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ANALYSIS AND QUANTITATION OF A METABOLITE OF DOXYCYCLINE IN MICE, RATS, AND HUMANS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. BÖCKER

Institut für Pharmakologie und Toxikologie der Universität Erlangen-Nürnberg, Universitätsstrasse 22, D-8520 Erlangen (G.F.R.)

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SUMMARY

A metabolite of doxycycline has not previously been isolated. In this paper it is demonstrated that doxycycline is metabolized in mice, rats, and humans. By means of high-performance liquid chromatography and consecutive gel chromatography one metabolite of doxycycline was isolated from animal organs and human urine. The metabolite was tentatively identified as N-monodemethyldoxycycline by mass spectral and spectrophotometric analyses. The rate of metabolism could be enhanced by pretreatment of the animals with phenobarbital, an inducing agent of the drug-metabolizing enzymes.

INTRODUCTION

Except for the lipophilic tetracycline derivative minocycline [1], metabolites of tetracycline antibiotics have not previously been isolated from humans or experimental animals. A decrease in the half-life of the lipophilic tetracycline derivative doxycycline was observed following coadministration of enzyme-inducing substances such as barbiturates [2], anti-epileptics [3, 4] and also of ethanol [5]. These findings could not be related to an increased metabolism of doxycycline since a metabolite of doxycycline could not be detected in human urine or feces [6].

In the present paper the analysis and isolation of a metabolite of doxycycline is described. It is performed by high-performance liquid chromatography (HPLC) and gel chromatography.

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MATERIALS AND METHODS

Animals

Female mice weighing 24–26 g and female albino Wistar rats weighing 180–220 g were used for the experiments. They were housed in plastic cages on soft wood bedding at 25° C room temperature under a 12-h dark–light rhythm. The animals had free access to standard laboratory diet (Herilan, Eggersmann, Rinteln, G.F.R.) and tap water.

Treatment of animals

To induce the drug-metabolizing enzymes the animals were pretreated intraperitoneally with phenobarbital (35 μg/g in 10 μl) twice a day for three days. Control animals received the same volume of saline. At the fourth day each animal received 50 μg/g doxycycline in 10 μl of 1.5 mmol/l $MgSO_4$. The mice were killed after 6 h (ten mice per group) and the rats after 10 h (four animals per group). Livers and kidneys were rapidly dissected and kept frozen at −70° C until preparation. The organs for HPLC analysis were prepared as described earlier [7, 8].

Human experiment

For analysis of doxycycline from human urine 2 × 100 mg doxycycline capsules were given to a healthy volunteer (male, 36 years, 72 kg) in the evening. Urine was collected for the following 16 h (980 ml); it was kept cold and freeze-dried. The residue of 200 ml urine was dissolved in 2 ml of water, filtered and prepared for HPLC analysis as described for the analysis of serum [8].

Chemicals

All chemicals used were of analytical grade except for acetonitrile which was of the grade "for residue analysis" (Merck, Darmstadt, G.F.R.). Doxycycline and 4-epi-doxycycline were kindly provided by Pfizer (Karlsruhe, G.F.R.).

Chromatography

For analytical HPLC a reversed-phase system as previously reported [8] was used with slight modifications. The HPLC apparatus consisted of a Waters 6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.) with the automatic sample injection system ASI 45 (Kontron Analytik, Eching, G.F.R.) with a 0.05-ml sample loop. The separation was performed on a column (25 × 0.4 cm) packed with Nucleosil C_8, 10 μm (Macherey & Nagel, Düren, G.F.R.) with a flow-rate of 1.9 ml/min (about 140 bars) at room temperature. The column was conditioned for at least 6 h with 3.5 mmol/l NaH_2PO_4 in water–acetonitrile (65:35) with the pH adjusted to 2.7 with H_3PO_4. The separation was performed with 3.5 mmol/l NaH_2PO_4 in water–acetonitrile (70:30) with the same pH and at the same flow-rate. After each separation the column was equilibrated again with the conditioning eluent for at least 10 min. The eluent was monitored in an LC 720 liquid chromatography ultraviolet (UV) detector (Kontron). The absorption was read at 344 nm (one absorption maximum of doxycycline), 370 nm, or 380 nm (67% and 41% of

the absorption at 344 nm, respectively) to eliminate the absorption of interfering endogenous substances from the organs and from urine. The absorption was automatically recorded on a C-RIA printer plotter (Shimadzu, Kyoto, Japan).

For semipreparative HPLC separations a column (25 × 1 cm) was packed with Nucleosil C_8, 10 μm. The column was conditioned and run with the same eluents as described for analytical separations at a flow-rate of 5 ml/min (about 120 bars). Samples of 1 ml were applied to the column.

Further purification of the docycycline metabolite was performed by gel chromatography (Sephadex G-15, 30 × 1.5 cm; Pharmacia, Uppsala, Sweden). Pooled fractions of doxycycline and its metabolite from 30—40 separations by the semipreparative HPLC were collected and freeze-dried. The residue was dissolved in 1—2 ml water and applied to the column. The column was conditioned and eluted with double-distilled water at 4°C with a flow-rate of 45 ml/h. The effluent was monitored at 280 nm in a UV absorbance monitor Model UA 5 (Isco, Lincoln, NE, U.S.A.) and automatically recorded. The peak fractions were combined and freeze-dried. The residue was used for UV analysis, fluorimetric analysis and for mass spectrometry.

UV analysis

A double-beam spectrophotometer (Beckman Model 5230; Beckman Instruments, Irvine, CA, U.S.A.) was used. UV spectra of doxycycline and its metabolite isolated from organs or urine were recorded in 0.1 mol/l HCl, alkali [addition of 0.05 ml of 32% (w/v) NaOH to 1 ml of the acidic solution] and alkali plus calcium ions [addition of 0.02 ml of a 1% (w/v) $CaCl_2$ solution to the basic solution].

Fluorimetry

Fluorimetric analyses were performed with an SFM 23 LC spectrofluorimeter (Kontron) fitted with a Phillips 150-W xenon arc lamp, and grating excitation and emission monochromators. Fluorescence spectra were obtained according to the method of Kohn [9].

Mass spectrometry

A Varian Model MAT 312 (Varian, Bremen, G.F.R.) was used to obtain direct probe mass spectra. In the electron impact mode the mass spectrometer was operated at 70 eV.

The residues of the freeze-dried fractions from gel chromatography were dissolved in small volumes of methanol and transferred into the analytical system. The mass spectrometric analyses were performed at the Institute of Organic Chemistry (Prof. Dr. G. Spiteller) of the University of Bayreuth (G.F.R.).

RESULTS AND DISCUSSION

In Fig. 1 the analytical separation of a liver extract of a control mouse (Fig. 1A) and a phenobarbital-pretreated mouse (Fig. 1B) is shown 6 h after application of 0.05 mg/g doxycycline. At 0.28 min prior to doxycycline a substance

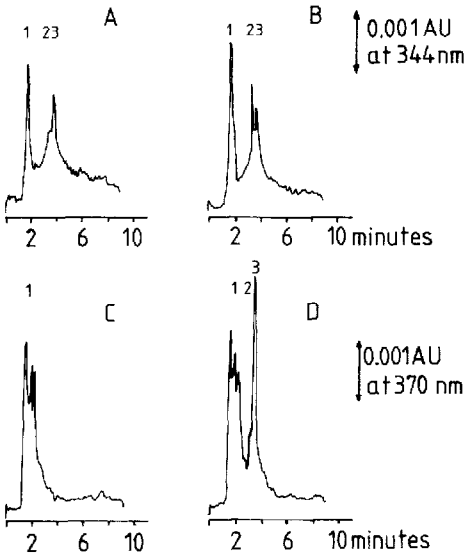

Fig. 1. Chromatographic analysis of doxycycline and its metabolite on RP C_8, 10 μm. Chromatographic conditions: column 25 × 0.4 cm; eluent, 3.5 mmol/l NaH_2PO_4 in water—acetontirile (70:30), pH 2.7; flow-rate, 1.9 ml/min; sample volume, 0.05 ml; detection, at 344 nm (A and B) and 370 nm (C and D); detection limit for doxycycline = 2—3 ng per injection. (A) Liver extract of a control mouse, 6 h after intravenous application of 50 μg/g doxycycline. (B) Liver extract of a phenobarbital pretreated mouse, 6 h after intravenous application of 50 μg/g doxycycline. (C) Human blank urine, collected before application of the drug. (D) Human urine, 16 h after ingestion of 2 × 100 mg doxycycline capsules. Peaks: 1 = solvent front; 2 = metabolite of doxycycline; 3 = doxycycline.

was eluted. Its amount increased ten times after phenobarbital pretreatment compared to the control. The mean values of the organ concentration are given in Table I. When female rats received doxycycline the metabolite could also be identified and its amount increased also after induction of the drug-metabolizing enzymes.

When human urine was analyzed after a single oral dose of 200 mg of doxycycline, 15.5 mg of doxycycline were excreted during the first 16 h. By HPLC analyses at different wavelengths (344 nm, 370 nm, 380 nm) the occurrence of the metabolite could be demonstrated (Fig. 1C and D).

The chromatographic and spectrophotometric behaviour of the doxycycline metabolite from mice, rats, and human urine were identical.

To isolate the metabolite of doxycycline the extracts of livers and kidneys of phenobarbital-pretreated mice were chromatographed on a semipreparative reversed-phase column. Doxycycline and its metabolite were eluted after 4.03 ± 0.08 min (doxycycline) and 3.77 ± 0.07 min (metabolite), respectively.

Since endogenous substances were eluted from the column together with doxycycline and its metabolite further purification was necessary for consecutive analyses. It was performed by gel chromatography (Fig. 2A). When re-chromatographed on the analytical HPLC system the substance of peak 3 of the gel chromatography coincided with doxycycline and the compound of peak 2 of the gel chromatography coincided with the metabolite (Fig. 2B).

TABLE I

CONCENTRATION OF DOXYCYCLINE AND ITS METABOLITE IN LIVER AND KIDNEY OF FEMALE NMRI MICE AND FEMALE WISTAR RATS AFTER INTRAVENOUS INJECTION OF 0.05 mg/g DOXYCYCLINE IN A CONTROL EXPERIMENT AND AFTER INDUCTION OF THE DRUG-METABOLIZING ENZYMES WITH PHENOBARBITAL

The data, expressed as mean ± S.D. (n = 10 for mice and n = 4 for rats), are corrected for the organ's blood content. The variance of the data is due to the biological variance because the method has a standard deviation of between 4 and 5% only [8]

		Doxycycline (μg/g)		Metabolite (μg/g)	
		Control	Phenobarbital	Control	Phenobarbital
Mouse, 6 h post injection	Liver	44.3 ± 16.5	33.6 ± 18.3	2.9 ± 2.8	48.1 ± 23.6
	Kidney	88.6 ± 21.3	43.4 ± 18.9	10.9 ± 2.1	100.8 ± 26.1
Rat, 10 h post injection	Liver	174.6 ± 32.4	92.2 ± 24.4	2.6 ± 2.5	7.8 ± 4.1
	Kidney	304 ± 62.9	168.3 ± 33.5	4.9 ± 3.8	70.7 ± 24.6

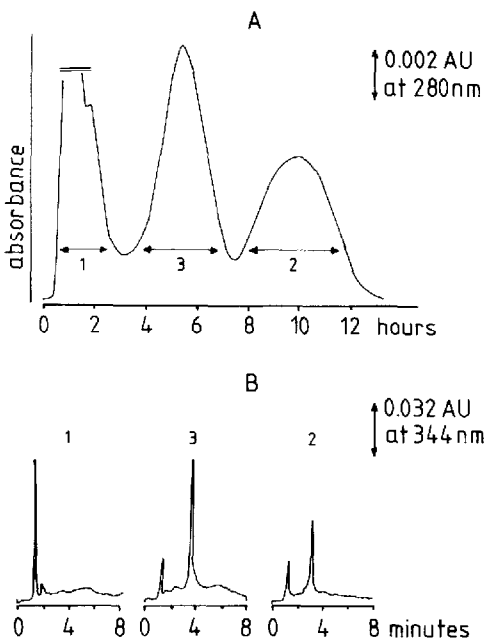

Fig. 2. Purification of doxycycline and its metabolite from livers of mice pretreated with phenobarbital, by gel chromatography (A) and analytical control by HPLC (B). (A) Gel chromatography: column 30 × 1.5 cm, packed with Sephadex G-15, particle size 40—120 μm; eluent, double-distilled water; flow-rate, 45 ml/h; detection at 280 nm. Separation of freeze-dried fractions of doxycycline and its metabolite after semipreparative HPLC of liver extracts of phenobarbital-pretreated mice, 6 h after application of 50 μg/g doxycycline. Peak 3 corresponds to 30 μg of doxycycline and peak 2 to 15 μg of metabolite (spectrophotometric quantitation after freeze-drying of the fractions indicated). (B) HPLC conditions were as described in the legend of Fig. 1 and in Materials and methods. The residues of peak 2 and peak 3 material were dissolved in water and 50 μl were applied to the analytical HPLC column.

The metabolite is not the 4-epi-doxycycline because this compound showed nearly the same retention time as doxycycline in this analytical system and a conversion between doxycycline and its metabolite did not occur in the acidic eluent even after 2 h. Additionally, the formation of the metabolite was enhanced by phenobarbital pretreatment, a typical inducing agent of the drug-metabolizing enzymes, and epimerization is normally not catalyzed by enzymes.

The metabolite displayed UV and fluorescence characteristics nearly identical with those of doxycycline (Table II). Addition of sodium hydroxide or calcium ions to the acidic solution of doxycycline and its metabolite, respectively, resulted in a bathochromic shift of the 344 nm peak. These effects are highly characteristic for tetracyclines. They are associated with enolization of the chromophore located on rings B, C and D (Fig. 3) [10, 11]. Both the metabolite and doxycycline itself showed an excitation maximum at 398 nm and an emission maximum at 536 nm in the fluorescence analysis.

TABLE II

UV CHARACTERISTICS OF DOXYCYCLINE AND ITS METABOLITE, EXTRACTED FROM LIVER AND KIDNEY OF MICE PRETREATED WITH PHENOBARBITAL

	λ_{max} (nm) in:		
	0.1 mol/l HCl	NaOH	NaOH—Ca^{2+}
Doxycycline*	267, 344	374	390
Metabolite	265, 344	371	380

*Reference standard as well as extracted from liver and kidneys.

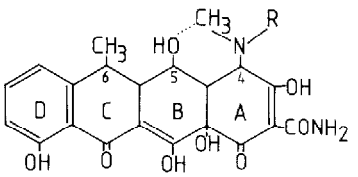

Fig. 3. Configuration of doxycycline (R = CH_3) and its metabolite (R = H). The methyl group (R = CH_3) becomes susceptible to enzymatic cleavage because the structure is fixed by a hydrogen bond between the hydroxyl group at position 5 and one methyl group of the dimethylamino group (indicated by the dotted line). By this means the methyl group at positon 6 is sterically hindered against enzymatic cleavage.

The metabolite is most probably a demethylated derivative of doxycycline as evidenced from its mass spectrum. The electron-impact (EI) spectrum (Fig. 4C) yielded a molecular ion (M^+) at *m/z* 430 compared to a molecular ion (M^+) at *m/z* 444 for the standard, which means loss of a methyl group. Nearly identical mass spectra were obtained from peak 3 material of the gel chromatography (Fig. 4B) and the doxycycline standard. The maxima of molecular ions were observed at the same temperature in the programme-controlled evaporation of the sample for doxycycline and its metabolite.

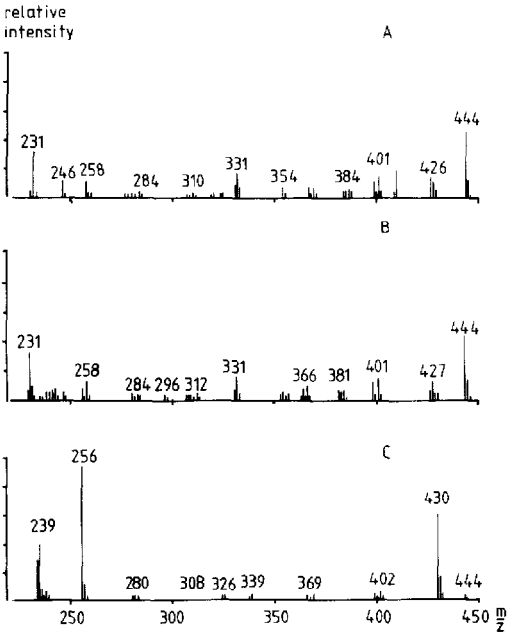

Fig. 4. EI mass spectral analysis of doxycycline standard (A), doxycycline extracted from liver (B), and the metabolite of doxycycline extracted from liver (C). The sensitivity of (C) is ten times the sensitivity of (A) and (B). The molecular ion M^+ *m/z* 444 of doxycycline accounts for about 25% relative intensity in (A) and (B) and about 3% in (C) attributable to impurities. For further details see Materials and methods.

Further analytical investigations were not possible because of the limited amounts of material available and the presence of impurities.

Since the UV and fluorescence characteristics of doxycycline and its metabolite were nearly the same, the absorption at 344 nm was taken for quantitation of the metabolite. Based on the assumption of comparable extinction coefficients the concentration of the metabolite was determined in livers and kidneys of mice and rats, and in human urine.

In the kidneys of mice the concentration of the metabolite was about twice the concentration in liver after phenobarbital pretreatment. In the kidneys of rats the concentration of the metabolite was about ten times the concentration in liver (Table I). In control mice about 5—10% of "doxycycline" was analyzed as metabolite while in phenobarbital-pretreated mice at the same time after application 50—70% of the totally analyzed drug was found as metabolite.

In human urine a total of 50 μg of metabolite could be detected together with 15.5 mg of unchanged doxycycline (i.e. 7.75% of the dose applied). From the present analytical data it is concluded that one of the methyl groups of the dimethylamino group is enzymatically cleaved off. The methyl group at position 6 is protected against enzymatic cleavage by steric hindrance because the configuration of the doxycycline molecule is stabilized by a hydrogen bond between the hydroxyl group at position 5 and one methyl group of the dimethylamino group [12] (Fig. 3). In this configuration one methyl group of the dimethylamino group becomes susceptible to enzymatic cleavage.

This appears to be the first time that a metabolite of doxycycline has been isolated. The results are in some contrast to observations by Nelis and De Leenheer [6] who stated that doxycycline was metabolically inert. In the chromatographic system used by these authors two peaks were analyzed as "unidentified by-products of doxycycline" and it might be possible that one of those peaks corresponds to N-monodemethyldoxycycline.

On the other hand, the occurrence of an N-monodemethyl derivative would be in good accordance with the recently described metabolism of minocycline, a tetracycline derivative which is nearly as lipophilic as doxycycline [1].

The method presented is suited for studies of doxycycline metabolism in animals and humans, especially to clarify the importance of doxycycline demethylation for shortening the half-life of doxycycline in man after premedication with drugs which induce the drug-metabolizing enzymes [2—4].

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