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Short communication

Age estimation of bloodstains: A preliminary report based on aspartic acid racemization rate

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ABSTRACT

This study describes an innovative application of a well-established method of age determination. The conventional method of aspartic acid racemization (AAR) is based on estimation of the D–L-aspartic acid ratio in slow turnover tissues, such as tooth tissue, to reflect the age of an individual. This method has been recently applied to age estimation in forensic investigations, and is also widely used for archeological dating of fossils. We suggest that the aspartic acid racemization method could be applied to a significant, although unresolved, forensic issue: that of bloodstain dating. Standard kinetic experiments were used to describe the characteristics of the racemization reaction in bloodstains, which were then employed to estimate the age of various samples. The soluble protein fraction of a bloodstain produced a stronger correlation between elapsed time and D-aspartic acid content than total amino acid fractions. According to our preliminary results, the time lapse after the creation of a bloodstain can be determined ex vivo by measuring the extent of aspartic acid racemization. Our analysis highlights the need for further study into the preservation and composition of bloodstains to assist in further development of this pioneering application.

1. Introduction

Determination of the age of bloodstains represents a demanding and crucial task in forensic practice. Pioneer approaches introduced decades ago differ in applied methodology, instruments and interpretation of the findings. Some of these methods were based on the observation of color changes in blood spots [1] or the examination of enzyme activity [2–4]. Other researchers attempted to correlate the elapsed time with the solubility of the bloodstain [5] or chloride transfer [6]. However, these techniques did not result in conclusive and accurate determination of the age of bloodstains due to methodological limitations and imprecise assessments. Moreover, sample quantity considered as a relevant factor during an investigation; therefore, an ideal method would require a small amount of blood (micro liter range) to allow for the possibility of repeated testing.

Subsequent efforts concentrated on the application of advanced technology and a better understanding of bloodstain ageing. Accordingly, the ratio of oxyhemoglobin to total hemoglobin [7], the increase in methemoglobin level [8], or the increase in the globin chains of hemoglobin [9] were investigated using various instruments, such as oxygen electrodes or HPLC. Despite their improved methodology, use of these methods in routine forensic investigation was problematic because of their high cost or their lack of adaptability to mobile crime scene investigations. Therefore, these attempts have not resulted in the development of a standard technique capable of rapid estimation of bloodstain age.

More recent publications in bloodstain ageing reported on state-of-the-art assays by force spectroscopy [10,11], and demonstrated promising results with bloodstains aged younger than 4 weeks. However, the article noted the possible effects of changes in the properties of erythrocytes (e.g. the age of the red blood cells) and high standard variation. A novel biochemical approach utilizing a real time PCR method [12] proved a linear correlation between the rate of two housekeeping genes (18S/β-actin) and the age of the bloodstain. This method greatly depends on RNA quality, an important factor since the integrity of RNA can easily be compromised by environmental conditions as slight as a temperature change during the bloodstain preservation period.

Understanding the biological process of erythrocyte ageing has been refined in the last two decades. The presence of post-biosynthetically modified protein structures in human tissues and the possibility of their gradual increase during ageing have been characterized in numerous low metabolism tissues [13,14] as well as in erythrocytes. The lifespan of human mature erythrocytes is around 120 days. Even during this relatively short period, non-enzymatic, spontaneous reactions, including

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deamidation, isomerization and racemization, lead to the accumulation of D-aspartyl derivatives (D-Asx) in proteins. Since circulating red blood cells are not capable of de novo protein synthesis, the accumulation of D-Asx described indicatively as "protein-fatigue" has been related to certain hematological diseases. Ingrosso and Perna [13] implicated "aspartyl-related fatigue damage" in pathological conditions such as chronic hemolytic anemia, hereditary spherocytosis and chronic renal failure. Furthermore, detailed descriptive data for erythrocyte proteins by Brunauer and Clarke [14] revealed that membrane and cytosol protein fractions accumulate D-Asx residues at different rates in vivo. Accordingly, in membrane proteins, particularly in intrinsic membrane fractions, racemization occurs approximately 15 times faster than in cytoplasm proteins, which reflects differences between protein configuration and potential repair mechanisms. Lowenson and Clarke [15] highlighted the fact that D-Asx in red blood cell proteins augmented 10–100 times faster than in some slow metabolism tissues, and also discussed non-enzymatic and enzymatic correction pathways for spontaneous protein modification.

Extensive international efforts on standardization and quality assurance [16–22] have established aspartic acid racemization (AAR) as a reliable approach for measuring ageing in forensic science by correlating the AAR rate with chronological age in tooth, bone, cartilage, and elastin [23–27]. However, AAR investigations into bloodstain ageing have not yet been performed. Therefore, we initially attempted to determine the racemization rate in bloodstain standards to gain an insight into the AAR reaction during the mechanism of ageing in bloodstains. Subsequently, we aimed to assess the elapsed time in aged bloodstain samples using an Arrhenius equation, defined by heating experiments of a standard bloodstain. The objective of this study is providing an introductory evaluation of the AAR method for dating bloodstains, considering recent advancements in the understanding of the AAR reaction.

2. Materials and methods

To analyze the racemization rate of amino acids in bloodstains we designed basic experiments using standard bloodstains from fresh human blood donated by a healthy male volunteer of Japanese origin. Five milliliters of blood was transferred directly into a Menault mortar and kept at 15 °C (the temperature was controlled in the sample room). After drying, the total amount of blood was pulverized homogenously with a pestle, and then the powdered blood was subjected to total amino acid fraction measurements to determine the rate of aspartic acid (Asx) racemization (D/L ratio). Heating experiments were employed to determine racemization kinetics of aspartic acid from the standard bloodstain powder. All the reagents used in this study were analytical grade and purchased from Wako, Japan. The amino acid analysis protocol has been detailed in previous reports [28]. Accordingly, 10 mg portions of powdered blood were placed in test tubes. The sealed tubes were placed in an aluminum block heater (Yamato HF-21, Japan) and heated (to simulate the ageing process) from 8 h to 216 h, at 90 °C, 100 °C, 110 °C and 120 °C. The standards were then subjected to hydrolysis using 6 M hydrochloric acid at 100 °C for 6 h, and then dried in an evaporator. Hydrolyzates were passed through Dowex 50W-X8 ion-exchange columns for desalting.

Various ages of bloodstain samples were prepared for age determination using aspartic acid racemization analysis. Samples ranging from one month to 20 years old were preserved on the surface of glass slides and kept in temperature ranged between 5 and 25 °C (temperature range in the laboratory room during years-long conservation periods). D/L Asx ratios as total amino acids, as well as its acid soluble fractions, were resolved. The acid soluble fraction (30 mg) was collected as the supernatant of acid extraction with 1 M HCl (1 ml) and was subsequently centrifuged at 4000 × g (1 h at 4 °C). Derivatization of the standards and other samples included esterification and trifluoroacylation before gas chromatography (GC) analysis. GC apparatus consisted of a gas chromatograph equipped with a FID detector (GC-11A, Shimadzu, Japan) using a capillary column coated with Chirasil-L-Val (30 m, 0.3 mm i.d.). D- and L-Asx ratios in the bloodstain samples were calculated from chromatogram peak areas. Each measurement was repeated three times. Statistical analysis consisted of linear regression analysis using average values.

As the racemization of Asx is considered to follow a first order reversible rate law reaction, the AAR rate constant was derived from kinetic heating experiments applying the Arrhenius-type equation (describes the quantitative relation between the reaction rate and temperature) [29]:

$$\ln k = \ln\left(\frac{A - E_a}{RT}\right)$$

(k, rate constant; A, frequency factor; E_a, activation energy; T, absolute temperature; R, gas constant). This formula was calculated by converting the velocity constant of each temperature to a logarithm and plotting it to a reciprocal of the reaction temperature. By assuming an annual mean air temperature to be 15 °C and substituting this temperature into the Arrhenius equation, the rate constants of Asx racemization were calculated to determine the age of the bloodstain samples.

3. Results

A representative of the gas chromatograms of the human bloodstain amino acid enantiomers is shown in Fig. 1. The quantity of enantiomers was calculated from the areas under the eluted peaks. D- and L-Asx are baseline separated, eluted after about 4 min, and detected after alanine, glycine, and proline peaks. In order to determine the racemization reaction velocity in the standard bloodstain, a heating experiment was conducted. D-Asx accumulated at a constant rate over time (up to nine days) with increasing velocity corresponding to higher temperatures. The linear least square regressions were constructed for the heating experiments and are shown in Fig. 2a. Rate constants and correlation coefficients of each temperature are summarized in Table 1.

The Arrhenius equation deduced from heating of standard bloodstain during kinetic analysis is shown in Fig. 2b. The equation

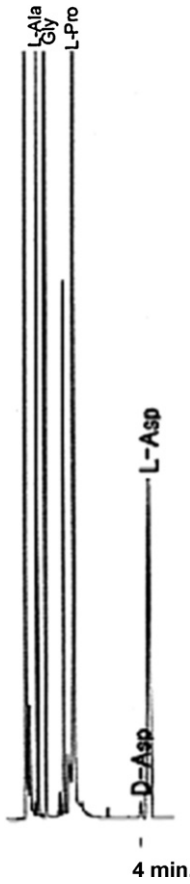

Fig. 1. D/L-Asx rates were calculated from elution peak areas of gas chromatograms. Samples of 0.2 μl were injected onto the chiral phase column and chromatographic conditions were controlled (injector temperature, 250 °C; detector temperature, 200 °C, initial column temperature, 100 °C, then increased to 180 °C [2 °C/min rate]). Abbreviations: Gly, Glycine; L-Ala, L-Alanine; L-Pro, L-Proline.