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Journal of Chromatography A, 1031 (2004) 295–301
JOURNAL OF CHROMATOGRAPHY A
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Rapid and simple determination of doxycycline in serum by high-performance liquid chromatography Application to particulate drug delivery systems

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Abstract

A simple, rapid and sensitive reversed-phase high-performance liquid chromatographic (HPLC) method for the measurement of doxycycline concentrations in both drug delivery systems (DDS) and serum extracted from mice after intraperitoneal (free drug) and intravenous (doxycycline administered in DDS) administration, has been developed. For the analysis of doxycycline in DDS, a known amount of particles was dissolved in chloroform and, after precipitating the polymer with methanol, the drug was assessed in the supernatant. For doxycycline quantification in microsamples of serum, proteins were precipitated with acetonitrile before chromatographic analysis. After centrifugation, the supernatant was mixed with a mixture of methanol and acetic acid (1:1) for analysis. The samples were chromatographed on a narrow-bore C_{18} column (Alltech Alltima 150 mm × 2.1 mm) using a mobile phase with 55% acetic acid (5%), 25% acetonitrile and 20% methanol. Doxycycline was detected 347 nm and the run time was 10 min. Linearity was confirmed in the concentration range 0.4–80 μg/ml for doxycycline quantification in serum and from 1 to 800 μg/ml for doxycycline extracted from DDS samples.

Keywords: Drug delivery systems; Doxycycline

1. Introduction

Doxycycline is a semisynthetic tetracycline antibiotic derived from oxytetracycline with a broad spectrum of activity against a wide range of Gram-positive and Gram-negative pathogens [1,2].

Doxycycline widely used in medicine is well tolerated at therapeutic doses [3], enter vertebrate cells by passive diffusion [4] and can be easily administrated (oral or intraperitoneally). Due to these characteristics, doxycycline has been recently chosen as a tool to control of gene expression in animal studies [5]. Indeed, doxycycline can be used to activate minimal promoters (tet on/off systems) that would limit the expression of a particular gene to a desired period of time [6,7]. Moreover, the amount of protein induced by these systems can be adjusted depending on the dose of doxycycline administrated. When studying gene expression regulated by the tet systems in the liver, specially for gene therapy applications, it would be preferable to target doxycycline to the hepatic cells to avoid side effects of the drug in other organs [8]. In this context, a drug delivery system (DDS) has been developed, loaded with doxycycline to target the drug to the liver and also to improve its sustained release, reducing the frequency of administrations. DDS may modify the distribution profile of encapsulated drug driving it to target organs or tissues and reducing both the given dose and the side effects [9]. Furthermore, the development of pharmacokinetic studies is a very useful tool to assess the properties of the developed dosage forms after their in vivo administration and requires sensitive analytical methods to allow the determination of low concentrations of drug in small samples.

Different analytical approaches have been employed for the determination of doxycycline in different biological samples. The antimicrobial properties of doxycycline have allowed the development of various biological assays [10,11] for the determination of doxycycline. Although

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doi:10.1016/j.chroma.2003.12.028

these assays have been applied in some pharmacokinetic and microbiological studies, there has recently been a move to replace by other methods. A further problem of the bioassay is that the degradation products or contaminants of doxycycline, which are present in the raw materials and the finished formulations, may also have antimicrobial properties. Thus, the results of bioassay could not necessary be a true representation of the antimicrobial potency of doxycycline alone [12].

Fluorimetric and spectrophotometric techniques has been used in the determination of doxycycline in pharmaceuticals. However, the specificity of these methods is very low when doxycycline are measured in biological samples, since interference from metabolites and endogenous components of the samples cannot always be excluded [13,14].

Thin-layer chromatography has reported to determine doxycycline in biological tissues using adsorbent layers of silica gel [15]. In general, thin-layer chromatography is simple and does not require special equipment. However, doxycycline shows extreme tailing spots when no prepared adsorbents are used, so the sensibility of the method is very low.

Capillary electrophoresis is recently applied for the determination of doxycycline in pharmaceuticals [16]. Nevertheless, despite of the many advantages that showed this technique, the low sample volume that can be injected in the capillary avoid the quantitation of this antibiotic in biological samples.

High-performance liquid chromatography (HPLC) has also been used to determine doxycycline and other tetracycline antibiotics in pharmaceuticals, and biological fluids, primarily in plasma, serum, urine or blood [17–25]. Doxycycline was also determined in isolated alveolar macrophages [26]. Nevertheless, the sensitivity of these analytical methods is enough insufficient when microsamples, as obtained from pharmacokinetic studies in mice, are analysed.

This paper describes the development and validation of a microanalytical high-performance liquid chromatographic technique for the determination of doxycycline in serial small serum samples obtained from DDS administered mice. This method is also suitable for the determination of this antibiotic in DDS formulations, without interference of other substances used in the preparation process of those systems.

2. Experimental

2.1. Chemicals, reagents and solutions

Doxycycline (Fig. 1A) was provided by Clontech (CA, USA) and piroxicam (I.S., Fig. 1B) was provided by CEPA Schwarz Pharma (Madrid, Spain). Acetonitrile, methanol, and acetic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ethyl acetate (purissimum) was supplied by Panreac (Barcelona, Spain) and chloroform by Sigma–Aldrich (Steinheim, Germany).

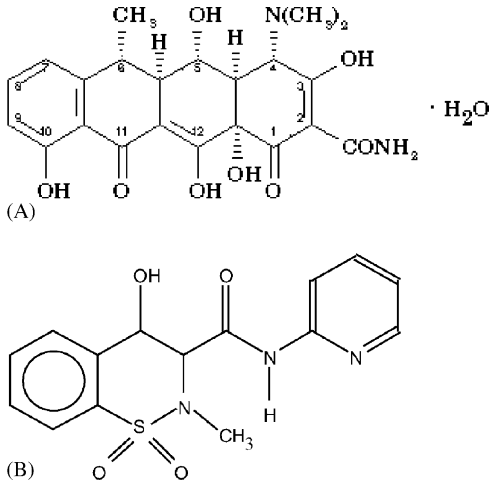

Fig. 1. Chemical structures of doxycycline (A) and piroxicam (B).

2.2. Standard solutions and samples

Stock solutions of doxycycline and piroxicam for serum, with a concentration of 320 and 100 μg/ml, respectively, were prepared separately by dissolving 8 mg of doxycycline in 25 ml of mobile phase and 5 mg of piroxicam in 50 ml of mobile phase. Finally, 10 standard solutions of doxycycline (8, 16, 32, 48, 80, 120, 160, 240, 280 and 320 μg/ml) were made by further dilution of the solutions with appropriate volumes of mobile phase. The standard solution of piroxicam (16 μg/ml) was similarly prepared.

For doxycycline quantification in DDS, stock solutions of doxycycline and piroxicam, with a concentration of 800 μg/ml and 2 mg/ml, respectively, were prepared separately by dissolving 8 mg of doxycycline in 10 ml of mobile phase and 10 mg of piroxicam in 5 ml of acetonitrile. Finally, 11 standard solutions of doxycycline (1, 5, 20, 60, 80, 160, 180, 300, 400, 640 and 800 μg/ml) were made by further dilution of the solutions with appropriate volumes of mobile phase. The standard solution of piroxicam (2 mg/ml) is the stock solution.

Standard and stock solutions of doxycycline and piroxicam were stored at 4 °C.

2.3. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a model 1100 series LC coupled with an UV diode array detector set at 347 nm (Hewlett-Packard, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA program. Separation was carried out at 10 °C on a reversed-phase, 150 mm × 2.1 mm column packed with C_{18}, 5 μm silica reversed-phase particles (Alltima) obtained from Alltech (Sedriano, Milan, Italy). This column was preceded by a reversed-phase C_{18}, 5 μm guard column (Kromasil, 20 mm × 4 mm, Symta, Spain). Mobile phase was a mixture of 5% acetic acid–methanol–acetonitrile (55:20:25, v/v/v).

Separation was achieved by isocratic solvent elution at a flow-rate of 0.25 ml/min.

2.4. Sample preparation

Serum samples (20 μl) were transferred to 13 mm × 100 mm conic tubes and spiked with the internal standard (10 μl of 16 μg/ml of piroxicam). Then, 20 μl acetonitrile was added to the tubes. The tubes were capped, vortex-mixed for 1 min., and centrifuged at 12,857 × *g* and 4 °C for 10 min. The supernatants were transferred to limited volume autosampler vials, diluted with a mixture of methanol–acetic acid (1:1), capped and placed on the HPLC autosampler. A 50 μl aliquot of the supernatant was injected onto HPLC column.

For the analysis of doxycycline in pharmaceutical formulations, a known amount of DDS was dissolved in 1 ml of chloroform. Then, 3 ml of methanol was added to precipitate the polymer. The samples were vortex-mixed for 1 min and centrifuged for 10 min at 9400 × *g*. A 500 μl aliquot of the supernatants with the internal standard (25 μl of 2 mg/ml of Piroxicam) were transferred to limited volume autosampler vials capped and placed on the HPLC autosampler. A 5 μl aliquot of the supernatant was injected onto HPLC column. The drug loading was expressed as μg of doxycycline/mg of DDS.

2.5. Quantitation

Calibration curves were determined by least square linear regression analysis (weighting $1/X^2$). Peak area ratio of doxycycline and piroxicam versus the corresponding doxycycline concentration was plotted. The linearity of the method was confirmed by comparing the slopes, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student's *t*-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones. Each calibration curve consisted of eight calibration points (1, 5, 20, 80, 160, 300, 400 and 800 μg/ml) for the determination of doxycycline in DDS. A calibration curve for doxycycline assay in mice serum was also constructed by spiking 15 μl samples of mice serum with increasing concentrations (16, 32, 80, 160, 240 and 320 μg/ml) of this antibiotic from 0.4 to 80 μg/ml.

2.6. Validation

The method was validated by analysis of mice whole-blood quality control samples prepared at four concentrations spanning the calibration range (for serum: 2, 12, 30 and 70 μg/ml; for DDS: 20, 60, 180 and 640 μg/ml). Four samples of each quality control pool, and calibration samples were analysed on three different days. On day 1, the number of samples each quality control pool was five. Precision and accuracy was determined. Precision of a method is expressed as the relative standard deviation (R.S.D., %) of the coefficient of variation of replicate measurements. Accuracy was measured according to the following equation:

$$\text{percentage difference from theoretical value} = \left(\frac{X - C_T}{C_T}\right) \times 100 \quad (1)$$

where X is the determined concentration of a quality control and C_T the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The overall recovery for doxycycline and piroxicam was calculated by comparing the peak area ratios of spiked samples with those obtained by direct injections of the same amount of both compounds.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix or other substances used in the preparation process of those particulate systems were compared with those of doxycycline and piroxicam.

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. The limit of quantitation (LOQ) was defined as the lowest drug concentration, which can be determined with an accuracy and precision <20%. In this work, LOD of the assay method was determined by analysis of the peak baseline noise in 10 blank samples.

2.7. Application of the method

This analytical HPLC method was applied to determine the doxycycline content in particulate dosage forms. The amount of doxycycline entrapped into the DDS was calculated as the difference between the initial drug and the amount of the active molecule recovered in DDS.

To demonstrate the reliability of this method for the study of doxycycline pharmacokinetics, this assay was applied to the determination of doxycycline in serum after administration of a doxycycline solution by the intraperitoneal route (1000 μg of doxycycline) and doxycycline loaded DDS to mice by the intravenous route (100 μg doxycycline encapsulated into 5 mg of DDS). Animals used were immunocompetent 6–8-week-old Balb/C females purchased from Harlan (Barcelona, Spain). To administer the DDS, the carriers were resuspended in 200 μl of physiological serum before tail vein injection. Blood samples were obtained at 0, 2, 10 and 24 h by retroorbital bleeding and the serum was subsequently recovered by centrifugation at 10,000 rpm for 5 min and stored frozen (−20 °C) until analysis.

3. Results and discussion

A number of assay methods have been published for the determination of doxycycline in biological fluids. Of these, the HPLC methods have been most frequently used in the pharmacokinetic studies of doxycycline because of

their simplicity, sensitivity and selectivity. However, in cases where microsample volumes are required, such as the case of mice pharmacokinetic studies, the HPLC methods previously developed for the determination of doxycycline are generally not considered sensitive enough due to the physicochemical characteristics of this molecule.

Doxycycline is an amphoteric compound (pK_a 3.4, 7.7 and 9.7) with characteristic physicochemical properties that have a strong influence in the chromatographic separation. This antibiotic, as the same form that other tetracyclines, forms chelate with divalent and trivalent cations including aluminium and calcium, which are present in the surface of silica gel particles employed in the packing of more chromatographic columns. Moreover, it also strongly interacts with the free charge silanols groups locating in the stationary phases and reversed-phase usually employed [27]. Therefore, doxycycline is eluted as tailing and band-broadening chromatographic peaks from the chromatographic column.

In order to avoid forming chelate complexes, different mobile phase additives such as, phosphoric acid, citric acid, tartaric acid, EDTA, and ion-pair reagents have been employed in the past with different results. In most cases, doxycycline still showed extreme tailing on the reversed-phase high-performance liquid chromatographic column obtaining unacceptable asymmetry coefficients and poor LOQs [28–30].

It well known that large differences in the chromatographic behaviour of doxycycline have been observed using different packing materials. Most C_8- and C_{18}-modified silica gel columns has been employed for analysing this compound with different results. In this work, different packing materials have been tested for the separation of doxycycline showing marked differences in chromatographic behaviour. In line with these observations, we also experienced poor chromatographic behaviour of doxycycline using Spherisorb C_8, Lichrospher C_8 Select B, and Ultrabase C_{18} packing materials (data not shown) despite of these packing materials showed and high end-capping of the stationary phase free silanol groups. On the other hand, chromatography using Alltima C_{18} stationary phase resulted in very acceptable peak shapes for doxycycline using a mobile phase with acetic acid, without the addition of any mobile phase additives. This column is a polimerically bonded C_{18} reversed-phase narrow-bore column packed with double-encapped spherical modified silica gel particles synthesised from pure silica gel. In order to further optimise the separation, methanol, an organic solvent that yields selectivity increase over chromatographic separation, was added to the mobile phase. The use of this well end-capped modified silica enabled us to determine doxycycline in small sample volumes, because of silanol groups have been mostly end-capped and the residual metals have been completely removed avoiding the formation of tailing chromatographic peaks. In addition the narrow bore of these columns (2 mm i.d.) produced an increased detector response than standard columns due to the decreased diffusion of the sample as it passes though the column.

The high selectivity degree of the method described here also allows us to simplify the extensive sample-preparation protocol applied in previous works prior chromatographic analysis. Several extraction methods have been described, but most involve complex extraction and time-consuming steps with great variability in the extraction ratio [24,25,31–34]. In this work, we have employed a single step extraction procedure in which acetonitrile, a deproteinising agent that disrupt the strong interaction between doxycycline and plasmatic proteins, was added to serum samples. It is interesting to note that with these extraction procedure we have been able to avoid the use of acidic solvents usually employed in these extraction procedures. Doxycycline is unstable in most of these solvents and form reversible epimers, being it responsible of the lower extraction ratios obtained [27]. These solvents also employed as deproteinising agents and, sometimes, interfere with the doxycycline peak in the chromatographic separation [28,35,36].

Under the previously described chromatographic conditions, chromatographic separation of the doxycycline and the internal standard was completed within 13 min. Fig. 2A displays the chromatogram of extracted serum from a mouse, which received a dose of DDS of 5 mg corresponding to 100 μg of doxycycline, and Fig. 2B is a similar chromatogram from blank sample of serum. The retention time

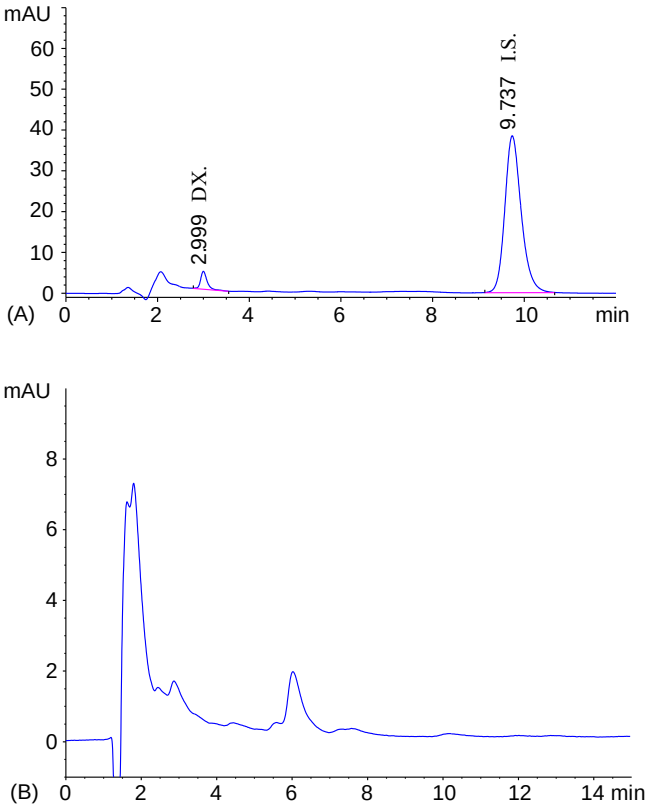

Fig. 2. Chromatogram from extraction of doxycycline of mouse serum sample obtained 10 h after doxycycline DDS administration (0.98 μg/ml doxycycline) (A) and chromatogram from analysis of blank mouse serum (B).

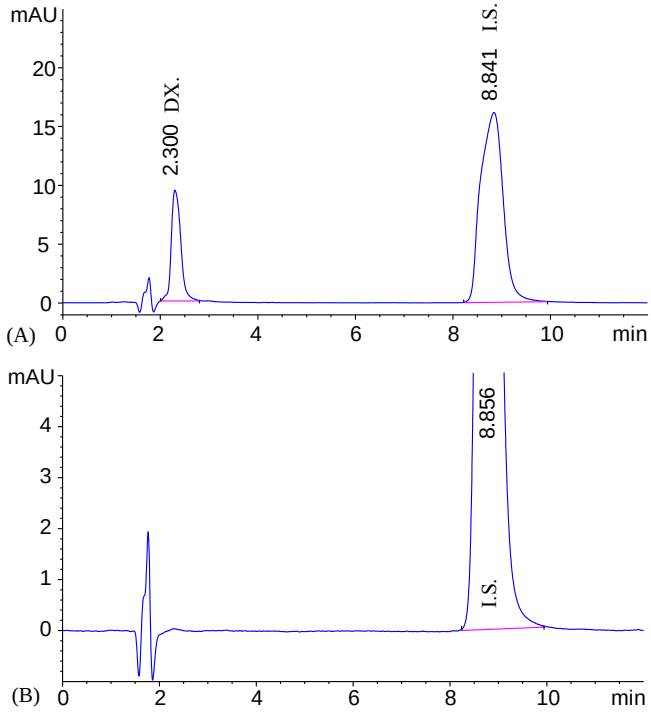

Fig. 3. Chromatogram from doxycycline extracted from DDS formulation (60 μg/ml doxycycline) (A) and chromatogram from analysis of blank DDS formulation with piroxicam (B).

for doxycycline was 2.2 ± 0.09 min and for piroxicam, the molecule selected as internal standard to have similar spectrophotometric properties to doxycycline, 9.9 ± 0.18 min. Both, doxycycline and piroxicam eluted as clear symmetrical peaks (asymmetry factors of 0.92 and 0.86 for doxycycline and piroxicam, respectively) which were well separated with minimal tailing and good separation between adjacent peaks (R_s value between doxycycline and endogenous compounds eluting before, 2.41). Fig. 3A display the chromatogram of doxycycline extracted from 10 mg of particles (22.8 μg doxycycline/mg of DDS), while Fig. 3B correspond to a mixture of the formulation compounds with piroxicam. All obtained chromatograms reflected the high degree of selectivity that shows the chromatographic developed method. No chromatographic interference was found either endogenous serum compounds or formulations components with doxycycline and internal standard.

Table 1
Accuracy of the method, expressed as relative error in percentage, for determining doxycycline concentrations

Concentration added (μg/ml)	Concentration found (mean ± S.D., μg/ml)	Accuracy (%)
DDS		
20	22.63 ± 0.12	13.13
60	59.92 ± 1.90	−0.13
180	168.46 ± 0.75	−6.41
640	575.00 ± 7.43	−10.16
Serum		
2	2.14 ± 0.16	6.86
12	11.93 ± 1.24	−0.57
30	29.26 ± 3.79	−2.46
70	68.19 ± 5.46	−2.59

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, stability, and applicability in pharmacokinetic studies and quality control of the DDS formulation development. The assays exhibited linearity between the response (y) and the corresponding concentration of doxycycline (x), over the 0.4–80 mg/ml range in biological samples and from 1 to 800 mg/ml for doxycycline extracted from DDS samples. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and intercept equal to 0 (Student's t-test). The extraction recoveries of doxycycline and piroxicam in serum were 65.7 ± 1.18, 66.2 ± 2.37%, respectively.

Accuracy values were within acceptable limits (Table 1). The results for intra-assay variability and between-day precision for our samples are presented in Table 2 and the values ranged in biological samples between 7.42 and 12.93,

Table 2
Between- and within-day variability of the HPLC method for determining doxycycline concentrations

Concentration added (μg/ml)	Within-day variability		Between-day variability	
	Concentration found (mean ± S.D., μg/ml)	R.S.D. (%)	Concentration found (mean ± S.D., μg/ml)	R.S.D. (%)
DDS				
20	22.63 ± 0.12	0.53	19.86 ± 2.38	12.01
60	59.92 ± 1.90	3.18	60.83 ± 2.18	3.59
180	168.46 ± 0.75	0.44	185.99 ± 14.38	7.73
640	575.00 ± 7.43	1.29	631.18 ± 43.84	6.95
Serum				
2	2.14 ± 0.16	7.42	2.18 ± 0.11	5.14
12	11.93 ± 1.24	10.43	11.86 ± 1.74	14.64
30	29.26 ± 3.79	12.93	28.11 ± 3.53	12.55
70	68.19 ± 5.46	8.00	69.46 ± 5.27	7.59

Table 3
Application of the method: influence of the doxycycline/polymer ratio and of the polymer type on the drug loading (μg/mg DDS)

Polymer	Doxycycline (mg)	Drug loading (μg/mg carrier)
PL 1	2	0.20
	10	1.27
	20	2.27
	30	3.90
	40	4.55
	65	22.8
PL 2	20	0.40
PL 3	20	1.20

and 5.14 and 14.64%, respectively. These values were lower when we have determined the amount of doxycycline in particulate formulations (intra-assay variability range: 0.44–3.18%; between-day precision range: 3.59–12.01%). Then, the obtained values for the precision were also acceptable.

The LOD of doxycycline was 20 ng/ml (S/N = 3) and the estimated LOQ was calculated as low as 80 ng/ml (S/N = 10). The last value was confirmed for our samples. The mean assay result was 80.32 ng/ml (n = 12), with R.S.D. < 5%. Limits of quantification varying according to method of detection. Studies using UV detection reported a limit of quantification of 25 ng/ml [20,24] while lower quantitation limits (near to 5 ng/ml) was also obtained when fluorescence detection was used [21,22]. However, the minimum quantificated amount of doxycycline injected onto the chromatographic column in our method (0.8 ng) is lower than the injected in the previously developed methods (2.5 ng for UV detection; 1 ng for fluorescence detection).

The applicability of this method has been demonstrated both in vitro and in vivo. The reported method was used for the determination of the drug content in DDS (Table 3). As can be observed in Table 3, depending on the polymer used in the formulation the drug content varied from 0.20 to 22.8 μg of doxycycline/mg of carriers.

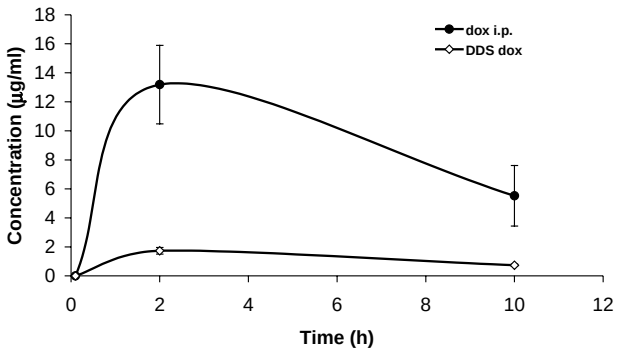

Fig. 4. Average doxycycline concentrations vs. time curve for mice after single intraperitoneal dose of doxycycline (50 μg/g) and after a single intravenous dose of doxycycline DDS (100 μg doxycycline per mouse).

On the other hand, the developed method was also applied for the determination of doxycycline in serum samples extracted from mice, after the administration of a doxycycline solution by the oral route and doxycycline loaded DDS administered intravenously (Fig. 4). The antibiotic dose administered intraperitoneally was 10-fold higher compared to the dose administered intravenously. A progressive slow reduction of the drug concentration value in the blood was found up for 10 h (Fig. 4).

4. Conclusion

A simple chromatographic method has been developed for the rapid and precise determination of doxycycline in both DDS and serum extracted from mice after the drug administration. The simplicity of the technique, the minimal volume requirement, the short analysis time and the high sensitivity makes this technique particularly attractive for this purpose.

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