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Hair analysis for nordiazepam and oxazepam by gas chromatography–negative-ion chemical ionization mass spectrometry

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Abstract

A procedure is presented for the identification of nordiazepam and its metabolite, oxazepam, in human hair. The method involves decontamination of hair with dichloromethane, incubation in phosphate buffer (pH 7.6) in the presence of deuterated internal standards, liquid–liquid extraction, derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane and gas chromatography–mass spectrometry using negative-ion chemical ionization with methane. Among thirty samples obtained from polydrug abusers, thirteen tested positive for nordiazepam, in the range 0.25–18.87 ng/mg. Five samples were also positive for oxazepam, in the range 0.11–0.50 ng/mg.

Keywords: Nordiazepam; Oxazepam

1. Introduction

Of the articles which address analyses of hair for the major drugs of abuse, those dealing with opiates or cocaine predominate in the available literature since 1979 [1]. The third most prevalent drugs tested in hair analysis studies are amphetamines. In the last months, the identification of cannabinoids was the major focus in hair testing simultaneously in France, Spain and the United States.

Surprisingly, the detection in human hair of benzodiazepines, the most abused pharmaceutical drugs in the world, appears not to be documented. To date, only one paper [2] reports their detection by radioimmunoassay. Diazepam was readily detected, but alprazolam and lorazepam were not found in subjects receiving therapeutic dosages.

Due to its sensitivity and specificity, GC–MS is now the state-of-the-art for the analysis of drugs of abuse in human hair. In the present report, an analytical procedure for testing nordiazepam (NOR) and oxazepam (OXA) in hair of chronic abusers is presented.

2. Experimental

2.1. Chemicals

Methanol, diethylether, chloroform and dichloromethane were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were analytical grade and supplied by Merck. NOR, NOR-d_5, OXA and

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OXA-d_5 were purchased from Radian (Austin, TX, USA). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) +1% trimethylchlorosilane (TMCS) was purchased from Interchim (Montluçon, France).

2.2. Material for examination

Hair samples were obtained from 30 subjects aged from 19 to 37 years, all of them were polydrug abusers. Hair samples weighing at least 100 mg were cut as close as possible to the skin from the posterior vertex. The hair was decontaminated by washing the specimen twice in 5 ml dichloromethane for 2 min at room temperature, which is the procedure also used for drugs of abuse.

2.3. Sample extraction

The hair was pulverized in a Retsch MM2-type ball mill (Haan, Germany). The matrix of the hair (about 50 mg) was incubated in Soerensen phosphate buffer, pH 7.6 (38.8 ml of KH_2PO_4 at 9.07 g/l and 61.2 ml of Na_2HPO_4 at 11.87 g/l) for 20 h at 40°C in the presence of 200 ng of deuterated internal standards (NOR and OXA). The homogenate was directly extracted with 5 ml diethyl ether–chloroform (80:20, v/v). After agitation (10 min) and centrifugation (10 min, 2000 *g*) the organic phase was removed and evaporated to dryness. The residue was derivatized by silylation using 35 μl BSTFA + 1% TMCS at 90°C for 20 min. After derivatization, a 1 μl portion was injected into the GC column, via a HP 7673 autosampler.

2.4. GC–MS method

The GC–MS system consisted of a Hewlett Packard (5890) chromatograph with a mass selective detector (Engine 5989B), with an ion source temperature of 200°C. The electron multiplier voltage was set at +400 V above the autotune (NCI tune) voltage. The flow-rate of carrier gas (helium, purity grade N55) through the column (HP-5 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 0.75 min.

A temperature column was programmed to rise from an initial temperature of 60°C kept for 1 min, to 290°C at 30°C/min and kept at 290°C for the final 2 min.

Mass spectra were recorded in the mass range *m/z* 170–450. Methane was used as reactant gas at an apparent pressure of 0.2 kPa in the ion source.

Analytes were identified and quantified on the basis of comparison of retention times and the abundance of two confirming ions relative to the deuterated internal standards.

Standard calibration curves were obtained by adding 5 (0.1 ng/mg), 25 (0.5 ng/mg), 50 (1.0 ng/mg), 100 (2.0 ng/mg), 500 (10 ng/mg) and 1000 (20.0 ng/mg) ng of pure standards prepared in methanol, to 50 mg of pulverized blank control hair (obtained from laboratory personnel and previously tested to be drug free).

Recovery ($n = 3$) and inter-day precision ($n = 8$) were determined by adding 100 ng of pure standards to 50 mg of pulverized blank control, corresponding to 2 ng/mg.

Due to the lack of suitable reference material, only a relative extraction recovery was measured by comparing the representative peak area of extracted spiked hair samples with the peak area of methanolic standards at the same concentration.

3. Results and discussion

The mass spectra generated by using NCI are presented in Fig. 1. Table 1 shows the ions monitored for NOR and OXA and the deuterated internal standards and retention times. Under the chromatographic conditions used, there was no interference of the drug tested with any extractable endogenous material present in hair. Fig. 2 and Fig. 3 are typical single ion chromatograms obtained after extraction of the hair of the same drug addict. NOR and OXA concentrations were 9.69 and 0.42 ng/mg, respectively.

Responses were linear in the range 0.1–20.0 ng/mg of hair with correlation coefficients of 0.995 and 0.993 for NOR and OXA, respectively.

The inter-day precision ($n = 8$), recovery and limit of detection for each drug are presented in Table 2.

Taking into account our previous considerations

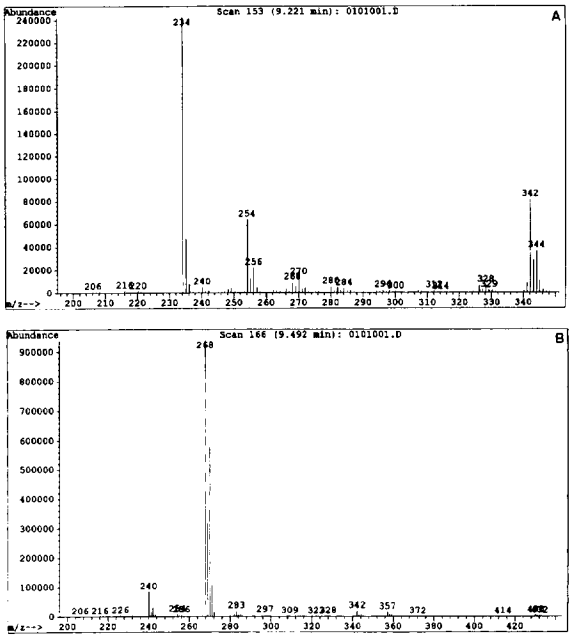

Fig. 1. (A) NCI mass spectrum of nordiazepam-TMS. (B) NCI mass spectrum of oxazepam-diTMS

Table 1
Selected ions and retention times

Compound	Retention time (min)	Ion (*m/z*)
Nordiazepam-TMS	9.05	*234*, 254, 342
Nordiazepam-TMS-d_5	9.04	*239*, 259, 347
Oxazepam-diTMS	9.28	*268*, 270, 357
Oxazepam-diTMS-d_5	9.27	*273*, 275, 362

The ions in italics were used for quantification.

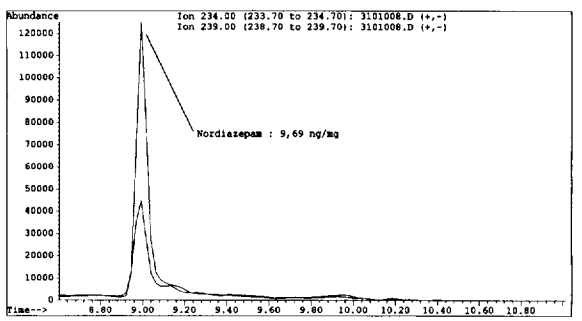

Fig. 2. Selected-ion monitoring (SIM) chromatogram of a hair extract. Nordiazepam concentration was 9.69 ng/mg.

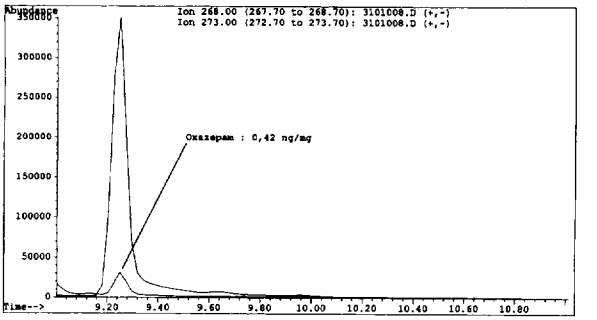

Fig. 3. SIM chromatogram of a hair extract. Oxazepam concentration was 0.42 ng/mg.

Table 2
Inter-day precision, recovery and limit of detection of each drug

Drug	Inter-day precision (%)	Recovery (%)	Limit of detection (ng/mg)
Nordiazepam	9.3	77.3	0.01
Oxazepam	13.6	68.6	0.005

[3], the positive cut-off value for NOR was set at 0.1 ng/mg, which consists of ten times the limit of detection.

Acid hydrolysis or alkaline hydrolysis were found unsuitable to extract the target drugs from the hair matrix, leading to decomposed compounds, including benzophenones.

At the initial stages of this work, methanol direct extraction after sonication was investigated. This procedure was not retained since the extracts obtained were dirty and the concentrations of the target drugs lower when compared with buffer incubation.

As compared with electron impact (EI) or positive chemical ionization (PCI), GC–MS coupled with negative chemical ionization (NCI) leads to increased sensitivity [4]. The NCI signal-to-noise ratio was several thousand times greater than that of EI, clearly demonstrating that NCI is the ionization mode of choice for the analysis of benzodiazepines.

Derivatization with BSTFA + 1% TMCS was essential to ensure no thermal degradation of OXA. The potential risk of deterioration of the GC column, which was sometimes mentioned after simultaneous injections of BSTFA samples and other samples (e.g. methanolic samples), was avoided by only using this GC–MS with silylating agents.

The NOR ion monitored for quantitative analysis (m/z) 234 at $[M-73]^-$ was probably formed by the loss of the trimethylsilyl group. OXA, which is silylated in two positions, has a spectra characterized by a peak at $[M-162]^-$, consistent with a loss of TMS-O-TMS [4].

Another advantage of this procedure, is that BSTFA does not have to be evaporated prior to injections, when compared with other derivatization agents, like trifluoroacetic or pentafluoropropianic anhydrides.

3.1. Applications

In 30 samples obtained from drug abusers, 13 tested positive for NOR (Table 3). Concentrations ranged from 0.25 to 18.87 ng/mg. The mean value was 4.16 ng/mg. In only five cases, OXA was simultaneously detected, ranging from 0.11 to 0.50 ng/mg with a mean value of 0.28 ng/mg. OXA was only detected when the concentration of NOR was higher than 4 ng/mg. As it is generally the case, the parent drug is in higher concentration than the metabolite [5,7].

Table 3
Nordiazepam and oxazepam concentrations in human hair

Subject	Nordiazepam (ng/mg)	Oxazepam (ng/mg)
1	1.34	ND
2	1.59	ND
3	4.30	0.24
4	1.77	ND
5	1.80	ND
6	18.87	0.50
7	8.24	0.11
8	0.44	ND
9	0.35	ND
10	0.81	ND
11	0.25	ND
12	9.69	0.42
13	4.65	0.15

ND, not detected.

As the pattern of drug use (identification of the drugs and amount consumed) by the addicts were unknown, one cannot exclude that the concentrations measured are representative of a diazepam, a NOR or an OXA exposure or the sum of all the possible situations.

In the absence of suitable literature, it was not possible to compare our data with other reported concentrations. Due to the absence of information on benzodiazepine dosage (generally the case for drug abusers) it was not possible to evaluate a correlation between concentration in hair and daily dose.

In conclusion, this preliminary study has the advantage of studying hair concentration of chronic benzodiazepine use. To our knowledge, this is the first report of NOR and OXA detection in human hair.

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