



Immunochemical detection of human hemoglobin from deteriorated bloodstains due to methamphetamine contamination, aging, and heating

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Abstract

Japanese police conduct highly sensitive and quick blood tests to detect human hemoglobin (Hb), because bloodstains left at a crime scene have probative value of circumstantial evidence in a criminal investigation. Although DNA detection from a bloodstain is a useful tool to identify an individual, doing so requires evidence that the bloodstain is of human origin. Stimulant drug abuse and dependence causes major social problems and crimes in Japan, and bloodstains are often found inside syringes seized from drug abusers. In this case, Hb often cannot be detected by conventional testing as high concentrations of stimulants, such as methamphetamine hydrochloride (MA), in blood trigger polymerization of Hb molecules, which become insoluble under non-reducing conditions and can no longer be detected by immunochemical detection kits. To overcome this problem, we analyzed methods to detect denatured Hb from bloodstains contaminated with MA. Reduction of polymerized Hb with a strong denaturing agent was required to solubilize polymers into monomers, suggesting that Hb aggregation is caused by aberrant formation of disulfide bonds. Based on these results, we established a pretreatment method, called Fukui's Reduction and Eiken's Dilution (FRED), that enables highly sensitive detection of human Hb from bloodstains mixed with MA by reducing and refolding of denatured Hb. This powerful method can be applied to blood that has been boiled or has otherwise deteriorated for over 20 years.

Keywords FRED method · Hemoglobin · Methamphetamine · Immunochemical test · Bloodstain · Forensic science

Introduction

DNA profiling as a means of human identification is a truly powerful forensic tool in criminal investigation, but it alone does not always provide sufficient evidence useful in the criminal justice system. Moreover, it is critical to determine the

origin of DNA extracted from samples left at a crime scene. For example, even if a man's DNA is detected within a victim's vagina, it does not constitute direct evidence of rape without identification of the suspect's semen. Bloodstains also are particularly valuable as personal identification. Serological enzymatic tests are currently popular for detecting of human hemoglobin (Hb) in forensic laboratories. For nearly 20 years, more simple and rapid immunochemical kits, such as OC-Hemocatch S (HC-S) 'Eiken' (Eiken Chemical, Tokyo, Japan) for fecal occult blood [1–3], or Hexagon OBTI (Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) [4] or RSID-Blood (Independent Forensics, Lombard, IL, USA) [5] kits have been used to identify human blood rather than the classical ring test method or counter current electrophoresis. Among these tests, HC-S is commonly used by the Japanese police to provide sensitive and specific detection of human Hb.

Drug abuse has emerged as a deep-rooted social problem spreading to teens, an urgent issue that demands prompt action. Serious abusers sometimes dissolve as much as 300 mg

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methamphetamine hydrochloride (MA) into their own blood and then re-inject the solubilized drug into their venous blood, so that bloodstains remain in the syringe. Since human Hb antigenicity is for unclear reasons lost once high concentrations of MA are added to blood, human Hb cannot be detected from these bloodstains by conventional immunochromatographic tests such as HC-S. Thus, without prior identification of their human origin, these types of bloodstain samples cannot be used for DNA typing.

A Hb molecule is a tetramer of four subunits. Adult human HbA consists of two α chain subunits (141 amino acids/subunit) and two β chains (146 amino acids/subunit). The α and β chains are structurally related with a sequence homology of 43%, and one heme group is bound to each subunit. The human Hb tetramer contains six cysteine residues, none of which forms disulfide bonds under normal conditions. Loss of Hb antigenicity severely decreases the reliability of detection methods: for example, a fecal occult blood test used for colorectal cancer screening [6, 7] occasionally gives false negative results due to degradation or denaturation of Hb by proteases and/or intestinal bacteria [8, 9]. These facts prompted us to investigate whether MA functions as a denaturing agent for Hb molecules, leading to negative results by immunologic tests. In the present study, we report evidence that it does and establish a rapid and sensitive method to detect MA-exposed human Hb and determine the mechanism for how MA inhibits interaction of Hb with anti-Hb antibodies.

Materials and methods

Chemicals and samples

Hiropon® (Sumitomo Dainippon Pharma, Osaka, Japan) was used as MA with appropriate permission. Fresh bodily fluids including blood, saliva, semen, urine, sweat, vaginal secretions ($n = 5$), and breast milk ($n = 2$) were obtained from volunteers (age 27–43 years old). Whole blood was centrifuged to remove plasma and leukocytes, and carbon tetrachloride was then added to saline-washed erythrocytes, which were lysed using a vortex mixer. After centrifugation at 3000 rpm for 10 min, only the top layer containing the hemolysate was carefully collected. Hemolysates, which normally contained ~10 g/dL Hb, were prepared and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Sweat was collected mainly from the face and arms after participants exercised or bathed. Vaginal secretions were collected by wiping the vaginal wall with a sterile cotton swab and were used as the supernatant obtained by leaching a 5-mm square of the cotton ball in 500 μL phosphate-buffered saline (PBS). Breast milk was prepared as a centrifuged supernatant. Those samples were labeled and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Old bloodstains were created, labeled, and stored at room temperature with light shielding for 4, 16, and 26 years.

Animal blood ($n = 37$ in total) was obtained from 10 species (Japanese macaque ($n = 1$), cattle and chickens (each $n = 5$), donkeys and horses (each $n = 1$), pigs, dogs, and cats (each $n = 6$), and rabbits and marmots (each $n = 3$)) and stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

Analysis of denatured Hb

One microliter whole blood and 2 μL hemolysate were diluted 1:10 and 1:5, respectively, with MA solutions (final concentrations ranging from 0 to 300 mg/mL), and incubated at $37\text{ }^{\circ}\text{C}$ for 3 days. Excess MA was removed using Amicon Ultra Centrifugal Filters with a 30 kDa cutoff (Millipore, Billerica, MA, USA) with PBS containing 1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS). Samples were then analyzed either by native-polyacrylamide gel electrophoresis (native-PAGE), non-reducing or reducing sodium dodecyl sulfate (SDS)-PAGE with Coomassie Brilliant Blue (CBB) staining, or western blotting with anti-human Hb α and β antibodies (Santa Cruz Biotechnology, Dallas, TX, USA).

To detect free thiol groups, MA-treated hemolysates were reduced by 30-min incubation in 50 mM dithiothreitol (DTT) at $60\text{ }^{\circ}\text{C}$. Reductants were removed using Amicon Ultra Centrifugal Filters with a 30 kDa cutoff with PBS containing 1% CHAPS. Samples were then reacted with 5 mM monobromo(trimethylammonio)bimane bromide (qBBr) (Thermo Fisher Scientific, Waltham, MA, USA) at $30\text{ }^{\circ}\text{C}$ for 1 h. Samples containing labeled proteins were analyzed by SDS-PAGE, and fluorescent signals were detected after irradiation at 400 nm.

Biacore antibody-antigen reaction

One microliter whole blood was diluted 1:10 with MA solutions (final concentration 300 mg/mL) and incubated at $37\text{ }^{\circ}\text{C}$ for 3 days. MA bloodstains of those solutions were prepared by air-drying at $37\text{ }^{\circ}\text{C}$ for 3 days. Hb was extracted from MA bloodstains using 100 μL 6 M guanidine hydrochloride (GuHCl) and diluted with 1400 μL of HBS-EP buffer (10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) [pH 7.0], 150 mM NaCl, 3 mM EDTA, and 0.005% detergent P-20). As a reducing agent, 25 μL of 1 M DTT or 1 M Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to 475 μL of the extractions and reacted at $4\text{ }^{\circ}\text{C}$ overnight. Solutions were then subjected to two rounds of gel filtration with HBS-EP buffer using a NAP-5 column (GE Healthcare, Buckinghamshire, UK).

Biosensor experiments were carried out using a Biacore 3000 instrument (GE Healthcare). Anti-mouse IgG and anti-rabbit IgG antibodies were immobilized on a CM5 biosensor chip (GE Healthcare) using the manufacturer's instructions for amine coupling chemistry. Anti-human Hb mouse

monoclonal antibodies (mAb) A and B, normal mouse globulin (Meridian Life Science, Inc., Memphis, TN, USA), anti-human Hb rabbit polyclonal antibodies (pAbs), and normal rabbit globulin (Dako, Carpinteria, CA, USA) were diluted to 100 µg/mL in HBS-EP buffer and then injected over the chip at a flow rate of 20 µL/min. After the surface plasmon resonance (SPR) signal reached a plateau, relevant antigens were sequentially injected over the chip at a flow rate of 20 µL/min. The chip was regenerated with 20 µL 10 mM glycine HCl (pH 3.0). The amount of each antigen bound to 1000 response units (RUs) of antibody was estimated. The amount of bound antigen (in RUs) for each antibody was calculated by subtracting the blank measurement value, which was the amount of bound antigen when normal globulin was applied.

Comparison of pretreatment methods to detect Hb from MA bloodstains

To test various pretreatment buffers, we diluted 1 µL whole blood 1:10 with MA solution (final concentration 300 mg/mL), incubated the sample at 37 °C for 3 days, and dried it at 37 °C for 1 week. We then incubated each MA bloodstain 30 min with 10 µL of one of the following buffers listed in Table 1. Each sample was then diluted 1:10 with 90 µL PBS, and then 1 µL of that solution was further diluted 1:1000 in PBS and subjected to the HC-S test. An additional sample (designated no. 7), which had been incubated 30 min with 10 µL buffer no. 6, was similarly diluted with 90 µL dilution buffer (50 mM phosphate [pH 6.6], 0.3% bovine serum albumin (BSA), 0.095% NaN₃, 0.005% Lipidure-BL1201). After an additional 30-min incubation, the mixture was further diluted 1:1000 in the same dilution buffer and subjected to the HC-S test. For that test, 50 µL of each sample was applied to the HC-S sample window, and 5 min later, the test line displayed as positive was quantified using the immunochromatographic reader C1066-10 (Hamamatsu Photonics, Shizuoka, Japan).

Detection of Hb from MA bloodstains using Fukui’s reduction and Eiken’s dilution method

As a novel pretreatment method, we applied 10 µL Fukui’s reduction and Eiken’s dilution (FRED) reduction buffer (50 mM HEPES [pH 7.2], 20 mM TCEP, 50 mM L-Arg, and 3 M GuHCl) to a fresh test sample of MA bloodstains and gently shook it for 30 min at room temperature. We then added FRED dilution buffer (50 mM phosphate [pH 6.6], 0.3% BSA, 0.095% NaN₃, 0.005% Lipidure-BL1201) to 100 µL (a 1:10 dilution) and incubated the sample 30 min at room temperature. Finally, we diluted the sample 1:1000 in FRED dilution buffer and performed HC-S to detect denatured Hb.

To conduct comparable analysis of MA bloodstains air-dried at 37 °C for 0–60 days, we resolubilized stains first in 10 µL reduction buffer and added 90 µL FRED dilution buffer. We then diluted 1 µL of each mixture 1:1000 in FRED dilution buffer and determined Hb concentration by HC-S using an immunochromatography reader. As controls (PBS method), we added 100 µL PBS to the original test samples, incubated them 30 min at room temperature with gentle shaking, and then diluted samples 1:1000 in PBS and determined Hb concentration.

We also undertook the FRED method as described but omitted GuHCl in the reduction buffer, which we designate “native-FRED.”

Preparation of forensic casework samples

Old bloodstains

A 1-cm piece of gauze was cut out from a bloodstain that had been kept at room temperature for 4–26 years and then pretreated using the FRED or PBS method. Since 5% NH₃ reportedly facilitates leaching of Hb from older body fluid stains [4, 10], 100 µL of 5% NH₃ was also added to other bloodstain samples rather than the FRED solution and the sample was shaken at room temperature for 30 min. After evaporation and drying, samples were resolubilized in

Table 1 Pretreatment buffers for comparative study

No.		Buffer conditions
1		PBS (pH 7.4)
2		50 mM HEPES (pH 7.2)
3		50 mM HEPES (pH 7.2), 3 M GuHCl
4		50 mM HEPES (pH 7.2), 20 mM TCEP
5		50 mM HEPES (pH 7.2), 3 M GuHCl, 20 mM TCEP
6		50 mM HEPES (pH 7.2), 3 M GuHCl, 20 mM TCEP, 50 mM L-Arg
7	(A)	50 mM HEPES (pH 7.2), 3 M GuHCl, 20 mM TCEP, 50 mM L-Arg
	(B)	50 mM phosphate (pH 6.6), 0.3% BSA, 0.095% NaN ₃ , 0.005% Lipidure-BL1201

100 μL of PBS (for the ammonia method) and subjected to the HC-S test. When both control and test lines appeared, samples were judged “positive.” Other samples were judged “negative.”

Heating of blood

Whole blood diluted 1:100 in PBS was heated either at 25 °C, 37 °C, 55 °C, 70 °C, 85 °C, or 99 °C for 30 min using a thermal cycler (GeneAmp® PCR System 9700, Thermo Fisher Scientific). Samples were then pretreated using either FRED, native-FRED, or PBS methods. For FRED treatment, 10 μL of heated samples was combined with 10 μL FRED reduction buffer, incubated for 30 min, and combined with 80 μL FRED dilution buffer. After a 30-min incubation, the sample was further diluted 1:100 in the same FRED dilution buffer and Hb concentration was detected by HC-S using an immunochromatography reader. Native-FRED samples were similarly treated using GuHCl-free reduction buffer rather than FRED reduction buffer. For the PBS method, 10 μL of heated samples was combined with 90 μL PBS and incubated for 30 min. After further dilution with PBS (1:100), Hb concentration was similarly determined.

Test compatibility with bodily fluids

To confirm that FRED pretreatment of bodily fluids (in the absence of blood) does not yield false-positive results in the HC-S test, 10 μL each of saliva, semen, vaginal secretions, urine, sweat, or breast milk were pretreated with either FRED or PBS (as a control) methods (as outlined above), and then Hb concentration was assessed by HC-S using an immunochromatography reader. We also performed comparable analysis of blood samples plus bodily fluids by combining 1 μL of whole blood that had been diluted 1:1000 in PBS with 9 μL of each bodily fluid.

Statistical analysis

Differences among groups were statistically analyzed by ordinary one-way ANOVA with Tukey’s post hoc-test using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). *p* values less than 0.05 were considered significant.

Results

MA mediates Hb polymerization via formation of disulfide bonds

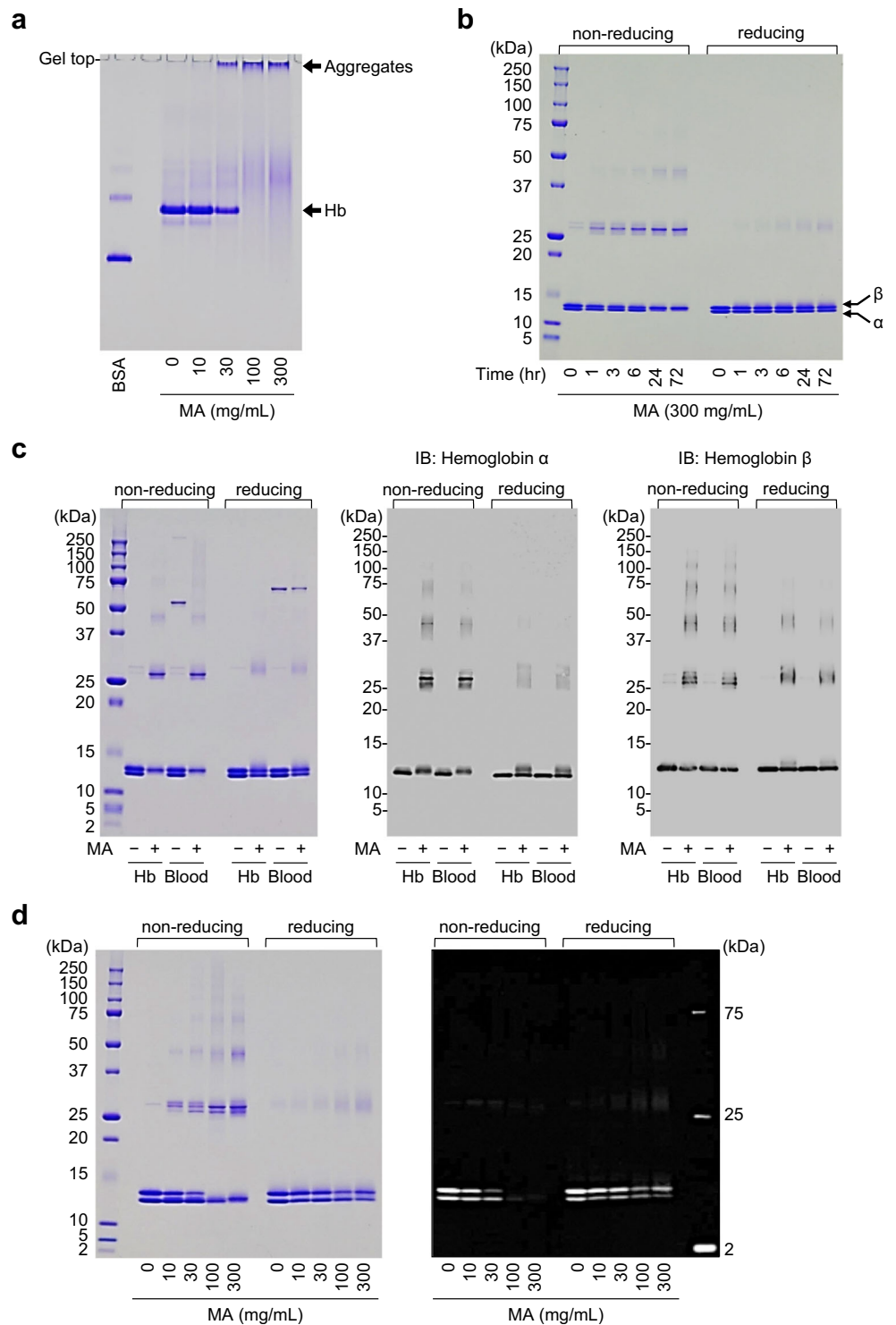
To determine why MA-treated human blood is not detected by standard immunochromatographic blood tests, we prepared

MA-blood mixtures for analysis. To do so, we mixed a freshly prepared hemolysate with MA at various concentrations and then analyzed samples by native-PAGE followed by Coomassie Brilliant Blue (CBB) staining. As MA concentrations increased, levels of native Hb tetramers decreased, while at the same time, we observed formation of Hb aggregates unable to enter the separating gel (Fig. 1a). We then conducted SDS-PAGE analysis under both non-reducing and reducing conditions of Hb incubated with 300 mg/mL MA for 0–72 h. As shown in Fig. 1b (left), under non-reducing conditions, we observed primarily Hb subunit monomers as well as formation over time of oligomers; the weight of which was integer multiple of the weight of individual Hb subunit. The longer the samples were incubated with MA, the greater the staining indicative of Hb oligomers; correspondingly, over time, we observed decreased levels of Hb subunit monomers. On the other hand, under reducing conditions (Fig. 1b, right), bands corresponding to Hb oligomers were largely absent. Moreover, western blot analysis of MA-blood mixtures with either anti-Hb α or β antibodies (Fig. 1c, middle and right) showed that both α and β subunits were aggregated by MA treatment, as evident from analysis in non-reducing conditions, and aggregates were largely absent when gels were run under reducing conditions (Fig. 1c). We then prepared hemolysate samples by treating them 72 h with various concentrations of MA. We then labeled Hb with a thiol group-specific fluorescent dye (qBBr) and performed SDS-PAGE analysis. As shown in Fig. 1d (left), under non-reducing conditions, levels of Hb subunit monomers decreased in a MA dose-dependent manner, and levels of corresponding Hb oligomers stained with CBB increased, as anticipated. However, when the same blot was monitored for fluorescence, fluorescent signals were seen only in monomers and decreased in a MA dose-dependent manner (Fig. 1d, right panel). In reducing conditions, evidence of oligomerization was negligible, regardless of MA concentration. These findings collectively suggest that MA induces Hb polymerization through formation of disulfide bonds and that reduction of samples improves the sensitivity and consequent accuracy of human blood tests, particularly in the case of MA-treated bloodstains.

Antigen-binding properties of three antibodies used in blood tests

Next, to determine whether HC-S test insensitivity to MA bloodstains is due to loss of sample antigenicity, we used Biacore analysis to assess reactivities of three anti-human Hb antibodies (mouse monoclonal antibodies A and B (mAb-A and mAb-B) and rabbit polyclonal antibodies (pAbs)) with human Hb, either with or without MA treatment or in the presence or absence of strong denaturing agents. We chose these antibodies as mAb-A and mAb-B are used in the HC-S protocol, and pAbs are used in OC-Hemodia Auto III

Fig. 1 MA-mediated Hb aggregation is due to formation of disulfide-linked oligomers. **a** Native-PAGE analysis of Hb aggregated with MA. **b** SDS-PAGE analysis of Hb aggregated with MA. MA-treated hemolysate was subjected to non-reducing (*left*) and reducing (*right*) SDS-PAGE, and visualized by CBB staining. (c) Western blot analysis of aggregated Hb α and β . MA-treated hemolysates (Hb) and MA whole blood (Blood) were resolved by non-reducing/reducing SDS-PAGE and stained by CBB (*left*) and western blotting with anti-Hb α antibody (*middle*) and anti-Hb β antibody (*right*). **d** Detection of thiol groups with qBBr. MA-hemolysate samples were reacted with qBBr. Labeled proteins were analyzed by non-reducing/reducing SDS-PAGE and detected by CBB (*left*) and fluorescence scanning (*right*)



‘Eiken’ test (HA-III) (Eiken Chemical). Table 2 shows the amount (in relative units (RU)) of antigen bound to 1000 RU antibody. While signals for mAb-A and mAb-B significantly decreased after denaturation of human Hb with GuHCl, pAb signals increased two-fold (compare rows 1 and 4 in Table 2).

MA-treated Hb did not react with mAb-A and mAb-B; however, stains of MA-treated Hb, which had been reduced by DTT or TCEP treatment, showed partial reactivity to mAb-A and mAb-B (rows 8 and 9, Table 2). On the other hand, Hb reacted with pAbs, regardless of MA treatment (row 7 in Table 2). Taken together, these findings suggest that mAb-A

Table 2 Biacore analysis of interactions of three antibodies with MA-treated Hb

No.	MA	Buffer solutions	Bound antigen (RU)			
			mAb-A	mAb-B	pAbs	
1	–	HBS-EP	–	59	303	45
2			DTT	15	182	61
3			TCEP	30	207	51
4	–	GuHCl	–	6	217	85
5			DTT	13	128	96
6			TCEP	38	226	77
7	+	GuHCl	–	1	2	75
8			DTT	9	99	97
9			TCEP	11	83	102

and mAb-B may recognize structural elements of human Hb, while pAbs recognize human Hb amino acid sequence.

The latex agglutination test with pAbs cannot detect MA-treated Hb

Biacore analysis suggests that MA-treated Hb can be detected by a human blood test using pAbs. Thus, we asked whether pAbs can detect MA-treated Hb in the absence of reducing agents. HA-III is a fecal occult blood test reagent used to detect human Hb with pAbs and is based on latex agglutination immunoturbidimetry. Here, we quantified human Hb in bloodstain samples, with or without MA treatment or in the presence or absence of strong denaturing agents, using the automatic analyzer OC-Sensor DIANA (Eiken Chemical). Table 3 shows values of Hb using HA-III and HA-mono, in which pAbs in HA-III were replaced with mAb-A and mAb-B. Hb levels detected in the presence of 6 M GuHCl using

HA-III and HA-mono (row 4 in Table 3) were 37.9% and 29.4%, respectively, of those seen in untreated Hb samples (row 1 in Table 3). On the other hand, levels of Hb that had been exposed to MA and denatured by 6 M GuHCl and then analyzed by HA-III and HA-mono (row 7 in Table 3) were 3.2% and 0.0%, respectively, of those seen in untreated Hb. Next, we treated MA bloodstains with buffers containing 6 M GuHCl and either DTT or TCEP, and analyzed them with HA-III and HA-mono. We observed that Hb levels after these treatments were 13.2% and 13.6%, respectively, those seen in untreated Hb samples using HA-III, and 18.8% and 18.3%, respectively, those seen in untreated Hb samples using HA-mono (rows 8 and 9 in Table 3). These results indicate that, in the absence of reducing agents, Hb in MA bloodstains is only minimally detected by HA-III and not detected by HA-mono. Moreover, while Hb detection sensitivity markedly improves in the presence of reducing agents, detection accuracy is far from satisfactory.

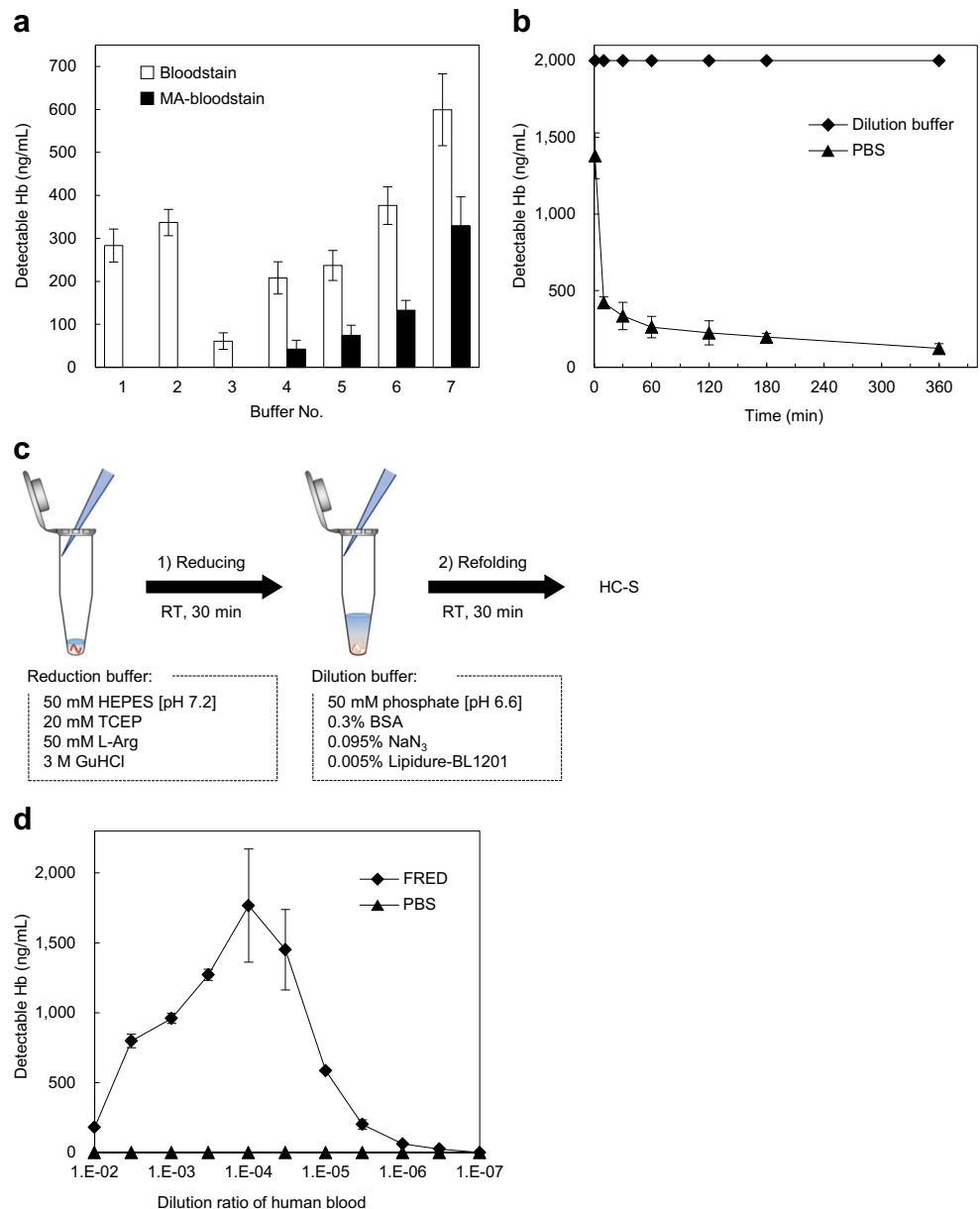
Development of a method to pretreat MA bloodstains for HC-S

Proper Hb folding is likely necessary for detection by mAb-A and mAb-B in HC-S. Fronticelli et al. reported that insoluble globin can be solubilized in 50 mM NaOH and then diluted in 40 mM borate buffer containing 1 mM EDTA and 1 mM DTT for refolding [11]. Although MA-treated Hb can be solubilized in NaOH solution, our HC-S test of NaOH-treated samples was negative even after dilution in borate buffer (data not shown). Thus, to detect Hb from MA bloodstains, we developed a pretreatment method to refold denatured Hb. To do so, we tested seven different buffers using not only MA bloodstains but also bloodstains that had not been exposed to MA (as controls). As shown in Fig. 2a, Hb was solubilized from MA bloodstains using GuHCl but could not be detected by HC-S (buffer 3). However, when we added the reducing agent

Table 3 Reactivities of HA-III and HA-mono with MA-treated Hb

No.	MA	Buffer solutions	HA-III		HA-mono		
			(ng/mL)	/No. 1	(ng/mL)	/No. 1	
1	–	HBS-EP	–	49,898	100.0%	56,760	100.0%
2			DTT	29,620	59.4%	45,092	79.4%
3			TCEP	17,654	35.4%	28,652	50.5%
4	–	GuHCl	–	18,915	37.9%	16,684	29.4%
5			DTT	15,733	31.5%	25,337	44.6%
6			TCEP	15,758	31.6%	24,001	42.3%
7	+	GuHCl	–	1572	3.2%	24	0.0%
8			DTT	6566	13.2%	10,699	18.8%
9			TCEP	6772	13.6%	10,401	18.3%

Fig. 2 Development of effective pretreatment for MA bloodstains. **a** Comparative study of pretreatment buffers. **b** Stabilization of Hb in dilution buffer. Whole blood diluted 1:100,000 with dilution buffer or PBS was shaken at 700 rpm at room temperature and then measured by HC-S. **c** Schematic protocol of the FRED method. For reduction, blood samples were dissolved in reduction buffer and incubated at room temperature for 30 min. For refolding of Hb proteins, samples were then diluted 1:10 in dilution buffer and incubated at room temperature for 30 min. Human Hb was detected by HC-S. **d** Effects of the FRED method on detection of human Hb from MA-treated blood compared with PBS



TCEP, we detected a positive signal based on HC-S from MA bloodstains (buffer 4). Inclusion of GuHCl (buffer 5) or both GuHCl and L-Arg (buffer 6) in buffer further improved detection sensitivity of Hb from MA bloodstains; however, compared with bloodstains without MA, detection sensitivity remained low. Thus, to avoid potential inhibitory effects of reducing or denaturing agents on refolding of denatured Hb, we devised an optimal “dilution buffer” (50 mM phosphate [pH 6.6], 0.3% BSA, 0.095% NaN₃, 0.005% Lipidure-BL1201). As a comparison, we found that if an Hb solution was shaken 10 min in PBS and then applied to HC-S, the apparent Hb concentration decreased to ~35% of the initial value (Fig. 2b). However, when we used our optimal dilution buffer rather than PBS, Hb concentration remained constant even after shaking for 6 h or more (Fig. 2b). We conclude that

detection sensitivity for human Hb from MA bloodstains is substantially improved by performing solubilization/reduction and dilution procedures that promote refolding (Fig. 2a, buffer 7). We call this novel pretreatment method FRED (for Fukui’s Reduction and Eiken’s Dilution) and define it as (1) treatment of samples in FRED reduction buffer (50 mM HEPES [pH 7.2], 3 M GuHCl, 20 mM TCEP, 50 mM L-Arg) and then (2) dilution in what we designate as FRED dilution buffer containing 50 mM phosphate [pH 6.6], 0.3% BSA, 0.095% NaN₃, and 0.005% Lipidure-BL1201 (shown stepwise in Fig. 2c). This method allows detection of human Hb from MA-treated blood diluted 1: 3000,000 (equivalent to 16.7 pL of blood). By contrast, we could not detect Hb from MA-treated blood diluted 1:100 with PBS (equivalent to 0.5 μL of blood) (Fig. 2d).

The FRED method detects Hb from time-elapsed MA bloodstains

Considerable time can elapse before bloodstains are found at incident scenes. To determine effects of elapsed time on detection sensitivity of HC-S with FRED pretreatment, we prepared samples by air-drying MA bloodstains for 0, 7, 30, and 60 days, and then performed either FRED, native-FRED, or PBS methods prior to performing the HC-S test. Figures 3a, b, c, and d show Hb concentrations detected from MA bloodstains air-dried for 0, 7, 30, and 60 days, respectively. As shown in all panels, use of the FRED method enabled us to detect Hb from dried MA bloodstains regardless of elapsed time. Moreover, in samples dried for long periods (7, 30, and 60 days; Fig. 3b–d), FRED sensitivity was significantly greater than that of a comparable FRED method performed without GuHCl (which we designate “native-FRED”). These observations suggest that the decreased sensitivity in Hb detection seen in dried bloodstains is likely due to Hb denaturation and aggregation.

Application of the FRED method to forensic casework samples

Old bloodstains

Under prolonged drying conditions, bloodstains become less soluble due to progression of coagulation and fibrin denaturation. To overcome this problem, we applied the FRED method to bloodstains acquired 4, 16, and 26 years ago. Interestingly, as shown in Table 4, Hb was detected even from 26-year-old bloodstains using FRED method, while Hb was not detected from 26-year-old bloodstains either by the conventional PBS method or by the ammonia method, which is effective in extracting Hb from old bloodstains.

Heated blood

In some cases, blood samples collected on a car bonnet in summer or at a fire scene test negative in blood tests using HC-S, likely due to heat. Thus, we asked whether FRED or the native-FRED method was useful to analyze samples that

Fig. 3 Detection of Hb from MA bloodstains. Samples were prepared by mixing blood with MA solution (300 mg/mL) at 37 °C for 3 days followed by air-drying at 37 °C for 0 days (a), 1 week (b), 1 month (c), and 2 months (d). Samples were pretreated using the FRED method and measured by HC-S. For comparison, PBS as well as native-FRED method (without GuHCl) was also tested. Data are presented as means with SD of triplicate determinations. $**p < 0.01$, $***p < 0.005$

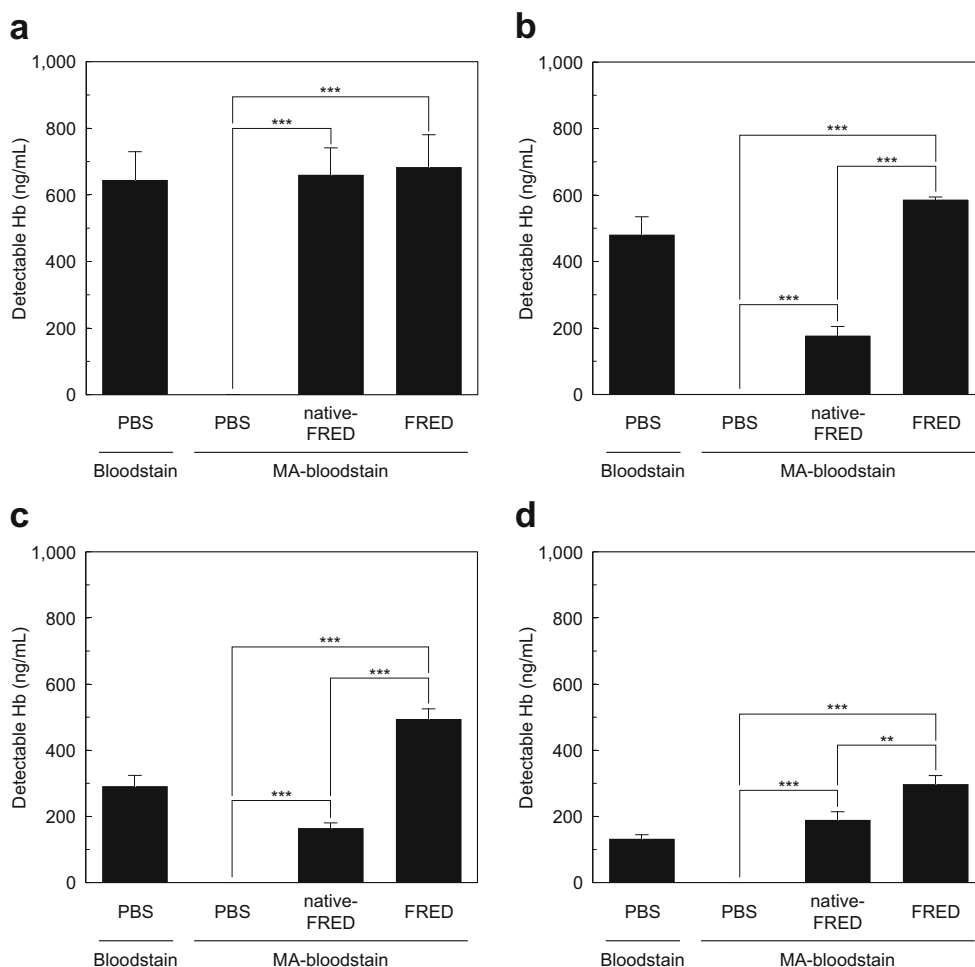


Table 4 Detection of Hb from old bloodstains

Bloodstain age	HC-S		
	PBS	5% NH ₃	FRED
4 years	+	+	+
16 years	ND	+	+
26 years	ND	ND	+

ND, not detected

had undergone heat denaturation. As shown in Fig. 4, we detected human Hb in blood heated to 99 °C when we used the FRED method, while Hb pretreated with PBS was detectable only from samples heated to 55 °C. Although not as effective as FRED, the native-FRED method was more effective in detecting Hb from heated samples than was PBS.

Compatibility with body fluids

To determine whether the presence of body fluids alters HC-S specificity, we applied the FRED method to saliva, semen, vaginal secretions, urine, sweat (n = 5), or breast milk (n = 2), with or without a trace amount of blood, prior to performing the HC-S blood test. Using the FRED method, HC-S testing of bodily fluids alone (without blood) yielded no false-positive signals in any sample examined (data not shown). However, in blood-containing samples, human Hb was accurately detected in the presence of all bodily fluids tested, and Hb concentrations measured in samples with FRED pretreatment were comparable with those observed when we used the conventional PBS method (Table 5).

Species specificity

We then tested species specificity of HC-S with FRED pretreatment using 37 blood samples from 10 different animals,

Table 5 Detection of Hb in the presence of bodily fluids

Bodily fluids + blood	n	HC-S (ng/mL)	
		PBS	FRED
–	5	109	782
Saliva	5	1124	1383
Semen	5	2000	1571
Vaginal secretions	5	1293	1415
Urine	5	605	1130
Sweat	5	901	989
Breast milk	2	1569	1070

n, number of samples tested

since anti-human Hb monoclonal antibodies used in the HC-S protocol (mAb-A and mAb-B) reportedly recognize PBS-solubilized Hb from *Mustelidae* and *Simiiformes* [3, 12]. As expected, like PBS treatment, the HC-S blood test with FRED pretreatment could not distinguish Japanese macaque Hb from human Hb. On the other hand, in blood samples from cattle, chickens, donkeys, horses, pigs, dogs, cats, rabbits, and marmots, Hb was not detected by the HC-S blood test with FRED pretreatment (data not shown).

Discussion

In forensic science, detection of human Hb is used to test for blood in red stains at crime scenes. However, in forensic case-work samples, Hb may not be detectable due to biochemical and physical damage resulting from not only chemicals such as stimulant drugs but also environmental factors such as dryness, heat, and degradation. In particular, it is common knowledge among forensic scientists the presence of stimulant drugs in a blood sample can give rise to a false negative in a blood test, which is a particular problem in criminal trials.

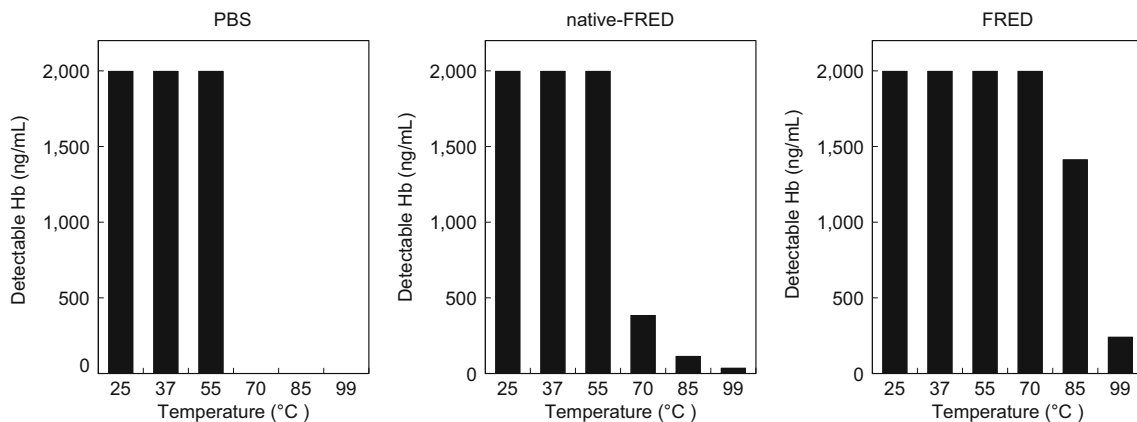


Fig. 4 Successful detection of Hb from heated blood. Samples were pretreated with PBS (left), native-FRED (middle), or the FRED method (right) and analyzed with HC-S

Bloodstains in syringes seized from a stimulant abuser are often insoluble. Even after attempted solubilization of such samples with denaturing agents or alkali, Hb in bloodstains is often still undetectable. Here, we conducted protein analysis revealing that MA potentially denatures Hb molecules, which undergo recovery following reduction (Fig. 1).

Since polyclonal antibodies generally recognize multiple epitopes, we assumed that aggregated Hb would be recognized by these antibodies. Thus, we tried to detect MA-treated Hb with polyclonal rather than monoclonal antibodies. Our Biacore analysis showed that two monoclonal antibodies (mAb-A and mAb-B) did not react with MA-treated Hb, although polyclonal antibodies (pAbs) did (Table 2). However, HA-III, which employs the same polyclonal antibodies, detected only a small fraction (3.2%) of MA-treated Hb relative to untreated Hb, most likely due to deformation of Hb structure (Table 3). Conversely, if specimens are adulterated or unknown, unpredicted interfering substances may alter the response of the HC-S test. Thus, it remains challenging to detect aggregated Hb by solubilization alone, even by polyclonal antibodies.

Treatment by reduction allowed even monoclonal antibodies to recognize MA-treated Hb (Table 2) and greatly improved detection sensitivities of both HA-III and HA-mono (Table 3). Moreover, treatment of MA-treated Hb with strong denaturing agents (such as GuHCl) and reductants (such as DTT or TCEP) also improved detection sensitivities. Note, however, that to avoid damage by those reagents, it was necessary to dilute samples with a buffer optimized for refolding after solubilization.

We also devised the two-step FRED pretreatment method based on reduction and then refolding in order to substantially increase Hb detection sensitivity (Figs. 2a and d and 3). The components of dilution buffers used for protein refolding play a pivotal role in maintaining a native Hb conformation. Although PBS is commonly used as a buffer in forensic testing, FRED dilution buffer contains stabilizing agents that makes it superior to PBS in detecting Hb (Fig. 2b). It should be reasonable to consider that the HC-S test was likely designed to function even in the presence of high protein amounts and/or a certain viscosity. Nonetheless, while having a room for optimization, FRED dilution buffer was found to increase the sensitivity of the HC-S test up to 100-fold when compared with PBS (data not shown). This approach could be beneficial to forensic biology, where often minute amounts of bloodstains in varying conditions are often inspected at crime scenes.

In blood tests performed with FRED pretreatment, Hb was detected with high sensitivity after mixing with body fluids, including semen (Table 5). This result suggests that some components in bodily fluids may actually stabilize Hb. On the other hand, when we performed the FRED method without including L-Arg, we did not detect

Hb in samples of blood and sweat (data not shown). Thus, unknown factors in sweat may promote Hb aggregation, leading to false negatives. Therefore, inclusion of L-Arg in the buffer is required to suppress Hb aggregation. Although reducing agents are generally avoided in analyses involving immunochromatography, in the case of FRED method, a small amount of reductant appears to break disulfide bonds formed in Hb aggregates without interfering with antigen-antibody reactions.

In the future, forensic scientists may have increased opportunities to re-examine very old samples due to the extension or abolition of statutes of limitations. Pretreatment with 5% NH₃ is effective for old bloodstains, but here we found that 16 years was the limit to detect Hb from bloodstains using this method. On the other hand, the FRED reagent successfully detected Hb in samples that were 26 years ago (Table 4). Moreover, the FRED method was useful to detect Hb from boiled blood samples (Fig. 4) and thus is particularly effective when bloodstains have become insoluble due to aging or thermal conditions.

A limitation is that we have not been able to detect Hb from some casework samples even by the FRED method when Hb has undergone degradation (data not shown). It is unlikely that MA promotes Hb degradation as Hb can be detected using the FRED method even after exposure for more than 3 years. Therefore, samples containing degraded Hb protein are likely undetectable by any method.

In recent years, real-time RT-PCR, which enables quantitative analysis of specific mRNAs, has been optimized to identify bodily fluids [13, 14]. Although this method has high detection sensitivity, blood markers still cannot be detected from stimulant-exposed bloodstains [15]. We find that the FRED method is an optimal pretreatment method for immunochromatographic tests and provides accurate and sensitive detection of human Hb from all forensic casework samples, not just stimulant bloodstains. We hope this report provides useful knowledge relevant to protein aggregation and fosters development of forensic science.

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Compliance with ethical standards

All procedures involving human volunteers were approved by the Human Subjects Ethics Committee of the Japanese Association of Forensic Science and Technology. Written informed consent was obtained from each participant.

Conflict of interest The authors declare that they have no conflict of interest.

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