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The correlation between concentrations of zolpidem and benzodiazepines in segmental hair samples and use patterns

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ABSTRACT

The aim of this study was to investigate the correlation between histories of zolpidem and benzodiazepines use and their concentrations in hair as determined by segmental hair analysis, that is, by analyzing hair samples taken 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm etc. and 0–3 cm from the scalp, and whole hair. Of the 23 hair samples examined, 18 were collected from patients in a rehabilitation program and five were from patients that had taken zolpidem only once by prescription. All 23 patients provided written informed consent after reviewing the research plan, described their zolpidem and benzodiazepines use histories accurately, and provided hair samples, which were weighed, washed, cut into lengths of <1 mm, and extracted in 100% methanol for 16 h (diazepam-d_5 was used as an internal standard). Extracts were evaporated under reduced pressure and reconstituted with aqueous methanol (1:1 v/v). These extracts (10 μL) were analyzed by Liquid Chromatography/Tandem Mass Spectrometry (LC–MS/MS). The method used was validated by determining LOD, LOQ, calibration curves, intra- and inter-accuracies, precisions, matrix effects, process efficiencies, extraction efficiencies, and processed sample stabilities. Five hundred and ninety-five 1 cm hair segments showed 61.59% positive probability and 86.71% negative probability of quality correlation between zolpidem and benzodiazepines use and concentrations in hair. Good qualitative correlations were observed between drug use and detection in hair. False positivity and false negativity were very low. Of the hair samples taken from patients in a rehabilitation program, subject nos. 4, 5, and 12 had correlation coefficients of 0.68, 0.54 and 0.71, respectively, for relationships between zolpidem use and concentration of zolpidem in hair. For the 5 patients taking only a single dose of zolpidem (10 mg), the average zolpidem concentrations in hair were 20, 15 and 40 pg/mg after 5, 30 and 60 days, respectively. This study shows a relationship between history of zolpidem and benzodiazepines use and their concentrations in 1 cm hair segment.

1. Introduction

Zolpidem is non-benzodiazepine, non-barbiturate hypnotic that has largely replaced benzodiazepines for the pharmacological treatment of insomnia and related diseases [1]. Benzodiazepines reduce anxiety and prevent panic-symptoms [2], but abuse and dependency have been reported [3,4]. In many countries, zolpidem and benzodiazepines are controlled narcotics and in Korea are viewed as psychotropic agents and controlled by the Narcotics Control ACT [5]. In Korea, zolpidem and benzodiazepines are usually prescribed as psychotropic drugs, but they are also misused and illegally overused [6].

The analysis of drugs in hair and segmental hair analysis are performed globally. However, it is difficult to predict and estimate history of drug abuse accurately based on the results of segmental

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hair analysis. Forensic scientists interpret concentrations of drugs in hair, and provide interpretations based on published literature to investigation agencies and courts.

Several analysis methods have been used to analyze drug concentrations in hair, for example, GC/MS [7–9]; LC–MS/MS [10–13], and MALDI-MS [14]. In addition, many papers have been published on the segmental hair analysis of zolpidem and benzodiazepines [6,15–18], of zolpidem and its metabolites [19,20]. In addition, correlation studies have been performed to investigate relations between histories of zolpidem and benzodiazepines use and concentrations in hair [21–32], and segmental hair analysis was performed on volunteers that had taken zolpidem or benzodiazepines on one occasion [14,33–35].

In this study, we evaluated correlations between histories of zolpidem and benzodiazepines use and concentrations of zolpidem and benzodiazepines in hair by segmental hair analysis. Drug users in rehabilitation programs are committed to quitting drugs and have no fear of legal punishment, and thus, they can be relied upon to report drug abuse accurately. The results obtained could be utilized in scientific investigations and by courts.

2. Method

2.1. Recruitment of subjects and survey

The study inclusion criteria were as follows: the administration of zolpidem or benzodiazepines during the previous year, a hair length of >4 cm, and the ability to write down an accurate history of psychotropic drug. Patients completed a questionnaire that included sex, date of birth, age, education, hair treatment (perm, bleach, dye), age of first use of psychotropic drugs, time of the most recent administration, and details of other drugs taken (e.g., MA, cannabis, MDMA, opiates, and cocaine) when they agreed to participate in this study.

2.2. Reagents and standards

7-Aminoflunitrazepam (the metabolite of flunitrazepam), α-hydroxyalprazolam (the metabolite of alprazolam) alprazolam, clonazepam, desalkylflurazepam (the metabolite of flurazepam), diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, nordiazepam (the metabolite of diazepam) temazepam, zolpidem, 7-aminoclonazepam (the metabolite of clonazepam), lorazepam glucuronide (the metabolite of lorazepam), zolpidem phenyl-4-carboxylic acid (the metabolite of zolpidem) and diazepam-d_5 (Internal standard) were obtained from Cerilliant Corporation (Round Rock, TX, USA).

Working solution of the internal standard diazepam-d_5 (1 μg/ml) and above all standards were diluted with methanol and stored in freezer at −20 °C. Methanols, distilled water, acetonitrile, ammonium formate, formic acid (99%) were HPLC grade.

2.3. Hair collection and hair analysis

Hair samples (about 100 strands) were collected from the vertex region of the scalps of the 23 study subjects, and total length of hair was measured. Five of the 23 subjects had dyed their hair brown; the others had natural dark brown to black hair. The study was approved by Duksung Women's University Institutional Review Board's (2015-3546). All subjects provided informed consent. Subjects recruited at a rehabilitation center (n = 18) wrote down drug doses and frequencies, using prescriptions with the assistance of doctors or professional counselors. The hair samples of zolpidem patients (n = 5, subject nos. 19–23) that had received a prescription for zolpidem only once to treat a psychotic disorder were collected, initially screened and confirmed to be negative results of zolpidem before administration, and hair samples at 5, 30, and 60 days after a single dose (10 mg) of zolpidem was collected and analyzed.

Hair samples were taken from the scalp and cut to obtain samples at 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm, etc. and 0–3 cm from the scalp, and whole hair (total length hair without cutting) and was analyzed. For LC–MS/MS analysis, hair samples (10 mg) were weighed, washed, cut into lengths of <1 mm, and extracted in 100% methanol for 16 h (diazepam-d_5 was used as an internal standard). Extracts were evaporated to dryness under reduced pressure and then reconstituted with aqueous methanol (1:1 v/v). 10 μL aliquots were injected in to the LC–MS/MS unit.

2.4. LC–MS/MS analysis

The method used in this study is a modification of previously published method [36,37]. However, the LC–MS/MS instrument, column, and the conditions used differed. The LC–MS/MS analysis was conducted on an Nexera X2 (SIMADZU) and MDS Sciex API 3200 Qtrap MS/MS. Zorbax Eclipse XDB-C18 (4.6 × 100 mm, 3.5 μm LC col) column and Eclipse XDB-C18 column (4.6 × 12.5 mm, 5 μm Grd Car) were used as the analytical and guard column, respectively. The mobile phase consisted of 2 mM ammonium formate/0.2% formic acid in distilled water (A) and 2 mM ammonium formate/0.2% formic acid in acetonitrile (B) at flow rate of 0.5 mL/min and injection volume was 10 μL. Column oven temperature was 40 °C. The initial gradient conditions were equilibrated with a mixture of 90% eluent A and 10% eluent B. The flow rate and gradient were programmed as follows: a flow rate increases from 500 μL/min to 1000 μL/min and a gradient increased to 90% eluent B from 0 to 8 min and then kept 10% eluent A and 90% eluent B from 8 to 11 min. Eventually, initial conditions were re-established from 11 to 15 min and the system was allowed time to equilibrate. Electrospray ionization (ESI) in positive mode to the MS system was used and the optimum condition of curtain gas was 20 psi, heated nebulizer temperature (TEM) was 650 °C, collision activated dissociation (CAD) was medium, nebulizing gas (GS1) was 50 psi and heater gas (GS2) was 40 psi. The conditions of LC–MS/MS such as precursor ion (m/z), Product ion (m/z), DP, EP, CEP and retention time (RT) were shown in Supplementary Table S1.

2.5. Method validation

The method was validated using intra/inter-assay precision and accuracy, matrix effect, process efficiency, extraction efficiency and stability of processed sample based on LOD, LOQ, and calibration curve. Blank hair samples (10 mg) spiked with 250, 1000 and 5000 pg of zolpidem and benzodiazepines were prepared to validate intra/inter-assay precision and accuracy at five sets of each sample on five different days. The stability of processed sample was investigated after 12, 24 and 36 h.

2.6. Statistical methods

For index of association, Pearson's correlation coefficient (r) was calculated. The correlation between zolpidem and benzodiazepines concentrations in hair and accumulative doses over time was performed by correlation analysis. Statistical analyses were performed under the SPSS (24 version) statistical software.

3. Results and discussion

3.1. Method validation

A method of hair analysis was established and validated (Supplementary Tables S2 and S3) according to previously published papers and guidelines [36,37]. The calibration curves were plotted using hair samples (10 mg) 10–5000 pg/mg for 7-aminoflunitrazepam, alprazolam, flurazepam, midazolam, zolpidem, 25–5000 pg/mg for 7-aminoclonazepam, α-hydroxyalprazolam, clonazepam, desalkylflurazepam, diazepam, flunitrazepam, lorazepam, nordiazepam and temazepam, 100–5000 pg/mg for lorazepam glucuronide, and zolpidem phenyl-4-carboxylic acid, respectively and R^2 was >0.9995.

Intra/inter-assay precision and inter-assay accuracy, matrix effect, process efficiency, extraction efficiency and stability of processed sample were satisfactory within 15%. Methods were successfully validated.

3.2. Characteristics of the study subjects

Table 1 shows the age, sexes, education levels, administration routes, ages at first use of zolpidem or benzodiazepines, hair treatment (dye, perm, etc.), use of other drugs, hair length and hair color of the 23 subjects. Most subjects had taken zolpidem or benzodiazepines for anxiety or sleeping disorders caused by the use of illegal drugs — primarily methamphetamine (MA). One subject was under treatment for bipolar disorder and dependence on zolpidem. Two subjects were being treated for lorazepam and alcohol addiction. Subject ages ranged from 29 to 61 years (average 41 years), and there were 17 males and 6 females. An education level, if subjects have graduated from high-school, it was indicated as twelve years, ranged from 8 to 21 years (average = 13). Most took drugs orally (n = 21); only two subject took drugs by injection and orally. Subjects generally took psychotropic drugs in 2014 or 2015 for the first time. Six subjects had dyed their hair. In addition to zolpidem and benzodiazepines, subjects used MA most frequently (n = 11); one used cannabis and sniffed glue. MA users at the rehabilitation center were frequently prescribed zolpidem or benzodiazepines.

3.3. Single doses, daily doses and monthly doses of zolpidem or benzodiazepines

Zolpidem or benzodiazepines users (n = 18) wrote down single, daily, and monthly doses of zolpidem or benzodiazepines with the assistance of doctors or counselors. Single doses ranged from 0.5 to 12.5 mg and frequencies ranged from once per month to daily. Single doses of zolpidem were highest (12.5 mg) and single doses of diazepam, alprazolam, clonazepam, or flunitrazepam prescribed was 0.5 mg.

3.4. Qualitative relation between a history of zolpidem or benzodiazepines use and concentrations of zolpidem or benzodiazepines in hair

Qualitative analysis of 1 cm hair segments (n = 595) showed a positive probability of 61.59% and a negative probability of 86.71% for qualitative relation between zolpidem or benzodiazepines use and concentrations in hair (Table 2). In detail, hair results were positive in 93 hair segments of 151 hair segments of subjects who reported taking zolpidem or benzodiazepines, and hair results were negative in 385 hair specimens of 444 hair segments of subjects that denied zolpidem or benzodiazepines administration during the preceding month. These results showed low false positivity and false negativity values and a good qualitative

Table 1
Characteristics of zolpidem and benzodiazepines users that provided hair samples (n = 23).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Code	b1	b2	b3	b4	b5	b6	b7	b8	b9	b10	b11	b12	b13	b14	b15	q1	q2	q3	sb1	sb2	sb3	sb4	sb5
Age	36	44	44	47	47	48	38	38	47	60	61	29	33	33	57	40	33	36	55	30	30	31	32
Sex	M	M	M	M	M	M	M	M	M	M	M	M	F	F	M	F	F	M	M	F	F	M	M
Education level	12	12	8	16	12	9	14	16	12	12	12	12	12	10	12	8	12	9	12	18	21	18	18
First administration	2012	2015	2015	2014	2014	2011	2010	2015	2013	2008	2014	2005	2013	2008	2007	2004	2008	2013	2015	2015	2015	2015	2015
Hair dyeing?	N	N	N	N	N	Y	N	N	N	Y	N	Y	Y	N	N	N	N	Y	N	N	Y	N	N
Other drugs	Y (MA)	N	Y (MA)	Y (MA)	Y (Cannabis)	Y (MA)	Y (MA)	N	N	Y (MA)	Y (MA)	Y (MA)	Y (MA)	N	N	N	Y (MA)	Y (MA)	N (bond)	N	N	N	N
Hair length (cm)	10.5	2	4	6	7.5	9.5	6.5	8.5	2.5	5	2.5	12	11.5	21	2.1	35	15	6	3.1	7.5	19	10.5	8.5
Hair color	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black

Table 2
Qualitative correlation between the history of zolpidem and benzodiazepine use and drug concentrations in hair.

Total	Admission		Denial		Total	
	N	%	N	%	N	%
Positive hair result	93	61.59[a]	59	13.29[b]	152	25.55
Negative hair result	58	38.41[c]	385	86.71[d]	443	74.45
Total	151	100	444	100	595	100

[a] Number of positive results/total number of positive self-reports × 100 (positive probability).
[b] Number of positive results/total number of negative self-reports × 100 (false positivity).
[c] Number of negative results/total number of positive self-reports × 100 (false negativity).
[d] Number of negative results/total number of negative self-reports × 100 (negative probability).

relation between zolpidem or benzodiazepines use and concentrations in 1 cm hair segments.

3.5. Quantitative correlation between zolpidem or benzodiazepines usage during the previous month and zolpidem or benzodiazepines concentrations in 1 cm long hair samples

One cm of segmental hair, 0–3 cm and whole hair analysis were performed on subjects nos. 1–18. All results are shown in supplementary data (Supplementary Tables S4–S27 and Figs. S1–S13), and the results of subjects nos. 4, 5, 7, and 12 are shown in Figs. 1–6. Some results of drug concentrations in hair for alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, and zolpidem are summarized in Tables 3–5.

In the case of alprazolam (Table 3), subject no. 7 took 45–118 mg (average = 57 mg) of alprazolam for six months before hair sampling and concentrations of alprazolam was 20–60 pg/mg in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair (Fig. 1). The concentrations of α-hydroxyalprazolam (its metabolite) ranged from ≤LOQ to 50 pg/mg (average = 37.5 pg/mg) in three segments (1–2, 2–3, and 3–4 cm) and whole hair.

In the case of clonazepam (Table 3), subject no. 1 took 9.5–55 mg (average = 27.25 mg) of clonazepam for six months before hair sampling. Clonazepam was not detected but the concentrations of 7-aminoclonazepam (its metabolite) ranged from 40–70 pg/mg (average = 47.5 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair.

In the case of diazepam (Table 3), subject no. 7 took 824 mg of diazepam over the recent one month before hair sampling, and concentrations of diazepam ranged from 170 to 350 pg/mg (average = 260 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair, and concentrations of nordiazepam (its metabolite) ranged from

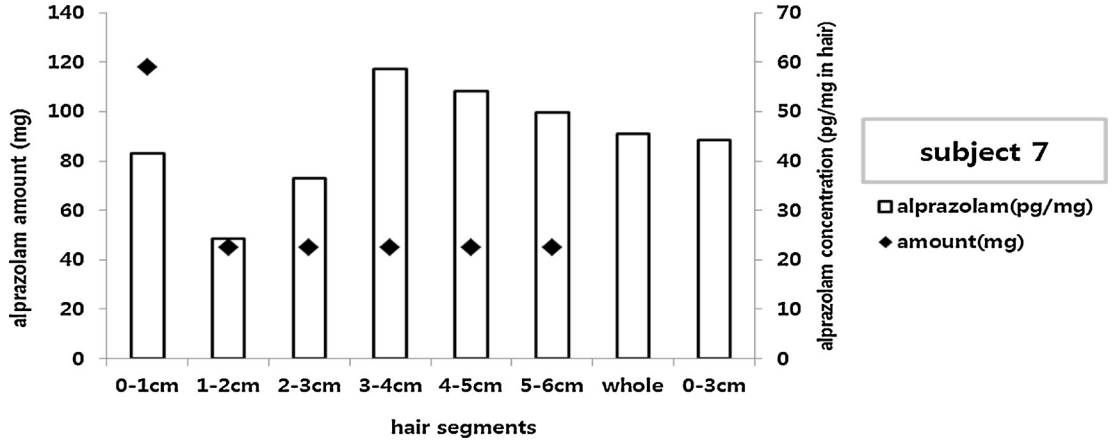


Fig. 1. Comparison between the history of alprazolam use and concentrations of alprazolam in hair from subject no. 7.

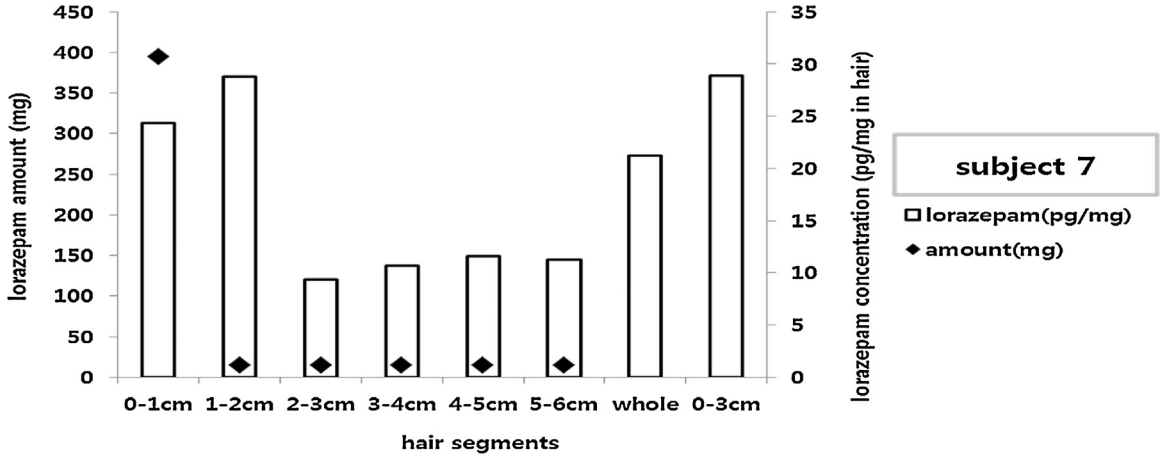


Fig. 2. Comparison between the history of lorazepam use and concentrations of lorazepam in hair from subject no. 7.

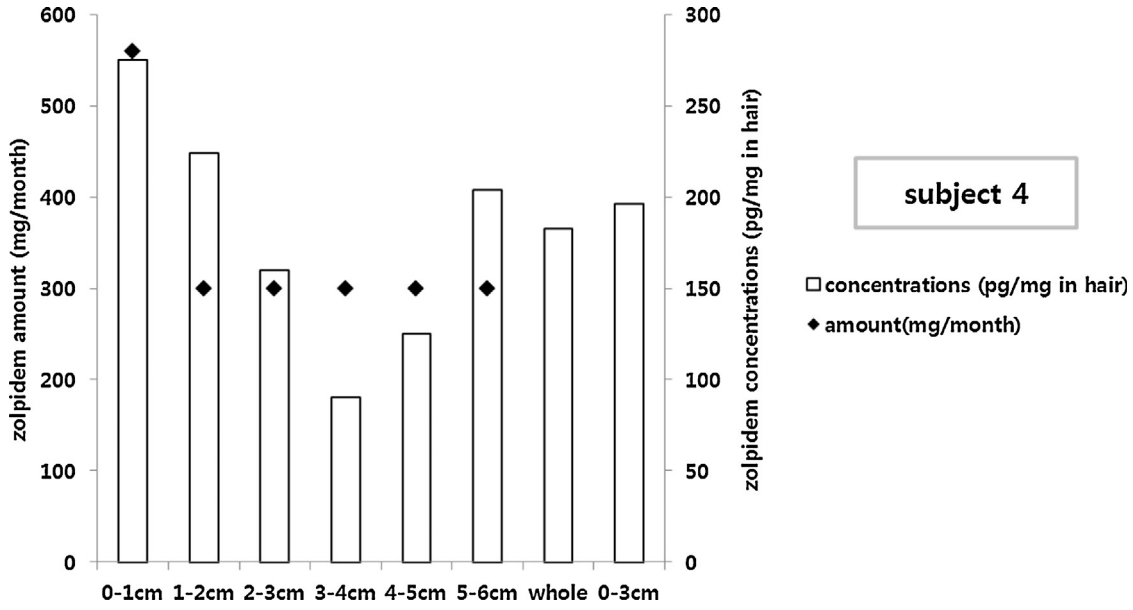


Fig. 3. Comparison between the history of zolpidem use and concentrations of zolpidem in hair from subject no. 4.

330 to 710 pg/mg (average = 520 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair.

In the case of flunitrazepam (Table 3), subject no. 11 took 30 mg of flunitrazepam for two month before hair sampling. Flunitrazepam was not detected but 7-aminoflunitrazepam (its metabolite) ranged from ≤LOQ to 50 pg/mg in two segmental hair (0–1, 1–2 cm).

In the case of lorazepam (Table 3), subject no. 7 took 15–395 mg (average = 173 mg) of lorazepam for six month before hair sampling and the concentrations of lorazepam ranged from ≤LOQ to 30 pg/mg in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair, and lorazepam-glucuronide (its metabolite) detected below LOQ (Fig. 2).

In the case of zolpidem (Table 4), subject no. 4 took 300–560 mg (average = 343 mg) of zolpidem for six month before hair sampling and zolpidem concentration ranged from 90 to 280 pg/mg (average = 182 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair (Fig. 3). Subject no. 5 took 227.5–487.5 mg (average = 395 mg) of zolpidem for six month before hair sampling and zolpidem concentration ranged from 150 to 220 pg/mg (average = 161.25 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair (Fig. 4). Subject no. 7 took 300–1290 mg (average = 465 mg) of zolpidem for six month before hair sampling and zolpidem concentration ranged from 480 to 1550 pg/mg (average = 976.25 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair (Fig. 5). Subject no. 12 took 60–300 mg (average = 260 mg) of zolpidem for six months (Table 4) and the concentrations of zolpidem ranged from 130 to 520 pg/mg (average = 325 pg/mg) in six segments (0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 cm), whole hair, and 0–3 cm segmental hair (Fig. 6).

A correlation was observed between a history of zolpidem use and concentration of zolpidem in hair of subject no. 4 (correlation

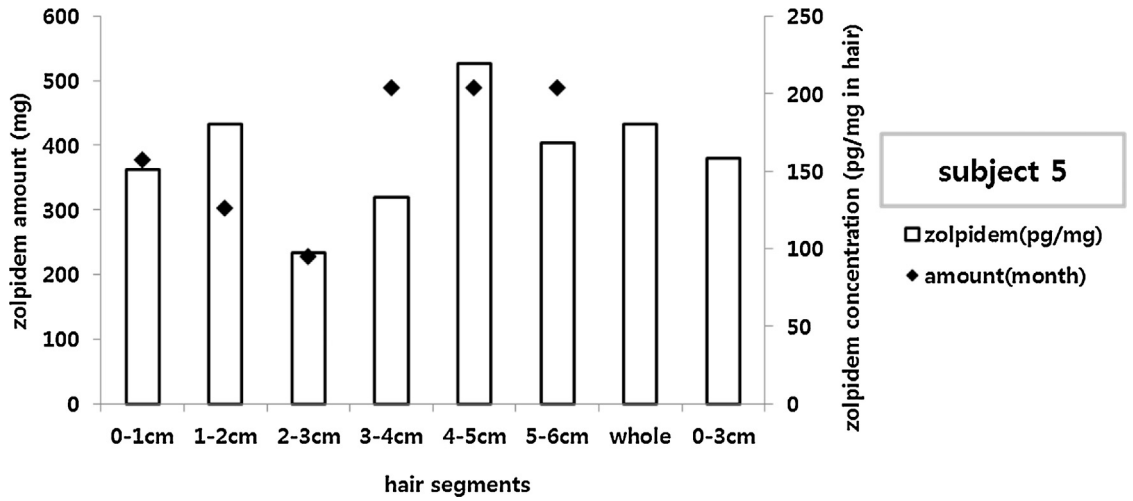


Fig. 4. Comparison between the history of zolpidem use and concentrations of zolpidem in hair from subject no. 5.

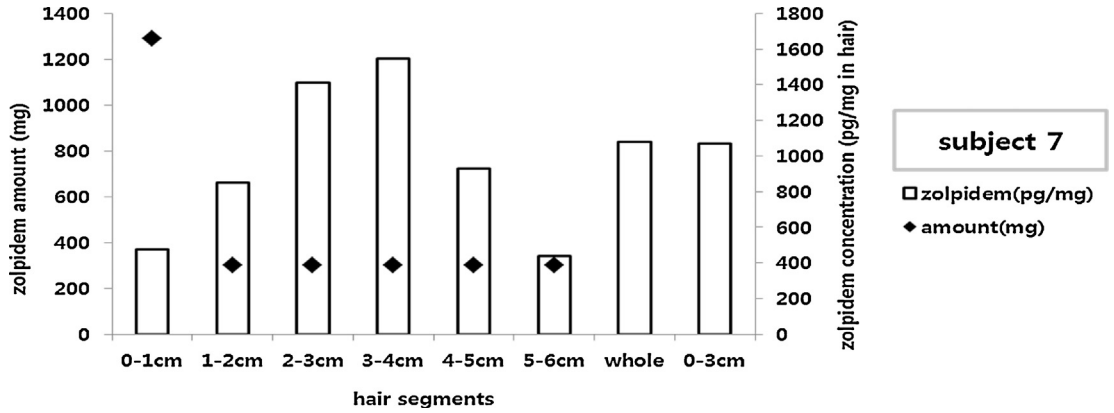


Fig. 5. Comparison between the history of zolpidem use and concentrations of zolpidem in hair from subject no. 7.

coefficient 0.68; Fig. 3), no. 5 (correlation coefficient 0.54; Fig. 4) and no. 7 (correlation coefficient 0.71; Fig. 5).

Total doses and drug concentrations in hair are summarized in Table 5. Zolpidem was taken at the highest dose (up to 2000 mg) in subject 14 who took 50 tablets of zolpidem (10 mg) once a week (4 times a month). Maximum concentration of zolpidem in hair in subject 14 was 6410 pg/mg, but zolpidem–PCA was only detected below its LOQ. Diazepam was taken at the second highest dose, and unlike that found for other drugs, nordiazepam, the metabolite of diazepam, was easily detected. Concentrations of alprazolam, clonazepam, and flunitrazepam in hair were low because dosages were low, whereas the concentrations of metabolites of diazepam, flunitrazepam were higher than those of parent drugs.

3.6. The detection in zolpidem in hair after a single dose

Five subjects (nos. 19–23) took only a single dose of zolpidem, and hair was collected 5, 30, and 60 days after administration and analyzed. Concentrations of zolpidem in hair samples are shown in Fig. 7. At 5 days after administration, concentrations of zolpidem in hair ranged from 10 to 30 pg/mg (average 20 pg/mg) in one segment (0–1 cm) from subjects nos. 19, 20, 22, and 23, and in subject 21 its concentration was 20 pg/mg in whole hair (Fig. 7a). At 30 days after administration, concentrations of zolpidem in two segments (0–1 and 1–2 cm) ranged from 10 to 20 pg/mg (average 15 pg/mg) in the five subjects, but zolpidem-PCA was not detected. The concentration of zolpidem in hair was 10 pg/mg in subject no. 22 (Fig. 7b). At 60 days after administration, the concentration of zolpidem in hair from subject nos. 19–23 ranged from 10 to 70 pg/mg (average 40 pg/mg) in two segments (1–2 and 2–3 cm) and was 10 pg/mg in whole hair from subject 23 (Fig. 7c).

4. Discussion

A number of studies have been conducted to investigate the correlation between zolpidem or benzodiazepines use and their concentrations in hair [1,38–46]. In a previous study, 18 zolpidem or benzodiazepines cases were investigated [36]; eight cases were related to zolpidem, seven to alprazolam, six to diazepam, and four to clonazepam. In the present study, the single-step methanol extraction method used was more efficient than previously described extraction methods of Refs. [34,47] (data was not shown), and qualitative relation were found between histories of zolpidem or benzodiazepines use and concentrations in hair segments.

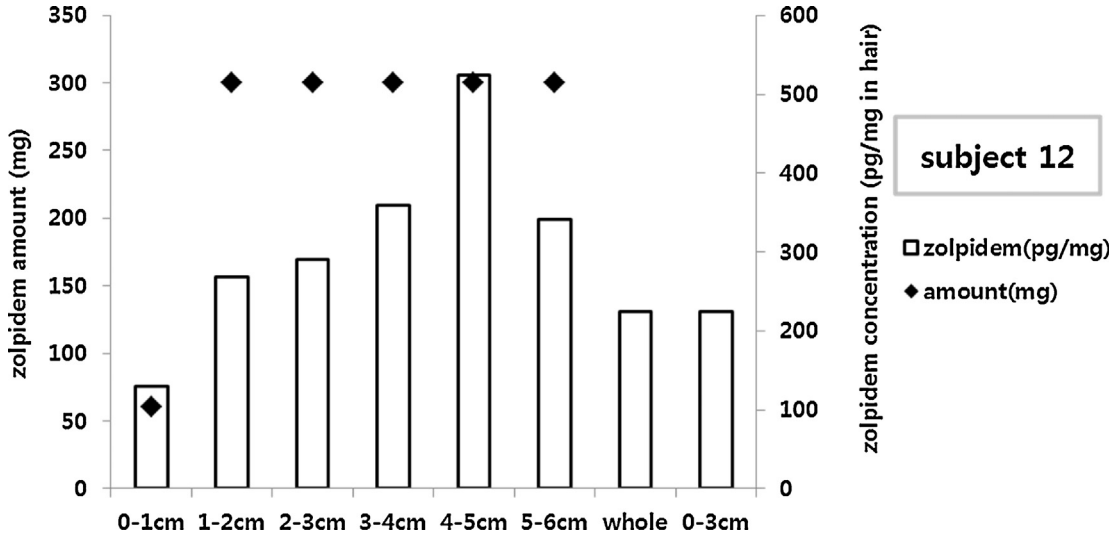


Fig. 6. Comparison between the history of zolpidem use and concentrations of zolpidem in hair from subject no. 12.

Table 3
Benzodiazepines concentrations in hair from subjects.

	Subjects	Month							
		1	2	3	4	5	6	Whole	0–3
Alprazolam	5	27.5[a]	1.5[a]						
						20[b]		10[b]	
	7	118[a]	45[a]	45[a]	45[a]	45[a]	45[a]		
		40[b]	20[b]	40[b]	60[b]	50[b]	50[b]	50[b]	40[b]
			50[c]	50[c]	40[c]			≤LOQ[c]	
	8	25[a]							
		20[b]							
	10	15[a]	15[a]	15[a]	15[a]				
		10[b]	20[b]						
	12	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]		
	15	29.5[a]	29[a]						
		20[b]	20[b]						10[b]
	17			37.5[a]	37.5[a]	30[a]	30[a]		
		30[b]	30[b]	30[b]	20[b]	30[b]	20[b]	30[b]	20[b]
						≤LOQ[c]	≤LOQ[c]		
Clonazepam	1	25[a]	11.5[a]	9.5[a]	17[a]	45.5[a]	55[a]		
		40[c]	30[c]	40[c]	60[c]	70[c]	50[c]	40[c]	50[c]
	3	36[a]	34[a]	17[a]	45[a]				
		≤LOQ[b]		≤LOQ[b]					≤LOQ[b]
		90[c]	70[c]	≤LOQ[c]	100[c]			50[c]	70[c]
	4	9[a]							
		≤LOQ[c]		≤LOQ[c]	≤LOQ[c]	30[c]	30[c]		≤LOQ[c]
	5	3.5[a]	4.5[a]						
			≤LOQ[c]	≤LOQ[c]					
		≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	280[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]
	14	17.5[a]	10.5[a]	10[a]					
		≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	30[c]
	16	24.5[a]	10[a]						
		30[b]							≤LOQ[b]
		40[c]	≤LOQ[c]	30[c]	50[c]	80[c]		≤LOQ[c]	50[c]
	18	15[a]	15[a]	15[a]	15[a]	15[a]	15[a]		
			≤LOQ[c]					≤LOQ[c]	
Diazepam	1	105[a]							
		≤LOQ[b]	40[b]	≤LOQ[b]				≤LOQ[b]	30[b]
		70[c]	50[c]	≤LOQ[c]			50[c]	≤LOQ[c]	30[c]
	7	824[a]							
		170[b]	200[b]	260[b]	350[b]	280[b]	230[b]	270[b]	210[b]
		330[c]	450[c]	530[c]	710[c]	670[c]	500[c]	550[c]	520[c]
	9	98[a]							
		100[b]	40[b]						80[b]
		60[c]	≤LOQ[c]						50[c]
	10	15[a]	15[a]	15[a]	15[a]				
		30[b]	30[b]	≤LOQ[b]	20[b]			≤LOQ[b]	≤LOQ[b]
		50[c]	50[c]	30[c]	30[c]			40[c]	≤LOQ[c]
	11	300[a]	300[a]						
		150[b]	200[b]						80[b]
		≤LOQ[c]	180[c]						100[c]
Flunitrazepam	1	3[a]	4[a]		7[a]	16.25[a]	105[a]	16.25[a]	12.5[a]
		≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	≤LOQ[c]
	7	30[a]							
		≤LOQ[b]							
	8	24[a]							
		≤LOQ[b]					≤LOQ[b]		
	11	30[a]	30[a]						
		50[c]	≤LOQ[c]						≤LOQ[c]
	14		1[a]						
	15		11[a]						
Lorazepam	1		2[a]						
	2	17.5[a]	17.5[a]						
									≤LOQ[c]
	3			8[a]	30[a]				
	5	48.5[a]		21[a]	45[a]	45[a]	45[a]		
		≤LOQ[b]	≤LOQ[b]		≤LOQ[b]	≤LOQ[b]	≤LOQ[b]		
				10[c]			10[c]		
	6	42[a]	16[a]	45[a]	45[a]	45[a]	45[a]		
				110[b]	50[b]	≤LOQ[b]	≤LOQ[b]	≤LOQ[b]	≤LOQ[b]
	7	395[a]	15[a]	15[a]	15[a]	300[a]	300[a]		
		≤LOQ[b]	30[b]	≤LOQ[b]	≤LOQ[b]	≤LOQ[b]	≤LOQ[b]	≤LOQ[b]	30[b]
		≤LOQ[c]	≤LOQ[c]	≤LOQ[c]		≤LOQ[c]	≤LOQ[c]	≤LOQ[c]	

Table 3 (*Continued*)

	Subjects	Month							
		1	2	3	4	5	6	Whole	0–3
	8	102.5[a] ≤LOQ[b]	60[a]	60[a]	60[a]	60[a]	60[a]		
	9	56[a] ≤LOQ[b]							140[b]
	13	4[a]		≤LOQ[b]					
	14		11[a] ≤LOQ[b]	16.5[a]	12[a] ≤LOQ[b]		≤LOQ[b]		
	15	76.5[a]	65[a]						

≤LOQ: detected but lower than the limit of quantification (LOQ).
[a] Amount (mg).
[b] Concentrations (pg/mg in hair).
[c] Metabolite concentrations (pg/mg in hair).

Table 4
Zolpidem concentrations in hair from subjects.

Subjects	Month							
	1	2	3	4	5	6	Whole	0–3
1	512.5[a] 60[b]	367.5[a] 60[b]	190[a] 40[b]	407.5[a] 60[b]	20[a] 70[b]	50[b]	40[b]	50[b]
3	10[b]	10[b]	10[b]	300[a] 10[b]			10[b]	10[b]
4	560[a] 280[b]	300[a] 220[b]	300[a] 160[b]	300[a] 90[b]	300[a] 130[b]	300[a] 200[b]	180[b]	200[b]
5	377.5[a] 150[b]	302.5[a] 180[b]	227.5[a] 100[b]	487.5[a] 130[b]	487.5[a] 220[b]	487.5[a] 170[b]	180[b]	160[b]
6	575[a] 620[b]	300[a] 790[b]	300[a] 180[b]	300[a] 1450[b]	300[a] 1220[b]	600[a] 1580[b]	1780[b]	870[b]
7	1290[a] 480[b]	300[a] 850[b]	300[a] 1410[b]	300[a] 1550[b]	300[a] 930[b]	300[a] 440[b]	1080[b]	1070[b]
8	250[a] 110[b]	10[b]		10[b]		20[b]	30[b]	40[b]
9	150[a] 140[b]	10[a] 40[b]						80[b]
10	410[a] 350[b]	440[b]	660[b]	1160[b]			620[b]	470[b]
12	60[a] 130[b]	300[a] 270[b]	300[a] 290[b]	300[a] 360[b]	300[a] 520[b]	300[a] 340[b]	220[b]	230[b]
13	50[b]	170[b]	320[b]	270[b]			150[b]	280[b]
14	2540[b] ≤LOQ[c]	2270[b] ≤LOQ[c]	2510[b] ≤LOQ[c]	10[a] 3360[b] ≤LOQ[c]	2000[a] 3320[b] ≤LOQ[c]	400[a] 4840[b] ≤LOQ[c]	6410[b] ≤LOQ[c]	1660[b] ≤LOQ[c]
15	470[a] 350[b]	257.5[a] 390[b]						510[b]
16	280[a] 2840[b] ≤LOQ[c]	840[a] 2260[b]	700[a] 2010[b]	700[a] 1970[b]	1630[b]		1800[b]	2700[b] ≤LOQ[c]
17	200[a] 530[b]	100[a] 880[b]	150[a] 870[b]	150[a] 640[b]	150[a] 740[b]	150[a] 830[b]	1140[b]	480[b]
18	300[a] 30[b]	300[a] 150[b]	300[a] 80[b]	300[a] 40[b]	300[a] 50[b]	300[a] 40[b]	50[b]	70[b]

≤LOQ: detected but lower than the limit of quantification (LOQ).
[a] Amount (mg).
[b] Zolpidem concentrations (pg/mg in hair).
[c] Zolpidem-PCA concentrations (pg/mg in hair).

Table 5
Comparison between the amount of drug use and concentrations in hair.

	Total dose/month (mg)	Concentrations in hair (pg/mg)
Alprazolam	1.5–118 (average: 28.70, median: 25)	10–60 α-Hydroxyalprazolam (metabolite) 0–30
Clonazepam	3.5–55 (average: 25.47, median: 24.5)	≤LOQ–30 7-Aminoclonazepam (metabolite) 0–≤LOQ
Diazepam	60–824 (average: 207.44, median: 98)	≤LOQ–350 Nordiazepam (metabolite) ≤LOQ–710
Flunitrazepam	1–30 (average: 15.34, median: 12.5)	≤LOQ 7-Aminoflunitrazepam (metabolite) ≤LOQ–50
Lorazepam	4–395 (average: 49, median: 38)	≤LOQ–140 Lorazepam-glucuronide (metabolite) ≤LOQ–70
Zolpidem	10–2000 (average: 362.79, median: 300)	10–6410 (average: 680, median: 220) Zolpidem-PCA (metabolite) ≤LOQ

In this study, though high concentrations of zolpidem were found in hair, zolpidem-PCA (its metabolite) was rarely detected. A small number of studies have detected zolpidem-PCA in urine [48,49], but the analysis of zolpidem-PCA in hair has not been previously reported. In this study, zolpidem-PCA was only detected at very low values and in a few cases, and no relation was found with zolpidem concentration. The usability of the consumption marker is necessary but parent drug, zolpidem is only confirmed in this study. Diazepam was administered at high doses, and nordiazepam (its metabolite) was easily detected. In fact the concentrations of nordiazepam and of 7-aminoflunitrazepam (metabolite of flunitrazepam) were higher than those of parent drugs. In this study, hair color of 23 subjects was all black and six of them (all Koreans) reported they had dyed their hair brown, which is of important because previously studies have reported concentrations of zolpidem and benzodiazepines in hair are dependent on race and hair color [15,33].

In this study, correlation coefficients between 0.54 and 0.71 were observed in only 3 of 18 zolpidem users, but nevertheless, the study represents a meaningful attempt to determine the nature of the correlation between zolpidem use and zolpidem concentration in 1 cm hair segment. Although subjects took drugs according to their prescriptions, only a few subjects showed a good qualitative correlation between zolpidem or benzodiazepines use and concentrations in hair. Evidently, factors related to transfer from blood to hair for zolpidem and benzodiazepines differ, a larger-scale study with samples other than hair samples is needed to investigate further the nature of the correlation between zolpidem and benzodiazepines use and their concentrations in hair.

Subjects 4 and 5 took similar doses of zolpidem, but concentrations of zolpidem in hair differed by a factor of two. Subject no. 7 took many different drugs (alprazolam, diazepam, flunitrazepam, lorazepam, and zolpidem) and parent drugs and the metabolites (α-hydroxyalprazolam, 7-aminoclonazepam, nordiazepam and lorazepam-glucuronide) were detected. Subject no. 13 reported taking only 4 mg of lorazepam over the previous one month before hair sampling but neither lorazepam nor lorazepam-glucuronide was detected. Subject no. 14 took highest dose of zolpidem (2000 mg) during one month. In other words, before 5 month, he took 200 tablets of zolpidem (10 mg) (50 tablets a week).

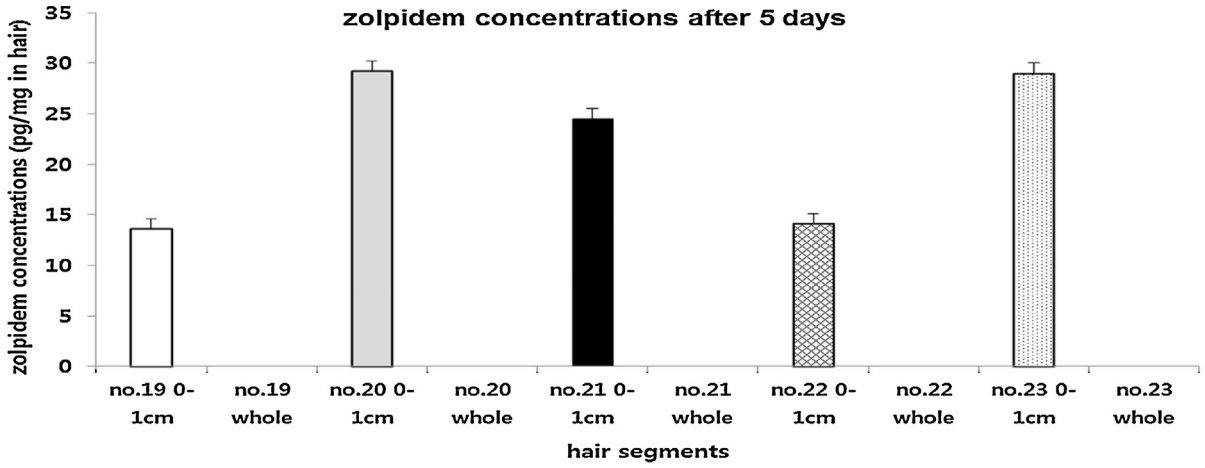
Some have performed controlled studies using volunteers that took a single dose 10 mg of zolpidem or benzodiazepines [14,33,34]. In the present study, subject nos. 19–23 took a single dose (10 mg) of zolpidem and concentrations of zolpidem in 0–1 cm hair segment after five days ranged from 10 to 30 pg/mg (average = 20 pg/mg), and these concentrations were 3.5–5 times higher and the average concentration of zolpidem was 3 times higher than that reported by Villain et al. [33]. In the present study, zolpidem was detected in 0–1 and 1–2 cm hair segments from all subjects and concentrations of zolpidem after 30 days ranged from 10 to 20 pg/mg (average 15 pg/mg) and the average concentration of zolpidem was 16 times lower than that reported by Shima et al. [47], but 3 times higher than that reported by Villain et al. [33].

This was the first study to provide reliable information for forensic investigations and trials by providing detail of the correlation between history of zolpidem or benzodiazepines use and zolpidem or benzodiazepines concentrations in 1 cm segment hair.

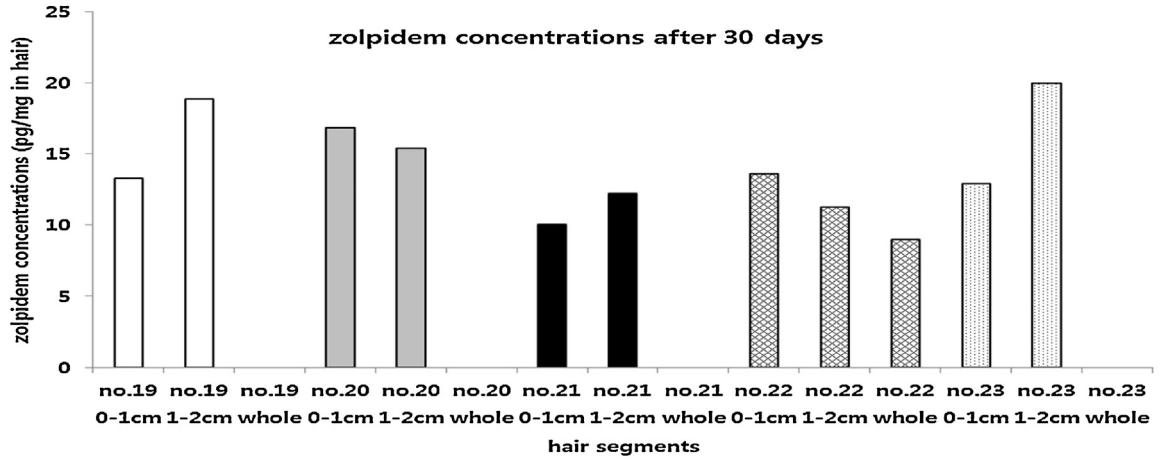
5. Conclusion

In this study, we sought to determine the nature of the relationship between zolpidem and benzodiazepines concentration in hair and cumulative dose in 18 Korean zolpidem and benzodiazepines users in a rehabilitation program. Most subjects took zolpidem or benzodiazepines in therapy. Psychotropic drug users in rehabilitation programs have the will to quit drug use and no fear of legal punishment, and thus, reliably report drug use. In addition, five of subjects took 10 mg of zolpidem on one occasion, and provided hair samples 5, 30, and 60 days later. The correlation found between drug use and drug concentration in hair provides objective information for scientific investigation and trial. Our greatest achievement was to obtain credible zolpidem and benzodiazepines administration records based on inputs from patients, doctors, and counselors and investigate the correlation study between drug consumption and concentrations by segmental hair analysis.

a)



b)



c)

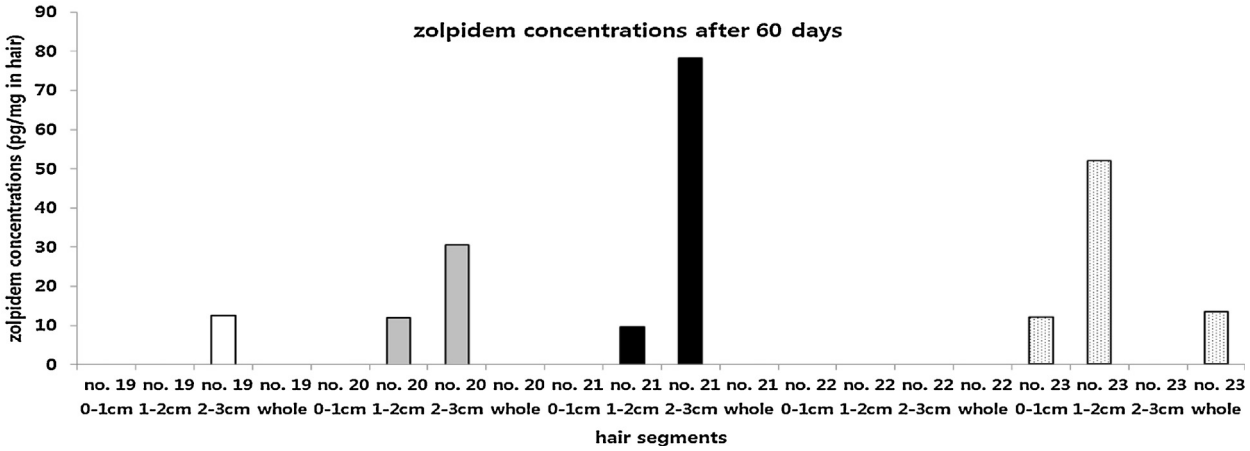


Fig. 7. Hair concentration of zolpidem following a single 10 mg dose of zolpidem after 5, 30 and 60 days.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2017.10.044.

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