

Amino Acid Racemization from Tooth for Age Estimation- An Overview

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ABSTRACT: Age estimation constitutes an important factor in identification of individual in forensic odontology. Out of the various methods used, Amino acid racemization (AAR) is the most reliable one. AAR occurs in tissues with low metabolic turn over. As age advances L-amino acid change to D-amino acid, and their ratio represents age. Amino acid racemization method has a historical background of over three decades. Since most of the methods used in forensic odontology age estimation were based on structural changes of teeth, racemization becomes the newer method by its chemical approach. In general racemization of amino acid follows a first order reversible rate law; $L\text{- amino acid } K_L/K_D$ D- amino acid where K_L and K_D are the first order rate constant of the interconversion of L and D amino acid enantiomers. Amino acid from dentin as well as from enamel is tried for racemization study. Several studies were conducted in this field and most of it had an age prediction range of less than ± 3 years. Advantage of using this method is that minute amount of tooth sample is well enough to perform this technique. This paper highlights an overall view of AAR method with relates to age.

Key words: Amino Acid Racemization, age estimation, forensic odontology

Introduction

Age can be estimated in children and in adolescents by means of development and eruption of deciduous and permanent teeth up to 14 years. For most age estimation methods the developing teeth are subjectively assessed on radiographs. After the age of 14, the third molar is the only remaining tooth that is still developing and consequently dental age estimation methods have to rely on the development of this tooth until the age of 20. After this period age determination is mainly done by visual examination, radiographic methods [1, 2] of structural changes in teeth and by means of chemical methods [3].

About 37 years back it was found that the extent of AAR could be used for dating of various biological materials. Later it was found that chronological age of an individual could also be estimated with a reasonably good precision [4]. At a temperature of 25 °C, it would take 100,000 years for all L-forms of amino acids present in living tissues to undergo complete racemization to the D-amino acid form [5]. This chemical method of age determination uses teeth from living as well as from deceased individuals. A gradual transformation of L-aspartic acid into its D-form (racemization) occurs during life in metabolically inactive tissues (eg. Tooth enamel, dentin, white brain matter and lens of the eye) and the ratio of the two forms is an expression of age [6].

AAR based on age-dependent, nonenzymtic changes of L- form amino acid to D-form amino acid is considered to be one of the most reliable and

accurate methods in estimation of age of an adult individual [7].

Review

Racemization of aspartic acid proceeds throughout lifetime and also after death, but probably at a reduced rate as a result of a presumed reduction in ambient temperature. In fresh cadavers or putrefied remains racemization of aspartic acid is applicable as long s the post-mortem interval does not exceed a few decades [8].

D/L ratio can be assessed by using either gas chromatography or by high performance liquid chromatography. Vanden Oetelaar [9] compared the separation efficiency of both and found that HPLC is preferred because of its higher reproducibility and convenience. But recent research point out that both method have its own advantages and draw backs [9-18]. The method has high sensitivity, contamination with proteins from other sources, such as blood, pulp or periodontal tissues may significantly increase the relative amount of the L- form, giving a too low estimate of the age. Also newly formed secondary dentin may decrease the relative amount of the D-form. Similarly contaminated with bacteria with D-form aspartic acid in their cell wall may cause an apparent too high age. The rate of racemization of amino acid in dentin in the living body is mainly determined by temperature, pH and water content [6].

As the post mortem temperature of cadavers rapidly reach the temperature of the preserving environment,

the racemization rate decreases. But the age estimation on cadavers indicted that post mortem preservation of upto 10 years had a negligible effect on the values estimated for the age of individuals at death [10].

In general racemization of amino acid follows a first order reversible rate law: L- amino acid K_L/K_D D-amino acid Where K_L and K_D are the first order rate constant of the interconversion of L and D amino acid enantiomers. If the ratios of the abundance of the D and L amino acid and K_L are known, the age of the tooth can be calculated by statistical regression analysis [5].

Many studies were done on age estimation using racemization of amino acids [5,6,10,11]. T. Ogino found that unerupted teeth with normal crown shape and size can be used for age estimation [5]. Susumn Ohtani showed not only total amino acid in dentin, but also of its fractionated and extracted substances, can lead to higher reliability in age assessment [6]. The author also showed that deciduous teeth can also be estimated by racemization technique [6]. Out of various aminoacids, aspartic acid was highly correlated with age [10].

There is broad consensus among experts about the best suited age estimation methods as described in the recommendations of the Study Group on Forensic Age Diagnostics [12]. Among the reliable methods, racemization of aspartic acid in dentin ranks first. Available reference works compiled to date result in an average regressive error of estimate of 2.1 years [12].

Shi-Jiang Fu et.al compared the effectiveness of GC and HPLC in separating aspartic acid and HPLC was reported to be better [13]. The highlights of the material and methods in AAR technique as mentioned by Shi-Jiang Fu et.al is as in (i) – (iv) below.

i. Apparatus

The HPLC system consisted of a 510 pump and 420 fluorescence detector (Waters), reverse phase column (250 x 4.6 mm).

ii. Chemicals

The chemicals used were D- and L-aspartic acids (BDH), N-acety-L-cysteine (NAC. SIGMA), O-phthalaldehyde (OPA. SIGMA), HPLC-grade water and methanol. All other chemicals were reagent grade.

iii. Sampling teeth

The first premolar was used in every case. Experimental teeth were extracted from cadavers and stored in 10% formalin. Before use, all the teeth were

washed under running water overnight and then allowed to dry naturally.

iv. Sample preparation

After storage, the tooth roots were removed, and the crowns were ground with a mortar and pestle. Dentin fragments (~40 mg) were separated by hand under ultraviolet light (they fluoresce and enamel does not). The dentin fragments were cleaned by ultrasonication in double-distilled water and dilute HCl, sealed in tubes, and hydrolyzed with 6 mol/L HCl for 6 h at 100°C. The hydrolyzates were dried under a flow of nitrogen gas, and desalted on a strong-acidity exchange resin. Amino acids were eluted with 2 mol/l ammonia solution, and specimens were once again dried by nitrogen gas, and 1 ml of double distilled water was added to the specimens.

v. Preparation of the OPA-NAC reagent

OPA (8.0 rag) was dissolved in 600 ml of methanol. The following were then added in the order indicated: 500 ml of 0.4 mol/l Na borate (pH 9.4); 800 ml of distilled water; and 120 ml of 1.0 mol/l NAC. The OPA-NAC reagent was stored at 4°C.

vi. Derivatization of amino acids for HPLC

Derivatization was accomplished by mixing 10 ml of prepared sample solution with 20 ml of OPA-NAC reagent in a small test tube. After 2.5 min, 200 µl of 50 mmol Na acetate (pH 5.2) was added, then 10 µl of the solution was taken for direct injection into the HPLC system. HPLC operating conditions: mobile phase was 92% of 50 mmol acetate (pH 5.7) and 8% of methanol; the fluorescence detector was equipped with a 300-400 nm excitation filter and a 400-700 nm emission filter; the flow rate was 1.0 ml/min

Ohtani et al [14] suggested that in elderly individuals racemization in teeth that have been situated deep in the oral cavity for a long time (and thus are exposed to higher ambient temperatures) are more influenced by the environment than by the period of tooth formation. Criteria for selecting tooth for racemization technique is effecting the age of the individual. The types of teeth best suited for racemization analysis are single rooted teeth such as mandibular incisors or mandibular premolars¹⁵. In these teeth, all the dentin can be easily collected, and test results have shown that analysis using whole dentin yields a more accurate age estimate than analysis using only part of the dentin [15].

Dentin is not a homogeneous substance. Initially primary dentin is formed at the crown portion followed by root portion. Throughout the life secondary and tertiary dentin are formed, their age will be definitely lower than primary dentin. Dentin formation is different in different teeth as the eruption dates [16]. Approximately 8-10 years are needed from start to completion for dentin; hence

degree of racemization may differ at different parts of teeth [16]. Degree of aspartic acid racemization (AAR) is expected to be higher in the earliest formed tooth like 1st molar. On contrary, 2nd molar shows high degree of racemization. It may be because molar teeth are deeper in the oral cavity. So, the teeth of older individual are more affected by environmental cause than the time since the moment of formation. AAR is highest in second molar and decrease in the following order >1st molar> 2nd premolar> central incisor> 1st premolar> lateral incisor> canine [14].

There is no variation of AAR of same teeth in right or left sides of jaw. There is a higher racemization rate in the lingual section of teeth crown than buccal section, because the lingual side may be exposed to high temperature in the mouth. Whereas no changes in root section of dentin were noticed [15,17]. It was suggested that AAR is more in crown and low in root apical portion in young individual, whereas it is affected in elderly as there is prolonged period of time the tooth apex remains there [18].

Dentin consists of approximately 91% of acid insoluble fraction (collagen) and 9% acid soluble fraction (non-collagen) [19, 20]. AAR is rapid in non-collagen protein but slow in collagen protein. Racemization of whole dentin is almost the same as racemization of collagen portion, since majority of dentin consist of collagen. Central incisor showed better correlation [18]. Standard specimen is prepared from D&L aspartic acids, which can be substituted for control teeth. This will be used as standard specimen in other laboratories as well; hence racemization can be measured with reproducibility [21]. It is preferred to do AAR technique in known ages of individual until the standard error of ± 3 years are obtained before starting the procedure in unknown age group [22].

Difference in specimen of dentin and difference in analytical methods have made the different results in various published studies. AAR is strongly influenced by the temperature [18]. Using a whole dentin is preferred to get an accurate result. And preferred tooth is ideally being incisors or premolars since they are single rooted and maximum dentin is easily attainable. HPLC coupled with fluorescence detection have improved the results of AAR [18]. Racemization reaction progresses more rapidly in the root than in the crown [23]. The effects of various experimental conditions [24], heating [25] and post-mortem changes [26] were also studied on the racemization reaction. Cases with prolonged post-mortem intervals (beyond decades), human remains with advanced degradation and burnt bodies should be treated with caution [27].

The fact is that age estimation by aspartic acid racemization from teeth requires specific technical

background which, in general, might not be available in all forensic laboratories and a great deal of standardization. Therefore, suggestions for an international standardization and recommendations for methodological approach were published [28]. The use of internal standards prevents experimental errors occurring from sample preparation, derivatization and separation. It is recommended for techniques where accuracy and reproducibility are of major concern. S. Ritz [23] concluded that the application of D-methionine as internal standard was proposed since it is properly separated as well as nearly eluted to Asp and provided calibration curves with excellent linearity. Once the standards are prepared it can be used for few months.

Griffin's [29] study showed a promising idea of using enamel as a source for AAR. The proteins of mature enamel are processed during enamel maturation by proteolytic enzymes to form low molecular weight peptides. During enamel formation the enamel proteins become entrapped in the growing crystalline structure of the enamel. As a result, enamel provides a much better source of intra-crystalline proteins than dentine. Moreover, enamel is much more resistant to change in the burial environment [30]. AAR by enamel sample is very rarely used as the levels of protein are so low that it presents a real analytical challenge [4, 30] and the correlation between racemization and age is not as strong as in dentine [17]. Previous work required large amounts of enamel, sometimes requiring destruction of an entire tooth for a single analysis. However, the introduction of reverse phase-high pressure liquid chromatography (RP-HPLC) with fluorescence detection has made it feasible to determine the extent of racemization of much smaller quantities of amino acids than had previously been possible. RPHPLC can detect amino acids at quantities as low as 0.9 pg [31]. The presence of dental caries in the tooth analyzed only has a small effect on the racemization of the tooth, and thus even carious teeth could be used in age estimation, albeit with slightly larger confidence intervals. Amino acid racemization in enamel can be used in cases where minimal sample destruction is required.

Jir et.al [32] made a study using non-collagenous protein from dentin for assessment of age. This method exploits the characteristic of staphylococcal protease V8, which specifically splits peptide bonds where Lglutamic or L-aspartic acid participate. Those peptide bonds where D-aspartic acid is present remain unsplit because of the stereospecificity of enzymes. In accordance with expectations, fewer peptide bonds are split by this protease at more advanced ages and larger peptide fragments are thus formed due to the higher content of D-amino acid residues in the proteins of older people. The samples of acid-extracted non-collagenous proteins from

dentin were separated using high performance liquid chromatography after enzymatic hydrolysis. A peak with a retention time of 45.3 min was chosen and his enlarging area showed a linear correlation with increasing age. Although the linear correlation with age was proved, the scattering of values decreases the usefulness of the proposed method for age estimation. Some of the present drawbacks may be eliminated by further research.

Conclusion

AAR is still the best method in estimation of age of an individual. Whole root dentin is used mostly as the source of amino acid. The best teeth selected are of single rooted tooth. Non-collagenous dentin is also tried for age assessment method, but needs further studies for its usefulness. Racemization from enamel is the new approach with a promising result, especially if the sample is of minute amount. Internal standard developed for the sampling stage improved the accuracy of AAR method.

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