J Forensic Sci, Sept. 2005, Vol. 50, No. 5
Paper ID JFS2004354
Available online at: www.astm.org

TECHNICAL NOTE

Susumu Ohtani,[1] *Ph.D.; and Toshiharu Yamamoto,*[2] *Ph.D.*

Strategy for the Estimation of Chronological Age Using the Aspartic Acid Racemization Method with Special Reference to Coefficient of Correlation Between D/L Ratios and Ages

ABSTRACT: The estimation of chronological age has been performed by various methods in forensic science. Among these, racemization methods, which are based on the age-dependent non-enzymatic changes of L-form amino acids to D-form mainly using aspartic acid, are one of the most reliable and accurate methods to date. Separation of enantiomers is generally performed by gas chromatography or high performance liquid chromatography. Various tissues with low metabolic rates have been applied for this purpose. In addition, single proteins purified from these target tissues are also applicable. In this brief review we describe this method in detail, noting points of caution, as well as the advantages and disadvantages of the different target tissues. In addition, special attention is given to the correlation rates obtained between chronological age and enantiomer ratios. Currently, based on accuracy of estimated age, simplicity of the method, time required, and reproducibility, tooth dentin is considered one of the best target tissues. Alternatively, analysis of osteocalcin and elastin have also provided accurate and reproducible results.

KEYWORDS: forensic science, age estimation, teeth, bone, lens, brain, racemization, D-aspartic acid

The estimation of chronological age in forensic science has long been made by using various methods (1). The most popular methods are those using morphological features such as evaluation of skeletal and dental morphological changes with age (1,2). In the adults, the age range estimated with those methods is rather wide, and the estimated and chronological ages often do not match (1,3). However, high concordance between tooth cementum annulations and chronological age was recently reported (4). Unlike the morphological methods, the racemization method does not have inter-laboratory differences. Analysis itself is more objective since the complete separation of D- and L-asp and data are indicated by numbers. For this racemization method, shorter specialist training and less thorough morphological knowledge seem to be sufficient to choose target organs and to prepare samples correctly.

There are several age-related changes that occur in proteins such as oxidation, isomerization, and racemization (5,6). Among these changes, racemization is the first-order chemical reaction from L-form amino acids to D-forms, and correlates highly with protein age. In the living body, newly synthesized proteins are normally composed of L-form amino acids, although there are some exceptional peptides which are biologically synthesized using D-form amino acids (7). In general, L-form amino acids within proteins are changed into D-forms by automatic chemical reactions whose rates are influenced by various factors such as temperature, humidity, pH, etc. Therefore, for the estimation of chronological ages, organs with low metabolic rates are better than those with high metabolic rates. In view of this, teeth are the best choice for a tissue to analyze for age estimation, although other organs such as bone, cartilage, white matter of the brain, and eye lens are also applicable. Using tooth enamel, Helfman and Bada (8) found a very high correlation ($r = 0.921$) between aspartic acid racemization ratios and age in 19 cases. Subsequently, from a study of 20 cases they reported that using dentin of the crown yielded better results ($r = 0.979$) than enamel (9). Likewise, the relation between the degree of racemization of teeth and age was also studied by Shimoyama and Harada (10), and Ogino et al. (11), and in agreement with Helfman and Bada, they also reported a high degree of correlation. Furthermore, these results have been double-checked and confirmed by others (12–21). Thus, the high reliability of the racemization method has been well established. At present, the method using the racemization reaction of aspartic acid (the degree of racemization) is one of the most accurate methods for the estimation of age (20). From 1987 to the present, the current authors and their co-workers studied the relationship between age and degree of aspartic acid racemization method, to the actual appraisal of age (22).

In this short review, the authors will describe the details of this technique (see Appendix) with key points for chronological age estimation for corpses and skeletons using the racemization method in order to spread and standardize the technique.

Choice of Organs

At present, teeth are the best organs for the estimation of chronological age, based on accuracy, simplicity, and time required.

[1] Institute for Frontier Oral Science and Department of Forensic Medicine, Kanagawa Dental College, 82 Inaoka-cho, Yokosuka, Kanagawa 238-8580, Japan.
[2] Department of Human Biology, Kanagawa Dental College, 82 Inaoka-cho, Yokosuka, Kanagawa 238-8580, Japan.

Received 2 Sept. 2004; and in revised form 10 Feb. and 23 April 2005; accepted 23 April 23 2005; published 3 Aug. 2005.

TABLE 1—*Coefficient of correlation between D/L ratios and ages in various teeth.*

Tissue	Location of Teeth	Fraction of Protein	Sample Nos.	Coefficient of Correlation	Reference
Enamel	Permanent & deciduous teeth	TAA	19	0.921	Helfman & Bada, 1975 (8)
	Central incisor	TAA	16	0.928	Ohtani & Yamamoto, 1992 (23)
Dentin	Central incisor (Whole)	TAA	16	0.995	
Enamel	Central incisor (Whole)	TAA	8	0.961	Ohtani et al., 1995 (25)
Dentin	Central incisor (Whole)	TAA	8	0.992	
Cementum	Central incisor (Whole)	TAA	8	0.988	
	Central incisor (Whole)	TAA	8	0.991	
	Lateral incisor (Cervical)	TAA	8	0.997	
	First premolar (Whole)	TAA	8	0.988	
	Second premolar (Whole)	TAA	8	0.984	
Dentin	Crown	TAA	20	0.979	Helfman & Bada, 1976 (9)
	Crown	TAA	61	0.991	Ogino et al., 1985 (11)
	Root	TAA		No correlation	Shimoyama & Harada 1984 (10)
	All of teeth	TAA	46	0.96	Ritz et al., 1990 (13)
	Central incisor (Cervical)	TAA	45	0.974	Ohtani & Yamamoto, 1987 (22)
	Lateral incisor (Cervical)	TAA	27	0.990	
	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 (Whole)	TAA	62	0.994	
	Canine (Whole)	TAA	28	0.991	
	First premolar (Whole)	TAA	14	0.997	
	Second premolar (Whole)	TAA	24	0.992	
	Central incisor (Whole)	TAA	13	0.996	Ohtani & Yamamoto, 1991 (52)
		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 et al., 1993 (14)
		SP	39	0.99	
		IP	39	0.96	
	First premolar, crown	TAA	28	0.9887	Fu et al., 1995 (18)
	Central incisor, Whole dentin	TAA	12	0.995	Ohtani, 1995 (58)
	Central incisor, Crown	TAA	12	0.986	
	Central incisor, upper of root	TAA	12	0.984	
	Central incisor, central of root	TAA	12	0.987	
	Central incisor, lower of root	TAA	12	0.984	
Deciduous teeth	Anterior teeth, whole dentin	TAA	24	0.941	Ohtani et al., 1994 (61)
	Molar teeth, whole dentin	TAA	16	0.919	

Abbreviations: IC: Insoluble collagen; IP: Insoluble protein; SP: Soluble peptide; TAA: Total amino acid.

However, depending on circumstances one can choose other organs such as bone, cartilage, and eye lens. These organs, however, show lower correlation rates between age and racemization rate than teeth (Tables 1 and 2). Alternatively, a single protein, such as osteocalcin or elastin, is also a good target molecule for the estimation of age. Regardless of the tissue selected, one should be aware of the range of error of the estimated ages.

Teeth

Among cementum, enamel, and dentin, dentin is the best for age estimation. When comparing the D/L ratios in these three structures, the order obtained was cementum > dentin > enamel (23–25). The highest D/L ratios in cementum may be due to the higher environmental temperature from being surrounded with root membranes and due to different protein composition. A high D-aspartic acid content provides easy detection of the enantiomers. However, the amount of sample obtained from one tooth is often insufficient (see below for explanation).

Dentin formation starts at the boundary between enamel and dentin, and gradually shifts toward the dental pulp and root apex regions. The period of dentin formation is variable, depending on the type of tooth as well as the individual. It is generally agreed that about 8–10 years or more are required from start to completion (26), indicating the possibility that the degree of racemization may differ in different parts of the dentin. For the formation of permanent teeth, it is generally agreed that dentin is formed earliest in the first molar and last in the second molar, with the exception of the third molar, in which dentin formation is very individual-dependent (26). If uniform conditions are maintained in the intraoral region, then the degree of racemization should be higher in the earliest formed and completed teeth. The degree of racemization in different kinds of teeth was found to be high in the first molar from middle-aged donors, corresponding to the time of its formation (27). In elderly donors, however, the degree of racemization tended to be higher in the second molar, which was a finally formed tooth (27). The molar region is deeper in the oral cavity than the region of the front teeth. Thus, it was suggested that the teeth of the elderly were more affected by the environment than the time of formation, since they had been in the oral cavity for a longer period of time (27).

TABLE 2—*Coefficient of correlation between D/L ratios and ages in various other tissues.*

Organ	Fraction or Protein	Sample Nos.	Coefficient of Correlation	References
Intervertebral discs				Ritz et al., 1993 (14)
Anterior peripheral annulus fibrosus	TAA	68	0.97	
Nucleus pulposus	TAA	19	0.89	
Skull	SP	25	0.96	Ritz et al., 1994 (62)
	TAA	10	Male, 0.977	Ohtani et al., 2002 (33)
	Osteocalcin	10	0.99	Ritz et al., 1994 (62)
	Osteocalcin	45	0.99	Ritz et al., 1996 (29)
Rib cartilage	SP	23	0.91	Pfeiffer et al., 1995 (63)
	IC	24	0.97	
	TAA	10	Male, 0.763	Ohtani et al., 2002 (33)
Cortical bone	SP	24	0.72	Pfeiffer et al., 1995 (64)
	IC	24	0.84	
Femur	TAA	21	Male, 0.947	Ohtani et al., 1998 (31)
	TAA	18	Female, 0.633	
	IC	21	Male, 0.914	
	IC	18	Female, 0.418	
	SP	21	Male, 0.969	
	SP	18	Female, 0.125	
	TAA	10	Male, 0.985	Ohtani et al., 2002 (33)
Sternum	TAA	10	Male, 0.974	
Lumbar spin	TAA	10	Male, 0.931	
Coxal bone	TAA	10	Male, 0.881	
Sacral spine	TAA	10	Male, 0.739	
Lumbar yellow ligaments	total tissue	46	$r = 0.84$–0.92	Ritz-Timme et al., 2003 (35)
	Purified elastin	24	$r = 0.96$–0.99	
Articular cartilage	Whole aggrecan	25	$r = 0.90$	Maroudas et al., 1998 (40)
	Large monomer aggrecan		no correlation	
Articular cartilage	Collagen		$r = 0.95$	Verzijl et al., 2000 (39)
Buttock skin	Collagen		$r = 0.78$	
Aorta	Elastin	12	age-related accumulation	Powell et al., 1992 (37)
	Collagen	10	no correlation	
Lung parenchyma	Elastin	14	$r = 0.98$	Shapiro et al., 1991 (36)
Lens	Central nucleus	17	$r = 0.912$	Masters et al., 1977 (44)
Cataractous lens grade I–II	Central nucleus	13	$r = 0.875$	
Cataractous lens grade IV	Central nucleus	5	no correlation	
Lens	Cortex water soluble	10	$r = 0.973$	Van den Oetelaar & Hoenders, 1989
	Cortex urea soluble	10	$r = 0.981$	
	Cortex urea insoluble	10	$r = 0.979$	
	Nucleus water soluble	10	$r = 0.982$	
	Nucleus urea soluble	10	$r = 0.987$	
	Nucleus urea insoluble	10	$r = 0.967$	
Cataractous lens	All above fractions		no correlation	
Lens	αA-crystallin		age-related accumulation	Fujii et al., 1999 (65)
Brain	White matter		age-related accumulation	Man et al., 1983 (45)
	Gray matter		no correlation	

Abbreviations: IC: Insoluble collagen; SP: Soluble peptide; TAA: Total amino acid.

With respect to the 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 (28), since the time of dentin formation is similar. When age was estimated using different parts of sagittal sections (labial-lingual direction) of the upper central incisor, the estimated ages were virtually identical among the sites of transection. Accordingly it was suggested that a slight deviation of transection from the medial to distal portion of the tooth would not significantly affect the estimated age (28). However, when lingual and labial (or buccal) 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. Furthermore, the lingual part of crown dentin tends to show a higher D/L ratio than the labial (or buccal) part of crown dentin; however, there are no significant differences in D/L ratios between the labial (or buccal) part of root dentin and the lingual part of root dentin, suggesting that roots have similar environmental temperature (28). In addition, when ages were estimated using different part of transverse sections of the same tooth, the younger donors tended to show higher values in the crown and lower values in the root apex. In the older donors, the degree of racemization showed a wave-like pattern waxing and waning from the crown toward the root apex (28). 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 donors. 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.

Bone and Adhering Tissues

Bone and adhering tissues may not be ideal organs for chronological age estimation because although the rate is slow, tissue turnover occurs, and is subject to influence by disease states (29). However, if teeth are not available, it becomes necessary to use other organs. Using bones, correlation rates between chronological age and racemization rate varied from 0.98 to 0.70 (Table 2). Racemization rates differed within the same tissue for example,

within intervertebral disks (30). Furthermore, gender differences in racemization rates are evident (31). When one chooses bone as the tissue for analysis, the diameter of pulverized samples should be similar because these sizes influence the D/L ratios (32). Therefore, special attention must be given to the portion, gender, disease history, pulverized size, fractionation (see below), and hydrolysis steps. Although the work was preliminary, Ohtani et al. (33), showed that correlation rates between age and D/L ratios varied depending on the kind of bone studied. This suggested that the ideal bone, with a much slower remodeling rate, needed to be identified.

One of the recent techniques for estimation of chronological age using racemization rate, is the application of a known single protein. Although the purification steps are rather complicated and extra-skills are required, the correlation between chronological age and D/L ratios is reasonably good (Table 2). Such proteins include osteocalcin from bone and elastin from yellow ligaments. Osteocalcin is a non-collagenous structural protein of the bone. The correlation rate obtained is 0.99, and it should be noted that there are no gender differences in the D/L ratios (29). Therefore, osteocalcin has good potential for the estimation of female age that is not possible using whole bone because the incidence of metabolic bone diseases is high in aged females, in general. However, even osteocalcin is subject to turnover due to disease and physiological conditions (34). This method is briefly as follows: 1) remove soft tissues from bones, pulverize, and stir overnight in a 15% NaCl solution; 2) extract each 15 g sample with formic acid for 5 h and desalt on a column of Sephadex G-25M; 3) gel filtration with a column of Sephadex G-50SF and twice ion exchange chromatography with an anion exchange column; 4) determination of osteocalcin by enzyme-linked immunosorbant assay and assess homogeneity by high-pressure liquid chromatography; 5) hydrolyze purified osteocalcin with 6N HCl for 6 h at 100° and separate D-and L- aspartic acid by gas-chromatography.

Another single protein from bone and adhering tissues for age estimation is elastin. Using this protein, excellent correlation rates of 0.96–0.99 were obtained (35,36). Elastin is a compositional protein comprised of elastic fibers, and is distributed throughout various parts of the body, suggesting that there may be organ differences in D/L ratios. In fact, D/L ratios of elastin from yellow ligaments (35), aorta (37), and lung parenchyma (36) are different (Table 2). In addition, it was demonstrated that different purification stages showed different D/L ratios (36). It should be also noted that some diseases influence elastin turnover as well. However, compared to purification of osteocalcin, procedures of elastin purification are relatively easier as shown in the following: 1) histological examination of yellow lagments ($1 \times 1 \times 0.3$ cm); 2) defat and digest with collagenase for 24 hrs; 3) histologically check the absence of collagen fibers; 4) amino acid analysis by ion-exchange chromatography to compare standard elastin; 5) hydrolyze samples in 6N HCl at 100° for 6 h and separate D-and L- aspartic acid by gas-chromatography.

Collagen is one of the main compositional proteins of teeth and bone. Therefore, collagen is the major contributing protein to D/L ratios determined from whole dentin and whole bone. Some studies have attempted to estimate age using purified collagen. Collagen is also a ubiquitous protein like elastin, suggesting organ diversity in D/L ratios. In fact, D/L ratios did vary between organs suggesting different turnover rates of collagen (38). However, in organs with relatively low turnover rates, such as auricular cartilage, purified collagen gave a reasonable correlation rate (39).

Another attempt using the auricular cartilage, aggrecan (a main component of the cartilage matrix) showed a reasonable correlation rate, $r = 0.90$ (40). However, when large monomer aggrecan instead of whole aggrecan was used for age estimation, there was no correlation between age and D/L ratios (40). In addition, aggrecan turnover is 2–3 times faster than collagen (41) suggesting that the aggrecan may not be an ideal protein for age estimation.

Eye Lens

The eye lens is a good organ for the estimation of age, because different from teeth, this organ is applicable to young people (Table 2). Within the lens, the central nucleus region shows a high correlation rate, but it is decreased in the cortex (42). Furthermore, partially purified protein samples also showed good correlation between D/L ratio and chronological age (43). Special care is required for analyzing the cataractous lens, as these lenses show remarkable changes of D-aspartic acid (D-asp) contents, resulting in low correlation rates between D/L ratio and chronological age (42–44). Therefore, it is important to distinguish normal lenses from cataractous lenses, and to demarcate the central nucleus from the cortex.

Brain White Matter

The brain is not an ideal organ for age estimation, although age-related increases in D-asp contents were reported in brain white matter (45). Attempts have been made to detect D-asp from purified myelin and myelin basic protein, which is a long-lived protein in the brain. The amount of D-asp is varied depending on the degree of purification (46) and on the myelin basic protein isoforms (47). However, there are no reports that these purified proteins show concrete correlations between age and D/L ratio. In the brain, studies of D-asp contents were focused on the relation with brain dysfunctions such as Alzheimer and Parkinson's disease, rather than age estimation (48).

Separation of L- and D-enantiomers of Amino Acids

To separate D- and L-forms, gas chromatography and high performance liquid chromatography are utilized in general. In both cases, this is one of the critical points in the racemization method to separate completely the D- and L-enantiomers of amino acids, and to obtain sharp peaks on the chromatogram. We recommend gas chromatography, as noted by Waite et al. (21) because a minute amount of the specimen seems to satisfactorily fit this purpose. The capillary column (30 m in length and 0.3 mm in internal diameter) coated with Chirasil Val (GL Science, Tokyo, Japan), with helium as the carrier gas under the following operating conditions: the injection port temperature was 220° the initial column temperature was 90°, which was raised after 4 min to 200° at a rate of 3°/min and then maintained at 200°. Figure 1 illustrates an ideal gas chromatogram of total amino acids in the whole dentin. From the total dentin large amounts of glycine, alanine, proline and hydroxyproline were detected, which indicates the composition of collagen amino acids. As shown, D-enantiomers were found for aspartic acid, glutamic acid, and alanine. In particular, D- and L-enantiomers of aspartic acid are clearly separate from each other. Complete separation of D- and L-amino acids in the chromatogram has been reported by only a few researchers other than the authors, and it is not mentioned in many other articles.

Factors Affecting D/L Ratios

Temperature—Racemization is a first-order chemical reaction. Accordingly, temperature has enormous influences on the D/L ratio. Therefore, standard samples should be stored at lower temperatures. When chronological age is estimated for unknown cadavers, it is assumed that the cadavers have been preserved at low temperatures

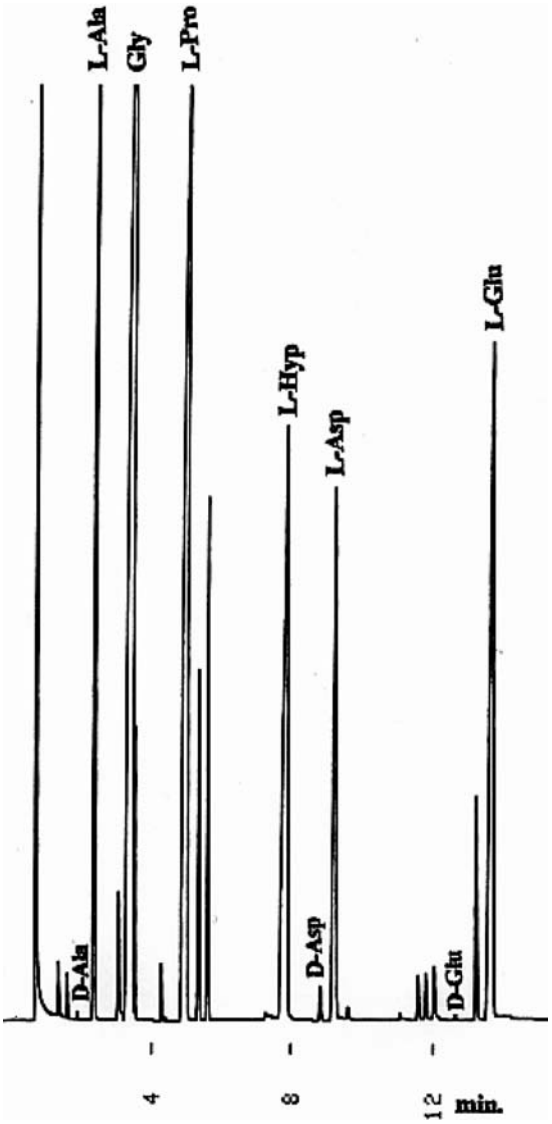

FIG. 1—*Gas chromatogram of N(0)-TFA isopropyl esters of amino acids in a single dentin specimen. Column temperature: 90°C, 4 min hold and then programmed to 180°C at 4°C /min; Injection temperature: 280°C; Carrier gas: He; Split ratio: 40/1.*

(below atmospheric temperature), and this racemization method is not fundamentally applicable to burned bodies.

Humidity and pH—We studied the extent to which racemization of aspartic acid in dentin proceeds when the tooth is stored in acidic (pH 4.0), alkaline (pH 9.0) and distilled water, and as well as in dry conditions. As the results demonstrate, the racemization rate was highest when the tooth was stored in alkaline solution (pH 9.0). Comparing the other conditions, in descending order, the rates of racemization were distilled water > acidic solution (pH 4.0) > dry condition. Thus, assuming that a tooth had been stored for 10 years at 16 °C (average atmospheric temperature in Japan), we converted the reaction rate to the age. As a result, we found that the estimated age was increased by 1.1 years in the acidic solution (pH 4.0), by 1.7 years in distilled water and by 6.0 years in the alkaline solution (pH 9.0). However, the estimated age was in creased by only 0.1 year in dry conditions (49). Therefore, these results suggest that age at the time of death of skeletonized bodies stored in dry conditions in the air could be estimated by calculation even if the death occurred 10 or more years previously. However, careful attention should be paid when the teeth have been stored in strong alkaline conditions as, for example, when cadavers were left in rivers running through volcanic regions.

Fixative—To accurately estimate age using teeth, homonymous teeth from the same jaw extracted from age-known cadavers are required as the standard teeth. We conducted heating experiments to study to what extent the degree of racemization of extracted control teeth is affected when they are stored in alcohol or formalin solution, by deriving the Arrhenius equation (50) which is based on plotted reciprocal numbers of reaction temperatures (absolute temperatures) on the x-axis and logarithms of reaction rates on the y-axis. Teeth were stored in a fixative of 95% ethanol, 10% formalin solution or 10% neutral formalin solution at various temperatures. The degree of racemization was highest in the teeth stored in 10% neutral formalin solution, second highest in those kept in 10% formalin solution and lowest in those kept in 95% ethanol. However, assuming that teeth had been stored at 15 °C, the racemization rates were almost not influenced by these fixatives, and the degree of racemization was almost the same as at the time of extraction even 10 years later (51).

Fractional Extraction and Size of Pulverized Powder—Most of our results were obtained from total amino acids. In addition, we also fractionated the total amino acids into acid-insoluble collagen and acid soluble peptides (51), and studied the correlation between the degree of racemization of each respective fraction and age. One ml of 1 N hydrochloric acid solution was added to 20 mg of dentin powder and the specimen was centrifuged (5,000 × g) for 1 h. The sediment was regarded as the acid-insoluble collagen and the supernatant as the acid-soluble peptides. Both fractions were dried in a rotary evaporator.

In 13 cases , the correlation between the degree of racemization of the lower central incisor and chronological age was ranked in the order of acid-soluble peptide > total amino acids > acid-insoluble collagen (52). Racemization reaction rates were high in these cases and also ranked in the same order as the correlation. Thus, the racemization rate was highest in the acid-soluble peptides. D-asp in the acid-soluble peptides is very unstable, and different values of the racemization rate were obtained depending on the amount and concentration of acid used for fractionation, as well as on how the specimen was stirred. Hence, it must be studied very cautiously. For this reason the author uses total amino acids for age estimation in the actual appraisal of age. Furthermore, when bones are utilized, the size of pulverized powder must be carefully controlled because different sizes resulted in different D/L ratios (31).

Protein Conformation—There are many papers discussing the mechanism of racemization (see reviews) (53,54). The rate of racemization of L-asp differs depending on not only the position of L-asp in an amino acid sequence, but also depends on the higher protein structures (55–57). Therefore, collection of similar parts of tissues as standard samples and test samples is very important. Application of single proteins implies an ideal material. However, it is still required that test samples and standard samples be the same type of tissue as well as the same purification grade since variation in these attributes can lead to varying D/L ratios.

Conclusions

In the appraisal of chronological age using the racemization method, currently teeth are the best available material based on accuracy, reproducibility, time required, and simplicity of the

methods. For the degree of racemization, whole dentin and total amino acids should be used. For the accurate estimation of age, it is recommended to study each time the test tooth along with 4 or more control teeth of known ages, which are homonymous teeth from the same jaw to the test tooth. This is analogous to the procedure used for DNA appraisal. In each individual case of age estimation, the authors derive the equation to calculate the age from the degree of racemization (a linear regression equation derived by the least squares method using standard teeth), and convert the degree of racemization of the test tooth to age in years. In practice, standard errors from control teeth should be within ± 3 years; and if not, it is suggested that analytical procedures are different from each other. For accuracy, we recommend pre-experiments using age-known teeth until the standard errors are within ± 3 years before applying the procedure to the appraisal of undetermined age.

Although many researchers obtain the specimens from only a part of the dentin, it is crucial to use the whole dentin from lingual-labial sagittal sections, in order to accurately determine the degree of racemization (58). As the selected tooth for the estimation of age, the incisor or premolar is the best, if possible, as both are single-rooted, small in size, and the whole dentin is easily attainable. When teeth are not available, bones and eye lenses are the next choice for the racemization method. Among bones, the skull or the femur is the better choice than other tested bones. When cadavers are female, bones may not be ideal organs. In this case, purification of osteocalcin from female bones is required. Alternatively, purified elastin from lumbar yellow ligaments, or from the lung parenchyma is a good choice for the method.

This racemization method is based on the constant accumulation of D-asp in organs with low metabolic rates at the body temperature. The second assumption for the method, that is supported by our and others' data, is that after death, the accumulation of D-asp is almost negligible under the postmortem conditions. Therefore, this method involves the possibility that these two assumptions are not effected on some materials. This may cause broad deviations as a result in rary cases. In the case that the assumptions are clearly not effected on cadavers, this method should not be applicable. For example, the organs assuming to have the possibility that metabolic rates are altered by disease, for example catalactous lenses may not be target organ for this method. Furthermore, the cadavers that have a possibility to be exposed to the conditions which cause acceleration of racemization after death may not be proper for this method. For example, burned bodies and cadavers left in an alkaline solution may not be ideal. Although we don't have data, cadavers left in a tropical rain forest might not be applicable, but cadavers in dry tropical areas may be possible. Further studies are required.

Even this method is one of the best techniques to estimate the age at death, not the passing time after death, we could not exclude the possibility that estimated ages are affected by accidental errors as in other methods. A crucial point is that one does not have the way to know whether unexpected errors happen or not. One way to address this problem, though still not perfect, is application of two or more methods to estimate ages.

In the appraisal of age of more than 100 cases conducted so far by the authors, the error has not exceeded ± 3 years in most of the cases (59). Among research institutes, however, different results have been recorded using the racemization method. This may be due to differences in the specimens of dentin, as well as to differences in the conditions of gas chromatography. However, we cannot say that the authors' method has been fully exploited in many cases of age estimation. The reason for this seems to be the laboriousness that this method requires to prepare sagittal sections of teeth. In addition, teeth of known ages are required as the control. As Ritz-Timme et al. (60) noted before, it is important to evaluate intralabratory quality of the method using mixtures of D- and L-asp, and age-known teeth. The authors have always stored teeth extracted due to alveolar pyorrhea and other reasons, and any remaining powdered specimens for the estimation of age. Currently we are trying to develop methods that do not require control teeth, and look forward to developing such methods in the near future.

Thus, the racemization method of aspartic acid seems to be highly reliable for the estimation of age when compared with conventional methods. We will continue to strive to improve this analysis method so that it will facilitate age estimation and identification of victims.

Appendix

Our Ordinal Procedures for Measuring D/L Ratios

1. Collect test samples and select appropriate standard samples.
   1a. In the case of teeth, prepare test tooth and 4 or more standard teeth of known ages
   1b. Cut into sagittal sections (labial-lingual or buccal-lingual direction) of 1 mm thickness with a low speed cutter*1.
2. Equalize standard and test samples in view of tissue composition.
   2a. In the case of teeth, completely remove enamel, cementum, and root soft tissues with an aid of a stereoscopic microscope (if necessary, stain by van Gieson to demarcate these structures).
3. Wash samples with 0.2 M HCl for 5 min.
4. Wash samples three times with distilled water for 5 min each.
5. Wash samples with ethanol for 5 min.
6. Wash samples with ether for 5 min.
7. Pulverize samples with a pulverizer*2.
8. Weigh 10 mg from each pulverized sample.
9. Hydrolyze powdered samples with 6N HCl for 6 h at 100°C.
10. Dry the powdered samples by evaporation.
11. Add 5 mL of distilled water.
12. Apply samples to ion-exchange resin (Dowex, 50W-X8, 50–100 mesh, Dow Chemical Company, USA) to collect amino acids.
13. Wash resin with 10 mL of distilled water.
14. Elute amino acids from resin with 10 mL of 2N NH_4OH.
15. Dry eluted fractions by evaporation.
16. Add 2 mL of a mixture of isopropyl alcohol and acetyl chloride (8:2 v/v).
17. Let samples stand for 30 min at 100°C.
18. Dry samples by nitrogen gas aeration.
19. Add 800 μL of dichloromethane.
20. Let samples stand for 30 min at room temperature.
21. Dry samples by nitrogen gas aeration.
22. Add 50 μL of ethyl acetate.
23. Apply samples to a gas chromatography*3 (to separate L- and D-aspartic acids).
24. Calculate D/L ratios and estimate age from a test sample by substitution to the regression line deduced from standard samples.

Asterisks indicate special instruments for the racemization method using teeth.

*1: Isomet low speed saw (BUEHLER, Chicago, USA), diamondo wafering blade, Series 15 LC diamond (102 mm × 0.3 mm).

*2: Vibratory Sieve Shaker (FRITSCH, Idar-Oberstein, Germany).

*3: See text.

References

1. Ritz-Timme S, Cattaneo C, Collins MJ, Waite ER, Schütz HW, Kaatsch H-J, Borrman HIM. Age estimation: The state of the art in relation to the specific demands of forensic practice. Int J Legal Med 2000;113: 129–36. [PubMed]
2. Sopher IM. Forensic dentistry. Springfield Illinois USA: Charles C Thomas, 1976; 113–24.
3. Ball J. A critique of age estimation using attrition as the sole indicator. J Forensic Odontostmatol 2002;20:38–42.
4. Wittwer-Backofen U, Gampe J, Vaupel W. Tooth cementum annulation for age estimation: results from a large known-age validation study. Am J Phys Anthropol 2004;123:119–29. [PubMed]
5. Balin AK, Allen RG. Molecular bases of biologic aging. Clin Geriatr Med 1989;5:1–21. [PubMed]
6. Stadtman ER. Protein modification in aging. J Gerontol Biological Sci 1988;43:B112–20.
7. Iida T, Santa T, Toriba A, Imai K. Amino acid sequence and D/L-configuration determination methods for D-amino acid-containing peptides in living organisms. Biomed Chromatogr 2001;15:319–27. [PubMed]
8. Helfman PM, Bada JL. Aspartic acid racemisation in tooth enamel from living humans. Proc Nat Acad Sci USA 1975;72:2891–4. [PubMed]
9. Helfman PM, Bada JL. Aspartic acid racemisation in dentine as a measure of ageing. Nature 1976;262:279–81. [PubMed]
10. Shimoyama A, Harada K. An age determination of an ancient burial mound man by apparent racemization reaction of aspartic acid in tooth dentin. Chem Lett 1984;1984:1661–4.
11. Ogino T, Ogino H, Nagy B. Application of aspartic acid racemization to forensic odontology: post mortem designation of age at death. Forensic Sci Int 1985;29:259–67. [PubMed]
12. Masters PM. Age at death determinations for autopsied remains based on aspartic acid racemization in tooth dentin: importance of postmortem conditions. Forensic Sci Int 1986;32:179–84. [PubMed]
13. Ritz S, Schütz HW, Schwarzer B. The extent of aspartic acid racemization in dentin: a possible method for a more accurate determination of age at death. Z Rechtsmed 1990;103:457–62. [PubMed]
14. Ritz S, Schütz HW, Peper C. Postmortem estimation of age at death based on aspartic acid racemization in dentin: its applicability for root dentin. Int J Legal Med 1993;105:289–93. [PubMed]
15. Saleh N, Deutsch D, Gil-Av E. Racemization of aspartic acid in the extracellular matrix proteins of primary and secondary dentin. Calcif Tissue Int 1993;53:103–10. [PubMed]
16. Mörnstad H, Pfeiffer H, Teivens A. Estimation of dental age using HPLC-technique to determine the degree of aspartic acid racemization. J Forensic Sci 1994;39:1425–31. [PubMed]
17. Ritz S, Stock R, Schütz HW, Kaatsch HJ. Age estimation in biopsy specimens of dentin. Int J Legal Med 1995;108:135–9. [PubMed]
18. Fu SJ, Fan CC, Song HW, Wei FQ. Age estimation using a modified HPLC determination of ratio of aspartic acid in dentin. Forensic Sci Int 1995;73:35–40. [PubMed]
19. Carolan VA, Gardner MLG, Lucy D, Pollard AM. Some considerations regarding the use of amino acid racemization in human dentine as an indicator of age at death. J Forensic Sci 1997;42:10–16. [PubMed]
20. Waite ER, Collins MJ, Ritz ST, Schutz HW, Cattaneo C, Borrman HIM. A review of the methodological aspects of aspartic acid racemization analysis for use in forensic science. Forensic Sci Int 1999;103:113–24. [PubMed]
21. Waite ER, Collins MJ, van Duin ACT. Hydroxyproline interference during the gas chromatographic analysis of D/L aspartic acid in human dentine. Int J Legal Med 1999;112:124–31. [PubMed]
22. Ohtani S, Yamamoto K. Age estimation using the racemization of amino acid in human dentin. Nippon Hoigaku Zasshi 1987;41:181–90 (in Japanese). [PubMed]
23. Ohtani S, Yamamoto K. Estimation of age from a tooth by means of racemization of an amino acid, especially aspartic acid–comparison of enamel and dentin. J Forensic Sci 1992;37:1061–7. [PubMed]
24. Ohtani S. Studies on age estimation using racemization of aspartic acid in cementum J Forensic Sci 1995;40:805–7. [PubMed]
25. Ohtani S, Sugimoto H, Sugeno H, Yamamoto S, Yamamoto K. Racemization of aspartic acid in human cementum with age. Arch Oral Biol 1995;40:91–5. [PubMed]
26. Logan WHG, Kronfeld R. Development of the human jaws and surrounding structures from birth to the age of fifteen years. J Am Dent Assoc 1933;20:379–427.
27. Ohtani S, Ito R, Yamamoto T. Differences in the D/L aspartic acid ratios in dentin among different types of teeth from the same individual and estimated age. Int J Legal Med 2003;117:149–52. [PubMed]
28. Ohtani S. Different racemization ratios in dentin from different locations within a tooth. Growth Develp Aging 1997;61:93–9.
29. Ritz S, Turzynski A, Schütz HW, Hollmann A, Rochholz G. Identification of osteocalcin as a permanent aging constituent of the bone matrix: basis for an accurate age at death determination. Forensic Sci Int 1996;770: 13–26.
30. Ritz S, Schütz HW. Aspartic acid racemization in intervertebral discs as an aid to postmortem estimation of age at death. J Forensic Sci 1993;38:633–40. [PubMed]
31. Ohtani S, Matsushima Y, Kobayashi Y, Kishi K. Evalution of aspartic acid racemization ratios in the human femur for age estimation. J Forensic Sci 1998;43:949–53. [PubMed]
32. Ohtani S. Technical notes for age estimation using the femur influence of various analytical conditions on D-aspartic acid contents. Int J Legal Med 2002;116:361–4. [PubMed]
33. Ohtani S, Matsushima Y, Kobayashi Y, Yamamoto T. Age estimation by measuring the racemization of aspartic acid from total acid content of several types of bone and rib cartilage: a preliminary account. J Forensic Sci 2002;47:32–6. [PubMed]
34. Lian JB, Gundberg CM. Osteocalcin. Biochemical considerations and clinical applications. Clin Orthop 1988;226:267–91.
35. Ritz-Timme S, Laumeier I, Collins. Age estimation based on aspartic acid racemization in elastin from the yellow ligaments. Int J Legal Med 2003;117:96–101. [PubMed]
36. Shapiro SD, Endicott SK, Province MA, Pierce JA, Campbell EJ. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. J Clin Invest 1991;87:1828–34. [PubMed]
37. Powell JT, Vine N, Crossman M. On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. Atherosclerosis 1992;97:201–8. [PubMed]
38. Gineyts E, Cloos PAC, Borel O, Grimaud L, Delmas PD, Garnero P. Racemization and isomerization of type I collagen C-telopeptides in human bone and soft tissues: assessment of tissue turnover. Biochem J 2000;345:481–5. [PubMed]
39. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. J Biol Chem 2000;275:39027–31. [PubMed]
40. Maroudas A, Bayliss MT, Uchitel-Kaushansky N, Schneiderman R, Gilav E. Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a Marker of molecular age. Arch Biochem Biophys 1998;350:61–71. [PubMed]
41. Maroudas A, Palla G, Gilav E. Racemization of aspartic acid in human articular cartilage. Connect Tissue Res 1992;28:161–9. [PubMed]
42. Masters PM, Bada JL, Zigler JSJ. Aspartic acid racemization in heavy molecular weight crystallins and water-insoluble protein from normal human lenses and cataracts. Proc Natl Acad Sci USA 1978;75: 1204–8. [PubMed]
43. Van den Oetelaar PJM, Hoenders HJ. Racemization of aspartyl residues in proteins from normal and cataractous human lenses: an aging process without involvement in cataract formation. Exp Eye Res 1989;48: 209–14. [PubMed]
44. Masters PM, Bada JL, Zigler JSJ. Aspartic acid racemization in the human lens during ageing and in cataract formation. Nature 1977;268: 71–3. [PubMed]
45. Man EH, Sandhouse ME, Burg J, Fisher GH. Accumulation of D-aspartic acid with age in the human brain. Science 1983;220:1407–8. [PubMed]
46. Fisher GH, Garcia NM, Payan IL, Cadilla-Perezrios R, Sheremata WA, Man EH. D-aspatic acid in purified myelin and myelin basic protein. Biochem Biophys Res Comm 1986;135:683–7. [PubMed]
47. Shapira R, Chou CHJ. Differental racemization of aspartate and serine in human myelin basic protein. Biochem Biophys Res Comm 1987;146: 1342–9. [PubMed]
48. Payan IL, Chou S-J, Fisher GH, Man EH, Emory C, Frey WH. Altered aspartate in Alzheimer neurofibrillary tangles. Neurochemical Res 1992;17:187–91.
49. Ohtani S. Estimation of age from dentin by utilizing the racemization of aspartic acid: influence of pH. Forensic Sci Int 1995;75:181–7. [PubMed]
50. Arrhenius S. Über die reaktionsgeschwindigkeit bei der inversion von rohrzucker durch säuren. Z Phys Chem 1889;4:226–48.

51. Ohtani S, Ohhira H, Watanabe A. Ogasawara A, Sugimoto H. Estimation of age from teeth by amino acid racemization: influence of fixative. J [PubMed] Forensic Sci 1997;42:137–9.
52. Ohtani S, Yamamoto K. Age estimation using the racemization of amino [PubMed] acid in human dentin. J Forensic Sci 1991;36:792–800.
53. Collins MJ, Waite ER, Van Duin ACT. Predicting protein decomposition: the case of aspartic-acid racemization kinetics. Phil T R Soc Lond B 1999;354:51–64.
54. Ritz-Timme S, Collins MJ. Racemization of aspartic acid in human proteins. Ageng Res Rev 2002;1:43–59.
55. Fujii N, Momose Y, Ishii N, Takita M, Akaboshi M, Kodama M. The mechanism of simultaneous stereoinversion, racemization, and isomerization at specific aspartyl residues of aged lens protein. Mech Ageing Develop 1999;107:347–58.
56. Groenen PJTA, von den Ijssel PRLA, Voorter CEM, Bloemendal H, De Jong WW. Site-specific racemization in aging αA-crystallin. FEBS 1990;269:109–12.
57. Shapira R, Wilkinson KD, Shapira G. Racemization of individual aspartate residues in human myelin basic protein. J Neurochemistry 1988;50:649–54.
58. Ohtani S. Estimation of age from dentin by using the racemization reaction of aspartic acid. Am J Forensic Med Pathol 1995;16: [PubMed] 158–61.
59. Ohtani S. Estimation of age from the teeth of unidentified corpses using the amino acid racemization method with reference to actual cases. Am [PubMed] J Forensic Med Pathol 1995;16:238–42.
60. Ritz-Timme S, Rochholz G, Schütz HW, Collins MJ, Waite ER, Cattaneo C, Kaatsch H-J. Quality assurance in age estimation based on aspartic acid racemisation. Int J Legal Med 2000;114:83–6. [PubMed]
61. Ohtani S. Age estimation by aspartic acid racemzation in dentin of deciduous teeth. Forensic Sci Int 1994;68:77–82. [PubMed]
62. Ritz S, Turzynski A, Schütz HW. Estimation of age at death based on aspartic acid racemization in noncollagenous bone proteins. Forensic Sci Int 1994;69:149–59. [PubMed]
63. Pfeiffer H, Mörnstad H, Teivens A. Estimation of chronologic age using the aspartic acid racemization method. 1. on human rib cartilage. Int J Legal Med 1995;108:19–23. [PubMed]
64. Pfeiffer H, Mörnstad H, Teivens A. Estimation of chronologic age using the aspartic acid racemization method. 2. on human rib cortical bone. Int J Legal Med 1995;108:24–6. [PubMed]
65. Fujii N, Takemoto LJ, Momose Y, Matsumoto S, Hiroki K, Akaboshi M. Formation of four isomers at the asp–151 residue of aged human αA-crystallin by natural aging. Biochem Biophys Res Comm 1999;265: 746–51. [PubMed]

Additional information and reprint requests:
Dr. Susumu Ohtani
Department of Forensic Medicine
Kanagawa Dental College
82 Inaoka-cho, Yokosuka, Kanagawa 238-8580
Japan
Fax: & Tel: +81-46-822-8801
E-mail: ohtanisu@kdcnet.ac.jp