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Determination of the enantiomers of zopiclone and its two chiral metabolites in urine using an automated coupled achiral–chiral chromatographic system

Christine Fernandez, François Gimenez*, Bruno Baune, Valérie Maradeix and Alain Thuillier

Hôpital Pitié Salpêtrière, Service Pharmacie, Unité de Dosage de Médicaments, 47 Boulevard de l'Hôpital, 75013 Paris (France)

Robert Farinotti

Faculté de Pharmacie, Université Paris XI, Département de Pharmacie Clinique, Rue J. B. Clément, 92290 Chatenay-Malabry (France) and Hôpital Bichat-Claude Bernard, Service de Pharmacie Clinique et Biomatériaux, 170 Boulevard Ney, 75018 Paris (France)

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ABSTRACT

The enantiomers of zopiclone and its two chiral N-desmethyl and N-oxide metabolites were determined in urine using a coupled achiral–chiral liquid chromatographic method. After liquid–liquid extraction, zopiclone and its two metabolites were quantified on a cyanopropyl column. After fluorimetric detection on the achiral system, the eluent was switched through a silica precolumn in order to trap and concentrate the analytes. Each fraction was then backflushed separately onto a carbamate cellulose chiral stationary phase in order to determine the enantiomeric ratios. The coupled system was automated with an autosampler and a switching valve programmed by an integrator. The method was validated, and a first trial was performed on urine samples of a volunteer treated with 15 mg of racemic zopiclone.

INTRODUCTION

Zopiclone (ZOP), (±)-6-(5-chloro-2-piridyl)-5-hydroxy-6,7-dichloro-5*H*-pyrrolo[3,4-*b*]pyrazin-7-ine, 4-methyl-1-piperazine carboxylate (ester, racemate) (Fig. 1), is a cyclopyrrolone used therapeutically as a hypnotic drug. In humans, ZOP is metabolized by three major pathways: decarboxylation, oxidation and demethylation. After oral administration, ZOP and its N-desmethyl (DMZOP) and N-oxide (OXZOP) metabolites (Fig. 1) are excreted in urine [1–3]. Like ZOP, DMZOP and OXZOP are chiral compounds.

After oral administration to humans, ZOP is the only one of the three compounds that can be quantified in plasma by liquid chromatography (LC) with spectrofluorimetric detection. Unchanged ZOP (5%), DMZOP and OXZOP (10–15% of the administered dose) can be quantified in urine [2,3].

Two liquid chromatographic (LC) methods have been developed for the determination of ZOP, DMZOP, and OXZOP in urine, but both were performed on the mixture of enantiomers

* Corresponding author.

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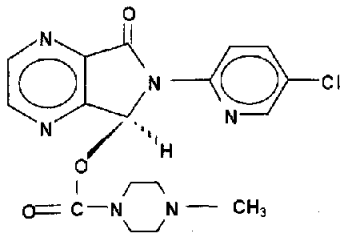

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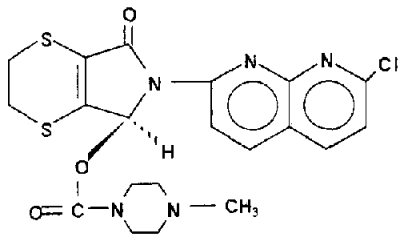

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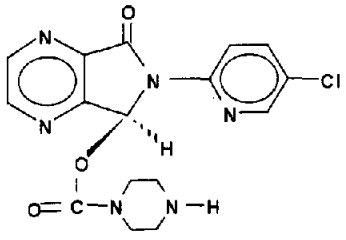

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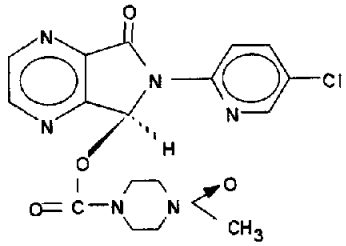

IV

Fig. 1. Structures of zopiclone (I), the internal standard suriclone (II), its N-desmethyl metabolite (III) and its N-oxide metabolite (IV).

[2,3]. We recently developed an LC method for the determination of plasma concentrations of ZOP enantiomers. This method, applied to a pilot study on one volunteer, suggested that the pharmacokinetics of ZOP could be stereoselective [4].

A method for the determination of the enantiomers of ZOP and its two chiral metabolites is necessary in order to investigate the stereoselectivity of metabolism and excretion. Achiral–chiral methods have been described in the literature [5,6]. We have developed an automated coupled achiral–chiral chromatographic system for that purpose.

EXPERIMENTAL

Chemicals

Racemic ZOP, the internal standard suriclone, and the two racemic metabolites DMZOP and OXZOP were kindly supplied by Rhône-Poulenc Rorer-Théraplix (Paris, France). Hexane (UV grade) was purchased from Carlo Erba (Paris La Défense, France). Ethanol, methanol and diethylamine were purchased from Merck (Strasbourg, France).

Stock solutions

Standard solutions of ZOP, DMZOP and OXZOP were prepared in acetonitrile at a concentration of 1 g/l. Because of its instability in acetonitrile, stock solutions of the internal standard suriclone were prepared in ethanol at a concentration of 1 g/l. All these solutions were stored at −20°C and were stable for at least five months.

Sequential dilutions of these four compounds were made daily in ethanol.

Apparatus

The coupled achiral–chiral method involved two chromatographic systems connected through an EPS 130 HP2P automatic switching system equipped with a Rheodyne 7000 switching valve and a 5-μm Kromasil silica guard column (60 mm × 4 mm I.D.) (Informatiques & Technologies, Le Blanc Mesnil, France) (Fig. 2).

Achiral chromatography

The achiral chromatographic system consisted of a Shimadzu LC-6A pump, an F535 Shimadzu detector, a Shimadzu C-R6A integrator (Touzart & Matignon, Vitry, France) and a Waters Wisp 720 autosampler (Millipore, Montigny le Bretonneux, France). Excitation and emission wavelengths were 305 and 470 nm, respectively. The

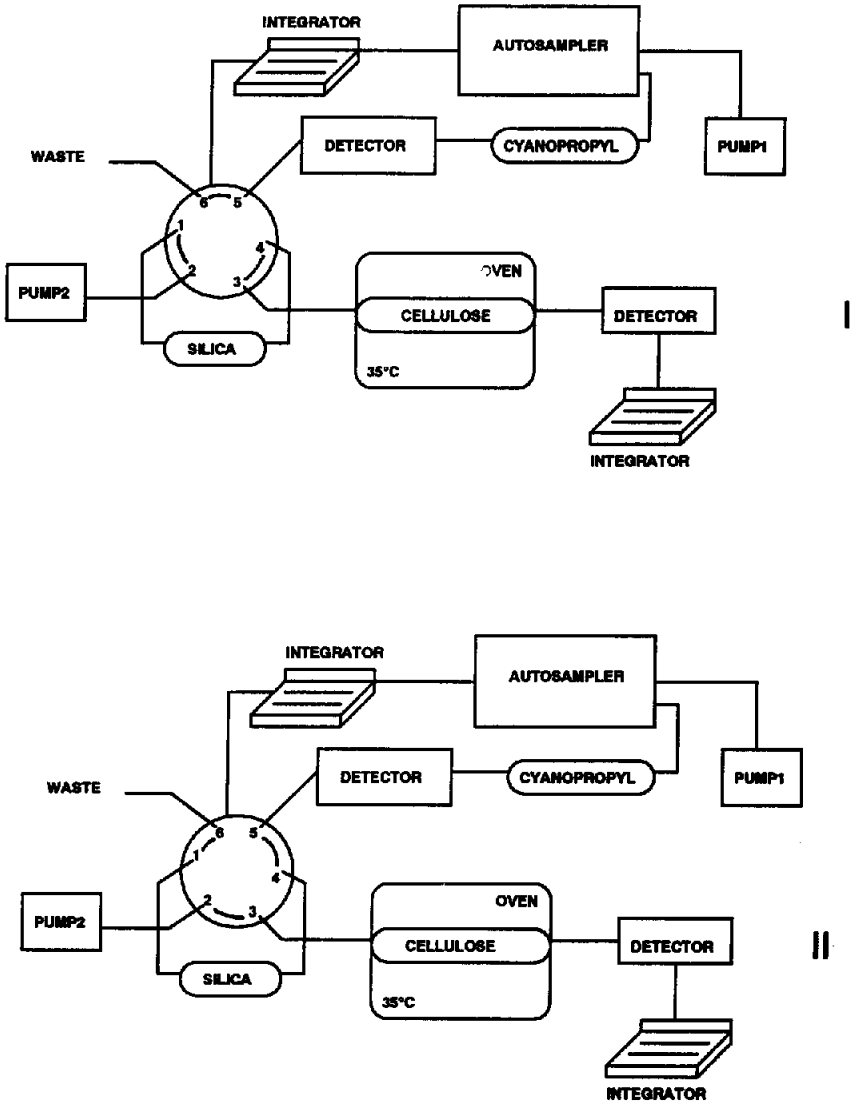

Fig. 2. The coupled achiral–chiral chromatographic system: (I) switching configuration for injection into the achiral chromatographic system 1 and for chiral chromatography on system 2 after re-injection from the guard column; (II) switching configuration for trapping the eluent fractions on the silica guard column just after detection with the achiral chromatographic system. For details, see text.

column used for the separation of racemic ZOP and the two racemic metabolites from biological matrices was a Nucleosil 5-μm cyanopropyl (150 mm × 4.6 mm I.D.) (Société Française Chromato Colonne/Shandon, Eragny, France). The mobile phase was hexane–ethanol–methanol (80:5:15, v/v/v), modified with 0.18% of a solution of diethylamine diluted in methanol (1:1000) and 0.05% water. Analyses were carried out at ambient temperature at a flow-rate of 0.7 ml/min.

Chiral chromatography

Chiral chromatography was performed with a Chromatem Beckman pump (Beckman, Gagny, France), an F535 Shimadzu detector, a Shimadzu C-R6A integrator and a column oven 850 LC (Dupont Instruments, Orsay, France). Excitation and emission wavelengths were 305 and 470 nm, respectively. The column used for the separation of the enantiomers of racemic ZOP and the two racemic metabolites was a 5-μm cellulose carbamate column (250 mm × 4.6 mm I.D.) equipped with a cellulose carbamate guard column (Société Française Chromato Colonne, Eragny, France). The mobile phase was hexane–ethanol–methanol (55:30:15, v/v/v) modified with 1% diethylamine. Analyses were carried out at 35°C at a flow-rate of 1.2 ml/min.

Extraction procedure

Racemic ZOP, DMZOP and OXZOP were extracted according to the Le Liboux method [7], modified as follows: urine samples were diluted five-fold with 0.05 *M* phosphate buffer (pH 8.4). To 1.0 ml of diluted urine, 100 μl of internal standard suriclone (20 μg/ml) and 10 ml of methylene chloride–2-propanol (95:5, v/v) were added. The mixture was gently shaken (10 min) and centrifuged (2000 *g*, 10 min). The organic phase was separated and evaporated under nitrogen at 37°C. The residue was reconstituted with 120 μl of ethanol, and 100 μl were injected into the achiral system.

Method validation

Achiral chromatography. Urine standards were prepared by supplementing normal human urine with ethanolic solutions of ZOP, DMZOP and OXZOP to achieve concentrations of 0, 50, 100, 250, 500, 750, 1000 and 1500 ng/ml for ZOP and OXZOP, and 0, 75, 100, 250, 500, 750, 1000 and 1500 ng/ml for DMZOP. Linearity was investigated on three calibration curves. Samples were analysed and evaluated by linear least-squares regression.

Reproducibility was studied by inter-day and intra-day validation. Five samples each of low (100 ng/ml) and high (1000 ng/ml) concentration were analysed in order to calculate the coefficients of variation.

Samples containing theoretical amounts of ZOP and its metabolites at the same concentrations as those used for the calibration curve, but spiked with other stock solutions, were analysed in order to determine the accuracy.

Chiral chromatography. The same samples were analysed on the chiral system. After analysis on the achiral column, each fraction of mixtures of enantiomers of ZOP, DMZOP and OXZOP was trapped on the guard column and injected into the chiral column. The enantiomeric ratios were determined.

RESULTS AND DISCUSSION

In order to protect chiral columns and increase their lifetime, the analysis of chiral compounds in biological matrices requires the use of two chromatographic systems. An achiral system may be used to separate chiral compounds from the biological matrix, and a chiral system can be used thereafter for the determination of the enantiomeric ratio. The injection of the chiral compounds after their separation from the biological matrix can be performed by sequential or coupled achiral–chiral systems [5,6]. In the sequential achiral–chiral method, the mobile phase containing the mixture of isomers is collected after detection and, after evaporation and retreatment, is reinjected into the chiral system. In the coupled method, both chromatographic systems are connected to a switching valve equipped with a precolumn in order to trap the mixture of isomers and inject it in the chiral system.

In the first method, the two systems are independent and the mobile phases can be different. In the second one, the mobile phases need to be similar and compatible. The phase used on the chiral system must have the same or higher elution strength than the one used on the achiral system. In the sequential method, the mobile phase of the achiral system is collected, evaporated and retreated, which is not always compatible with unstable compounds. This operation is avoided in the coupled method.

For the determination of ZOP enantiomers in plasma, we used a sequential achiral–chiral method with a silica gel as the achiral stationary phase and a cellulose carbamate as the chiral stationary phase [4].

We tried to use a similar method for the determination of the enantiomers of ZOP, DMZOP and OXZOP in urine. The silica gel could not be used as achiral stationary phase because DMZOP and OXZOP were too polar to be analysed on that phase. The three compounds were separated from the biological matrix with good resolution factors on a cyanopropyl column.

However, both metabolites were very unstable and were destroyed during evaporation and retreatment for reinjection in the chiral system. This is why the on-line coupled achiral–chiral system was the only suitable method for the simultaneous determination of the enantiomers of these three compounds.

TABLE I

ANALYSIS PARAMETERS EVALUATED BY LINEAR LEAST-SQUARES REGRESSION IN SPIKED URINE SAMPLES ON THE ACHIRAL SYSTEM

Compound	Correlation coefficient	Intercept	Slope
ZOP	0.9996	0.9	787
DMZOP	0.9989	12.7	1792
OXZOP	0.9998	9.3	926

Achiral chromatography

Under the chromatographic conditions used, the capacity factors of ZOP, the internal standard suriclone, DMZOP and OXZOP were 5, 7, 9 and 23, respectively. Standard curves for ZOP, DMZOP and OXZOP were linear over the range investigated. Analysis parameters are presented in Table I. The intra-day and inter-day reproducibility and precision are given in Table II. The accuracy is given in Table III.

TABLE II

REPRODUCIBILITY AND PRECISION

Sample	Intra-day validation			Inter-day validation		
	Spiked urine concentration (ng/ml)	Mean determined concentration (ng/ml)	C.V. (n = 5) (%)	Spiked urine concentration (ng/ml)	Mean determined concentration (ng/ml)	C.V. (n = 5) (%)
ZOP						
Low	100	97	5.5	100	100	12.6
High	1000	985	4.6	1000	1005	9.3
DMZOP						
Low	100	97	10.6	100	89	13.3
High	1000	1026	7.0	1000	1024	11.7
OXZOP						
Low	100	103	6.5	100	100	9.9
High	1000	1014	7.0	1000	1011	9.8

TABLE III

ACCURACY OF THE DETERMINATION IN SPIKED URINE SAMPLES ON THE ACHIRAL SYSTEM

Theoretical concentration (ng/ml)	Concentration found (ng/ml)			Error (%)		
	ZOP	DMZOP	OXZOP	ZOP	DMZOP	OXZOP
250	249	262	242	0.4	4.8	3.2
500	472	520	482	5.6	4.0	3.6
750	800	752	801	6.7	0.3	6.8
1500	1482	1520	1478	1.2	1.3	1.5

The limits of quantification for ZOP, DMZOP and OXZOP were 10, 15 and 10 ng, respectively. For these respective amounts, the coefficients of variation (C.V.) were 4.4, 10.2 and 13.9.%

The recoveries for ZOP, DMZOP and OXZOP were 96 ± 6.5, 56 ± 7.5 and 71 ± 7.5%, respectively. For the internal standard suriclone, the recovery was 67.4 ± 3.3%.

The chromatograms obtained from a blank urine, a spiked urine and a urine sample from a volunteer treated with 15 mg of racemic zopiclone are presented in Fig. 3.

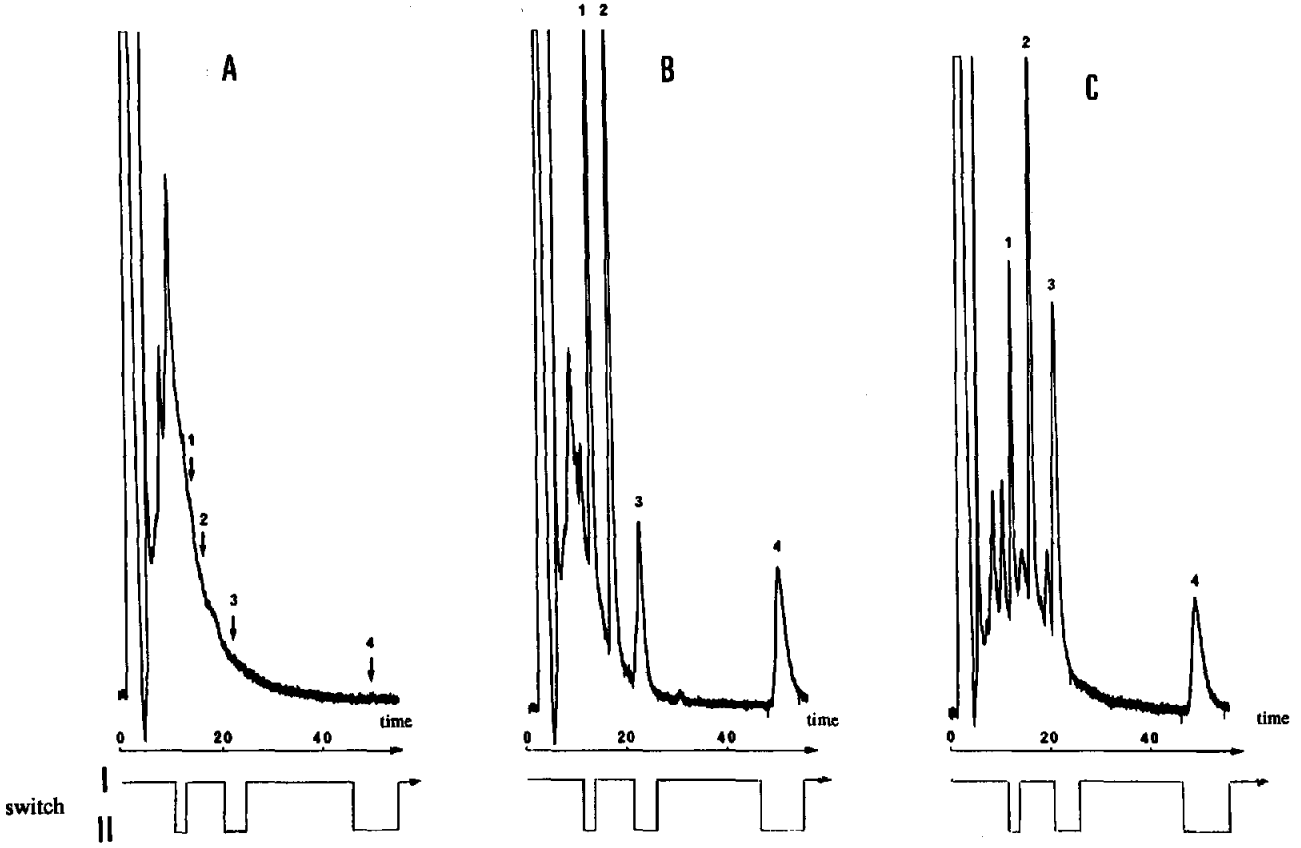

Fig. 3. Chromatograms on the achiral cyano column of (A) a blank urine, (B) a spiked urine with 750 ng/ml racemic zopiclone and its two racemic metabolites, and (C) an urine sample collected from a volunteer 12–24 h after drug administration. Peaks: 1 = racemic zopiclone; 2 = internal standard suriclone; 3 = N-desmethylzopiclone; 4 = zopiclone N-oxide. I and II: switching positions.

Coupled achiral–chiral system

The switching system was programmed from the Shimadzu C-R6A integrator. For each of the three peaks (ZOP, DMZOP and OXZOP) on the achiral column, the fractions were trapped onto the silica gel pre-column and re-injected separately into the chiral column.

Injection into the achiral system was made in position I (Fig. 2). The switch to position II was made 1 min before detection of the peak of the mixture of enantiomers on the achiral column. The switch from position II to position I was made after trapping ZOP, DMZOP and OXZOP during 2.5, 5 and 9 min, respectively, into the silica guard column in order to concentrate the mixtures of enantiomers and inject them into the chiral column. The time windows (I → II, II → I) programmed on the integrator for switching were (11.5, 13), (20.5, 25.5) and (46.5, 55.5) for ZOP, DMZOP and OXZOP, respectively (Fig. 3).

Chiral chromatography

Under the chromatographic conditions used on the cellulose carbamate column in this study, the enantiomers of ZOP, DMZOP and OXZOP were separated after direct injection on the chiral column with capacity factors of the first eluted enantiomer and selectivity factors ($k'1$, α) of (0.85, 1.85), (1.63, 1.32) and (1.58, 1.36), respectively. Compared with direct injection, the coupled column system resulted in a band broadening, which decreased the resolution factors on the chiral column from 3.98, 2.46 and 1.97 to 3.23, 2.15 and 1.77 for ZOP, DMZOP and OXZOP, respectively.

The elution order (+)-ZOP and (−)-ZOP was determined by injection of the separated enantiomers into the chiral column [4]. The elution order of the DMZOP and OXZOP enantiomers is not known.

Enantiomeric ratios of ZOP, DMZOP and OXZOP were determined by dividing the areas of each enantiomer. As we did not obtain a baseline separation for ZOP, DMZOP and OXZOP on the cellulose column, the drift parameter of the integrator was programmed in order to modify the baseline.

During the validation and analysis of the racemic mixtures, the enantiomeric ratios varied by the following intervals: 0.98 ± 0.04, 1.02 ± 0.04 and 0.96 ± 0.03 for (−)ZOP/(+)ZOP, DMZOP1/DMZOP2 and OXZOP1/OXZOP2, respectively. This confirms that both enantiomers are extracted from urine with similar efficiency.

The chromatograms obtained from a blank urine, a spiked urine and a urine sample from a

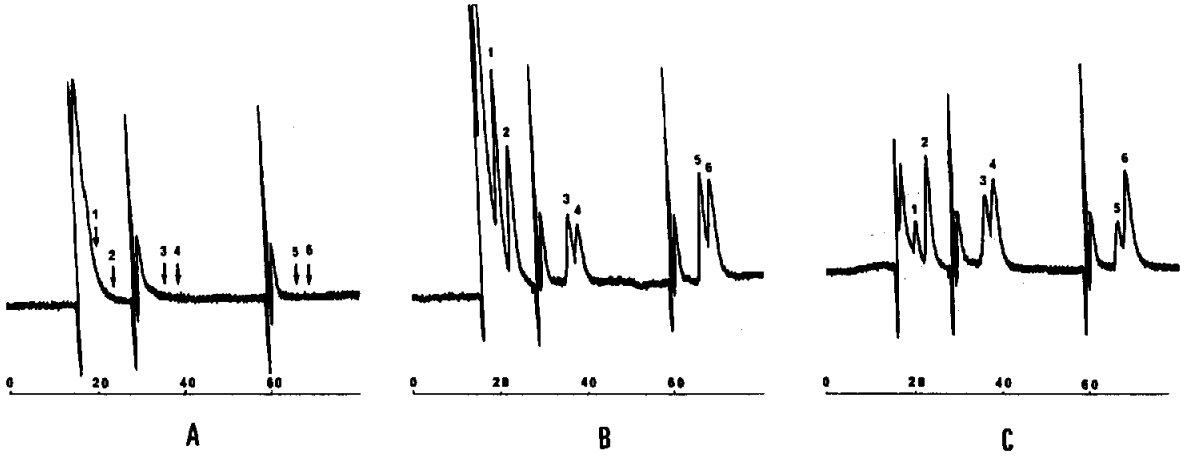

Fig. 4. Chromatograms obtained after switching from the achiral column and re-injection into the cellulose carbamate column of (A) a blank urine, (B) a spiked urine with 750 ng/ml racemic zopiclone and its two racemic metabolites, and (C) a urine sample collected from a volunteer 12–24 h after drug administration. Peaks: 1 = (−)-zopiclone; 2 = (+)-zopiclone; 3 = first eluted enantiomer of N-desmethylzopiclone; 4 = second eluted enantiomer of N-desmethylzopiclone; 5 = first eluted enantiomer of zopiclone N-oxide; 6 = second eluted enantiomer of zopiclone N-oxide.

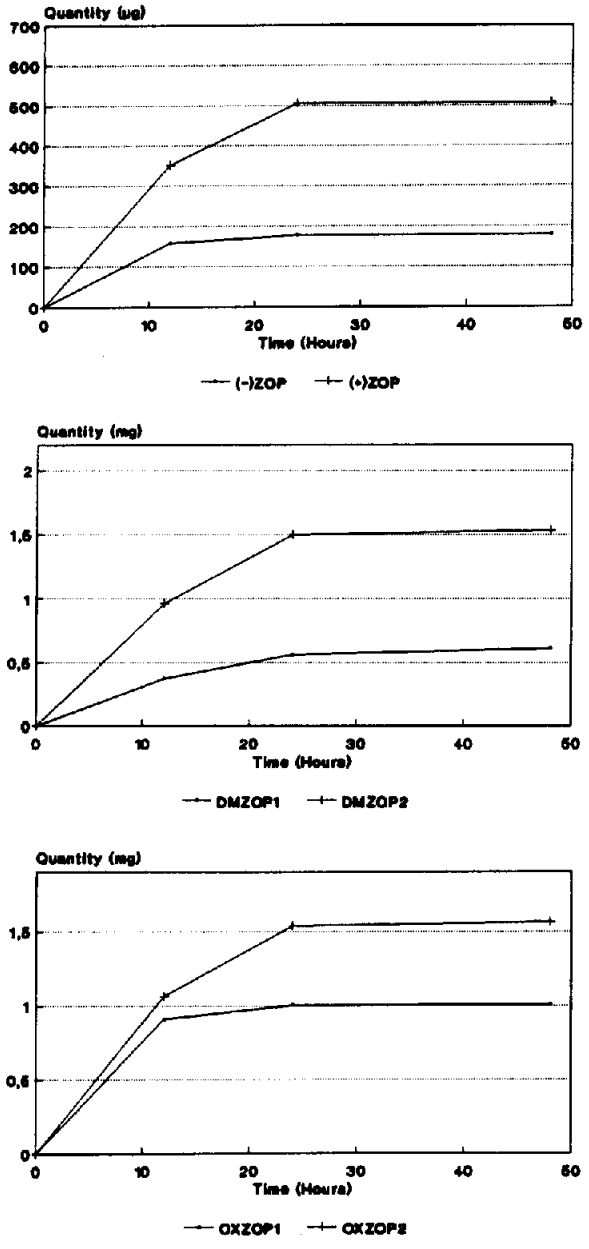

Fig. 5. Cumulative excreted amounts of the enantiomers of zopiclone [(−)-ZOP and (+)-ZOP)], its N-desmethyl metabolite (DMZOP1 and DMZOP2) and its N-oxide metabolite (OXZOP1 and OXZOP2).

volunteer treated with 15 mg of racemic ZOP after switching and reinjection into the chiral column are shown in Fig. 4.

Pharmacokinetic pilot study

After validation, this method was applied to a pharmacokinetic pilot study, in which 15 mg of racemic ZOP (Imovane, 7.5 mg, two tablets, Rhône-Poulenc Rorer Théraplix, France) were administered to a male caucasian volunteer. Urine samples were collected at the following intervals: 15 min before, and 0–12, 12–24 and 24–48 h after administration. The concentrations of each enantiomer of ZOP, DMZOP and OXZOP are represented in Fig. 5.

These first results do not allow us to draw conclusions about the stereoselectivity of ZOP pharmacokinetics, which must be investigated with a significant number of patients.

CONCLUSION

This method is efficient and suitable for the determination of enantiomer concentrations of zopiclone and its two chiral metabolites in urine. It is sensitive enough for the concentrations found in urine up to 48 h after a single dose treatment. Although the analysis time is long, the system can be automated.

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