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Short communication

Homogeneity and stability of a candidate certified reference material for the determination of methamphetamine and amphetamine in hair

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ABSTRACT

In the preparation of a reference material (RM) for quality assurance, both homogeneity and stability studies are integral parts. In the present study, both homogeneity and stability of a candidate RM for the determination of methamphetamine and amphetamine in hair were examined by an isotope dilution gas chromatography/mass spectrometry (GC/MS) method, which is not only one of the analytical methods validated in our previous study but also one of the primary methods for the preparation of a certified reference material (CRM). Additionally, homogeneity was monitored using a different method: micropulverized extraction followed by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), which was fully validated in the previous study. In order to demonstrate the suitability of the method as an isotope dilution with mass spectrometry (IDMS), the extraction efficiency was also determined according to time. Our results showed that the current method, *i.e.*, agitating hair with isotope internal standards in the extraction solvent for 20 h followed by GC–MS, was accepted as an IDMS. No significant difference was observed between bottles of the candidate CRM. The statistical results also showed no significant trends in stability for 92 days at room temperature and 4 °C. An inter-laboratory quality assurance program was also performed successfully using this material. The candidate CRM developed in the present study demonstrated its suitability for quality assurance in hair drug analysis. Even though a RM is necessity as a quality control tool, it is not always easy to have an authentic RM containing target drugs and metabolites. Even when an in-house quality control material is used, both homogeneity and stability should be investigated.

1. Introduction

The demand for a reference material (RM) has increased in forensic and clinical toxicology laboratories as quality assurance has been emphasized more and more. The RM is necessary in method development and validation, estimation of measurement uncertainty, internal quality control, proficiency tests and training, etc. In forensic and clinical toxicology laboratories, RMs in diverse matrixes (blood, urine, hair, etc.) with different concentrations of various analytes, such as drugs and poisons, are required; however, few appropriate RMs are available. Therefore, some laboratories prepare their own quality control samples to apply to their analysis.

Both homogeneity and stability are essential in the preparation and certification of RMs. The homogeneity and stability of in-house quality control samples should be also examined during preparation. Particularly, those RMs used for proficiency testing should be checked before sample distribution. In a solid type of RM such as hair, the homogeneity is a major concern, so various homogenizing techniques such as pulverizing, mixing and sieving are used during preparation in order to ensure the homogeneity. The stability of a RM should be also considered carefully and proper shelf-life should be set [1,2].

Hair is a useful specimen as probative evidence of drug use in forensic toxicology. Drug analysis in hair can provide information on previous drug use and demonstrate the drug-use period according to the rate of hair growth (ca. 1 cm/month). In Korea, hair analysis is often applied to demonstrate illegal methamphetamine use, and its results are accepted to facilitate the court's decision regarding specific circumstances surrounding drug-related crimes [3]. Therefore, the quality control of hair drug analysis is of great importance.

In previous studies, two RMs for the determination of methamphetamine (MA) and amphetamine (AP) in hair were prepared with authentic and spiked hair and used in a pilot-proficiency testing

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program. Two different extraction methods, agitation and ultrasonication, were used for quantification after full validation and comparison of extraction efficiency [4,5]. However, some limitations were recognized. Firstly, the homogeneity of those materials was determined but the stability was not because it was considered that hair is stable indefinitely and little influenced by preservation, storage and transportation conditions. However, it is necessary to examine the stability of analytes in cut or pulverized hair since its surface area increases significantly compared to that of intact hair strands. Secondly, even though the previous hair RMs were evaluated by two different extraction methods, both methods included methanol extraction followed by gas chromatography/mass spectrometry (GC/MS). However, NIST Special Publication 260-136 [6] recommends that two totally different analytical methods at two different laboratories be used as one mode of value-assignment of reference materials for chemical measurement. Furthermore, in contrast to other traditional biological specimens, such as blood and urine, hair is a complicated solid matrix in which drugs are firmly incorporated. Therefore, application of two totally different analytical methods is necessary for the evaluation of a hair RM. Thirdly, all participating laboratories in a pilot-proficiency testing program reported in a previous study [5] used methanol extraction (either agitation or ultrasonication) followed by GC–MS. Nevertheless, various decontamination, extraction and purification methods as well as a wide range of instruments are currently used in the field of hair analysis in practice [7].

The National Institute of Standards and Technology (NIST) previously developed two standard reference materials for drugs of abuse in human hair, SRM 2379 and 2380, by soaking drug-free hair in solutions containing drugs and metabolites [8]. However, the Society of Hair Testing recommended that authentic standard hair specimens be used in a proficiency testing program for external quality control [9] because the condition of drug incorporation into hair of the spiked hair is different from that in real cases. Therefore, a candidate certified reference material (CRM) was prepared from a pool of MA abusers' hair samples in the present study. Both homogeneity and stability were examined by the isotope dilution GC/MS method, which is not only one of the analytical methods validated in our previous study [4] but also one of the primary methods for the preparation of a CRM recommended by the Consultative Committee on the Quantity of Material (CCQM) [10]. In order to demonstrate the suitability of the method as an isotope dilution with mass spectrometry (IDMS), the extraction efficiency was also determined according to time. Furthermore, the prepared hair samples were distributed to 13 laboratories for an inter-laboratory quality assurance program. The results were then evaluated statistically.

2. Experimental

2.1. Preparation of a RM

Hair samples were collected from about 200 suspected drug addicts and submitted to the National Institute of Scientific Investigation by the police. The specimens were first analyzed to report results to the police and then the remaining hair of each specimen was used to make a hair pool. All the subjects were Korean with originally black hair. Dyed hair samples were excluded and the roots of the hair were removed. The specimens in which the MA concentrations ranged from 10 to 20 ng/mg, were mixed to create a pool. The candidate hair CRM was prepared as described in the previous study [4]. Briefly, the hair was washed in dichloromethane and homogenized in distilled water. After that, it was cut into about 1-mm pieces, sieved, blended and finally bottled (92 bottles, ca. 100 mg each).

2.2. Hair analysis

MA and AP in hair were analyzed using the fully validated method described previously [4]. The hair sample was accurately weighed (ca. 10 mg) without washing and cutting because it had already been washed and cut completely during preparation. MA-d_5 and AP-d_5 were then added as internal standards and the hair sample was agitated with 3 ml of 1% HCl in methanol for 20 h at 38 °C. The hair extract was evaporated to dryness at 45 °C under N_2 gas and then the residue was derivatized with 100 μl of a mixture of trifluoroacetic anhydride (TFAA) and ethyl acetate (1:1) at 65 °C for 15 min. Excess derivatizing reagent was removed under N_2 gas at 45 °C and the residue was reconstituted in ethanol.

Analysis was performed using an Agilent 6890N/5975 GC–MS system. For the quantification of MA and AP, the MS was operated in selected ion monitoring (SIM) mode. The TFAA-derivatized ions for MA, AP, MA-d_5 and AP-d_5 were as follows: MA, m/z 154, 118, 91; AP, m/z 140, 118, 91; MA-d_5, 158, 122; AP-d_5, 144, 122.

2.3. Evaluation of the extraction efficiency

In order to evaluate the extraction efficiency of the method according to extraction time, MA and AP in the candidate CRM were analyzed using the same method described above, but at six different extraction times, *i.e.*, 30 min, 1, 3, 5, 10 and 20 h. Six bottles were randomly chosen after a homogeneity test and analyzed in triplicate simultaneously. The extraction solutions of three aliquots of hair samples from each bottle were removed after 30 min, 1, 3, 5, 10 and 20 h, respectively, in order to prevent further drug extraction.

Table 1
Results of the homogeneity test of MA and AP in the candidate reference material.

Bottle no.	GC/MS		Bottle no.	HPLC–MS/MS	
	Concentration (mean ± S.D., ng/mg, n = 3)			Concentration (mean ± S.D., ng/mg, n = 3)	
	MA	AP		MA	AP
7	4.22 ± 0.01	0.30 ± 0.00	5	4.14 ± 0.01	0.28 ± 0.00
13	4.13 ± 0.04	0.28 ± 0.00	12	3.88 ± 0.06	0.27 ± 0.00
22	4.19 ± 0.02	0.31 ± 0.00	23	4.48 ± 0.08	0.29 ± 0.00
30	4.10 ± 0.00	0.29 ± 0.00	33	4.16 ± 0.01	0.30 ± 0.00
37	4.05 ± 0.01	0.28 ± 0.00	43	4.28 ± 0.32	0.27 ± 0.00
45	4.01 ± 0.00	0.28 ± 0.00	52	4.31 ± 0.08	0.28 ± 0.00
53	3.92 ± 0.01	0.28 ± 0.00	60	4.48 ± 0.09	0.30 ± 0.00
62	4.15 ± 0.05	0.29 ± 0.00	67	4.76 ± 0.16	0.31 ± 0.00
69	3.95 ± 0.04	0.28 ± 0.00	73	3.89 ± 0.02	0.28 ± 0.00
80	4.06 ± 0.09	0.30 ± 0.00	82	4.12 ± 0.15	0.28 ± 0.00
F_{calc}	1.1	1.0	F_{calc}	2.3	0.7
F_{crit}	2.4	2.4	F_{crit}	2.4	2.4
u_h (%)	0.03 (0.8)	0.00 (0.2)	u_h (%)	0.10 (2.4)	0.00 (0.0)

F_{calc}: calculated F-value; F_{crit}: critical F-value of α = 5%; u_h: uncertainty of homogeneity.

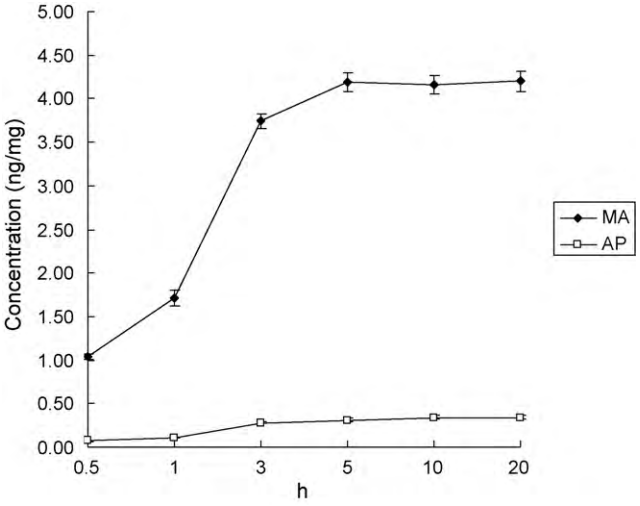

Fig. 1. MA and AP concentrations in hair at various extraction times.

2.4. Homogeneity test

To evaluate the homogeneity among the bottles, the variation of the concentrations of MA and AP was examined. Three portions from 10 randomly selected bottles for each were taken and analyzed using the method described above.

MA and AP in hair were also assessed using a different method at a different laboratory: micropulverized extraction followed by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), which was fully validated in a previous study [11]. Briefly, 2 mg of hair was micropulverized for 5 min in a polypropylene tube together with 10 μl of acetonitrile, 10 μl of 1 M trifluoroacetic acid (TFA), 20 μl of an aqueous solution of the internal standards comprised of 500 ng/ml MA-d_5 and 100 ng/ml AP-d_5 and 60 μl of water. After filtering the suspension with a membrane-filter unit, the clear filtrate was directly analyzed by HPLC–MS/MS. Ten different bottles were randomly chosen and analyzed in triplicate.

Statistical analysis was carried out and the uncertainties of homogeneity for both MA and AP were calculated using a one-way analysis of variance (ANOVA) [1,12].

2.5. Stability test

The stability of MA and AP in hair was evaluated for three months, including the period of the inter-laboratory quality assurance program, under two different storage conditions; at room temperature and at 4 °C. MA and AP in hair were analyzed in triplicate using the method described in Section 2.2. Statistical analysis was carried out and the uncertainties of stability for both MA and AP were calculated using regression analysis [2,12].

Table 2
Results of the stability test of MA and AP in the candidate reference material.

	Room temperature		4 °C	
	MA	AP	MA	AP
b_1	0.0002	0.0001	−0.0006	0.0001
s_b	0.0017	0.0002	0.0008	0.0002
u_s, ng/mg (%)	0.15 (3.7)	0.02 (7.1)	0.07 (1.7)	0.03 (10.7)

b_1: slope; s_b: standard deviation in b_1; u_s: uncertainty of stability.

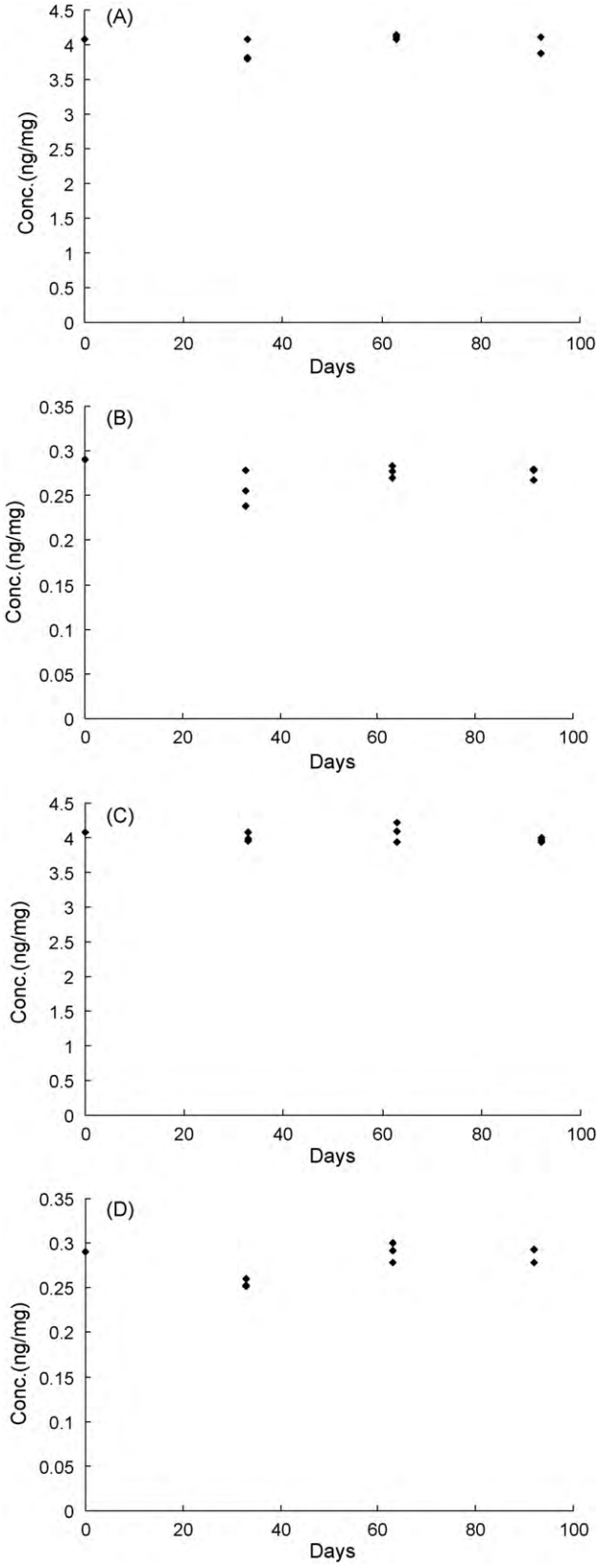

Fig. 2. MA and AP concentrations in the candidate reference material stored at room temperature or 4 °C for 92 days. (A) MA at room temperature, (B) AP at room temperature, (C) MA at 4 °C and (D) AP at 4 °C. The concentrations on day 0 are the mean values of the results in the homogeneity test using GC–MS.

Table 3
Results of the proficiency test using the candidate RM.

Identification no.	Bottle no.	Extraction method	Analytical instrument	Concentration (ng/mg)		Robust *Z*-scores	
				MA	AP	MA	AP
1	18	Micropulverization in acetonitrile–TFA (1 M)–water (1:1:8, v/v/v) followed by filtering	HPLC–MS/MS	4.512	0.323	1.48	0.42
2	16	Digestion in 1 M NaOH at 70 °C for 20 min followed by LLE	GC–MS	4.3	0.2	0.79	−1.14
3	86	Agitation in 1% HCl in MeOH for 36 h	GC–MS	4.26	0.91	0.65	7.85
4	56	Agitation in 1% HCl in MeOH for 20 h	GC–MS	3.77	0.09	−0.96	−2.53
5	92	Agitation in 1% HCl in MeOH at 38 °C for 20 h	GC–MS	4.11	0.28	0.16	−0.13
6	66	Agitation in 1% HCl in MeOH for 16 h	GC–MS	4	0.616	−0.20	4.13
7	8	Wash with water and acetone followed by sonication in MeOH for 1 h	GC–MS	2.69	0.17	−4.51	−1.52
8	26	Incubation in 0.1 M HCl at 45 °C overnight followed by LLE	HPLC–MS/MS	4.9	0.37	2.76	1.01
9	28	Sonication in ammonium formate (0.01 M):acetonitrile (85:15, v/v) (pH 3) for 2 h followed by filtering	HPLC–MS/MS	3.71	0.29	−1.15	0.00
10	36	Incubation in 1 M HCl at 60 °C for 1 h followed by SPME	GC–MS	4.73	Negative	2.20	–
11	76	Agitation in 1% HCl in MeOH for 20 h	GC–MS	4.012	0.305	−0.16	0.19
12	46	Incubation in 0.1 M HCl at 50 °C overnight followed by SPE	GC–MS	Detected[a]	Not detected[b]	–	–
13	6	Extraction using 0.1 M HCl followed by SPE	HPLC–MS/MS	4.0	0.28	−0.20	−0.13
Number of quantitative results				12	11		
Mean (ng/mg)				4.08	0.35		
Median (ng/mg)				4.06	0.29		
NIQR (ng/mg)				0.30	0.08		
Robust CV (%)				7.5	27.2		
Minimum (ng/mg)				2.69	0.09		
Maximum (ng/mg)				4.90	0.91		
Range (ng/mg)				2.21	0.82		

LLE: liquid–liquid extraction; SPME: solid-phase microextraction; SPE: solid-phase extraction; NIQR: normalized inter quartile range; CV: coefficient of variation.
[a] Limit of detection = 0.2 ng/mg.
[b] Limit of detection = 0.5 ng/mg.

3. Results and discussion

Even though the GC–MS method in the current study was fully validated previously [4], its eligibility as an IDMS needed to be investigated because hair is a solid matrix in which drugs and metabolites are incorporated. Therefore, the MA and AP concentrations in hair were determined as the extraction time increased. As shown in Fig. 1, MA was extracted to 89% after 3 h and completely after 5 h. AP was extracted to 82% after 3 h, to 91% after 5 h, and completely after 10 h. Both analytes were neither degraded nor elevated significantly after up to 20 h. Therefore, the current method, agitating hair with isotope internal standards in the extraction solvent for 20 h followed by GC–MS, was accepted as an IDMS. At least 10 h was required for the thorough extraction of both MA and AP from hair.

In the process of the preparation of a RM, homogeneity is the first consideration. The homogeneity of a liquid or gas type of RM can be easily obtained by repetitive shaking. However, it is more difficult to homogenize a solid type of RM. Therefore, two totally different analytical methods, the GC–MS and HPLC–MS/MS methods, were used in order to confirm the homogeneity of the candidate CRM. No significant differences were found for the concentrations of MA and AP by the two methods. The calculated *F*-values of both analytes were lower than the critical *F*-values in both methods (Table 1). The uncertainties of homogeneity (u_h) were calculated using the following expression:

$$u_h = s_{bb} = \sqrt{\frac{M_{among} - M_{within}}{n}}$$

where s_{bb} is between-bottle homogeneity standard deviation, M is mean square (ANOVA) and n is number of observations. Consequently, the uncertainties were 0.08 ng/mg (0.8%) and 0.00 ng/mg (0.2%) for MA and AP by the GC–MS method and 0.10 ng/mg (2.4%) and 0.00 ng/mg (0.0%) by the HPLC–MS/MS method, respectively (Table 1). The uncertainties of the homogeneity of the candidate CRM prepared with a hair pool of 10–20 ng/mg of MA in the current study were lower than those of the RM with a hair pool of 0.5–50 ng/mg of MA in the previous study [4].

The homogeneity of a RM also depends on the sample quantity. Due to improvement in analytical instruments like HPLC–MS/MS, the sampling amount of hair has become smaller. In this study, the GC–MS method used about 10 mg of hair while the HPLC–MS/MS method used about 2 mg. The uncertainty of the homogeneity of MA by the HPLC–MS/MS method was much higher than that by the GC–MS method, but the statistical results showed that the prepared candidate CRM was homogeneous. Since the smaller sampling quantity can cause heterogeneity of a RM, it is necessary to inform the minimum sampling quantity at which homogeneity is ensured. The prepared RM in the current study is suitable for a minimum sampling quantity of 2 mg.

In forensic and clinical laboratories, biological samples are usually preserved in a refrigerator at 4 °C, while hair is more commonly stored at room temperature. Therefore, the stability test was performed for 92 days under both conditions. The statistical results showed no significant trends in stability because the $|b_1|$ values of MA and AP in both conditions were lower than $t_{0.95,n-2} \cdot s_b$ (Fig. 2 and Table 2), where b_1 is the slope, $t_{0.95,n-2}$ is the Student's t-factor at the degree of freedom of $n-2$ and a confidence level of 95%, and s_b is the standard deviation in b_1. The uncertainty of stability (u_s) was calculated using the following expression:

$$u_s = s_b t$$

where t is time of observation. In our results, the uncertainties were 0.15 ng/mg (3.7%) and 0.02 ng/mg (7.1%) for MA and AP at room temperature and 0.07 ng/mg (1.7%) and 0.03 ng/mg (10.7%) at 4 °C, respectively (Table 2).

Table 3 shows the results from the proficiency test using the candidate RM. A total of 13 laboratories reported either quantitative or qualitative results. A statistical evaluation was performed using 12 and 11 quantitative results for MA and AP, respectively. The mean and median concentrations of MA were 4.08 and 4.06 ng/mg and those of AP were 0.35 and 0.29 ng/mg, respectively. The Robust coefficients of variation (CVs), derived by (normalized inter quartile range (NIQR)/median) × 100, of MA and AP were 7.5 and 27.2%, which means the spread of the AP results is greater than that of the MA results. According to the Robust Z-score result, most results were acceptable, except the MA concentration in Lab no. 7 and the AP concentration in Lab nos. 3 and 6. Until now, analytical methods for drugs in hair have not been standardized. Therefore, the participating laboratories applied diverse extraction methods followed by either HPLC–MS/MS or GC–MS. It is therefore not surprising that one or more laboratory gave slightly different results. Even though a washing step was not recommended, Lab no. 7 washed the hair sample with water and acetone, which might have caused the comparatively lower concentrations of MA and AP.

4. Conclusions

The candidate RM developed in the present study is suitable for quality assurance in hair drug analysis. It shows both satisfactory homogeneity and stability. Even though a RM is a necessity as a quality control tool, it is not always easy to have an authentic RM containing target drugs and metabolites. Even when an in-house quality control material is used, both homogeneity and stability should be monitored.

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