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Research Article

Development and Validation of a Stability-Indicating RP-HPLC Method for Rapid Determination of Doxycycline in Pharmaceutical Bulk and Dosage Forms

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ABSTRACT

Background: A rapid stability-indicating RP-HPLC method for analysis of doxycycline in the presence of its degradation products was developed and validated.
Methods: Forced degradation studies were carried out on bulk samples and capsule dosage forms of doxycycline using acid, base, H_2O_2, heat, and UV light as described by ICH for stress conditions to demonstrate the stability-indicating power of the method. Separations were performed on a Perfectsil® Target ODS column (3-5µm, 125 mm×4 mm), using a mobile phase consisting of methanol-50 mM ammonium acetate buffer (containing 0.1% v/v trifluoroacetic acid and 0.1% v/v triethylamine, pH 2.5) (50:50 v/v) at room temperature. The flow rate was 0.8 mL/min.
Results: The method linearity was investigated in the range of 25–500 µg/mL (r > 0.9999). The LOD and LOQ were 5 and 25 µg/mL, respectively. The method selectivity was evaluated by peak purity test using a diode array detector. There was no interference among detection of doxycycline and its stressed degradation products. Total peak purity numbers were in the range of 0.94-0.99, indicating the homogeneity of DOX peaks.
Conclusion: These data show the stability-indicating nature of the method for quality control of doxycycline in bulk samples and capsule dosage forms.

Introduction

Doxycycline (DOX), [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-naphtacene carboxamide monohydrate (Figure 1), is a semi-synthetic broad spectrum tetracycline antibiotic.[1]

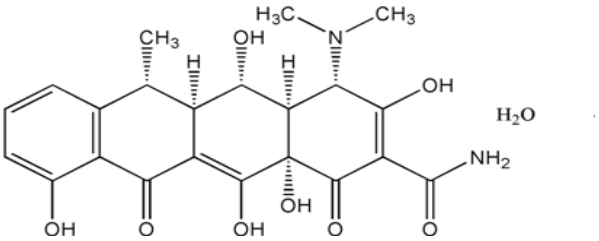

Figure 1. Doxycycline monohydrate structure; (molecular weight = 462.5 g/mol).

Tetracyclines are mainly bacteriostatic, with a broad spectrum of antimicrobial activity against many aerobic and anaerobic bacteria and some protozoa. DOX is more active than many other tetracyclines against bacterial species like *Streptococccus pyogenes*, enterococci, *Nocardia* spp., various anaerobes and *Plasmodium* species.[2] Recently DOX is being used for its inhibition effect on a group of enzymes named matrix metalloproteinase's (MMP's) that are involved in a number of immune and inflammatory responses such as periodentitis, gingivitis, degenerative rheumatic diseases and degenerative vascular disorders.[3,4] DOX can undergo different degradation pathways during storage, making a range of degradation products.[5,6] Stability is a major concern in pharmaceutical quality control and analytical methods are preferred to be stability-indicating.[7] The United States Pharmacopeia (USP), European Pharmacopeia (EU) and Hoogmartens and colleagues described the same method for determination of DOX in bulk or pharmaceutical preparations.[6-9] That method is one of the first HPLC methods for analysis of DOX in the presence of its degradation products and is based on styrene divinylbenzene copolymer stationary phase and a mixture of phosphate buffer, tetrabutylammonium hydrogen sulphate, ethylenediaminetetraacetic acid (EDTA) and tertiary butyl alcohol as the mobile phase. Separation temperature at 60 °C on a specific column and method's long run time (20 minutes) are some of the downsides of this method. Furthermore, there is not enough evidence about its stability-indicating nature.

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Two other studies developed HPLC methods for determination of DOX in the presence of its common degradation products, 6-epidoxycycline and metacycline.[4,10] These studies used mixture of DOX with these degradation products for HPLC method development, while a reliable stability-indicating HPLC method should be able to separate any possible degradation products that may emerge during storage under different destructive conditions. Forced degradation is the best way for obtaining such compounds for stability-indicating analytical method development.[11-15] In the study conducted by Injac and co-workers thermal degraded samples were used for HPLC method development, which means the method cannot essentially be stability-indicating for degradation products which are produced under other instability conditions.[5] Srilekha and colleagues reported a stability-indicating HPLC method for simultaneous determination of doxycycline and ornidazole in pharmaceuticals.[16] However, there is some evidence indicating that the stress tests were not performed correctly in that study. In order to develop a stability-indicating method for determination of a drug substance, it is critical to perform the stress testing under suitable stress condition for both bulk and dosage form. In the mentioned above article, the stress testing was performed only for bulk form of doxycycline. Also in that study oxidative (using H_2O_2) and thermal stress studies were performed at high temperatures (60, 105°C) which were not recommended by ICH. Under the high temperature, drug degradation kinetics does not follow the Arrhenius equation. In conclusion, regarding our knowledge in pharmaceutical stress testing studies, the temperature increment (> 70°C) may change the mechanisms and the nature of degradation products which were not formed in long term stability studies. So, it is not a good prediction of drug degradation pathways and the drug shelf life in quality control procedures.[11,12,14,17]

In the present study a simple, precise, accurate and rapid stability-indicating RP-HPLC method for the determination of DOX in the presence of its degradation products in bulk and capsule dosage forms using forced degraded samples was developed and validated. Forced degradation studies including oxidative degradation, acid hydrolysis, base hydrolysis, thermolysis and photodegradation were performed on DOX bulk and capsule dosage forms in both solid and solution forms. Peak purity was calculated for untreated and forced degraded doxycycline samples in order to investigate selectivity and stability-indicating nature of the method.

Materials and Methods

Chemicals and standard solutions

Doxycycline monohydrate (99.8% purity) was purchased from Changzhou Pharmaceutical Factory (Changzhou, Jiangsu, China) and doxycycline capsules (batch number 91-010, expiry date 07-2017) containing doxycycline monohydrate equivalent to 100 mg doxycycline were obtained from RAZAK LABORATORIES (Tehran, Iran). HPLC-grade methanol, ammonium acetate, glacial acetic acid, hydrochloric acid, sodium hydroxide, hydrogen peroxide, trifluoroacetic acid (TFA) and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany). Ionic liquid (IL), 1-Butyl-3-methylimidazolium tetrafluoroborate, was purchased from Kimia Exir Chemical Company (Tehran, Iran). All chemicals and reagents were analytical grade and used as received. All solutions were prepared by HPLC grade water obtained from a Milli-Q system (Millipore Milford, MA, USA). Buffer and all solutions were filtered through 0.45 µm nitrocellulose membrane filters (Schleicher & Schuell, Germany) before use. Stock solution of DOX (500 µg/mL) was prepared in water and stored in refrigerator. Aliquots of the stock solution were transferred into 10 mL A-grade volumetric flasks and diluted with mobile phase to obtain standard solutions with final concentrations of 5, 10, 25, 50, 100 and 200 µg/mL. Contents of twenty 100 mg doxycycline capsules were well mixed in a mortar. A quantity of powder equal to 100 mg doxycycline was transferred into a 200 mL volumetric flask and dissolved using 15 min sonication and adjusted to volume by water to obtain a 500 µg/mL solution. In order to perform the assay experiments, aliquot of this solution was diluted with mobile phase to obtain the concentration of 50 µg/mL.

Chromatographic conditions

HPLC system consisted of two k-1001 HPLC pumps, injection system, solvent degasser and a k-2600 UV detector (KNAUER, Berlin, Germany). Data record and analysis was performed by ChromGate® Chromatographic Data System, version 3.1.7 (Berlin, Germany). All analyses were performed on a Perfectsil® Target ODS (3-5µm, 125 mm×4 mm) column (MZ-Analysentechnik, Mainz, Germany) using a mobile phase consisting of methanol-50 mM ammonium acetate buffer (containing 0.1%, v/v TFA and 0.1%,v/v TEA, pH 2.5) (50:50, v/v), at a flow rate of 0.8 mL/min. The injection volume was 50 µL and column was exposed to ambient temperature (25°C). UV detection was optimized at 269 nm. Peak purity results were obtained using k-2800 Well Chrome Diode Array Detector (KNAUER, Berlin, Germany).

Forced degradation studies of DOX bulk and capsule contents

Stress tests were performed on DOX bulk and capsule content in both solid and solution forms in order to obtain degradation products. The certain amounts of bulk and capsule content were dissolved in deionized water under described condition to obtain 500 µg/mL solutions. Aliquots of these solutions were diluted by stress agents including deionized water, H_2O_2, HCl and NaOH solutions to obtain the concentration of 250

µg/mL. Bulk and capsule content were also exposed to UV-C irradiation and dry heat and then dissolved in deionized water to 250 µg/mL solutions. Degraded samples were collected at appropriate intervals, diluted by mobile phase (1:10, v/v) and subjected to HPLC analysis using diode array detector until the optimum amount of degradation was reached. HPLC method development was performed using untreated and forced degraded samples to ensure the specificity and stability-indicating nature of the method.

Oxidative degradation studies

Certain amounts of bulk and capsule powder were dissolved in deionized water to obtain 500 µg/mL under described condition. These solutions were diluted by 5, 10 and 30% H_2O_2 (1:1, v/v) to obtain 250 µg/mL solutions. No degradation occurred using 5 and 10% H_2O_2 after 5 hours. Therefore, oxidative degradation was conducted using 30% H_2O_2. After stirring for 60 minutes at ambient temperature (25 °C) and further dilution with mobile phase (25 µg/mL), samples were analyzed.

Acid degradation studies

Certain amounts of bulk and capsule powder were dissolved in deionized water to obtain 500 µg/mL. These solutions were diluted by 0.1 N hydrochloric acid (1:1 v/v) to obtain the solutions in concentration of 250 µg/mL and stored at 70 °C for 48 hours. The resulting solutions were analyzed after 6, 24 and 48 hours, after neutralizing by 0.1 N sodium hydroxide and then diluting by mobile phase to 25 µg/mL.

Base degradation studies

Certain amounts of bulk and capsule powder were dissolved in deionized water to 500 µg/mL. These solutions were diluted by 0.1 N sodium hydroxide (1:1 v/v) to obtain 250 µg/mL and stored at 50 °C for 30 minutes. The resulting solutions were analyzed after 10, 20 and 30 minutes, after neutralizingby 0.1N hydrochloric acid and then diluting by mobile phase to 25 µg/mL.

Aqueous hydrolysis studies

Certain volumes of aqueous DOX bulk and capsule solutions (250 µg/mL) were stored at 70 °C for 48 hours. The resulting solutions were analyzed after 6, 24 and 48 hours after diluting by mobile phase to 25 µg/mL.

Photodegradation studies

Certain volumes of aqueous DOX bulk and capsule solutions (250 µg/mL) were exposed to UV-C irradiation (30W, OSRAM®, Germany) for 5 hours. The resulting solutions were analyzed every 1 hour after diluting by mobile phase to 25 µg/mL.

Solid samples of DOX bulk and capsule content were also placed under UV-C irradiation until one week. Every 24 hours, certain amounts of solid samples were dissolved in deionized water to obtain 250 µg/mL solutions and analyzed after further dilution to 25 µg/mL by mobile phase.

Thermal degradation studies

Solid samples of DOX bulk and capsule content were exposed to 70 °C dry heat in a convection oven for three weeks. Every week, certain amounts of solid samples were dissolved in deionized water to obtain 250 µg/mL solutions and analyzed after further dilution to 25 µg/mL by mobile phase.

Results and Discussion

HPLC method development and optimization

RP-HPLC method was developed using a Perfectsil Target ODS column at ambient temperature (25 °C). Several analytical parameters including percentage of methanol and ammonium acetate buffer in mobile phase composition, buffer pH, buffer additives and flow rate were optimized in order to analyze DOX in degraded samples under various stress conditions.

Buffer pH is a critical parameter because of its effect on the ionization state of DOX molecule. Tetracycline antibiotics have four ionization equilibriums at approximate pH values of 3.2, 7.6, 9.6 and 12 and five possible protonation states, H_4Tc^+, H_3Tc, H_2Tc^-, HTc^{2-} and Tc^{3-}.[18] Ammonium acetate buffer was adjusted to pH 2.5 in this study because at this pH DOX molecule is present in the form of a cation with good elution from the column. However, increasing the buffer pH to 3.7 changed DOX molecule to a zwitterion with total charge of zero which is probably responsible for its slow elution from the column, long retention time and peak broadness. Different percentages of methanol in mobile phase composition including 30, 40, 50 and 60% were investigated. Increasing the methanol portion from 30% to 40% and then 50%, decreased the retention time from 20 minutes to a more reasonable time. However further increase to 60% disturbed the resolution and to obtain a balance between retention time and resolution, 50% was chosen as the optimum amount of methanol in mobile phase composition. Decreasing the flow rate from 1 to 0.8 mL/min improved resolution between DOX peak and its degradation products, but caused an increase of about 1 minute in retention time. This retention time (RT=6.1 min, RSD = 1.15%) is still much better than DOX retention time by pharmacopeial methods (RT = 20 min) that are not even based on forced degraded samples under various stress conditions.

Tailing and band broadening of the chromatographic peaks are common problems in HPLC analysis of DOX and other tetracycline antibiotics. Tetracyclines form chelates with divalent and trivalent cations like calcium and aluminum which are present in the surface of silica gel particles of chromatographic columns. Their positively charged amino groups also strongly interact with free charge silanol groups that are usually employed in reversed stationary phases. In order to

reduce these interactions, different mobile phase additives such as phosphoric acid, citric acid, EDTA, ion pair reagents and silanol masking agents can be employed.[19,20] TFA is a commonly used ion pairing reagent that can sharpen peaks and improve their resolution by suppressing the interaction of zwitterionic and basic compounds with active silanol sites on silica-based HPLC columns.[21] TFA is also known as an acceptable pH stabilizer when mobile phase pH is lower than 2.5.[22] In this study, 0.1% v/v TFA was added to the ammonium acetate buffer which improved peak sharpness as could be predicted. We also evaluated and compared the effect of TEA and an Ionic Liquid (IL), as silanol screening agents, on peak shape and symmetry. 0.1% v/v TEA and 20 mM IL were added to the ammonium acetate buffer, separately. Results showed that TEA is more efficient in sharpening the peaks and reducing their tailing. TEA was not used at higher concentrations.[23] The higher concentration of TEA caused to decrease the retention time by blocking DOX interaction with active silanol groups and caused to decrease in resolution.

Validation of the method

The HPLC method was validated according to ICH guidelines.[24] Parameters considered for method validation were linearity and range, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and selectivity. Recovery of DOX from capsule dosage form content was also examined.

Linearity and Range

Plots of peak area (Y) against concentration (X, µg/mL) were drawn for DOX standard solutions with 25, 50, 100, 200 and 500 µg/mL concentrations, by three different analysts on three consequent days. The resultant levels of relative standard deviation (RSD%) were between 0.06% and 2.57%. Regression equations and coefficients of determination amounts (R^2) were calculated by the method of least squares and are presented in Table 1. Visual inspection and data obtained from regression plots indicate that all plots are linear in the range of 25-500 µg/mL.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Since this method determines DOX in bulk and pharmaceutical dosage forms, LOD and LOQ are not essential validation parameters.[22] However, the LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injection. The LOQ that produced the requisite precision and accuracy was 25 µg/mL (RSD<1.78%, RE<1.68%). The LOD was determined based on signal-to-noise ratio using an analytical response of three times of background noise. The LOD for DOX was found to be 5 µg/mL.

Precision

The intra and inter-day precision data are summarized in Table 2. Intra-day precision was obtained by analyzing three replicate samples of DOX solutions with five different concentrations in three different days. Inter-day precision was assessed by analyzing results of the same five samples over three days. As shown in Table 2, the intra- and inter-day assays have RSD values below 2.57% and 1.75%, respectively.

Accuracy

Accuracy of the method was assessed using relative error (RE %) values, calculated by interpolating the peak area of three replicates of DOX standard solutions of five different concentrations from the regression equations in three different days.

Table 1. Data of Calibration Plots.

Regression plot	Regression equation[a]	R^2	RSD[b] (%) range
1	Y = 121,559.43X-593,606.52	0.99998	0.06-2.57
2	Y = 119,711.81X-1,259,526.34	0.99991	0.95-2.15
3	Y = 121,740.24X-936,581.71	0.99996	0.33-0.95

[a]Y is peak area and X is DOX standard solution concentration (µg/mL).
[b]RSD: Relative standard deviation.

Table 2. Intra- and Inter-Day Precision and Accuracy Data.

Actual concentration (µg/mL)	Measured concentration (µg/mL), RSD[a] (%), RE[b] (%)			
	Intra-day (*n* = 3)			Inter-day (*n* = 9)
	Day 1	Day 2	Day 3	
25	24.58, 1.78, - 1.68	25.13, 1.42, 0.52	24.97, 0.33, -0.12	24.89, 1.29, -0.44
50	50.37, 2.57, 0.74	50.31, 2.15, 0.62	50.04, 0.95, 0.08	50.19, 1.53, 0.38
100	101.63, 0.06, 1.63	98.86, 1.26, -1.14	98.32, 0.47, -1.68	99.69, 1.75, -0.31
200	199.27, 1.18, - 0.36	196.45, 0.95, -1.77	198.8, 0.76, -0.6	198.17, 1.05, -0.91
500	500.42, 0.58, 0.08	501.47, 1.18, 0.29	500.86, 0.33,0.17	500.92, 0.68, 0.18

[a] RSD: Relative standard deviation
[b] RE: Relative error

Inter-day relative error was assessed by analyzing results of the same five samples over three days. Table 2 shows the mean calculated amounts for DOX standard solutions, their RSD and percent of relative error. According to these data, accuracy of the method in intra-day analysis is between 98.29-99.92% and in inter-day analysis is between 99.09-99.82% for different concentrations.

Selectivity

In this study, method development was performed using DOX bulk and capsule content samples that had undergone forced degradation conditions. HPLC method parameters were optimized in order to obtain good resolution between DOX and its degradation products peaks. Peak purity was investigated by analyzing untreated and forced degraded DOX samples by a diode array detector in the range of 200-400 nm. Total peak purity numbers were between 0.94-0.99, indicating the homogeneity of DOX peaks. These data show that the method is stability-indicating.

Recovery

This test was performed in order to investigate the possible interactions of capsule content matrix with analysis of the drug substance. Since the concentration of 50 µg/mL of doxycycline is target concentration for analysis of capsule content, different amounts of standard powder equal to 80, 100 and 120% of the target concentration were added to DOX capsule content and dissolved in deionized water to yield added concentrations of 40, 50 and 60 µg/mL. Each sample was analyzed using the present method in three replicate. The resultant percent of recovery and RSD% are shown in Table 3. These results indicate that the method is specific for analysis of DOX without any interference from the excipients in the formulation of capsules.

System suitability parameters

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system.[22] System suitability parameters including resolution, repeatability, capacity factor, tailing factor and number of theoretical plates were calculated through three replicate injections of a 50 µg/mL DOX capsule content solution. Results are shown in Table 4.

Forced degradation studies

As mentioned in introduction, in order to develop a stability-indicating method for determination of a drug substance, it is critical to perform the stress testing under suitable stress condition for both bulk and dosage form. It was not obtained in previously reported methods. In the present study, forced degradation studies were performed on DOX bulk and capsule content in both solid and solution forms, under standard conditions. Samples were collected at appropriate intervals and analyzed using the HPLC method until degradation had proceeded to the desired extent (>5-20% degradation[12]). Degraded samples were then used for stability-indicating HPLC method development. Performing the degradation studies under standard conditions led to obtain the degradation products that may be produced during the storage. Subsequently, a more reliable method will be developed.

DOX in both API bulk samples and capsule dosage forms degraded to the same extent and followed the same degradation profile under various stress conditions. During forced degradation studies, samples were monitored for any changes in their appearance, as well. DOX bulk and capsule content showed same physical changes during various stress conditions. DOX forms yellow solution in water, which gets intensified in basic pH values. Hydrolytic degradation changed the solution color to dark yellow, while a pink color was observed after photolytic degradation.

The quantity percent of DOX decomposition under the stress conditions are shown in Table 5 for both API and capsule content samples.

Basic hydrolysis found to be the main instability condition that rapidly degraded DOX to the maximum number of degradation products compared with other degradation pathways.

In Figure 2, chromatograms of basic stressed sample of DOX capsule content analyzed at two wavelengths 269 and 354 nm show these degradation products. Hydrolysis in acidic and neutral solutions occurred only after 48 hours and using maximum allowed temperature for forced degradation studies (70 °C).

Solid form of DOX was stable under both UV-C irradiation and heat after 1 and 3 weeks, respectively. However, DOX solution is unstable under UV-C irradiation and 30.4% and 26.7% degradation was achieved after 5 hours of exposure for DOX bulk and capsule content aqueous solutions. Oxidative degradation did not occur using 5% and 10% H_2O_2, but 30% H_2O_2 degraded DOX up to 19% after 60 minutes. Figure 3 shows HPLC chromatograms of degraded samples of DOX capsule content under various stress conditions. Our method seems better than previously reported method[16] because of the separation of more degradation products under the suitable stress conditions and performing the stress testing for both bulk and dosage forms.

Assay

The validated stability indicating RP-HPLC method was applied for analysis of DOX in commercially available RAZAK® 100 mg DOX capsules. Figure 4 displays two typical HPLC chromatograms obtained from assay of DOX in capsule and API bulk samples. The assay amount of DOX in RAZAK® 100 mg capsules was calculated using the regression equation: $Y=121,740.24X-936$ ($R^2=0.9999$)

and found to be 101.36% the label claimed amount (n = 3, RSD = 0.68%) which is equivalent to 101.36 mg DOX. Mean retention time of DOX was 6.1 min (n = 15, RSD = 1.15%).

Table 3. Recovery Data (n = 3).

Level of addition to target concentration (%)	Added concentrations (µg/mL)	Found concentrations (µg/mL)	Recovery %, RSD[a]%
80	40	38.84	97.09, 0.68
100	50	49.8	99.6, 0.45
120	60	60.12	100.19, 0.63

[a]RSD: Relative standard deviation.

Table 4. System Suitability Data (n=3).

Parameter	Found amounts
Resolution	2
Repeatability	0.81%
Capacity factor	2.8
Tailing factor	1.4
Number of theoretical plates	2549

Table 5. Decomposition Percent of Doxycycline in API Bulk and Capsule Dosage Forms Under Various Stress Conditions.

Stress Condition	Time, temperature	API Decomposition %	Capsule Decomposition %
UV Photolysis (solution)	5 hours, 25°C	30.4	26.7
UV Photolysis (solid)	1 week, 25°C	1.9	3.1
Acid Hydrolysis	48 hours, 70°C	36	44.5
Basic Hydrolysis	30 minutes, 50°C	41.2	45.5
neutral Hydrolysis	48 hours, 70°C	40.9	42.2
H_2O_2 Oxidation	60 minutes, 25°C	19	19.2
Thermolysis (solid)	3 weeks, 70°C	ND[a]	ND[a]

[a] ND = no significant decomposition.

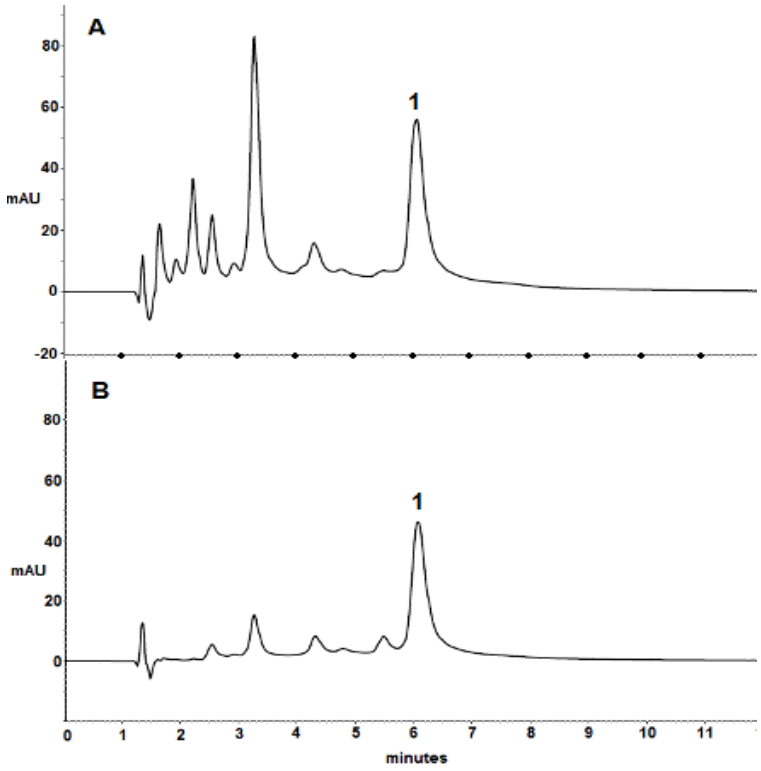

Figure 2. HPLC chromatograms of basic hydrolyzed doxycycline, analyzed at wavelengths (A): 269 nm and (B): 354 nm; (peak 1 shows doxycycline).

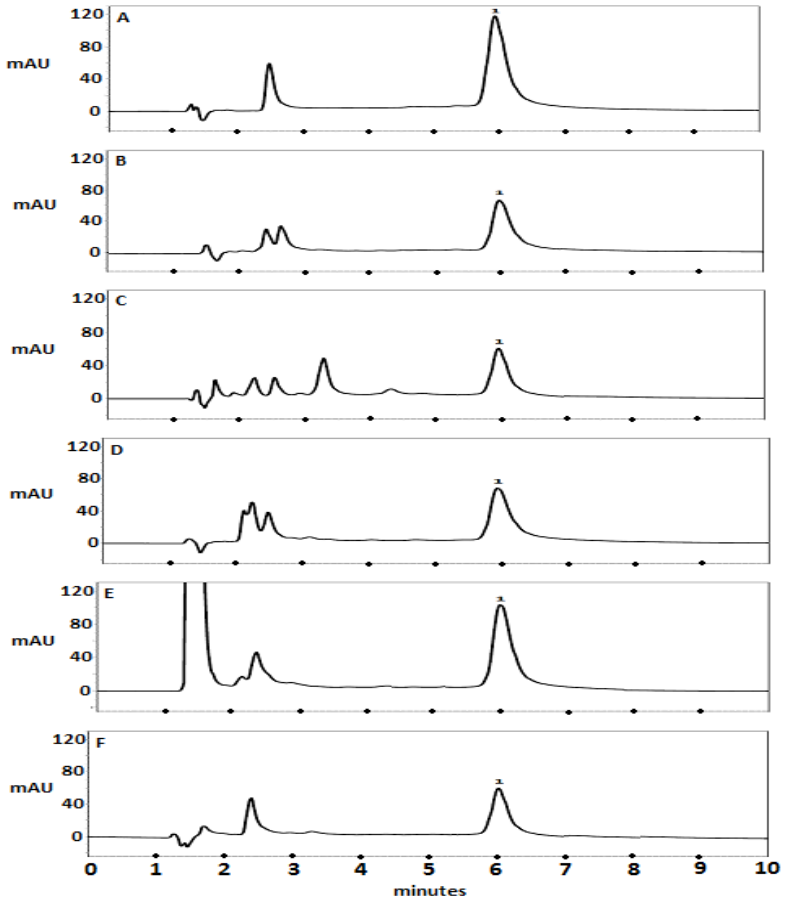

Figure 3. HPLC chromatograms of untreated and forced degraded doxycycline capsule contents; (A): untreated doxycycline; (B): acid hydrolysis; (C): base hydrolysis; (D): neutral hydrolysis; (E): oxidation; (F): photolytic degradation of solution. Dry-heated and UV-C irradiated samples are not shown because there was not any significant change compared with the untreated sample; (peak 1 shows doxycycline).

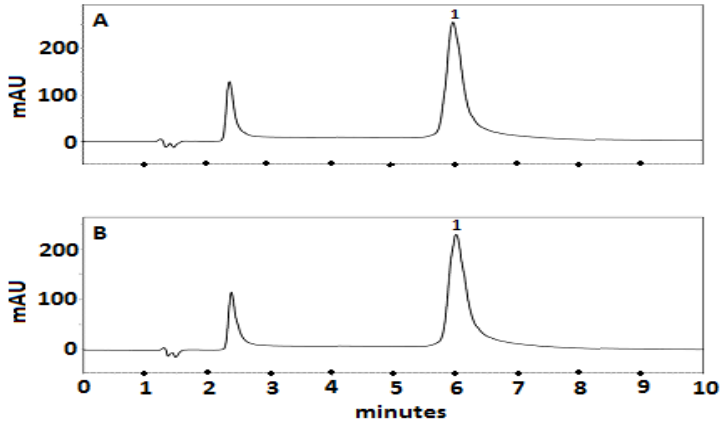

Figure 4. HPLC chromatograms of 50 µg/mL doxycycline in (A): capsule dosage form and (B): API bulk sample; (peak 1 shows doxycycline).

Conclusion

A validated stability-indicating RP-HPLC method has been developed for the determination of DOX in bulk and capsule dosage forms. The proposed method is accurate, precise, and selective. This method can separate DOX from its degradation products formed under various stress conditions. Results of stress testing reveal that the method is selective and stability-indicating and can be applied for analysis of DOX in either bulk powder or capsule dosage form in routine quality control or accelerated stability studies. Simplicity of this HPLC-UV method with short run time is the main advantage in comparison to other complicated, costly and time consuming methods like LC–MS or GC–MS.

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Conflict of interests
The authors claim that there is no conflict of interest.

References
1. O'Neil MJ, Budavari S. The merck index: An encyclopedia of chemicals, drugs and biological. NJ: Merck Whitehouse Station; 2001.
2. Sweetman SC. Martindale: The complete drug reference. London: Pharmaceutical Press; 2011.
3. Axisa B, Naylor A, Bell P, Thompson M. Simple and reliable method of doxycycline determination in human plasma and biological tissues. J Chromatogr B Biomed Sci Appl. 2000;744(2):359-65. doi:10.1016/s0378-4347(00)00261-9
4. Skulason S, Ingolfsson E, Kristmundsdottir T. Development of a simple HPLC method for separation of doxycycline and its degradation products. J Pharm Biomed Anal. 2003;33(4):667-72. doi:10.1016/s0731-7085(03)00316-9
5. Injac R, Djordjevic-Milic V, Srdjenovic B. Thermostability testing and degradation profiles of doxycycline in bulk, tablets, and capsules by HPLC. J Chromatogr Sci. 2007;45(9):623-8. doi:10.1093/chromsci/45.9.623
6. Hoogmartens J, Khan NH, Vanderhaeghe H, Van der Leeden A, Oosterbaan M, Veld-Tulp G, et al. A collaborative study of the analysis of doxycycline hyclate by high-performance liquid chromatography on polystyrene-divinylbenzene packing materials. J Pharm Biomed Anal. 1989;7(5):601-10. doi:10.1016/0731-7085(89)80226-2
7. Mohammadi A, Esmaeili F, Dinarvand R, Atyabi F, Walker RB. Development and validation of a stability-indicating method for the quantitation of paclitaxel in pharmaceutical dosage forms. J Chromatogr Sci. 2009;47(7):599-604. doi:10.1093/chromsci/47.7.599
8. The United States Pharmacopeia. 34th ed. Rockville: United States Pharmacopeia Convention; 2011.
9. Committee CoEEPH. European pharmacopoeia. 6th ed. Maisonneuve: Commission EP; 1994.
10. Fiori J, Grassigli G, Filippi P, Gotti R, Cavrini V. HPLC-DAD and LC-ESI-MS analysis of doxycycline and related impurities in doxipan mix, a medicated premix for incorporation in medicated feedstuff. J Pharm Biomed Anal. 2005;37(5):979-85. doi:10.1016/j.jpba.2004.06.017
11. Yaripour S, Nezami Rashid S, Alibakhshi H, Mohammadi A. Development and validation of a stability-indicating reversed phase HPLC method for the quality control of zolpidem in bulk and tablet dosage forms. J Anal Chem. 2015;70(6):738-43. doi:10.1134/s1061934815060143
12. Baertschi SW, Alsante KM, Reed RA. Pharmaceutical stress testing: Predicting drug degradation. 2nd ed. Boca Raton : CRC Press; 2011. p. 78-92.
13. El-Houssini O. Study of forced degradation behavior of idrocilamide and development of stability indicating lc method. J Anal Chem. 2014;69(10):1003-8. doi:10.1134/s1061934814100050
14. Mohammadi A, Rezanour N, Ansari Dogaheh M, GhorbaniBidkorbeh F, Hashem M, Walker R. A stability-indicating high performance liquid chromatographic (HPLC) assay for the simultaneous determination of atorvastatin and amlodipine in commercial tablets. J Chromatogr B. 2007;846(1-2):215-21. doi:10.1016/j.jchromb.2006.09.007
15. Mohammadi A, Haririan I, Rezanour N, Ghiasi L, Walker R. A stability-indicating high performance liquid chromatographic assay for the determination of orlistat in capsules. J Chromatogr A. 2006;1116(1-2):153-7. doi:10.1016/j.chroma.2006.03.038
16. Srilekha A, Pavani P, Sreedhar B. Stability indicating RP-HPLC method development and validation for simultaneous estimation of doxycycline monohydrate and ornidazole in bulk and pharmaceutical dosage form. Int J Pharm Sci Res. 2015;6(5):2228-39.
17. Stability Testing of New Drug Substances and Products (Q1AR2), International Conference on Harmonization, Geneva: IFPMA; 2003.
18. Jin L, Amaya-Mazo X, Apel ME, Sankisa SS, Johnson E, Zbyszynska MA, et al. $Ca^{2+}$and Mg^{2+} bind tetracycline with distinct stoichiometries and linked deprotonation. Biophys Chem. 2007;128(2):185-96. doi:10.1016/j.bpc.2007.04.005
19. Ruz N, Zabala M, Kramer MG, Campanero MA, Dios-Viéitez MC, Blanco-PrietoMaJ. Rapid and simple determination of doxycycline in serum by high-performance liquid chromatography: Application to particulate drug delivery systems. J Chromatogr A. 2004;1031(1-2):295-301. doi:10.1016/j.chroma.2003.12.028
20. Ruiz-Angel M, Carda-Broch S, Berthod A. Ionic liquids versus triethylamine as mobile phase additives in the analysis of β-blockers. J Chromatogr A. 2006;1119(1):202-8. doi:10.1016/j.chroma.2005.11.132
21. Guan K, Palmer DC. Effects of trifluoroacetic acid concentrations in mobile phases on hplc retention of zwitterionic and weakly basic triazole derivatives. J Liq Chromatogr Relat Technol. 2006;29(3):415-30. doi:10.1080/10826070500452077
22. Cai B, Li J. Evaluation of trifluoroacetic acid as an ion-pair reagent in the separation of small ionizable molecules by reversed-phase liquid chromatography. Anal Chim Act. 1999;399(3):249-58. doi:10.1016/s0003-2670(99)00461-4

23. Li H, Krieger RI, Li QX. Improved HPLC method for analysis of 1-hydroxypyrene in human urine specimens of cigarette smokers. Sci Total Environ. 2000;257(2-3):147-53. doi:10.1016/s0048-9697(00)00504-0

24. I.C.H. Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1), Geneva: International Conference on Harmonization; 2005.