Liquid Chromatographic Determination of Minocycline in Human Serum

A. P. De LEENHEER ˣ and H. J. C. F. NELIS

Abstract □ A rapid, specific, and sensitive liquid chromatographic assay for minocycline in human serum is described. The drug and an internal standard (oxytetracycline) were extracted into ethyl acetate from 0.5 ml of buffered serum (pH 6.5). Chromatographic separation was achieved on a 10-μm Lichrospher 100 CH 8 column with acetonitrile–citric acid (0.1 M) as the eluent. The column effluent was monitored at 352 nm. The assay was linear up to 3 μg/ml with a mean coefficient of variation of 3.3% (n = 6). An extraction recovery of 89.4 ± 3.2% (mean ± SD, n = 17) was obtained over the 0.5–2.6-μg/ml range. The detection limit averaged 50 ng/ml. A serum concentration–time profile in humans after oral intake is presented.

Keyphrases □ Minocycline—analysis, liquid chromatography, human serum □ Antibiotics—minocycline, liquid chromatographic analysis, human serum □ Liquid chromatography—analysis, minocycline in human serum

A new generation of semisynthetic tetracyclines has almost completely replaced the older group members in antibiotic therapy. Removal of the hydroxyl group at C-6 of the molecule yields acid-stable 6-deoxytetracyclines (1), which now are amenable to substitution at C-7 (2). A new series of highly active, alkylated 7-amino-6-demethyl-6-deoxytetracyclines was developed (3), of which minocycline (I) showed the most favorable properties. The additional dimethylamino group at C-7 accounts for better lipid solubility (4) and, consequently, for improved pharmacokinetics in humans (5). This compound has the highest biological activity among the tetracyclines and still occupies a unique position in broad spectrum chemotherapy (6). In addition, because the uptake mechanism of minocycline in bacteria is different from that of other tetracyclines (7), it remains active against tetracycline-resistant pathogens, including staphylococci (3, 6, 8).

BACKGROUND

Long-term treatment of patients with minocycline requires monitoring of serum levels (9). Therefore, the possibility of metabolism *in vivo* was suggested (10, 11). Existing microbiological (12) and fluorometric (13–15) assays have limited use because they lack specificity.

A few TLC methods for various tetracyclines in pure solutions include the separation of minocycline from its analogs (16, 17) but lack the sensitivity required for application to biological materials. Liquid chromatography offers a valuable alternative; it allows separate quantitation of the parent drug and its metabolites (including those devoid of antimicrobial and/or fluorescent activity). Only two papers have dealt with liquid chromatography of minocycline. The first employed pellicular ion-exchange column systems (18) with poor efficiency and sensitivity. The other described a complex mobile phase and gradient elution (19).

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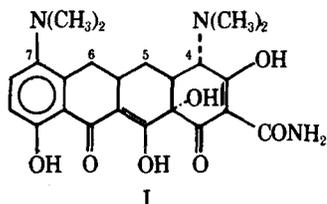
A major disadvantage is the considerable time (25–44 min) required to separate minocycline from its analogs.

Some high-pressure liquid chromatographic (HPLC) procedures for the determination of three classical tetracyclines in biological fluids were reported but were not applied to minocycline (20–23). A similar assay for doxycycline in serum and urine (24) undoubtedly could be adapted to tetracyclines other than minocycline.

This paper presents a new and efficient reversed-phase system for the specific and sensitive determination of minocycline in human serum.

EXPERIMENTAL

Reagents—All chemicals[1] were analytical grade and were used as received. Distilled water was used to prepare the solutions. A pH 6.5 phosphate–sulfite buffer consisted of 1.2 M monobasic sodium phosphate and 2.0 M sodium sulfite. Standard minocycline[2] solutions (10 and 30 μg/ml) were prepared in water adjusted to pH 4.2 with 0.1 M HCl. This optimal pH (4) provided stable solutions for at least 1 week when stored at 4° and protected from light.

Oxytetracycline[3] was dissolved in 0.03 M HCl (50 μg/ml) and was stable for several months (25). Methanolic solutions of ascorbic acid (0.2% w/v) and cysteine (0.1% w/v) were prepared. A 10% (v/v) solution of hexamethyldisilazane in toluene was used to silanize the glassware.

Apparatus and Operating Conditions—The liquid chromatograph was equipped with a constant-flow pump[4], a sampling valve[5] with a 20-μl loop, and a variable-wavelength detector[6]. A 15 × 0.32-cm i.d. column was packed with 10-μm Lichrospher 100 CH 8[1] under the following conditions: carbon tetrachloride slurry, 10% (w/v); packing pressure, 34.47 MPa; and pressurizing liquid, acetonitrile. Elution was performed with 9% (v/v) acetonitrile in 0.1 m citric acid at a flow rate of 1 ml/min (3.45 MPa). The column effluent was monitored at 352 nm.

Extraction—Serum, 0.5 ml, 50 μl of the internal standard solution, and 1 ml of phosphate–sulfite buffer were mixed[7] thoroughly and extracted with 6 ml of ethyl acetate on a rotating mixer[8]. After centrifugation at 3000 rpm, the organic layer was transferred to a conical tube containing 100 μl of ascorbic acid and cysteine in methanol. The mixture was evaporated to dryness at room temperature under vacuum[9]. The residue was dissolved immediately in 200 μl of 9:91 (v/v) acetonitrile–hydrochloric acid (0.1 M), and a 20-μl aliquot was injected on top of the liquid chromatographic column.

The same procedure was applied to urine samples (100–200 μl), using 0.4 ml of buffer solution and 2.5 ml of ethyl acetate.

Standard Curves—Standardization samples were prepared by adding 26–51 μl of the minocycline stock solutions to 0.5 ml of a blank serum pool. Calibration curves were constructed by plotting peak height ratios (minocycline to internal standard) *versus* the corresponding minocycline concentration.

Blood Concentrations—A human volunteer received two 100-mg capsules po just after a light breakfast. Venous blood samples were withdrawn at 0.5, 1, 1.5, 2, 3, 4, 8, 12, 25, 49, and 78 hr after dosage. Blood serum was isolated and stored at −20° until analyzed.

RESULTS AND DISCUSSION

Chromatography—A suitable chromatographic system for minocycline was critical in the development of a specific assay. During earlier

[1] E. Merck, Darmstadt, West Germany.
[2] Lederle Laboratories, Brussels, Belgium.
[3] Pfizer Inc., Brussels, Belgium.
[4] Model 8500, Varian Associates, Palo Alto, Calif.
[5] Model CV-6-UHPa-N60, Valco Instruments Co., Houston, Tex.
[6] Varichrom, Varian Associates, Palo Alto, Calif.
[7] Super mixer 1291, Lab-Line Instruments, Melrose Park, Ill.
[8] Cenco Instruments, Breda, The Netherlands.
[9] Rotary Evapo-Mix, Büchler Instruments, Fort Lee, N.J.

Compound	k'
Minocycline	2.2
Lymecycline by-product	3.2
Lymecycline	4.9
4-Epitetracycline (quatrimycin)	6.3
Oxytetracycline	6.6
Rolitetracycline	7.9
Tetracycline	10.0
4-Epidemeclocycline	10.6
Demeclocycline	16.6
Chlortetracycline	33.7
Methacycline	60.1
Doxycycline	76.2
Anhydrotetracycline	>80.0
4-Epianhydrotetracycline	>80.0

[a] The eluent was acetonitrile–0.1 M citric acid (9:91 v/v), and the temperature was ambient.

studies, efficient and reproducible reversed-phase systems were established for all tetracyclines except minocycline (24, 26). Minocycline always eluted as a severely tailing peak, useless for quantitation. This chromatographic behavior, atypical for tetracyclines, is associated with the presence of two dimethylamino groups, affording strongly different physicochemical properties.

Lichrospher 100 CH 8, a recently marketed reversed-phase packing material, consists of spherical particles with a large pore size, 100 Å instead of the usual 60 Å. The potential of larger pore particles for the chromatography of relatively large molecules was suggested previously[10]. Although its smaller surface area and carbon content predicted smaller
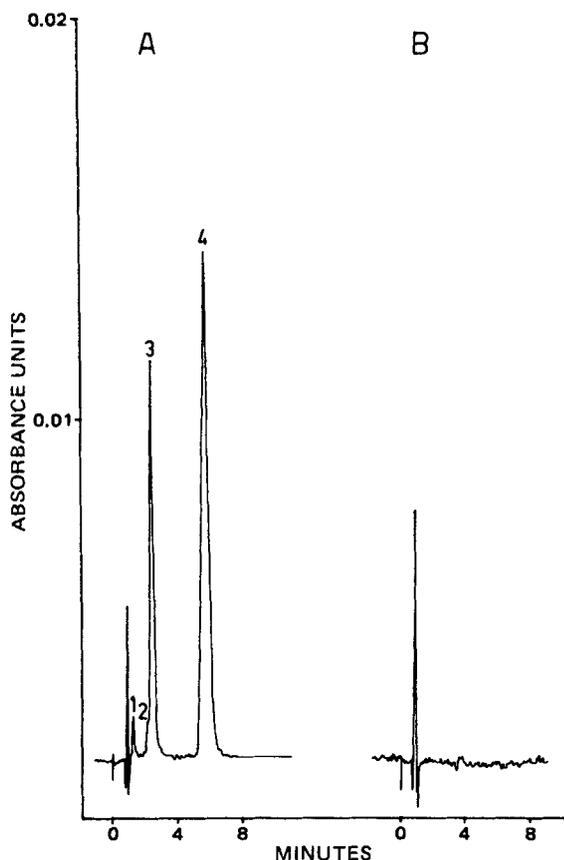
Figure 1—*Chromatograms of serum containing 1.5 μg of minocycline/ml (A) and of blank serum (B). Key: 1 and 2, possible minocycline metabolites; 3, minocycline; and 4, oxytetracycline (internal standard). The flow rate was 1 ml/min, the temperature was ambient, and the chart speed was 0.5 cm/min.*

[10] J. H. M. Van den Berg, Institute of Technology, Eindhoven, The Netherlands, personal communication.

Table II—Minocycline Extraction Recovery from Human Serum

Added, μg/ml	Measured, μg/ml	Recovery, %
0.51	0.45	87.4
0.51	0.43	84.8
0.91	0.81	89.3
0.91	0.81	89.3
1.44	1.27	88.5
1.44	1.26	87.8
1.44	1.32	91.7
1.44	1.31	91.1
1.44	1.23	85.7
1.44	1.33	92.5
2.00	1.83	91.5
2.00	1.67	83.6
2.55	2.27	89.1
2.55	2.38	93.6
2.55	2.19	86.0
2.55	2.39	93.8
2.55	2.38	93.6
Mean ± SD		89.4 ± 3.2

capacity ratios, the material showed a unique selectivity for the different tetracyclines. With an acidic mobile phase, which is essential for the chromatography of other tetracyclines (26), good peak shape (asymmetry factor 1.3) and reasonable column efficiencies (h = 10 at a flow rate of 1 ml/min) were obtained.

Table I lists the k' values of different tetracyclines on the Lichrospher column. The elution sequence of tetracyclines on this packing material essentially could be related to their partition behavior at pH 2.1, i.e., the mobile phase pH, in a two-phase in vitro model (27). High partition coefficients (octanol–aqueous buffer) correspond to strong retention. Although minocycline was the most lipophilic of the tetracyclines (at neutral pH), its low capacity ratio was not surprising. As a result of its two dimethylamino cationic groups, its apparent partition coefficient at pH 2.1 is zero (27). Consequently, no other commonly used tetracyclines will interfere in this system.

The described elution pattern also excluded several derivatives from use as an internal standard. Only three early eluting compounds were considered. Lymecycline was unsuitable because it is very unstable (three decomposition peaks in the chromatogram). Epitetracycline (quatrimycin) gave poor extraction yields, as expected from its stronger basic character (28). However, oxytetracycline fulfilled the conditions of stability and extraction yield and was chosen as the internal standard.

Extraction—Several general problems involved in tetracycline extraction were discussed elsewhere (24). The direct application of a doxycycline extraction procedure (24) to minocycline resulted in unsatisfactory extraction yields. The acid-base characteristics of minocycline differ markedly from those of other tetracyclines. A fourth pKa value (4), assigned to the dimethylamino group at C-7, accounts for a higher isoelectric point of 6.5. The partition into an organic solvent such as ethyl acetate is maximal around this pH. Thus, a pH 6.5 buffer system produced significantly higher extraction yields. A phosphate–sulfite buffer provided cleaner extracts than a previously described phosphate buffer (14). The advantages of concentrated sulfite-containing buffers for tetracycline extraction were noted previously (13, 24).

Minocycline is more susceptible to oxidation than the other tetracyclines (4). For example, thiol-containing substances were reported to possess tetracycline-stabilizing properties (29). Therefore, antioxidants

Table III—Calibration Curve Linearity and Precision for 0.56–2.83 μg/ml (n = 6)

Day	Mean Normalized PHR[a], (μg/ml)$^{-1}$	SD, (μg/ml)$^{-1}$	CV, %
1	0.539	0.008	1.5
2	0.513	0.014	2.7
3	0.503	0.024	4.7
4	0.545	0.016	2.9
5	0.512	0.032	6.2
6	0.508	0.008	1.6
Average	0.520	0.017	3.3

[a] Normalized PHR equals the peak height ratio of each minocycline standard divided by the corresponding concentration.

Table IV—Day-to-Day Precision Data over a Period of 6 Days (n = 6)

Concentration, μg/ml	Mean Normalized PHR^a, $(\mu g/ml)^{-1}$	SD, $(\mu g/ml)^{-1}$	CV, %
0.56	0.507	0.039	7.6
0.92	0.508	0.019	3.7
1.44	0.528	0.020	3.8
2.00	0.527	0.022	4.1
2.55	0.520	0.020	3.9
2.83	0.529	0.013	2.6

a See footnote to Table III.

such as cysteine and ascorbic acid were added routinely. In addition, ascorbic acid should have a protecting effect against epimerization at C-4 of certain tetracyclines (24). In general, slightly higher extraction yields. were obtained by including these preservatives.

Considerable response loss occurred when the extraction residue was dissolved in a small volume of chromatographic solvent. The use of stronger acids, e.g., 0.1 M HCl or H_2SO_4, overcame the problem. Solution filtration before injection is recommended but is not necessary. When filtration is employed, silanized filters should be used to avoid adsorption losses (5–10%). Without filtration, no considerable column efficiency decrease was observed after the injection of several hundred serum extracts.

Extraction Recovery—Extraction recovery was evaluated by analyzing spiked serum samples. In these experiments, the internal standard was added after transfer of the organic phase. Calibration data were obtained by carrying out the same analysis, without extraction, of minocycline and internal standard solutions in ethyl acetate, starting from the evaporation step. The results (Table II) indicate reasonably constant extraction yields (84.8–93.8%) over the entire therapeutic range (0–2.8 μg/ml).

Linearity, Precision, and Sensitivity—A linear relationship was
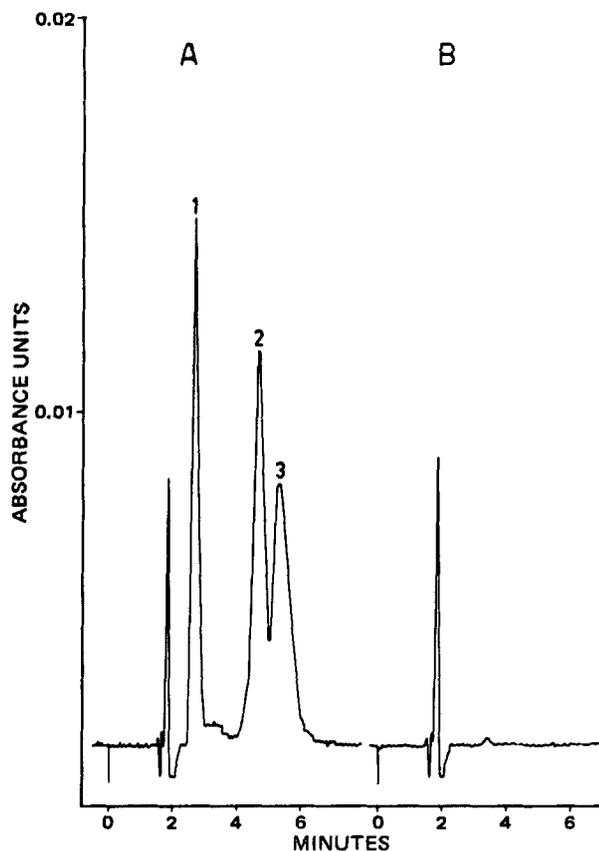
Figure 2—*Chromatograms of urine from a patient after 200 mg of minocycline po (A) and of blank urine (B). Key: 1 and 2, unidentified metabolites; and 3, minocycline. The flow rate was 0.5 ml/min, and the chart speed was 1 cm/min.*
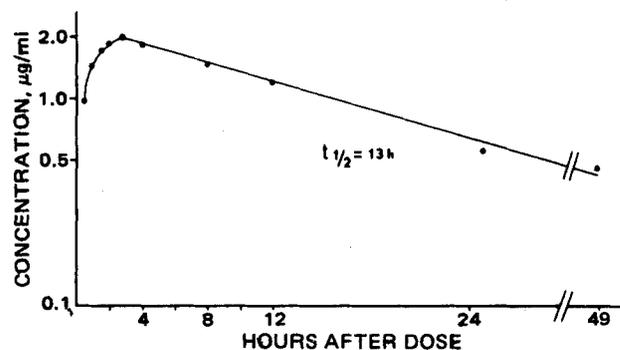
Figure 3—*Serum minocycline levels in a human volunteer following a single oral 200-mg dose.*

found over the 0–3-μg/ml range. Calibration data obtained on 6 consecutive days are summarized in Table III. Precision was evaluated by calculating variations on normalized peak height ratios (peak height ratio of each standard divided by the corresponding minocycline concentration). A pooled standard curve of mean peak height ratios versus concentrations based on all data was calculated by linear regression fitting ($y = 0.531x - 0.012$, $r = 0.9998$). Day-to-day variations (CV) on normalized peak height ratios for six concentrations ranged from 2.6 to 7.6% (Table IV).

The detection limit with a 0.5-ml sample was ~50 ng/ml. This limit corresponds to a serum concentration resulting in a peak height equal to four times the signal-to-noise ratio.

Applicability—The overall procedure is very convenient for routine determinations because of its simplicity and speed (one analysis may be completed in <45 min). A typical chromatogram of a serum extract from a patient who received 200 mg of minocycline po is presented in Fig. 1A.

As shown by a blank serum chromatogram (Fig. 1B), no interfering peaks were observed. Two small additional peaks (1 and 2) eluted before minocycline. They possibly were associated with some polar metabolites. This assumption was supported by the analysis of a urine extract from the same patient (Fig. 2). Two large peaks with the same retention times were visible, representing >50% of the total drug content in the urine sample. Their isolation and identification are now under study.

As an illustration, serum minocycline levels as a function of time were studied in a human volunteer following a single oral 200-mg dose. Serum levels were plotted versus time and yielded the profile shown in Fig. 3. A peak serum level of 2.1 μg/ml was reached after 3 hr; 45 hr later, 0.22 μg/ml still could be measured accurately. A very small peak was detected 78 hr after dosage and corresponded to ~50 ng/ml, the detection limit.

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Use of an Iodide-Specific Electrode to Study Lactoperoxidase-Catalyzed Iodination of l-Tyrosine

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Abstract ◻ An *in vitro* method employing an iodide-specific electrode for monitoring lactoperoxidase-catalyzed iodination is described. The method utilized lactoperoxidase, potassium iodide, and a glucose–glucose oxidase system for the generation of hydrogen peroxide and l-tyrosine. As iodination of l-tyrosine proceeded, the free iodide concentration in solution decreased and was monitored by an iodide-specific electrode. The iodide electrode was reliable when compared to a ^{131}I-method for measuring free iodide changes in solution. Increasing concentrations of resorcinol, a well-known inhibitor of thyroid peroxidase-catalyzed iodination, in the reaction mixture resulted in graded inhibition of the initial rate of lactoperoxidase-catalyzed l-tyrosine iodination. This *in vitro* system can be used to assess inhibitory activity of various antithyroid substances.

Keyphrases ◻ l-Tyrosine—iodination, lactoperoxidase catalysis, kinetics, *in vitro*, iodide-specific electrode ◻ Lactoperoxidase—l-tyrosine iodination, catalysis, kinetics, *in vitro* ◻ Resorcinol—inhibitory activity, kinetics, iodide-specific electrode ◻ Iodination—l-tyrosine, lactoperoxidase catalysis, kinetics, *in vitro*, iodide-specific electrode

Several recent investigations were conducted to study the kinetics of thyroid peroxidase-catalyzed iodination, the reaction responsible for thyroid hormone synthesis (1–4). Since thyroid peroxidase is not unique in its ability to catalyze iodination and thyroxine formation, *in vitro* iodination studies have employed a wide variety of peroxidases (5–10).

Numerous procedures have been utilized to study peroxidase-catalyzed iodination *in vitro* (7, 9, 11–15). The most widely used procedure involves isolation of the iodinated organic compounds and determination of the iodide incorporated into these products. Spectrophotometry is also used to monitor iodination *via* the changing spectral properties of the iodinated products (12, 13). A potentiometric procedure employing various substrates also has been used to follow the kinetics of iodination catalyzed by peroxidase (7, 9, 15).

The objective of the present study was to determine the optimal conditions for the use of an iodide-specific electrode for monitoring lactoperoxidase-catalyzed iodination of l-tyrosine. It was determined that lactoperoxidase is an acceptable peroxidase for investigating iodination kinetics (6, 10). Since several of the well-known antithyroid agents used clinically are general peroxidase inhibitors (6, 16–18), the present study also was intended to determine whether the iodide electrode could be used to study the inhibition kinetics of the antithyroid compound resorcinol.

EXPERIMENTAL

Procedure—An iodide-specific electrode[1], a pH expanded-scale millivolt meter[2], and a strip-chart recorder[3] were used to measure the free iodide concentration. As the peroxidase enzyme catalyzed the iodination of an appropriate iodide acceptor at a known initial iodide concentration, the iodide concentration decreased. The reduction in iodide concentration resulted in a potential change, which was related to the iodide-ion activity by the Nernst equation (19). To obtain the actual iodide concentration decrease per unit time (iodination rate), each millivolt change was converted to the corresponding iodide concentration by calibrating the iodide electrode in the concentration range of interest $(1 \times 10^{-5}-10 \times 10^{-5}\,M)$, using appropriate dilutions of a 0.1 M sodium iodide standard solution[4].

Lactoperoxidase[5] was quantified by activity units; 1 unit of lactoper-