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# Investigation of the stereoselective metabolism of praziquantel after incubation with rat liver microsomes by capillary electrophoresis and liquid chromatography–mass spectrometry

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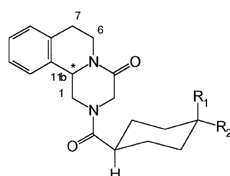
**Abstract**

Two different separation methods for the antischistosomal drug praziquantel and its metabolites by capillary electrophoresis are described. Achiral separation was obtained by micellar electrokinetic capillary chromatography using sodium dodecyl sulfate as micelle-forming surfactant. On the other hand, the negatively charged sulfobutylether-β-cyclodextrin as a chiral selector enabled the separation of the drug and its metabolites as well as their enantioseparation. Based on this separation, the enantioselectivity of the metabolism of praziquantel was studied by incubation of the drug with rat liver microsomes. Whereas *trans*- and *cis*-4-hydroxypraziquantel were mainly formed from the *R*-(−)-enantiomer, another, different monohydroxylated metabolite was only formed from the *S*-(+)-enantiomer. Information about the structure of these metabolites was obtained, using LC–MS. © 1998 Elsevier Science B.V.

*Keywords:* Enantiomer separation; Praziquantel; 4-Hydroxypraziquantel

## 1. Introduction

Praziquantel (Fig. 1), an anthelminthic drug, exhibits broad activity against trematodes and cestodes. It is used in therapy as the racemate although the anthelminthic activity is mainly associated with the *R*-(−)-enantiomer [1,2].




| | $R_1$ | $R_2$ |
|---|---|---|
| praziquantel (1) | H | H |
| trans-4-hydroxypraziquantel (2) | H | OH |
| cis-4-hydroxypraziquantel (3) | OH | H |

Fig. 1. Structure of praziquantel (1), *trans*- (2) and *cis*-4 hydroxypraziquantel (3).

The drug undergoes extensive metabolism. After in vitro incubation with rat liver microsomes several monohydroxylated metabolites are formed, with *cis*-4-hydroxypraziquantel being the main metabolite [3,4]. By incubation of each praziquantel enantiomer separately with rat liver microsomes, followed by achiral high-performance liquid chromatography (HPLC) analysis of the resulting metabolites, metabolism was shown to be highly stereoselective. However, chiral separation and analyzing the enantiomeric ratio directly could only be examined for praziquantel and its metabolite *trans*-4-hydroxypraziquantel [5].

Capillary electrophoresis (CE) has become a

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powerful separation technique for the analysis of the biotransformation of drugs [6]. The development of micellar electrokinetic capillary electrophoresis (MECC) allows the separation of neutral compounds [7]. In addition to achiral analysis, CE is also suitable for the separation of enantiomers using cyclodextrins (CDs) as chiral selectors because of the high separation efficiency [8]. Charged CDs allow the analysis of even neutral components [9].

For information about the structure of metabolites liquid chromatography–mass spectrometry (LC–MS) is a powerful method. The use of HPLC in combination with a mass spectrometer in the $MS^n$ scan mode enables the analysis of different metabolites in biological samples in one step.

This paper describes a method for the separation of praziquantel and its main metabolites, *cis*- and *trans*-4-hydroxypraziquantel, by MECC and the simultaneous enantioselective separation of praziquantel and its metabolites after incubation with rat liver microsomes by using sulfobutylether-β-CD (SBE-β-CD) as a chiral selector. For the identification of these metabolites a LC–MS method was developed.

## 2. Experimental

### 2.1. Chemicals

Racemic praziquantel was obtained from Merck (Darmstadt, Germany). *Trans*- and *cis*-4-hydroxypraziquantel were synthesized and characterized in our laboratory [11]. The enantiomers were separated by low-pressure LC on a cellulose triacetate (CTA) column. The optical purity of the enantiomers was determined on a Chiracel-OD column and amounts to 99.9% for praziquantel and >99% for *trans*-4-hydroxypraziquantel, respectively [12].

Sodium dodecyl sulfate (SDS) was purchased from Fluka (Buchs, Switzerland). SBE-β-CD, substitution degree approx. 4.0, was a gift from Prof. J.F. Stobaugh and Prof. V.J. Stella (Center for Drug Delivery Research, The University of Kansas, Lawrence, KS, USA) whereas carboxymethyl-β-cyclodextrin (CM-β-CD) was acquired from Wacker Chemie (Munich, Germany). Analytical grade $Na_2HPO_4$, $KH_2PO_4$, NaOH, $H_3PO_4$, tris(hydroxymethyl)aminomethane, $MgCl_2$, NADPH, urea and ethyl acetate were purchased from Merck. Analytical grade tris(hydroxymethyl)aminomethane hydrochloride was obtained from Sigma (Deisenhofen, Germany). The source of HPLC-grade methanol was Merck and HPLC-grade acetonitrile was Scharlau (Barcelona, Spain). The solvents were used without further purification.

### 2.2. Buffer and sample preparation

Stock solutions of 50 m*M* $KH_2PO_4$ were prepared in double-distilled water. The pH was adjusted to 5.25 with 0.1 *M* NaOH. Stock solutions of 50 m*M* $Na_2HPO_4$ were prepared in double-distilled water and adjusted to pH 7.0 with 50 m*M* $H_3PO_4$. The running buffers were prepared accordingly after the addition of appropriate amounts of the micelle-forming surfactant or the chiral selector. All solutions were filtered and degassed by sonication before use.

Standard solutions (0.1 *M*) of the racemic drugs were prepared in methanol and stored at 4°C. For sample preparation, aliquots of the solution were evaporated to dryness under a stream of nitrogen and dissolved in the $Na_2HPO_4$ buffer for achiral separation and in 0.1 *M* solution of urea for chiral separation.

### 2.3. Apparatus

#### 2.3.1. CE

Achiral separation was performed on a P/ACE 2100 instrument (Beckman Instruments, Munich, Germany) with an untreated fused-silica capillary (Grom, Herrenberg, Germany) of 40 cm (effective length)×50 μm I.D. The samples were introduced by low pressure for 2 s. The electric field strength was 500 V/cm and the temperature was 25°C. The anode and cathode buffers had the same pH and molarity as the running buffer. Detection was carried out at a wavelength of 214 nm at the cathodic end.

Chiral separation was performed on a Grom CE system 100, equipped with a Linear Instruments (Reno, NV, USA) UVIS 200 detector and a HP 3396 A integrator (Hewlett-Packard, Avondale, PA, USA) with an untreated fused-silica capillary (Grom) of 43 cm (effective length)×50 μm I.D. The samples were introduced hydrostatically (10 cm) for 10 s. The

electric field strength was 300 V/cm and the temperature was 21°C. The anode and cathode buffers had the same pH and molarity as the running buffer. Only the running buffer in the capillary contained the CD. Detection was carried out at a wavelength of 210 nm at the cathodic end.

### *2.3.2. HPLC*

The chromatographic system for the fractionation of the metabolites was composed of a Merck–Hitachi L-6200 Intelligent pump equipped with a Rheodyne 7125 injector with a 50-μl loop, a Merck–Hitachi 655A variable-wavelength UV monitor and a Merck–Hitachi D-2500 Chromato-integrator. Separation was performed on a LiChrospher 100 RP-18 column 125×4.6 mm I.D., 5 μm (Merck) with a LiChrospher 100 RP-18 guard column 40×4.6 mm, 7 μm.

The mobile phase consisted of acetonitrile (ACN)–double-distilled water. The gradient started at ACN–water (16:84, v/v) and acetonitrile was increased continuously up to 38% in 40 min.

### *2.3.3. LC–MS*

The LC–MS system was composed of a Varian 5000 pump equipped with a Rheodyne 7125 injector with a 20-μl loop and a Finnigan MAT LCQ mass spectrometer (San Jose, CA, USA). Separation was performed on a LiChrospher 100 RP-18 column 125×4.6 mm I.D., 5 μm (Merck) with a LiChrospher 100 RP-18 guard column 40×4.6 mm, 7 μm. The mobile phase consisted of methanol–double-distilled water. The initial composition of 45% methanol was changed linearly to 60% methanol over 40 min. The mass spectrometer was set to a positive $MS^n$ scan mode with electrospray ionisation. The data were collected and analyzed by the Navigator software (version 1.0, copyright Finnigan 1995/96).

### *2.4. In vitro biotransformation with rat liver microsomes*

Livers from male Sprague–Dawley rats pretreated for six days with 50 mg/kg phenobarbital were used. The fresh livers were homogenized and prepared by a standard sedimentation procedure according to the method of Dayer et al. [13]. The protein concentration was determined as described by Bradford [14]. The incubation mixture contained 31 μg (0.1 μmol) praziquantel (racemate or enantiomers), 100 μl microsomal preparation with a protein concentration of 4.1 mg/ml and 400 μg (0.5 μmol) NADPH in 100 μl incubation buffer. This incubation buffer consisted of 0.05 *M* Tris buffer pH 7.4 and a 0.1 *M* solution of $MgCl_2$ in a ratio of 20:1. The incubations were carried out with stirring in a water bath at 37°C. After 30 min the incubation was stopped by cooling on ice. For LC–MS analysis 100-μl iced acetonitrile was added and the sample was centrifuged at 2500 *g* for 10 min. The supernatant was directly injected and analyzed. For CE analysis the samples were extracted twice with 2 ml ethyl acetate. The organic phase was separated by centrifugation at 2500 *g* for 5 min, removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 35 μl 1 *M* urea solution and analyzed by CE.

## 3. Results and discussion

### *3.1. Achiral separation using SDS as surfactant in MECC*

Praziquantel and its two metabolites *cis*- and *trans*-4-hydroxypraziquantel, as uncharged compounds, were separated by MECC using 20 m*M* SDS as anionic surfactant. A typical electropherogram is shown in Fig. 2. However, the migration times were very long. Finally, this method proved to be unsuitable for the analysis of the in vitro metabolism of praziquantel because of interfering components from the microsomes.

### *3.2. Separation of praziquantel and its metabolites with simultaneous enantiomeric separation*

The separation of praziquantel and its neutral metabolites, including enantiomeric separation of all analytes, was achieved by using a 50 m*M* phosphate buffer pH 5.25 and SBE-β-CD as a chiral selector.

In the absence of any selector, praziquantel and its metabolites migrated as uncharged analytes with the

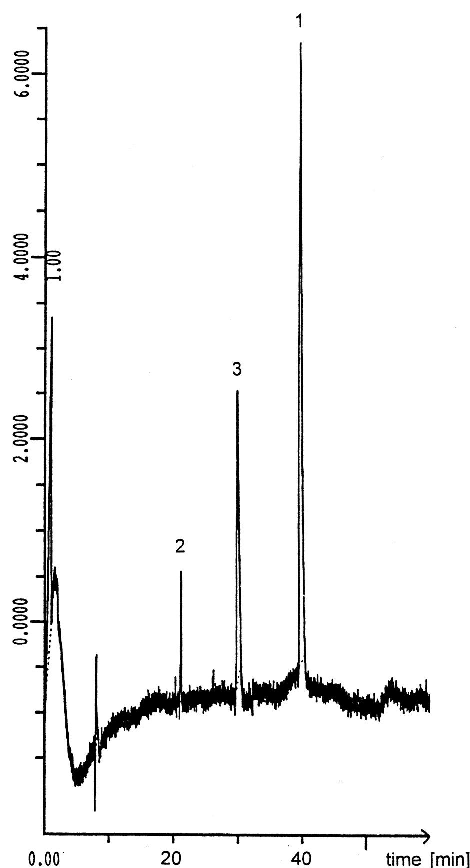


Fig. 2. MECC separation of praziquantel (1), *trans*- (2) and *cis*-4-hydroxypraziquantel (3). Conditions: 20 m*M* SDS, 50 m*M* phosphate buffer pH 7.0, 500 V/cm; detection: UV 214 nm.

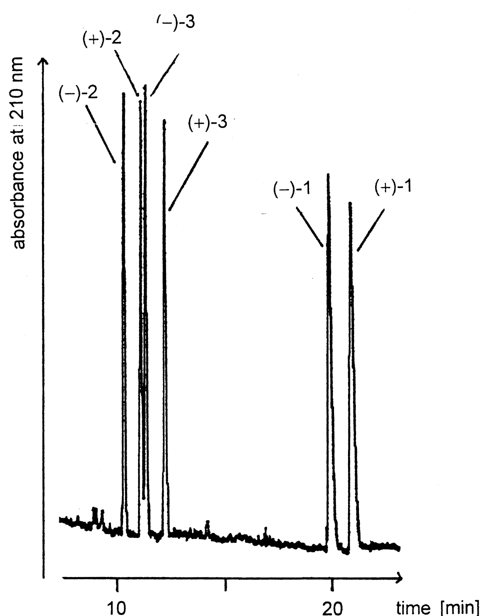


Fig. 3. Separation of the enantiomers of praziquantel (1), *trans*- (2) and *cis*-4-hydroxypraziquantel (3). Conditions: 4 m*M* SBE-β-CD, 50 m*M* phosphate buffer pH 5.25, 300 V/cm; detection UV 210 nm; $\alpha$-values; 1.050, 1.068 and 1.072 for 1, 2 and 3, respectively.

electroosmotic flow (EOF), resulting in one peak and no separation at all. However, SBE-β-CD is negatively charged at any pH [16] and it obtained its own mobility opposite to the EOF. As an additive to the running buffer, it acted as a mobile pseudophase, which enabled a simultaneous separation and chiral resolution of praziquantel and its metabolites.

A typical electropherogram for praziquantel, *cis*- and *trans*-4-hydroxypraziquantel is shown in Fig. 3. The (−)-enantiomer of each compound migrated before the (+)-enantiomer, respectively. To achieve the simultaneous enantiomeric separation of praziquantel, *cis*- and *trans*-4-hydroxypraziquantel a concentration of 4 m*M* SBE-β-CD was required. Decreasing concentrations of SBE-β-CD deteriorated the separation. A concentration of 2 m*M* SBE-β-CD enabled the enantiomeric separation of each analyte, whereas (+)-*trans*-4-hydroxypraziquantel and (−)-*cis*-4-hydroxypraziquantel comigrated. Lower concentrations resulted in no enantiomeric separation at all. A higher concentration than 4 m*M* of SBE-β-CD increased the retention time, but only slightly affected the separation factors, especially for (+)-*trans*-4-hydroxypraziquantel and (−)-*cis*-4-hydroxypraziquantel.

In addition to SBE-β-CD, another charged CD derivate, CM-β-CD, was tested for enantiomeric separation of praziquantel and its metabolites. In a concentration of 10 m*M* at pH 6.0 it was proved to be suitable for the separation of the enantiomers of *trans*- and *cis*-4-hydroxypraziquantel, respectively, although this could not be attained simultaneously and enantiomeric separation of praziquantel could not be achieved at all.

### 3.3. In vitro metabolism

The SBE-β-CD method was used to study the stereoselectivity of the metabolism of praziquantel after incubation of racemic praziquantel with rat liver microsomes. Praziquantel and its metabolites were resolved from interfering components of the microsomal matrix as blank runs showed.

A representative electropherogram of the rat liver microsomal incubation is shown in Fig. 4.

In addition to *trans*- and *cis*-4-hydroxypraziquantel, four other metabolites were detected, M1, M2, M3 and M4.

To identify the peaks of these metabolites, M1 to M4, a microsomal incubation sample was analyzed by an achiral HPLC method and the detected peaks of the metabolites were collected in separate fractions. Microsomal incubation samples were spiked with each of these fractions and these spiked samples were analyzed by the chiral CE method.

Additionally, to identify whether a metabolite was formed from the *R*-(−)- or the *S*-(+)-enantiomer both praziquantel enantiomers were incubated separately and analyzed by CE. The pure enantiomers of praziquantel, *trans*- and *cis*-4-hydroxypraziquantel were proved to be stable under the conditions of this method. Because no reference standards for the metabolites, M1–M4, were available, no further validation was possible. The quantification was done by analyzing the ratio of the peak area of each enantiomer (−/+).

For the stereoselective metabolism of racemic praziquantel the following results were obtained:

More *S*-(+)- than *R*-(−)-praziquantel was metabo-

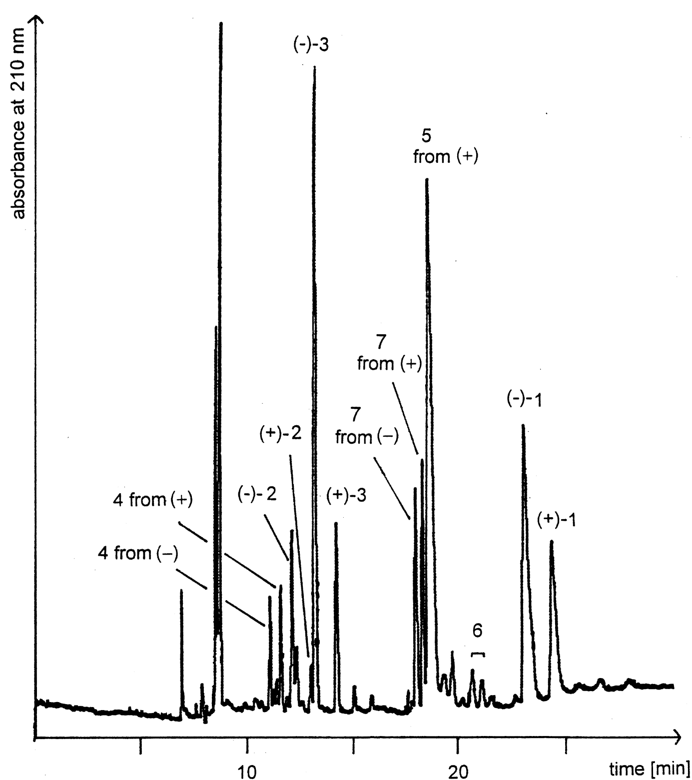


Fig. 4. Simultaneous enantioselective separation of praziquantel (1) and its metabolites after incubation with rat liver microsomes. Conditions: see Fig. 3. *trans*-4-Hydroxypraziquantel (2), *cis*-4-hydroxypraziquantel (3), M1 (4), M2 (5), M3 (6) and M4 (7).

lized. *Cis*-4-hydroxypraziquantel represented the main metabolite. It was mainly formed from the *R*-(−)-praziquantel with a peak area ratio of 77/23 [(−)/(+) enantiomer]. In contrast, the second main metabolite, M2, was only formed from the *S*-(+)-praziquantel. *trans*-4-Hydroxypraziquantel was mainly formed from the *R*-(−)-enantiomer with a peak area ratio of 83/17 [(−)/(+) enantiomer], whereas the formation of the other metabolites, M1, M3 and M4, showed little stereoselectivity.

These results are in agreement with those obtained by Westhoff [10]. Westhoff studied the stereoselective metabolism of praziquantel by incubating the praziquantel-enantiomers separately with rat liver microsomes followed by analysis using an achiral HPLC method. This method described an acetonitrile–water step-gradient on a $C_{18}$ column and resulted in enantiomeric ratios (−/+) of the metabolites, *trans*-, *cis*-4-hydroxypraziquantel, M2 and M5, of 72/28, 75/25, 3/97 and 24/76, respectively. The main difference was found for the metabolite M5. Whereas Westhoff obtained a ratio of 24/76 for the formation of M5 from *R*-(−)-praziquantel/M5 from *S*-(+)-praziquantel our result was 51/49. The reason for this discrepancy may be, that further metabolism of M5 occurs due to the use of different incubation conditions. M5 was converted to the metabolite M1 [15], but Westhoff provided no HPLC data for this pathway for comparison.

For further identification of these metabolites, M1–M4, a HPLC–MS method was developed, which allowed the simultaneous analysis of all metabolites obtained by incubation of praziquantel with rat liver microsomes. Therefore the HPLC was linked with a quadrupole ion-trap mass spectrometer by an atmospheric pressure ionization interface. The ionization of the sample was done by electrospray in a positive $MS^n$ scan mode. This means, that after the first ionization and fragmentation of the sample, one mass peak was isolated and further fragmented. For the MS–MS fragmentation, the specified mass of the metabolite was selected for this procedure and for all further $MS^n$ fragmentations, the base peak of the previous mass spectrum was isolated and fragmented automatically. With this method all metabolites from the incubation, even those with different fragmentation patterns, could be analyzed in one run.

It was evident that the metabolite M2 was a monohydroxylated product of praziquantel due to the peak at $m/z$ 329 (Fig. 5a) being 16 mass units higher (corresponding to an oxygen) than the known value of $m/z$ 313 for praziquantel. The fragmentation pattern of M2 was similar to that of praziquantel. All peaks had $m/z$ masses of two units lower than praziquantel. This could be explained by subsequent loss of water (−18 mass units) following hydroxylation (+16 mass units). For example the peak at $m/z$ 130 occurring as a fragment of the $MS^4$ spectrum (Fig. 5c), could have been due to a previous hydroxylation in position 6 or 7 of the praziquantel molecule (Fig. 1). The fragments of M2 with $m/z$ 283 and $m/z$ 173 (Fig. 5c) could not be analyzed for praziquantel under the same conditions.

M3 was a monohydroxylated metabolite with the same fragmentation pattern as praziquantel only having $m/z$ mass peaks of 16 units higher than the parent compound. The hydroxylation was most likely at the tetrahydroisoquinoline part of the molecule.

The mass spectrum of the metabolite M4 (Fig. 6) was almost identical to that of 1,11b-dehydropraziquantel, a reference standard (Fig. 7). However the peak at $m/z$ 311 was not the molecule peak but the MS–MS peak from $m/z$ 329 (Fig. 6a). This could occur due to a spontaneous loss of water following initial hydroxylation in position 1 or 11b. It was confirmed by further studies of samples obtained by chemical oxidation of praziquantel and GC–MS analysis [15] that the hydroxy group was located in position 1 (Fig. 1).

The metabolite M1 was a dihydroxylated product of praziquantel with $m/z$ 345 for MS and $m/z$ 327 for MS–MS. It was evident the hydroxy groups were located in position 1 and position 4 of the cyclohexyl part of the molecule because M1 was proved to be formed from M4 as well as from *cis*-4-hydroxypraziquantel [15].

## 4. Conclusions

An achiral MECC method using SDS was developed for the separation of the uncharged drug praziquantel and its metabolites *cis*- and *trans*-4-hydroxypraziquantel.

With the negatively charged SBE-β-CD used as a chiral additive, a simultaneous separation and enan-

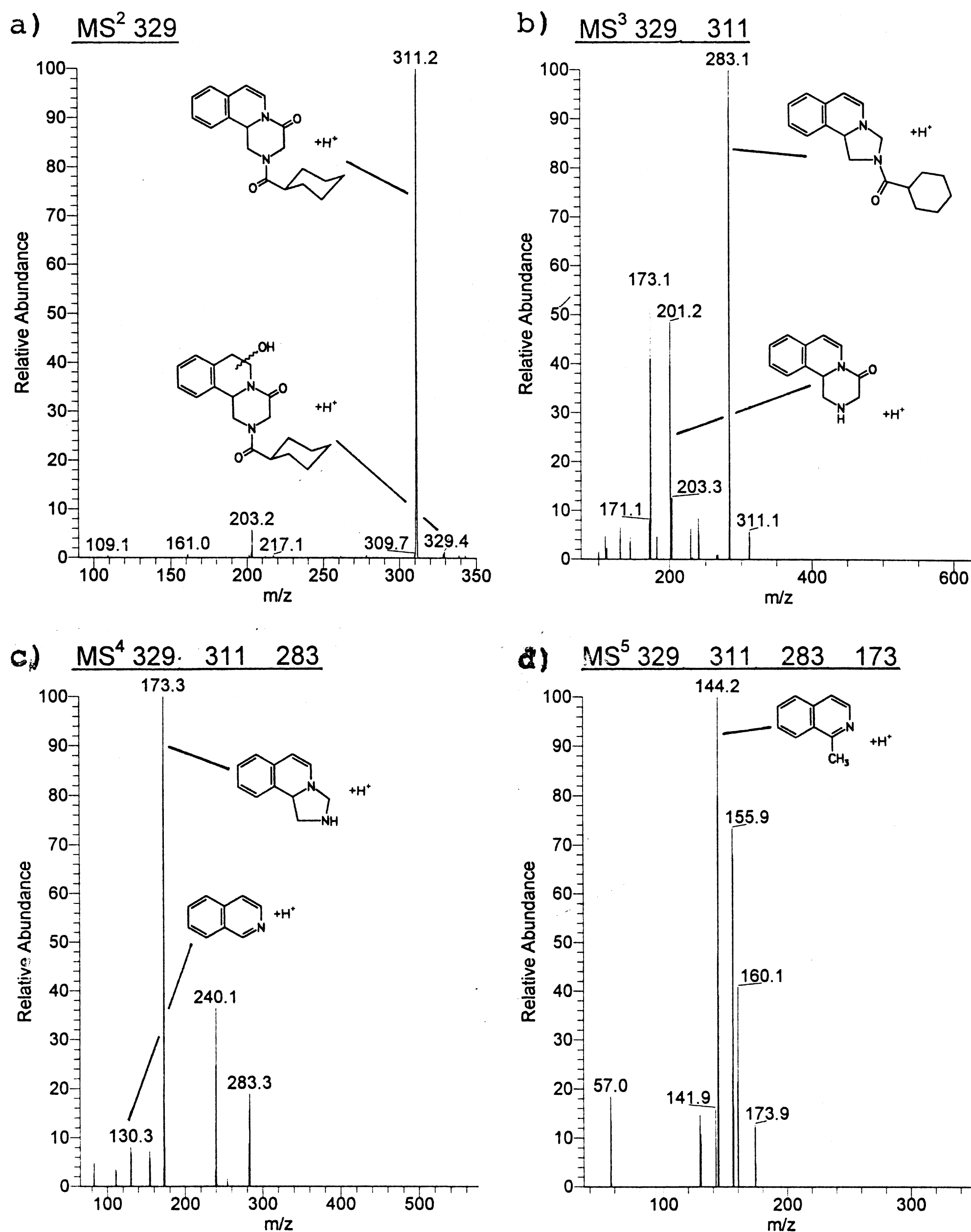


Fig. 5. Mass spectrum of M2 following five consecutive fragmentations (a–d).

tioselective resolution of praziquantel and its metabolites was possible.

The use of a HPLC method allowed the study of the stereoselective metabolism of praziquantel only after incubation of each enantiomer separately [10], whereas with this CE method the full stereoselective metabolism could be studied directly after incubation of racemic praziquantel.

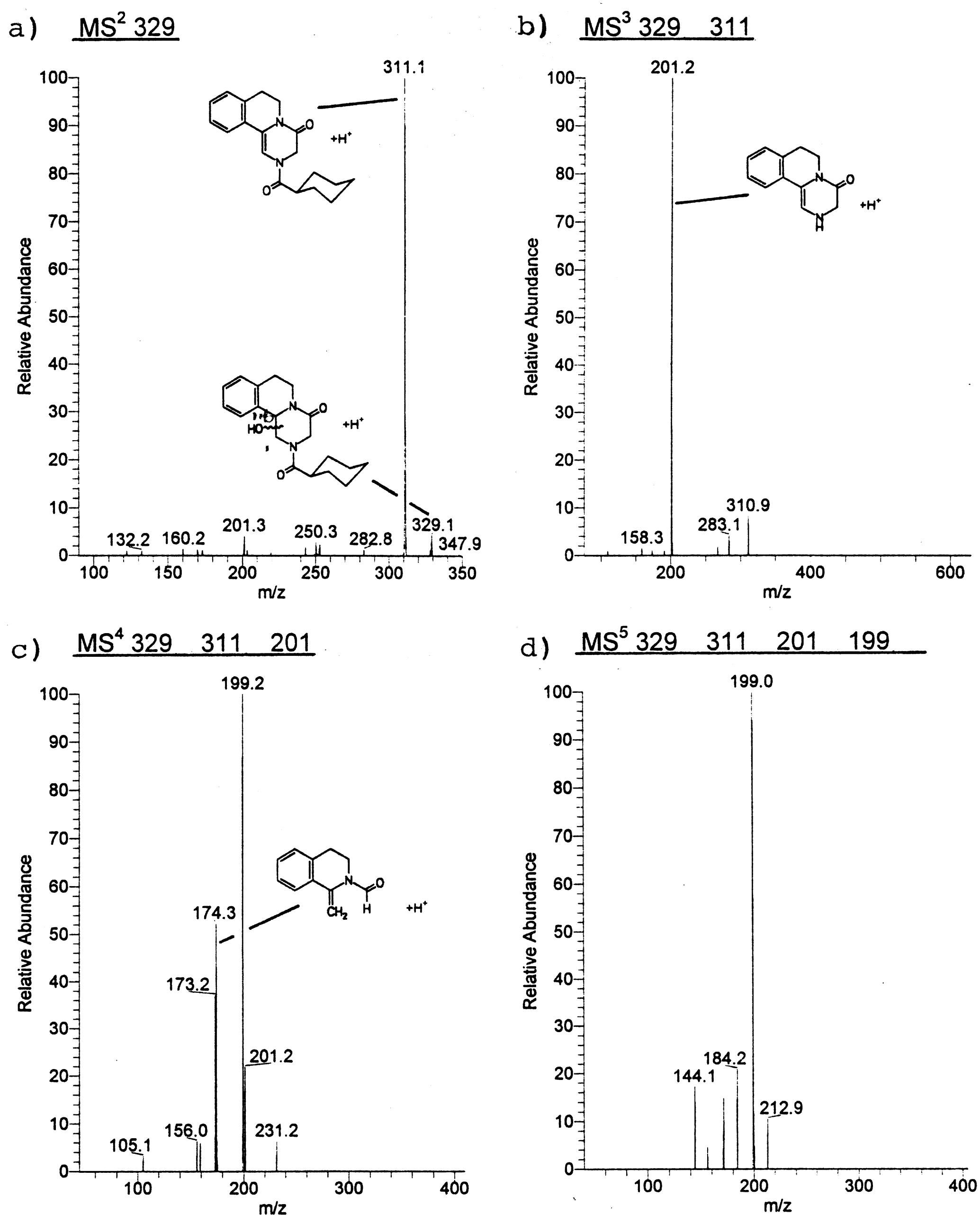


Fig. 6. Mass spectrum of M4 following five consecutive fragmentations (a–d)

The previous results obtained by HPLC were supported.

By use of LC–MS with a $MS^n$ scan mode it was possible to analyze the structure of all these metabolites obtained after in vitro metabolism with even different fragmentation patterns in one run.

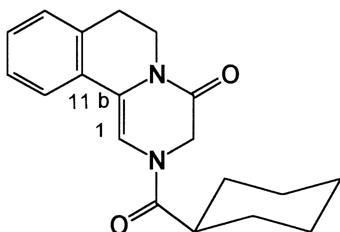


Fig. 7. Structure of 1,11b-dehydroxypraziquantel.

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