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Direct determination of zolpidem and its main metabolites in urine using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Zolpidem is a new sleep inducer belonging to the imidazopyridine class. We wish to report a method for the determination of zolpidem and its main metabolites in urine without extraction using capillary electrophoresis with UV laser-induced fluorescence detection with a He-Cd laser. A 10-nl sample of urine can be directly applied to the capillary. The separation is carried out within 10 min, and the limit of detection is 2 ng/ml. This procedure is very simple and fast. No organic solvents are necessary.

Keywords: Zolpidem

1. Introduction

Zolpidem (Fig. 1) is a non-benzodiazepine hypnotic drug which acts at the ω_1-receptor subtype of the benzodiazepine binding site [1]. After oral administration the drug is rapidly and completely absorbed. The mean elimination half-life is about 2 h. Zolpidem is extensively metabolized. Methyl substituents on the phenyl and the imidazopyridine moiety are oxidized to hydroxy and carboxylic acid derivatives (Fig. 1, metabolites I–IV). About 60% of the administered dose is excreted as metabolites in urine [2]. Only trace amounts of unmetabolized zolpidem are excreted. For the simultaneous determination of zolpidem and its main metabolites in biological fluids, a method using high-performance liquid chromatography (HPLC) with a column-switching technique has been described [3]. The analytes are quantified by fluorescence detection, and the detection limit is about 0.2 ng/ml. However, the method requires two HPLC pumps and organic solvents.

Capillary electrophoresis (CE) is becoming a very useful technique for the determination of drugs in body fluids because of its high resolution, mass sensitivity and speed [4]. However, using UV, the detection limit frequently is not sufficient for drugs and metabolites in biological fluids due to the low sensitivity. Sample preparations including extraction and preconcentration steps are often necessary.

In order to improve the sensitivity, we are using UV laser-induced fluorescence detection

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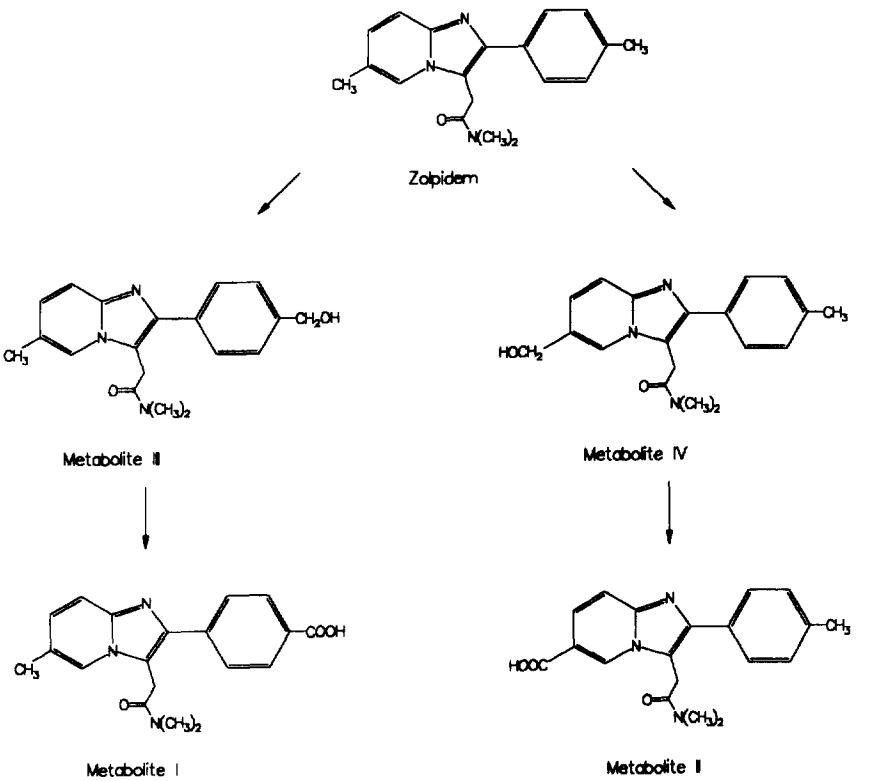

Fig. 1. Metabolism of zolpidem.

(UV–LIF) [5] with a He-Cd laser emitting in the UV region at 325 nm. With this method we can determine zolpidem and its main metabolites directly in 10 nl of human urine. No sample preparation is necessary. The method has been applied to study the metabolism of zolpidem in urine from two volunteers treated orally with 10 mg zolpidem tartrate.

2. Experimental

2.1. Capillary electrophoresis

A Beckman P/ACE system 2100 (Beckman Instruments, Munich, Germany) was used with an uncoated fused-silica capillary (50 μm I.D., 60 cm effective length, 67 cm total length). Separation was carried out with 50 m*M* phosphate buffer pH 5.6 (prepared by mixing aqueous solutions of Na_2HPO_4 and KH_2PO_4). Between each analysis the capillary was rinsed with 0.1 *M* sodium hydroxide for 1 min followed by buffer pH 5.6. Samples were introduced into the capillary by pressure injection at 0.5 psi for 10 s, so that the sample volume is about 10 nl. A run potential of 20 kV was used. Temperature was maintained at 20°C. Detection was carried out with a He-Cd laser (Omnichrome 3056-10M, 20 mW) operated at an excitation wavelength of 325 nm. Emission was measured with a Beckman LIF detector at 405 nm. The corrected peak areas were calculated using the Beckman System Gold software 7.11.

2.2. Chemicals

Zolpidem and the metabolites I (SL 84.0589), II (SL 84.0853), III (SL 84.0877) and IV (SL 84.0904) were kindly supplied by Synthelabo Recherche (L.E.R.S.), Bagneux Cedex, France. β-Glucuronidase (from *Helix pomatia*) was from

Sigma Chemicals, Deisenhofen, Germany. Stock solutions (0.2 mg/ml) of zolpidem and its metabolites were prepared in methanol (HPLC grade). Standard solutions were prepared from the stock solutions by dilution with urine. Stock solutions were stored at −18°C and were found to be stable for at least three months. Standard solutions were freshly prepared from stock solutions every week by dilution with urine.

2.3. Deconjugation with glucuronidase

A 1-ml sample of urine was incubated for 24 h at 37°C together with 0.9 ml 100 m*M* sodium acetate buffer pH 4.6 containing 477 U β-glucuronidase. The reaction was stopped by cooling with ice.

3. Results and discussion

3.1. Separation

Fig. 2 shows an electropherogram of urine spiked with zolpidem and its metabolites. At this pH zolpidem and the metabolites III and IV are positively charged and move towards the cathode. In contrast, the acidic metabolites I and II are negatively charged and migrate slower than the electroosmotic flow. Separation was achieved in less than 10 min. At the corresponding migration times of zolpidem and its metabolites, no interfering peaks from urine were observed. Experiments with sodium dodecyl sulfate (SDS) were carried out to try to improve the peak shape. The addition of different concentrations (10 m*M* up to 50 m*M*) SDS did not improve the separation but resulted in longer migration times.

3.2. Calibration

Standard solutions with five different concentrations in the range between 8 ng/ml and 4000 ng/ml for metabolite I, and 2 ng/ml to 1000 ng/ml for zolpidem and metabolites II, III and IV were analyzed. The corrected peak areas (peak area × capillary length/migration time)

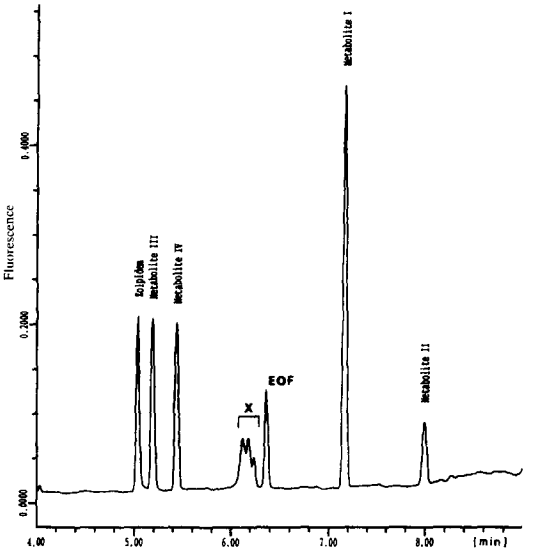

Fig. 2. Electropherogram of urine spiked with zolpidem and its metabolites (1 μg/ml) and 4 μg/ml of metabolite I. In blank urine no interfering peaks were observed. X = Fluorescent components from urine; EOF = electroosmotic flow determined under the same conditions with mesityl oxide.

were calculated using the Beckman System Gold Software 7.11. A linear correlation was found between the concentration and the corrected peak areas. The parameters of the linear regression for zolpidem and its metabolites are given in Table 1. No internal standard was necessary, because the samples could be analyzed without any pretreatment. However, fluctuations in the intensity of the laser emission of up to 50% from day to day appeared sometimes. The supplier of the laser (Omnichrome) guarantees fluctuations in the light intensity of less than ±5% per hour. In our experiments these fluctuations were probably higher sometimes. Thus, the calibration has to be repeated every 6 h with these standard solutions.

3.3. Reproducibility and accuracy

Urine spiked with three different concentrations of the analytes was investigated with five repetitions. The results are summarized in Table 2. For the determination of drugs in biological fluids, precision and accuracy should always be

Table 1
Typical calibration for zolpidem and its metabolites

Compound	Slope (Mean ± S.D.)	Intercept (Mean ± S.D.)	r
Zolpidem	0.018618 ± 0.00048	0.020467 ± 0.06662	0.9933
Metabolite III	0.022118 ± 0.00070	0.083432 ± 0.09075	0.9902
Metabolite IV	0.032141 ± 0.00074	0.163800 ± 0.10750	0.9947
Metabolite I	0.010254 ± 0.00027	0.109262 ± 0.22874	0.9933
Metabolite II	0.008145 ± 0.000193	−0.000748 ± 0.05168	0.9944

Table 2
Reproducibility of the method ($n = 5$)

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	S.D. (%)
Zolpidem	536.50	549.7	6.03
	53.65	53.1	8.46
	10.73	10.0	19.66
Metabolite III	505.50	544.5	6.81
	47.00	48.5	14.06
	11.80	10.8	9.38
Metabolite IV	565.50	555.6	9.55
	56.55	48.7	4.67
	11.31	11.3	19.69
Metabolite I	3340.85	3527.8	8.37
	334.09	331.0	8.04
	66.82	71.7	3.56
Metabolite II	1066.81	1144.8	5.29
	100.56	94.7	5.17
	20.70	19.8	15.67

within ±15% except at the lower limit of quantification (LOQ), where they should not deviate by more than ±20% [6]. In our experiments the relative standard deviations were usually 3.5%, and up to 19.7% at the LOQ. However, in some instances the deviations of the mean values found in the concentration added were higher than 10% (metabolite IV at 56.5 ng/ml). These deviations seem to be due to the fluctuations in the light energy of the laser. In general, the deviations were less than 3%, so that we can conclude that the method is suitable for the determination of drugs in biological fluids.

The LOQ in human urine without extraction, defined as the lowest concentration which can be measured with acceptable precision, accuracy and variability [6], was 10 ng/ml for zolpidem and metabolites III and IV and 20 ng/ml for metabolites I and II. The detection limits for zolpidem and the metabolites are summarized in Table 3. Fig. 3 shows an electropherogram of urine spiked with 2 ng/ml of zolpidem and

Table 3
Detection limits of zolpidem and its main metabolites in human urine

Analyte	Detection limit (ng/ml)	Absolute amount (pg)
Zolpidem	2	0.02
Metabolite I	10	0.1
Metabolite II	10	0.1
Metabolite III	2	0.02
Metabolite IV	2	0.02

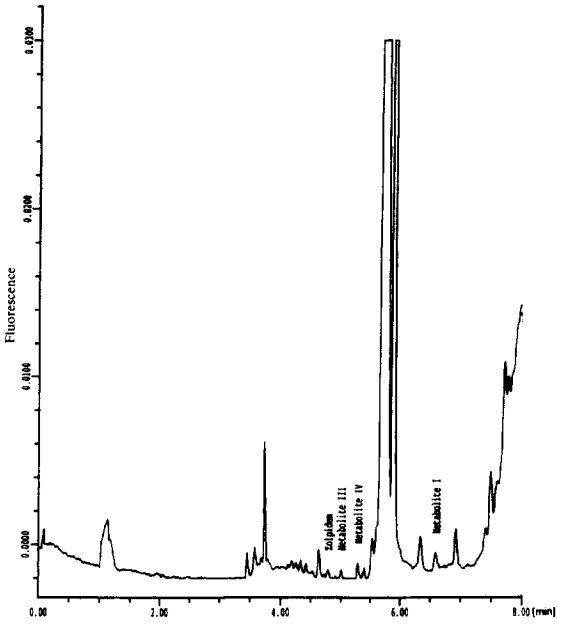

Fig. 3. Electropherogram of urine spiked with 2 ng/ml of zolpidem and the metabolites III, IV and II and 8 ng/ml metabolite I.

metabolites III, IV and II and 8 ng/ml of metabolite I, at its detection limit. However, metabolite II could not be detected at this level.

3.4. *In vivo analysis*

Urine samples of two male Caucasian volunteers treated orally with 10 mg of zolpidem tartrate were collected at time intervals of 3 h for at least 24 h. These fractions were analysed directly and after deconjugation with glucuronidase. During the incubation the analytes were found to be stable. A typical electropherogram is shown in Fig. 4. The results are summarized in Table 4.

To date, only very few data are found in the literature about the urinary excretion of zolpidem. In accordance with other studies using HPLC [2,3], metabolite III was found to be undetectable in human urine. Metabolite I is the main metabolite (43.8% and 32.4%, respectively). Only small amounts of unchanged zolpidem (0.2% and 1.3%) and metabolite II (1.3% and

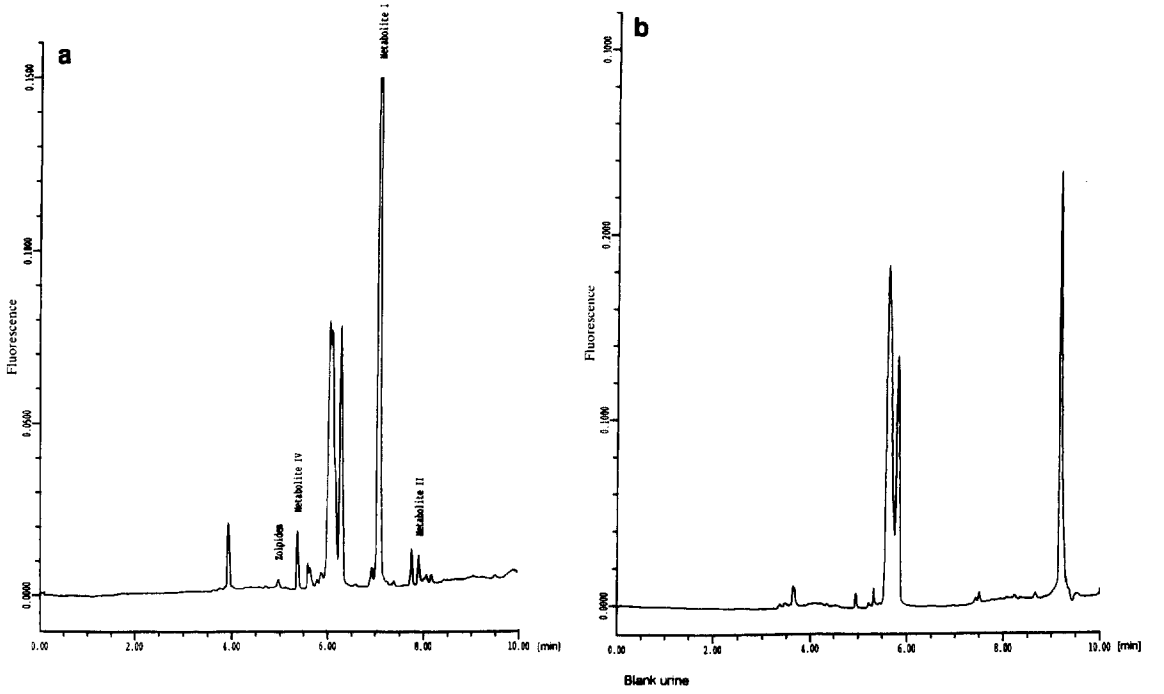

Fig. 4. Electropherogram of urine 3 h after oral administration of zolpidem and deconjugation with β-glucuronidase (a), and electropherogram of blank urine (b).

Table 4
Total urinary excretion of zolpidem and its metabolites (μg) after oral administration of 10 mg zolpidem tartrate

Time (h)	Volunteer 1 (in parentheses volunteer 2)			
	Zolpidem	Metabolite I	Metabolite II	Metabolite IV
0– 3	10 (20)	1920 (191)	59	2 (3)
3– 6	6 (18)	989 (329)	33 (28)	3 (4)
6– 9	2 (35)	278 (831)	5 (19)	(11)
9–12	(20)	115 (757)	5 (33)	(6)
12–15	(9)	83 (320)	4	(51)
15–18	(2)	130 (105)		(20)
18–24	(71)			
Total amount	18 (105)	3515 (2604)	107 (80)	5 (95)
Percentage of dose	0.2 (1.3)	43.8 (32.4)	1.3 (1.0)	0.1 (1.0)

1.0%) were found. Metabolite IV, a primary alcohol, was partially conjugated with glucuronic acid. In order to determine this glucuronide, an aliquot of the urine samples was deconjugated with β-glucuronidase. Fig. 5 shows the amount of metabolite IV found in urine of volunteer 2 before and after deconjugation with β-glucuronidase. After deconjugation the concentration of metabolite IV is increased, indicating the presence of varying amounts of the glucuronide. Similar results were obtained from the deconjugated urine samples of volunteer 1. Our experiments confirm the results from an earlier experiment [3], where the data of only one patient is given.

4. Conclusion

The analysis of drugs in biological fluids by capillary electrophoresis is limited by high detection limits when using UV detection due to the small inner diameter of the capillary (path length of absorption 50 μm) and additionally to the very small sample volume applied to the capillary. With UV laser-induced fluorescence detec-

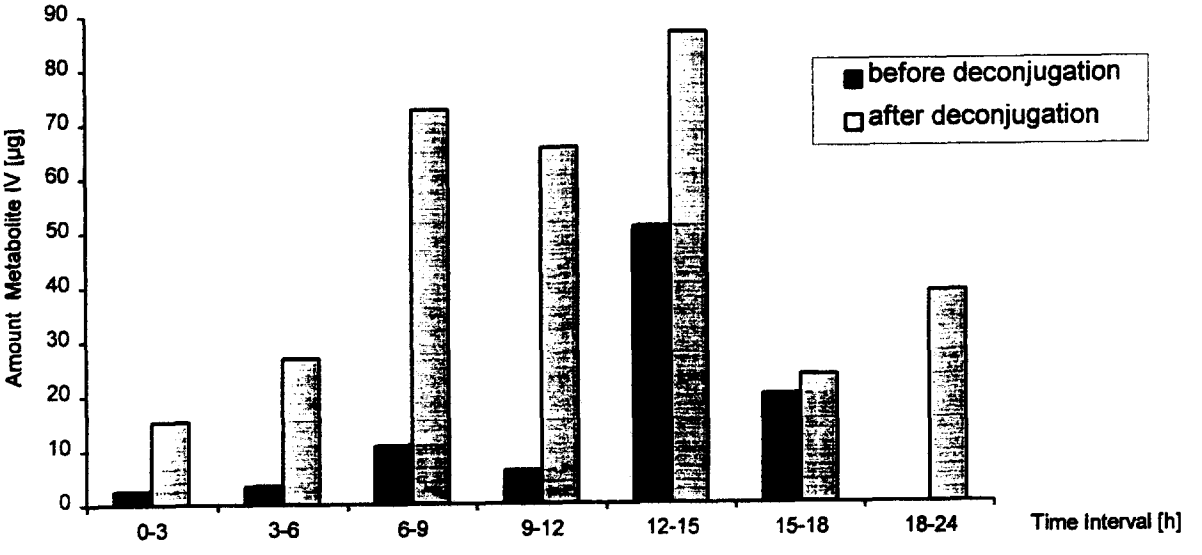

Fig. 5. Total amount of metabolite IV in urine of volunteer 2 before and after deconjugation with β-glucuronidase.

tion (UV–LIF), an amount as low as 0.02 pg ($7 \cdot 10^{-17}$ mol) zolpidem is detectable. The direct analysis of 10 nl urine without any extraction or preconcentration was achieved. In comparison with HPLC only a very small sample volume is necessary. Because of the high resolution the analysis time is short, and no sample clean-up is necessary. However, improvements in the stability of the light emission of the laser are necessary.

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