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Determination of zolpidem, a new sleep-inducing agent, and its metabolites in biological fluids: pharmacokinetics, drug metabolism and overdosing investigations in humans

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ABSTRACT

For the determination of zolpidem, a new sleep inducer, and its metabolites in human plasma and urine, three methods were developed that are suitable for pharmacokinetics, drug metabolism and overdosing investigations. The methods used for pharmacokinetic and drug metabolism studies are based on column-switching high-performance liquid chromatography; they do not require any sample manipulation because the plasma or diluted urine is injected into a pre-column where clean-up and preconcentration take place. The analytes are transferred by valve-switching to the C_{18} analytical column for chromatography. To investigate overdose cases, urine samples only are used: the method is simple, because the diluted urine can be injected directly into the analytical column (phenyl type). This allows the identification and quantification of the principal urinary metabolite of zolpidem, the unchanged drug being practically undetectable. All the methods use fluorescence detection, which affords high sensitivity and selectivity. It is necessary to use a method capable of the determination of metabolites even if these are apparently pharmacologically inactive, because in different physiopathological populations the qualitative and quantitative metabolic profiles of zolpidem could be different. The method designed for the investigation of (accidental or deliberate) overdose cases is, as required on such occasions, simple and rapid, with good selectivity with respect to commonly prescribed psychotropic drugs.

INTRODUCTION

Zolpidem, an imidazopyridine, 2-(4-methylphenyl)-N,N,6-trimethylimidazo[1,2-*a*]pyridine-3-acetamide, is a new sleep inducer, which acts in the brain principally at receptors of the ω_1,-receptor subtype belonging to GABAergic system. The drug has a rapid onset of action and short elimination half-life and, unlike benzodiazepines, it has weak myorelaxant and anticonvulsant effects [1–3].

At the clinical level, zolpidem is a potent, short-acting hypnotic devoid of residual effects on day-time activities [4–7]. After oral administration the drug is rapidly and completely absorbed. The absolute bioavailability is *ca.* 70% [8], and the C_{max} value is usually observed between 30 and 60 min. The drug is extensively metabolized both in animals and in humans, and the metabolites are excreted into the urine and faeces ($\geq$ 90%); unchanged zolpidem has been observed only in trace amounts in urine [8,9]. The

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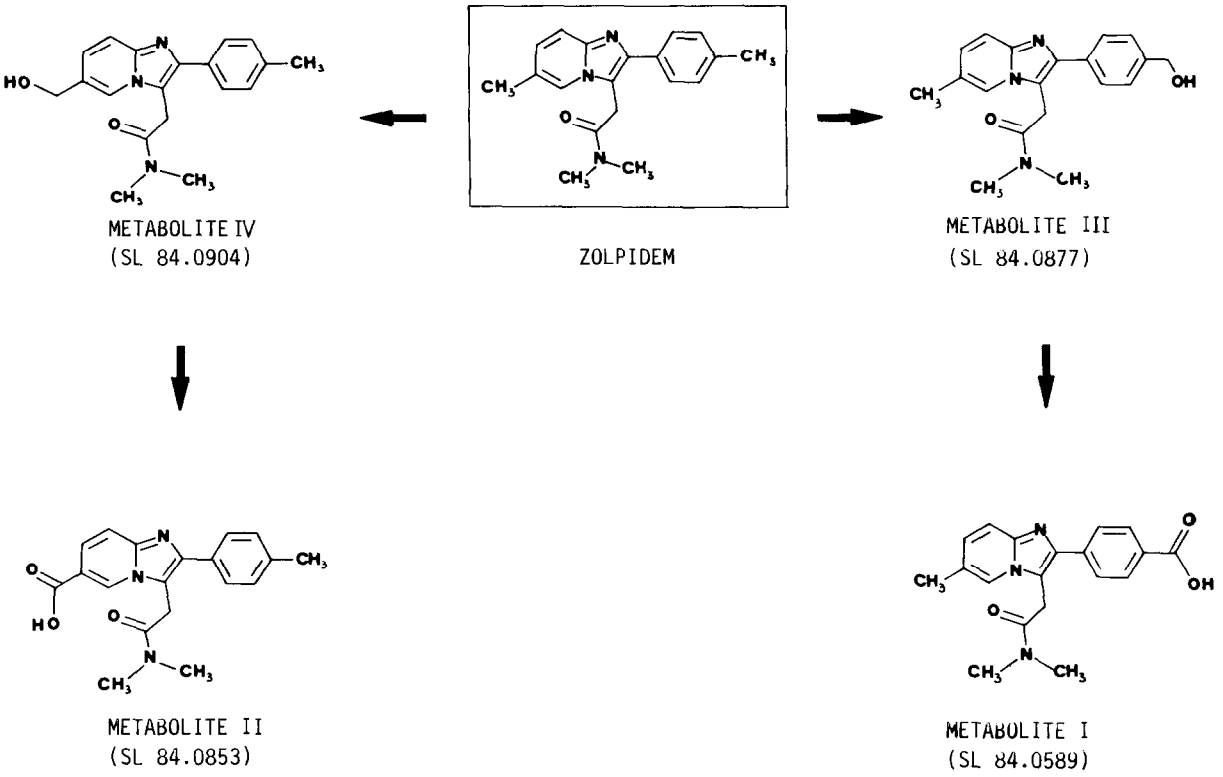

Fig. 1. Metabolism of zolpidem in humans.

principal metabolites have been identified, and they are all pharmacologically inactive [1–3,8]. There are four routes of biotransformation (Fig. 1): oxidation of each of the three different methyl groups on the phenyl moieties and hydroxylation of the imidazopyridine moiety. In humans, metabolites can be detected in plasma and urine.

A method is currently available for the quantification of zolpidem [10], based on the extraction of the drug from alkalinized biological fluids with diethyl ether, followed by high-performance liquid chromatography (HPLC) with fluorimetric detection. However, this method does not allow the determination of any of the metabolites, and it requires the evaporation of the diethyl ether extract and re-dissolution of the residue in a suitable solvent for HPLC. Other published methods deal with the determination of zolpidem by capillary gas chromatography (GC) with thermoionic detection [11] and HPLC with UV detection [12]. The GC method is not sensitive enough for pharmacokinetic studies and does not allow determination of the metabolites. The HPLC method is used for emergency toxicological investigations, having been used in a case of deliberate intoxication of zolpidem in combination with prothipendyl and ethanol; this method is also not suitable for metabolite determination.

Different clinical or experimental cases have different analytical requirements. For pharmacokinetic studies in humans, it is necessary to use a method that is suitable for the rapid determination of unchanged zolpidem with high sensitivity. For drug metabolism studies in different physiopathological populations, a method is required for the simultaneous determination of zolpidem and its principal metabolites in plasma and urine. In hospital emergency laboratories, where biological fluids are obtained after (accidental or deliberate) overdosing, a very simple and rapid method is necessary to analyse mainly urine samples. For these reasons three different methods were developed.

EXPERIMENTAL

Reagents, chemicals and standards

Methanol and acetonitrile were HPLC grade (E. Merck, Darmstadt, Germany), potassium dihydrogenphosphate and phosphoric acid were analytical grade (E. Merck), potassium hydroxide was analytical grade (E. Merck), the water used for the preparation of the buffer solution and chromatographic eluent was HPLC grade, produced by the Milli Q-4 system (Millipore, Bedford, MA, USA), and triethylamine was analytical grade (Carlo Erba, Milan, Italy).

The phosphate buffer, used for the eluent mixture (for both column-switching HPLC methods), was prepared from 0.05 *M* potassium dihydrogenphosphate adjusted to pH 6.0 with 1 *M* potassium hydroxide. The buffer used for preparing the eluent for toxicological analysis of urine was 0.025 *M* potassium dihydrogenphosphate adjusted to pH 3.0 with phosphoric acid. Zolpidem (SL 80.0750-00) was pharmaceutical grade and was provided with the synthetic metabolites, metabolite I (SL 84.0589), metabolite II (SL 84.0853), metabolite III (SL 84.0877), metabolite IV (SL 84.0904) (see Fig. 1 for the structures), by Synthélabo Recherche, Chemistry Department (Paris, France), as were the internal standard for the pharmacokinetic analysis, N,6-dimethyl-2-(4-methylphenyl)-N-propylimidazo[1,2-*a*]pyridine-3-acetamide (SL 81.0010, I.S.-1), and that for the drug metabolism and toxicological investigations, 6-chloro-2-(4-chlorophenyl)-N,2-hydroxypropyl-3-imidazo[1,2-*a*]pyridine-acetamide (SL 83.0725, I.S.-2) (Fig. 2).

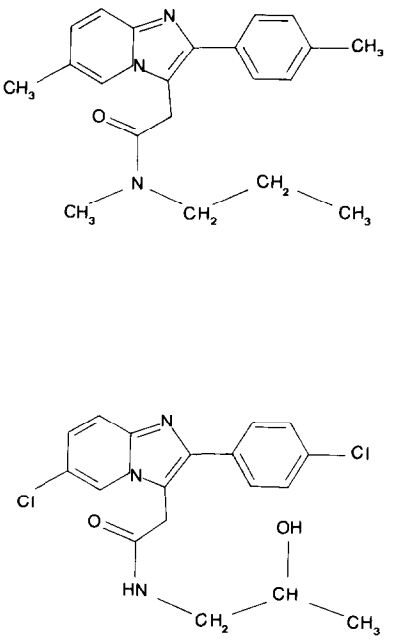

Fig. 2. Structures of the internal standards: upper, I.S.-1; lower I.S.-2.

Standard solutions

Stock solutions (0.5 mg/ml) of zolpidem, its metabolites and the internal standards were prepared in methanol. Standard solutions were prepared from stock solutions by suitable dilution with methanol (Tables I–IV), and used for the preparation of biological standards. Stock solutions were stable for at least one month and standard solutions for at least one week if stored at 0–5°C.

The standard solutions were added to human plasma or urine from untreated subjects for the preparation of the biological standards used during daily calibration for different purposes.

Chromatographic systems

Pharmacokinetic studies. The chromatographic system consisted of a constant-flow pump (Model 420, Kontron, Milan, Italy), a Model SFM 23/B spectrofluorimetric HPLC detector (Kon-

TABLE I

STANDARD SOLUTIONS USED FOR THE ANALYSIS OF ZOLPIDEM IN HUMAN PLASMA (PHARMACOKINETIC STUDIES)

Standard solution No.	Zolpidem (ng per 20 μl)	Internal standard I.S.-1 (ng per 30 μl)
1	240	–
2	60	–
3	20	–
4	10	–
5	5	–
6	–	30

TABLE II

STANDARD SOLUTIONS USED FOR THE ANALYSIS OF ZOLPIDEM AND METABOLITES IN HUMAN PLASMA (DRUG METABOLISM STUDIES)

Standard solution No.	Metabolite I (ng per 20 μl)	Metabolites I, II, III and IV (ng per 20 μl)	Zolpidem (ng per 20 μl)	Internal standard I.S.-2 (ng per 20 μl)
7	400	20	240	–
8	200	10	60	–
9	80	5	20	–
10	40	2.5	10	–
11	10	1.0	5	–
12	–	–	–	150

TABLE III

STANDARD SOLUTIONS USED FOR THE ANALYSIS OF ZOLPIDEM AND METABOLITES IN HUMAN URINE (DRUG METABOLISM STUDIES)

Standard solution no.	Metabolite I (ng per 20 μl)	Metabolite II (ng per 20 μl)	Metabolite III[a] (ng per 20 μl)	Metabolite IV[b] (ng per 20 μl)	Zolpidem (ng per 20 μl)	Internal standard I.S.-2 (ng per 20 μl)
13	5000	500	–	500	20	–
14	1000	100	–	100	10	–
15	500	50	–	50	5	–
16	100	25	–	25	2.5	–
17	50	10	–	5	–	–
18	–	–	–	–	–	150

[a] Not detected in human urine.

[b] This range is suitable for the determination of the total metabolite (free plus conjugated form); for the free form determination, the range 2–20 is more suitable.

tron) operating at excitation and emission wavelengths of 254 and 390 nm, respectively, Model 460 automatic sample injector (Kontron), with a six-port automatic valve and an external 200-μl loop, an analytical column (15 cm × 0.46 cm I.D.) of 5-μm Supelcosil LC 18-DB, and a guard column (2 cm × 0.46 cm I.D.) of 40-μm Pelliguard (Supelchem, Milan, Italy). The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 6.0) (60:40, v/v) containing 0.75% methanol. The flow-rate was 1.0 ml/min.

TABLE IV

STANDARD SOLUTIONS FOR THE RAPID SCREENING OF ZOLPIDEM IN HUMAN URINE (OVERDOSING INVESTIGATIONS)

Standard solution No.	Metabolite I (ng per 20 μl)	Internal standard I.S.-2 (ng per 20 μl)
19	10 000	–
20	5000	–
21	1000	–
22	500	–
23	100	–
24	–	100

Drug metabolism studies. The chromatographic apparatus was similar to that described above, with the following modifications: the analytical column was longer (25 cm × 0.46 cm I.D.); the mobile phase was acetonitrile–methanol–0.05 *M* phosphate buffer (30:10:60, v/v) to which was added 0.2% triethylamine (final pH 6.0).

Overdosing investigations. The chromatographic apparatus was similar to that described

above, with some modifications: the analytical column was packed with Hypersil-5 Phenyl material (15 cm × 0.46 cm I.D.) (Shandon, Runcorn, UK); the mobile phase was acetonitrile–0.025 *M* phosphate buffer (pH 3) (30:70, v/v); the flow-rate was 1.5 ml/min; the excitation and emission wavelengths of the detector were 315 and 360 nm, respectively.

Extended chromatographic system for pharmacokinetic and drug metabolism studies. This extension is not necessary for the analytical method dedicated to overdosing investigations. For the on-line liquid–solid clean-up and pre-concentration of the biological fluids, the basic apparatus was supplemented with a pre-column (7.5 cm × 0.21 cm I.D.) dry-filled with Perisorb C_{18}, 30–40 μm (E. Merck) or a pre-concentration column packed with a reversed-phase material supplied for this purpose by Chrompack (Middelburg, Netherlands), a service pump, a Model 414 constant-flow pump (Kontron) for pumping the necessary solvents for the clean-up of biological fluids on the pre-column, a Tracer MCS-670 compact column-switching device provided with six-way high-pressure valves and a six-way solvent selector and a Model 200 programmer (Kontron) for the automation of the switching apparatus and the control of the service pump (see Fig. 3).

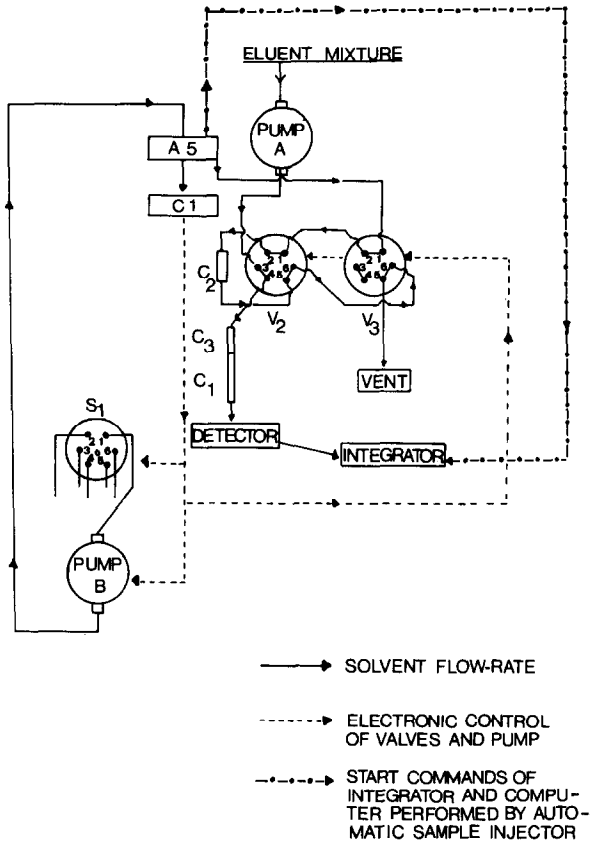

Fig. 3. Scheme of the chromatographic apparatus used for automatic on-line clean-up of samples and HPLC with column switching. C_1 = analytical column; C_2 = pre-column; C_3 = guard-column; V_2 = valve connecting pre-column to analytical column) V_3 = valve for back-flushing of pre-column; S_1 = solvent selector; A5 = automatic sample injector; C1 = computer.

Operating conditions for on-line clean-up and column-switching HPLC (pharmacokinetic and drug metabolism studies)

The automatic operations of fluid sampling, clean-up and enrichment on the pre-column, transfer of the analytes from the pre-column to the analytical column and, finally, analytical chromatography with simultaneous pre-column back-flushing and regeneration are depicted in Figs. 4–7.

The programmes for on-line clean-up and column-switching for the pharmacokinetic and drug metabolism analysis can be summarized respectively as follows.

After injection of a human plasma sample (80–200 μl) on the pre-column, the pre-column was flushed for 2 min with pure water at 2.0 ml/min, then connected, after valve switching, to the analytical column where the analytes were transferred by the HPLC mobile phase; the pre-column was disconnected after 1.5 min and then, while the chromatography took place on the analytical column, it was back-flushed with different solvents such as acetonitrile–water (1:1, v/v) and methanol–water (1:1, v/v) and finally reequilibrated with water.

In the case of the HPLC of zolpidem and its metabolites, the sample clean-up conditions were modified in order to improve the recovery of metabolite I from plasma and urine; for the pre-column clean-up and enrichment, water was replaced by 0.025 *M* KH_2PO_4 (pH6) at 1.0 ml/min

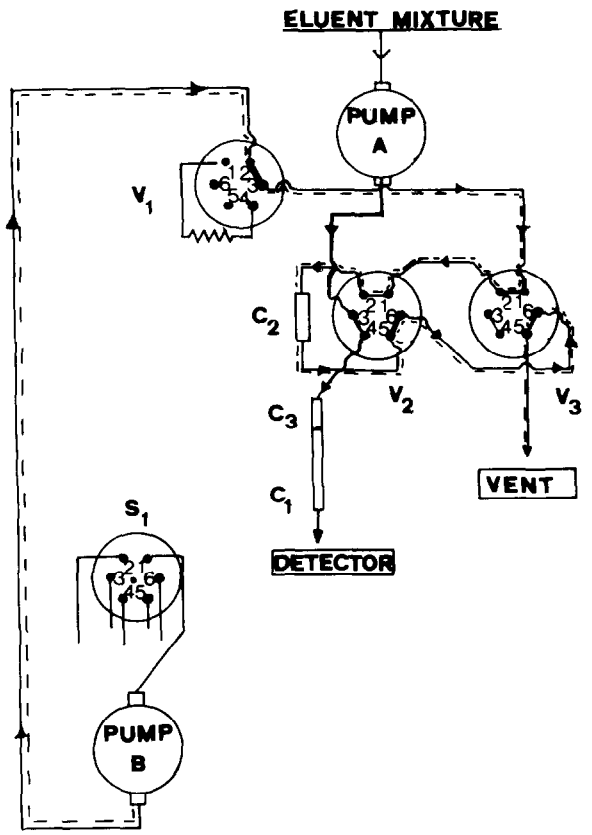

Fig. 4. Equilibrium preceding sample injection.

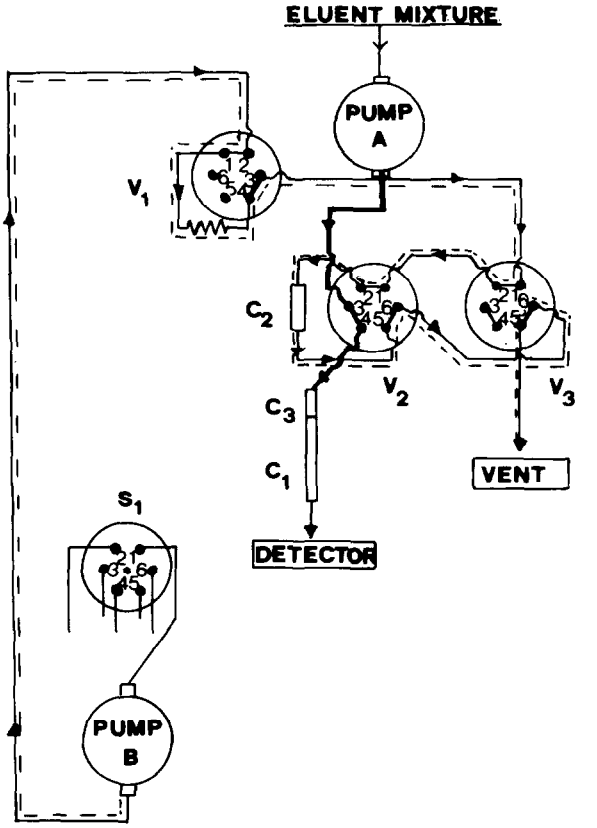

Fig. 5. Loading and clean-up of the sample on the pre-column.

for 2 min, the other conditions being similar to those previously described.

Procedure for plasma and urine samples (pharmacokinetic and drug metabolism studies)

Aliquots (20 μl) of standard solutions (Table I, II or III) were added to 1 ml of either pre-dose plasma or urine diluted ten-fold with water. To all the samples (standards and unknowns) 20 μl of internal standard were added (Table I, II or III). All samples were then vortex-mixed and centrifuged in Eppendorf plastic tubes at 11 000 *g* for *ca.* 3 min (on an Eppendorf or similar centrifuge). The clear upper phase was transferred to conical vials for automatic sample injection. A 100-μl aliquot of plasma supernatant or 50 μl of diluted urine supernatant were injected automatically into the column-switching HPLC apparatus.

Procedure for urine (overdose investigations)

Aliquots (20 μl) of standard solutions (Table IV) were added to the pre-dose urine samples, then diluted 1:10 with 0.025 *M* phosphate buffer (pH 3); to 1 ml of the pre-dose and unknown diluted samples, 20 μl of internal standard (I.S.-2) (Table IV) were added.

Samples were vortex-mixed, and 50 or 100 μl were processed by HPLC by direct automatic injection.

RESULTS

Linearity

Pharmacokinetics. A linear correlation was found between the ratio of the peak heights of zolpidem and the internal standard (I.S.-1) *versus* the concentration of the drug, in the range 1–300 ng/ml zolpidem in human plasma. The linear

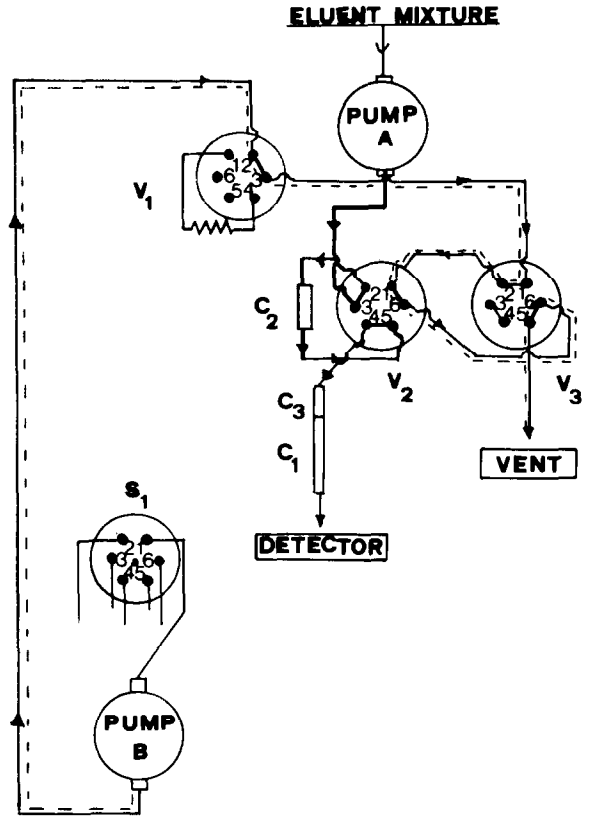

Fig. 6. Switching of valve V_2 and elution of sample from the pre-column to the top of analytical column.

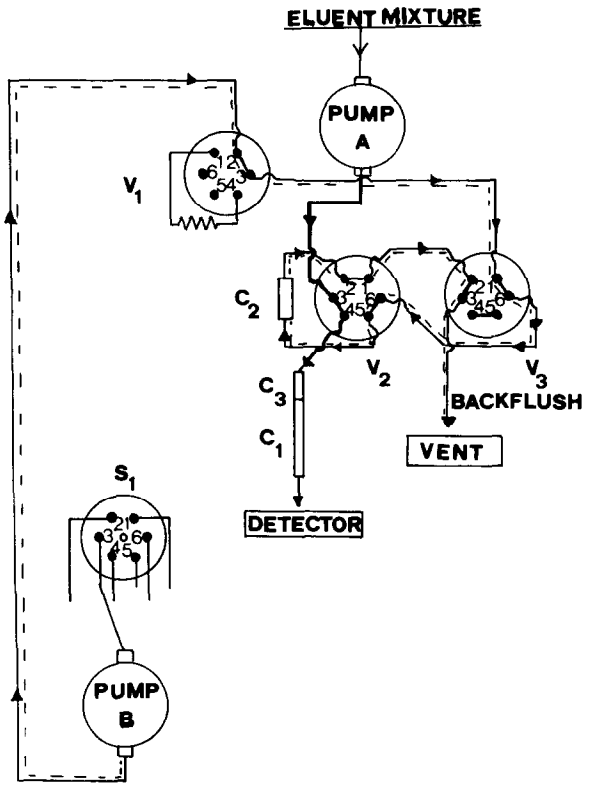

Fig. 7. Chromatography of the sample on the analytical column and back-flush of the pre-column.

least-squares regression, performed on the peak-height ratios *versus* the concentrations of zolpidem, gave the following equation: $y = 24.0x - 0.75$ ($r = 0.9994$).

Drug metabolism. A linear correlation was found between peak-height ratio of zolpidem or its metabolites and the internal standard (I.S.-2) *versus* the concentration of the drug or metabolites. It was decribed by the following equations: $y = 40.1x - 2.9$ ($r = 0.9996$) for metabolite I in the range 4–400 ng/ml; $y = 32.2x - 1.0$ ($r = 0.9996$), $y = 13.4x - 0.26$ ($r = 0.9995$), $y = 20.8x - 0.13$ ($r = 0.9996$) for metabolites II, III and IV, respectively, in the range 1–100 ng/ml; and $y = 36.7x + 0.02$ ($r = 0.9996$) for zolpidem in the range 5–240 ng/ml in human plasma. Linearity was also demonstrated for metabolite I in the range 50–5000 ng/ml, for metabolite II in the range 10–500 ng/ml, for metabolite IV in the range 5–500 ng/ml and for zolpidem in the range 2.5–20 ng/ml in human urine. Metabolite III was not detected in human urine after an oral dose of 10 mg of zolpidem.

Overdose method of analysis. A linear correlation was found between peak-height ratio of the metabolite I and the internal standard (I.S.-2) *versus* the concentration of the metabolite I in the range 10–1000 ng/ml (of 1:10 diluted urine, representing an *in vivo* range of 100–10 000 ng/ml of metabolite I in urine).

Recovery

The absolute recovery of zolpidem and its metabolites from human plasma was evaluated as follows.

(1) Standard solutions of zolpidem, its metabolites and the internal standard (in water or in buffer at pH 6, in the pharmacokinetic and drug

metabolism methods, respectively) were directly injected into the HPLC column (by-passing the pre-column) in order to obtain the linear least-squares regression line (calibration equation).

(2) Plasma standards (pre-dose plasma samples spiked with the drug, its metabolites and the internal standard) at the same nominal concentrations as the aqueous standards, were processed according to the described column-switching methods.

The chromatographic response of the internal standard was identical in the aqueous and plasma samples (100% recovery). The concentrations in plasma samples of the substances of interest were calculated by interpolation from the calibration equation using peak-height ratios of zolpidem or its metabolites to the internal standard obtained from plasma samples (considered as unknowns). The determination was confirmed by directly comparing the chromatographic response of each substance in the aqueous and plasma samples.

The relative recovery was obtained by preparing a calibration curve with plasma standards according to the described methods, and analysing plasma samples of known concentrations of zolpidem and its metabolites (considered as unknowns) according to the internal standard method. The relative recovery is expressed as (amount found/amount added) × 100, and it is designated also by the term "accuracy".

Pharmacokinetics. The absolute recovery of zolpidem from human plasma was *ca.* 90% in a wide range of concentrations; the mean relative recovery in the range 5–300 ng/ml was 100.9 ± 5.3% (coefficient of variation, C.V.) (n = 6).

Pharmacokinetics and drug metabolism. The absolute recovery of zolpidem and its metabolites from human plasma in a wide range of concentrations was as follows: *ca.* 60% for metabolite I, *ca.* 72% for metabolite II, *ca.* 93% for metabolite III, *ca.* 90% for metabolite IV and *ca.* 70% for zolpidem. The mean relative recoveries for zolpidem and its metabolites from human plasma were as follows: 100 ± 2.4% for zolpidem in the range 4–200 ng/ml; 98 ± 3.5% for metabolite I in the range 10–300 ng/ml; 101 ± 2.7% for metabolite II in the range 4–100 ng/ml; 102 ± 3.7% for metabolite III in the range 4–100 ng/ml; 97 ± 4.2% for metabolite IV in the range 4–100 ng/ml.

Overdose method of analysis. No difference was obtained between absolute and relative recoveries in the range 100–10 000 ng/ml of metabolite

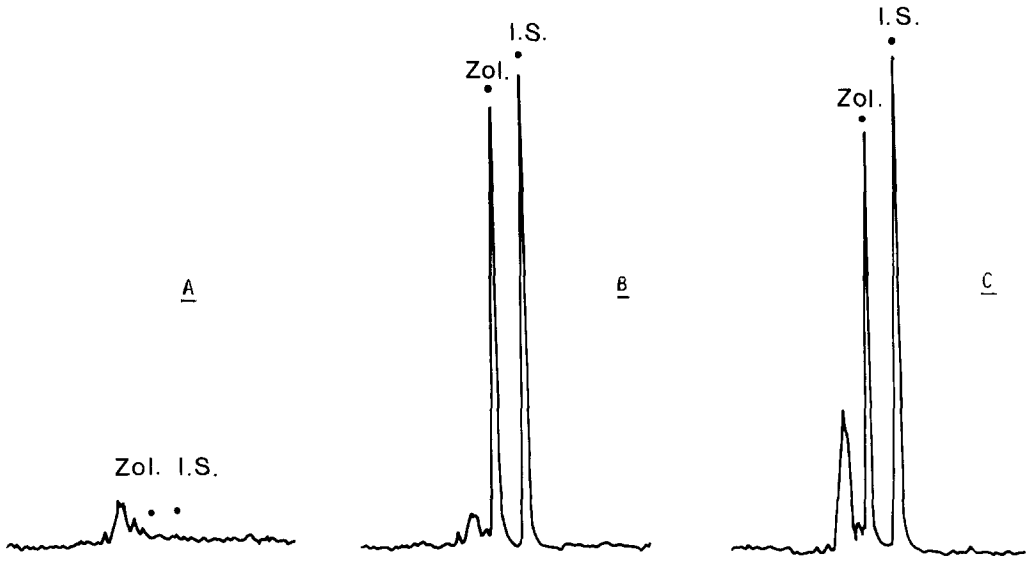

Fig. 8. (A) Chromatogram of pre-dose plasma spiked with I.S.-1; (B) chromatogram of authentic standards recovered from pre-dose plasma spiked with zolpidem (10 ng/ml) and I.S.-1; (C) chromatogram of plasma sample obtained from a patient administered orally with 10 mg of zolpidem; sample taken 1 h after administration; value found: 8.8 ng/ml of plasma. Peaks: Zol = zolpidem; I.S. = I.S.-1.

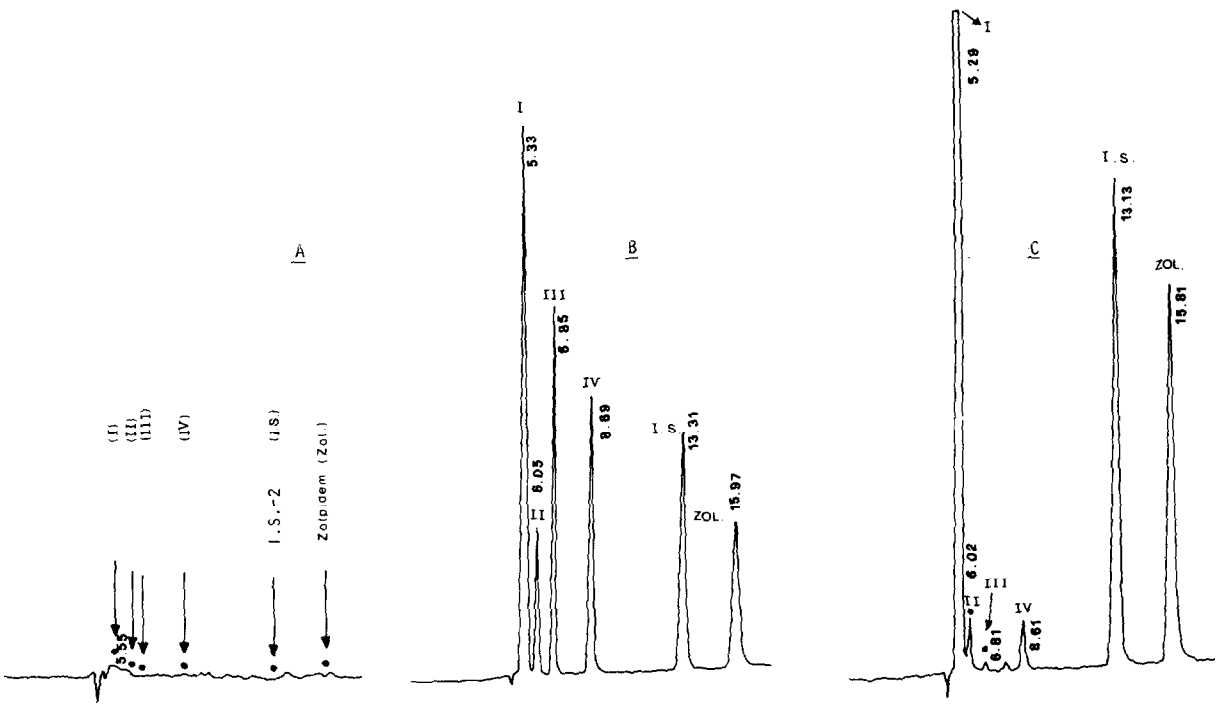

Fig. 9. (A) Chromatogram of pre-dose plasma; (B) chromatogram of authentic standards recovered from pre-dose plasma spiked with metabolite I (80 ng/ml) and zolpidem and metabolites II, III and IV (20 ng/ml); (C) Chromatogram of plasma sample from a subject administered orally with 10 mg of zolpidem; sample taken 6 h after drug intake on 14th day of treatment; values found: I = 139.9 ng/ml; II = 2.9 g ng/ml; IV = 1.7 ng/ml; zolpidem = 26.0 ng/ml.

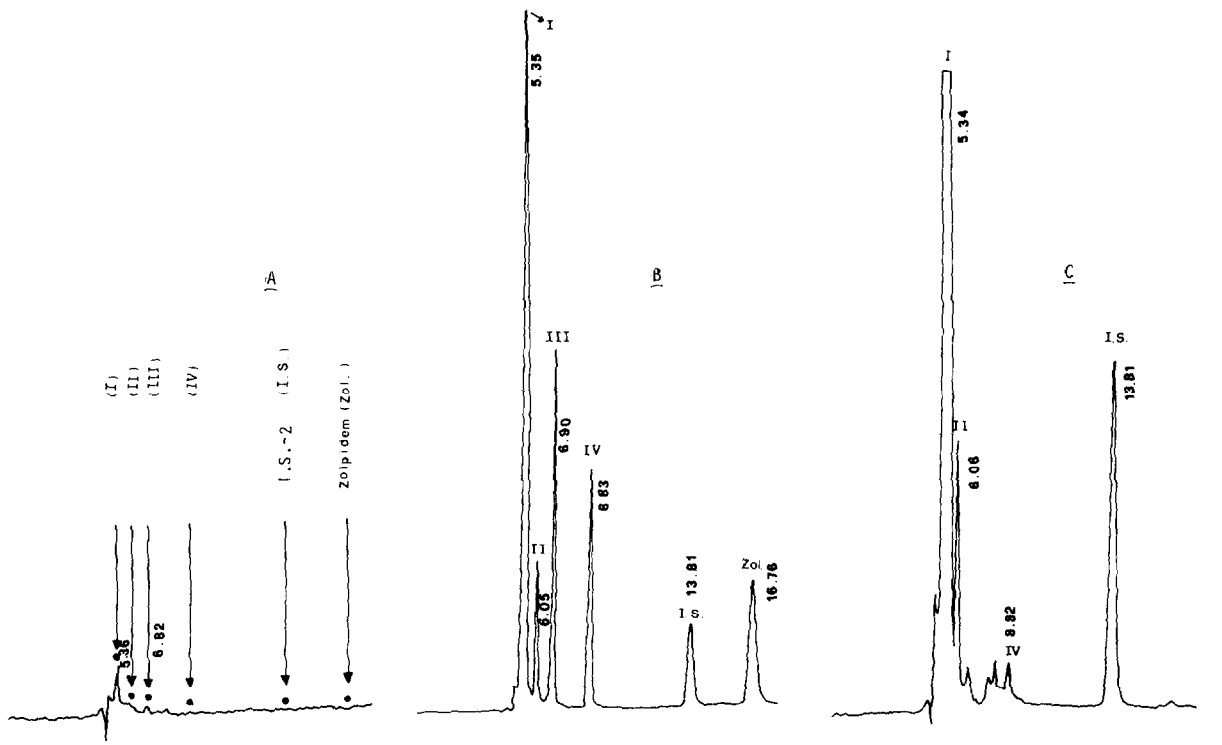

Fig. 10. (A) Chromatogram of pre-dose urine; (B) chromatogram of authentic standards recovered from pre-dose urine spiked with the metabolite I (2000 ng/ml) and zolpidem and metabolites II, III and IV (500 ng/ml); (C) chromatogram of a urine sample from a subject administered orally with 10 mg of zolpidem; sample taken 8.30 h after drug intake; values found: I = 3691 ng/ml; II = 210.8 ng/ml; IV = 6.6 ng/ml (all urine samples were diluted 1 to 10 ml).

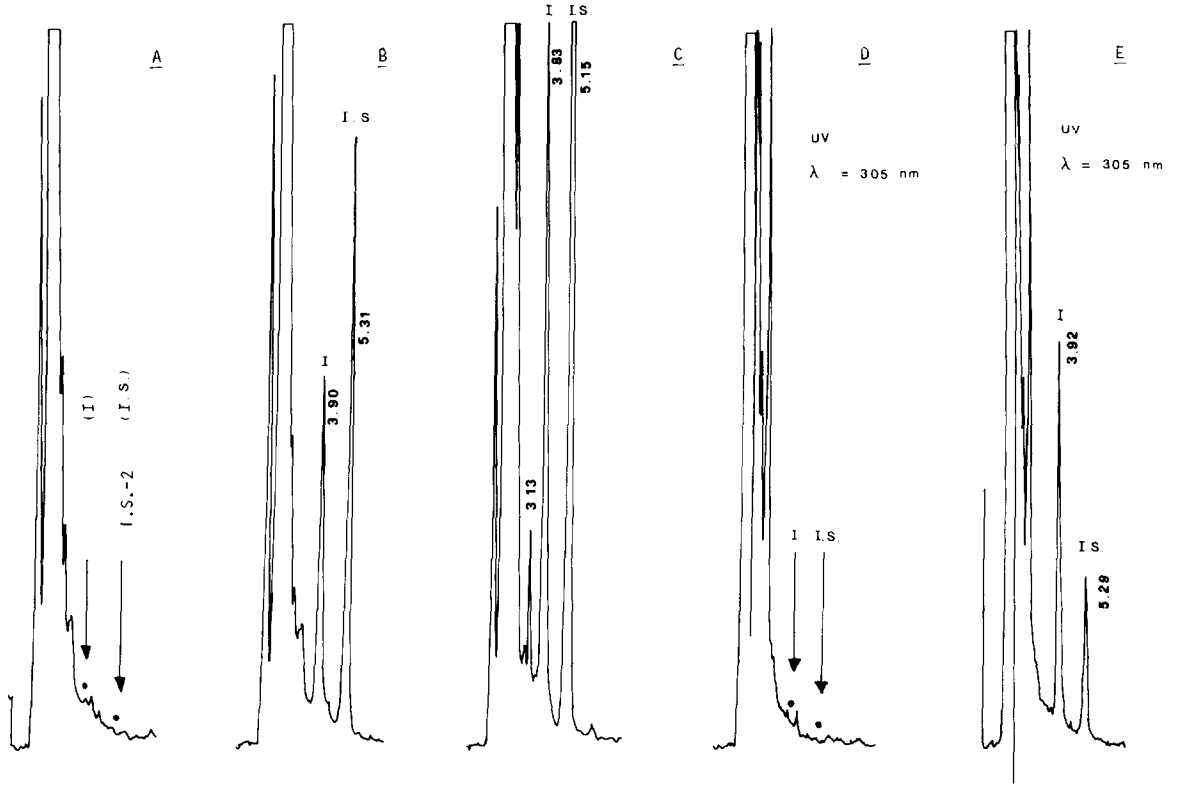

Fig. 11. (A) Chromatogram of pre-dose urine (1 ml diluted to 10 ml before HPLC); (B) chromatogram of metabolite I recovered from pre-dose human urine spiked with 2000 ng of metabolite I (1 ml diluted to 10 ml before HPLC); (C) chromatogram of urine sample from a subject administered orally with 10 mg of zolpidem (1 ml diluted to 10 ml before HPLC; the concentration of the internal standard is twice that used in other cases); samples taken 8 h after drug intake; concentration found, 4500 ng/ml; (D) chromatogram of pre-dose urine (1 ml diluted to 10 ml before HPLC with UV detector set at 305 nm); (E) chromatogram of urine sample from a subject administered orally with 10 mg of zolpidem (1 ml diluted to 10 ml before HPLC with UV detector); sample taken 7 h after drug intake; concentration found, 3980 ng/ml.

I in human urine, because the diluted samples was directly processed by HPLC without any preliminary pre-concentration or clean-up step; the recovery was 103 ± 5% over a wide range of concentrations.

Selectivity

Several pre-dose samples of human plasma and urine were tested for the absence of interfering endogenous compounds. No significant chromatographic interference was found at the retention times of any of the compounds of interest in any of the three methods (Figs. 8A, 9A, 10A, 11A and 11D, from drug-free sample chromato-

TABLE V

REPRODUCIBILITY STUDY FOR URINE SAMPLES SPIKED WITH METABOLITE I (OVERDOSING METHOD)

Amount added (ng/ml)	*n*	Accuracy (%)	C.V. (%)
Intra-day			
2000	11	94.5	2.1
8000	11	97.0	1.0
Inter-day			
2000	11	91.6	0.5
8000	11	96.0	0.2

TABLE VI

REPRODUCIBILITY STUDY FOR PLASMA SAMPLES SPIKED WITH ZOLPIDEM (PHARMACOKINETIC METHOD)

Amount added (ng/ml)	*n*	Accuracy (%)	C.V. (%)
Intra-day			
300.0	4	95.6	0.5
40.0	4	95.1	3.7
5.0	4	101.0	3.2
Inter-day			
120.0	10	102.9	3.4
20.0	11	97.9	4.5
1.2	10	100.0	6.9

grams). However, in the case of pre-dose urine (Fig. 10A), a small but negligible interference was found at the retention time of metabolite I, which is generally present at a very high concentration. In the assay designed for pharmacokinetic studies, the metabolites did not interfere with the determination of zolpidem: they were all eluted before it (Fig. 8C).

Detection limit

The detection limit for zolpidem in human plasma in the pharmacokinetic method was *ca.* 0.2 ng/ml. When the method suitable for drug metabolism studies was used, the detection limits for zolpidem and its metabolites were, respectively, 0.5 and 0.25 ng/ml. In urine the detection limit was *ca.* 1 ng/ml for zolpidem and *ca.* 5 ng/ml for the metabolites (we were obliged to dilute urine samples to minimize the effect of the interfering peak eluting close to metabolite I). When diluted urine was injected directly into the column, the detection limit for metabolite I was *ca.* 50 ng/ml.

TABLE VII

REPRODUCIBILITY STUDY FOR PLASMA SAMPLES SPIKED WITH ZOLPIDEM AND METABOLITES (DRUG METABOLISM METHOD) (INTRA-DAY)

Compound	Amount added (ng/ml)	*n*	Accuracy (%)	C.V. (%)
Zolpidem	100.0	4	98.4	2.4
	20.0	4	100.7	1.4
Metabolite I	250.0	3	103.5	1.7
	50.0	3	98.1	2.3
Metabolite II	20.0	3	104.2	4.5
	4.0	4	101.0	3.4
Metabolite III	20.0	4	101.5	3.3
	4.0	4	95.0	2.6
Metabolite IV	20.0	3	99.0	1.7
	4.0	4	91.0	1.0

Stability of the compounds in human plasma and urine

The stability of zolpidem and its metabolites was investigated and assessed in human plasma and urine during long-term storage at −20°C for six months. Two freeze–thaw cycles were performed on plasma and urine samples spiked with zolpidem and its metabolites without causing any appreciable degradation. No significant variations were found in the stability of zolpidem and its metabolites maintained at 37°C for 6 h, in human plasma, in comparison with similar freshly prepared samples. For metabolite I only, a degradation of *ca.* 10% was observed after 6 h of incubation. The stabilities of zolpidem and its metabolites in urine were very similar to those in plasma.

Validation of the methods

Intra- and inter-assay reproducibility studies were performed on control plasma and urine samples spiked with different amounts of zolpidem and its metabolites, which were processed according to the described methods. The results (Tables VI–VIII) from the plasma samples were obtained with the column-switching methods. The results (Table V) for the urine samples were obtained with the direct injection of diluted sample into the HPLC column and concern only metabolite I. The drug metabolism method for urine samples has not been extensively validated. The results demonstrated an acceptable precision and accuracy of the methods at the investigated concentrations. Typical chromatograms of plasma and urine standards are shown in Figs. 8B, 9B, 10B and 11B.

TABLE VIII

REPRODUCIBILITY STUDY FOR PLASMA SAMPLES SPIKED WITH ZOLPIDEM AND METABOLITES (DRUG METABOLISM METHOD) (INTER-DAY)

Compound	Amount added (ng/ml)	*n*	Accuracy (%)	C.V. (%)
Zolpidem	100.0	6	100.1	2.2
	20.0	6	100.7	3.8
Metabolite I	200.0	6	100.6	1.3
	40.0	6	100.4	4.5
Metabolite II	20.0	6	100.2	2.4
	2.5	6	94.1	7.3
Metabolite III	20.0	6	105.0	2.2
	2.5	6	98.0	3.1
Metabolite IV	20.0	6	101.5	2.6
	2.5	6	98.9	2.4

TABLE IX

URINARY LEVELS OF ZOLPIDEM AND METABOLITES IN A SUBJECT ADMINISTERED ORALLY WITH 10 mg OF DRUG

Time (h)	Urinary level (ng/ml)				
	I	II	III	IV[a]	Zolpidem
3.5	3492.8	142.5	n.d.	5.6 (131.0)	8.7
6.5	3761.7	272.2	n.d.	15.9 (245.0)	8.0
8.5	3691.9	210.8	n.d.	6.6 (174.6)	7.2
16.0	600.0	92.0	n.d.	2.3 (150.0)	n.d.
30.0	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Values in brackets refer to total metabolite (free plus conjugated form) obtained after urine deconjugation performed by means of β-glucuronidase.

Application of the methods to biological specimens (from in vivo studies)

The described methods were applied to the determination of zolpidem and its metabolites in plasma and urine of subjects treated orally with 10 mg of zolpidem. Figs. 8C, 9C, 10C and 11C show chromatograms from plasma and urine samples taken at various times. As shown in Fig. 12, metabolite I has the highest concentration in human plasma, and metabolites II, III and IV occur at low concentrations. The concentrations of the metabolites in urine samples range from not detectable (metabolite III) to very high (*ca.*

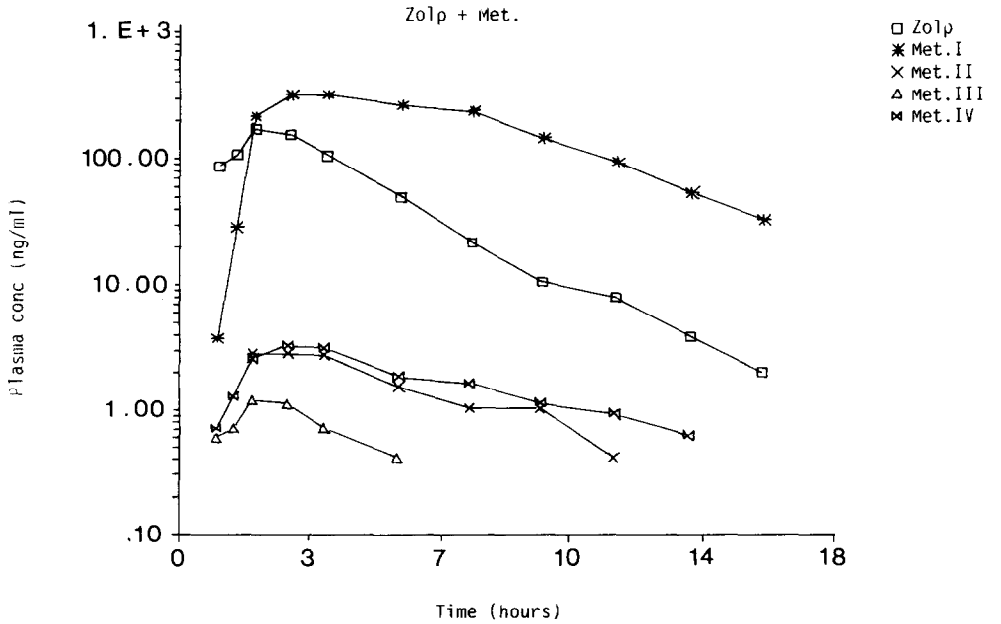

Fig. 12. Plasma concentration–time course of zolpidem and its metabolites in a volunteer administered orally with 10 mg of drug.

3.5 μg/ml of metabolite I) (Table IX). The concentration of zolpidem in urine is negligible because, as already reported, the drug is extensively metabolized. Metabolite IV shows negligible concentrations in the free form, but after deconjugation, it is present at appreciable levels (Table IX).

DISCUSSION

It would be ideal to have available an analytical method suitable for pharmacokinetics, drug metabolism studies and overdosing investigations. As far as pharmacokinetic studies are concerned, there is no interest in knowing the plasma levels of the metabolites because they are inactive in humans. It could be very interesting, however, to know the qualitative and quantitative metabolic profiles in populations under different physiopathological conditions, and for overdosing analysis of zolpidem it is useful to have a very rapid method for analysing urine. Accordingly, we have developed three HPLC methods with different features: the method dedicated to pharmacokinetic studies is very simple, rapid and sensitive; the method used in drug metabolism studies is also very simple, the chromatography takes *ca.* 16 min and is completely automated; the method dedicated to overdosing investigation is extremely rapid because diluted urine is chromatographed directly. The methods, which are based on column-switching HPLC, do not require any sample manipulation because the clean-up and preconcentration steps are performed automatically on-line.

In the past few years, interest in column-switching HPLC performed on drugs and metabolites in biological fluids has grown greatly, as demonstrated by some recent reviews [13,14]. Such a methodological approach offers a simple solution to the complex problem of sample purification and pre-concentration of xenobiotic traces in biological matrices.

The fluorescence properties of zolpidem and its metabolites make them very sensitive and the amount of plasma required for column-switching HPLC does not exceed 100 μl. As a consequence, the pre-column can be used for more than 200 samples before being replaced (in our laboratory, such replacement is performed as a precaution and out of real necessity).

In the overdose method, the HPLC analysis is based on the determination of only metabolite I (in urine the amount of zolpidem is negligible) whose urinary concentration is generally high, so the sample must be diluted before direct HPLC injection. However, in the case of a positive result, it is advisable to confirm the findings by using the drug metabolism method, which gives more information on the levels of metabolites II and IV. The assay can be performed without using the internal standard (I.S.-2, Fig. 2) because no urine manipulation or extraction is required.

UV detection at 305 nm can also be used in the overdose method, as shown in Fig. 11D and E. However, the sensitivity is *ca.* 50% lower than that with fluorescence detection, and the selectivity, with regard to other drugs, should be inferior; however, no method validation has been performed with UV detection.

The use of a phenyl-bonded phase in the overdose method is necessary to obtain a selectivity with respect to endogenous urinary compounds, which is not achieved with a C_{18} phase or liquid–liquid extraction. In fact, the presence of porphyrins in urine is a source of interferences in the analysis of metabolite I on C_{18} and C_8 columns, even with prior liquid–liquid extraction.

Finally, we have checked that some central nervous system drugs that could be present in urine samples from subjects overdosed with zolpidem do not interfere with its identification. Diazepam, nordiazepam, lorazepam, oxazepam, triazolam, and flunitrazepam are not detectable under the conditions used for zolpidem. Unlike these drugs, trazodone, which shows fluorescence properties, could be identified, at a concentration of 200 ng/ml, because it is well separated from other substances.

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