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Simple and reliable method of doxycycline determination in human plasma and biological tissues

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Abstract

Over recent years there has been a resurgence in the use of doxycycline in clinical practice, which does not depend on its antibacterial properties. This paper describes a method of determination of doxycycline in human plasma and atheromatous tissue using high-performance liquid chromatography (HPLC), and a cheap commercially available extraction system. Doxycycline is extracted in the mobile phase and injected directly into the HPLC system, avoiding time consuming drying up steps. A limit of detection of 0.125 μg/ml of plasma, and a relative standard deviation of 3% was achieved, making the method very reliable and useful for assays within the usual therapeutic range. The method has also been applied to the extraction of a mixture of tetracyclines from plasma and atherma with equal efficacy, making it useful for assays of this class of drugs in veterinary practice and assays of food contaminants.

Keywords: Doxycycline

1. Introduction

Doxycycline is a broad spectrum antibiotic, with activity against a wide range of gram positive and gram negative organisms. It is the drug of choice in the treatment of Lyme disease, Brucellosis and several Rickettsial infections, and it is also utilised in the treatment of sexually transmitted diseases [1,2]. In recent years there has been increasing interest in its potential therapeutic role as an inhibitor of a group of enzymes called matrix metalloproteinases (MMPs), which are involved in a number of pathological processes such as periodontitis, degenerative rheumatic diseases and degenerative vascular disorders. A number of trials are currently underway to evaluate its role in these situations [3–5].

Several methods have been used to determine doxycycline concentrations in biological tissues. These include microbiological [6,7] and fluorimetric techniques [8], gas–liquid chromatography [9], thin-layer chromatography [10], and high-performance liquid chromatography (HPLC) techniques [11–13]. Spectrophotometric [14] and combined procedures for example electrospray HPLC–tandem mass spectrometry [15] have also been described.

Microbiological methods are still used quite extensively, but they are non specific and time consuming, relying on inhibition of a microorganism (usually *Bacillus cereus* or *Bacillus subtilis*) in a special culture medium as a means of detection. The inhibition of growth can occur with any antibiotic in the

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test solution, and so the specificity of the method is very low. Fluorimetric and spectrophotometric techniques are sensitive, but interference from other material cannot always be excluded. In view of this, several methods of chromatographic determination of tetracycline levels in biological tissues have been described. HPLC has a very high sensitivity, specificity and speed of assay determinations. This paper describes a simple, quick and sensitive assay for determination of doxycycline concentrations in biological tissues, requiring only basic HPLC apparatus which is available in most assay laboratories, and making use of a commercially available extraction pack which considerably reduces the variability if extraction associated with traditional extraction methods.

2. Experimental

2.1. Apparatus

An Iso-Chrom liquid chromatography constant flow pump (Spectra Physics, Oxford, UK) was used and was set at a flow-rate of 1.25 ml/min. An injection valve with a 200-μl loop was fitted between the pump and the column. A Spectra 100 variable-wavelength detector (Spectra Physics) set at 350 nm was used for sample detection. A Hypersil ODS C_{18} column (Jones Chromatography, Mid Glamorgan, UK) of 100 mm×4.6 mm I.D. was used. A Hypersil ODS guard column (Jones Chromatography) of 30 mm×4.6 mm I.D. was fitted in series to protect the separation column. A Shimadzu C-R1B integrator (Shimadzu, MD, USA), was used to record the data. All solutions were analysed at room temperature.

2.2. Reagents

All chemicals were of analytical grade. Doxycycline, demeclocycline, oxytetracycline and methacycline were purchased from Sigma (Poole, UK). Acetonitrile, trifluoroacetic acid, methanol and 85% phosphoric acid were all HPLC grade and were also purchased from Sigma. Double distilled water from an Elgastat water purifier was used in the preparation of the stock solutions.

2.3. Stock solutions

Solutions of doxycycline, demeclocycline, oxytetracycline and methacycline were prepared by dissolving in double distilled water and made up to a concentration of 1 mg/ml and stored at 4°C away from light. The solutions were stable under these conditions for 5 days.

2.4. Mobile phase

This consisted of a solution of 50% (v/v) acetonitrile in water, and 0.15% trifluoroacetic acid. The solution was degassed with helium for 10 min before use to prevent interference from gas bubbles in the HPLC system.

2.5. Calibration

The system was calibrated by preparing serial dilutions of stock solutions from 0.25 to 5.00 μg/ml. A 200-μl volume of the stock solutions was then injected into the chromatography column and the sample detected at 350 nm. A calibration curve was constructed by plotting the peak column height in mV against the relevant doxycycline concentration. An external calibration curve for assays in human serum was also constructed by spiking 1-ml samples of human serum with increasing concentrations of both drugs from 0.25 to 5.0 μg/ml. These were extracted using the method described below, and the eluate assayed under the experimental conditions described.

2.6. Collection of plasma samples

2.0-ml blood samples from patients taking doxycycline at a dose of 200 mg daily were collected in EDTA bottles. These were centrifuged at 3000 *g* for 5 min and the plasma collected and stored at −85°C until the assays were carried out. Prior to being assayed, the samples were spiked with 2.0 μg/ml of internal standard (oxytetracycline) and 1% phosphoric acid. The latter disrupts the drug protein interactions and considerably improves the extraction. The plasma was then vortex-mixed for 2 min before extraction. Prior studies had shown that the drug was stable under these conditions, and could be stored at

−85°C for up to 4 months with no deterioration in the assays (data not shown).

2.7. Tissue samples

Carotid endarerectomy was performed in a series of patients taking doxycycline (200 mg per day). The carotid plaques were collected at operation and snap frozen in liquid nitrogen. The samples were stored at −80°C until the assays were carried out. Prior to extraction the plaques were weighed and homogenised for 10 min in homogenising solution using a mechanical homogeniser set at 24 000 rpm. During this process the samples were kept cold by surrounding the homogenising tubes with ice to prevent any effect of heat degradation on the doxycycline.The homogenised plaques were then spun at 11 000 rpm for 1 h and the supernatant used for doxycycline determination. The supernatant was then spiked with internal standard and the drugs was then extracted from the solution using the method described below.

2.8. Extraction

A commercially available extraction cartridge (Oasis HLB Extraction cartridge, 1 ml/30 mg, Waters Associates, UK), was utilised. This consists of a solid-phase extraction medium which retains both polar and non-polar compounds, which are then eluted from the extraction cartridge. After conditioning the extraction cartridge with 1 ml of methanol and 1 ml of water, the drugs were extracted into the cartridge by injecting 1 ml of serum. This was followed by three consecutive washes using 1 ml of 5% methanol in water in order to wash off unbound substances in the cartridge and reduce any interfering bands in the chromatograms. The drug was finally eluted off the extraction cartridge using 1 ml of 50% acetonitrile in water (the mobile phase) and a 200-μl sample was injected into the HPLC system at 1.25 ml/min and detected at 350 nm. During extraction, flow through the extraction cartridges was kept at 1.0 ml/min.

Sample-to-sample variability was determined by repeated assays on five samples from the same batch on the same day. Variability at the limit of quantification was determined in a similar manner using plasma spiked with 0.125 mg/ml of oxytetracycline and doxycycline. Day-to-day variability was estimated on the basis of five assays on the same batch of plasma on different days.

3. Results and discussion

3.1. Chromatographic separation

Chromatographic separation of the doxycycline and the internal standard was completed within 4 min. The retention time for doxycycline was 2.5±0.12 min and for the internal standard 1.7 min. The slight variation in the retention time of doxycycline was due to the fact that the integrator identified peaks at the point of reversal of the direction of current. Hence the slightly rounded peaks of the doxycycline curves produce some variation while the perfectly sharp peaks of oxytetracycline produced no variation at all. A chromatogram showing separation of doxycycline and oxytetracycline in stock solutions is shown in Fig. 1. The different substances eluted as clear symmetrical peaks which were well separated with minimal tailing and good separation between adjacent peaks.

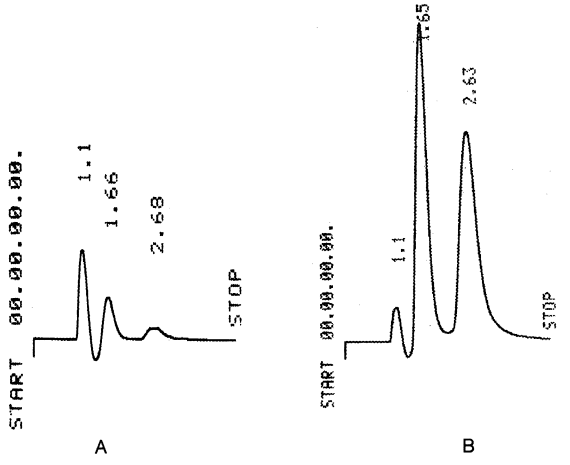

Fig. 1. Chromatogram showing separation of oxytetracycline (1.6 min) and doxycycline (2.6 min) at the limit of detection (A) and at 2.0 μg/ml (B).

3.2. Calibration

Separate calibration curves were constructed using dilutions of the stock solutions in distilled water and the mobile phase, as well as an external calibration curve using human plasma spiked with different concentrations of oxytetracycline and doxycycline. These showed a linear correlation between the peak column height (mV), and the plasma concentration of doxycycline and internal standard, with a correlation coefficient (r^2) greater than of 0.998 for all curves. The curve from the stock solutions in distilled water had a slope of 7355±42.32 SD for doxycycline and 16 410±236.3 SD for oxytetracycline, compared with a slope of 7319±172.7 SD and 12 870±147.2 SD for the solutions in the mobile phase, confirming the greater sensitivity of extraction in the mobile phase as compared to water. The lower limit of quantification for both drugs was 125 ng/ml from plasma samples (Fig. 1B).

3.3. Plasma samples and tissue homogenates

Fig. 2A is a chromatogram of extracted plasma from a patient on a standard dose of doxycycline of 200 mg/day, and Fig. 2B is a similar chromatogram from a blank sample of serum. The extraction ratio for the sample in Fig. 1A was 0.78, and corresponds to a plasma concentration of 3.8 μg/ml 2 h after the last dose. The mean serum concentrations of doxycycline varied from 2.2 to 5.2 μg/ml (mean 3.7 μg/ml) 3 h after ingestion and fell to 1.2 to 2.6

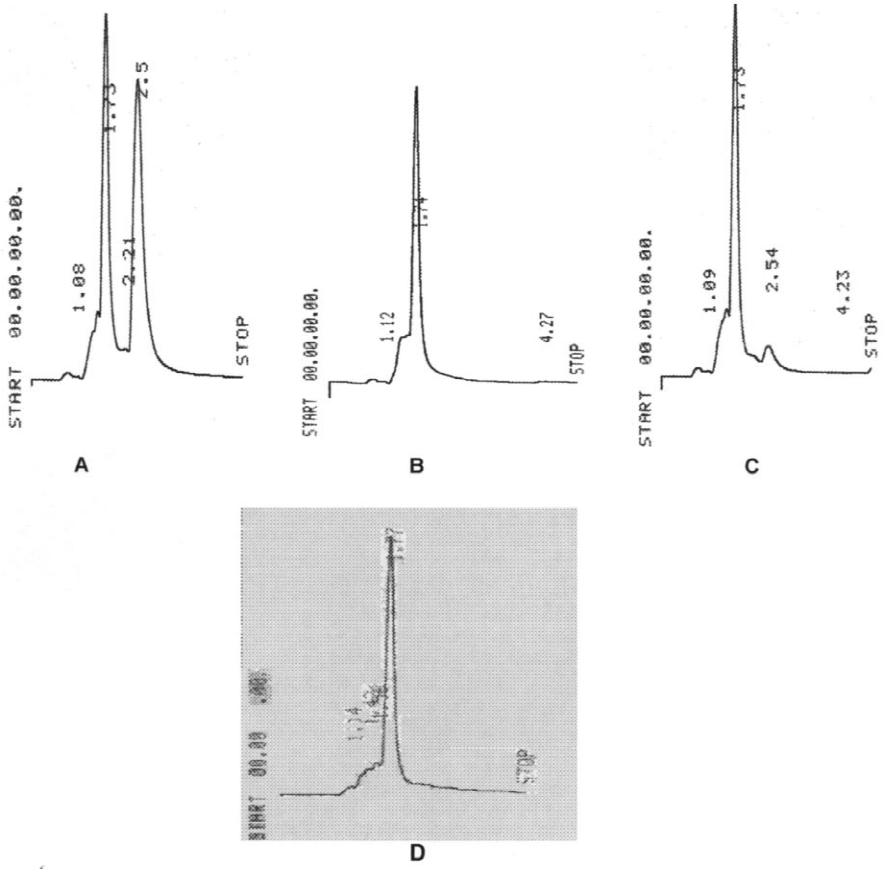

Fig. 2. Chromatogram from a patient on 100 mg of doxycycline daily (A), chromatogram of a blank sample of serum (B), chromatogram from tissue homogenate (C). Oxytetracycline peak at 1.7 s, doxycyline peak at 2.5 s. Chromatogram from plaque homogenate from a patient on placebo (D).

Table 1
Data showing relative standard deviations (RSDs) based on five replicate analyses from each batch[a]

						RSD (%)
Intra-sample variability	28.3	27.8	27.0	26.8	27.6	2.2
Day-to-day variability	31.6	30.6	28.3	31.3	29.6	4.4
Variability at limit of quantification	14.8	15.3	16.9	16.6	11.7	13.7

[a] Units shown are peak column height of internal standard in volts.

μg/ml (mean 1.9 μg/ml) 12 h after the last dose. The sensitivity of this method is therefore well within the limits required for estimation of plasma doxycycline.

A chromatogram from a sample of carotid plaque homogenate from a patient on a standard dose of 200 mg of doxycycline daily is shown in Fig. 2C. The effect of the homogenising process on the stability of doxycycline was tested using stock solutions which were submitted to all the homogenising and quantification process and no significant effect was noted on the drug. Mean concentrations achieved in atherosclerotic tissue were found to be very low and varied between 0.195 and 0.389 μg per ml. (mean=0.292 μg/ml), corresponding to a tissue concentration of 1.9 to 3.9 μg/g of tissue (wet mass).

Plasma levels of doxycycline as determined using this assay correspond well with similar levels found in the literature [1,2]. No work has been carried using HPLC to quantify levels of doxycycline in atherosclerotic tissue, but the levels we found correlate with similar levels obtained by biological assays performed by another group using atherosclerotic tissue in human aorta [16].

3.4. Variability and limit of quantification

Intra-assay variability was 2.23%. This was estimated on the basis of five different samples from the same batch of fetal calf serum spiked with a known concentration of doxycycline (Table 1). Day-to-day variation of assays on the same batch was 4.43% (Table 1). The limit of quantification was defined as the lowest concentration producing a reproducible positive deflection with a relative standard deviation (RSD) less than 15% between estimated levels. Variability at the limit of quantification was 13.23% (Table 1), and was also estimated on the basis of five replicate analyses on the same batch. The limit of detection was 50 ng/ml, producing a positive deflection of around 1000 mV on the chromatogram, however at these levels the RSD was above 15% and precluded its use for reliable quantification. Extraction ratios from human plasma samples varied between 73 and 93%, with a mean of 82.7% and an RSD of 7.69% (Table 2). Acidifying the sample prior to extraction considerably improved the extraction ratio and reproducibility of the system, presumably because all of the drug was extracted from protein, and all data supplied here is reported was determined using acidified samples as described previously.

The limits of quantification in the literature vary with the method used for detection. Using UV detection, Mulders and Van de Lagemaat [17] reported a limit of quantification of 0.5–1 μg/g of tissue, while Prevosto et al. [18] were able to quantify down to 25 ng/ml of plasma. Studies using fluoroscopic detection report lower limits of quantification varying between 5 and 50 ng/ml [19,20]. Limits of detection in the literature again vary according to method of detection, varying from 50 ng/ml using UV detection to 1.38 ng/g of doxy-

Table 2
Extraction ratios from eight consecutive plasma samples and carotid plaque homogenates from patients on doxycycline (100 mg/day)

	Extraction ratios								RSD (%)
Plasma	0.73	0.85	0.87	0.78	0.93	0.8	0.79	0.85	7.6
Tissue homogenate	0.64	0.49	0.72	0.48	0.53	0.71	0.58	0.67	15.9

cycline in porcine muscle using fluoresence detection [19,21].

The problems associated with tetracycline assays have been reviewed by Ashworth [22]. Several extraction methods have been described, but most involve complex extraction steps [23–25]. The main problem with these procedures is that most are time consuming but most importantly result in a great variability in the extraction ratio between different samples and even in the same sample. In view of this we tested a commercially available extraction pack (Oasis HLB Extraction cartridge, 1 ml/30 mg). This consists of a solid-phase extraction medium which retains both polar and non-polar compounds, which are then eluted from the extraction pack. Using the method suggested by the manufacturer extraction ratios of 30±5% were obtained. This is because tetracyclines are very polar compounds and attempted extraction with methanol as described resulted in a very poor yield. However different extraction solvents were tested at different concentrations and pH values. These included butanol, acetonitrile and ethyl acetate. Mean extraction ratios above 80% were obtained using 50% acetonitrile in water (the mobile phase), which was therefore used as described previously. The eluent obtained after the washing up steps with 5% methanol was chromatographed several times at the setting up stage and it was found that no doxycycline was removed by the washing solution (data not shown).

When using one of the above methods, a drying up step is usually involved before a solution of the extracted substance is then made up in the mobile phase and analysed in the HPLC apparatus. By using the mobile phase as the extracting solution, we have been able to avoid a drying up step, thereby simplifying and speeding up the assay procedure. The sensitivity of the system is adequate enough to enable this to be carried out without the need for concentrating the drug in the eluent solution.

Doxycycline is very highly protein bound (82–93%), and both hydrophobic as well as charge transfer mechanisms are involved at physiological pH [26]. Addition of phosphoric acid to a sample disrupts this drug protein interaction and greatly improves the extraction yield. Other authors have advocated the use of phenylbutazone during the extraction in order to displace the drug from protein and improve extraction yield [24]. Ascorbic acid has also been used to suppress epimerization of tetracyclines and improve the extraction of the internal standard [14], however this was not necessary under our conditions, as long as the samples are injected on the same day as extracted. If the samples are kept at 4°C and protected from light the rate of epimerization is significantly reduced, and they can be injected the next day; provided they are quantitated and compared with a standard prepared at the same time and kept under the same conditions [27].

Tetracyclines are very polar substances, and their dissociation is very highly influenced by pH changes. Hence varying the pH during different phases of extraction and elution has a great influence on the sensitivity of the assays, as discussed fully by De Leener [28]. In the set up experiments on our system we found that reducing the pH of the mobile phase to 2.0 greatly affected the sensitivity of the system, and also resulted in symmetrical and well defined peaks. When calibrating the system, 1% phosphoric acid was added to the stock solutions, (as in the case of the plasma), as the presence of phosphoric acid in the sample resulted in up to a twofold increase in peak height (sensitivity) per unit concentration.

Tetracyclines are potent chelators of divalent cations like calcium and magnesium, which affect the way the drug elutes. Some authors have suggested the addition of EDTA to the mobile phase in order to improve resolution, while others pretreat the column with a chelating agent prior to elution [14,29]. This problem was avoided in our case as human sera were collected in EDTA bottles which chelate any divalent cations and prevent interaction with the drug.

The interposition of a 30 mm guard column between the injection valve and the separating column had the beneficial effect of further sharpening the peaks and improving separation as well as its main function of protecting the main column from excessive debris. This is because in effect the guard column adds a further 3 cm to the separating system. We tried to use other tetracyclines including demeclocycline and methacycline as internal standards, but the clearest separation between the peaks was obtained using oxytetracycline as this elutes well before doxycycline giving clear separation between bands.

The method we have described is very simple, rapid, and has good reproducibility, and we have used it to assay doxycycline levels in patients on standard therapeutic doses. A blood sample can be assayed in less than 20 min and the sensitivity of the system is well within the range of values in the therapeutic range.

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