a diseased animal is consumed. Therefore, antibiotics are often used to treat diseases, prevent infections, and as growth promoters in poultry production (18, 19). However, these drugs can accumulate in animal-derived foods. In addition, residues of these drugs may also be transmitted by human resistant bacteria through the consumption of animal foods. Knowing those crucial information was the motive behind this work and behind studying veterinary medicine. In addition to this, responsibility towards our environment guided a very cautious solvent choice, and that the methods were evaluated by three different greenness assessment tools.

Method Development

(a) *Mixture A.*—The two drugs under investigation in mixture A (FLR and FLU) were co-formulated in the Megluflor injection, a widely used veterinary antibiotic used to treat various diseases in poultry and also large animals. There were no reported spectrophotometric methods for their simultaneous determination in their dosage form, which motivated the application of simple, accurate, precise, cost- and time-effective spectrophotometric methods for their simultaneous determination.

The first challenge in this mixture was the overlap of the zero-order absorption spectra of FLR with FLU over the wavelength region 200–283 nm, as shown in Figure 2a, which hindered the direct estimation of FLU at its λ_{max} 280.1 nm The second challenge was the dosage form ratio of FLU: FLR was found to be 16.5:300 which hindered the accurate estimation of both drugs within their proposed linearity ranges. Therefore, standard addition was used in order to delete the deviation from Beer's law that results in cases of low concentrations. Deviations occur when transmittance values are almost close to 100%, in which the incident light approaches the transmitted one, aiding deviations. As a result, the enrichment technique had to be introduced to the mixtures. Optimum concentration range choice depended on the spectral characteristics of the compound, ratio and absorptivity, without changing its concentration in the mixture.This may lead to Beer's law deviation, which is a result of electrostatic attraction between the ions. The standard additions technique helps overcome the problem of matching the linearity range of the standard with that of the sample. A known amount of drug is added to the working aliquot of the dosage form and measured. To get the actual concentration of the drug present in the dosage form, the amount of standard added drug is subtracted from the total concentration of the drug found in the analyzed sample.

With the intention of applying green chemistry principles, to avoid increased pollution and hazardous reagents, distilled water has been used exclusively as a solvent, and the spectra were found to be accurate, reproducible, and selective.

(1) *Approaches.*—Three approaches were applied on this mixture. The first approach based on the extended region, CC-MS, and CNV methods, were applied to obtain the concentration FLU and FLR. The second approach dealt with both drugs in their severely overlapped region and each drug was obtained by either RD or DD^1 methods (20).

The third approach was based on the presence of an isoabsorptive point at 216.2 nm which was retained in their ratio spectra, so two progressive techniques were applied for the determination of both drugs named AS and AM.

(2) *Constant multiplication-spectrum subtraction method (CM-SS).*—It was a successive spectrophotometric resolution technique used to resolve individually the zero-order absorption spectra of both FLU and FLR, where the absorbance at λ_{max} (280.1 nm) or at λ_{max} (225 nm) for FLU and FLR respectively was substituted in the corresponding regression equation to obtain the concentration of either FLU or FLR present in the mixture (21).

The resolution technique CM-SS depend upon the presence of one component (FLU) whose spectrum was more extended than the other component in mixture (FLR). Normalized FLU as a divisor was chosen as it represented the absorptivity over all the wavelengths region. The constant obtained from the plateau parallel to the *x*-axis in the extended region (284–310 nm) was

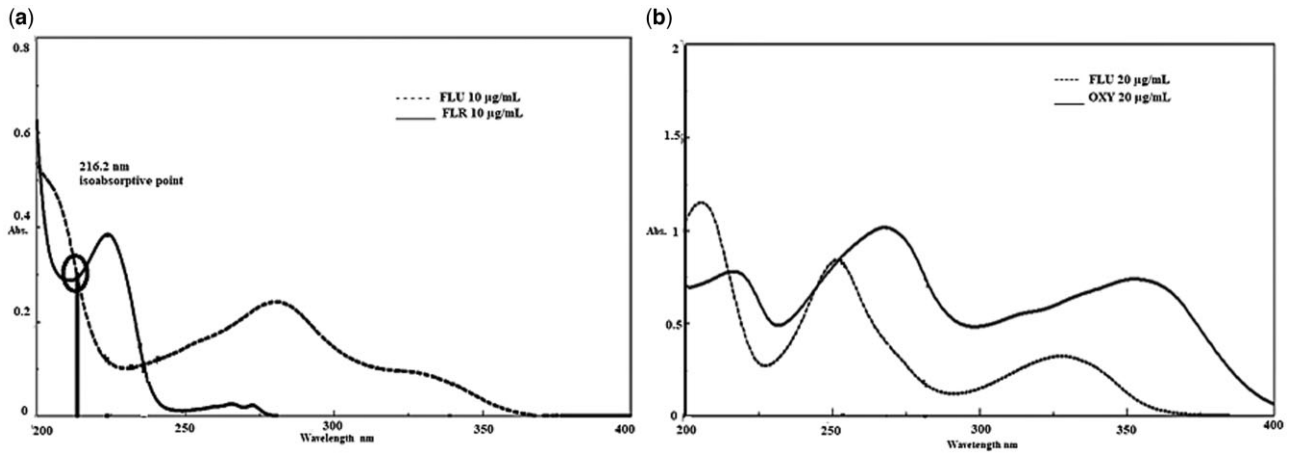

Figure 2. (a) Zero-order absorption spectrum of FLR 10 µg/mL (___) and FLU 10 µg/mL (——). (b) Zero-order absorption spectrum of FLU 20 µg/mL (—) and OXY 20 µg/mL (_____).

multiplied by the normalized FLU spectrum to obtain the zero-order absorption spectrum of FLU in the mixture, allowing its measurement at λ_{max} (280.1 nm). Spectrum subtraction was a very simple method to subtract the resolved FLU spectrum from the spectrum of the laboratory mixture and obtain the zero-order absorption spectrum of FLR where the absorbance at its λ_{max} (225 nm) was substituted in the corresponding regression equation to obtain its concentration.

(3) *RD and DD*.—Those methods were applied on the severely overlapped region of the two drugs in the wavelength region 200–280 nm to determine both the concentrations of FLU and FLR in the mixture. The ratio difference method was applied twice, the first time for the determination of FLU using normalized FLR as a divisor, where the contribution of FLR was cancelled upon determination of $\Delta P_{207\text{-}224}$ as shown in Figure 3(a), and the concentration of FLU was obtained by substitution in the corresponding regression equation. The same steps were repeated for the determination of FLR using normalized FLU as divisor and determining $\Delta P_{226\text{-}237}$ as shown in Figure 3(b), where the ratio spectrum of FLU showed zero difference, while FLR, the component of interest, showed significant difference at $\Delta P_{226\text{-}237}$. The derivative ratio method was applied twice. The first derivative of the stored ratio spectra of FLR as shown in Figure 4(a) and FLU as shown in Figure 4(b), using either normalized FLU spectrum or normalized FLR spectrum as a divisor. The concentration was obtained by substitution in the regression equations relating the amplitudes of the D^1 of the division spectra at 232.7 nm to the corresponding FLR concentrations, and the amplitudes at 213 nm to the corresponding FLU concentrations.

The overlaid spectra of mixture A components (Figure 2a) revealed two isoabsorptive points (points of equal absorptivity of both drugs), 216.2 and 234.6 nm. Trials for both wavelengths were done and found that 216.2 nm gave better results. The isoabsorptive point made it possible to apply the two methods, absorbance subtraction and amplitude modulation.

(4) *Absorbance subtraction (AS)*.—Absorbance subtraction was applied depending upon the presence of an isoabsorptive point 216.2 nm and an extended region where only FLU had a contribution as shown in Figure 5(a). The absorbances at 333 nm where only the extended drug FLU had a contribution, and the absorbance at isoabsorptive point 216.2 nm were used to calculate the response factor. The response factor [$F = A_{216.2}/A_{333}$] was calculated and found to be 2.9. Next, absorbances at 216.2 nm were used to construct the unified regression equation for both drugs. To obtain the concentration of FLU in mixture, the absorbance of FLU at λ_{iso} (216.2 nm) in laboratory mixtures was calculated by multiplying the absorbance at 333 nm where only FLU had a contribution by the previously calculated response factor, and then substituted in the unified regression equation. Subsequently, to get FLR; the postulated absorbance of FLU at λ_{iso} (216.2 nm) was subtracted from the recorded absorbance at λ_{iso} (216.2 nm) from the laboratory mixture spectrum, and substituted in the unified regression equation.

(5) *Amplitude modulation (AM)*.—This method also depended on the same isosbestic point, 216.2 nm present in the zero-order absorption spectra of FLU and FLR, that was preserved in their ratio spectra using normalized FLU as a divisor as shown in Figure 5(b). The amplitude in the extended region (284–310 nm) of the mixture's ratio spectrum was substituted in the unified regression equation to obtain the FLU concentration. Then the postulated amplitude of FLR at λ_{iso} (216.2 nm) was obtained by subtracting the postulated amplitude of FLU at λ_{iso} (216.2 nm) from the recorded amplitude of the mixture's ratio spectrum at λ_{iso} (216.2 nm). Finally, the result of this subtraction was substituted in the unified regression equation, attaining the FLR concentration. Both drugs were determined over a common linearity range (5–40 μg/mL).

From the ratio spectrum of the laboratory mixture using normalized FLU as a divisor, the peak amplitude in the extended region (284–310 nm) graphically represented the FLU concentration without substitution in a regression equation. As the recorded peak amplitude at the λ_{iso} (216.2 nm) from the laboratory mixture ratio spectrum represented the total concentration of two components in mixture (FLU+FLR), so by subtracting the concentration of FLU from the total concentration of the laboratory mixture, the FLR concentration was obtained by the CNV method.

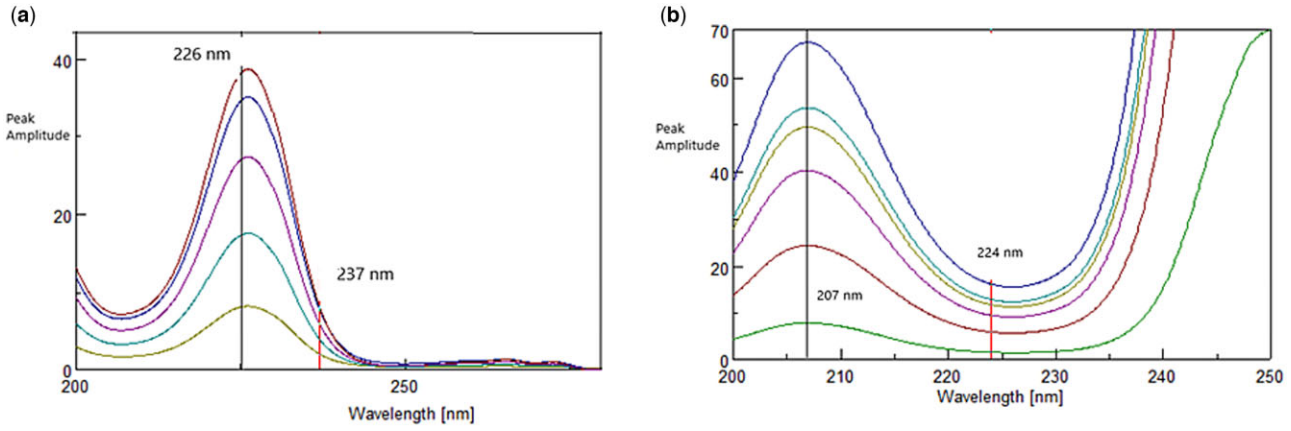

Figure 3. (a) Ratio spectra of FLR (3–40 μg/mL) using normalized FLU as a divisor. (b) Ratio spectra of FLU (5–40 μg/mL) using normalized FLR as a divisor.

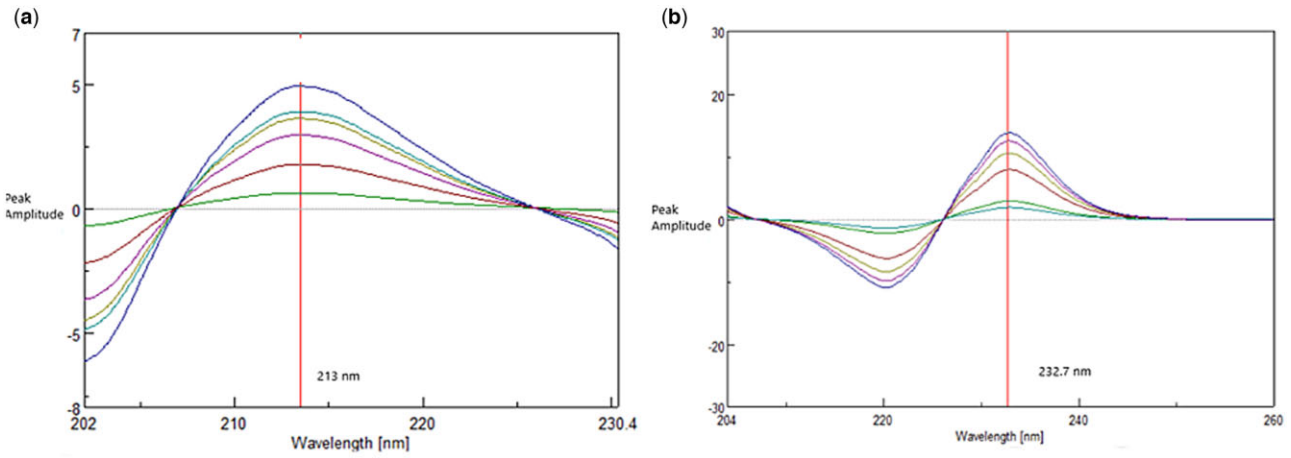

Figure 4. (a) The first derivative of the ratio spectra of FLU (5–40 μg/mL) using normalized FLR as a divisor. (b) The first derivative of the ratio spectra of FLR (3–40 μg/mL) using normalized FLU as a divisor.

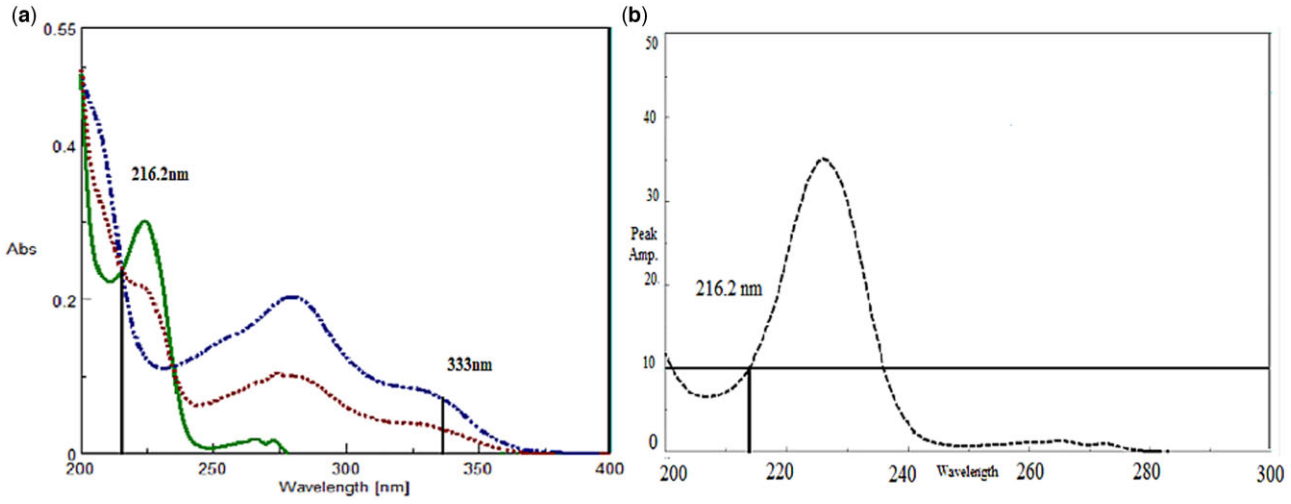

Figure 5. (a) Ratio spectrum of FLR 8 μg/mL (____),FLU 8 μg/mL (. . . . .), and total laboratory mixture 8 μg/mL (- - - -) of both FLU and FLR. (b) Ratio spectrum of FLU 20 μg/mL (- - -) using normalized FLU spectrum as a divisor, overlaid with ratio spectrum of FLR 10 μg/mL (___).

(b) *Mixture B.*—Both FLU and OXY were co-formulated in Floxon, a veterinary antibiotic injection indicated for treatment of bovine respiratory disease (BRD) in cattle associated with *Pasteurella multocida*, *Pasteurella haemolytica*, and *Mycoplasma bovis*. The zero-order absorption spectra of OXY with FLU were severely overlapped in the wavelength region 250–380 nm as shown in Figure 2b. As the UV bands of both drugs were severely overlapped conventional spectrophotometric approaches could not be applied for the simultaneous determination of this mixture.

In mixture B, using distilled water as a solvent was not possible because solutions in water become turbid on standing, owing to precipitation of OXY .Instead, 0.1 N HCL was used.

The literature revealed conventional spectrophotometric methods for the determination of a FLU and OXY mixture (13) with severely overlapped peaks such as derivative ratio, dual wavelength and ratio difference. The current study present a more sensitive method analyzing FLU over a linearity range 2.5–24 μg/mL with the ability to mathematically filter each drug alone in its zero-order spectrum, thus achieving the spectral profile of each drug, and allowing the measurement of each drug at its λ_{max}.

(1) Approaches.—Four approaches, all of which were performed using the same constant, were applied for the determination of both OXY and FLU.

Methods such as CV, CNV, CM, and RS/AD-SS are well known and could be applied to partially overlapping spectra, where they all depend upon getting a divisor spectrum of the extended component. After division, a plateau region appears in the extended part of the spectrum from which a constant can be obtained, and the four methods depend on that constant. Since the mixture of FLU and OXY was severely overlapped showing no extended region, manipulation steps depending on amplitude difference (AD) were performed to help obtain a constant representing either FLU/FLU' or OXY/OXY' that is required for applying CV, CNV, CM, and RS-SS after using the amplitude difference step, where the methods are now called: CV-AD, ACV, CC, and RS/AD-SS. Several trials were made to choose the best possible divisor, finding that a

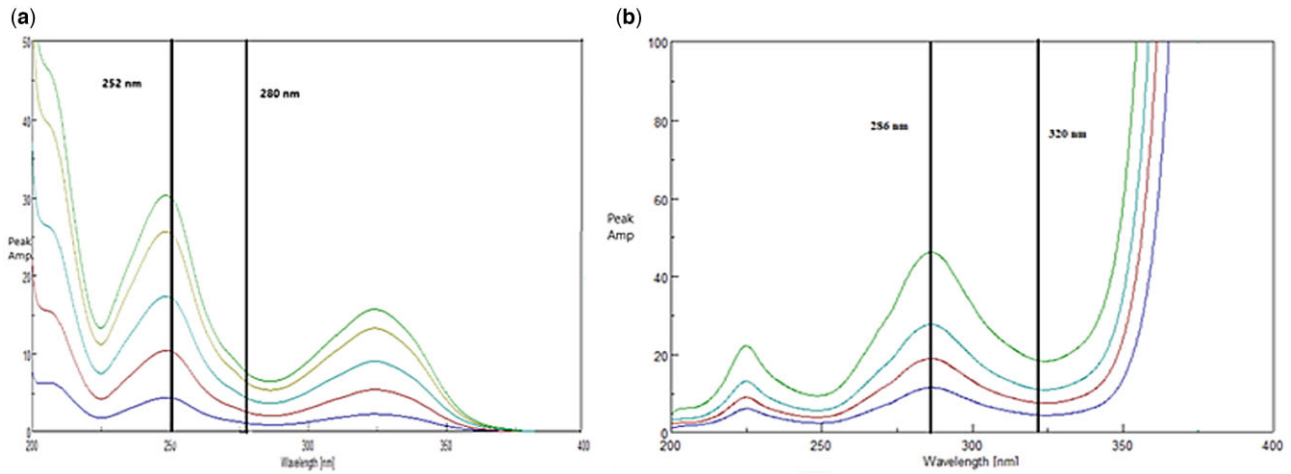

Figure 6. (a) Ratio spectra of FLU using normalized OXY spectrum as a divisor showing peak amplitudes at 252 and 280 nm. (b) Ratio spectra of OXY using normalized FLU spectrum as a divisor showing peak amplitudes at 286 and 320 nm.

normalized divisor in both OXY and FLU gave the best results. In comparison to other traditional ratio manipulating spectrophotometric methods, normalized spectra-manipulating methods are characterized by being simple and modest procedures. Using a normalized divisor has an advantage of eliminating errors due to using different concentrations as it represents absorptivity. Moreover, when utilized as a divisor, the importance of the normalized spectrum was highlighted because it produced a constant line in the ratio spectra which represents the concentration of the component having the extended spectrum. Different concentrations of FLU were separately divided by the normalized OXY spectrum as divisor, and different concentrations of OXY were separately divided by normalized spectrum of FLU as a divisor, then amplitudes at 252 and 280 nm and 286 and 320 nm, respectively, were subtracted to obtain ΔP_1 and ΔP_2 (Figure 6a and b); ΔP_1 versus P_{252} yielded the regression equation for different concentrations of FLU, and ΔP_2 versus P_{286} the regression equation for different concentrations of OXY. ΔP_1 obtained from the ratio spectra of the laboratory mixtures using normalized OXY as a divisor was substituted in the corresponding regression equation to obtain the peak amplitude due to FLU in the mixture. The amplitude difference obtained by subtracting this postulated peak amplitude at P_{252} from the laboratory mixture recorded P_{252} represented the constant (OXY/OXY'). ΔP_2 obtained from the ratio spectra of the laboratory mixtures using normalized FLU as a divisor was substituted in the corresponding regression equation to obtain the peak amplitude due to OXY in the mixture. The amplitude difference obtained by subtracting this postulated peak amplitude at P_{286} from the laboratory mixture recorded P_{286} represented the constant (FLU/FLU').

(2) *The constant obtained for each drug was used as follows.*— First, the advanced concentration value (ACV) method was applied where the constant obtained was directly recorded representing the actual concentration without prior substitution in the regression equation. The method's advantage over the concentration value was that it could be applied to the binary mixture spectra showing partial and severe overlap, where the constant was obtained via amplitude difference not from the extended region.As previously known, the concentration value (CNV) was restricted for the partially overlapped spectra with extension.

The second method applied was constant value via amplitude difference (CV-AD) method. The constant OXY/OXY' or FLU/FLU' was replaced in the corresponding regression equation obtaining either OXY or FLU concentration, respectively.

The third method applied was the constant enter (CC) method, which resolved the zero-order absorption spectrum of each drug alone, allowing measurement of the absorbance of OXY or FLU at their λ_{max}, 267.6 or 252 nm. The constant representing FLU/FLU' was multiplied by normalized FLU to obtain the zero-order absorption spectrum of FLU, and the absorbance at 267.6 nm was substituted in the corresponding regression equation. The same steps were done for OXY; the constant representing (OXY/OXY') was multiplied by normalized OXY' resulting in the zero-order absorption spectrum of OXY. The absorbance obtained at 252 nm was substituted in the corresponding regression equation to obtain the OXY concentration.

The fourth method applied was ratio subtraction via amplitude difference-coupled spectrum subtraction (RS/AD-SS). For determination of OXY, laboratory mixture spectra were divided by normalized FLU. Then the constant representing FLU/FLU' was subtracted from the resulting spectra followed by multiplication with the normalized FLU spectrum. The zero-order absorption spectrum of OXY was obtained. and the absorbance at 267.6 nm was substituted in the corresponding regression equation to obtain the concentration of OXY in the mixture. For determination of FLU, the laboratory mixture spectrum was subtracted from the resolved OXY spectrum to obtain the zero-order absorption spectrum of FLU where measurements were conducted at its λ_{max} 252 nm (22, 23).

Method Validation

Method validation parameters for the proposed methods are presented in Tables 1 and 2 and the validation was performed according to ICH guidelines (24).

Table 1. Assay parameters and method validation obtained by applying the proposed spectrophotometric methods for the binary mixture FLR and FLU

	FLR					FLU					
Parameter	D^0 225 nm	$DD^1_{232.7\ nm}$	$RD^{\Delta P}{}_{226-237\ nm}$	AS 216.2 nm	AM 216.2 nm	D^0 280.1 nm	$DD^1_{213\ nm}$	$RD^{\Delta P}{}_{207-224\ nm}$	CNV 280 nm	AS 216.2 nm	AM 216.2 nm
Range, μg/mL		3–40						5–40			
Slope	0.0354	0.3409	0.2533	0.0269	1.0104	0.0252	0.0274	1.2871	—[a]	0.0269	1.0104
Intercept	0.019	0.1240	0.0912	0.0166	−0.0648	−0.01	0.0091	0.3708	—	0.0166	−0.0648
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9998	0.9999	0.9999	0.9998	0.9999	—	0.9998	0.9999
Accuracy % Mean ±SD	100.42 ±1.02	100 ± 1.89	100.59 ± 0.83	100.353 ± 1.47	99.87 ±0.49	100.15 1.13	101.07 ±1.47	100.82 ± 1.05	100.47 ±0.61	100.353 ± 1.47	99.87 ±0.49
Repeatability (RSD)	0.738	0.762	0.819	0.200	0.330	0.153	1.185	0.304	0.231	0.200	0.330
Intermediate precision (RSD)	1.961	0.999	1.322	0.624	0.535	0.948	1.361	1.631	0.271	0.624	0.535
Selectivity % Mean ±SD	100.26 ±1.048	100.29 ±0.708	99.524 ±1.000	100.65 ±1.153	100.67 ±0.935	101.1 ±0.937	100.24 ±0.642	101.32 ±1.042	100.96 ±0.502	99.71 ±1.594	100.07 ±1.189

[a]— = No value.

Table 2. Assay parameters and method validation obtained by applying the proposed spectrophotometric methods for binary mixture OXY and FLU

	OXY				FLU			
	ACV $P_{252-280}$	CV-AD 252 nm	D^0 267.6 nm		ACV $P_{286-320}$	CV-AD 286 nm	D^0 252 nm	
Range, μg/mL		4–28				2.5–24		
Slope	—[a]	0.9995	0.0505		—	1.0071	0.0433	
Intercept	—	0.1662	0.0005		—	−0.1036	−0.003	
Correlation coefficient (r)	—	0.9998	0.9999		—	0.9998	1	
Accuracy	101	100.934	99.89		99.86	100.345	99.4	
Mean ±SD	± 1.55	± 1.05	±0.999		±0.67	± 1.06	±0.89	
Repeatability (RSD)	0.214	0.432	0.164		0.049	0.232	0.593	
Intermediate Precision (RSD)	0.501	0.645	0.754		0.496	0.900	0.672	
			CC-MS:	RS/AD-SS:			CC-MS:	RS/AD-SS:
Selectivity	100.82	99.93	99.10	99.84	100.44	98.80	99.05	101.200
Mean ±SD	± 0.582	± 1.106	±0.999	±1.469	± 1.042	± 1.290	± 1.071	±0.964

[a]— = No value.

(a) *Linearity*.—The linearity of the methods were evaluated by constructing the graphs of the concentrations of all drugs versus the response. Each concentration was repeated three times.

(b) *Range*.—Linearity ranges were found to be 5–40 and 3–40 μg/mL for FLU and FLR, respectively, in distilled water, while 4–28 and 2.5–24 μg/mL for OXY and FLU, respectively, in 0.1 N HCl.

(c) *Accuracy*,—To study the accuracy of the proposed methods, procedures under (a) for the drugs were repeated three times for the determination of three different concentrations of pure OXY, FLR, and FLU. The accuracy is expressed as recovery %.

(d) *Precision*.—Repeatability and intermediate precision were determined by the analysis of three different concentrations of the proposed drugs within the linearity range three times for three pure samples of the drugs on a single day and on three consecutive. The results are expressed as RSD.

(e) *Specificity*—This parameter was evaluated by analyzing multiple mixtures that contain the drugs in different ratios within the mentioned ranges. Satisfactory percentage recoveries with low standard deviation were obtained.

Application to the Veterinary Formulation

Table 3 shows satisfactory recovery percentages when the proposed methods were applied to veterinary formulations of Megluflor and Floxon injections.

Statistical Analysis

Tables 4 and 5 show statistical comparison of the results obtained by the proposed methods and official methods or reported methods (3, 7) and that the calculated *t*- and *F*-values were less than the theoretical ones, indicating that there was no significant difference between the proposed and the official methods or reported methods with respect to accuracy and precision.

Table 3. Analysis of FLR and FLU in Megluflur injection, and analysis of OXY and FLU in Floxon injection.

Megluflur injection (batch no. 191264)												
Drug	FLR						FLU					
Method	CM-SS	DD[1]	RD	CNV	AS	AM	CM-SS	DD[1]	RD	CNV	AS	AM
Claimed concn	37.5 μg/mL						5.06 μg/mL					
Found, %	101.1	100.50	99.92	100.8	100.79	101.33%	99.99[a]	101.1[a]	100.20[a]	99.00[a]	100.79[a]	101.02[a]
± SD		±1.00	±1.53	±1.31	±1.01	±1.00	±1.00	±0.89	±1.03	±0.9	±1.01	±0.66

Floxon injection (batch no. 180362)								
Drug	OXY				FLU			
Method	ACV	CV-AD	CC	RS/AD-SS	ACV	CV-AD	CC	RS/AD-SS
Claimed concn	21.6 μg/mL				6.6 μg/mL			
Found, %	101.14	100.25	99.66	100.80	101.75	102	100.75	102
± SD	±1.00	±1.53	±1.00	± 0.54	±0.63	±0.75	±1.00	± 1.32

[a] After subtraction of 3 μg/mL FLU for enrichment.

Table 4. Statistical comparison between results obtained by the proposed methods, the official method, and the reported method for the determination of FLR and FLU in pure powder form

	FLR							FLU						
Parameters	Reported method[a]	D^0	DD^1	RD	AS	AM	CNV	USP method[b]	D^0	DD^1	RD	AS	AM	CNV
Mean	100.10	100.25	100	100	99.62	99.89	100.12	100.08	100.17	100.03	100.20	99.64	99.87	99.87
SD	0.67	0.97	1.52	1.30	1.44	1.15	1.630	1.26	1.26	0.55	1.03	1.78	0.49	0.49
N	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Variance	0.82	0.98	1.23	1.14	1.14	1.07	1.63	1.12	1.01	0.75	1.01	1.33	0.70	1.28
F-test (5.05)[c]	—[d]	1.20	1.50	1.40	1.46	1.31	1.99	—	1.11	1.50	1.11	1.19	1.61	1.14
Student's *t*-test (1.81)[c]	—	0.801	0.80	0.83	0.73	0.37	0.04	—	0.15	0.16	0.18	0.49	0.38	0.56

[a] Derivative spectrophotometric method.
[b] Titrimetric method.
[c] The figures in parentheses are the corresponding theoretical values at $P = 0.05$.
[d] No value.

Table 5. Statistical comparison between results obtained by the proposed methods and the official methods for the determination of OXY and FLU in pure powder form

	OXY				FLU			
Parameters	D^0 267 nm	ACV $P_{252–280}$	CV-AD P_{252} nm	USP method[a]	D^0 252 nm	ACV $P_{286–320}$	CV-AD 255 nm	USP method[b]
Mean	99.95	100.95	99.63	99.48	100.22	99.86	100.25	100.08
SD	0.93	1.55	1.68	1.43	0.89	0.68	1.36	1.26
N	6	6	6	6	6	6	6	6
Variance	0.96	1.25	1.29	1.19	0.94	0.82	1.16	1.12
F-test (5.05)[c]	1.23	1.29	1.34	—[d]	1.19	1.37	1.04	—
Student's *t*-test (1.81)[c]	0.68	1.41	0.18	—	0.37	0.38	0.23	—

[a] Direct spectrophotometric determination at 353 nm.
[b] Titrimetric method.
[c] The figures in parentheses are the corresponding theoretical values at $P = 0.05$.
[d] No value.

Greenness Assessment

It was also critical for us to consider environmental safety and health risks, thus our top priority was to develop a green process that used environmentally friendly solvents. The term "green analytical chemistry" (GAC) was brought forward at the beginning of 1990s. The GAC concept aims to either reduce or eliminate hazardous chemicals from proposed analytical processes in order to increase the environmental friendliness without compromising the overall method overall. Greening an analytical procedure, as well as obtaining acceptable figures of merit, can be achieved by different strategies, such as reducing the number of solvents, and trying to choose a safe

solvent over hazardous ones, and also trying to reduce the amount of waste. In the proposed methods, we attempted to reduce the number of solvents used and chose the least hazardous ones. Evaluation of greenness is mandatory, so we evaluated our method using the most common methods of assessment: National Environmental Methods Index (NEMI) labeling, the Analytical Eco-Scale, and the Green Analytical Procedure Index (GAPI). The evaluation of analytical techniques in the name of green chemistry is rather challenging due to the vast diversity of utilized analytes and methods, sample matrix complexity, and the particular analytical criteria that need to be considered (25).

National Environmental Methods Index (NEMI) Labeling

This method is considered the oldest tool for assessing analytical method greenness. It simply represents a pictogram that is divided to four equal sectors where each represents the waste and solvents utilized in the proposed method: (1) PBT (persistent, bio-accumulative, and toxic); (2) hazardous; (3) corrosive (pH <2 or >12); (4) waste volume is >50 mL.

Each quadrant may be green or blank, depending whether if each previous point is fit with the proposed method. The first proposed method was found to be fully green, and the second proposed method possessed only one unshaded quadrant corresponding to hazardous.

The major drawback in NEMI is that it just gives a qualitative evaluation of the tested method; it does not include much information about the quantity of hazardous material, so we used another assessment method.

Table 6. The penalty points for the proposed methods for both binary mixtures FLR and FLU, and OXY and FLU

FLR and FLU	
Hazard	Penalty points
Reagents:	
H_20	0
Instruments:	
Waste	1
Energy and occupational hazard (UV spectrometry <0.1 kWh per sample)	0
Total penalty points	1
Analytical Eco-Scale for the proposed method	99
OXY and FLU	
Reagents:	
HCl	4
Instruments:	
Waste	1
Energy and occupational hazard (UV spectrometry <0.1 kWh per sample)	0
Total penalty points	5
Analytical Eco-Scale for the proposed method	95

Analytical Eco-Scale

This assessment method involves more environmental impact parameters than NEMI. It is based on penalty points assigned to different factors (such as reagent type and amount, amount of energy consumed by electrical devices used, waste, and occupational hazards) and summed up, then finally subtracted from a base of 100. A score greater than 75 is considered excellent green analysis, a score higher than 50 represents an acceptable green method, while a score less than 50 signifies an inadequate green method. This method has an advantage that it discovers the weak points in the proposed method. All penalty points and scores are shown in Table 6. All of the proposed methods were found to be excellent green analyses.

Greenness Analytical Procedure Index (GAPI)

This method is one of the most recent tools for evaluating the greenness of an analytical method. It has a major advantage that it evaluates the greenness of the whole method starting from sample collection until determination, giving us a whole overview of the proposed method. It is made up of five pentagrams with three different color scales to indicate each stage, with green indicating low environmental damage, yellow indicating medium-to-low environmental impact, and red indicating significant environmental impact, as shown in Figure 7a. GAPI pentagram interpretation regarding the proposed methods revealed that the first mixture, illustrated in Figure 7b, had nine green-shaded fields, five yellow-shaded fields, and only one red-shaded region corresponding to no waste treatment, as there is no opportunity to apply this phase.Yet, to compromise

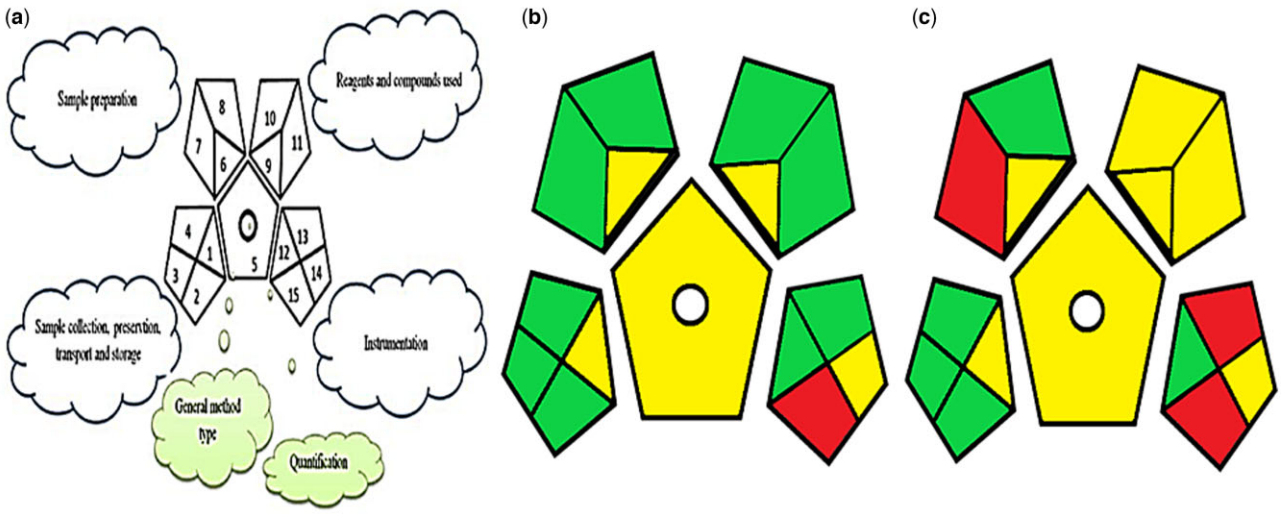

Figure 7. (a) GAPI original pentagram. (b) GAPI pentagram for binary mixture FLR and FLU. (c) GAPI pentagram for binary mixture OXY and FLU.

this, our system produces modest amounts of waste. The second mixture, illustrated in Figure 7c, contained five green-shaded regions, four yellow-shaded regions, and three red regions; the first was due to non-green solvents/reagents used, the second was due to emission of vapors to the atmosphere, and the third was due to no waste treatment, which is mitigated by the low amounts of waste produced in the method.

Conclusions

This work introduced simple, sensitive, accurate, and rapid spectrophotometric methods for the analysis of binary drug mixtures without their prior separation, and without the need for specially purchased software. The methods were applied by using simple mathematical manipulation steps via the built-in spectrophotometer software.

The proposed methods allowed FLU, FLR, and OXY measurements to be done at their λ_{max} with maximum accuracy and precision. These resolution techniques were considered as mathematical filtration methods to obtain the zero-order absorption spectrum of each drug alone whether from a severely overlapping mixture spectrum (mixture A) by the aid of CM-SS, or a partially overlapping mixture spectrum (mixture B) by the aid of CC-SS, and RS/AD-SS.

Graphical representation of the concentration by CNV and ACV methods allowed quantitation of the three drugs under investigation from their mixtures without the aid of regression equations.

Also, progressive methods based on isoabsorptive points were applied as AS and AM where the two components in the mixture were estimated using a single divisor and a unified regression equation showing very high accuracy and precision, besides the few manipulation steps.

The methods obeyed green principles and did not require complicated treatment, sophisticated experimental setup, or excessive usage of organic solvents that is usually associated with HPLC and HPTLC methods of analysis. The applicability of the developed methods was evaluated through the determination of a FLR and FLU mixture in pharmaceutical dosage form (Megluflor), which had good accuracy and precision, so they could be considered an alternative tool for the routine analysis of this mixture with minimum sample preparation and without hazardous solvents.

The second mixture was successfully determined by four methods all of which depended on one constant, which made the steps easier and time effective. The applicability of the developed methods was evaluated through the determination of a FLU and OXY mixture in pharmaceutical dosage form (Floxon) with good accuracy and precision, allowing routine analysis in QC laboratories with simple sample preparation and cost-effective solvents.

Finally, the whole process was evaluated by three green assessment tools, and found to be of excellent greenness with no toxic wastes or hazards to the environment.

Conflict of Interest

All authors declare no conflict of interest.

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