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Estimation of Dental Age Using HPLC-Technique to Determine the Degree of Aspartic Acid Racemization

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ABSTRACT: Age determinations of adult individuals are of great importance within the forensic sciences. Presently, age changes in the teeth provide good means for this. The best method up to now has been "Gustafson's method" with modifications. Over the last 10 years a new chemical method, based on the racemization of aspartic acid in enamel and dentine, has been developed. Enamel and dentine belong to the very few tissues that do not have metabolic turn-over after formation. Both reliability and validity have been shown to be high, and this method therefore seems to be the best choice for the future. However, most studies to date have been using expensive and special instruments (gas chromatographs or amino acid analyzers) for these determinations. This study was performed to explore if the same determinations can be carried out with ordinary HPLC technique. It was shown that there is a good correlation between the ratio of D- and L-forms of the aspartic acid and age of the tooth. The correlation coefficient was 0.97, which is close to those presented with the other techniques, where r has been 0.95 − 0.99. The prediction of an individual age can be made with a 95% confidence interval of about ± 12 years.

KEYWORDS: odontology, age determination by teeth, aspartic acid, high pressure liquid chromatography

Age estimation by teeth is one of the best methods available for the determination of chronological age of individuals with uncertain birth records. In adult individuals these determinations most often have been carried out on ground sections of extracted teeth. Most of these methods are based on morphological changes, and the relation of these changes to chronological age. Since Gustafson [*1*] published his original work, several studies have refined the method, and the dispersion around a prediction is now reported to be ± 10–15 years [*2–11*]. However, the use of these methods has restrictions in living individuals, since one or more teeth have to be extracted and ground into thin sections for microscopical examination.

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About 25 years ago it was found that the extent of amino acid racemization could be used for dating of various biological materials [*12,13*]. Later it was found that also chronological age of an individual could be estimated with a reasonably good precision [*14,15*]. 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, and the ratio of the two forms is an expression of age.

A number of studies have shown the possibility of age estimation by aspartic acid racemization in both enamel [*14*] and dentine [*15–29*]. These authors have also tested a series of factors that could be expected to influence the precision of the age determination. This latter "amino acid method" seems to be more exact and to have a reproducibility superior to the earlier methods. The correlation coefficient between the degree of racemization and age has been reported to be up to 0.99, and the error of the estimated age ± 2–3 years.

After the initial studies by Bada *et al.* [*12*] and Helfman and Bada [*14*], which were carried out on amino acid analyzers, the subsequent authors have been using gas chromatographic technique. Since the separation of D- and L- forms of aspartic acid with amino acid analyzer or with gas chromatograph requires expensive and special equipment, it was found of practical significance to explore the possibility to develop a cheap and simple method for age estimation by separation of D- and L-forms of aspartic acid with ordinary HPLC-technique.

Materials and Methods

Fifty-one teeth extracted from 32 individuals aged 3.0–86.7 yr (mean 43.3 ± 24.0 yr) of both sexes (36 males' and 15 females' teeth) were collected immediately after extraction and stored in 10% neutral formalin at 4°C for less than 2 weeks. All types of teeth were used, and coronal caries was accepted. The teeth were washed in distilled water, briefly dried in the air and frozen at -18°C until used. The cementum was removed with a dental diamond disc, the apical 4 mm of the root were separated and the pulp tissue removed. This apical dentine was washed in 15% NaCl at 4°C for 1 h, in ethanol/ether (3:1) for 15 min, in 2% SDS for 1 h, in distilled water for 15 min, followed by a brief drying in the air. The dentine was then broken into small pieces between two metal plates and subsequently ground in a porcelain mortar. The fragments were divided into 10 mg aliquotes and an acid soluble (peptide) fraction was extracted in 1 mL of 1M HCl under centrifugation at 5000 xg at 4°C for 1 h. The supernatant was secured and dried under reduced pressure. After redissolving with 1 mL of 6M HCl, the sample was placed in a hydrolysis tube, which was washed several times with argon, evacuated, sealed and heated to 100°C for 6 h. After cooling, the hydrolysate was evaporated to dryness under reduced pressure and finally redissolved in 1 mL of 0.06 M HCl.

To remove particulate matter and inorganic salt the sample was passed through a BIORAD AG 50W-X8, 100 to 200 mesh, cation exchange column. The column was washed with 6 mL of water, after which the amino acids were eluted between 2 and 3.5 mL of 8 M NH_4OH. The eluate was evaporated to dryness under reduced pressure, and the residue was finally redissolved in 1 mL of water.

A 100 μL aliquote of each sample was derived with a chiral fluorogen according to Aswad [*30*]. Derivatization was accomplished by thoroughly mixing the sample with 5 μL of OPA-NAC (o-phthaldialdehyde adducted with *N*-acetyl-L-cysteine) reagent in a polyethylene microfuge tube. After 2 to 3 min, 475 μL of 50 mM Na-acetate, pH 5.2, was added. 50 μL of this solution was taken for direct injection into the HPLC system.

A Waters HPLC system (Millipore Co, Milford, MA) consisting of two Model 510 pumps and one model 470 scanning fluorescence detector was used throughout this study. The diastereomeric dipeptides were separated on a reversed phase HPLC column, Kromasil C8, 25 cm and 4.6 mm i.d. (Eka Nobel AB, Bohus, Sweden), placed after guard and scavenger

columns. Isocratic eluation with 90% solvent A and 10% solvent B was caried out for 5 min. Solvent B was then increased linearly to 100% over a period of 5 min. Solvent A was 50 mM sodium acetate, pH 5.9, and solvent B was 80% (v/v) methanol and 20% (v/v) solvent A. Flow rate was 1.0 mL/min throughout. The excitation wavelength was set to 340 nm, and the emission wavelength to 420 nm. The data from the detector was collected and processed by Waters' Baseline system.

The ratio of D- and L-aspartic acid was calculated from the areas under the eluted peaks (mV*sec). The racemization of amino acids follows a first-order reversible rate law, where the racemization equation is

$$\ln[(1 + D/L)/(1 - D/L)]_t - \ln[(1 + D/L)/(1 - D/L)]_{t=0} = 2k_t{*}t$$

A linear regression model was constructed using this expression, where D/L is the ratio of D- and L-aspartic acids, t is any given time during racemization and the logarithmic term at $t = 0$ describes the amount of D-aspartic acid formed during hydrolysis. t (toothage) was expressed as the age of the individual when the root was fully formed (Rc), but apex not closed (Ac) according to the developmental tables by Haavikko [*31*] for each individual tooth.

Results

Figure 1 shows a chromatogram of the hydrolysate of the acid soluble fraction of apical dentine. D- and L-aspartic acid peaks were distinctly separated at about 8 and 9.5 min, respectively, and the remaining compounds were collected between 15 and 20 min.

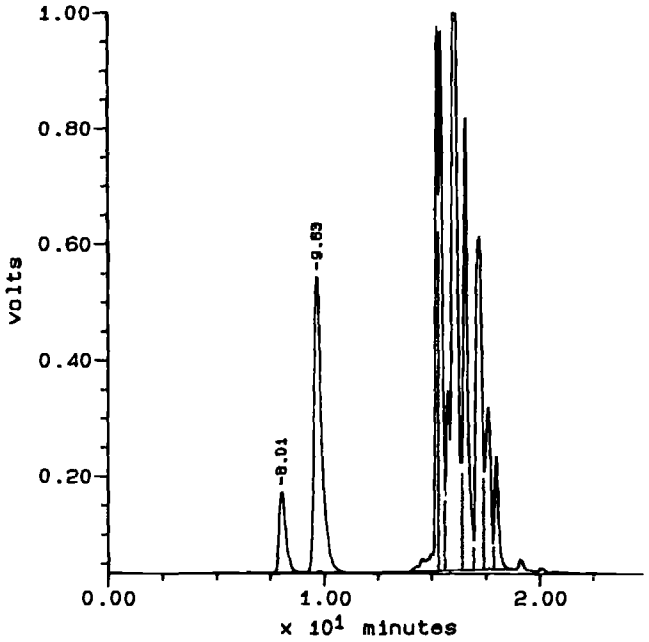

FIG. 1—*Reversed phase HPLC separation of OPA-NAC derivatives of D- and L-aspartic acid, at 8.01 and 9.63 min, respectively. The peaks between 15 and 20 min contain the rest of the injected material. Isocratic eluation with 90% solvent A and 10% solvent B was carried out for 5 min. The content of solvent B was then increased linearly to 100% over a 5 min period. The flowrate was 1 mL/min.*

A plot of ln[(1 + D/L)/(1 − D/L)] of aspartic acid against ages of the teeth (t_{Rc}) is shown in Fig. 2. The expression obtained by the linear regression with the ratio of D- and L-forms as the dependent variable was as follows:

$$\ln[(1 + D/L)/(1 - D/L)] = 0.043\,43 + 0.005\,30 * t_{Rc}.$$

The lines surrounding the regression line delineate a 95% prognostic limit around an individual prediction. The correlation coefficient (r) was 0.97, $N = 51$. The standard error of the regression coefficient was $1.97 * 10^4$, and the standard deviation of the residuals 0.032. The degree of explanation (r^2) was 93.7%, giving a methodological error of 6.3%.

To calculate age of the apical dentine from the D/L ratio, the following equation was derived:

$$t_{Rc} = \ln[(1 + D/L)/(1 - D/L)] * 174.46 - 4.55$$

The standard deviation around an individual prediction was ±6.1 to 6.3 yr, giving a 95% confidence interval of ±12.1 to 12.5 yr (Fig. 2). The standard deviation around a mean prediction was 0.9 to 1.9, giving a 95% confidence interval of ±1.7 to 3.7 yr.

Finally, chronological age of the individual was calculated by adding the age at which the root of the particular tooth was fully formed (Rc) to the calculated t_{Rc}.

Discussion

Age determination of teeth with the aid of the racemization of aspartic acid has been shown to be valuable for the estimation of chronological age of individuals with unknown birth records [*17,19,20*]. A series of studies over the last decade [*15–29*] has shown that both the precision and the reliability of the method are high. Although no comparative study between the "Gustafson method" and the "amino acid method" has been published,

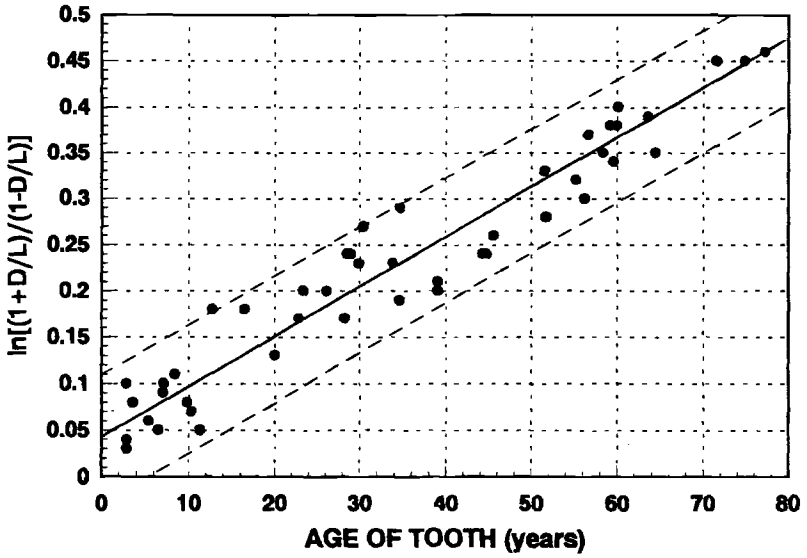

FIG. 2—*Plot of ln[(1 + D/L)/(1 − D/L)] of aspartic acid against the age of the apical part of the root dentine (Rc) of various kinds of teeth. The slope defines the rate constant (2k) of racemization. The surrounding lines denote a 95% confidence interval of an individual prediction.*

the latter method seems to give a more precise result. After some refinement of the Gustafson method, Johanson [*3*] reported a multiple correlation coefficient (r) of 0.92 and a standard deviation around the regression line of ±5.16 yr. Recently Solheim [*11*] reported a correlation coefficient of 0.89, and a standard error of the estimate of ± 7.9 yr. However, the authors did not give any further information about the statistics, and it is therefore not possible to calculate a 95% confidence interval around an estimated age. The various studies with racemization of aspartic acid have shown correlation coefficients of 0.95 to 0.99, and errors of estimates between 2 and 4 yr. However, it is not quite clear what is meant with the different values of dispersion that are presented. The present study gave a somewhat lower correlation coefficient, $r = 0.97$, and a 95% confidence interval around an estimated individual age of between 12.1 and 12.5 yr. None of the earlier studies has presented this kind of confidence interval around an estimated age, and direct comparisons are therefore not possible.

This study was carried out to explore whether ordinary HPLC technique could be used instead of the predominant gas chromatographic (GC) technique, which needs expensive equipment and special chiral columns. The result has shown that HPLC technique can be used with fairly good result, even if most of the studies that have used GC technique seem to have a higher reliability, up to $r = 0.99$ *vs* $r = 0.97$ in this study. It is presently not known if the reason for this descrepancy is differences in the techniques *per se*, or if the HPLC technique needs further refinements to make full use if its capacity. van den Oetelaar et al. [*32*] compared the separation efficiency of chiral capillary gas chromatographic and diastermeric high performance liquid chromatographic methods and found that the latter was preferred because of its higher reproducibility and convenience. Only the resolution was lower with HPLC technique. We have modified the technique, and thereafter demonstrated a very high resolution.

The method has high sensitivity, and when small samples (less than 0.5 mg of dentine) are tested 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 dentine may decrease the relative amount of the D-form. Similarly, contaminated with bacteria with D-form aspartic acid in their cell walls may cause an apparent too high age.

The rate of racemization of amino acids in dentine in the living body is mainly determined by temperature, pH and water content. Normal differences in the body temperature (36.2 to 37.6) may influence the rate of racemization to such a degree that age determinations of old individuals may differ up to 20 yr [*15*]. The rate of racemization also seems to be higher in the lingual (warmer) than the buccal (cooler) coronal dentine of anterior teeth [*26*].

Another explanation for a somewhat lower correlation coefficient in the present study may be that all kinds of teeth were used, and that all kinds of pathology was allowed, provided the apical part of the root was unaffected. Some of the earlier studies have been using only one kind of teeth [*26,29*].

It seems as if the "amino acid method" is the method of choice for age determination of adult teeth. This method also has the advantage of being usable on teeth *in situ*, since less than 0.05 mg of dentine is needed for the analysis. This amount can be taken from a viable tooth, which can then be restored with a small filling.

Conclusion

It has been shown that reversed phase HPLC technique can be used for the separation of D- and L-forms of aspartic acid in minute amounts of dentine, and that the sensitivity is high enough for practical use. However, further studies will show if the reliability can be increased.

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