Quantification of Doxycycline Hyclate in Different Pharmaceutical Samples by UV Assay

Kazi Md Mahmudul Hasan1*, Md Anwarul Haque1, Sm Alamgir Kobir1, Mobarak Hossain2

1Department of Biotechnology and Genetic Engineering, Faculty of Applied Science and Technology, Islamic University, Kushtia, 7003, Bangladesh.
2Department of Chemistry, Faculty of Physical Sciences, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh.

Available Online: 1st December, 2016

ABSTRACT

Objective: Doxycycline hyclate is a broad spectrum antibiotic with activity against a wide range of gram-positive and gram-negative bacteria and it is widely used as a pharmacological agent. A simple, selective, linear, precise and accurate ultraviolet detection (UV) method has been developed and applied for the determination of doxycycline hyclate in different pharmaceutical samples. Methods: Acid-base analysis and titrimetric method were utilized to determine the value of pH and moisture content of purchased pharmaceutical samples. A mixture of methanol and hydrochloric acid (0.01N Methanolic HCl) was used to determine the biochemical properties of doxycycline hyclate. UV detector set at 349 nm was used to monitor the effluent. The purified water was used as solvent. Results: In 1% aqueous solution of doxycycline, three samples (4th, 5th and 7th) showed lower pH values of 1.97, 1.98, and 1.99 respectively. Furthermore, the same samples indicated the additional moisture contents of 2.81%, 2.85% and 2.83% respectively while considering the acceptance level (1.4% to 2.8%). The method proved to be linear (R2=0.993), precise (RSD=0.79% for inter-day precision), accurate (Recovery=100.59%) and selective regarding possible impurities and excipients of the samples. The doxycycline content obtained in the sample analysis was within the range of 84.05% to 85.80%. Conclusion: The optimized and validated method may be successfully employed to perform routine quality control analyses. Investigation of the pH, moisture content and potency of doxycycline hyclate in different samples give a general view of local pharmacies trade and ensure that the method applied here was validated for this kind of analysis.

Keywords: Doxycycline hyclate; Methanolic HCl; Moisture content; Ultraviolet detection; Method validation.

INTRODUCTION

Doxycycline is a broad-spectrum antibiotic of the tetracycline class that is used in the treatment of infections caused by bacteria and protozoa1. Like other agents of this class it kills bacteria and protozoa by inhibiting protein synthesis2. Researchers show that tetracycline antibiotics are most extensively used antimicrobials worldwide3. Tetracyclines are one of the cheapest antibiotics, hence extensively used in countries with limited health care budgets4. In gram-negative bacteria, transporta-tion of the doxycycline into the cell occurs dependent active transport system. The later system is either by passive diffusion or through an energy also believed to exist in gram-positive bacteria. Doxycycline is more lipophilic than the other tetracyclines, which allows it to pass easily through the lipid bilayer of bacteria. Doxycycline penetrates the bacterial cell and interferes with the protein biosynthesis, stopping the process of bacterial reproduction. It is the drug of choice in the treatment of sexually transmitted diseases3. It is preferred to other tetracyclines in the treatment of specific infections because of its fairly reliable absorption and its long half-life, which permits less frequent dosage. Doxycycline presents itself in three forms: hyclate, monohydrate and hydrochloride. The molecular formula of doxycycline hyclate is C22H26N2Ox HCl. ½C2H2O. ½H2O. From doxycycline hyclate, it is possible to obtain other forms. The hyclate dissolved in water and neutralized with sodium hydroxide, then becomes doxycycline monohydrate which undergoes doxycycline hydrochloride with the addition of hydrochloric acid. Doxycycline hyclate is much more soluble than doxycycline monohydrate, which is one of the main reasons for it more frequent uses as antibacterial drug. Various methods for quantification of doxycycline in vitro and in vivo have been reported. These include in vitro experimental model6, fluorimetry7, TLC-fluorescence scanning densitometry8 and HPLC9-19 for the quantification of doxycycline in biological materials. HPLC was also applied for the determination of doxycycline in pharmaceutical formulations20. Various chromatographic methods have also been reported for the determination of doxycycline in human tissues21 and foods22,23. Sequential injection chromatograph(SIC) for pharmaceutical preparations24 and derivative spectrophotometry for the determination of doxycycline in pharmaceuticals, urine and honey have

*Author for Correspondence: knmhr89@gmail.com
additionally been developed. Many researchers reported that there are a few methods available till date for the determination of doxycycline hyclate in pharmaceutical samples. However, the procedure provided by British Pharmacopoeia is widely used for the determination of doxycycline hyclate in pharmaceutical samples till date. The aim of the present study was to establish a standard procedure to quantify doxycycline hyclate in pharmaceutical samples and determine the biochemical properties of doxycycline hyclate. In addition, investigation of the pH, moisture content, purities and potency of this active ingredient in different samples collected from local pharmacies in Bangladesh have been performed. Furthermore, analytical method validation was done to check whether this method could pass the performance characteristics such as specificity, linearity, repeatability, intermediate precision and accuracy. Due to the extensive market value and clinical applications of this drug, product quality, shelf life, other active ingredients and excipients should be analyzed and justified for different brands.

MATERIALS AND METHODS
Reagents and Materials
Chemicals such as hydrochloric acid (HCl), Karl Fischer reagent and methanol were purchased from Merck (Darmstadt, Germany). Doxycycline hyclate reference standard (PromoChem, Teddington, United Kingdom) and HPLC grade solvent were used here for all analytical purposes. Ten doxycycline hyclate capsules in concentration with 100 mg/ml were collected from local pharmacies.

Instrumentation and Methodologies
Balance Sartorius LE225D, pH meter BT-600, Shimadzu UV-1650 visible spectrophotometer, KF Titrando were utilized. UV spectrophotometric method was applied here for the determination of doxycycline hyclate. Several performance characteristics were also analyzed such as pH, water content and impurities. To perform this analysis, the following methods were applied:

**pH analysis**
Doxycycline hyclate is considered as soluble in water. Aqueous solution of doxycycline hyclate, containing 1% doxycycline, has a pH of 2.39. For pH analysis, 1.0g test sample transferred in a 100ml volumetric flask and dissolved. Then volume with water up to the mark and shaken well. Rinsed and clean the electrode with deionized water. Wiped the electrode with tissue paper and immersed it into the sample solution. When the reading stabilized, pH value recorded.

**Water content determination**
Titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions. The water content should be not less than 1.4% and not more than 2.80%. For this analysis, weighed about 35 to 40 mg of the test sample and transferred it into a Karl Fischer’s vassal. Afterwards KF Titrando automatically showed the results on the print out in percentage.

**Light Absorbing Impurities Detection**
Dissolved 0.25g in a mixture of 1 volume of 1M HCl and 99 volumes of methanol and diluted to 25.0ml with the same mixture of solvents. The absorbance of the filtrate determined at 490 nm is not greater than 0.07 (anhydrous and ethanol-free substance) and carried out the measurement within 1 hour of preparing the solution.

**UV Assay**

**Standard preparation A (System Suitability) and B (Assay Calculation)**
According to the Code of Federal Regulations (Title 21, Food and Drugs), 50mg of doxycycline hyclate was taken in 5ml volumetric flask and bring to the volume with 0.05N methanolic hydrochloric acid. Here, 50mg of doxycycline hyclate was transferred in a 50ml volumetric flask dissolved with 0.01N methanolic HCl and volume up to the mark and sonicated. Then 1ml of this solution added to the 100ml volumetric flask, volume up to the mark with 0.01N methanolic HCl.

**Sample preparation**
Same as Standard preparation.

**Procedure**
Concomitantly measured the absorbance of the standard and sample solution in a 1cm quartz cell at the wavelength of 349 nanometers (nm) with a suitable spectrophotometer using 0.01N methanolic HCl as blank.

**Standard Concordance**

**Assay**
Sample abs. × Standard weight in mg × potency of standard(%)

**Method Validation**

**Specificity**
The specificity of the method can be determined with the addition of impurities and degradation products, obtained experimentally or by inducing their formation.

**Standard preparation**
Weighed and transferred accurately about 50 mg of doxycycline hyclate working standard into a 100 ml volumetric flask.

**Placebo preparation**
Placebo is a substance having no pharmacological effect but administered as a control in testing the efficacy of a biologically active preparation. Weighed and transferred accurately about 108.81 mg of placebo into a 100 ml volumetric flask. For both standard and placebo preparation, 60 ml of diluent added and sonicated for 15 minutes to dissolve. Cooled it and diluted it with same solvent. Diluted 2 ml of this solution to 50 ml with same solvent.

**Linearity**
Linearity was determined by linear regression analysis by the method of least squares. Health Canada (HC) states that...
the coefficient of determination for active ingredients should be $\geq 0.997$, for impurities 0.98 and for biologics 0.9545.

**Preparation**

Weighed and transferred accurately about 40 mg, 45 mg, 50 mg, 55 and 60 mg of doxycycline hyclate standard into five 100 ml volumetric flask. Added 60 ml of diluent into each 100 ml volumetric flask and sonicated to dissolve. Cooled it and dilute it with same solvent. Diluted 2 ml of this solution to 50 ml with same solvent. Plotted a graph of concentration versus absorbance (Abs).

**Repeatability**

This parameter determines the repeatability of assay results under the same operating conditions over a short period of time. HC states that the RSD should be 1% for drug substances and 2% for drug products46.

**Preparation of standard**

Weighed and transferred accurately about 50 mg of doxycycline hyclate standard into a 100 ml volumetric flask.

**Preparation of sample**

Prepared accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as mentioned above.

**Accuracy**

Samples were prepared at three concentrations levels over the range of 80 to 120% of the target concentration. Three individually prepared replicates at each concentration were analyzed. For the U. S. pharmaceutical industry, 100 ± 2% is typical for an assay of an active ingredient in a drug product over the range of 80 to 120% of the target concentration44.

**Preparation of standard**

Weighed and transferred accurately about 50 mg of doxycycline hyclate working standard into a 100 ml volumetric flask.

**Preparation of sample for 80% recovery**

Weighed accurately about 40 mg of doxycycline hyclate standard and 87.04 mg placebo and transferred into a 100 ml volumetric flask.

**Preparation of sample for 100% recovery**

Weighed accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask.

**Preparation of sample for 120% recovery**

Weighed accurately about 60 mg of doxycycline hyclate standard and 130.57 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as other parameters analysis. Intermediate precision of an analytical procedure expresses intra-laboratory variations of the repeatability test performed by a different analyst, on a different day. The assay results obtained by two operators on different days should have a statistical RSD ≤ 2%44,45.

**Preparation of standard**

Weighed and transferred accurately about 50 mg of doxycycline hyclate standard into a 100 ml volumetric flask.

**Preparation of sample**

Prepared accurately about 50 mg of doxycycline hyclate standard and 108.81 mg placebo and transferred into a 100 ml volumetric flask. For both standard and sample preparation, same procedures were performed as it was done for specificity, linearity, repeatability and accuracy analysis.

**RESULTS**

**Method optimization**

According to the United States Pharmacopoeia (USP), Doxycycline hyclate has potency not less than 80% and not more than 92%33 whereas it should be approximately 84.7% declared in British Pharmacopoeia (BP)44. For UV assay of sample-1, Weight of SS standard = 0.05084g, Weight of C standard = 0.05085g, Sample weight = 0.05093g, Average area of SS standard = 0.668, Average area of C standard = 0.666, Sample absorbance = 0.664. By putting these values into the calculating formula, assay result for sample-1 was 85.80 % on dried basis and repeated the formula for rest of the samples.

**Assay validation**

**Specificity**

The specificity of the method can be determined with the addition of impurities and degradation products, obtained experimentally or by inducing their formation42. Here, the specificity of the chromatographic method was determined by the screening of a placebo solution and the assay solution. The placebo solution was prepared in the same manner as the investigated solution but without doxycycline hyclate. It would be investigated by injecting of the extracted placebo to demonstrate the absence of interference with the elution of analyte.

**Linearity**

The linearity of an assay method is its ability to elicit test results, which are directly proportional to the

![Figure 1: Structure of doxycycline hyclate.](image)

![Figure 2: Conversion of doxycycline hyclate into doxycycline monohydrate and doxycycline hydrochloride.](image)
III) chemicals were used.

The % RSD due to doxycycline hyclate concentration for the determination of doxycycline in bulk drug and in different concentrations ranged 80, 100 and 120 % of recovery values. Series of solution were made containing concentrations of drug actives in samples in a given range.

The accuracy of the method was checked by determining area, and represents concentration in ppm, or absorbance of standard drug actives in samples in a given range. The obtained results are given in Table 5, together with the calculated values of their standard deviation, SD, and relative standard deviation, RSD.

Intermediate precision

In line with ICH guidelines, precision is subdivided into short term (within-run precision or intrabatch) and intermediate precisions (between run or interbatch) which measure precision with time, and may involve different analysts, equipment and reagents. Intrabatch precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV). For interbatch precision, intrabatch experiments are repeated on four different days by different analysts. Precision from the four-day experiments is compared with the intrabatch precision.

Table 1: Summary report of pH, water content, impurities and UV assay.

| Sample | Water by KF | pH at 1% aqueous | Light absorbing | Assay at 349nm | Impurities |
|--------|-------------|------------------|----------------|----------------|------------|
| 1      | 2.41%       | 2.15             | 0.018          | 85.80%         |
| 2      | 2.16%       | 2.32             | 0.020          | 85.24%         |
| 3      | 2.08%       | 2.39             | 0.019          | 85.53%         |
| 4      | 2.81%       | 1.97             | 0.022          | 84.31%         |
| 5      | 2.85%       | 1.98             | 0.017          | 84.03%         |
| 6      | 2.11%       | 2.31             | 0.022          | 85.76%         |
| 7      | 2.83%       | 1.99             | 0.019          | 84.52%         |
| 8      | 2.07%       | 2.02             | 0.016          | 84.71%         |
| 9      | 2.19%       | 2.29             | 0.017          | 84.95%         |
| 10     | 2.05%       | 2.25             | 0.020          | 84.05%         |

Table 2: Placebo interference/Specificity study.

| S. No. | Weight of Placebo (mg) | Absorbance of Standard (mg) | Absorbance of Standard (% | Placebo Interference |
|--------|------------------------|------------------------------|----------------------------|----------------------|
| 1      | 108.72                 | 0.00                         | 10                         | 0.60                 |
| 2      | 108.75                 | 0.00                         | 5                          | 0.34                 |
| 3      | 108.90                 | 0.00                         | 2                          | 0.13                 |
| Average|                        |                              |                            | 0.36                 |

Table 3: Linearity study.

| S. No. | Concentration % | Sample weight (mg) | Concentration (ppm) | Absorbance |
|--------|-----------------|--------------------|---------------------|------------|
| 1      | 80%             | 47.94              | 16.06               | 0.566      |
| 2      | 90%             | 54.04              | 18.10               | 0.635      |
| 3      | 100%            | 60.55              | 20.28               | 0.697      |
| 4      | 110%            | 65.99              | 22.10               | 0.784      |
| 5      | 120%            | 71.53              | 23.95               | 0.848      |

Concentrations of drug actives in samples in a given range. Proof of linearity justifies the use of single-point calibrations. The linearity was checked on samples of standard doxycycline hydrochloride at five different concentrations (16.06-23.95 ppm). A regression curve was constructed: y = 0.036x - 0.016, with R² = 0.993; where x represents concentration in ppm, y represents the peak area, and R is the correlation coefficient.

Accuracy

The accuracy of the method was checked by determining recovery values. Series of solution were made containing 80, 100 and 120 % of doxycycline hyclate. Recoveries for different concentrations ranged from 99.58 to 101.93% for the determination of doxycycline in bulk drug and in tablets.

Repeatability

The % RSD due to doxycycline hyclate concentration for the six samples was found to be less than 2.0%. Six separated sample preparations were analysed according to the method of analysis. The % of RSD due to doxycycline hyclate concentration for the assay meets the requirements.

DISCUSSION

In this study, there was no residual effect that could be harmful to humans. In 2001, Zarghi et al. developed a method for determination of doxycycline in human plasma using HPLC assay based on a mbondapak C18 column and a lambda (347 nm) UV detector. Reagents like actonitrile, potassium dihydrogen phosphate, perchloric acid aqueous solution were utilized there. In 2011, Ramesh et al. developed and validated three cost-effective spectrophotometric methods for the determination of doxycycline in bulk drug and in tablets where HCl, NaOH, H₂SO₄ medium and iron(III) chemicals were used. In 2012, Kagawa et al. developed and validated an accurate, sensitive, precise and rapid gradient reversed-phase high-performance liquid chromatographic method for the determination of doxycycline hyclate in bulk drug and tablets. CN Luna column at 360 nm was utilized as stationary phase and water, 0.1% TFA-acetonitrile and 0.1% TFA were used as the mobile phase at a flow rate of 1.0 mL/min. In 2015, Kagawa et al. developed and validated an eco-friendly method of infrared spectroscopy for quantification of doxycycline in raw material. The proposed spectrophotometric methods do not require any expensive equipment and specialized technicians when compared alongside HPLC and bioassay. Besides, other characteristics of these methods are the short time and less space required for performance and handling respectively than other methods. Although there is not enough evidence of pH determination of antibiotics and drug products, here it was performed because doxycycline hyclate causes ulcer in stomach due to acid reflux and heartburn. For this reason, pH level should be within the range of acceptance. Here, sample 4th, 5th and 7th had slightly higher acidic pH levels that would be a concern of health safety issues. Beside, stability of doxycycline hyclate in aqueous solutions depends on the pH value. On the other hand, Karl Fischer titration method in analytical chemistry to determine trace amounts of water in a sample. In the environmental science, it is used to test the percent (% of water in a sample).
water content in soil and cosmetic industry to test the percent (%) of moisture content in cosmetics like soap, detergents, cream etc. After the analysis of ten different samples of doxycycline hyclate, sample no. 4th, 5th and 7th showed results those had some differences considering the specification limit. Due to the high moisture content value, these samples potency might be lost before the given expiry date. A linear regression curve was constructed and the correlation coefficient (R²) and assessment value calculated. Here, a plot of concentration against peak area shows a straight line where R² value is 0.993. Precision determined the repeatability of assay results under the same operating conditions over a short period of time. The % RSD due to doxycycline hyclate concentration for the

Table 4: Accuracy study.

| Sample No. | Level | Sample Weight (mg) | A (mg) | B (mg) | B/A×100 (% Recovered) | Mean Value % | %RSD |
|------------|-------|--------------------|--------|--------|-----------------------|-------------|-------|
| 1.         | 80%   | 166.24             | 80.66  | 80.82  | 100.20                | 101.22      | 0.91  |
| 2.         | 100%  | 165.93             | 98.92  | 100.36 | 101.11                | 101.11      | 0.59  |
| 3.         | 120%  | 168.30             | 119.82 | 119.47 | 99.71                 | 99.43       | 0.26  |

A=Theoretical concentration, B=Concentration recovered, RSD=Relative standard deviation

Table 5: Repeatability study.

| Injection No. | Level | Sample Weight (mg) | Absorbance | Assay (%) | Mean Assay (%) | Standard Deviation | %RSD |
|---------------|-------|--------------------|------------|-----------|----------------|--------------------|-------|
| 1.            | 100%  | 166.36             | 0.705      | 98.70     | 99.80          | 0.72               | 0.65  |
| 2.            | 166.83| 0.715              | 100.10     | 99.68     | 100.42         |                    |       |
| 3.            | 166.76| 0.712              | 99.68      | 100.52    |                |                    |       |
| 4.            | 167.06| 0.718              | 100.52     | 99.54     |                |                    |       |
| 5.            | 166.65| 0.711              | 99.54      | 100.24    |                |                    |       |
| 6.            | 165.92| 0.716              | 100.24     | 99.80     |                |                    |       |

Table 6: Intermediate precision study.

| S. No. | Weight of sample (mg) | Absorbance | Assay % | Weight of sample (mg) | Absorbance | Assay % |
|--------|-----------------------|------------|---------|-----------------------|------------|---------|
| 1.     | 166.36                | 0.705      | 98.70   | 167.80                | 0.705      | 100.55  |
| 2.     | 166.83                | 0.715      | 100.10  | 168.40                | 0.711      | 101.40  |
| 3.     | 166.76                | 0.712      | 99.68   | 168.21                | 0.707      | 100.83  |
| 4.     | 167.06                | 0.718      | 100.52  | 167.50                | 0.700      | 99.83   |
| 5.     | 166.65                | 0.711      | 99.54   | 168.60                | 0.712      | 101.55  |
| 6.     | 165.92                | 0.716      | 100.24  | 167.30                | 0.704      | 100.41  |

Mean assay (n=6) 99.80 Mean assay (n=6) 100.76
Standard deviation (n=6) 0.72 Standard deviation (n=6) 0.43
Relative standard deviation (n=6) 0.65 Relative standard deviation (n=6) 0.63
Mean assay (Analyst 1 & Analyst 2) n=12 100.28
Standard deviation n=12 0.72
Relative standard deviation n=12 0.79

Table 7: Summary report of method validation.

| S. No. | Analytical performance parameter | Acceptance limit | Result |
|--------|----------------------------------|-----------------|--------|
| 1.     | Specificity/Placebo interference | NMT 2%          | 0.36   |
| 2.     | Accuracy                         | 80%-102%        | 101.22 |
| 3.     | Repeatability                    | % RSD NMT 2%    | 0.65   |
| 4.     | Linearity (Correlation Coefficient) | R² Value NLT 0.99 | 0.99   |
| 5.     | Intermediate Precision           | % RSD NMT 2%    | 0.79   |

NMT=Not more than, NLT=Not less than
Figure 3: Ultraviolet spectrum of ten samples and standards.
six samples was 0.65%. From the accuracy results, the percentage recovery values for doxycycline hyclate satisfy the acceptance criteria (98% - 102%) for accuracy across the range of 80-120%. A comparison of the proposed method with other methods is given in Table - 8. The results obtained by the proposed method have an RSD of 0.65 %, better than that reported by Šatínský et al.24, Salinas et al.25 and Lopez-Paz et al.26. The first two methods24,25 have relatively high RSD values, higher than RSD max. This indicates that the proposed method is more precise and accurate than some of the aforetime published methods. Mahrous and Abdel-Khalek27 described a long pre-treatment of the drug through mixing with acetic acid and sodium cobalt nitrite, then boiling the mixture, followed by cooling. In addition, the same author28 described a determination employing ammonium vanadate but the later has less recovery percentage than the earlier one. In comparison of both methods27,28, this proposed method have the higher recovery rate (100.59%). Here, the validated methods yielded good results and suggest their application in the quality control laboratories where the modern and expensive instruments are not available.

CONCLUSION
In this work, an analytical UV method was developed for the quantitative determination of doxycycline hyclate in samples. Its advantages over other existing method include its simplicity, speed and low cost. The proposed method not only provides a linear relation between absorbance and concentration in 349nm wavelength, but also ensures a simple, sensitive, accurate, and repeatable determination of doxycycline hyclate in pharmaceutical samples. Doxycycline was shown to be stable during all the procedure. Thus, the result parameters demonstrated that the spectrophotometric method could be applied for the analysis of the pharmaceutical formulations assuring the quality and efficacy of the doxycycline hyclate under investigation.

ACKNOWLEDGEMENTS
This research was supported by Islamic University fund. All authors are contributed for conception of the work, data collection, data analysis, data interpretation, drafting the article and critical revision of the article. The final manuscript has been read and approved by all authors.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES
1. Cinquina A, Longo F, Anastasi G, Giannetti L, Cozzani R. Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlorotetracycline and doxycycline in bovine milk and muscle. Journal of Chromatography A. 2003; 987: 227-233.
2. Nelson M, Levy S. The history of the tetracyclines. Annals of the New York Academy of Sciences. 2011; 1241: 17-32.
3. Lian F, Song Z, Liu Z, Zhu L, Xing B. Mechanistic under-standing of tetracycline sorption on waste tire powder and its chars as affected by Cu²⁺ and pH. Environmental Pollution. 2013; 178: 264-270.
4. Roberts M. Tetracycline therapy: update. Clinical Infectious Diseases. 2003; 36: 462-467.
5. Joshi N, Miller DQ. Doxycycline revisited. Archives of Internal Medicine. 1997; 157: 1421-1428.
6. Cars O, Ryan DM. Concentrations of doxycycline in muscle tissue and muscle tissue fluid. Scandinavian Journal of Infectious Diseases. Supplementum. 1988; 53:18-21.
7. Van Bogert C, Kroox A. Fluorometric Determination of tetracyclines in small blood and tissue samples. Journal of Pharmaceutical Sciences. 1981; 70: 186-189.
8. Xie H, Dong C, Fan Y, Liu C. Determination of doxycycline, tetracycline and oxytetracycline simultaneously by Tlc-Fluorescence scanning densitometry. Analytical Letters. 1997; 30: 79-90.
9. Klimeš J, Dohnalová M, Sedláček J. Microcolumn high-performance liquid chromatographic assay for doxycycline in isolated alveolar macrophages. Journal of Chromatography A. 1999; 846: 181-184.
10. Lavda M, Clausnitzer C, Walters J. Distribution of systemic ciprofloxacin and doxycycline to gingiva and gingival crevicular fluid. Journal of Periodontology. 2004; 75: 1663-1667.
11. Prevost JM, Beraud B, Cheminel V, Gaillard Y, Mounier C, Chaulet JF. Determination of doxycycline in human plasma and urine samples by high performance liquid chromatography. Application for drug monitoring in malaria chemoprophylaxis. Annales de Biologie Clinique. 1995; 53: 29-32.

12. Riond J, Hedeen K, Tyczkowska K, Riviere J. Determination of doxycycline in bovine tissues and body fluids by high-performance liquid chromatography using photodiode array ultraviolet-visible detection. Journal of Pharmaceutical Sciences. 1989; 78: 44-47.

13. Ruiz N, Zabala M, Kramer M, Campanero M, Dios-Vieitez M, Blanco-Prieto M. Rapid and simple determination of doxycycline in serum by high-performance liquid chromatography. Journal of Chromatography A. 2004; 1031: 295-301.

14. Farin D, Piva G, Gozlan I, Kitzés R. High performance liquid chromatography method for the determination of doxycycline in human plasma. Chromatographia. 1998; 47: 547-549.

15. Axasa B, Naylor A, Bell P, Thompson M. Simple and reliable method of doxycycline determination in human plasma and biological tissues. Journal of Chromatography B: Biomedical Sciences and Applications. 2000; 744: 359-362.

16. Santos MD, Vermeersch H, Remon JP, Schelkens M, De Backer P, Ducatelle R et al. Validation of a high-performance liquid chromatographic method for the determination of doxycycline in turkey plasma. Journal of Chromatography B: Biomedical Sciences and Applications. 1996; 682: 301-308.

17. Alsarra IA, Niazy EM, Al-Sayed YM, Al-Khamis KI, El-Awad Ibrahim M. High performance liquid chromatographic method for determination of doxycycline in human plasma and its application in pharmacokinetic studies. Saudi Pharmaceutical Journal; 2005, 13: 42-47.

18. Newton PN, Chaulet JF, Brockman A, Cheriauk W, Dondorp A, Rangeverayuth R et al. Pharmacokinetics of oral doxy-cycline during combination treatment of severe falciparum malaria, Antimicrobial Agents and Chemotherapy, 2005, 49:1622-1625.

19. Monser L, Darghouth F. Rapid liquid chromatographic method for simultaneous determination of tetracyclines antibiotics and 6-Epi-doxycycline in pharmaceutical products using porous graphic carbon column. Journal of Pharmaceutical and Bio-medical Analysis. 2000; 23: 353-362.

20. De Leenheer A, Nelis H. Doxycycline determination in human serum and urine by high-performance liquid chromatography. Journal of Pharmaceutical Sciences. 1979; 68: 999-1002.

21. Oka H, Ito Y, Matsumoto H. Chromatographic analysis of tetracycline antibiotics in foods. Journal of Chromatography A. 2000; 882: 109-133.

22. Ding X, Mou S. Ion chromatographic analysis of tetracyclines using polymeric column and acidic eluent. Journal of Chromatography A. 2000; 897: 205-214.

23. Šatinský D, Dos Santos L, Sklenárová H, Solich P, Montenegro M, Araújo A. Sequential injection chromatographic determination of ambroxol hydrochloride and doxycycline in pharmaceutical preparations. Talanta. 2005; 68: 214-218.

24. Salinas F, Nevado J, Espinosa A. Determination of oxytetra-cycline and doxycycline in pharmaceutical compounds, urine and honey by derivative spectrophotometry. The Analyst. 1989; 114: 1141.

25. Paz J, Calatayud J. Copper carbonate as a solid-bed reactor for spectrophotometric determination of doxycycline and oxytetracycline in an unsegmented continuous flow assembly. Journal of Pharmaceutical and Biomedical Analysis. 1993; 11: 1093-1098.

26. Mahrous MS, Abdel-Khalek MM. Spectrophotometric determination of phenothiazines, tetracyclines and chloramphenicol with sodium cobaltinitrite. Talanta. 1984; 31: 289-291.

27. Abdel-Khalek MM, Mahrous MS. Spectrophotometric determination of tetracyclines and cephalosporins with ammonium vanadate. Talanta. 1983; 30: 792-794.

28. British Pharmacopoeia 98/34/EEC, The Stationary Office, London, 2005.

29. ICH Q2A, Validation of Analytical Procedures: Definitions and Terminology, Geneva, 1995, in 2005 incorporated in Q2(R1).

30. ICH Q2B, Validation of Analytical Procedures: Methodology, adopted in 1996, Geneva Q2B, in 2005 incorporated in Q2(R1).

31. Fischer K. Neues Verfahren zur maßanalytischen Bestimmung des Wassergehaltes von Flüssigkeiten und festen Körpern. Angewandte Chemie. 1935; 48: 394-396.

32. US Pharmacopoeia USP29-NF24, US Pharmacopeial Convention, Rockville, MD, 2006, (MDANT05) Monograph Development-Antibiotics, pp. 764.

33. US Pharmacopoeia USP29-NF24, US Pharmacopeial Convention, Rockville, MD, 2006, General Chapter 05, pp. 2785.

34. US Pharmacopoeia USP29-NF24, US Pharmacopeial Convention, Rockville, MD, 2006, General Chapter 05, pp. 2730.

35. The Merck Index. 13th ed. New Jersey, Merck Research Labora-tories, 2005.

36. Ramesh P, Basavaiah K, Divya M, Rajendraprasad N, Vinay K, Revanasiddappa H. Simple UV and visible spectrophotometric methods for the determination of doxycycline hyclate in pharmaceuticals. Journal of Analytical Chemistry. 2011; 66: 482-489.

37. The European Pharmacopoeia. Strasbourg: European Direc-torate for the Quality of Medicines, Council of Europe; 2005.

38. United States Pharmacopeial Convention. USP 32 NF 27. Rockville, MD: United States Pharmacopeial Convention; 2009.

39. WHO Expert Committee on Specification for Pharmacopeial Preparations. Geneva, Switzerland: World Health Organization; 2008.

40. The Code of Federal Regulations of the United States of America [Internet], Google Books. 2016 [cited 2016]
41. Ermer J. Validation in pharmaceutical analysis. Part I: A integrated approach. Journal of Pharmaceutical and Biomedical Analysis. 2001; 24(5-6): 755-767.
42. Green J. Peer Reviewed: A Practical Guide to Analytical Method Validation. Analytical Chemistry. 1996; 68: 305A-309A.
43. European pharmacopoeia 7.0. Strasbourg: European Directorate for the Quality of Medicines & HealthCare, Council of Europe; 2010.
44. Shabir G. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Journal of Chromatography A. 2003; 987: 57-66.
45. Product Master Files. Ottawa: Drugs Directorate, Health Protection Branch, Health Canada; 1994.
46. Kogawa A, Salgado H. Quantification of doxycycline hyclate in tablets by HPLC-UV method. Journal of Chromatographic Science. 2012; 51(10): 919-925.
47. Kogawa AC, de Mello N. Quantification of doxycycline in raw material by an eco-friendly method of infrared spectroscopy. Pharm Anal Acta. 2015; 07.