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Relationship between methamphetamine concentrations in hair root, blood and urine samples in postmortem cases

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Abstract

Urine and blood samples are normally used to determine methamphetamine (MA) level in forensic and clinical toxicology for assessment of illicit MA use. In cases in which both samples are unavailable or contaminated, hair root has been proposed as an alternative for evaluation of recent MA use. The goal of this study was to examine the correlations of MA concentrations in hair root, blood and urine samples in postmortem cases. Samples were collected from 45 deceased persons with MA-positive urine samples in a screening test using MA strips. MA concentrations in hair root, blood and urine samples were detected by gas chromatography coupled with a triple quadrupole mass spectrometry. The results showed that MA concentrations were linearly correlated in hair root vs. blood, hair root vs. urine, and urine vs. blood, with correlation coefficients (r) of 0.939 (p <0.001), 0.579 (p = 0.001), and 0.597 (p = 0.007), respectively.

Keywords: Methamphetamine, Blood, Hair root, Urine

Introduction

Methamphetamine (MA) is classified as a Schedule II controlled substance under the Controlled Substance Act of 1970 [1]. The widespread and addictive use of MA is currently a major concern in many countries. Determination of MA in biological samples (urine and blood) is used to assess illicit MA use in forensic and clinical toxicology. However, the compound can only be detected in blood and urine for limited periods. The maximal detection time that MA can be found in urine is approximately 6 days after the last exposure, with a mean detection time of 87 ± 51 hours; and MA can be detected in blood up to 48 hours after exposure [2].

Practically, analysis of MA in urine samples from deceased persons or suspects comprises two steps: preliminary screening using an immunoassay, followed by further analysis of amphetamine-positive samples using chromatographic techniques. This second step requires liquid-liquid or solid-phase extraction, after which MA and its metabolites are determined by gas chromatography or liquid chromatography [3-6]. MA in urine samples is used as evidence by law enforcement, while the concentration of MA in blood samples is useful for

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interpretation of MA toxicity for clinical or forensic purposes. However, in some circumstances urine and blood samples are not available or may be contaminated. In these cases, oral fluid, sweat and hair have been proposed as alternative specimens [7].

In postmortem cases, hair samples are useful alternative specimens when urine or blood samples cannot be used, such as in cases of severe trauma, charring or putrefaction of dead bodies. Use of hair is also noninvasive, has a longer detection window for longer-term drug use, is relatively free from contamination, and the specimen is stable [8]. Since hair grows at an average of 1 cm per month [9], segmental hair analysis also provides an invaluable source of information related to antecedent drug use history in the months prior to death [8]. In addition, collection and analysis of drug in hair roots may provide information relating to acute poisoning. MA can be detected in hair root as early as 5-30 minutes after MA administration in rats, and in humans after acute MA poisoning [10]. Use of hair root also permits a longer detection time for MA (up to 14 days) compared to plasma (about 1 day) after MA administration in rats [11].

In this study, we assessed the relationships among MA concentrations in hair roots, blood and urine, with the goal of determining whether hair root is an appropriate alternative specimen in cases in which blood and urine samples are not available.

Materials and Methods

Chemicals and reagents: *d*-Methamphetamine HCl was purchased from Alltech (PA, USA). Trimipramine maleate was selected as the internal standard (IS) and was purchased from Sigma-Aldrich (MO, USA). Chloroform (AR grade) was purchased from RCI Labscan (Samudsakorn, Thailand). Diethyl ether (GR grade), methanol (GR grade) and potassium hydroxide (pellets, GR grade) were purchased from Merck (Darmstadt, Germany). Orange Test Methamphetamine Strip® was purchased from IND Diagnostic Inc. (Delta, BC, Canada).

Hair root, blood and urine samples: The 45 subjects were deceased persons whose bodies were sent for autopsy at the Institute of Forensic Medicine, Police General Hospital, The Royal Thai Police, and had MA-positive urine samples in a screening test using Orange Test Methamphetamine Strip®. Hair root samples were plucked from the posterior vertex region of the head and kept in well-sealed plastic bags. Blood samples were kept in sodium fluoride tubes. Urine samples were kept in well-sealed plastic containers. All samples were stored at 2-4°C until analysis.

Sample preparations: Hair samples were washed once by vortexing with methanol for 1 min. After drying, the samples were cut into 0.5 cm lengths from the root for collecting hair root and 1 mg of the sample was transferred to an injection vial for a Thermal Separation Probe (TSP, Agilent Technologies, USA), using a method modified from that of Wainhaus et al. [12]. Then, 1 µL of 100 ng/mL trimipramine (IS) in methanol was added and the hair root sample with IS in the TSP vial was injected into a GC/MS/MS instrument via the TSP interface.

Analysis of MA in blood and urine samples was performed using a method modified from that of Marquet et al. [13]. Blood and urine samples (500 µL) were extracted via liquid/liquid extraction with 1 mL of chloroform and diethyl ether, respectively, after adjustment to pH 10-11 with 200 mM potassium hydroxide. Then, 200 µL of 250 ng/mL trimipramine in methanol was added and the mixture was vortexed for 5 min and centrifuged at 5000 rpm for 5 min. The organic layer was transferred to an injection vial and 2 µL of the solvent was injected into GC/MS/MS. Each sample was measured in duplicate.

Gas Chromatography coupled with Triple Quadrupole Mass Spectrometry (GC/MS/MS): A GC/MS/MS (Agilent 7890A GC with a 7000 Series Triple Quadrupole MS; Agilent Technologies, USA) was used in constant pressure mode with splitless injection. The analysis was performed in multi-reaction monitoring (MRM) mode for compound confirmation. The MRM transition ions and collision energy used in this analysis are shown in Table 1. The instrument was fitted with a DB-5MS UI column (15 m, 0.25 mm, 0.25 µm film thickness (Agilent Technologies, USA) and helium was used as the carrier gas. The GC oven temperature was programmed from 80°C as the initial temperature and ramped to 240°C at 20°C/min. The injector and transfer lines were set at 280°C.

Calibration curves: Calibration curves were constructed using MA-spiked blood at 50, 100, 150, 250, 500 and 1000 ng/mL and MA-spiked urine at 100, 250, 500, 1000 and 2000 ng/mL, using serial dilution from a MA stock solution of 1 mg/mL in methanol. A volume of 500 µL of each MA-spiked sample was analyzed in

Table 1 MRM transition ions and collision energy for detection of methamphetamine and trimipramine

Compound	RT (min)	Transition ion	Collision energy (eV)
Methamphetamine	4.95	91.0 > 65.0	15
		58.0 > 43.0	15
Trimipramine	11.3	128.5 > 59.6	15
		101.6 > 59.6	15
		101.6 > 42.7	15
		59.6 > 42.7	15

Table 2 Within-day precision, between-day precision, and accuracy of the method for determination of MA concentrations in hair root, blood and urine samples

Biological samples	MA concentrations	Accuracy [a] (%)	Precision (% CV)	
			Within-day [b]	Between-day [c]
Hair root	1 ng/mg	112.83 ± 5.91	5.30	2.51
	10 ng/mg	109.52 ± 2.44	2.19	
	50 ng/mg	101.71 ± 2.66	2.62	
Blood	50 ng/mL	99.92 ± 2.53	2.54	2.04
	250 ng/mL	95.51 ± 1.21	1.26	
	1000 ng/mL	98.00 ± 2.13	2.18	
Urine	100 ng/mL	102.51 ± 1.28	1.25	1.38
	500 ng/mL	100.01 ± 0.51	0.51	
	2000 ng/mL	112.31 ± 2.56	2.28	

[a] Data are shown as the mean ± S.D. of n = 5
[b] Data calculated from the mean and S.D. of n = 5 within one day
[c] Data calculated from the mean and S.D. of n = 4 (4 days). Experiments were performed in triplicate in each day

triplicate using the methods described above. A calibration curve was also prepared with MA-spiked hair root samples, using MA standard solutions in methanol of 1, 5, 10, 25 and 50 µg/mL prepared by serial dilution of the MA stock solution of 1 mg/mL in methanol. A volume of 1 µL of each solution was pipetted into a TSP vial filled with 1 mg of blank hair root to obtain MA-spiked hair root concentrations of 1, 5, 10, 25 and 50 ng/mg, respectively. After addition of 1 µL of 100 ng/mL trimipramine in methanol, the vial was transferred to GC/MS/MS via the TSP interface. Each MA-spiked blank hair root sample was analyzed in triplicate. Calibration curves for MA in each matrix were prepared from the MA concentrations and corresponding peak area ratios of MA and the internal standard.

Method validation:

Linearity: MA standard solutions were spiked into blank blood, urine and hair root samples for preparation of calibration curves. The MA concentration in each sample was measured five times using the method described above. The relationship between the actual and measured MA concentrations was assessed by Pearson correlation and simple linear regression analysis.

Limit of detection (LOD) and quantification (LOQ): LOD was assessed by determination of the lowest concentrations of MA in blank hair root, blood and urine samples that produced a signal-to-noise ratio of 3:1 to 2:1. LOQ was determined from blank hair root, blood and urine containing various concentrations of MA, as described above, until samples were obtained with the lowest concentration of MA that could be measured with accuracy and precision not exceeding 20 % [14]. A signal-to-noise ratio of 10 : 1 is recommended for determining the quantitation limit [15].

Precision**:** The assay was evaluated for within-day and between-day precision based on the coefficient of variation (% CV). To evaluate within-day precision, blank hair root samples of 1 mg with 1 µL of 1, 10 and 50 µg/mL MA were analyzed five times by GC/MS/MS. Blood samples containing 50, 250 and 1000 ng/mL MA and urine samples containing 100, 500 and 2000 ng/mL MA were similarly analyzed 5 times by GC/MS/MS. To evaluate between-day precision, hair root, blood and urine samples containing 10 ng/mg and 250 and 500 ng/mL MA, respectively, were analyzed in triplicate for four consecutive days.

Accuracy**:** Blank hair root samples of 1 mg with 1 µL of 1, 10 and 50 µg/mL MA were analyzed five times by GC/MS/MS. Blood samples containing 50, 250 and 1000 ng/mL MA and urine samples containing 100, 500 and 2000 ng/mL MA were similarly analyzed five times by GC/MS/MS. Percentage accuracy was calculated from the measured MA concentrations and the corresponding actual MA concentrations.

Statistical analysis: Results are presented as mean ± standard deviation (S.D.) or standard error of the mean (S.E.M.). Relationships between MA concentrations in hair root, blood and urine samples were analyzed by Pearson correlation and simple linear regression using SPSS for Windows, ver.16.0. A *p-value* of less than 0.05 was considered significant**.**

Results

Method validation: The linearity of the method was shown by the close linear relationship between measured and actual MA concentrations in blank hair root samples (R^2 = 0.998, p <0.001), blood samples (R^2 = 0.993, p <0.001), and urine samples (R^2 = 0.998, p <0.001). Within-day and between-day precision and the accuracy of the method for determination of MA concentrations in hair root, blood and urine samples are shown in Table 2. The % CV for within-day and between-day precision of all concentrations of MA in all types of samples did not

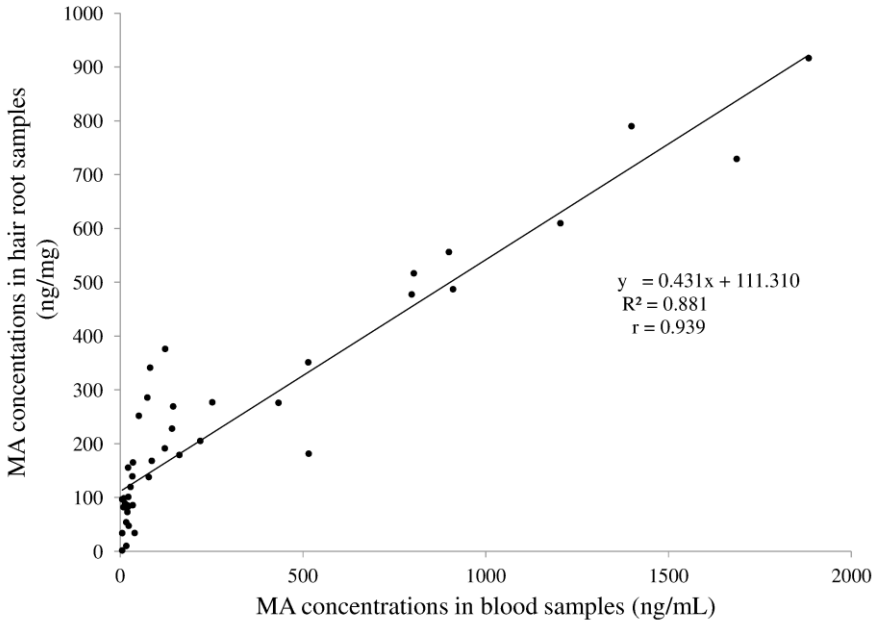

Figure 1 Relationship between MA concentrations in hair root and blood samples (n = 45)

exceed 15 %. Regarding accuracy, the mean measured values were all within 15% of the actual values, indicating % accuracy within the range of 85-115 %. Based on a signal-to-noise ratio of 3:1, the LOD of the method for determining MA in hair root, blood, and urine samples was 0.125, 40, and 40 ng/mL, respectively. The quantification limit based on a signal-to-noise ratio of 10 : 1 and accuracy and precision < 20 % showed that the LOQ of the method for determining MA in hair root, blood, and urine sample was 0.2 ng/mg, 50 ng/mL, and 50 ng/mL, respectively.

Relationships between MA concentrations in hair root, blood and urine samples: The samples examined in the study were obtained from 45 deceased persons (43 males, 95.6%) with mean (± S.E.M.) ages of 42 ± 12 years (range = 17-61 years). The mean (± S.E.M.) MA concentrations in hair root, blood and urine samples were 236 ± 32 ng/mg, 289 ± 71 ng/mL, and 17618 ± 3931 ng/mL, respectively. The MA concentrations in the three sample types were fitted to linear regression equations for hair root (y) and blood (x) ($y = 0.431x + 111.310$; $R^2 = 0.881$, $r = 0.939$, $p < 0.001$; Figure 1); hair root (y) and

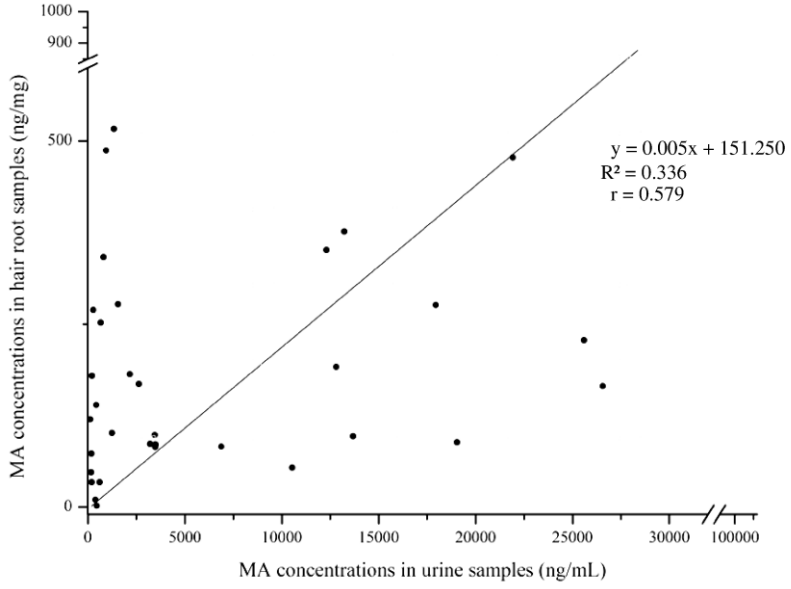

Figure 2 Relationship between MA concentrations in hair root and urine samples (n = 45)

urine (x) (y = 0.005x + 151.250; R^2 = 0.336, r = 0.579, p = 0.001; Figure 2); and urine (y) and blood (x) (y = 33.174x + 8036.800; R^2 = 0.357, r = 0.597, p = 0.007; Figure 3).

Discussion

Method validation: Validation of the GC/MS/MS method to determine MA in hair root, blood and urine samples indicated linearity based on R^2 >0.99 in Pearson correlation tests for all samples. Based on a signal-to-noise ratio of 3 : 1, the LOD was 0.125 ng/mg, 40 ng/mL and 40 ng/mL in the respective sample types, and LOQ determined based on a signal-to-noise ratio of 10:1 [15] and accuracy and precision < 20 % [14] was 0.2 ng/mg, 50 ng/mL and 50 ng/mL, respectively. The cutoff concentration of MA in hair using tests mandated by the Federal Drug Testing Program, Department of Health and Human Services, Substance Abuse Mental Health Service Administration (SAMHSA) is 300 pg/mg (0.3 ng/mg) [7]. Thus, both LOD and LOQ in the hair root analysis in this study were lower than the MA cutoffs in hair root proposed by SAMHSA. In detection of MA and methylenedioxymethamphetamine in hair roots using HPLC-chemiluminescence, with derivatization of MA with 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) to increase sensitivity, Wada et al. [11] obtained LOD and LOQ values of 0.005-0.058 and 0.017-0.191 ng/mg, respectively. Our method has lower sensitivity, but the simplicity of the method is advantageous and LOD and LOQ are still satisfactory since they are lower than the cutoff values for MA confirmation proposed by SAMHSA.

Determination of MA concentrations in blood and urine samples was modified from the published method [13] with different organic solvents used for extraction. Chloroform was used as an organic solvent for liquid-liquid extraction of MA from blood samples, instead of diethyl ether, because higher recovery of amphetamines can be achieved using chloroform [16]. However, diethyl ether was used for extraction of MA from urine samples because transfer of the upper layer of diethyl ether for GC/MS/MS analysis was simpler and less open to contamination compared to use of chloroform, which forms the bottom layer. The LOD and LOQ reported by Marquet et al. [13] for analysis of MA in blood samples were 2 and 20 ng/mL, respectively. The greater sensitivity compared to our method may be due to inclusion of an evaporation step and derivatization with heptafluorobutyric anhydride to enhance GC/MS detection [13]. These steps were not performed in the current study.

From a regulatory perspective, the cutoff value for MA in blood samples has not been defined by SAMHSA in the Federal Drug Testing Program. The toxic level of MA in blood is 200-1000 ng/mL [17] or 600-5000 ng/mL [18]. Thus, the sensitivity of our method is sufficient to detect MA at concentrations that are much lower than the toxic level of MA in humans. In urine samples, LOD and LOQ were 40 and 50 ng/mL, respectively, which are both much lower than the cutoff values mandated in the Federal Drug Testing Program. The cutoff concentration of MA detected by GC/MS is 250 ng/mL [19]. Using GC/ion-trap MS, Wu et al. [20] found LOD and LOQ values for determination of MA in urine of 50 and 100 µg/L (ng/mL), respectively, which were higher than those in the current study. Using solid phase extraction, derivatization, and analysis by GC/positive chemical ionization MS, Oyler et al. obtained a LOQ of 2.5 ng/mL. Derivatization enhances GC/MS detection, which may explain the lower LOQ in Oyler et al. [21].

The within-day and between-day precision of our method are shown by the % CV of <15%, and the accuracy is indicated by the measured MA being within 15 % of the actual value. These results are within the acceptable ranges for bioanalytical method validation [14].

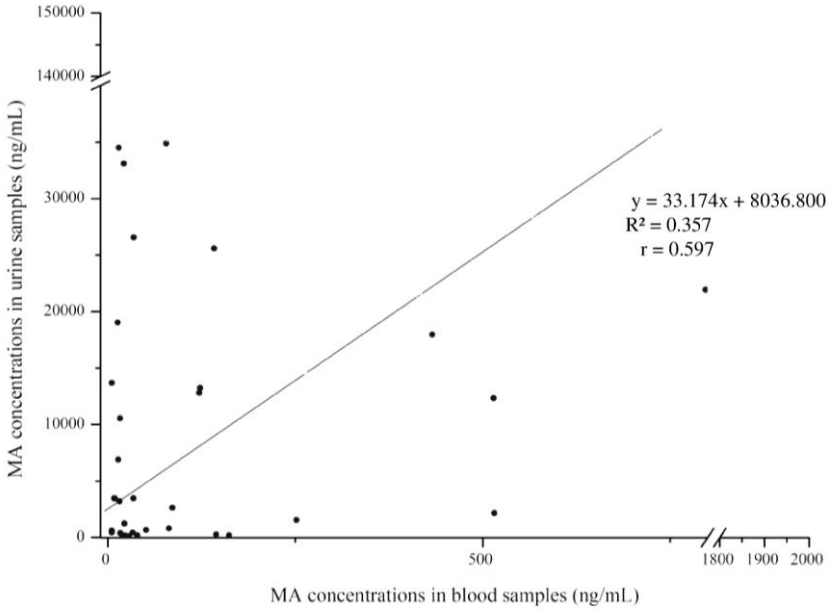

Figure 3 Relationship between MA concentrations in urine and blood samples (n = 45)

Relationships between MA concentrations in hair root, blood and urine samples: MA levels in hair root, blood and urine samples were found to be linearly correlated, with correlation coefficients of 0.939 (hair root vs. blood), 0.579 (hair root vs. urine), and 0.597 (urine vs. blood). Thus, concentrations of MA in hair root samples were more strongly correlated with those in blood samples than with those in urine samples. This is consistent with the finding that MA is detected in hair roots as early as 5 min after single MA administration in rats and was detectable in humans who died due to acute MA poisoning [10]. MA was found in rat hair root at concentrations of 11.1, 12.3 and 13.2 ng/mg at 5 min after administration of MA at 20, 40 and 60 mg/kg, respectively [10]. These data suggest that MA reaches the hair root rapidly after entering the blood and that hair root is useful for demonstrating acute MA poisoning. MA concentrations in hair root and urine samples were less strongly correlated, although the correlations were significant. Several factors can influence urinary excretion of MA, including urinary pH, frequency of emptying the bladder, volume of urine, and time elapsed after use of MA [22]. Jones and Karlsson [22] obtained a correlation coefficient (r) of 0.53 (n = 48) for amphetamine concentrations in urine and blood, which is similar to the value of r = 0.597 in the current study (Figure 3).

In conclusion, MA concentrations in hair root, blood and urine samples collected from 45 deceased persons were linearly correlated with correlation coefficients (r) of 0.939 (hair root vs. blood), 0.579 (hair root vs. urine), and 0.597 (urine vs. blood). These findings suggest that hair root can be used as an alternative specimen in a case in which blood and urine samples are not available.

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