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Hair analysis for drugs of abuse VIII. Effective extraction and determination of 6-acetylmorphine and morphine in hair with trifluoroacetic acid–methanol for the confirmation of retrospective heroin use by gas chromatography–mass spectrometry

Yuji Nakahara*, Ruri Kikura, Kazunori Takahashi

National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

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Abstract

A procedure for the detection and determination of 6-acetylmorphine (6AM) and morphine in rat and human hair is described. The efficiency of extraction of 6AM and morphine from hair was compared between five extraction methods using methanol, 0.1 *M* hydrochloric acid, methanol–5 *M* hydrochloric acid (20:1), helicase and methanol–trifluoroacetic acid (TFA) (9:1). Methanol–TFA was found to be the best solvent for extracting 6AM and morphine with minimum hydrolysis and maximum extraction efficiency of 6AM. The extraction rates of 6AM and morphine from heroin abuser's hair with methanol–TFA reached a plateau after 8–10 h. After the extraction from hair with deuterated internal standards added, the extract was purified by Bond Elut Certify in the usual manner. The 6AM and morphine were then derivatized with bis(trimethylsilyl)acetamide, which were characterized by GC–MS with electron impact ionization. Confirmation was accomplished by comparing their retention times and the relative abundances of major four selected ions with those of the standards. Quantification was based on the deuterated internal standards. Linearity was obtained in the concentration range 0.1–50 ng/mg. The overall recoveries after solvent extraction and solid-phase purification were 95% for 6AM and 97% for morphine. The limit of detection for confirmation was 0.2 ng/mg. The coefficients of variations for 6AM and morphine at concentrations of 4 ng/mg were generally less than 8%. 6AM was stable during extraction with methanol–TFA (9:1) at room temperature. This method was applied to the elucidation of heroin drug history by using hair sectional analysis.

1. Introduction

In recent years, forensic toxicologists have been strongly concerned about the distinction between heroin use and the use of morphine, codeine and poppy seed food by drug testing. In the urine of a person who takes such a morphine-like drug, morphine and its glucronides are commonly seen except for 6-acetylmorphine in a limited period after heroin use. Drug testing for opiates has often involved a hydrolysis procedure under enzymatic or acidic conditions.

* Corresponding author.

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Therefore, it has been not easy for urine drug testing to prove the use of heroin. In a modern laboratory with the sophisticated analytical instruments, it has been required routinely to distinguish heroin use from other opiate use in various cases. It is very important to be able to identify a unique metabolite of heroin, 6-acetylmorphine (6AM), by drug testing. Recently, several identification methods for 6AM in urine have been reported [1–9]. However, because of the very short lifetime of 6AM in urine, it is difficult to detect it 12 h or more after the last use. It has been suggested that hair might have some advantages for detecting some unstable compounds such as 6AM [10–12]. Although many studies on the detection of morphine in hair have been reported [13–19], the effective extraction of 6AM from hair has not yet been studied.

This paper describes the effective extraction of 6AM from hair with minimum hydrolysis using methanol–trifluoroacetic acid (TFA) and its application to the elucidation of retrospective heroin abuse history over 1 year.

2. Experimental

2.1. Chemicals

Morphine hydrochloride was purchased from Takeda Pharmaceutical (Osaka, Japan). 6-Acetylmorphine (6AM), normorphine, morphine-d_3 and 6AM-d_3 were synthesized as described previously [11]. Bond Elut Certify was purchased from Varian (Harbor City, CA, USA); it contains a mixture of bonded silica gel and a cation exchanger.

2.2. Hair samples

Animal hair samples

Three Dark-Agouti rats (male, aged 5 weeks, 90–120 g) were administered heroin, 6AM and morphine independently at 2.5 mg/kg once a day for 10 days. Before drug administration, the back hair of the animals was cut with an animal electric shaver and used as control hair. Back hair newly grown on the same area was collected 4 weeks after the first administration.

Human hair samples

Hair samples were collected from the posterior vertex of the scalp by cutting near the scalp. The root sides of the hair samples obtained were bundled with a rubber band and the samples were wrapped in aluminium foil.

Scalp hair sampales from heroin abusers, ST-2 (male, age 27), ST-5 (male, age 30) and ST-12 (female, age 32), were collected and their drug histories were recorded by Dr. A. Saitoh of Kanagawa Psychiatry Serigaya Hospital. The heroin abuse histories of three abusers are described under Results and discussion.

2.3. Preparation of the standard hair samples

To 10 mg of finely cut control human hair in a 10-ml glass tube was added 0.1 ml of methanolic solutions of 6AM and morphine (0.01–10 μg/ml) to give concentrations of 0.1–50 ng/mg hair, respectively. The hair sample was mixed occasionally with a vortex mixer while the solvent was naturally evaporated at room temperature. This sample obtained in the glass tube was used as a standard hair sample (positive control).

2.4. Effect of five solvents on the efficiency of extraction of 6AM and morphine from hair

The efficiency of extraction of 6AM and morphine from rat hair was investigated with five solvents: methanol, 0.1 *M* hydrochloric acid, methanol–hydrochloric acid (20:1), helicase solid phase extraction and methanol–TFA (9:1). Extraction was carried out by keeping the sample in 2 ml of solvent overnight following ultrasonication for the first hour, except for ultrasonication for 14 h in methanol. The pretreatment using helicase, guanidine and 2-mercaptoethanol in phosphate buffer (pH 7.0) at 45°C for 4 h and extraction with Bond Elut Certify

were carried out according to Ahrens *et al.*'s method [12].

2.5. Standard analytical methods

The hair samples were washed three times with 10 ml of 0.1% sodium dodecylsulphate (SDS) and 10 ml of distilled water under ultrasonication for 1 min. After the hair had been dried and finely cut, to each section of hair (4–8 mg) were added 100 μl of internal standard aqueous solution containing 6AM-d_3 and morphine-d_3 at 400 n g/ml and the mixture was extracted with 2 ml of methanol–TFA (9:1) in a 10-ml stoppered glass tube at room temperature for 1 h under ultrasonication and allowed to stand overnight. The hair was filtered off and the solvent was evaporated under a nitrogen stream. The residue was dissolved in 2 ml of 0.1 *M* phosphate buffer (pH 6.0) and applied to Bond Elut Certify, followed by rinsing with water (2 ml), 0.1 *M* acetic acid (2 ml) and water (2 ml). After drying the column with an aspirator for 5 min, it was rinsed again with methanol (2 ml) followed by drying for 5 min. The fraction containing the drug was eluted with methanol–chloroform–ammonia (80:20:2). The combined extract was evaporated to dryness under a nitrogen stream and the residue was dissolved in 50 μl of bis(trimethylsilyl)acetamide and heated at 80°C for 20 min. A 1-μl volume of the reaction mixture was analysed by GC–MS using a Hewlett-Packard Model 5890/MSD597 instrument and a TC-1 capillary column (GL Science, Tokyo, Japan) (15 m × 0.25 mm I.D.) with a 0.25-μm film thickness, injection temperature 200°C and column temperature programmed from 60°C (0.5-min hold) to 280°C (5-min hold) at 20°C/min. The multimode inlet was operated in the splitless mode and the carrier gas was helium at a pressure of 31 kPa. The mass spectrometer was operated in the selected-ion monitoring (SIM) mode for TMS-6AM at *m/z* 399.15, 340.00 and 287.00, for TMS-6AM-d_3 at *m/z* 402.10, for TMS_2-morphine at *m/z* 429.15, 414.00 and 287.00 and for TMS_2-morphine-d_3 at *m/z* 432.15.

2.6. Recovery of 6AM from hair sample

A standard solution of morphine in methanol was added to the control human hair at a concentration of 4 ng/mg and then the hair samples were mixed well with a vortex mixer and naturally dried at room temperature. A 10-mg hair sample was extracted with 2 ml of methanol–TFA (9:1) and analysed as described above.

2.7. Application to analysis of heroin abuser's hair

For the exact arrangement of the length from the root of 40–50 hairs, the samples were pasted straight on adhesive paper from the root side, then 2-cm sections from the root side were cut with scissors. At 10 or 20 min after each section was soaked in 0.1% SDS, the hair tips were peeled off from the paper. After removing the paper, the hair tips were washed three times with 10 ml of 0.1% SDS and 10 ml of distilled water under ultrasonication for 1 min. After the hair had dried, each section of hair (4–8 mg) with 100 μl of the I.S. aqueous solution added was pretreated and analysed as described above.

3. Results and discussion

3.1. Time course of extraction of 6AM from heroin abuser's hair

Using a heroin abuser's hair, the optimum time for extraction of 6AM and morphine from the hair was investigated with methanol–TFA (9:1). The extraction of the two drugs from the hair almost reached a maximum after 8 h, as shown in Fig. 1.

3.2. Recovery of 6AM from spiked hair samples

The recoveries of 6AM from hair samples were more than 94.7% and the coefficients of variation (C.V.) were less than 7.94% in six analyses (Table 1). During the extraction of 6AM from the spiked hair with methanol–TFA,

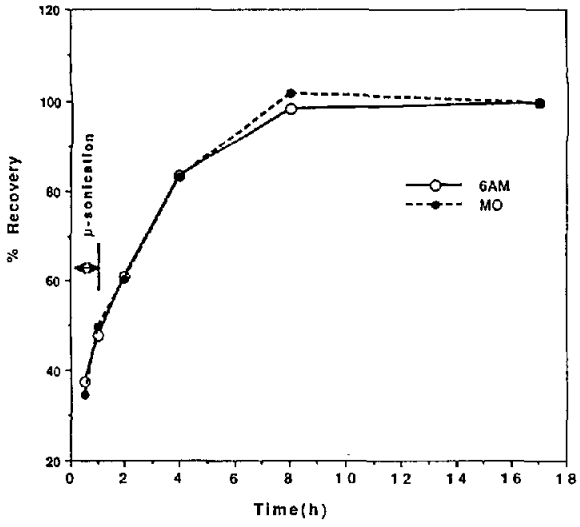

Fig. 1. Time course of extraction rate of (○) 6AM and (●) morphine from heroin abuser's hair. Hair samples of 50 mg were extracted with 5 ml of methanol–TFA (9:1) and 500 μl of each extract were analysed to determine the concentrations of the drugs extracted for 0.5, 1, 2, 4, 8 and 17 h. The results are means of three experiments.

a small amount (1.27 ng) of morphine was produced. Therefore, the amount of morphine extracted from the spiked hair with 4AM and morphine apparently exceeded 100% of the original amount of morphine. The degradation of 6AM to morphine by our method, however, is not so high and the accurate determination of 6AM and morphine in hair can be achieved by using deuterated 6AM.

3.3. Confirmation of 6AM in rat and human hair

To a 10-ml tube containing washed hair (20 mg) was added 2 ml of methanol–TFA (9:1) and extracted as described above. The SIM chromatograms of the extract from rat hair contaminated with heroin and the control rat hair are shown in Fig. 2.

3.4. Limit of detection for confirmation

The limit of detection for 6AM in spiked hair was determined by confirmation of the three major ions (*m/z* 399, 340 and 287) on the GC–MS–SIM trace. Although the limit of detection was less than 0.05 ng/mg hair with S/N > 3 for the highest single ion (*m/z* 399.15) for 6AM extracted from a hair specimen, the limit of confirmation was determined to be 0.2 ng/mg, which allows the distinction of three major ions (*m/z* 399, 340 and 287 for 6AM; *m/z* 429, 414 and 287 for morphine) from the noise level, as shown in Fig. 3.

3.5. Calibration graphs

Linear calibration lines were obtained when the *m/z* 399/402 and 429/432 peak-area ratios were plotted against concentration (0.1, 0.5, 1, 5, 20, 50 ng/mg) of 6AM and morphine in spiked control hairs. In the concentration range 0.1–50 ng/mg, the regression equations where $y = 0.039x - 0.0103$ (correlation coefficient $r =$

Table 1
Recoveries of 6-acetylmorphine and morphine from spiked samples ($n = 6$)

Drug	Amount added to hair (ng)	Amount recovered (mean ± S.D.) (ng)	R.S.D. (%)	Recovery (%)
6AM	40	38.16 ± 3.03[a]	7.94	95.40
6AM plus	40	37.89 ± 1.98	5.23	94.72
Morphine	40	40.62 ± 0.49	1.21	101.55
Morphine	40	38.76 ± 0.60	1.55	96.90

[a] 1.27 ng of morphine was produced.

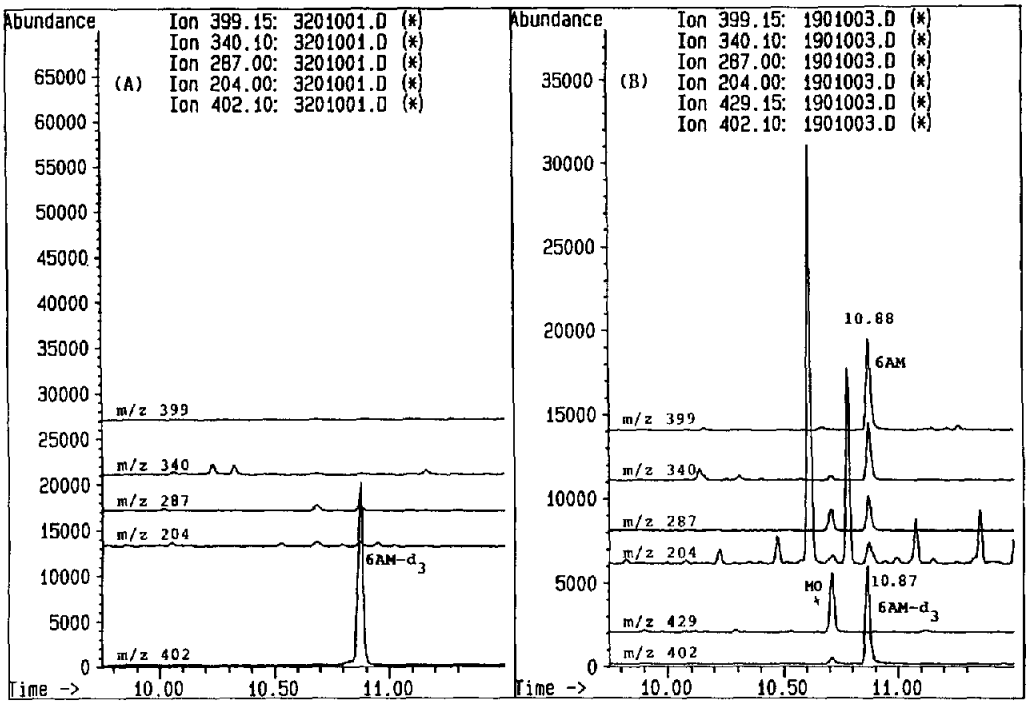

Fig. 2. GC–MS–SIM of the TMS-derivatized extract from (A) rat control hair and (B) rat hair administered heroin.

0.999) for TMS-6AM and $y = 0.042x + 0.0087$ ($r = 1.000$) for TMS_2-morphine, where y = peak-area ratio and x = concentration in ng/mg.

3.6. *Efficiency of extraction of 6AM and morphine from hair*

The efficiency of extraction of 6AM and morphine from rat hair was investigated by using methanol, 10% hydrochloric acid, helicase, methanol–hydrochloric acid (20:1) and methanol–TFA (9:1) as extraction solvents. A comparison of the extractive efficiency of 6AM and morphine from animal hair containing heroin and 6AM is shown in Table 2. We consider that the recovery of morphine from hair with 10% hydrochloric acid was almost quantitative, because no more morphine was found in the residual hair. The sum of 6AM and morphine extracted from the rat hair with methanol was only 7–8% of the total morphine extracted with 10% hydrochloric acid. Extraction with methanol–hydrochloric acid also caused considerable hydrolysis of 6AM to morphine. The use of methanol–TFA resulted in minimum hydrolysis of 6AM and maximized extraction of 6AM and morphine from hair. The sum of 6AM and morphine that was extracted from hair with methanol–TFA was almost the same as the total morphine extracted with 10% hydrochloric acid from rat hair (Table 2). The pretreatment with helicase folowed by solid-phase extraction seemed to suppress the degradation of 6AM, but the recovery was 71–87% of the total morphine obtained with 10% hydrochloric acid from rat hair.

Goldberger *et al.* [10] reported that heroin was detected in heroin abuser's hair in some cases. In our study, no heroin was detected in animal or human hair. It is considered that the amount of heroin in hair samples was very low and/or heroin may have been degraded to 6AM during

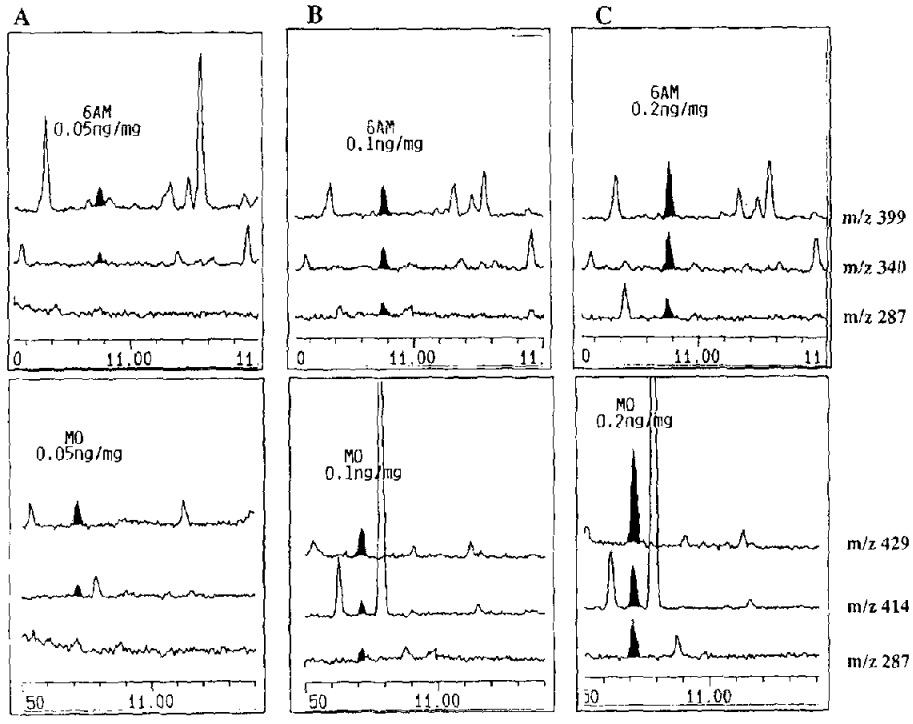

Fig. 3. Investigation of limit of detection using spiked human hair samples containing (A) 0.05, (B) 0.1 and (C) 0.2 ng/mg of 6AM (top) and morphine (bottom).

Table 2
Comparison of efficiency of extraction of 6AM and morphine from hair

Drug administered	Extraction efficiency (mean ± S.D., $n = 3$) (%)				
	MeOH	MeOH–5*M* HCl (20:1)	Helicase	MeOH–TFA (9:1)	10% HCl
Heroin-1[a]					
6AM	0.98 ± 0.04	4.56 ± 0.49	5.89 ± 0.52	7.54 ± 0.21	–
Morphine	Trace	7.94 ± 0.74	3.22 ± 0.35	3.98 ± 0.11	12.59 ± 0.25
Heroin-2[a]					
6AM	0.80 ± 0.03	5.03 ± 0.64	5.67 ± 0.48	6.56 ± 0.18	–
Morphine	Trace	5.39 ± 0.72	3.38 ± 0.41	3.90 ± 0.20	10.45 ± 0.51
6AM-1[b]					
6AM	1.23 ± 0.06	6.95 ± 0.86	8.06 ± 0.75	9.73 ± 0.38	–
Morphine	Trace	8.66 ± 1.08	3.62 ± 0.47	5.75 ± 0.50	15.68 ± 1.22
6AM-2[b]					
6AM	1.29 ± 0.05	6.39 ± 0.76	8.83 ± 0.91	10.58 ± 0.39	–
Morphine	Trace	90.5 ± 1.16	4.71 ± 0.70	6.15 ± 0.12	15.27 ± 0.66

[a] Heroin-1 and heroin-2 represent hair sample from two rats independently administered heroin.
[b] 6AM-1 and 4AM-2 represent hair samples from two rats independently administered 6AM.

extraction. For forensic cases, we consider that it would be sufficient confirmation of heroin use only to detect 6AM, which is more stable than heroin.

3.7. Stability of 6AM during extraction with methanol–TFA (9:1)

The stability of 6AM in rat hair during extraction with methanol–TFA were investigated. It was found that 3.6% of 6AM was degraded to morphine during the extraction and its recovery was 94.7–95.4%. In comparison with methanol–hydrochloric acid, methanol–TFA minimized hydrolysis and maximized the extraction of 6AM and morphine from hair.

3.8. Application to analysis of herion abuser's hair

The three heroin abusers' hair samples were analysed by the proposed method. Fig. 4 shows the two chromatograms, which indicated 14.1 ng/mg of 6AM and 3.2 ng/mg of morphine in section 7 of ST-2 and 6.2 ng/mg of 6AM and 1.7 ng/mg of morphine in section 6 of ST-5. In all instances the 6AM level was *ca.* 4–5 times higher than that of morphine in hair.

We applied the method to the elucidation of heroin abuse history by using the sectional analysis of heroin abuser's hair. An abuser (ST-2) had been seeking heroin around the world. About 1 year before hair collection, he was in Nairobi and New York. Sometimes he used up to 1 g/day

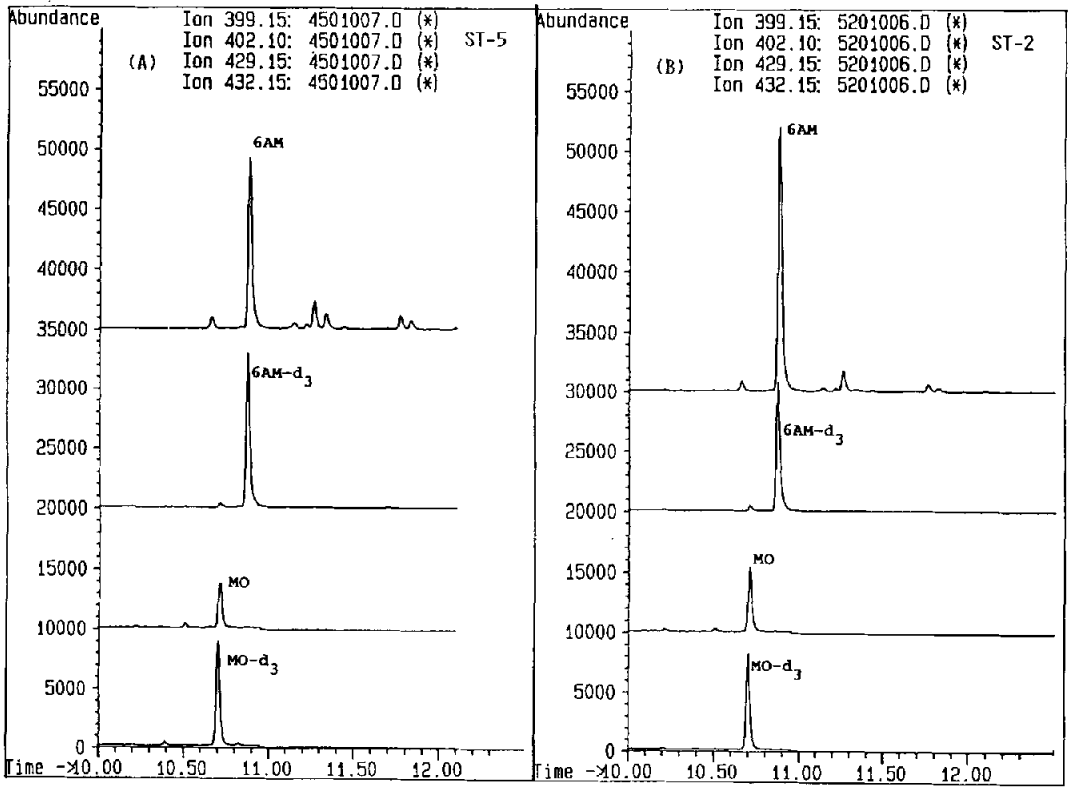

Fig. 4. GC–MS–SIM of the TMS-derivatized extracts from heroin abusers' hair. Detection of (A) 6AM (6.2 ng/mg) and morphine (1.8 ng/mg) in section 6 (14–16 cm from the root) of ST-5 hair; (B) 6AM (14.1 ng/mg) and morphine (3.2 ng/mg) in section 7 (16–18 cm from the root) of ST-2 hair.

Table 3
Relationship between drug distribution in hair and drug history

Distance from root (cm)	ST-2			ST-5			ST-12		
	6AM (ng/mg)	Morphine (ng/mg)	Drug history	6AM (ng/mg)	Morphine (ng/mg)	Drug history	6AM (ng/mg)	Morphine (ng/mg)	Drug history
0–2	10.8	1.6	Used every day	1.2	1.3	Gradually decreased	ND	ND	In
2–4	6.9	1.3	up to 1 g per day	2.0	1.4	dose for	ND	ND	hospital
4–6	4.1	0.5	In Thailand, Nairobi	3.4	1.6	hospitalization	ND	ND	
6–8	ND	0.5	Drug-free in Japan	4.8	1.6		ND	ND	
8–10	7.2	1.6	Used every day	5.4	1.7	Everyday use	1.0	1.2	Frequently used
10–12	11.0	1.9	up to 1 g per day	6.2	1.7	up to 250 mg per day	2.3	1.1	as long as
12–14	14.1	3.2	In New York,	8.9	1.9	in New York	3.1	1.3	heroin available
14–16	17.3	2.6	Nairobi				2.8	1.2	
16–18	22.8	4.1					3.2	1.0	

Route of administration: ST-2, smoking and snorting; ST-5, smoking; ST-12, snorting.

of heroin by smoking or snorting. For a while, he returned to Japan and was drug free in Japan. Subsequently, he went to Tahiland and Nairobi to obtain heroin. The distribution of 6AM in his hair corresponded well with his heroin abuse history when the time scale was fitted to a rate of hair growth of 1.2 cm/month from root to tip (Table 3). As shown in Table 3, the sectional analysis for ST-5 and ST-12 also disclosed their drug abuse history.

A large amount of 6AM (22.8 ng/mg) was detected in the 1.3-year-old section of heroin abuser's hair. This shows that it is possible to establish retrospective drug use for more than 1 year by hair analysis and 6AM in living hair was comparatively stable for years.

4. Conclusion

The extraction of 6AM from hair with methanol–TFA (9:1) has various merits, such as good extraction efficiency, less hair ingredient, slight degradation of 6AM and a simple procedure. In order to determine 6AM and morphine accurately in hair, it is very important to choose a solvent that does not hydrolyse it and has an effective extraction potential. Methanol–TFA minimizes hydrolysis of 6AM and maximizes the extraction of 6AM and morphine from hair.

In conclusion, we have developed a highly sensitive and accurate method for the analysis of hair for confirmation of heroin use. The sensitivity was as low as 0.2 ng/mg for 6AM and morphine in hair. Moreover, it was demonstrated that 6AM in living hair was comparatively stable for years. It is possible to establish retrospective heroin use for more than 1 year with this method.

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