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Development of a dual test procedure for DNA typing and methamphetamine detection using a trace amount of stimulant-containing blood

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ABSTRACT

Investigation of drug-related crimes, such as violation of the Stimulant Drug Control Law, requires identifying the used drug (mainly stimulant drugs, methamphetamine hydrochloride) from a drug solution and the DNA type of the drug user from a trace of blood left in the syringe used to inject the drug. In current standard test procedures, DNA typing and methamphetamine detection are performed as independent tests that use two separate portions of a precious sample. The sample can be entirely used up by either analysis. Therefore, we developed a new procedure involving partial lysis of a stimulant-containing blood sample followed by separation of the lysate into a precipitate for DNA typing and a liquid-phase fraction for methamphetamine detection. The method enables these two tests to be run in parallel using a single portion of sample. Samples were prepared by adding methamphetamine hydrochloride water solution to blood. Samples were lysed with Proteinase K in PBS at 56 °C for 20 min, cooled at −20 °C after adding methanol, and then centrifuged at 15,000 rpm. Based on the biopolymer-precipitating ability of alcohol, the precipitate was used for DNA typing and the liquid-phase fraction for methamphetamine detection. For DNA typing, the precipitate was dissolved and DNA was extracted, quantified, and subjected to STR analysis using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. For methamphetamine detection, the liquid-phase fraction was evaporated with N_2 gas after adding 20 μL acetic acid and passed through an extraction column; the substances captured in the column were eluted with a solvent, derivatized, and quantitatively detected using gas chromatograph/mass spectrometry. This method was simple and could be completed in approximately 2 h. Both DNA typing and methamphetamine detection were possible, which suggests that this method may be valuable for use in criminal investigations.

1. Introduction

In Japan, ⩾10,000 people are arrested each year for violating the Stimulant Drug Control Law, representing >80% of all drug cases [1]. Major methods for using stimulant drugs in Japan include intravenous injection, absorption, and ingestion [2]. When investigating drug-related crimes involving intravenous injection of a stimulant, the drug can be identified from the residual solution in the syringe, and the drug user's DNA type can be determined by analyzing trace amounts of blood in the syringe after injecting the drug.

In current standard test procedures, DNA typing and drug analysis are independently performed. In the personal identity by DNA analysis, the personal difference with the target person was performed by examinations for short tandem repeat (STR) analysis with various multiplex STR amplification kit [3–11]. The result of the STR analysis can also clarify that trace blood in syringe was from a single person or multi-person because of syringe sharing [12]. The proof of using the stimulant by the recognition of stimulant detection was performed using thin-layer chromatography (TLC), gas chromatography–mass spectrometry (GC/MS), liquid chromatography–mass spectrometry (LC/MS) and high performance liquid chromatography (HPLC) [13–15].

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In addition, these analysis methods have been performed for not only investigating drug-related crimes but also investigating anti-doping [16,17].

In DNA typing and drug analysis, each test procedure requires two separate portions of the sample. In cases where sample volumes are extremely small, a sample may be completely used up by only one of these tests. In addition, the formation of solid blood clots in a syringe makes it difficult to collect a test sample by just washing with distilled water, and the clots should be dissolved prior to analysis (Fig. 1).

To overcome these problems, we developed and tested a new procedure involving partial lysis of a stimulant-containing blood sample followed by separation of the lysate into a precipitate for DNA typing and a liquid-phase fraction for drug testing. In other words, this method divides washings including blood into components derived from a blood corpuscle and liquid. Nakazono et al. have reported the separation of steeping urine stain using a filtration device [18]. This method may aid in separating a part of the components derived from the blood corpuscle and liquid. However, we used a different approach because blood cells in our sample were lysed using protease.

We selected methanol for high volatility so that the organic solvent could be removed from the liquid-phase fraction for methamphetamine detection. According to the general method of ethanol precipitation, we tried using methanol instead of ethanol because methanol had the biopolymer-precipitating ability as it was an alcohol and polar solvent. In addition, in treatment with Proteinase K, PBS contained salts such as NaCl, KH_2PO_4, and Na_2HPO_4. Although the biopolymer-precipitating ability of methanol was inferior to ethanol, isopropanol, and polyethylene glycol, we considered that the amount of DNA yield from 1 μL blood using this method was sufficient for STR analysis.

Thus, on the basis of the biopolymer-precipitating ability of alcohol, the precipitate was used for DNA typing and the liquid-phase fraction for methamphetamine detection. This method allows the two tests to be simultaneously run in a simple manner using only a single portion of sample. In this study, we examined the validity of this method by comparing the control sample without this method and limits of sensitivity. In addition, this method was performed on syringe samples as an application of forensic samples.

2. Material and methods

2.1. Test samples

Blood samples were provided by eight adult volunteers (four males, four females; mean age = 32.6 ± 6.9 years) who were not taking any medications. This study was approved by the Ethics Committee of The Jikei University School of Medicine for Biomedical Research (25–112). All volunteers provided written informed consent.

Fig. 1. Blood clots into a syringe.

The stimulant used in this study was methamphetamine hydrochloride (MA), (Sumitomo Dainippon Pharma Co., Ltd., Tokyo, Japan), which is the primary ingredient of a drug used illegally in Japan [14]. All test samples were prepared by adding 1 μL MA water solution to 1 μL blood that was either undiluted or diluted using phosphate-buffered saline (PBS; pH 7.2; Thermo Fisher Scientific Inc., Waltham, MA). The concentration of MA water solution and dilution ratio of blood for each test samples are shown in Table 1.

2.2. Sample separation

According to the general method of ethanol precipitation, samples were lysed with Proteinase K (20 mg/ml; QIAGEN, Hilden, Germany) in PBS at 56 °C for 20 min, cooled at −20 °C for 1 h after adding methanol, and then centrifuged at 15,000 rpm (Fig. 2). Using this method, the lysate was separated into a blood cell-derived fraction as a precipitate for DNA typing and a liquid-phase fraction for methamphetamine detection. In addition, the enzyme protein of Proteinase K in the lysate was collected as a part of the precipitate by denaturation and precipitation using methanol.

2.3. DNA typing

From the precipitate, DNA was extracted using a QIAamp DNA Investigator Kit (QIAGEN) according to the manufacturer's protocol [19]. Final elution was performed by adding 50 μL buffer ATE to ensure that the membrane was completely covered.

DNA was quantified by real-time PCR using the D17Z1 locus, which generated an amplicon of 207 bp [20]. Real-time PCR was performed using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc.). The lower limit was set at 0.001 ng/μL.

DNA typing was performed by STR analysis using an AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific Inc.). The procedure was followed as per a manual of kit, using 25 μL of the PCR reaction mix and 1 ng of the DNA template, 28 cycles were run [21]. The minimum quantity of DNA template that full profiles were detected in 28 cycles was 0.125–0.25 ng [5,21,22]. The maximum volume of the template DNA solution which could be added in a PCR reaction mix was 10 μL; therefore, if the concentration of the provided DNA solution did not reach 0.1 ng/μL, a DNA solution of 10 μL was used for PCR amplification. Electrophoresis of PCR products was performed using an ABI PRISM 3130 XL Genetic Analyzer (Thermo Fisher Scientific Inc.). DNA type was determined with the minimum peak height set at 150 RFU.

Table 1
The concentration of MA water solution and dilution ratio of blood for preparing each test sample.

Sample name	Concentration of MA water solution (ng/μL)	Dilution ratio of blood	The subsection number using samples
Sample 1	500	Undiluted	2.5.1–2.5.5.
Sample 2	500	×5	2.5.2.
Sample 3	100	Undiluted	2.5.3. and 2.5.4.
Sample 4	100	×5	2.5.5.
Sample 5	50	×10	2.5.5.
Sample 6	25	×20	2.5.5.

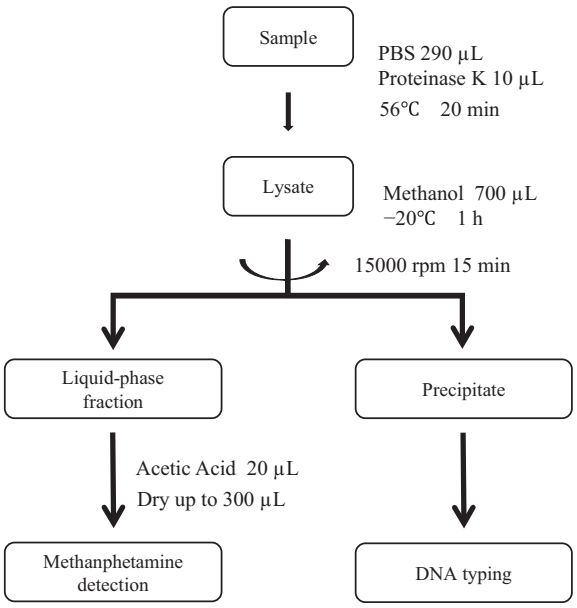

Fig. 2. Sample separation method.

2.4. Methamphetamine detection by GC/MS

Drug analysis (methamphetamine detection) was performed by gas chromatography/mass spectrometry (GC/MS). The liquid-phase fraction was separated using a Liquid–Liquid Extraction with Extrelut® NT3 Kit (Merck Ltd, Darmstadt, Germany) [23,24]. In some reports, the Extrelut® column was used to simultaneously extract and derive [25–27]; however, in this study, these procedures were separately performed.

The liquid-phase fraction was evaporated with N_2 gas to ⩽300 μL for methanol extraction after adding 20 μL acetic acid. To each sample, 200 ng *N*-ethyl benzylamine (NEBA, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (as 10 μg/mL of 1% EtOH solution 20 μL) was added as an internal standard. The extraction and derivatization of methamphetamine was performed according to the procedure [28]. Derivatized samples were evaporated to dryness using N_2 gas, dissolved in 100 μL ethyl acetate, and 1 μL of sample was injected into GC/MS.

The apparatus used was a 7890A GC combined with a 5975C MS (Agilent Technologies, Santa Clara, CA) with software provided by Agilent Technologies. An HP-5-ms-fused silica capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Agilent Technologies) was used. The splitless injection mode was selected. GC/MS conditions were as follows: 60 °C for 2 min, which was then increased by 20 °C/min to reach 300 °C. The injection port and transfer line temperatures were 250 °C and 280 °C, respectively. Helium was used as a carrier gas. The electron impact ionization energy was set to 70 eV. Full-scan mode (scanning range: *m*/*z* 50–550) was used. Ions selected for monitoring were MA-TFA (*m*/*z* 154) and NEBA-TFA (internal standard, *m*/*z* 231).

2.5. Method testing and validation

2.5.1. Optimal conditions for precipitation by methanol

Sample 1 was used in this section ($n = 8$). To each sample, either 88 μL PBS or 288 μL PBS and 10 μL Proteinase K solutions were added. The negative control contained 2 μL of PBS without blood or methamphetamine hydrochloride. Samples were incubated at 56 °C for 20 min. Furthermore, methanol was added at 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (90% omitted in the final volume of 300 μL) of the total volume and cooled at −20 °C for 1 h. Next, each sample was centrifuged at 15,000 rpm and both the precipitate and supernatant were collected. DNA was extracted using a column kit and quantified as previously described. Positive controls were prepared without adding methanol and separation.

2.5.2. Comparison of DNA typing

Preparation of test samples (Sample 1 and 2) and methods for DNA typing were performed as previously described ($n = 8$). The precipitate of each test sample was collected and analyzed for DNA typing. For each control sample, DNA typing was performed without separation. The number of detected loci and peaks in STR analysis were compared.

2.5.3. Influence of Proteinase K and temperature on methamphetamine detection on GC/MS

To each test sample (Samples 1 and 3), 288 μL PBS and 10 μL Proteinase K solution were added, and samples were incubated at room temperature or 56 °C for 20 min ($n = 5$). Control samples did not contain Proteinase K solution and were not heated. Each sample was analyzed by GC/MS as previously described.

2.5.4. Comparison of methamphetamine detection

The liquid-phase fraction of each test sample (Samples 1 and 3) was collected ($n = 5$). Methamphetamine detection was performed on the entire liquid phase (approximately 1 mL) or a liquid phase that was evaporated with N_2 gas to approximately 300 μL after adding 20 μL acetic acid as previously described. Control samples were analyzed without separation.

2.5.5. Analysis of mixed samples

Preparation of each mixed sample (Samples 1 and 4–6) and sample separation were performed as previously described ($n = 8$). Each mixed sample was analyzed for DNA typing and methamphetamine detection using the separating method. Control samples were run without separation.

2.5.6. Sensitivity of the DNA typing method

Test samples were prepared by adding 1 μL blood to PBS (2, 4, 8, 16, 32, 64, 128, and 256-fold dilutions). 1 μL of MA water solution (500 ng/μL) was added to each dilution. DNA typing was performed as previously described. Control samples were analyzed without separation.

2.5.7. Sensitivity of methamphetamine detection

Test samples were prepared by mixing 1 μL undiluted blood and 1 μL MA water solution (10–1 ng/μL). Methamphetamine was analyzed by GC/MS as previously described. Control samples were analyzed without separation.

2.5.8. Syringe samples

Test samples (Syringe 1–3) were prepared by mixing 1 μL undiluted blood with MA water solution into a syringe ($n = 5$). A 1-mL syringe [sterile single-use syringes with needle for insulin, MYJECTOR® 29G × 1/2″ (0.33 × 13 mm), Terumo Corporation, Tokyo, Japan] was used for a sample. Each sample was prepared after absorbing 500 μL of MA water solution (Syringe 1: 2 μg/μL, Syringe 2: 20 μg/μL, and Syringe 3: 100 μg/μL) and having spitted it out, a share was to absorb 1 μL blood from a needle. Samples were kept in a cool and dark space and processed 10 days later.

Each sample was washed by absorbing a mixture of 290 μL PBS and 10 μL Proteinase K in a 1.5 mL tube warming to 56 °C beforehand. Each blood in the syringe was washed and collected together with MA water solution heated at 56 °C for 20 min. Each washing solution was analyzed for DNA typing and methamphetamine detection as previously described. Control samples were washed

using 50 μL PBS for DNA typing or 300 μL distilled water for methamphetamine detection and analyzed without separation.

In methamphetamine detection, to fix quantity range of nearly 1 μg in GC/MS, the liquid phase that was evaporated with N_2 gas to approximately 300 μL was divided as follows; 150 μL (1/2 vol) from Syringe 1, 15 μL (1/20 vol) from Syringe 2, and 3 μL (1/100 vol) from Syringe 3. As an internal standard, 2 μg NEBA was added.

3. Results

3.1. Optimal conditions for precipitation by methanol

At both the final volumes of 100 μL and 300 μL, the concentration of eluted DNA from the precipitate after adding ⩾40% methanol (volume ratio) was similar to the positive control. In addition, when DNA solution obtained from the supernatant was less than the lower limit, the quantity of methanol was ⩾60% (Fig. 3).

3.2. DNA typing

For STR analysis, all test samples and each control sample had complete STR profiles. In addition, the peak in the STR profile of each sample was equal to that of each control (Fig. 4).

3.3. Methamphetamine detection on GC/MS

Methamphetamine was detected in all samples under all conditions (Fig. 5).

Adding Proteinase K had an influence on increasing the noise peak in total ion chromatogram. However, methamphetamine could be detected without any problem using mass chromatogram and mass spectra.

Methanol removal had an influence on reducing the noise peak in total ion chromatogram.

3.4. Analysis of mixed samples

For DNA typing of each mixed sample, Samples 1 and 4 and each control sample had complete STR profiles. The STR profile of Sample 5 lost one loci in one sample of eight samples by both sample with this method and the control sample. In the other seven samples, test samples and control samples had complete STR profiles. The STR profile of Sample 6 lost a few loci in five of eight samples. This also occurred in four of eight control samples. The peak in the STR profile of Sample 6 that contained the least amount of blood was comparable with that of the control sample.

Methamphetamine was detected in all samples. The results of Sample 6 that contained the least amount of methamphetamine are shown in Fig. 5.

3.5. Sensitivity of the DNA typing

During the separating method, the sample diluted 256 times in PBS (equal to 0.00390625 μL undiluted blood) was not quantified because it was below the lower limit of detection (0.001 ng/μL) (Fig. 6).

For STR analysis, samples diluted 2-fold (equal to 0.5 μL undiluted blood) and 4-fold (equal to 0.25 μL undiluted blood) in PBS had complete STR profiles. Samples diluted ⩽8-fold in PBS (equal to ⩽0.125 μL undiluted blood) lost some or many loci. Samples that had complete STR profiles were in six of eight samples diluted 8-fold (equal to 0.125 μL undiluted blood), in four of eight samples diluted 16-fold (equal to 0.0625 μL undiluted blood). In addition, samples diluted 256-fold lost all loci. The number of loci detected with this method was slightly less than that of the control samples (Table 2).

3.6. Sensitivity of methamphetamine detection

The detection limit was calculated to be >3 based on the result of the signal vs. noise ratio of *m*/*z* 154 as calculated by the SIM method.

The detection limit was 5 ng methamphetamine in samples and 1 ng in controls.

3.7. Syringe sample

For DNA typing of each syringe sample, concentrations of eluted DNA were quantified (Fig. 7A). As a result of STR analysis, a few loci were lost in some samples and controls. The number of loci detected with this method was slightly less than that of the control samples (Table 3).

Methamphetamine was detected in all samples. In addition, the quantity of methamphetamine was determined by internal standard method, with a standard curve prepared from samples of known quantity that were used in a separating method (Fig. 7B).

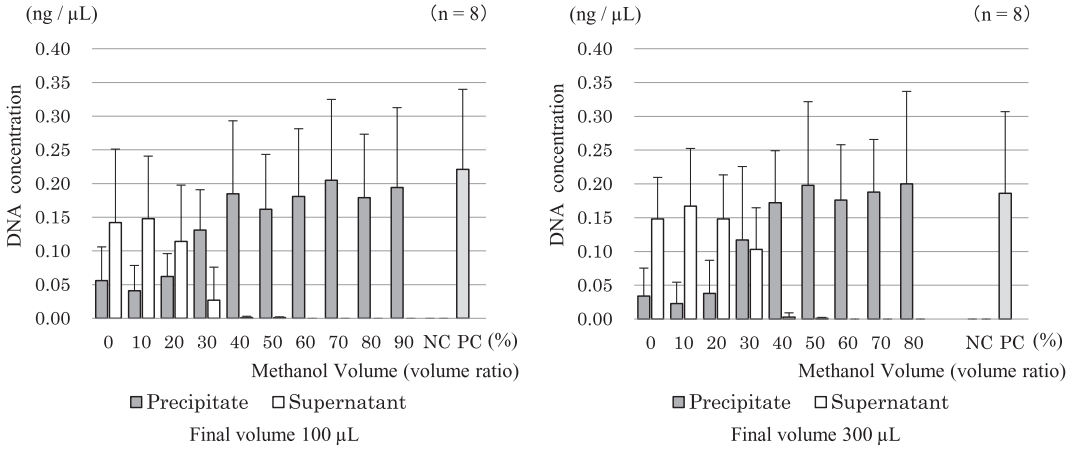

Fig. 3. Concentrations of eluted DNA in precipitate (gray) and supernatant (white). PC = positive control and NC = negative control.

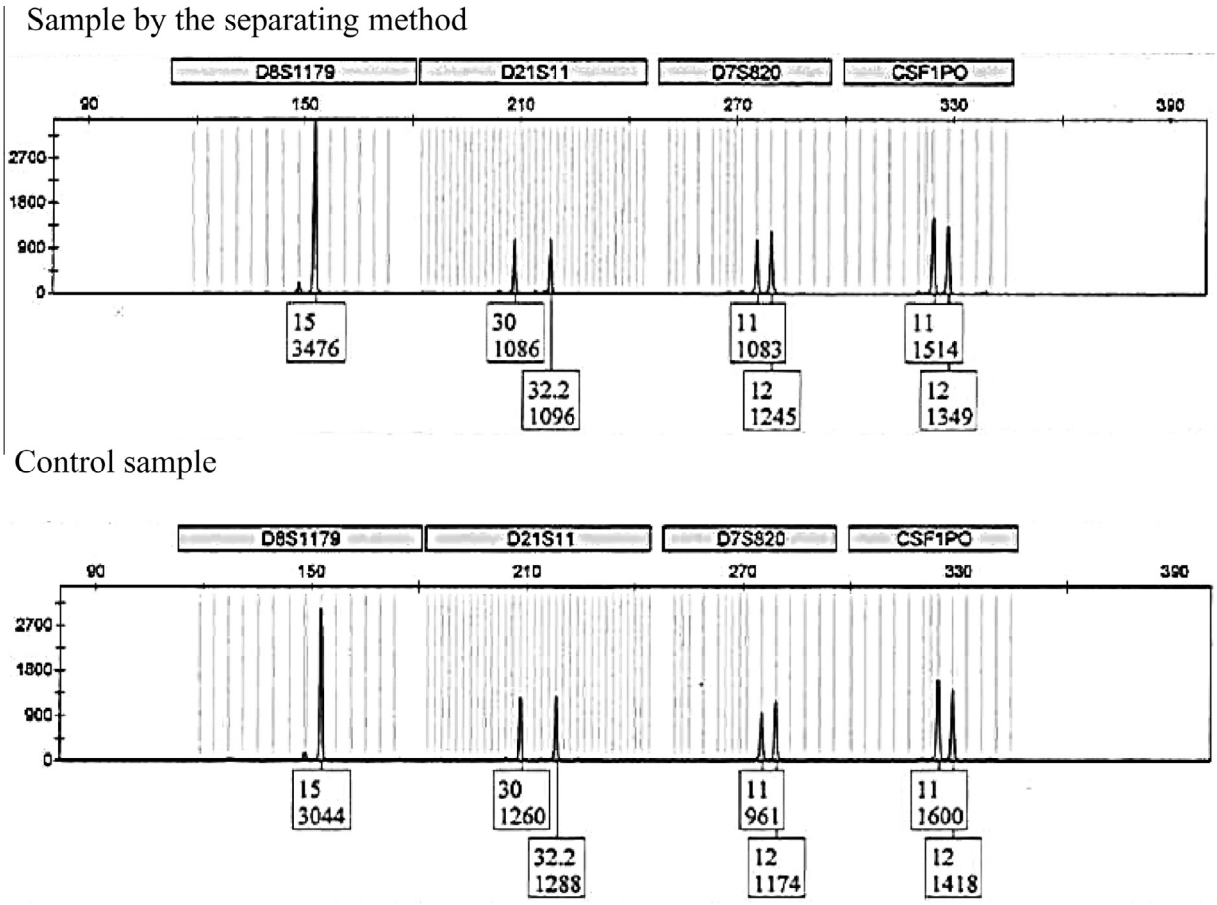

Fig. 4. STR profiles of a sample prepared with the separating method vs. control (Sample 1). STR profile of a sample prepared using the separating method (top) compared with the control (bottom).

4. Discussion

Optimal conditions for this method required ⩾60% methanol (volume ratio) for effective precipitation. Therefore, we decided to add 70% methanol (volume ratio) to samples containing 290 μL PBS and 10 μL Proteinase K solution (final volume of 300 μL). We found that this addition resulted in precipitation sufficient for STR analysis.

Because a difference in baseline noise and peak between the alleles in the electropherogram was not observed, STR analysis was performed equal to the control samples. These results showed that this method did not affect DNA typing.

During the detection of methamphetamine, Proteinase K solution affected recovery and increased the noise peak. An oily residual substance was observed in the sample containing Proteinase K after evaporation and drying with N_2 gas during the derivatization. Because this residual substance was not observed in the control sample, we assumed that it was caused by Proteinase K solution. In addition, in samples where methanol was removed by this method, a very small amount of oily residual substance was also observed.

Removal of methanol was necessary because it resulted in reducing the noise peak in total ion chromatogram. In analysis of mixed samples, methamphetamine was detected in samples that contained a trace amount of methamphetamine (Fig. 5).

The detection limit for DNA typing was dependent on the individual difference of white blood-cell count in blood. If 1 μL blood diluted four times (equal to 0.25 μL undiluted blood) was left in syringe, DNA typing was completely detectable. In addition, from the result of concentration of eluted DNA solution, 1 μL blood diluted 16 times (equal to 0.0625 μL undiluted blood) was possible to detect DNA typing for identification, total DNA template satisfied the minimum quantity of DNA template (0.125 ng–0.25 ng) [5,21,22].

Similarly, because the detection limit for methamphetamine was 5 ng, it should be possible to use our method to detect methamphetamine from a syringe used for intravenous injection. On the other hand, the detection limit for methamphetamine was greater than that of the control samples. This may have been caused by a decrease in methamphetamine extraction efficiency or an increase in noise peaks as a result of performing this method. We recommend that this noise peak and detection limit of the instrument are checked before performing this method.

As an application of forensic samples, this method was used to perform DNA typing and methamphetamine detection in syringe samples. In DNA typing of syringe samples, the number of loci detected with this method had little difference with that of the control samples. As shown in Table 3, the number of loci detected in Syringe 3 was less than that detected in Syringes 1 and 2 in both the samples and in controls using this separating method. In addition, the concentration of eluted DNA in a precipitate of Syringe 3 was less than half of that in precipitates of Syringes 1 and 2 (Fig. 7A). We considered that exposing blood to a high concentration of MA water solution deteriorated the condition of the blood cells.

In methamphetamine detection, the quantity of methamphetamine in each syringe sample was determined in the range that residual solution into the syringe was supposed to be

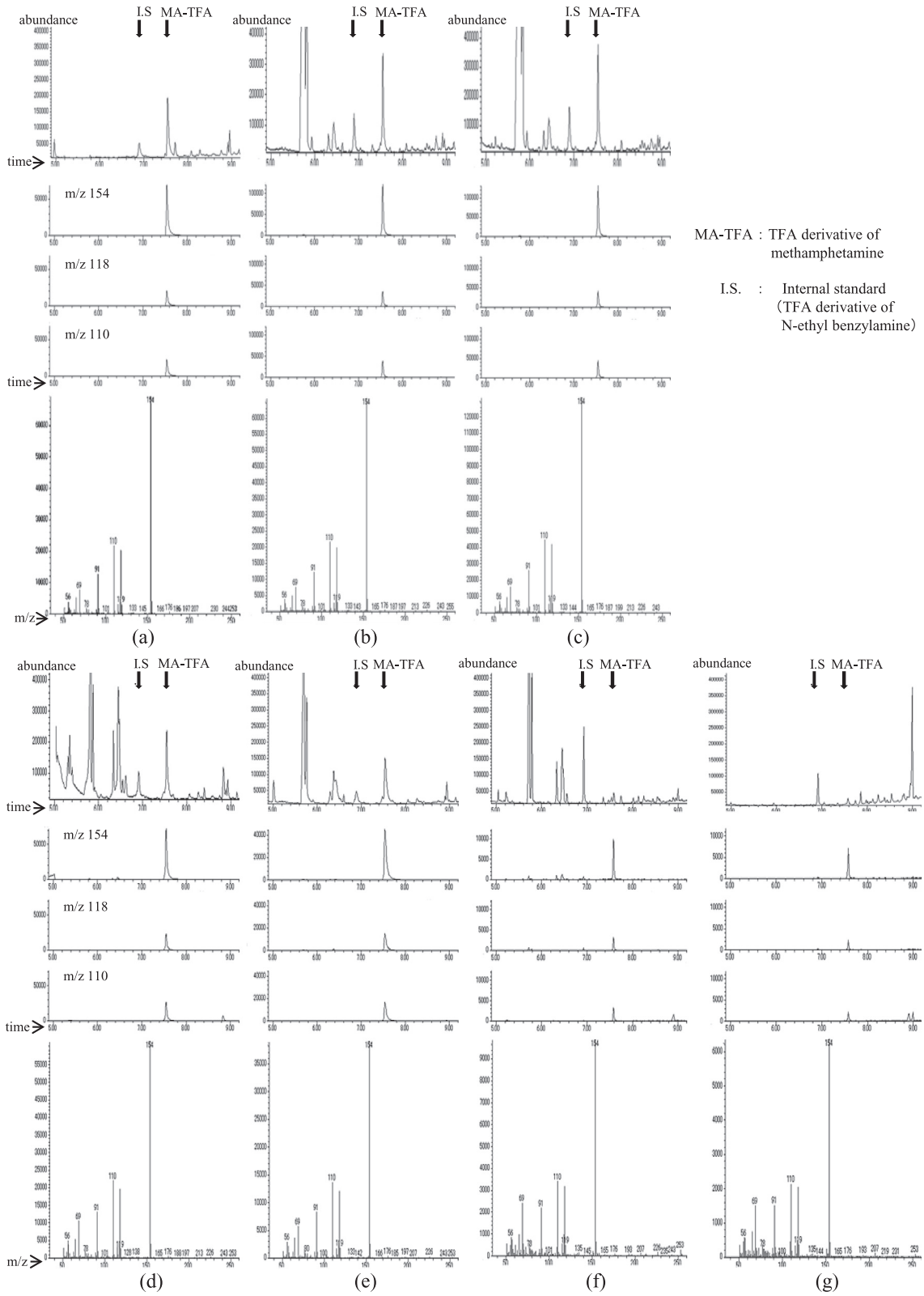

Fig. 5. Total ion chromatogram, mass chromatogram, and mass spectra (*m*/*z* 154,118,110) of methamphetamine detected in the liquid phase of samples. (a)–(e): Test samples contained 500 ng methamphetamine hydrochloride (Sample 1). To determine the influence of temperature and Proteinase K on methamphetamine detection, 288 μL PBS and 10 μL Proteinase K solution were added to each sample and incubated for 20 min under the following conditions: (a) control; (b) sample incubated at room temperature; (c) sample incubated at 56 °C. To determine the influence of methanol on methamphetamine detection: (d) the entire 1 mL whole liquid phase; (e) liquid phase evaporated using N_2 gas to approximately 300 μL after adding 20 μL acetic acid. (f), (g): Total ion chromatogram, mass chromatogram, and mass spectra (*m*/*z* 154,118,110) of methamphetamine detected in the liquid phases of Sample 6 (f) and the control sample (g).

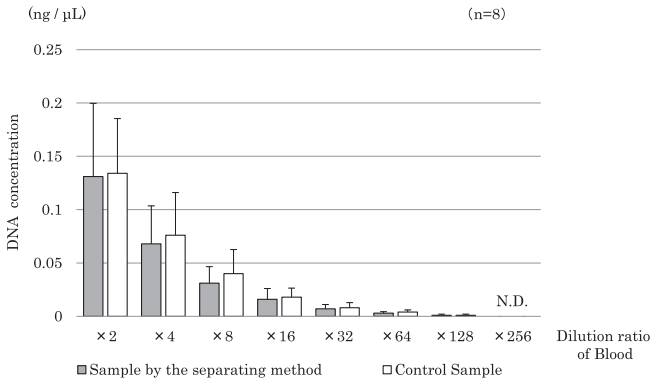

Fig. 6. Limits of sensitivity for DNA typing. Concentrations of DNA eluted using the separating method (gray) and that of the control samples (white). N.D. = Not detected.

1–5 μL. For example, Syringe 1 quantified approximately 2–10 μg methamphetamine so that it could absorb 2 μg/μL MA water solution. In medical applications, the amount of solution left in a syringe after injection is generally <10 μL. In Japan, this quantity is also supported by Japanese Industrial Standards (JIS) for sterile single-use syringes with needles for drugs, (e.g., insulin) that are frequently injected [29].

5. Conclusion

Our method allows for DNA typing and methamphetamine detection from a single sample; therefore, personal identity and stimulant use can be determined using a very small quantity of sample left at the crime scene. The method is simple, easy to perform, and can be completed in a short amount of time (approximately 2 h). In addition, this method can be performed on syringe samples as an application of forensic samples.

Table 2
Number of loci detected in DNA subjected to sensitivity testing.

	Dilution ratio of blood	Sample								Average number of detected loci
		A	B	C	D	E	F	G	H	
Sample by the separating method	×8	16	16	16	15	11	16	16	16	15.25
	×16	16	16	10	8	5	16	14	16	12.625
	×32	4	7	4	5	3	16	6	14	7.375
	×64	2	2	2	1	0	2	4	4	2.125
	×128	0	0	0	0	0	1	0	1	0.25
	×256	0	0	0	0	0	0	0	0	0
Control sample	×8	16	16	16	16	16	16	16	16	16
	×16	16	16	15	5	2	16	16	16	12.75
	×32	11	14	1	3	1	16	8	15	8.625
	×64	6	1	1	1	0	7	6	6	3.5
	×128	1	0	0	0	0	2	3	0	0.75
	×256	0	0	0	0	0	0	0	0	0

The AmpF*ℓ*STR® Identifiler® Plus PCR Amplification Kit used in this study has 16 loci (15 tetranucleotide repeat loci and the Amelogenin gender-determining marker).

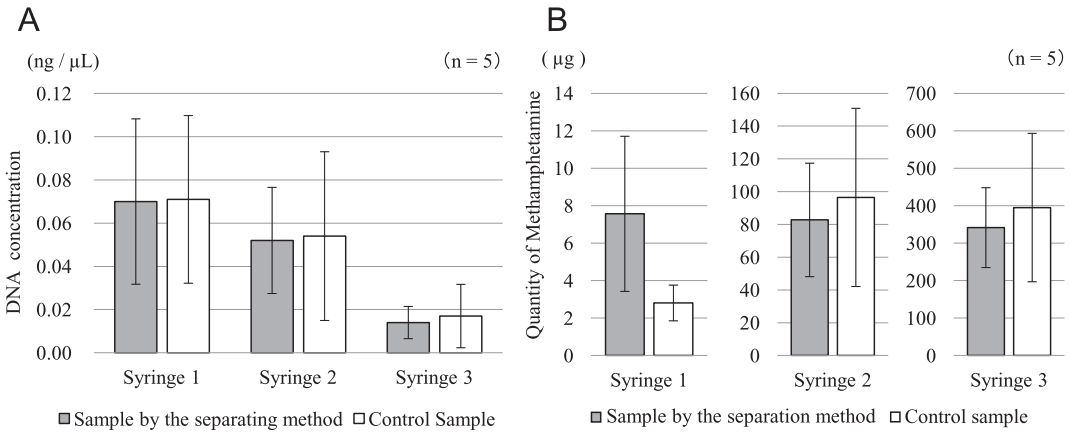

Fig. 7. DNA typing and methamphetamine detection of Syringe samples. (A) Concentration of eluted DNA in the precipitates of Syringe 1–3. Concentration of DNA eluted using the separating method (gray) and that of the control samples (white). (B) Quantity of methamphetamine in liquid fractions of Syringe 1–3. Quantity of methamphetamine in liquid fractions using the separating method (gray) and control samples (white).

Table 3
Number of loci detected in DNA subjected to syringe samples.

	Syringe	Sample					Average number of detected loci
		A	B	C	D	E	
Sample by the separating method	Syringe 1	16	15	16	16	16	15.8
	Syringe 2	16	10	16	16	16	14.8
	Syringe 3	11	4	16	10	14	11
Control sample	Syringe 1	16	14	16	16	16	15.6
	Syringe 2	16	16	16	16	16	16
	Syringe 3	16	4	15	16	11	12.4

In future, we will examine various approaches pertaining to the dual test procedure for DNA typing and methamphetamine detection, including other methods for DNA typing and methamphetamine detection. We hope that our method will be considered for use in actual criminal cases.

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