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Screening for forensically relevant benzodiazepines in human hair by gas chromatography–negative ion chemical ionization-mass spectrometry

V. Cirimele*, P. Kintz, B. Ludes

Institut de Médecine Légale, 11, rue Humann, 67085 Strasbourg Cédex, France

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Abstract

A procedure is presented for the detection in human hair of forensically relevant benzodiazepines, i.e. nordiazepam, oxazepam, bromazepam, diazepam, lorazepam, flunitrazepam, alprazolam and triazolam. The method involves decontamination of hair with methylene chloride, pulverization in a ball mill, incubation of 50 mg powdered hair in Soerensen buffer (pH 7.6) in the presence of prazepam-d_5 used as internal standard, liquid–liquid extraction with diethyl ether–chloroform (80:20, v/v) and gas chromatography–mass spectrometry using negative chemical ionization after derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane. The limits of detection for all benzodiazepines ranged from 1 to 20 pg/mg using a 50-mg hair sample. Coefficients of variation and extraction recoveries, ranging from 7.4 to 25.4% and 47.6 to 90%, respectively, were found suitable for a screening procedure. One hundred and fifteen samples were submitted to this screening procedure, and specimens tested positive for nordiazepam (0.20–18.87 ng/mg, $n=42$) and its major metabolite oxazepam (0.10–0.50 ng/mg, $n=14$), flunitrazepam (19–148 pg/mg, $n=31$), lorazepam (31–49 pg/mg, $n=4$) and alprazolam (0.3–1.24 ng/mg, $n=2$). Bromazepam, diazepam and triazolam were not detected.

Keywords: Hair analysis; Benzodiazepines; Nordiazepam; Oxazepam; Bromazepam; Diazepam; Lorazepam; Flunitrazepam; Alprazolam; Triazolam

1. Introduction

Benzodiazepines, alone or in conjunction with alcohol, morphinomimetics, antidepressants, sedatives or neuroleptics, are frequently involved in traffic accidents [1–3], or often misused by polydrug users.

Generally, drug testing is based on blood or urine measurement, but blood concentration may only reflect dosage at the time of sampling. To assess compliance over longer periods, it would be an advantage to sample a readily accessible tissue, which provided a more permanent marker of drug intake. To obtain data on individual past history of dosage, drug analysis in hair was recently suggested as being useful [4].

In the past decade, particular attention has been given to the use of hair for the detection of drugs of abuse such as cocaine, heroin, amphetamines and cannabis. However, the detection in human hair of

*Corresponding author.

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benzodiazepines, the most abused pharmaceutical drug in the world, appears not to be well documented. One paper reports their detection by radioimmunoassay [5]. Diazepam was readily detected, but alprazolam and lorazepam were not found in subjects receiving therapeutic dosages. In 1995, Couper et al. [6] established a procedure for the detection of psychotic drugs in hair by high-performance liquid chromatography (HPLC), but diazepam, nitrazepam and oxazepam were not detected in hair samples from subjects under treatment. HPLC was certainly inadequate for the detection of benzodiazepines in human hair due to a lack of sensitivity.

More recently, the identification of benzodiazepines has been a major focus in hair testing [3,7–11]. In these papers, gas chromatography coupled to mass spectrometry (GC–MS) was always used because of its sensitivity and specificity, in electronic impact (EI) or in negative chemical ionization (NCI) modes of detection, with or without derivatization step (*N,O*-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilan or heptafluorobutyric anhydride).

Nordiazepam and its major metabolite, oxazepam, were identified by Kintz et al. [7] using GC–NCI-MS after derivatization by silylation. In 1996, Gaillard and Pépin [8] tested a hair sample positive for alprazolam by GC–EI-MS. This compound was also identified by Höld et al. [10] in hair of treated rats using GC–NCI-MS. In 1997, we established an analytical procedure for the detection of flunitrazepam and its major metabolite, 7-amino-flunitrazepam, using GC–NCI-MS after derivatization with heptafluorobutyric anhydride [11]. Finally, lorazepam was detected in human hair using GC–NCI-MS after silylation [3]. All these procedures were able to determine a chronic consumption of one or two benzodiazepines only. However, Yegles et al. [9] reported the identification of several benzodiazepines in hair specimens, without validation of the analytical procedure.

The purpose of this work was to establish a screening procedure for the simultaneous detection in human hair of eight forensically relevant benzodiazepines: nordiazepam, oxazepam, bromazepam, diazepam, lorazepam, flunitrazepam, alprazolam and triazolam.

2. Experimental

2.1. Chemicals

Methylene chloride (Carlo Erba, Milan, Italy), diethyl ether (Prolabo, Fontenay, France) and chloroform (Carlo Erba) were HPLC grade.

Sodium hydroxide (Merck, Darmstadt, Germany) was analytical grade.

Standard solutions of nordiazepam (Winsthrop Laboratories), oxazepam (Wyeth Laboratories), bromazepam (Roche Laboratories), diazepam (Roche Laboratories), lorazepam (Wyeth Laboratories), flunitrazepam (Roche Laboratories), alprazolam (Upjohn Laboratories) and triazolam (Upjohn Laboratories) were prepared in methanol at concentrations of 0.1 and 1 mg/l.

Deuterated prazepam (Praz-d_5) was purchased from Promochem (Molsheim, France).

N,O-Bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA–TMCS) was purchased from Pierce (Rockford, IL, USA).

Soerensen buffer was prepared by adding 38.8 ml KH_2PO_4 buffer (9.07 g/l) to 61.2 ml Na_2HPO_4 (11.87 g/l). The pH was adjusted to a value of 7.6 with sodium hydroxide.

2.2. Materials for examination

Hair samples were obtained from subjects who had died from fatal heroin overdosage and from living persons.

Strands of hair were cut closely as possible to the skin, in the vertex posterior region, dried and stored in tubes at room temperature.

Before analysis, samples (approximately 100 mg) were twice decontaminated in 5 ml of methylene chloride, for 2 min, at room temperature and pulverized in a ball mill (Retsch MM2 type, Haan, Germany).

2.3. Sample extraction

Fifty mg of powdered hair were incubated in 1 ml of Soerensen buffer (pH 7.6), for 2 h at 40°C, in the presence of 25 ng Praz-d_5 used as internal standard. Then, the homogenate was directly extracted with 5 ml of diethyl ether–chloroform (80:20, v/v). After

horizontal agitation (20 min at 100 cycles/min) and centrifugation (15 min at 2200 *g*), the organic phase was removed and evaporated to dryness. The residue was derivatized using 35 μl BSTFA–TMCS, for 20 min at 70°C.

2.4. Gas chromatography–mass spectrometry

A 1.5-μl portion of the derivatized extract was injected into the column of a Hewlett-Packard (5890) gas chromatograph via a Hewlett-Packard (7673) autosampler. The flow-rate of carrier gas (helium, purity grade N55) through the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m×0.25 mm I.D.×0.25 μm film thickness) was 1.0 ml/min.

Injector temperature was 250°C and splitless injection was employed with a split valve off-time of 1.0 min. The column oven temperature was programmed to rise from an initial temperature of 60°C, kept for 1 min, to 295°C at 30°C/min and maintained at 295°C for the final 6 min.

The detector was a Hewlett-Packard (5989 B) Engine operated in negative chemical ionization (NCI) detection mode.

The ion source and quadrupole temperature were 200 and 100°C, respectively. The electron multiplier voltage was set at +400 V above the NCI-tune voltage. Methane (purity grade N55) was used as reactant gas at an apparent pressure of 1.3 Torr in the ion source.

Mass spectra were recorded in single ion monitoring (SIM) mode. Analytes were identified on the basis of comparison of retention indices and the abundance of two ions with those of the methanolic solutions. Concentrations were evaluated after determination of the response factor (R_F) for each benzodiazepine against Praz-d_5.

For nordiazepam, oxazepam, bromazepam, diazepam, triazolam and alprazolam, standard calibration curves were obtained by adding 0.5 (0.01 ng/mg), 2.5 (0.05 ng/mg), 5 (0.1 ng/mg), 12.5 (0.25 ng/mg), 25 (0.5 ng/mg), 50 (1 ng/mg), 500 (10 ng/mg) and 1000 ng (20 ng/mg) of each benzodiazepine prepared in methanol (0.1 and 1 mg/l) to 50 mg of pulverized blank control hair (obtained from laboratory personnel and previously tested to be free of benzodiazepines).

For flunitrazepam and lorazepam, standard calibration curves were obtained by adding 0.5 (0.01 ng/mg), 2.5 (0.05 ng/mg), 5 (0.1 ng/mg) and 12.5 ng (0.25 ng/mg) to 50 mg of pulverized blank control hair.

Recovery and within-run precision for nordiazepam, oxazepam, diazepam, bromazepam, triazolam and alprazolam, were determined by adding 50 ng of each benzodiazepine to 50 mg of powdered blank control, corresponding to a final concentration of 1 ng/mg hair.

Recovery and within-run precision for flunitrazepam and lorazepam were determined by adding 5 ng of each benzodiazepine to 50 mg of powdered blank control, corresponding to a final concentration of 0.1 ng/mg hair.

3. Results and discussion

Table 1 shows the ions monitored for each benzodiazepine and for the deuterated internal standard Praz-d_5, the retention times (t_R) and the response factors (R_F).

Mass spectra obtained for nordiazepam, oxazepam, bromazepam, diazepam, lorazepam, flunitrazepam, alprazolam, triazolam and Praz-d_5 using NCI detection are shown in Figs. 1–9.

Under the chromatographic conditions used, there was no interference with the target benzodiazepines or Praz-d_5 by any extractable endogenous materials present in hair.

Correlation coefficients, within run precision (n=

Table 1
Selected ions (m/z), retention times (t_R) and response factors (R_F) for each benzodiazepine and Praz-d_5

Benzodiazepines	t_R (min)	m/z	R_F
Nordiazepam (TMS)	9.10	234–342	3.65
Oxazepam (2TMS)	9.36	268–357	0.17
Bromazepam (TMS)	9.65	389–317	28.96
Diazepam	9.75	284–268	0.63
Lorazepam (2TMS)	9.79	302–464	0.28
Flunitrazepam	10.48	313–297	13.16
Alprazolam	12.56	308–310	0.61
Triazolam	13.47	306–342	1.41
Prazepam-d_5	10.59	329	–

The underlined ions were used for quantification. TMS= trimethylsilyl derivative group.

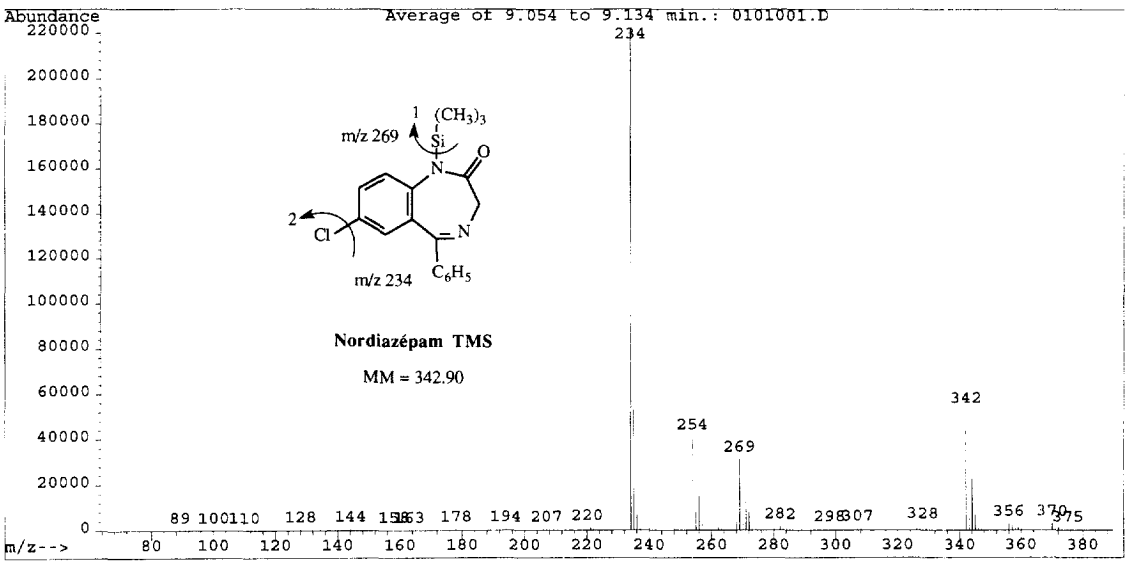

Fig. 1. Mass spectra of nordiazepam derivative (TMS) using NCI detection with methane.

8), extraction recoveries ($n=8$) and limits of detection are presented in Table 2.

The limits of detection (calculated for a signal-to-noise ratio of 3), using a 50-mg hair sample, were in the pg/mg range. GC–MS represents the state of the art for hair analysis. Benzodiazepines possess halogen groups (electronegative functional groups) located on aromatic rings with high negative density

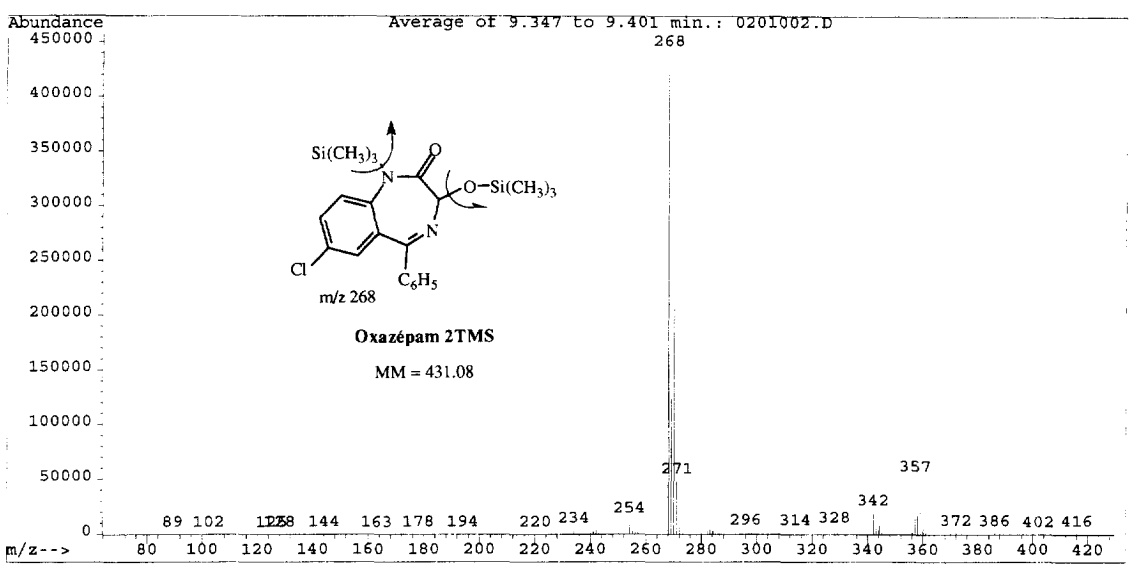

Fig. 2. Mass spectra of oxazepam derivative (2TMS) using NCI detection with methane.

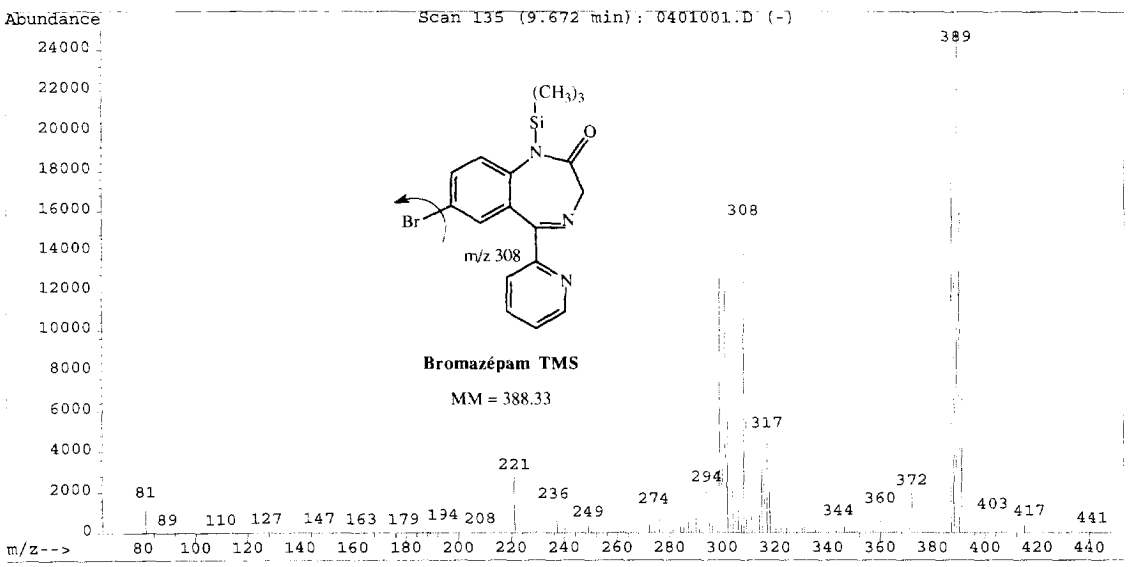

Fig. 3. Mass spectra of bromazepam derivative (TMS) using NCI detection with methane.

that will give more stability to the anions formed in the ion source. GC–NCI-MS seems to be the technique of choice to detect low benzodiazepine concentrations in hair.

The within-run precision of the method was assessed by testing eight replicate samples through the entire procedure in one analysis day. The values ranged from 7.4 to 25.4% and were found acceptable

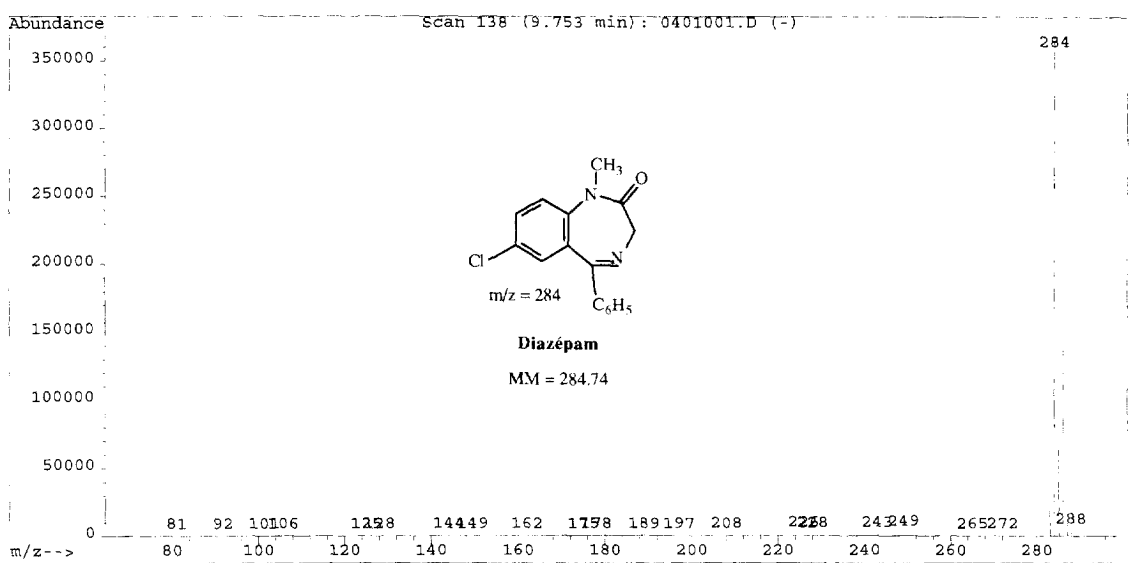

Fig. 4. Mass spectra of diazepam using NCI detection with methane.

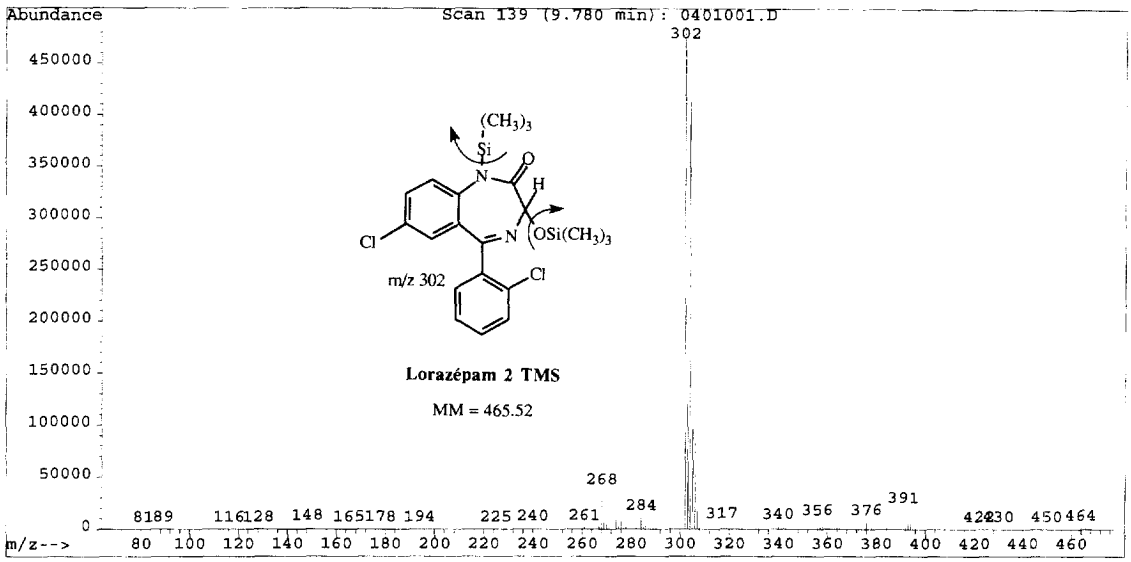

Fig. 5. Mass spectra of lorazepam derivative (2TMS) using NCI detection with methane.

for a screening procedure. However, one cannot exclude that the use of a deuterated analog of each benzodiazepine would enhance the performance of the method. Due to high cost, this was not retained.

4. Applications

One hundred and fifteen hair samples, obtained from addicts who had died from fatal heroin over-

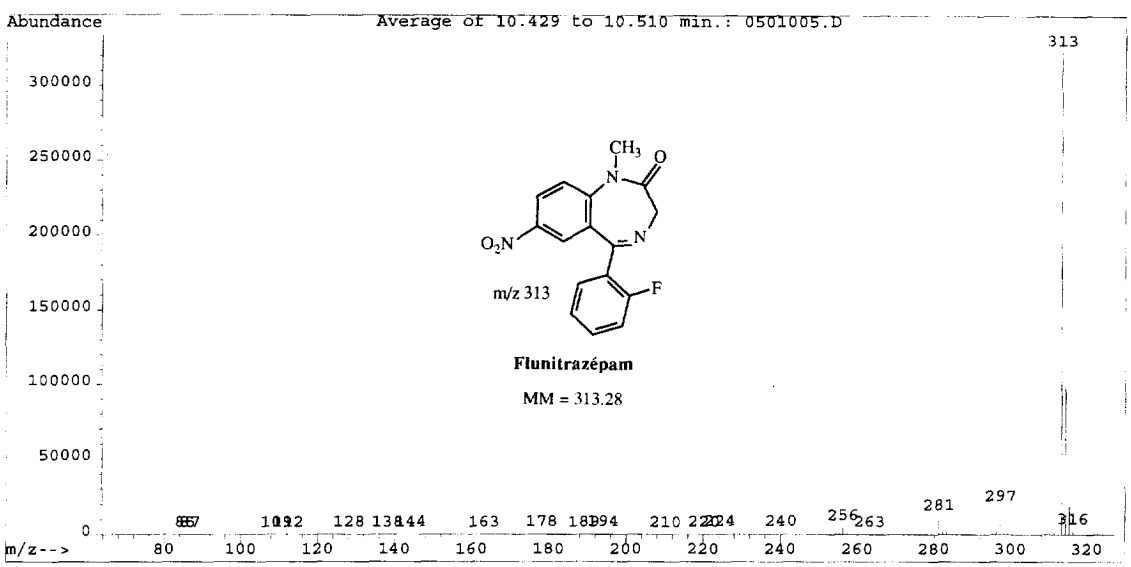

Fig. 6. Mass spectra of flunitrazepam using NCI detection with methane.

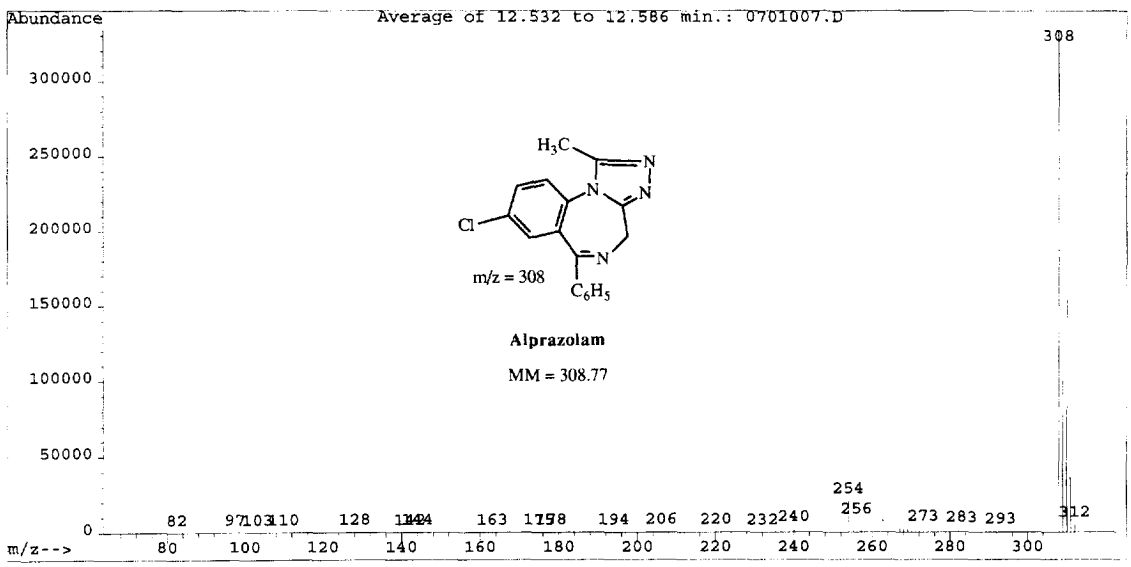

Fig. 7. Mass spectra of alprazolam using NCI detection with methane.

dose or from living persons, were tested for benzodiazepines by the established procedure. Results are presented in Table 3.

Forty-two specimens were positive for nordiazepam and 14 for oxazepam, with concentrations ranging from 0.20 to 18.87 ng/mg for the parent nordiazepam and from 0.10 to 0.50 ng/mg for the metabolite. Our concentrations for nordiazepam are

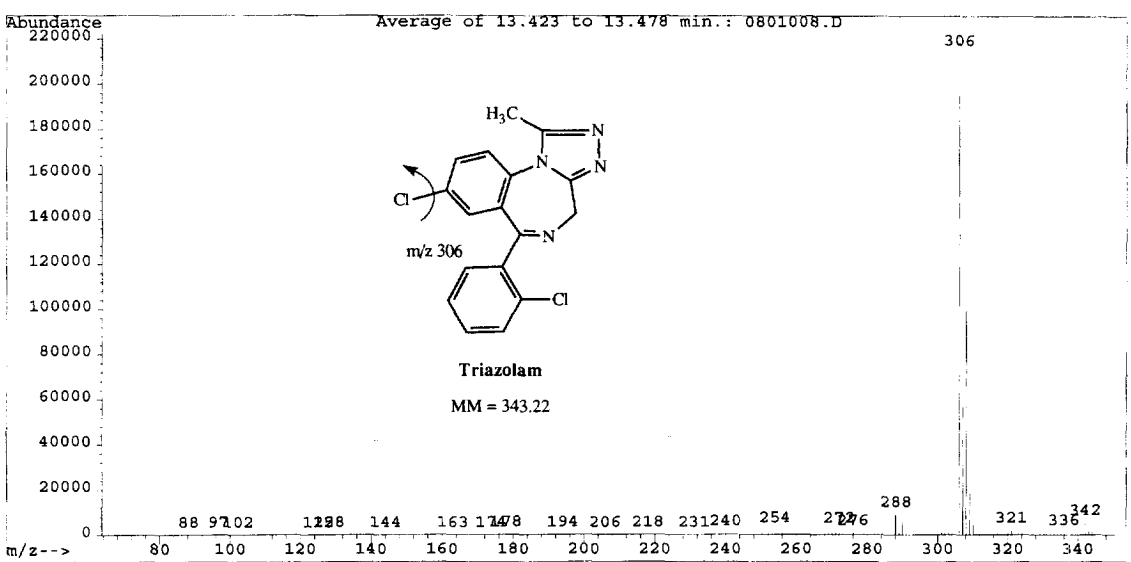

Fig. 8. Mass spectra of triazolam using NCI detection with methane.

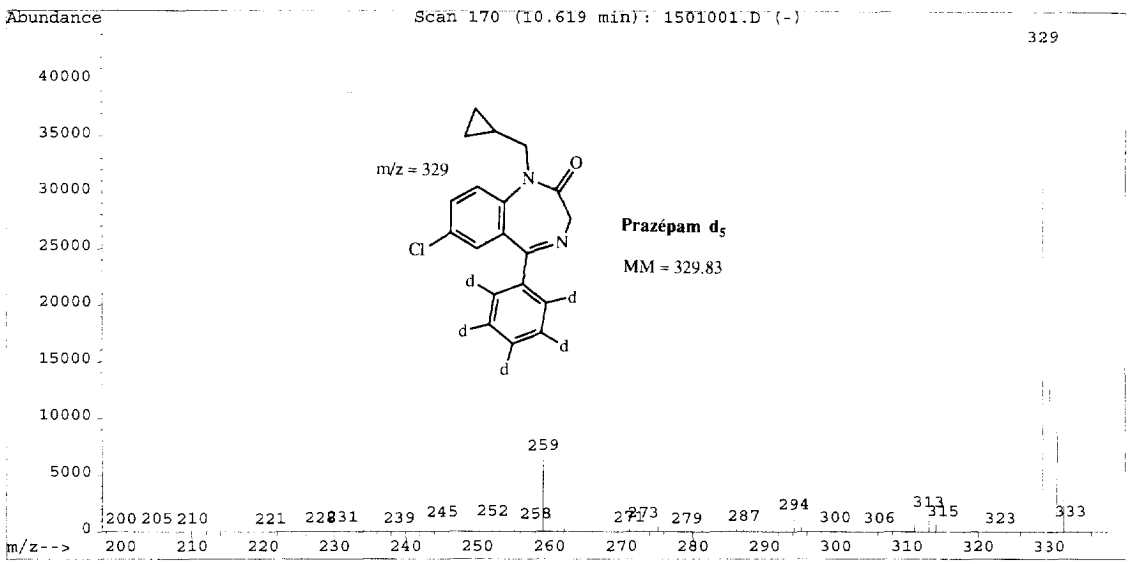

Fig. 9. Mass spectra of Praz-d_5 using NCI detection with methane.

Table 2
Correlation coefficients, within-run precision ($n=8$), extraction recoveries ($n=8$) and limits of detection for each benzodiazepine

Benzodiazepines	R (linearity ranges, ng/mg)	Within-run precision (C.V., %)	Limit of detection (pg/mg)	Recovery (%)
Nordiazepam	0.995 (0.01–20.00)	9.3	4	77
Oxazepam	0.993 (0.01–20.00)	13.6	1	69
Bromazepam	0.970 (0.01–20.00)	25.4	20	50
Diazepam	0.930 (0.01–20.00)	19.8	11	48
Lorazepam	0.980 (0.01–0.25)	7.4	1	84
Flunitrazepam	1.000 (0.01–0.25)	15.5	15	90
Alprazolam	0.998 (0.01–20.00)	15.7	1	51
Triazolam	0.996 (0.01–20.00)	16.8	1	50

higher than those observed by Yegles et al. [9], but oxazepam concentrations are lower than those found by the same authors. It was observed, as is generally the case for the other drugs like heroin or cocaine, that the parent drug is present in hair in higher concentrations than the metabolite. In contrast,

Table 3
Benzodiazepine concentrations in 115 hair specimens

Benzodiazepines	No. of positive samples	Concentrations
Nordiazepam	42	0.20–18.87 ng/mg
Oxazepam	14	0.10–0.50 ng/mg
Flunitrazepam	31	19–148 pg/mg
Lorazepam	4	31–49 pg/mg
Alprazolam	2	0.30–1.24 ng/mg

Yegles et al. [9] observed quite similar concentrations for both nordiazepam and oxazepam. However, as oxazepam is sold as a pharmaceutical compound, one cannot exclude that the measured oxazepam concentrations represent the sum of the metabolite of nordiazepam and its own consumption.

To get sedation, a chronic administration of benzodiazepine to a 4-year-old child was suspected. After extraction of his hair by the established procedure, nordiazepam was detected at a concentration 0.29 ng/mg (Fig. 10).

Flunitrazepam was detected in 31 hair samples with concentrations ranging from 19 to 148 pg/mg. These concentrations are largely lower than those reported by Yegles et al. [9] (0.02–9.51 ng/mg, $n=8$).

Finally, alprazolam was determined in two specimens with concentrations of 0.3 and 1.24 ng/mg. Fig. 11 shows the SIM chromatogram (m/z 308) obtained by GC–NCI-MS after extraction of a hair strand collected from a subject treated with alprazolam for chronic insomnia. The concentration of alprazolam was 1.24 ng/mg.

Bromazepam, triazolam and diazepam were never

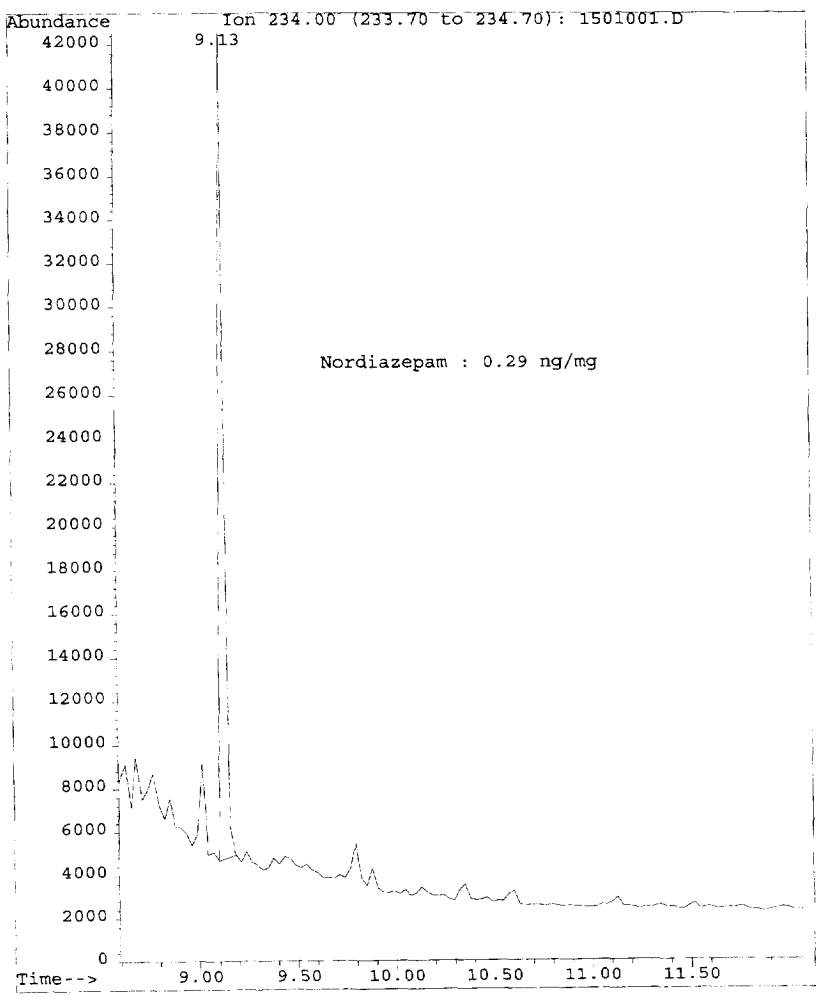

Fig. 10. Chromatogram in SIM mode (m/z 234) of a hair sample positive for nordiazepam. The concentration determined at the retention time 9.13 min was 0.29 ng/mg of hair.

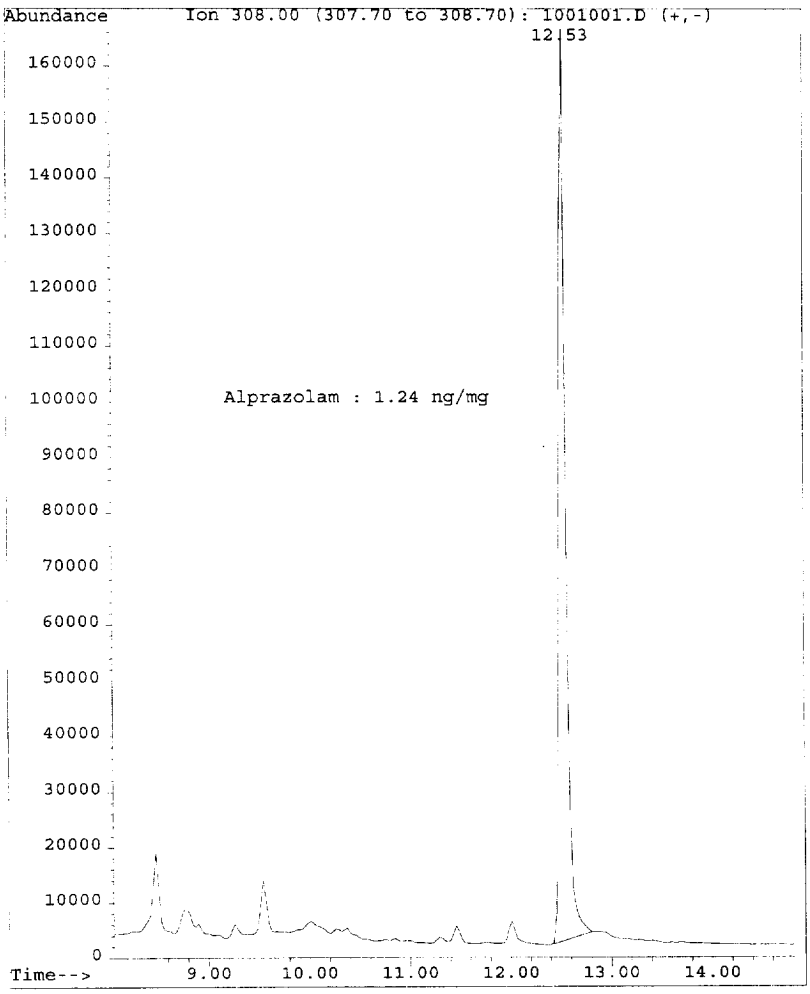

Fig. 11. Chromatogram in SIM mode (m/z 308) of a hair sample positive for alprazolam. The concentration determined at the retention time 12.53 min was 1.24 ng/mg of hair.

detected in the hair samples tested. This can be explained by the fact that the studied population did not use these drugs.

5. Conclusions

In the future, more experiments are necessary to enhance the number of positive cases in order to evaluate accurately the concentration ranges for each benzodiazepine in human hair. Efforts must be made to extend hair analysis to other benzodiazepines.

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