Journal of Chromatography, 616 (1993) 95–103
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6834

High-performance liquid chromatographic assay with diode-array detection for toxicological screening of zopiclone, zolpidem, suriclone and alpidem in human plasma

A. Tracqui*, P. Kintz and P. Mangin

Institut de Médecine Légale, Faculté de Médecine de Strasbourg, 11 Rue Humann, 67085 Strasbourg (France)

(First received October 30th, 1992; revised manuscript received March 1st, 1993)

ABSTRACT

A high-performance liquid chromatographic assay with diode-array detection has been developed for the toxicological screening of the newly developed non-benzodiazepine hypnotics and anxiolytics zopiclone, zolpidem, suriclone and alpidem. After single-step liquid–liquid extraction of plasma at pH 9.5 using chloroform–2-propanol–*n*-heptane (60:14:26, v/v), the substances are separated on a Nova-Pak C_{18} 4-μm column (300 mm × 3.9 mm, I.D.), with methanol–tetrahydrofuran–pH 2.6 phosphate buffer (65:5:30, v/v) as the mobile phase (flow-rate 0.8 ml/min). Full ultraviolet spectra from 200 to 400 nm are recorded on-line during the entire analysis and may be automatically compared to spectra stored in a library. The retention times of the four drugs are 4.05 min (zopiclone), 4.66 min (zolpidem), 6.74 min (suriclone) and 10.97 min (alpidem). The analysis is performed in 15 min. The method is simple, rapid and highly specific. It is the first assay to be described for convenient screening of cyclopyrrolones and imidazopyridines.

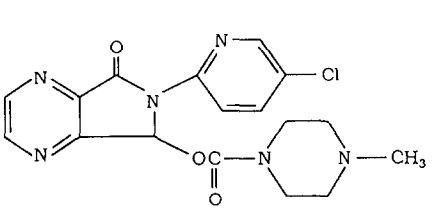
INTRODUCTION

Zopiclone (ZOP) and zolpidem (ZOL) are two non-benzodiazepine (non-BZD) hypnosedative drugs with, respectively, cyclopyrrolone and imidazopyridine structure (Fig. 1), which have been shown in insomniac patients to exhibit rapid onset of action, short elimination half-life (*ca.* 5.5 and 2.0 h, respectively) and few associated side-effects [1,2]. Suriclone (SUR) and alpidem (ALP), the anxiolytic counterparts of ZOP and ZOL (Fig. 1), are claimed to be of equivalent potency to BZDs for the long-term therapy of anxiety, with significant reduction of the side-effects (daytime sedation, dependence potential and withdrawal phenomena) encountered with those drugs [3–6].

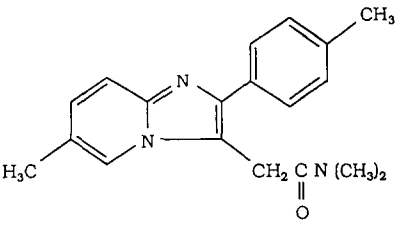
Several analytical methods, using either high-performance liquid chromatography (HPLC) or gas chromatography (GC), have been proposed for the individual determination of ZOP [7–12], ZOL [13–17] and ALP [18]. Up to now, however, no screening method was available for rapid identification of cyclopyrrolones and imidazopyridines in human biofluids, which was particularly awkward, considering (1) that the prescription of these drugs is continuously gaining in importance and (2) that numerous acute intoxications with ZOP, ZOL and ALP have been already described [15,19–22]. In particular, the present immunoassays that are employed worldwide for toxicological screening of biological samples in

* Corresponding author.

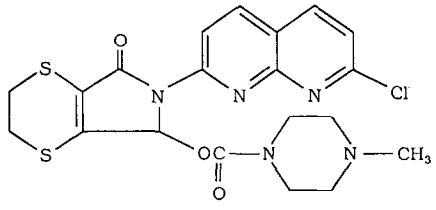
0378-4347/93/$06.00



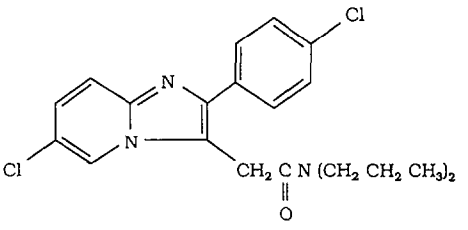
ZOPICLONE



ZOLPIDEM



SURICLONE



ALPIDEM

Fig. 1. Structures of zopiclone, zolpidem, suriclone and alpidem.

forensic or emergency-room situations are unable to detect these new compounds.

This paper presents a simple, rapid and highly selective method for screening of ZOP, ZOL, SUR and ALP in human plasma, by means of HPLC with diode-array detection (DAD).

EXPERIMENTAL

Materials

ZOP (free base, MW = 388.82), ZOL (free base, MW = 307.40), SUR (free base, MW = 477.98) and ALP (free base, MW = 404.34) were kindly donated by their manufacturers, namely Théraplix Labs., Paris, France (ZOP), Rhône-Poulenc Rorer Labs., Vitry-Alfortville, France (SUR) and Synthélabo Labs., Paris, France (ZOL and ALP). Methanol, tetrahydrofuran, chloroform, 2-propanol and *n*-heptane were HPLC grade (Merck, Darmstadt, Germany). All other chemicals and reagents were analytical grade and purchased from Merck and Prolabo (Paris, France).

Stock solutions of each drug tested were prepared in methanol at a concentration of 100.0 μg/ml. All drugs could be stored at 4°C in the dark without significant decay for at least two months, except for ZOP that showed relative instability and had to be prepared weekly. For each compound, working solutions at concentrations of 10.0, 1.0 and 0.1 μg/ml were prepared daily by appropriate methanolic dilutions.

The pH 9.5 buffer solution was prepared using a satured ammonium chloride solution, diluted 25% with deionized water and adjusted to the desired pH by appropriate addition of 25% diluted ammonia solution. The pH 2.6 buffer was prepared with a 10^{-2} *M* potassium dihydrogenphosphate solution, adjusted to the desired pH by appropriate addition of concentrated orthophosphoric acid.

Chromatography

The HPLC system consisted of a quaternary low-pressure pump (Waters 600-E, Milford, MA, USA) and a 200-μl loop volume automatic sample injection module (Waters 715 Ultra Wisp), which were connected to a UV–VIS diode-array

spectrophotometer (Waters 991) with a wavelength range of 190–800 nm and maximal spectral resolution of 1.3 nm.

The system was monitored by a computer (PowerMate SX Plus, NEC, Boxborough, MA, USA) with software (Water PDA) allowing the creation of a personal database, and automatic comparison of current analytical data (retention times and UV spectra) with references previously stored in the library. Drug identification was carried out, first using a time window parameter that restricts the library search to a definite slice around the retention time of an unknown peak, then by point-to-point comparison of the unknown spectrum to spectra of all reference compounds contained within this time window, and calculation in each case of a fit value (degree of similarity) ranging from 0 to 1000. Time window and fit threshold were set at ±0.5 min and 900/1000, respectively.

A Nova-Pak C_{18} (Waters) 4-μm column (300 mm × 3.9 mm I.D.) was used at the constant temperature of 30°C. The elution was achieved isocratically (flow-rate 0.8 ml/min, average operating pressure 15.97 MPa) with a mobile phase of methanol–tetrahydrofuran–pH 2.6 phosphate buffer (65:5:30, v/v); before analysis, this mobile phase was degassed and filtered through 0.45-μm filters (Durapore GVWP 047, Bedford, MA, USA) with a Pyrex filter holder (Millipore, Bedford, MA, USA). The equilibration time of the system was 30 min before analysis; at the end of each chromatographic session, the column was washed with deionized water (0.8 ml/min for 1 h) then methanol (0.8 ml/min for 1 h).

Procedure

To 2.0 ml of plasma in a 15-ml Pyrex centrifuge tube were added 2.0 ml of the pH 9.5 ammonium chloride buffer and 5.0 ml of the extracting solvent (chloroform–2-propanol–*n*-heptane, 60:14:26, v/v). The mixture was gently shaken on a horizontal agitator for 10 min, then centrifuged at 2800 *g* for 10 min. The lower organic phase was removed and evaporated to dryness at 45°C in a rotary evaporator (Speed Vac concentrator A290, Savant Instruments, Hicksville, NY, USA). The residue was dissolved in 100 μl of the mobile phase, from which 50 μl were injected into the chromatographic system.

The eluent was monitored at 210 nm, while full UV spectra (200–400 nm) were recorded on-line during the whole chromatographic run. The spectral resolution and the sampling interval (delay between acquisition of two successive spectra) were set at 1.3 nm and 1.0 s, respectively.

RESULTS AND DISCUSSION

Fig. 2 shows the chromatogram obtained following extraction of a blank plasma spiked to contain the four drugs tested at the concentration of 1.0 μg/ml each. The separation was judged adequate, with retention times of 4.05, 4.66, 6.74 and 10.97 min for ZOP, ZOL, SUR and ALP, respectively [capacity factor (k'): 0.36, 0.56, 1.26, 2.68, respectively]. The typical UV spectra of the four drugs are represented in Fig. 3. Owing to the diode-array detection the method was found to be extremely selective, since no interference (*i.e.* identity based on retention time *and* UV spectrum) was noticed between any of the four drugs tested and more than 300 pharmaceuticals and drugs of abuse that were assayed under the same chromatographic conditions; the most likely troublemakers are listed in Table I: when compared to the drugs tested, none of these compounds was found to present fit values above the cut-off chosen for identification (900/1000).

Fig. 4 represents the chromatogram obtained from the post-mortem blood sample of a 28-year-old female in a fatality involving zolpidem, acepromazine and desmethyldiazepam [23], showing that the method also proved to be reliable, without any modification, in screening of haemolysed blood.

Chloroform–2-propanol–*n*-heptane (60:14:26, v/v) was chosen as the extraction solvent since it is little prone to emulsion formation and was found to allow good recoveries not only for the four drugs tested, but also for a great variety of pharmaceuticals and drugs of abuse [24–26]; this non-selectivity of the extraction step is of prime necessity, since the present technique is intended

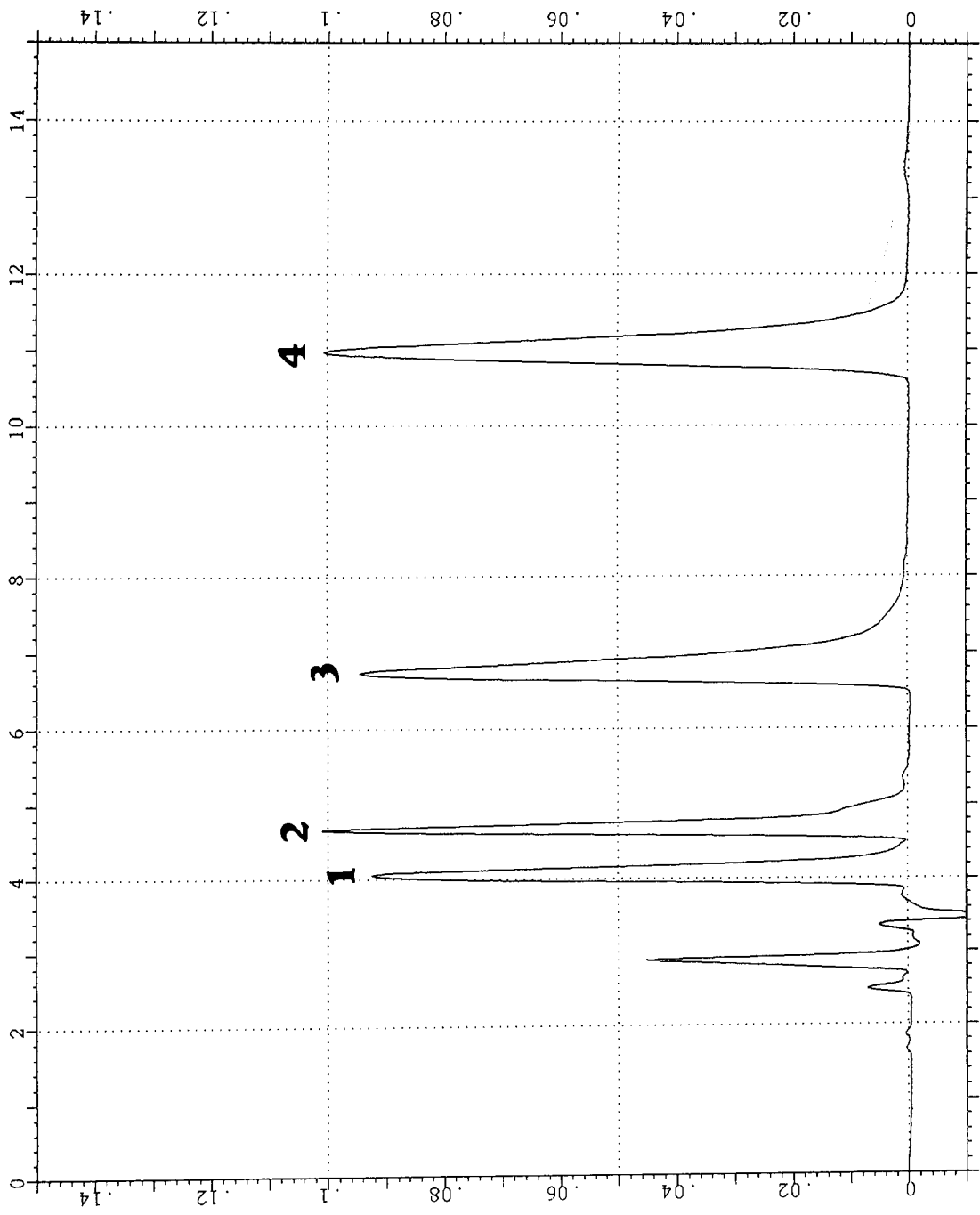


Fig. 2. Chromatogram (displayed at 210 nm) of a blank plasma sample spiked with a mixture of the four drugs tested (1.0 μg/ml each). Peaks: 1 = zopiclone (4.05 min); 2 = zolpidem (4.66 min); 3 = suriclone (6.74 min); 4 = alpidem (10.97 min).

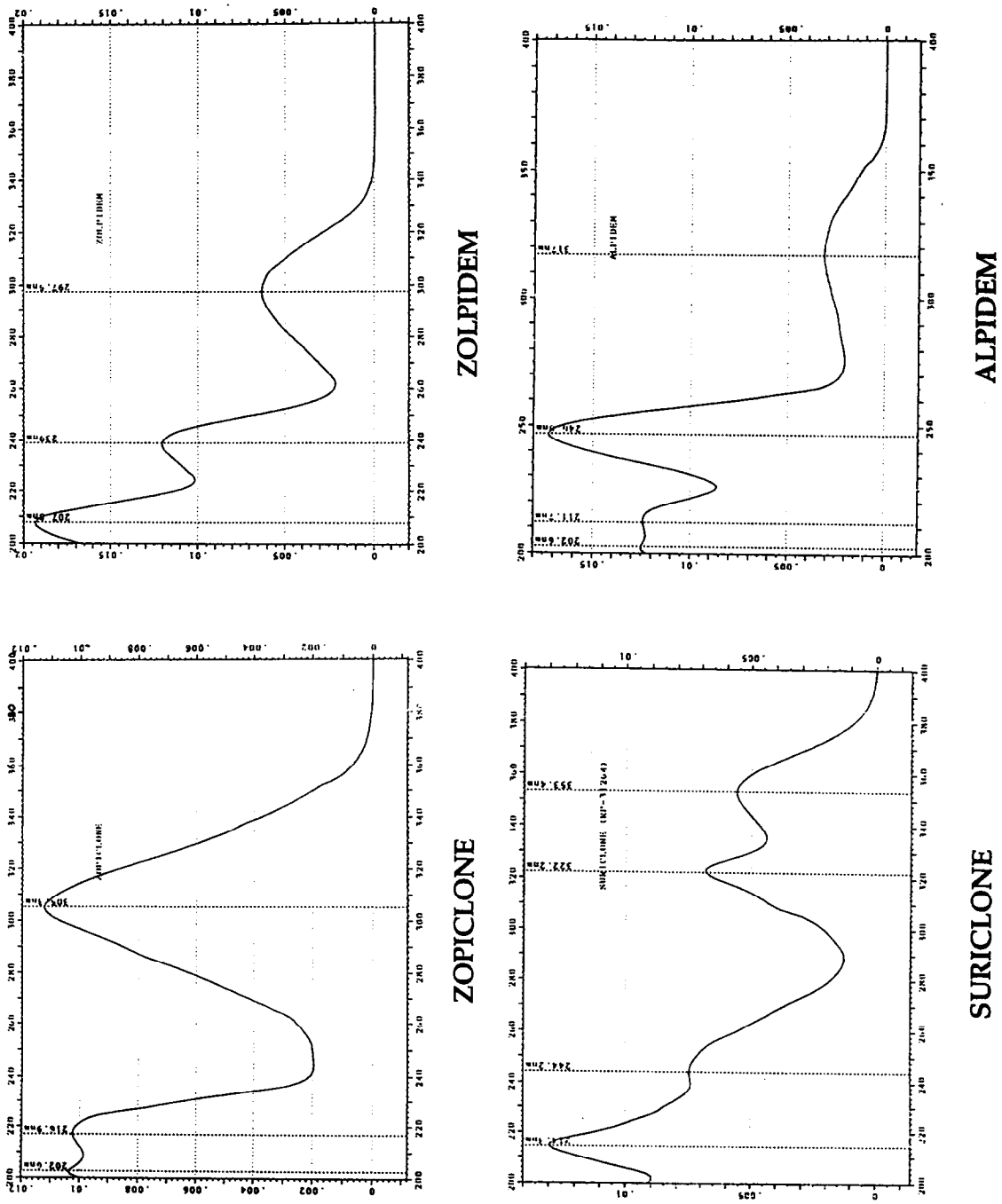


Fig. 3. Typical UV spectra (200–400 nm) at pH 2.6 for zopiclone, zolpidem, suriclone and alpidem.

TABLE I

COMPOUNDS POSSIBLY CAUSING CONFUSION WITH ZOPICLONE, ZOLPIDEM, SURICLONE OR ALPIDEM

Time window: ±0.5 min; only compounds with fit values above 800/1000 have been listed

Compound	Reference drug	t_R (min)	Fit value (*versus* drug tested)
Zopiclone (t_R = 4.05 min)	*p*-Nitrophenol	3.66	827
	Ketotifen	4.40	827
	Tiaprofenic acid	4.34	812
Zolpidem (t_R = 4.66 min)	Vincristine	5.06	832
	Ketotifen	4.40	811
	Sultopride	4.23	809
	Pyrimethamine	5.00	802
Suriclone (t_R = 6.74 min)	Nimodipine	6.36	808
Alpidem (t_R = 10.97 min)	–	–	–

to be incorporated, in the end, into a general HPLC–DAD method with single-step liquid–liquid extraction for rapid screening of 300–400 toxicologically relevant compounds in human biofluids. Absolute recoveries were determined for ZOP, ZOL, SUR and ALP by extracting and assaying blank plasma samples loaded with the drugs at the concentrations of 0.1 and 1.0 μg/ml, and by comparing the representative peak areas of these extracted samples with those of unextracted methanolic standards at the same concentrations; these experiments were carried out in triplicate for each drug at each concentration. Results are given in Table II.

The HPLC analysis is performed in 15 min, and the entire screening procedure (including single-step extraction, evaporation and chromatographic run) may be achieved in less than 120 min, making our method convenient not only for forensic determinations, but also for emergency-room cases.

The lower limits of detection were determined for each drug by extracting and assaying pure plasma samples spiked with decreasing concentrations of the compound tested, until a reponse equivalent to three times the background noise was obtained; for this determination, each drug was monitored (1) at 210 nm and (2) at its wave-

TABLE II

ABSOLUTE RECOVERIES AND DETECTION LIMITS FOR ZOPICLONE, ZOLPIDEM, SURICLONE AND ALPIDEM

Compound	Absolute recovery[a] (%)		Detection limits[a] (ng/ml)	
	0.1 μg/ml	1.0 μg/ml	At 210 nm	At λ_{max}[b]
Zopiclone	76.5 ± 4.4	83.8 ± 5.0	24.5 ± 5.9	24.8 ± 4.6
Zolpidem	86.9 ± 3.7	86.0 ± 5.7	24.6 ± 4.8	23.1 ± 5.7
Suriclone	84.5 ± 9.1	92.3 ± 5.1	32.3 ± 6.7	33.9 ± 6.4
Alpidem	80.8 ± 5.5	84.1 ± 5.7	34.8 ± 5.1	19.2 ± 3.2

[a] All results are the mean ± S.D. of three separate experiments.

[b] Wavelength of maximal absorbance (ZOP = 305 nm; ZOL = 207 nm; SUR = 214 nm; ALP = 247 nm).

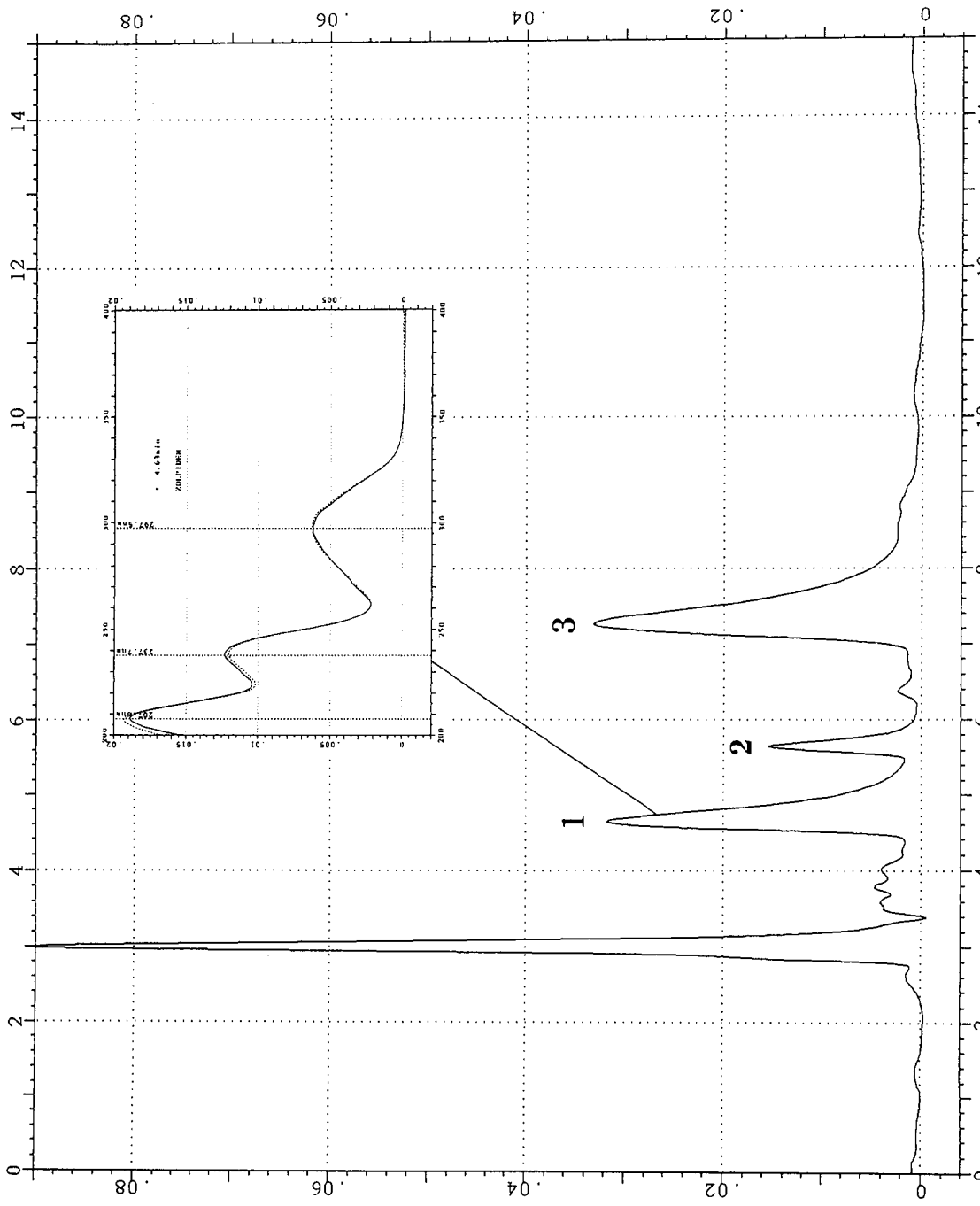


Fig. 4. Chromatogram of the blood sample in a fatality involving zolpidem, acepromazine and desmethyldiazepam [20]. Peaks: 1 = zolpidem; 2 = desmethyldiazepam; 3 = acepromazine. Window: superposition of UV spectra (200–400 nm) of peak 1 (full line) and zolpidem (dotted line; spectrum from the library); similarity 986/1000.

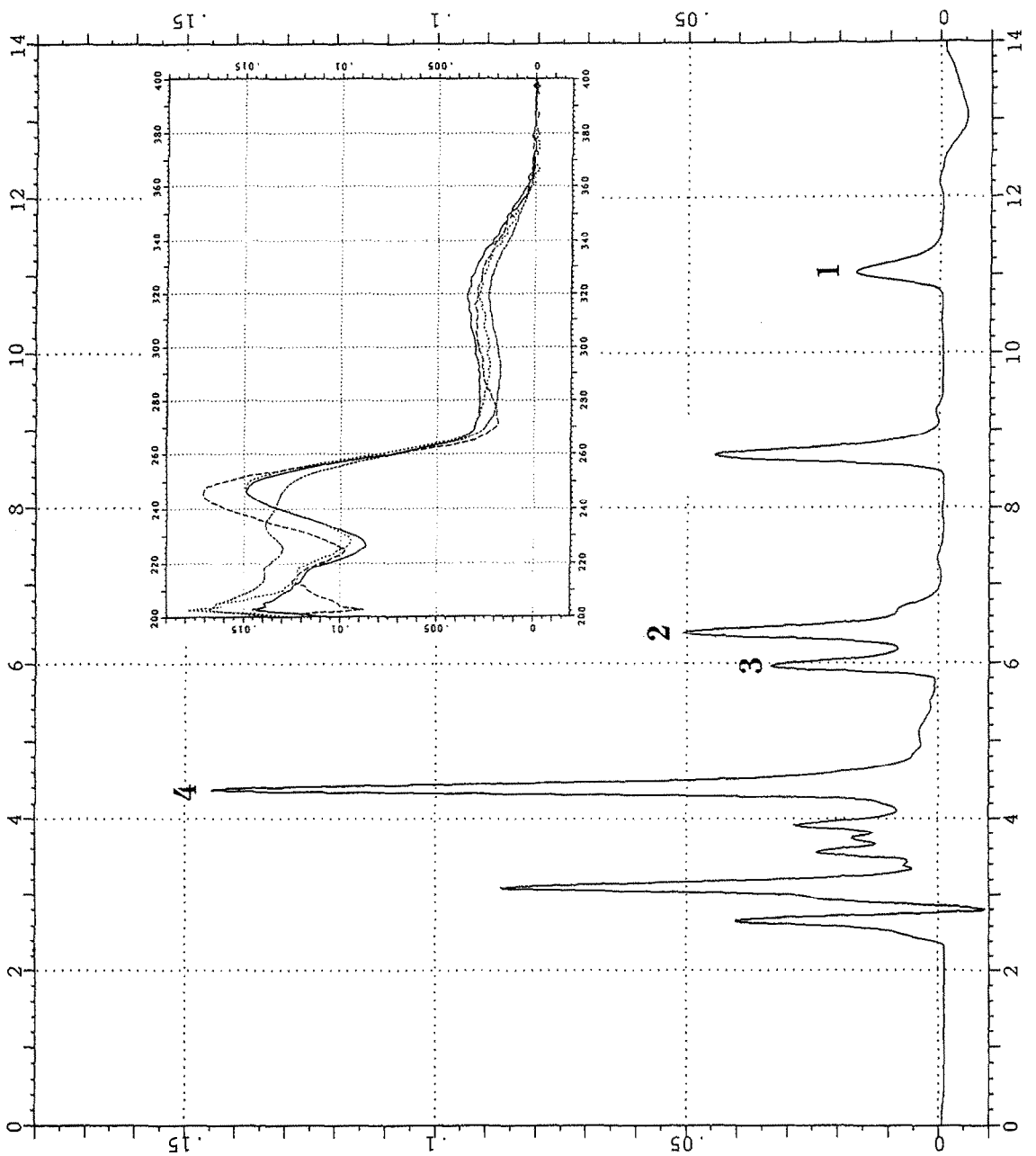


Fig. 5. Chromatogram of the plasma sample in a non-fatal self-poisoning involving alpidem. Peaks: 1 = alpidem; 2–4 = alpidem circulating metabolites. Window: superposition of the UV spectra (200–400 nm) of peaks 1–4.

length of maximal absorbance, *i.e.* 305, 207, 214 and 247 nm for ZOP, ZOL, SUR and ALP, respectively. Results are given in Table II. Considering that therapeutic plasma levels of ZOP, ZOL and ALP are reported to be 60–70, 139 and 53 ng/ml, respectively (mean values of peak plasma concentrations after intake of one 7.5-, one 10- and one 50-mg tablet, respectively) [1,27,28], these results make our method convenient for simultaneous identification of the three drugs presently marketed, at least at therapeutic levels, and *a fortiori* in poisoning cases.

The intra-laboratory day-to-day variation of the retention times (t_R) was investigated for the four drugs by carrying out weekly analyses of the 1.0 μg/ml methanolic standards over a period of four months. The coefficients of variation of the retention times were found to be 2.1% (ZOP), 2.0% (ZOL), 3.9% (SUR) and 4.9% (ALP).

Since the UV spectra of one compound and its metabolites frequently exhibit great similarities, drug screening by HPLC–DAD may be of value for substances that are present in blood mainly in the form of metabolites. This is the case for alpidem, whose three circulating metabolites are responsible for majority of the pharmacological activity [18,29]. As an example, Fig. 5 shows the chromatogram of a 47-year-old female's plasma, taken 4 h after suicidal ingestion of an undetermined amount of alpidem; alpidem metabolites (t_R = 4.37, 5.96, and 6.38 min) were clearly identified owing to the similarity of their UV spectra with that of the parent drug (see inset).

CONCLUSION

The present method is the first described for convenient screening of the newly developed non-BZD drugs ZOP, ZOL, SUR and ALP. Owing to single-step liquid–liquid extraction and photodiode-array detection, it is particularly simple, rapid and highly specific. It proved to be useful in both forensic (Fig. 4) and clinical (Fig. 5) cases.

REFERENCES

1 K. L. Goa and R. C. Heel, *Drugs*, 32 (1986) 48.
2 H. D. Langtry and P. Benfield, *Drugs*, 40 (1990) 291.
3 D. Wheatley, *Psychother. Psychosom.*, 49 (1988) 63.
4 D. J. Sanger, G. Perrault, E. Morel, D. Joly and B. Zivkovic, *Prog. Neuro-Psychopharmacol. Biol. Psychiatry*, 15 (1991) 205.
5 B. Saletu, R. Frey, J. Grünberger, M. Krupka, P. Anderer and B. Musch, *Br. J. Clin. Pharmacol.*, 30 (1990) 703.
6 P. L. Morselli, *Pharmacopsychiatry*, 23, Suppl. 3 (1990) 129.
7 C. Stanley, P. Mitchell and C. M. Kaye, *Analyst*, 110 (1985) 83.
8 L. G. Miller, B. W. Leduc and D. J. Greenblatt, *J. Chromatogr.*, 380 (1986) 211.
9 A. L. Liboux, A. Frydman and J. Gaillot, *J. Chromatogr.*, 417 (1987) 151.
10 S. Kennel, P. Kintz, A. Tracqui, P. Mangin, A. A. Lugnier and A. J. Chaumont, *J. Chromatogr.*, 527 (1990) 169.
11 C. Fernandez, B. Baune, F. Gimenez, A. Thuillier and R. Farinotti, *J. Chromatogr.*, 572 (1991) 195.
12 M. J. Royer-Morrot, M. Rambourg, I. Jacob, P. Bauer and R. J. Royer, *J. Chromatogr.*, 581 (1992) 297.
13 P. Guinebault, C. Dubruc, P. Hermann and J. P. Thenot, *J. Chromatogr.*, 383 (1986) 206.
14 S. Vajta, J. P. Thenot, F. de Maack, G. Devant and M. Lesieur, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 223.
15 G. Debailleul, F. Abi Khalil and P. Lheureux, *J. Anal. Toxicol.*, 15 (1991) 35.
16 D. Debruyne, J. Lacotte, B. Hurault de Ligny and M. Moulin, *J. Pharm. Sci.*, 80 (1991) 71.
17 V. Ascalone, L. Flaminio, P. Guinebault, J. P. Thenot and P. L. Morselli, *J. Chromatogr.*, 581 (1992) 237.
18 V. Ascalone, P. Catalini, L. Dal Bo', N. Deschamps and P. Guinebault, *J. Chromatogr.*, 414 (1987) 101.
19 D. Méram and J. Descotes, *Rev. Med. Interne*, 10 (1989) 466.
20 P. Lheureux, G. Debailleul, O. De Witte and R. Askenasi, *Hum. Exp. Toxicol.*, 9 (1990) 105.
21 Y. Regouby, G. Delomez and A. Tisserant, *Thérapie*, 45 (1990) 161.
22 A. Tracqui, P. Kintz and P. Mangin, unpublished results.
23 A. Tracqui, P. Kintz and P. Mangin, *Am. J. Forensic Med. Pathol.*, (1993) in press.
24 A. Tracqui, P. Kintz, P. Kreissig and P. Mangin, *J. Liq. Chromatogr.*, 15 (1992) 1381.
25 P. Kintz, A. Tracqui, A. A. Lugnier, P. Mangin and A. J. Chaumont, *Methods Find. Exp. Clin. Pharmacol.*, 12 (1990) 193.
26 A. Tracqui, P. Kintz, P. Kreissig and P. Mangin, *Ann. Biol. Clin.*, 50 (1992) 639.
27 *Stilnox (Zolpidem)*, technical documentation, Synthélabo Labs., Paris, 1988.
28 *Ananxyl (Alpidem)*, technical documentation, Synthélabo Labs., Paris, 1991.
29 D. Garrigou-Gadenne, A. Durand, J. P. Thenot and P. L. Morselli, *Drug Metab. Dispos.*, 19 (1991) 574.