*Journal of Chromatography*, 417 (1987) 151–158
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3606

# SIMULTANEOUS DETERMINATION OF ZOPICLONE AND ITS TWO MAJOR METABOLITES (N-OXIDE AND N-DESMETHYL) IN HUMAN BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AIMÉ LE LIBOUX*, ARMAND FRYDMAN and JEAN GAILLOT

*Département de Biodynamique, Institut de Biopharmacie Rhône-Poulenc, 20 Rue Raymond Aron, 92160 Antony (France)*

(First received August 14th, 1986; revised manuscript received January 14th, 1987)

## SUMMARY

A high-performance liquid chromatographic method has been developed for the simultaneous determination of zopiclone and its main metabolites (N-oxide and N-desmethyl derivatives) in biological fluids. After selective extraction (dichloromethane–2-propanol) these compounds are chromatographed on a column packed with Spherisorb ODS-2 (5 μm) using monobasic sodium phosphate–methanol (45:55, v/v). The eluted compounds are measured by fluorescence detection. The limit of detection of the method is 5 ng/ml for zopiclone in plasma and urine and 10 ng/ml for its two main metabolites (coefficient of variation less than 10%). This method has been successfully applied to pharmacokinetic studies of zopiclone and its two main metabolites in healthy subjects and patients with chronic renal failure.

## INTRODUCTION

Zopiclone, 6-(5-chloro-2-pyridyl)-7-(4-methyl-1-piperazinyl)carbonyloxy-6,7-dihydro[5*H*]pyrrolo[3,4-*b*]pyrazin-5-one (I, Fig. 1), is the first compound of the new pyrrolopyrazine series with potent hypnotic properties[1]. In clinical investigations, it exhibits the same hypnotic activity as flurazepam [2,3] but has a less marked residual effect on vigilance [4]. In healthy subjects, 20–30% of the dose administered occurs in the urine as the two principal metabolites of zopiclone, the N-oxide (II) and N-desmethyl derivatives (III) (Fig. 1) [5,6]. Unchanged zopiclone is also excreted in urine (5%).

Two methods have been described [7,8] for the determination of zopiclone and its two metabolites in biological samples. However, the first method [7] is time-consuming because zopiclone and each metabolite have to be determined sepa-

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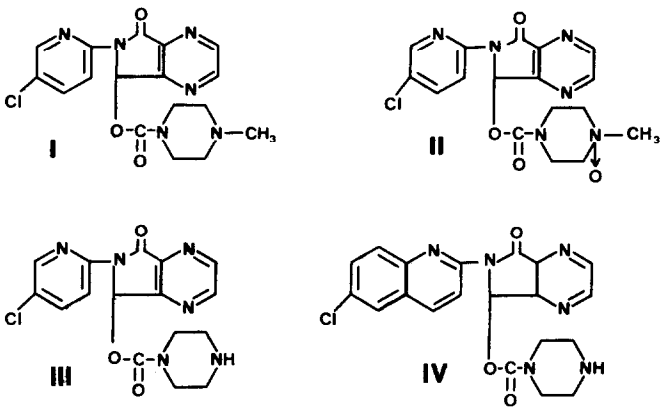



Fig. 1. Chemical structures of: zopiclone, 6-(5-chloro-2-pyridyl)-7-[(4-methyl-1-piperazinyl)carbonyloxy]-6,7-dihydro[5*H*]pyrrolo[3,4-*b*]pyrazin-5-one (I); its N-oxide derivative, 6-(5-chloro-2-pyridyl)-7-[4-oxo-4-methyl-1-piperazinyl)carbonyloxy]-6,7-dihydro[5*H*]pyrrolo[3,4-*b*]pyrazin-5-one (II); its N-desmethyl derivative, 6-(5-chloro-2-pyridyl)-7-[(1-piperazinyl)carbonyloxy]-6,7-dihydro[5*H*]pyrrolo[3,4-*b*]pyrazin-5-one (III); and the internal standard, 6-(7-chloro-2-quinolyl)-7-[(4-methyl-1-piperazinyl)carbonyloxy]-6,7-dihydropyrrolo[3,4-*b*]pyrazin-5-one (IV).

rately. In the second method [8], the N-desmethyl compound interferes with zopiclone during chromatography when this metabolite is present in large amounts.

Recently, Miller et al. [9] proposed a method of measure zopiclone in plasma, but it is not suitable for the N-oxide and N-desmethyl derivatives in urine because these metabolites are not quantitatively extracted in acidic media. Miller et al. [9] also confirmed that, for high concentrations of the N-desmethyl metabolite, "some interference might occur".

Although these metabolites are not present in plasma, the amounts excreted in urine vary according to liver and kidney functions. Indeed, preliminary results have shown that urinary excretion of these metabolites decreased when liver and kidney functions are impaired (20–30% in healthy subjects, ca. 5% in patients with impaired renal function). As the N-oxide metabolite is also pharmacologically active, an extensive pharmacokinetic study of these metabolites in patients with liver and renal impaired functions could be of interest. For this purpose, we have developed a single and sensitive method using reversed-phase high-performance liquid chromatography (HPLC) and spectrofluorimetric detection, which can be used for the simultaneous measurement of plasma and urine concentrations of zopiclone and its N-oxide and N-desmethyl metabolites in humans given therapeutic doses.

## EXPERIMENTAL

### *Materials*

Zopiclone, its two major metabolites II and III and the internal standard IV (Fig. 1) were supplied by Rhône-Poulenc (Vitry-sur-Seine, France). The sol-

vents and reagents used were all of analytical grade: dichloromethane, 2-propanol and methanol (Merck, Darmstadt, F.R.G.), hydrochloric acid and disodium hydrogen phosphate (Prolabo, Paris, France). The pH 8 borate–hydrochloric acid buffer (boric acid, 6.928 g/l; sodium hydroxide, 2.234 g/l; hydrochloric acid, 1.61 g/l), Titrisol Merck of twice the usual strength (one bottle in 500 ml instead of 1000 ml), was selected to optimize extraction yields.

*Apparatus and chromatographic conditions*

The HPLC system consisted of a fluorescence detector (Shimadzu RF 530), a pump (Chromatem 380), a recorder (Kipp and Zonen Model BD 40) (Touzart Matignon, Vitry-sur-Seine, France) and an automatic sample injector (Model WISP 710, Waters, Montigny le Bretonneux, France). The detector was connected to a computer (Hewlett-Packard HP 1000 with a HP 150 terminal) in order to measure peak-height ratios (via the Hewlett-Packard H 3350 L.A.S. data acquisition program).

The chromatographic column was a stainless-steel tube (20 cm×4.6 mm I.D.) packed with Spherisorb ODS-2 (5-$\mu$m particles) (Prolabo).

The fluorescence detector was operated at excitation 300 nm and emission 470 nm. The column was eluted with 0.025 *M* monobasic sodium phosphate–methanol (45:55, v/v) at room temperature. The flow-rate was 1.0 ml/min.

*Preparation of plasma and urine samples*

Plasma and urine standards were prepared by supplementing 1.0 ml of normal human plasma or urine with a solution containing zopiclone and its two metabolites to achieve concentrations of 5, 10, 20, 50, 100, 250 and 500 ng/ml for zopiclone and of 10, 20, 50, 100, 250 and 500 ng/ml for the N-oxide and N-desmethyl metabolites.

The calibration curves were obtained by linear regressions of the peak-height ratios of zopiclone or metabolites/internal standard (I.S.) versus the concentration of each compound. Four, five or six replicate extractions were made at each concentration level over the calibration range. An unweighted least-squares regression relationship between concentration and the peak-height ratio of each compound to the I.S. was calculated by the HP 3350A L.A.S. system. The peak-height ratios of unknown samples were compared with the standard curve in urine or plasma.

Quality-control samples were also prepared as above, in multiples, on receipt of the clinical samples and were stored with the samples under identical conditions to ensure the integrity of sample storage and analytical methodology.

The recoveries (%) of the extraction procedure for the drug, the metabolites and the I.S. were determined by comparing the peak heights of zopiclone, the metabolites and the I.S. obtained from extracted samples with those obtained by direct injection.

*Assay procedure*

To 1 ml of plasma or urine (or dilution 1:10 for healthy subject) in a 20-ml glass centrifuge tube were added 1 ml of buffer solution pH 8, 200 ng of I.S. and

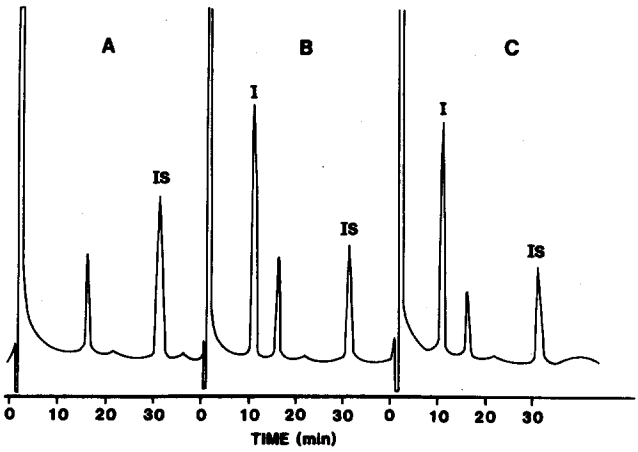



Fig. 2. Typical chromatograms of extracts from (A) blank plasma with 200 ng/ml internal standard (IS), (B) spiked plasma with 50 ng/ml zopiclone (I) and 200 ng/ml IS and (C) plasma sample from patient with renal failure, obtained 3 h after a 7.5-mg oral dose of zopiclone, spiked with 200 ng/ml IS.

10 ml of dichloromethane–2-propanol (5:0.5, v/v). The tube was stoppered and mechanically shaken for 10 min at 350 rpm with an Infors shaker, then centrifuged for 15 min at 3000 *g*. The organic phase was transferred to a 10-ml conical glass tube and then evaporated to dryness under a stream of nitrogen. The sample residue was reconstituted into 50–200 $\mu$l of a 0.01 *M* hydrochloric acid solution, agitated ultrasonically and injected into the column via the automatic sampler.

*In vivo studies*

The described procedure was used in the quantitative assay of zopiclone and its two main metabolites in the plasma and urine of fourteen patients (aged 18–65 years) with chronic renal failure (creatinine clearance less than 15 ml/min) and of 23 healthy subjets (sixteen young, seven elderly), all given a single oral dose of 7.5 mg of zopiclone in tablet form. Blood samples were collected before and after (10, 20, 30 and 45 min and 1, 2, 3, 4, 6, 8, 10, 22 and 30 h) medication. Urine samples were collected for 24 h before medication and afterwards in the following in fractions: 0–6, 6–12, 12–24, 24–36, 36–48 and 48–72 h. Plasma and urine samples were immediately frozen until analysis.

## RESULTS

*Method validation*

Fig. 2 includes chromatograms of blank plasma, plasma spiked with 50 ng/ml zopiclone and 200 ng/ml I.S., and plasma (3 h) samples from a patient with renal failure given 7.5 mg of zopiclone orally.

Likewise, Fig. 3 shows chromatograms of blank urine, urine spiked with 50 ng/ml zopiclone, 50 ng/ml N-oxide metabolite and 50 ng/ml N-desmethyl metabolite, a typical urine (0–24 h) from a patient with renal failure and a typical urine

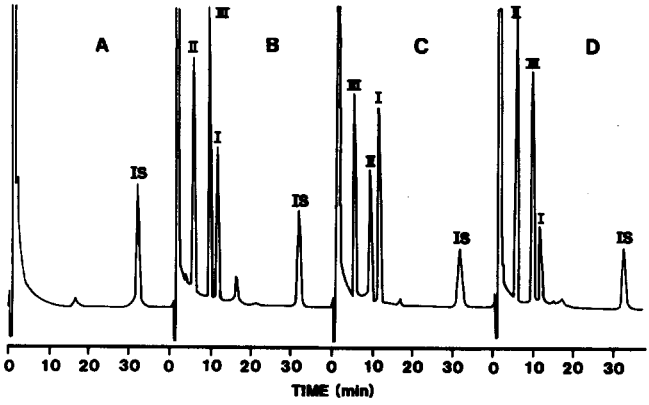



Fig. 3. Typical chromatograms of extracts from (A) blank urine with 200 ng/ml internal standard (IS), (B) spiked urine with 50 ng/ml zopiclone (I), 50 ng/ml N-oxide metabolite (II), 50 ng/ml N-desmethyl metabolite (III) and 200 ng/ml IS, (C) typical urine sample (0–24 h) from a patient with renal failure and (D) typical urine sample (0–24 h) from a young healthy subject. Both subjects were given 7.5 mg of zopiclone orally.

(0–24 h) from a young healthy subject, both given 7.5 mg of zopiclone orally.

No interfering peaks from endogenous compounds were observed when blank plasma or urine were assayed. The retention times of N-oxide, N-desmethyl, zopiclone and I.S. were 6, 10, 12 and 32 min in plasma and urine, respectively.

Under these conditions, the rate of HPLC analysis is 30–32 samples per day.

The accuracy and precision of the method were studied by analysing five identically spiked plasma samples (Table I) or five or six identically spiked urine samples (Table II) at each concentration level.

The variability of the assay over the whole concentration range (5–100 ng/ml zopiclone in spiked plasma and urine and 10–100 ng/ml N-oxide and N-desmethyl metabolites in spiked urine), as evidenced by the coefficient of variation (C.V.) for peak-height ratios, was always less than 10%. Repeated determination of five plasma or urine samples at different concentrations yielded C.V. values from 6.92 to 3.70% for zopiclone in plasma (Table I), from 5.57 to 3.89% for zopiclone in urine, from 3.42 to 2.43% for the N-oxide metabolite in urine and

TABLE I

REPRODUCIBILITY AND ACCURACY IN THE DETERMINATION OF ZOPICLONE IN SPIKED HUMAN PLASMA SAMPLES

| Added (ng/ml) | Found (mean ± S.D., $n=5$) (ng/ml) | Coefficient of variation (%) |
|---|---|---|
| 5 | 4.80 ± 0.33 | 6.92 |
| 10 | 9.98 ± 0.85 | 8.48 |
| 20 | 18.89 ± 1.83 | 9.68 |
| 50 | 52.30 ± 2.32 | 4.43 |
| 100 | 99.19 ± 3.87 | 3.90 |

TABLE II

REPRODUCIBILITY AND ACCURACY IN THE DETERMINATION OF ZOPICLONE AND ITS TWO MAIN METABOLITES IN SPIKED URINE SAMPLES

| Compound | Added (ng/ml) | $n$ | Found (mean ± S.D.) (ng/ml) | Coefficient of variation (%) |
|---|---|---|---|---|
| Zopiclone | 5 | 4 | 4.50 ± 0.25 | 5.57 |
| | 10 | 4 | 9.57 ± 0.20 | 2.10 |
| | 20 | 5 | 19.72 ± 0.50 | 2.54 |
| | 50 | 5 | 51.83 ± 2.07 | 4.00 |
| | 100 | 5 | 99.19 ± 3.32 | 3.35 |
| | 250 | 5 | 256.40 ± 7.32 | 2.85 |
| | 500 | 5 | 497.60 ± 19.37 | 3.89 |
| N-Oxide | 10 | 4 | 9.14 ± 0.31 | 3.42 |
| | 20 | 5 | 19.33 ± 0.70 | 3.65 |
| | 50 | 6 | 51.91 ± 1.60 | 3.08 |
| | 100 | 6 | 99.21 ± 2.49 | 2.51 |
| | 250 | 5 | 254.68 ± 7.28 | 2.86 |
| | 500 | 5 | 498.24 ± 12.12 | 2.43 |
| N-Desmethyl | 10 | 5 | 9.73 ± 0.96 | 9.84 |
| | 20 | 5 | 20.64 ± 0.95 | 4.59 |
| | 50 | 5 | 49.45 ± 0.87 | 1.75 |
| | 100 | 6 | 100.14 ± 1.77 | 1.77 |
| | 250 | 5 | 254.52 ± 8.03 | 3.15 |
| | 500 | 5 | 498.30 ± 8.14 | 1.63 |

from 9.84 to 1.63% for the N-desmethyl metabolite in urine (Table II).

Plots of the peak-height ratio of drug to I.S. versus concentrations for the plasma and urine standards were linear over the range 5–100 ng/ml zopiclone in plasma and urine (typically $y=0.0422x+0.0624$, $r=0.9978$ in plasma; $y=0.0308x+0.0759$, $r=0.9985$ in urine) and over the range 10–100 ng/ml in urine ($y=0.05x+0.0035$, $r=0.9985$ for N-oxide; $y=0.0473x+0.3537$, $r=0.9989$ for N-desmethyl) where $y$ is the peak-height ratio of zopiclone to I.S., $x$ is the concentration of zopiclone and $r$ is the correlation coefficient.

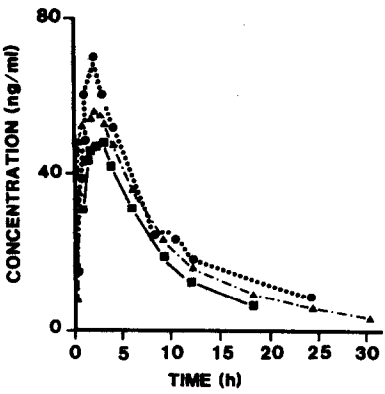
The threshold of sensitivity of this technique (defined as a double-high signal in comparison with background noise) was 5 ng/ml (zopiclone in plasma and urine) and 10 ng/ml (N-oxide and N-desmethyl metabolites).

The recovery was 94.5 ± 5% for zopiclone, 90 ± 6% for the N-oxide metabolite and 91.6 ± 4% for the N-desmethyl metabolite.

The stability of zopiclone and its two metabolites in plasma and urine stored at −20 °C was evaluated by analysing pooled plasma and urine standard samples against fresh standards. There was no loss in potency after two months of storage.

*Application to human clinical samples*

The application of this method to human plasma from healthy subjets and patients with renal failure clearly shows that the two main metabolites are not detectable in plasma when the administered doses remain in the therapeutic range.



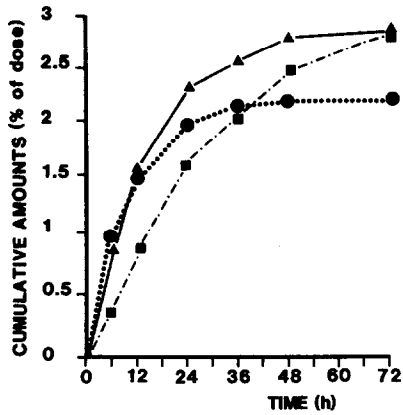




Fig. 4. Kinetics of the mean plasma concentration of zopiclone in young (■) and elderly (▲) healthy volunteers, and in patients with severe renal failure (●).

Fig. 5. Urinary excretion rate of zopiclone (●) and its two main metabolites, N-oxide (▲) and N-desmethyl (■) derivatives, expressed as percentage of the dose in patients with chronic renal failure.

Nevertheless, the method is applicable to the determination of unchanged drug in plasma and to simultaneous measurement of the renal excretion of unchanged drug and the N-oxide and N-desmethyl metabolites because no interference occurs.

The mean plasma concentration–time curve of zopiclone for the patients with chronic renal failure who were given a single dose (7.5 mg) of zopiclone is shown in Fig. 4. Two other curves illustrate administration of the same dose to healthy subjects (sixteen young and seven elderly volunteers).

The cumulative urinary excretion from patients with renal failure of zopiclone and its N-oxide and N-desmethyl metabolites is given in Fig. 5. The amount of zopiclone excreted unchanged in the urine is ca. 2.4% of the administered dose; excretion of the N-oxide and N-desmethyl metabolites represents 3.2 and 3.3% of the dose, respectively. For healthy subjects, these percentages are higher: Fig. 3 shows the urinary excretion of zopiclone (5.7%) and the N-oxide (14.4%) and N-desmethyl (11.2%) metabolites after a 7.5-mg oral dose of zopiclone.

## DISCUSSION

This paper describes a reliable and selective method for the simultaneous assay of zopiclone and two main metabolites (N-oxide and N-desmethyl) in plasma and urine. As the other known metabolites of zopiclone (to date twelve compounds) represent less than 5% of the dose each, the N-oxide and N-desmethyl derivatives, which correspond to the main metabolic pathways, remain the most interesting compounds to study in humans, from a pharmacokinetic point of view.

The alkaline (pH 8) extraction from plasma and urine allows the simultaneous analysis of the unchanged compound and the two main metabolites, and produces blank plasma samples that are consistently free of contaminants in the areas corresponding to the retention times for the three compounds and the I.S. The method is faster than previously published techniques [6–9] because only one analytical run is needed for all the compounds of interest.

In spite of its retention time of 32 min, the I.S. proposed here was selected for two main reasons: firstly, it is a structural analogue of zopiclone and the two main metabolites and so has a similar fluorimetric spectrum, and secondly, it has a good recovery (ca. 90%) under the extraction conditions used in this assay. The retention time does not significantly slow down the analysis.

The first results of the application study confirm that the urinary excretion of the N-oxide and N-desmethyl metabolites is decreased in patients with renal impairment. Because healthy subjects and patients with renal failure show the same differences in rate and extent of excretion of the main metabolites, the method described here can be considered as suitable for further pharmacokinetic studies in this field (i.e. liver insufficiency/healthy subjects).

ACKNOWLEDGEMENTS

J. Hetre and P. Delplanque are thanked for skilled technical assistance.

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