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Enantioselective determination of zopiclone and its metabolites in urine by capillary electrophoresis

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Abstract

A method has been developed for the stereoselective determination of zopiclone and its main metabolites in urine. After the addition of the internal standard zolpidem the urine samples were extracted at pH 8 with chloroform–isopropanol (9:1). Analyses were carried out using capillary electrophoresis (CE) with β-cyclodextrin as the chiral selector. The analytes were detected using UV laser-induced fluorescence detection with a He–Cd laser operated at 325 nm. Urine samples of two volunteers after oral administration of 7.5 mg zopiclone were investigated. The *S*-(+)-enantiomers of zopiclone and its metabolites were always excreted in higher amounts than the *R*-(−)-enantiomers. With the same method the zopiclone enantiomers were quantified in saliva. Compared to high-performance liquid chromatography, the CE method is very fast and simple.

Keywords: Zopiclone; N-Desmethylzopiclone; Zopiclone N-oxide

1. Introduction

Zopiclone (Fig. 1) is a chiral nonbenzodiazepine hypnotic drug of the cyclopyrrolone class. The drug is marketed as a racemate. Recently it was shown, that the *S*-(+)-enantiomer exhibits a fifty times higher affinity towards the benzodiazepine receptor binding site than the *R*-(−)-enantiomer [1]. After oral administration the drug is extensively metabolized. The main metabolites in urine are the chiral N-desmethylzopiclone and zopiclone N-oxide (Fig. 1), which is pharmacologically active. These metabolites are not detectable in human plasma [2].

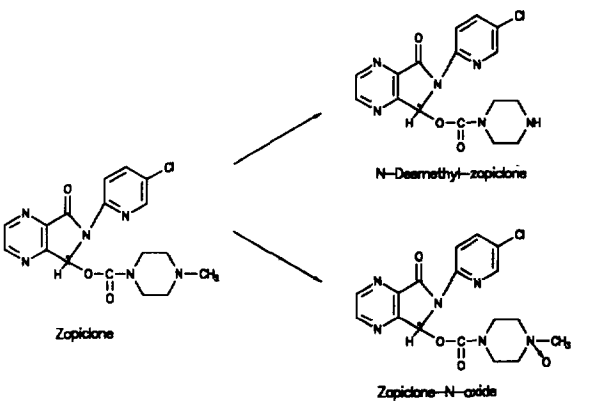

Fig. 1. Metabolism of zopiclone.

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About 50% of the dose are decarboxylated to unidentified metabolites.

Two methods have been described to quantify the zopiclone enantiomers in plasma using high-performance liquid chromatography (HPLC) with a chiral cellulose trisphenylcarbamate column [3,4]. For the simultaneous enantioselective determination of zopiclone and its main metabolites in urine a HPLC method with a column-switching system has been reported [5]. However, the analysis time was long and the separation of the enantiomers incomplete.

Capillary electrophoresis (CE) is a very useful technique for the determination of drugs in body fluids because of its high resolution and speed [6]. We have developed an enantioselective method for the determination of zopiclone and its main metabolites in biological fluids using β-cyclodextrin as the chiral selector in the run buffer. In order to improve the sensitivity we used laser-induced fluorescence detection (UV–LIF) with a He–Cd laser. The method has been applied to study the biotransformation of zopiclone in urine from two volunteers after oral administration of 7.5 mg zopiclone.

2. Experimental

2.1. Capillary electrophoresis

A Beckman P/ACE System 2100 was used with an uncoated fused-silica capillary (50 μm I.D., 40 cm effective length, 47 cm total length). The separation was carried out with 100 m*M* phosphate buffer pH 2.75 (100 m*M* phosphoric acid was adjusted to pH 2.75 by addition of triethanolamine) and 16.3 m*M* β-cyclodextrin as

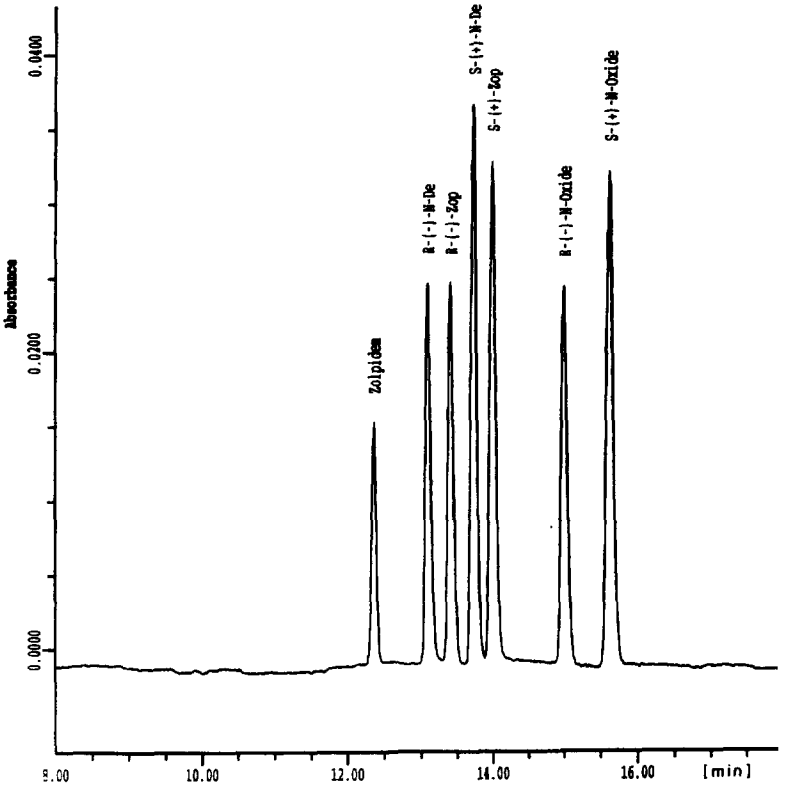

Fig. 2. Electropherogram of the racemates of zopiclone (Zop), its metabolites N-desmethylzopiclone (N-De) and zopiclone N-oxide (N-oxide) and the internal standard zolpidem.

the chiral selector. A run potential of 18 kV was applied. With triethanolamine the migration times were more constant in comparison to a buffer prepared from phosphoric acid and potassium dihydrogen phosphate. Between each analysis the capillary was rinsed with 100 m*M* sodium hydroxide for 1 min followed by a rinse with buffer pH 2.75 containing β-cyclodextrin. Samples were introduced into the capillary by pressure injection with 0.5 p.s.i. for 10 s. The temperature was maintained at 20°C. The detection was carried out with a Beckman LIF-detector equipped with a He–Cd laser (Omnichrome 3056-10M, 20 mW) operated at an excitation wavelength of 325 nm. Emission was measured at 450 nm. The data were collected and integrated with Beckman System Gold software 7.11 and the corrected peak-area ratios were calculated.

2.2. Chemicals

Zopiclone and its metabolites zopiclone N-oxide and N-desmethylzopiclone were kindly supplied by Rhône Poulenc Rorer (Köln, Germany). The enantiomers of zopiclone were obtained by fractional crystallisation described previously [1]. The absolute configuration of (+)-zopiclone was determined by Dr. Weckert, University of Karlsruhe, Germany, using X-ray analysis (unpublished results). The migration orders of the metabolites were determined using enriched enantiomer standards prepared as follows: the pure metabolites were separated by chiral HPLC according to the method of Fernandez [5] and the fractions with the enantiomers were collected from three runs. These fractions were evaporated to dryness. The residues were dissolved in ethanol and the rotation values were determined with a polarimeter (Perkin-Elmer 241).

The internal standard zolpidem was kindly supplied by Synthelabo Recherche (L.E.R.S.), Bagneux Cedex, France. All stock solutions of zopiclone and its metabolites were prepared in acetonitrile (HPLC grade) and were stored at −18°C. The other solvents were of analytical grade.

2.3. Extraction from urine samples

The method of Le Liboux et al. [7] was modified. 1 ml of urine, 2 ml of 100 m*M* borate buffer pH 8, 50 μl of a solution of zolpidem (1 μg/ml) in acetonitrile and 5 ml of chloroform–isopropanol (9:1) were shaken for 10 min and centrifuged for 10 min (3000 rpm, 1410 g). The organic phase was separated and evaporated

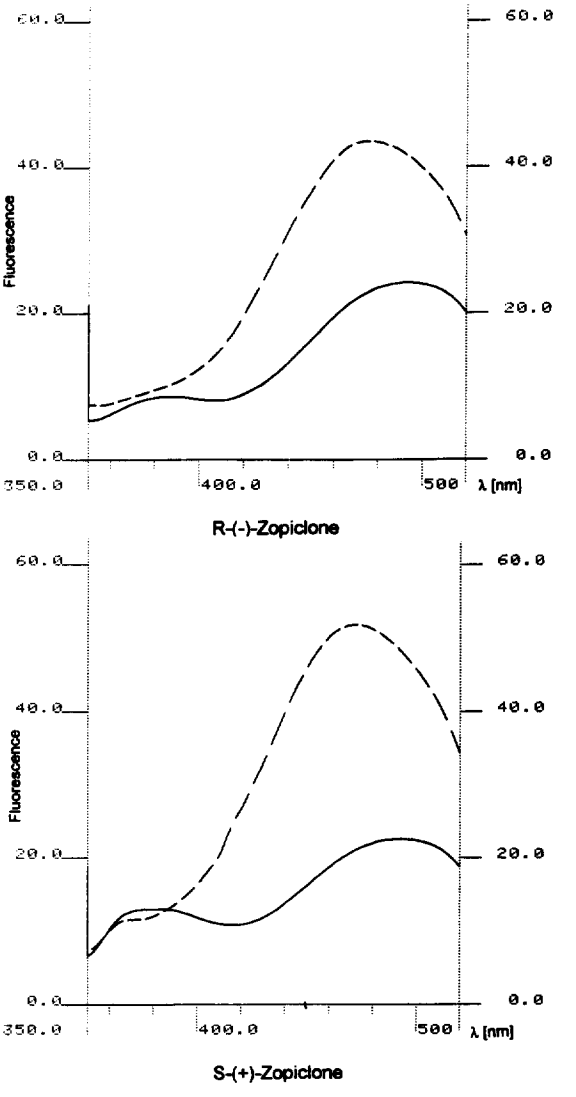

Fig. 3. Fluorescence spectra of the enantiomers of zopiclone (each 10 μg/ml). Conditions: phosphate buffer pH 2.75, 50 m*M*; dashed lines: the same buffer with 16.3 m*M* β-cyclodextrin.

under nitrogen at 20°C. The residue was reconstituted in 50 μl of acetonitrile–water (1:1).

Fluorescence spectra were accomplished with a Shimadzu RF 540 fluorescence spectrometer.

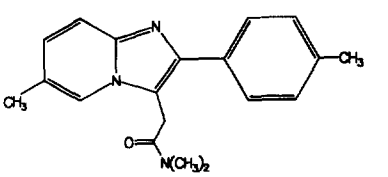

Fig. 4. Zolpidem.

3. Results and discussion

3.1. Development of the chiral assay

The separation of the enantiomers of zopiclone by capillary electrophoresis with β-cyclodextrin as the chiral additive [8] was modified to simultaneously separate zopiclone and its two chiral metabolites by reducing the applied voltage and changing the buffer composition. The best results were achieved with a saturated solution of β-cyclodextrin (i.e. 16.3 m*M*). Experiments with derivatized cyclodextrins like hydroxypropyl-β-cyclodextrin or hydroxymethyl-β-cyclodextrin did not improve the separation (data not shown).

Fig. 2 shows an electropherogram of zopiclone and its metabolites. By injecting the racemates the peaks from the second eluting enantiomers of the analytes are greater than the peaks of the first eluting enantiomer. There are two reasons for this observation: one reason is the slower migration velocity of the second peak in the detection window. This is a common observation in CE [9] and was also observed by UV-detection, but this cannot lead to different peak heights. The more important reason is an enhancement of the fluorescence by complexation. Fig. 3 shows the fluorescence spectra of the enantiomers of zopiclone under the same conditions used for the CE separation. The zopiclone–cyclodextrine complex has a much higher fluorescence intensity than zopiclone itself. Since the cyclodextrine complex of the slower migrating *S*-(+)-zopiclone is more stable than the complex with the *R*-(−)-enantiomer, the *S*-(+)-enantiomer will pass the detector cell to a higher degree as a cyclodextrin complex than the *R*-(−)-enantiomer. Thus, the peak of the *S*-(+)-enantiomer is much larger than the peak of the corresponding *R*-(−)-enantiomer when the racemate is applied. Although we have proven this fluorescence enhancement only for zopiclone, we conclude that these differences are similar for the metabolites.

Zolpidem (Fig. 4) was chosen as the internal standard because of its similar structure and the high fluorescence intensity at the same wavelength. Preliminary experiments were carried out with UV-detection, but the sensitivity was not sufficient for the analysis in biological fluids. With LIF-detection the sensitivity was at least fifteen times higher.

Table 1
Typical parameters of the calibration for the enantiomers of zopiclone (Zop) and its metabolites N-desmethylzopiclone (N-Des) and zopiclone N-oxide (N-Ox)

Analyte	Slope, *m* (mean ± S.D.)	Intercept, *b* (mean ± S.D.)	Correlation coefficient, *r*
R-(−)-Zop	0.057407 ± 0.000448	0.003545 ± 0.003072	0.9998
S-(+)-Zop	0.059305 ± 0.000663	0.000941 ± 0.004547	0.9996
R-(−)-N-Des	0.049107 ± 0.000582	0.005072 ± 0.003777	0.9996
S-(+)-N-Des	0.050953 ± 0.001218	0.006273 ± 0.007907	0.9983
R-(−)-N-Ox	0.050066 ± 0.000308	0.004633 ± 0.001920	0.9998
S-(+)-N-Ox	0.050346 ± 0.000740	0.001716 ± 0.004611	0.9994

3.2. Validation of the assay

Recovery

Urine spiked with three different concentrations of zopiclone and its metabolites (2, 11 and 22.6 μg/ml) was extracted and analysed, each five-fold. The recovery was determined by comparison of the peak areas of each enantiomer with the peak areas of standard solutions. For zopiclone the recovery was calculated to be 94.8 ± 6.5%, for N-desmethylzopiclone 84.3 ± 7.8% and for zopiclone N-oxide 62.0 ± 4.3%. No differences were found in the recovery of the enantiomers and no concentration-related bias in the recoveries were observed.

Linearity

Standard solutions with five different concentrations of the analytes (from 0.57 to 30 μg/ml of the racemates) were analysed three times. A linear correlation was found between the concentration of the analytes and the ratios of the peak areas of each enantiomer and the peak area of the internal standard. Typical parameters of the calibration are summarized in Table 1. All calculations were carried out using the corrected peak areas calculated with the System Gold Software (peak area × effective capillary length/migration time) to correct differences from varying migration times. The calibration was repeated every 6 h due to fluctuations of the light

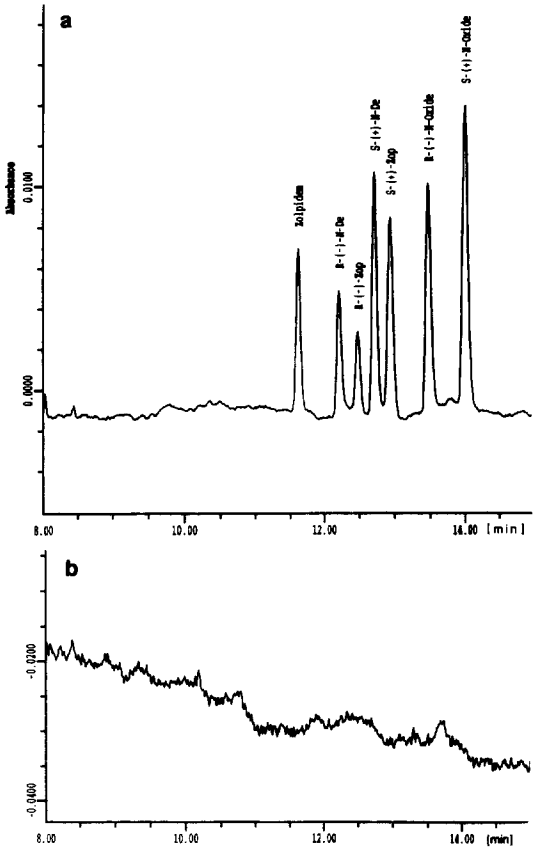

Fig. 5. Electropherogram of (a) human urine 6 h after oral administration of zopiclone and (b) blank urine.

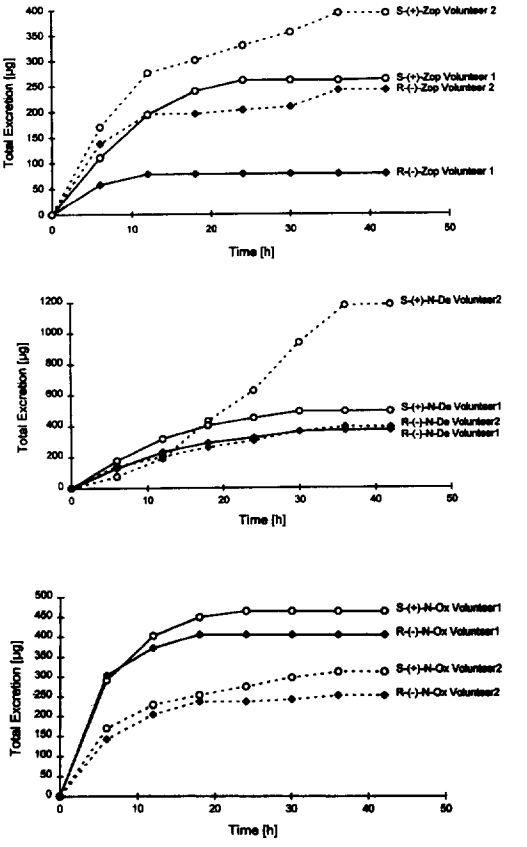

Fig. 6. Cumulative urinary excretion of zopiclone (Zop) and its metabolites N-desmethylzopiclone (N-De) and zopiclone N-oxide (N-Ox) in two volunteers.

Table 2
Reproducibility of the assay

Analyte	Concentration added (ng/ml)	Calculated concentration (ng/ml)	R.S.D. (%)
R-(−)-Zop	566.20	509.5	5.5
	283.10	299.0	1.7
	56.62	62.9	17.0
S-(+)-Zop	566.20	501.3	6.6
	283.10	293.6	9.4
	56.62	52.3	6.8
R-(−)-N-Des	536.40	541.3	5.4
	268.20	284.3	11.5
	53.64	50.2	18.1
S-(+)-N-Des	536.40	564.1	6.9
	268.20	272.1	11.5
	53.64	44.0	18.2
R-(−)-N-Ox	515.00	493.1	6.4
	257.50	260.3	0.7
	51.50	53.3	9.7
S-(+)-N-Ox	515.00	513.5	12.4
	257.50	272.4	5.1
	51.50	60.4	17.1

energy of the laser. These fluctuations were not compensated by the laser system used in this experiment.

Selectivity

Several human urine samples were extracted and analysed. No interference was observed at the migration times of the analytes and the internal standard (Fig. 5).

Precision and accuracy

Blank urine spiked with three different concentrations of each analyte was extracted and analysed three times. The concentrations were calculated for each enantiomer from the calibration curves. The results are given in Table 2. For the determination of drugs in biological fluids precision and accuracy should always be within ±15% except at the lower limit of quantification, where they should not deviate by more than ±20% [10]. In our experiments the results were always within this range. However, in comparison to HPLC the deviations of the calculated concentrations to the real concentrations are higher. This could be due to the fluctuations of the light energy of the laser. The detection limit was about 6 ng/ml for each enantiomer.

3.3. Stability of the analytes

In our experiments no racemization or hydrolysis has been observed under the conditions applied. A systematic study about the racemization of zopiclone has been carried out by Fernandez [11], who found no racemization under similar conditions (pH 8.4).

3.4. Application for human urine samples

Following oral administration of 7.5 mg of zopiclone (one tablet of Ximovan) urine of two male Caucasian volunteers was collected for at least 42 h in fractions of 6 h. A typical electropherogram 6 h after the oral administration of zopiclone is shown in Fig. 5. The cumulative urinary excretion of each enantiomer was calculated from the concentrations in each fraction. The results for both volunteers are shown in Fig. 6.

About 7% of the applied dose were excreted

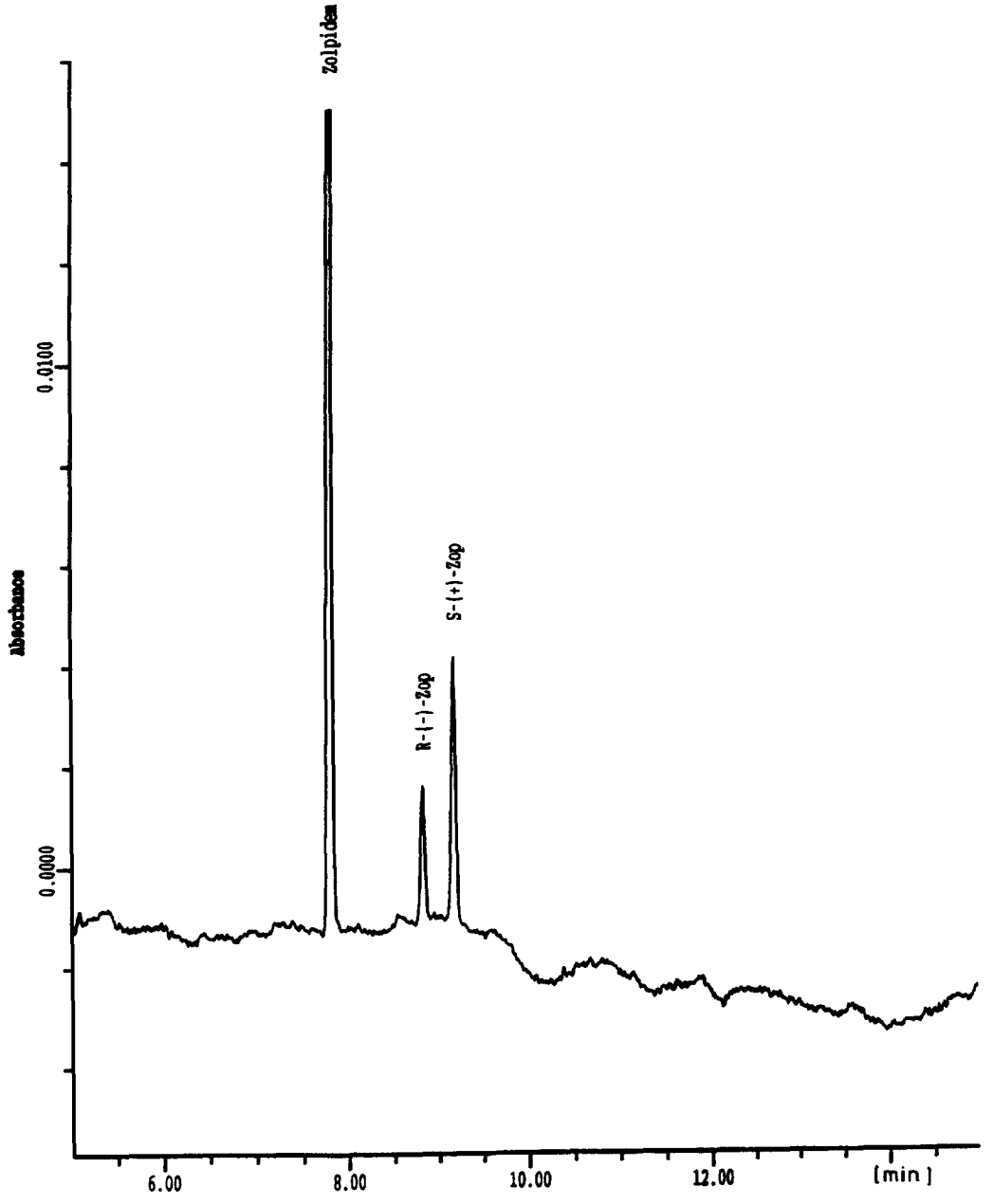

Fig. 7. Electropherogram of saliva 16 h after oral administration of zopiclone. Applied voltage: 20 kV.

unchanged in urine. In contrast, large intersubject variabilities were observed in the excretion of the metabolites. A dose percentage of 11.5 was found as N-desmethylzopiclone in urine of volunteer 1 compared with 21% of volunteer 2. Zopiclone N-oxide was excreted in higher amounts from volunteer 1 (11.6% vs. 7.5%).

The urinary excretion of the *S*-(+)-enantiomers of zopiclone and its chiral metabolites was always higher than the excretion of the respec-

tive *R*-(−)-enantiomers. These results are in accordance to the study of Fernandez [12], who had investigated the urinary excretion of zopiclone and its metabolites of twelve volunteers treated with 15 mg of zopiclone.

Our experiments confirm the stereoselective pharmacokinetics of zopiclone. Effects involved in the stereoselectivity might be plasma protein binding and a stereoselective metabolism. In vivo experiments with rat liver microsomes [13] indicate that *R*-(−)-zopiclone is preferentially metabolized.

3.5. Zopiclone enantiomers in saliva

A bitter taste often occurs after administration of zopiclone [14]. This is due to the excretion of zopiclone in the saliva. 16 h after administration saliva of volunteer 2 was collected. The sample was extracted and analysed in the same procedure as the urine samples. An electropherogram is shown in Fig. 7. The concentration of *S*-(+)-zopiclone was about twice as high as the concentration of *R*-(−)-zopiclone (154.7 ng/ml vs. 80.0 ng/ml). Metabolites could not be detected in saliva. However, there was no validation work done with saliva.

4. Conclusions

This study demonstrates that capillary electrophoresis is a very useful technique to analyse the metabolism of chiral compounds in biological fluids. The major advantage of CE in comparison to HPLC is the high resolution factor resulting in short migration times and the absence of interferences from endogenous compounds. With UV–LIF-detection of fluorescent drugs like zopiclone and its metabolites the sensitivity is highly improved.

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