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Note

Improved high-performance liquid chromatographic determination of doxycycline in serum and urine using solid-phase extraction columns

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Doxycycline (Fig. 1), 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrate, is a tetracycline antibiotic with a wide spectrum of activity. Several methods have been described for the analysis of doxycycline in serum and urine which require either (a) liquid extraction [1], (b) protein precipitation followed by liquid extraction [2] or (c) ion-pair formation between the ionizable groups of doxycycline and phenylbutazone followed by liquid extraction [3,4]. These methods involve several involved manipulations which make them less suitable for automation. Another report makes use of solid-phase C_{18} cartridges for the sample preparation in the analysis of tetracycline residues in animal liver [5]. We now report a high-performance liquid chromatographic (HPLC) method for the analysis of doxycycline in serum and urine using disposable solid-phase extraction columns which represents a significant improvement over previous techniques in speed of sample preparation and analysis time, whilst retaining good recoveries and precision. Use of these columns also offers semi-automated sample preparation which affords protection of the doxycycline from light. The recovery of doxycycline from the solid-phase extraction columns is such that it obviates the requirement for an internal standard.

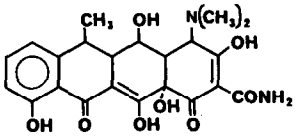

Fig. 1. Structure of doxycycline.

EXPERIMENTAL

Materials

Doxycycline was purchased as doxycycline hyclate. All solvents were HPLC grade from BDH (Poole, U.K.). All water used in this method was glass-distilled. Bond-Elut C_{18} (Cat. No. 607303) cartridges were purchased from Jones Chromatography (Mid Glamorgan, U.K.). McIlvaines buffer was prepared by mixing 0.1 *M* citric acid and 0.2 *M* disodium hydrogen phosphate in the ratio 61.4:38.6 [6].

Apparatus

HPLC separations were performed using a Beckman 114M pump with a flow-rate of 1.0 ml min^{-1}. A Kontron (Watford, U.K.) Model 660 MSI autosampler with 100 μl injection size was used for sample processing. Chromatography was performed at ambient temperature using a Waters Nova-Pak C_{18} 4-μm (15 cm × 3.9 mm I.D.) reversed-phase HPLC column and a LiChrosorb RP-18 guard column (BDH). A Waters μBondapak C_{18} guard column could not be used for this analysis as doxycycline strongly binds to this packing material. The eluent was monitored for UV absorption using a Kratos Spectroflow 773 detector (Applied Biosystems, Warrington, U.K.) operating at 340 nm and consisted of acetonitrile–acetic acid–0.1 *M* potassium dihydrogenphosphate in the ratios 75:150:125 and 65:150:125 for serum and urine, respectively. Amber glassware was used throughout this study as doxycycline is sensitive to photodegradation.

Standard solutions

A stock solution of doxycycline hyclate was prepared weekly in 0.1 *M* phosphoric acid (1 mg ml^{-1}) and stored at 4°C. Dilutions of this stock solution were prepared daily to contain 1000 ng ml^{-1} in 0.1 *M* phosphoric acid.

Sample preparation procedure

Serum. Sample (1 ml) was mixed thoroughly with 5 ml of 0.1 *M* disodium EDTA–McIlvaine buffer (pH 4.0) and applied to a Bond-Elut C_{18} cartridge activated with one volume of methanol and two volumes of water. The cartridge was further washed with 10 ml of water and the doxycycline was eluted and collected from the cartridge with 10 ml of 0.01 *M* phosphoric acid in acetonitrile. The sample was evaporated to dryness in a heating block at 50°C under a gentle stream of nitrogen and the dried residue resuspended in 1 ml water. The sample was then centrifuged for 1 min at approximately 10 000 *g* to remove small particles presumed to be silica from the solid-phase extraction cartridges and which must be removed to prevent blocking of the analytical column filter. The sample was then injected onto the HPLC system for analysis.

Urine. The procedure for urine was exactly the same as for serum except that the cartridge was washed with 10 ml of acetonitrile prior to elution and collection of the sample with 10 ml of 0.01 *M* phosphoric acid in acetonitrile as before.

Recovery

Recovery of doxycycline was determined by comparison of the peak height of the standard solution to that of blank human serum or urine samples to which doxycycline was added over a range of concentrations.

RESULTS AND DISCUSSION

This method was developed to support a study of doxycycline in humans and at least 5 ml of serum or urine was available for assay.

Fig. 2 shows typical chromatograms for (a) standard doxycycline, 500 ng ml^{-1}, (b) an extracted sample of blank human serum and (c) an extracted sample of serum from the same subject following doxycycline administration. Fig. 3 shows typical chromatograms for (a) standard doxycycline, 1000 ng ml^{-1}, (b) an extracted sample of blank human urine and (c) an extracted sample of urine from the same subject following doxycycline administration. The organic modifier

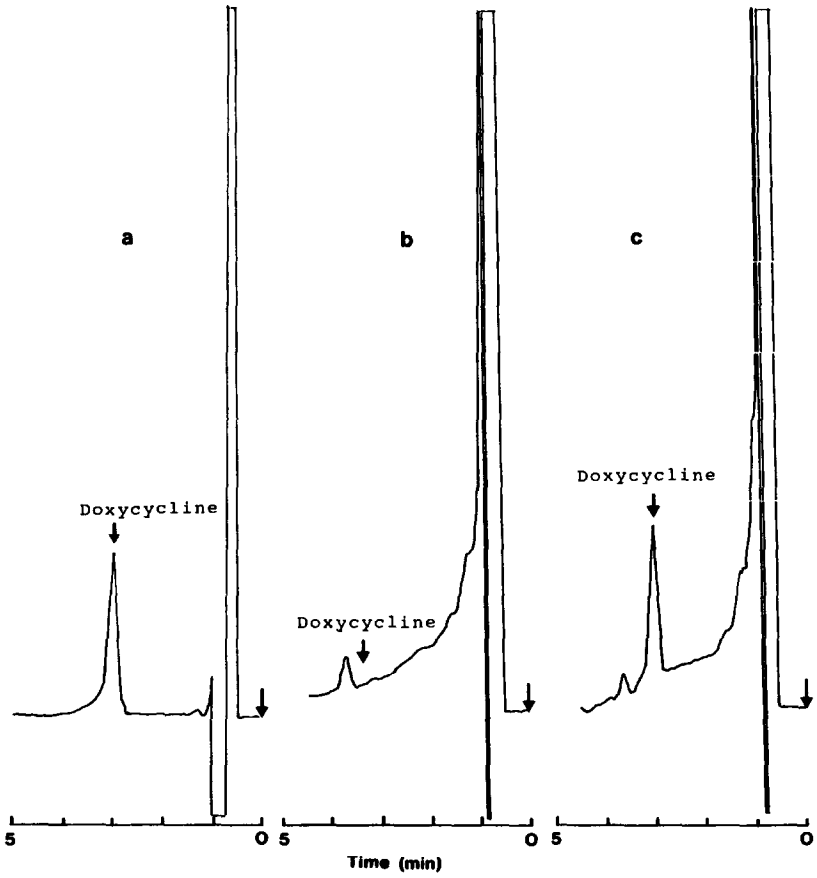

Fig. 2. Typical chromatograms for (a) standard doxycycline, 500 ng ml^{-1}, (b) human serum prior to dosing with doxycycline and (c) human serum from the same subject following doxycycline administration. Detector sensitivity was 0.01 a.u.f.s. Injection volume was 100 μl.

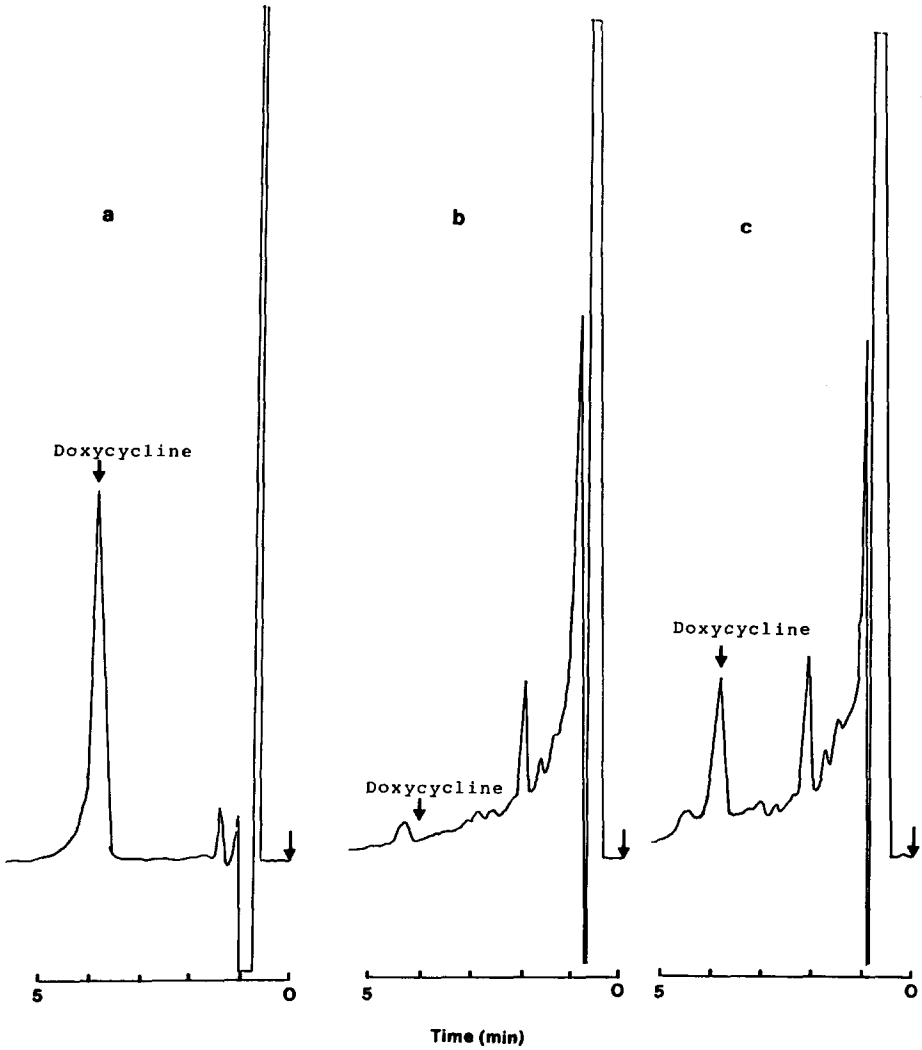

Fig. 3. Typical chromatograms for (a) standard doxycycline, 1000 ng ml^{-1}, (b) human urine prior to dosing with doxycycline and (c) human urine from the same subject following doxycycline administration. Detector sensitivity was 0.01 a.u.f.s. Injection volume was 100 μl.

component of the mobile phase was reduced for urine samples, to retain the doxycycline on the column longer and thus improve resolution from endogenous interfering components. Under the chromatographic conditions described, the Waters Nova-Pak was the only analytical column found to give adequate efficiency and resolution for the analysis. Using Hypersil ODS, Waters μBondapak C_{18} and Spherisorb C_8, doxycycline was not eluted from the column.

A linear regression standard was constructed for doxycycline over the range 0–2500 ng/ml and a linear response determined ($r=0.999$), with an intercept of -3.85 ± 5.65 (95% probability).

To determine the precision and accuracy of the assay methodology, replicate

TABLE I

PRECISION AND ACCURACY OF DOXYCYCLINE ASSAY

Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Recovery (%)	Relative standard deviation (%)
Serum			
250	215.9	86.4	6.5
500	487.5	97.5	2.3
750	715.9	95.5	2.2
1000	939.0	93.9	2.4
1500	1340.9	89.4	2.1
Urine			
250	213.5	85.4	15.5
500	420.0	84.0	5.7
750	602.3	80.3	2.0
1000	795.0	79.5	2.2
1500	1290.0	86.0	0.5

samples (two to eleven) were analysed over the range 250–1500 ng ml^{-1} for both matrices and the results are summarized in Table I.

The relative standard deviation of the recoveries ranged from 2.1 to 6.5 and 0.5 to 15.5 for serum and urine, respectively. The lower limit of detection was approximately 25 ng ml^{-1} using 1 ml of serum or urine (2×baseline noise). As recoveries of doxycycline were high (typically 86–98% for serum and 80–86% for urine), an internal standard was unnecessary. The standards were prepared in 0.1 *M* phosphoric acid as degradation of the doxycycline was observed when the standard was prepared in water. The working standard prepared in 0.1 *M* phosphoric acid was stable for several days but was diluted daily as a precautionary measure.

The assay of tetracyclines by HPLC has often proved to be notoriously difficult. Several methods are available for the assay of doxycycline in biological materials, however, the method described herein combines simple isocratic chromatography with solid-phase sample extraction which is amenable to semi-automation. This allows the rapid and sensitive analysis of samples and has been used to determine the pharmacokinetics of doxycycline when administered orally. It is envisaged that this assay could be readily further automated by using a dedicated or multi-tasking sample processing unit such as the Zymate robot [7]. This enables a continuous stream of samples to be sequentially prepared and either automatically injected onto an HPLC system or to be stored in an HPLC autosampler rack for analysis at a later time. The use of solid-phase extraction also makes the method easily transferable to systems such as the AASP (Varian) which can also achieve high sample throughput by direct injection from the solid-phase cartridge.

Although attempts have been made to extend this method to the tetracyclines demeclocycline and chlortetracycline, these were unsuccessful due to their elu-

tion from the solid-phase extraction column in a fraction other than that which elutes doxycycline.

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