Incorporation of Zolpidem into Hair and Its Distribution after a Single Administration


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ABSTRACT

To obtain fundamental information on the drug incorporation into hair, time-course changes in drug distribution along single-strand hair were observed after a single oral administration of zolpidem (ZP), one of the most frequently used hypnotic agents. Quantitative sectional hair analyses of 1-mm segments were performed for each single-strand hair using a validated LC-MS/MS procedure. ZP was detected in all specimens plucked at 10 and 24 hours after a single dose, and the distribution ranged over the whole hair root (4–5 mm in length). A significantly high concentration of ZP was detected in the hair bulb region, whereas much lower concentrations were widely observed in the upper part of the hair root of those samples; this suggested that the incorporation of ZP occurred in two regions, mainly in the hair bulb and to a lesser extent in the upper dermis. The ZP-positive area formed lengths of up to 10–12 mm after a single administration, indicating that its incorporation from the hair bulb would continue for about 2 weeks. Time-course changes in the ZP concentration in the hair root additionally revealed that only a small portion of ZP that initially concentrated in the bulb was successively incorporated into the hair matrix and moved toward the keratinized region as hair grew. These findings should be taken into account upon discussing individual drug-use history based on hair analysis. The matrix-assisted laser desorption/ionization mass spectrometry imaging of ZP in the same kinds of hair specimens was also successfully achieved.

Introduction

Disposition of drugs into hair can provide firm evidence and chronological information on individual drug use, which are often indispensable evidence of crimes and can also be used for therapeutic drug monitoring and drug adherence in the field of clinical medicine. In fact, hair drug tests have recently appeared more frequently in the cases of drug-related crimes, such as drug-facilitated sexual assault and drug abuse (Wada et al., 2010; Johansen and Dahl-Sörensen, 2012; Salomone et al., 2012; Kim et al., 2013; Vincenti et al., 2013; Wille et al., 2014; Kintz, 2016), and in the assessment of medical care (e.g., for HIV patients and alcoholic patients) (Agius et al., 2012; Crunelle et al., 2014; Hickey et al., 2015). It is believed that observation of drug distribution along the hair shaft can provide information about the long-term drug-use history because hair strands grow at a rate of about 1.0–1.2 cm/month, incorporating ingested drugs.

Since the 1990s, the mechanisms of drug incorporation into hair have been intensively investigated to interpret the results of hair analysis properly (Miyazawa and Uematsu, 1992; Henderson, 1993; Cone, 1996; Nakahara, 1999; Beumer et al., 2001). However, the exact mechanisms are still under much discussion despite the growing application of hair tests. It has been generally accepted that the passive-diffusion route from the bloodstream at the base of the hair bulb predominantly contributes to drug incorporation into hair (Baumgartner et al., 1995). However, direct incorporation of drugs from the bloodstream alone cannot explain several experimental results (Henderson, 1993; Potsch et al., 1997; Pragt et al., 1998; Cooper, 2011; Schräder et al., 2012), which suggests the indirect incorporation via sebum and sweat, containing the ingested drugs, by soaking the hair root. Such ambiguity over drug incorporation can mislead the estimation of intake history (e.g., chronic, occasional, or single ingestion) when hair analysis is performed at a high time resolution. With conventional segmental hair analysis (typically divided into 10- or 20-mm segments), temporal resolution in the estimated intake history has been constrained to about 1–2 months (Kintz, 2013), having only a limited effect on the confirmation of facts. Thus, detailed information about drug incorporation pathways into hair, as well as further improvements in the resolution of hair analysis, is absolutely required to overcome such limitations.

To clarify the incorporation pathways of drugs into hair, several researchers have analyzed substance/metabolite in daily shaved beard hairs over a few weeks after a single dose of drug/substance. Kintz et al. (1993) showed that meprobamate, a moderately lipophilic drug, detected in shaved beard hair peaked at approximately 7–9 days after intake, implying that its incorporation predominantly occurred at the lower part of the hair bulb.

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ABBREVIATIONS: ESI, electrospray ionization; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-FTICR MS, matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometer; MSI, mass spectrometry imaging; SRM, selected reaction monitoring; ZP, zolpidem.
of the hair root, including the hair bulb. Contrastingly, Bernert et al. (2011) and Schräder et al. (2012) demonstrated that nicotine/cotinine and ethyl glucuronide, a highly polar minor metabolite of ethanol, were mainly incorporated into hair from the upper part of the hair root (upper dermis zone), not from the hair bulb. However, the following drawbacks exist in their experiment that obscure their results: (1) unavoidable contamination of shaved powdered beard hair with skin scurf that may contain the drug/metabolite; and (2) variability in the growth rate of each hair strand. Recently, Kamata et al. (2015) reported on the time-course changes in the distribution of methoxyphenamine, a nonregulated analog of methamphetamine, in single intact hairs plated at appropriate intervals over 14 days after a single dose. We concluded that there are two major drug incorporation sites, the hair bulb and the upper part of the hair root. These findings suggest that the incorporation pathways and distribution profiles of drugs in single hairs vary considerably depending on the properties of substances/drugs and metabolites.

In the present study, to investigate the incorporation sites of lipophilic zolpidem (ZP) into hair, time-course changes in the distribution along the hair shaft were carefully observed from 10 hours to 70 days after a single oral administration of ZP. Sectional hair analysis of ZP using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedure previously optimized by Shima et al. (2015) was further improved to allow analyses of 1-mm sectioned single hairs and was applied to the investigation of its detailed distribution. Based on these results, the incorporation pathways of ZP into hair are discussed and compared with those of other types of drugs. In addition, mass spectrometry imaging (MSI) of ZP on the longitudinal sections of single-strand hair specimens, one of the most practical methods for monitoring drug distribution, was successfully achieved by matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometer (MALDI-FTICR MS).

Materials and Methods

Study Drug

Sleep medication tablets (containing 10 mg of ZP tartrate/tablet; Myslee) were purchased from Astellas Pharma (Tokyo, Japan). Analytical standards of ZP and ZP-d6, used as an internal standard (IS), were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Subjects and Single-Hair Specimens

Three volunteers (subjects A–C), two males in their 30s and 40s, and one female in her 30s, with straight, black hair of Asian ethnicity, 60-80 μm in thickness who had never received treatment with hypnotic agents before the present study were engaged in this study. They orally ingested a tablet (containing 10 mg of ZP tartrate) once before bedtime. All subjects washed their scalp hair with shampoo every night. Hair specimens were collected from the posterior vertex region at appropriate time intervals. For subject A, hair specimens were collected by plucking with the roots intact using a narrow conductive adhesive tape had been attached lengthwise. The hair was longitudinally cut with a retraction system (Nippon Microtome Laboratory) to the mixture (1:20, v/v; 20 l of chloroform-isopropanol (3:1, v/v). These processes were successively performed in the 2 ml plastic tube that also contained a round-bottomed-test tube

Results

Method Validation. An optimized LC-MS/MS procedure for the sectional analysis of ZP in 1-mm single-hair segments in this study was

| TABLE 1 |
| Optimized SRM parameters of ZP and ZP-d6 (IS) |

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1 m/z</th>
<th>Q3 m/z</th>
<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP</td>
<td>308.2</td>
<td>235.1</td>
<td>91</td>
<td>10</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td>ZP-d6 (IS)</td>
<td>314.1</td>
<td>235.1</td>
<td>91</td>
<td>10</td>
<td>47</td>
<td>13</td>
</tr>
</tbody>
</table>

The transition for quantification is underlined. Q1, quadrupole 1; Q3, quadrupole 3; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.
validated by analyzing various concentrations of ZP spiked into a single drug-free 1-mm hair strand, where ZP was spiked in the extraction buffer (50 mM borate buffer, pH 8.4, 0.2 ml) described in the Materials and Methods section. Table 2 summarizes the validation data. The lower limit of detection was 50 fg ZP/1-mm single hair, and the intraday accuracy and precision at 5 and 50 pg ZP/1-mm single hair were both less than 5%. To assess any potential suppression of ionization from components present in the extracted hair matrix, a decrease in the response was calculated by comparing the ion intensity of ZP (1 pg/ml, n = 5) spiked in distilled water with that of the hair extract from three different hair samples. Consequently, no significant changes in responses were observed (less than 3%; data not shown). The carryover effects were not observed in our range concentration (0.1–200 pg/1-mm single hair). These results guaranteed the satisfactory sensitivity, accuracy, and precision of the procedure.

**Distributions along Hair Shaft Sampled 1 and 2 Months after Intake.** To investigate the ZP distributions along hair shafts after a single administration, single-strand specimens sampled from three volunteers were analyzed using the above-mentioned LC-MS/MS procedure. Figure 1 shows the results obtained from specimens 1–6 (two samples per subject) collected by cutting at 30 or 35 days after intake. ZP was detected in the 8–12 segments located within 15 mm from the root end. Despite single administration, distribution ranged over approximately 10 mm in length. The ZP concentration-segment profiles (hereafter called “the ZP profile”) exhibited a large peak (root side) and a small peak (tip side), with no significant difference in the peak shapes or patterns among all six specimens tested. The main peaks have a local maximum point of about 5–20 pg ZP/1-mm single hair and the tip-seek peaks have a local maximum point of about 1 pg ZP/1-mm single hair (Fig. 1). The total amount of ZP detected in a single-hair specimen ranged from 14 to 43 pg (average, 34 pg; n = 6). Specimen 4, which had the lowest total amount of ZP (14 pg), was characterized by a lower local maximum point of 5 pg in the main peak. In contrast, that of about 1 pg was commonly detected for the tip-side peak, and no significant difference in the ZP amount was noticed among these specimens.

Figure 2 shows the results obtained from specimens 7, 8, and 9 (one sample per subject) collected at 57, 67, or 70 days after intake (from subjects A, B, and C, respectively). ZP was detected across approximately 10 segments, and the two-peak profiles, which were similar to those of the 1-month specimens (Fig. 1), were commonly observed. Also, no significant difference was noticed in the total amount of ZP in each single hair between 1-month specimens (range, 14–43 pg; average, 34 pg; n = 6) and 2-month specimens (range, 37–66 pg; average, 53 pg; n = 3). The ZP-positive segments were located at around 23–25, 18–20, and 11–13 mm from the root side for subjects A, B, and C, respectively. In comparison with the 1-month specimens, each positive band shifted toward the tip side by about 15 mm in 35 days (subject A), 12 mm in 32 days (subject B), and 9 mm in 27 days (subject C).

**Time-Course Changes over 35 Days after Intake.** To investigate the formation process of such two-peak profiles, time-course sectional hair analysis focusing on the hair root was additionally performed using single-hair specimens (two specimens each), which were carefully collected by plucking with the roots intact at appropriate time intervals over 10 hours to 35 days after single administration. The results are shown in Fig. 3. In the 10-hour specimens (plucked 10 hours after intake; specimens 10 and 11 in Fig. 3A), ZP was detected in the 0- to 4-mm segments, which correspond to the whole hair root, but was not detected in the 4- to 10-mm segments that correspond to the hair shaft (outside of the scalp surface). The most ZP-abundant area (about 20 and 50 pg) was the 0- to 1-mm segment including the hair bulb. Much lower concentrations of ZP were commonly observed in the upper part of the hair root (the 1- to 4-mm segments) including the already matured keratinized region, where the ZP profiles exhibited a local maximum point of about 1 pg ZP. In the 24-hour specimens (specimens 12 and 13 in Fig. 3B), ZP was also detected in the 0- to 5-mm segments, and significant amounts of ZP were detected in the 0- to 1-mm segments (about 50 and 100 pg), suggesting that ZP concentration in the hair bulb gradually increased between 10 and 24 hours after intake. In the 1- to 5-mm segments, lower concentrations of ZP were widely observed, and the profiles were similar to those of the 10-hour specimens. In the 72-hour specimens (specimens 14 and 15 in Fig. 3C), ZP was detectable in the 0- to 5-mm segments. The amounts of ZP in the 0- to 1-mm segment (about 30 and 50 pg) in the 72-hour specimens halved from those in the 24-hour specimens, and the small peak in the upper part of hair root slightly shifted toward the tip side (in the 2- to 5-mm segments), while maintaining its peak shape. In the 7-day specimens (plucked 7 days after intake; specimens 16 and 17 in Fig. 3D), ZP was detected in the five or six segments from the root end. The amounts of ZP in the 0- to 1-mm segments (about 16 and 7 pg) were lower than those of the 72-hour specimens, and the tip-side peak appeared to shift toward the tip side (in the 3- to 6-mm or 4- to 7-mm segments). In the 14-day specimens (specimens 18 and 19 in Fig. 3E), the ZP-positive area further spread, forming a 2- to 4-mm distinctive positive area with tailing. The tip-side peak also shifted toward the tip side, located in the 5- to 8-mm or 7- to 10-mm segments. These migration speeds were calculated to be 0.29–0.43 mm/d (4–6 mm/14 days), which is nearly equal to the commonly accepted hair growth rate. Interestingly, the main peak having a local maximum point of about 16 or 8 pg developed 2 weeks after intake. This peak pattern in the profile was significantly similar to those of the 1- and 2-month specimens. In the 35-day specimens (specimens 20 and 21 in Fig. 3F), ZP was detected in the 10 or 11 segments within the range of 7–18 mm from the root end and was not detected in the hair root containing the hair bulb. The ZP profiles correlated well with those of the 1-month specimens.

**MSI of ZP in a Single-Strand Hair.** The 1-mm segmental single-hair analysis by the LC-MS/MS procedure performed in this study requires skilled and time-consuming steps for segmentation, extraction, and instrumentation. A more practical method is necessary in the fields of forensic science and clinical medicine because analysts in such fields often encounter a large number of samples and emergency situations. In this study, MSI of ZP, one of the most practical methods, was examined with single-strand scalp hairs collected after a single administration. Figure 4 shows the imaging results obtained from the 24-hour and 1-month specimens by monitoring the protonated ZP ([C19H22N3O]+) in the m/z window of 308.1757 Da (±0.0001%). In the 24-hour specimen, a notable ZP-positive area was observed in the range just

| Table 2 Validation data of the LC-MS/MS procedure for ZP in 1-mm hair segments |
|------------------|------------------|
| LOD (fg/1-mm hair) | 50               |
| Recoveryb (%) | 20 pg/1-mm hair, n = 10 | 53 |
| Linearityb (r2; 0.1-200 pg/1-mm hair) | 0.999 |
| Intraday accuracy (%) | 5 pg/1-mm hair, n = 10 | 2.4 |
| 50 pg/1-mm hair, n = 10 | 4.1 |
| Precision RSD (%) | 5 pg/1-mm hair, n = 10 | 1.5 |
| 50 pg/1-mm hair, n = 10 | 3.2 |

aLOD, limit of detection; RSD, relative S.D.
bRecovery was calculated by comparing peak areas of ZP extracted from spiked samples with those spiked in hair extract.
cLinearity ranges were tested with seven different concentrations.
under 1mm from the hair root, including the hair bulb. For the 1-month specimen, the positive area was detected in the 2-3 mm range located at the center, 1 cm from the root-side end. The positive areas of both specimens were significantly narrower compared with those detected by LC-MS/MS (about 5 or 10 mm in length). This difference may be attributed to the lower sensitivity of MSI to ZP than that of LC-MS/MS; thus, it is reasonable that only high-concentration regions (more than about 5–10 pg/segment) were detectable by MSI.

**Fig. 1.** Amount of ZP in each 1-mm segment along single-strand hairs collected by cutting from subjects A–C (specimens 1–6) (A–C) about 1 month after a single oral administration of 10 mg of ZP tartrate.
Discussion

Distribution along the Hair Shaft. Distribution of ZP along single-strand hair shafts ranged over approximately 10 mm in length at both 1 and 2 months after a single administration, and the lengths were equivalent to about 1 month in terms of the time required for hair growth. The total amounts of ZP detected in single-strand hair ranged from 14 to 66 pg (average, 40 pg; n = 9; Figs. 1 and 2). We previously analyzed single-strand black hair specimens (n = 15) collected 35 days after a single 10-mg dose of ZP tartrate and demonstrated that ZP was detected in 14 of 15 tested specimens, and the amounts of the drug incorporated into each single hair ranged from 27 to 63 pg (average, 43 pg; n = 14) (Shima et al., 2015). The amounts of ZP obtained here are very close to the previous results. In an earlier study, Cui et al. (2013) quantitated the concentrations of ZP incorporated in black scalp hair sampled about 1 month after a single 10-mg dose of ZP. The concentration of ZP ranged over 135.0–554.6 pg/mg hair (mean, 250 pg/mg hair) in the ZP-positive 2-cm segments. Because an average weight of 0.15 mg was measured for each 2-cm-sectioned single-hair specimen in this study, the average ZP concentration of 40 pg/single-strand hair obtained here was calculated to be about 267 pg/mg hair (40 pg/0.15 mg hair), which was also similar to the results reported by Cui et al. (2013).

The two-peak ZP profiles were observed in almost all of the specimens tested, except for specimen 7 in Fig. 2. A commonly detectable small peak appearing in the tip side has a low local maximum of around 1 pg, and a large peak on the root side has a high local maximum ranging from 5 to 25 pg. In the latter peaks, relatively large individual differences in the local maximum value of ZP exist among samples, which appear to reflect such individual differences in the total amounts of ZP in single hairs. The total amounts of ZP in the 2-month specimens did not appear to decrease compared with those in the 1-month specimens, suggesting that ZP was not severely washed out of the hair shaft through actions like shampooing for at least 2 months after intake. As for the location of the ZP-positive band in the hair shaft, when compared with the 1-month specimens, the positive band in the 2-month specimens moved toward the tip side, by about 15 mm in 35 days (subject A), 12 mm in 32 days (subject B), and 9 mm in 27 days (subject C), which corresponds to the movement rates of 0.43, 0.38, and 0.33 mm/d, respectively, roughly comparable to the generally accepted hair-growth rates. These data revealed that there was no symptom of spreading of the ZP-positive area and no remarkable change in the concentration, whereas the positive area shifted toward the tip side as hair grows.

Incorporation Pathways of ZP into Hair. Time-course changes in the ZP distribution in the hair root at a shorter time frame after intake would be the key in unveiling the mechanism of its incorporation into hair. At 10 and 24 hours after intake, although ZP was detected over the whole hair root (4- to 5-mm lengths), significant amounts of ZP was localized in the 0- to 1-mm segment, including the hair bulb, whereas much lower concentrations (about 1 pg) were commonly found over the 1- to 5-mm segments. Similar two-peak profiles were also found in those of methoxyphenamine, which were reported previously by the authors. Based on a commonly established growth rate of 0.3-0.4 mm/d, the incorporation of ZP occurred through multiple pathways, in the hair bulb and the upper part of the hair root. It was estimated that highly lipophilic ZP was predominantly incorporated into the 0- to 1-mm segment, including the hair bulb, directly from the bloodstream and was slightly but widely distributed over the whole hair root, probably through sweat and/or sebum, which contain ingested ZP, by soaking the hair root near the scalp surface. The exposure time of the hair bulb region to ZP in blood should have an essential effect on its incorporation. Although the time changes in ZP level in the blood of the subjects was not monitored, Weinling et al. (2006) reported that a single oral dose in 24 healthy men attained peak plasma ZP concentrations at approximately 1.6 hours after intake, declining with an average elimination half-life of 2.8 hours. In this study, ZP in the 0- to 1-mm segment, including the bulb, was found to be more abundant at 24 hours than at 10 hours after intake. Thus, ZP in the hair bulb took a much longer time to achieve the peak level than ZP in plasma, suggesting that ZP was gradually accumulated in the hair bulb.

Fig. 2. Amount of ZP in each 1-mm segment along a single-strand hairs collected by cutting from subjects A–C (specimens 7–9) (A–C) about 2 months after a single oral administration of 10 mg of ZP tartrate.
region for roughly 24 hours (range, 10–72 hours) after a single administration, independent from blood concentrations. Interestingly, the concentrations of ZP in the 0- to 1-mm segments of 24-hour specimens were about twice as high as the average of the total ZP amounts detected in 1-month specimens as well as those found in our previous study (Shima et al., 2015) and were approximately four to five times higher than the highest concentrations detected in the hair shafts of the 1- and 2-month specimens. These results suggest that only a small portion of ZP detected in the hair bulb was incorporated into the keratinized hair tissue and that the positive band generated moved...
toward the tip side as hair grew. The subsequent changes in the ZP profiles during the period from several days to 35 days after intake additionally revealed that incorporation from the hair bulb continued for about 2 weeks.

In the upper part of the hair root, ZP was also detected in the 1- to 4-mm and 1-to 5-mm segments of the 10- and 24-hour specimens, and the ZP profiles appeared to be maintained in all the specimens sampled 3–35 days after intake, whereas the positive area shifted toward the tip side. The small peak at the tip side with a local maximum point of about 1 pg was widely and stably detected in all specimens, suggesting that ZP in the upper dermis area was incorporated within 10 hours after intake through separate pathways (e.g., via sweat and/or sebaceous glands), not from the hair bulb.

The ratios of ZP incorporated via the hair bulb to that via the upper part of hair root in the 7-day specimens were 24 for specimen 16 (0- to 3-mm segments/3- to 6-mm segments) and 19 for specimen 17 (0- to 4-mm segments/4- to 7-mm segments), as shown in Fig. 3d. These ratios were significantly higher than those of methoxyphen-amine (0.39–1.6; n = 3) reported previously by Kamata et al. (2015) and also indicate that highly lipophilic ZP was predominantly incorporated through the hair bulb. On the contrary, Schräder et al. (2012) reported that ethyl glucuronide, highly polar minor metabolite of ethanol, was predominantly incorporated into the upper part of the hair root, instead of the hair bulb. These findings indicate that the incorporation pathways of drugs into hair depend heavily on the properties of the drug, such as low/high polarity and pH (base/acid). Drugs with less polar, and basic properties tend to be incorporated predominantly through the hair bulb region taking a passive diffusion route from the bloodstream.

**Practical Application of MSI.** Recently, the application of MSI has been expanded to visualize the localization of drugs in hair, such as methamphetamine, cocaine, and ketamine (Miki et al., 2011a,b; Porta et al., 2011; Poetzsch et al., 2014; Shen et al., 2014; Flinders et al., 2015; Rosen et al., 2016). The authors previously achieved (Shima et al., 2015) the direct detection of ZP incorporated into mustache hair 24 or 32 hours after a single administration and its MSI by MALDI-FTICR MS. In the present study, MSI was further applied to scalp hair specimens collected 24 hours and 1 month after a single dose of ZP and successfully detected ZP signals. The locations of ZP in single-hair specimens were satisfactorily consistent with those detected by LC-MS/MS for 1-mm single-hair segments, although detectable ZP-positive areas were narrower for MSI compared with LC-MS/MS. MSI appears to be inferior in detection sensitivity and quantitative accuracy to LC-MS/MS but is advantageous in spatial resolution and greatly reduces time required for sample preparation and analyses. Although MSI may limit the detectable areas of the drug, it enables a drug history estimation and can distinguish among single, occasional, and multiple ingestions by detecting high-concentration areas. Thus, MSI of drugs in hair is a promising methodology to investigate chronological information of drug ingestion, not only for forensic purposes, but also for clinical purposes.

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Authorship Contributions

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Performed data analysis: Shima
Wrote or contributed to the writing of the manuscript: Shima, Miki, and Katagi

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