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# Optimization and validation of a new chromatographic method for the assay of veterinary formulation

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ABSTRACT

New, validated and accurate reversed phase HPLC method with UV detection has been established for simultaneous determination of a veterinary binary mixture of doxycycline hydrochloride (DOX) and tylosin tartrate (TYT). The stationary phase was ACE- 126-2546 AQ C-18 (250 × 4.6 mm i.d., 5 µm particle size) column at 25 °C, in an isocratic mode, using mobile phase containing a mixture of methanol: acetonitrile: distilled water in the ratio of 60:20:20 (*v*:*v*:*v*), with 0.01% trichloroacetic acid at the flow rate of 0.8 mL/min and UV detection was performed at 270 nm. The retention times were 4.02±0.01 and 5.62±0.01 mins for DOX and TYT, respectively. Selective determination of the cited veterinary drugs has been developed in their formulation. The method was found to be linear over 1-50 µg/mL for DOX and TYT with mean percentage recoveries 99.62±1.220 and 100.09±1.104%. The method was proven to be accurate, precise and specific. The obtained results were statistically compared with those of the official and reported methods; using Student's *t* test, *F* test and one-way ANOVA, showing no significant difference with high accuracy. Specificity of the applied method was assessed by analysing the laboratory-prepared mixtures and their combined dosage form. The developed method was confirmed according to ICH guidelines. The validated method can be considered as alternative and basic method for the routine determination of this fixed dose combination with minimum sample preparation.

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## 1. Introduction

Peptic ulcers are hurting lesions triggered by infection with a *Helicobacter pylori* or extra acid in the stomach. For microbial infection, the most current management is a mixture of two drugs (e.g. Doxycycline hydrochloride and Tylosin tartrate) [1]. Both drugs have been co-formulated and widely used for the cure of gastrointestinal infections triggered by tylosin and/or doxycycline sensitive microorganisms like *Escherichia coli* and *Salmonella* in poultry, calves and lambs. The examined drugs are formally listed in British Pharmacopeia (BP) [2].

Doxycycline hydrochloride, (4*S*, 4*aR*, 5*S*, 5*aR*, 6*R*,1 2*aR*)-4-(dimethylamino)-1,5,10,11,12*a*-pentahydroxy-6-methyl-3, 12-dioxo-4*a*, 5, 5*a*, 6-tetrahydro-4*H*-tetracene-2-carboxamide; hydrochloride (Figure 1a) is a tetracycline derivative which is bacteriostatic with a broad spectrum of antimicrobial activity against aerobic and anaerobic Gram-positive and Gram-negative pathogenic bacteria and some protozoa [3]. It blocks binding of aminoacyl-tRNA to the mRNA-ribosome complex, thereby inhibiting protein synthesis. In addition, this agent has exhibited inhibition of collagenase activity.

Tylosin tartrate, (2*R*,3*R*)-2,3-dihydroxybutanedioic acid;2-[(4*R*, 5*S*, 6*S*, 7*R*, 9*R*, 11*E*, 13*E*, 15*R*, 16*R*)-6-[(2*R*,3*R*,4*R*,5*S*,6*R*)-5-[(2*S*, 4*R*, 5*S*, 6*S*)-4, 5-dihydroxy-4, 6-dimethyloxan-2-yl]oxy-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-16-ethyl-4-hydroxy-15-[[(2*R*,3*R*,4*R*,5*R*,6*R*)-5-hydroxy-3,4-dimethoxy-6-methyloxan-2-yl]oxymethyl]-5, 9, 13-trimethyl-2, 10-dioxo-1-oxacyclohexadeca-11, 13-dien-7-yl]acetaldehyde (Figure 1b) belongs to the group of broad-spectrum macrolide antibiotics and is approved for the control of mycoplasmosis in poultry. It is used widely in veterinary medicine as anticoccidial feed additives in poultry and livestock, as growth promoters, for improved feed efficiency in ruminants and due to their antibiotic activities against gram-positive microorganisms also acts as inhibitor of bacterial protein synthesis. Due to its anti-inflammatory properties, it is prescribed for pets in inflammatory issues of the bowel [4,5].


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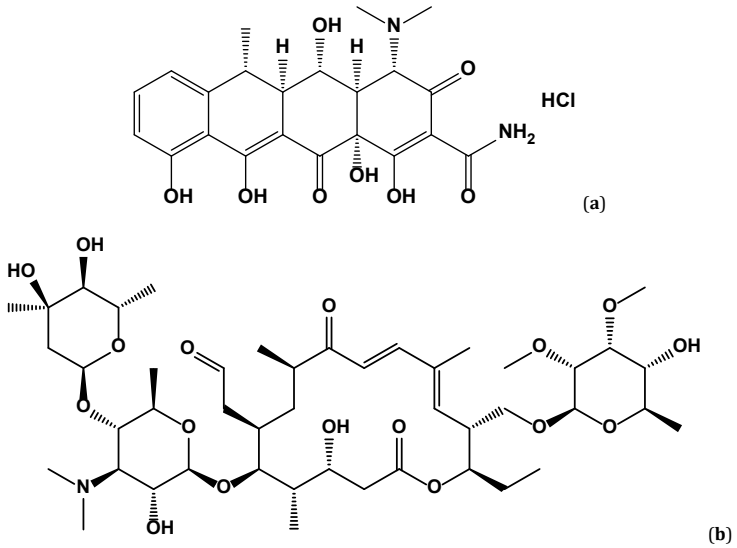


**Figure 1.** Chemical structure of doxycycline HCl (**a**) and tylosin tartrate (**b**).

Various methods have been reported for the quantitative determination of DOX both in pharmaceutical preparations and biological samples based on UV-Visible spectrophotometry [6-10], spectrofluorimetry [11-13], chromatographic methods [14-22] and electrochemical methods [23,24]. Several analytical procedures have been reported for the quantitative determination of TYT including spectrophotometric method [25] and chromatographic methods [26-30].

To the best of our knowledge, the survey of literature shows that no RP-HPLC method has been published for the assay of DOX and TYT in their veterinary formulation, so there is a need for method that permits simple, accurate and precise simultaneous quantification of the proposed drugs in their solid dosage form. The aim of this work was to develop and validate new RP-HPLC method for resolving this binary mixture. Furthermore, the separation and quantification of the proposed method HPLC was simple, rapid, selective, accurate and less time-consuming (only about 6 min for a single run). The new proposed method is simple and allows rapid analysis of drugs in bulk and dosage form for quality control laboratories.

## 2. Experimental

### *2.1. Apparatus and software*

Analysis was performed on a chromatographic system Agilent1200 equipped with multi-wave detector (G1365B), Quat pump (G1311A) and 1260 HiP degasser (G1322A). A chromatographic separation was achieved by ACE-126-2546 AQ C-18 (250×4.6 mm i.d., 5 µm particle size) analytical column. Data acquisitions were made with ChromNAV software C.

### *2.2. Materials*

#### *2.2.1. Samples*

DOX was kindly supplied by Pharma Swede Veterinary Company, Egypt. Its purity was found to be 99.72±0.92 according to BP 2013 [2]. TYT was kindly supplied by Pharma Swede Veterinary Company, Egypt. Its purity was found to be 99.17±0.55 according to BP 2013 [2].

#### *2.2.2. Veterinary dosage form*

Tydovet® powder was manufactured by Pharma Swede Veterinary Company, the industrial zone in the 10th of Ramadan city, Egypt, One gram of Tydovet® powder contains 133 mg doxycycline hydrochloride and 110 mg tylosin tartrate.

#### *2.2.3. Chemicals*

Water and methanol (99.9%) (HPLC grade) were purchased by E. Merck, Darmstadt, Germany. Acetonitrile (99.9%) (HPLC grade) was supplied from Lab Scan Limited, Dublin, Ireland. Trichloroacetic acid was supplied from (Adwic-El Nasr Pharmaceutical Chemicals Co. Egypt).

### *2.3. Solutions*

#### *2.3.1. Standard stock solutions*

Accurately weighed 50 mg of each pure drug was dissolved in methanol in 100 mL volumetric flasks, and then the volume was accomplished to the mark with the same solvent (each, 500 µg/mL). The final prepared solutions were put in storage in a fridge at 4 °C.

#### *2.3.2. Standard working solutions*

Aliquots of the prepared stock solutions were further diluted with methanol to a final volume of 100 mL. The obtained diluted solutions were used as the working solutions for DOX and TYT (each, 100 µg/mL). All solutions were put in storage in a fridge at 4 °C.

### *2.4. Procedures*

#### *2.4.1. Spectral characteristics*

DOX and TYT were scanned separately using methanol as blank in a wavelength range of 200-400 nm and maxima for each component were measured in zero-order absorption spectra. It was found that 270 nm is best wavelength for UV detection.

**Table 1.** Statistical analysis of parameters required for system suitability of RP-HPLC chromatographic method.

| Parameter | RP- HPLC method | | Reference value [31] |
|---|---|---|---|
| | DOX | TYT | |
| $t_R$ (Retention time) | 4.02±0.01 | 5.62±0.01 | - |
| N (Column efficiency) (Theoretical plate count) | 8989 | 17651 | N > 2000 Increases with efficiency of the separation |
| HETP (Height equivalent to theoretical plates) | 0.0028 | 0.0014 | The smaller the value, the higher the column efficiency |
| T (tailing factor) | 1.33 | 1.5 | T = 1 for symmetric peak |
| Rs (Experimental Resolution) | 9.45 | | Rs > 1.5 |

#### *2.4.2. Chromatographic conditions*

RP-HPLC was carried out at ambient temperature on ACE AQ-C18 column. The mobile phase consisted of a mixture of methanol: acetonitrile: distilled water in the ratio of 60:20:20 (*v*:*v*:*v*), with 0.01% trichloroacetic acid in an isocratic mode. The mobile phase was filtered using 0.45 µm Millipore membrane filter (Billerica, MA) and delivered at a flow rate of 0.8 mL/min. The injection volume was 50 µL and the detection was done at 270 nm.

#### *2.4.3. System suitability*

Fifty microliters of the working solutions were injected and applied to the chromatographic conditions. The system suitability parameters including tailing factor (*T*), theoretical plate count (*N*), height equivalent to theoretical plates (HETP), and resolution (*R*s) were calculated according to USP guidelines [31].

#### *2.4.4. Construction of calibration graphs*

Aliquots equivalent to 1-50 µg of DOX and 1-50 µg of TYT were accurately transferred from their working solutions (each, 100 µg/mL) into separate series of 10 mL volumetric flasks, and then the volume was completed to the mark with methanol. The corresponding chromatographic conditions were applied for these solutions and the chromatograms were recorded. The calibration graphs of DOX and TYT were constructed by plotting the relative peak area (the recorded peak area of each concentration of DOX and TYT to that of an external standard 10 µg/mL of DOX or 10 µg/mL of TYT, respectively) against the corresponding concentration at 270 nm and the regression equations were computed. The validation parameters as accuracy, precision and specificity were assessed according to ICH guidelines.

#### *2.4.5. Analysis of laboratory-prepared mixtures*

For the preparation of laboratory mixtures, aliquots equivalent to 1-50 µg of DOX and 1-50 µg of TYT were accurately transferred from their working solutions (each, 100 µg/mL) with different ratios of the two drugs into a series of 10 mL volumetric flasks, and the volume was completed to the mark with methanol and mixed well to prepare different mixtures of DOX and TYT. The chromatographic conditions of the developed method were adopted for each laboratory-prepared mixture and the concentrations of each drug were calculated from the corresponding regression equation. Each concentration was conducted from the average of three experiments.

#### *2.4.6. Application to veterinary formulation*

Ten grams of Tydovet® powder pack were mixed thoroughly, and then one gram of it equivalent to 133 mg DOX and 110 mg TYT was accurately weighed. The weighed amount was quantitatively transferred into 100 mL beaker. The powder was extracted with 30 mL methanol for 20 min by vortex shaker then the solution was filtered using through Whatman® filter paper No. 41 into a 100 mL volumetric flask. The volume was completed with methanol. Further dilution was applied from the filtrate to obtain a final concentration claimed to be 13.3 µg/mL of DOX and 11 µg/mL of TYT.

The proposed method was used for the analysis of the cited drugs in the previously prepared solution in three replicates. The concentrations of the studied drugs were calculated from the corresponding regression equations.

The corresponding chromatographic conditions of the applied method were adopted for each working solution and the average of three replicates was calculated for each concentration. The concentrations of each studied drug were calculated via substitution in the corresponding regression equation.

## 3. Results

The literature review shows that no published analytical method has been established for simultaneous determination of DOX and TYT in their bulk powders and/or their veterinary formulation. The main goal of this work is to establish new, simple, sensitive and accurate analytical method for simultaneous analysis of DOX and TYT in their bulk powders, laboratory-prepared mixtures and veterinary dosage form with satisfactory accuracy and specificity.

A simple isocratic RP-HPLC method was developed for the determination of DOX and TYT in their mixtures. The best resolution was obtained by using mobile phase containing a mixture of methanol: acetonitrile: distilled water in the ratio of 60:20:20 (*v*:*v*:*v*), with 0.01% trichloroacetic acid as shown in Figure 2. To optimize the UV maxima, various HPLC experiments were performed at various wavelengths starting from 250 to 320 nm. The best response has achieved with UV detection at 270 nm.

This HPLC method can represent a good analytical tool for the simultaneous analysis of DOX and TYT, especially there is no any technique has been published in the literature review.

The newly proposed RP-HPLC applied a simple isocratic mobile phase in which, less time-consuming (only about 6 min for a single run). System suitability parameters of the proposed method was calculated and listed in Table 1.

### *3.1. Optimization of the developed method*

The chromatographic conditions were optimized to afford robust performance so to optimize the RP-HPLC method, it was necessary to test the effect of different variables since different developing systems with different ratios were tried especially there is no published paper on this mixture.

### *3.2. Solvent effect*

Different types of solvents were tried including distilled water, methanol and acetonitrile. The developed system was the best mobile phase and showed satisfactory results for DOX and TYT regarding selectivity as well as it gave good linearity with high correlation coefficient. Higher methanol and acetonitrile concentrations (>85%) in the mobile phase caused DOX and TYT peaks to be superimposed with inadequate separation. At lower methanol and acetonitrile concentrations (<75%), the retention time of the drugs increased.

**Table 2.** Assay parameters and validation sheet obtained by applying RP-HPLC chromatographic method.

| Parameters | RP-HPLC method | | Literature | |
|---|---|---|---|---|
| | DOX | TYT | DOX [7] | TYT [29] |
| Linearity [a] | | | | |
| Slope | 0.1025 | 0.0985 | 0.0387 | 1652.1 |
| Intercept | -0.017 | 0.0201 | -0.0253 | 30311 |
| Correlation coefficient (r) | 0.9998 | 0.9999 | 0.9997 | 0.9996 |
| Range (µg/mL) | 1-50 | 1-50 | 6-21 | 10-250 |
| Mean±SD [a] | 99.62±1.22 | 100.09±1.11 | 100.23±1.43 | 102.36±1.56 |
| RSD | 1.220 | 1.104 | 1.432 | 1.561 |
| Accuracy [b] | 100.02±0.998 | 99.76±0.736 | 99.02±0.240 | 100.23±0.456 |
| Repeatability [c] | 100.42±1.099 | 99.91±0.962 | 100.21±1.211 | 99.45±1.244 |
| Intermediate precision [c] | 100.49±1.131 | 99.82±1.553 | 101.22±1.321 | 100.45±1.456 |
| Specificity [d] (laboratory-prepared mixtures) mean±SD | 98.99±1.34 | 99.59±1.29 | 100.32±1.342 | - |
| Tydovet® mean±SD | 98.78±0.55 | 100.21±0.95 | 110.68±0.26 | 98.36±0.45 |

[a] Six calibration points, average of three experiments.
[b] Mean±RSD.
[c] Relative standard deviation (RSD) % of three different concentrations.
[d] For five laboratory-prepared mixtures.

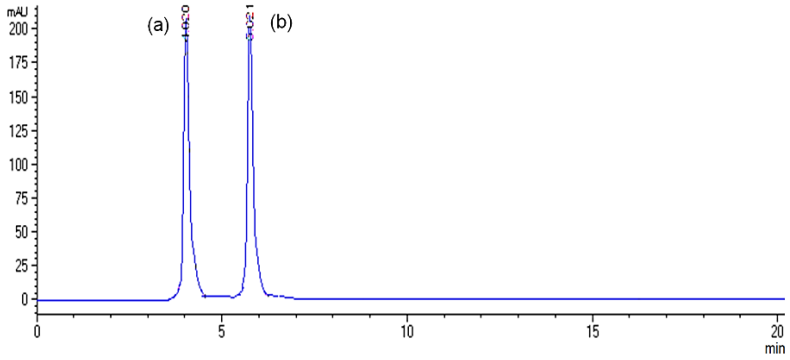


**Figure 2.** RP-HPLC chromatogram of 20 µg/mL of DOX at 4.02 min (a) and 40 µg/mL of TYT at 5.62 min (b) using a mixture of methanol: acetonitrile: distilled water in the ratio of 60:20:20 (*v*:*v*:*v*), with 0.01% trichloroacetic acid at 270 nm.

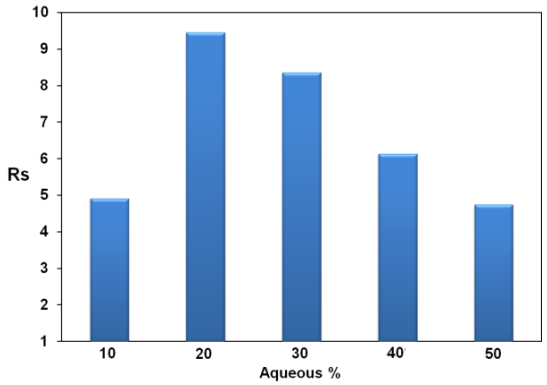


**Figure 3.** The effect of aqueous percent on the resolution of the separated drugs.

Different mobile phase systems were investigated for appropriate chromatographic separation. The mobile phase selection was based on peak shape parameters, ease of preparation and run time. Acetonitrile and methanol were tried as the organic part and gave better separation and high resolution efficiency. Water containing trichloroacetic acid of different ratio was tried as the aqueous part. By varying the concentration of aqueous part in the mobile phase, it was observed that 20% of it provided the optimum resolution and gave better results as shown in Figure 3.

The mobile phase was composed of a mixture of methanol: acetonitrile: distilled water in the ratio of 60:20:20 (*v*:*v*:*v*), with 0.01% trichloroacetic acid at the flow rate 0.8 mL/min and UV detection was performed at 270 nm and the separation was completed in 6 minutes.

### *3.3. Method validation*

The proposed method was applied for the determination of pure drugs since the statistical parameters of the regression equations, the concentration ranges and the linear equations were summarized and the satisfactory results were obtained. ICH parameters [32] are represented in Table 2.

#### *3.3.1. Linearity*

The linearity of the method was evaluated by analyzing the concentrations of DOX and TYT. Each concentration was repeated three times. The concentrations of DOX and TYT range from 1-50 µg/mL. The assay was performed according to the experimental conditions previously mentioned.

**Table 3.** Statistical comparison between the results obtained by the proposed and the official methods for the determination of DOX and TYT, respectively in pure powder form.

| Parameters | RP-HPLC method | | Literature | | Official methods [b] | |
|---|---|---|---|---|---|---|
| | DOX | TYT | DOX | TYT | DOX | TYT |
| Mean±SD | 99.62±1.22 | 100.09±1.11 | 100.23±1.43 | 102.36±1.56 | 99.72±0.92 | 99.17±0.55 |
| No. of experiments | 6 | 6 | 6 | 6 | 6 | 6 |
| Student's t test (2.571) [a] | 0.9035 | 0.0861 | 1.9800 | 0.1254 | | |
| F test (5.050) [a] | 1.7633 | 3.9846 | 2.6700 | 1.2654 | | |

[a] Figures between parentheses represent the corresponding tabulated values of t and F at p = 0.05.
[b] The official methods for DOX & TYT are HPLC methods. British Pharmacopoeia, Her Majesty's Stationary Office, London, vol. I & II (pp. 552) and vol. V (pp. 6) (2013) [2].

**Table 4.** One way ANOVA testing for the different proposed and the official methods used for the determination of DOX and TYT in pure powdered form at the 0.05 level, the population means are not significantly different.

| | Source | DF | Sum of squares | Mean square | F value | F critical |
|---|---|---|---|---|---|---|
| DOX | Between exp. | 1 | 0.028 | 0.028 | 0.024 | 4.965 |
| | Within exp. | 10 | 11.570 | 1.157 | | |
| TYT | Between exp. | 1 | 2.553 | 2.553 | 3.340 | 4.965 |
| | Within exp. | 10 | 7.643 | 0.764 | | |

**Table 5.** One way ANOVA testing for the different proposed and the reported method used for the determination of DOX and TYT in their dosage form *.

| | Source | DF | Sum of squares | Mean square | F value | F critical |
|---|---|---|---|---|---|---|
| DOX | Between exp. | 1 | 0.005 | 0.005 | 0.029 | 7.709 |
| | Within exp. | 4 | 0.669 | 0.167 | | |
| | Between exp. | 1 | 9.282 | 9.282 | 4.736 | 7.709 |
| TYT | Within exp. | 4 | 7.839 | 1.959 | | |

* At the 0.05 level, the population means are not significantly different.

#### *3.3.2. Range*

The calibration range was established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of DOX and TYT present in the veterinary preparations to give accurate precise and linear results.

#### *3.3.3. Accuracy*

Accuracy of the obtained results was examined by using the developed method for analysis of another concentrations of more different blind samples of DOX and TYT in between those of regression equation to clarify that the point in between are fitted on the line of regression equation with satisfactory recovery percentage thus good mean and low standard deviation. The concentrations were obtained from the corresponding regression equations, from which the percentage recoveries suggested good accuracy of the proposed method.

#### *3.3.4. Precision*

Repeatability and intermediate precision express the precision under the same operating conditions over a short interval of time. They were determined using three concentrations of each of DOX and TYT, which were analyzed three times intra-daily and inter-daily on three different days using the proposed method. The relative standard deviations were calculated.

#### *3.3.5. Specificity*

Specificity of the proposed method was successfully evaluated by the analysis of different laboratory-prepared mixtures of DOX and TYT within the linearity range. Satisfactory results were shown in Table 2.

#### *3.3.6. Stability*

DOX and TYT working solutions in methanol showed no spectrophotometric changes up to 4 weeks when stored at 4 °C.

### *3.4. Application of the methods in assay of Tydovet® powder*

The proposed UV methods were applied for the determination of DOX and TYT in their combined formulation Tydovet® powder and the results were shown in Table 2. The good percentage recoveries confirm the suitability of the proposed methods for the routine determination of these components in their dosage form.

### *3.5. Statistical analysis*

Statistical comparison between the results obtained by the proposed method and those obtained by the official methods [2] showed no significant difference as given in Table 3. In order to compare the ability of the proposed methods for the determination of DOX and TYT, the results obtained by applying the proposed method were subjected to statistical analysis using one way ANOVA test, there was no significant difference among the proposed method and those obtained by the official methods [2] as shown in Table 4 and the reported method [7,29] as shown in Table 5.

## 4. Conclusion

This work represents the first trial to analyze the veterinary binary mixture of DOX and TYT by simple, rapid and accurate RP-HPLC in their pure powdered form and laboratory-prepared mixtures with good accuracy and precision. A simple optimized study was done to determine the run time, the resolution and the validation of the studied method. RP-HPLC method provided rapid analysis and better separation efficiency and resolution. The linearity ranges were the same. The described method was applied successfully to determine the studied drugs in the veterinary formulation. It can be considered as an alternative tool for the routine analysis of this fixed dose combination with minimum sample preparation so it could be easily applied in quality control laboratories for the simultaneous determination of DOX and TYT without any tedious separation steps.

Overall, the adopted methodology is readily transferable to quality control laboratories with advantage of rapid analysis showing high robustness using simple isocratic mobile system with no buffer solution, unlike the complicated spectrophotometric methods [33,34] or tedious chromatographic method

[35]. The final separation conditions yielded separations that were complete in 6 min for a single run, thus resulting in minimal analytical dead time and save the reset of the system computer necessary to reset the next injection.

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**References**

[1]. University of Michigan Health System Peptic Ulcer Disease. Accessed June 01, 2019. at: http://www.med.umich.edu/1info/FHP/practiceguides/newpud/pud.pdf
[2]. British Pharmacopoeia, Her Majesty's Stationery Office, London, vol. I & II (P. 552) and vol. V (P. 6), 2013.
[3]. Sweetman, S. C., Martindale, The complete drug reference, 33rd ed. Pharmaceutical Press, London, 2002.
[4]. Watanabe, W.; Harter, T.; Bergamaschi, B. *J. Environ. Quality* **2008**, *37*, 78-85.
[5]. Sun, P.; Barmaz, D.; Cabrera, M.; Pavlostathis and Huang, Ch. *J. Chromatogr. A* **2013**, *1312*, 10-17.
[6]. Lotfy, H. M.; Maha, A. H.; Tawakkol, S.; Mohamed, E. H. *Spectrochim. Acta A* **2016**, *153*, 321-332.
[7]. Kogawa, A. C.; Salgado, H. R. N. *World Res. J. Pharm. Res.* **2013**, *1(2)*, 021-024.
[8]. Lopez, P. J. L.; Martinez, C. J. *J. Pharma. Biomed. Anal.* **1993**, *11*, 1093-1098.
[9]. Salinas, F.; Berzas Nevado, J. J.; Espinosa, A. Analyst **1989**, *114*, 1141-1145.
[10]. Mahrous, M. S.; Abdel-Khalek, M. M. *Talanta* **1984**, *31*, 289-291.
[11]. Attia, M. S.; Mahmoud, W. H.; Ramsis, M. N.; Khalil, L. H.; Othman, A. M.; Hashem, S. G.; Mostafa, M. *J. Fluores.* **2011**, *21(4)*, 1739-1748.
[12]. Van den Bogert, C.; Kroon, A. M. *J. Pharm. Sci.* **1981**, *70*, 186-189.
[13]. Salinas, F.; Pena, A. M.; Duran, M. I. *Anal. Lett.* **1990**, *23*, 863-876.
[14]. Croubels, S.; Vermeersch, H.; Backeer, P.; Santos, M. D. F.; Remon, J. P.; Van Peteyhem, C. *J. Chromatogr. B* **1998**, *708*, 145-152.
[15]. Klimes, J. D. M. *J. Chromatogr. A* **1999**, *846*, 181-184.
[16]. Gajda, A.; Posyniak, A.; Pietruszka, K. *Bull. Vet. Inst. Pulawy.* **2008**, *52*, 417-420.
[17]. Gajda, A.; Posyniak, A.; Zmudzki, J.; Tomczyk, G. *J. Chromatogr. B* **2013**, *928*, 113-120.
[18]. Pavagada, J. R.; Kanakapura, B.; Kalsang, T.; Kanakapura, B. V.; Hosakere, D. R. *J. Pre-Clin. Clin. Res.* **2010**, *4(2)*, 101-107.
[19]. Ruz, N.; Zabala, M.; Kramer, M. G.; Campanero, M. A.; Dios-Vieitez, M. C.; Blanco-Prieto, M. J. *J. Chromatogr.* **2004**, *1031*, 295-301.
[20]. Cinquina, A. L.; Longo, F.; Anastasi, G.; Giannetti, L.; Cozzani, R. *J. Chromatogr. A* **2003**, *987*, 227-33.
[21]. Gastearena, I.; Dios-Vieitez, M. C.; Segura, E.; Goni, M. M.; Renedo, M. J.; Fos, D. *Chromatographia* **1993**, *35*, 524-526.
[22]. Zarghi, A.; Kebriaeezadeh, A.; Ahmadkhaniha, R. *Bull. Chim. Farm.* **2001**, *140*, 112-114.
[23]. Shoukry, A. F.; Badawy, S. S. *Microchem. J.* **1987**, *36*, 107-112.
[24]. Issa, Y. M.; Abdel-Fattah, H. M.; Abdel-Moniem, N. B. *Int. J. Electrochem. Sci.* **2013**, *8*, 9578-9592.
[25]. Vargas, A., BSc Thesis, Faculty of L'Ecole Nationale Supérieure des Industries Chimiques and Worcester Polytechnic Institute, Nancy, France, 1-88, 2011.
[26]. Ghanem, M.; Saleh, A. *J. Appl. Pharm. Sci.* **2015**, *5(1)*, 094-098.
[27]. Pietron, W.; Cybulski, W.; Kos, K.; Mitura, A. *Bull. Vet. Inst. Pulawy* **2011**, *55*, 725-729.
[28]. Ahmed, M. B. M.; Sree, Y. H. A.; Abdel-Fattah, S. M.; Hassan, N. S.; Saad, M. M. E. *J. Planar Chromat.* **2013**, *26(5)*, 409-416.
[29]. Kotha, S.; Sunitha, N.; Manoharbabu, S.; *Inter. J. Pharm. Anal. Res.* **2014**, *3(2)*, 214-221.
[30]. Stan, G.; Badea, I. A.; Hassan, Y. A. *J. Chromatogr. Sci.* **2016**, *54(9)*, 1567-1572.
[31]. United States Pharmacopoeia Commission, United States pharmacopeia-National formulary. United States Pharmacopoeial Inc., 2280-2282, 2004.
[32]. International Conference on Harmonization (ICH), Q2 (R1): Validation of Analytical Procedures: Text and Methodology, US FDA, Federal Register, ICH, 2005.
[33]. Lotfy, H. M.; Fayez, Y. M.; Michael, A. M.; Nessim, C. K. *Spectrochim. Acta A* **2016**, *155*, 11-20.
[34]. Hegazy, M. A.; Lotfy, H. M.; Rezk, M. R.; Omran, Y. R. *Spectrochim. Acta A* **2015**, *140*, 600-613.
[35]. Michael, A. M.; Shalliker, R. A. *Anal. Met.* **2017**, *9(31)*, 4514-4519.