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Measurement Of Doxycycline Plasma Concentrations By Calibration Curve Method With Aid Of Hplc-Esi-Ms/Ms

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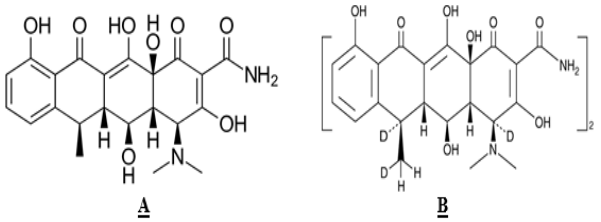
Abstract
The validated protein precipitation method was applied for the estimation of Doxycycline in human plasma with Doxycycline-d_3 as an internal standard by using HPLC-ESI-MS/MS. The chromatographic separation was achieved with Methanol: Acetonitrile(pH: 6.5 Adjusted with diluted ammonia solution) (20:80%, v/v) using the Discovery® C18 HPLC Column, 2 cm × 2.1 mm, 5 µm. The total analysis time was 3.0 min and the flow rate was set to 0.7 ml/min. The mass transitions of Doxycycline and Doxycycline-d3 obtained were m/z 445.20 →428.10 and 895.11→428.23. Using the linear regression model, the standard curve shows a correlation coefficient (r2) greater than 0.9983 with a range of 6.00-768.00 pg/ml.

Keywords: Doxycycline; Human plasma; HPLC-ESI-MS/MS; Bioanalysis

INTRODUCTION

Doxycycline is a semisynthetic tetracycline broad-spectrum antibiotic frequently used to treat chronic prostatitis, syphilis, sinusitis, chlamydia, pelvic inflammatory diseases, rickettsial infections, and sexually transmitted diseases. Doxycycline chemically is [4S-(4α,4aα,5α,5aα,6α,12aα)-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a–pentahydroxy-6-methyl-1,11-dioxo-2-naphthalene carboxamide monohydrate[1]. Doxycycline is a yellow crystalline powder with a molecular weight of 444.45 g/mol. Its empirical formula is $C_{22}H_{24}N_2O_8$ and is sparingly soluble in water, slightly soluble in alcohol and in methylene chloride.

Very few HPLC[2,3,4], LC-MS methods[5] have been applied for the determination of doxycycline in plasma, and in other biological matters in different methods[6,7,8,9,10,11,12], but no method has been found and performed for doxycycline by LC-MS/MS in human plasma. Hence, the authors have proposed, developed and validated a simple, rapid, specific, sensitive LC-MS/MS method to determine doxycycline in human plasma.



A B

Fig.1. Chemical Structures of A) Doxycycline (DV) B) Doxycycline-d_3

Review of Literature

Various Pharmacokinetics studies [6-17] have been reported for Doxycycline and none of the methods were reported for estimation of Doxycycline in human plasma by LC-MS/MS using Doxycycline-d3 as internal standard.

Thus, this study aimed to simplify the sample preparation step using protein precipitation and simultaneously to shorten the chromatographic run time with a more selective LC-MS/MS procedure. Further, to improve the precision and accuracy of the method isotopically labeled Doxycycline was used (Doxycycline-d3) to reduce matrix effect and reproducibility. These improvements enabled development of a rapid, selective and sensitive LC-MS/MS method for determination of Doxycycline in human plasma.

It is important to develop the superior bio analytical method with proper deuterated or analogue based internal standards interms of reduce matrix effect and improve reproducibility.

The present study describes, the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive, simple extraction, good linear method for quantitative determination of Doxycycline in

human plasma with the small amount of plasma usage as per bio analytical FDA guideline[13].

MATERIALS AND METHODS

Materials:

Chemical Resources

Doxycycline and Doxycycline-d3 was obtained from Alsachim, France. Water (HPLC Grade). Methanol and Acetontrile (HPLC Grade) were obtained from J.T. Baker, India and Formic acid (AR grade) were obtained from Merck, India. Human plasma was procured from Doctors labs Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.

Instrument Resources

An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

Methods:

Chromatographic conditions

The chromatographic separation was achieved with Methanol: Acetontile (pH: 6.5, Adjusted with diluted ammonia) (20: 80, v/v), gave the best peak shape and low baseline noise was observed using the Discovery® C18 HPLC Column, 2 cm × 2.1 mm, 5 µm. The total analysis time was 3 min and flow rate was set to 0.7 ml/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

Detection

The pure drug of Doxycycline and Doxycycline-d3 were prepared in acetontrile (200.00 pg/mL) and injected with a flow rate of 10 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of 445.20→428.10 and 895.11→428.23 m/z for Doxycycline and Doxycycline-d3. The mass spectrums of parent, product ions were depicted in figure 2 and 3.

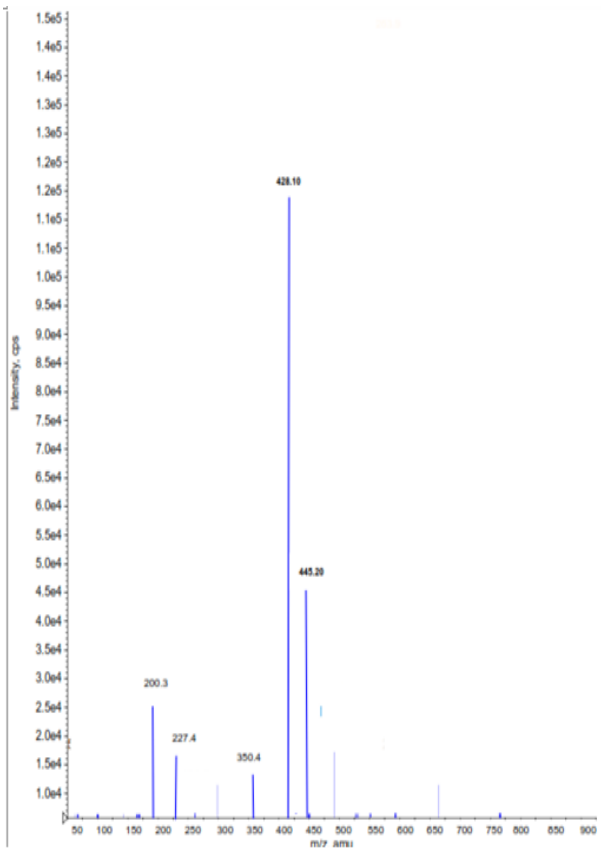


Figure.2: Parent ion mass spectra (Q1) and (Q3) of Doxycycline

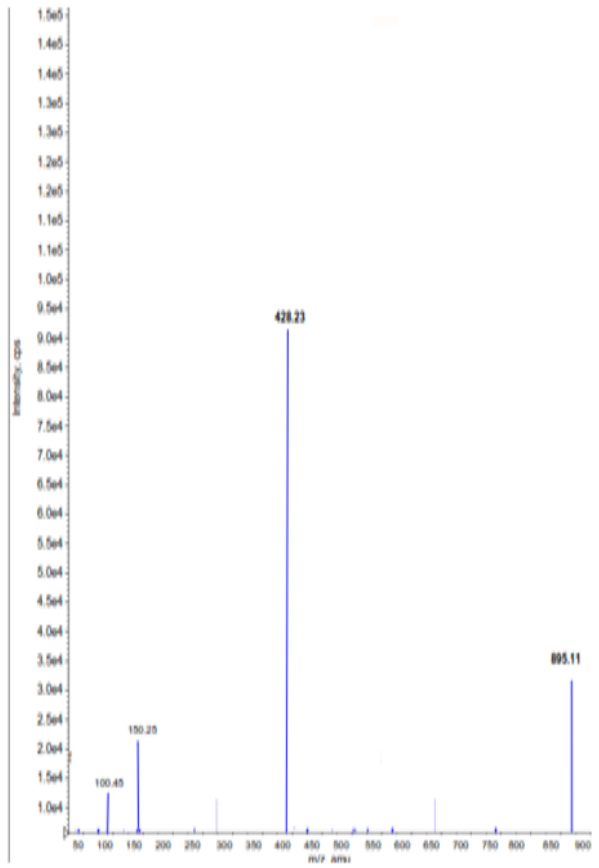


Figure.3: Parent ion mass spectra (Q1) and (Q3) of Doxycycline-d3

Standard calibration and quality control samples preparation

Standard stock solutions of Doxycycline (10.0mg/mL) and Doxycycline-d3 (10.0 mg/mL) were prepared in Acetontrile. The IS spiking solution (200.0 pg/mL) was prepared in mobile phase solution (Methanol: Acetontrile(pH: 6.5 Adjusted with diluted ammnia solution) (20:80%, v/v)) from Doxycycline-d3 stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8ºC until analysis. Standard stock solutions of Doxycycline (10.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 6, 12, 24, 48, 96, 192, 384 and 768 pg/mL for analytical standards and 6 (LLOQ), 18 (LQC), 360 (MQC) and 720 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at -30ºC until analysis. The aqueous standards were prepared in a mobile phase solution (Methanol: Acetontrile (pH: 6.5 Adjusted with diluted ammnia solution) (20:80%, v/v)) and stored in the refrigerator at 2–8ºC until analysis.

Sample extraction

The protein precipitation method was used to isolate Doxycycline and Doxycycline-d3 from human plasma. For this purpose, 10 µL of Doxycycline-d3 (200 pg/mL) and 150 µL of plasma sample were added to the labelled polypropylene tubes and vortexed briefly for about 10 min. Thereafter, 20µL of 0.1% Formic acid and vortexed for 30 sec. Then 1mL of acetonrile extraction solvent was added and vortexed for about 10 min.

Next, the samples were centrifuged at 15000 rpm for approximately 5 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 45ºC briefly, and then reconstituted with a mobile phase solution Methanol: Acetontile (pH: 6.5, Adjusted with diluted ammonia) (20: 80, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS/MS for study.

Method validation

The developed method was validated over a linear concentration range of 6.0–768.0 pg/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under the validation section.

Selectivity and Specificity

Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of Doxycycline retention time and less than 5% for Doxycycline-d3 retention time.

Limit of Quantification (LOQ)

Six LLOQ standards were prepared in screened plasma lot along with IS (6.00 pg/ml) and signal to noise ratio (S/N) was calculated using analyst software.

Linearity

Calibration standards were prepared to obtain linearity range of 6, 12, 24, 48, 96, 192, 384 and 768 pg/ml and assayed in five replicates on five different days.

Precision & Accuracy

One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (6.00 pg/ml), Low QC (18.00 pg/ml), Mid QC (360.00 pg/ml) and High QC (720.00 pg/ml) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix Effect

Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (360.00 pg/ml) and compared with un-extracted standards of the same concentration.

Recovery

The recovery of samples was performed by protein precipitation method.The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (18.00 pg/ml), medium (360.00 pg/ml), high (720.00 pg/ml).

Stability studies

Bench top Stability (Room Temperature Stability, 48 h)

Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 48 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Freeze and thaw stability (after 3rd cycle at -30°C)

Six replicates of low and high concentrations (FT stability samples) were frozen at -30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler stability/ Processed Stability (2-8°C, 70 h)

Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 70 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-30°C, 90 Days)

After completion of the stability period stored at -30 °C (90 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

RESULTS AND DISCUSSION

Method development

On the way to develop a simple and easy applicable method for determination of Doxycycline in human plasma, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the Doxycycline was performed with different branded RP-HPLC C18 columns. Initial separation was performed with isocratic elution of formic acid, ammonium acetate, ammonium formate combined with organic phases like methanol and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of Methanol: Acetontile (20: 80, v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of Methanol: Acetontile in varying combinations were tried. Using a mobile phase containing Methanol: Acetontile (pH: 6.5, Adjusted with diluted ammonia) (20: 80, v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Discovery® C18 HPLC Column, 2 cm × 2.1 mm, 5 µm analytical column with a flow rate of 0.7 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 38°C and the temperature of auto sampler was maintained at 4°C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography. For selection of internal standard, amoxicillin, cephalexin, ciprofloxacin, clindamycin, metronidazole, azithromycin, sulfamethoxazole, trimethoprim, amoxicillin and levofloxacin were tried with optimized mobile phase and column conditions. Finally Doxycycline-d3 was selected as an internal standard in terms of better chromatography and extractability.

The retention times of analyte (Doxycycline) and internal standard (Doxycycline-d3) were eluted at 1.38±0.02 min and 1.39±0.02 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the LLE was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.

Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Doxycycline gave more response in positive ion mode as compare to the negative ion mode.

To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500°C.

The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for Doxycycline and Doxycycline-d3 , respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for Doxycycline and Doxycycline-d3 .

The predominant peaks in the primary ESI spectra of Doxycycline and Doxycycline-d3 correspond to the MH+ ions at m/z 445.20 m/z (parent ion) and 895.11 m/z respectively. Productions of Doxycycline and Doxycycline-d3 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 428.10 m/z (parent ion) and 428.23 m/z (product ion), respectively. The parent and productions mass spectrums of Doxycycline and Doxycycline-d3 were shown in Figure 2 & 3.

Method validation

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed at retention times of Doxycycline and Doxycycline-d3 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 6.0 pg/ml. Represent chromatograms were shown in Figure 4 and 5.

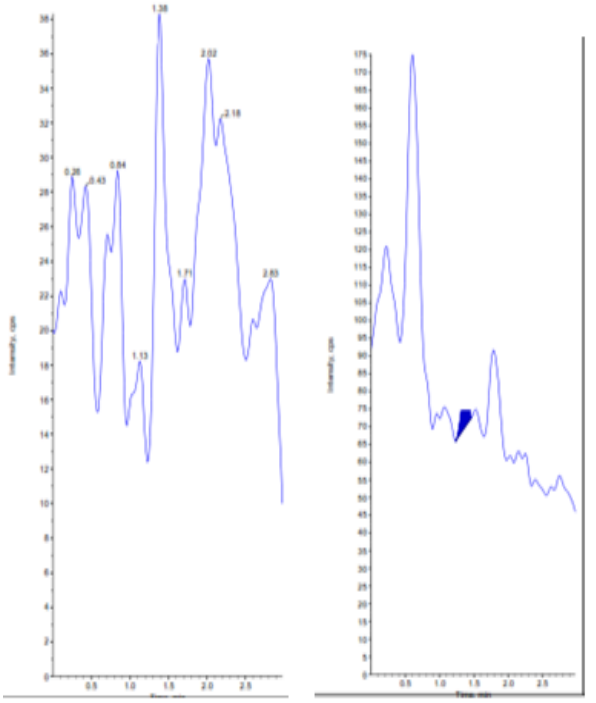


Fig.4-Blank plasma chromatogram for interference free Doxycycline and Doxycycline-d3

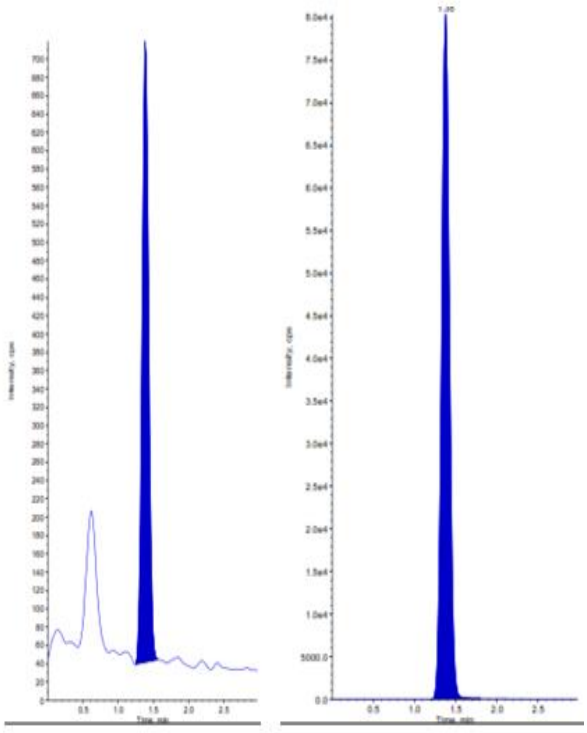


Fig.5- Chromatogram of LLOQ sample (Doxycycline and Doxycycline-d3).

Linearity

Linearity was plotted as a peak area ratio (Doxycycline peak area / Doxycycline-d3 peak area) on the y-axis against Doxycycline concentration (pg/ml) on the x-axis. Calibration

curves were found to be consistently accurate and precise for Doxycycline over a linearity range of 6 to 768.00 pg/ml. The correlation coefficient was greater than 0.99980 for Doxycycline. The %CV was less than 15% and mean %accuracy was ranged between 96.83 – 100.52%. Results were presented in Table 1.

Table. 1 - Calibration curve details of Doxycycline

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
6.00	5.81± 0.62	10.68	96.83
12.00	11.64 ± 1.11	9.57	96.97
24.00	23.56 ± 1.27	5.40	98.17
48.00	46.99 ± 1.00	2.12	97.90
96.00	96.49 ± 1.17	1.21	100.52
192.00	191.13 ± 1.98	1.03	99.55
384.00	383.33 ± 1.01	0.26	99.83
768.00	768.16 ± 0.30	0.004	100.02

Precision & Accuracy

Intra and inter batch %accuracy for Doxycycline was ranged between 97.33-99.58 and 100.48 to 104.27. %CV is 0.39 to 3.68 and 0.28% - 3.18%. Results are presented in Table 2.

Table.2-Precision and accuracy (Analysis with spiked samples at three different concentrations) of Doxycycline

Spiked Plasma Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy
18.00	17.52±0.65	3.68	97.33	18.77±0.60	3.18	104.27
360.00	366.62±6.40	1.75	101.84	361.73±1.01	0.28	100.48
720.00	716.94±2.78	0.39	99.58	721.72±5.31	0.74	103.24

Recovery

The mean %recovery for LQC, MQC, HQC samples of Doxycycline were 98.12%, 99.27%, 97.24%, respectively. The overall mean % recovery and %CV of Doxycycline across QC levels is 98.21% and 2.30%. For the Doxycycline-d3 (internal standard) the mean % recovery and %CV is 94.31% and 4.82%.

Matrix Effect

No significant matrix effect found in different sources of rat plasma tested for Doxycycline, Doxycycline-d3 . The %CV was found to be 1.98.

Stability (freeze–thaw, auto sampler, bench top, long term)

Quantification of the Doxycycline in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

Table. 3 - Stability studies of Doxycycline in spiked plasma samples

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	48h		70h		90 days		Cycle (48h)	
	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)
18.00	18.44±0.90	4.88	18.33±0.48	2.63	18.67±0.71	3.82	18.03±0.88	4.87
720.00	721.28±1.71	0.24	720.33±0.39	0.05	723.22±2.90	0.40	722.4±2.15	0.30

CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 6.0–768.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 6.0% and %accuracy ranged from 98.9%–102.4%. The overall %recovery for Doxycycline and Doxycycline-d3 was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid protein precipitation extraction with less run time of 4.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Doxycycline.

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CONFLICT OF INTEREST: Authors declare that, there is no conflict of interest.

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