Derivative Spectrophotometric Determination of Florfenicol in Chicken Samples

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A simple, rapid, and reliable derivative spectrophotometric method for determination of florfenicol was developed. Florfenicol belongs to amphenicol group antibiotics, the most widespread antibiotics used in poultry. Antibiotic residues in edible tissues of food-producing animals contribute to several reasons, such as a withdrawal period, overdose and long acting drugs. The analysis of the derivative spectra was done through the determination of the derivative values, using one of the four graphical methods, which included peak–to-baseline (peak height), peak-to-peak, and area under peak methods, chicken tissues.

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1. INTRODUCTION

There are various purposes for using antibiotics in the animal farms, such as prevention and control of disease, prophylactic, and as growth promoter (Shankar et al., 2010, Hind et al., 2014). Drugs can be easily absorbed and distributed in the animal tissues, accumulate and transferred into their products; milk, egg, and meat when the withdrawal periods is not recommended. So animals products may contain the drug residues (Salman et al., 2013), which may lead to cause several health problems in humans, such as allergies (Khalil et al., 2012), spreading of drug-resistant microorganisms, carcinogenic effect, reproductive effects and potential harmful effect on human intestinal micro flora (Renew and Huang, 2004, Xie et al., 2011).

The oversight of veterinary drug residue is an important measure in consumer protection assurance due to its harmful health effects on human life. Several countries were strictly adjusted the use of veterinary drugs in...
food animal. Some of these drugs can be allowed with specific circumstances but under rigorous control, administration by a veterinarian and withdrawal time observance (Shankar et al., 2010).

Florfenicol, as shown in Figure 1, [D-D-threo-3-fluoro-2-dichloroacetamido-1-(4-methylsulfonyl)-1-propanol], belongs to the amphenicol family of antibiotics with a broad antibacterial spectrum. It was developed by Schering-Plough Corp (El-Banna and El-Zorba, 2011).

![Figure 1: Structure of florfenicol](image)

The fenicol group of antibiotic is one of the vastly used drugs which are preferred to treat diseases relating to digestive and respiratory systems. This group of antibiotics are used in veterinary medicine effectively in poultry, against both Gram-positive and Gram-negative bacteria like Escherichia coli, Corynebacterium pyogenes, Streptococcus spp., Haemophilus somnus, Clostridium spp., Pasteurella haemolytica, Staphylococcus spp., and Actinobacillus pleuropneumoniae. Chloramphenicol tiamfenicol, azidamfenicol, and florfenicol are examples of the most commonly fenicol group antibiotics (El-Banna and El-Zorba, 2011, Ekici and Yarsan, 2014).

Derivative spectroscopy has important role in UV-VIS spectra interpretation and quantitative analysis. It has a great advantage to resolve the overlapping spectra problems especially in those methods which lack selectivity making them suitable for application (Hadkar, 2014).

The derivative spectra methods allow using the wavelength of highest value of maxima or minima signals. Furthermore, the presence of a large number of maxima and minima wavelengths gives an opportunity to select a particular wavelength for determination of active compounds without interference (Attimarad et al., 2012).

An additional property of derivative spectrophotometry is the dependence of derivatization results on the shape (i.e. geometrical characteristic) of zero-order spectra. So, analyte signals, which are in zero-order spectra narrow, undergo amplification and quenched in higher derivative orders. This property allows reducing or eliminating the effect of background and provide higher sensitivity and/or selectivity of determination (Allassaf, 2016).

Several methods have been reported for determination of florfenicol, like, spectrophotometric method (Sadeghi and Jahani, 2013) high-performance liquid chromatography (Guo et al., 2015, Peng, 2015, Barreto et al., 2016, Nasim et al., 2016), gas chromatography (Azzouz et al., 2011a, Azzouz et al., 2011b), flow injection analysis (Ge et al., 2010).

The present work describes a simple, sensitive and selective method for determination of florfenicol in chicken tissues by first-derivative spectrophotometric method.

2. Experimental

2.1 Instruments and Chemicals

A double beam UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) connected to computer loaded with UV Probe software, was employed with spectral bandwidth of 1nm and a pair of 10 mm matched quartz cells. All spectra were recorded from 200 nm to 400 nm. Weights were taken on electronic balance (Denver, Germany). Sorvall RC-5B Superspeed Centrifuge (at 25°C) was used for centrifugation.
Hexane (Scharlau), ethyl acetate, methanol, were all analytical grade, sodium chloride, Sodium sulfate were obtained from Merk, and were used as received without any further purification.

2.2 Preparation of Solutions

Standard stock solutions of florfenicol (100 μg/ml): 0.010 g of florfenicol was dissolved and diluted in 100mL methanol. The solution was monthly prepared and was diluted to obtain working solutions for the preparation of calibration curves.

Phosphate buffer solution (pH =6.88) was prepared from the mixture of 0.1 M KH₂PO₄ and 0.1 M Na₂HPO₄ solutions.

2.3 Derivative Spectra of Florfenicol Solution

To study the derivative order, aliquots of florfenicol standard stock solutions were accurately transferred in to 10 ml volumetric flasks, and volumes were completed with distilled water. All the working solutions were scanned from 200 to 400 nm in the spectrum mode.

2.4 Extraction Procedure

Thirty grams of the chicken samples were mixed with 40 mL phosphate buffer solution (pH = 6.88), and then 10 g sodium sulfate, 20 ml ethyl acetate were added. The mixture was shaken vigorously for 10 min. After centrifugation for 5 min at 5500 rpm, the supernatant was transferred into another flask. The extracts were evaporated to dryness under nitrogen stream in a water bath at 45–50°C. Ten milliliters of methanol was added, and the tube then vortexes for about 30 seconds. Thereafter, 10 ml of 4% NaCl and 30 mL of hexane was added into the tube. The mixture was shaken for about one minute. Then the hexane layer was discarded. The de-fatting step was repeated and the extract was again evaporated to dryness with a gentle stream of nitrogen in a water bath at 45–50 °C. The dried sample was dissolved in 5.0ml methanol(Khalil et al., 2012) and was used for analysis by the first derivative method.

The chicken samples were treated according to Khalil method, briefly, 30g of chicken tissues were mixed with 40 mL phosphate buffer solution (pH = 6.88), and then 10 g sodium sulfate, 20 ml ethyl acetate were added. The mixture was shaken vigorously for 10 min. After centrifugation for 5 min at 5500 rpm, the supernatant was transferred into another flask. The extracts were evaporated to dryness under nitrogen stream in a water bath at 45–50°C. Ten milliliters of methanol was added, and the tube then vortexes for about 30 seconds. Thereafter, 10 ml of 4% NaCl and 30 mL of hexane was added into the tube. The mixture was shaken for about one minute. Then the hexane layer was discarded. The de-fatting step was repeated and the extract was again evaporated to dryness with a gentle stream of nitrogen in a water bath at 45–50 °C. The dried sample was dissolved in 5.0ml methanol, and was used for analysis by the first derivative method.

3. RESULTS

Antibiotics are being excessively used in the different animal farms for various purposes: such as growth stimulant, prophylactic, prevention and control of disease. When with drawl periods of drug is not applied, antibiotic residues may appear in the animal products such as milk, eggs, meat, so it is necessary to monitoring the antibiotic residue in animal products due to the side effects on human health (Hind et al., 2014).

3.1 Stability of the Analytical Solutions

The stability of the florfenicol solution was evaluated by measuring absorbance of freshly prepared standard solution, and
repeating the measurement after 24, 48, 72 hours, one, two, three and four weeks, no changes in the absorbance were observed when the solution was stored at 4°C for one month.

3.2 Zero-Order Derivative Spectrophotometry

The drug solution was scanned (200-400 nm) against reagent blank, the absorption spectrum (Fig. 2) was recorded. The absorption maximum (λmax) was observed at 224 nm, and the absorbance of all the sample solutions (0.3-53 μg/ml) was recorded at that λmax. A graph was plotted by taking the concentration of the solutions on the x-axis and the corresponding absorbance values on the y-axis. Fig. 3 shows the calibration curve of zero-order derivative spectrophotometry (normal spectrum) of florfenicol.

3.3 Derivative Spectra of Florfenicol Solution

To study the derivative order, aliquots of florfenicol standard stock solutions were accurately transferred in to 10 ml volumetric flasks, and volumes were completed with distilled water. All the working solutions were scanned from 200 to 400 nm in the spectrum mode. Thus, the obtained absorption spectra were derivatized from first to fourth order as shown in Fig. 4-7. The study shows that first order spectra were simple and gave results of highest accuracy and detection limits.
3.4 First Derivative Spectra of Florfenicol Solution

The first derivative spectrum of florfenicol solution has maximum absorption at 218.5 nm and 233.7 nm as shown in figure 4, in which a good proportionality between the amplitude and the concentration of florfenicol was found, so it was selected for the present study.

Different optimizations were done on the 1st order derivative spectra such as smoothing, data interval, and scan speed. Finally smoothing of 2 and 25 nm of data interval at scanning speed of 10 nm/sec in the wavelength range of 200-400 nm, were selected for further working.

The analysis of the derivative spectra was done through the determination of the derivative values, which was carried out by one of the four graphical methods, which included area under peak, baseline-to-peak (peak height), and peak-to-peak techniques.

In area under peak technique, the area of a peak or a valley is measured. In the baseline to peak technique, the span of the peak is measured from a maximum to the zero line or from a minimum to the zero line. In the
peak-to-peak technique, the determination is carried out by measuring the amplitude from a maximum to a minimum of the curve.

Graphically, basing upon different techniques such as peak-to-baseline, peak-to-peak, and peak area of 1st derivative spectra more than one relation between the concentration of florfenicol and signals of 1st derivative spectra of florfenicol were been obtained.

3.4.1 Calibration Graph of 1st Derivative Spectrophotometric Determination of Florfenicol Using Peak-to-Baseline Technique

Under the optimum experimental conditions for the 1st derivative spectrophotometric method using peak-to-baseline technique a good proportionality was found between florfenicol concentration and peak amplitude at 218.6 and 233.8 nm as shown in Fig. 8 and 9, respectively. Florfenicol was determined over the concentration range of 1.0-53 μg/ml with detection limit of 0.7 μg/ml, \( r^2 = 0.9996 \) and 0.5 μg/ml detection limit, \( r^2 = 0.9997 \) at 218.5 and 233.7nm respectively.

3.4.2 Calibration Graph of 1st Derivative Spectrophotometric Determination of Florfenicol Using Peak-to-Peak Technique

Application of the peak-to-peak technique in the 1st derivative spectra of florfenicol leads to find linearity between the florfenicol concentration and peak-to-peak amplitude which can be used for direct determination of florfenicol in the concentration range of 0.5-53 μg/ml, with detection limit of 0.3 μg/ml and \( r^2 = 0.9995 \) as shown in Fig. 10.

3.4.3 Calibration Graph of 1st Derivative Spectrophotometric Determination of Florfenicol Using Peak Area Technique

Peak area technique is another technique that can be applied at selected wavelength intervals to find relation between
the concentration and area under peak. Therefore, different wavelength intervals were studied. The results showed that at the intervals of 211.2-224 nm and 224-249.2 nm, good relation between florfenicol concentration range of 0.7-53 μg/ml, with detection limit of 0.435 μg/ml and r²= 0.9999, and concentration range of 0.3-53 μg/ml, with detection limit of 0.112 μg/ml and r²=0.9998 were determined as shown in Fig.11 and 12, respectively.

Figure 11: Calibration curve of 1st derivative spectrophotometric determination of florfenicol using peak area from 211.2-224 nm

Figure 12: Calibration curve of 1st derivative spectrophotometric determination of florfenicol using peak area from 224-249.2 nm

3.5 Precision and Accuracy

The precision and accuracy of 1st derivative spectrophotometric determination of florfenicol were studied based on the values of the relative standard deviation percentage (RSD %) and the relative error percentage (Error %) for five replicate measurements of three different concentrations, respectively. Table 1 shows the results.

| Technique of analysis                  | Concentration of florfenicol | RSD%  | Error% |
|----------------------------------------|------------------------------|-------|--------|
| peak-to-baseline at 218.5nm 1          | 1                            | 3.01  | -3.60  |
|                                       | 25                           | 2.50  | -0.79  |
|                                       | 53                           | 1.32  | 0.69   |
| peak-to-baseline at 233.7nm 1          | 1                            | 2.43  | -3.98  |
|                                       | 25                           | 1.13  | 2.75   |
|                                       | 53                           | 0.45  | -0.45  |
| peak-to-peak                           | 0.5                          | 1.12  | -4.72  |
|                                       | 25                           | 0.84  | 3.88   |
|                                       | 53                           | 0.41  | 0.31   |
| peak area from 211.2-224nm 0.5         | 0.5                          | 1.22  | -4.72  |
|                                       | 25                           | 0.84  | 3.88   |
|                                       | 53                           | 0.31  | 0.31   |
| peak area from 224-249.2nm 0.3         | 0.3                          | 3.42  | 4.33   |
|                                       | 25                           | 0.11  | 1.79   |
|                                       | 53                           | 0.22  | -0.89  |

3.6 Interferences Study

The effect of tylosin and enrofloxacin; the most used antibiotics in chickens were studied as interfering materials on the determination of 25 μg/mL of florfenicol with the proposed method. A species considered as an interferent, when it caused a relative error percentage greater than ± 5.0% in the peak height of the signals of the sample. Results indicated that the commonly encountered excipients did not interfere in the examined method as shown in Table 2

| Interfering compound | Interfering concentration, μg/ml | Relative Error% |
|----------------------|----------------------------------|-----------------|
| Tylosin              | 35                               | +4.67           |
Enrofloxacin | 15 | -4.88

3.7 Application of the Method

The proposed method (namely first derivative – peak area from 224-249.2 nm) was successfully applied with the aid of standard addition method for direct determination of florfenicol in the chicken tissues. The samples include muscles (thigh, arm), kidney and liver in three different weights of chicken; 2.30, 3.10 and 4.0 kg. Table (3) summarizes the results of application and recovery study.

Table 3: Determination and recovery of florfenicol in chicken tissues

| Target tissue* | Chicken weight, Kg | Conc. Of florfenicol, found, µg/ml | Recovery % |
|----------------|--------------------|-----------------------------------|------------|
| Thigh and arm  | 2.3                | 0.4101                            | 97.15      |
|                | 3.1                | 0.4763                            | 101.64     |
|                | 4.0                | 0.5986                            | 102.62     |
| Kidney         | 2.3                | 2.133                             | 116.87     |
|                | 3.1                | 4.988                             | 95.81      |
|                | 4.0                | 6.215                             | 109.59     |
| Liver          | 2.3                | 9.011                             | 100.73     |
|                | 3.1                | 14.725                            | 102.04     |
|                | 4.0                | 19.503                            | 97.88      |

*Each target tissue is a mean of 10 chicken

4. DISCUSSION

Derivative spectrophotometry method has been widely used to enhance the signal and resolve the overlapped peak-signals due to its advantages in differentiating closely adjacent peaks, and identifying weak peaks obscured by sharp peaks. Elimination of spectral background matrix interferences and Enhancement of sensitivity and specificity in mixtures analysis(Ojeda and Rojas, 2013)

The additional property of derivative spectrophotometry, as compared with the classical method, is the dependence of derivatisation result on the shape of zero-order spectra. Signals of analyte which are in basic spectrum narrow, undergo amplification, whereas broad even intense zero order signals undergo flattening and in the end derivatization leads to their zeroing. This property allows eliminating the influence of the background and increases selectivity of determination(Zhang et al., 2009, Parmar and Sharma, 2016).

Derivative spectrophotometry offers a convenient solution to a number of analytical problems, such as resolution and analysis of multicomponent systems and matrix background without any separation procedures such as solvent extraction, leading to avoid the use of toxic and ozone depleting organic solvents, thus DS is regarded as a tool for green analytical chemistry and economic techniques(Dinç and Onur, 1998).

The use of antibiotics cannot be avoided; however, it is important to ensure the safety of food and food products for human consumption, so the Committee for Veterinary Medicine Products (CVMP) of the European Agency for the Evaluation of Medicinal Products were set the maximum residue limits (MRLs) of florfenicol in the chicken muscle, kidney and liver as 100 µg/kg, 750 µg/kg and 2500 µg/kg respectively.

In this study, mean muscle, kidney and liver florfenicol level was not above the MRLs in 2 Kg and 3Kg chicken's weight. While in muscle tissue, kidney and liver of 4.0 kilograms chicken's weight, kidney and liver of three kilograms chicken's weight, the florfenicol level was higher than the MRLs. The results of liver and kidney show that they contain considerable level of target drug, so, it must be forbidden to consumption, as long as they contain residue of florfenicol in all cases. Therefore, for safe life it is better to consume two kilograms (and less) chicken weight in human food, and not to use organs like liver, kidney in the food.
Florfenicol concentration was found in chicken's liver due to the fact that liver is a critical organ which contains most of the accumulated antibiotics and where toxic effects can be expected. Moreover, liver is the main and important detoxifying organ and is essential for both the metabolism and the excretion of drugs in the body, causing histological changes in the liver (Van Dyk et al. 2007). The main reason that concentration of florfenicol in the chicken organs all over the MRL due to the fact that the poultry industries use antibiotics in excess for both of disease control and as growth promoter purposes and does not respect the withdrawal time of each drug for trading purposes. Florfenicol is considered safe for human consumption after 5 days withdrawal time when used at a level of 30 mg/kg body weight and after 7 days withdrawal time when used at a level of 60 mg/kg body weight.

5. CONCLUSION

The most consumed edible tissues (muscles, liver and kidney) could have significantly different residue concentrations. Based on the maximum residue limit, florfenicol should deplete from chicken at least 5 days before marketing to ensure that it is excreted from the tissues following dosing to be safe for human consumption. This study suggested widespread misuse of florfenicol in farm and lack of implementation of recommended withdrawal times, so the poultry industries must be awareness and educated for the use of antimicrobial drugs, in addition to investigate the antibiotics residue in chicken prior to marketing and respect the withdrawal times of the drugs.

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Conflict of Interest

There is no conflict of interest.

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