Thermostability Testing and Degradation Profiles of Doxycycline in Bulk, Tablets, and Capsules by HPLC

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Abstract

A high-performance liquid chromatography (HPLC) method for the quantitation of doxycycline in bulk, tablets, and capsules after storage at –20, 5, 25, 40, 50, 60, and 70°C, has been developed and validated. The samples are eluted from a µ-Bondapak C8 column (4.6 × 150-mm, i.d., 5-µm particle size) at 27°C, with a mobile phase of acetonitrile–water–THF (29.5:70:0.5, v/v/v), adjusted to pH 2.5 with 1.0M HCl. The flow rate is 1.0 mL/min and detection by UV is at 350 nm. The stability of doxycycline in bulk and in pharmaceuticals is checked over 90 days. Doxycycline shows thermo-degradation after exposure to high temperature; tablets are more stable than capsules. The shelf lives ($t_{90\%}$) are determined to be 1.00, 2.84, and 5.26 years in bulk, capsules, and tablets, respectively, at 25°C. Metacycline and 6-epidoxycyline are identified as degradation products at high temperatures. Amounts of doxycycline, metacycline, and 6-epidoxycycline in all samples are determined by HPLC, and the results compare with those from micellar electrokinetic capillary chromatography. After 90 days, metacycline and 6-epidoxycyline are almost equal in test samples from standard bulk form, tablets, and capsules. It is 27.8 ± 0.3%, 13.7 ± 0.1%, and 18.8 ± 0.2%, respectively.

Introduction

Doxycycline (DOX), 1-dimethylamino-2,4a,5,7,12-pentahydroxy-11-methyl-4,6-dioxo-1,4a,11,11a,12,12a-hexahydrotetracene-3-carboxamide (Figure 1), is a semi-synthetic broad spectrum tetracycline antibiotic, widely used in human and veterinary medicine and as an animal feed supplement to prevent disease. It is the drug of choice in the treatment of Lyme disease, Brucellosis, and several Rickettsial infections, and it is also utilized in the treatment of sexually transmitted diseases (1–3). It is derived from oxytetracycline and the synthetic pathway of DOX involved metacycline (MET) as an intermediate. During this process 6-epidoxycyline (6-EPO) can be formed as a side product (1,3), and European Pharmacopoeia (Ph Eur) (4) and United States Pharmacopoeia (USP) (5) set a limit of 2% for MET and 6-EPO, and 0.5% for any other impurity. Both Ph Eur and USP prescribe high-performance liquid chromatography (HPLC) for the analysis of DOX and related compounds.

The tetracyclines (TCs) are amphoteric compounds, forming salts with either bases or acids. In neutral solutions, these substances exist mainly as zwitterions. The acid salts, which are formed through protonation of the enol group on C-2, exist as crystalline compounds that are very soluble in water. Doxycycline is available as a hydrate salt, a hydrochloride salt solvated as the hemiethanolate hemihydrate, and monohydrate. The hydrate form is sparingly soluble in water and is used in capsule and tablet dosage forms (usually as doxycycline hyclate). The monohydrate is water insoluble and is used for aqueous suspensions, which are stable for two weeks (6).

Many tetracyclines are available commercially and permitted for human administration, such as oxytetracycline, metacycline, chlortetracycline, and doxycycline. Under abnormal conditions (heat, pH, and humidity), tetracyclines undergo reversible epimerization at positions C-4 and C-6 to form a mixture of degradation products. These degradation products or contaminants have very low antibiotic activity; in addition, some of them can be toxic (7).

Tetracyclines exhibit general poor stability and during storage in animal feeds and premixes may be subjected to extensive degradation, such as epimerization (8).

Recently, many feasible and rapid HPLC methods for analysis of doxycycline, impurities, and degradation products have been

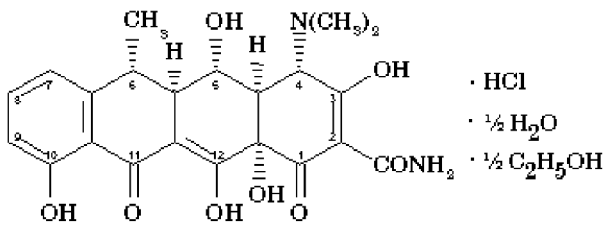

Figure 1. Chemical structure of doxycycline hyclate.

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developed. An HPLC–diode array detector (DAD) system was used for determining TCs (including DOX) in milk, using a solid-phase extracting column and water eluent (9), serum (10), alveolar macrophages (11), turkey tissue (12), residues in honey (13), pharmaceuticals (14,15), and human plasma and biological tissues (16). HPLC–DAD and LC–ESI–MS analysis of doxycycline and related impurities in doxipan mix were developed (17), as well as dissipation of oxytetracycline, chlortetracycline, tetracycline, and doxycycline using HPLC–UV and LC–MS–MS under aqueous semi-field microcosm conditions (18). A simple HPLC method for the separation of DOX and its degradation products was also developed (19), but with a long analysis time (30 min).

This paper describes a rapid and selective HPLC method for the simultaneous separation and determination of DOX and its degradation products after storage at different temperatures. The method was compared with micellar electrokinetic capillary chromatography (MEKC) (20), as a means of assessing the stability of DOX.

The purpose of this study was to further characterize the thermostability and the degradation mechanisms and products of free DOX standard and DOX in pharmaceuticals, capsules, and tablets.

Materials and Methods

Materials

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Milli-Q-quality). Doxycycline hyclate, metacycline, and 6-epidoxycycline were obtained from Jugoremedija (Zrenjanin, Serbia). The quality of the DOX was to EP5 requirements (98%).

Acetonitrile, tetrahydrofuran (THF), and methanol (HPLC-grade) were obtained from Sigma (Deisenhofen, Germany). Hydrated sodium tetraborate ($Na_2B_4O_7 \times 10H_2O$) was p.a. from Kemika (Zagreb, Croatia). Sodium dodecylsulphate (SDS) was from Riedel-de Haën AG (Seelze, Germany).

The pharmaceutical formulations Doksiciklin (tablets: serial No 28160, exp. date 12.2007) and Doxiciklin (capsules: serial No 0951105, exp. date 11.2008.) containing 115 mg of doxycycline hyclate corresponding to 100 mg of doxycycline free base, were obtained from Jugoremedija (Zrenjanin, Serbia) and Panfarma (Belgrade, Serbia), respectively.

HPLC assay

An HPLC–DAD model Agilent HP 1100 system equipped with an autosampler (Waldbronn, Germany), was used with a µ-Bondapak C_8 column (4.6 × 150-mm, i.d., 5-µm particle size). The mobile phase was acetonitrile–water–THF (29.5:70:0.5 v/v/v), adjusted to pH 2.5 with 1.0M HCl and filtered (0.45-µm nylon filter). The determination took 4 min, with a flow rate of 1.00 mL/min and a column temperature of 27°C. UV detection was at 350 nm.

MEKC

An Agilent 3D-CE capillary electrophoresis system (Waldbronn, Germany), with a diode-array detector and controlled by HP ChemStation software, was used to perform MEKC. Compounds were determined on a 56 cm (50 cm to the detector) × 50 µm i.d. fused silica capillary (with bubble cell, 150 µm) (Agilent, Waldbronn, Germany).

The background electrolyte (BGE) was 30mM borate buffer, pH 9.0, containing 60mM SDS and 5% (v/v) methanol. Samples were analyzed at 30 kV and 25°C (under an applied pressure of 15 mbar) in 8 min; under these conditions, the current was 59–60 µA. UV detection was at 350 nm. This was followed by hydrodynamic sample injection at 600 mbars.

Preparation of standard stock solutions

Standard stock solutions of DOX, MET, and 6-EPO were prepared by weighing 50 mg of the drugs and dissolving in 50 mL acetonitrile–methanol (30:70, v/v) for HPLC and 50 mL methanol for MEKC. Solutions were stored at 4°C until use. The stock solutions for MEKC were diluted with running buffer in the concentration range of 5–100 mg/L to obtain the concentrations required.

Sample preparation and extraction

Doxycycline was extracted from the tablets and powder from capsules using the following procedure. First, 20 tablets (average weight 208.46 ± 1.63 mg) or powders from 20 capsules (average weight 304.72 ± 1.45 mg) were accurately weighed, finely ground to powder, and thoroughly mixed. Aliquots of this powder corresponding to 25 mg of declared active principle (calculated as doxycycline free base) were weighed and extracted with methanol (5 × 10 mL), first by shaking, then in an ultrasonic bath for 10 min. The extracts from each powder (tablets and capsules) were combined, filtered (0.45-µm nylon filter), transferred to four 50 mL volumetric flasks (two with extract from tablets and two with extract from capsules), and diluted with acetonitrile–methanol (30:70, v/v) for HPLC and with methanol for MEKC. These solutions were placed in 1.5-mL auto-sampler vials and diluted with mobile phase (HPLC) or with running buffer (MEKC), by automatic pipette, to a concentration of 0.1 mg/mL. One microliter was injected into the HPLC system.

Experimental procedures

In accordance with ICH stability testing of drug substances and products (21), DOX standard, pharmaceuticals, capsules, and tablets were stored at –20, 5, 25, 40, 50, 60, and 70°C. Stress testing of a drug, in this case DOX, can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecules, and validate the stability-indicating power of the analytical procedures used. The nature of the stress testing depends on the chemical and physical characteristic of DOX and the type of formulation. Stress testing according to ICH is likely to be carried out on a single batch of the drug substance. It should include the effect of temperature (in 10°C increments; for accelerated testing 50°C, 60°C, etc.), humidity (e.g., 75% RH or greater), oxidation, and photolysis.

In this work, thermostability was determined under dry and dark conditions. At the accelerated storage condition, a minimum of three time points, including the initial and final time points for a six-month study, is recommended. For stress testing,

it could be three months. Samples were taken at 0, 7, 15, 30, 45, 60, 75, and 90 days after being stored at –20, 5, 25, 40, 50, 60, and 70°C, and analyzed by HPLC and MEKC.

Data analysis

The temperature dependence of the rate of chemical degradation of DOX in bulk and in pharmaceuticals was investigated by determining the concentration of active principal (DOX) by HPLC and MEKC as a function of time. A linear correlation was obtained at each temperature, and the observed rate constant (k) was calculated as the slope of these curves. The activation energy (E_a) was derived from the slope of the natural logarithm of the observed rate constants (ln k) plotted against the inverse absolute temperature ($1/T$) using the Arrhenius equation

$$\ln k = \ln A - E_a/RT \qquad \text{Eq. 1}$$

where the constant (A) is the frequency factor; E_a is the energy of activation; R is the universal gas constant; and T is the absolute temperature.

Validation of the methods

To validate the HPLC and MEKC methods, a series of tests were made using the most promising conditions. The linearity of the assay was determined by analysis of a series of standards at five different concentrations that span at least 80–120% of the expected working range (22,23).

The limits of detection (LOD) and quantitation (LOQ) were estimated by the baseline noise method. Baseline noise was evaluated by recording the detector response over a period of ten times the peak width. LOD and LOQ, respectively, were defined as the analyte concentrations resulting in heights of peaks three and ten times the baseline noise level (24).

The accuracy of the methods was determined by analyzing a standard solution of known concentration. Repeatability of assays was checked to determine intraday variation of corrected areas and migration times.

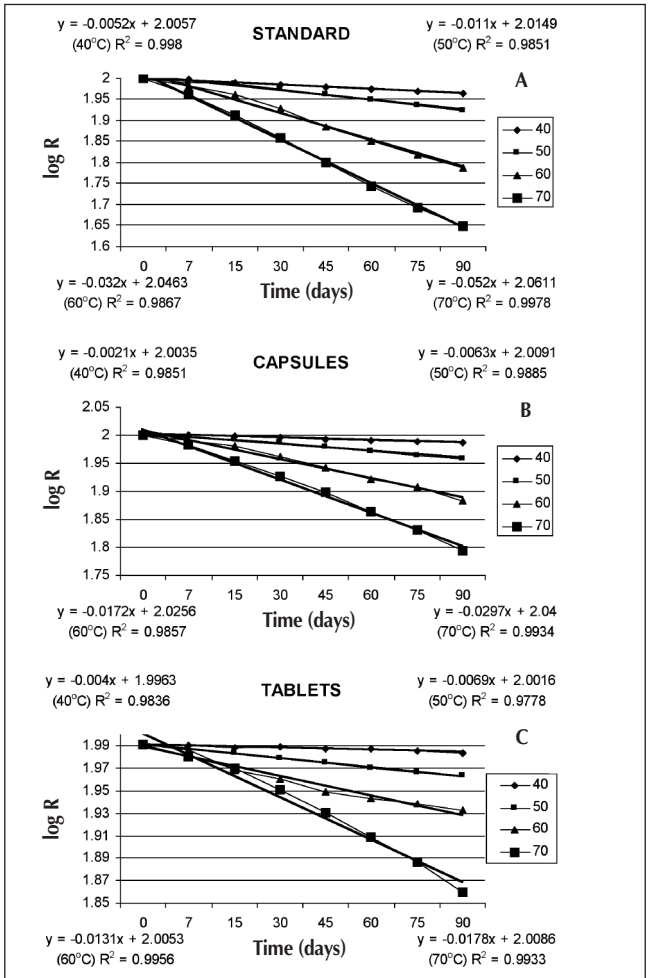

Figure 2. Apparent first order plots for degradation of DOX in bulk (A), capsules (B), and tablets (C) at 40, 50, 60, and 70°C.

Results and Discussion

The thermostability of doxycycline in bulk, tablets, and capsules was determined by HPLC and MEKC.

Appearance

Colors of the DOX standard, tablets, and capsules were recorded as a function of time and temperature (Table I). The color of capsules changed faster than that of tablets. At –20°C, no change of color was observed for tablets, capsules, or standard.

Assay of DOX

The content of authentic DOX was determined in samples as a function of time and temperature (Table II). All starting samples gave results between 95% and 105% of the label claim, which is within content limit.

No significant change was observed in any of the samples stored for three months at –20, 5, and 25°C, except for the standard at –20°C. Normally, active principals have to be stored at 4 to 8°C. All products with DOX are to be stored at controlled room temperature of 15°C to maximum 30°C and dispensed in tight, light-resistant containers (5).

For our work, it is more important to determine the stability

Table I. Visual Examination of the Samples

Temperature	0 time	30 days	90 days
Standard			
–20°C	yellow	yellow	yellow
40°C	yellow	yellow	green
70°C	yellow	green	dark green
Tablets			
–20°C	white	white	white
40°C	white	white	off-white
70°C	white	slightly beige	mottled yellow
Capsules			
–20°C	green	green	green
40°C	green	green-white	yellow
70°C	green	yellow	yellow, sticky

of DOX in bulk and in pharmaceuticals at temperatures which are higher then 40°C. Also, identification and quantitation of degradation products have to be reported.

After 30 days at 70°C, all samples exhibited a significant decrease in DOX content below 95%, and the DOX standard was reduced to an average value of 84.3%. After 90 days, DOX content was dramatically reduced at 70°C for all samples, but less in pharmaceuticals. DOX content in at 40°C was less than 95%.

Thermo-kinetics of degradation

The effect of high temperature on dry material in the dark was studied between 40 and 70°C. Degradation of DOX was monitored by HPLC and MEKC. No significant degradation of DOX was observed at 40°C over a period of 30 days. At 70°C, the content of DOX decreased from 10–15% in 30 days, and from 27–55% in 90 days of the initial amount (Table II).

A linear correlation between recoveries and the time of the temperature exposure is shown in Figure 2. For all samples, there was a linear relationship between storage time and the logarithmic content of DOX (Figure 2), and the degradation of DOX followed first order kinetics in bulk and in pharmaceuticals. The observed first order rate constant (k) was determined from the slope of the graph by statistical regression analysis method according to equation:

$$\ln [C] = \ln [C_o] - kt \qquad \text{Eq. 2}$$

where C is the concentration at the time t (days), and Co is concentration before exposed the high temperature.

According to equations 1 and 2, E_a was calculated. The activation energy for loss of DOX was 67.72 kJ mol^{-1}, 80.41 kJ mol^{-1}, and 85 kJ mol^{-1} for DOX in bulk, capsules, and tablets, respectively. The relationship between ln k and 1/T was determined by linear regression and shown in Figure 3. The correlation coefficients (R^2) were 0.9854 for standard ($y = -7.87x + 18.211$), 0.9777 for capsules ($y = -9.14x + 21.422$), and 0.9806 for tablets ($y = -9.36x + 21.543$).

The shelf life ($t_{90\%}$) is a value that indicates the period of storage of a product without loss of potency. It is described by ICH as the time taken for 10% of the product to decompose at given temperature. To determine the shelf life of DOX at room temperature (25°C ± 2°C for climatic zone III and IV), the following equation was used:

$$t_{90\%} = 0.105/k_{25} \qquad \text{Eq. 3}$$

The shelf life for DOX at 25°C was determined to be 1.00, 2.84, and 5.26 years in bulk, capsules, and tablets, respectively. This indicates that DOX is stable at room temperature and conformed to the shelf life given by the manufacturer as 3 years for capsules and 5 years for tablets, at 15 to 30°C, when protected from light and humidity.

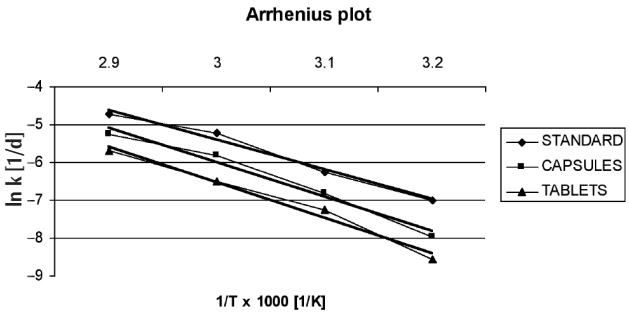

Figure 3. Arrhenius plot of DOX in bulk, capsules, and tablets.

Table II. Assay Results Presented as Percentage of Label Claim

Temperature	0 time		30 days		90 days	
	HPLC	MEKC	HPLC	MEKC	HPLC	MEKC
Standard						
–20°C			98.65	98.69	95.05	94.89
5°C			99.31	99.08	98.95	99.00
25°C	99.95	99.92	99.85	99.83	99.77	99.80
40°C			96.32	96.24	92.01	91.98
70°C			85.13	83.39	44.46	45.18
Tablets						
–20°C			97.90	97.75	97.71	97.62
5°C			97.97	98.02	97.88	97.98
25°C	98.02	98.11	98.00	98.07	97.93	97.89
40°C			97.51	97.53	96.34	96.23
70°C			90.16	91.01	72.59	71.99
Capsules						
–20°C			99.17	99.11	97.23	97.19
5°C			99.12	99.28	98.35	98.49
25°C	100.07	100.58	100.00	100.39	99.98	100.10
40°C			98.94	99.02	96.97	96.83
70°C			87.32	87.14	62.41	61.96

Thermal degradation product of DOX

The stability-indicating characteristic of the HPLC assay is demonstrated in Figure 4. A typical chromatogram of DOX in tablets is shown in Figure 4A, with retention time 3.234 ± 0.005 min. Chromatogram of degradation products of DOX in capsules is presented in Figure 4B. Resolution for MET/6-EPO was 1.1 and for 6-EPO/DOX 2.9. MET and 6-EPO exhibited with retention times of 2.105 ± 0.002 min and 2.266 ± 0.002 min, respectively. The degradation products were identified by a comparison of retention times on HPLC chromatograms, MEKC electropherograms, and HPLC and MEKC spectra with those authentic compounds. A good agreement was obtained between the spectrum of each degradation compound and its corresponding authentic compound. The degradation products observed for powder from capsules, with retention times of 1.677 and

3.982 min, were not identified as tetracyclines. They could be degradation products from some other compound from the excipient. Some of the excipient in capsules, such as yellow iron oxide (E172), indigo carmine (E132), titanium dioxide (E171), gelatin, shellac, and *N*-butyl alcohol, could be unstable at high temperature or humidity (25). Caviglioli et al. (26), in a stability study of hard gelatin capsules containing retinoic acid, showed that the shelf life for these capsules stored at room temperature in light-resistant containers was 678 days. Tablets are formed by direct compression of a powdered form of the active ingredient. Many "inactive" ingredients, primarily sugars, starches, and other fillers, are often added to tablets to better enable efficient and stable tablet formation. Dry gelatin has an almost infinite shelf life as long as the moisture content is such as to ensure that the product is stored below the glass transition temperature. The stability of gelatin depends on temperature and humidity. Generally, to minimize loss of gel strength and viscosity with time, the temperature and humidity would be kept as low as possible (27).

After 90 days, amounts of MET and 6-EPO are almost equal in each of the test samples from standard, tablets, and capsules, and it was 27.76 ± 0.26%, 13.71 ± 0.13%, and 18.79 ± 0.18%, respectively.

The main advantage of our method compared with those utilized in the European Pharmacopoeia (4) and in the literature (19) is that it is faster and simpler to carry out. The retention time of the compounds with this method is approximately eight times faster than in methods previously used (4,19). The validation parameters determined here (linearity, selectivity, precision, and accuracy) do not significantly differ from those of the previous methods (19). Peak resolution for MET/6-EPO is almost the same as with the method presented by Skulason et al. (19). On the other hand, resolution for 6-EPO/DOX is better (2.9) with this method [1.9 (19)]. The other advantage of this method, and the MEKC method over the HPLC method described in literature (10–13,15,19), is its lower running costs and higher environmental friendliness. An HPLC analysis (19) with flow-rate of 1.0 mL/min and analysis time of 30 min, requires 30 mL of acetonitrile–water–perchloric acid as the mobile phase, while in our case with the same flow-rate and an analysis time of 4 min, 7.5 times less mobile phase is used and the perchloric acid is replaced by THF. Twenty to thirty analyses with MEKC require 3 mL of borate buffer containing SDS and 5% (v/v) methanol, while twenty analyses by HPLC require 80 mL of acetonitrile–water–THF or 600 mL of acetonitrile–water–perchloric acid (19). No significant difference in selectivity of this HPLC method over the MEKC method was observed.

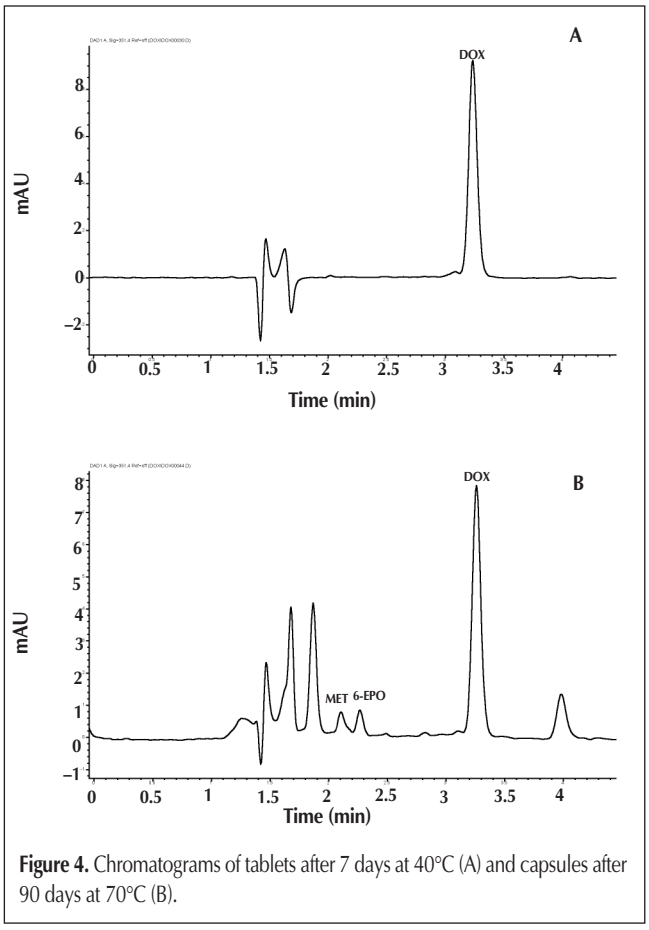

Figure 4. Chromatograms of tablets after 7 days at 40°C (A) and capsules after 90 days at 70°C (B).

Validation parameters

A calibration curve was made for each of the three compounds. The concentrations examined were between 0.5 and 100 µg/mL for DOX and 0.5 to 10 µg/mL for MET and 6-EPO. The correlation coefficients (R^2) were 0.9999 for DOX, 0.9997 for MET, and 0.9995 for 6-EPO, for HPLC analysis.

The selectivity of the method was investigated by observing any interference from excipient present in the pharmaceuticals. The capsules include lactose, magnesium stearate, sodium lauryl sulphate, maize starch, and alginic acid. Tablets contain anhydrous colloidal silica, microcrystalline cellulose, and magnesium stearate. There was no interference in HPLC and MEKC results from the excipient, which indicates that the reported methods are selective.

The limits of detection (LOD) and limits of quantitation (LOQ) were 0.2–1.5 and 0.7–5.0 µg/mL, respectively.

The intraday precision (expressed as the relative standard deviation RSD) for the area under the curve (AUC) and retention times, was determined for all three substances by repeated analysis (n = 7). Average intraday RSD values obtained for retention times were 1.17% and, for areas under the curve, 1.34%. The average RSD values for between-day precision obtained for AUC were 0.99%.

Stability of analytical solutions

The stability of DOX, MET, and 6-EPO in methanol and acetonitrile–methanol (30:70, v/v) solutions was checked at room temperature for 72 h and the recoveries were 98.9 ± 0.2%, 99.6 ± 0.3%, and 99.8 ± 0.5%, respectively. The stability was also checked at 72 h at 4ºC (refrigerator). Recovery was ≥ 99.9 ± 0.1%, indicating good stability of analytical solutions in all cases.

Conclusion

The stability of doxycycline in bulk, tablets, and capsules was checked by testing real samples during 90 days by HPLC and compared with MEKC results. Doxycycline showed thermal

degradation after storage at high temperature. It is more stable in tablets than in capsules, although more than 80% of the oral dosage form of doxycycline hyclate on the market is in the form of capsules. This study further demonstrates that DOX is stable in pharmaceutical formulations, but some of capsule's excipient are also unstable at high temperatures, indicating almost half the shelf life of the tablet formulation. Pharmaceuticals with DOX can not be used after the expiration date indicated on the package because of potential toxicity. The stability of DOX capsules could be improved by coating them with a thermo-stable substance. A new condition for the rapid HPLC analysis of DOX could be also used for quality control of pharmaceuticals.

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