Doxycycline Determination in Human Serum and Urine by High-Performance Liquid Chromatography

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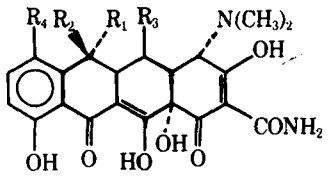
Abstract □ A reversed-phase high-performance liquid chromatographic method for quantitative doxycycline determination in human serum and urine is described. The drug was extracted from buffered (pH 6.1) serum or urine into ethyl acetate. A structural analog, demeclocycline, was added as the internal standard. A 10-cm × 2-mm i.d., 5-μm Lichrosorb RP8 column with acetonitrile–0.1 *M* citric acid as the eluent was used. The effluent was monitored at 350 nm. The extraction recovery from spiked serum was 87.8 ± 4.3% (mean ± *SD*, $n = 11$); for urine, a value of 92.2 ± 2.0% (mean ± *SD*, $n = 10$) was found. Within-run and within-day relative standard deviations averaged 1.80% ($\bar{x} = 2.5$ μg/ml, $n = 10$) and 4.75% ($\bar{x} = 2.6$ μg/ml, $n = 9$), respectively. The detection limit was estimated at 50 ng/ml of serum. No significant extra peaks were observed in chromatograms obtained on serum or urine extracts, suggesting the probable absence of metabolic processes *in vivo*.

Keyphrases □ Doxycycline—analysis, human serum and urine, high-performance liquid chromatography □ High-performance liquid chromatography—analysis, doxycycline in human serum and urine □ Antibiotics—doxycycline, high-performance liquid chromatographic analysis, human serum and urine



	R_1	R_2	R_3	R_4
oxytetracycline	CH_3	OH	OH	H
tetracycline	CH_3	OH	H	H
demeclocycline	H	OH	H	Cl
chlortetracycline	CH_3	OH	H	Cl
methacycline	$=CH_2$		OH	H
doxycycline	CH_3	H	OH	H

The disposition of the antibiotic doxycycline in the human body has been the subject of numerous investigations. Owing to its stronger lipophilicity, this drug has a higher absorption rate and a longer half-life than the other tetracyclines (I). Although the absence of metabolites *in vivo* has been postulated (1), this has not been proven due to the lack of specific assay methods.

The existing fluorometric (2–4) and microbiological (5, 6) procedures provide good sensitivity but are unable to differentiate doxycycline from its analogs, including possible metabolites. In addition, the latter technique only allows estimation of total biological activity without correlation to the chemical structure of the substance. It also overlooks possible metabolites without antimicrobial activity.

Therefore, the incorporation of a suitable chromatographic step in the analysis is highly desirable. Several TLC methods allow the separation and densitometric quantitation of tetracyclines in pure solutions (7–10). However, no attempt has been made to investigate their potential for analysis in biological materials.

High-performance liquid chromatography (HPLC) combines a fast and reliable chromatographic technique with the relatively high sensitivity of on-line UV detection. A few papers (11–13) report its use for tetracycline, oxytetracycline, and chlortetracycline determination in serum and urine. However, none of these systems was applied to doxycycline. Disadvantages include the poor efficiency of some chromatographic separations (11, 13), the lack of an internal standard (11, 12), and bad detection limits (12, 13).

During some preliminary work, an HPLC system capable of separating doxycycline from several analogs in pure solutions was developed (14). A new system is now reported for quantitative doxycycline determination in human serum and urine. The procedure described involves a simple one-step solvent extraction followed by reversed-phase HPLC, using a structural analog as an internal standard.

EXPERIMENTAL

Reagents—All chemicals were analytical grade[1]. Acetonitrile and distilled water were used without further purification for the preparation of the chromatographic eluent. A pH 6.1 phosphate sulfite buffer was prepared by dissolving 27.6 g of monobasic sodium phosphate and 25.2 g of sodium sulfite in 100 ml of water to give a final concentration of 2 *M* for each salt. Stock solutions of doxycycline hydrochloride[2] and demeclocycline (demethylchlortetracycline) hydrochloride[3] were prepared by dissolving the compounds in methanol–0.1 *M* HCl. All glassware was silanized using a 10% (v/v) solution of hexamethyldisilazane in toluene.

Apparatus and Operating Conditions—The basic liquid chromatographic unit consisted of a single pumping system[4] and a sampling valve[5] with a 20-μl loop. A variable-wavelength detector[6] was used to monitor the column effluent at 350 nm. Chromatography was performed on a homemade 10-cm × 2-mm i.d., 5-μm Lichrosorb RP8[1] column with acetonitrile–0.1 *M* citric acid (24:76 v/v) as the mobile phase. The flow rate was 0.5 ml/min (9.65 MPa or 1400 psi). A filtration device was assembled by pressing a filter disk[7] in a pasteur pipet.

Column Preparation—The column was slurry packed using a modified version of the viscosity method (15). A 9% (w/v) slurry was prepared in glycerol–methanol (20:80 v/v) and introduced into a 4.1-ml reservoir directly assembled on top of the column. A pressure of 34.47 MPa (5000 psi) was applied by pumping acetonitrile through the column, using the maximum flow rate setting of the pump[4]. This procedure allowed reproducible packing of some 30 columns, which were all of the required standard, *i.e.*, 2000 theoretical plates or more under the working conditions described.

Extraction Procedure—A 0.5-ml sample of human serum, 50 μl of a 6% (w/v) aqueous ascorbic acid solution, 50 μl of the internal standard stock solution, and 1 ml of the pH 6.1 phosphate sulfite buffer were combined in a screw-capped polytef-lined tube. After mixing[8] for 30 sec, 6 ml of ethyl acetate was added. The tube was placed for 10 min on a rotating mixer[9] and centrifuged for 6 min at 3000 rpm.

[1] E. Merck Co., Darmstadt, West Germany.
[2] Pfizer Inc., Brussels, Belgium.
[3] Lederle Laboratories, Brussels, Belgium.
[4] Model 8500, Varian Associates, Palo Alto, Calif.
[5] Model CV-6-UHPa-N60, Valco Instruments Co., Houston, Tex.
[6] Varichrom, Varian, Palo Alto, Calif.
[7] Type AP 40, pore size "prefilter," Millipore Corp., Bedford, Mass.
[8] Super mixer 1291, Lab-Line Instruments, Melrose Park, Ill.
[9] Rotary mixer, Cenco Instruments, Breda, The Netherlands.

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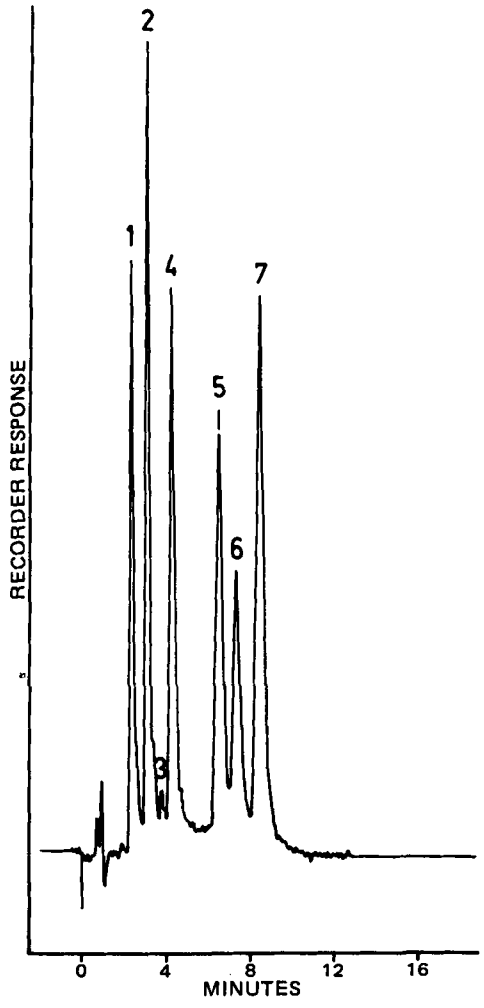

Figure 1—*Separation of seven tetracyclines. Key: 1, oxytetracycline; 2, tetracycline; 3, unidentified by-product of doxycycline; 4, demeclocycline; 5, chlortetracycline; 6, methacycline; and 7, doxycycline. The column was 10-cm × 2-mm Lichrosorb RP8 (5 μm), the mobile phase was acetonitrile–0.1 M citric acid (24:76 v/v), the flow rate was 0.5 ml/min, and the temperature was ambient.*

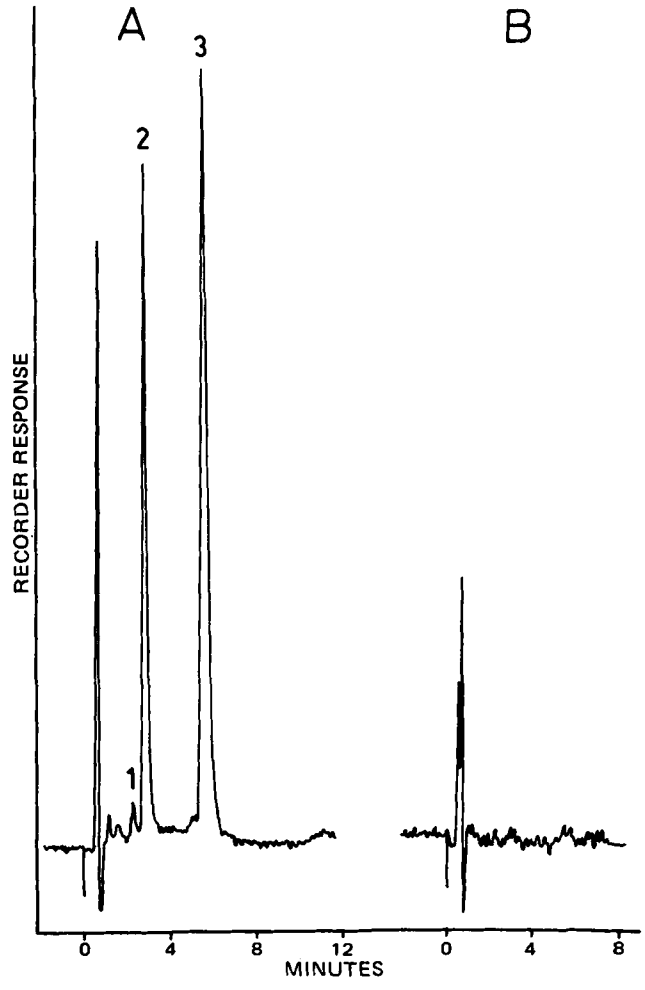

Figure 2—*Chromatograms of serum containing 3 μg of doxycycline/ml (dose of 200 mg iv) (A) and of blank serum (B). Key: 1, unidentified by-product of doxycycline; 2, demeclocycline (internal standard); and 3, doxycycline. Conditions were as in Fig. 1 except for the temperature (30°); detector sensitivity was 0.02 aufs.*

The organic layer was transferred to a conical evaporation tube containing 100 μl of a 0.2% (w/v) methanolic ascorbic acid solution. The mixture was evaporated[10] to dryness at room temperature by applying vacuum under continuous vortexing of the tubes. The residue was finally dissolved in 200 μl of the chromatographic eluent. After mixing[8], the solution was filtered and kept in ice until a 20-μl aliquot was injected into the chromatograph.

A procedure identical to the plasma assay was followed for urine extraction, except that a 100-μl sample, 0.4 ml of buffer, and 3 ml of ethyl acetate were used.

Calculations—Standard curves were constructed by analyzing serum samples containing known drug amounts and plotting peak height ratios (doxycycline/internal standard) *versus* the corresponding doxycycline concentration.

RESULTS AND DISCUSSION

Chromatography—The excellent selectivity of the chromatographic system for the separation of seven tetracyclines is illustrated in Fig. 1. A previously used 25-cm × 4.6-mm i.d. RP8 column (14) was replaced by a short narrow-bore column to improve the detection limit. The extremely small void volume reduces dilution in the column (16) but requires severe precautions to minimize extracolumn band broadening, which especially occurs in the connecting tubes and the detector cell. In addition, injection of components in a solvent that is stronger than the eluent, *e.g.*, methanol, must be strictly avoided since such solvents cause a severe loss of column efficiency due to a secondary solvent effect.

On the basis of this elution pattern, as well as its structural analogy, demeclocycline was chosen as the internal standard. Both other closely eluting derivatives, *i.e.*, chlortetracycline and methacycline, were unsuitable. The former showed a pronounced chemical instability, and low extraction yields (5–10%) were obtained for the latter.

Extraction—The development of a suitable extraction step for this very polar, amphoteric, and labile compound involved various problems. A few techniques, used as a part of some fluorometric assay methods for tetracyclines in general, have been reported (2, 3, 17, 18). Only medium polar organic solvents, *i.e.*, ethyl acetate, amyl acetate, *n*-butanol, and 1-pentanol, can be considered for the isolation of these compounds from a weakly acidic medium (2, 17). Several authors also used chelate extraction performed with divalent cations in a strong alkaline medium (3, 4, 12, 13, 18). However, subsequent concentration of the organic extract proved inconvenient; a dense salt residue prevented redissolution of the compounds in a limited aqueous solvent volume.

The isolation procedure is a modification of a previously described method (2). Amyl acetate was replaced by ethyl acetate to accelerate the final evaporation step. Partition into the organic solvent was particularly favored by salting out the compounds in the nearly saturated phosphate sulfite buffer. Three different buffers consisting of 3, 2, and 1 *M* sodium dihydrogen phosphate and 1, 2, and 2 *M* sodium sulfite gave corresponding pH values of ~5.4, 6.1, and 6.5, respectively. The second buffer system was chosen because it provided the least aggressive medium and gave slightly superior extraction yields. In fact, all tetracyclines are subject to a reversible epimerization process of the C_4-dimethylamino group, occurring preferentially in various buffers over a pH 2–6 range (19, 20).

[10] Rotary Evapo-Mix, Büchler Instruments, Fort Lee, N.J.

Table I—Analytical Recovery of Doxycycline from Human Serum

Added, μg/ml	Measured, μg/ml	Recovery[a], %
3.07	2.69	87.6
3.07	2.73	88.9
3.07	2.80	91.2
3.07	2.63	85.7
3.07	2.60	84.7
6.18	5.27	85.3
6.18	5.55	89.8
6.18	5.25	85.0
6.18	5.75	93.0
6.18	5.93	96.0
6.18	5.40	87.4
6.18	4.92	79.6

[a] Mean ± *SD* = 87.8 ± 4.3.

Despite these precautions, the internal standard, unlike doxycycline, initially deteriorated when serum extracts in ethyl acetate were evaporated at 60°. The additional peak appearing in the chromatogram was identified as 4-epidemethylchlortetracycline on the basis of its retention time and UV characteristics (absorption rate method). No degradation occurred when a standard solution of demeclocycline in ethyl acetate was taken to dryness at 60°. However, considerable epimerization took place even when the compound was added to the organic layer after the extraction of a blank serum sample. Apparently, one or more coextracted

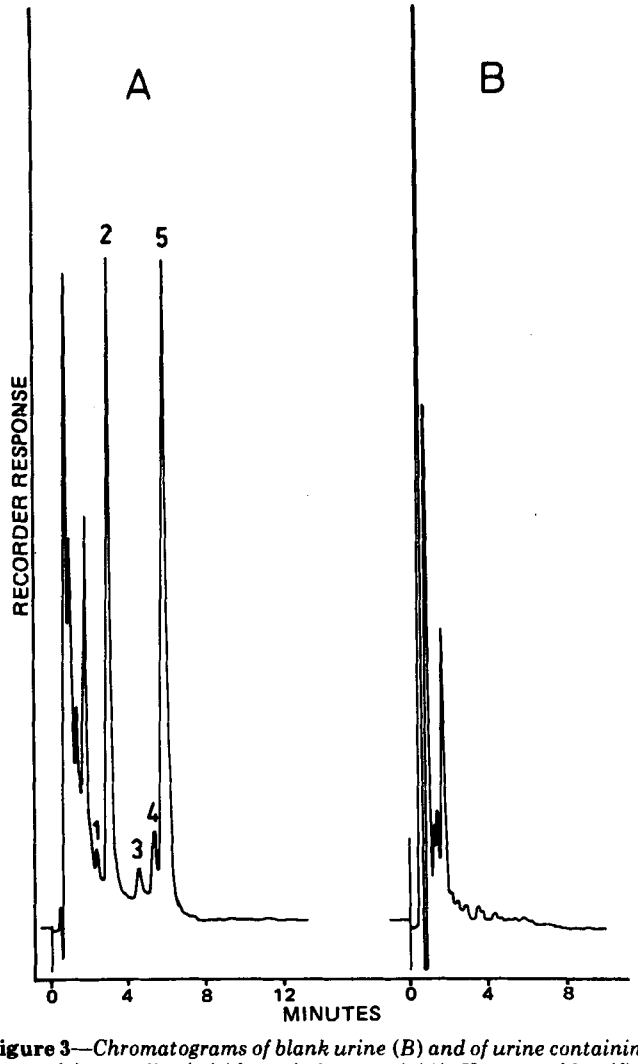

Figure 3—*Chromatograms of blank urine (B) and of urine containing 15 μg of doxycycline/ml (dose of 100 mg po) (A). Key: 1, unidentified by-product of doxycycline; 2, demeclocycline (internal standard); 3 and 4, unidentified peaks; and 5, doxycycline. Conditions were as in Fig. 2; detector sensitivity was 0.02 aufs.*

Table II—Analytical Recovery of Doxycycline from Human Urine

Added, μg/ml	Measured, μg/ml	Recovery[a], %
26.15	24.35	93.1
26.15	23.85	91.2
26.15	25.20	96.4
26.15	24.70	94.5
26.15	23.91	91.4
52.30	47.40	90.6
52.30	46.70	89.3
52.30	48.10	92.0
52.30	48.10	92.0
52.30	48.00	91.8

[a] Mean ± *SD* = 92.2 ± 2.0.

serum component(s) act as a catalyst to this effect. Doxycycline itself was more stable under the same conditions. As previously suggested (20), a hydroxyl group at position 5 of the molecule should prevent epimerization by forming an intramolecular hydrogen bond with the dimethylamino group at carbon 4.

Mild evaporation conditions (room temperature, vacuum) and addition of a little ascorbic acid to the ethyl acetate eliminated the epimerization reaction. Although other tetracycline-stabilizing properties of ascorbic acid are reported (21), its action in suppressing the epimerization was somewhat surprising.

Dissolution of the residue in the chromatographic eluent was adopted for column efficiency. It also served as an ultimate purification step. Most of the coextracted lipids and yellow serum pigments finally remained undissolved in this medium and were filtered off. Colorless and cleaner extracts were thus injected. This practice contributed markedly in prolonging the column life. If loss of column efficiency is occasionally noticed, a simple replacement of the PTFE inlet filter will restore most of the original efficiency.

Extraction Recovery—Recovery studies were carried out by extracting samples containing known drug amounts and adding the internal standard after the extraction was completed. An external calibration curve for this experiment was constructed from the direct analysis (without extraction) of mixtures of doxycycline and the internal standard. Results for serum and urine are summarized in Tables I and II.

Linearity, Sensitivity, and Reproducibility—Linear relationships were observed up to concentrations of 6 μg/ml for serum and 100 μg/ml for urine. The amounts covered the entire anticipated therapeutic range. A pooled standard curve for serum, based on 30 values (0–6 μg/ml) obtained on 12 different days, had a slope of 0.305, an intercept of +0.008, and a correlation coefficient of 0.9995. A similar curve constructed for urine (0–100 μg/ml) yielded 0.073, + 0.002, and 0.9999, respectively. The detection limit in serum is estimated at 50 ng/ml, a value much lower than the concentrations occurring at usual dosage regimens. A within-run relative standard deviation for serum at the 2.5-μg/ml level of 1.8% (n = 10) was obtained. Long-term reproducibility was evaluated over a 20-day period (n = 9) at a concentration of 2.6 μg/ml and yielded a value of 4.75%.

Applications—The assay method presented has been used routinely in this laboratory to monitor serum drug levels in patients receiving doxycycline therapy (130 samples, 38 subjects). Levels after 2–3 hr ranged from 2 to 9 μg/ml after a single intravenous dose of 200 mg.

Figure 2A shows a typical HPLC record of a human serum extract containing doxycycline and the internal standard. Capacity ratios (k') of both compounds were 4.5 and 9.6, respectively. No interfering peaks were observed in a blank serum extract (Fig. 2B).

Chromatograms of a blank urine extract (B) and of a urine extract (A) from a patient who received 100 mg of doxycycline are shown in Fig. 3.

No large extra peaks were present in serum or in urine extracts. The small unidentified peak (Figs. 2 and 3) was probably due to a doxycycline degradation product since it was found in freshly prepared standard solutions and in serum and urine samples from patients receiving doxycycline therapy. Because metabolites are generally more polar than the parent compound, they should elute earlier than doxycycline from a reversed-phase column. Therefore, it is noteworthy that no such metabolites could be detected.

Furthermore, the method can be valuable for determining the pharmacokinetic parameters of the drug to optimize its dosage scheme. Its application to other biological materials requires a few modifications and is presently under study.

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Examination of Blood Clobazam Levels and Several Pupillary Measures in Humans

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Abstract □ The State–Trait Anxiety Inventory was administered to 15 subjects before initiation of the experiment. Three subgroups of five subjects were defined by computing the unweighted sum of the state and trait anxiety scores. A 40-mg dose of clobazam, a 1,5-benzodiazepine, was administered to each subject and repeated with two additional dosage forms following a 2-week washout period. Blood samples were withdrawn, and blood levels were determined by fluorometric analysis. Additionally, pupillary measures of critical flicker fusion, constriction, and dilation in response to a cognitive task were obtained at 0, 2, 4, and 6 hr. A repeated measures analysis of variance revealed that blood levels were, as expected, statistically different over time and dosage form. The pupillary constriction mirrored the blood levels in statistical patterns. The pupillary measure of cognition related to the anxiety state after the performance effects of the cognitive task were statistically removed. The results suggest that clobazam has less immediate human effect than does diazepam.

Keyphrases □ Clobazam—analysis, fluorometry, blood levels, effect on pupillary constriction, and anxiety level □ Pupillary constriction—clobazam effect, blood level, and anxiety level □ Tranquilizers, minor—clobazam, fluorometric analysis, blood levels, effect on pupillary constriction, and anxiety level

Clobazam, a 1,5-benzodiazepine, is effective in the treatment of anxiety neurosis (1, 2). The immediate and long-term effects of the benzodiazepines on human performance ability likewise have been demonstrated. Generally, the benzodiazepines decrease human ability to perform complex tasks (3–5). Additionally, the benzodiazepine drug class affects physiological measures such as auditory reaction times and complex visual reaction times (6–8).

Pupillometrics is defined as the aspect of psychology that deals with the assessment of pupillary alterations elicited by any stimulus other than light (9). Pupil dilation in response to complex cognitive tasks can be observed with proper instrumentation and experimental controls (10). A sensitive means of generating pupillary cognition curves by verbally presenting randomized digits to subjects was described (11, 12). Furthermore, diazepam consumption was shown to alter the pupil cognition curves and recall ability (12).

The objective of this research was to determine if clobazam consumption related to pupillometry involving human cognition and two noncognitive pupillary measures. Critical flicker fusion and miotic effect of the drug were selected as noncognitive measures.

EXPERIMENTAL

Fifteen male volunteers were recruited and subjected to the State–Trait Anxiety Inventory (13). A high anxiety subgroup, consisting of five members, was defined operationally by a combined state–trait anxiety score of >72.0. A five-membered, low anxiety group was defined by a combined score of <64.0. The remaining five subjects were assigned to the middle anxiety group.

A randomized Latin-square design was employed to assign the subjects to a three-level dosage form treatment group. Each subject received separate 40-mg doses by tablet, solution, and capsule dosage forms in randomized and matched sequences. Blood samples were obtained for up to 144 hr following ingestion (14). The blood levels were determined by a fluorometric assay, which did not distinguish between the drug and its active major metabolite, the *N*-desmethyl compound (15).

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