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Validation of HPLC Method for Determination of Doxycycline Hyclate in Turkey Serum

Nahla S. Elshater*., Fatma Hussein*., Mai A. Fadel*., Marwa Ragab*., Heba Hassan* and Maha Sabry**

*Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute,Dokki &**Chemistry Department, Animal Health Research Institute,Dokki

Abstract

An accurate, sensitive and reproducible high performance liquid chromatographic (HPLC) method for the quantitation of doxycycline in turkey serum has been developed and validated. The doxycycline standard was eluted from a C18 column (4.6 mm i.d., 250 mm, 5 µm particle size) at 15 ^{0}C with a mobile phase consisting of 5% acetic acid : methanol : acetonitrile (55:20:25, v/v/v). A UV detector set at 347 nm was used to monitor the effluent. Retention time of doxycycline was approximately at 4 min and the run time was 10 min. The column temperature adjusted at 15 ^{0}C with a flow rate 1ml/min and injection volume 25 µl. Doxycycline was extracted from serum by ACN. Linearity was confirmed in the concentration range 0.1-2 µg/ml, had regression coefficients (R^2) 0.9999 for doxycycline quantification in turkey serum. The analytical conditions gave a recoveries in a range of 97.57% to 99.83%. The limit of detection (LOD) was 0.035 µg/ mL and limit of quantification (LOQ) was 0.108 µg/ml. The relative standard deviation (RSD %) was less than 2% in all cases. The developed method was accurate, precise and rapid for simultaneous estimation of doxycycline in turkey serum samples.

Keywords: Validation, Doxycycline, Serum, HPLC

Introduction

Doxycycline is a semisynthetic tetracycline antibiotic derived from oxytetracycline with a broad spectrum of activity against a wide range of Gram-positive and Gram-negative pathogens **Joshi and Miller (1997), Dollery (1999)**. Doxycycline widely used in medicine is well tolerated at therapeutic doses **Klein and Cunha (1995)**, enter vertebrate cells by passive diffusion **Kringstein *et al*, (1998)** and can be easily administrated (oral or intraperitoneally). Due to these characteristics, doxycycline has been recently chosen as a tool to control of gene expression in animal studies **Kistner *et al*, (1996)**. Drug delivery system (DDS) has been developed, loaded with doxycycline to target the drug to the liver and also to improve its sustained release, reducing the frequency of administrations. DDS may modify the distribution profile of encapsulated drug driving it to target organs or tissues and reducing both the given dose and the side effects **Hans and Lowman (2002)**. Furthermore, the development of pharmacokinetic studies is a very useful tool to assess the properties of the developed dosage forms after their in vivo administration and requires sensitive analytical methods to allow the determination of low concentrations of drug in small

samples. Different analytical approaches have been employed for the determination of doxycycline in different biological samples. The antimicrobial properties of doxycycline have allowed the development of various biological assays **Cars and Ryan (1996), Yoshimura *et al*, (1991)** for the determination of doxycycline. Although these assays have been applied in some pharmacokinetic and microbiological studies, there has recently been a move to replace by other methods **Ray and Harris (1989)**. Fluorimetric and spectrophotometric techniques has been used in the determination of doxycycline in pharmaceuticals. However, the specificity of these methods is very low when doxycycline are measured in biological samples, since interference from metabolites and endogenous components of the samples cannot always be excluded **Van den Bogert and Kroon (1981), Lopez Paz *et al.*, (1993)**. Thin-layer chromatography has reported to determine doxycycline in biological tissues using adsorbent layers of silica gel **Naidong *et al.*, (1990**). In general, thin-layer chromatography is simple and does not require special equipment. However, doxycycline shows extreme tailing spots when no prepared adsorbents are used, so the sensibility of the method is very low. Capillary electrophoresis is recently applied for the determination of doxycycline in pharmaceuticals **Gil, (2000)**. Nevertheless, despite of the many advantages that showed this technique, the low sample volume that can be injected in the capillary avoid the quantitation of this antibiotic in biological samples. High-performance liquid chromatography (HPLC) has also been used to determine doxycycline and other tetracycline antibiotics in pharmaceuticals, and biological fluids, primarily in plasma, serum, urine or blood**. Tsai and Kondo (1994), De Leenheer and Nelis (1979), Mulders and Van de Lagemaat (1989), Prevosto *et al.*, (1995), MacNeil *et al.*, (1996), Iwaki *et al.*, (1993), Farin *et al.*, (1998),** Gastearena *et al.*, (1993), Axisa et al., (2000). This paper describes the development and validation of a high-performance liquid chromatographic technique for the determination of doxycycline in serial small serum samples obtained from turkey.

Material and Methods

Material

Doxycycline hyclate (lot # D9891, purity > 98%) was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Methanol and acetonitrile were of HPLC grade, while other chemicals and reagents analytical grade were obtained from BDH Laboratories Supplies (BDH Chemical Ltd., Poole, U.K.).

Deionized, Milli Q water (Millipore, Bedford, MA, USA) was used to prepare the mobile phase and diluent solutions.

Apparatus

Agilent Series 1200 quaternary pump, Series 1200 auto sampler, Series 1200 UV Vis detector and HPLC 2D Chemstation Software (Hewlett-Packard, Les Ulis,France)

Chromatographic column: was reversed-phase column C18 (4.6 mm, i.d., 250 mm, 5 µm), (Teknokroma Co.)

Cooling centrifuge, ultrasonic bath (Buhler, Germany), vortex mixer (Inc., N.Y., USA)

Standards solutions

Stock standard solution of doxycycline: amount equivalent to 10 mg of doxycycline was dissolved in 10 ml of methanol to obtain a final concentration of 1 mg/ml. Stock standard solution was put in amber glass to prevent the photo-degradation and stored at -20°C and was stable for at least four weeks. Stock solution was diluted with methanol to give a series of working standard solution freshly prepared daily. **Shalaby *et al.*, (2011)**

Preparation of the samples for analysis

The collected blood samples in centrifuge tubes were left to coagulate and were centrifuged at 3000 rpm for 15 minutes to obtain clear serum. The serum was then transferred immediately to sterile tubes and stored at -20°C until analysis using High Performance Liquid chromatography (HPLC). **Said *et al.*, (2015)**

Assay procedure

To 200 µl of serum previously spiked with doxycycline, 2 µl (10.0 µg/ml) of internal standard for preparation of 0.1 µg/ml spiked serum sample, then 200 µl of acetonitrile were added. The mixture was vortexed for 30 seconds and then centrifuged for 10 min at 12,857 Xg and 4 °C. Then 200 µl of supernatants were taken for evaporation by nitrogen evaporator at 40 °C. The dried residues were reconstituted by 200 µl of mobile phase and 25 µl aliquot samples were injected to HPLC. **Ruza *et al.*, (2004), Farin *et al.*, (1998), Wang *et al.*, (2009).**

Chromatographic separation

Isocratic mobile phase consisted of 55% acetic acid (5%), 25% acetonitrile and 20% methanol.

Chromatographic column: was reversed-phase column C18 (4.6 mm, i.d., 250 mm, 5 µm)

Adjusted at 15^0C.

Flow rate of 1 ml/min

Injection volume: 25 µL. **Ruza *et al.*, (2004)**.

Detection and quantitation

Detection and quantitation: using UV detector at 347 nm, quantitation was integrated by HPLC 2D Chemstation software interfaced to a personal computer. **Ruza *et al.*, (2004)**.

Method validation

It is the procedure by which the performance characteristics of the method meet the requirements for the intended analytical application. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines. **ICH, Q2 (R1), Harmonised tripartite guideline, Validation of analytical procedures: text and methodology International Conference Harmonization ICH, Geneva, Nov 2005.**

1.Precision

1.A. The intraday precision

Precision is the closeness of agreement among a set of results. The intraday precision (Repeatability) of the assay were measured by analyzing six replicate of spiked turkey serum sample with doxycycline (2 μg/mL) on the same day. The percent of relative standard deviation (% RSD) for peak responses was calculated.

1. B. Intermediate precision:

Intermediate precision (within-laboratory variation) determined by estimation of six replicates of spiked serum samples with doxycycline (2 μg/mL) daily for 6 times over a period of one week (interday precision). The %RSD for peak responses was calculated.

2. Linearity and range

Linearity was performed by preparing a six different concentrations of drug standard at squared correlation coefficient of 0.99 (r^2) according to ICH. Calibration lines of peak area ratios (peak area analyte/peak area internal standard) versus concentration were determined by single level calibration curve (linear regression equation, Y = A X +B), where: Y = peak area ratio, A = slope, X = concentration and B = intercept.

1. Specificity:

Solutions of standard and spiked serum samples were prepared as per the test method and injected into the chromatographic system, the chromatograms were recorded.

4. Accuracy and Recovery:

The accuracy of an analytical method is the closeness of test results (theoretical value) obtained by method to the assay value. Accuracy must be established across the specified range of the analytical procedure. Accuracy was determined over the range of 50%, 100% and 150% of the sample concentration.

The accuracy was then calculated as the percentage of analyte recovered by the assay.

% Recovery = [Theoretical value / Assay value] × 100

Accepted criteria of recovery ranged from 85-110%

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5. Robustness:

It was determined by detecting how a method stands up to slight variations in normal operating conditions.

To determine the robustness of the method, small deliberate changes in the chromatographic conditions, such as the effect of mobile phase composition was assessed at (5 % acetic acid: methanol : acetonitrile (55:25:20, v/v/v) and 5 % acetic acid: methanol : acetonitrile (53:26:21, v/v/v) instead of 5 % acetic acid: methanol : acetonitrile (55:20:25, v/v/v). The effect of wavelength detection at 346 nm and 348 nm instead of 347nm. The effect of column temperature was studied at 17 and 13◦C instead of 15◦C. The %RSD of robustness testing under these conditions was calculated in all cases.

6. Limits of detection (LOD)

It is considered to be the quantity yielding a detector response which gives signal to noise ratio 3:1 according to (ICH)

7. Limits of quantification (LOQ)

It is the lowest amount that can be analyzed within acceptable precision and accuracy which give signal to noise ratio 10 : 1 according to (ICH)

Results and Discussion

1. Precision

The values of %RSD for intraday and inter day variation are given in **Table (1, 2)**. In both cases, %RSD values were found well within 2% limit, indicating that the current method is repeatable.

Table (1): Intraday precision value:

Sr. no.	Concentration (µg/ml)	Peak area (In the same day)
1	2	102.65
2	2	101.64
3	2	102.73
4	2	103.55
5	2	104.31
6	2	102.24
Mean		102.8533333
SD		0.949961403
RSD%		0.923607794

Table (2): Inter day precision value:

Sr. no.	Concentration (µg/ml)	Peak area (In six days)
1	2	102.65
2	2	102.48
3	2	103.34
4	2	104.25
5	2	103.51
6	2	103.63
Mean		103.31
SD		0.655957316
RSD%		0.634940776

2. linearity and range

Standard curves were constructed by spiking of six blank serum turkey samples with various volumes of doxycycline stock solution to yield a concentration range of 0.1, 0.2, 0.4, 0.8, 1.0 and 2.0 µg/ml.

The Standard curves were shown linearity from 0.1 to 2µg/ml for doxycycline hyclate in turkey serum figure (1).

The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were y = 0.0516x - 0.5924 with correlation coefficient (r^2) = 0.9999

Linearity values and peaks area were shown in **table (3).** Analytical performance parameters were shown in **table (4).**

Table (3): linearity of doxycycline hyclate in turkey serum sample

RT	Level	concentration (µg/ml)	Peak area
4.344	1	0.1	4.6961
	2	0.2	9.947
	3	0.4	19.84
	4	0.8	40.13
	5	1	51.34
	6	2	102.65

*RT: Retention Time

Table (4): Analytical performance parameters

Parameters	Value
Linearity range	0.1-2 µg/ml
Correlation coefficient (r^2)	0.9999
Slope (a)	0.0516
Intercept (b)	-0.5924
Regression equation	y= 0.0516x - 0.5924

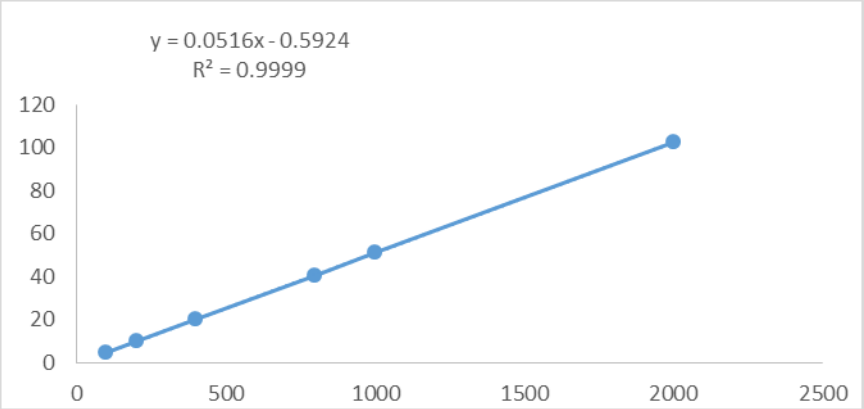

Figure 1: Standered curve of doxycycline hyclate in turkey serum

3. Specificity

The chromatograms compared to know that there is no excipient compound interference between peaks of the pure standard and peaks of spiked serum samples. Figure 2.

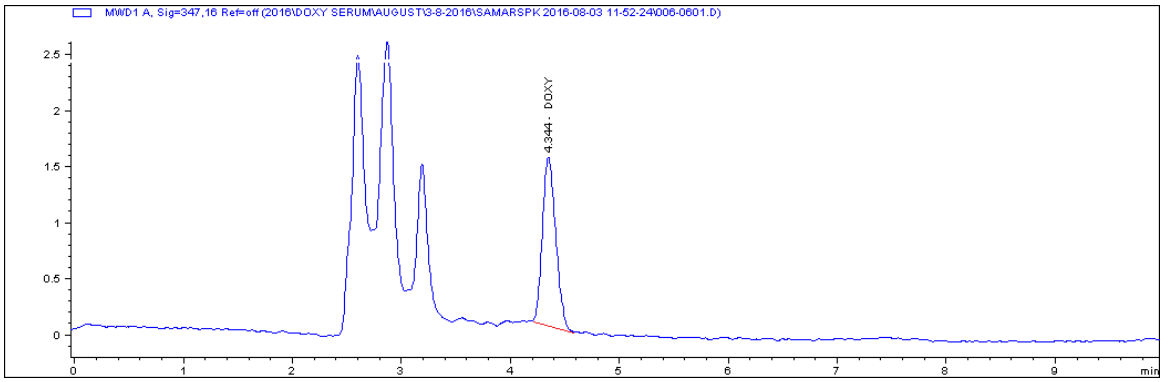

Figure 2: Chromatogram of spiked turkey serum sample with doxycycline hyclate (0.2µg/ml)

4. Accuracy and Recovery

Spiked samples will be prepared at three concentrations of 50, 100 and 150 % which represented by 0.1, 0.2, and 0.4 µg/ml respectively. Each spiked sample was prepared in triplicate at each level and injected. The absolute recovery of doxycycline were shown in table (5).

Table (5) Recovery studies

Concentration level (µg/ml)	Found concentration (µg/ml)	Recovery %	Mean	SD	RSD%	Average % Recovery±SD
0.1 (50%)	0.09674	96.74	0.097723	0.000905	0.925636	97.06 ±0.000905
	0.09852	98.52				
	0.09791	95.91				
0.2 (100%)	0.20235	101.175	0.204237	0.001755	0.859187	102.118 ±0.001755
	0.20582	102.91				
	0.20454	102.27				
0.4 (200%)	0.39644	99.11	0.39402	0.003775	0.958116	98.505 ±0.003775
	0.38967	97.4175				
	0.39595	98.9875				

5. Robustness:

The results of robustness indicated that changing the mobile phase composition and changing the detection wavelength had slight effect on the chromatographic behavior of doxycycline. However, the alteration in the column temperature had no significant effect. The RSD % of robustness testing under different altered conditions are given in Table (6), indicating that the current method is robust.

Table (6) Results of Robustness study (Doxycycline concentration, 2 µg/ mL, n=3)

Chromatographic parameter	Modification	Peak Area Precision (n=3)	Mean area ± SD	RSD%
Change in Mobile phase composition	5%Acitic Acid : MEOH : CAN 55 : 25 : 20 55 : 20 : 25 53 : 26: 21	101.85 102.65 100.26	101.5867 ± 1.216566	1.19
Change in wavelength (nm)	346 347 348	103.03 102.65 100.68	102.12 ± 1.261	1.235
Change in Column temperature (°C)	17 15 13	102.644 102.65 102.78	102.69 ± 0.0768	0.0748

6. Limit of detection and limit of quantification

LOD and LOQ were calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where, σ is the standard deviation of intercept S is slope of the calibration curve. So, LOD of doxycycline in serum was 0.035 µg/ mL and LOQ was 0. 108 µg/ mL

Conclusion

In the present investigation simple, sensitive and economical new analytical method was developed for estimation of doxycycline in turkey serum by HPLC. The developed and validated HPLC method was found to be more economical. The result of analysis of formulation and recovery studies obtained by HPLC method were statistically validated and high percentage of recovery studies suggest that the developed methods were free from interference of excipients used in formulation. The HPLC method was statistically validated in terms of accuracy, precision, linearity and reproducibility. Hence above methods can be employed in laboratories to estimate doxycycline in turkey serum.

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