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Development and validation of RP-HPLC method for the determination of doxycycline hyclate in spiked human urine and pharmaceuticals

Pavagada J. Ramesh, Kanakapura Basavaiah, Kalsang Tharpa, Kanakapura Basavaiah Vinay, Hosakere Doddarevanna Revanasiddappa

Department of Chemistry, University of Mysore, Manasagangothri, India

Abstract: An accurate, sensitive, precise, rapid, isocratic reversed phase HPLC (RP-HPLC) method for the determination of doxycycline hyclate in bulk drug and in tablets, and also in spiked human urine, was developed and validated. The best separation was achieved on a 250 mm × 4.0 mm i.d, 5.0 µm particle size C_8 reversed phase thermo column with acetonitrile-potassium dihydrogenorthophosphate buffer (pH 4.0), 40:60 (v/v) as mobile phase at a flow rate of 1.0 mL/min. UV detection was performed at 325 nm at ambient column temperature (25 °C). The method was linear over the concentration range of 30-300 µg/ mL (r=0.9994) with limits of detection and quantification of 0.02 and 0.1 µg/ mL, respectively. The drug was subjected to oxidation, acid and base hydrolysis, photolysis and heat as stress conditions. Degradation products were found interfering with the assay of doxycycline hyclate only in oxidation and base hydrolysis, therefore the method can be regarded as indicating stability in the case of acid hydrolysis, photolysis and heat stress conditions. The method was applied for determination of doxycycline hyclate in pharmaceuticals and spiked human urine with excellent recoveries. The method can be used for the quality control of doxycycline hyclate.

Key words: doxycycline assay, stability, RP-HPLC, determination, pharmaceuticals

INTRODUCTION

Doxycycline hyclate (DOX), (Figure 1) ($C_{22}H_{24}N_2O_8$ HCl· $0.5C_2H_5OH$·0.5 H_2O, molecular mass 512.94 g/mol, CAS number: 24390-14-5) is the hydrochloride hemiethanol hemihydrate of doxycycline. The synonym for DOX is doxycycline hydrochloride. DOX is much more soluble than doxycycline monohydrate, which is one of the main reasons for its more frequent use in pharmaceutical samples. Doxycycline is preferred to other tetracyclines in the treatment of specific infections because of its fairly reliable absorption and its long half-life, which permits less frequent dosage. It is frequently used to treat chronic prostatitis, sinusitis, syphilis, chlamdydia, and pelvic inflammatory disease [1-4].

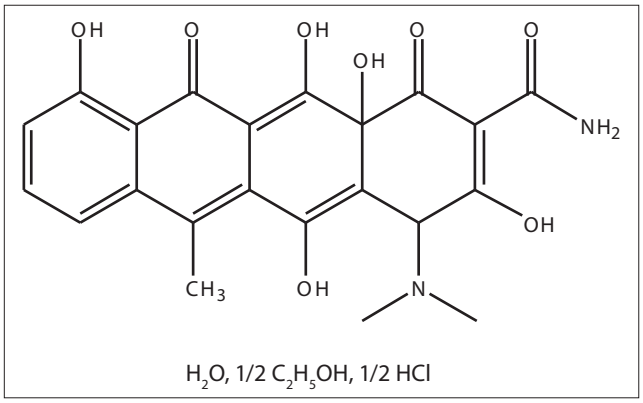

Figure 1 Structure of doxycycline hyclate.

The liquid chromatographic method for the determination of DOX is the choice of some Pharmacopoeias [5-7]. Several techniques have been reported for the *in vitro* and *in vivo* determination of DOX and include microbiology [8], fluorimetry [9], lanthanide sensitized luminescence [10], chemiluminescence [11], optical fibre sensor [12], solid surface phosphorescence [13], ion selective electrode (ISE)-potentiometry [14], cyclodextrin based fluorosensor [15], internal solid contact sensor based on a conducting polypyrrole [16], thin-layer chromatography (TLC) [17], TLC-fluorescence scanning densitometry (TLC-FSD) [18], derivative spectrophotometry [19] and HPLC for body fluids [20-26]. HPLC has also been applied for the determination of DOX in turkey liver and muscle [27], bovine tissue [22], bovine milk and muscle [28], animal tissues [29], human tissue [30], alveolar macrophages [31] and in milk [32]. Various other chromatographic methods have also been reported for the determination of DOX in milk and milk powder [33], human plasma [34], human urine [35], human serum, urine, semen, tears and saliva [36], and foods [33, 37]. DOX in pharmaceuticals has been assayed by derivative spectrophotometry [19], visible spectrophotometry [38-42], capillary electrophoresis [43] and micellar electrokinetic chromatography [35].

HPLC offers several advantages over other techniques, including minimal sample manipulation before chromato-

Corresponding author: Prof. Kanakapura Basavaiah, Department of Chemistry, University of Mysore, Manasagangothri, Mysore-570 006, India.
E-mail: basavaiahk@yahoo.co.in

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graphy, rapid analysis and the simultaneous analysis of multi-component mixtures with good specificity, precision and accuracy. There are only six reports on the HPLC determination of DOX in pharmaceuticals. The method of Snezana et al., [44] has been applied for veterinary pharmaceutical samples, whereas Dihuidi et al., [45] have applied the isocratic HPLC method for the separation of DOX from its related substances. Simultaneous determination of five tetracyclines, including DOX and the impurity, 6-epi-doxycycline, has been achieved using a porous graphite carbon column [46]. Two methods [47, 48] have also been presented for the separation of DOX from its analogs and for its determination in powder and tablets. HPLC analysis of DOX in bulk drug and in dosage forms using a polymeric column has been studied by Bryan and Stewart [49]. However, the reported methods for the determination of DOX in pharmaceuticals have disadvantages (Table 1).

The stability of a drug substance or drug product is defined as its capacity to remain within established specifications, i.e., to maintain its identity, strength, quality and purity until the retest or expiry date [50]. Stability testing of an active substance or finished product provides evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, e.g. temperature, humidity and light. Knowledge from stability studies is used in the development of manufacturing processes, selection of proper packaging and storage conditions, and determination of product shelf-life [51, 52].

There is no reported stability-indicating analytical method for the determination of DOX in the presence of its degradation products. The objective of this work therefore was to develop a simple, economical, rapid, precise and accurate stability-indicating HPLC method for quantitative determination of DOX in pharmaceuticals, and to validate the method in accordance with ICH guidelines [53]. The developed method was applied for determination of DOX in spiked human urine.

MATERIALS AND METHODS

Materials and standards. HPLC grade acetonitrile (Labscan Asia Co. Ltd, Bangkok, Thailand), analytical reagent grade potassium dihydrogenorthophosphate (Rankem, Bangalore, India) and potassium hydroxide

Table 1 Comparison of the performance characteristics of the proposed method with reported spectrophotometric and HPLC methods.

	A. Spectrophotometric methods.						
Sl. No.	Reagent/Reaction	λ, nm	Linear range, µg/ mL	LOD µg/ mL	RSD	Remarks	Reference
1	Derivative spectrophotometry	–	–	–	–	Used for assay of binary mixture	19
2	Copper carbonate as a solid bed reactor	395	10-80 mg/ mL	–	>2%	Flow injection analysis required, least sensitive	38
3	Uranyl acetate in DMF medium	405	0-135	–	>2%	Expensive reagent and anhydrous reaction medium required	39
4	Thorium (IV) as a coloring reagent	–	–	–	–	–	40
5	Sodium cobaltinitrite	243	10-30	–	>2%	Requires boiling in CH_3COOH medium; narrow linear dynamic range.	41
6	Copper (II)-H_2O_2, kinetic spectrophotometry	510	2.97-17.78	0.57	3.8%	Rigorous control of experimental variables required	42
	B. HPLC Methods						
Sl. No.	HPLC Conditions	UV detection	Linear range, µg/ mL	LOD µg/ mL	RSD	Remarks	Reference
7	Lichrosorb RP-8 (250 mm'4.6 mm, 10 mm particle size); methanol:acetonitrile: 0.01M oxalic acid (2:3:5, v/v); flow rate: 1.25 mL/ min	350	25.20-252.0	1.15	>2%	Three component mobile phase, applied to veterinary pharmaceutical samples	44
8	Hamilton RP-1 (25 x 0.46, cm, i.d.); tetrahydrofuran:0.2M phosphate buffer (pH 8.0):0.2M tetrabutylammonium hydrogen sulphate (pH 8.0): 0.1M sodium acetate (pH 8.0): water (6:10:5:1:78), flow rate: 1.00	254	60	–	>2%	Multi- constituents of mobile phase, elevated temperature	45
9	Porous graphitic carbon column; 0.05M potassium phosphate buffer (pH 2.0):acetonitrile (40:60), flow rate: 1.00	268	5.0-50	2.00	>2%	Applied to assay in a multicomponent mixture	46
10	Chromolith flash RP-18e, 25-4.6 mm; acetronitrile:water (20:90, v/v) pH 2.5 adjusted with 98% H_3PO_4, flow rate: 0.48	213	0.5-2.0		>2%	Narrow range, sequential injection setup required	47
11	A polystyrene-divinylbenzene column and a polymethaxrylate column with octadecyl ligands. Acetonitrile-0.02 M sodium perchlorate (pH 2.0)	–	–	–	–	–	49
11	Hypersil BDS C_8 (250 mm x 4.0 mm i.d, 5.0 µm particle size) thermo column; potassium dihydrogen orthophosphate buffer (pH-4.0)-acetonitrile (60:40) (v/v), flow rate: 1.00	325	30.0-300	0.1	<1%	Wide linear dynamic range, low LOD, low RSD (%), simple mobile phase and first application to stability-indicating assay	Present method

(Rankem, Bangalore, India) were used. Deionised, Milli Q water (Millipore, Bangalore, India) was used to prepare the mobile phase and diluent solutions. Doxycycline hyclate was obtained from Lotus Pharmaceuticals Ltd., Bangalore, India. Dosage forms were purchased from local commercial sources. Mobile phase was prepared by mixing 0.01 M potassium dihyrogenorthophosphate buffer adjusted to pH 4.0 with 0.1 M potassium hydroxide and acetonitrile in the ratio 60:40 (v/v), and filtered through 0.4 micron membrane filter. Diluent was a 1:1 mixture of potassium dihydrogenorthophosphate buffer and acetonitrile. A stock standard solution equivalent to 1,000 µg/mL DOX was prepared by dissolving an accurately weighed amount of pure drug in the diluent solution.

Equipment. HPLC analysis was performed on an Alliance Waters HPLC system equipped with Alliances 2657 series low pressure quaternary pump, a programmable variable wavelength UV-visible detector, Waters 2996 photodiode array detector and auto sampler. Data were collected and processed using Waters Empower 2.0 software.

Chromatographic conditions. Chromatographic analysis was carried out at ambient temperature (25 °C) on a hypersil BDS C_8 (250 mm × 4.0 mm i.d, 5.0 µm particle size) thermo column. The mobile phase was a mixture of 0.01 M potassium dihyrdogenorthophosphate, pH adjusted to 4.0 with 0.1 M potassium hydroxide and acetonitrile (60: 40, v/v). Flow rate was 1.0 mL/min. The detector wavelength was set at 325 nm, with injection volume at 10 µL.

MATERIALS AND METHOD

Calibration graph. Ten µL of working standard solutions (30-300 µg/mL DOX) were injected automatically onto the column in triplicate and the chromatograms recorded. Concentration of the unknown was computed from the regression equation derived using the mean peak area and concentration data.

Urine sample. Five mg of pure DOX was added to 1 mL of drug-free urine, followed by 3 mL of acetonitrile, and the solution was allowed to stand for 2 min. Five mL of mobile phase were added and the solution then diluted with water to 10 mL. The content was allowed to stand for 5 min and then centrifuged for 15 min at 4000 rpm. Finally, the solution was filtered through 0.2 µm cellulose acetate syringe filter and the filtrate made upto 25 mL with mobile phase. The resulting 200 µg/mL DOX and 100 µg/mL (on dilution with same solvent) solutions were subjected to analysis.

Pharmaceutical preparations. Twenty tablets were accurately weighed and crushed into a fine powder and mixed using a mortar and pestle. A quantity of tablet powder equivalent to 100 mg of DOX was weighed accurately into a 100 mL calibrated flask; 50 mL of diluent solution was added and sonicated for 20 min to complete dissolution of the DOX; the mixture was then diluted to the mark with the diluent and mixed well. A small portion of the resulting mixture (ca. 10 mL) was withdrawn and filtered through a 0.2 µm filter to ensure the absence of particulate matter. The filtrate was appropriately diluted with the diluent before injection onto the column.

Forced degradation procedure. Two portions of pure DOX each weighing 5 mg were separately spread uniformly on two Petri dishes. One portion was kept in an oven at 105 °C and the other exposed to UV radiation of 360 nm, for 48 hours each. At the end of the stipulated time period, the powders were transferred to two separate 25 mL calibrated flasks, dissolved in and diluted to the mark with the diluent before analysis. Into three separate 25 mL calibrated flasks, 5 mg of pure DOX was weighed, and 5 mL of 1 M HCl, 1 M NaOH or 10% H_2O_2 was added to the flasks. All three flasks were kept in a hot water bath at 80 °C for 3 h. After cooling, the volume in each flask was made up to the mark with diluent before being subjected to analysis.

RESULTS

Under the stated chromatographic conditions, the mean retention time was 3.110 min (n=10). A model chromatogram is shown in Figure 2. Further, the optimized chromatographic

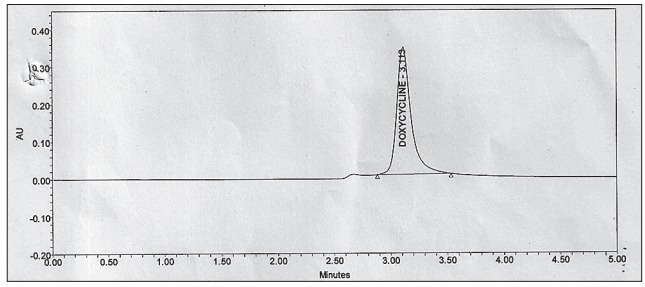

Figure 2 Typical Chromatogram (Pure DOX, 300 µg/mL).

conditions were used to study the effect of forced degradation of DOX after subjecting to various experimental conditions. Upon treatment with 1 M NaOH, 1 M HCl or 1% H_2O_2, for 3 hrs at 80 °C, separately, there was no change in the retention time and mean peak area in 1 M HCl, whereas considerable deviation from the above parameters was observed in 1 M NaOH (Figure 3) and 1% H_2O_2 (Figure 4). There was no effect upon exposure to UV light at 1200 K flux and thermal treatment at 105 °C, both for 48 hrs. All three chromatograms of DOX, after acid, light and heat induced degradation, were very similar to the typical chromatogram of pure DOX (Figure 2). Further, the optimized chromatographic conditions were used to study the effect of forced degradation of DOX after subjecting to various experimental conditions. Upon treatment with 1 M NaOH, 1 M HCl or 1% H_2O_2, for 3 hrs at 80 °C, separately, there was no change in the retention time and mean peak

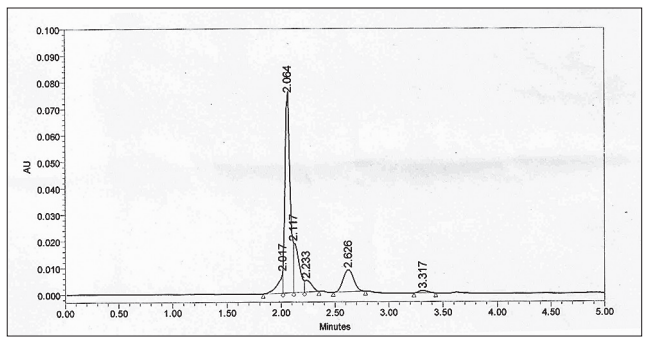

Figure 3 Chromatogram after treatment with 1 M NaOH (Pure DOX, 200 µg/mL).

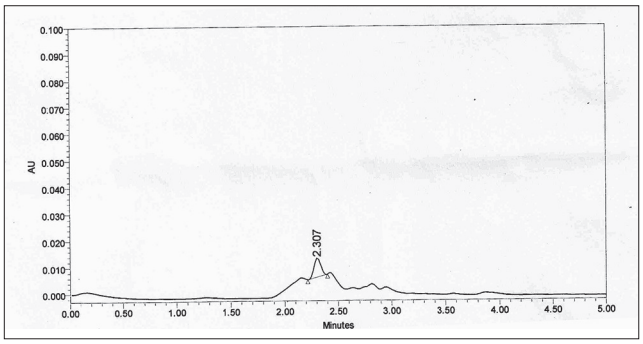

Figure 4 Chromatogram after treatment with 1% H_2O_2 (Pure DOX, 200 µg/mL).

area in 1 M HCl, whereas considerable deviation from the above parameters was observed in 1 M NaOH (Figure 3) and 1% H_2O_2 (Figure 4). There was no effect upon exposure to UV light at 1200 K flux and thermal treatment at 105 °C, both for 48 hrs. All the three chromatograms of DOX after acid, light and heat induced degradation were very similar to the typical chromatogram of pure DOX (Figure 2).

METHOD VALIDATION

Linearity. Working standard solution of DOX (1000 µg/mL) was appropriately diluted with the diluent solution to obtain solutions in the concentration range 30-300 µg/mL DOX. Ten µL of each solution was injected in triplicate into the column under the operating chromatographic conditions described above. The least squares method was used to calculate the slope, intercept and correlation coefficient (r) of the regression line. The relation between mean peak area Y (n=3) and concentration, X expressed by the equation Y = -10691.69 + 9811.60X, was linear. A plot of log peak area *Vs* log concentration was a straight line with a slope of 0.9974, indicating excellent linearity between the mean peak area and concentration in the range 30-300 µg/mL DOX.

Limits of detection (LOD) and quantification (LOQ). LOD and LOQ were estimated from the signal-to-noise ratio. The LOD, defined as the lowest concentration that gave a peak area with signal-to-noise ratio greater than 3:1, was found to be 0.02 µg/mL. The lowest concentration that provided a peak-area with a signal-to-noise ratio 9.78, which is called LOQ, was found to be 0.1 µg/mL.

Specificity. Method specificity was checked by comparing the chromatograms obtained for pure DOX solution, synthetic mixture, tablet solution and placebo blank. An examination of the chromatograms of the above solutions revealed the absence of peaks due to additives present in tablet preparations.

Precision and accuracy. Method precision was evaluated from the results of seven independent determinations of DOX at three different concentrations, 50, 100 and 150 µg/mL, on the same day. The inter-day and intra-day relative standard deviation (RSD) values for peak area and retention time for the selected concentration of DOX were less than 1.3 and 0.5%, respectively (Table 2).

The method accuracy, expressed as relative error (%), was determined by calculating the percent deviation found between concentrations of DOX injected and concentrations found from the peak area. This study was performed by taking the same three concentrations of DOX used for precision estimation. The intra-day and inter-day accuracy (expressed as % RE) was better than 3.2% and the values compiled (Table 3).

Table 2 Regression and Sensitivity parameters.

Parameters	Value
Linearity range, µg/mL	30-300
Slope (b)	9,811.60
Intercept (a)	-10,691.69
Standard deviation of intercept (S_a)	± 36,996.04
Standard deviation of Slope (S_b)	± 148.04
Correlation co-efficient (r)	0.9994
Limit of detection (LOD, µg/mL)	0.02
Limit of quantification (LOQ, µg/mL)	0.10
Variance (Sa^2)	6,996.04
$\pm tS_a/\sqrt{n}$	41,836.88
$\pm tS_b/\sqrt{n}$	168.33

**Y = a + bX, where Y is the area and X concentration in µg/mL.
$\pm tS_a/\sqrt{n}$ = confidence limit for intercept,
$\pm tS_b/\sqrt{n}$ = confidence limit for slope.

Table 3 Accuracy and precision study of intra-day and inter-day analysis.

DOX Injected,	Intra-day accuracy and precision				Inter-day accuracy and precision			
µg/ mL	DOX found[a], µg/mL	RE, %	RSD[b], %	RSD[c], %	DOX, found[a] µg/mL	RE, %	RSD[b], %	RSD[c], %
50.0	51.0	2.0	0.75	0.05	51.3	2.6	1.04	0.26
100.0	102.5	2.5	0.30	0.49	103.2	3.2	0.96	0.32
300.0	303.0	1.0	0.52	0.16	307.2	2.4	1.24	0.46

[a] Mean value of seven determinations
[b] Based on peak area.
[c] Based on retention time
RE: Relative error
RSD: Relative standard deviation

Robustness. To determine the robustness of the method, small deliberate changes in the chromatographic conditions, such as detection wavelength and column temperature, were made and the results compared with those of the optimized chromatographic conditions. The results indicated that changing the detection wavelength (±1 nm) had some effect on the chromatographic behaviour of DOX. However, the alteration in the column temperature (±1 °C) had no significant effect. The results of this study expressed as % RSD are summarized in Table 4.

System suitability. System suitability parameters were measured to verify the system performance and the values of retention time; the number of theoretical plates and tailing factor were 3.113 ± 0.0049, 5.180 per column and 1.2, respectively. All the values were within the acceptable range.

Solution stability. The stability of standard and sample solutions was determined by monitoring the peak area and retention time over a period of 24 hrs by injecting the solutions every 8 h. The standard and sample solutions were stored at ambient temperature (25 °C), and protected from light during

Table 4 Results of Robustness study (DOX concentration, 200 µg/ mL, n=3).

Chromatographic Condition	Modification	Peak Area Precision (n=3)				Retention Time Precision (n=3)			
		Area	Mean area ± SD	Standard error of mean	RSD, %	Retention time, min	Mean R_T ± SD, (min)	Standard error, min	RSD, %
Wavelength (nm)	324	2255108				2.823			
	325	2200112	2202238.3 ± 173866.8	29929.39	0.0231	2.819	2.818 ± 0.005	0.0029	0.0018
	326	2151495				2.813			
Column temperature	26	2373643				2.822			
(°C)	25	2371524	2371667.3 ± 1907.89	1101.61	0.0008	2.823	2.823 ± 0.001	0.0006	0.0004
	24	2369835				2.824			

the stability study. No changes in drug concentrations were observed over a period of 24 h as shown by the small % RSD values. The % RSDs for peak area (n = 4) was 1.4% for pure drug solution, and the value for retention time (n = 4) was 0.14% (Table 5). No significant changes in concentration of the active ingredient were also not observed in the tablet solution.

Table 5 Solution stability (DOX concentration was 200 µg/ mL).

Time, h	Peak area	Retention time (min)
0	2138277	3.11
8	2145671	3.12
16	2246830	3.10
24	2378480	3.09

Method application. The developed and validated method was successfully applied to determine DOX in spiked urine sample with satisfactory recovery (Table 6). The results

Table 6 Determination of doxycycline hyclate in spiked urine sample.

Spiked concentration (µg/ mL)	Found ± S.D[a]	% Recovery ± RSD[a]
100.0	101.4 ± 0.005	101.4 ± 0.005
200.0	206.4 ± 0.012	103.2 ± 0.033

[a] Mean for five determinations.

obtained tallied closely with the labelled amount in the case of tablets (Table 7), thus indicating the utility of the method for content uniformity evaluation. The results were statistically compared with those obtained by the official method [6] for accuracy and precision by applying the Student's t-test and variance ratio F-test. The official method consisted of liquid chromatography with UV detection. The calculated t- and F- values were less than the tabulated values of 2.77 and 6.39 at the 95% confidence level and for four degrees of freedom, suggesting that there was no significant difference between the proposed method and the reference method with respect to accuracy and precision.

Table 7 Results of determination of DOX in tablets and statistical comparison with the reference method.

Tablet brand name**	Nominal amount, mg	Found* (Percent of label claim ± SD)			
		Reference method	Proposed method	Student's t-value (2.77)	F-value (6.39)
Doxy[1]	100	104.8 ± 0.67	106.0 ± 1.22	2.00	3.31
Doxy[2]	100	98.46 ± 0.83	99.38 ± 1.42	1.29	2.93

* Mean value of five determinations
** Marketed by: 1. MicroLabs Pvt. Ltd., Bangalore, India
2. Dr. Reddy's Laboratory, Bangalore, India.
Figure in the parenthesis are the tabulated values for four degree of freedom at 95% confidence level.

Recovery study. To further assess the accuracy and reliability of the method, recovery studies *via* the standard addition method was performed. To the pre-analyzed tablet powder, pure DOX was added at three levels and the total was found by the proposed method. Each test was performed in triplicate. When the test was performed on 100 mg tablets, the percent recovery of pure DOX was 106.0 with standard deviation of 0.12. The results indicated that the method is very accurate and that common excipients found in tablet preparations did not interfere (Table 8).

Table 8 Results of recovery study by standard addition method.

Tablet	DOX in tablet, µg/mL	Pure DOX added, µg/mL	Total found, µg/mL	Pure DOX recovered*, Percent ± SD
Doxy	106.0	50	159.95	107.90 ± 0.56
100 mg	106.0	100	212.95	106.95 ± 0.72
	106.0	150	265.98	106.65 ± 0.86
Doxt	99.38	50	150.03	101.30 ± 0.64
100 mg	99.38	100	201.88	102.50 ± 0.46
	99.38	150	250.28	100.60 ± 0.92

* Mean value of three determinations

DISCUSSION

Mobile phase containing only acetonitrile or methanol was tried without success. Sodium acetate and potassium dihydrogenorthophosphate in combination with either acetonitrile or methanol in different volume ratios as organic modifiers were also tried. The mobile phase consisting of 0.01 M potassium dihyrdogenorthophosphate with pH adjusted to 4.0 using potassium hydroxide and acetonitrile (60:40, v/v) was found to be ideal. The selected mobile phase produced a well defined and resolved peak almost free from tailing (tailing factor 1.2). The analysis was carried out at ambient temperature which, besides being economical, offers many advantages, e.g. low column back pressure, good chromatographic peak shape, improved column efficiency, higher theoretical plates and consistency in retention time.

The proposed method uses a simple mobile phase compared to the multi-component mobile phase in many reported methods. The separation and determination was achieved at an ambient temperature relative to elevated temperatures in a couple of reported HPLC methods. This itself offers the advantages of low column back pressure, good peak shape, improved column efficiency, higher theoretical plates and consistent retention time. Furthermore, to date, this is the first stability-indicating method reported for doxycycline. In addition to solution stability as a function of time, forced degradation under a variety of stress-conditions has been studied, thereby widening the application. The small retention time (ca. 3 min) and runtime (5 min) enable rapid determination which is important in routine analysis. Simple mobile phase and low flow rate (1 mL/ min) make the method attractive since these features help in saving cost and time of analysis.

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