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Development of a method for the determination of zopiclone in whole blood

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ABSTRACT

Mass spectrometry in both electron-impact and positive chemical ionisation modes has been used to elucidate the structures of the decomposition products of zopiclone after gas chromatography. A high-performance liquid chromatographic method has been developed for the determination of zopiclone in whole blood. After selective extraction (butyl chloride) the extracts are chromatographed on Spherisorb ODS-5 (5 μm) using dibasic ammonium phosphate–acetonitrile (40:60). The zopiclone is measured by ultraviolet detection with a limit of quantitation of 4 ng/ml. This method has been successfully applied to the determination of zopiclone in post-mortem blood. Zopiclone levels found in five post-mortem cases are presented.

INTRODUCTION

Zopiclone, 6-(5-chloro-2-pyridyl)-7-[(4-methyl-1-piperazinyl)carbonyloxy]-6,7-dihydro[5*H*]-pyrrolo[3,4-*b*]pyrazine-5-one, is a pyrrolopyrazine drug with hypnosedative effects that has been shown in insomniac patients to possess rapid onset of action and few associated side-effects [1–3].

A recent paper [4] described a gas chromatographic (GC) procedure with nitrogen–phosphorus detection (NPD) for the characterisation of zopiclone. This was of interest to us because in our laboratory there also arose a need for the quantitation of this drug from a post-mortem examination of a suspected zopiclone overdose. A GC approach with NPD or electron-capture detection (ECD) gave good response and selectivity particularly with ECD. However, examination of the chromatogram revealed the detection of more than one significant peak, the ratios of which depended on the injector port temperature, the condition of the liner and the injection mode. The target drug zopiclone appeared to decompose under the thermal conditions of the GC analysis making it unsuitable for quantitative work.

As a GC technique was not suitable for the quantitation of zopiclone, we turned to a high-performance liquid chromatographic (HPLC) technique. This method was based on earlier method developed for droperidol [5]. Several liquid chromatographic methods have been described for the detection of zopiclone in plasma and/or urine [6–10]. However the first method [6] is time-consuming as zopiclone and each metabolite must be analysed separately. In the second method [7] the N-desmethyl compound interferes with zopiclone during chromatography when it is present in high concentration. The method of Miller *et al.* [8] is not suitable for the N-oxide and N-desmethyl metabolites because these metabolites are not quantitatively extracted in acidic

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media. The method of Fernandez *et al.* [10] is used for determining zopiclone enantiomers in plasma and is not suitable for the metabolites.

All the previous methods [6–10] are designed for plasma or urine rather than whole blood and use fluorometric detectors rather than the more widely used ultraviolet detectors. We describe here a selective and reproducible HPLC method for the determination of zopiclone in whole blood. The method uses ultraviolet detection with a limit of quantitation of 4 ng/ml. Attempts were also made to elucidate the structures of the GC peaks obtained from the injection of zopiclone standards, using mass spectrometry (MS) in both the electron-impact (EI) mode and the positive chemical ionisation (PCI) mode.

EXPERIMENTAL

Reagents and standards

Zopiclone (I) and its two principal metabolites zopiclone N-oxide (II) and N-desmethylzopiclone (III) were gifts of Rhone-Poulenc (Vitry Sur Seine, France) while the internal standard (IV) (29481 R.P.) was a gift of Rhone-Poulenc (Dagenham, UK) (Fig. 1). Acetonitrile (HPLC grade) was purchased from May and Baker (Dagenham, UK). *n*-Butyl chloride (synthesis grade, Merck) was distilled prior to use. All other chemicals were analytical reagent grade obtained from usual commercial sources.

Both zopiclone and the internal standard are unstable in nucleophilic solvents such as methanol or ethanol. Stock standard solutions of zopiclone and the internal standard for quantitation were therefore prepared in non-nucleophilic solvents such as acetonitrile and stored at −15°C. In acetonitrile, zopiclone was stable for at least two months [8].

Phosphate buffer was prepared by dissolution of 1.15 g of ammonium dihydrogenphosphate in 1.0 l of distilled water. Triethylamine (1.0 ml) was added giving a final unadjusted pH of about 6.8. This buffer was used in the preparation of the HPLC mobile phase and was prepared fresh each day.

Blood zopiclone standards, over a concentration range of 6.6–66 ng/ml, were prepared by supplementing blank transfusion blood with appropriate volumes of 0.66 and 6.6 μg/ml zopiclone solutions. These solutions were prepared by diluting stock standard solution with acetonitrile.

Apparatus

GC was performed on a 15 m × 0.2 mm I.D. DB 17 column, 0.25 μm film thickness (J & W Scientific, Folsom, CA, USA) installed in a Hew-

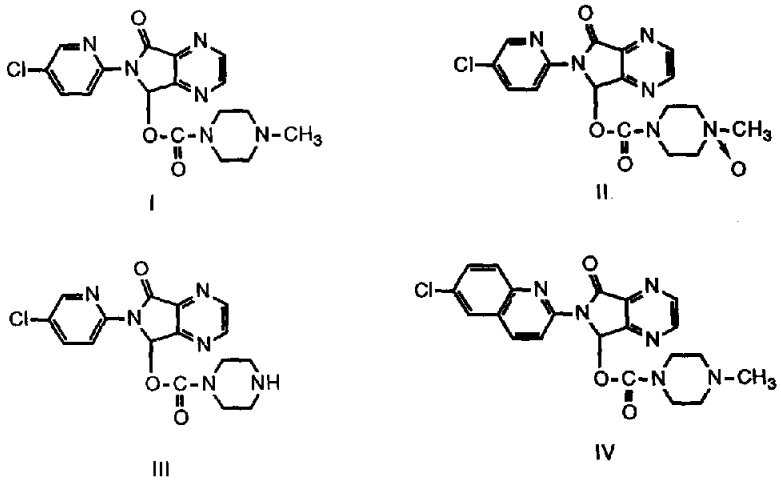


Fig. 1. Structures of zopiclone and its metabolites.

lett Packard 5890 gas chromatograph (Avondale, PA, USA) equipped with an electron-capture detector and a 3393A integrator. Hydrogen was used as a carrier gas and the GC oven was temperature-programmed from 220°C (5 min hold) to 280°C (11 min hold) at 10°C/min. Injector port and detector temperatures were 250 and 300°C, respectively.

Combined GC–MS analyses were carried out on a Hewlett Packard 5890/5970 gas chromatograph–mass-selective detector (Palo Alto, CA, USA) using a 25 m × 0.2 mm I.D. HP-1 methyl silicone column, 0.11 μm film thickness, for EI experiments. PCI experiments were carried out on a Hewlett Packard 5890/5989A GC–MS instrument using a 12 m × 0.2 mm I.D. HP-1 methyl silicone column, 0.11 μm film thickness.

The Hewlett Packard 1050 HPLC system (Waldbronn, Germany) consisted of a quaternary pump, a multiple-wavelength detector, an autosampler with a high-pressure sampling valve (1–100 μl) and a 220 mm × 4.6 mm I.D., 5-μm silica-bonded C_{18} reversed-phase column (Brownlee Spheri 5) operated at room temperature. The retention times (min) and peak heights were measured using Hewlett Packard HPLC ChemStation (DOS Series) software on an IBM compatible 386 computer. The flow-rate was 1.0 ml/min and the UV detector was set at λ = 305 nm. The mobile phase consisted of 40% (v/v) phosphate buffer in acetonitrile. The overall performance of the system was verified periodically by injection of the standards. Quantitation was effected using authentic spikes in blank transfusion blood and the given internal standard in the preparation of a calibration curve. Unknown concentrations were calculated using linear regression.

Procedure

A 1-ml volume of blood (samples, blood standards or blanks) and 85 ng of internal standard (10 μl of a 8.5 μg/ml solution) were added in succession to PTFE-lined screw-top glass test tubes, mixed and allowed to stand for 5 min. Saturated borate buffer (0.3 ml, pH 9.2) was added, followed by 3 ml of *n*-butyl chloride. The resultant mixture was vortex-mixed for 2 min, centrifuged for 10 min at 2140 *g* and the upper organic phase transferred to a clean test tube. The extraction was repeated with a further 3 ml of *n*-butyl chloride. The combined organic layers were evaporated to dryness under nitrogen at 40°C and reconstituted in 100 μl of acetonitrile. A 10–30 μl portion of the reconstituted extracts was subjected to chromatographic analysis.

RESULTS AND DISCUSSION

Gas chromatography

Preliminary work was performed using GC as described by Kennel *et al.* [4] with minor modification. A capillary fused-silica column was used in place of a packed column and, as the target drug contained a chlorine atom, an electron-capture detector was used in place of a nitrogen–phosphorus detector. This may afford a higher degree of selectivity and sensitivity towards a chlorinated drug as opposed to other endogenous substances which may be present in the sample extract.

The chromatogram of zopiclone obtained from a GC–ECD analysis is displayed in Fig. 2a. A major peak (peak 2), eluting slightly before diazepam, corresponded to that given by Kennel *et al.* [4] for zopiclone. However, two additional peaks occur in our chromatogram, one eluting slightly before (peak 1) and the other after (peak 3). Consideration of the molecular mass of zopiclone (*i.e.* molecular mass of 388 *cf.* the diazepam molecular mass of 284) suggests that zopiclone may be peak 3. The implication of this is that the target drug zopiclone may be decomposed under the thermal conditions of the GC analysis to give smaller decomposition products. Subsequent investigation demonstrated that the ratios were dependent on the injector port temperature, the condition of the liner and the injection mode (*i.e.* split or splitless). Furthermore, the ratios were also dependent on the injection solvent, as zopiclone appears to be unstable in nucleophilic solvents such as methanol or ethanol (see Fig. 2). Owing to these problems acetonitrile was chosen as the solvent for the preparation of zopiclone standards.

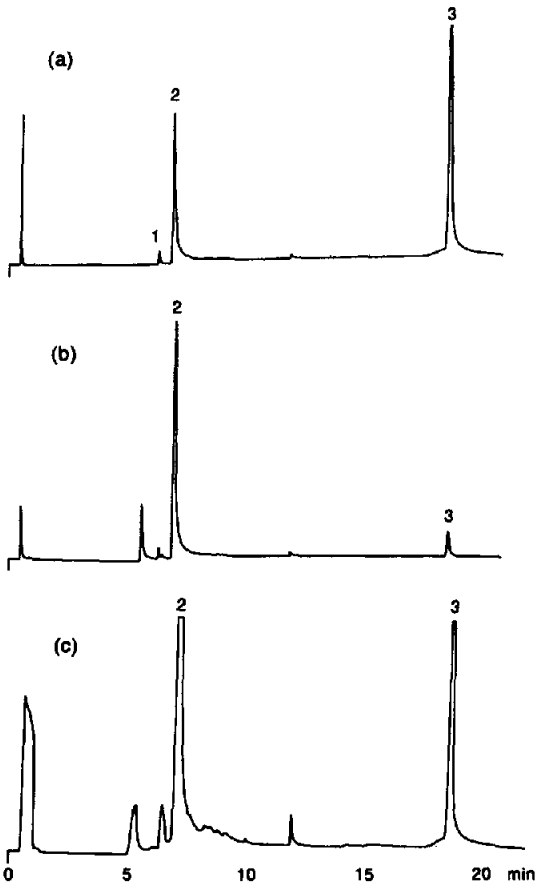


Fig. 2. GC–ECD profile of 35 ng of zopiclone standard in (a) acetonitrile split injection mode (30:1 split ratio), (b) methanol split injection mode (30:1 split ratio) and (c) acetonitrile splitless injection mode. For peak identifications, see text. GC conditions: 15 m × 0.2 mm I.D. DB17 column; hydrogen as carrier gas; 220°C (5 min hold), up 10°C/min to 280°C (11 min hold).

Mass spectrometry

Having established that zopiclone is sensitive to the GC conditions, we then sought to determine unequivocally the identity of peaks 1, 2 and 3. However, the total ion current (TIC) from GC–MS experiments only exhibited two peaks using the EI or PCI mode. The first peak elutes at the retention time which corresponds to peak 2 of the GC trace while the second elutes at the retention time which corresponds to peak 3. Peak 1, a minor peak in the GC–ECD trace, was not detected by the GC–mass-selective detection run, presumably because it was too weak to detect in scan mode.

The mass spectra of peaks 2 and 3 recorded in the EI and PCI modes are presented in Fig. 3. In the EI mode the highest observable ions of the mass spectrum for peak 2 are *m/z* 246 and 248 at an intensity ratio of 3:1. This pattern is typical for a compound with one chlorine substituent. The mass spectrum appears to correspond to an M-OR fragment, probably the result of a disproportionate thermal rearrangement, where OR (the substituent on the pyrrolidone ring) could conceivably be a carbamate, with structure V (Fig. 4) proposed for M. On the other hand, the highest observable ions for peak 3 were *m/z* 245 and 247, again a typical pattern for a compound with one chlorine substituent.

PCI of peak 2, with methane as ionising gas, displays the MH^+ (M + 1) quasimolecular ion cluster at *m/z* 247 and 249 at a ratio of 3:1 together with a weaker ion adduct cluster at *m/z* 275 and 277 ($[M + C_2H_5]^+$) and *m/z* 287 and 289 ($[M + C_3H_5]^+$) consistent with our postulated structure (V). Similarly peak 3 displays the (M + 1) quasimolecular ion cluster at *m/z* 389 and 391 along with a weaker ion adduct cluster at *m/z* 417 and 419 ($[M + C_2H_5]^+$). This mass spectrum is consistent with peak 3 being zopiclone.

In a later publication, Kintz *et al.* [11] extended the GC–NPD of zopiclone work to include the application of MS. Mass spectra of zopiclone recorded in both the EI and the PCI mode were also included in this work. However, there appear to be major differences between the mass spectra of zopiclone recorded by Kintz *et al.* [11] and those recorded by us on our mass spectrometers in both the EI and PCI mode. The base peak in the EI mode in the published mass spectrum was *m/z* 149 with a strong *m/z* 167. There is no indication of chlorine isotope pattern. The base peak of zopiclone recorded by us was at *m/z* 143 with strong ions at *m/z* 245 and 247 at an intensity ratio of 3:1, typically observed for compounds with one chlorine substituent. The EI mass spectrum recorded by Kintz *et al.* [11] for zopiclone is similar to that recorded for diisooctylphthalate, molecular mass 390 [12].

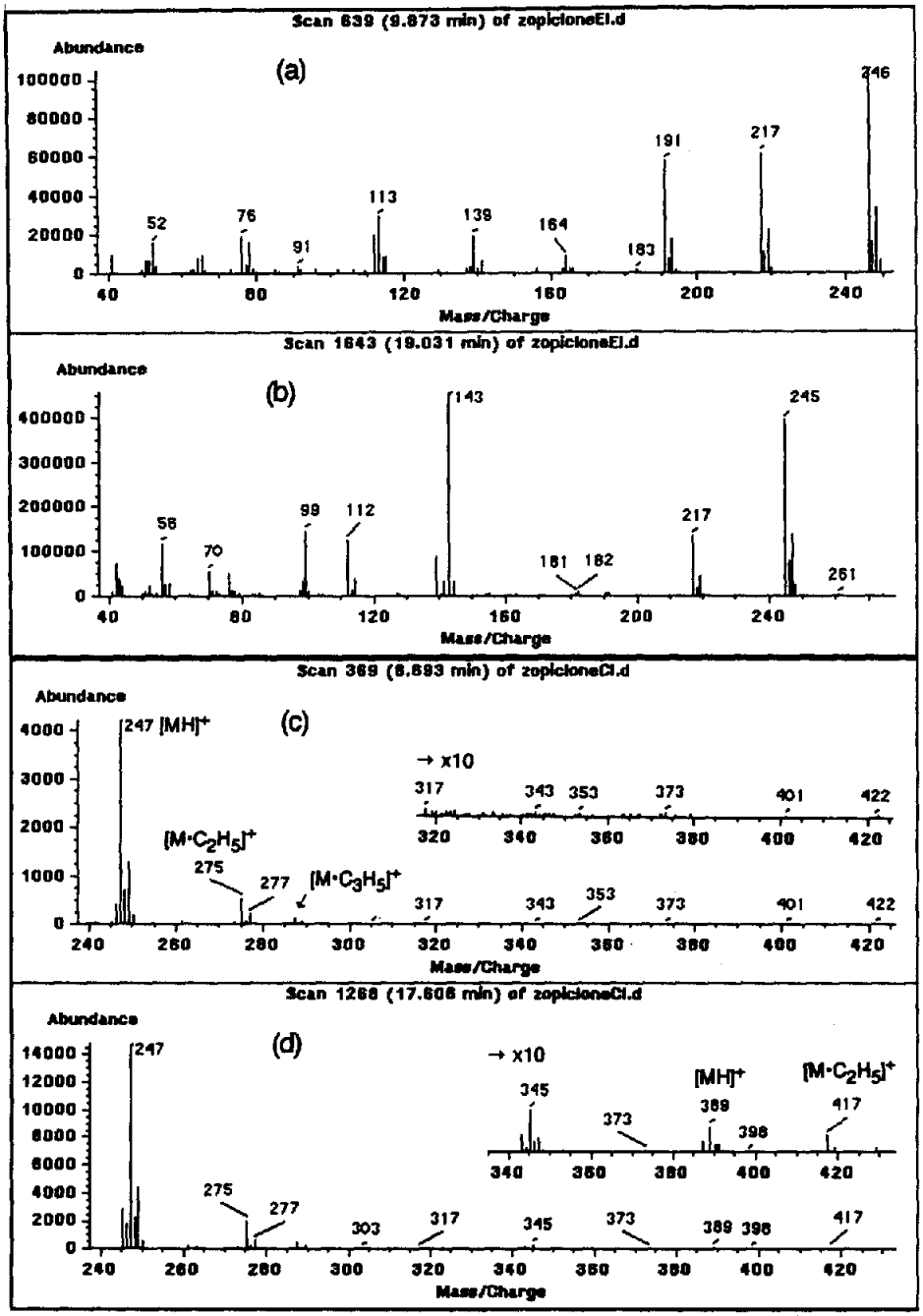
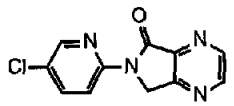


Fig. 3. Mass spectra of peaks 2 and 3 recorded in the EI (a and b) and the PCI (c and d) mode.

HPLC method

Chromatography conditions were optimised to achieve the elution of zopiclone in a reasonable time, to separate it from interferences and to obtain good peak shape using an isocratic system. The addition of a small amount of triethylamine (0.1%) to the mobile phase improved the chromatography markedly and resulted in both rapid elution and a symmetrical peak shape for zopiclone.



V

Fig. 4. Structure of compound V.

The effect of pH, over the range 3.75–7.50, on the chromatography was studied. At pH 7.5 the separation of the internal standard and zopiclone was excellent and the peak shapes symmetrical. Conversely at low pH, the separation was not completely resolved. The working pH of approximately 6.8 was not too high to damage the column but still gave good resolution and excellent peak shape. Higher percentages of triethylamine do not improve the separation between zopiclone and the internal standard.

Method evaluation

Fig. 5 shows the chromatograms obtained from a blood blank, a blood blank spiked at 6.6 ng/ml zopiclone and a patient's blood. The separation of zopiclone and the internal standard, from endogenous substances and the N-oxide metabolite (II) of zopiclone, was good. The separation between zopiclone and its N-desmethyl metabolite (III) was not fully resolved. However, better separation can be achieved by reducing the phosphate buffer in acetonitrile to 32% (v/v) (Fig. 5d). It is noteworthy that these metabolites were not detected in plasma [9] and do not appear to be found in whole blood. The metabolites are present in urine but the amounts vary depending on liver and kidney function (20–30% in healthy subjects, *ca.* 5% in patients with impaired renal function) [9].

The method of internal standardisation was used to determine unknown concentrations of zopiclone. A graph of the peak height of zopiclone relative to that of the internal standard against drug concentration was linear over the ranges 6.6–198 and 250–2000 ng/ml with a correlation coefficient of 0.99 in both cases. The recoveries were excellent (mean 92%) for both zopiclone and the internal standard. Low concentrations gave lower recoveries as expected (see Table I). The precision and accuracy of the method are presented in Table II.

TABLE I

EFFICIENCY OF EXTRACTION RECOVERY

Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%)	*n*
9.9	85.4	10	6
66.0	100.2	1.2	6

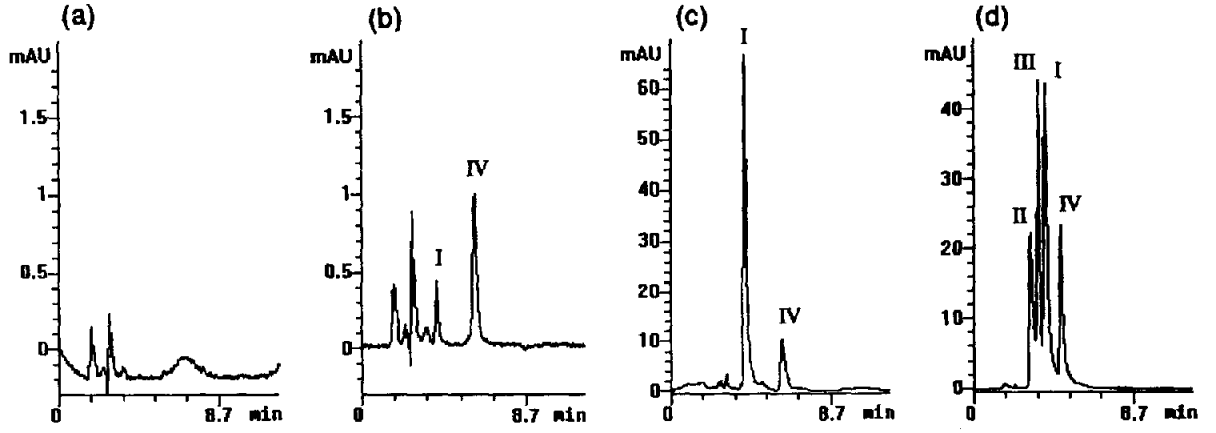


Fig. 5. HPLC of (a) blank blood, (b) 6.6 ng/ml zopiclone in blood, (c) patient's blood and (d) zopiclone, its metabolites and internal standard in a mixture of 32% (v/v) phosphate buffer in acetonitrile. Peaks: I = zopiclone; II = N-oxide metabolite of zopiclone; III = N-desmethyl metabolite of zopiclone; IV = internal standard.

TABLE II

PRECISION AND ACCURACY OF THE METHOD

Precision type	Concentration added (ng/ml)	Concentration found (ng/ml)	n	Coefficient of variation (%)	Relative error (%)
Intra-assay	9.9	10.3	6	10	+4
Within-day	66.0	63.3	6	8	−4.1
Inter-assay	9.9	9.8	6	3	−1.0
Day-to-day	66.0	64.7	6	5	−2.0

The sensitivity of a method is often expressed as the limit of detection (LOD). The LOD for spectrometric analysis is defined as the standard deviation of the baseline noise [13]. The measurement of the baseline noise can be ambiguous and is useful only if there is no interference from the matrix. For example, when blood samples are analysed, there are usually peaks from endogenous compounds present, the number and intensity varying between samples. This results in poor repeatability at amounts near the LOD.

A more useful way to describe the sensitivity could be to calculate both the instrumental limit of detection (ILOD) and the limit of quantitation (LOQ) [13]. The ILOD is the amount of an analyte giving a peak height three times the standard deviation of the baseline noise without any matrix interference, whereas the LOQ is the concentration of an analyte in the matrix that could be determined with a reasonable precision under the given analytical procedure. In our case the ILOD for zopiclone was 1 ng and the LOQ was approximately 4.0 ng/ml for a 1.0-ml blood sample.

TABLE III

POST-MORTEM BLOOD LEVELS OF ZOPICLONE FOUND

Case No.	Zopiclone level (μg/ml)	Comments[a]
1	0.62	Trichloroethanol (F); took thirty 7.5-mg zopiclone tablets 24 h before death
2	1.16	Dothiepin (F); procyclidine (F)
3	0.40	Temazepam (Th)
4	N.D.	Died 24 h after taking one 7.5-mg zopiclone tablet
5	1.70	Amylobarbitone (F); took 60–100 7.5-mg zopiclone tablets

[a] Th and F indicate therapeutic and fatal levels, respectively.

Post-mortem blood levels

Therapeutic plasma levels in five subjects receiving 7.5 mg of zopiclone ranged from 18.4 to 35.2 ng/ml at 9 h post-dosage. The level of zopiclone was not detectable at 24 h post-dosage [8]. In five subjects receiving 15 mg per dose of zopiclone, the plasma levels ranged from 25.8 to 68.1 ng/ml at 9 h, and were undetectable to 15.9 ng/ml at 24 h post-dosage [8].

The zopiclone levels found in five post-mortem examinations are indicated in Table III. To our knowledge, no therapeutic whole blood levels for zopiclone have been reported. In case 1 the deceased took thirty 7.5-mg Imovane tablets approximately 24 h before death while in case 4 the deceased took his normal 7.5-mg tablet and died 24 h later of unrelated causes. In cases 2 and 3 the amount of zopiclone taken is unknown. It seems that 24 h after a 7.5-mg dose of zopiclone (compare case 4 to plasma levels) blood levels are similar to plasma levels. In all four overdose cases, the zopiclone levels are significantly higher than therapeutic plasma levels. However, in the cases 1, 2 and 5, other drugs were also found at fatal levels. In case 3 the cause of death was determined as overdose of zopiclone by the coroner's court. There is, however, no clear evidence as to what a fatal level of zopiclone may be.

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