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Separation and identification of zaleplon metabolites in human urine using capillary electrophoresis with laser-induced fluorescence detection and liquid chromatography–mass spectrometry

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Abstract

A capillary electrophoresis (CE) method using laser-induced fluorescence (LIF) detection for the determination of the hypnotic drug zaleplon and its metabolites in human urine could be developed using carboxymethyl-β-cyclodextrin as a charged carrier. By the help of a complementary HPLC method coupled to mass spectrometry, three metabolites present in human urine could be identified as 5-oxozaleplon, 5-oxo-*N*-deethylzaleplon and 5-oxozaleplon glucuronide. *N*-Deethylzaleplon, a previously described zaleplon metabolite, as well as zaleplon itself could not be detected in human urine by the CE–LIF assay. The results were confirmed by spiking with reference compounds of the phase I metabolites. The metabolites differed very much concerning their fluorescence intensities, thus the 5-oxo metabolites present as lactam tautomer fluoresced tenfold lower than the unchanged drug zaleplon and its *N*-deethylated metabolite. The glucuronide of the 5-oxozaleplon, however, showed high fluorescence due to its lactim structure. Limits of quantification yielded by the CE–LIF assay including a ten-fold preconcentration step by solid-phase extraction were 10 ng/ml for zaleplon and *N*-deethylzaleplon and 100 ng/ml for 5-oxozaleplon and 5-oxo-*N*-deethylzaleplon.

Keywords: Zaleplon; Deethylzaleplon; Oxozaleplon; Oxodeethylzaleplon; Oxozaleplon glucuronide

1. Introduction

Zaleplon (ZAL), a pyrazolopyrimidine derivative, is an ultra-short-acting sleep-inducing drug with a prompt onset of action. Although not structurally related to the benzodiazepines, zaleplon acts through binding to the γ-aminobutyric acid ($GABA_A$)–benzodiazepine receptor complex producing sedative and hypnotic effects similar to those of benzodiazepines [1–3]. The drug is reported to have relative selectivity for the Ω_1-subtype of the benzodiazepine binding site [4] implementing less side effects than benzodiazepines [5].

After oral administration zaleplon is rapidly and nearly completely absorbed with peak plasma concentrations occurring within 1 h after dosing [6]. Due to an extensive presystemic metabolism, however, the absolute oral bioavailability of the drug is only 30% [7]. A terminal elimination half-life time of 1 h indicates rapid clearance from the body [6].

The proposed phase I metabolism is presented in Fig. 1. Zaleplon has been found to be a substrate for both aldehyde oxidase and the cytochrome P450 system [8]. In man (and in monkey), the aldehyde oxidase system predominates with the major metabolite 5-oxozaleplon (5-OZ) [9]. In contrast, in mouse,

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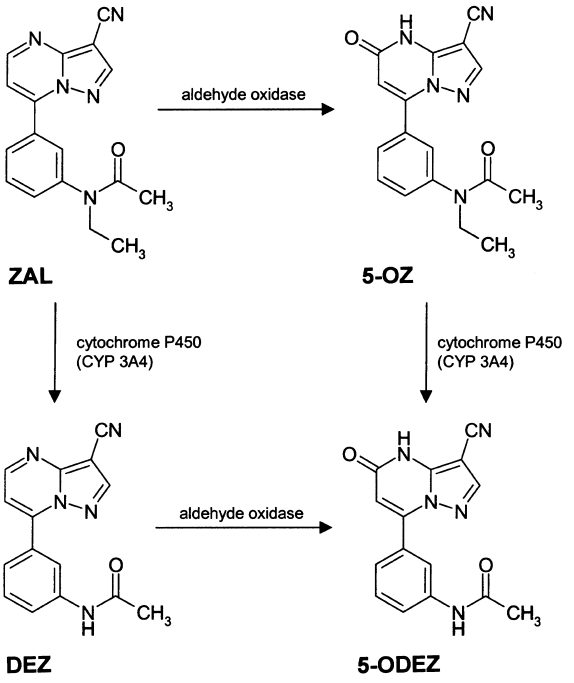

Fig. 1. Metabolic pathways of the phase I metabolism of zaleplon.

rat and dog, the cytochrome P450 system predominates and *N*-deethylzaleplon (DEZ) is formed as the major metabolite [9]. In vitro CYP 3A4 could be identified as the isoform mainly involved into the *N*-deethylation process [8]. Both, 5-oxozaleplon and *N*-deethylzaleplon are further converted to 5-oxo-*N*-deethylzaleplon (5-ODEZ). Beyond this, glucuronide conjugates of 5-oxozaleplon and 5-oxo-*N*-deethylzaleplon have been described as phase II metabolites [8,10]. All zaleplon metabolites are pharmacologically inactive [11].

For the simultaneous determination of zaleplon and its metabolites, different high-performance liquid chromatography methods with both UV and fluorescence detection have been described [1,9]. However, one of these methods [1] only considered ZAL and its minor metabolite DEZ, and by none of these methods glucuronide conjugates were detected.

Capillary electrophoresis (CE) is becoming a useful technique for the determination of drugs and drug metabolites in biological fluids because of its high resolution, mass sensitivity and the small sample volumes required. However, using conventional UV absorbance detection, concentration sensitivity and detection limit are frequently not sufficient because of the short optical path length in the capillary. In order to improve sensitivity, laser-induced fluorescence (LIF) detection can be used, if the analytes can be excited to natural fluorescence at the emission wavelength of the employed laser light source [12,13]. In previous investigations, the non-benzodiazepine hypnotics zolpidem, an imidazopyridine, and zopiclone, a cyclopyrrolone, and their metabolites have been successfully separated and detected by CE with LIF detection using a HeCd-laser with an excitation wavelength of 325 nm [14,15].

In the present publication we present a CE–LIF method for the simultaneous separation of ZAL and its phase I and phase II metabolites in human urine. As at the beginning of our work reference compounds of the metabolites were not available to us, peaks were identified via a complementary HPLC method and LC–electrospray ionization (ESI)-MS coupling. Later on, the obtained results could be confirmed and extended by comparison with the reference compounds of ZAL and its phase I metabolites. Furthermore, by a simple calibration experiment the limits of quantification (LOQs) for all compounds were determined as well as the concentrations of the phase I metabolites present in urine of a human volunteer.

2. Experimental

2.1. Chemicals and reagents

Zaleplon {3′-(3-cyanpyrazolo[1,5-*a*]pyrimidin-7-yl)-*N*-ethylacetanilide}, 5-oxozaleplon {3′-(3-cyan-5-oxo-4,5-dihydropyrazolo[1,5-*a*]pyrimidin-7-yl)-*N*-ethylacetanilide}, *N*-deethylzaleplon {3′-(3-cyan-pyrazolo[1,5-*a*]pyrimidin-7-yl)-acetanilide} and 5-oxo-*N*-deethylzaleplon {3′-(3-cyan-5-oxo-4,5-dihydropyrazolo[1,5-*a*]pyrimidin-7-yl)-acetanilide} were obtained as a gift from Wyeth (Pearl River, NY, USA). Sonata 10 mg capsules were supplied from Wyeth-Pharma (Münster, Germany).

All reagents used were of analytical grade unless otherwise specified. Boric acid, dimethyl sulfoxide (DMSO), methanol, tris(hydroxymethyl)aminomethane (Tris, base and hydrochloride) and 0.1 *M*

sodium hydroxide solution were obtained from E. Merck (Darmstadt, Germany). Acetonitrile (HPLC gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands) and ammonium acetate by J.T. Baker (Deventer, The Netherlands). Carboxymethyl-β-cyclodextrin (average degree of substitution 0.5; molecular mass 1415.1 g/mol) was supplied by Wacker (Burghausen, Germany). Uridine-5′-diphosphoglucuronic acid (UDPGA, as triammonium salt) was purchased from Fluka (Buchs, Switzerland).

2.2. Sample collection and pretreatment

Urine samples in the time intervals 0–2, 2–4, 4–6, 6–9 and 9–12 h after oral administration of 10 mg zaleplon (Sonata) were collected from a human volunteer and stored at −18 °C until analysis.

In order to obtain sufficient limits of detection, a preconcentration step was necessary and carried out by solid-phase extraction (SPE) using a BakerBond spe Octadecyl (C_{18}) column (No. 7020-03, J.T. Baker, Phillipsburg, NJ, USA). The SPE columns were preconditioned with methanol (2×3 ml) and equilibrated with double-distilled water (2×3 ml). After loading 10 ml of the urine sample, salts and other hydrophilic matrix compounds were washed out with double-distilled water (2×3 ml). After drying the SPE material by applying a reduced pressure for about 15 min, the analytes were eluted with 4×250 μl methanol.

The methanol was evaporated in a gentle nitrogen stream at about 60 °C and the residue was reconstituted in 1.0 ml of double-distilled water.

Blank urine collected from the same volunteer directly before dosing was pretreated the same way.

2.3. Preparation of spiked urine samples

As the solubility of the analytes, in particular of 5-ODEZ, in water was poor, stock solutions of ZAL, DEZ, 5-OZ and 5-ODEZ were prepared by dissolving about 1 mg of each compound (0.5 mg of 5-ODEZ) in 300 μl (500 μl in case of 5-ODEZ) DMSO. Afterwards the solution was diluted with double-distilled water to a final volume of 10.0 ml.

To obtain urine samples with known content, blank urine was spiked with these stock solutions in the concentrations required. The spiked urine samples were extracted as described before.

2.4. Fluorescence spectra

Fluorescence emission spectra (λ_{ex}=325 nm) of ZAL, DEZ, 5-OZ and 5-ODEZ were recorded on a Shimadzu spectrofluorometer RF-540 equipped with a Shimadzu data recorder DR-3 (Shimadzu, Kyoto, Japan). Aqueous stock solutions prepared as described before were further diluted with double-distilled water to a concentration of 1.0 μg/ml each. Fluorescence spectra were recorded in the emission range from 350 to 600 nm using a quartz glass cuvette with a diameter of 1 cm.

2.5. Separation by capillary electrophoresis

A Beckman P/ACE system 2100 equipped with a LIF detector (Beckman Instruments, Fullerton, CA, USA) was used with an untreated fused-silica capillary of 50 μm I.D.×365 μm O.D., 20 cm effective length and 27 cm total length (Polymicro Technologies, Phoenix, AZ, USA).

The electrophoresis buffer was prepared by dissolving 12.37 mg/ml boric acid (0.2 *M*) and 121.14 mg/ml tris(hydroxymethyl)aminomethane (1.0 *M*) in double-distilled water. Afterwards 707.6 mg of carboxymethyl-β-cyclodextrin (50 m*M*) were dissolved in this buffer to a final volume of 10.0 ml. The pH value of the buffer was at about 9.4.

Separation was carried out by applying a voltage of 15 kV (electric field strength=556 V/cm) resulting in a current of approximately 100 μA. The temperature was maintained at 20 °C during electrophoresis.

Detection was performed by laser-induced fluorescence using a HeCd-laser (Omnichrome, Series 74, Laser 2000, Wessling, Germany) with a power of 20 mW and a wavelength of 325 nm. The laser was connected to the LIF detector of the CE system by an optical fiber (Omnichrome POS FDS-A$\frac{1}{2}$, Laser 2000). Emission was measured at 450 nm using an interference filter with a half bandwidth of about 13 nm. Data collection and processing were performed using System Gold software 7.11 (Beckman Instruments).

At the beginning of a series the capillary was rinsed with 0.1 *M* sodium hydroxide solution for 30 min to equilibrate the capillary. Rinsing steps were always carried out applying a pressure of 20 p.s.i. (=1379 hPa). Before each run the capillary was flushed with electrophoresis buffer for 2 min. Samples were introduced into the capillary by pressure injection with 0.5 p.s.i. (=34.5 hPa) for 10 s, equivalent to an injected sample volume of about 19.5 nl. After each run the capillary was washed with 0.1 *M* sodium hydroxide solution for 1.5 min and with double-distilled water for 1 min. At the end of a day the capillary was always rinsed with 0.1 *M* sodium hydroxide solution and double-distilled water for 5 min each before drying with nitrogen for 5 min.

2.6. Separation and identification by HPLC–ESI-MS

A complementary liquid-chromatographic separation of the zaleplon metabolites in human urine was carried out on a LiChrospher RP 8e column (particle size 5 μm, dimensions 125×4 mm) (Merck–Hitachi, Darmstadt, Germany). The mobile phase consisted of acetonitrile (ACN) and 50 m*M* ammonium acetate buffer (3,8545 g ammonium acetate dissolved in 1.0 l double-distilled water). The following binary gradient was applied at a flow-rate of 1 ml/min: 0 min, ACN 10%; 0–10 min, ACN linear from 10 to 20%; 10–13 min, ACN at 20%; 13–23 min, ACN linear from 20 to 45%; 23–25 min, ACN at 45%.

The chromatographic system consisting of a Waters 2690 separations module with an automatic sample injection and a Waters 486 tunable absorbance detector (Waters, Milford, MA, USA) was coupled on-line to a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) via a common electrospray interface. The injection volume of the urine extracts (0–2 h and blank) prepared as described in Section 2.2 was 100 μl.

The wavelength of the UV absorbance detector was set to 245 nm. The ESI voltage of the mass spectrometer was set to 4.2 kV, the sheath gas flow was adjusted to 80 arbitrary units and the auxiliary gas flow was set to 25 arbitrary units. The temperature of the heated capillary was 200 °C. Mass spectra (m/z 100–1000) were recorded in positive ion mode.

2.7. Identification of the unknown peaks in the CE–LIF assay

To identify the unknown peaks obtained by the CE separation (Fig. 2), zaleplon metabolites were isolated by semi-preparative HPLC under the same chromatographic conditions as described above.

The chromatographic system used consisted of a L-6200A intelligent pump, a L-4000 UV detector and a D-2500 chromato-integrator (Merck–Hitachi). Sample injection was performed by a Rheodyne 7125 with a 100 μl loop (Rheodyne, Cotati, CA, USA). For each run, 100 μl of the preconcentrated urine extract collected 0–2 h after oral administration of 10 mg zaleplon were injected. About 12 runs were carried out. The fractions at the retention times of the three peaks of interest were collected manually and combined before the eluent was evaporated under reduced pressure until a residue of about 1 ml. Finally, the isolated metabolites obtained were used for spiking experiments in the CE–LIF assay.

Later on, the obtained results could be confirmed by spiking with the reference compounds of the metabolites 5-OZ, 5-ODEZ and DEZ.

2.8. Enzymatic glucuronidation of 5-oxozaleplon

To confirm the formation of the glucuronide of

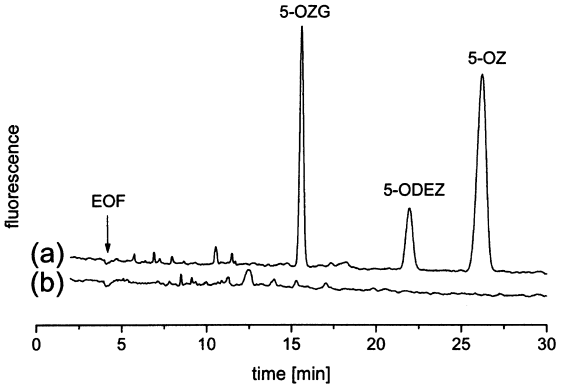

Fig. 2. Electropherograms of a urine sample obtained from a human volunteer 2 h after dosing with 10 mg zaleplon (a) and blank urine, (b) electrophoretic conditions: 27 cm (effective length 20 cm)×50 μm I.D. capillary, applied voltage: 15 kV, running buffer: 0.2 *M* boric acid, 1.0 *M* Tris, 50 m*M* carboxymethyl-β-cyclodextrin, pH 9.4, LIF detection (λ_{ex} = 325 nm, λ_{em} = 450 nm), EOF = electroosmotic flow.

5-OZ, enzymatic glucuronidation of this phase I metabolite was performed. Therefore, 200 μl of a solution of 5-OZ in acetonitrile (0.3 mg/ml) was evaporated. The residue was dissolved in 800 μl 0.05 *M* Tris buffer pH 7.4, and 200 μl of a suspension of phenobarbital-induced rat liver microsomes and 20 μl of 0.5 *M* UDPGA solution (315 mg/ml in 0.05 *M* Tris buffer pH 7.4) were added. The mixture was incubated for 4 h at 37 °C. Afterwards the reaction was stopped by addition of 1 ml of about −18 °C cold acetonitrile. The protein precipitate was removed by centrifugation and the supernatant was directly injected into the CE system and analyzed. A blind experiment without addition of UDPGA solution was carried out under the same conditions.

2.9. Calibration of the CE–LIF assay and in vivo analysis

A simple calibration experiment was done for the CE–LIF determination of ZAL, DEZ, 5-OZ and 5-ODEZ. Spiked standard samples at eight (respectively, seven for 5-ODEZ) different concentration levels covering the range from 10 ng/ml to 2 μg/ml for ZAL and DEZ (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/ml), from 100 ng/ml to 20 μg/ml for 5-OZ (0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg/ml) and from 100 ng/ml to 7 μg/ml for 5-ODEZ (0.1, 0.2, 0.5, 1, 2, 5 and 7 μg/ml) were prepared and extracted according to Section 2.3.

Originally it was intended to use zolpidem, that exhibits excellent fluorescence properties [14] and is well separated under the electrophoretic conditions applied (Fig. 7) as an internal standard (I.S.), but as the recovery from the extraction procedure for this substance was not reproducible, regression lines were calculated without internal standard basing upon the absolute corrected peak areas (=peak area/electrophoretic velocity) of the analytes. Recoveries of the analytes ZAL, DEZ, 5-OZ and 5-ODEZ were assessed by comparing the corrected peak areas of an extracted standard sample with an unextracted standard prepared at the same concentration in blank urine representing 100% recovery.

Human urine samples collected as mentioned in Section 2.2 were extracted and analyzed in the same way.

3. Results and discussion

3.1. Capillary electrophoretic conditions

As in the beginning of our work only the drug zaleplon itself was available as reference compound, electrophoretic conditions were optimized in particular with regard to this analyte. Zaleplon was not chargeable at all even at very low pH values (pH 2.0), thus charged buffer additives implementing a carrier function for this analyte were necessary for the separation. Different anionic cyclodextrins as well as micelle-forming surfactants (sodium dodecyl sulphate) were tested under various pH conditions for this purpose, and finally carboxymethyl-β-cyclodextrin as a 50 m*M* solution in an alkaline borate–Tris buffer was chosen. Applying these conditions, analytes were separated as negatively charged cyclodextrin complexes and migrated slower than the electroosmotic flow (EOF) towards the detection window at the cathodic side of the capillary.

The addition of the anionic carrier, however, led to prolonged analysis times, therefore the capillary length was chosen as short as possible and the applied voltage as high as possible. At a voltage higher than 15 kV, however, implementing a resulting current higher than 100 μA current breakdowns very often occurred in the system.

The electropherogram obtained with an extracted urine sample from a human volunteer collected 2 h after oral administration of 10 mg zaleplon is presented in Fig. 2. Three peaks obviously corresponding to zaleplon metabolites could be observed in comparison to blank urine; zaleplon itself migrating at about 11 min under these conditions was not detected.

As a result, the developed assay was capable of distinguishing between the unchanged drug and three initially unknown metabolites present in human urine.

3.2. Identification of the metabolites

3.2.1. Identification of zaleplon metabolites in human urine by LC–ESI-MS

In order to identify the zaleplon metabolites observed in human urine, a complementary HPLC method with simultaneous UV and MS detection was

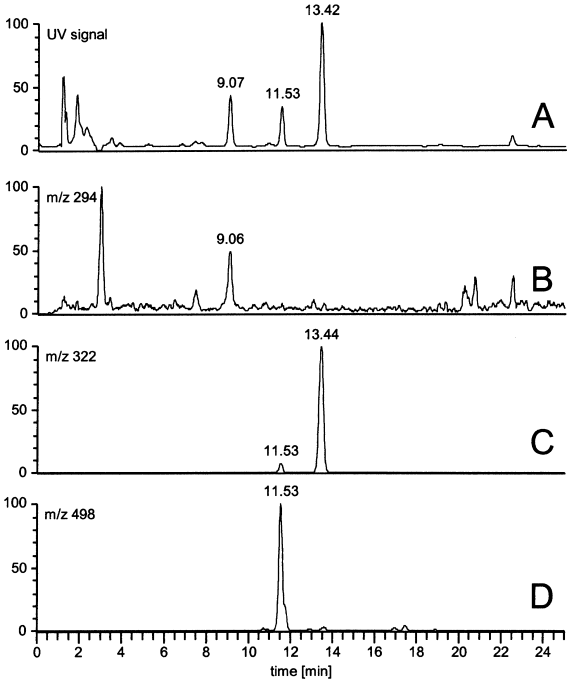

Fig. 3. HPLC–ESI-MS chromatogram of a solid-phase extracted urine sample (0–2 h) after oral administration of 10 mg zaleplon: (A) UV signal, (B) selected mass track m/z 294, (C) selected mass track m/z 322 and (D) selected mass track m/z 498. Experimental conditions: column: LiChrospher RP 8e (5 μm) 4×125 mm, mobile phase: acetonitrile–50 m*M* ammonium acetate buffer, gradient elution, flow-rate: 1 ml/min, injection volume: 100 μl, detection: UV (245 nm) and ESI-MS.

developed. The separation based on a previously published HPLC method [9] that was slightly modified. The resulting chromatogram (UV signal) of the 0–2 h urine extract is presented in Fig. 3A. Mass spectra have been recorded at the maxima of the three peaks appearing at 9.06, 11.53, and 13.44 min (Table 1).

The peak at 13.44 min could be identified as 5-OZ ($M_r = 321$). The corresponding mass spectrum (Fig. 4A) shows m/z ratios of 322 (5-OZ + H^+) and 339 (5-OZ + NH_4^+). Furthermore, dimeric adducts were observed (m/z 643 (2× 5-OZ + H^+) and m/z 660 (2× 5-OZ + NH_4^+)). Ammonium adducts were formed under the applied conditions as ammonium acetate was used in the mobile phase.

The peak at 9.06 min (Fig. 4B) referred to 5-ODEZ ($M_r = 293$). The m/z ratios of 294 (5-ODEZ + H^+), 311 (5-ODEZ + NH_4^+), 587 (2× 5-ODEZ + H^+) and 604 (2× 5-ODEZ + NH_4^+) could be observed in the corresponding mass spectrum.

Finally, the peak with the retention time of 11.53 min could be identified as a glucuronide of 5-OZ (5-OZG, $M_r = 497$). The mass spectrum (Fig. 4C) shows m/z ratios of 498 (5-OZG + H^+) and 515 (5-OZG + NH_4^+). The peak at m/z 322 (5-OZ + H^+) can be explained as the result of a partial fragmentation of the glucuronide in the ion-trap.

In Fig. 3B–D the mass tracks for the M + H^+ adducts of the metabolites 5-ODEZ, 5-OZ and 5-

Table 1
Results of the LC–ESI-MS investigation of a human urine sample obtained after oral administration of 10 mg zaleplon: measured data and proposed compounds

Retention time (min)	Measured m/z	Proposed compound	Calculated monoisotopic mass	Peak identified as
13.44	322.3	$[C_{17}H_{15}N_5O_2+H]^+$	322.1	5-OZ
	339.1	$[C_{17}H_{15}N_5O_2+NH_4]^+$	339.2	
	642.9	$[2\times C_{17}H_{15}N_5O_2+H]^+$	643.3	
	659.7	$[2\times C_{17}H_{15}N_5O_2+NH_4]^+$	660.3	
9.06	294.3	$[C_{15}H_{11}N_5O_2+H]^+$	294.1	5-ODEZ
	311.0	$[C_{15}H_{11}N_5O_2+NH_4]^+$	311.1	
	587.1	$[2\times C_{15}H_{11}N_5O_2+H]^+$	587.2	
	603.6	$[2\times C_{15}H_{11}N_5O_2+NH_4]^+$	604.2	
11.53	322.3	$[C_{17}H_{15}N_5O_2+H]^+$	322.1	5-OZG
	498.0	$[C_{23}H_{23}N_5O_8+H]^+$	498.2	
	514.6	$[C_{23}H_{23}N_5O_8+NH_4]^+$	515.2	

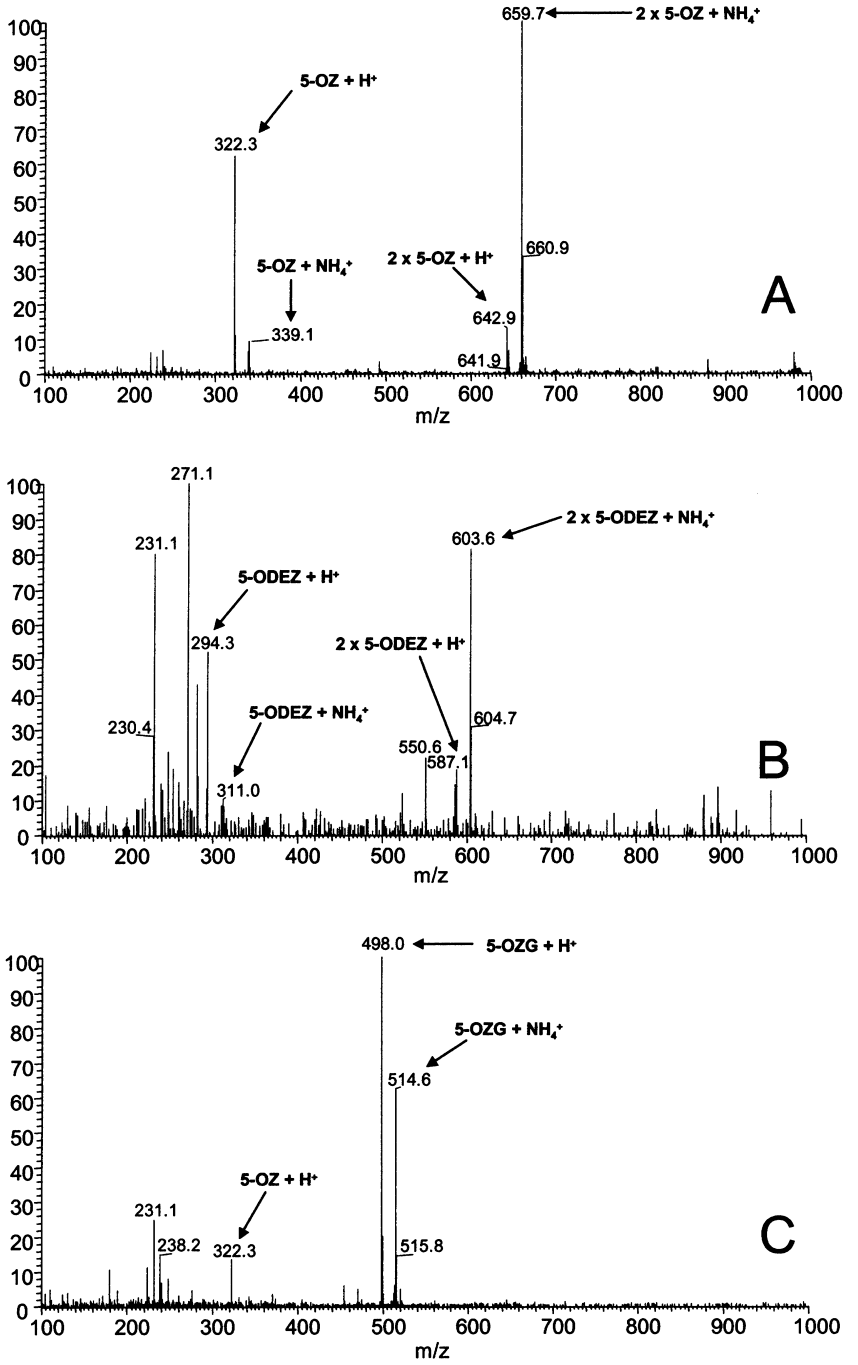

Fig. 4. Mass spectra of the peaks at 13.44 min (A), 9.06 min (B) and 11.53 min (C). Experimental conditions: positive ion mode, ESI voltage: 4.2 kV, sheath gas flow: 80 arbitrary units, auxiliary gas flow: 25 arbitrary units, capillary temperature: 200 °C, full scan, mass range: m/z 100–1000.

OZG have been selected, the observed peaks are corresponding to the peaks in the UV signal (Fig. 3A). The proposed partial fragmentation of the glucuronide in the electrospray can be observed again, as m/z 322 does not only appear at the retention time of 5-OZ but also at a minor extension at the retention time of the 5-OZG.

3.2.2. Peak identification of the CE assay

After the identification of the three appearing metabolites by LC–ESI-MS, they were isolated by HPLC fractionation. About 12 runs and evaporation of the eluent of the combined fractions of each analyte to about 1 ml were necessary for spiking experiments in the CE–LIF assay. The results were like presented in Fig. 2. The first peak at about 16 min was the glucuronide of 5-OZ, the second peak (22 min) was corresponding to 5-ODEZ and the third peak (27 min) was identified as 5-OZ. In contrast to the chromatogram presented in Fig. 3A, in the CE–LIF assay the glucuronide peak is higher than the peak of 5-OZ indicating better fluorescence of this conjugate. This will be further discussed in Section 3.3.

The identification of the peaks of 5-OZ and 5-ODEZ could be confirmed later by spiking with the corresponding reference compounds.

3.2.3. Confirmation of the identity of 5-OZG by enzymatic glucuronidation

The result of the enzymatic glucuronidation of 5-OZ is presented in Fig. 5. It could be shown that the glucuronide appearing at about 16 min in the CE–LIF assay is derived from the phase I metabolite 5-OZ by enzymatic incubation with ‘‘active glucuronic acid’’ (UDPGA). Thus, the identity of 5-OZG is confirmed by this experiment.

3.3. Fluorescence emission spectra

Fluorescence emission spectra ($\lambda_{ex} = 325$ nm) recorded with the reference compounds of ZAL and the phase I metabolites DEZ, 5-OZ and 5-ODEZ (Fig. 6) revealed great differences of the fluorescence intensities of ZAL and DEZ on the one hand,

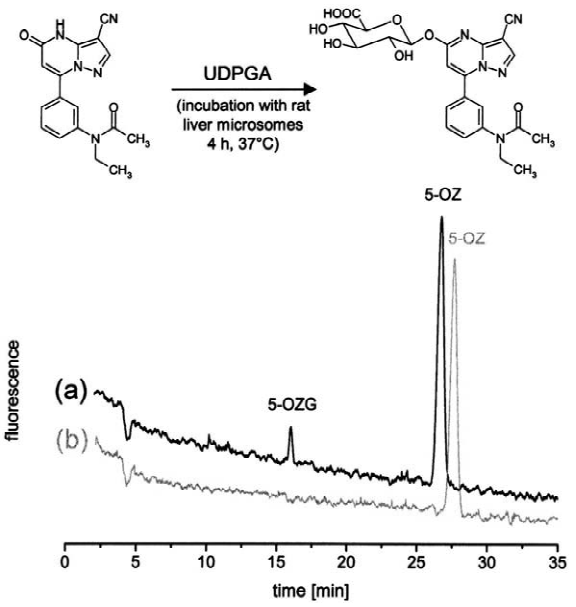

Fig. 5. Enzymatic glucuronidation of 5-oxozaleplon: electropherograms of a microsomal incubation with UDPGA (a) in comparison to an incubation without UDPGA (b). For electrophoretic conditions: see Fig. 2.

and of the 5-oxo metabolites 5-OZ and 5-ODEZ on the other hand. The fluorescence maxima were at about 460 nm for all compounds investigated. Fluorescence intensity of a substance is strongly correlated with the planarity of the molecule. By the formal hydroxylation of ZAL in position 5 and the subsequent tautomerisation into the more favored lactam form, the planarity of the aromatic pyrazolopyrimidine structure is disturbed resulting in a decrease of fluorescence intensity of these metabolites.

In order to predict the fluorescence of the glucuronide conjugate of the 5-oxo metabolite 5-OZ, it has to be considered, that for the glucuronidation the lactim form has to be realized, that is planar again and should exhibit fluorescence intensities in the order of magnitude of the non-5-oxo compounds.

Hereby it can be explained that the signal of 5-OZG in the urine extract is the highest peak in the CE–LIF assay (Fig. 2) while it is a minor peak in the HPLC determination with UV absorbance detection applied (Fig. 3A).

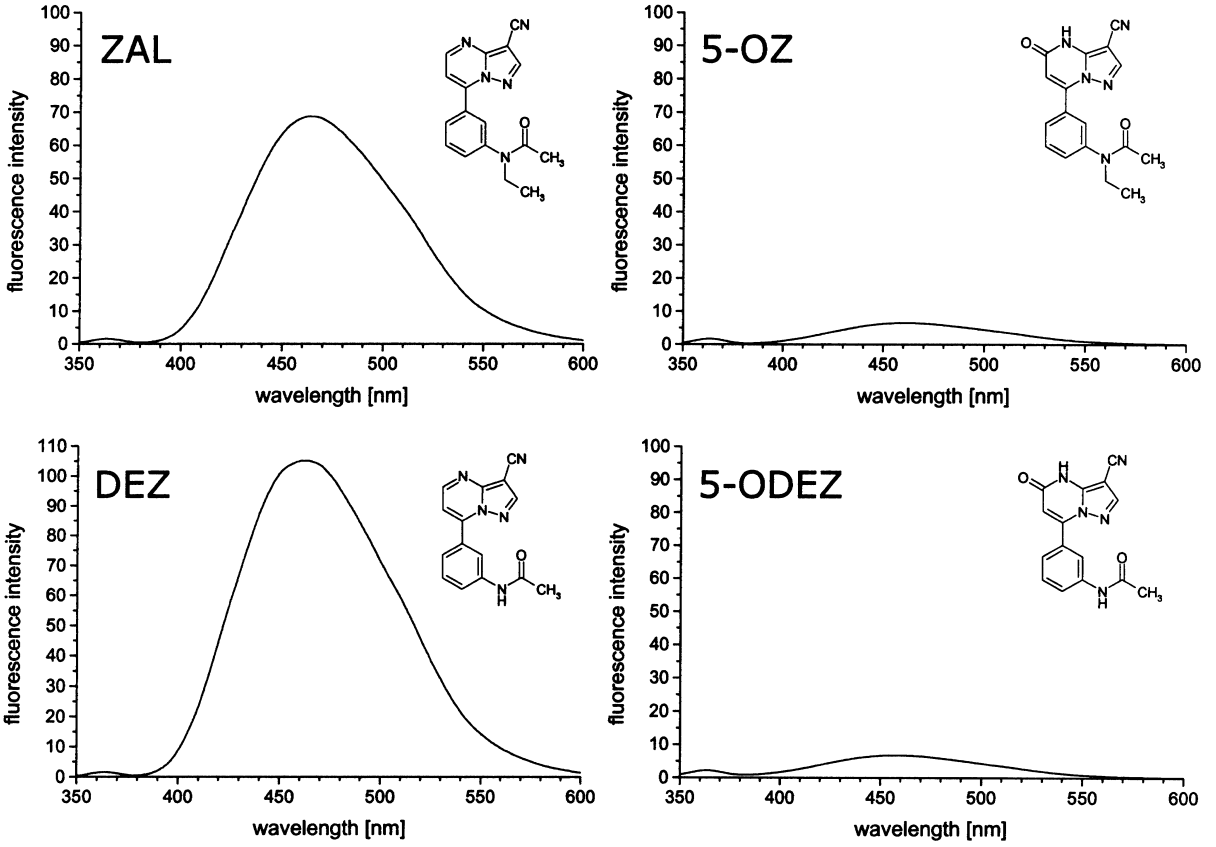

Fig. 6. Fluorescence emission spectra (λ_{ex} = 325 nm) of ZAL, DEZ, 5-OZ and 5-ODEZ.

3.4. Calibration data

The linearity and limits of quantification of the CE–LIF assay with a preceding solid-phase extraction from human urine for the compounds ZAL, DEZ, 5-OZ and 5-ODEZ were assessed by a calibration in the concentration range between 10 ng/ml and 2 μg/ml for ZAL and DEZ, between 100 ng/ml and 20 μg/ml for 5-OZ and between 100 ng/ml and 7 μg/ml for 5-ODEZ. As the intended I.S. compound zolpidem was not reproducibly recovered from the SPE, slope and intercept were calculated directly with the absolute corrected peak areas using a $1/x^2$-weighted regression model in order to minimize the residuals over the entire calibration range. The results are summarized in Table 2. A typical electropherogram of an extracted human urine sample spiked with ZAL, DEZ, 5-OZ and 5-ODEZ (and zolpidem) (Fig. 7) demonstrates in particular the location of those compounds (ZAL and DEZ) in the electropherogram that were not present in the native urine samples.

3.5. In vivo analysis

The CE–LIF assay was applied to the urine samples collected from a human volunteer in certain time intervals after oral administration of 10 mg zaleplon. The measured urinary concentrations of 5-OZ and 5-ODEZ (Table 3) demonstrate the necessity of the preconcentration procedure by SPE, as without a tenfold preconcentration only the peaks of the first urine fraction (0–2 h) would have been higher than the LOQs of the assay. On the assump-

Table 2
Calibration data, limits of quantification and recovery values of the CE–LIF assay

	Slope	Intercept	Correlation coefficient	Limit of quantification (ng/ml)	Recovery (%)
ZAL	10.6023	−0.0237	0.9998	10	94
DEZ	14.5941	0.0419	0.9995	10	97
5-OZ	1.1172	−0.0296	0.9997	100	96
5-ODEZ	1.2621	0.0285	0.9993	100	85

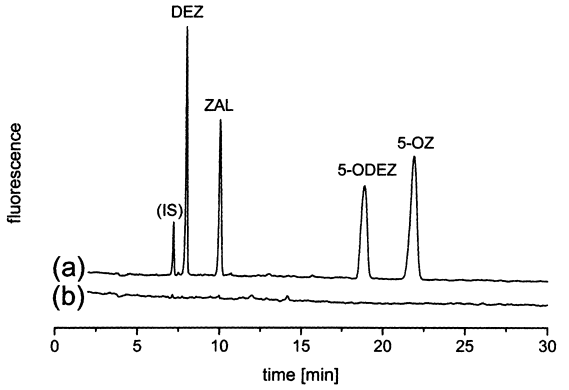

Fig. 7. Electropherograms of a urine sample spiked with 1 μg/ml of ZAL and DEZ, 10 μg/ml of 5-OZ and 7 μg/ml of 5-ODEZ (a) and blank urine (b). For electrophoretic conditions: see Fig. 2.

tion that 5-OZG exhibits the same molar fluorescence intensity as ZAL itself (as discussed in Section 3.3) and furthermore shows equal recovery from the extraction procedure, urinary concentrations of 5-OZG can be estimated as shown in Table 3.

4. Conclusion

Regarding all previously reported zaleplon metabolites, three phase I metabolites (5-OZ, DEZ and 5-ODEZ) and two possible glucuronides (of 5-OZ and 5-ODEZ), only three of them (5-OZ, 5-ODEZ and the glucuronide of 5-OZ) have been detected in human urine by the presented CE–LIF assay.

Although laser-induced fluorescence detection using a HeCd-laser with an excitation wavelength of 325 nm proved excellent sensitivity for the compounds zaleplon and *N*-deethylzaleplon (not present in human urine), it showed no notable advantage in the detection of 5-oxozaleplon and 5-oxo-*N*-deethylzaleplon, that were present in human urine. Merely the glucuronide of 5-OZ revealed high fluorescence intensity under the applied conditions, but could not be exactly quantified as no reference standard was available of this compound.

Table 3
Urinary concentrations of zaleplon metabolites after oral administration of 10 mg zaleplon to a human volunteer

Time (h)	Concentration (μg/ml)		
	5-OZ	5-ODEZ	5-OZG*
0–2	8.88	2.13	[1.33]
2–4	2.57	0.90	[1.41]
4–6	0.57	0.30	[0.34]
6–9	0.36	0.28	[0.23]
9–12	Not detectable	Not detectable	Not detectable

*Values in square brackets are estimated (as described in Section 3.5).

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