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# Identification and determination of the two principal metabolites of minocycline in humans

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ABSTRACT

A chromatographic method has been developed for the quantification of minocycline in human serum and urine. The chromatographically determined concentration of minocycline correlated well with the microbiologically active concentration in serum. Two metabolites, 9-hydroxyminocycline and N-demethylated minocycline, could be isolated and identified as the principal metabolites of this tetracycline antibiotic. The structure of the 9-hydroxy compund was proved by nuclear magnetic resonance analysis for the first time. About 15% of the drug was actively converted in the body into a substance less microbiologically active than the parent compound and excreted in the urine within 96 h after the application.

## INTRODUCTION

Minocycline and doxycycline are unique tetracycline derivatives which are metabolized in humans [1–5]. Although 30–50% of doxycycline is excreted unchanged in the urine [6–9], only *ca.* 10% of minocycline can be analysed from this body fluid as a microbiologically active compound [9–11]. From animal experiments with radiolabelled minocycline it was concluded that this tetracycline antibiotic might be metabolized, because the microbiologically determined amount of recovered minocycline was somewhat less than the recovered radioactivity [12].

A method has recently been described by which seven tentative metabolites of minocycline were analysed in human urine [1,2,13] without a direct proof of the structures or a quantitation of the metabolically converted compounds. This paper describes a multiple-step liquid chromatographic method for the isolation

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and purification of the two principal metabolites of minocycline from human urine, and the quantification of minocycline and its metabolites in urine. The analytical method described is demonstrated as a simple and fast tool for the determination of minocycline in serum and urine, and also for the determination of its metabolites.

## EXPERIMENTAL

### *Chemicals*

Reference standards of minocycline and 4-epiminocycline hydrochloride were donated by Lederle Cyanamid (Wolfratshausen, Germany). Hymecromone was donated by LIPHA-Arzneimittel (Essen, Germany). Solvents for chromatography were of high-performance liquid chromatography (HPLC) grade (Chromasolve, Riedel de Haen, Seelze, Germany). All other chemicals were of the purest quality available.

### *Biological samples*

Serum samples were taken from three male volunteers (23, 26 and 31 years old; height: 180, 175 and 171 cm; weight: 77, 60 and 80 kg, respectively) up to 60 h after a single 100-mg dose of minocycline (Klinomycin) given either intravenously or orally. After an overnight fast the subjects were given the drug together with 250 ml of fluid. Four hours later they had the first food intake. Urine was collected quantitatively for up to 96 h after drug intake in several pre-specified fractions.

### *Serum extraction procedure*

Serum samples (1 ml) were mixed with 0.1 ml of 0.025 m*M* $Na_2EDTA$ and developed on a $C_{18}$ extraction column (Bond Elut, Analytichem, Harbor City, CA, USA) (100 mg), which had been pre-treated consecutively with 1 ml of methanol and 1 ml of 1% formic acid. Minocycline was extracted from the column by 1 ml of methanol. The organic solvent was evaporated to dryness under nitrogen, and the residue was redissolved in water.

### *Urine extraction procedure*

Urine samples (2 ml) were mixed with 0.1 ml of 3 *M* $Na_2HPO_4$ (pH 6.5) and 5 μg of hymecromone to control the extraction procedure, and extracted twice with 2 ml of dichloromethane (stored under an argon atmosphere to prevent oxygen-mediated reactions of the solvent). The combined organic layers were evaporated to dryness (under nitrogen), and the residue was redissolved in 1 ml of water.

### *High-performance liquid chromatography*

The HPLC instrument used was equipped with two solvent-delivery pumps (Model T414), an injection valve with a 0.3-ml loop (Model 7125, Rheodyne), and a variable-wavelength detector (Uvicon 720) set at 352 nm. The detector

signal was automatically recorded by the integrating–controller unit (Anacomp 220). All chromatographic apparatus were supplied by Kontron, Eching, Germany.

To analyse the absorption properties of minocycline and its metabolites on line, some of the analyses were run using a diode-array detector (HP 1040A with data station, Hewlett-Packard, Bad Homburg, Germany).

The analyses were performed on a $C_{18}$reversed-phase column (25 cm × 0.46 cm I.D., 10 μm particle size; Macherey & Nagel, Düren, Germany) equipped with a $C_{18}$ guard column (3 cm × 0.46 cm I.D., 10 μm particle size; Macherey & Nagel). After injection of the sample a gradient was run from 10% to 40% acetonitrile (in 0.2% formic acid in water) within 5 min and held at 40% acetonitrile for 8 min. After that the column was readjusted to 10% acetonitrile (within 0.1 min) and held for another 5 min. The flow-rate was 1.2 ml/min (at *ca.* 100 bar).

For the semi-preparative separation of the different fractions, a $C_{18}$ column (25 cm × 1 cm I.D., 7 μm particle size; Macherey & Nagel) equipped with a guard column was used, and eluted with a gradient of 10–40% acetonitrile (in 0.2% formic acid) within 5 min after the start of the run at a flow-rate of 4.2 ml/min (at *ca.* 120 bar), held at 40% acetonitrile for another 8 min and readjusted to 10% acetonitrile.

For analysis of the 4-epimer of minocycline a $C_{18}$ column (25 cm × 0.46 cm I.D., 10 μm particle size; Macherey & Nagel) with an isocratic eluent (25% dimethylacetamide in 0.1 *M* oxalic acid, pH adjusted to 4.2 with concentrated ammonia) at a flow-rate of 1.0 ml/min (at *ca.* 250 bar) was used.

For the analysis of minocycline by the ion-exchange method a Nucleosil SA column (25 cm × 0.46 cm I.D., 5 μm particle size, Macherey & Nagel) was used and eluted isocratically with 50% methanol in 0.1 *M* citric acid (pH 4.6) at a flow-rate of 1.2 ml/min (150 bar).

Minocycline and its metabolites were also chromatographed on some other $C_8$, $C_2$ and phenyl-modified silica material (all from Macherey & Nagel), which could be run under isocratic conditions and in the absence of ions known to modify the tetracycline structure. But there were no additional peaks seen in the chromatograms of the metabolite analysis. The peak shapes and the sensitivity of the method were best using the above-mentioned $C_{18}$ material.

*Isolation and purification of the metabolites*

A 1-l volume of urine from the combined 0–48 h collection period was extracted twice with 0.5 l of dichloromethane according to the analytical method at pH 6.0. Fractions corresponding to the peaks of the metabolites of minocycline and of minocycline itself were eluted from the semi-preparative HPLC column (detected at 352 nm) and collected. The solvent of the combined fractions was evaporated to dryness under reduced pressure at 35°C. The residues were redissolved in water and rechromatographed twice on the same semi-preparative column.

*In vitro hydroxylation of minocycline*

The hydroxylation of minocycline was performed as described in the literature [14,15], and the reaction products were extracted with dichloromethane at pH 6.0. The residue was dissolved in methanol and filtered. The clear methanol solution was reduced to dryness. This residue was dissolved in water containing 10% acetonitrile and chromatographed on a silica gel column (30 cm × 3 cm I.D., 100–200 mesh, ICN Biomedicals, Eschwege, Germany) to remove most of the complex-bound iron ions. The appropriate fraction absorbing at 346 nm was collected and reduced to dryness. This compound exhibited the same retention time, mass spectral and absorption behaviour as the metabolite of minocycline isolated from urine and eluted as the first one.

*Spectrometry*

A double-beam spectrophotometer (Model 5230, Beckmann, Munich, Germany) was used for the spectral analyses of minocycline and its metabolites in hydrochloric acid, alkali, and alkali plus calcium ions, according to the general procedures described in the literature [3,4,16,17].

*Mass spectrometry (MS)*

A Hewlett Packard Model 5878A instrument was used to obtain LC–MS spectra. The spectrometer was installed on-line with an isocratic HPLC system equipped with an additional injection valve between the column and the spectrometer adapter. The isocratic analysis was performed on an RP $C_{18}$ column (25 cm × 0.46 cm I.D., 10 μm particle size; Macherey & Nagel), at a flow-rate of 1 ml/min (20% acetonitrile in 0.2% formic acid). The eluent was split at a ratio of 1:10 and this part was introduced directly into the mass spectrometer (direct liquid introduction). The ion source was set at constant 250°C and 70 eV. The system worked either in the positive (PCI) or negative (NCI) chemical ionization mode. Only prepurified and concentrated samples were applied. Alternatively, the sample could be injected into the eluent stream prior to the removal of contaminating material before the eluent entered the MS apparatus (columnless LC–MS coupling).

*NMR analysis*

A 400 MHz NMR instrument (Model Jeol-GX400, Jeol, Tokyo, Japan, 9.4 Tesla) was used to obtain one- and two-dimensional NMR spectra of the hydroxylated metabolite. The metabolite of minocycline that eluted just prior to the parent compound could not be analysed by NMR because insufficient amounts of this compound were purified. The amount of this sample was sufficient only for carrying out the spectral, mass spectral, and microbiological analyses.

*Microbiological assay*

The microbiological activity of minocycline in serum was tested against *Bacil-*

*lus cereus* (Difco 0959-36) according to the method described for doxycycline [18] and related to the activity of the reference compound. The microbiological activities of the metabolites of minocycline were determined by W. Hillen, Institute of Microbiology, The University of Erlangen-Nürnberg, and compared with the biological activity of the reference compound.

*Other assays*

To compare the lipophilicity of minocycline and of its metabolites, the general procedure for the determination of the partition ratio in an octanol–buffered aqueous system was used [19,20] and the concentration of minocycline or that of its metabolites was determined in both phases by the analytical HPLC method.

*Calculation*

The variation of the method was determined from the analysis of ten serum or ten urine samples for five concentrations of minocycline (ranging from 0.5 to 200 μg/ml) or its metabolites (only at two different concentrations corresponding to 0.1 and 10 μg/ml for each of the two metabolites) using the one-way analysis of variance on a HP 97 calculator (Hewlett-Packard). This program was also used to determine the mean (± S.D.) concentration values of minocycline and its metabolites in the serum and urine of the three male volunteers.

## RESULTS AND DISCUSSION

Minocycline can be separated by the HPLC method described as a single symmetrical peak (Fig. 1). The detection limit for minocycline was below 0.05 μg/ml in serum and below 0.1 μg/ml in urine (signal-to-noise ratio of 3:1). The deviation was between 3.5% (for concentrations above 0.3 μg/ml) and 35% (for concentrations below 0.1 μg/ml), for both serum and urine samples. The recovery of minocycline from serum was 90 ± 6.3% ($n$ = 50) and from urine 70 ± 11.6% ($n$ = 50). Similar recoveries were obtained for the demethylated compound, whereas that of the hydroxylated metabolite was only 60 ± 16% ($n$ = 20), possibly owing to the lower lipophilicity of the hydroxylated compound (the partition coefficient was 0.1 for the hydroxylated metabolite and 0.7 for the demethylated metabolite, compared with 1.4 for the parent compound).

The detector response was linear with the minocycline concentration in serum (and urine) up to 200 μg/ml. The concentration of minocycline in serum determined by the HPLC method correlated well with the microbiologically determined concentration ($r^2$ = 0.92; $y = -0.04 + 0.72x$). The metabolites of minocycline could not be detected in serum, again possibly owing to the lower lipophilicity of the metabolites.

When urine was analysed for minocycline, two additional peaks were observed in the chromatograms eluted prior to the parent compound (Fig. 1), even in the first urine samples (0–2 h). These additional peaks were not apparent when mino-

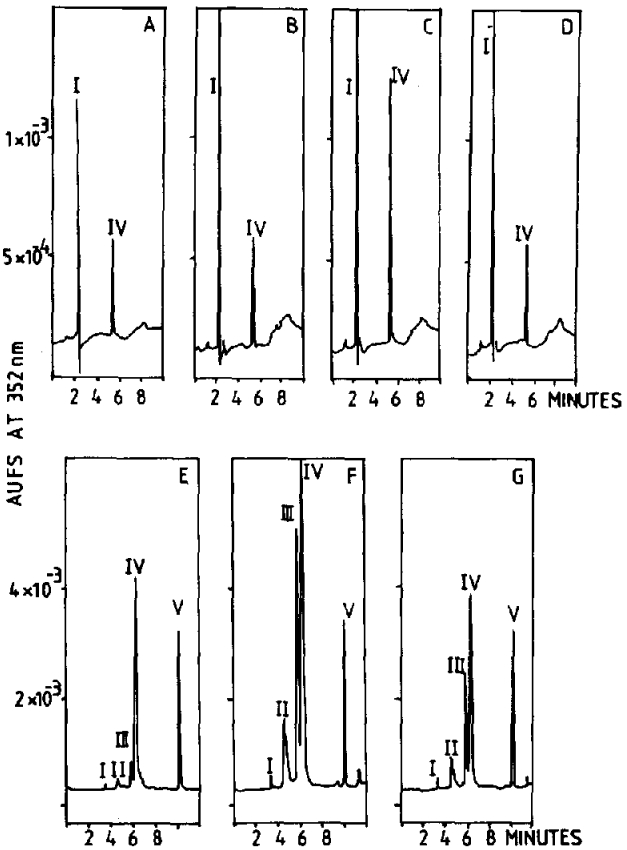


Fig. 1. HPLC determination of minocycline in human serum and urine. Minocycline was extracted from serum and urine as described in Experimental. The aqueous solution of the residue was chromatographed on a $C_{18}$ reversed-phase column (10 μm, 25 cm × 0.46 cm I.D.) with a gradient from 10 to 40% acetonitrile in the first 5 min after the start of a run at a flow-rate of 1.2 ml/min. The eluent was monitored at 352 nm. (A) Blank serum spiked with 1 μg/ml minocycline; (B) serum at 0.5 h; (C) serum at 1.5 h; (D) serum at 24 h after the oral application of 100 mg of minocycline (subject 1); (E) urine at 0–12 h; (F) urine at 12–24 h; (G) urine at 24–48 h (subject 1). Peaks: I = solvent; II = hydroxylated minocycline; III = demethylated minocycline; IV = minocycline; V = hymecromone (5 μg/ml).

cycline was incubated in urine for up to 36 h at 37°C. Because the semi-preparative separation of a 0–48 h urine sample displayed the same elution profile as the analytical system, the two fractions could be collected and developed as described in Experimental.

After repeated purification, the isolated compounds of these two peaks were eluted in a single peak in all analytical HPLC systems (on $C_{18}$, $C_8$, $C_2$ and phenyl-substituted silica) (Fig. 2). When ion-exchange separation of these compounds (as of minocycline itself) was carried out prior to the analysis by enantiomer-specific analysis, some additional compounds were found, possibly

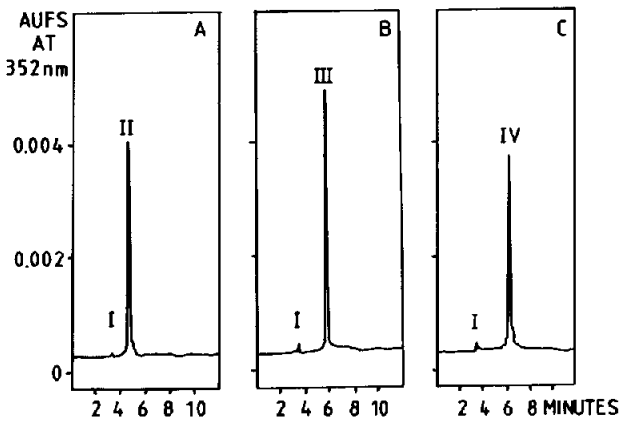


Fig. 2. HPLC of minocycline and its purified metabolites. After repeated chromatography as described in Experimental, the purified metabolites were determined as described in the legend of Fig. 1. The amounts injected onto the column were 0.1 $\mu$g of hydroxylated minocycline (A), 0.08 $\mu$g of demethylated minocycline (B) and 0.05 $\mu$g of minocycline (C).

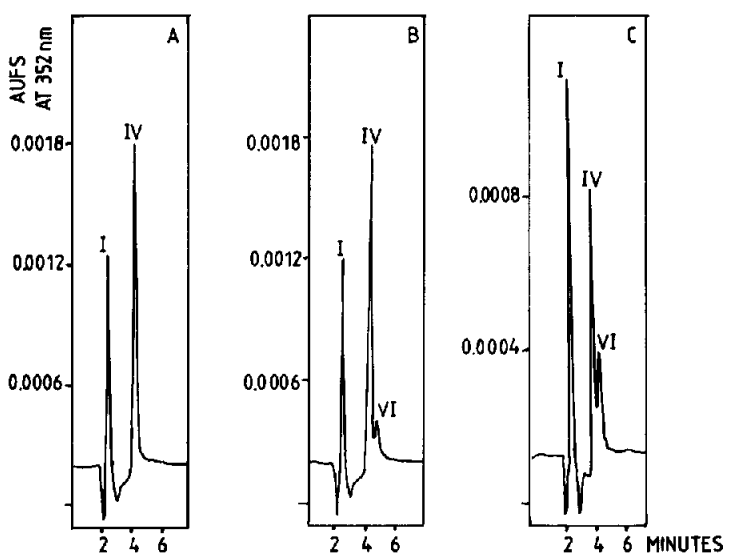


Fig. 3. HPLC of minocycline and its 4-epimer. Minocycline (IV) and its 4-epimer (VI) were analysed on a reversed-phase $C_{18}$ column (10 $\mu$m, 25 cm × 0.46 cm I.D.) with an isocratic eluent (25% dimethylacetamide in 0.1 *M* oxalic acid, pH 4.2, adjusted with concentrated ammonia) at a flow-rate of 1.0 ml/min (*ca.* 250 bar). (A) 0.25 $\mu$g of minocycline; (B) 0.25 $\mu$g of minocycline and 0.1 $\mu$g of 4-epiminocycline; (C) analysis of minocycline after passage through an acidic ion-exchange column (Nucleosil SA) as described in the literature [2] and in Experimental after extraction from the eluent by dichloromethane; chromatography of minocycline extracted from an aqueous solution by dichloromethane yielded the same chromatogram as shown in A.

enantiomers or degradation products formed under the very acidic conditions of the Nucleosil SA material (the structures of these compounds were not further investigated) (Fig. 3).

After all the purification steps and intensive drying under high vacuum at ambient temperature, the initially eluted compound (which was also synthesized by the Udenfriend reaction) gave a dark brown powder, soluble in water, with an absorption maximum at 344 nm and a broader peak at 270 nm. The ratio of the maximal absorbancies was 1.1 (270/344).

The second fraction eluted just prior to the parent compound could be isolated only from urine samples, and very small amounts were purified as a material eluting as a single peak. The absorption behaviour of this metabolite (the two maxima at 270 nm and 352 nm, respectively, and the ratio of the maximal absorbancies) was identical with that of minocycline itself. From the absorption data the extinction coefficient was calculated in comparison to the parent compound

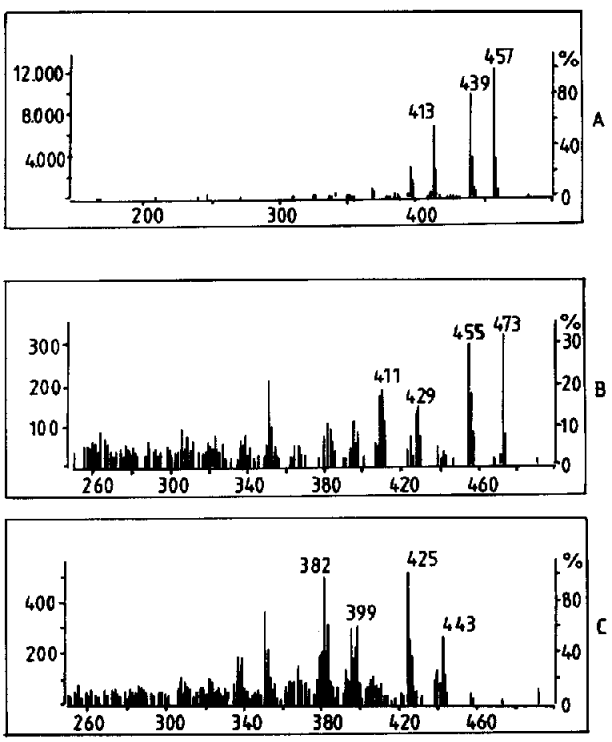


Fig. 4. Mass spectral analysis of minocycline and its metabolites. The analysis was performed by the LC–MS coupled system described in Experimental, and the spectra were recorded in the NCI mode. The left axis shows the absolute count number and the right axis the percentage in relation to the most intense signal. (A) Minocycline hydrochloride (reference compound); (B) metabolite 1: the additional mass of 16 indicates the introduction of an oxygen atom; (C) metabolite 2: the loss of mass 14 indicates the abstraction of a methyl group.

and used to quantify this metabolite (as done in the previous quantification method for the metabolite of doxycycline [3]).

MS data for the metabolites and for minocycline itself could be obtained only from LC–MS coupled analyses. In the direct probe inlet mode, the compounds decomposed during the heating period (under electron-impact or chemical-ionization mode) and a mass corresponding to the molecular ion could not be obtained. The best results were seen in the NCI mode of the LC–MS method. The PCI mode could also be employed for mass analysis but with a sensitivity about ten-fold less than that of the NCI mode. The molecular ion of metabolite 1 (Fig. 4B) was 473 compared with 457 for minocycline (Fig. 4A), with some characteristic fragments (mass 455, loss of water, mass 429, loss of a dimethylamino moiety; mass 411, loss of water and the dimethylamino moiety). This additional mass of 16 can be explained by the addition of oxygen to give the aromatic hydroxylated product. Metabolite 2 (eluted just prior to minocycline) displayed a molecular ion of 443 due to loss of a methyl group and also peaks due to some characteristic losses (mass 425, loss of water; 399, loss of a dimethylamino moiety; 382, loss of both groups) (Fig. 4C). This metabolite might be N-demethylated minocycline

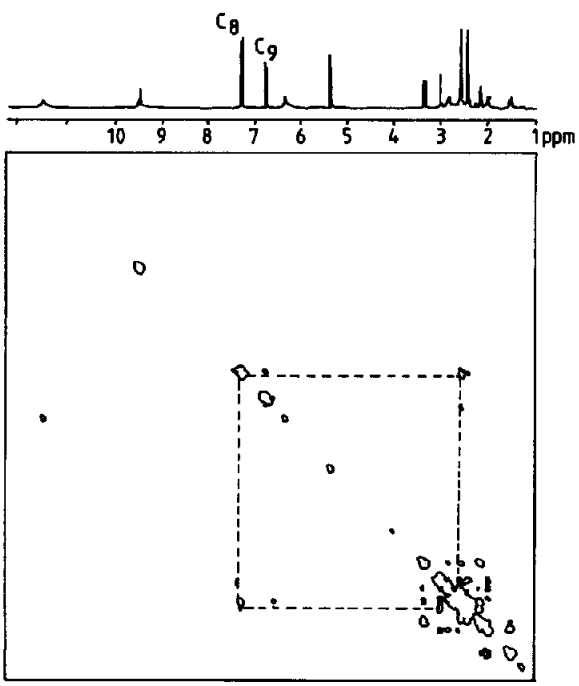


Fig. 5. $^1$H NMR analysis of minocycline (NOESY). Minocycline (*ca.* 2 mg) was dissolved in 0.4 ml of $CD_2Cl_2$ and measured on a 400-MHz instrument. The signals of the two aromatic protons appear at *ca.* 6.8 and 7.2 ppm. The NOESY contour plot indicates close spatial contacts of the aromatic proton with nearby methyl groups (indicated by off-diagonal cross peaks).

(demethylation at the 4 position of the dimethylamino moiety), because it was not microbiologically active and it is known that the intact 4-dimethylamino moiety is necessary for the microbiological activity of tetracyclines [17,21] as well as its normal configuration [22]. Metabolite 1 showed a residual microbiological activity of 12–15% (in comparison with the parent drug).

The $^1$H NMR spectrum (400 MHz) of minocycline obtained in $CD_2Cl_2$ shows the presence of two aromatic protons, resonating at *ca.* 6.8 and 7.2 ppm (Fig. 5). In the corresponding NOESY spectrum, which is indicative of close spatial contacts, one of these signals exhibits a cross peak to the signals of the spatially close $N(CH_3)_2$ group at 2.5 ppm (Fig. 5). By contrast, only one aromatic proton at *ca.* 7.0 ppm is observed for the hydroxylated compound (Fig. 6). The NOESY spectrum of this species shows a cross peak between the aromatic proton and neighbouring dimethylamine protons at 2.6 ppm. This is in good agreement with the structural data in the literature for other tetracycline structures [23]. Thus, as suggested by Nelis and De Leenheer [1,2], position 9 of the molecule is proved to carry the OH substituent, which can now form the chinone structure under alkaline conditions, as seen in the investigations of the absorption behaviour.

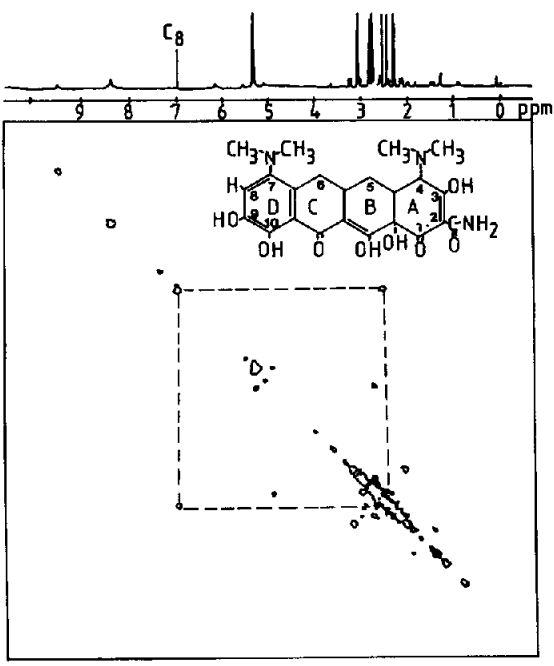


Fig. 6. $^1$H NMR analysis of 9-hydroxyminocycline (NOESY). Hydroxyminocycline (*ca.* 1 mng) was dissolved in $CD_2Cl_2$ and analysed as in Fig. 4. The signal of the atomatic proton at 7 ppm exhibits a cross peak to the signal of the $N(CH_3)_2$ group at 2.5 ppm (NOESY contour plot). This indicates close proximity of the involved nuclei.

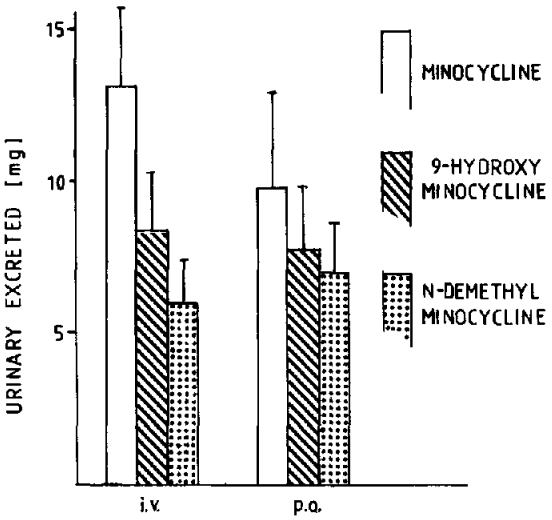


Fig. 7. Renal excretion of minocycline and its metabolites within 96 h after intravenous and peroral application. A dose of 100 mg of minocycline was given either intravenously or orally to three male volunteers (after a wash-out period of ten days). The mean amounts (± S.D., $n = 3$) excreted are shown for the unchanged drug and its two principal metabolites.

Within 96 h after the oral or intravenous administration of 100 mg of minocycline, 10–12% of the unchanged drug was renally excreted in three male volunteers, which is in good agreement with previous results [9,24–26]. An additional 8% of minocycline was found as the hydroxylated form, and 6–8% as the demethylated form, in urine collected up to 96 h after the administration of minocycline (Fig. 7). Therefore a total of up to 28% of the dose applied was renally excreted. As incubation of minocycline in urine stored at 37°C for up to 36 h did not result in any changes of the chromatographic or absorbance behaviour, the metabolites detected must have arisen from an active metabolic conversion in the body. The additional 16% of actively metabolized minocycline (with lower microbiological activity) might explain the lack of recovery of this tetracycline described in former publications [9,24–26] when the analyses were done with the microbiological assay.

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