Journal of Chromatography B, 878 (2010) 2845–2851

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

The dependence of the incorporation of methamphetamine into rat hair on dose, frequency of administration and hair pigmentation

Eunyoung Han[a,b], Yonghoon Park[a], Eunmi Kim[a], Sooyeun Lee[a], Hwakyung Choi[a], Heesun Chung[a], Joon Myong Song[b,*]

[a] National Institute of Scientific Investigation, 331-1 Shinwol 7-Dong, Yang-Chun Gu, Seoul, South Korea
[b] Research Institute of Pharmaceutical Sciences and College of Pharmacy, Seoul National University, Seoul 151-742, South Korea

ARTICLE INFO

Article history:
Received 15 March 2010
Accepted 28 August 2010
Available online 20 September 2010

Keywords:
Hair analysis
Methamphetamine
Incorporation
GC/MS

ABSTRACT

In this paper, the incorporation of methamphetamine (MA) into rat hair was studied. The main purpose of this study was to investigate whether MA can be detected or positive hair results can be obtained in hair of rats administered a single dose of MA. The relationship between dose and frequency of administration and the concentrations of MA and its metabolite, amphetamine (AP), in rat hair were evaluated and the MA and AP concentrations in white and pigmented hair were compared. MA was administered to rats as follows: low dose (0.5 mg/kg/day), medium dose (2 mg/kg/day) and high dose (10 mg/kg/day). The frequency of administration was one time per day for 1, 2, 3, 4, 5, 15 and 30 days. Hair and urine samples were collected from rats and analyzed by gas chromatography/mass spectrometry (GC/MS). MA could be identified in pigmented rat hair when MA was administered for 4 or more days at low daily dose and on day 1 following administration of medium and high daily doses. Positive results for MA were obtained from pigmented rat hair when MA was administered for 30 days at low daily dose, for 4 or more days at medium daily dose, or for 2 or more days at high daily dose. The concentrations of MA and AP found in rat hair were proportional to the dose and frequency of administration. The concentrations of MA and AP in pigmented rat hair were 2–10 times higher than those in white rat hair. The results of this study on the incorporation of MA into rat hair can serve as a model to better understand the incorporation of MA into human hair even though there are differences between animal models and human hair.

1. Introduction

Methamphetamine (MA) is the most abused drug in our country. Hair analysis can provide information regarding long-term MA use from weeks to years and be used as a supplementary specimen to blood and/or urine. However, the direct relationship of MA concentrations in hair to doses and frequency remains a debate. Most MA abusers tend to deny their consumption or claim only one-time use although they have used MA repeatedly. In this paper, we investigated whether MA can be detected or positive hair results be obtained from hair of rat administered a single dose. We also evaluated the relationship between dose and frequency of administration and the amount of MA and its metabolite, amphetamine (AP), in rat hair.

The research on drug incorporation into hair has focused mainly on cocaine [1–3] and morphine [3–5]. Nevertheless, studies on MA in rat [6–8], monkey [9–10] and human hair [9–11] have been reported. Nakahara et al. [12] reported on methoxyphenamine incorporation into human beard because methoxyphenamine is similar in structure to MA. The studies on MA incorporation into human hair are difficult to perform because of ethical issues and limitations in controlling possible variables [4]. Therefore, animal models such as rats have been used [6–8]. Researchers studied MA incorporation into rat hair but the administered doses were high or the frequency was limited. In acute poisoning research [13], rats were administered MA with variations in dose and frequency of administration and the doses were high (20, 40 and 60 mg/kg). But, in this study, it was necessary to approach the most commonly used dose of MA (30 mg/60 kg/day in human) for MA abusers in our country. There are few studies on the most commonly used dose of MA and the detection threshold in hair. Meanwhile, hair results of animal model have to be confirmed in humans. Jancovic et al. [14] reported that there are differences between animal models and human hair follicles in terms of endogenous substances that affect hair growth. Poet et al. [15] found that there was a significant variation in the amount of cocaine incorporated into hair of similarly treated and genetically identical animals, while Cone et al. [16] reported that, in general, the drug concentrations in animal hair are similar to those found in human hair, and the ratios

* Corresponding author. Tel.: +82 2 2600 4938; fax: +82 2 2600 4939.
E-mail address: homepage2600@hanmail.net (J.M. Song).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.08.040

Table 1
Each group according to dose and frequency of administration.

Dose	Frequency						
	1 day	2 days	3 days	4 days	5 days	15 days	30 days
Low dose (0.5 mg/kg/day)	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Medium dose (2 mg/kg/day)	Group 8	Group 9	Group 10	Group 11	Group 12	Group 13	Group 14
High dose (10 mg/kg/day)	Group 15	Group 16	Group 17	Group 18	Group 19	Group 20	Group 21

of cocaine to its metabolites appear to be similar as well. But few studies [9,10] have demonstrated the correlation between animal models and human hair for MA, and those compared MA concentrations in hair of humans with self-reported drug histories and monkeys.

This paper mainly dealt with rat hair, but the concentrations in rat urine according to dose and frequency of administration of MA and the metabolite-to-parent ratios (AP/MA) were also briefly reported. The concentrations of MA and AP in rat hair and urine were quantified using GC/MS, which is the most effective and easiest way to detect MA and AP [17–19]. An understanding of the incorporation of MA into rat hair is critical for forensic scientists in order to properly interpret the results of hair analysis. The results of animal experiments could be used as a reference when results from human hair are interpreted, although there are differences between animal models and human hair.

2. Materials and methods

2.1. Reagents and standards

MA, AP, MA-d_5 and AP-d_5 were purchased from Radian International LLC (Austin, TX, USA). Methanol, hydrochloric acid, ethylacetate, ethanol, and sodium hydroxide were of analytical grade. Methoxyphenamine, trifluoroacetic anhydride (TFAA) and pentafluoroacetic anhydride (PFPA) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Working standard solutions of MA and AP (1 μg/ml) and of the internal standards AP-d_5 and MA-d_5 (1 μg/ml) were prepared in methanol for hair analysis. Working standard solutions of MA and AP (10 μg/ml) and of the internal standard methoxyphenamine (10 μg/ml) were prepared in methanol for urinalysis. All solutions were stored at −20 °C in the dark until use. MA solutions, which were administered to rats, were made in 0.9% saline. 0.5 mg/ml (low dose), 2 mg/ml (medium dose) and 10 mg/ml (high dose) solutions were prepared and injected intraperitoneally with 0.1 ml solution per 100 g of body weight. MA saline solutions were stored at −4 °C in the dark until use.

2.2. Sample collection

Zucker lean rats were purchased at 6 weeks of age from Charles River Laboratories (Kanagawa, Japan) and acclimated for 2 weeks prior to the study. Rats were allowed free access to water and food (5L79 Formula 18% rat/mouse, Brentwood, MO, USA) for 7 weeks. The experiments were performed according to the National Institutes of Scientific Investigation Guide for the Care and Use of Laboratory Animals, and were approved by the National Institute of Scientific Investigation Animal Care and Use Committee.

The rat hair was shaved with an animal electric shaver 5 days before administration of MA. Zucker lean rats have both white and pigmented hair and before administration each hair was used as a blank hair sample. MA was administered to male rats ($n = 3$ in each group) as follows: low dose (0.5 mg/kg/day), medium dose (2 mg/kg/day) and high dose (10 mg/kg/day). The frequency of administration was one dose daily for 1, 2, 3, 4, 5, 15 and 30 days. Rats were thus divided into 21 different groups ($n = 3$ in each group) that received low, medium and high doses for 1, 2, 3, 4, 5, 15 and 30 days. Each group is shown in Table 1. The newly grown hair was cut 30 days after first administration of MA in each group. The hair samples were stored at room temperature in the dark until analysis.

Rat urine was collected at 32 h after last administration of MA from group 1, 6, 7, 8, 13, 14, 15, 20 and 21. The urine samples were stored at −20 °C in the dark until analysis.

2.3. Sample preparation

The hair analysis was preformed as previously described [17–19]. Approximately 10 mg of hair was weighed and washed. Washing procedure included two washes with water (3 ml) for 5 min followed by two 5-min washes with methanol (3 ml). Then, the hair was cut into small pieces of less than 1 mm. The hair was incubated for 20 h in 1 ml methanol containing 1% hydrochloric acid in the presence of 50 ng of each of the following internal standards: MA-d_5 and AP-d_5. Hair extracts were evaporated under reduced pressure at 45 °C. Thirty microliters of ethylacetate and 30 μl of TFAA were added to the residue, and the mixture was incubated at 65 °C for 30 min. Excess TFAA was removed under a stream of dry nitrogen for 4 min and reconstituted with ethanol (40 μl) prior to GC/MS analysis.

Rat urine samples (1 ml) were extracted by adding 0.5 μg of methoxyphenamine (internal standard), 200 μl of 6 N NaOH and 3 ml of ethylacetate. Samples were shaken for 30 min on a mechanical shaker. Then, samples were centrifuged for 5 min at 2500 rpm to achieve adequate phase separation and the organic phase was transferred into a clean test tube. The extracts were evaporated under reduced pressure at 45 °C and residue was derivatized with 30 μl PFPA and 30 μl ethylacetate at 65 °C for 15 min. Excess PFPA was removed under a stream of dry nitrogen for 4 min and reconstituted with ethanol (40 μl) prior to GC/MS analysis.

For internal standard, different internal standard was used for different matrix. Deuterated compounds were used for accuracy of hair analysis in which the concentrations of MA in hair are ng/mg unit and are about 1000-fold lower than those in urine while methoxyphenamine was used for urine analysis, in which methoxyphetamine is stable, has different retention time with MA and is easily derivatized with pentafluoroacetic anhydride.

2.4. GC/MS analysis

For hair analysis, the GC–MS system consisted of a Hewlett Packard 7683 series injector, HP 6890N series GC system (Wilmington, DE, USA), and HP 5975 inert XL mass selective detector. The column used (Agilent Technologies, Foster, CA, USA) was a fused silica capillary column (HP-5 MS capillary column, 30.0 m × 250 μm × 0.25 μm). The injector was operated in the splitless mode, the injection volume was 1 μl, the injector temperature was 250 °C, the ionization energy was 70 eV and the transfer line temperature was 280 °C. Initial oven temperature was 100 °C,

Table 2
Validation data of MA and AP in white and pigmented rat hair.

Parameters	Concentration targeted (ng/mg)	MA in white hair	AP in white hair	MA in pigmented hair	AP in pigmented hair
LOD (ng/mg)[a]		0.05	0.1	0.05	0.1
Accuracy (%)[b]					
Intra-day ($n=3$)	4	−3.63	0.35	−3.89	1.28
	8	−5.09	1.03	0.28	−0.01
	16	−0.16	2.61	1.21	1.46
Inter-day ($n=18$)	4	−6.29	0.95	0.04	2.42
	8	−4.65	0.63	1.38	1.68
	16	−0.1	2.34	1.87	0.67
Precision (%)[c]					
Intra-day ($n=3$)	4	8.07	4.45	2.91	3.14
	8	2.62	3.15	2.94	1.05
	16	3.76	2.19	1.77	3.66
Inter-day ($n=18$)	4	8.85	4.13	3.79	2.92
	8	3.37	3.70	4.18	2.09
	16	4.8	2.62	3.39	3.99
Extraction efficiency (%)					
($n=3$)	2.5	73	69	105	105
	5	102	98	108	84
	10	109	107	109	107

MA = methamphetamine, AP = amphetamine.
[a] Limit of detection.
[b] Calculated as [(mean calculated concentration − nominal concentration)/nominal concentration] × 100 (% bias).
[c] The coefficient of variance (% CV): SD/mean × 100%.

maintained for 1 min, increasing at 20 °C/min to 270 °C and maintained at this temperature for 10 min. The GC/MS was operated in selective ion monitoring (SIM). The quantification of MA and AP was based on peak area ratios. The *m/z* of TFAA-derivatized MA, AP, MA-d_5 (internal standard), and AP-d_5 (internal standard) was as follows: MA, *m/z* 154, 118, 110, 91; AP, *m/z* 140, 118, 91; MA-d_5, *m/z* 158, 122; AP-d_5, m/z 144, 122 (the underlined ions were used for quantitation). The cut-off values for MA in rat hair were 0.1 ng/mg by GC–MS confirmation, and AP (metabolite of MA) must also be identified above the detection limit.

For urinalysis, the GC–MS system consisted of a Hewlett Packard 7683 series injector, HP 6890N series GC system (Wilmington, DE, USA), and HP 5973N mass selective detector. The column, injection mode, injector temperature and transfer line temperature were the same as those for hair analysis. The oven was programmed to operate at an initial temperature of 100 °C for 1 min, to increase the temperature to 160 °C at a heating rate of 15 °C/min for 3 min and to increase the temperature to 280 °C at a heating rate of 30 °C/min for 10 min. The GC/MS was operated in full scan mode. The PFPA-derivatized ions for MA, AP and methoxyphenamine (internal standard) were as follows: MA, *m/z* <u>240</u>, 160, 119; AP, *m/z* <u>190</u>, 119, 91; methoxyphenamine, *m/z* <u>148</u>, 204, 91 (the underlined ions were used for quantitation).

2.5. Method validation

For hair analysis, method validation was carried out by establishing linearity, limit of detection (LOD), intra- and inter-assay accuracy and precision, and extraction efficiency. In rat hair, six sets of calibrators with MA at concentrations between 0.10 and 50 ng/mg and with AP at concentration between 0.15 and 20 ng/mg were prepared using 10 mg of blank white and pigmented hair, respectively. The LOD was estimated from extracted samples spiked with decreasing concentrations of the compounds, where the response of qualifying ions was equivalent to three times the background noise. Drug-free white and pigmented rat hair samples ($n=3$) were spiked with 4, 8 and 16 ng/mg MA and AP to assess the intra-assay accuracy and precision. For the inter-assay accuracy and precision, drug-free white and pigmented rat hair samples ($n=3$) were spiked with 4, 8, and 16 ng/mg MA and AP, and examined in series on six consecutive days. Extraction efficiencies were determined by adding 25, 50 and 100 ng of standards to 10 mg of pulverized white and pigmented drug-free hair samples, corresponding to 2.5, 5 and 10 ng/mg hair.

For urinalysis, method validation was carried out by establishing linearity, LOD, intra- and inter-assay accuracy and precision, and recovery. In rat urine, six sets of calibrators with MA and AP at concentrations between 0.1 and 50 μg/ml were prepared using 1 ml of blank rat urine. The LOD was estimated from extracted samples spiked with decreasing concentrations of the compounds, where the response of qualifying ions was equivalent to three times the background noise. Intra/inter-assay accuracy and precision were determined by replicate analysis ($n=3$) at three different concentrations (0.2, 2 and 8 μg/ml for MA and AP). The extraction procedure for inter-assay accuracy and precision was repeated independently on six successive days. The recovery of MA and AP was also determined by comparing the analysis of extracted and non-extracted spiked samples at three different concentrations (0.2, 2 and 8 μg/ml).

3. Results

3.1. Method validation for MA and AP in rat hair and urine

Table 2 shows the results of method validation for MA and AP in white and pigmented rat hair. The detection limit of MA and AP in rat hair was 0.05 and 0.1 ng/mg, respectively, using 10 mg of hair. The calibration curves of MA and AP were linear in the concentration range of 0.10–50 and 0.15–20 ng/mg and R^2 was 0.9966 and 0.9994, respectively, in white rat hair, and 0.9989 and 0.9989, respectively, in pigmented rat hair. The inter-assay accuracy and precision were determined using all calibration points analyzed during the study. Accuracy is expressed as % bias of the estimated concentrations. Precision is expressed as % CV. The intra- and inter-assay accuracy and precision for MA and AP in rat hair were all less than 9% at three different concentrations. The extraction efficien-

Table 3
Validation data of MA and AP in rat urine.

Parameters	Concentration targeted (μg/ml)	MA in rat urine	AP in rat urine
LOD (ng/ml)[a]		1	5
Accuracy (%)[b]			
Intra-day (n = 3)	0.2	4.32	12.89
	2	6.36	-10.77
	8	-6.54	9.47
Inter-day (n = 18)	0.2	-5.67	14.99
	2	10.87	2.22
	8	0.92	18.42
Precision (%)[c]			
Intra-day (n = 3)	0.2	14.88	0.96
	2	13.34	8.73
	8	17.12	1.02
Inter-day (n = 18)	0.2	9.81	6.33
	2	11.47	10.59
	8	14.61	10.76
Recovery (%)			
(n = 3)	0.2	84	117
	2	115	89
	8	93	108

MA = methamphetamine, AP = amphetamine.
[a] Limit of detection.
[b] Calculated as [(mean calculated concentration − nominal concentration)/nominal concentration] × 100 (% bias).
[c] The coefficient of variance (% CV): SD/mean × 100%.

cies of MA and AP in rat hair were more than 73% and 69% at three different concentrations, respectively.

Table 3 shows the results of method validation for MA and AP in rat urine. The detection limit of MA and AP in rat urine was 1 and 5 ng/ml using 1 ml of urine, respectively. The calibration curves of MA and AP were linear in the concentration range of 0.1–50 μg/ml and R^2 was 0.9952 and 0.9987, respectively, in rat urine. The intra- and inter-assay accuracy for MA and AP were less than 19%, and the intra- and inter-assay precision for MA and AP were less than 18% at three different concentrations. The recoveries of MA and AP were more than 84% and 89% at three different concentrations, respectively.

3.2. MA and AP concentrations and the AP/MA ratios in rat hair according to dose and frequency

Figs. 1 and 2 show MA and AP concentrations in white and pigmented rat hair after administration of MA to rats at various doses and frequencies. Rats were divided into 21 different groups (n = 3 in each group) that received low, medium and high doses for 1, 2, 3, 4, 5, 15 and 30 days as described in Table 1.

As shown in Fig. 1, the concentrations of MA and AP ranged from 0.18 to 2.82 ng/mg (mean 1.99 ng/mg) and from 0.26 to 0.75 ng/mg (mean 0.51 ng/mg), respectively, in white rat hair. The AP/MA ratios ranged from 0.22 to 0.27 (mean 0.25) in white rat hair. MA was detected in white rat hair when MA was administered for at least 15 days at low daily dose, for 4 or more days at medium daily dose, or for 2 or more days at high daily dose. When the cut-off level (0.1 ng/mg) was applied to our results from rat hair, positive results for MA in white rat hair were obtained only when MA was administered for 15 and 30 days at high daily dose although the cut-off level (0.5 ng/mg) from human hair in our country was generally applied to MA abusers for determination of MA consumption. AP was detected in white rat hair only when MA was administered for 15 and 30 days at high daily dose.

As shown in Fig. 2, the concentrations of MA and AP ranged from 0.22 to 27.61 ng/mg (mean 3.86 ng/mg) and from 0.15 to 4.46 ng/mg

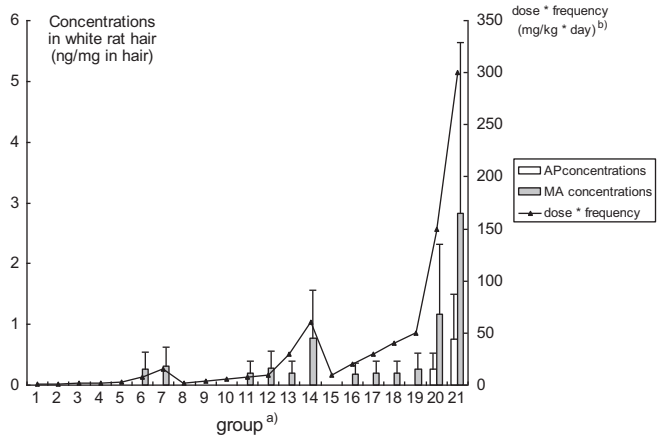

Fig. 1. The concentrations of MA and AP in white rat hair according to dose and frequency of administration (n = 3 rats in each group. a)Each group was described previously in Table 1. b)Dose × frequency (mg/kg day) unit means amount administered in a dose of 0.5 mg/kg/day (low dose), 2 mg/kg/day (medium dose) and 10 mg/kg/day (high dose) multiplied by frequency of 1, 2, 3, 4, 5, 15 and 30 days.

(mean 1.02 ng/mg), respectively, in pigmented rat hair. The AP/MA ratios ranged from 0.08 to 0.36 (mean 0.17) in pigmented rat hair. MA could be identified in pigmented rat hair when MA was administered for 4 or more days at low daily dose and on day 1 following administration of medium and high daily doses. But, positive results for MA in pigmented rat hair were obtained when MA was administered for 30 days at low daily dose, for 4 or more days at medium daily dose, or for 2 or more days at high daily dose. AP was detected in pigmented rat hair when MA was administered for 30 days at low daily dose, for 4 or more days at medium daily dose, or for 2 or more days at high daily dose.

3.3. MA and AP concentrations and the AP/MA ratios in rat urine according to dose and frequency

Rat urine was collected at 32 h after last administration of MA from group 1, group 6, group 7, group 8, group 13, group 14, group 15, group 20 and group 21. The concentrations of MA and AP ranged from 1.12 to 121.09 μg/ml (mean 24.80 μg/ml) and from 1.22 to 57.56 μg/ml (mean 20.00 μg/ml) in rat urine, respectively.

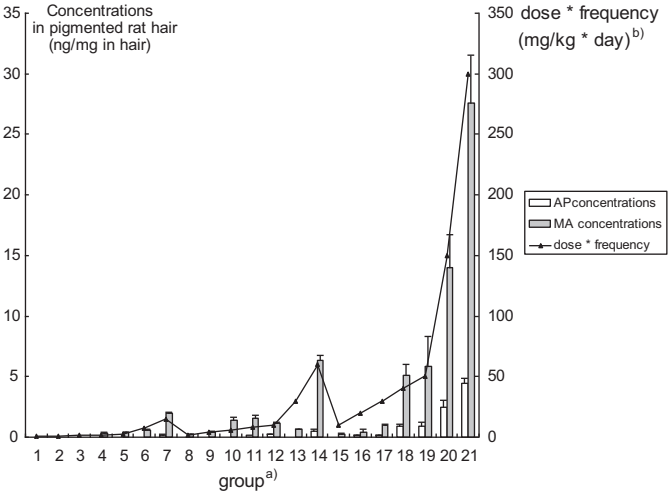

Fig. 2. The concentrations of MA and AP in pigmented rat hair according to dose and frequency of administration (n = 3 rats in each group. a)Each group was described previously in Table 1. b)Dose × frequency (mg/kg day) was described previously in Fig. 1.

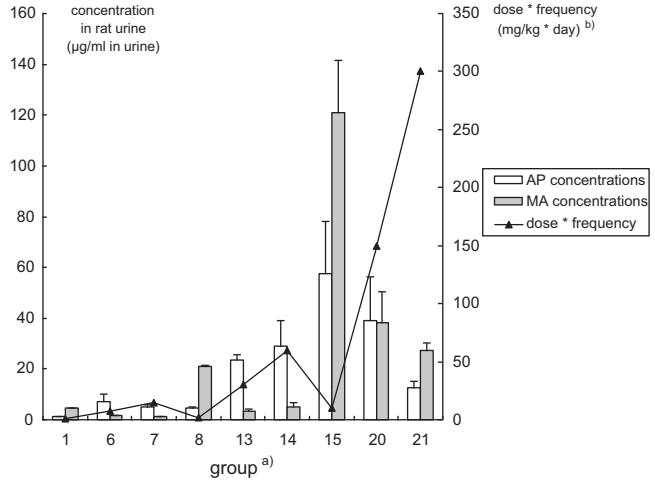

Fig. 3. The concentrations of MA and AP in rat urine according to dose and frequency of administration ($n = 3$ rats in each group. a)Each group was described previously in Table 1. b)Dose × frequency (mg/kg day) was described previously in Fig. 1.

The AP/MA ratio in rat urine ranged from 0.23 to 6.93 (mean 2.71) (Fig. 3).

4. Discussion

4.1. MA and AP concentrations and the AP/MA ratios in rat hair according to dose and frequency

Police records show that 30 mg/60 kg is the dose most frequently used by MA users in our country. However, a dose that is most frequently abused by individuals cannot be equivalent to the dose administered to rats. Shannon et al. [20] suggested that the animal dose should not be extrapolated to a human equivalent dose (HED) by a simple conversion based on body weight and advocated the use of the body surface area (BSA). Therefore, when applied to the conversion equation based on BSA as suggested by Shannon et al., the 30 mg/60 kg human dose is equivalent to 3.13 mg/kg for rats. This dose (0.5 mg/kg) was referred to as "low dose" in the present study using rats. With respect to the manipulating dose, this study was designed to compare three different doses: low dose (0.5 mg/kg/day), medium dose (2 mg/kg/day) and high dose (10 mg/kg/day). Various frequencies, short-term frequency (1–5 days) and long-term frequency (15–30 days), were designed because MA abusers appear to vary in their dosage/frequency of use from beginner to chronic users.

In this study, we investigated whether MA can be detected or positive hair results obtained in hair of rats administered a single dose. Researchers studied MA incorporation into rat hair, but the administered doses were high or the frequency was limited [8,12,13]. MA and AP levels and the AP/MA ratios in rat hair in this study were consistent with our previously published results [18,21] for human hair. Previously published results (drug concentrations in animal hair) were compared to the present results after conversion into dose × frequency unit as shown in Figs. 1 and 2 because of different doses and frequencies of administration among papers. When codeine was administered by intraperitoneal injection (10, 20, 40, or 60 mg/kg/day) daily for 5 days, the mean concentrations of codeine and morphine in rat hair for each group were much lower [4] and when three monkeys were administered with morphine at 10 mg/kg for 10 days, the concentrations of morphine in monkey hair was lower than the present results [22]. However, after the rats were intraperitoneally (i.p.) administered with cocaine hydrochloride at 5 mg/kg for 5 days, the concentrations of cocaine and its metabolite were much higher [2,23] and when the rats were intraperitoneally administered with MA at 5 mg/kg for 10 successive days and the concentrations of MA were approximately 2 times higher than the present results [6,24].

The relationship between dose and frequency of administration and the concentrations of MA and AP in rat hair was also evaluated. Several researchers have demonstrated a relationship between dose and frequency and drug concentrations in hair. Some authors have found that there is a positive relationship between them [1–5,8,9], but others have reported no correlation between them in self-reported human experiments [25,26]. In this study, there was a positive exponential relationship among dose, frequency of administration and MA concentration in rat hair. This has also been observed with codeine in an experimental model [27]. In the present study, there was little change in MA concentrations in rat hair in the low and medium dose groups according to frequency, but some changes were seen in high dose groups according to frequency. Generally, there was little change in MA concentrations in rat hair according to frequency while there was much change according to dose; therefore, this study indicated that the dose more strongly affected MA and AP concentrations in rat hair than the frequency.

This study also determined the AP/MA ratios in pigmented rat hair. AP was mostly below the detection limit in white rat hair, and therefore the AP/MA ratios in white rat hair were not discussed. The AP/MA ratio (0.36) in pigmented rat hair was highest in group 16 (for 2 days at high dose). In groups 17–21 among high dose groups, the AP/MA ratios (0.15–0.18) remained constant. Because both MA and AP concentration in rat hair in high dose groups increased at the same rate according to frequency, the AP/MA ratios were constant. In our previous studies [18,21], the AP/MA ratios decreased as the MA concentrations increased; therefore, we concluded that the drug metabolism of heavy drug users is saturated. However, in this study on rat hair, the significant patterns and the results were inconsistent with our previous results. Enzyme polymorphisms and conversion of MA to hydroxymethamphetamine might have contributed to different ratios in rodents compared to man. MA is included in the cytochrome P450-mediated "debrisoquine oxidation polymorphism" panel. The human gene (CYP2D6) is responsible for the "poor metabolizer" (PM) and "extensive metabolizer" (EM) phenotypes: a similar polymorphism (the CYP2D1 gene) exists in rats and each rat shows the PM or EM trait [28]. Caldwell et al. [29] reported that for the metabolism of MA according to species, the percentage of unchanged MA excreted in urine was lower and that of 4-hydroxymethamphetamine was higher in rats compared to humans [30]. Sample size can be an important factor in determining the AP/MA ratios. In this study, we analyzed hair from 3 rats in each group, while previous studies dealt with hair samples from more than 2000 suspects.

Some papers have compared drug concentrations in pigmented and non-pigmented hair [6,7,27,31–33]; in this study, Zucker lean rats, which have both white and pigmented hair, were used to compare MA and AP concentrations in these hair types. The presence of greater MA concentrations in pigmented hair than in white hair in this study is consistent with some reports [6,7,27,31–33]. Drugs rapidly incorporate into melanin-rich structures and slowly incorporate into melanin-deficient structures [8,34,35]. Likewise, Nakahara and Kikura [24] reported that drug incorporation rates were high in melanin-rich structures. In the present study, even after a 30-day repeated administration of MA at 10 mg/kg/day, the concentration of MA in the white hair was 2.82 ng/mg, and this result was consistent with previous results [8] in which animal experiments using white hairy rat administered MA have been reported. Also, Nakahara et al. [9,10] reported the incorporation of MA and AP into pigmented hair of animals based on stable isotope dilution gas chromatography/mass spectrometry. In the present study, the difference between MA concentrations in white and

pigmented rat hair was relatively small in low dose groups, but was significant in high dose and frequency groups. There was little change in MA concentrations in white rat hair according to dose and frequency in comparison to pigmented rat hair, and AP was hardly detected in white rat hair. Based on these results, white hair is an important parameter in the evaluation of MA concentrations in suspects' hair.

4.2. MA and AP concentrations and the AP/MA ratios in rat urine according to dose and frequency

MA and AP concentrations and the AP/MA ratios in rat urine were investigated according to dose and frequency. These results were compared with rat hair results. The concentrations of MA and AP (121.09 and 57.56 μg/ml respectively) in rat urine were highest in group 15 (one time at high dose) among all groups. The concentrations of MA and AP in rat urine were proportional to dose (low, medium and high dose). But MA concentrations in rat urine were inversely proportional to the frequency in each dose group. AP concentrations in rat urine increased in low and medium dose groups, but decreased in high dose group according to frequency. Therefore, the relationship between MA and AP concentrations in rat urine and frequency was inconsistent with the results from rat hair. High dose administration more strongly affected MA and AP concentrations in rat urine than frequency, which was consistent with the hair results. AP concentrations in rat urine were 10,000–14,000 times higher than those in rat hair and MA concentrations in rat urine were 4000–5000 times higher than those in rat hair.

The AP/MA ratios in rat urine were also evaluated. The AP/MA ratios in rat urine increased in low and medium dose groups according to frequency but were constant in high dose groups. Because both MA and AP concentrations in rat urine in high dose groups decreased at the same rate according to frequency, the AP/MA ratios (0.46-1.02) were constant. The AP/MA ratio in group 13 (for 15 days at medium dose) was highest, followed by that in group 14 (for 30 days at medium dose). The AP/MA ratios in rat urine after administration of MA one time at low, medium and high doses were extremely low. The high frequency caused high AP/MA ratios in rat urine because MA gradually decreased according to frequency. The AP/MA ratios in urine were 10–20 times higher than hair, which was consistent with the report of Kim et al. [36]. In their report, they suggested some possible factors for consideration. The differences of matrix type (solid and liquid) and metabolism (absorption and elimination) are possible factors.

5. Conclusions

In most drug incorporation studies using animals, administration of a high dose of drug has been investigated and frequencies of administration were limited or fixed in each experiment, but in this study we designed various methodologies such as administration of low, medium and high doses of MA and administration of a single dose, or repeatedly up 30 days using rats, which simulated administration of human MA users.

From this investigation, the following conclusions can be drawn:

(1) MA was detected in white rat hair when MA was administered for at least 15 days at low daily dose, for 4 or more days at medium daily dose, or for or more 2 days at high daily dose. Positive results for MA in white rat hair were obtained only when MA was administered for 15 and 30 days at high daily dose.
(2) MA could be identified in pigmented rat hair when MA was administered for 4 or more days at low daily dose and on day 1 following administration of medium and high daily doses. Positive results for MA in pigmented rat hair were obtained when MA was administered for 30 days at low daily dose, for 4 or more days at medium daily dose and for 2 or more days at high daily dose.
(3) There was a positive relationship among dose, frequency of administration and MA concentration in rat hair. This study indicated that the dose more strongly affected MA and AP concentrations in rat hair than the frequency.
(4) MA and AP concentrations in pigmented rat hair were higher than those in white rat hair in all groups and 2–10 times higher than those in white rat hair, and therefore white hair is an important parameter in the evaluation of MA concentrations in suspects' hair.
(5) The concentrations of MA and AP in rat urine were proportional to dose. But, MA concentrations in rat urine were inversely proportional to the frequency in each dose group.

MA and AP concentrations in rat hair were consistent with those found in human hair, and the AP/MA ratios were within the range of those found in human hair. The results of the rat model can not be directly extrapolated to man and experiments in humans are necessary to determine the relevance of animal data to the incorporation of MA into human hair, but this preliminary animal study was important because of ethical issues and limitations in controlling possible variables in human experiments.

Acknowledgements

This study was supported by funding from National R&D Program of Ministry of Education, Science and Technology (MEST), Korea. The authors would like to thank ChunSam Kim, Boram Kang and Seewun Cho for their technical skills and help.

References

[1] A.P. Ferko, E.J. Barbieri, G.J. DiGregorio, E.K. Ruch, Life Sci. 51 (1992) 1823.
[2] Y. Nakahara, T. Oehiai, R. Kikura, Arch Toxicol. 66 (1992) 446.
[3] Y. Nakahara, R. Kikura, K. Takahashi, K. Konum, Adv. Chem. Diagn. Metab. Dis. (1994) 187.
[4] S.P. Gygil, F. Colon, R.B. Raftogianis, R.E. Galinsky, D.G. Wilkins, D.E. Rollins, Drug Metab. Dispos. (1996) 282.
[5] S.P. Gygi, D.G. Wilkins, D.E. Rollins, J. Anal. Toxicol. 19 (6) (1995) 387.
[6] Y. Nakahara, R. Hanajiri, Life Sci. 66 (7) (2000) 563.
[7] Y. Nakahara, R. Kikura, K. Takahashi, Life Sci. 63 (10) (1998) 883.
[8] T. Niwaguchi, S. Suzuki, T. Inoue, Arch. Toxicol. 52 (1983) 157.
[9] Y. Nakahara, K. Takahashi, M. Shimamine, Y. Takeda, J. Forensic Sci. 36 (1991) 70.
[10] Y. Nakahara, K. Takahashi, Y. Takeda, K. Konuma, S. Fukui, T. Tokui, Forensic Sci. Int. 46 (1990) 243.
[11] G.A. Cooper, D.L. Allen, K.S. Scott, J.S. Oliver, J. Ditton, I.D. Smith, J. Forensic Sci. 45 (2) (2000) 400.
[12] Y. Nakahara, K. Takahashi, K. Konumab, Forensic Sci. Int. 63 (1993) 109.
[13] Y. Nakahara, R. Kikura, M. Yasuhara, T. Mukai, Forensic Sci. Int. 84 (1997) 157.
[14] S.M. Jankovic, S.V. Jankovic, Dermatology 4 (1) (1998) 2.
[15] T. Poet, F. Martinez, R.R. Watson, Forensic Sci. Int. 54 (1992) 29.
[16] E.J. Cone, M.J. Welch, M.B.G. Babecki, NIH Publication No. 95-3727, 1995, p. 91.
[17] E. Han, W. Yang, J. Lee, Y. Park, E. Kim, M. Lim, H. Chung, Forensic Sci. Int. 147 (2005) 21.
[18] E. Han, Y. Park, W. Yang, J. Lee, S. Lee, E. Kim, M. Lim, H. Chung, Forensic Sci. Int. 161 (2006) 124.
[19] E. Han, E. Miller, J. Lee, Y. Park, M. Lim, H. Chung, F.M. Wylie, J.S. Oliver, J. Anal. Toxicol. 30 (2006) 383.
[20] R.S. Shannon, N. Minakshi, A. Nihal, FASEB 22 (2007) 6591.
[21] S. Lee, E. Han, Y. Park, H. Choi, H. Chung, Forensic Sci. Int. 190 (1–3) (2009) 16.
[22] Y. Nakahara, K. Takahashi, M. Shimamine, A. Saitoh, Arch. Toxicol. 66 (1992) 669.
[23] Y. Nakahara, R. Kikura, Arch. Toxicol. 68 (1994) 54.
[24] Y. Nakahara, R. Kikura, Arch. Toxicol. 70 (1996) 841.
[25] K. Tassiopoulos, J. Bernstein, T.H. SuzetteLevenson, R. Hingson, E. Bernstein, Addiction 99 (2004) 590.
[26] F. Musshoff, F. Driever, K. Lachenmeier, D.W. Lachenmeier, M. Banger, B. Madea, Forensic Sci. Int. 156 (2006) 118.
[27] L. Potsch, G. Skopp, M.R. Moeller, J. Forensic Sci. 42 (1997) 1095.
[28] C.V. Vorhees, T.M. Reed, M.A. Schilling, J.E. Fisher, M.S. Moran, G.D. Cappon, D.W. Nebert, Neurotoxicol. Teratol. 20 (1998) 265.

[29] J. Caldwell, L.G. Dring, R.T. Williams, Biochem. J. 129 (1972) 11.
[30] NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Amphetamine and Methamphetamine, National Toxicology Program US department of Health and Human Services, March 2005, http://cerhr.niehs.nih.gov/chemicals/stimulants/amphetamines/Amphetamine_final.pdf.
[31] B. Gerstenberg, G. Schepers, P. Voncken, H. Volkel, Drug Metab. Dispos. 23 (1995) 143.
[32] P.R. Stout, J.A. Ruth, Drug Metab. Dispos. 27 (1999) 731.
[33] P.R. Sout, D.J. Claffey, J.A. Ruth, Drug Metab. Dispos. 28 (2000) 286.
[34] T. Uematsu, N. Miyazawa, O. Okazaki, M. Nakashima, J. Pharm. Sci. 81 (1992) 45.
[35] A. Mizuno, T. Uematsu, A. Oshima, M. Nakamura, M. Nakashima, Ther. Drug Monit. 15 (1993) 99.
[36] E. Kim, J. Lee, H. Choi, E. Han, Y. Park, H. Choi, H. Chung, Ann. Toxicol. Anal. 20 (3) (2008) 145.