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Capillary electrophoresis–mass spectrometry, liquid chromatography–mass spectrometry and nanoelectrospray-mass spectrometry of praziquantel metabolites

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Abstract

Capillary electrophoresis–mass spectrometry (CE–MS) and liquid chromatography–mass spectrometry (LC–MS) coupling was used for the investigation of metabolites of the anthelmintic drug praziquantel. Human urine samples and microsomal incubation mixtures were investigated after preparation by solid-phase extraction. CE– and LC–MS coupling was performed using an electrospray ionization interface. An ion trap mass spectrometer equipped with a laboratory-made nanoelectrospray ion source was used for the investigation of the glucuronide conjugates by consecutive fragmentations. The nanoelectrospray interface offers the opportunity to perform complicated MS^n spectrometric investigations. Different phase I metabolites of praziquantel and their glucuronidated and sulfated conjugates were detected.

Keywords: Mass spectrometry; Nanoelectrospray ion source; Praziquantel

1. Introduction

In the last few years the on-line combination of liquid chromatography (LC) and mass spectrometry (MS) has developed rapidly. LC–MS and $LC–MS^n$ has become the method of choice in many stages of drug development and has attracted more and more attention in forensic and clinical toxicology, environmental and biochemical analysis [1–4].

On the other hand, capillary electrophoresis (CE) has been established as a powerful method for the analysis of drugs and their metabolites in biological samples. In particular, charged phase II metabolites like glucuronides or sulfates which are difficult to determine with other techniques due to their high polarity can be analyzed in biological samples by CE [5].

CE–MS coupling is a promising combination of two analytical techniques even though it is still not generally considered for routine analysis at present [6,7]. The usefulness of CE–MS coupling was demonstrated for the direct investigation of drugs like paracetamol, acetylsalicylic acid, antipyrine and other nonopioid analgesics and their metabolites in urine samples [8,9].

Praziquantel (PZQ) (Fig. 1A) is the drug of choice for the treatment of human schistosomiasis. It is estimated that about 200 million people in the world are currently affected by this tropical disease. The drug undergoes extensive metabolism. No unmetabolized drug was found in serum, urine or feces of rats after oral administration of praziquantel [10].

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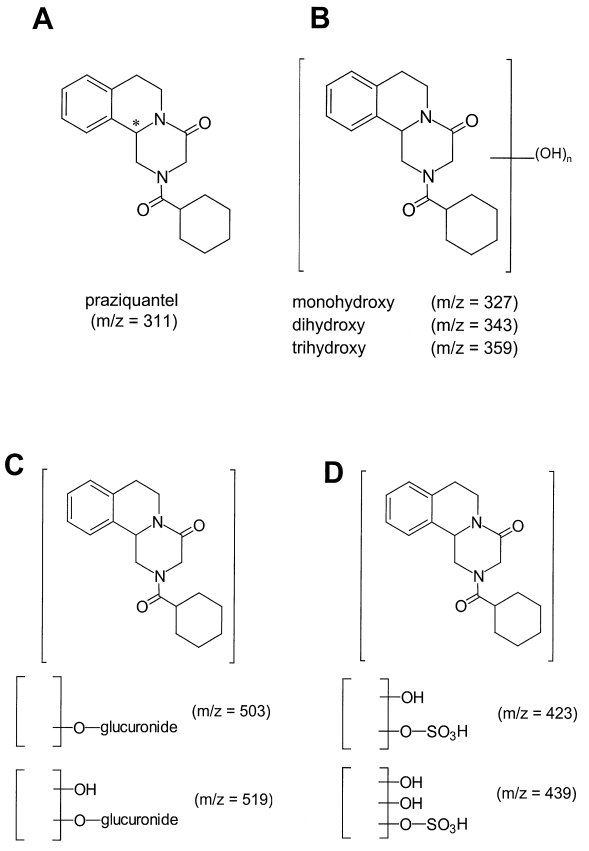

Fig. 1. Structure of praziquantel (PZQ) (A); phase I metabolites of praziquantel detected in a human urine sample (B), glucuronide conjugates of praziquantel detected in a human urine sample (C), sulfate conjugates of praziquantel detected in a human urine sample (D). The molecular masses represent the deprotonated species ($[M-H]^-$).

The major metabolites in serum and urine are hydroxylated products [11]. The exact structures of the metabolites are unknown except for *cis*- and *trans*-4-hydroxypraziquantel. Additionally, glucuronide and/or sulfate conjugates were identified by enzymatic hydrolysis of polar fractions after thin-layer chromatographic separation [10].

In this study we report the investigation of metabolites of the anthelmintic drug praziquantel using CE– and LC–MS coupling. Additionally, the usefulness of a simple laboratory-made nanoelectrospray device is demonstrated. Different hydroxylated metabolites of PZQ as well as their glucuronidated and sulfated conjugates were detected in a human urine sample. The polar glucuronide and sulfate conjugates of praziquantel were identified directly without enzymatic hydrolysis prior to the analysis. A LCQ ion trap mass spectrometer equipped with an electrospray ion source was used for LC– and CE–MS coupling and nanoelectrospray MS^n investigations.

2. Experimental

2.1. Chemicals and reagents

Racemic praziquantel (Cesol) was purchased from Merck (Darmstadt, Germany). Acetonitrile, ammonium acetate, hydrochloric acid, ammonia, methanol and trifluoroacetic acid were from different commercial sources and at least of analytical grade. Uridine-5′-diphosphoglucuronic acid and β-glucuronidase from *Helix pomatia* were purchased from Sigma–Aldrich (Deisenhofen, Germany). Water was deionized and bidistilled. Bakerbond Octadecyl solid-phase extraction (SPE) columns were purchased from Mallinckrodt Baker (Griesheim, Germany).

2.2. Apparatus and methods

2.2.1. CE–electrospray ionization (ESI)-MS set-up

Separations in CE were performed using commercially available CE equipment, HP^{3D} (Hewlett-Packard, Waldbronn, Germany). The total as well as the effective length of the separation capillary was 48.5 cm. An LCQ ion trap mass spectrometer (Thermoquest, San Jose, CA, USA) equipped with a sheath flow electrospray interface was used [7]. The following mass spectrometric conditions were used: capillary temperature 200°C, source voltage −3.0 kV, full scan (m/z=150–700), negative ion mode, sheath gas 20 arbitrary units nitrogen. The sheath liquid consisting of methanol–water–ammonia (50:49:1) was delivered at a flow-rate of 6 μl min^{-1} using a syringe pump. Uncoated fused-silica capillaries of 50 μm I.D.×375 μm O.D. from Polymicro Technologies (Phoenix, AZ, USA) were used for the capillary electrophoresis separations. The volatile buffer consisted of 25 m*M* ammonium acetate adjusted to pH 8.5 with 0.1 *M* ammonium hydroxide containing 10% methanol. A separation voltage of 17 kV was applied. Due to the electrospray voltage of

−3 kV, the effective separation voltage was 20 kV. Samples were injected hydrodynamically with a pressure of 50 mbar for 5 s.

2.2.2. LC–ESI-MS set-up

The chromatographic separation was performed on a 250×2.1 mm column packed with Superspher RP-18 (Merck) using a Waters 2690 separation module (Waters, Eschborn, Germany). The initial mixture of the mobile phase consisted of 17% acetonitrile (A) and 83% 0.01% trifluoroacetic acid (B) at a flow-rate of 0.18 ml min^{-1}. The gradient was changed linearly to A–B (19:81) at $t=10$ min and this solvent composition was held until $t=35$ min. Then the gradient was changed to A–B (25:75) in 10 min. After isocratic elution of the column for 15 min the gradient was changed linearly to A–B (60:40) until $t=75$ min. The eluent was introduced into the LCQ ion trap mass spectrometer without a split. The following mass spectrometric conditions were used: capillary temperature 250°C, source voltage 6.0 kV, full-scan ($m/z=290–600$), positive ion mode, sheath gas 45 arbitrary units nitrogen.

2.2.3. Nanoelectrospray-MS set-up

For nanoelectrospray ionization investigations a laboratory-made nanoelectrospray ion source as described by Wilm and Mann [15] was used. Pulled glass capillaries were positioned 1–2 mm in front of the orifice of the mass spectrometer. The tip of the spray capillary and the heated capillary of the mass spectrometer were viewed by magnifying glasses. The nanoelectrospray ion source could be manipulated in *x*, *y* and *z* directions via micrometer screws. After loading the capillary with 5 μl sample solution a thin stainless steel wire was inserted into the tip of the capillary to provide the contact of the sample solution with the electrospray voltage. To prepare for spraying, the capillary was slightly touched against the heated capillary of the mass spectrometer and then centered in front of the mass spectrometer's orifice. A potential of 800 V was applied to the spray capillary. If necessary, a static air pressure was applied to the spray capillary using a syringe to start the electrospray process. Then the spray voltage was adjusted to achieve maximum ion intensities.

2.2.4. Sample preparation for CE– and LC–MS coupling

Urine samples were collected from a healthy human volunteer 2–4 h after oral administration of 600 mg racemic praziquantel (Cesol). Prior to the analysis SPE was performed using a 100 mg C_{18} cartridge to remove matrix components and to preconcentrate the phase I and phase II metabolites. The cartridge was preconditioned with 4 ml methanol and 2 ml distilled water adjusted to pH 3 with 0.5 *M* hydrochloric acid. A 1-ml volume of urine, also adjusted to pH 3 with 0.5 *M* hydrochloric acid was slowly passed through the cartridge and then rinsed again with 1 ml water–hydrochloric acid, pH 3. The sorbent was dried for 10 min by applying a vacuum. In the next step, the analytes were eluted by 200 μl methanol and the extract was evaporated at ambient temperature under a steady stream of nitrogen. The residue was reconstituted in 100 μl of the separation buffer. For LC–ESI-MS analysis the residue was reconstituted in acetonitrile–water (50:50).

2.2.5. Sample preparation for nanoelectrospray investigations

Monohydroxypraziquantel glucuronides from urine samples (see Section 2.2.4) were collected in fractions using LC. SPE was performed as described in Section 2.2.4 to remove matrix components. The incubation mixtures were desalted by the same SPE procedure as described in Section 2.2.4.

2.2.6. In vitro biotransformation with rat liver microsomes

Livers from male Sprague–Dawley rats pretreated for six days with 50 mg kg^{-1} phenobarbital were used for the preparation of microsomes. The fresh livers were homogenized and prepared by a standard sedimentation procedure according to the method of Dayer et al. [12]. The protein concentration was determined as described by Bradford [13]. The incubation mixture contained each 66 μg (0.2 μmol) *cis*-4-hydroxypraziquantel or *trans*-4-hydroxypraziquantel, 200 μl microsomal preparation with a protein concentration of 14.75 mg ml^{-1} and 650 μg (1 μmol) uridine-5′-diphosphoglucuronic acid in 500 μl incubation buffer. This incubation buffer consisted of 50 m*M* Tris buffer, pH 7.8 and 5 m*M*

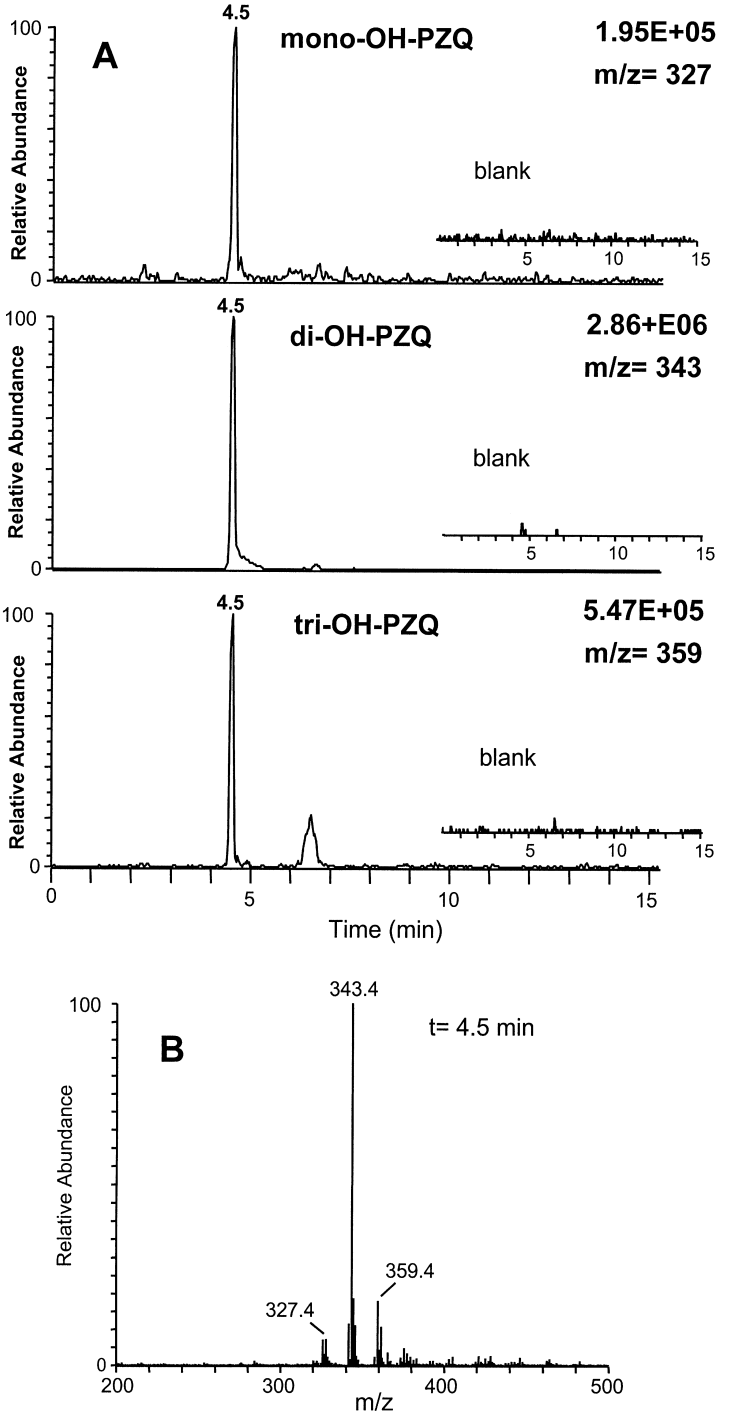

Fig. 2. CE–ESI-MS analysis of a urine extract after oral administration of 600 mg praziquantel and a blank urine extract, reconstructed ion electropherograms (A), mass spectrum recorded in the apex of the peak (B); CE and ESI-MS conditions as described in Section 2.2.1.

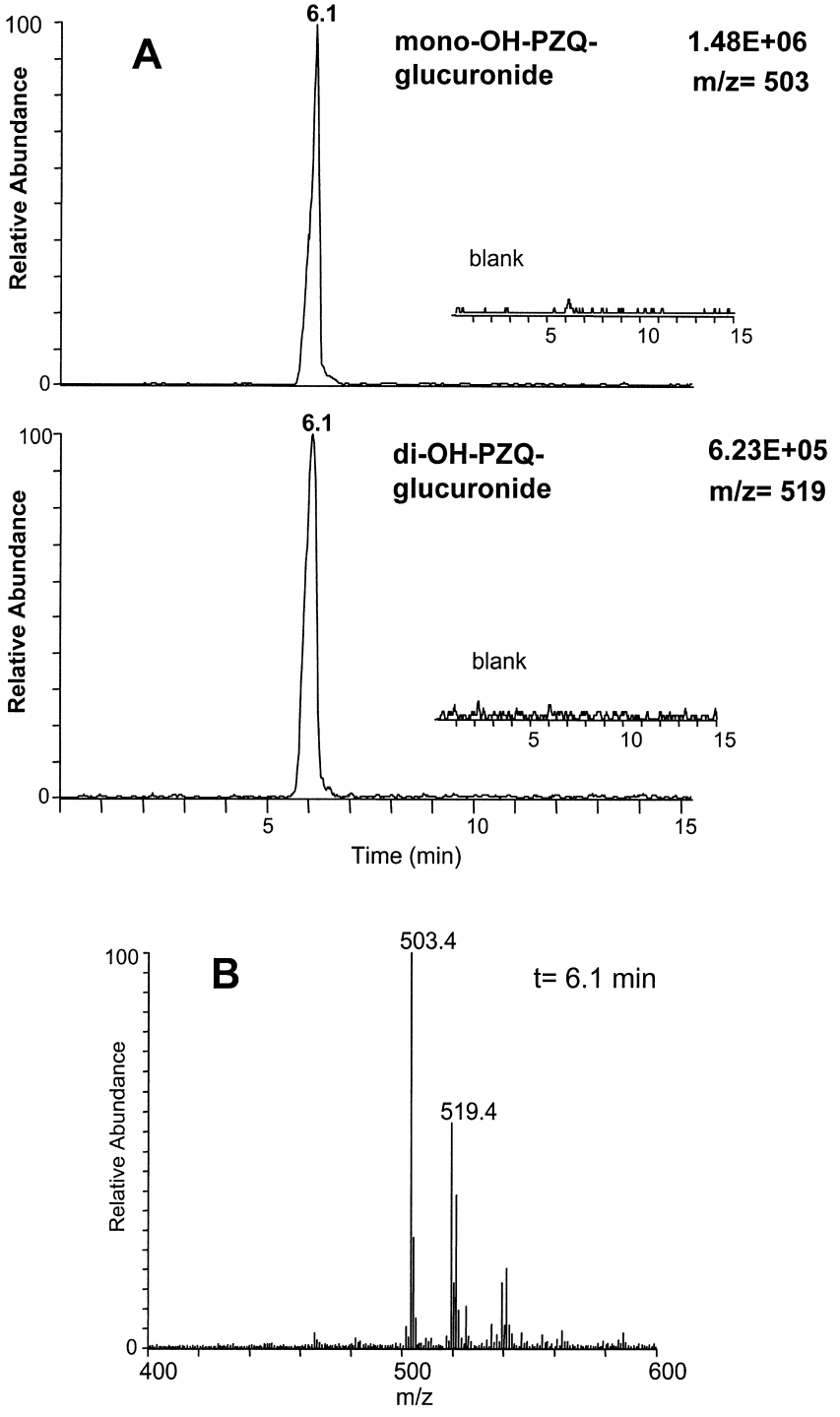

Fig. 3. CE–ESI-MS analysis of a urine extract after administration of 600 mg praziquantel and a blank urine extract, reconstructed ion electropherograms (A), mass spectrum recorded in the apex of the peak (B); CE and ESI-MS conditions as described in Section 2.2.1.

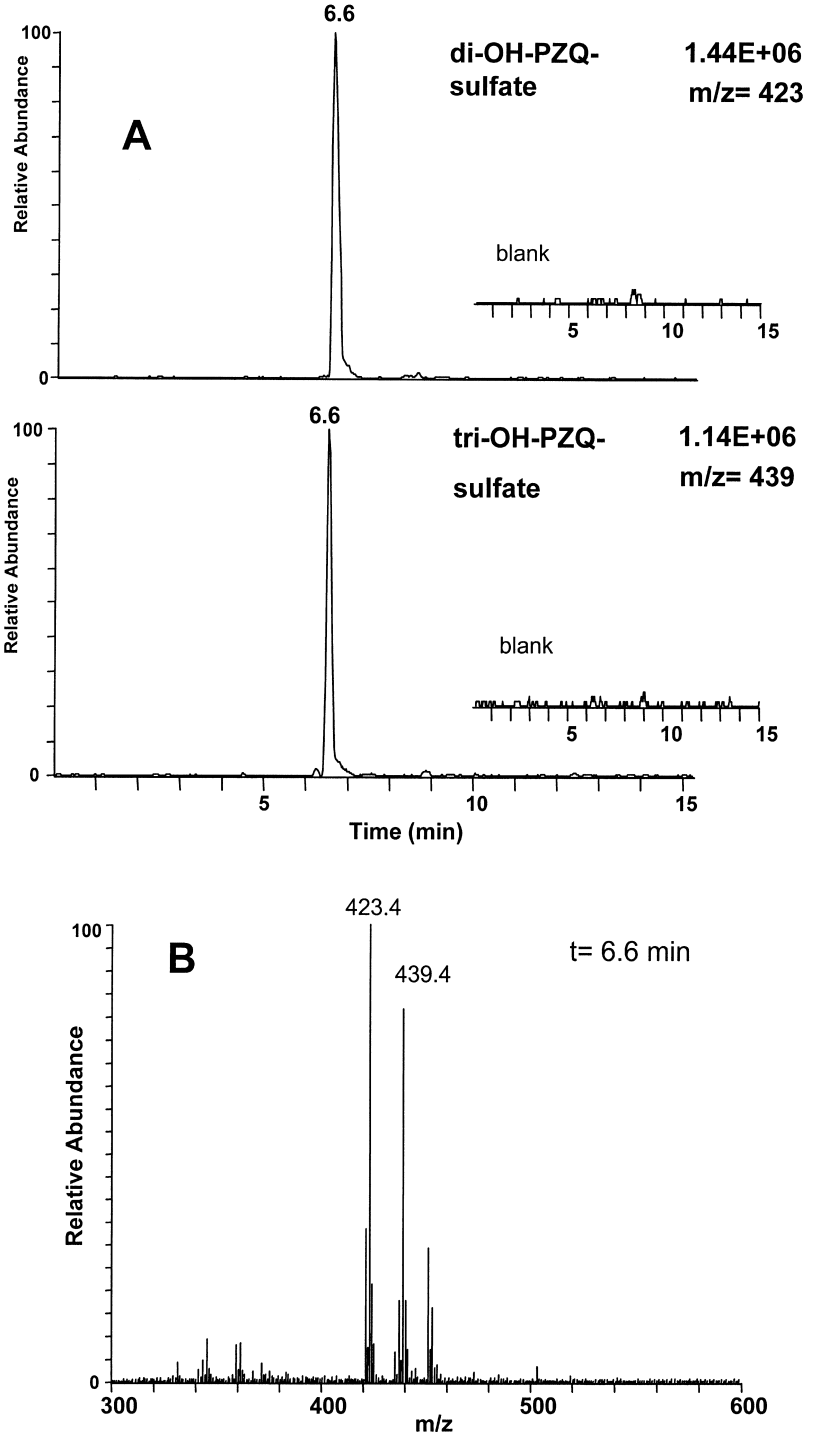

Fig. 4. CE–ESI-MS analysis of a urine extract after administration of 600 mg praziquantel and a blank urine extract, reconstructed ion electropherograms (A), mass spectrum recorded in the apex of the peak (B); CE and ESI-MS conditions as described in Section 2.2.1.

$MgCl_2$. The incubations were carried out with stirring in a water bath at 37°C. After 4 h the incubation was stopped by adding 500 μl iced acetonitrile. The samples were centrifuged at 2500 *g* for 10 min and the supernatant was used directly for LC–MS investigations.

3. Results and discussion

3.1. CE–MS coupling

Successful coupling of CE and MS demands the use of a volatile separation buffer. Therefore 25 m*M* ammonium acetate adjusted to pH 8.5 with 0.1 *M* ammonium hydroxide was selected as running buffer. Methanol was added to the running buffer to lower its surface tension and thus improving its electrospray characteristics [14]. In Fig. 2, the reconstructed ion electropherograms of different praziquantel metabolites detected in a urine sample after oral administration of 600 mg praziquantel and a blank urine sample are presented. The run was carried out in an alkaline separation media in order to obtain both, a strong electroosmotic flow (EOF) and the dissociation of any acidic analytes. Under the CE conditions used in our investigations the uncharged analytes comigrate with the EOF towards the cathode. However, because of the selective mass spectrometric detection we were able to distinguish three different degrees of hydroxylation products within this peak (Fig. 1B). The first peak (t=4.5 min) represents the EOF including the uncharged phase I metabolites mono-OH-PZQ (m/z=327.4), di-OH-PZQ (m/z=343.4) and tri-OH-PZQ (m/z=359.4). The corresponding mass spectrum was recorded in the apex of the peak. The precise position of the hydroxyl groups is under investigation. Unmetabolized PZQ was not detectable. The reconstructed ion electropherograms of the monohydroxypraziquantel glucuronides and dihydroxypraziquantel glucuronides are presented in Fig. 3. Two different types of PZQ glucuronides were detected at t=6.1 min (Fig. 1C). Additionally, two different types of sulfate conjugates were detected. In Fig. 4 the reconstructed ion electropherograms of dihydroxypraziquantel sulfate and trihydroxypraziquantel sulfate are presented (Fig. 1D). Both types of praziquantel conjugates, praziquantel glucuronides and praziquantel sulfates, were negatively charged under CE conditions used, i.e., they reach the detector after the EOF.

3.2. LC–MS coupling and nanoelectrospray investigations

For further investigations of the praziquantel glucuronides the LC–MS method described in Section 2.2.2 was used, which allowed the separation of different monohydroxypraziquantel glucuronides. The liquid chromatograph was linked with the ion trap mass spectrometer by an atmospheric pressure ionization interface. The ionization of the sample was done by electrospray in the positive ion mode. The corresponding glucuronides of *cis*- and *trans*-4-hydroxypraziquantel were obtained after microsomal incubation of *cis*- and *trans*-4-hydroxypraziquantel with rat liver microsomes (Fig. 5). The glucuronides of *cis*- and *trans*-4-hydroxypraziquantel were collected in fractions and spiked to a human urine sample. After confirmation of the structures of the conjugates by cleaving experiments with β-glucuronidase and their migration behavior we were able to identify two monohydroxypraziquantel glucuronides in a human urine sample as *trans*-4-hy-

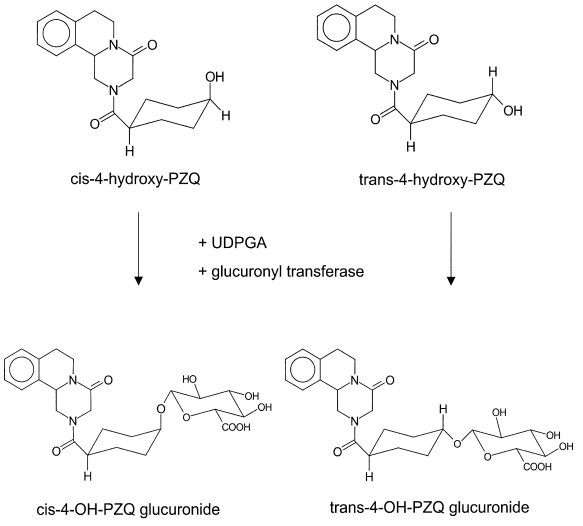

Fig. 5. Conjugation of *trans*- and *cis*-4-hydroxypraziquantel with uridine-5′-diphosphoglucuronic acid (UDPGA) and microsomes containing glucuronyl transferase.

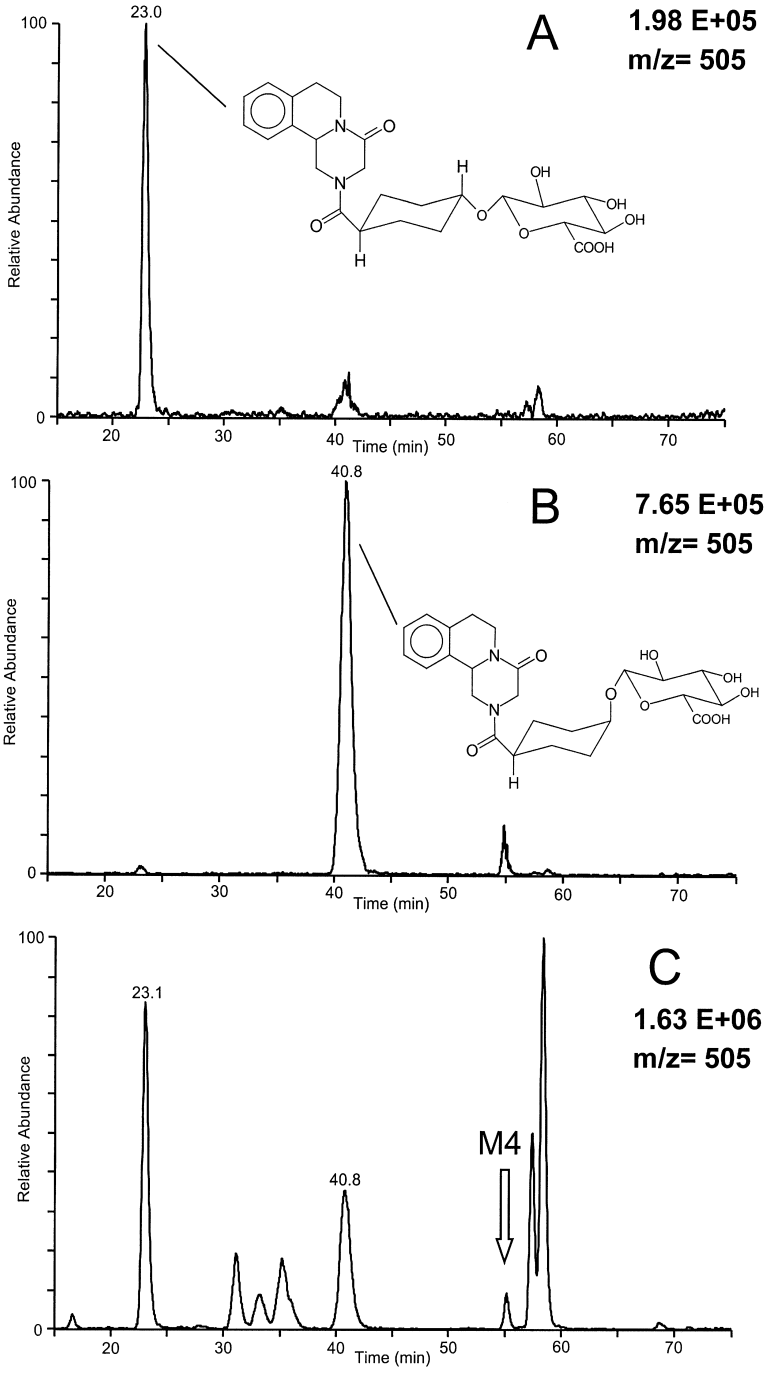

Fig. 6. LC–ESI-MS analysis of glucuronide conjugates after incubations of *trans*-4-hydroxypraziquantel (A) and *cis*-4-hydroxypraziquantel (B) (see Section 2.2.6) and a urine sample extract (C) (see Section 2.2.4). Injection volume: 5 μl; LC and ESI-MS conditions as described in Section 2.2.2.

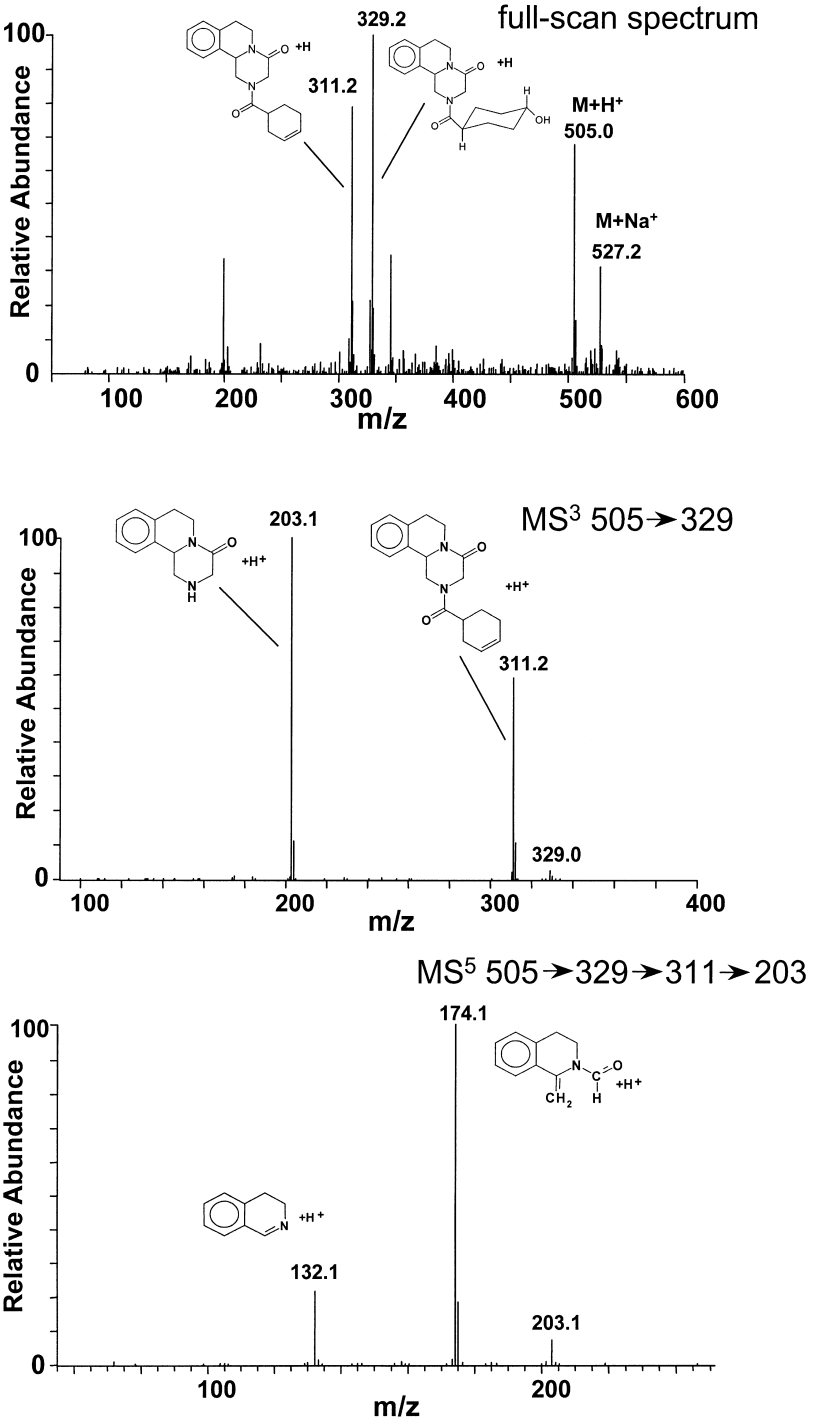

Fig. 7. Mass spectra of *trans*-4-hydroxypraziquantel glucuronide after consecutive fragmentations. MS^n conditions: m/z=505: isolation width 3 u, collision energy 12% of maximum; m/z=329: isolation width 3 u, collision energy 11% of maximum; m/z=311: isolation width 3 u, collision energy 11% of maximum; m/z=203: isolation width 3 u, collision energy 12% of maximum.

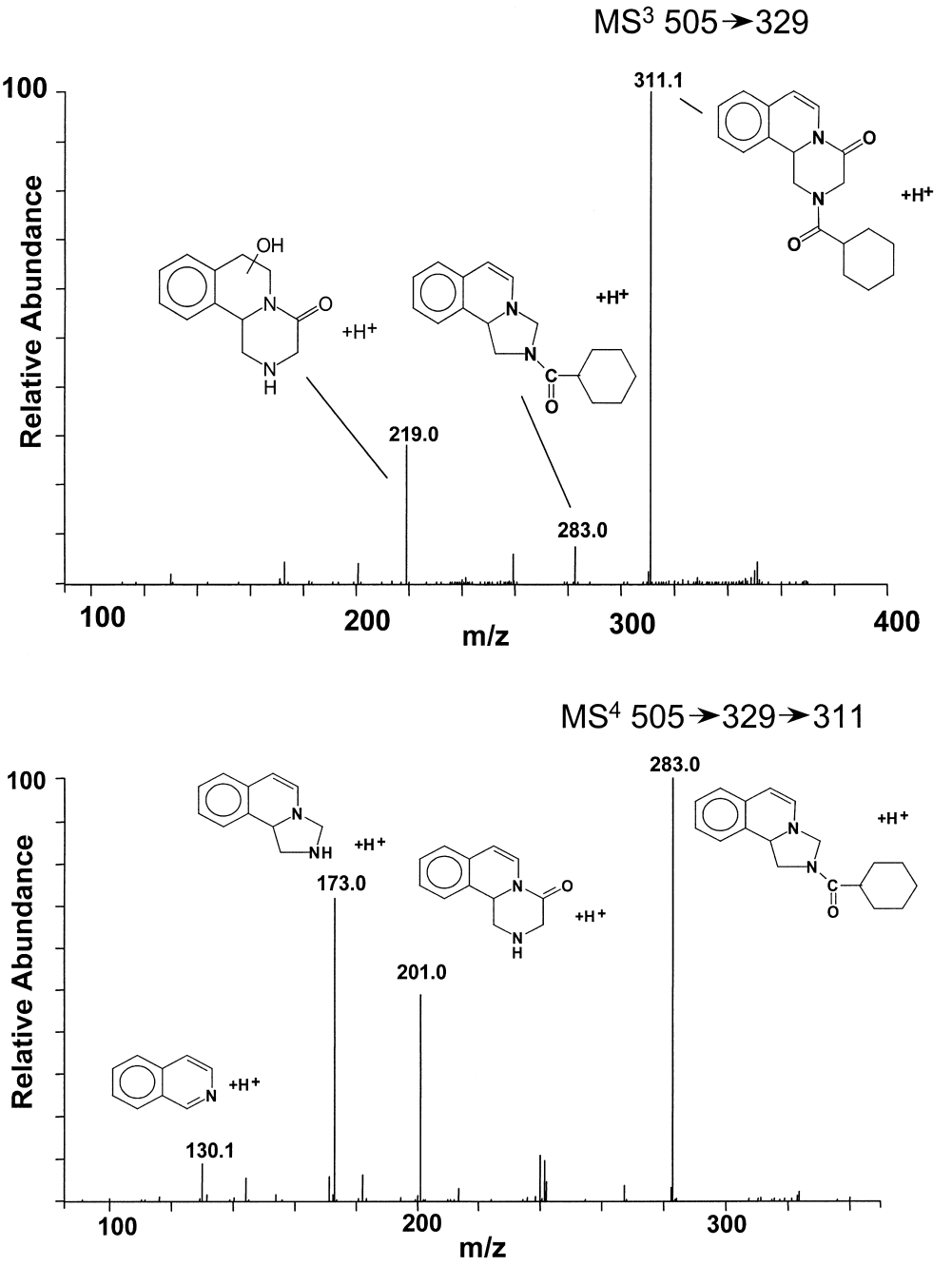

Fig. 8. Mass spectra of M4 after consecutive fragmentations. MS^n conditions: m/z=505: isolation width 3 u, collision energy 11% of maximum; m/z=329: isolation width 3 u, collision energy 10% of maximum; m/z=311: isolation width 3 u, collision energy 12% of maximum.

droxypraziquantel glucuronide and *cis*-4-hydroxypraziquantel glucuronide (Fig. 6). As demonstrated in Fig. 6, other monohydroxypraziquantel glucuronides of unknown structure are present in human urine samples resulting of the conjugation of different monohydroxypraziquantel metabolites with

glucuronic acid. We collected the peaks by LC in separate fractions and investigated their fragmentation pattern using the laboratory-made nanoelectrospray interface. The nanoelectrospray device employs pulled capillaries with a very small spraying orifice at a very small flow-rate. It operates at the flow-rate generated by the electrospray process itself. In contrast to online coupled systems there is almost unlimited time for MS and MS^n spectrometric investigations due to the very low flow-rates [15,16]. Two different fragmentation patterns were obtained after fragmentation of the monohydroxypraziquantel glucuronides collected in separate fractions. In Fig. 7 the full-scan spectrum of *trans*-4-hydroxypraziquantel glucuronide obtained by microsomal incubation of *trans*-4-hydroxypraziquantel after consecutive fragmentation is presented. The nanoelectrospray device was used as ion source. The signal $m/z=329$ results of the deconjugated product and the fragment $m/z=311$ can be explained by the loss of water. The signal $m/z=203$ representing the pyrazinoisoquinoline-4-one moiety demonstrates that hydroxylation and following glucuronidation occurred in the cyclohexyl part of the molecule. The same fragmentation pattern was obtained after consecutive fragmentation of *cis*-4-hydroxypraziquantel glucuronide (data not shown). In contrast to the *trans*-4-hydroxypraziquantel glucuronide, a different fragmentation pattern was obtained by consecutive fragmentation of M4 (Fig. 8). M4 was chosen as an example to demonstrate a different fragmentation pattern in contrast to *trans*-4-hydroxypraziquantel glucuronide. Especially the fragments $m/z=219$, $m/z=201$ and $m/z=130$ indicate that hydroxylation and following glucuronidation occurred in position 6 or 7 of PZQ. These results are in agreement with those obtained by Lerch and Blaschke who investigated phase I metabolites of praziquantel after incubation with rat liver microsomes [17].

4. Conclusions

The usefulness of LC–MS and CE–MS coupling as complementary techniques in the investigation of drug metabolism was demonstrated. With CE–MS coupling we were able to detect different hydroxylated praziquantel metabolites as well as their glucuronide and sulfate conjugates within a single run, whereas sulfate conjugates could not be detected with the developed LC–MS method. The polar glucuronide and sulfate conjugates of praziquantel were identified directly without enzymatic hydrolysis prior to the analysis for the first time. Due to the formation of different positional isomers the determination of the exact structure of the hydroxylated praziquantel metabolites and their conjugates only by their molecular mass is not possible. Therefore the nanoelectrospray ion source was proved to be very useful. Because of its low flow-rates and sample consumption it offers the opportunity to perform even time consuming MS^n spectrometric investigations.

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