ความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในรากผม เลือด และปัสสาวะหลังการเสียชีวิต

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THE CORRELATION OF METHAMPHETAMINE CONCENTRATIONS IN HAIR ROOT, BLOOD AND URINE IN POSTMORTEM CASES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
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Thesis Title	THE CORRELATION OF METHAMPHETAMINE CONCENTRATIONS IN HAIR ROOT, BLOOD AND URINE IN POSTMORTEM CASES
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Field of Study	Pharmacology
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ศิริรัตน์ พรหมหิตาธร : ความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในรากผม เลือด และปัสสาวะหลังการเสียชีวิต (THE CORRELATION OF METHAMPHETAMINE CONCENTRATIONS IN HAIR ROOT, BLOOD AND URINE IN POSTMORTEM CASES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. พ.ต.ท.หญิง ดร.สมทรง ลาวัณย์ประเสริฐ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: พ.ต.ท.หญิง ดร.ธีรินทร์ สินไชย, 62 หน้า.

เมทแอมเฟตามีนจัดเป็นยาเสพติดประเภทที่ 1 ตามพระราชบัญญัติยาเสพติดให้โทษ พ.ศ. 2522 ปัจจุบันการติดยาเสพติดชนิดเมทแอมเฟตามีนเกิดขึ้นอย่างแพร่หลาย เป็นปัญหาสำคัญของหลายประเทศ การตรวจเมทแอมเฟตามีนในตัวอย่างชีววัตถุ (ส่วนใหญ่ใช้ ปัสสาวะ และเลือด) เป็นการประเมินผู้เสพเมทแอมเฟตามีนทั้งในด้านนิติเวชวิทยาและด้านคลินิก ในบางกรณีไม่สามารถเก็บตัวอย่างชีววัตถุทั้งสองชนิดได้หรือตัวอย่างทั้งสองชนิดมีการปนเปื้อน การใช้ตัวอย่างรากผมอาจเป็นทางเลือกที่ใช้ประเมินการเสพเมทแอมเฟตามีนก่อนเสียชีวิต งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างความเข้มข้นของเมทแอมเฟตามีนในรากผม และเลือด หรือปัสสาวะของผู้เสียชีวิต ตัวอย่างรากผม เลือด และปัสสาวะเก็บจากผู้เสียชีวิต จำนวน 30 รายซึ่งมีปัสสาวะที่ได้ผลบวกเมทแอมเฟตามีนจากการตรวจด้วยชุดทดสอบเมทแอมเฟตามีนสำเร็จรูป นำรากผมมาผ่านกระบวนการล้างแล้วตัดรากผมปริมาณ 1 มิลลิกรัมมาตรวจวัดเมทแอมเฟตามีนด้วยเครื่องแกสโครมาโตกราฟี/แมสสเปกโตรเมทรี ทริปเปิลควอดรูโพล (GC/MS/MS) นำตัวอย่างเลือด และปัสสาวะ มาสกัดด้วยด้วยวิธีลิควิด/ลิควิด เอกซ์แทรกชัน แล้วตรวจวิเคราะห์ด้วยเครื่อง GC/MS/MS ผลการศึกษาพบว่า ความเข้มข้นของเมทแอมเฟตามีนในรากผมและเลือด รากผมและปัสสาวะ และ ปัสสาวะและเลือด มีความสัมพันธ์กันในเชิงเส้นตรง โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ 0.904 (p<0.001), 0.572 (p=0.001) และ 0.690 (p<0.001) ตามลำดับ สมการถดถอยเชิงเส้นของความสัมพันธ์ของความเข้มข้นของเมทแอมเฟตามีนในรากผมและเลือด รากผมและปัสสาวะ และ ปัสสาวะและเลือด คือ y = 1.997x - 162.620, y = 77.618x - 683.460 และ y = 0.011x + 130.210 ตามลำดับ ทำการทดสอบสมการถดถอยโดยการใช้ค่าความเข้มข้นของเมทแอมเฟตามีนในรากผมของผู้เสียชีวิต 20 ราย แทนค่าในสมการถดถอยแล้วคำนวณค่าความเข้มข้นของเมทแอมเฟตามีนในเลือดและปัสสาวะ ผลการทดสอบพบว่าความเข้มข้นของเมทแอมเฟตามีนที่วัดได้จริงและความเข้มข้นของเมทแอมเฟตามีนที่คำนวณจากสมการมีค่าไม่แตกต่างกัน ผลจากการศึกษานี้สนับสนุนการใช้รากผมเป็นตัวอย่างชีววัตถุทางเลือกในการตรวจวิเคราะห์ความเข้มข้นของเมทแอมเฟตามีนในกรณีที่ไม่มีตัวอย่างเลือดหรือปัสสาวะ

ภาควิชา เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชวิทยา	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา 2556	ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SIRIRAT PHOMHITATORN : THE CORRELATION OF METHAMPHETAMINE CONCENTRATIONS IN HAIR ROOT, BLOOD AND URINE IN POSTMORTEM CASES. ADVISOR : ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR : POL. LT. COL. THEERIN SINCHAI, PhD., 62 pp.

Methamphetamine (MA) is classified as the Schedule I Narcotic drugs according to Thai Narcotic Act B.E. 2522. Its widespread and addictive uses are currently a national threatening issue in many countries. Determination of MA in biological samples (mostly urine and blood) is used to assess illicit MA uses in forensic as well as in clinical purposes. In some circumstance, both samples are not available or contaminated; hair root is purposed as an alternative sample representing the recent MA uses before death. This study aimed to assess the correlation of MA concentrations in hair root and blood/urine samples in postmortem cases. Hair root, blood and urine samples were collected from 30 Thai deceased whose urine samples were MA positive in the screening test with MA strips test. After the washing process, hair root of 1 mg was detected by Gas chromatography/mass spectrometry triple quadrupole (GC/MS/MS). Blood and urine samples were extracted by liquid/liquid extraction and detected by GC/MS/MS. The results showed that MA concentrations in hair root vs blood, hair root vs urine, and urine vs blood were linearly correlated with the correlation coefficient (r) of 0.904 (p<0.001), 0.572 (p=0.001), and 0.690 (p<0.001), respectively. The corresponding linear regression equations were y = 1.997x - 162.620, y = 77.618x - 683.460, and y = 0.011x + 130.210, respectively. Verification of the regression equations was performed using MA concentrations in hair root of an additional 20 decease to calculate MA concentrations in blood and urine. It was shown that calculated and measured concentrations in both samples were not statistically different. Results from this study suggested hair root as an alternative specimen in case that blood or urine are not available.

Department : Pharmacology and Physiology
Field of Study : Pharmacology
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Student's Signature……..
Advisor's Signature……
Co- advisor's Signature……

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Finally, I would like to dedicate this work express my appreciation to the persons whose samples were used in this study.

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LIST OF ABBREVIATIONS

%	= percent
ºC	= degree Celcius
µg/l	= microgram per liter
µg/ml	= microgram per milliliter
µl	= microliter
5-HT	= serotonin
AP	= amphetamine
AUC	= area under the curve
B.E.	= Buddist Era
CNS	= Central Nervous System
CV	= coefficient of variation
DA	= dopamine
DSI	= direct sample introduction
et al.	= et alii (and others)
etc.	= etcetera (and so forth, and so on)
g	= gram
GC/MS	= gas chromatography/ mass spectrometry
GC/MS/MS	= gas chromatography/ mass spectrometry/ mass spectrometry
GC/CI/MS	= gas chromatography/ chemical ionization/ mass spectrometry
g/kg	= gram per kilogram
g/mol	= gram per mole
HPLC	= high performance liquid chromatography
i.p.	= intraperitoneal
IS	= internal standard
KOH	= potassium hydroxide
LC/MS	= liquid chromatography/ mass spectrometry
LC/MS/MS	= liquid chromatography/ mass spectrometry/ mass spectrometry
LOD	= limit of detection

LOQ	= limit of quantitation
L/kg	= liter per kilogram
min	= minute
mg	= milligram
ml	= milliliter
mm	= millimeter
mg/kg	= milligram per kilogram
MA	= methamphetamine
MA HCl	= methamphetamine hydrochloride
MDA	= 3, 4-methylenedioxyamphetamine
MDEA	= 3, 4-methylenedioxyethylamphetamine
MDMA	= 3, 4-methylenedioxymethamphetamine
NA	= not available
NaOH	= sodium hydroxide
ND	= not detectable
NE	= norepinephrine
ng/mg	= nanogram per milligram
ng/ml	= nanogram per milliliter
no.	= number
pg/mg	= pictogram per milligram
r	= correlation coefficient
R^2	= coefficient of determination
rpm	= revolutions per minute
RSD	= relative standard deviation
RT	= retention time
SAMHSA	= Substance Abuse and Mental Health Services Administration
SNR	= signal-to-noise ratio
SOFT/AAFS	= The Society of Forensic Toxicologists/The Toxicology Section of the American Academy of Forensic Sciences

S.D.	= standard deviation
TFA	= trifluoroacetyl
TSP	= Thermal Seperation Probe
vs	= versus
U.S.FDA	= The United States Food and Drug Administration

CHAPTER I

INTRODUCTION

Methamphetamine (MA) is classified as the Schedule I Narcotic drugs according to Thai Narcotic Act B.E. 2522. The punishment for the use of narcotic drugs of Schedule I includes imprisonment for six months to three years or being fined from ten thousand baht to sixty thousand baht or both (Office of Narcotic Control Board, 2009: online). In the United States, MA is classified as a Schedule II controlled substance under the Controlled Substance Act of 1970 (The Comprehensive Drug Abuse Prevention and Control Act of 1970, The United States federal law, 1970). Its widespread and addictive uses are currently a national threatening issue in many countries. Determination of MA in biological samples (mostly urine and blood) is used to assess illicit MA uses in forensic as well as in clinical purposes. However, there is limitation of time that the compound can be found in blood and urine. The maximum of time that MA could be found in urines was approximately 6 days after last exposure with the generally detection time of 48 hours while MA could be detected in blood up to 2 days after exposure (Verstraete, 2004).

Practically, analysis of MA in urine samples of deceased or suspected persons comprises two steps. The first step is preliminary screening using immunoassay. The samples with positive amphetamine results are further analysed by chromatographic techniques, which need the extraction processes such as liquid-liquid extraction or solid phase extraction. After the extraction process, MA and its metabolites are determined by gas chromatography/mass spectrometry (GC/MS) (Ishiyama, Nagai, and Toshida 1983; Nakahara et al., 1991; Broussard, 2008) or liquid chromatography/mass spectrometry (LC/MS) (Kronstrad et al., 2004). While MA in urine samples is used as an evidence according to the law enforcement, concentration of MA in blood samples is useful for interpretation of MA toxicity in clinical or forensic purposes. However, in some circumstance both urine and blood samples are not available or contaminated.

Alternative specimens that are suggested include oral fluid, sweat and hair (Kwong, 2008).

In postmortem cases, hair samples are useful alternate specimens. Urine or blood samples may not be available or contaminated such as severe trauma, charring or putrefaction of the dead bodies. In addition, the window of detection of MA and its metabolites is limited. The advantages of using hair include specimen collection is noninvasive; detection window is for longer term drug use, specimen contamination is less and the hair specimen is stable (Kwong, 2008). As hair grows at an average rate of 1 cm each month (Cooper, Kronstrand, and Kintz, 2012), segmental hair analysis provides an invaluable source of information relating to antecedent drug use history in the months prior to death (Xiang et al., 2011). In addition, collection and analysis of hair roots has the potential to provide information relating to acute poisoning. It is reported that MA could be detected in hair root as early as 0.5 hours after MA administration in rats and in men who died due to acute MA poisoning (Nakahara et al., 1997). Moreover, hair root was found to possess longer detection time for MA (1-4 days) than the plasma (about 1 day) after MA administration in rats (Wada et al., 2012). Therefore, this study aimed to assess the relationship between MA concentrations in hair roots and blood/urine so as to propose hair root as an alternative specimen in case that both samples are not available.

Hypothesis

There are relationships between MA concentrations in hair root and blood/urine samples.

Objective

To investigate the relationships between MA concentrations in hair root and blood/urine samples in postmortem cases.

Benefit gained from the study

This study will provide the information of the relationships between MA concentrations in hair root and blood/urine samples in postmortem cases for considering hair root as an alternative specimen.

CHAPTER II

LITERATURE REVIEWS

Methamphetamine (MA) is classified as schedule I narcotic drug in the Thai Narcotic Act 1979 (B.E. 2522). MA or R, S-N-methyl-1-phenyl-propanamine is an amphetamine compound with the structure of secondary amine (Figure1). Its molecular formula and weight are $C_{10}H_{15}N$ and 149.2 g/mole, respectively. Physical property of methamphetamine hydrochloride is solid white crystalline, but colorless volatile oil, insoluble in water in free base form. It melts between 171 -175°C. It is odorless with a bitter taste (Remington's, 1985). MA has been sold illegally as powder, and pure crystalline hydrochloride form which is called “ice”.

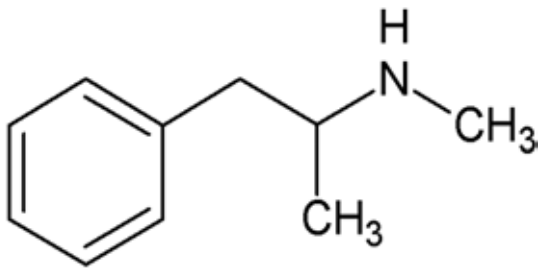

Figure1. Chemical structure of MA ($C_{10}H_{15}N$)

Pharmacodynamics (Moore, 2003)

MA increases synaptic levels of the neurotransmitters dopamine (DA), serotonin (5-HT) and norepinephrine (NE), and has α and β adrenergic agonist effects. NE is responsible for MA’s alerting, anorectic, locomotor and sympathomimetic effects; dopamine stimulates locomotor effects, psychosis, and perception disturbances; and 5-HT is responsible for delusions and psychosis. Racemic amphetamine (AP) and d-AP have similar chemical properties and actions to MA but are less potent. MA stimulates the CNS by displacing dopamine from nerve terminal storage vesicle. This release causes the hyperstimulation of dopaminergic receptor neurons in the synaptic cleft.

AP and MA are substrates for 5-HT, NE, and DA transporters and lead to transmitter release by process of transport-mediated exchange. Upon entry to the cytoplasm, the amphetamines further reduce accumulation of NE/DA in the synaptic vesicles. Catecholaminenergic vesicles use an interior-acidic proton gradient for transmitter uptake. These drugs compete for protons with neurotransmitter already present in the granules. The resulting unchaged neurotransmitter then diffuses out of the granules down its concentration gradient. This mechanism causes a continuous release of neurotransmitter at low doses of stimulant, accounting for locomotor stimulant and reinforcing effects of this compound. Direct peripheral and organ stimulation at the various α and β adrenergic receptors also occur, resulting in elevation of systolic and diastolic blood pressures and weak bronchodilator and respiratory stimulant action. At therapeutic doses, the heart rate may be slow, at large doses may produce cardiac arrhythmias.

Pharmacokinetics (Moore, 2003)

1. Absorption

MA is highly lipid soluble and well absorbed orally with a bioavailability of approximately 67% and a volume of distribution of 3-7 L/kg. Oral MA produces a peak plasma concentration at 2.6-3.6 hours with approximate half-life 10.1 hours. The peak plasma concentration is 3.1-6.3 hours by insufflation and 2.5$\pm$0.5 hours by inhalation. The plasma half-life is 12.2 hours by intraveneous injection.

2. Distribution

Because MA is high lipophilic, it is able to pass blood-brain barrier into brain and pass into breast milk for a few minutes after taking the compound by intraveneous route. It can also across placenta.

3. Metabolism

MA is excreted in urine largely as unchanged parent compound, under normal conditions, up to 45% of the dose in 24 hours. Approximately 7% of an administered dose undergoes N-demetylation to AP as shown in Figure 2. AP is also metabolized in liver via aromatic hydroxylation. Hydroxylated metabolites accumulation can develop amphetamine psychosis. Urinary acidification to pH less than 5.6 decreases the plasma half-life from 11-12 hours to 7-8 hours. Alkalization increases half-life to 18-34 hours.

4. Excretion

MA is excreted primarily in urine, with little biliary excretion of the parent compound or metabolites (Caldwell, 1976). In normal urine (pH 6–8), 37–54% of a dose is excreted as parent drug and 4–7% as AP (Cook et al., 1992).

Each unit increase or decrease in urinary pH produces a respective 7 hours increase or decrease in the MA plasma half-life. Therefore, the percentage of the dose excreted as parent compound can range from as low as 2% in alkaline (pH $\geq$8.0) to 76% in acidic urine (pH $\leq$5.0).

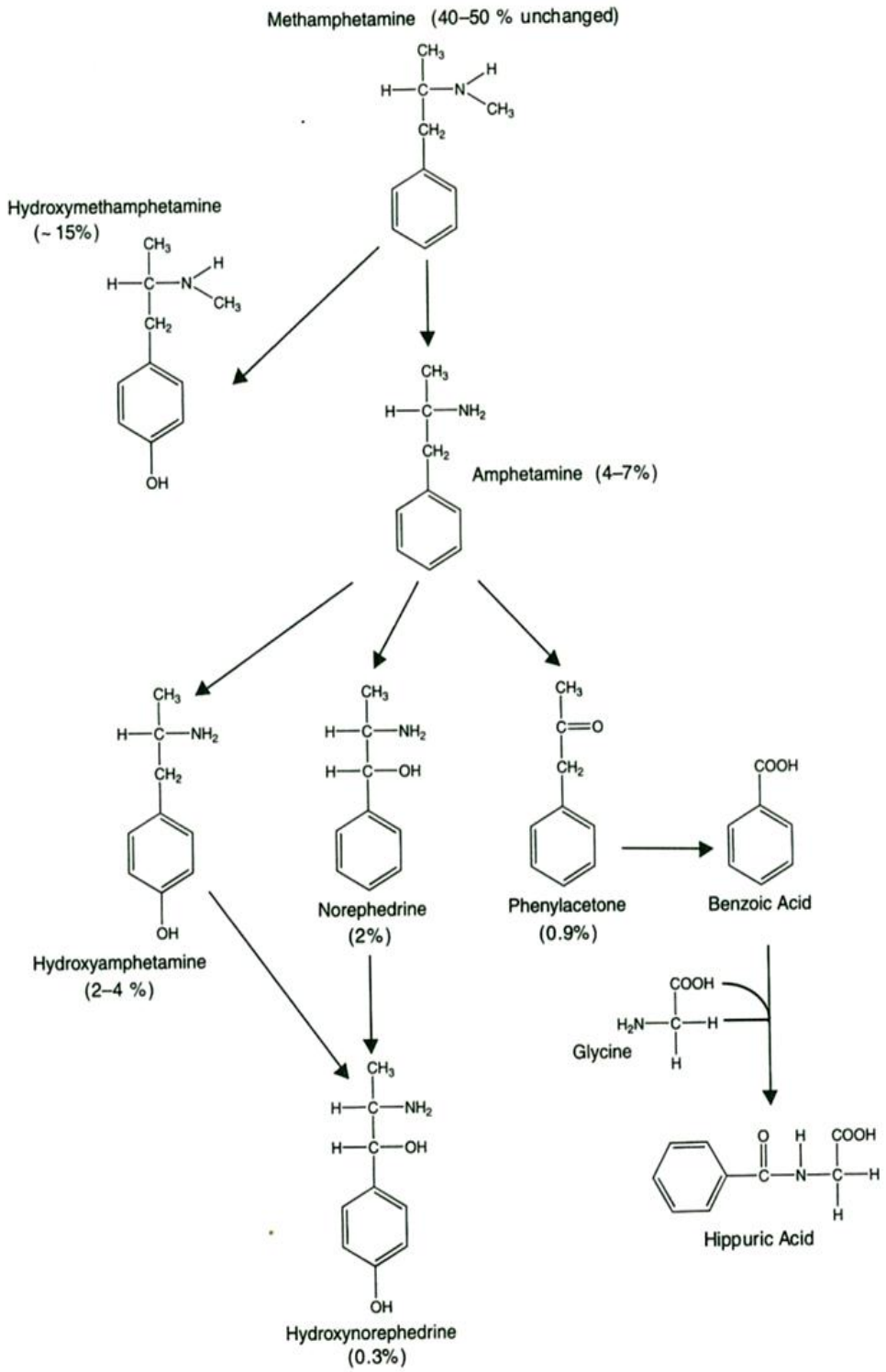

Figure 2 Metabolic pathway of MA (Moore, 2003)

Detection of methamphetamine in biological samples

MA is one of the most popular drugs of abuse in many countries. Detection of MA in biological samples comprises two steps. The first step is primary screening with immunoassay technique. The biological samples that are positive of MA will be analyzed in the second step, the confirmatory tests, by Gas Chromatography/Mass Spectrometry (GC/MS) technique. The Substance Abuse Mental Health Services Administration (SAMHSA) has proposed cut-off values for drugs of abuse in biological samples which are listed in Table 1. Currently, cutoff concentrations of drugs of abuse have been revised (Table 2).

Table 1 Cutoff concentrations mandated by Federal Drug Testing Programs, Department of Health and Human Services, Substance Abuse Mental Health Services Administration (SAMHSA) (Kwong, 2008).

Drug or drug class	*Immunoassay (ng/ml)*	*GC-MS confirmation*	*(ng/ml)*
Amphetamines	1000	Amphetamine	500
		Methamphetamine	500[a]
Cannabinoids	50	THC-COOH[b]	15
Cocaine metabolites	300	Benzoylecgonine	150
Opiates	2000	Morphine	2000
		Codeine	2000
		6-Acetylmorphine	10[c]
Phencyclidine	25	Phencyclidine	25

[a] Department of Health and Human Services, Substance Abuse Mental Health Services Administration (SAMHSA). Federal Regist 1988; 53:11970; Federal Regist 1994;59:29908; Federal Regist 1997; 62:51118. Department of Transportation (DOT). Federal Regist 2000; 65:79462.

[a] Amphetamine must be present ≥200 ng/ml.

[b] THC-COOH, 11-nor-delta 9-tetrahydrocannabinol-9-carboxylic acid.

[c] Test for 6-acetlmorphine when morphine concentration >2000 ng/ml.

Table 2 Cutoff concentrations mandated by Federal Drug Testing Programs, Department of Health and Human Services, Substance Abuse Mental Health Services Administration (SAMHSA) (Department of Health and Human Services, SAMSHA, 2008)

Initial test analyte	Initial test cutoff concentration	Confirmatory test analyte	Confirmatory test cutoff concentration
Marijuana metabolites	50 ng/mL	THCA[1]	15 ng/mL
Cocaine metabolites	150 ng/mL	Benzoylecgonine	100 ng/mL
Opiate metabolites Codeine/Morphine[2]	2000 ng/mL	Codeine Morphine	2000 ng/mL 2000ng/mL
6-Acetylmorphine	10 ng/mL	6-Acetylmorphine	10 ng/mL
Phencyclidine	25 ng/mL	Phencyclidine	25 ng/mL
Amphetamines[3] AMP/MAMP[4]	500 ng/mL	Amphetamine Methamphetamine[5]	250 ng/mL 250 ng/mL
MDMA[6]	500 ng/mL	MDMA MDA[7] MDEA[8]	250 ng/mL 250 ng/mL 250 ng/mL

[1] Delta-9-tetrahydrocannabinol-9-carboxylic acid (THCA).
[2] Morphine is the target analyte for codeine/morphine testing.
[3] Either a single initial test kit or multiple initial test kits may be used provided the single test kit detects each target analyte independently at the specified cutoff.
[4] Methamphetamine is the target analyte for amphetamine/methamphetamine testing.
[5] To be reported as positive for methamphetamine, a specimen must also contain amphetamine at a concentration equal to or greater than 100 ng/mL.
[6] Methylenedioxymethamphetamine (MDMA).
[7] Methylenedioxyamphetamine (MDA).
[8] Methylenedioxyethylamphetamine (MDEA).

There is limitation of time that the compound can be found in blood and urine that show in Table 2 and 3. While MA in urine samples is used as an evidence according to the law enforcement, concentration of MA in blood samples is useful for interpretation of MA toxicity in clinical or forensic purposes. It was reported that methamphetamine remained detectable in blood (~3ng/ml) for 48 hours and in urine (~300 ng/ml) for 60 hours after smoking 22 mg of methamphetamine (Cook et al., 1993).

Table 3 Typical detection times of drugs of abuse in blood or serum or plasma (Verstraete, 2004)

Drug	Dose (mg)/Route	Analyte	Cutoff (ng/mL)	Detection Time (hours)	Reference
Amphetamine	6/PO	Amphetamine	4	46	8
Methamphetamine	22/SM	Methamphetamine	3	48	13
MDMA	100/PO	MDMA	20	24	16
Cannabis	34/SM	THC	10	5	21
		THCCOOH	10	36	
Cocaine	100/IN	Cocaine	10	12	32
		Benzoylecgonine	10	48	
Heroin	12–20/SM	Morphine	1	20	44
GHB	4680/PO	GHB	5000	5	54

PO, oral; SM, smoked; IN, intranasal.

Table 4 Typical and maximal detection times of drugs of abuse in urine (Verstraete, 2004)

Drug	Dose (mg Unless Noted Otherwise)/Route	Analyte	Cutoff (ng/mL)	Detection Time (hours)	Reference	Maximal Detection Time (days)
Amphetamine						9
Methamphetamine	10/PO	Methamphetamine	2.5	87 ± 51	14	6
MDMA	100/PO	MDMA	20	48	16	
Cannabis	1.75%	THCCOOH	15	34	23	95
	3.50%/SM	THCCOOH	15	87		
Cocaine	100/IN	Benzoylecgonine	1000	48–72	34	22
LSD	0.28/PO	LSD	0.2	36	42	4
		2-Oxo-3OH-LSD	0.2	96		
Heroin	10–15 IV/SM	Morphine		11–54	47	11.3
GHB	100 mg/kg PO	GHB	10000	12	53	

PO, oral; SM, smoked; IN, intranasal; IV, intravenous.

There are other biological samples that can be used as an alternative biological samples for drug of abuse testing, if urine samples cannot be collected, such as bile (Drummer, 2002), vitreous humor (Drummer, 2004), hair, saliva and sweat (Kwong, 2008). The cutoff values in other alternative biological samples are presented in Table 4

Table 5 Proposed cutoff concentrations of amphetamine (AMP), methamphetamine (MAMP), and designer amphetamine in urine and other matrices (Broussard, 2008)

	Proposed test cutoff concentrations					
	AMPS[a]	*AMP*	*MAMP*	*MDMA*	*MDA*	*MDEA*
Hair initial (pg/mg)	500			500		
Hair confirmatory (pg/mg)		300	300[b]	300	300	300
Oral fluid initial (ng/mL)	50			50		
Oral fluid confirm (ng/mL)			50[c]	50	50	50
Sweat initial (ng/patch)	25			25		
Sweat confirm (ng/patch)			25[c]	25	25	25
Urine initial (ng/mL)	500			500		
Urine confirmatory (ng/mL)		250	250[d]	250	250	250

MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine.

[a] Methamphetamine is the target analyte.

[b] Specimen must also contain amphetamine at a concentration ≥50 pg/mg.

[c] Specimen must also contain amphetamine at a concentration ≥ limit of detection.

[d] Specimen must also contain amphetamine at a concentration ≥100 ng/mL.

Hair is a biological specimen that is useful in forensic toxicology. In the case of death for a long time that cause putrefaction of urine and blood samples, head hair can be used as an alternative biological samples. The advantage of hair as a testing matrix is its ability to provide historical information of an individual's exposure to drugs following chronic use or even a single exposure. Hair has an average growth rate of 1 cm per month (Cooper, Kronstrand and Kintz, 2012). Hair analysis provides an invaluable source of information relating to antecedent drug use history in the months prior to death (Xiang et al., 2011). The analysis of hair root is reported to provide information relating to acute poisoning (Nakahara et al., 1997). Methoxyphenamine, a bronchodilator which possesses the structure related to MA, is used to study the movement of a compound along the hair. It was shown that methoxyphenamine moved along hair shaft at the rate of 2.8-3.2 mm/week and the level of this drug was highest in the root side, but lowest in distal side. And it was also found that the drug level in hair decreased approximately 50% in 5 months later (Nakahara, Shimamine and Takahashi, 1992).

AP, MA (Nakahara et al., 1997) and codeine (Takayama et al., 1997) were detected in hair roots 30 minutes after administration. Likewise, 3,4-methylenedioxymethamphetamine was identified in the root bulb of rat hair five minutes after administration (Nakahara et al., 1997). In short-term monitoring (from 0–455 minutes after administration) experiments in rats administered MA at 10 mg/kg, i.p., concentrations of MA in plasma increased immediately after administration, and then decreased rapidly but could not detect after 360 minutes (Wada et al., 2012). The concentration of MA in hair root samples was determined from 30 minutes after administration, and reached a maximum at 120 minutes, remaining relatively constant through 455 minutes (Wada et al., 2012).

Herkey and Henderson (1989) reported that hair is best collected from posterior vertex. In this area, follicles in the growing phase are more constant. There are more long terminal hairs and the hairs are less influenced by age and sex. Nakahara et al. (1990) also found that the vertex hair of most MA abusers had minimal variation of drug levels when compared with the other region.

In hair analysis, it is difficult to know if a detected compound had existed inside or outside the hair, even if the hair sample is washed. It is very hard to isolate compounds that exist only within the hair bulb, because plucking hair bulb is bare and drug inside hair is also susceptible to effect of wash. However, it was considered that it would be possible to remove any blood adhered to the hair bulb by several rapid washes. It seems likely that any blood which existed on the hair bulb as a result of surface adhesion of the capillary vein onto the hair bulb, however, a portion of the compound which exists inside the hair may also have been affected by the washings. Nevertheless, to compare the concentration of various compounds incorporated inside the hair shaft, it presumably would be necessary to wash the hair samples before extraction (Nakahara, 1998).

Nakahara et al. (1997) also found that, upon intraperitoneal administration of MA, methylenedioxymethamphetamine (MDMA) or phencyclidine into rats, the

compounds reached the rat hair root within 5 minutes after administration. Therefore, it would be possible to monitor compounds in the hair root shortly after a drug is administered, similar to drug monitoring in the plasma.

Nagata et al. (1983) extensively measured MA concentration in blood obtained from actual cases and presented a criterion on MA intoxication as a function of its blood levels: 4.5 to 6.0 ng/ml, fatal; 3.0 to 4.5 ng/ml, severe; 0.4 to 3.0 ng/ml, moderate; and < 0.3 ng/ml, weak. In comparison with these blood levels, it seems likely that MA in blood is actively accumulated in hair, since its levels in hair of some samples in the study of Suzuki et al. (1984) was far exceed the fatal blood level.

The concentrations of MA and AP in black hairs were higher than those in white hairs taken from the same person (Takayama, 1999; Al-Dirbashi et al., 2000). The concentrations of MA in brown hairs were higher than those in blond and red hairs (Mieczkowski and Newel, 2000). Higher concentrations of AP in black and brown hairs were also reported (Kelly et al., 2000). It was reported that concentration of AP in black hairs was higher than that in white hairs of rat (Borges et al., 2001). Many studies also examined the effects of hair color on other illegal drugs such as cocaine (Joseph et al., 1996; Kelly et al., 2000). The permanent treatment broke disulfide bonds that strengthen keratins in the hair by one of the permanent treatment ingredients, such as ammonium thioglycolate. Because MA has an affinity for melanin or amino acids containing SH groups, MA might be eluted from hair into the permanent treatment solution that breaks the disulfide bonds. The decrease of MA and AP by the hair dye and bleach treatments was considered to be due to destruction of melanin in hair by hydrogen peroxide and ammonia. It was reported that MA changed to *o*-, *m*- and *p*-OHMA isomers in the presence of hydrogen peroxide at 39°C using LC/MS/MS (Tanaka et al., 2001). The effects of permanent and bleach treatments on other illegal drugs such as morphine, codeine (Skopp et al., 1997; Jurado et al., 1997; Yegles et al., 2000), cocaine (Jurado et al., 1997; Yegles et al., 2000), and tetrahydrocannabinol (Jurado et al., 1997) were examined in many studies. The ionic interaction of basic drug

with melanin which is an acidic compound containing carboxyl and semiquinone groups may have an effect on its transformation and retention in black hair (Kikura and Nakahara, 1998).

Hair structure

Hair is cylindrical structure, shafts, made of tightly compacted cell follicles. The diameters of human hair shafts range from 15 to 120 μm depend on hair types and located area. Hair follicle embedded in the dermis, 3-4 mm. below the skin surface that are close to sebaceous and apocrine glands. It is composed of 65 to 95% proteins, 1 to 9% lipids, 0.1 to 5% pigments (melanin), and small amounts of trace elements, polysaccharides, and water (Harkey, 1993). Human hair contains at least two cell types: the cuticle composed of overlapping scale cells and the cortex composed of spindle-shaped cortical cells. In the core of the cortex there may be condensed cells forming the medulla, which might be continuous or interspersed with air spaces. The main features of the hair follicle are shown in Figure 3.

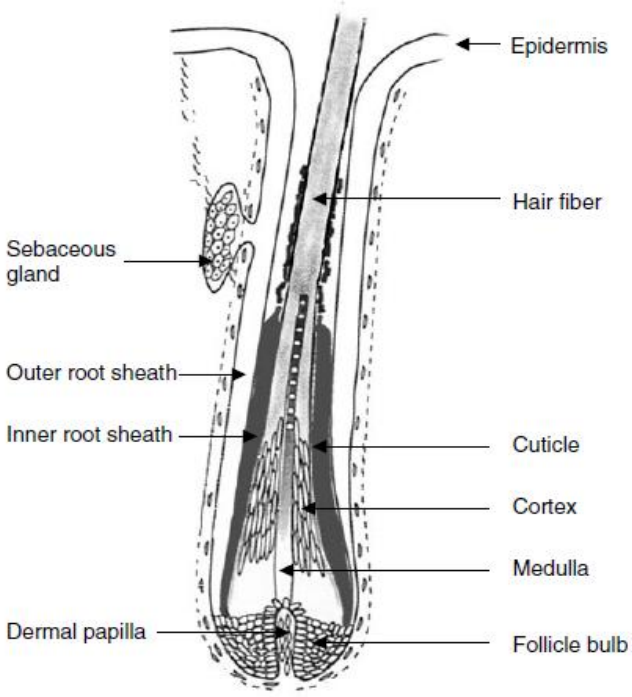

Figure 3 Hair follicle structure (Powell et.al., 1997)

Routes of incorporation (Kronstrand, 2007)

The pathway of drug incorporation into hair and the mechanisms by which they bind to hair constituents have been discussed in the scientific literatures. The schematic view of pathways for incorporation of drugs into hair is shown in Figure 4. There are three pathways of drug incorporation into hair. The first pathway is active or passive diffusion from the bloodstream feeding the dermal papilla. The second pathway is diffusion from sweat and other secretions bathing the growing or mature hair fiber. The third pathway is external drug from vapors or powders that diffuse into the mature hair fiber.

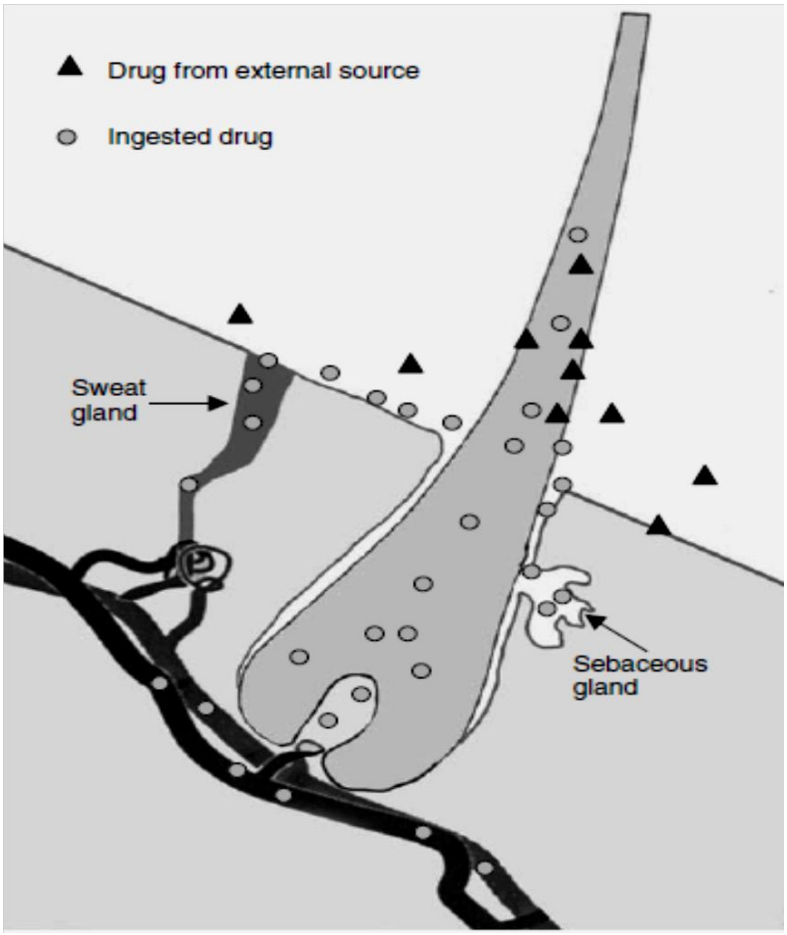

Figure 4 Three pathway of drug incorporation into hair (Kronstrand, 2007)

MA in hair analyses by gas chromatography/mass spectrometry (GC/MS) (Musshoff et al., 2002; Nakahara et al., 1991), liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) (Wood et al., 2003), and direct sample introduction (DSI) that is added to GC/MS/MS in order to analyse faster because of reduction of extraction steps (Amirav and Dagan, 1997). Then, DSI instrument is developed and called Thermal Seperation Probe (TSP).

Niwaguchi et al. (1983) detected MA and AP using GC/MS in rat hairs given MA orally. MA was also detected in the hairs of MA addicts by GC/MS (Ishiyama et al., 1983) and immunoassay (Aoki and Kuroiwa, 1983). Several studies reported the detections of illegal drugs in hair such as cocaine (Cone et al., 1991; Mieczkowski, 1992, 1994; Marques et al., 1993), morphine (Cone, 1990), codeine (Mieczkowski and Newel, 1994), cannabinoids (Han et al., 2011), MA and its related compounds (AP,3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyethylamphetamine) (Nakahara, 1990, 1995; Moeller, 1992; Kikura, 1998; Kikura and Nakahara, 1998.

The purpose of clean-up of hair is to remove the external contamination. Takayama et al. (2003) reviewed the clean-up, extraction, analytic methods and distribution of MA and it metabolites in hairs. Several washing solvents have been reported, including water (Aoki and Kuroiwa, 1983; Kajitani et al., 1989; Cassani and Spiehler, 1993; Kimura et al., 1999), dichloromethane and water (Kintz et al., 1992, 1995; Skender et al., 2002; Musshoff et al., 2002), methanol and water (Suzuki et al., 1984; Moriya et al., 1992; Takayama et al., 1997, 1999; Kronstrand et al., 1998), ethanol and/or acetone and water (Takahashi, 1984; Takahashi et al., 1984; Kintz et al., 1992;), Tween 20 and water (Scarcella et al., 1997), sodium dodecylsulfate (SDS) and methanol/water (Nakahara et al., 1992, 1993, 1997, 1998; Kikura and Nakahara, 1995; Nakahara and Kikura, 1996, 1997; Al-Dirbashi et al., 1999, 2000; Cooper et al., 2000; Yamada et al., 2001; Kalasinsky et al., 2001), SDS and diluted hydrochloric acid (Ishiyama et al., 1983; Kimura et al., 1999), diluted hydrochloric acid and water

(Niwaguchi et al., 1983). Other procedures such as incubation, sonication and vortex mixing were also used to increase the efficiency of these washing solvents. Among these, the most common clean-up method is to wash the hair with 1% SDS and water alternately 3-5 times each. Kintz and Cirimele (1997) compared four different clean-up procedures for MDA, AP and MDMA including methanol sonication, acid hydrolysis, alkaline hydrolysis and enzymatic hydrolysis.

GC/MS is the method that combines the feature of gas chromatograph and mass spectrometry. Gas chromatograph utilizes a capillary column which depends on the column (length, diameter, film thickness) and stationary phase properties. The difference in the chemical properties between different molecules in matrix will separate the molecules as the sample moves through the column. The molecules are retained by the column and then elute from the column at different times, the retention times (RT) and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments according to their mass charge ratio. Basically, mass spectrometry involves first, the production of ion from the sample eluted from the column. The ion can be molecular ions, ion fragments or ion complexes, depends on the type of ionization process that is employed. Then accelerated to high velocities in a vacuum and, by arranging for them to pass through either electric or magnetic fields, the ions can be separated from one another on the basis of their individual masses. By means of suitable scanning procedure, each individual ion mass is then sensed and its mass is identified (Scott, 2012: online).

Thermal Separation Probe or TSP is one of the direct sample introduction devices. The direct sample introduction devices was designed and built by Professor Aviv Amirav and Shai Dagan and patented in the United States and Japan in 1997. The TSP is based on sample introduction into the sample inlet of GC in a small disposable

glass vial (Aviv et al., 2011: online). The structure and components of TSP are shown in Figure 5.

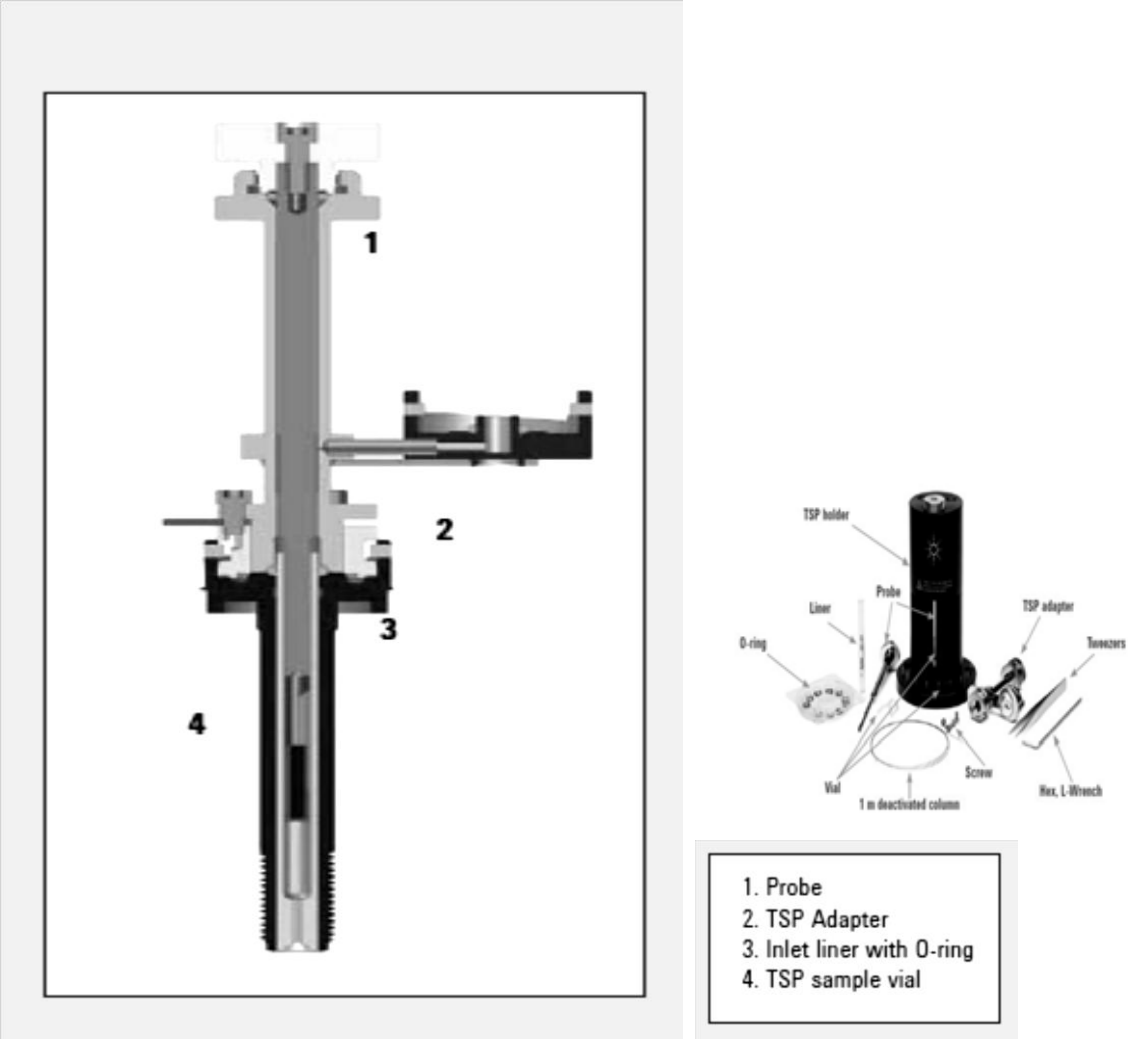

Figure 5 The structure and components of TSP (Aviv et al., 2011: online) (http://www.tau.ac.il/chemistry/amirav/dsi.shtml)

The TSP can be used in two main modes of operate. The first, sample introduction for mass spectrometry, the TSP is connected to a short capillary column. This transforms a GC injector into an effective alternative to the standard direct insertion probe. The second main mode is extract-free sample introduction for GC/MS. The sampling is performed in the small disposable vial that retains non-volatile matrix residue thus eliminates the sample clean-up steps. Accordingly, the TSP serves as low cost manual thermal desorption unit with excellent GC integrity (Amirav, 1997).

GC/MS method is the most popular method for analyzing a very small amount of drugs in hair. Ishiyama et al. (1983) analyzed MA and AP in hair by trifluoroacetyl (TFA) derivatization with N-ethylbenzylamine as an internal standard. The concentrations of AP in MA abusers' hair ranged from 4 to 120 ng/mg. Niwaguchi et al. (1983) detected MA in Wistar rat hair after single, 5-day and 14-day repeated administration of 20 mg/kg/day of MA. The concentrations of MA in the hair were quite low (0.5-1.9 ng/mg). Suzuki et al. (1984) demonstrated that MA and AP in a single hair could be detected by methane GC/CI/MS (Gas chromatography/chemical ionization/mass spectrometry). Moriya et al. (1992) sectionally analyzed the scalp and pubic hair of a deceased habitual abuser of MA. They obtained results for the root- to 0.2-cm section of the scalp which were 1.06 and 2.52 ng/mg, respectively, and which were apparently higher than the other sections.

Nakahara et al. (1990) demonstrated that distribution of MA in 1 or 2 cm sectional hair mostly corresponded to drug histories which abusers reported to their doctors. They also reported the movement of methoxyphenamine along human hair shaft with hair growth and studied the excretion of methoxyphenamine into human beard hair by stable isotope dilution-GC/MS. It was found that drug concentration in the next day's beard following the last use of 7 days-doses was at a peak and the drug was continuously excreted into beard for more than 7 days after quitting drug use. Moeller et al. (1992) reported the detection of MDMA and AP in human hair by GC/MS-SIM (selected ion monitoring).

It has been reported the detection in human hair by GC/MS of AP, MA, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), and N-methy-1-1-(1,3-benzodioxol-5-yl)-2-butylamine (MBDB) (Ishiyama, Nagai, and Toshida, 1983; Suzuki, Hattori, and Asano, 1984). It was also performed the detection of MA and AP in a single hair by HPLC-chemiluminescence (Takayama, Tanaka, and Hayakawa, 1997). Sectional hair analysis for MA was shown to correspond to the reported drug history. Moreover, five minutes after administration of MDMA in rats, MDMA and MDA could be detected in hair root

samples (Nakahara 1999). The detection limit of MDMA and MDA in hair was approximately 0.125 ng/mg of hair (Han et al., 2005).

Stability test of MA was performed in non-sterile samples. There was not changed in concentrations of MDMA and MA after 3 and 7 days of storage at 37 ˚C, compared with keeping in -20 ºC (Jim´enez et al., 2006).

Hughes et al. reported the stability of amphetamine and methamphetamine in spiked urine samples stored at 4 ˚C for up to 6 months (Hughes, 1991). Nakata et al. reported that there was not changed in concentrations of MA in postmortem rabbit tissues and blood under 25ºC for more than 24 months. Butzbatch reported that concentration of MA and primary metabolites did not change in various conditions (Butzbatch, 2010).

Stability of MA in hair stored at 4°C compared to hair kept at room temperature for 92 days, there was no significant difference in the concentration of MA between the two conditions (Lee et al., 2010).

CHAPTER III

METHODOLOGY

1. Chemicals and Instruments

1. Chloroform, ether, methanol, potassium hydroxide (KOH), sodium hydroxide (NaOH) (Merck KGaA Co. Ltd., Germany)

2. Methamphetamine HCl (Sigma-Aldrich Corporation, United States) was prepared as stock solution 10 mg/ml, trimipramine (Sigma-Aldrich Corporation, United States) was prepared as stock solution of 100 ng/ml

3. Orange Test Methamphetamine Strip® (True line Med. Co. Ltd., Switzerland)

4. Beakers, cylinder, micropipettes, micro centrifuge tubes 2 ml, injection vial for GC/MS/MS with lids (National Scientific Co. Ltd., United States), pipette tips, Thermal Seperation Probe (TSP, Agilent Technologies Co. Ltd., Germany)

5. Gray-top vacutainer tube, 3 ml containing 2.5 mg/ml of sodium fluoride (NaF) and 2.0 mg/ml of potassium oxalate for collecting blood samples (Cangzhou Yongkang Medical Devices Co. Ltd., China)

6. Plastic container, 60 ml with lid for collecting urine samples, plastic bag for collecting hair roots

7. GC/MS/MS comprises GC (7890A, Agilent Technologies Co. Ltd., Germany) MS/MS (7000, Agilent Technologies Co. Ltd., Germany)

2. Subjects

Thai deceased whose bodies were sent for autopsy at the Institute of Forensic Medicine, Police General Hospital, The Royal Thai Police Headquarter during 2012, January-December. The number of subjects was not less than 30.

2.1 Sample size calculation (Zou et al., 2003)

$$N = \frac{(Z_{\alpha} + Z_{\beta})}{\frac{1}{4}\log_e\left[\frac{(1+\rho)}{(1-\rho)}\right]}^2 + 3$$

N = sample size

α = type I error and β = type II error

ρ = correlation coefficient

If $\rho = 0.5$, $\alpha = 0.05$ then $Z_{\alpha} = 1.96$; If power = 0.8 then $Z_{\beta} = 0.84$

$$N = \frac{(1.96 + 0.84)^2}{\frac{1}{4}\log_e\left[\frac{(1+0.5)}{(1-0.5)}\right]}^2 + 3$$

N = 29

Therefore, the number of samples not less than 30 cases was used to assess the correlations. Additional 20 samples were used to test the correlation equations.

2.2 Inclusion criteria

Thai deceased whose bodies were sent for autopsy at the Institute of Forensic Medicine, Police General Hospital, The Royal Thai Police Headquarter. Orange Test Methamphetamine Strip® was used to exclude the subjects whose urine samples were MA negative. The deceased whose urine samples were MA positive by the strip tests, their urine, blood and hair root samples were collected for analysis of MA by GC/MS/MS.

2.3 Exclusion criteria

The deceased according to 2.2 whose urine samples were MA negative by Orange Test Methamphetamine Strip®.

3. Procedure

3.1 Hair root, blood, and urine samples collections

Hair root samples were plucked from the posterior vertex region of the head and kept in well closed plastic bags. Blood samples were collected from basal artery and kept in sodium fluoride tubes. Urine samples were collected from urinary bladder and kept in well closed plastic containers. All samples were stored at 2-4 °c until analysis.

3.2 Sample preparations

3.2.1 Hair root samples were washed once by vortexing with methanol for 1 minute. After drying, they were cut into 0.5 cm in length and 1 mg of the samples was transferred to an injection vial for TSP according to the method modified from Wainhaus et al. (1998). Then, 1 µl of 100 ng/ml trimipramine in methanol was added. The content in the vial was injected to GC/MS/MS via TSP interface.

3.2.2 Analysis of MA in blood samples was modified from Marquet et al. (1997). Blood samples, 500 µl, were extracted via liquid/liquid extraction with 1 ml of chloroform after adjusted to pH 10-11 with 200 mM potassium hydroxide. Then, 200 µl of 100 ng/ml trimipramine in methanol was added. The mixture was vortexed for 5 minutes, centrifuged at 5000 rpm for 5 minutes. The supernatant of 1000 µl was transferred to an injection vial and the vapor was injected to GC/MS/MS. Each sample was performed in duplicate.

3.2.3 Preparation of urine samples was modified from Marquet et al. (1997). Urine samples of 500 µl was extracted via liquid/liquid extraction with 1 ml of diethyl ether after adjusted to pH 10-11 with 200 mM potassium hydroxide. Then, 200 µl of 100 ng/ml trimipramine in methanol was added. The mixture was vortexed for 5 minutes, centrifuged at 5000 rpm for 5 minutes. The

supernatant of 1000 µl was transferred to an injection vial and the vapor was injected to GC/MS/MS. Each sample was performed in duplicate.

Instrument condition

GC/MS/MS triple quadrupole was operated in multi reaction monitoring mode with helium gas type. The instrument was operated with a DB-5MS UI column (15 m, 0.25 mm, 0.25 µm film thickness, Agilent part number 122-5512 UI) using helium as the carrier gas at flow rate of 50 ml/min. The GC oven temperature programmed from 80°c to 240°c (at 20°c/min).The injector and transfer lines were set at 280°c.

3.3 MA standard curves

3.3.1 MA stock standard solution preparations

MA HCl of 0.94 mg was dissolved in 940 µl of methanol to obtain the MA concentration of 1 mg/ml.

3.3.2 MA standard curve of blood samples

Working MA standard solutions of 50, 100, 150, 250, 500, and 1000 ng/ml were prepared by serial dilution from MA stock standard solution as following:

1. 5 µl of 1 mg/ml MA was diluted with 5 ml of blank blood sample to obtain 5 ml of 1000 ng/ml MA.
2. 2.5 ml of 1000 ng/ml MA was diluted with 2.5 ml of blank blood sample to obtain 5 ml of 500 ng/ml MA.
3. 2.5 ml of 500 ng/ml MA was diluted with 2.5 ml of blank blood sample to obtain 5 ml of 250 ng/ml MA.
4. 1.5 ml of 500 ng/ml MA was diluted with 3.5 ml of blank blood sample to obtain 5 ml of 150 ng/ml MA.
5. 2 ml of 250 ng/ml MA was diluted with 3 ml of blank blood sample to obtain 5 ml of 100 ng/ml MA.

6. 2.5 ml of 100 ng/ml MA was diluted with 2.5 ml of blank blood sample to obtain 5 ml of 50 ng/ml MA.

Each concentration of MA standard solutions was analyzed by GC/MS/MS in triplicate.

3.3.3 MA standard curve of urine samples

Working MA standard solutions of 100, 250, 500, 1000, and 2000 ng/ml were prepared by serial dilution from MA stock standard solution as following:

1. 10 µl of 1 mg/ml MA was diluted with 5 ml of blank urine sample to obtain 5 ml of 2000 ng/ml MA.
2. 2.5 ml of 2000 ng/ml MA was diluted with 2.5 ml of blank urine sample to obtain 5 ml of 1000 ng/ml MA.
3. 2.5 ml of 1000 ng/ml MA was diluted with 2.5 ml of blank urine sample to obtain 5 ml of 500 ng/ml MA.
4. 2.5 ml of 500 ng/ml MA was diluted with 2.5 ml of blank urine sample to obtain 5 ml of 250 ng/ml MA.
5. 2 ml of 250 ng/ml MA was diluted with 3 ml of blank urine sample to obtain 5 ml of 100 ng/ml MA.

Each concentration of MA standard solution was analyzed by GC/MS/MS in triplicate.

3.3.4 MA standard curve of hair root samples

Working MA standard concentrations in blank hair of 1, 5, 10, 25, and 50 µg/ml were prepared by serial dilution from MA stock standard solution as following:

1. 100 µl of 10 mg/ml MA was diluted by addition of 10 ml MeOH to obtain 10 ml of 100 µg/ml MA.
2. 500 µl of 100 µg/ml MA was diluted by addition of MeOH to obtain 1000 µl of 50 µg/ml, then 1 µl of this solution was transfered to TSP that filled with 1 mg of blank hair root samples to obtain 50 ng/mg of MA standard.

3. 500 µl of 50 µg/ml MA was diluted by addition of MeOH to obtain 1000 µl of 25 µg/ml, then 1 µl of this solution was transfered to TSP that filled with 1 mg of blank hair root samples to obtain 25 ng/mg of MA standard.
4. 400 µl of 25 µg/ml MA was diluted by addition of MeOH to obtain 1000 µl of 10 µg/ml, then 1 µl of this solution was transfered to TSP that filled with 1 mg of blank hair root samples to obtain 10 ng/mg of MA standard.
5. 500 µl of 10 µg/ml MA was diluted by addition of MeOH to obtain 1000 µl of 5 µg/ml, then 1 µl of this solution was transfered to TSP that filled with 1 mg of blank hair root samples to obtain 5 ng/mg of MA standard.
6. 200 µl of 5 µg/ml MA was diluted by addition of MeOH to obtain 1000 µl of 1 µg/ml, then 1 µl of this solution was transfered to TSP that filled with 1 mg of blank hair root samples to obtain 1 ng/mg of MA standard.

Each concentration of MA standard solution was analyzed by GC/MS/MS in triplicate.

3.4 Method validation

3.4.1 Accuracy test

Blank hair root samples of 1 mg with 1 µl of 1, 10, 50 µg/ml of MA were transferred to TSP and analyzed according to sample preparations mentioned above followed by GC/MS/MS technique. Each concentration was performed for 5 times. Blood samples containing 50, 250, 1000 ng/ml of MA were analyzed according to the sample preparations mentioned above followed by GC/MS/MS technique. Each concentration was performed for 5 times. Urine samples containing 100, 500, 2000 ng/ml of MA were performed in the same manner for 5 times for each concentration. Accuracy was evaluated by the percentage of recovery by the following equation

$$\% \text{ Recovery} = \frac{\text{Measured MA concentration}}{\text{Actual MA concentration}} \times 100$$

The mean value of % recovery should be within 15% (U.S. FDA, 2001)

3.4.2 Precision test

Precision of the assay was evaluated by the percentage of coefficient of variation (%CV). Percentage of CV was calculated as following:

$$\% \text{ CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The % CV should not exceed 15% (U.S. FDA, 2001)

1) Within-day precision

To evaluate within-day precision, blank hair root samples of 1 mg with 1 µl of 1, 10, 50 µg/ml of MA were transferred to TSP and analyzed according to sample preparations mentioned above followed by GC/MS/MS technique. Each concentration was performed 5 times. Blood samples containing 50, 250, 1000 ng/ml of MA were analyzed according to the sample preparations mentioned above followed by GC/MS/MS technique. Each concentration was performed 5 times. Urine samples containing 100, 500, 2000 ng/ml of MA were performed in the same manner for 5 times for each concentration within 24 hours.

2) Between-day precision

To evaluate between-day precision, hair root, blood and urine samples containing 10 ng/mg, 250 and 500 ng/ml of MA, respectively were analyzed with 3 replicate analyses. The experiments were performed for four consecutive days.

3.4.3 Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD was determined from hair root, blood and urine containing various concentrations of MA according to the method mentioned above until obtained the samples with lowest concentration of MA that showed the peak with a signal-to-noise ratio of at least 3 or 2:1 (U.S. FDA, 1996).

LOQ was determined from hair root, blood and urine containing various concentrations of MA according to the method mentioned above until obtained the samples with lowest concentration of MA that could be analyzed with the accuracy and precision not exceed 20% (U.S. FDA, 2001). Based on signal to noise, the ratio of 10:1 is recommended for determining the quantitation limit (U.S. FDA, 1996).

3.5 Relationships between MA concentration in hair root, blood and urine samples

Hair root, blood and urine samples of 30 deceased (Group I samples) were prepared and quantitated for MA concentrations according to the method as mentioned above. Each sample was analyzed in duplicate.

The results were presented as mean ($\bar{X}$) $\pm$ standard deviation (S.D.). Relationships between MA concentrations in hair root, blood and urine samples was analyzed by Pearson's correlation and simple linear regression using SPSS for windows, version 16.0. A *p-value* of less than 0.05 was considered statistically significant.

3.6 Verification of the linear regression equations

Hair root, blood and urine samples of 20 deceased (Group II samples) were prepared and quantitated for MA concentrations according to the method as mentioned above. MA concentration in hair root of each subject was used to calculate for MA concentration in blood and urine samples using the regression equations obtained from the results of group I samples. The differences between measured and calculated MA concentration in blood or urine samples were statistically analyzed by Paired t-test. A difference was considered to be significant at $p < 0.05$.

CHAPTER IV

RESULTS

Method validation

The standard calibration curves of peak area ratio of MA to internal standard (trimipramine) and MA concentrations in blank hair root, blood and urine samples were shown in Figure 6-8.

The method validation was reported by linearity, precision, accuracy, LOD and LOQ. Linearity was shown by the closely linear relationship between measured MA concentrations and actual MA concentrations in hair root samples (R^2 = 0.998, p <0.000, Figure 9), blood samples (R^2 = 0.993, p = 0.000, Figure 10) and urine samples (R^2 = 0.998, p = 0.000, Figure 11). Within-day and between-day precision as well as accuracy of the method for determination of MA concentrations in hair root, blood and urine samples were shown in Table 3. It was shown that both % recovery and % CV of the assay at all MA concentrations in all specimens were within 15%. Based on the signal-to-noise ratio of 3:1, the LOD of the method for determining MA concentrations in hair root, blood, and urine sample was 0.125 ng/mg, 40 ng/ml, 40 ng/ml, respectively (Figure 12-14). Quantitation limit based on signal-to-noise ratio of 10:1 and the accuracy as well as precision of less than 20%, it was shown that LOQ of the method for determining of MA concentrations in hair root, blood, and urine sample was 0.2 ng/mg, 50 ng/ml, 50 ng/ml, respectively (Figure 15-17, Table 4-6).

Demographic profile

Demographic profile of 50 MA subjects was shown in Table 7. The postmortem cases included 48 male (96%) and 2 female (4%) corpses with ages range from 17-61 years. Mean $\pm$ S.D. of their ages was 42 $\pm$ 11.41 years. Range of MA concentrations in hair root, blood and urine samples were 1.59-916.34 ng/mg, 4.00-1883.23 ng/ml and 52.89-129823.48 ng/ml, respectively. The causes of death were mostly unknown cases.

Relationships between MA concentrations in hair root, blood, and urine samples

MA concentrations in hair root, blood and urine samples of 30 postmortem cases (Group I samples) were shown in Table 8. The relationship between MA concentrations in hair root and blood samples was shown by the linear regression equation of y = 1.997x - 162.620 (R^2 = 0.818, r = 0.904, p < 0.001) (Figure 18). Those of hair root vs urine samples was y = 77.618x - 683.460 (R^2 = 0.327, r = 0.572, p = 0.001) (Figure 19) and urine vs blood samples was y = 0.011x + 130.210 (R^2 = 0.477, r = 0.690, p < 0.001) (Figure 20).

Verification of the linear regression equations

MA concentration in hair root samples of each 20 deceased was used to calculate MA concentrations in blood and urine samples using the regression equation of y = 1.997x - 162.620, y = 77.618x - 683.460, respectively. The differences between calculated and measured MA concentrations in blood or urine samples were analyzed by Paired t-test. It was shown that calculated and measured MA concentrations in both samples were not statistically different (Table 9).

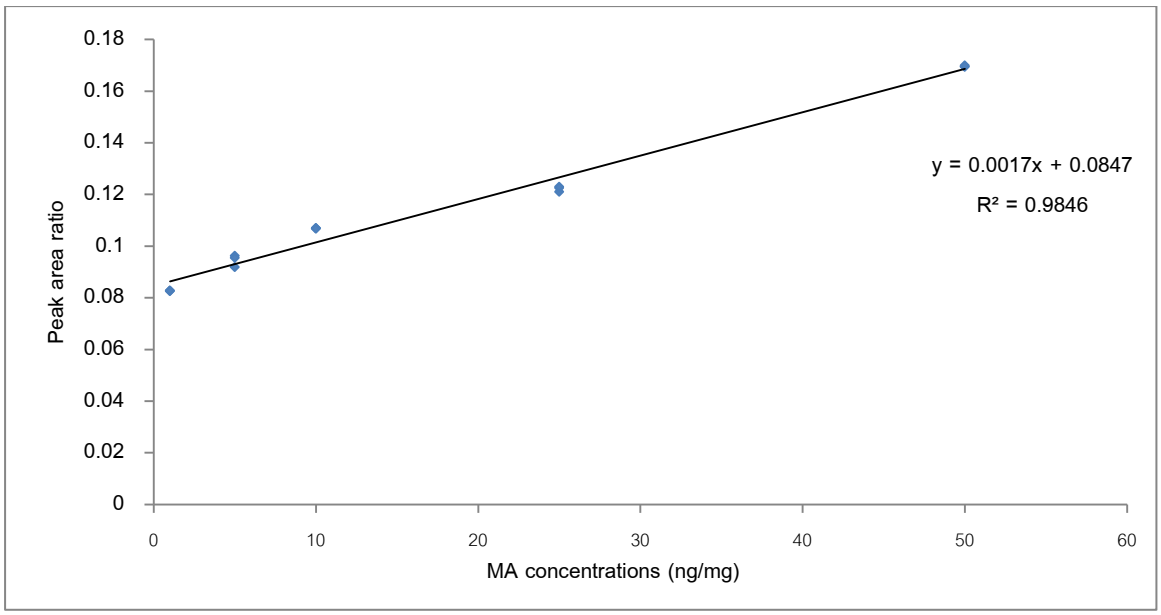

Figure 6 Standard calibration curve of MA in hair root

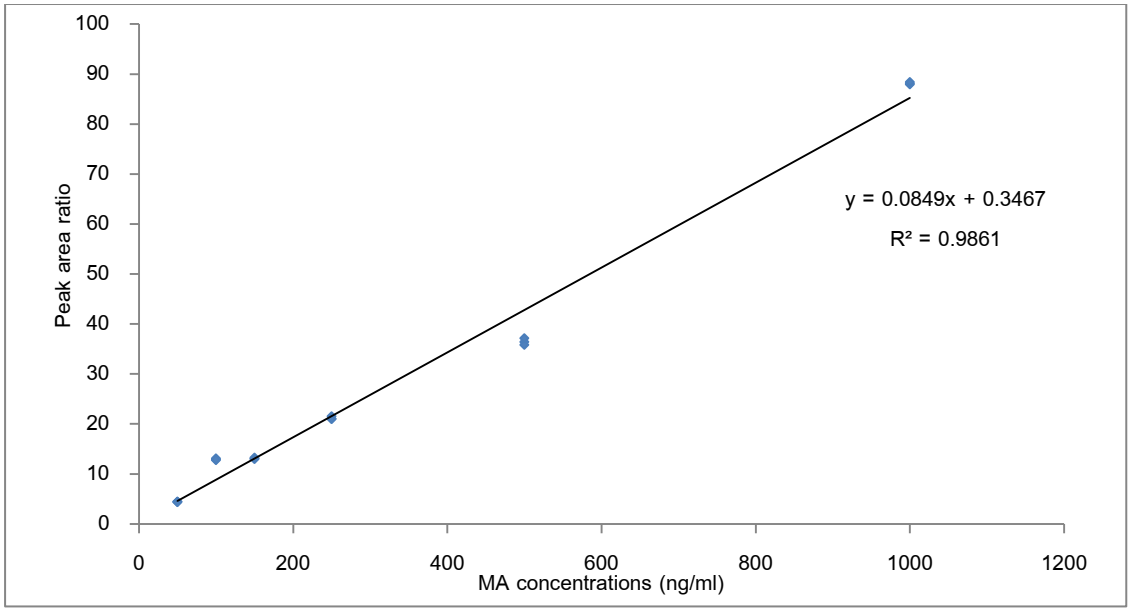

Figure 7 Standard calibration curve of MA in blood

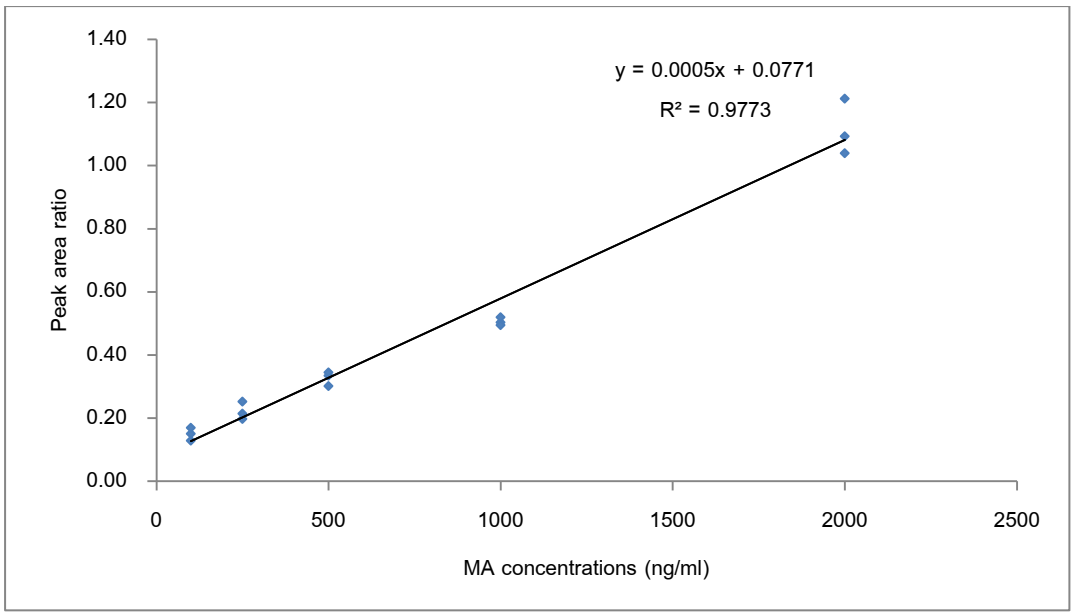

Figure 8 Standard calibration curve of MA in urine

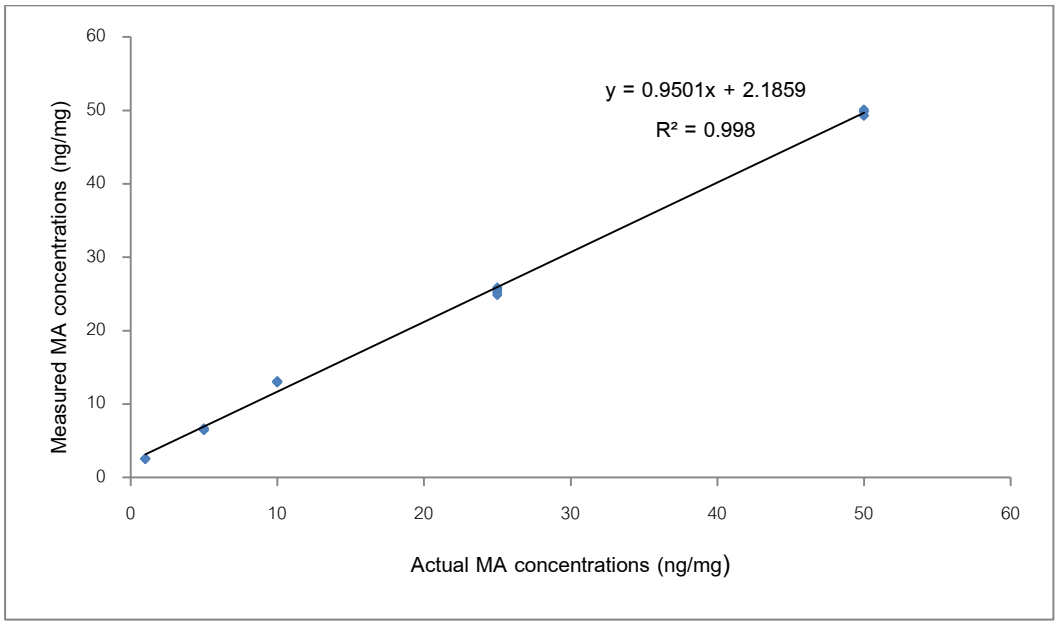

Figure 9 Linearity of the method for determination of MA in hair root samples

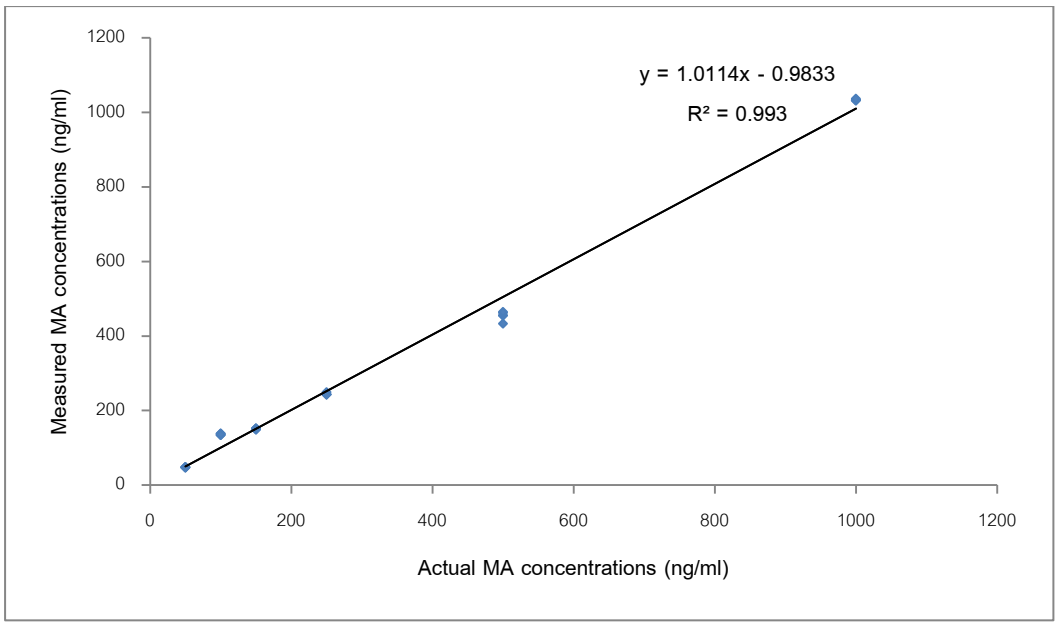

Figure 10 Linearity of the method for determination of MA in blood samples

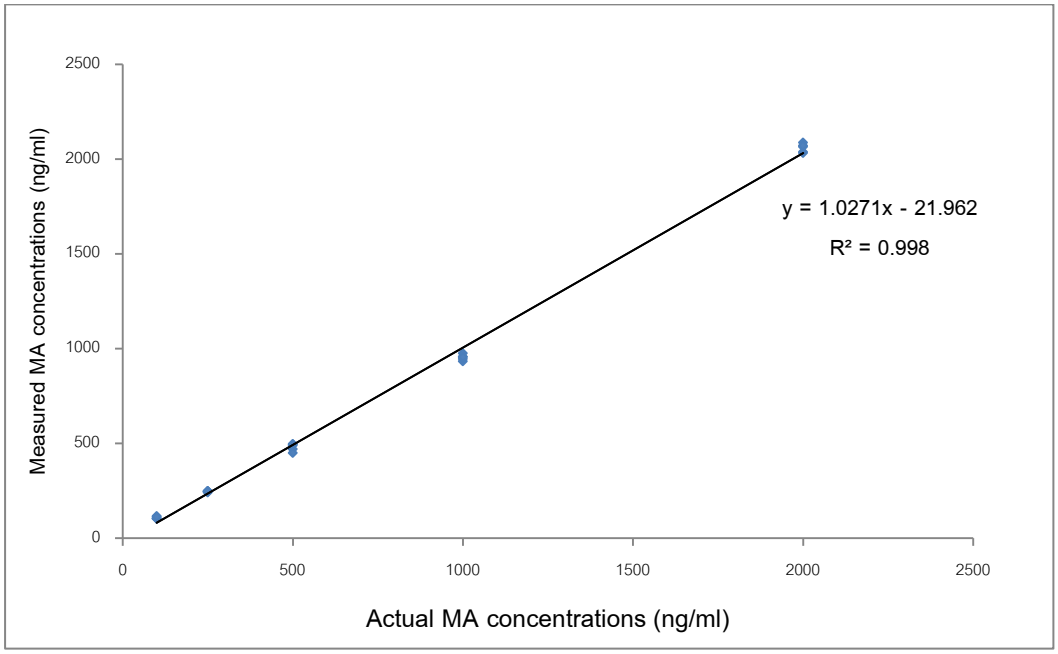

Figure 11 Linearity of the method for determination of MA in urine samples

Table 6 Within-day, between-day precision, and accuracy of the method for determination of MA concentrations in hair root, blood and urine samples

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

[a] The data shown were mean ± S.D. of n = 5

[b] The data shown were calculated from mean and S.D. of n = 5 within one day.

[c] The data shown were calculated from mean and S.D. of n = 4 (4 days). The experiments were performed in triplicate in each day.

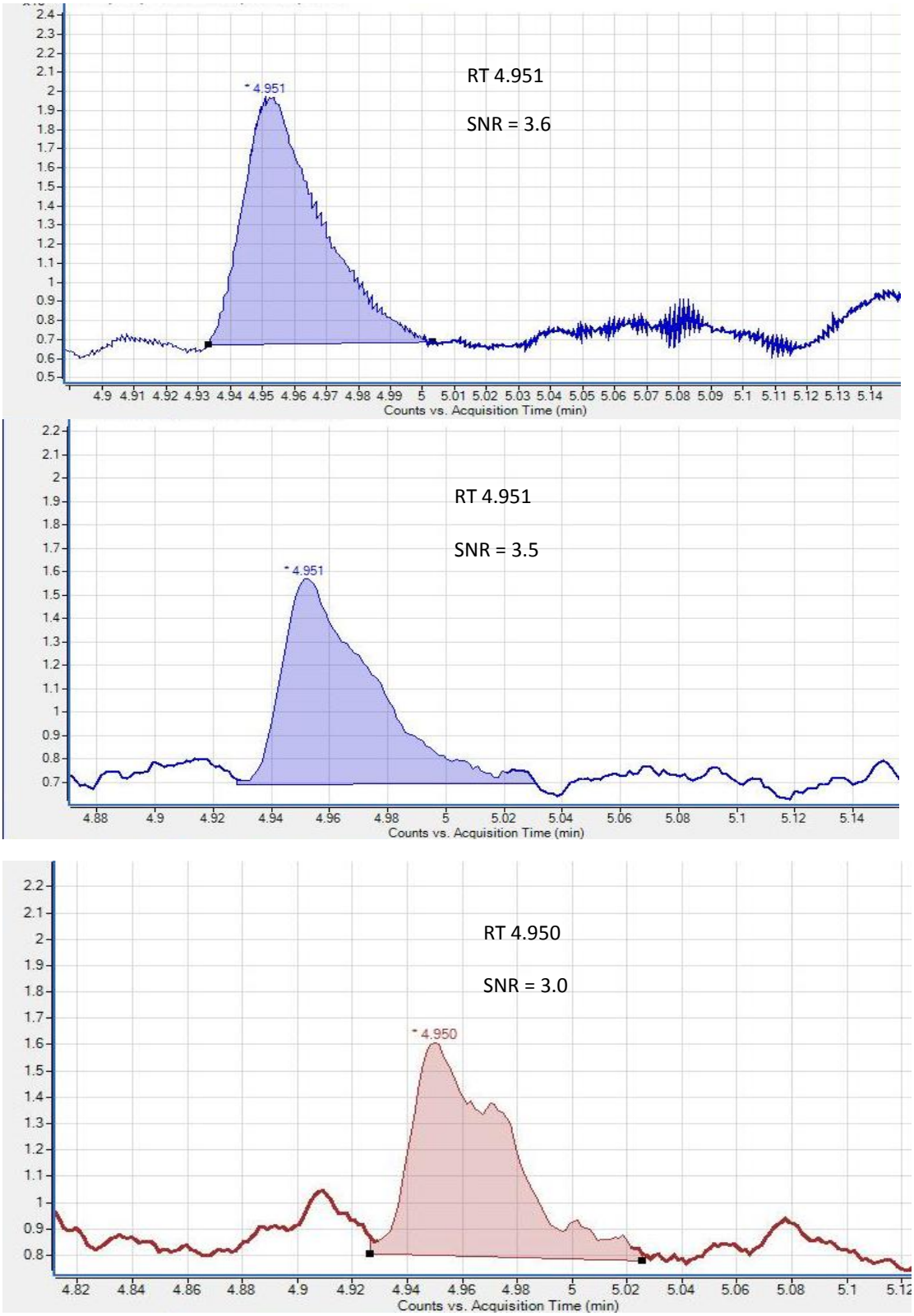

Figure 12 Chromatogram demonstrating LOD of the method for determination of MA in hair root samples (MA concentration = 0.125 ng/mg)

Figure 13 Chromatogram demonstrating LOD of the method for determination MA in blood samples (MA concentration = 40 ng/ml)

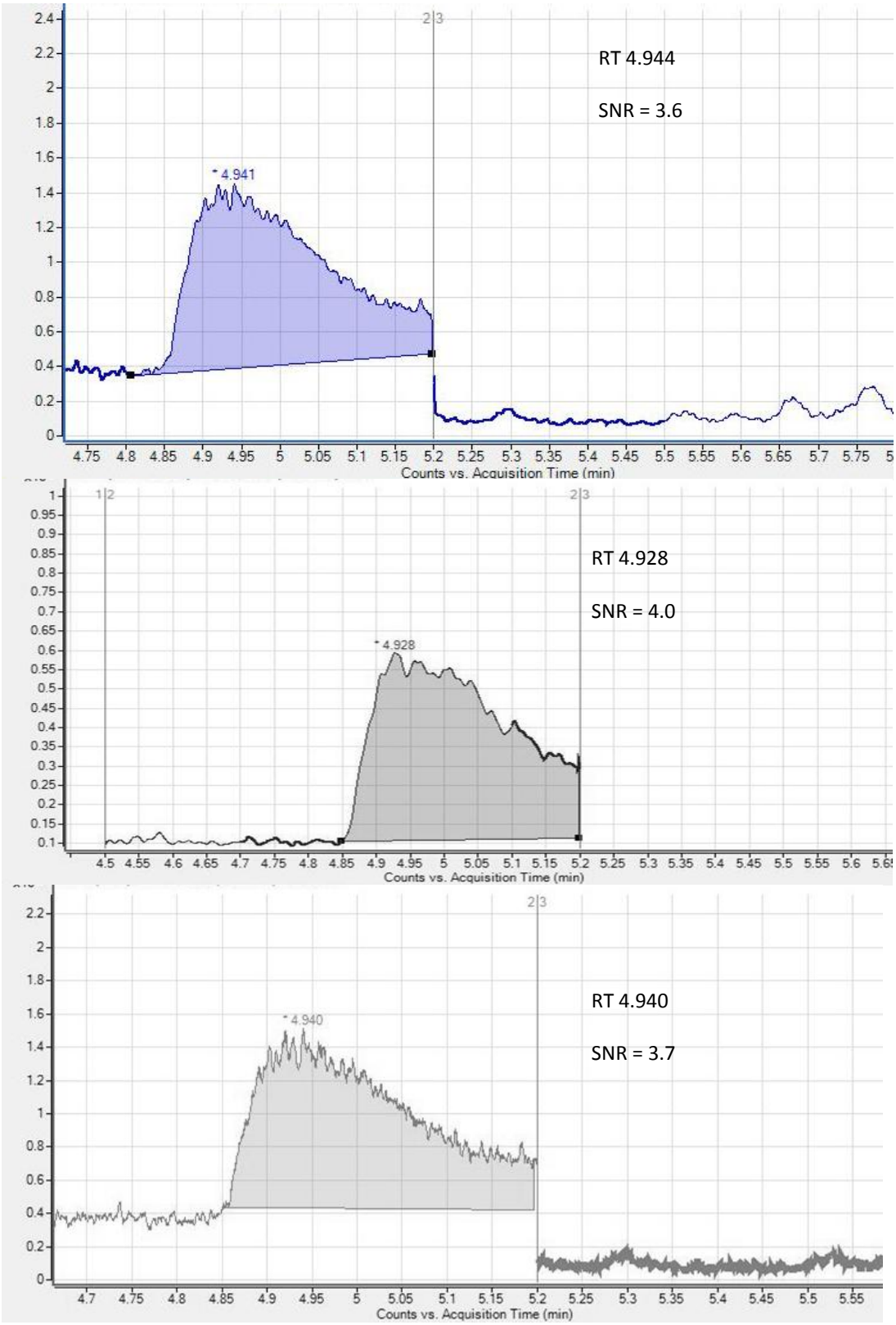

Figure 14 Chromatogram demonstrating LOD of method for determination MA in urine samples (MA concentration = 40 ng/ml)

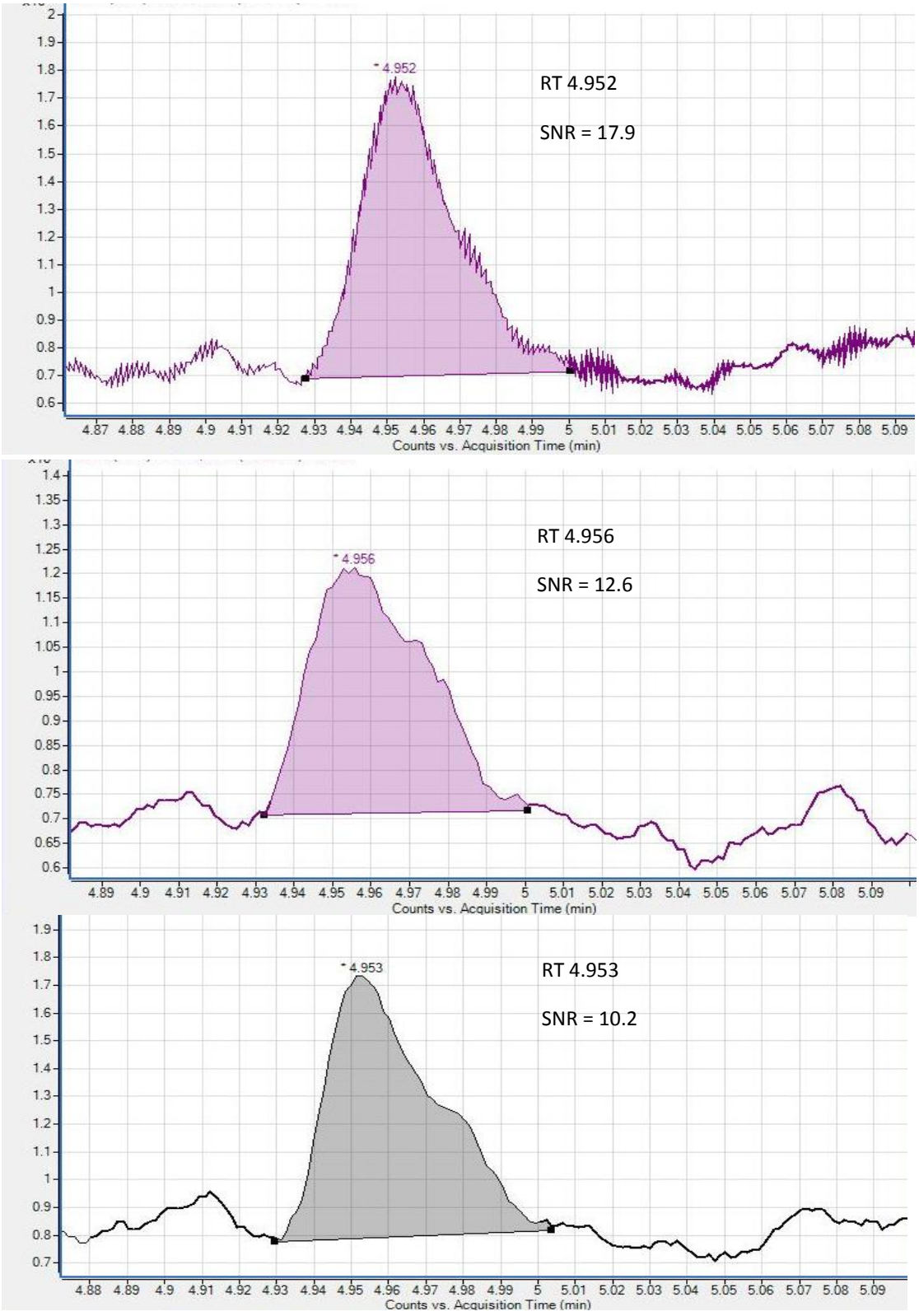

Figure 15 Chromatogram demonstrating LOQ of the method for determination MA in hair root samples (MA concentration = 0.2 ng/mg)

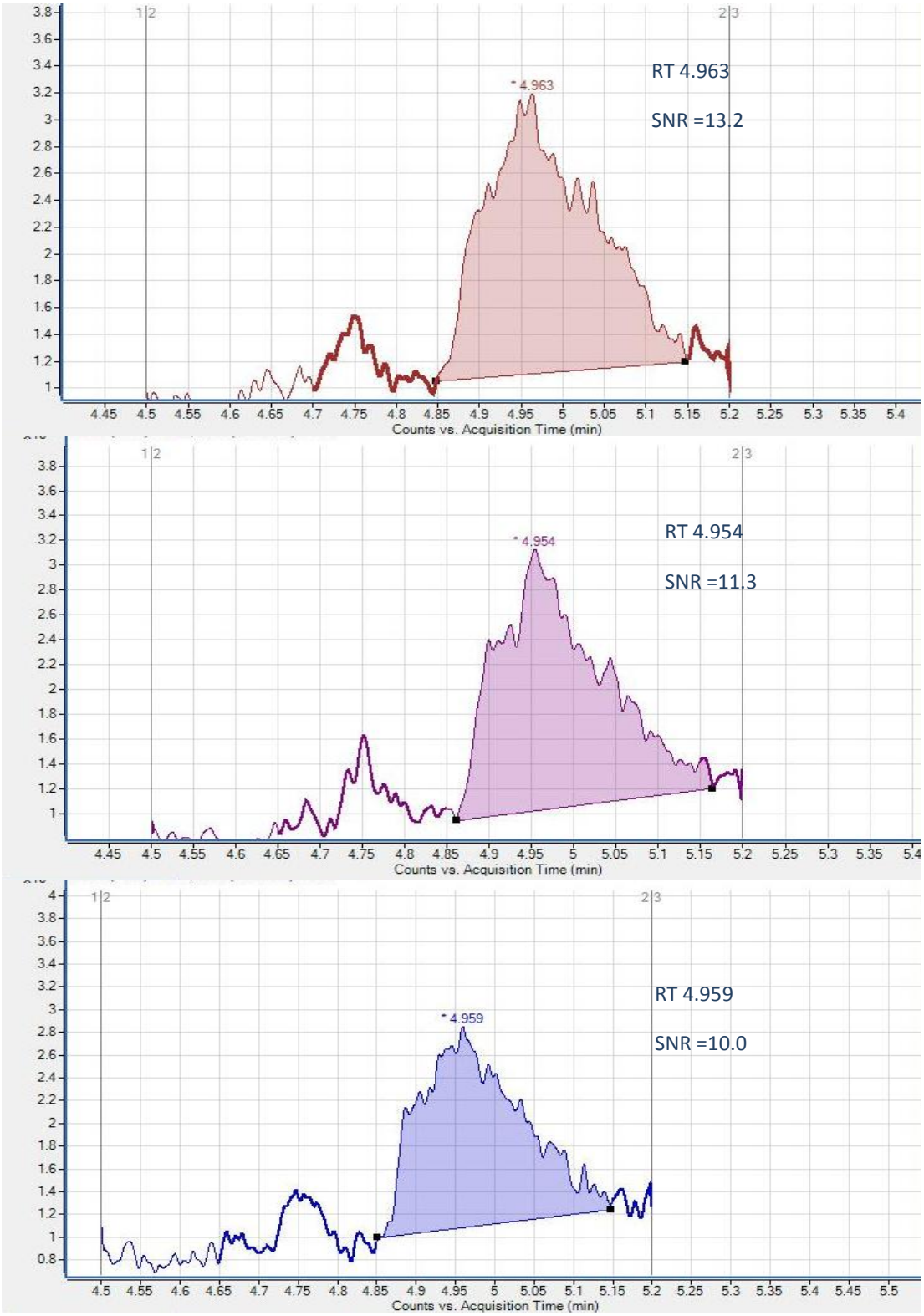

Figure 16 Chromatogram demonstrating LOQ of the method for determination MA in blood samples (MA concentration = 50 ng/ml)

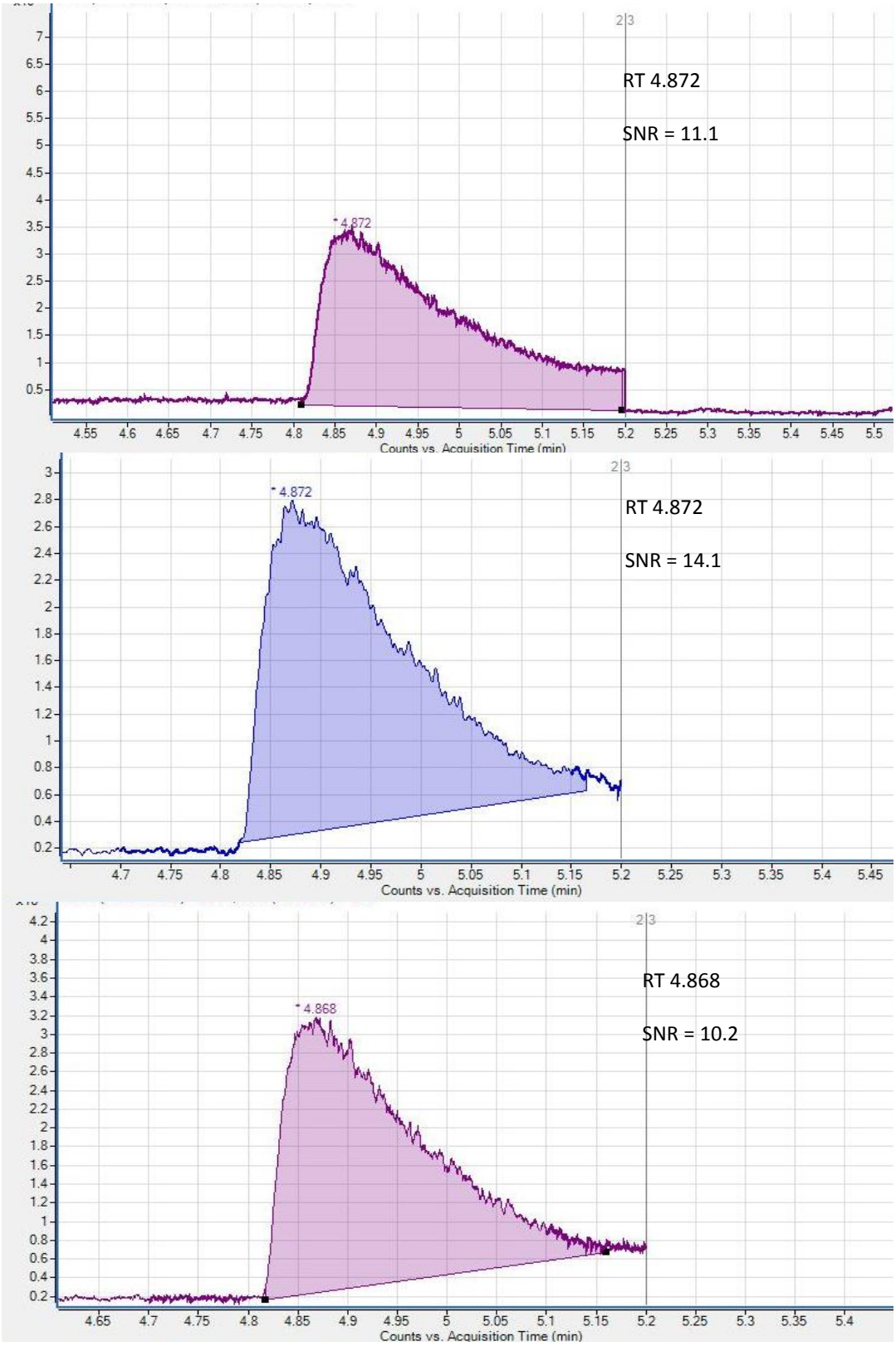

Figure 17 Chromatogram demonstrating LOQ of the method for determination MA in urine samples (MA concentration = 50 ng/ml)

Table 7 LOD and LOQ of the method for determination of MA in hair root samples

MA concentrations (ng/mg)		% recovery	% CV
LOD	0.125	101.93	14.47
		86.12	
		71.79	
		102.02	
		100.75	
		Mean ± SD 92.52 ± 13.39	
LOQ	0.20	97.41	4.73
		88.59	
		86.18	
		89.08	
		91.77	
		Mean ± SD 90.60 ± 4.29	

Table 8 LOD and LOQ of the method for determination of MA in blood samples

MA concentrations (ng/ml)		% recovery	% CV
LOD	40	101.72	3.85
		104.67	
		101.68	
		98.68	
		94.47	
		Mean ± SD 100.24 ± 3.86	
LOQ	50	99.47	2.53
		103.99	
		97.82	
		97.87	
		100.45	
		Mean ± SD 99.52 ± 2.53	

Table 9 LOD and LOQ of the method for determination of MA in urine samples

MA concentrations (ng/ml)		% recovery	% CV
LOD	40	101.86	3.43
		110.00	
		108.67	
		110.13	
		104.57	
		Mean ± SD 107.05 ± 3.67	
LOQ	50	96.80	3.70
		100.52	
		91.52	
		95.48	
		99.64	
		Mean ± SD 96.79 ± 3.59	

Table 10 Demographic profile of the subjects (n = 50)

No.	Sex	Age (years)	Causes of death
1	Female	24	Accident
2	Male	21	Gunshot
3	Male	N/A	Homicide
4	Male	27	Unknown
5	Male	53	Die of disease
6	Male	17	Homicide
7	Male	35	Unknown
8	Male	33	Gunshot
9	Male	45	Homicide
10	Male	35	Unknown
11	Male	37	Accident
12	Male	31	Unknown
13	Male	N/A	Unknown
14	Male	18	Homicide
15	Male	N/A	Electrocution
16	Male	43	Electrocution
17	Male	44	Homicide
18	Male	45	Unknown
19	Male	46	Drowning
20	Male	33	Unknown
21	Male	61	Unknown
22	Female	30	Gun shot
23	Male	35	Unknown
24	Male	55	Unknown
25	Male	N/A	Unknown
26	Male	N/A	Unknown
27	Male	N/A	Gunshot
28	Male	N/A	Accident
29	Male	22	Unknown
30	Male	36	Unknown
31	Male	N/A	Gunshot
32	Male	26	Unknown
33	Male	22	Gunshot
34	Male	N/A	Unknown

No.	Sex	Age (years)	Causes of death
35	Male	26	Gun shot
36	Male	27	Homicide
37	Male	N/A	Gunshot
38	Male	N/A	Accident
39	Male	N/A	Unknown
40	Male	19	Hanging
41	Male	37	Unknown
42	Male	N/A	Unknown
43	Male	20	Accident
44	Male	33	Unknown
45	Male	25	Gunshot
46	Male	50	Hanging
47	Male	N/A	Unknown
48	Male	42	Gunshot
49	Male	N/A	Unknown
50	Male	N/A	Unknown
N = 50	Male = 48 (96%) Female = 2 (4%)	Range = 17-61 Mean $\pm$ S.D. = 42 $\pm$ 11.41	Unknown = 23

N/A = not available

Table 11 MA concentrations in hair root, blood, and urine samples of 30 postmortem cases

No.	MA in hair root (ng/mg)	MA in blood (ng/ml)	MA in urine (ng/ml)
1	82.50 ± 9.19	20.97 ± 1.55	1139.61 ± 3.17
2	729.04 ± 76.42	1686.27 ± 161.17	129823.48 ± 3691.46
3	609.54 ± 8.20	1204.08 ± 96.97	53143.62 ± 1099.35
4	33.76 ± 3.15	5.25 ± 2.11	606.63 ± 33.09
5	53.95 ± 3.03	16.25 ± 2.98	10543.36 ± 4376.64
6	285.62 ± 51.41	74.05 ± 2.38	70067.23 ± 5770.27
7	82.72 ± 9.53	13.69 ± 0.70	6882.62 ± 709.53
8	916.34 ± 6.72	1883.23 ± 66.83	46525.60 ± 4836.97
9	167.06 ± 45.46	649.06 ± 87.59	104799.87 ± 12881.73
10	276.85 ± 30.96	251.74 ± 20.71	1547.49 ± 316.88
11	486.95 ± 104.85	910.33 ± 82.49	929.40 ± 39.60
12	341.16 ± 14.64	81.58 ± 7.70	800.13 ± 45.40
13	139.28 ± 7.98	33.10 ± 4.46	427.79 ± 43.38
14	99.57 ± 1.90	34.07 ± 6.31	10776.20 ± 309.57
15	556.02 ± 14.20	899.03 ± 42.02	53734.64 ± 15816.04
16	119.53 ± 1.75	27.98 ± 3.87	112.67 ± 2.05
17	789.90 ± 28.85	1398.46 ± 41.30	50010.35 ± 1197.87
18	72.98 ± 0.03	19.38 ± 5.26	176.60 ± 33.26
19	101.07 ± 4.32	21.93 ± 7.03	1239.16 ± 106.93
20	426.06 ± 7.09	4.00 ± 0.57	52.89 ± 6.32
21	1.59 ± 0.60	5.20 ± 0.78	456.92 ± 14.25
22	34.01 ± 7.07	39.34 ± 2.87	184.63 ± 6.84
23	269.18 ± 36.18	144.63 ± 3.88	265.20 ± 36.26
24	251.81 ± 69.54	50.82 ± 7.87	652.09 ± 160.20
25	178.97 ± 4.29	161.60 ± 24.85	195.02 ± 1.50
26	181.36 ± 39.80	515.41 ± 12.13	2157.82 ± 244.84
27	88.61 ± 2.26	13.05 ± 1.19	19033.73 ± 706.73
28	47.40 ± 6.04	22.92 ± 0.23	166.46 ± 10.53
29	168.03 ± 16.94	86.12 ± 3.13	2627.34 ± 349.35
30	10.02 ± 1.43	16.51 ± 0.33	383.25 ± 95.20
$\bar{X}$	253.36 ± 248.33	336.29 ± 541.78	18982.06 ± 33714.47
Range	1.59-916.34	4.00-1883.23	52.89-129823.48
N	30	30	30

Data shown were in mean ± S.D. of duplicated experiments

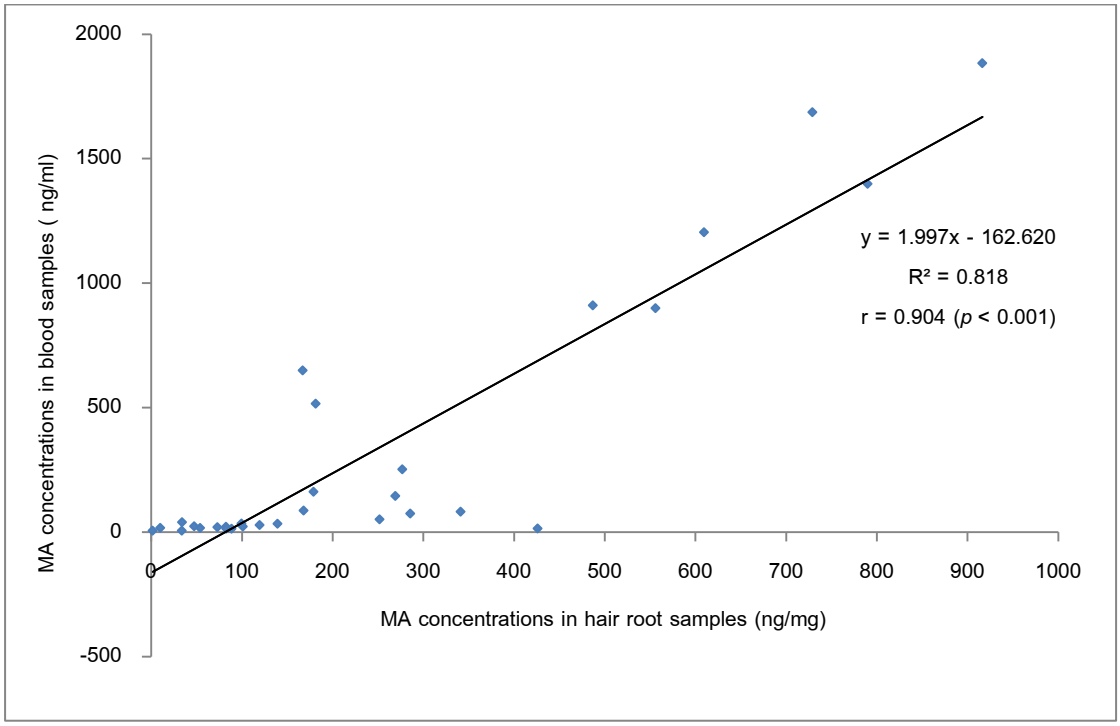

Figure 18 Relationship between MA concentrations in hair root and blood samples (n = 30)

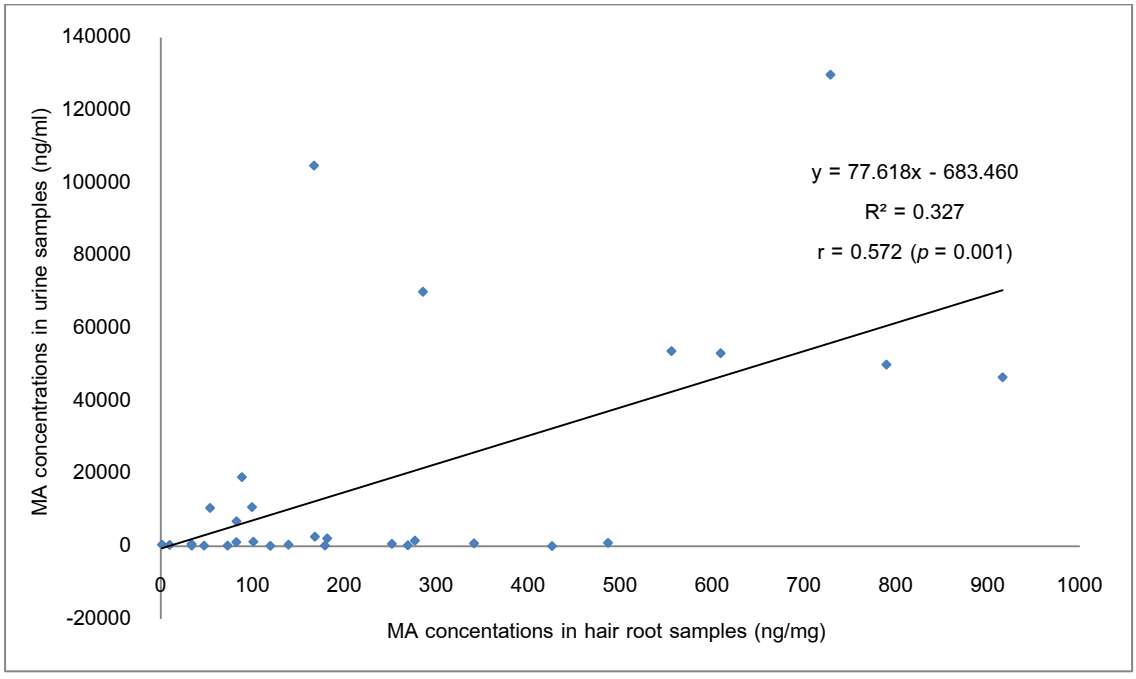

Figure 19 Relationship between MA concentrations in hair root and urine samples (n = 30)

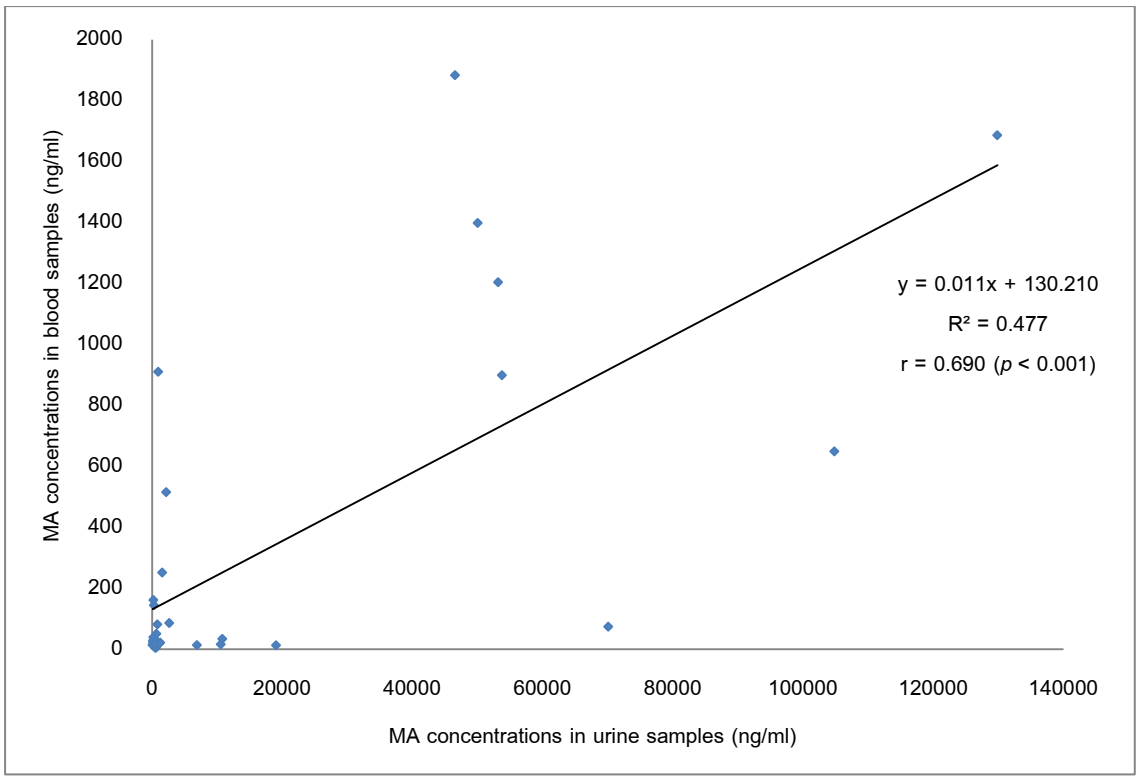

Figure 20 Relationship between MA concentrations in urine and blood samples (n = 30)

Table 12 Verification of the linear regression equations of MA concentrations in hair root vs blood and hair root vs urine samples of 20 deceased

Sample no.	MA concentrations in hair root (ng/mg)	MA concentrations in blood (ng/ml)		MA concentrations in urine (ng/ml)	
		Calculated	Measured	Calculated	Measured
1	275.90	388.35	432.82	20731.10	17951.19
2	191.22	219.24	121.84	14158.28	12808.51
3	477.28	790.51	797.27	36361.96	21926.37
4	351.11	538.54	514.28	26568.78	12314.51
5	937.24	1709.04	27.64	72062.88	70240.14
6	516.50	868.83	803.04	39406.24	1334.76
7	441.37	718.79	78.05	33574.64	14772.48
8	137.94	112.84	77.96	10022.93	34879.87
9	376.00	588.24	122.83	28500.55	13232.38
10	227.92	292.54	141.57	17007.24	25588.33
11	165.10	167.07	34.66	12130.87	26567.48
12	82.07	1.28	14.43	5687.01	34506.75
13	96.59	30.26	5.23	6813.38	13683.18
14	155.46	147.82	21.29	11382.45	33104.99
15	85.69	8.51	33.99	5967.93	3467.42
16	98.39	33.86	9.57	6953.37	3445.50
17	82.02	1.17	8.49	5682.71	3478.31
18	86.27	9.65	15.52	6012.36	3211.76
19	85.02	7.16	20.62	5915.62	3503.82
20	205.06	246.88	218.81	15232.56	73990.42
Range	82.07-937.24	1.17-1709.04	5.23-803.04	5682.71-72062.88	1334.77-73990.42
Paired t-test		t (19) =1.915, *p*-value = 0.071		t (19) = -0.475, *p*-value = 0.640	

CHAPTER V

DISCUSSION AND CONCLUSION

Method validation

Method validation was performed before using the methods for determination of MA concentration in hair root, blood and urine samples. Linearity, LOD, LOQ, accuracy and precision of both within- and between-day were tested. Regarding linearity, this study showed that linearity of the method for determination of MA in hair root, blood and urine samples as shown by the R^2 were all more than 0.99 using Pearson's correlation test. Based on the signal-to-noise ratio of 3:1, LOD of the procedure for determination of MA in hair root, blood and urine samples were 0.125 ng/mg, 40 ng/ml and 40 ng/ml, respectively. For LOQ, if determined based on the signal-to-noise ratio of 10:1, LOQ of the procedure for determination of MA hair root, blood and urine samples were 0.2 ng/mg, 50 ng/ml and 50 ng/ml, respectively while determination according to the precision and accuracy of not less than 20%, the LOQ of the method for hair root, blood and urine samples were 0.2 ng/mg (MA concentration at 0.125 ng/mg, the accuracy as shown by the %recovery was not met the criteria of not less than 20% while the precision as shown by the %CV was within the range), 40 ng/ml and 40 ng/ml, respectively.

MA concentration in hair root samples was analyzed according to the method modified from the method of Wainhaus et al. (1998). Cutoff concentration of MA in hair using confirmatory testing mandated by Federal Drug Testing Program, SAMHSA was 300 pg/mg or 0.3 ng/mg (Broussard, 2008). Thus, both LOD and LOQ of the method used for hair root in this study were lower than the proposed cutoff by SAMHSA.

Recently, Wada et al. (2012) detected MA and MDMA in hair roots using HPLC-chemiluminescence method. To increase sensitivity of the method, they derivertized MA with 4-(N,N-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F). They found

that LOD and LOQ of the method to detect MA in hair roots were in the ranges of 0.005-0.058 ng/mg and 0.017-0.191 ng/mg, respectively. Even though the present study possesses lower sensitivity than the method of Wada et al. (2012), simplicity of the method is satisfactorily advantageous with the LOD and LOQ that are still lower than the cutoff value of MA confirmation by SAMSHA.

Determination of MA concentrations in blood samples was modified from the method of Marquet et al. (1997). LOD and LOQ of the method reported by Marquet et al. (1997) were 2 ng/ml and 20 ng/ml, respectively. Both LOD and LOQ of the method in this study were higher than those reported by Marquet et al. (1997). Less sensitivity of the method used in this study could be explained by 2 reasons. Marquet et al. (1997) performed the evaporation step and the residue was derivertized with heptafluorobutyric anhydride to enhance the capacity of GC/MS detection. Cutoff value of MA in blood samples is not proposed in the Federal Drug Testing Program, SAMHSA.

Determination of MA concentrations in urine samples was modified from the method of Marquet et al. (1997) that determined MA concentration in blood. LOD and LOQ shown in this present study were 40 and 50 ng/ml, respectively which both values were much lower than the cutoff value mandated by Federal Drug Testing Program, SAMHSA. Actually, the cutoff concentration of MA detected by GC/MS mandated by this Institution is 250 ng/ml (Department of Health and Human Services, SAMSHA, 2008). Wu et al. (1992) reported the LOD and LOQ of the method for determination of MA in urine as 50 and 100 μg/L (or ng/ml), respectively which were higher than this present study. In the study of Wu et al. (1992), they used GC/ion-trap MS. However, in the study of Oyler et al. (2002), they demonstrated the LOQ of the method as 2.5 ng/ml In that study, they performed the extraction by solid phase extraction, derivertization then analyzation by GC/positive chemical ionization MS. The derivertization could enhance the capacity of GC/MS detection thus LOQ in Oyler et al. (2002) was lower than that found in this present study.

The accuracy and precision of the method for MA detection in hair root, blood and urine samples in this study as shown by the % recovery and % CV of both within- and between-day precision were not more than 15% which are suggested by the guidelines (U.S.FDA, 2001).

Relationships between MA concentrations in hair root, blood and urine samples and verification of the linear regression equations

In this study, it was shown that MA in hair root and blood or urine samples were linearly correlated with the correlation coefficient of 0.904 (hair root vs blood), 0.572 (hair root vs urine), 0.690 (urine vs blood). Thus, hair root was potentially to be proposed as an alternative specimen in case that blood or urine samples were not available. The concentration ranges of MA in hair root, blood and urine samples were 1.59-916.34 ng/mg, 4.00-1883.23 ng/ml and 52.89-129823.48 ng/ml, respectively. It was shown that concentrations of MA in urine were mostly far higher than MA concentrations in blood and hair root samples. And the correlation coefficient of MA concentration in hair root vs urine as well as blood vs urine was lower than the correlation coefficient of MA concentration in hair root vs blood. Jones and Karlson (2005) also reported the correlation coefficient of AP concentrations in urine and blood as 0.53. The concentrations of MA or AP in urines that were higher than in blood samples might be related to many factors such as the time elapsed since use of the drug, the route of administration and the frequency of emptying the bladder. The half-life of AP and MA in urines is longer than in blood samples (Jones and Karlson, 2005). However, the correlation of MA in hair root vs blood of this present report was 0.904 because drugs were rapidly transported from the blood to the hair root, where they accumulate (Wada et al., 2012). Nakahara et al. (1997) also reported that MA could be found in the rat hair root within 5 minutes after administration as 11.1, 12.3 and 13.2 ng/mg at 20, 40 and 60 mg/kg dosages, respectively. They suggested that MA reached

the hair root very rapidly after entering the blood and hair root was useful for demonstrating acute poisonings.

To verify the linear regression equations, unrelated subjects of group II deceased of n = 20 were used to test the equations. It was shown that adding the MA concentrations in hair root into the regression equations, the calculated MA concentrations in both blood and urine samples were not significant difference from the measured MA concentrations in both corresponding specimens. Even though this verification supported the proposed information of using hair root as alternative specimen for blood and urine samples, MA concentrations in blood and urine samples should not be absolutely inferred from that measured in the hair root. Several factors could affect MA concentrations in hair root, blood and urine samples such as time interval between MA exposure to death, acute or chronic use, urine volume, etc.

In conclusion, MA concentrations in hair root and blood or urine samples collected from 30 Thai deceased were linear correlated with correlation coefficient (r) of 0.904 (hair root vs blood), 0.572 (hair root vs urine) and 0.690 (urine vs blood). The corresponding linear regression equations were $y = 1.997x - 162.620$, $y = 77.618x - 683.460$, and $y = 0.011x + 130.210$, respectively. This relationship is preliminarily advantage for prediction of MA concentrations in blood or urine samples from MA concentrations in hair root samples while urine and blood samples are not available. This study suggested that hair root can be used as an alternative specimen in case that blood and urine are not available.

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