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Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography–mass spectrometry/mass spectrometry

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Abstract

A procedure is presented for the screening of 16 benzodiazepines and hypnotics in human hair by LC–MS/MS (alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, bromazepam, clobazam, diazepam, lorazepam, lormetazepam, midazolam, nordiazepam, oxazepam, temazepam, tetrazepam, triazolam, zaleplon and zolpidem). The method involves decontamination of hair with methylene chloride, hair cut into small pieces, incubation of 20 mg in phosphate buffer (pH 8.4) in the presence of 1 ng diazepam-d_5 used as internal standard, liquid–liquid extraction with diethyl ether/methylene chloride (10/90) and separation using liquid chromatography–tandem mass spectrometry. The limits of quantification for all benzodiazepines and hypnotics range from 0.5 to 5 pg/mg using a 20-mg hair sample. Linearity is observed from the limit of quantification of each compound to 200 pg/mg ($r^2 > 0.99$). Coefficients of variation measured on six points and at two concentrations (10 and 50 pg/mg) range from 5 to 20% for all drugs but one. Extraction recovery, measured at the two same concentrations range from 32 to 76%. These results were found suitable to screen for 16 benzodiazepines in hair and detect them at very low concentrations, making this method suitable to monitor single dose.

Keywords: Benzodiazepines; Hypnotics; Hair; LC–MS/MS

1. Introduction

Hair testing is widely employed to evaluate drug use. In the past decades, particular attention has been given to the use of hair for the detection of drugs of abuse, on chronic bases. However, the detection in human hair of benzodiazepines, the most abused pharmaceutical drugs in the world, appears not to be well documented. One old paper reports their detection by radioimmunoassay [1]. Diazepam was readily detected, but alprazolam and lorazepam were not found in subjects receiving therapeutic dosages. In 1995, Couper et al. [2] established a procedure for the detection of psychotic drugs in hair by HPLC, but diazepam, nitrazepam and oxazepam were not detected in hair samples from subjects under treatment.

Nordiazepam and its major metabolite, oxazepam, were identified by Kintz et al. [3] using GC–NCI–MS after derivatization by silylation. In 1996, Gaillard and Pépin [4] identified alprazolam by GC–EI–MS. In 1997, Cirimele et al. [5] published two different analytical procedures for the detection of flunitrazepam and its major metabolite, 7-amino-flunitrazepam, using GC–NCI–MS after derivatization with heptafluorobutyric anhydride and for the detection of lorazepam after silylation [6]. In 2000, Negrusz et al. [7] presented an analytical procedure for the detection of clonazepam and 7-amino-clonazepam, using GC–NCI–MS after derivatization with heptafluorobutyric anhydride.

All these procedures were able to test for one or two benzodiazepines only.

In 1997, Cirimele et al. [8] established the first screening procedure for the simultaneous detection in human hair of eight forensically relevant benzodiazepines using

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GC–NCI–MS after silylation. Limits of detection were in the range 1–20 pg/mg, using a 50 mg sample.

El Mahjoub and Staub [9] published in 2001 a screening method for five benzodiazepines in human hair by on-line high-performance liquid chromatography using a restricted access extraction column, with limits of detection of about 200 pg/mg.

More recently, Kronstrand et al. [10] established an analytical procedure for the screening of seven benzodiazepines and metabolites, using LC–MS–MS, in hair of psychiatric patients. Typical limits of quantification were 25–125 pg/mg.

The purpose of our work was to establish a screening method for the simultaneous detection of 16 benzodiazepines and hypnotics present in hair at pg/mg level.

2. Materials and methods

2.1. Specimens

Blank hair samples (blond) were obtained from laboratory personnel, through verbal consent.

2.2. Chemicals and reagents

Alprazolam, 7-amino-clonazepam, 7-amino-flunitrazepam, bromazepam, clobazam, diazepam, lorazepam, lormetazepam, midazolam, nordiazepam, oxazepam, temazepam, triazolam, zolpidem and diazepam-d_5 were obtained from Promochem (Molsheim, France) and tetrazepam from Euromedex (Souffelweyersheim, France).

Table 1
MRM transitions for the detection of 16 benzodiazepines and hypnotics and IS by LC–MS/MS

Compound	Retention time (min)	Parent ion (*m*/*z*)	Daughter ions (*m*/*z*)	Cone (V)	Collision energy (eV)
Alprazolam	10.9	309.1	281.3	45	40
			274.2	45	26
7-Aminoclonazepam	7.5	286.1	222.2	40	25
			250.2	40	20
7-Aminoflunitrazepam	8.2	284.2	135.1	40	28
			227.2	40	25
Bromazepam	9.6	316.0	182.3	35	30
			209.3	35	25
Clobazam	11.7	301.1	224.2	30	33
			259.1	30	20
Diazepam	12.0	285.2	154.2	40	25
			193.3	40	30
Lorazepam	11.0	321.1	229.1	30	27
			275.1	30	22
Lormetazepam	11.7	335.1	177.1	28	40
			289.1	28	20
Midazolam	9.3	326.1	244.1	44	25
			291.2	44	28
Nordiazepam	11.1	271.2	140.1	40	25
			165.1	40	28
Oxazepam	10.8	269.1	163.1	45	32
			241.2	45	20
Temazepam	11.5	301.1	283.1	30	40
			255.2	30	20
Tetrazepam	11.1	289.2	225.2	40	26
			253.2	40	22
Triazolam	11.0	343.1	308.1	45	26
			315.1	45	27
Zaleplon	10.4	306.2	236.2	40	28
			264.2	40	20
Zolpidem	8.4	308.2	235.3	40	35
			263.2	40	26
Diazepam-d_5	12.0	290.2	154.1	40	30
			198.3	40	30

The transition for quantification is underlined.

Zaleplon was provided by Wyeth laboratories (Paris, France). Formic acid was from Prolabo (Paris, France). Acetonitrile, methanol and methylene chloride HPLC grade were obtained from Merck (Darmstadt, Germany). Diethyl ether was from SDS (Peypin, France). Chemicals for the saturated phosphate buffer—$(NH_4)_2HPO_4$ adjusted to pH 8.4 with hydrochloric acid 1N—were purchased from Merck (Darmstadt, Germany).

Drugs and diazepam-d_5 were prepared in methanol after appropriate dilutions and were stored at +4 °C.

2.3. Extraction

After decontamination of the hair strand with methylene chloride (2 × 5 ml for 2 min), hair was segmented, if necessary, and cut into small pieces. About 20 mg were incubated overnight in 1 ml of phosphate buffer pH 8.4, in the presence of 1 ng of diazepam-d_5 used as internal standard (IS), and extracted by 5 ml methylene chloride/diethylether (90/10, v/v). After horizontal agitation (15 min) and centrifugation (10,000 × *g* for 15 min), the organic phase was collected and evaporated to dryness using a SpeedVac®. The residue was reconstituted by adding 60 μl of methanol.

2.4. LC–MS/MS procedure

A 10 μl aliquot of the extract was injected onto the column (XTerra MS C18 3.5 μm, 100 mm × 2.1 mm i.d.), protected by a 1 mm C18 frit. Each 20 min chromatographic run was carried out with a gradient (5% acetonitrile–95% formic acid 0.1% to a ratio 80–20% at 10 min), at a flow rate of 200 μl/min. The HPLC system was a Waters Alliance 2695.

Detection was carried out by a Micromass Quattro Micro tandem mass spectrometer equipped with an ionspray atmospheric pressure interface. The instrument was operated in the positive ionization mode. Best results were obtained with a capillary voltage of 1 kV, source block temperature of 120 °C and desolvatation gas (nitrogen) heated to 350 °C and delivered at 550 l/h. Collison cell pressure was 4 mbar of argon.

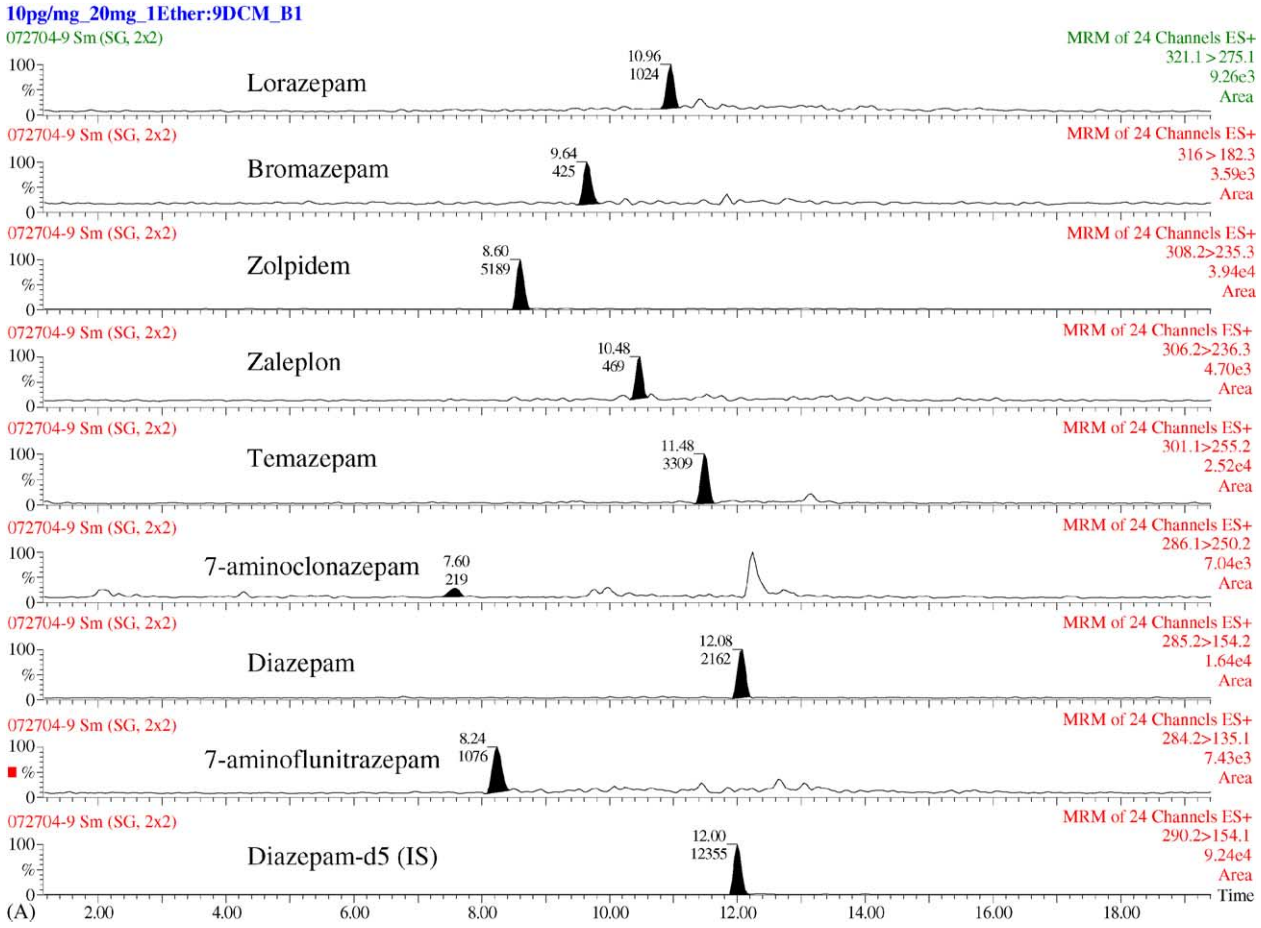

Fig. 1. Chromatogram of a blank hair (20 mg) spiked at a final concentration of 10 pg/mg with, from the top to the bottom, the quantification ions of (A) lorazepam, bromazepam, zolpidem, zaleplon, temazepam, 7-amino-clonazepam, diazepam, 7-amino-flunitrazepam and the IS (50 pg/mg) and (B) triazolam, lormetazepam, midazolam, alprazolam, clobazam, tetrazepam, nordiazepam, oxazepam and the IS (50 pg/mg).

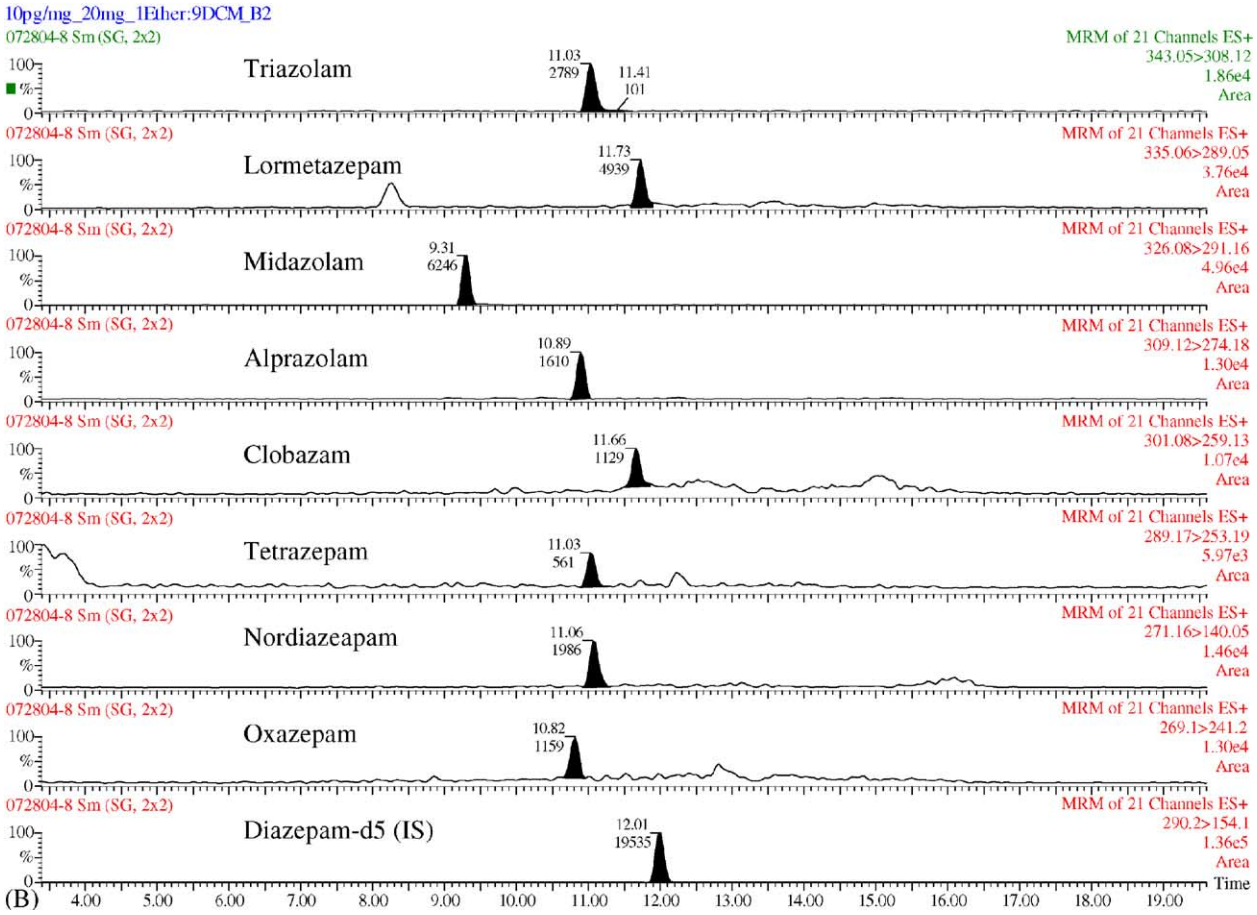

Fig. 1. (*Continued*.)

Table 2
Validation parameters for the different compounds

Compound	Linearity	Extraction recovery (%)	Within batch precision (C.V., $n = 6$)	Within batch accuracy (%Bias, $n = 6$)	LOQ (pg/mg)
Alprazolam	1–200 pg/mg, $r^2 = 0.9999$	59.2 (10 pg/mg) 69.5 (50 pg/mg)	12.9 (10 pg/mg) 9.9 (50 pg/mg)	10.5 (10pg/mg) 3.9 (50 pg/mg)	1
7-Aminoclonazepam	5–200 pg/mg, $r^2 = 0.9976$	38.5 (10 pg/mg) 46.1 (50 pg/mg)	28.6 (10 pg/mg) 14.9 (50 pg/mg)	0.8 (10 pg/mg) 12.8 (50 pg/mg)	5
7-Aminoflunitrazepam	2–200 pg/mg, $r^2 = 0.9972$	56.7 (10 pg/mg) 48.4 (50 pg/mg)	12.1 (10 pg/mg) 19.7 (50 pg/mg)	3.0 (10 pg/mg) 0.9 (50 pg/mg)	2
Bromazepam	2–200 pg/mg, $r^2 = 0.9977$	51.7 (10 pg/mg) 45.3 (50 pg/mg)	9.9 (10 pg/mg) 11.6 (50 pg/mg)	1.3 (10 pg/mg) 2.4 (50 pg/mg)	2
Clobazam	2–200 pg/mg, $r^2 = 0.9985$	56.5 (10 pg/mg) 75.8 (50 pg/mg)	11.4 (10 pg/mg) 10.1 (50 pg/mg)	10.7 (10 pg/mg) 6.2 (50 pg/mg)	2
Diazepam	1–200 pg/mg, $r^2 = 0.9994$	54.5 (10 pg/mg) 59.9 (50 pg/mg)	5.3 (10 pg/mg) 8.5 (50 pg/mg)	0.8 (10 pg/mg) 11.2 (50 pg/mg)	1
Lorazepam	5–200 pg/mg, $r^2 = 0.9996$	32.2 (10 pg/mg) 38.0 (50 pg/mg)	6.1 (10 pg/mg) 9.1 (50 pg/mg)	17.7 (10 pg/mg) 12.2 (50 pg/mg)	5
Lormetazepam	2–200 pg/mg, $r^2 = 0.9978$	59.3 (10 pg/mg) 63.5 (50 pg/mg)	6.6 (10 pg/mg) 9.7 (50 pg/mg)	7.9 (10 pg/mg) 3.3 (50 pg/mg)	2
Midazolam	0.5–200 pg/mg, $r^2 = 0.9989$	54.5 (10 pg/mg) 63.8 (50 pg/mg)	17.9 (10 pg/mg) 7.2 (50 pg/mg)	4.7 (10 pg/mg) 1.8 (50 pg/mg)	0.5

Table 2 (*Continued*)

Compound	Linearity	Extraction recovery (%)	Within batch precision (C.V., $n = 6$)	Within batch accuracy (%Bias, $n = 6$)	LOQ (pg/mg)
Nordiazepam	2–200 pg/mg, $r^2 = 0.9999$	49.2 (10 pg/mg) 60.1 (50 pg/mg)	9.9 (10 pg/mg) 8.1 (50 pg/mg)	1.1 (10 pg/mg) 1.7 (50 pg/mg)	2
Oxazepam	1–200 pg/mg, $r^2 = 0.9996$	38.6 (10 pg/mg) 50.3 (50 pg/mg)	13.0 (10 pg/mg) 7.5 (50 pg/mg)	8.6 (10 pg/mg) 1.1 (50 pg/mg)	1
Temazepam	1–200 pg/mg, $r^2 = 0.9997$	52.5 (10 pg/mg) 54.4 (50 pg/mg)	5.8 (10 pg/mg) 8.3 (50 pg/mg)	5.8 (10 pg/mg) 3.8 (50 pg/mg)	1
Tetrazepam	5–200 pg/mg, $r^2 = 0.9999$	35.9 (10 pg/mg) 41.7 (50 pg/mg)	13.1 (10 pg/mg) 7.3 (50 pg/mg)	20.2 (10 pg/mg) 3.0 (50 pg/mg)	5
Triazolam	0.5–200 pg/mg, $r^2 = 0.9991$	55.0 (10 pg/mg) 63.0 (50 pg/mg)	9.2 (10 pg/mg) 6.4 (50 pg/mg)	11.0 (10 pg/mg) 4.6 (50 pg/mg)	0.5
Zaleplon	1–200 pg/mg, $r^2 = 0.9970$	58.7 (10 pg/mg) 52.7 (50 pg/mg)	9.3 (10 pg/mg) 9.4 (50 pg/mg)	3.9 (10 pg/mg) 4.5 (50 pg/mg)	1
Zolpidem	0.5–200 pg/mg, $r^2 = 0.9993$	45.7 (10 pg/mg) 40.9 (50 pg/mg)	4.6 (10 pg/mg) 19.6 (50 pg/mg)	4.3 (10 pg/mg) 20.4 (50 pg/mg)	0.5

Data were recorded in the multiple reaction monitoring (MRM) mode. Parent ions, the corresponding daughter ions, retention time, cone voltage and collision energy optimized for the 16 benzodiazepines and IS are presented in Table 1.

2.5. Method validation

Standard calibration curves were obtained by preparing authentic spiked blank hair (20 mg) containing final 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 pg/mg of the 16 compounds. Within

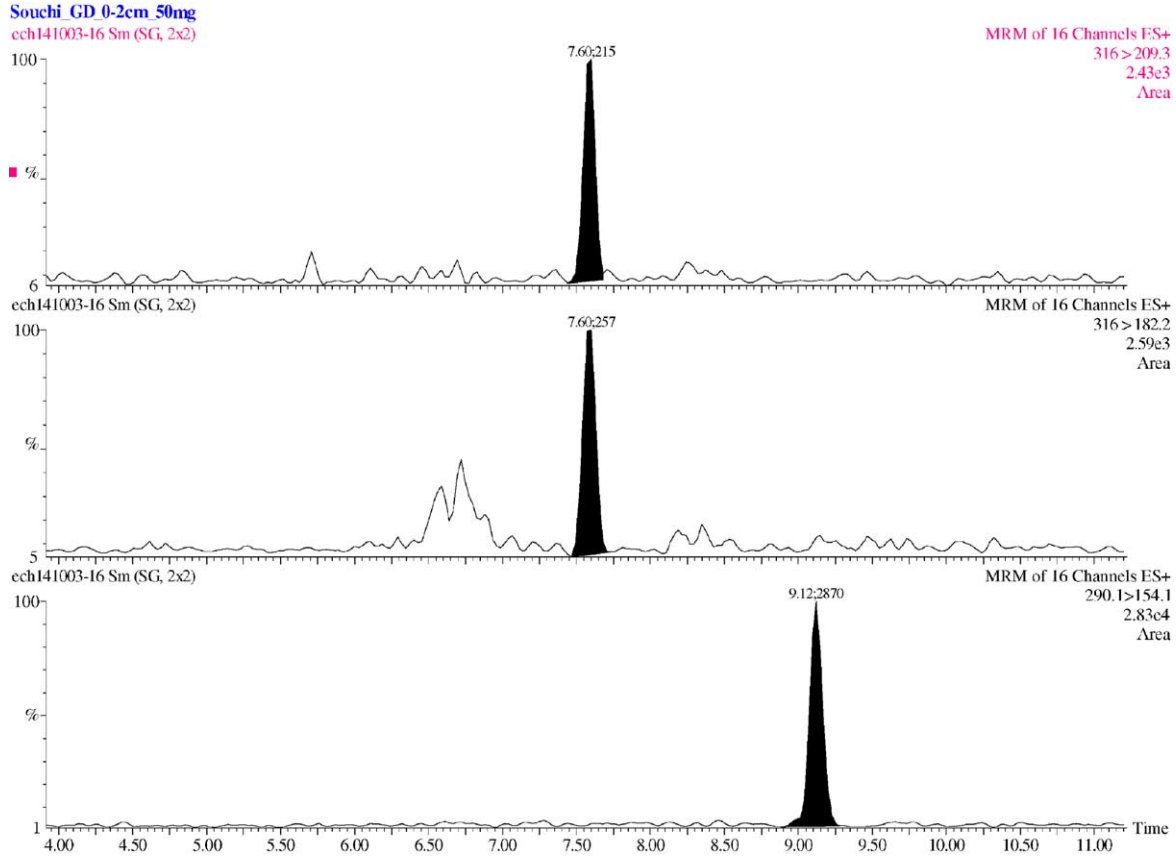

Fig. 2. Chromatogram obtained after analysis of the root segment of the hair of a volunteer who was administered a single dose of 10 mg of zolpidem 1 month before. On the top, the two daughter ions of zolpidem, on the bottom, the daughter ion of the IS. Concentration was 1.8 pg/mg.

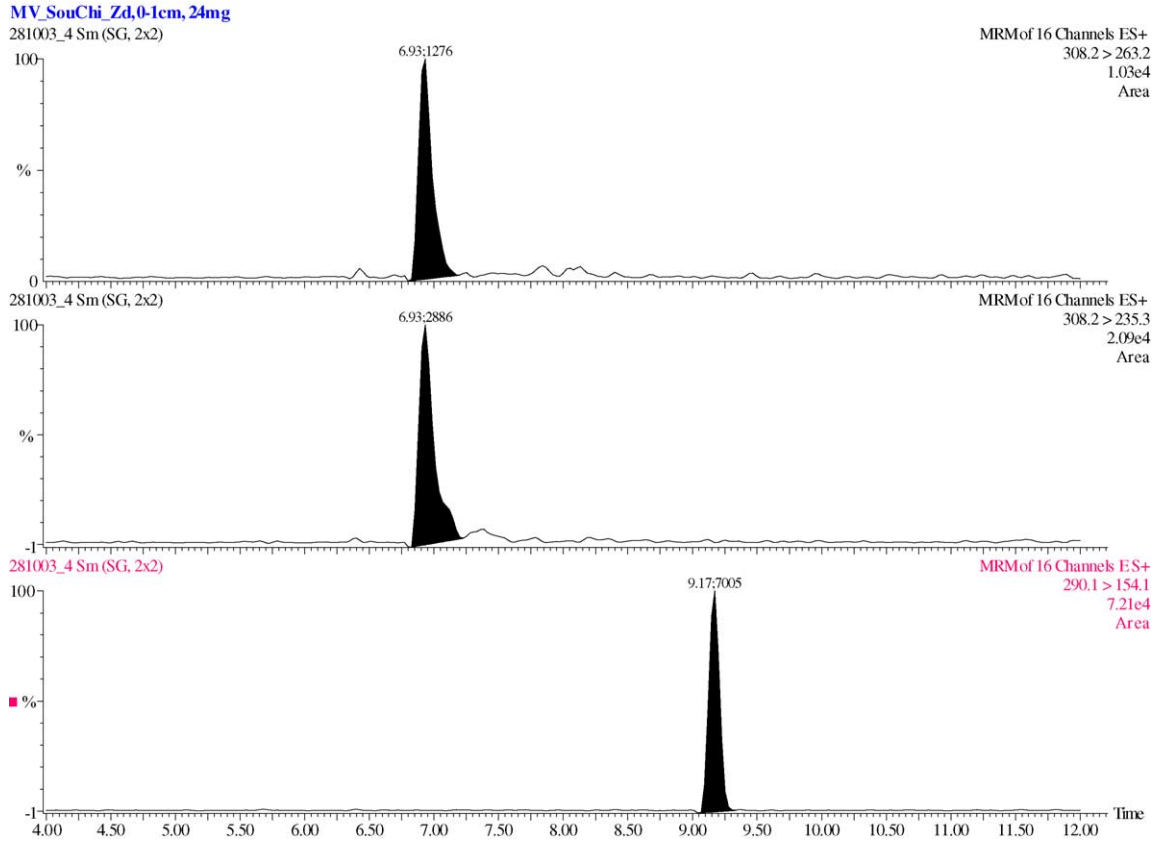

Fig. 3. Chromatogram obtained after analysis of the root segment of the hair of a volunteer who was administered a single dose of 6 mg of bromazepam 1 month before. On the top, the two daughter ions of bromazepam, on the bottom, the daughter ion of the IS. Concentration was 4.7 pg/mg.

batch precisions and accuracies ($n = 6$) were determined using blank hair spiked with the 16 compounds at 10 and 50 pg/mg. Recovery was established, at 10 and 50 pg/mg, by comparing the analyte peak areas of three extracted samples with those of three extracted samples blanks spiked with the same amounts of the analyte after extraction.

The limit of quantification (LOQ) is the lowest concentration of analyte that could be measured specifically with the presence of the two transitions and corresponds to the lowest point of the linearity for each molecule.

3. Results and discussion

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in hair. There were no blank effects.

The chromatogram of a blank hair spiked with the 16 benzodiazepines and hypnotics at 10 pg/mg is shown Fig. 1A and B. The method provides good resolution of the different drugs.

Validation data are presented in Table 2.

The limit of quantification for all benzodiazepines and hypnotics range from 0.5 to 5 pg/mg using a 20-mg hair sample. The method was linear in hair for each compound, from the limit of quantification to 200 pg/mg ($r^2 > 0.99$). Precisions and accuracies, at 10 and 50 pg/mg, were <20% in all cases but one. Extraction recovery, measured at the two same concentrations, range from 32 to 76%, that is suitable for a screening procedure.

As proposed by several authors [5,7], in case of nitro-benzodiazepines, the target compound is the 7-amino-metabolite. Due to their stability in alkaline medium, in contrast with other benzodiazepines, it is possible to lower their LOQ about five times with a specific extraction after sodium hydroxide hydrolysis [11].

To demonstrate the applicability of this procedure, Figs. 2 and 3 represent the chromatograms obtained after analysis of the root segment of volunteers who have been administered a single dose of 10 mg of zolpidem (women, 28-year-old, 60 kg) or 6 mg of bromazepam (women, 26-year-old, 50 kg), 1 month before. These findings demonstrate that it is possible to track a single drug exposure using an ultra-sensitive procedure. Fig. 4 is the chromatogram obtained after analysis of the hair of a 12-year-old girl sexually abused by her father since she was 6. During the last months, she was sometimes administered half a white tablet of Xanax 0.5 mg, to be more willing. The chromatogram represents the analysis of the root segment (0–2 cm) and relate the exposition to alprazolam during the 2 last months.

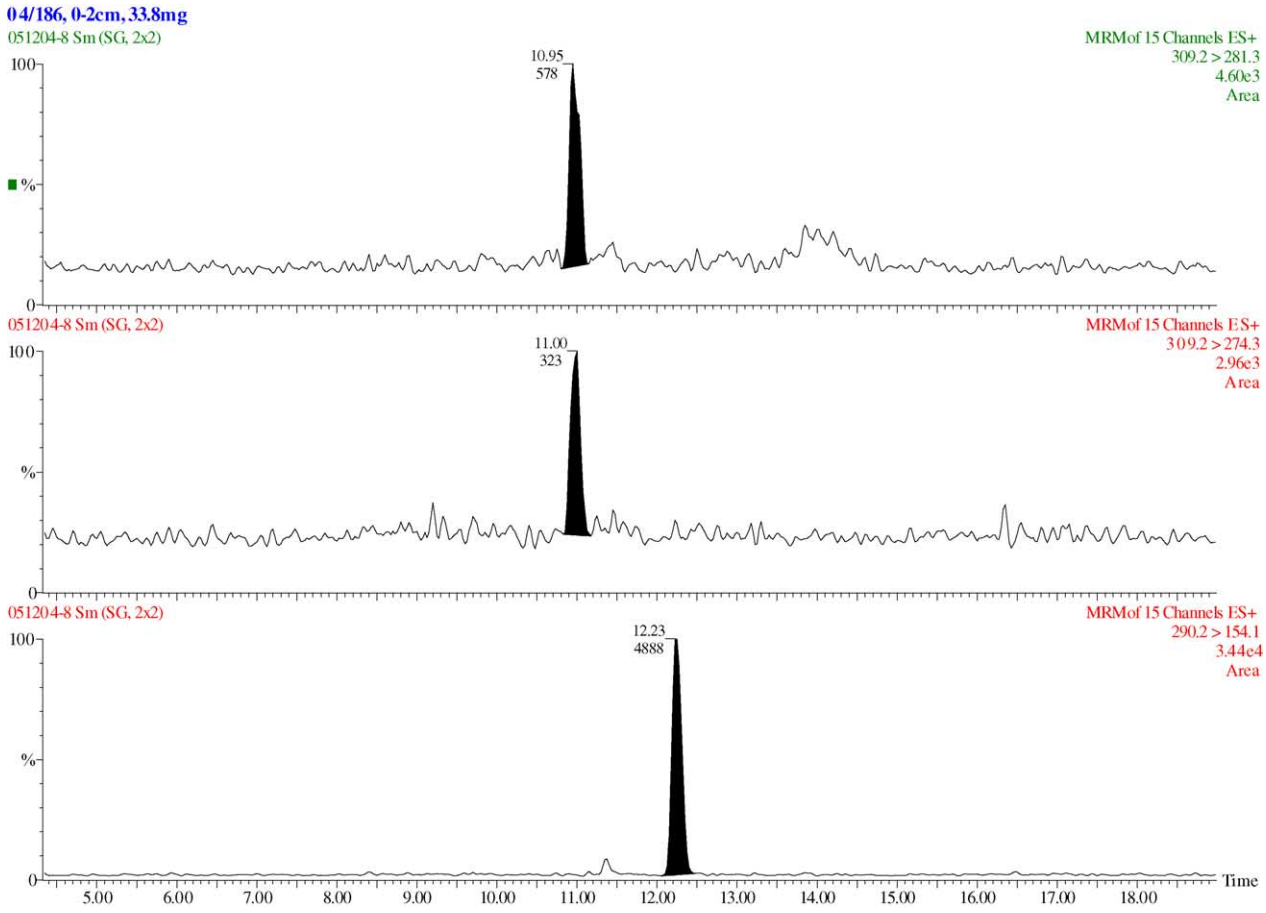

Fig. 4. Chromatogram obtained after the analysis of the hair of a girl sexually abused by her father under the influence of alprazolam. Alprazolam tested positive at 4.9 pg/mg.

This method appears rapid and simple, detecting all compounds from an aliquot. Therefore, it can be used for the analysis of a large number of hair specimens. Specificity is observed by using two daughter ions for each compound. Finally, the procedure is flexible, as it is always possible to include new compounds, such as drug coming from foreign countries (e.g. phenazepam used in east Europe, . . .).

4. Conclusion

This sensitive, specific and reproducible method developed is suitable for the detection and quantification of 16 benzodiazepines and hypnotics in human hair.

This technology may find useful applications, such as the monitoring of a single dose or to document exposure in forensic cases involving drug-facilitated crimes.

References

[1] J.J. Sramek, W.A. Baumgartner, T.N. Ahrens, V.A. Hill, N.R. Cutler, Ann. Pharmacother. 26 (1992) 469.
[2] F.J. Couper, I.M. McIntyre, O.H. Drummer, J. Forensic Sci. 40 (1995) 83.
[3] P. Kintz, V. Cirimele, F. Vaysette, P. Mangin, J. Chromatogr. B 677 (1995) 241.
[4] Y. Gaillard, G. Pépin, Toxicorama 8 (1996) 29.
[5] V. Cirimele, P. Kintz, C. Staub, P. Mangin, Forensic Sci. Int. 84 (1997) 189.
[6] V. Cirimele, P. Kintz, P. Mangin, Int. J. Leg. Med. 108 (1996) 265.
[7] A. Negrusz, C.M. Moore, J.L. Kern, P.G. Janicak, M.J. Strong, N.A. Levy, J. Anal. Toxicol. 24 (2000) 614.
[8] V. Cirimele, P. Kintz, B. Ludes, J. Chromatogr. B 700 (1997) 119.
[9] A. El Mahjoub, C. Staub, Forensic Sci. Int. 123 (2001) 17.
[10] R. Kronstrand, I. Nyström, M. Josefsson, S. Hodgins, J. Anal. Toxicol. 26 (2002) 479.
[11] M. Chèze, M. Villain, G. Pépin, Forensic Sci. Int. 145 (2004) 123.