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Forensic Science International 159S (2006) S89–S94
Forensic Science International
www.elsevier.com/locate/forsciint

Racemization of aspartic acid from human dentin in the estimation of chronological age

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Available online 22 March 2006

Abstract

The estimation of chronological age in cadavers, human remains and in living human beings by various methods is discussed. These methods, which are based on the age dependent non-enzymatic changes of L-form amino acids to D-form amino acids, mainly aspartic acid, are among the most reliable and accurate methods to date. Most of these methods use gas chromatography (GC). In this review, results of aspartic acid racemization in dentin at different targets are discussed. In addition, pre-considerations and guidelines are given for the selection of dentin from teeth. A pilot project was run to evaluate the efficiency of high performance liquid chromatography (HPLC) coupled with fluorescence detection. New buffer conditions were found to obtain stable derivatives of aspartic acid enantiomers for the estimation of racemization.
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Keywords: Age estimation; Dentin; Teeth; Racemization; D-aspartic acid; Forensic science; Forensic odontology

1. Introduction

The estimation of chronological age in cadavers, human remains and in living human beings has been investigated by using various methods [1–4]. Among all, limited numbers of methods are recommended to reach specific needs of legal and forensic issues in childhood, adolescence and adults [1]. In childhood and adolescence, morphological methods and radiological examination of dental and skeletal development are recommended. In adults, the age range estimated with those methods is rather wide, and the estimated and chronological ages often differ [5]. There are several age related changes that occur in amino acids, which constitute proteins such as oxidation, isomerisation and racemization [6,7]. Among these changes, racemization of aspartic acid is a first order chemical reaction [8,9]. In the living body, amino acids that constitute biological proteins are normally composed of the L-form of amino acids, although there are some exceptional peptides which are biologically synthesized using D-form amino acids [10]. With increasing age, a gradual transformation of the L-form of amino acids in proteins into the D-forms (racemization) occurs and this chemical reaction is influenced by various factors such as temperature, humidity, pH, etc., and this racemization also occurs in metabolically inactive or bradytrophic tissues such as teeth, eye lens, vertebral discs and parts of the brain. It has also become clear that racemization of aspartic acid is a significant phenomenon in non-bradytrophic tissues such as bone or lung parenchyma [11] and even in cellular proteins [12]. So, racemization of aspartic acid may also occur as a secondary phenomenon caused by protein degradation, e.g. under pathological conditions racemization is used to investigate tissue turnover and pathogenesis of typical disease of old age as the chronic emphysema of the lung, atherosclerosis or degenerative disorders of articular cartilage [11,13–16].

The 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 as the post-mortem interval does not exceed a few decades [1].

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doi:10.1016/j.forsciint.2006.02.022

Finding single permanent ageing proteins exhibiting racemization can only be achieved by the analysis of defined, purified proteins from individuals and not from total tissue specimens. The very close relationship between racemization of aspartic acid in such permanent proteins and age proves that racemization of aspartic acid obviously is an autonomic, inevitable process during the natural ageing of proteins.

In 1976, Helfman and Bada found the highest correlation ($r = 0.979$) between chronogical age and racemization ratio of aspartic acid compared to the other amino acids studied in dentin of teeth [17]. Subsequently this correlation was examined and confirmed by a number of researchers. It was further reported that the racemization method using aspartic acid in dentin was able to estimate the chronological age more accurately than conventional methods [18–41]. At present, the method using racemization of aspartic acid is one of the most accurate methods for the estimation of age [3].

All these age estimation methods use gas chromatography (GC). As an alternative to GC, very few estimations used high performance liquid chromatography (HPLC) coupled with ultraviolet detection [35,36] and with fluorescence detection [37–40].

Any method used for age estimation in forensic sciences may clarify issues with significant legal and social ramifications for individuals as well as for the community. In such cases methods for estimating age should fulfill the following specific demands: (1) they must have been presented to the scientific community, as the rule by publication in peer-reviewed journals, (2) clear information concerning accuracy of age estimation by the method should be available, (3) the analytical methods need to be sufficiently accurate themselves and (4) in cases of age estimation in living individuals principles of medical ethics and legal regulations have to be considered [1].

Dentin is not a homogeneous substance. At tooth eruption, the formation of primary dentin is completed but since the coronal part develops first, there is a gradient in age of several years from the crown to the root. Throughout lifetime, secondary and tertiary dentin is formed and their age is obviously lower than that of the primary dentin. The formation of dentin is very individual and follows the pattern of growth, earliest in the first molar, central incisive, canine, first premolar, second premolar and last in the second molar with the exception of the third molar [42]. It is known that about 8–10 years or more are required from start to completion, indicating the possibility that the degree of racemization may differ in different parts of the dentin [42].

If uniform conditions are maintained in the intraoral region, then the degree of aspartic acid racemization is expected to be higher in the earliest formed tooth, i.e., the first molar. The degree of aspartic acid racemization in different kinds of teeth was found to be high in the first molar from middle-aged individuals and corresponding to the time of formation. On the contrary, a higher degree of aspartic acid racemization was found in the second molar of elderly individuals, which is a tooth formed at a later stage in life (max of ± 5 years age difference in one out of four second molars was estimated) [31]. The molar region is deeper in the oral cavity than the region of the other teeth. Thus, it was suggested that the teeth of the elder individuals were more affected by environment than the time since the moment of formation, since they were in the oral cavity for a longer period of time [31]. Among the different types of teeth examined, the rate of aspartic acid racemization (k) is the highest in the second molar and decreases in the following order: first molar > second premolar > central incisor > first premolar > lateral incisor > canine [31].

In homonymous teeth of the same jaw (i.e., teeth on the left and right sides), nearly the same degree of racemization was found using whole dentin [43], as the time of formation is similar. When different parts of sagittal sections (labial-lingual direction) of the upper central incisor are taken, the estimated ages were almost identical among the sites of transsection. It was suggested that a slight deviation of transsection from the medial to distal portion of the tooth would not significantly affect the estimated age [2,43]. However, when lingual and labial parts of dentin were compared, the lingual part tended to show higher D/L ratios, suggesting that the lingual part may be exposed to a higher environmental temperature. In addition, the lingual part of crown dentin tends to show a higher D/L ratio than the labial part of crown dentin; there are however no significant differences in D/L ratios between the labial part of root dentin and the lingual part of root dentin, suggesting that roots have similar environmental temperature [2,43]. When ages were estimated using different parts of transverse sections of the same tooth, the younger individuals tended to show higher values in the crown and lower values in the root apex. In the older individuals, the degree of racemization showed a wave-like pattern waxing and waning from the crown towards the root apex [2,43]. Based on the process of dentin formation, the degree of racemization should be high in the crown and low in the root apex as seen in the younger individuals. However, it was suggested that in the elderly, the environmental temperature around the root apex might have affected the degree of racemization, since a prolonged period of time has passed since their tooth formation.

The organic matrix of dentin consists of approximately 91% acid-insoluble fraction (collagen) and 9% acid-soluble fraction (non-collagen) [44,45]. Aspartic acid racemization is rapid in non-collagen proteins but proceeds slowly in collagen proteins. Since total dentin consists mostly of collagen, racemization of aspartic acid in total dentin and in collagen is nearly the same. Better results for coefficients of correlation were observed with central incisor (Table 1).

In this review paper the method using racemization of aspartic acid is discussed and special attention is given to the analytical method, which uses HPLC.

2. Materials and methods

2.1. Reagents

Reference materials of D- and L-aspartic acid were purchased from Sigma (St. Louis, USA). *o*-Phthaldialdehyde (OPA) and *N*-acetyl-L-cysteine (NAC) were purchased from Acros Organics (Geel, Belgium). Methanol, boric acid, hydrochloric

Table 1
Coefficient of correlation between *D*/*L* ratios and age in various teeth [2]

Location of dentin	Fraction of protein	Samples nos.	Coefficient of correlation	References
Crown	TAA	20	0.979	Helfman and Bada [17]
Crown	TAA	61	0.991	Ogino et al. [19]
Central incisor (cervical)	TAA	45	0.974	Ohtani and Yamamoto [21]
Lateral incisor (cervical)	TAA	27	0.99	
Canine (cervical)	TAA	19	0.995	
First premolar (cervical)	TAA	31	0.992	
Second premolar (cervical)	TAA	27	0.969	
First molar (cervical)	TAA	17	0.984	
Second molar (cervical)	TAA	21	0.976	
Third molar (cervical)	TAA	34	0.971	
Central incisor (whole)	TAA	38	0.996	
Lateral incisor (cervical)	TAA	62	0.994	
Canine (whole)	TAA	28	0.991	
First premolar (whole)	TAA	14	0.997	
Second premolar (whole)	TAA	24	0.992	
First premolar (crown)	TAA	28	0.9887[a]	Fu et al. [39]
Central incisor (whole)	TAA	13	0.996	Ohtani and Yamamoto [23]
	IC	13	0.988	
	SP	13	0.997	
First premolar (whole)	TAA	14	0.991	
	IC	14	0.988	
	SP	14	0.994	
Third molar (root)	TAA	70	0.99	Ritz-Timme et al. [25]
	IC	39	0.99	
	IP	39	0.96	
Central incisor (whole)	TAA	16	0.995	Ohtani and Yamamoto [24]
Central incisor (whole)	TAA	8	0.992	Ohtani et al. [41]
Central incisor (whole)	TAA	12	0.995	Ohtani [28]
Central incisor (crown)	TAA	12	0.986	
Central incisor (upper root)	TAA	12	0.984	
Central incisor (central root)	TAA	12	0.987	
Central incisor (lower root)	TAA	12	0.984	
Canine (vestibular root)	IC	71	0.93	Pilin et al. [34]
		NS	0.96	
NS	IC	9	0.982[a]	Benešová et al. [40]
		9	0.968	
Root	TAA	NS	NS	Shimoyama and Harada [18]
All of teeth	TAA	46	0.96	Ritz et al. [22]

IC: insoluble collagen; IP: insoluble protein; SP: soluble peptide; TAA: total amino acids; NS: not specified.
[a] Obtained by HPLC.

acid and phosphoric acid were purchased from Fisher Scientific (Loughborough, UK). Sodium azide was purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and sodium hydroxide were purchased from Riedel-de Haën (Seelze, Germany). Disodium hydrogen phosphate and disodium edetate (Na_2EDTA) were purchased from Acros Organics.

2.2. Instrumentation and operating procedures

The HPLC system consisted of a Spectra SYSTEM P1000XR pump (Thermo Separations Products, San Jose, USA), an ASI-100 automated sample injector (Dionex, München, Germany) and an F-1050 Fluorescence spectrophotometer (Merck Hitachi, Darmstadt, Germany). The fluorescence detection was performed at λ_{ex} 337 nm and λ_{em} 442 nm. Data were processed by ChromPerfect® Spirit software (Justice Laboratory Software, Fife, UK). The column was kept in a water bath at 25 °C. A Nova-Pak® C8 5 μm column (15 cm × 3.9 mm i.d.) (Waters, Milford, Massachusetts, USA) was used. The mobile phase consisted of methanol-30 mM sodium phosphate buffer pH 5.5 (A; 10:90, v/v) (B; 90:10, v/v). The best separation was obtained between OPA-NAC-D-aspartic acid and OPA-NAC-L-aspartic acid using the gradient: 0–8 min (0–100% B), 8–13 min (100% B), 13–15 min (100–0% B) and 15–35 min (0% B). The flow rate was 0.5 ml/min. The injection volume was 225 μl.

2.3. Sample preparation and derivatization

The collected premolar teeth ($n = 3$) were extracted from living individuals because of periodontal reasons. Bucco-lingual longitudinal sections of 1 mm thickness were prepared using a slow-speed diamond saw (Leica SP1600, Leica Microsystems, Bensheim, Germany) cutting the extracted tooth. Afterwards the outerlayer of cementum and enamel were

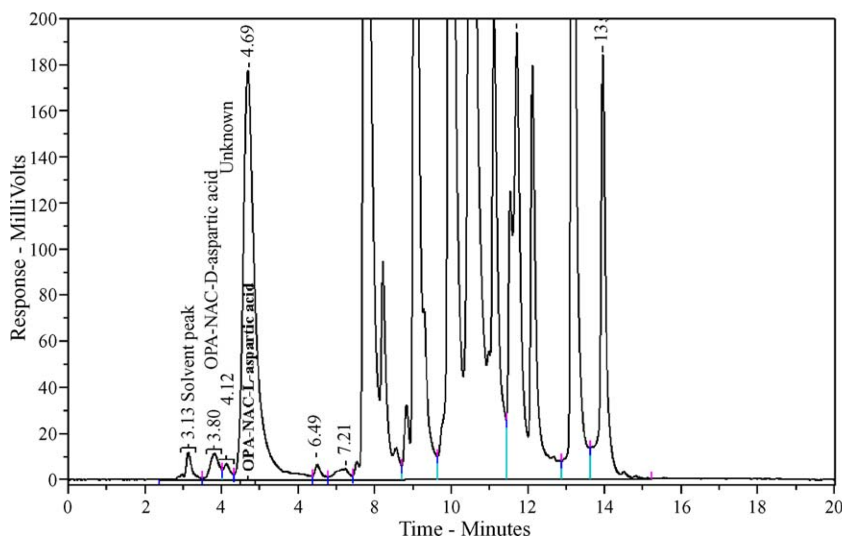

Fig. 1. Typical HPLC chromatogram of derivatized amino acids obtained from human premolar dentin (conditions: see instrumentation and operating procedures).

removed using a high speed diamond bur. Both procedures were carried out under constant cooling with water in order to avoid heat related racemization of aspartic acid. The samples of dentin were precrushed into small shards and then powdered in a vibratory mill during 15 s. The obtained dentin samples (5 mg) were demineralised in 2 ml solution consisting of Na_2EDTA (0.5 M, pH 7.4 adjusted with 2 M NaOH) stabilized with NaN_3 (0.05 mM) in centrifuge tubes. Samples were shaken intensively for 2 h, centrifuged (5000 rpm for 5 min), and the supernatant was discarded. The sediment was washed with water (2 ml each time) and centrifuged (5000 rpm for 5 min) three times to remove residues of Ca- and Mg-EDTA complexes and free EDTA. Approximately 1 mg of sediment was obtained and was transferred to a hydrolyzing tube. About 6 M hydrochloric acid (600 μl) was added, the tubes were flame sealed and heated at 100 °C for 6 h. After hydrolysis, samples were evaporated to dryness at 70 °C under vacuum. Three different dentin samples of known age were derivatized according to the procedure previously described [40,46,47]. OPA-NAC derivatization reagent was prepared by dissolving *o*-phthaldialdehyde (5.5 mg) in methanol (420 μl) and adding *N*-acetyl-L-cysteine (13.4 mg). The total volume of reagent was adjusted to 10 ml with 0.4 M sodium borate buffer (pH 9.4). The reagent stock solution was stored at 4 °C. Prior to derivatization a sample of hydrolyzate (approximately 0.5 mg of amino acid mixture) was dissolved in 0.1 M hydrochloric acid (1 ml) and diluted to 10 ml with the same solvent. Sample solution (100 μl) was mixed with OPA-NAC reagent (200 μl) and left for 5 min. Then 0.3 M sodium phosphate buffer pH 7.5 (200 μl) was added and, after the mixture had stood for 5 min, OPA-NAC amino acid derivatives (225 μl) were injected into the HPLC system.

3. Results

All samples were analyzed in triplicate. A typical chromatogram of derivatized amino acids obtained from human premolar tooth dentin under above described conditions is shown in Fig. 1. In this study, a Nova-Pak® C8 (15 cm × 3.9 mm i.d.) 5 μm column was used for method development. The mobile phase composition was slightly adapted to have a minimum amount of buffer in mobile phase A to avoid mixing problems and the gradient was adapted accordingly. The pH of the buffer used for sample preparation before injection was also found to have significant influence on sample stability [40]. The buffer (0.3 M sodium phosphate) pH 2.0 used by Benešová et al. [40] was found to be inadequate in this respect for the derivatization of D- and L-aspartic acid. Hence it was decided to investigate different pH values: 2.0, 4.0 and 7.5. With the 0.3 M sodium phosphate buffer pH 7.5 the D- and L-aspartic acid derivatives were found to be stable. Various gradient methods were performed to examine the separation between OPA-NAC-D-aspartic acid and OPA-NAC-L-aspartic acid. Conditions mentioned above were found to be the best. A system peak always eluted with the OPA-NAC-L-aspartic acid. The area of the system peak is subtracted from the area of OPA-NAC-L-aspartic acid for the calculation of racemization constant ($k = \ln[1 + (D/L)/(1-(D/L)]$; D and L are integrated peak areas of the respective enantiomer) and the results obtained using this method are summarized (Table 2). Higher D/L ratios and coefficients of aspartic acid racemization were found for all known ages when compared with the results by Benešová et al. [40].

Table 2
Relation between coefficients of racemization (k) of aspartic acid in premolar dentin and age

Age (years)	D/L ratio	k
13	0.017875	0.035752 ± 0.001
15	0.018085	0.036173 ± 0.001
70	0.043005	0.087736 ± 0.009

4. Discussion

The estimation of chronological age in cadavers, human remains and in living human beings has been performed in forensic science practise for almost more than 25 years. During the last decade, numbers of papers were published with both accurate and reproducible results of aspartic acid racemization for age determination in reasonable time. Most of these methods use gas chromatography (GC). However, the HPLC pilot project revealed that higher ratios of aspartic acid racemization were found for all known ages when compared to those obtained by Benešová et al. [40], which can lead to differences in age estimation.

Also the type of tooth chosen may influence the results because, as was already mentioned above, the racemization rate differs in different regions of dentin. The dentin region analysed has to be clearly defined and prepared according to a very rigid protocol and standardization of the method enables quality assurance within and between laboratories. For the accurate estimation of age, it is recommended to study each time the test tooth along with four or more control teeth of known ages, which are homonymous teeth from the same jaw to the test tooth. However, a recent study has demonstrated that in the estimation of chronological age the control teeth can be substituted by standard specimens that are prepared from mixtures of D- and L-aspartic acids. This shows the possibility of using the standard specimens also in other laboratories, so that the racemization ratio can be measured with sufficient reproducibility, which is always an issue [32]. In most cases, age estimation is calculated from the linear regression equation derived by the least squares method using standard teeth. In order to relay on the evaluated unknown ages of teeth, it is necessary to perform a set of experiments using known age teeth until the standard errors are within ± 3 years before applying the procedure to the evaluation of undetermined age [29]. Among the papers published, different results have been observed using the racemization method. This may be due to a difference in the specimens of dentin, as well as the difference in analytical conditions used in gas chromatography and other analytical techniques. The racemization rate is strongly affected by the temperature [48].

Although most of the investigations were done by using only a part of dentin specimens, it is important to use whole dentin from lingual-labial sagittal sections, in order to determine the degree of racemization accurately [28,32]. It is best to select incisor or premolar for the estimation of age, as both are single rooted, small in size, and the maximum dentin is easily attainable.

5. Conclusion

In this article different approaches are discussed towards the investigation of aspartic acid racemization rate, which is a relevant parameter in chronological age determination. Besides the review part, efforts were put into the improvement of a previously described protocol for analysis of aspartic acid enantiomers by HPLC with fluorescence detection. In particular, the stability of derivatized samples was enhanced by the addition of a neutral buffer just after derivatization. The samples were analysed by HPLC, and the results confirm the usefulness of this method in the investigation of aspartic acid racemization.

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