— Minireview —

Recent Improvements in Forensic Hair Analysis for Illicit Drugs

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This review summarizes technical and administrative improvements in forensic hair analysis for methamphetamine (MA) and designer drugs, recently developed by the present authors' team. These improvements include the establishment of three types of screening methods, and a column-switching liquid chromatographic-mass spectrometric (LC-MS) method for determining such drugs and their metabolites to serve as an alternative confirmation technique. The screening methods include ion mobility spectrometry-based, enzyme multiplied immunoassay technique (EMIT)-based, and immunochromatography-based methods. The column-switching LC-MS method proved to be effective in clearly determining MA and its metabolites, amphetamine and *p*-hydroxy-MA, which provides indisputable proof of MA intake. Quality assurance measures in forensic hair analysis, such as semiquantitative cross-checkings between the results of different analyses, are also discussed.

Key words —— hair, methamphetamine, designer drug, liquid chromatography-mass spectrometry, immunoassay, quality assurance

INTRODUCTION

Hair analysis for drugs is increasingly drawing attention, especially in the forensic field.[1,2] Of the various drugs sold on the street, methamphetamine (MA) and its analogs are among the most prevalent of illicit drugs used throughout Asian and Pacific countries, including the United States.[3] To combat the use of such drugs, hair analysis, in addition to conventional urinalysis, is becoming an indispensable tool of drug enforcement procedures in Japan. In fact, the detection of a substantial amount of MA in hair (typically 5 ng/mg equivalent or more) is increasingly employed as evidence of chronic MA use in Japan's courts of law.

Hair analysis for such drugs is primarily conducted using GC-MS,[1,2,4] owing to its high specificity and sensitivity. However, such GC-MS procedures involve tedious sample preparation: appropriate purification of hair extracts, followed by derivatization under anhydrous conditions is necessary.[4] Also, Japan's authorities highly recommend that forensic hair analysis have a combination of two different specific analyses based on different separation principles when it is introduced as legal evidence. Thus, there is a need for a sensitive screening method which eliminates unnecessary GC-MS analysis to cope with an increasing number of samples, and for a specific second method to determine the presence of drugs and metabolites in hair.

In order to meet these requirements, the authors have developed three types of screening methods: ion mobility spectrometry (IMS)-based,[5–8] enzyme multiplied immunoassay technique (EMIT)-based,[9] and an immunochromatography-based method.[10] Also, an automated column-switching high performance liquid chromatographic-mass spectrometric (LC-MS) method has been established for the determination of amphetamines (APs) in hair extracts.[11,12] This review summarizes these improvements recently achieved by the authors' team at the Forensic Science Laboratory of Osaka Prefectural Police Headquarters. Quality assurance measures, which must be strictly enforced in forensic hair analysis, are also discussed.

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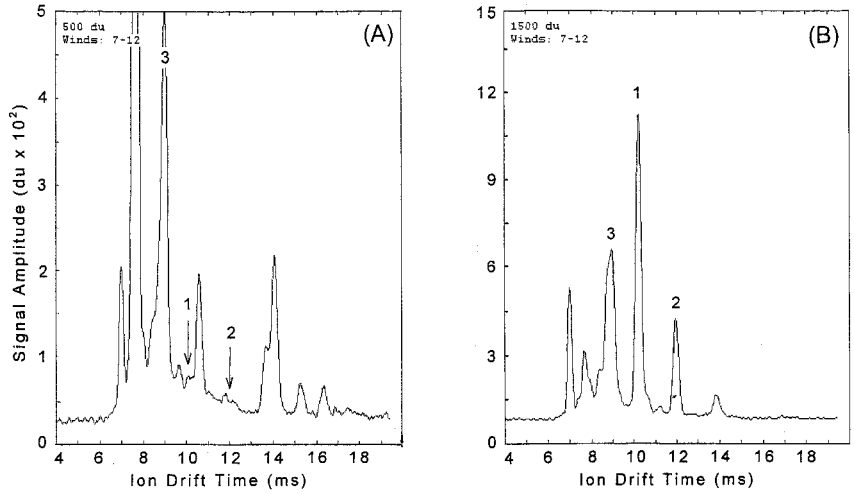

Fig. 1. Plasmagrams Obtained from a Drug-Free Hair Sample (A) and an MA User's Hair Sample (B)

Peaks: 1 = MA; 2 = dibenzylamine (IS); 3 = nicotinamide (reactant/calibrant). The amount of MA detected by GC-MS from the MA user's hair was 27.4 ng/mg.

SCREENING METHODS

Semiquantitative Screening of APs in Hair Using Ion Mobility Spectrometry

IMS is a mass-selective technique in which the analytes are vaporized, ionized, and with given initial velocity are moved along a drift region towards a collector electrode under atmospheric pressure conditions.[13] The drift time needed to reach the collector electrode is proportional to the masses of the ions.[14] The primary advantages of IMS include the very short analysis time (around 5 sec), detection limits in the ng to pg range, and various options for sample introduction including applying a solution on the filter and trapping particulate matter on the filter.[15] IMS is increasingly utilized as a powerful tool for detecting trace amounts of drugs on postal items, imported articles, and on a suspect's clothing, luggage, fingernail dirt, and so on.

The authors had initially analyzed hair samples directly by IMS without any sample pretreatment. Those experiments presented sufficient sensitivity only to externally contaminated hair (usually found on drug dealers), but poor results were obtained for drugs incorporated in the hair samples of drug users. Thus, the authors devised a simple and rapid sample preparation process: a 2-mg portion of hair sample was digested with 5 M NaOH (methanol-water, 4 : 1; 200 μl), which sufficiently unbinds drugs from hair matrices to achieve their effective vaporization.[5] Ultrasonication at 45°C for 20 min resulted in the complete digestion of hair, and a 50 μl portion of the digest was then applied on a Teflon membrane filter. After drying it with a gentle stream of warm air for 60 sec, the sample was immediately measured using nicotinamide added automatically as the reactant and calibrant in the IMS instrument (IONSCAN model 250 ion mobility spectrometer; Barringer, Rexdale, Canada).

MA in the hair was semiquantitatively detected by monitoring the digested hair sample with dibenzylamine added as an internal standard (IS).[6,7] Figure 1 shows plasmagrams obtained from a drug-free hair sample (A) and an MA user's hair sample (B). The semiquantitative screening was possible over the concentration range from 0.5 to 120 ng/mg hair, and the lower limit of detection (LLOD) of MA in hair was 0.5 ng/mg. The relative standard deviation of MA/IS ratio was 5.2% at an MA concentration of 1.0 ng/mg. The IMS results were in good agreement with those of GC-MS determination. This method was also applicable to the sectional analysis of MA even in a limited amount of hair specimen. For the screening of designer drugs, trihexylamine was used as an IS. The LLODs of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) were 3 ng/mg and 2 ng/mg, respectively.[8] It should be noted that the LLODs can be affected by coexisting drugs or substances which may compete with target drugs and the IS in the ionization process, though the authors have not experienced a noticeable decrease in sensitivity due to this. The most significant advantages of this IMS method include its quali-

tative efficiency, small sample size requirement, and very short analysis time; the sort of drugs contained at pg levels could be determined based on the drift times of individual drugs, while requiring only a 2-mg hair specimen, within some 40 min: 10 min for decontamination, 20 min for digestion, and 5 min for sample application and IMS measurement.

EMIT for the Semiquantitative Screening of MA and AP in Hair

Because the IMS method requires a sophisticated and expensive instrument, much attention was focused on the development of an inexpensive semiquantitative screening method for drugs in hair that could be employed as a standard method among analytical engineers. Various immunoassay methods have been developed for the screening of drugs in hair. ELISA (enzyme-linked immunosorbent assay) and coated-plate 96 well microplate enzyme immunoassays have primarily been employed for screening hair digests or extracts for drugs.[16,17] In forensic laboratories, however, EMIT serves as a requisite screening tool for drugs/metabolites in urine.[18–20] The primary advantages of EMIT include a short assay time (around 1 min), LLODs down to the 100 ng/ml level, semiquantitative screening ability, and its user-friendly properties. The present writers' team has established a fairly sensitive semiquantitative screening method for MA and AP in hair, by modifying the EMIT® d.a.u.™ assay for APs in urine, and combining it with a simple sample preparation: drugs in a 10 mg portion of satisfactorily-decontaminated hair extracted into 5 M HCl-methanol (1 : 20, v/v).[9] The extract was then evaporated to dryness, reconstituted in 100 μl water, and assayed with double-concentrated Emit® d.a.u.™ Amphetamine Class Assay reagents (Syva Company Dade Behring; Cupertino, CA, U.S.A.). Measurements were automatically performed with a Syva® ETS® Plus System; the absorbance change rates (ΔA) at 340 nm during a 30 sec assay period at 30°C were measured with three repetitions utilizing a 17.5 μl sample per run. To examine the optimal cutoff concentration of MA in hair, receiver operating characteristic analysis[21] was performed by assaying 82 hair specimens collected from a population of possible MA users. The optimal cutoff was found to be 1.0 ng/mg hair, while the LLOD was calculated to be 0.5 ng/mg. The semiquantitative screening was possible over the concentration range from 1.0 to 200 ng/mg, and the results were in good agreement with those by GC-MS determination.

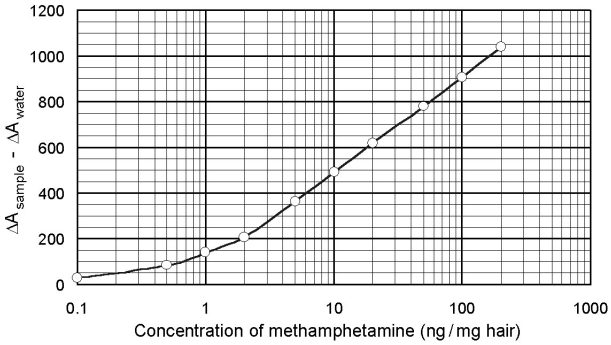

Fig. 2. Calibration Curve of the EMIT Semiquantitative Screening for MA in Hair

Figure 2 shows a calibration curve for *d*-MA obtained using drug-free hair extracts spiked with *d*-MA at known concentrations. The LLODs of *l*-MA, *dl*-AP, *dl*-MDA, *dl*-MDMA, and *dl*-MDEA in hair were 1.0, 1.0, 15, 7.5, and 3.0 ng/mg, respectively. Since cross reactivities of *dl*-MDMA and *dl*-MDA were not sufficient, the present method may fail to provide a sensitive general screening for a wide range of APs in hair. Colored extracts were obtained from colored or dyed hair specimens, and these hair treatments resulted in slight positive interference in the EMIT screening. It is well established that bleaching and/or dyeing would reduce drug levels incorporated in hair, but this EMIT screening is still applicable for colored, bleached, and/or dyed hair, when the cutoff is set at 1.0 ng/mg. Although all positive results must be confirmed by either GC-MS or a specific alternative methodology, this method provides user-friendly yet reliable screening of MA, the most prevalent illicit drug in Japan, and AP in hair.

Immunochromatography-Based Screening of MA and Designer Drugs in Hair

A variety of immunochromatography-based drug tests serve as convenient screening tools for drugs and metabolites in biological fluid samples.[22] This section describes the application of the immunochromatography-based saliva drug test ORAL•screen™ for the screening of drugs in hair, without the use of any instruments, combining it with the simple sample preparation mentioned above in the EMIT section.[10] This immunoassay device is intended for use in the highly sensitive screening of the following drugs and/or their metabolites in human oral fluid: cannabinoids, cocaine, opiates, and

MA-type drugs. Among these categories, the authors focused on its application to the screening of MA and designer drugs in hair. This immunoassay provided high sensitivities to *d*-MA, *dl*-MDMA, and *dl*-MDEA. To the contrary, it showed low cross-reactivities (10 ng/mg hair or higher) to *dl*-MDA, *dl*-AP, as well as to common over the counter drug ingredients such as methylephedrine. A low cross-reactivity was also observed for the *l*-isomer of MA that is used as an over the counter drug in the United States. The LLODs of drugs in hair were 0.5 ng/mg for *d*-MA, 0.8 ng/mg for *dl*-MDMA, and 1.0 ng/mg for *dl*-MDEA. Thus, this method proved a fairly sensitive screening for *d*-MA, *dl*-MDMA, and *dl*-MDEA which are among the most frequently-encountered illicit drugs in many countries and regions. Because opiates, cocaine, and their metabolites are detectable in the same manner, this method would also be useful for the screening of cocaine and heroin addicts *via* possible drug users' hair analysis.

LC-MS METHOD

Column-Switching LC-MS for the Determination of APs and Metabolites in Hair

Recently LC-MS has been increasingly utilized as a promising analytical tool to identify a wide range of substances and metabolites in biological samples.[23] Few papers,[24,25] however, had previously described the use of LC-MS for hair analysis of drugs, and such methods still require sample preparation that is as tedious as those for GC-MS. In the analytical field, column-switching on-line solid phase extraction is becoming a popular technique to directly determine with an enhanced sensitivity various analytes in biological matrices such as blood and urine.[26–29] By combining this methodology with LC-MS, the authors' team has established rapid and sensitive methods for determining APs in hair extracts.[11,12] The use of an *N*-vinylacetamide-containing, hydrophilic polymer on-line extraction column, an SCX semi-micro LC column, and a high-organic mobile phase (12.5 mM NH_4OAc, adjusted to pH 4.0 with formic acid — acetonitrile; 40 : 60, v/v) provided the successful concentration, separations, and highly sensitive electrospray ionization (ESI)-MS determinations of such analytes, without tedious sample pretreatments.[26,28,30] The calibration curves for MA and its possible metabolites, AP and *p*-hydroxymethamphetamine (*p*-OH-MA), were linear ($r > 0.9995$) over the range of 0.02–30 ng/mg. The LLODs of these analytes were 0.02 ng/mg and 0.1–0.2 ng/mg in the SIM and full-scan modes, respectively, using a 100 μl hair extract sample that corresponds to a 2.5 mg sample of hair.

Common urinary metabolites of MA include AP and *p*-OH-MA, and AP has usually been detected in MA users' hair samples.[4] Although the existence of *p*-OH-MA could have been expected in MA users' hair, the clear determination of *p*-OH-MA in hair had not been previously reported. As a result of the authors' study, *p*-OH-MA was detectable in all of 22 MA users' hair samples/sections that were positive in the EMIT screening (the cutoff being 1.0 ng/mg hair as MA).[11,12] Figure 3 shows extracted mass chromatograms obtained from an MA user's hair sample taken in the full-scan and SIM modes. The ratio of *p*-OH-MA to MA ranged from 0.0067 to 0.11 (mean = 0.041), while that of AP to MA ranged from 0.015 to 0.15 (mean = 0.050). Because the LLODs of *p*-OH-MA and AP are both 0.02 ng/mg in the SIM mode, we concluded that *p*-OH-MA is mostly detectable, in addition to AP, in MA users' hair samples from which 1.0 ng/mg or more of MA is detectable. Thus, this method proved to serve as an effective second analytical tool meeting previously mentioned forensic requirements.

The most significant advantages of hair analysis for drugs include its capability for investigating individual drug-use history *via* sectional analysis. The determination of drugs in each hair section has usually been carried out by GC-MS. However, the purification and derivatization processes after extraction are almost mandatory before GC-MS,[4] which makes sectional analysis increasingly labor-intensive. Owing to the automated on-line extraction methodology, the present method does not require such tedious pretreatment. This method also provides a cleaner background for hair samples compared with those by GC-MS. Thus, this LC-MS methodology proved to be an effective alternative for determining drug concentrations in each section of hair.

To demonstrate the forensic utility of the method, the detection of MA and its metabolites in a single 10-mm hair strand of an MA addict was also attempted. Although clear detection of *p*-OH-MA was unsuccessful in this case, MA and AP were clearly detected; the amounts of these analytes detected per mg hair were estimated to be about 50 ng/mg and 4 ng/mg equivalents for MA and AP, respectively.

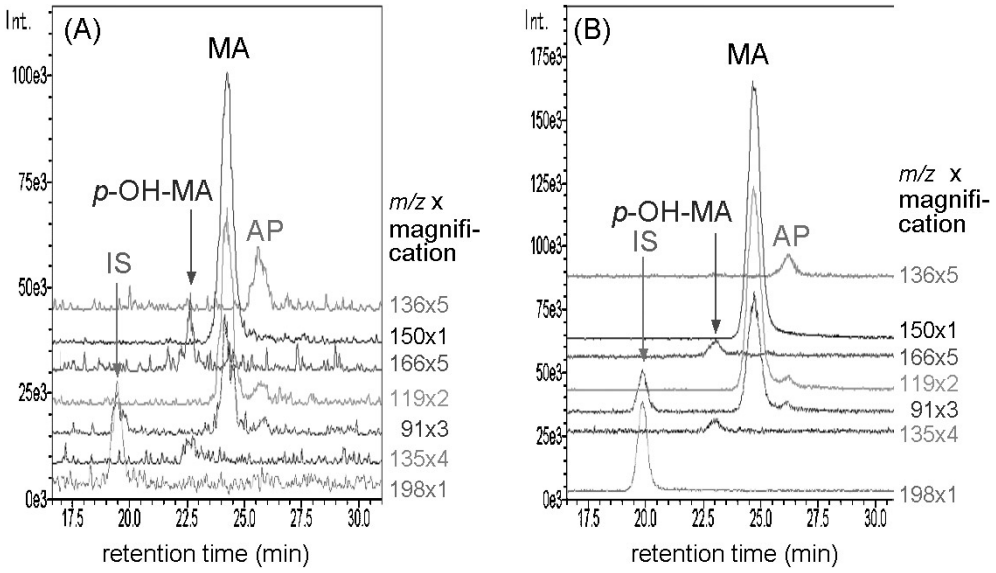

Fig. 3. Extracted Mass Chromatograms Obtained from an MA User's Hair Sample, Taken in the Full-Scan (A) and SIM (B) Modes
The amounts of MA, AP, and *p*-OH-MA detected per mg hair were 40.3, 3.5, and 0.73 ng, respectively.

QUALITY ASSURANCE MEASURES

As mentioned above, quality assurance measures must be carefully undertaken in forensic hair analysis for illicit drugs when the result is to be employed as legal evidence of drug use. In this section, quality assurance issues in forensic hair analysis are discussed.

Cross-Checkings between the Results of Different Analyses

As the authorities highly recommend, forensic hair analysis for illicit drugs should utilize a combination of two different specific techniques. To fulfill these requirements, we used to adopt thin-layer chromatography and now utilize the LC-MS method as a second analytical technique, in addition to the essential tool GC-MS and the semiquantitative EMIT screening. Because hair specimen sets are composed of non-uniform fragments, the amounts of a drug detected in equally subdivided sample portions may differ somewhat even where the specimen had been cut into 2–3 mm lengths and mixed thoroughly before subdividing. However, satisfactory agreements between such different analyses (using individually prepared extracts) contribute to the indisputable proof of drug use *via* hair analysis. Table 1 demonstrates semiquantitative cross-checkings between the results of the EMIT screening, GC-MS, and LC-MS determination, usually performed at the authors' laboratory to ensure quality assurance.

Detection of Relevant Metabolites for Distinguishing Endogenous Drug Incorporation from External Contamination

In hair analysis for illicit drugs, the endogenous incorporation by the intake of the drug should be clearly distinguished from the external contamination (*e.g.*, surface contact).[31] Thus, other quality assurance measures should be addressed by determining relevant metabolites, in addition to the parent drug. For this purpose, AP has been a commonly monitored metabolite in hair analysis for proving MA intake. However, even if AP is detectable in hair, it is a legitimate question whether it originated from the metabolism of MA, the intake of AP itself, or the thermoconversion of MA to AP during the smoking use of MA. (Passive exposure of hair in the environment) To the contrary, *p*-OH-MA is not usually produced in the thermoconversion of MA.[32,33] Therefore, the authors concluded that the detection of both metabolites, AP and *p*-OH-MA, in addition to the parent drug MA with reasonable ratios, provides indisputable proof of MA use by means of hair analysis. For MDMA user hair samples, the detection of its relevant metabolite MDA, with a ratio of MDA to MDMA of around 0.01–0.03, seems to be reasonable for reporting MDMA use via hair analysis.[1,10]

Administrative Cutoff Setting

As mentioned above, the detection of MA and even its metabolite AP in a single 10-mm hair strand

Table 1. Application of the LC-MS Method for the Sectional Analysis of MA Users' Hair Samples: Comparison with the GC-MS Determination after Trifluoroacetylation and the EMIT Screening

User / Section No.[a)]	EMIT screening Pos./Neg.[b)]; ng/mg as MA	GC-MS (ng/mg hair)			LC-MS (ng/mg hair)		
		MA	AP	*p*-OH-MA	MA	AP	*p*-OH-MA
User A							
1	Neg.; *ca.* 0.3	0.14	ND	ND	0.12	ND	ND
2	Pos.; *ca.* 5	3.4	0.14	0.12	3.0	0.12	0.10
3	Pos.; *ca.* 7	6.2	0.27	0.20	5.9	0.18	0.17
4	Pos.; *ca.* 8	5.7	0.30	0.20	6.3	0.24	0.14
5	Pos.; *ca.* 5	2.0	0.16	0.07	2.4	0.11	0.10
6	Neg.; *ca.* 2	1.1	0.04	tr.	1.2	0.06	0.02
7	Neg.; *ca.* 2.5	1.1	0.05	tr.	1.1	0.02	0.02
User B							
1	Pos.; *ca.* 15	14	1.1	0.65	14	1.3	0.52
2	Pos.; *ca.* 10	10	0.66	0.20	12	0.53	0.23

a) 20 mm sections from the scalp. *b*) An administrative cutoff level was set at 5 ng/mg hair, while GC-MS and LC-MS analyses were performed for every specimen for research purposes.

from an MA addict is possible by the column-switching LC-MS method.[12)] Also, the previously mentioned screening methods can detect MA and some related drugs even at around 1.0 ng/mg hair. Kintz[34,35)] proposed a cutoff concentration of 0.5 ng/mg in the GC-MS confirmation of APs in hair to exclude external contamination. This value is also considered to be near the typical LLOD of APs in the full-scan GC-MS confirmation when some 10 mg portion of hair specimen is used. In the authors' opinion, however, the administrative cutoff level of MA in hair should be set at a higher value (*e.g.*, 5 ng/mg equivalent) than the technical cutoff (for research or therapeutic purposes) when the result of hair analysis is to be used as legal evidence of chronic MA use. This is because the clear detection of AP and *p*-OH-MA, with their concentrations typically being 1–15% of MA, is highly recommended. Also, a higher administrative cutoff setting will contribute to clearly distinguishing internal MA incorporation from external contamination, which eliminates a defender's excuses, such as passive exposure of hair to MA in the environment.

CONCLUSION

The analysis of drugs in hair is drawing increasing attention, especially in the forensic field. To cope with a growing number of hair samples, the authors' team has developed several types of screening methods for drugs in hair. Also, an automated column-switching LC-MS method has been established for the highly sensitive determination of MA, its metabolites, and analogs in hair extracts. Using this methodology, the detection of trace-level drugs and metabolites even in a single 10-mm hair strand has become possible. In this era of ultra-sensitive analytical technologies, however, the authors stress that maximum quality assurance measures must be taken in the forensic hair analysis for drugs when the result is to be employed as legal evidence of drug use.

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