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Isolation and identification of 8-hydroxypraziquantel as a metabolite of the antischistosomal drug praziquantel

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Abstract

Praziquantel, a broad spectrum anthelmintic drug, is extensively metabolized in the liver, yielding mainly monohydroxylated and dihydroxylated phase-I metabolites. However, the exact chemical structure of most metabolites is still unknown. One of these unidentified phase-I metabolites was isolated from human urine by high performance liquid chromatography using an isocratic separation method. This metabolite was identified as 8-hydroxypraziquantel. For the structure elucidation, electrospray ionization–mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy have been used.

Keywords: 8-Hydroxypraziquantel; Praziquantel; Phase-I metabolites; Electrospray ionization–mass spectrometry; NMR

1. Introduction

Praziquantel (PZQ), 2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]*iso*-quinoline-4-one (Fig. 1), is an anthelmintic drug with a broad activity against trematodes and schistosomes. It is included in the WHO Model List of Essential Drugs and it is the drug of first choice in the treatment of schistosomiasis. According to the WHO, an estimated of 200 million people are infected with schistosomiasis [1].

PZQ is used in therapy as its racemate although the anthelmintic activity is mainly associated with the *R*-(−)-enantiomer [2,3]. The drug undergoes extensive metabolism, yielding numerous monohydroxylated and dihydroxylated metabolites. Also, polyhydroxylated metabolites have been described [4–6]. The hydroxylated metabolites are also excreted in the urine as conjugates with glucuronic acid and sulphuric acid [6,7].

Up to now, only the monohydroxylated metabolites *trans*-4′-hydroxypraziquantel and *cis*-4′-hydroxypraziquantel have been identified. *Trans*-4′-hydroxypraziquantel is the main metabolite in humans. Recently, MEIER [6] and LERCH [8] made proposals for the chemical structure of other metabolites but their exact chemical constitution is still unknown.

One of these unidentified phase-I metabolites was isolated from human urine by using a semipreparative RP-18 column. A solid phase extraction was performed to preconcentrate the

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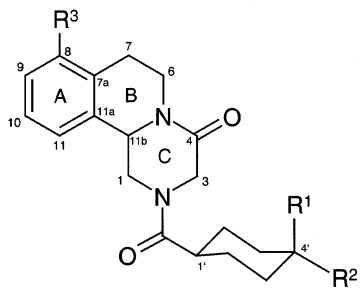
sample and remove salts and urinary matrix compounds. We were able to identify the metabolite as 8-hydroxypraziquantel (8-OH-PZQ) by electrospray ionization–mass spectrometry (ESI–MS), ^{1}H and ^{13}C NMR spectroscopy.



	R^1	R^2	R^3
praziquantel	H	H	H
trans-4'-hydroxypraziquantel	H	OH	H
cis-4'-hydroxypraziquantel	OH	H	H
8-hydroxypraziquantel	H	H	OH

Fig. 1. Structures of praziquantel (PZQ) and its metabolites *trans*-4′-hydroxypraziquantel, *cis*-4′-hydroxypraziquantel and 8-hydroxypraziquantel (8-OH-PZQ).

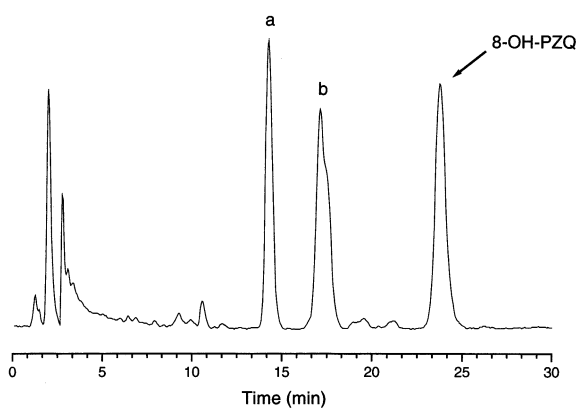


Fig. 2. HPLC chromatogram of a human urine sample (1–6 h) after oral administration of 900 mg racemic PZQ. Column: LiChrospher® 100 RP-18 (5 μm), 4 × 250 mm; mobile phase: acetonitrile/water (28/72 v/v); flow: 1.0 ml/min; injection: 20 μl; UV detection (210 nm).

2. Experimental

2.1. Chemicals and reagents

Racemic PZQ and PZQ tablets (Cesol®) were obtained from Merck (Darmstadt, Germany). Acetonitrile, methanol (both of HPLC grade) and hydrochloric acid were from different commercial sources and used without further purification.

LiChrospher® 100 RP-18 (5 μm) material used for the filling of the columns and LiChroprep® RP-18 (40–63 μm) material used for the solid phase extraction were gifts from Merck (Darmstadt, Germany). $CDCl_3$-d_1 was also purchased from the same company.

2.2. Sample pretreatment

The urine sample was collected 1–6 h after oral administration of 900 mg racemic PZQ (Cesol®). To obtain the phase-I metabolites of PZQ, the urine sample (≈ 400 ml) was hydrolyzed with 1 N HCl at 80 °C for 1 h.

The solid phase extraction was performed with LiChroprep® RP-18 (40–63 μm) material. The RP-18 material was conditioned with methanol and equilibrated with double-distilled water. After loading the urine sample, salts and hydrophilic urinary matrix compounds were washed from the column with double-distilled water. The analytes were eluted with different methanol/water mixtures (100 ml each) of increasing eluotropic strength (10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20, and 100/0 v/v). The fraction eluted with 60% methanol and 40% water was analyzed and used for the isolation of 8-OH-PZQ. Prior to the isolation, the methanol was evaporated under reduced pressure.

2.3. Analytical separation with LC–ESI–MS-coupling

The analytical separation of the PZQ phase-I metabolites was carried out on a LiChrospher® 100 RP-18 column (particle size 5 μm, dimension 250 × 4 mm) connected with a LiChrospher® 100 RP-18 guard column (particle size 5 μm, dimension 4 × 4 mm). The mobile phase consisted of

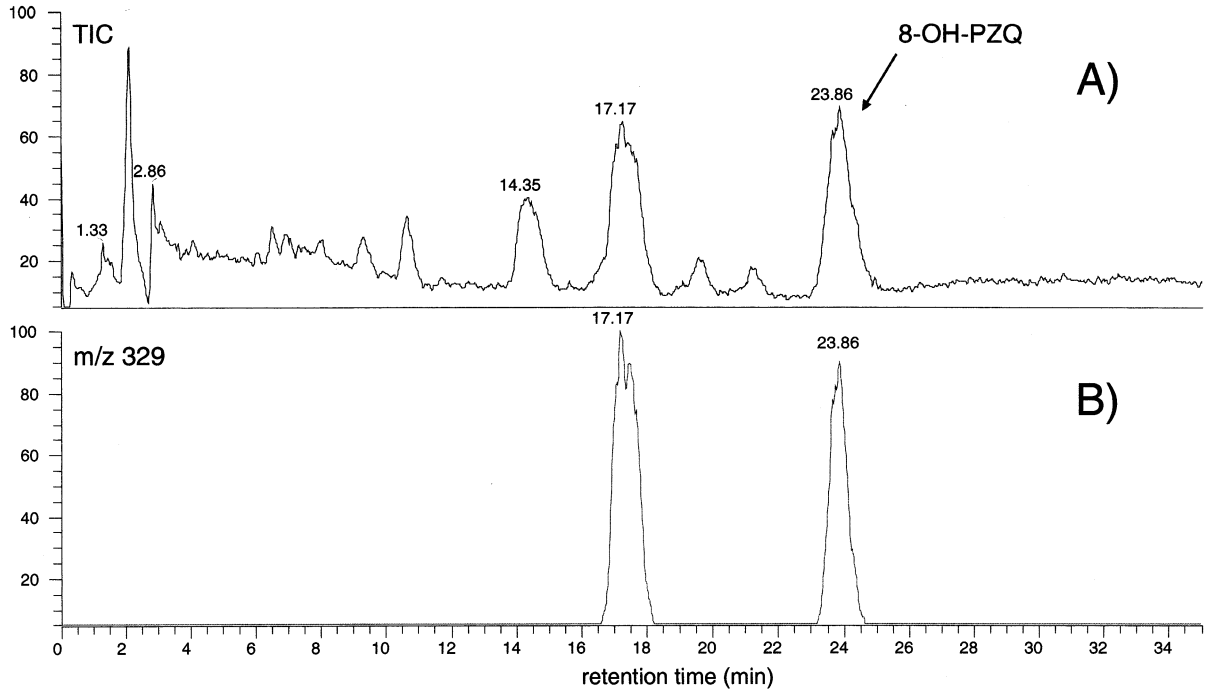


Fig. 3. HPLC–ESI–MS chromatogram of a human urine sample (1–6 h) after administration of 900 mg racemic PZQ. (A) Total-ion-current (TIC), and (B) selected mass track $m/z = 329$. Column: LiChrospher® 100 RP-18 (5 μm), 4 × 250 mm; mobile phase: acetonitrile/water (28/72 v/v); flow: 1.0 ml/min; injection: 20 μl; detection: ESI–MS; conditions: positive polarity; source voltage: 4 kV; sheath gas flow: 80 arbitrary units; auxiliary gas flow: 20 arbitrary units; heated capillary: 250 °C; mass range: $m/z = 200$ to 600.

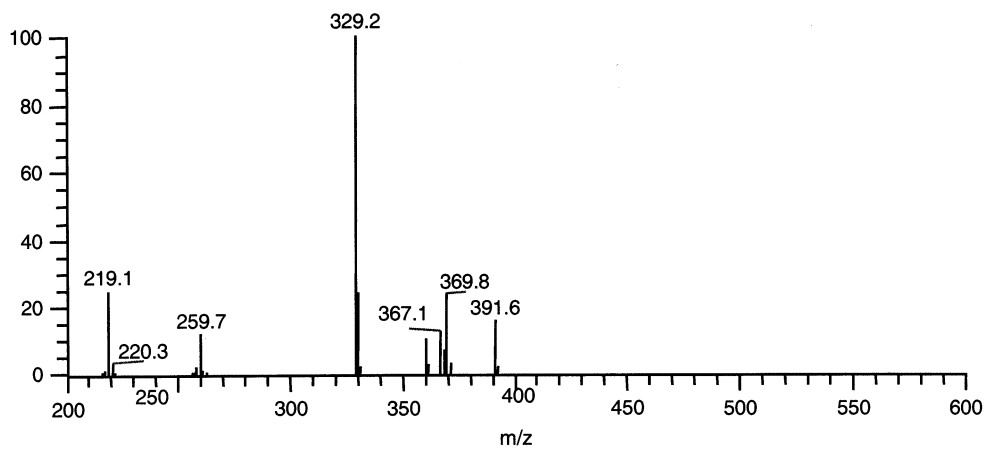


Fig. 4. Mass spectrum of the peak at 23.86 min. Conditions: see Fig. 3.

acetonitrile and double-distilled water (28/72 v/v) at a flow-rate of 1.0 ml/min. The injected volume was 20 μl. The chromatographic system consisting of a Waters 2690 separations module (Waters, Milford, MA) was coupled on-line to the LCQ® ion trap mass spectrometer (Thermo-Finnigan,

San Jose, CA) via an ESI-interface. The ESI voltage was set to 4 kV, the sheath gas flow was adjusted to 80 arbitrary units and the auxiliary gas flow was set to 20 arbitrary units. The temperature of the heated capillary was 250 °C. The mass spectrum ($m/z = 200$ to 600) was recorded in positive ion mode. For the simultaneous UV detection, the Waters 486 tunable absorbance detector (Waters) was used. The detection wavelength was set to 210 nm.

2.4. Isolation of 8-hydroxypraziquantel

The isolation of the metabolite was performed on a LiChrospher® 100 RP-18 column (particle size 5 μm, dimension 250×7 mm^2), connected with a LiChrospher® 100 RP-18 guard column. The chromatographic system consisted of a L6200-A intelligent pump, a 655-A variable wavelength UV detector and a D-2500 chromato-integrator (all devices Merck Hitachi, Tokyo, Japan). The mobile phase consisted of acetonitrile and double-distilled water (28/72 v/v) at a flow-rate of 2.5 ml/min. The detection wavelength was set to 210 nm. For each run, 1000 μl of the aqueous sample solution were injected. The fraction at a retention time of 28–33 min from ≈ 50 runs was collected and the eluent was evaporated under light vacuum. The obtained raw fraction was further purified by performing another ten runs on the same column under identical chromatographic conditions.

The ten purified fractions were combined and the acetonitrile was removed under light vacuum. The resulting aqueous sample solution was lyophilized with a Lyovac® GT 2 freeze–dryer (Finn-Aqua, Santasalo-Sohlberg Corp., Tuusula, Finland).

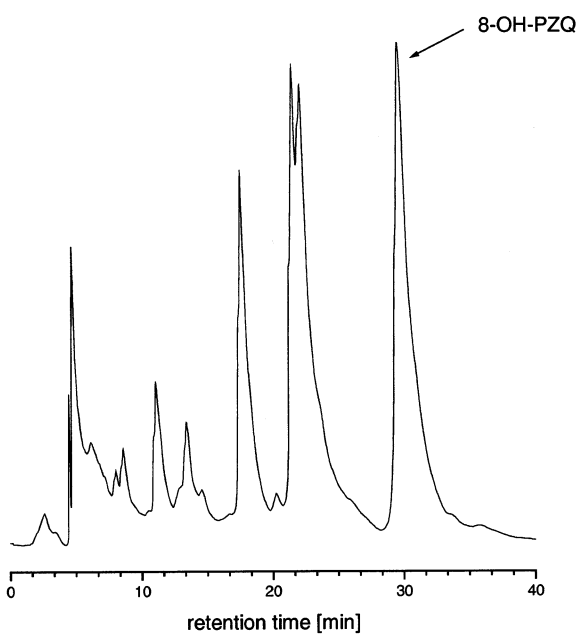


Fig. 5. HPLC chromatogram of a human urine sample (1–6 h) after oral administration of 900 mg racemic PZQ. Column: LiChrospher® 100 RP-18 (5 μm), 7×250 mm; mobile phase: acetonitrile/water (28/72 v/v); flow: 2.5 ml/min; injection: 1000 μl; UV detection (210 nm).

2.5. Characterization of 8-hydroxypraziquantel by nanospray-ESI–MS

For the recording of the mass spectrum and mass fragmentation pattern of 8-OH-PZQ, a laboratory-made nanospray interface and the LCQ® ion trap mass spectrometer (Thermo-Finnigan) were used. Approximately, 10 μl of the purified fraction of the metabolite were filled into the nanospray capillary. The electrical contact between the sample solution and the electrospray voltage was established with a thin stainless steel wire, which was introduced into the tip of the capillary. The capillary was positioned 2 mm in front of the orifice of the mass spectrometer inlet with micrometer screws. An ESI voltage of 800 V was then applied and a full scan mass spectrum (mass range 100–900) in positive ion mode was recorded.

The fragmentation pattern of 8-OH-PZQ was analyzed using the MS^n-mode of the LCQ® ion trap mass spectrometer. After the isolation and first fragmentation of the mass peak corresponding to 8-OH-PZQ ($m/z = 329$) the peak with the highest intensity (base peak) of the resulting mass spectrum was isolated and fragmented again. For the fragmentation experiments, a relative collision energy of 8–10% was used.

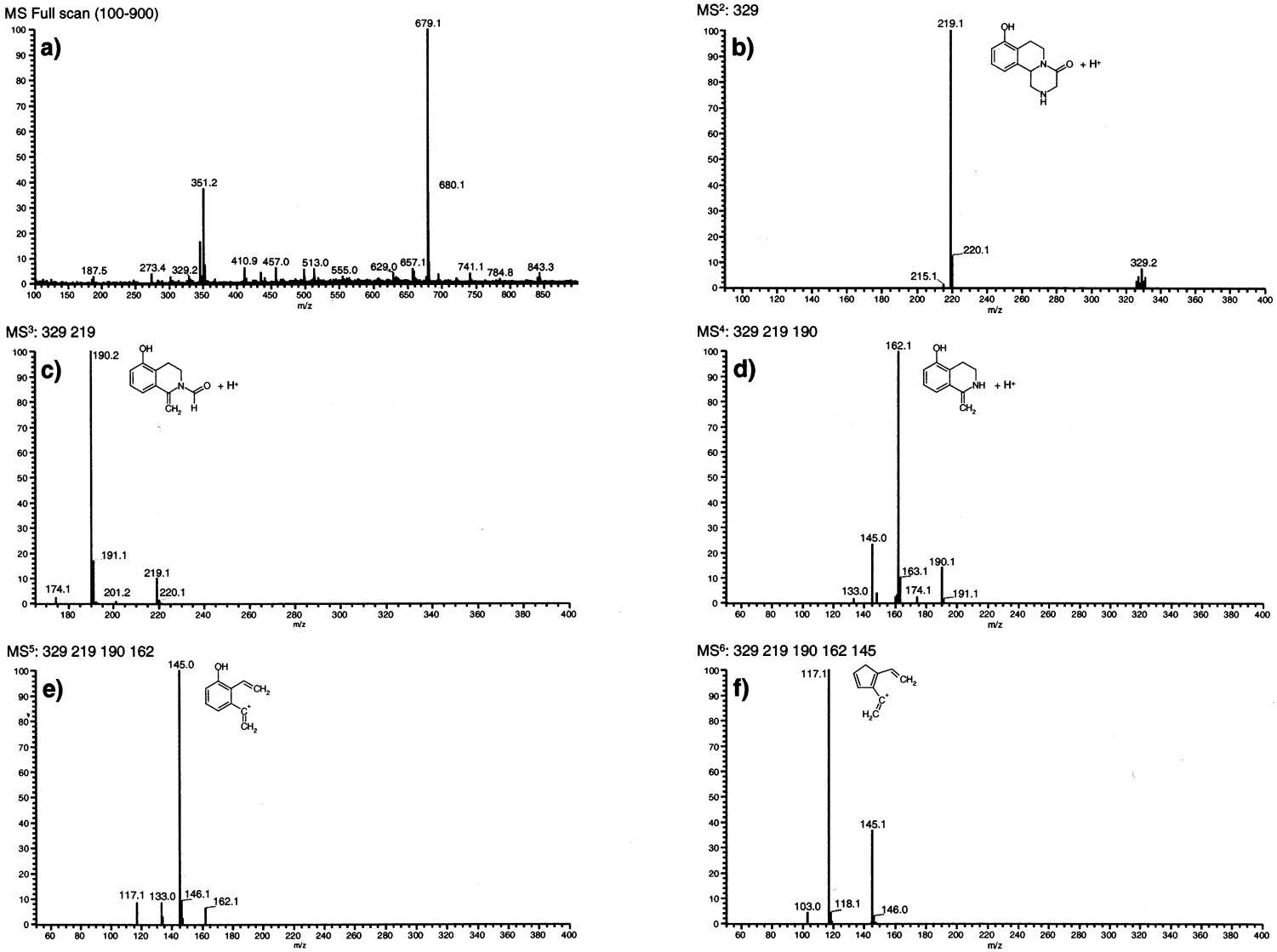


Fig. 6. Mass fragmentation pattern of 8-OH-PZQ. Interface: laboratory-made nanospray interface. ESI–MS conditions: positive polarity; source voltage: 800 V; heated capillary: 100 °C; mass range: $m/z = 100$ to 900.

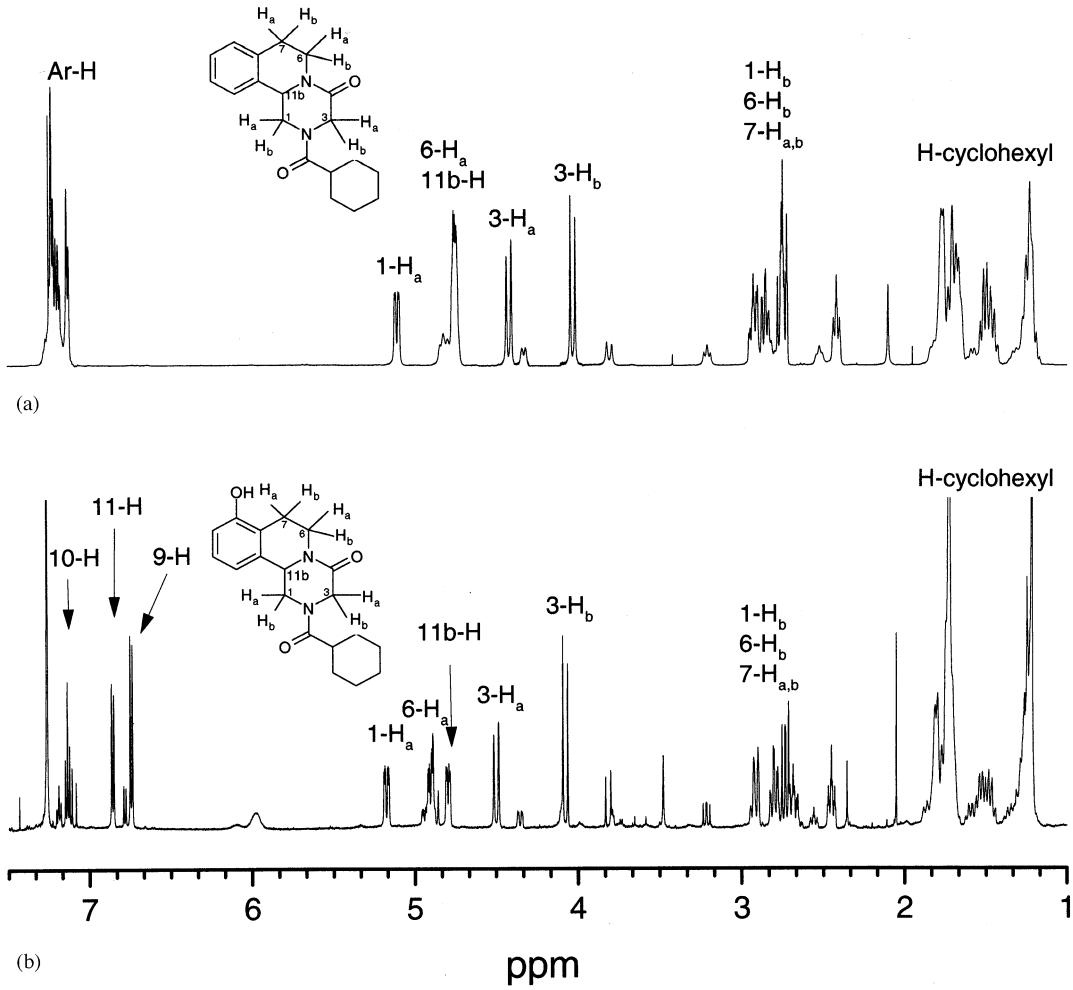


Fig. 7. ^{1}H NMR spectrum (600 MHz) of (A) PZQ, and (B) 8-OH-PZQ in chloroform-d_1.

2.6. Characterization of 8-hydroxypraziquantel by NMR spectroscopy

For the NMR experiments, a Varian Unity Plus 600 (Varian, Palo Alto, CA) was used. The ^{1}H NMR spectrum was recorded at 600 MHz and the ^{13}C NMR spectrum at 150 MHz. The whole amount of the isolated metabolite (about 0.8 mg) was dissolved in chloroform-d_1.

With this sample, a ^{1}H NMR spectrum, ^{13}C spectrum and ^{1}H, ^{13}C gradient heteronuclear multiple bond correlation (GHMBC) spectrum were recorded at a temperature of 253 K.

3. Results and discussion

3.1. Analytical separation with LC–ESI–MS-coupling

For the isolation of 8-OH-PZQ, an isocratic HPLC separation method on an analytical RP-18 column was developed. The best result was obtained with a mobile phase consisting of 28% acetonitrile and 72% double-distilled water. Thus, several unknown phase-I metabolites of PZQ could be separated in less than 25 min (Fig. 2).

Using the ion track mode of the LCQ® ion trap

mass spectrometer, it was possible to identify the peaks at the retention times of 17.17 and 23.8 min as monohydroxylated metabolites of PZQ. Their m/z of 329 ($328 + H^+$) is 16 MU higher than the m/z of 313 ($312 + H^+$) of the unchanged drug PZQ (Fig. 3).

The peak at 17.17 min (peak b) is consisting of two co-eluting metabolites. Under the chromatographic conditions used it was not possible to separate these two analytes. They both have a m/z of 329 ($328 + H^+$) so they are most likely monohydroxylated metabolites with the same retention factors under given conditions.

Fig. 4 shows the mass spectrum of the peak at 23.86 min, which is corresponding to 8-OH-PZQ. It can be seen that the peak purity is sufficient for a semipreparative isolation. The peak at $m/z = 219$ is caused by a fragmentation of the metabolite in the ESI source (cleavage of the cyclohexylcarbonyl function). The peaks at $m/z = 367$ and $m/z = 369$ are most likely adducts of the metabolite with potassium ($328 + K^+$) and acetonitrile ($328 + MeCN + H^+$), respectively.

3.2. Isolation of 8-hydroxypraziquantel

For the isolation of 8-OH-PZQ, the isocratic analytical separation method was transferred to a semipreparative HPLC-column with a diameter of 7 mm. For each run, 1000 μl of the aqueous sample solution were injected. The isocratic separation is illustrated in Fig. 5. Except for the retention times there are no significant differences to the analytical separation described above. The fraction consisting

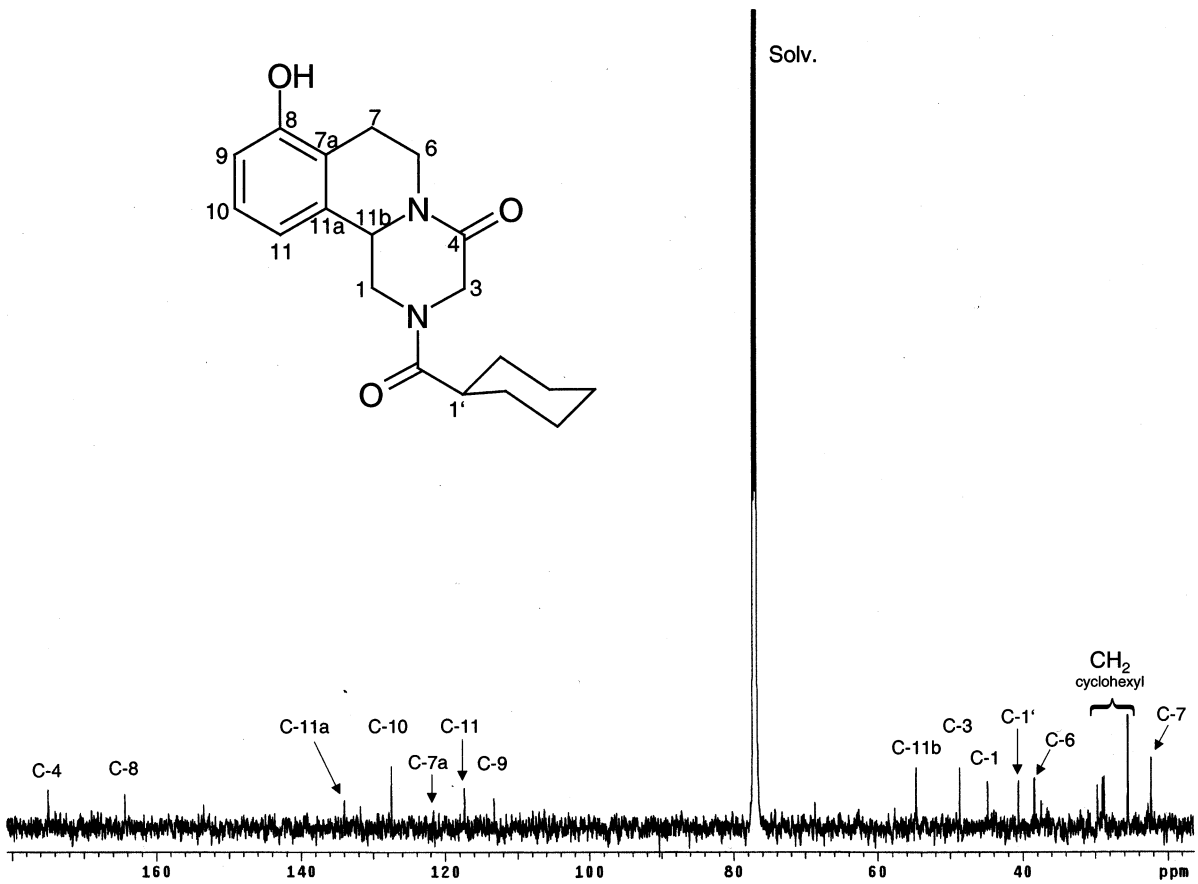


Fig. 8. ^{13}C NMR spectrum (150 MHz) of 8-OH-PZQ in chloroform-d_1.

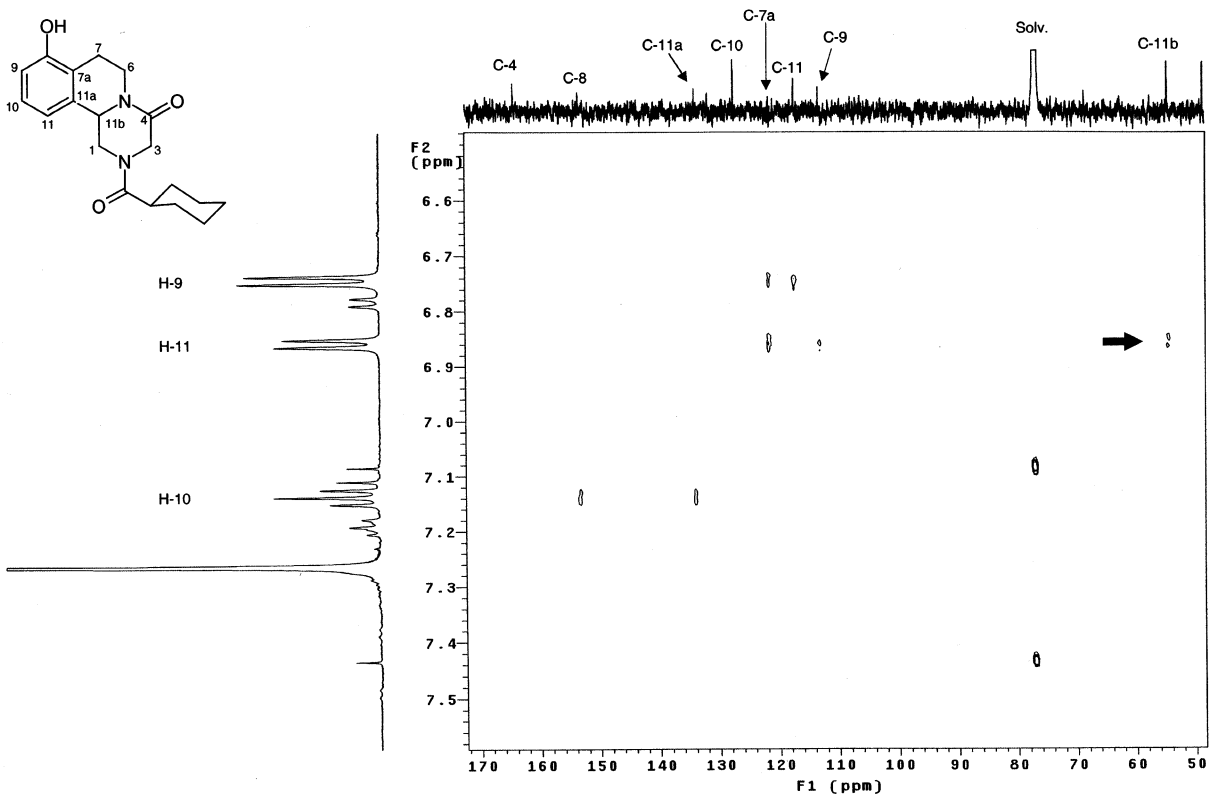


Fig. 9. GHMBC (^{1}H, ^{13}C gradient heteronuclear multiple bond correlation) spectrum of 8-OH-PZQ in chloroform-d_1.

of 8-OH-PZQ (retention time 28–33 min) from ≈ 50 runs was collected. The collected raw fraction was then further purified using the same chromatographic method. After evaporating the solvent and lyophilisation of the sample, a small amount (about 0.8 mg) of a colorless substance was obtained.

3.3. Characterization of 8-hydroxypraziquantel by nanospray-ESI–MS

A very small amount of the purified fraction was analyzed by a laboratory-made nanospray-ESI–MS interface. The resulting mass spectrum of the fractionated metabolite is depicted in Fig. 6(a). The mass peak at $m/z = 329$ is corresponding to 8-OH-PZQ. The peak at $m/z = 351$ is the sodium adduct of 8-OH-PZQ ($M + Na^+$) and the mass peak at $m/z = 679$ can be assigned to the single positively charged sodium adduct of the dimer of the molecule ($M_2 + Na^+$).

Using the MS^n-mode of the LCQ ion trap mass spectrometer, it was possible to analyze the fragmentation pattern of 8-OH-PZQ. The mass of the molecular ion ($m/z = 329$) was selected and fragmented by controlled collision with helium molecules in the ion trap (collision induced fragmentation). The peak with the highest intensity (base peak) of the resulting mass spectrum was isolated and fragmentated again. With this method, the metabolite was gradually fragmentated in five steps (MS^6) and further information about the chemical constitution of the metabolite could be obtained. The observed fragmentation products and their proposed structures are illustrated in Fig. 6(b–f).

The first fragmentation step is the cleavage of the cyclohexylcarbonyl function of the molecule (Fig. 6(b)). The mass of the remaining ring system ($m/z = 219$) is still 16 MU higher than the unchanged ring system of PZQ ($m/z = 203$). So the

hydroxyl group must be located in the heterocyclic ring system and not in the cyclohexyl ring of the molecule. The mass spectra in Fig. 6(c) and (d) show that the hydroxylation did not occur in ring C of the metabolite. The fragmentation step in Fig. 6(f) (loss of 28 amu) can be explained with an elimination of a CO group, which is often observed in mass spectra of phenolic substances [9]. This indicates that the hydroxylation most likely occurred in the aromatic ring of the molecule.

3.4. Characterization of 8-hydroxypraziquantel by NMR spectroscopy

The confirmation for the chemical constitution of 8-OH-PZQ was achieved by NMR spectroscopy. The ^{1}H NMR spectra of the unchanged drug PZQ and 8-OH-PZQ are shown in Fig. 7.

The two doublets at $\delta = 6.74$ and 6.86 in the spectrum of 8-OH-PZQ are corresponding to the protons 9-H and 11-H at the aromatic ring. The coupling constants of the doublets show a typical value for an aromatic *ortho* coupling ($^{3}J = 8$ Hz). The triplet at $\delta = 7.14$ can be assigned to the aromatic proton 10-H.

The ^{13}C NMR spectrum of 8-OH-PZQ is depicted in Fig. 8. The signal assignment (in $CDCl_3$) is as follows: δ (ppm) = 22.3 (C-7); 38.3 (C-6); 44.8 (C-1); 48.6 (C-3); 54.6 (C-11b); 113.5 (C-9); 117.4 (C-11); 121.8 (C-7a); 127.3 (C-10); 134.1 (C-11a); 164.2 (C-8); 174.8 (C-4). The signals for the C-atoms in the cyclohexyl ring appear between 24 and 30 ppm. The strong signal around $\delta = 77$ ppm is from the solvent.

The hydroxylation at position 8 of the molecule was confirmed through a GHMBC spectrum.

The GHMBC spectrum (Fig. 9) shows a correlation signal (denoted by an arrow) which results from a long-range coupling of the aromatic proton 11-H with the C-11b ($^{3}J_{HC}$). This effect is only possible, if PZQ is hydroxylated in position 8.

4. Conclusions

We were able to identify 8-OH-PZQ as a monohydroxylated phase-I metabolite of the anthelmintic drug PZQ by mass spectrometry and NMR spectroscopy. It could also be demonstrated that the step-by-step fragmentation of molecules by using the MS^n-scan mode of the ion trap mass spectrometer is a very versatile tool for the structure elucidation of unknown compounds or metabolites in bioanalysis.

Acknowledgements

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