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Validation of a high-performance liquid chromatographic method for the determination of doxycycline in turkey plasma

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Abstract

A high-performance liquid chromatographic method for the analysis of doxycycline in turkey plasma samples using demeclocycline hydrochloride as the internal standard was developed, optimized and validated. A one-step extraction procedure and an isocratic HPLC method with UV detection were used. No interferences with endogenous compounds or with the anticoagulant were observed. Linear calibration curves (r^2>0.99) were obtained in water and plasma between 0 and 600 μg ml^{-1}. Good recoveries for doxycycline (>66%) and demeclocycline (>72%) were seen both in water and in plasma. The coefficient of variation was <9.86% for within-day reproducibility and <7.53% for the between-day reproducibility. The deviation between the mean value found and the true value was <14.5% (accuracy). The limit of detection was 0.1 μg ml^{-1} in plasma samples. A good stability of doxycycline was observed in water and in plasma samples after storage for six months at −20°C (recovery >91%).

Keywords: Doxycycline

1. Introduction

Several papers dealing with the liquid chromatographic determination of tetracyclines and their degradation products have been published. Liquid chromatography is preferred above microbiological and spectrophotometric methods due to its higher selectivity and/or sensitivity. Most current liquid chromatographic methods for the determination of tetracyclines in biological materials use solid-phase extraction (SPE) for the isolation of the analyte [1–8]. However, a troublesome feature of the SPE of tetracyclines is the poor reproducibility.

For the isolation of tetracyclines from biological materials, other methods were described: dilution of the sample, followed by direct injection [9–12], simple deproteinization [13–15] and liquid–liquid extraction [16]. For lower concentrations (sub-microgram levels) liquid–liquid extraction is needed.

Most papers describe the use of reversed-phase

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systems, based on modified silica as stationary phase. The use of silica-based materials incur two severe problems: the poor chromatographic efficiency due to complexation of metal ions present in the chromatographic system with tetracyclines and an apparently irreversible adsorption of small amounts of tetracyclines, presumably onto residual silanol groups of the column material [17–19]. The former problem can be reduced by adding EDTA to the mobile phase. In order to reduce the adsorption onto the silanol groups some investigators apply "capped" reversed-phase materials by trialkysilylation [19], while others use systems based on ion-pair formation [18,20,21] or the use of ion-exchange materials [22,23]. Other investigators add another member of the tetracycline group to the mobile phase in order to "deactivate" the packing material [12]. Efficiency however remains rather poor. The first reports [12,24,25] on a successful and sensitive HPLC method for doxycycline in body fluids showed a good agreement with the results of the microbiological techniques [26]. However, the peak shapes were poor with marked tailing. Some HPLC methods for the determination of tetracyclines in serum and urine [27–29], require large sample volumes. This is an important drawback in experiments with small animals such as turkeys with a low blood volume.

Since tetracycline antibiotics are often used to treat infectious diseases in poultry [30–32] and doxycycline is preferred because of its advantageous pharmacokinetic properties [33,34], a selective, sensitive and fully validated analytical method is required for pharmacokinetic studies of doxycycline. The present paper describes the validation of an HPLC method for the determination of doxycycline in plasma samples of turkeys. The isolation of doxycycline from the plasma is performed using a liquid-liquid extraction procedure adapted from the one described by De Leenheer and Nelis [16].

2. Experimental

2.1. Chemicals

Doxycycline Hyclas was purchased from Alpha-Pharma (Zwevegem, Belgium). Demeclocycline hydrochloride (Fluka, Buchs, Switzerland) was used as the internal standard. Acetonitrile (Lab-Scan, Dublin, Ireland) was of HPLC grade. Na_2EDTA (Federa, Brussels, Belgium), 70% perchloric acid (UCB, Leuven, Belgium), oxalic acid dihydrate (UCB) and sodium hydroxide (Janssen Chimica, Geel, Belgium) were of analytical grade. Ascorbic acid was purchased from Alpha Pharma. Ethyl acetate (Lab Chemistry, Haneffe, Belgium), *n*-hexane (UCB), methanol (Lab-Scan), sodium sulphite anhydrous (Federa), sodium dihydrogenphosphate dihydrate (UCB) were of analytical grade.

2.2. Methods

2.2.1. HPLC assay

The HPLC system consisted of an isocratic HPLC pump (L-6000 Merck-Hitachi, Darmstadt, Germany), a septumless syringe-loaded injector (Valco six-channel injector, Valco Instruments, Houston, TX, USA) and a loop of 100 μl, a reversed-phase column (5-μm particles LiChrospher RP-18; 125×4 mm, Merck) equipped with a precolumn (5-μm particles LiChrospher RP-18; 4×4 mm, Merck) and a variable-wavelength UV detector (L-4000 Merck-Hitachi). The mobile phase consisted of water–acetonitrile–70% $HClO_4$ (699:298.5:2.5, v/v), Na_2EDTA (0.6 m*M*) and oxalic acid (5 m*M*). The pH was adjusted to 2.5 using 1 *M* NaOH. The mobile phase was degassed before use. The flow-rate was 1 ml min^{-1} and an ambient column temperature was used.

2.2.2. Sample preparation

Stock solutions of doxycycline·HCl (10 mg ml^{-1}) and democlocycline·HCl (0.5 mg ml^{-1}) were prepared in methanol–0.01 *M* HCl. These solutions were stored at −20°C. Working solutions were made by diluting the stock solutions with aqueous 0.001 *M* HCl. All preparations were made in borosilicate glass tubes (Corning Glass Works, Corning, NY, USA). For the preparation of the calibration samples, doxycycline·HCl was used at a concentration of 0, 0.2, 0.5, 1, 2.5, 5, 7.5 and 10 μg ml^{-1} and 0, 25, 50, 75, 100, 150, 200, 250, 300 and 600 μg ml^{-1} in water (reference matrix) and turkey plasma. Concentrations of the internal standard (democlocycline·

HCl) were 5 μg ml^{-1} for doxycycline·HCl concentrations ranging between 0 and 10 μg ml^{-1} and 135 μg ml^{-1} for doxycycline·HCl concentrations ranging between 0 and 300 μg ml^{-1}. A 250-μl volume of blank turkey plasma or water were used. Then 50 μl of the 0.001 *M* HCl solutions containing doxycycline and demeclocycline were added to the tubes. Next 50 μl of an aqueous ascorbic acid solution (6%, w/v) were added. After the addition of 1 ml phosphate–sulphite buffer pH 6 (25.2 g sodium sulphite–36.3 g $NaH_2PO_4 \cdot 2H_2O$, added to 100 ml of bidistilled water) and 6 ml of ethyl acetate, the mixture was vortex-mixed for 2 min. After centrifugation for 10 min at 2600 *g*, 5 ml of the organic phase were transferred to another tube containing 100 μl of a methanolic ascorbic acid solution [0.2% (w/v) in methanol] and evaporated to dryness at room temperature under nitrogen. The samples were redissolved in 300 μl of the mobile phase and 4 ml of *n*-hexane were added. Next, the tubes were vortex-mixed for 1 min and centrifuged for 5 min at 2600 *g*. After the removal of the hexane layer by aspiration, 100 μl of the aqueous phase were injected onto the chromatographic system.

2.2.3. Stability of doxycycline in water and plasma

Known amounts of doxycycline·HCl were added to distilled water and blank plasma in order to obtain concentrations of 0.5, 5, 50 and 250 μg ml^{-1}. These samples were stored at room temperature and −20°C, and analyzed after two and six months. The stability of the samples was expressed as the residual concentration related to the freshly prepared samples.

2.2.4. Calculations

The calibration curves were calculated by linear regression of the peak-area ratio versus the concentration.

3. Results and discussion

Doxycycline, one of the newer tetracyclines is a lipophilic molecule with acidic, enol and phenol groups, a strongly basic tertiary amine and a dipolar amide function (Fig. 1). Its determination in plasma samples using an HPLC method requires a previous extraction. The extraction procedure described by De Leenheer and Nelis [16] was modified. The volume of the plasma sample was reduced to 250 μl and the residue was redissolved in 300 μl of mobile phase. A final purification step was introduced by the addition of *n*-hexane to the residue. This purification step is probably required to extract residual plasma lipids. A minimal of 4 ml of *n*-hexane was needed in order to obtain a clear solution. However, the recovery of doxycycline from plasma samples decreased from 85% using 1 ml of *n*-hexane to 65% using 4 ml of the solvent. As a good reproducibility was found (coefficient of variation, C.V. <10%), the use of 4 ml of *n*-hexane was adopted in order to obtain a longer column life and cleaner chromatograms (Fig. 2). If less than 4 ml of *n*-hexane was used, the retention times decreased very quickly and the peaks tended to collapse (Fig. 3). Democlocycline·HCl was preferred to other tetracyclines as the internal standard since it showed a similar chromatographic behaviour as doxycycline and at the same time it was clearly separated from doxycycline and its degradation products.

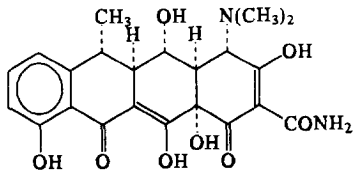

Fig. 1. Structure of doxycycline.

The chromatographic conditions were based on those used by Nieder and Jaeger [35]. The use of sodium dihydrogen phosphate reported by these authors resulted in precipitation problems. It was replaced by 5 m*M* oxalic acid which was seen to be essential for a good peak shape. The addition of tetrahydrofuran to the mobile phase, reducing the assymetry factor in the analysis of oxytetracycline in *Artemia* [36], was tried without success in this study. The mobile phase was adjusted to pH 2.5 with 1 *M* NaOH. At this pH, less tailing and better resolution factors were obtained. A pressure of 95 to 105 bar was recorded using these HPLC conditions. Two absorption maxima were found for doxycycline (260 and 350 nm) and demeclocycline (267 and 360 nm). At 260 nm interference was observed with endogenous substances of the blank plasma. Hence the wavelength of 350 nm was chosen.

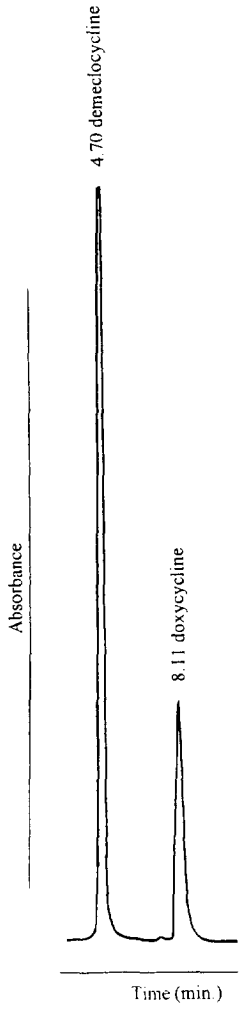

Fig. 2. Chromatograms of turkey plasma containing 25 μg ml^{-1} doxycycline·HCl and 135 μg ml^{-1} demeclocycline·HCl. Extraction was performed using 4 ml of *n*-hexane.

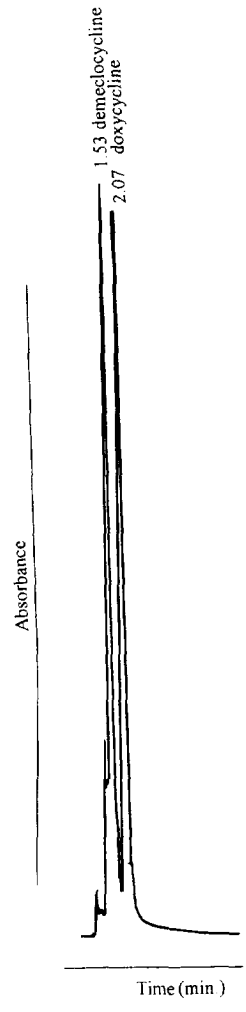

Fig. 3. Chromatograms of turkey plasma containing 25 μg ml^{-1} doxycycline·HCl and 135 μg ml^{-1} demeclocycline·HCl. Extraction was performed using 1 ml of *n*-hexane.

3.1. Selectivity

Fig. 4 shows typical chromatograms of blank turkey plasma after extraction (Fig. 4A), plasma spiked with doxycycline (10 μg ml^{-1}) and demeclocycline (5 μg ml^{-1}) after extraction (Fig. 4B), and plasma obtained after intravenous administration of 25 mg doxycycline·HCl per kg body weight in turkeys (Fig. 4C). No interference of endogenous compounds or the anticoagulant (10% (v/v), citrate solution 4% (w/v)) was detected. Interference of doxycycline with the internal standard or the degradation and by-products (metacycline, 6-epidoxycycline and 4-epidoxycycline) was not observed. The retention times of doxycycline and demeclocycline were 7.15±0.17 (7.11–8.17) and 4.28±0.14 (4.21–4.73) min, respectively.

The purity of the demeclocycline and doxycycline peak was verified using a diode-array detector (Kontron diode-array detector 440, pump system 32 X, data system DS 450-MT2/DAD series). Samples obtained at different time intervals from turkeys intravenously given doxycycline·HCl at a dose of 25 mg kg^{-1} body weight and samples of the calibration

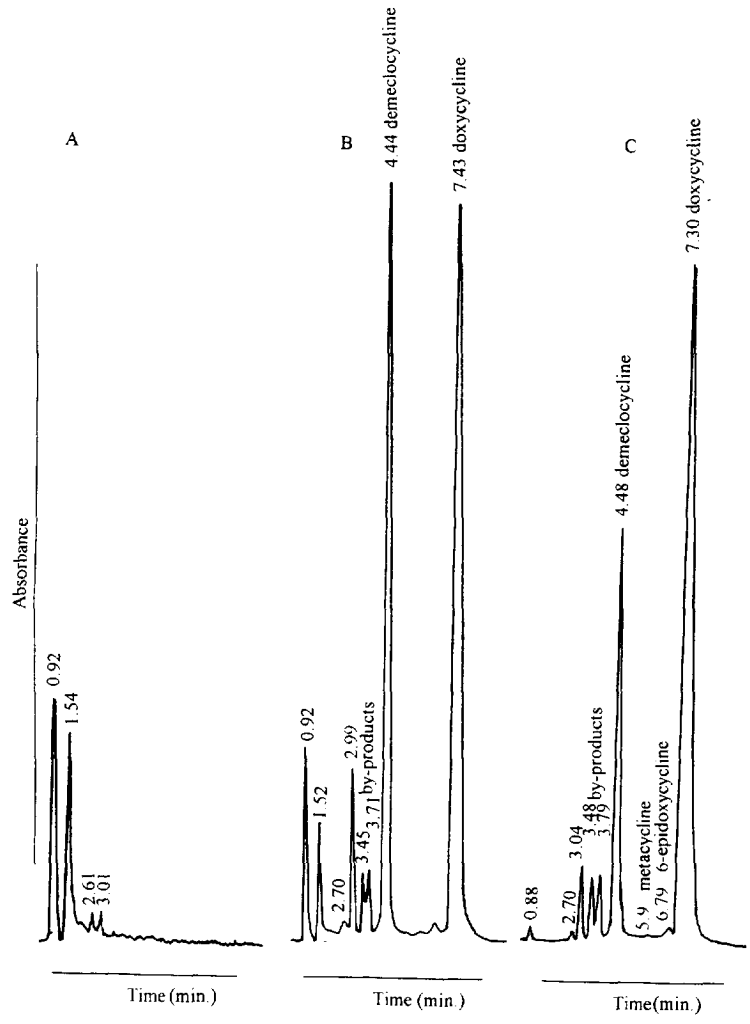

Fig. 4. (A) Chromatogram of turkey blank plasma after extraction. (B) Chromatogram after extraction of plasma spiked with doxycycline·HCl (10 μg ml^{-1}) and demeclocycline·HCl (5 μg ml^{-1}). (C) Chromatogram of extracted plasma after intravenous administration of 25 mg doxycycline·HCl per kg body weight.

curve were used. In both cases, doxycyline and demeclocycline peaks were checked for identity by comparison of the UV spectra (250–400 nm) and for purity by comparison of the chromatograms at two different wavelengths. Doxycyline and demeclocycline peaks revealed to be pure in the chromatograms of all samples. The UV scan between 250 and 400 nm of the doxycycline samples revealed the same characteristics when the drug was added to the blank plasma as when doxycycline was administered intravenously to turkeys. It can be concluded that the proposed method is selective for doxycycline.

3.2. Stability of doxycycline in the plasma and water samples

It is well known that drugs may be degraded in plasma during storage or analysis due to temperature, light, air and enzymes [37]. Doxycycline and demeclocycline were stable (95–98%) during extraction and analysis, both performed at room temperature. Plasma and water samples were stored over a 6-month period at room temperature and at −20°C. Doxycycline·HCl concentrations of 0.5, 5, 50, and 250 μg ml^{-1} were used. Results of the study indi-

cated the good stability of doxycycline in plasma samples when stored over a six-month period at −20°C but not at room temperature. After a period of two months, the measured concentrations in the turkey plasma samples ranged from 80–126% and 63–64% for samples kept at −20°C and room temperature, respectively. For water samples the concentration range was within 93–105% for samples kept at −20°C, and 60–76% for room temperature samples. The measured concentrations after six months storage of these samples ranged from 91 to 113% and 3 to 30% for plasma samples kept at −20°C and at room temperature, respectively, and 102 to 105% and 18 to 48% for water samples under the same conditions.

3.3. Reference standard

The doxycycline·HCl content of the commercially available (Alpha Pharma) product was determined by comparison to the reference standard of the European Pharmacopeia. The doxycycline·HCl content of the commercial available hyclate was 92.30±1.97%.

3.4. Calibration and linearity

Water was used as a reference matrix next to blank turkey plasma. To prepare the calibration curve samples, doxycycline and demeclocycline were added from a dilution of the methanolic stock solutions in aqueous 0.001 *M* HCl. Blank samples were included in the calibration curves to assure no interfering components were co-eluted. In Table 1 the slope and correlation coefficients are shown for calibration curves in water and in turkey plasma, respectively. The calibration curves were linear over the entire concentration range (0.2–600 $\mu g\ ml^{-1}$).

3.5. Precision

Within-day and between-day reproducibilities were calculated for doxycycline·HCl added to the two matrices at different concentrations ($n=3$). C.V. values for the within-day test were within a 0.13–9.86% and a 0.06–7.53% range in water and plasma samples, respectively. For the between-day reproducibility variations of 0.13–10.30% and 0.24–7.08%, in water and plasma, respectively, were obtained.

3.6. Accuracy

For the determination of the accuracy, the closeness of agreement between the value accepted as the conventional true value and the mean value obtained by applying the test procedure six times was calculated [38]. The concentrations used were 0.2, 0.5, 1, 5, 10, 50, 100 and 250 $\mu g\ ml^{-1}$ doxycycline·HCl in plasma and water samples. The C.V. for these measured concentrations were 14.5, 8.3, 0.3, 1.3, 4.5, 0.68, 0.71 and 6.39% for the plasma samples and 9.0, 2.6, 4.34, 2.0, 0.33, 1.64, 1.78 and 3.28% for the water samples. The acceptance criterium for the accuracy is set at a C.V. value not greater than 15% [39].

Table 1
Slopes and correlation coefficients for the calibration curves ($n=5$; mean±S.D.) in water and plasma

Concentration ($\mu g\ ml^{-1}$)	Water		Turkey plasma	
	Slope	r^2	Slope	r^2
0–10	4.07±0.36	$0.9994\pm6.3\cdot10^{-4}$	4.41±0.18	$0.9983\pm1.8\cdot10^{-3}$
0–50	31.29±2.73	$0.9994\pm2.4\cdot10^{-4}$	27.93±1.83	$0.9994\pm1.8\cdot10^{-3}$
0–300	103.53±1.37	$0.9998\pm9.9\cdot10^{-5}$	109.74±6.56	$0.9994\pm9.8\cdot10^{-4}$
0–600	–	–	96.15±2.70	$0.9994\pm8.7\cdot10^{-4}$

Calibration curves were made for doxycycline·HCl concentrations between 0–10, 0–50, 0–300 and 0–600 $\mu g\ ml^{-1}$ in both matrices. Concentrations of the internal standard (demeclocycline·HCl) were 5 $\mu g\ ml^{-1}$ for doxycycline·HCl concentrations ranging between 0 and 10 $\mu g\ ml^{-1}$, 40 $\mu g\ ml^{-1}$ for doxycycline·HCl concentrations ranging between 0 and 50 $\mu g\ ml^{-1}$ and 135 $\mu g\ ml^{-1}$ for doxycycline·HCl concentrations ranging between 0 and 300 and 0 and 600 $\mu g\ ml^{-1}$.

3.7. Analytical recovery

Sample preparation procedures often cause a loss of drug substance. Recovery is defined as the concentration of drug found in spiked control samples expressed as the percentage of the known or true drug concentration [40].

The experiments were performed at different concentrations of doxycycline·HCl and at two different concentrations of demeclocycline (corresponding to the concentrations used in the two different calibrations curves) in plasma and water. The analytical recovery data are presented in Table 2 for doxycycline and Table 3 for demeclocycline. The recovery of doxycycline was higher than 66% from plasma, and higher than 73% from water. Demeclocycline recovery from plasma was higher than 79% and higher than 72% from water. These values for the recovery of doxycycline from turkey plasma were somewhat lower than those reported with other extractions in other HPLC methods for human plasma samples: 87.8% [15] and 100% (SPE) [37]. The relatively low recovery was due to the increased volume of *n*-hexane added to the residue after the final extraction in order to eliminate lipids, since a recovery of 85% was obtained when 1 ml of *n*-hexane was used. The addition of phenylbutazone reported by Sharma and Bevill [41] as a contributing factor for the improvement of recovery was not successful.

Table 2
Recovery of doxycycline from water and plasma matrices (mean±S.D.; $n=3$)

Concentration ($\mu g\ ml^{-1}$)	Recovery (%)
Water	
0.2	74.3±7.4
0.5	77.0±3.5
1	79.8±2.9
5	83.1±4.0
10	73.2±6.7
25	77.0±6.8
50	84.9±2.8
100	85.6±0.7
250	85.2±1.0
Plasma	
0.2	66.1±8.2
0.5	69.2±3.5
1	69.4±2.3
5	72.3±8.0
10	69.0±4.4
25	66.4±7.7
50	69.1±1.5
100	68.3±1.7
250	68.0±1.9

Table 3
Recovery of demeclocycline from water and plasma matrices (mean±S.D.; $n=3$)

Matrix	Concentration ($\mu g\ ml^{-1}$)	Recovery (%)
Water	5	79.45±0.04
	135	74.73±0.02
Plasma	5	71.55±0.11
	135	82.73±0.01

3.8. Limit of detection and limit of quantification

The limit of detection is the lowest concentration of an analyte that the analytical process can reliably differentiate from background levels [39]. The quantification limit is the lowest amount of an analyte which can be quantitatively determined with defined precision and accuracy under the given experimental conditions. Mathematically, the detection limit is defined as mean analyte blank signal plus three times the standard deviation [38], and the quantification limit as mean analyte signal plus ten times the standard deviation. Both values are calculated as concentrations (CPMP working party EEC; 1989). The concentration used as the mean blank signal was the concentration having a coefficient of variation still less than 20% after ten injections of the same concentration (0.1 $\mu g\ ml^{-1}$ in water and in plasma). The values obtained were 0.1 and 0.2 $\mu g\ ml^{-1}$, respectively for detection and quantification limit, both in water and plasma samples.

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